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Progesterone Receptor Membrane Component 1 Promotes Cell Proliferation and Tamoxifen Resistance through Alteration of Sexual Receptors in Breast Cancer

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Abbreviations

AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
APS	ammonium persulfate
ATAC	arimidex, tamoxifen, alone or in combination
ATCC	american type culture collection
CYP	cytochrome P450
E_2	17-β estradiol
EtOH	ethanol
ER	estrogen receptor
ER-α	estrogen receptor-alpha
ERE	estrogen response element
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
Hpr6	heme-1 domain protein
HR	hormonal receptor
HRP	horseradish peroxidase
HRT	hormone replacement therapy
Kd	dissociation constant
kD	kilodalton
M	mole per liter

miRNA	microRNA
P_4	progesterone
PAIRBP1	plasminogen activator inhibitor 1 RNA-binding protein
PRE	progesterone response element
MAPR	membrane-associated progesterone receptor
MPA	medroxyprogesterone acetate
MTT	methyl thiazoletetrazolium
NET	norethisterone
PAGE	polyacrylamide gel electrophoresis
PEPI-MDS	postmenopausal estrogen/progestin interventions mammographic density
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween
PGRMC1	progesterone receptor membrane component 1
PR	progesterone receptor
RNAi	RNA interference
SERM	selective estrogen receptor modulator
SD	standard deviation
SDS	sodium dodecyl sulphate
SM	stripped medium
TAM	tamoxifen
TEMED	tetramethylenediamine
Tris	tris(hydroxymethyl)aminomethane

V	volt
WHI	women's health initiative

1. Introduction

1.1. Epidemiology of women breast cancer

Breast cancer remains the leading malignancy in women around the world and ranks the 5th among all cancerous causes of death (1). Globally 1 in 14 women develops breast cancer between birth and age 79 years (2). According to GLOBOCAN database (3), it is estimated that approximately 2.3 million new cases were diagnosed and 684,996 deaths attributed to breast cancer occurred women worldwide in 2020. In the United States, approximately 281,550 new invasive cases and 43,600 deaths of breast cancer were estimated among females in 2021 (4). In the European Union, breast represents the most commen cancer site in females, with 523,000 new cases and 138,000 deaths in 2018 (5). In China, breast cancer cases account for 12.2% of all newly diagnosed breast cancers and 9.6% of all breast cancer deaths worldwide (6).

Epidemiological distribution varies among regions all over the world. According to the statistics of World Cancer Research Fund (7), developed regions have higher incidences than developing regions, being 27 per 100,000 in Middle Africa and Eastern Asia, and 92 per 100,000 in Northern America. However, disparities in mortality rate appear negligible among different regions in that deaths account for 14.3% and 15.4% of total population in developing and developed regions, respectively. This is partly due to the more favorable survival of invasive cases in developed regions. For example, 5- and 10-year relative survival rates for invasive breast cancer have achieved 90% and 84% in the United (8) compared with 40% of 5-year survival rate in South Africa (9). Another factor is the sparse coverage of the population-based registries in developing regions, which leads to the underestimation of cancer incidence rate (1).

Increasing age is an important risk factor for all types of breast cancers (10). It is less frequently (<10%) diagnosed in younger women below 40 years old than the elderly (11). Perimenopausal and postmenopausal women are the two age groups with the highest incidence rates (12). In addition, the rapid increase after 1980s and the sharp decline of the breast cancer rate in those age groups between 2002 to 2003 are repectively in conformity with the introducted and decreased use of menopause

hormones (13). This suggests a close correlation between hormone replacement therapy (HRT) and breast cancer risk.

Breast cancers are categorized into five subtypes according to the St. Gallen classification based on the expression of hormonal receptor (HR), human epidermal growth factor receptor 2 (HER2), and Ki-67 (14-16): luminal A (high estrogen receptor (ER)/progesterone receptor (PR), HER2 negative, Ki-67<14%, T1/2, and N0/1); luminal B/HER2 negative (low ER/PR, HER2 negative, Ki-67≥20%, N2/3, T3, histologic grade 3, and extensive lymphovascular invasion); luminal B/HER2 positive (HR positive and HER2 positive); HER2 (HR negative and HER2 positive); and triple negative (HR negative and HER2 negative). Molecular characteristics of these subtypes are summarized in Table 1. HR positive cancers account for over two thirds among all the subtypes (11,7,18) and ER is more frequently expressed in postmenopausal patients than younger patients (19).

Table 1. Molecular classification of breast carcinoma

Classification	Immunoprofile	Other characteristics	Example cell lines
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Ki-67 low, endocrine responsive, often chemotherapy responsive	MCF-7, T47D, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^{+/-}$	Ki-67 high, usually endocrine responsive, variable to chemotherapy, $HER2^+$ are trastusumab responsive	BT474, ZR-75
Triple negative (Claudin-low)	ER^-, PR^-, $HER2^-$	Ki-67 high, E-cadherin, claudin-3, claudinin-4 and claudinin-7 low, intermediate response to chemotherapy	BT549, MDA-MB-231, Hs578T, SUM1315
Triple negative (Basal)	ER^-, PR^-, $HER2^-$	$EGFR^+$ and/or cytokeratin $5/6^+$, Ki-67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468, SUM190
HER2	ER^-, PR^-, $HER2^+$	Ki-67 high, trastusumab responsive, chemotherapy responsive	SKBR3, MDA-MB-453

(Soure: adapted from Holliday DL et al (20).)

1.2. Effects of estrogen signaling in breast cancer

1.2.1. Multiple estrogen signaling pathways

Estrogen alone has no boosting effect or even significantly reduces breast cancer risk in postmenopausal women (21). Normal proliferating breast epithelial cells are steroid hormone receptors negative (22). 17β-estradiol (E_2) is the most biologically active sexual hormone in breast tissue that stimulates breast development at puberty and during sexual maturity (23-26). Apart from this, E_2 also plays a major role in breast tumorigenesis and progression. Considerable evidence revealed a strong association between higher E_2 exposure and higher breast cancer risk (27-32). Among the established risk factors for breast cancer, early age of menarche and late onset of menopause are strongly correlated with higher circulating E_2 levels (33-35). In postmenopausal women, elevation of serum levels of total and non-sex hormone-binding globulin-bound E_2 increases breast cancer risk. Especially for non-sex hormone-binding globulin-bound E_2, compared with women in the lowest quartile, the relative risk is 5- to 6-fold higher for women in the upper three quartiles (27). The mean serum E_2 level in postmenopausal breast cancer women is 21-28% higher than those free of cancer, and the mean free E_2 percentage is 6-7% higher in the breast cancer patients (36). In addition, despite the significant difference in plasma levels of estrogen between pre- and postmenopausal women, local estrogen levels in breast cancer tissues remain similar between the two age groups where *de novo* biosynthesis catalyzed by aromatase inhibitors (AIs) plays a major role (37-44).

ER belongs to the nuclear receptor superfamily of transcriptional regulators (45). In human breast cancer cells, for example in MCF-7 cells, approximately ~85% of the total cellular estrogen receptors are in the nuclear, ~10% are in the mitochondria and 5% are at the plasma membrane (46). There are two subtypes of estrogen receptor, i.e. ER-α and ER-β, which share 56% of homology in their ligand-binding domains. Although DNA-binding sites of them overlap substantially (47-49), their downstream gene expressions differ dramatically (48-53). ER-α is found upregulated in most breast cancers and has been proved a hallmark of hormone-dependent growth. However, despite decreased levels of ER-β in some breast cancers (54-59), no definitive evidence of ER-β's linkage to clinical parameters in breast cancer has been determined (60). Currently existing carcinogenic effects of estrogen includes classic ER-α signaling

mediated proliferation, direct genotoxic effects mediated by cytochrome P450, and induction of aneuploidy (61). Among them, ER-α signaling pathway is the principle and most acknowledged mechanism by which estrogen regulates gene expression. Previous studies shows that ER-α is significantly upregulated in the majority of breast cancers (62) and elevation of ER-α level in benign breast epithelium appears to indicate an increased risk of breast cancer (63). These suggest an essential role for ER-α in breast cancer initiation and progression. However, the definitive map of an ER-α signaling with its downstream cascades, bypasses, and regulating factors has yet to be fully described. In the clinic setting, ER-α level is also a usual marker to predict hormone responsiveness in breast cancers. ER positive patients have longer disease-free intervals and overall survivals than ER negative patients (64).

The two distinct types of signaling mediated by ER-α referred to as genomic and non-genomic pathways, respectively, have been identified. Schematic model is shown in Figure 1. In the genomic pathway, external E_2 diffuses through the plasma and nuclear membranes into the nucleus. Upon E_2's binding to ER-α in the nucleus, a conformational change occurs in the receptors and the chaperones dissociate from the E_2-ER-α complex, followed by dimerization and association of dimeric ER to the estrogen response elements (EREs) of the target genes that eventually induce or inhibit gene transcription (65-70). Non-genomic pathway mediated by plasma membrane associated ER is another important mechanism of estrogen actions (71-75) which is much more rapid than genomic pathway (approximately 15 seconds versus at least 2 hours in the genomic pathway). Despite of the term “non-genomic”, non-genomic pathway actually induces both cytoplasmic alterations and effects on gene expression (75-79). However, compared with nuclear ER, regulation of transcription mediated by plasma membrane associated ER may be more complex which involves direct and indirect association of ER to DNA. As for the rapid events of the non-genomic pathway, at least four main downstream signaling cascades are activated upon stimulation: phospholipase C (PLC)/protein kinase C (PKC) (80-86), Ras/Raf/MAPK (87-93), phosphatidyl inositol 3 kinase (PI3K)/AKT (71,75,94-100), and cAMP/protein kinase A (PKA) (84,101-105). Moreover, several studies suggest that proliferative effect of E_2 appears to be mediated exclusively by non-genomic pathway (81,82,87,89,93,95,97,105) and that different cell line types and conditions may influence the rapid action of E_2 (71,82,90,97,106,107).

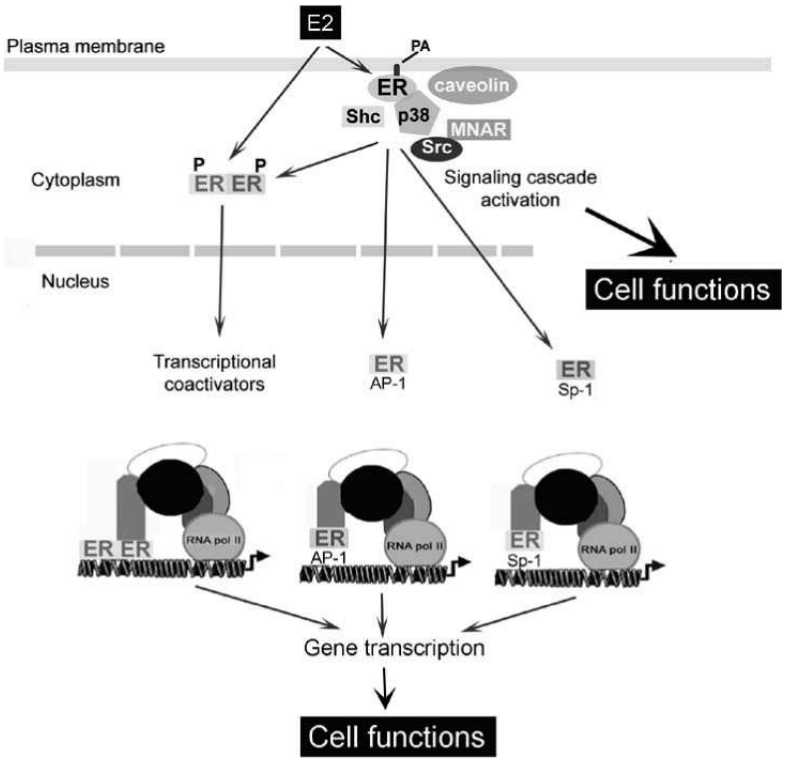


Figure 1. Schematic model illustrating the relationship between rapid, intermediate, and long term actions of E_2 on target cells.
(Source: adapted from Marino M et al (70).)

1.2.2. Antiestrogenic treatments in breast cancer

As proliferation is mainly mediated via ER-α in breast tissue and over two third of breast cancers are ER-α positive (18), antagonists targeting ER-α or inhibiting E_2 production remain the basic and primary strategy for endocrine therapy (108). Currently available drugs for the treatment of breast cancer that act on the ER signaling generally include three classes (109): the selective estrogen receptor modulators (SERMs) which bind to and block the signaling of the estrogen receptors, aromatase inhibitors (AIs) which block the activity of the key enzyme called aromatase during the production of estrogen, and gonadotropin-releasing hormone agonists which inhibit E_2 biosynthesis through blocking gonadotropin production.

Tamoxifen (TAM) represents the most commonly used and the "gold standard" treatment for ER positive breast cancer (110-112). Since the introduction of tamoxifen to ER positive breast cancer treatment more than 40 years ago (113), indications have expanded to include treatments of breast cancers of all stages, and ductal carcinoma in

situ; chemoprevention of breast cancer in pre- and postmenopausal women at high risk (114,115). Despite the dramatic decrease of mortality in breast cancer with adjuncvant TAM treatment (116), ~30% of the ER positive breast cancer population demonstrate minimal benefit (111,117). Even among patients with similar prognostic factors at diagnosis, treatment responses vary substantially (112). Currently understood mechanisms of hormone resistance include enhanced agonist action of antagonists and upregulation of cross-talk signaling, like growth factor signaling (118). In addition to the suppressive activity, TAM has also been reported to exert agonist activity. Some estrogen upregulated genes involved in cell proliferation and progression, such as androgen receptors (AR), TAF9B-like RNA polymerase II, TATA box binding protein (TBP)-associated factor (TAF9B, formerly TAF9L), carbonic anhydrase XII (CA12), Down syndrome cell adhesion molecule (DSCAM) and pre-B-cell leukemia homeobox (PBX1) are found to be upregulated by tamoxifen (65,119,120).

AIs are divided into two groups: steroidal AIs like exemestane that irreversibly inactivate aromatase, and nonsteroidal AIs like letrozole and anastrozole that reversibly bind and inactivate aromatase. AIs demonstrated superiority of efficacy over TAM in the first-line treatment for metastatic cancers (120-124) or as adjuvant therapy (125-136) in multiple randomized control trials and showed a significant effect for breast cancer prevention among women with increased risk (137-139). In the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial, at a median follow-up of 100 months, postmenopausal breast cancer women received anastrozole treatment demonstrated superiority over those who received TAM treatment in terms of disease-free survival, time to recurrence, incidence of new contralateral breast cancer and time to distant recurrence (125). However, due to the deprival of total body estrogen production, usage of AIs is limited at least beyond younger women.

1.3. Effects of progestogens in breast cancer

Progestogens refer to a group of compounds that bind to progesterone receptor and exhibit progestational activities. Progestogens are generally divided into two classes: natural progesterone (P_4) and synthetic progestins. P_4 is the only natural progestogen, and progestins comprise a variety of synthetic progestogens (140). According to the structural similarities to progesterone and testosterone, progestins are further constituted of two subclasses: medroxyprogesterone acetate (MPA) is structurally related to

progesterone, and norethisterone (NET) is structurally related to testosterone, respectively.

Roles of progestogens in breast cancer growth and progression remain controversial. Epidemiological and clinical evidence have linked increased breast cancer risk to the combined estrogen plus progestogen therapy (141-148). This increase is thought to be attributed to the usage of synthetic progestins rather than progesterone (149,150). Results from the Women's Health Initiative (WHI) trial have revealed an increased breast cancer risk with conjugated equine estrogens plus MPA (151), but not with single estrogen therapy (152,153). However, in the Postmenopausal Estrogen/Progestin Interventions Mammographic Density Study (PEPI-MDS), increased serum progestogen levels including progesterone level following estrogen plus progestogen treatment is positively associated with greater increase in mammographic density which has been considered a strong predictor for breast cancer (154).

PR belongs to the steroid hormone family of nuclear receptors and it regulates gene transciption through ligand activation. Isoforms of PR include a full length PR-B which is necessary for mammary gland development and expansion, an N-terminally truncated PR-A (the -NH_2 terminus of 164 amino acids encoding a transativation function (155-158) which is predominantly responsible for uterine and ovary actions, and a non-functional PR-C which only consists of a hinge region and a hormone binding domain without the capability of DNA binding. Expressions of PR-B and PR-A are in an 1:1 ratio manner, whereas PR-C expression is limited. Upon ligand binding, PRs dimerize to form both homo- and heterodimers which consist of A:A, B:B and A:B. In normal mammary gland development, only 10-15% of mammary luminal cells express PR, and for those steroid receptor negative cells, progestogens might act through a paracrine action mechanism (159). In mammary gland, PR exerts a mitogenic role in concert with ER as opposed to an antiproliferative action in endometrium and ovary. Therefore, PR expression is usually considered as a biomarker of ER-α function and a predictor of breast cancer prognosis (160). However, induction of PR expression highly requires activation of ER-α signaling pathway in ER positive breast cancer cells (161-166), which causes a huge challenge for researchers to investigate the PR actions separately.

The mechanisms by which progestogens increase breast cancer risk are thought to be via genomic and/or non-genomic actions. Classical mechanism of progestogens is

generally mediated via PR. In addition, direct role of PR in human breast cancer has been well demonstrated in previous clinical studies. Similar to estrogen, the mechanism of progestogen in cancerous proliferation consists of both genomic and non-genomic actions (167). Progesterone enters the cell by diffusion and bind to the hormone binding domain of PR. Upon ligand-PR interaction, conformation of PR changes and promotes nuclear localization, dimerization, and DNA binding. PR typically binds to the Progesterone Response Elements (PREs) of the target genes to initiate gene transcription. Besides classical mechanism, non-classical mechanism, also known as non-genomic mechanism plays an additionally important role in stimulating cell proliferation which does not involve a direct binding of ligand-receptor comlex to DNA to regulate gene transcription but via activation of various protein-kinase cascades initiated by progestogen's binding to membrane associated PRs to elicit quick actions. Up till now, two types of membrane associated PR have been identified which include progesterone membrane receptors (mPRs) and Progesterone Receptor Membrane Component 1 (PGRMC1)/sigma-2 receptor. The structure of mPR contains seven trans-membrane domains and has a high binding affinity for progesterone (dissociation constant, Kd ~ 5 nM) (168,169). PGRMC1 contains one trans-membrane domain and binding affinity for progesterone measured in Kd is approximately ~ 11 nM (170).

1.4. Characteristics of PCRMC1 mechanism

1.4.1. Structure and location of PGRMC1

PGRMC1 belongs to the membrane-associated progesterone receptor (MAPR) protein family. It was firstly purified in sufficient amount from porcine liver membranes by Meyer et al (170), and human PGRMC1 cDNA was cloned by Gerdes et al (171). Human PGRMC1 gene (Gene ID: 10857) is located on X chromosome and contains 3 exons that encode the 195 amino acid residues of 22 kD PGRMC1 protein (172,173). Structure of PGRMC1 contains a short luminal peptide, a single N-terminal transmembrane helix, and a C-terminal cytochrome b5-related heme-binding domain (Figure 2) (174,175). PGRMC1 is anchored to the membrane through the single N-terminal transmembrane helix. The C-terminal cytochrome b5-related heme-binding domain works as a functional domain to participate in the signaling pathways and cholesterol biosynthesis (176,177).

PGRMC1 resides widely but diversely throughout the body. It is abundant in tissues of high cytochrome P450 enzyme activity like liver, kidney and adrenal gland (178), and is detected of increased expression in many tumor types (179-185). Subcellular locations of PGRMC1 vary among different cell line types, and may include but not limited to the endoplasmic reticulum, plasma membrane, nucleus, endosomes, Golgi, and cytoplasm (172,186-190). For exemple, PGRMC1 is mainly located in the cytoplasm and nuecleus in Ovcar-3 overy cancer cells, and mainly located in the perinucleus and endoplasmic reticulum in MCF-7 breast cancer cells (191). While endoplasmic reticulum is the most common subcellular location, PGRMC1 can transfer from membrane-associated status into cytoplasm and nucleus to exert different functions when microenvironment changes (172).

1.4.2. Active form and binding characteristics

Monomers of PGRMC1 are functionally inactive. Dimerization of PGRMC1 is required for most of its functions, and such dimerization exhibits a haem-dependent manner. In the presence of haem, one PGRMC1 and one haem iron form a pentacoordinate compound and two coordinated compounds dimerize through hydrophobic interactions between the two haem moieties (174). Some conserved amino acid residuals among MAPR family members are found essential for the coordination between PGRMC1 and haem, including Tyr113 or Tyr 164 and Tyr 107 (174,192,193). Besides, PGRMC1 dimer appears to be more stable than other transmembrane protein dimers like Toll like receptor 9 and plexin A2 receptor, with dimerization Kd of < 3.5 mmol/L versus 20 mmol/L and > 300 mmol/L (174). Carbon monoxide is an inhibitor for such haem-dependent PGRMC1 dimerization by binding to the sixth coordination site of the haem, which at physiological levels can induce dissociation of the PGRMC1 dimers (174).

PGRMC1 is a high-affinity progesterone binding protein, and this binding shows a reversible, rapid, selective and saturable feature (170). Chemical and structural analyses (194) have been conducted to identify the amino acid residues of functional significance for progesterone binding. Amino residues of histidine, arginine, cysteine and tyrosine do not have a significant role in the binding of progesterone. In contrast, carboxyl, tryptophan and methionine residues are involved in a direct or indirect manner. In addition, diassociation of PGRMC1 dimer results in significant reduction of progesterone binding, and this implies that PGRMC1 dimer is the active form to exert

nongenomic progesterone actions. Other steroids also exhibit differential binding activities to PGRMC1. For example, corticosterone has 25% and testosterone has 16% affinities for PGRMC1 relative to progesterone, whereas β-estradiol and aldosterone only have negligible affinities of < 0.5% (170). However, clinical precautions have to be taken since physiological levels of these steroids may be hundreds of folds higher than that of progesterone (195), and this may negate the impact of affinity differences on the amount of receptor activated by PGRMC1 among the above steroids.

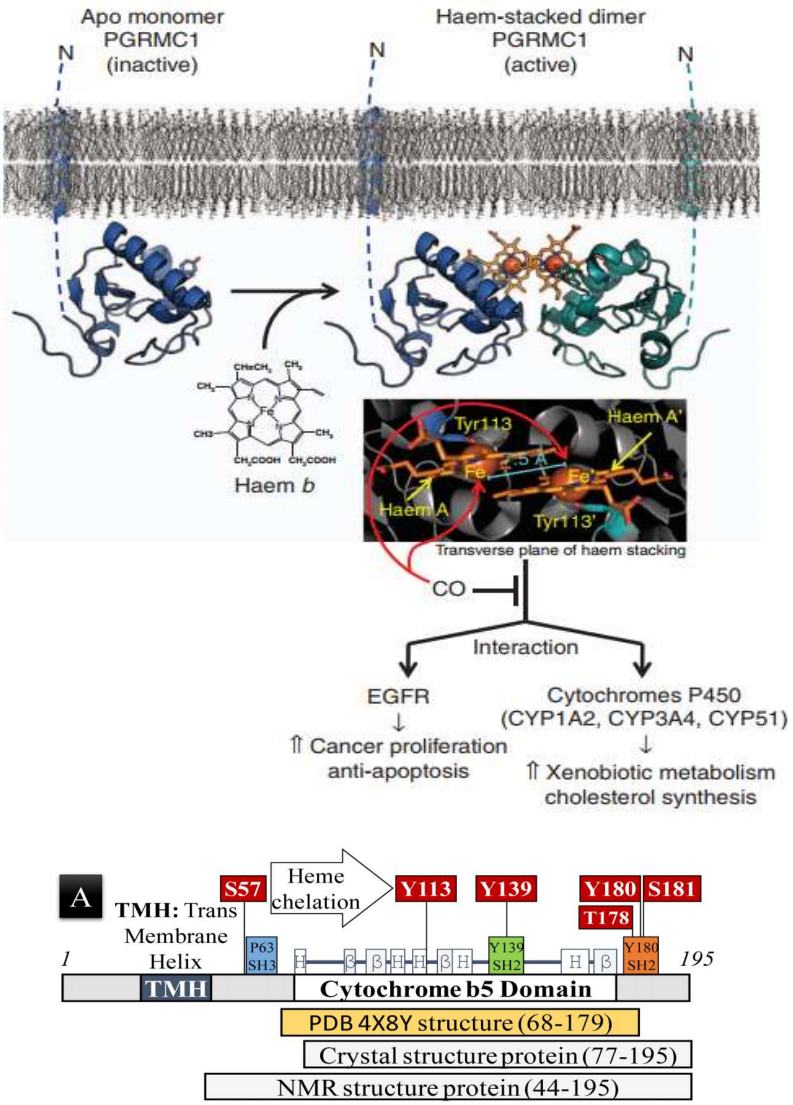


Figure 2. Schematic diagram of the structure and function of PGRMC1 protein. Structure of PGRMC1 contains a short luminal peptide, a single N-terminal transmembrane helix, and a C-terminal cytochrome b5-related heme-binding domain. Monomers of PGRMC1 are functionally inactive. Upon haem binding, PGRMC1 forms a dimer to induce interactions with EGFR and cytochromes P450, leading to an enhanced proliferation and chemoresistance of cancer cells.
(Source: adaped from Kabe et al (174) and Michael et al (175).)

1.4.3. Regulatory factors of PGRMC1 expression

Regulation of PGRMC1 expression has not been fully understood, with only a minority of factors being identified. Several aryl hydrocarbon receptor (AhR) response elements are hypothesized to be located in the promoter region of human PGRMC1 and excert regulatory funtions by binding with corresponding ligands (172). 2,3,7,8-tetrachloro dibenzodioxine is a strong ligand for AhR. In an early study (196), Selmin et al have found a dose-dependent upregulation of *Pgrmc1* gene in the liver of Sprague-Dawley male rats treated with 2,3,7,8-tetrachloro dibenzodioxine. Similarly, other xenobiotic inducers like omeprazol and carcinogens such as doxorubicin, dioxin and heavy metals are also found to promote PGRMC1 expression through activation of AhR signaling pathway, and in contrast, flavone AhR ligands surpress this pathway (197-199) .

Steroid hormones have also been found to be possible regulatory factors of PGRMC1 expression. In one animal experiment conducted by Kreb et al (200), ovariectomized Sprague-Dawley rats were treated with 12.5 μg E_2 benzoate subcutaneous injection followed by 500 μg P_4 intraperitoneal injection 24 hours later, or E_2 benzoate or vehicle alone. Three hours following the last injection, PGRMC1 expression in the ventromedial hypothalamus was enhanced by nearly 40% with E_2 benzoate treatment as compared to a repression to the control level with the combined treatment of E_2 benzoate followed by P_4 as measured by *in situ* hybridizations. In the same experiment, knochout of PR increased PGRMC1 expression in the ventromedial hypothalamus compared with wild type mice. These indicate a repressive action of P_4 combined with PR in the regulation of PGRMC1 expression. However, in another experiment conducted by Bali et al (201), ovariectomized adult rats were treated with E_2 and P_4 alone or in combination in two simulated schedules of 4-day estrous cycle and 30-day postmenopausal hormone therapy. Results revealed a general induction of *Pgrmc1* gene with treatments of E_2 and/or P_4 throughout the hippocampus in CA1, CA3, and DG neurons. In addition, Nilsson et al used ovaries from Sprague–Dawley rat pups to conduct an *in vitro* experiment (202) and found an increase of 1.5-fold in PGRMC1 expression by 10^{-6} M P_4 treatment despite the lack of statistical significance. Unfortunately, up to the present time, studies to assess steroidal regulations of PGRMC1 expression in breast tissues have not been conducted.

Solid tumors including breast cancers are generally at uncontrollably rapid metabolism

and proliferation, making a hypoxic microenvironment with limited oxygen and nutrition supply. Hypoxia is of great regulatory value in tumor growth and tumor progression, and is a critical predictive factor for responses to radiation and chemotherapy (183-187,203). In hypoxic conditions, hypoxia-inducible factor 1α escapes hydroxylation and subsequent proteasome mediated destruction, which allows its heterodimer formation with hypoxia-inducible factor 1β in the nucleus and initiate the transcription of related genes of which most are related to angiogenesis, glycolytic pathway, apoptosis. Oxygen supply has been linked to PGRMC1 expression in some species. In one *in vitro* experiment conducted by Hughes et al (176), cells of *Schizosaccharomyces pombe* were cultured under anaerobic conditions for 1.5 hours, expression of DAP1, a fission yeast homology of PGRMC1, was increased by approximately 2.6 folds compared with control. *Dap1* was considered a target gene of an oxygen-sensing functioning sterol regulatory element binding protein, i.e. Sre1p, and upregulation of DAP1 showed a sre1p-dependent manner (176,204). Neubauer et al (205) used the differential two dimensional polyacrylamide gel electrophoresis and immune fluorescence analysis to analyze the proteomics of cryopreserved human breast cancer tumors and found that PGRMC1 was expressed in cells surrounding the tumor necrotic centre and that the PGRMC1 expressing cells were presumably in the hypoxic zone. Furthermore, a ffymetrix U133 Plus 2.0 GeneChip arrays was performed by Dressman HK et al (206) to analyze the gene expression of locally advanced breast cancer, and PGRMC1 was found to be correlated with hypoxia status and was considered to be one of the predictors of tumor hypoxia. All these findings imply a probable inducing effect of hypoxia for PGRMC1 upregulation though the exact regulatory mechanism warrants further investigations.

MicroRNAs (miRNA) are a group of conservated, single-stranded and noncoding RNAs of approximately 22 nucleotides that through inhibiting mRNA translation or promoting mRNA degradation regulate various biological processes including gene expression at the post-transcription level. Several miRNAs have been found to regulate PGRMC1 expression at this level. In human ovarian granulosa tumor KGN cells and rat primary granulosa cells, hyaluronic acid at 100 μg/ml, 200 μg/ml and 500 μg/ml treating for 24 or 48 hours significantly upregulated PGRMC1 expression, whereas transfection of miR-139-5p before hyaluronic acid treatment suppressed such induction (207). In additon, the same research has identified additional miRNAs including miR-98, miR-

let-7i and miR-7 to target PGRMC1. In endometrial cancer Ishikawa cells, transfection of miR-98 significantly repressed PGRMC1 expression to reduce cell proliferation (208). In ovarian cancer OVCAR-3 cells, the mucin 1 aptamer-let-7i miRNA chimera released let-7i miRNA was found to reverse paclitaxel-induced chemoresistance through downregulation of PGRMC1 expressions (209). In human ovary cancer SKOV-3 cells, transfection of miRNAs let-7/miR-98 resulted in significant supression of *pgrmc1* transcription (210). In the same experiment, binding sites of high conservancy for let-7/ miR-98, miR-141/200a and some nonconserved sites for more miRNAs were identified in the 3'-untranslated region of PGRMC1 messenger RNA by in silico analysis. This funding provides a theoretical explanation of miRNAs' intervention in PGRMC1 expression.

1.4.4. Interaction of PGRMC1 with cytochrome P450 enzymes

Cytochrome P450 (CYP450) enzymes as heme-dependent monoxygenases participate in various physiological processes including biosynthesis of cholesterol, steroids, bile acids, vitamin D_3 and eicosanoids, as well as detoxification of xenobiotics and metabolism of pharmaceutical drugs (211). Reduced activity of cytochrome P450 enzymes results in abnormalities in the above processes, including carcinogenesis. PGRMC1 shares a key structural motif with cytochrome b5 and is hyphthesized to interact with CYP450 enzymes. In an early study conducted by Min et al (193), rat adrenal inner zone antigen (IZA)/PGRMC1 was proved to bind heme as evaluated by spectrophotometric analysis and electron paramagnetic resonance measurements. In addition, two Tyr residues, Tyr107 and Tyr113, and a peptide stretch, D99-K102, were found to play a key role in the anchoring of heme into a hydrophobic pocket. In another study conducted by Ghosh et al (212), Yeast damage-associated response protein and mouse PGRMC-1 protein were also reported to bind heme, and such binding showed a noncovalent manner with Tyr138 most likely being the axial ligand to the heme in Yeast damage-associated response protein.

Functional interactions between PGRMC1 and CYP450 enzymes' activities have demonstrated to cause alterations in various biological processes, and such regulations are broadly conserved among eukaryotes. For example, in the *in vitro* studies (213,214), *Saccharomyces cerevisiae* DAP1, an *Schizosaccharomyces pombe* homology of mammalian PGRMC1 was found to increase the activities of Erg11/Cyp51A1 and Erg5/

Cyp61A1 which are required for sterol biosynthesis. In rat adrenal microsomes, cells overexpressing PGRMC1 posessed increased activity of progesterone hydroxylation catalyzed by CYP21 (215). Coexpression of IZA/PGRMC1 with CYP21A2, CYP11B1 or CYP17 in monkey fibroblast COS-7 cells was found to enhance progesterone 21-hydroxylation but not 11β- or 17α-hydroxylation, and activation of the CYP17-catalyzed 17–20 lyase reaction (216). Knockdown of human PGRMC1 by RNA interference (RNAi) reduced Cyp51A1 activity, resulting in suppression of cholesterol synthesis and increased production of toxic sterol intermediates (176). Recently, Piel et al demonstrated that PGRMC1 interacts as a regulator with ferrochelatase in the pathway of heme synthesis to shuttle newly synthesized heme from the mitochondrion to CYP450 enzymes (217). Further detailed summary of the interaction between PGRMC1 and CYP450 system is provided in Table 2.

Table 2. Functional interactions between PGRMC1 and CYP450 system

PGRMC1	CYP	System	Enzymatic activity	Direction of influence
IZA/PGRMC1 (rat)	CYP21A2 (rat)	Inhibition by anti-IZA monoclonal antibody	Progesterone 21-hydroxylase	Activation
IZA/PGRMC1 (rat)	CYP21A2 (rat)	COS-7 cell coexpression	Progesterone 21-hydroxylase	Activation
IZA/PGRMC1 (rat)	CYP17 (guinea pig)	COS-7 cell coexpression	Progesterone 17α-hydroxylase	Little or no influence
IZA/PGRMC1 (rat)	CYP17 (guinea pig)	COS-7 cell coexpression	17α-Hydroxyprogesterone 17–20 lyase	Activation
IZA/PGRMC1 (rat)	CYP11B1 (rat)	COS-7 cell coexpression	Progesterone 11β-hydroxylase	Little or no influence
PGRMC1 (human)	CYP19A1 (human)	CYP19-engineered MCF-7 human breast cancer cells RNAi knockdown	Androst-4-ene-3,17-dione conversion	Activation
Dap1/PGRMC1 (*S. cerevisiae*)	CYP51A1 (*S. cerevisiae*)	*S. cerevisiae* genetics	Lanosterol-14-demethylase	Activation
Dap1/PGRMC1 (*S. pombe*)	CYP51A1 (*S. pombe*)	*S. pombe* strain lacking DAP1	Lanosterol-14-demethylase	Activation

(to be continued)

Table 2. Functional interactions between PGRMC1 and CYP450 system (continued)

PGRMC1	CYP	System	Enzymatic activity	Direction of influence
Dap1/PGRMC1 (*S. pombe*)	CYP61A1 (*S. pombe*)	*S. pombe* strain lacking DAP1	Lanosterol-22-desaturase	Activation
PGRMC1 (rabbit)	CYP2C2 (rabbit)	HEK293, HepG2 cell coexpression	Luciferin 6′ methyl ether O-demethylation	Inhibition
PGRMC1 (human)	CYP2C8 (human)	HEK293, HepG2 cell coexpression	Luciferin 6′ methyl ether O-demethylation	Inhibition
PGRMC1 (human)	CYP3A4 (human)	HEK293, HepG2 cell coexpression	Luciferin 6′ pentafluorobenzyl ether depentafluorobenzylation	Inhibition
PGRMC1 (human)	CYP2C9 (human)	HepG2 coexpression, human hepatocytes RNAi knockdown	S-Warfarin 7-hydroxylase and diclofenac 4′-hydroxylase	Inhibition
PGRMC1 (human)	CYP3A4 (human)	HepG2 coexpression, human hepatocytes RNAi knockdown	Testosterone 6β-hydroxylase and midazolam 1′-hydroxylase	Inhibition
PGRMC1 (human)	CYP2E1 (human)	HepG2 coexpression, human hepatocytes RNAi knockdown	Chlorzoxazone 6-hydroxylase and 7-ethoxycoumarin O-deethylase	No influence

(Soure: adapted from Ryu et al (218).)

1.4.5. Correlation of PGRMC1 with neoplasms

PGRMC1 as a potential regulator of various aspects of tumorigenesis including alterations of CYP450 activity, progesterone metabolism, steroid biosynthesis and genotoxic agents resistance has been broadly examined in clinical tumor samples and cancer cell lines. Elevated PGRMC1 expression has been detected in a broad spectrum of neoplasms, and in most tumors, including breast cancer, its expression appears to increase in more advanced stages and thus is considered as an independent prognostic factor.

In the renal tissues, Zhang et al (219) compared PGRMC1 expressions using 135 pairs of cancerous and para-cancerous tissues from renal cell carcinoma patients. PGRMC1 protein was found to significantly increase by 3.91-fold in cancerous tissues compared with para-cancerous tissues as measured by a quantitative proteome identification. By immunohistochemical staining, 86 out of 135 renal cell carcinoma samples showed a higher level of PGRMC1 staining compared with the noncancerous counterparts. In the clinical aspect, higher PGRMC1 level showed a significant correlation with more advanced malignancy degree, and patients with lower PGRMC1 level had significantly longer overall survival time compared with those with higher PGRMC1 level.

In the pulmonary tissues, Mir et al (184) compared PGRMC1 levels of squamous cell lung cancers and lung adenocarcinomas to corresponding nonmalignant tissue. PGRMC1 levels were found to increase significantly in most tumors, especially poorly differentiated samples. Inhibition of PGRMC1 with siRNA knockdown and AG-205 both significantly inhibited tumor cell survival. Ahmed et al (220) used A549 non-small cell lung cancer cells to establish PGRMC1 RNA interference cells with a lentiviral-based shRNA approach to investigate the role of PGRMC1 in tumorigenesis. Athymic nude female mice were injected subcutaneously with A549/control or A549/RNAi cells. Three weeks later, the excised tumor weight of A549/control tumors was found to be 2.9-fold more than that of A549/RNAi tumors, and the tumor volume of A549/control tumors was found to be 8.1-fold larger than that of A549/RNAi tumors.

In the ovarian tissues, Peluso et al (181) used archival tissues and cDNAs of ovarian cancers to conduct the expression studies, PGRMC1 mRNA levels were found to remain relatively constant in early stages and significantly increased with advancing

stages.

In female breast tissues, PGRMC1 is immunohistochemically undetectable in normal mammary gland (221) and positively expressed in breast cancer tissues (205). In one study conducted by Crudden et al (203), Hpr6/PGRMC1 expression was reported to be significantly upregulated in breast tumors compared with matched nonmalignant tissues as measure by Western blot. In the clinical settings, increased expression of PGRMC1 is significantly correlated to increased lymph node metastasis, tumor size and TNM stage as well as reduced overall survival rate and tumor-free survival. In the clinical setting, Ji et al (221) used an immunohistochemical method to investigate the association of PGRMC1 expression with the clinicopathological features of breast cancer in 60 surgical specimens of breast cancer. PGRMC1 expression was strongly associated with cancer progression in terms of tumor size, TNM stage, lymph node metastasis, overall survival rate, and tumor-free survival. PGRMC1 was identified to be an independent prognostic factor and thus a useful prognostic indicator of breast malignancy.

In addition, PGRMC1 has also been found to mediate resistance to chemotherapeutic agents. In the MES-SA uterine sarcoma cells, Lin et al (222) used knockdown and overexpression approaches to evaluate the role of PGRMC1 in chemoresistance and found that PGRMC1 overexpression exhibited an anti-apoptotic effect and repressed doxorubicin-induced cytotoxicity; furthermore, it promoted cell proliferation, cell cycle progression to the S phase, epithelial-mesenchymal transition, thus facilitating tumor migration and invasion. In the P-glycoprotein-overexpressing doxorubicin-resistant MES-SA cells, PGRMC1 knockdown combined with P-glycoprotein inhibitor verapamil significantly decreased cell viability after doxorubicin treatment. The authors concluded that PGRMC1 contributed to chemoresistance through the effects of anti-apoptosis, cell proliferation and epithelial-mesenchymal transition induction.

In endometrial cancers, Friel et al (182) conducted both *in vitro* and *in vivo* studies to evaluate the impact of PGRMC1 on endometrial cancer cell viability in response to chemotherapy. They used a lentiviral-based shRNA knockdown approach to generate stable PGRMC1-intact and PGRMC1-deplete Ishikawa endometrial cancer cell lines. PGRMC1-intact cells treated with 2 μg/ml doxorubicin for 48 hours showed a significant increase in cell death than PGRMC1-deplete cells. Similarly, in the mouse xenograft models established with the above cells, tumors derived from PGRMC1-

deplete cells showed slower growth and approximately 4-fold higer tumor volume decrease following 15 mg/kg paclitaxel intraperitoneal injection than that from PGRMC1-intact cells.

In female breast tissues, Crudden et al (198) found that MDA-MB-231 ER negative human breast cancer cells with Hpr6 (heme-1 domain protein)/PGRMC1 expression suppressed by RNAi demonstrated significantly increased sensitivity to chemotherapeutic drugs of topoisomerase II inhibitor doxorubicin and topoisomerase I inhibitor camptothecin. The author also used an adenovirus encoding aspartate 120-to-glycine (D120G) mutant of Hpr6, i.e. Ad-Hpr6^{hbd} adenovirus which suffered a loss of heme binding activicy to infect MDA-MB-231 ER cells. Ad-Hpr6^{hbd} adenovirus-infected cells showed a significant loss of viability after doxorubicin and camptothecin treatments compared with control virus infected cells. The author considered Hpr6/PGRMC1 as a promising target for cancer theapy. In another aspect, Willibald et al (223) used tissue biopsies of 69 breast cancer patients were analyzed by immunohistochemistry for expression levels of PGRMC1 and phosphorylated PGRMC1 and found that patients with higher PGRMC1 tumor levels showed worse response to anthracycline-based therapy. These suggest that PGRMC1 could take an important part in breast cancer progression and treatment resistance and might offer an optional target for future anticancer therapy.

1.5. Aims of the study

Based on the emerging observations, the present study was undertaken in empty vector or PGRMC1 transfected estrogen receptor positive breast cancer cells, ie. MCF-7ECV, T47DEVC, MCF-7WT12, and T47DWT3 cells (1) to evaluate the impact of PGRMC1 overexpression on the regulation of cell proliferation by E_2 and progestogens; (2) to evaluate the impact of PGRMC1 overexpression on the response to TAM treatment; (3) to evaluate the impact of PGRMC1 overexpression on the regulation of ER-α, PR and intrinsic PGRMC1 expression by E_2 and progestogens so as to discover the possible correlation between PGRMC1 and sexual hormone signaling pathways in breast cancer, as well as to investigate a tentative role of PGRMC1 in endocrine resistance.

2. Materials and Methods

2.1. Materials and equipment

Media, Sera and Reagents	Manufacturers
Albumin Standard	(Thermo Fisher Scientific, USA)
DMEM medium	(Thermo Fisher Scientific, USA)
Dimethyl sulfoxide	(Sigma-Aldrich, USA)
Ethanol	(Sigma-Aldrich, USA)
Fetal bovine serum	(Thermo Fisher Scientific, USA)
HEPES buffer solution	(Thermo Fisher Scientific, USA)
Hyclone® charcoal-stripped fetal bovine serum	(Thermo Fisher Scientific, USA)
Hygromycin B	(Invitrogen, Carlsbad, USA)
Methanol	(Sigma-Aldrich, USA)
Methyl thiazoletetrazolium	(Sigma-Aldrich, USA)
Nonfat milk	(Sucofin, Germany)
Pageruler prestained protein ladder	(Fermentas, Germany)
PBS	(Thermo Fisher Scientific, USA)
Penicillin/streptomycin	(Sigma-Aldrich, USA)
RIPA	(Sigma-Aldrich, USA)
RPMI medium 1640	(Thermo Fisher Scientific, USA)
Sodium pyruvate	(Thermo Fisher Scientific, USA)
TEMED	(Sigma-Aldrich, USA)
Tris-HCl	(Sigma-Aldrich, USA)
Trypan blue stain (0.4%)	(Thermo Fisher Scientific, USA)
Trypsin-EDTA	(Sigma-Aldrich, USA)
TWEEN-20	(Sigma-Aldrich, USA)

Drugs	**Manufacturers**
Estradiol	(Sigma-Aldrich, USA)
MPA	(Sigma-Aldrich, USA)
NET	(Sigma-Aldrich, USA)
P_4	(Sigma-Aldrich, USA)
TAM	(Sigma-Aldrich, USA)

Antibiotics	**Manufacturers**
Actin (I-19)-antibody	(Santa Cruz Biotechnology, USA)
ER-α antibody (H-184)	(Santa Cruz Biotechnology, USA)
PR antibody (AB-400)	(AAT Bioquest, USA)
Goat anti-rabbit IgG-HRP	(Santa Cruz Biotechnology, USA)
PGRMC1 antibody (G-21)	(Santa Cruz Biotechnology, USA)

kits	**Manufacturers**
Pierce® BCA protein assay kit	(Thermo Fisher Scientific, USA)
ECL western blotting analysis system	(Amersham, UK)
Protease inhibitor cocktail kit	(Thermo Fisher Scientific, USA)
PVDF blot membrane	(Amersham Life Science, Sweden)

Equipment	**Manufacturers**
-20°C refrigerator	(Bosch, Germany)
-80°C refrigerator	(Heraeus, Germany)
0.22 µm filter	(Whatman, UK)
0.45 µm PVDF blot membrane	(Amersham Life Science, Sweden)
96-well plate	(Corning, Germany)
8-well plate	(Corning, Germany)

Bag sealer	(Krups, Germany)
Balance CP323S-OCE	(Sartorius, Germany)
Biological safety cabinet	(Heraeus, Germany)
Cell culture flask	(Greiner Bio-One, Germany)
Cell freezing tube	(Greiner Bio-One, Germany)
Cell incubator	(Binder, Germany)
Centrifuge 5417R	(Eppendorf, Germany)
ELISA Reader	(Tecan Sunrise, Germany)
Electrophoresis system	(Biorad, USA)
Eppendorf® thermomixer compact	(Sigma-Aldrich, USA)
Filter paper	(Whatman, UK)
Gel electrophoreses and blotting equipment	(Biorad, USA)
Heraeus Biofuge Pico microlitre centrifuge	(Heraeus, Germany)
Ice maker	(Scotsman, USA)
Inverted microscope	(Leica, Germany)
Magnetic stirrer	(Uniequip, Germany)
Mini Trans-Blot® Electrophoretic Transfer system	(Biorad, USA)
MS1 Minishaker	(IKA, Germany)
Odyssey® Fc Imaging System	(LI-COR Biosciences, USA)
PH meter	(Mettler Toledo, Germany)
Pipette	(Abimed, Germany)
Pipetus® Electronic pipette	(Hirschmann, Germany)
Shaker	(Heidolph, Germany)
Transferpette® micropipette	(Eppendorf, Germany)
Vortex	(Heidolph, Germany)
Vortex-Genie	(Heidolph, Germany)
Waterbath	(Heidolph, Germany)

2.2. Drug dilution

E_2, P_4, MPA and NET were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). All drugs were diluted with ethanol (EtOH) to 10^{-3} M (mol/l) and stored at -20°C. Serial dilution was performed with charcoal-stripped fetal bovine serum (SM) for each drug to corresponding working concentrations which included 10^{-9} M for E_2, and 10^{-6} M and 10^{-7} M for the remaining drugs. As a result, EtOH levels in all working solutions were at most 0.1%. For the present study, 0.1% of EtOH in SM was use as control.

2.3. Methyl thiazoletetrazolium (MTT) dilution

MTT was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). MTT was dissolved in PBS to a concentration of 1 mg/ml as stock solution. After sterilization with 0.22 μm filter, stock solution was stored at -20°C. Working solution was made by diluting stock solution at 1:4 with culture medium without phenol red.

2.4. Cells and cell culture

MCF-7 and T47D cells were both purchased from American Type Culture Collection (ATCC). MCF-7EVC and MCF-7WT12 cells were PGRMC1 expression plasmid and empty vectors stably transfected MCF-7 cells, respectively. T47DEVC and T47DWT3 cells were PGRMC1 expression plasmid and empty vectors stably transfected T47D cells, respectively. All cells used for the present study were within passage 10. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml) in sterile conditions at 37˚C with humidified atmosphere of 5% CO_2. Trypsinization and inoculation with sterile 96-well plate were performed at a confluence of approximately 85%. Trypan blue exclusion test was performed concomitantly with inoculation to ensure proper and consistent inoculum density of living cells. Initial incubation included 1 day's incubation with medium containing 10% FBS and 2 days' incubation with medium containing 10% SM instead of FBS to deplete intrinsic hormones.

2.5. MTT test

Following the above 3 days' incubation in 96-well plate, cells in each well were treated with drugs at corresponding working concentrations for 5 days. Medium was changed every 48 hours. MTT test was performed on the 9th day. Old medium was aspirated and 100 μl MTT was added to each well. After 4 hours' incubation, supernatant was aspirated and 100 μl dimethyl sulfoxide (DMSO) was added, following 5 minutes' mixing. OD value was measured with ELISA Reader at a wavelength of 550 nm. All MTT tests were performed in triplicate and results were expressed as percentage of mean adjusted OD value in each treatment group relative to that of SM group.

2.6. Western blot test

Protein extraction. Following the above 3 days' incubation in 8-well plates, cells in each dish were treated with drugs at corresponding concentrations for 5 days. Medium was changed every 48 hours. Adherent cells were washed with cold PBS for 3 times. 100 μl RIPA buffer plus 1 μl PMSF and 1 μl protease inhibitor cocktail was added to each dish and homogenized. Adherent cells were scraped off quickly and lysed on ice for 30 minutes with vortex every 10 minutes. Lysates were transferred to centrifuge tubes and centrifuged at 1300 rpm, 4℃ for 20 minutes. Supernatants were collected for further analysis.

Protein analysis and denaturation. Total protein contents were analyzed according to Pierce® BCA protein assay kit protocol. All samples within one western blotting were adjusted to equal total protein level. Loading dye (6x) was added to the protein extracts at the ratio of 1:5. Denaturation was at 100℃ for 5 minutes.

Western blot. Total protein of 100 μg was loaded to each sample lane. 5% stacking gel and 12% separating gel were used. SDS-PAGE electrophoresis was performed at constant voltage of 90 volts (V) on ice. Proteins were transferred to a 0.45 μm PVDF membrane at constant voltage of 100 V for 120 minutes. PVDF membrane was blocked with 5% nonfat milk in TBST for 2 hours at room temperature. Primary antibody incubation (for PGRMC1, ER-α, PR and actin, 1:400, 1:400, 1:400 and 1:1000) was carried out at 4℃ overnight and secondary antibody incubation (1:1000) was carried out at room temperature for 2 hours. PVDF membrane was washed in TBST for 10 minutes and 3 times following each incubation. ECL western blotting analysis system was used to detect and quantify target proteins.

2.7. Statistical analysis

All experiments were repeated three times. Quantitative data was expressed as mean±standard deviation (SD). SPSS statistical software 23.0 (SPSS Inc., Chicago, USA) was used for quantitative analysis. Multi-group comparison was carried out using ANVOA followed by Bonferroni test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Proliferation results measured by MTT

3.1.1. Proliferations in MCF-7EVC and MCF-7WT12 cells treated with single E_2 or progestogens

As shown in Figure 3, 10^{-9} M E_2 significantly increased cell proliferation by 100.7% ($P<0.001$) and 171.2% ($P<0.001$) in MCF-7EVC and MCF-7WT12 cells, respectively.

Neither at the concentration of 10^{-6} M nor at that of 10^{-7} M did P_4 show any significant effects on cell proliferation in MCF-7EVC cells ($P=0.965$, 10^{-6} M P_4; $P=0.055$, 10^{-7} M P_4); whereas in MCF-7WT12 cells, cell proliferation significantly increased by 100.8% ($P<0.001$) and 87.6% ($P<0.001$) with P_4 treatments at the concentrations of 10^{-6} M and 10^{-7} M, respectively.

With 10^{-6} M and 10^{-7} M NET treatments, MCF-7EVC cells significantly increased cell proliferation by 60.6% ($P<0.001$) and 40.6% ($P<0.001$), respectively; and MCF-7WT12 cells significantly increased by 281.4% ($P<0.001$) and 250.0% ($P<0.001$), respectively.

At the concentrations of 10^{-6} M and 10^{-7} M, MPA significantly increased cell proliferation by 171.8% ($P<0.001$) and 149.4% ($P<0.001$), respectively in MCF-7EVC cells; and by 350.1% ($P<0.001$) and 310.6% ($P<0.001$), respectively in MCF-7WT12 cells.

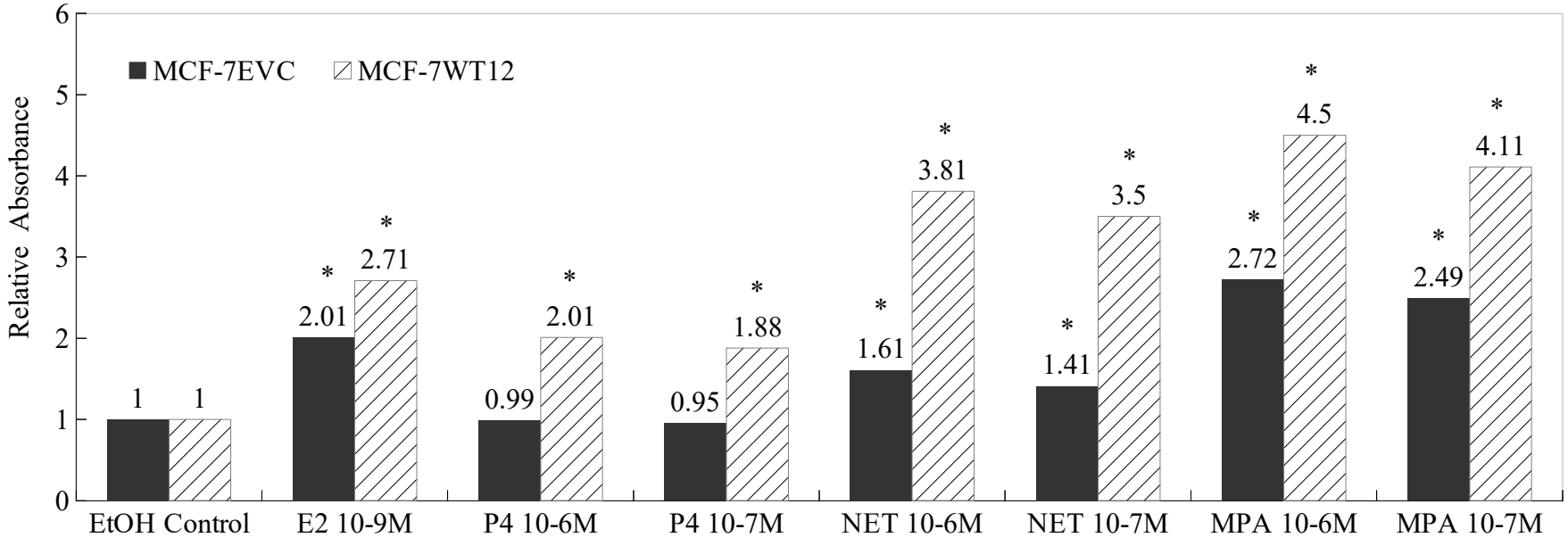


Figure 3. Relative absorbances measured by MTT in MCF-7EVC and MCF-7WT12 cells after each single hormen treatment. Treatments included 10^{-9} M EtOH, 10^{-9} M E_2, 10^{-6} M and 10^{-7} M P_4, 10^{-6} M and 10^{-7} M NET, 10^{-6} M and 10^{-7} M MPA for 5 days.
* $P<0.05$.

3.1.2. Proliferations in T47DEVC and T47DWT3 cells treated with single E_2 or progestogens

As shown in Figure 4, 10^{-9} M E_2 significantly increased cell proliferation by 122.9% ($P<0.001$) and 175.6% ($P<0.001$) in T47DEVC and T47DWT3 cells, respectively.

Neither at the concentration of 10^{-6} M nor at that of 10^{-7} M did P_4 show any significant effects on cell proliferation in T47DEVC cells ($P=0.750$, 10^{-6} M P_4; $P=0.611$, 10^{-7} M P_4); whereas in T47DWT3 cells, cell proliferation significantly increased by 106.9% ($P<0.001$) and 187.0% ($P<0.001$) with 10^{-6} M and 10^{-7} M P_4 treatment.

With 10^{-6} M and 10^{-7} M NET treatments, T47DEVC cells significantly increased cell proliferation by 51.7% ($P<0.001$) and 45.0% ($P<0.001$), respectively; and T47DWT3 cells significantly increased by 287.0% ($P<0.001$) and 249.6% ($P<0.001$), respectively.

At the concentrations of 10^{-6} M and 10^{-7} M, MPA significantly increased cell proliferation by 177.7% ($P<0.001$) and 155.0% ($P<0.001$), respectively in T47DEVC cells; and by 354.3% ($P<0.001$) and 334.3% ($P<0.001$), respectively in T47DWT3 cells.

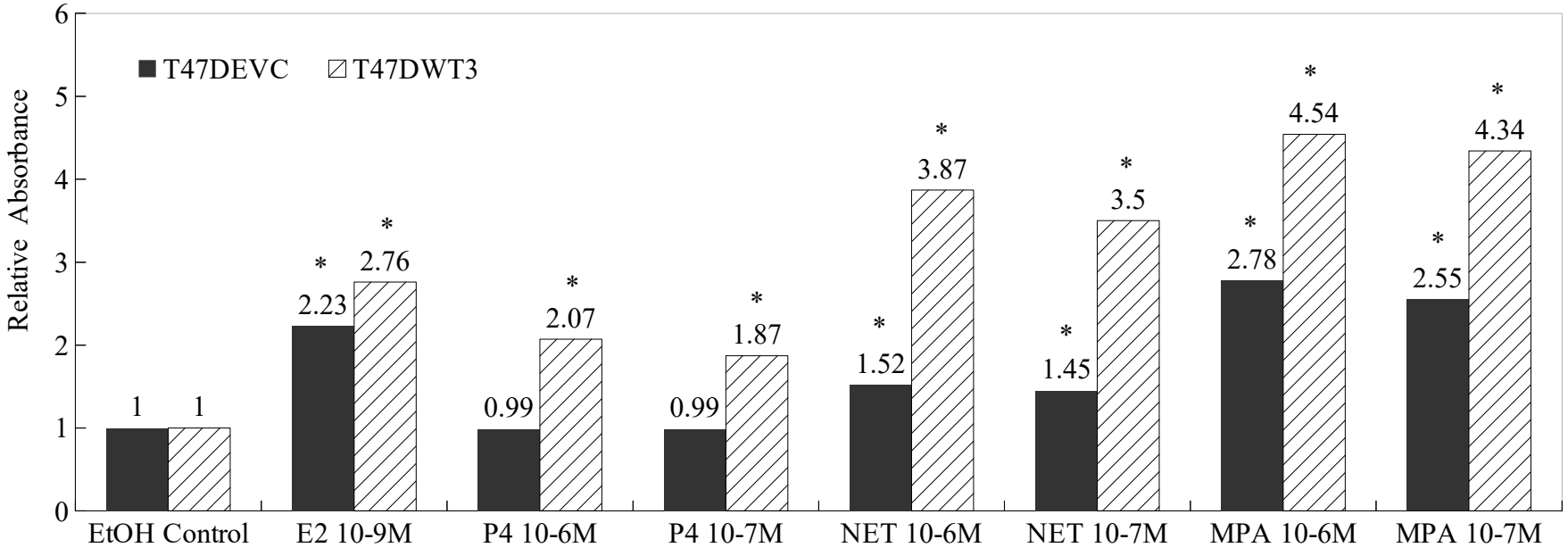


Figure 4. Relative absorbances measured by MTT in T47DEVC and T47DWT3 cells after each single hormen treatment. Treatments included10^{-9} M EtOH, 10^{-9} M E_2, 10^{-6} M and 10^{-7} M P_4, 10^{-6} M and 10^{-7} M NET, 10^{-6} M and 10^{-7} M MPA for 5 days.
* $P<0.05$.

3.1.3. Proliferations in MCF-7EVC and MCF-7WT12 cells treated with TAM

As shown in Figure 5, 10^{-6} M and 10^{-7} M TAM had no significant impact on cell proliferation in either MCF-7EVC or MCF-7WT12 cells. Relative proliferation ratios versus EtOH treatment were 0.968 (P=0.241) with 10^{-6} M TAM treatment and 1.013 (P=0.622) with 10^{-7} M TAM treatment in MCF-7EVC cells; and 1.001 (P=0.944) with 10^{-6} M TAM treatment and 1.016 (P=0.348) with 10^{-7} M TAM treatment in MCF-7WT12 cells.

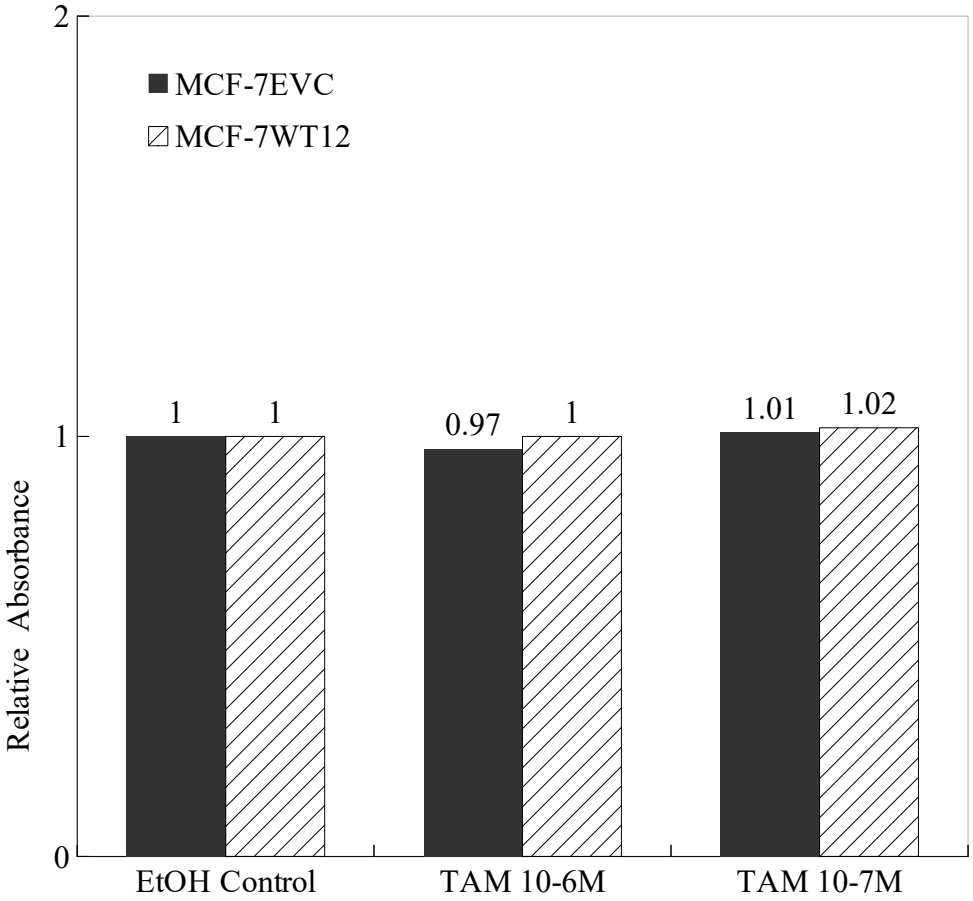


Figure 5. Relative absorbances measured by MTT in MCF-7EVC and MCF-7WT12 cells after single antiestrogenic treatment. Treatments included 10^{-9} M EtOH, 10^{-6} M and 10^{-7} M TAM for 5 days. * $P<0.05$.

3.1.4. Proliferations in T47DEVC and T47DWT3 cells treated with TAM

As shown in Figure 6, 10^{-6} M and 10^{-7} M TAM had no significant impact on cell proliferation in either T47DEVC or T47DWT3 cells. Relative proliferation ratios versus EtOH treatment were 1.002 (P=0.968) with 10^{-6} M TAM treatment and 1.020 (P=0.350) with 10^{-7} M TAM treatment in T47DEVC cells; and 0.989 (P=0.524) with 10^{-6} M TAM treatment and 0.967 (P=0.089) with 10^{-7} M TAM treatment in T47DWT3 cells.

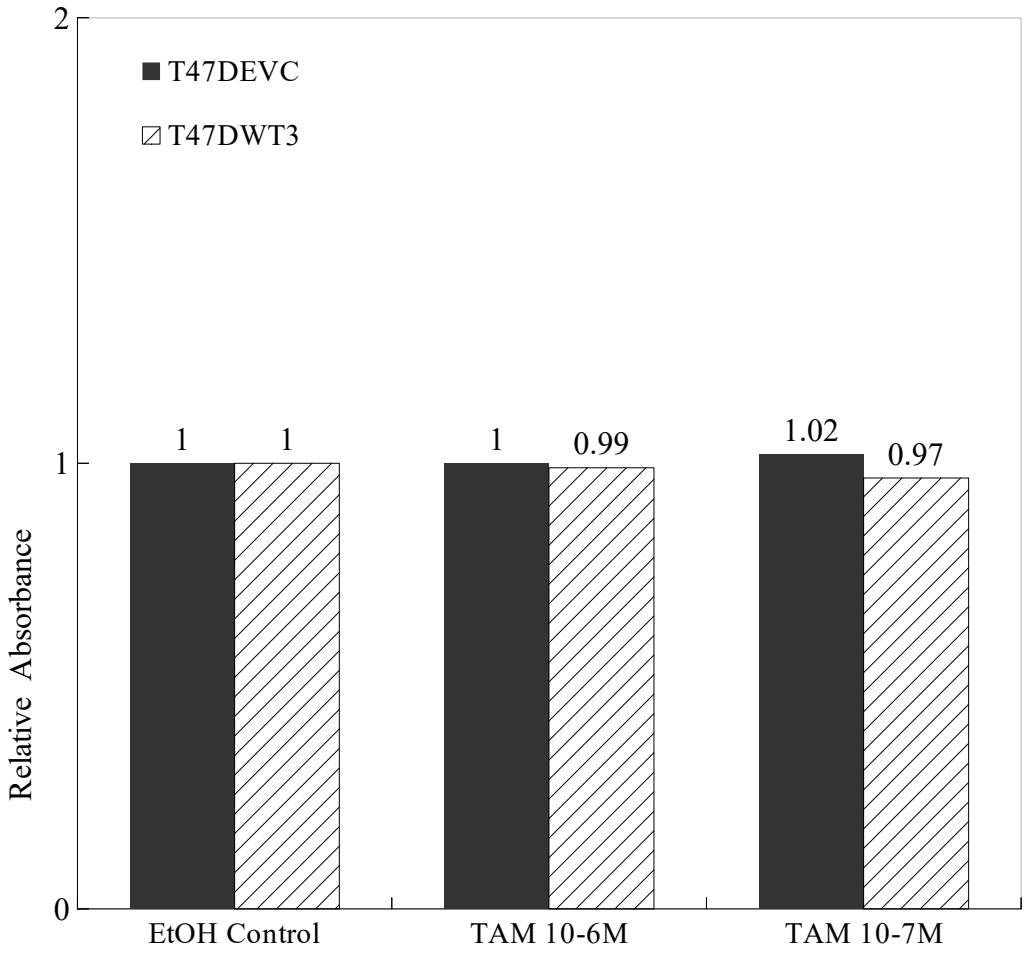


Figure 6. Relative absorbances measured by MTT in T47DEVC and T47DWT3 cells after single antiestrogenic treatment. Treatments included 10^{-9} M EtOH, 10^{-6} M and 10^{-7} M TAM for 5 days. * $P<0.05$.

3.1.5. Proliferations in MCF-7EVC and MCF-7WT12 cells treated with TAM plus E_2

As shown in Figure 7, treatments of E_2 plus TAM showed no significant promoting effect on cell proliferation in MCF-7EVC cells (P=0.253, 10^{-6} M TAM; P=0.136, 10^{-7} M TAM), whereas significant proliferation was observed in MCF-7WT12 cells, with relative proliferation ratios versus control treatment being 166.1% (P<0.001, 10^{-6} M TAM) and 174.4% (P<0.001, 10^{-7} M TAM), respectively.

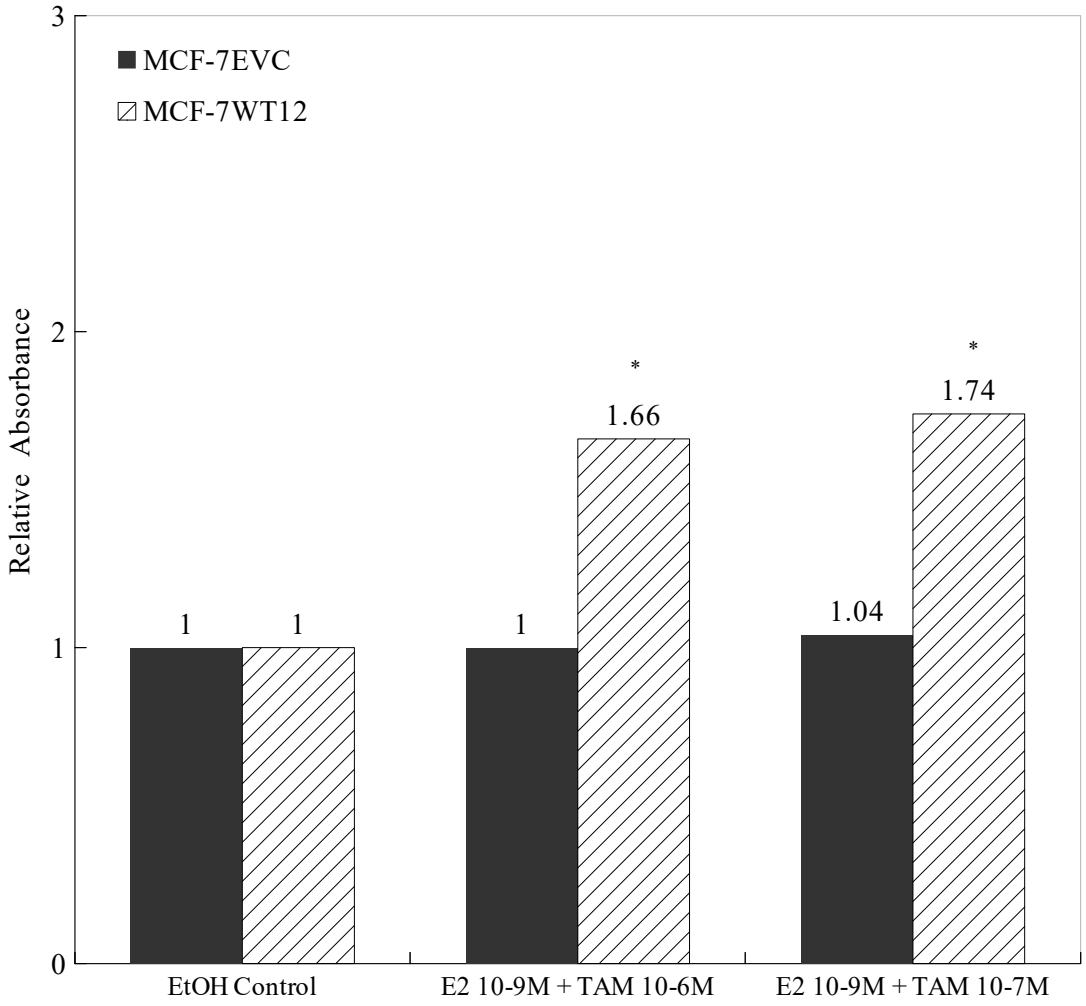


Figure 7. Relative absorbances measured by MTT in MCF-7EVC and MCF-7WT12 cells after treatments of TAM plus E_2. Treatments included 10^{-9} M EtOH, 10^{-9} M E_2+10^{-6} M TAM and 10^{-9} M E_2+10^{-7} M TAM for 5 days. * $P<0.05$.

3.1.6. Proliferations in T47DEVC and T47DWT3 cells treated with TAM plus E_2

As shown in Figure 8, treatments of E_2 plus TAM showed no significant promoting effect on cell proliferation in T47DEVC cells (P=0.533, 10^{-6} M TAM; P=0.144, 10^{-7} M TAM), whereas significant proliferation was observed in T47DWT3 cells, with relative proliferation ratios versus control treatment being 159.5% (P<0.001, 10^{-6} M TAM) and 173.7% (P<0.001, 10^{-7} M TAM), respectively.

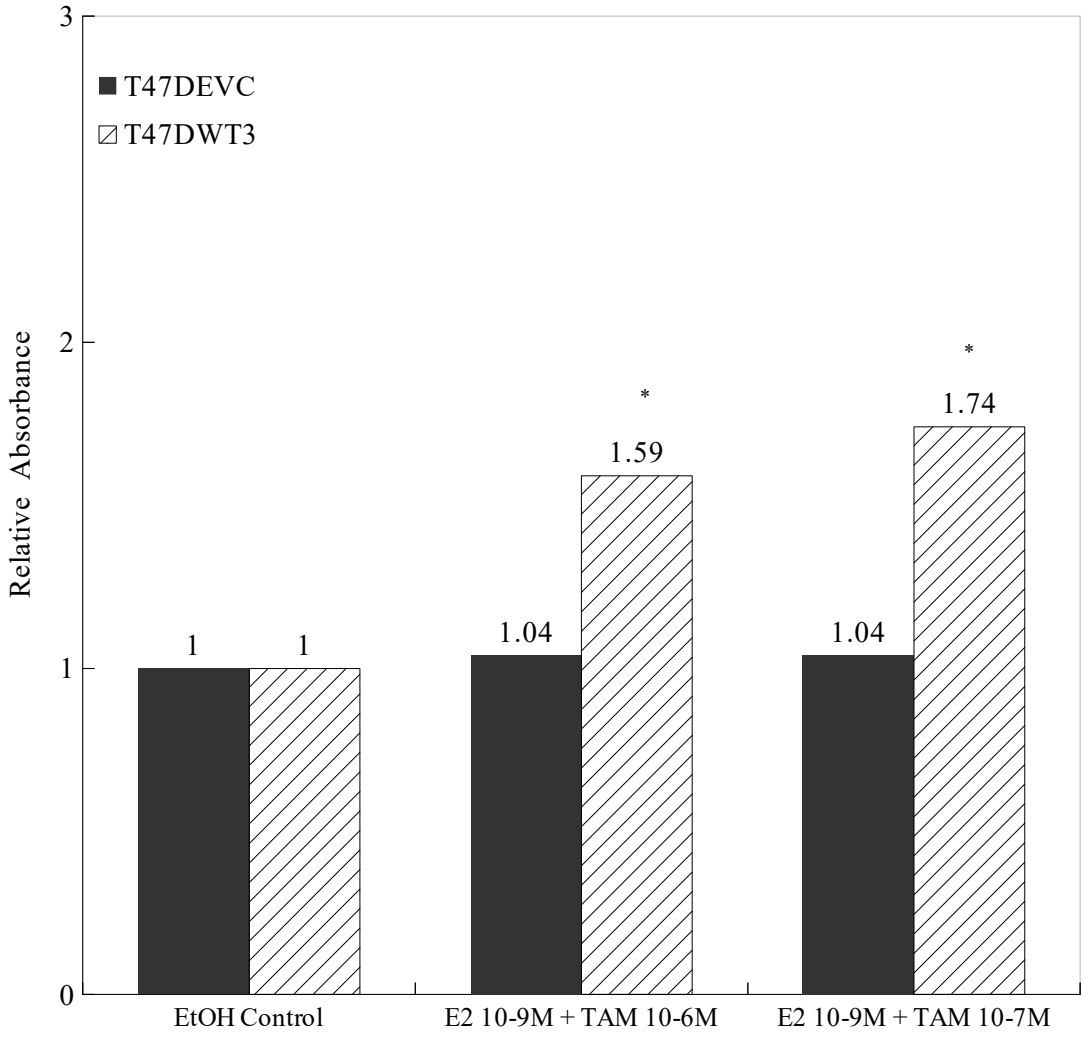


Figure 8. Relative absorbances measured by MTT in T47DEVC and T47DWT3 cells after treatments of TAM plus E_2. Treatments included 10^{-9} M EtOH, 10^{-9} M E_2+10^{-6} M TAM and 10^{-9} M E_2+10^{-7} M TAM for 5 days. * $P<0.05$.

3.2. ER-α, PR and PGRMC1 expressions measured by western blot

3.2.1. ER-α, PR and PGRMC1 expressions in MCF-7EVC and MCF-7WT12 cells treated with single E_2 and progestogens

As shown in Figure 9-11, 10^{-9} M E_2 significantly upregulated ER-α expression by 108.6% (P<0.001) in MCF-7EVC cells and by 62.1% (P<0.001) in MCF-7WT12 cells; significantly upregulated PGRMC1 expression by 33.1% (P<0.001) in MCF-7EVC cells, and by 56.7% (P<0.001) in MCF-7WT12 cells. PRA expression was significantly upregulated by 90.3% (P<0.001) and PRB expression was significantly upregulated by 58.9% (P<0.001) in MCF-7EVC cells, whereas in MCF-7WT12 cells, no significant PRA and PRB expressions were observed either before or after 10^{-9} M E_2 treatment.

10^{-6} M and 10^{-7} M P_4 significantly downregulated ER-α expression by 87.5% (P<0.001) and 86.0% (P<0.001) in MCF-7EVC cells, and by 83.6% (P<0.001) and 83.3% (P<0.001) in MCF-7WT12 cells; significantly downregulated intrinsic PGRMC1 expression by 58.0% (P<0.001) and 56.1% (P<0.001) in MCF-7EVC cells, whereas significantly upregulated intrinsic PGRMC1 expression by 10.2% (P<0.001) and 10.8% (P<0.001) in MCF-7WT12 cells. With the treatments of 10^{-6} M and 10^{-7} M P_4, PRA expression was significantly downregulated by 55.7% (P<0.001) and 54.7% (P<0.001) in MCF-7EVC cells, PRB expression was significantly downregulated by 49.7% (P<0.001) and 47.9% (P<0.001) in MCF-7EVC cells, whereas in MCF-7WT12 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M P_4 treatments.

With the treatments of 10^{-6} M and 10^{-7} M NET, ER-α expression was significantly downregulated by 76.0% (P<0.001) and 72.4% (P<0.001) in MCF-7EVC cells, and by 77.7% (P<0.001) and 77.2% (P<0.001) in MCF-7WT12 cells; PGRMC1 expression was significantly downregulated by 45.2% (P<0.001) and 45.2% (P<0.001) in MCF-7EVC cells, whereas was significantly upregulated by 52.9% (P<0.001) and 52.9% (P<0.001) in MCF-7WT12 cells; PRA expression was significantly upregulated by 38.6% (P<0.001) and 36.9% (P<0.001) in MCF-7EVC cells, PRB expression was significantly upregulated by 103.8% (P<0.001) and 100.7% (P<0.001) in MCF-7EVC cells, whereas in MCF-7WT12 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M NET treatments.

With the treatments of 10^{-6} M and 10^{-7} M MPA, ER-α expression was significantly downregulated by 69.9% ($P<0.001$) and 69.8% ($P<0.001$) and by 77.1% ($P<0.001$) and 76.9% ($P<0.001$) in MCF-7WT12 cells; PGRMC1 expression was significantly downregulated by 49.0% ($P<0.001$) and 48.4% ($P<0.001$) in MCF-7EVC cells, whereas was significantly upregulated by 49.7% ($P<0.001$) and 49.7% ($P<0.001$) in MCF-7WT12 cells; PRA expression was significantly upregulated by 38.9% ($P<0.001$) and 36.6% ($P<0.001$) in MCF-7EVC cells, PRB expression was significantly upregulated by 115.4% ($P<0.001$) and 104.5% ($P<0.001$) in MCF-7EVC cells, whereas in MCF-7WT12 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M MPA treatments.

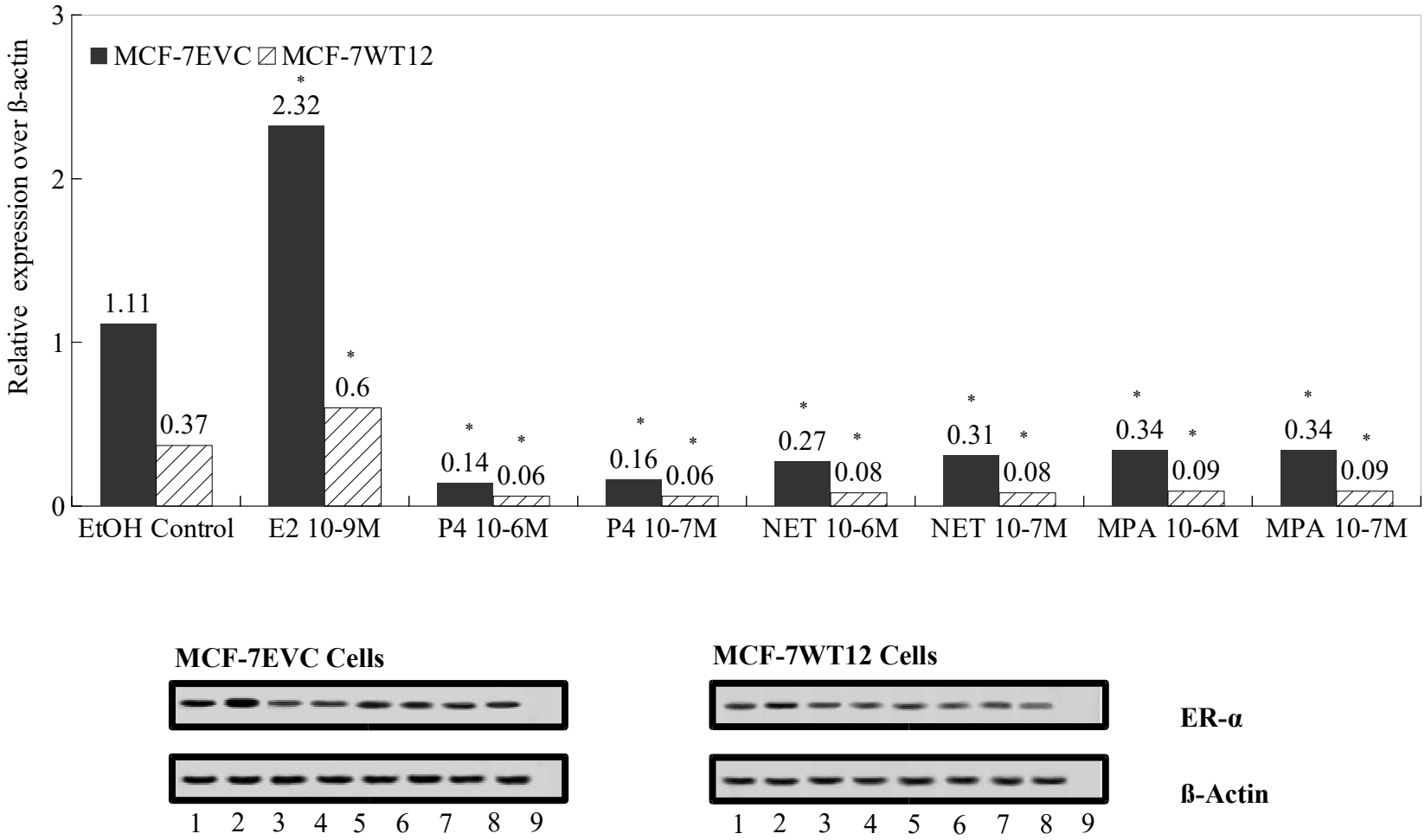


Figure 9. ER-α expressions measured by western blot in MCF-7EVC and MCF-7WT12 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days. * $P<0.05$.

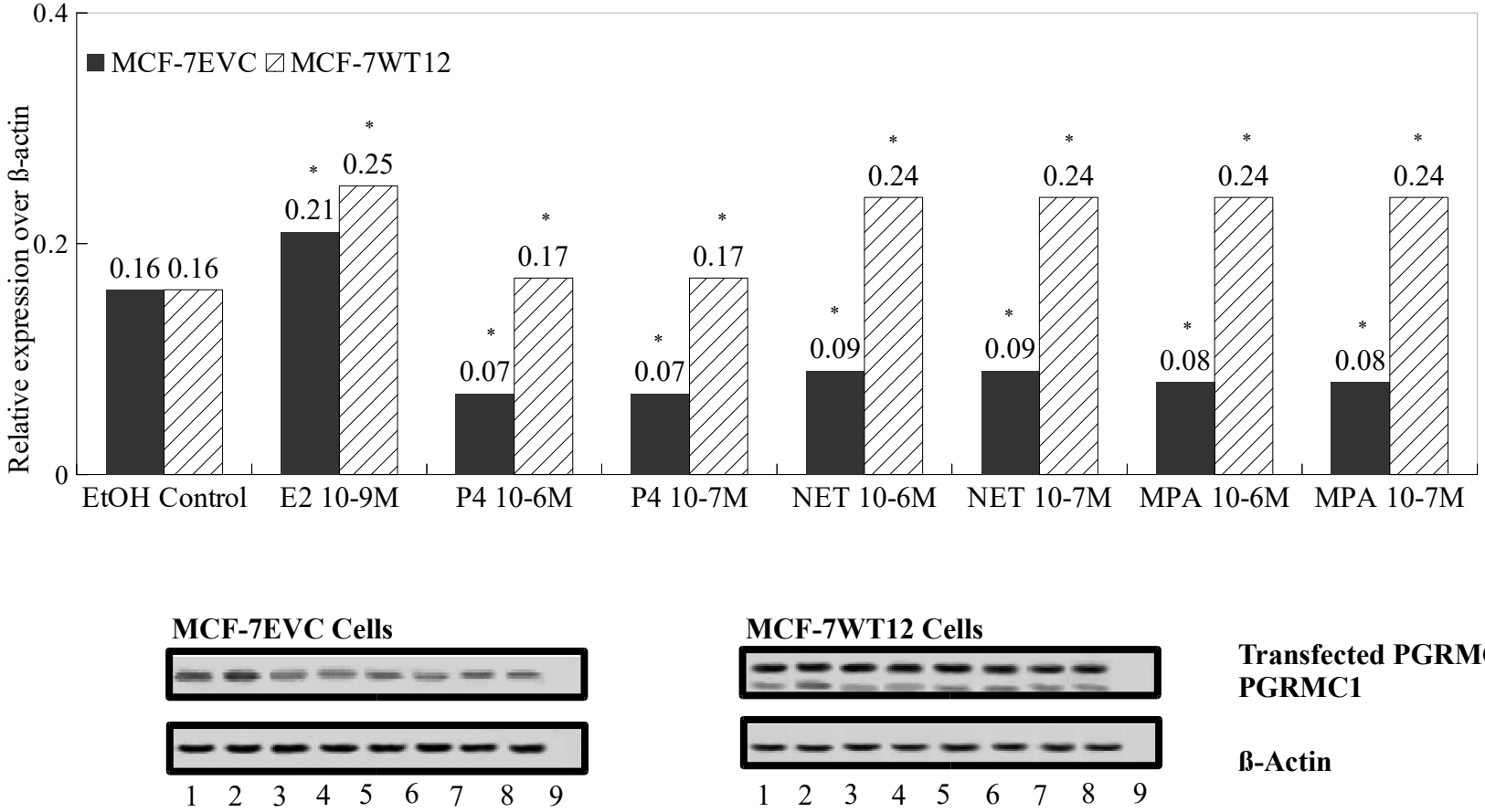


Figure 10. PGRMC1 expressions measured by western blot in MCF-7EVC and MCF-7WT12 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days.
* $P<0.05$.

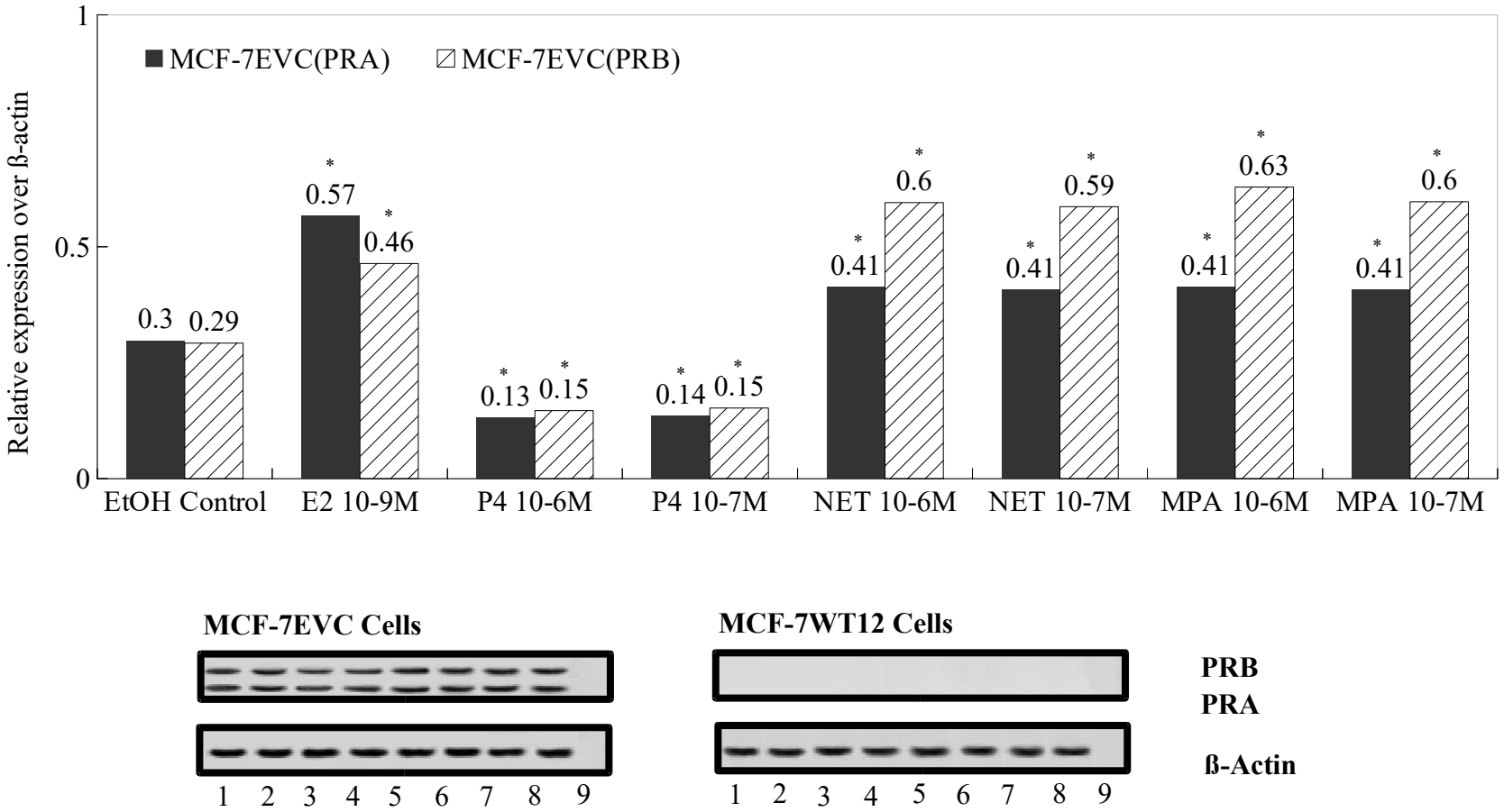


Figure 11. PRA and PRB expressions measured by western blot in WCF-7EVC and WCF-7WT12 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days.
* $P<0.05$.

3.2.2. ER-α, PR and PGRMC1 expressions in T47DEVC and T47DWT3 cells treated with single E_2 and progestogens

As shown in Figure 12-14, 10^{-9} M E_2 significantly upregulated ER-α expression by 101.8% (P<0.001) in T47DEVC cells and by 88.4% (P<0.001) in T47DWT3 cells; significantly upregulated PGRMC1 expression by 38.5% (P<0.001) in T47DEVC cells, and by 38.7% (P<0.001) in T47DWT3 cells. With 10^{-9} M E_2 treatment, PRA expression was significantly upregulated by 85.1% (P<0.001) and PRB expression was significantly upregulated by 53.3% (P<0.001) in T47DEVC cells, whereas in T47DWT3 cells, no significant PRA and PRB expressions were observed either before or after 10^{-9} M E_2 treatment.

10^{-6} M and 10^{-7} M P_4 significantly downregulated ER-α expression by 80.0% (P<0.001) and 76.9% (P<0.001) in T47DEVC cells, and by 77.2% (P<0.001) and 73.7% (P<0.001) in T47DWT3 cells; significantly downregulated intrinsic PGRMC1 expression by 49.1% (P<0.001) and 49.1% (P<0.001) in T47DEVC cells, whereas significantly upregulated intrinsic PGRMC1 expression by 7.7% (P=0.037) and 8.8% (P=0.017) in T47DWT3 cells. With the treatments of 10^{-6} M and 10^{-7} M P_4, PRA expression was significantly downregulated by 73.2% (P<0.001) and 70.6% (P<0.001) in T47DEVC cells, PRB expression was significantly downregulated by 76.2% (P<0.001) and 74.9% (P<0.001) in T47DEVC cells, whereas in T47DWT3 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M P_4 treatments.

With the treatments of 10^{-6} M and 10^{-7} M NET, ER-α expression was significantly downregulated by 73.6% (P<0.001) and 70.3% (P<0.001) in T47DEVC cells, and by 72.8% (P<0.001) and 63.4% (P<0.001) in T47DWT3 cells; PGRMC1 expression was significantly downregulated by 46.6% (P<0.001) and 48.4% (P<0.001) in T47DEVC cells, whereas was significantly upregulated by 36.5% (P<0.001) and 37.0% (P<0.001) in T47DWT3 cells; PRA expression was significantly upregulated by 117.7% (P<0.001) and 117.8% (P<0.001) in T47DEVC cells, PRB expression was significantly upregulated by 168.7% (P<0.001) and 168.7% (P<0.001) in T47DEVC cells, whereas in T47DWT3 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M NET treatments.

With the treatments of 10^{-6} M and 10^{-7} M MPA, ER-α expression was significantly

downregulated by 69.6% (P<0.001) and 71.5% (P<0.001) and by 64.7% (P<0.001) and 63.4% (P<0.001) in T47DWT3 cells; PGRMC1 expression was significantly downregulated by 48.4% (P<0.001) and 47.8% (P<0.001) in T47DEVC cells, whereas was significantly upregulated by 33.1% (P<0.001) and 32.0% (P<0.001) in T47DWT3 cells; PRA expression was significantly upregulated by 118.9% (P<0.001) and 117.4% (P<0.001) in T47DEVC cells, PRB expression was significantly upregulated by 170.4% (P<0.001) and 169.2% (P<0.001) in T47DEVC cells, whereas in T47DWT3 cells, no significant PRA and PRB expressions were observed either before or after 10^{-6} M and 10^{-7} M MPA treatments.

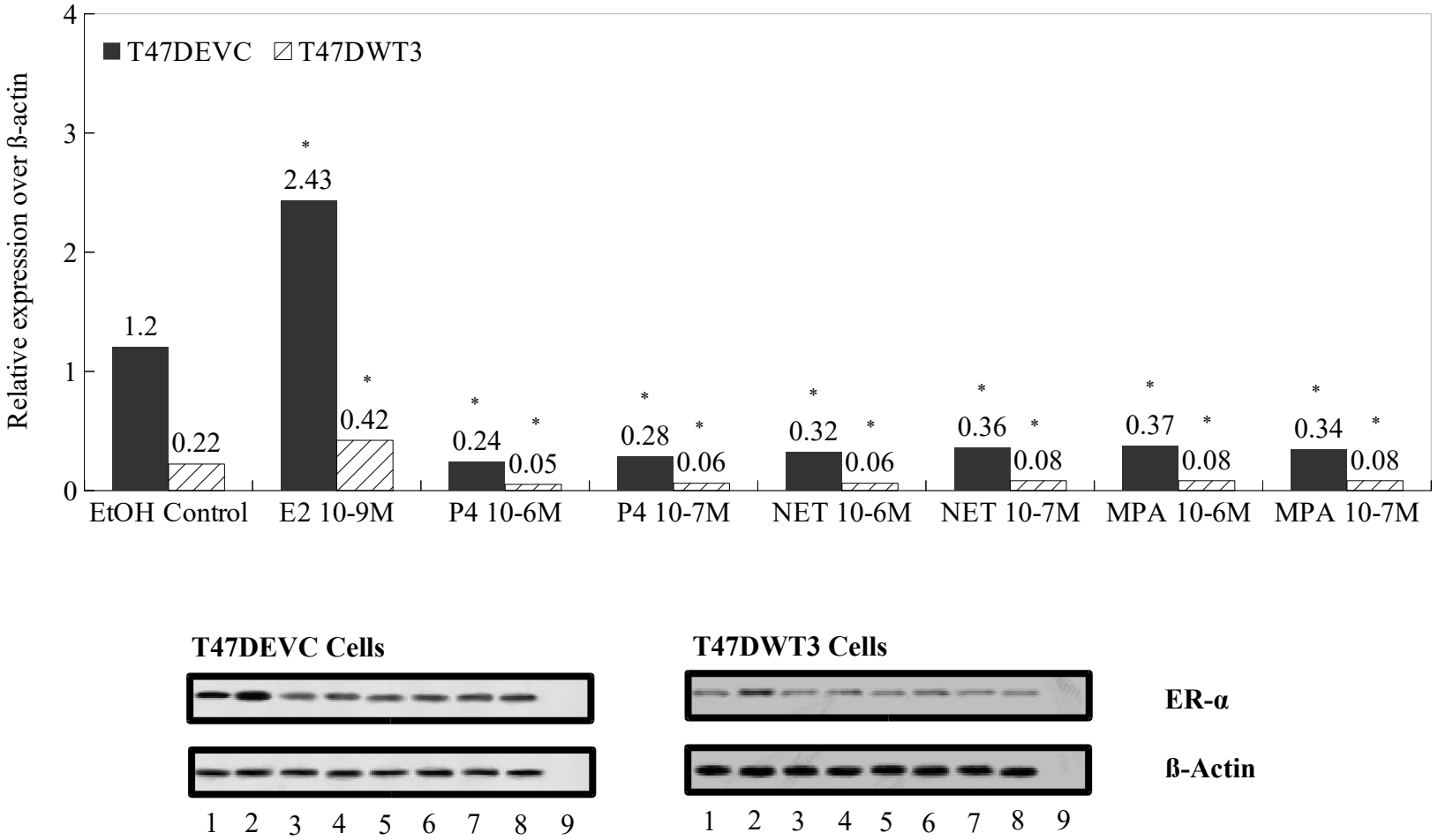


Figure 12. ER-α expressions measured by western blot in T47DEVC and T47DWT3 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days. * $P<0.05$.

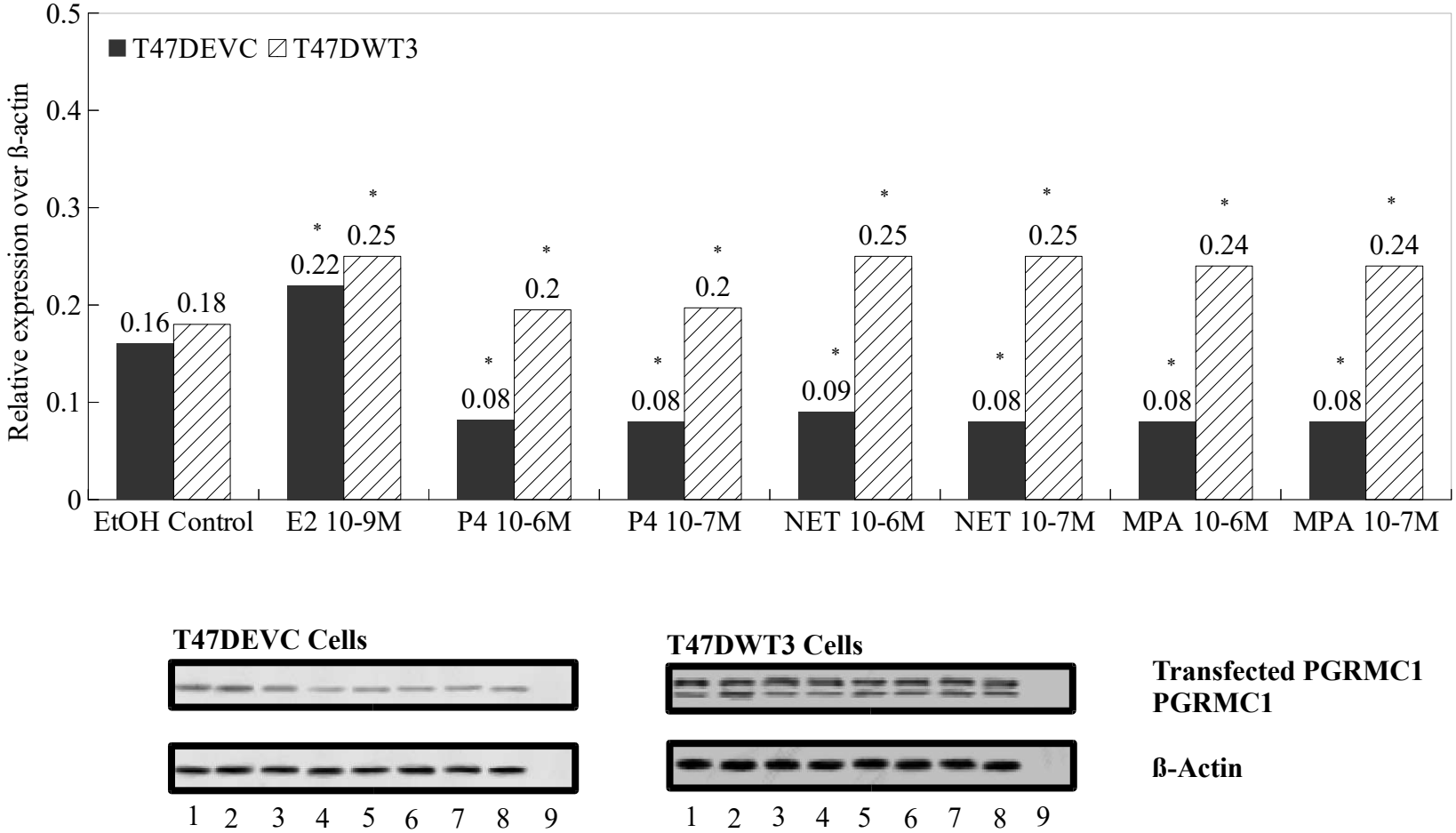


Figure 13. PGRMC1 expressions measured by western blot in T47DEVC and T47DWT3 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days.
* $P<0.05$.

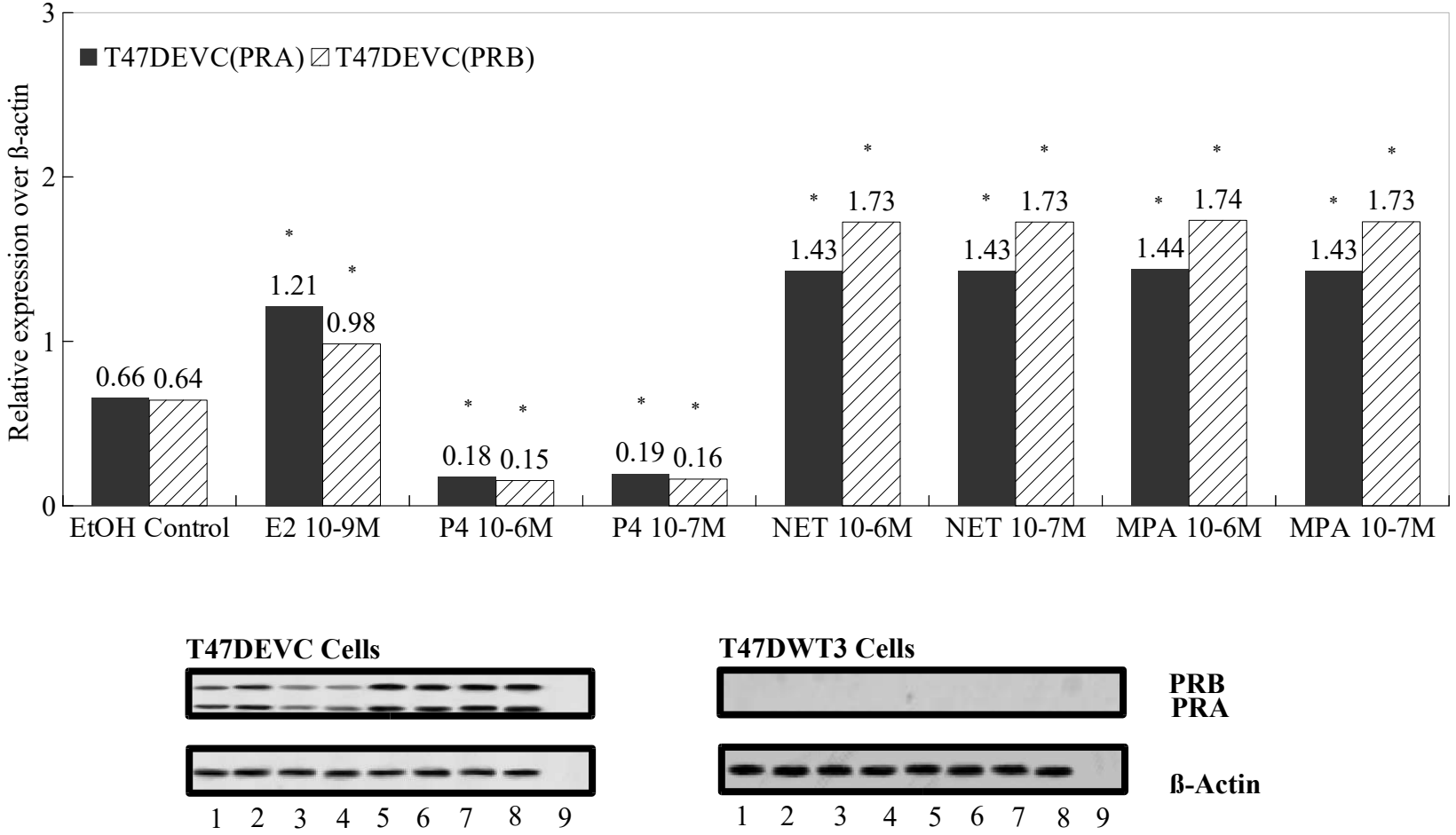


Figure 14. PRA and PRB expressions measured by western blot in T47DEVC and T47DWT3 cells after each single hormone treatment. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2; Lane 3=10^{-6} M P_4; Lane 4=10^{-7} M P_4; Lane 5=10^{-6} M NET; Lane 6=10^{-7} M NET; Lane 7=10^{-6} M MPA; Lane 8=10^{-7} M MPA; Lane 9=blank for 5 days.
* $P<0.05$.

3.2.3. ER-α, PR and PGRMC1 expressions in MCF-7EVC and MCF-7WT12 cells treated with TAM plus E_2

As shown in Figure 15-17, addition of E_2 plus TAM significantly upregulated ER-α expression by 234.3% ($P<0.001$, 10^{-6} M TAM) and 233.0% ($P<0.001$, 10^{-7} M TAM) in MCF-7EVC cells, whereas no significant change of ER-α expression was observed in MCF-7WT12 cells ($P=0.871$, 10^{-6} M TAM; $P=0.418$, 10^{-7} M TAM).

PGRMC1 expression was significantly downregulated by 55.8% ($P<0.001$, 10^{-6} M TAM) and 55.0% ($P<0.001$, 10^{-7} M TAM) in MCF-7EVC cells, whereas was significantly upregulated by 44.9% ($P=0.002$, 10^{-6} M TAM) and 45.1% ($P=0.025$, 10^{-7} M TAM) in MCF-7WT12 cells.

PRA expression was significantly downregulated by 19.1% ($P<0.001$, 10^{-6} M TAM) and 39.6% ($P<0.001$, 10^{-7} M TAM) in MCF-7EVC cells, PRB expression was significantly downregulated by 20.6% ($P<0.001$, 10^{-6} M TAM) and 20.1% ($P<0.001$, 10^{-7} M TAM) in MCF-7EVC cells, whereas in MCF-7WT12 cells, no significant PRA and PRB expressions were observed either before or after any treatment.

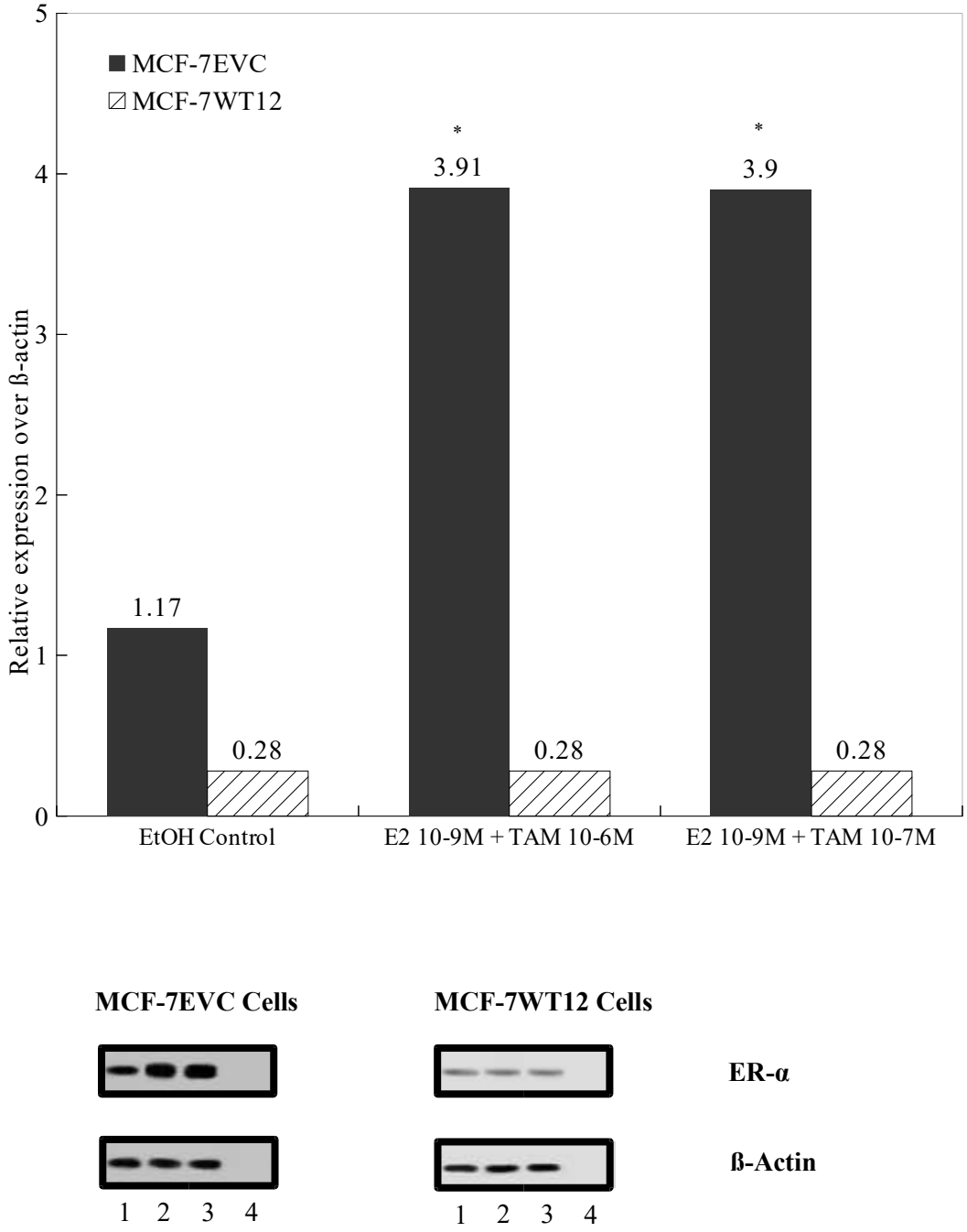


Figure 15. ER-α expressions measured by western blot in MCF-7EVC and MCF-7WT12 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.

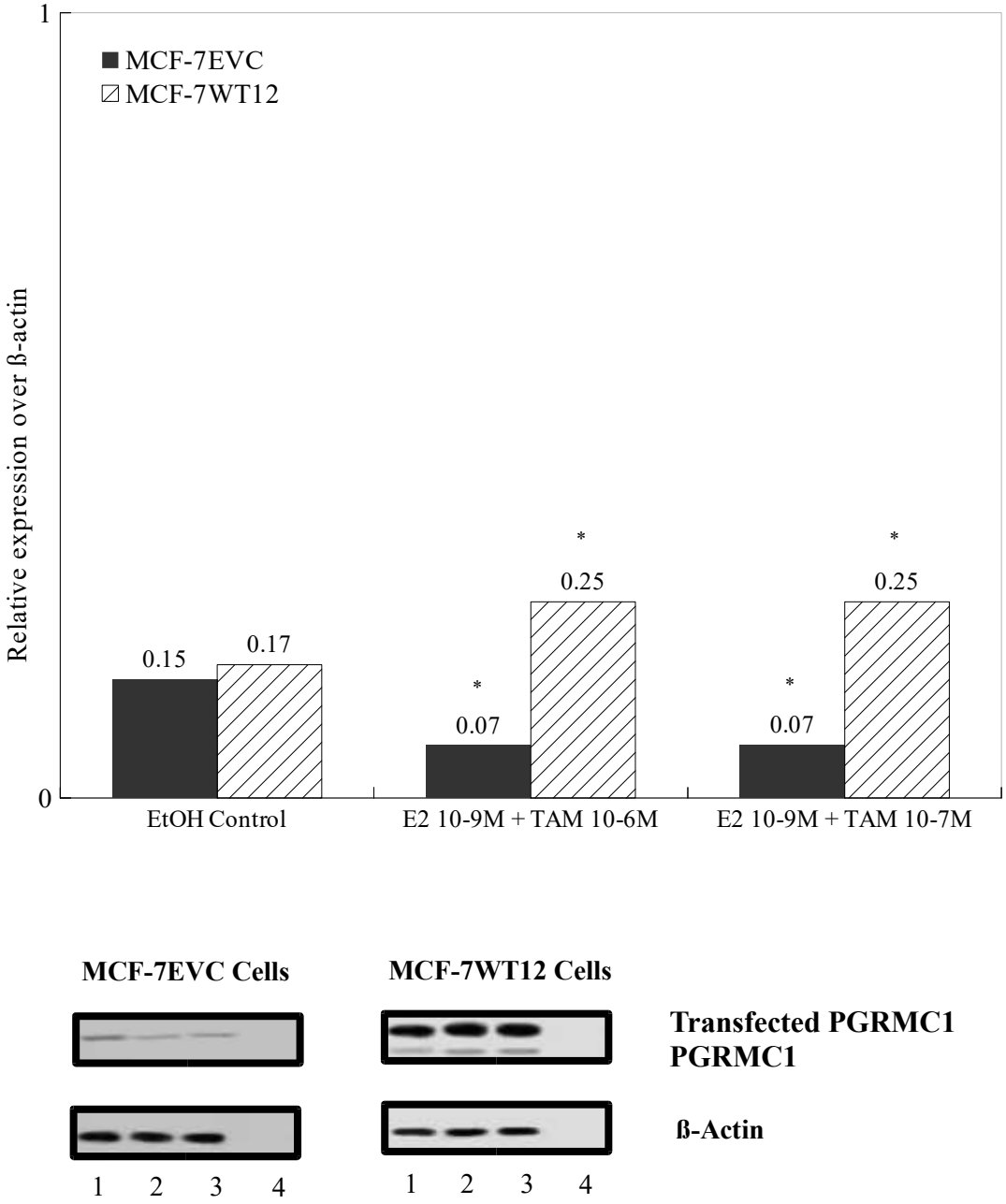


Figure 16. PGRMC1 expressions measured by western blot in MCF-7EVC and MCF-7WT12 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.

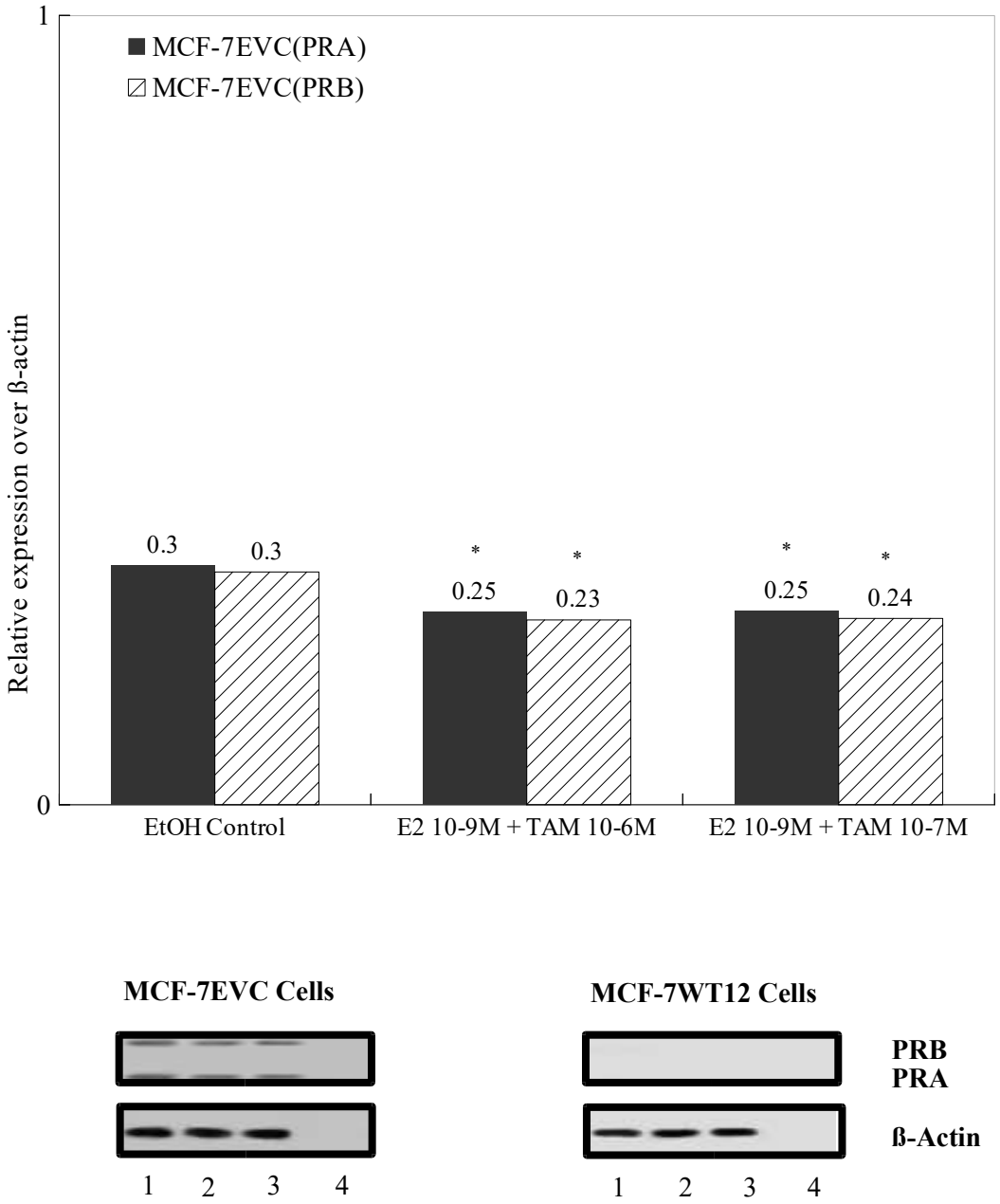


Figure 17. PRA and PRB expressions measured by western blot in MCF-7EVC and MCF-7EVCWT12 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.

3.2.4. ER-α, PR and PGRMC1 expression in T47DEVC and T47DWT3 cells treated with TAM plus E_2

As shown in Figure 18-20, addition of E_2 plus TAM significantly upregulated ER-α expression by 275.4% ($P<0.001$, 10^{-6} TAM) and 212.0% ($P<0.001$, 10^{-7} TAM) in T47DEVC cells, whereas no significant change of ER-α expression was observed in T47DWT3 cells ($P=0.345$, 10^{-6} M TAM; $P=0.849$, 10^{-7} M TAM).

PGRMC1 expression was significantly downregulated by 68.4% ($P<0.001$, 10^{-6} M TAM) and 68.5% ($P<0.001$, 10^{-7} M TAM) in T47DEVC cells, whereas was significantly upregulated by 16.9% ($P<0.001$, 10^{-6} M TAM) and 15.9% ($P<0.001$, 10^{-7} M TAM) in T47DWT3 cells.

PRA expression was significantly downregulated by 21.6% ($P<0.001$, 10^{-6} M TAM) and 21.3% ($P<0.001$, 10^{-7} M TAM) in T47DEVC cells, PRB expression was significantly downregulated by 38.2% ($P<0.001$, 10^{-6} M TAM) and 37.8% ($P<0.001$, 10^{-7} M TAM) in T47DEVC cells, whereas in T47DWT3 cells, no significant PRA and PRB expressions were observed either before or after any treatment.

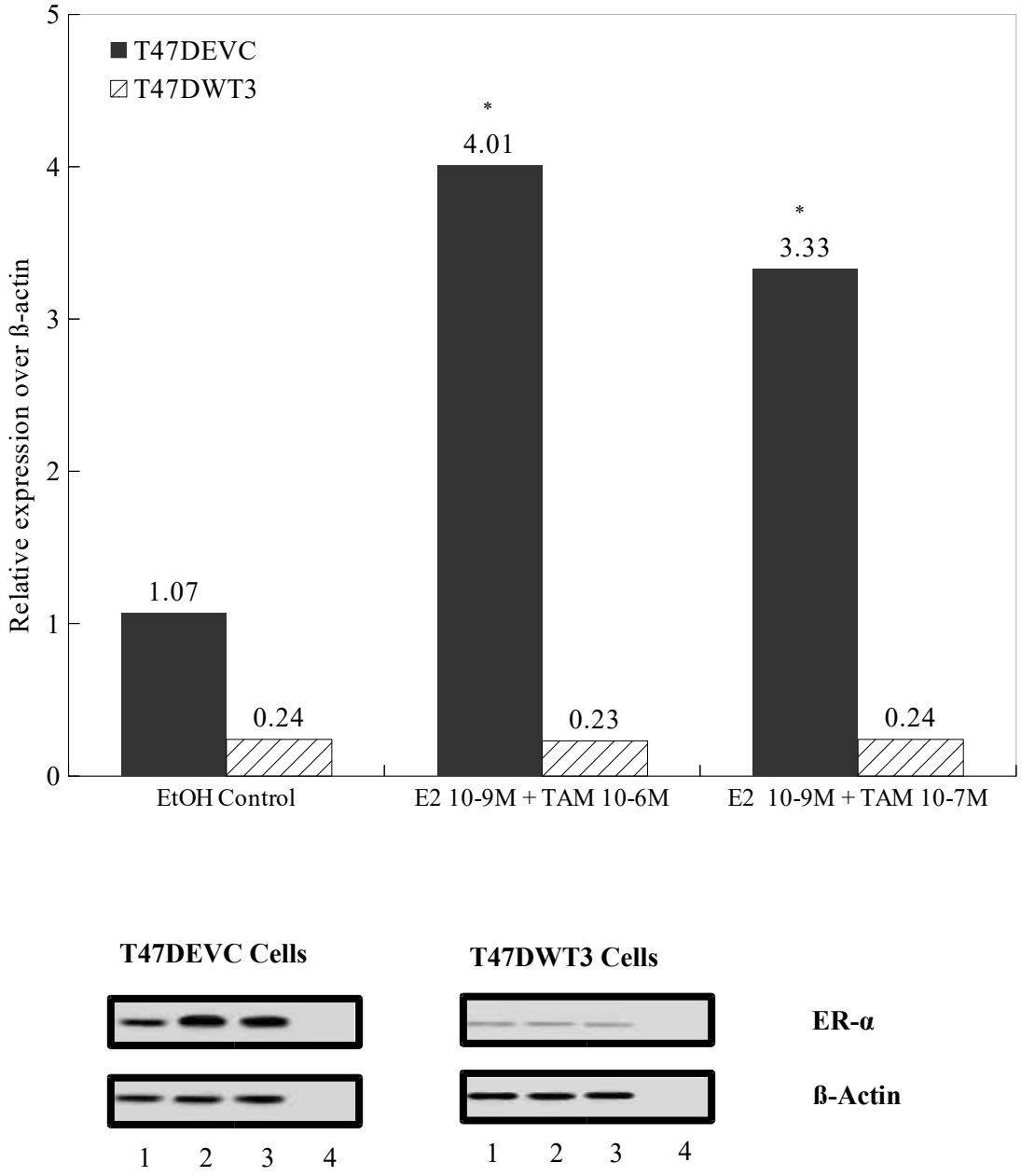
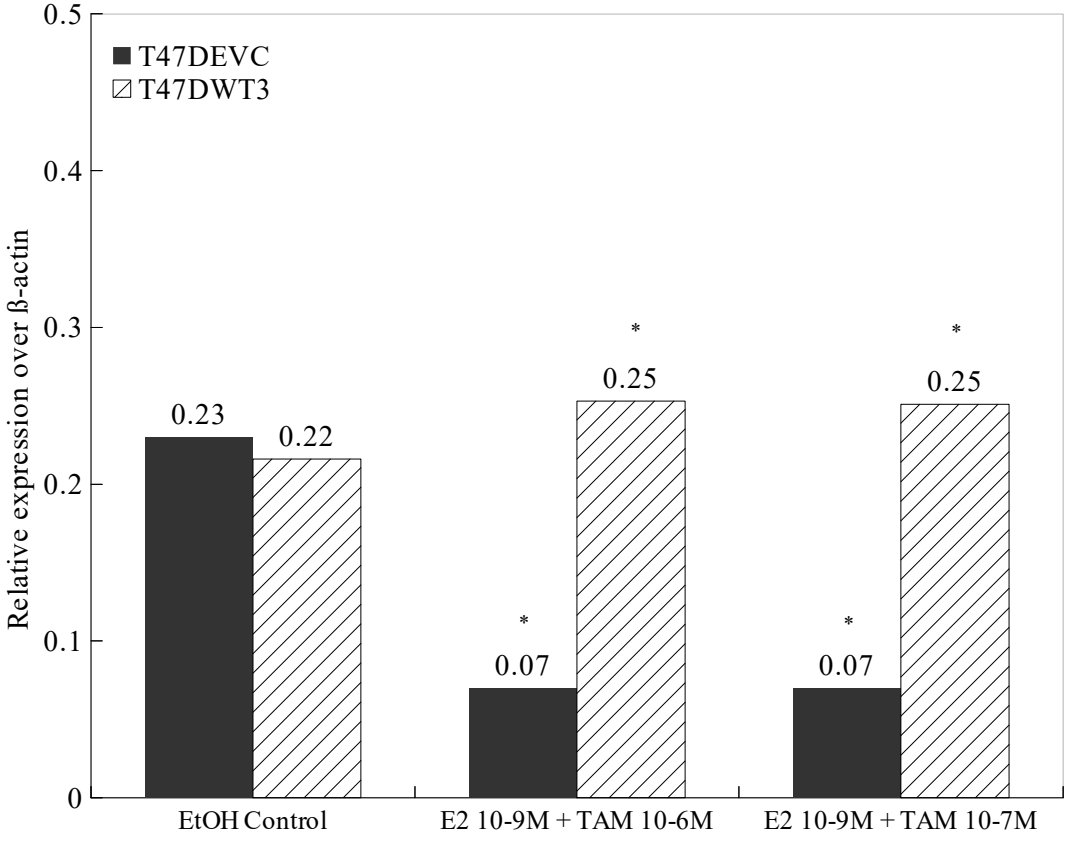


Figure 18. ER-α expression measured by western blot in T47DEVC and T47DWT3 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.



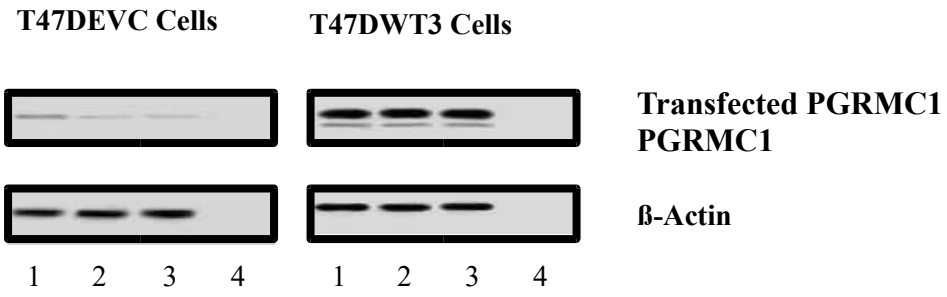


Figure 19. PGRMC1 expression measured by western blot in T47DEVC and T47DWT3 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.

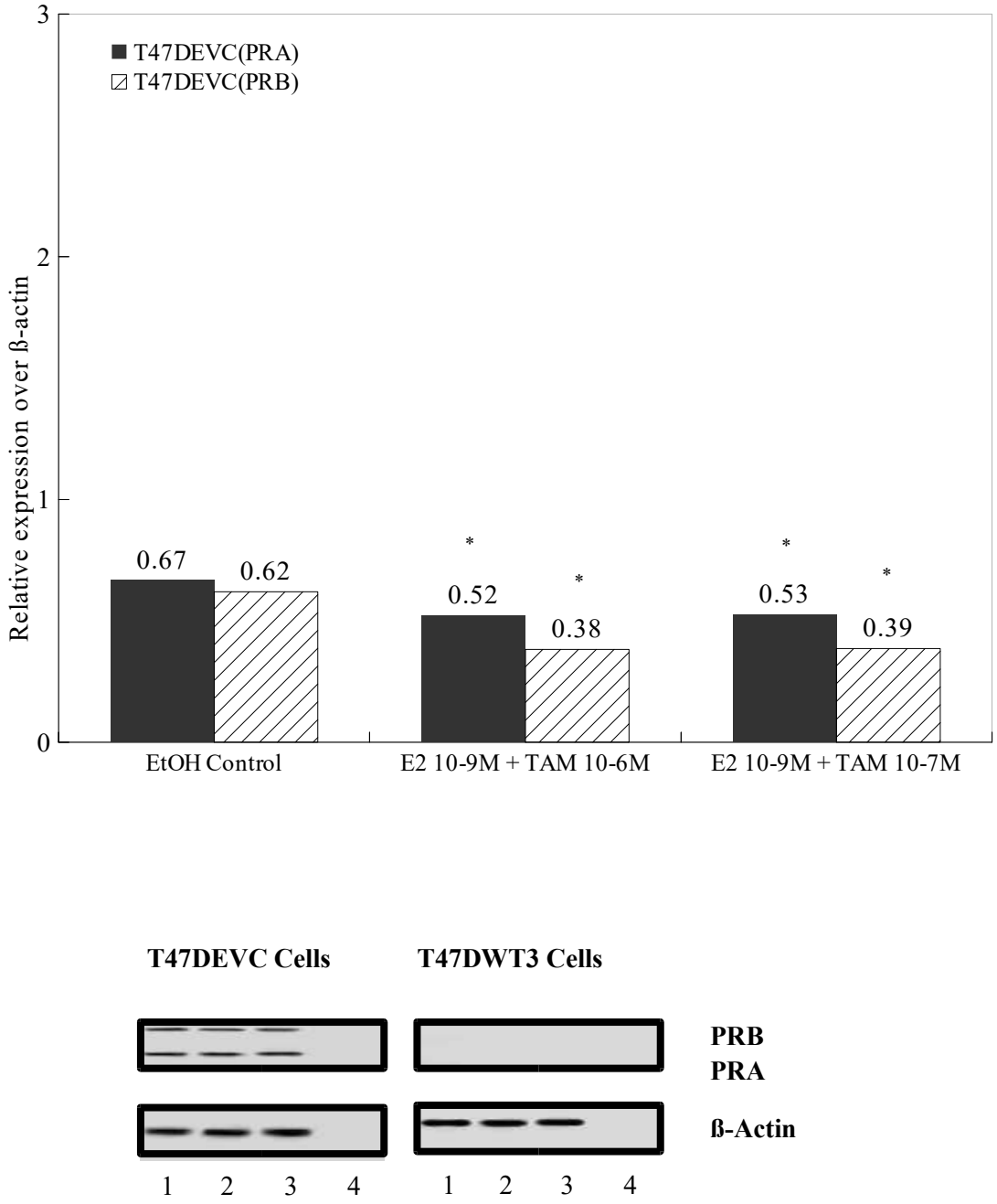


Figure 20. PRA and PRB expressions measured by western blot in T47DEVC and T47DWT3 cells after treatments of TAM plus E_2. Treatments included Lane 1=10^{-9} M EtOH; Lane 2=10^{-9} M E_2+10^{-6} M TAM; Lane 3=10^{-9} M E_2+10^{-7} M TAM; Lane 4=blank for 5 days. * P<0.05.

4. Discussion

Occurrence of malignancy from benign breast and its aggression is a more highly hormone responsive event than any other tissue else. Postmenopausal women are a group of frequent consumers for whom HRT is the general strategy against climacteric syndromes (224) which, without any medical intervention, would give rise to the hassle systemic disturbances, leaving unpleasant memories in a woman's life. Typical hormone therapies are comprised of supplement of the reduced estrogen production and addition of a progestogen to prevent the endometrium from hyperplasia in an otherwise unopposed estrogen condition. Women at high risk of or with existing breast cancer are contraindicated to HRT due to the definite correlation between HRT and breast cancer risk (224,225). However, individual assessment is a tough and complicated task, and the risk may be underestimated or neglected in a portion of women who have underlying risks at the screening. Therefore, to investigate the risks of HRT is of great value to facilitate healthcare professionals' decision making. Currently, out of safety concerns, studies in terms of direct responses to HRT are only limited to experimental researches.

Cells used in the present study are derived from MCF-7 and T47D cells, which are the two subtypes of breast cancer cell lines that express both ER-α and PR but absent of HER2. However, some differences exist between the two subtypes. For example, MCF-7 cells habor wild-type p53 gene and T47D cells habor mutant p53 gene (226,227). Compared with MCF-7 cells, T47D cells express lower ER-α/ER-ß ratio and higher level of PR (228). Besides ER and PR, MCF-7 cells also express receptors for glucocorticoids and androgens. Sucrose density gradient experiments (229) showed that ER, PR, 5α-dihydrotestosterone receptor and glucocorticoid receptor in MCF-7 cytosol are approximately 100 fm/mg protein, over 300 fm/mg protein, about 40 fm/mg protein and 800 fm/mg, respectively; Among these hormone receptors, no cross competition for estrogen receptor binding exists; However, progestins compete for androgen and glucocorticoid binding; Furthermore, androgens, but not glucocorticoids, partially compete for progestin binding. In this respect, these cells exhibit exquisite hormone sensitivity while avoiding additional HER2 signaling confounder, therefore, are ideal models to study hormone responses regarding estrogen and/or progestogen signalings exclusively (20). MCF-7EVC and MCF-7WT12 cells were empty vector and PGRMC1 expressing plasmid stably transfected MCF-7 cells, respectively. Correpondingly, T47DEVC and T47DWT3 cells were empty vector and PGRMC1 expressing plasmid

stably transfected T47D cells, respectively. Clinical observation has revealed the association between elevated PGRMC1 level and aggressive phenotype as well as poor prognosis in breast cancer (230). To investigate the impact of PGRMC1 on cell responses to sexual hormones and on TAM treatment responses in breast cancer cells, preliminary comparisons between relatively lower and higher expression of PGRMC1 using MCF-7EVC and MCF-7WT12 as well as T47DEVC and T47DWT3 cells were thus made. Results of the present study are discussed in separate aspects below.

4.1. Interaction between sexual hormones and PGRMC1 expression

Regulation of sexual hormones on PGRMC1 expression has not been largely investigated. In the porcine follicular granulosa cells, using RT-qPCR analysis, PGRMC1 and PGR transcriptions significantly increased from 48 to 120 hours following 1.0 μg/ml E_2 treatment (231). Another study conducted in porcine luminal epithelial cells also revealed that 500 pg/ml E_2 upregulated the expressions of both PR and PGRMC1 (232). The present study used 10^{-9} M E_2, 10^{-6} M and 10^{-7} M of different progestogens to investigate the sexual hormonal regulations of PGRMC1 expresssion. Results showed that E_2 significantly upregulated intrinsic PGRMC1 expression in all cell lines. In addition, TAM antagonized E_2-induced upregulating effect of PGRMC1 expression in MCF-7EVC and T47DEVC cells, but not in MCF-7WT12 and T47DWT3 cells. E_2 might be another direct or indirect positive regulatory factor of PGRMC1 expression although currently no robust evidence is available. Furthermore, according to the results of the present study, it is worth noting that the massive presence of extrinsic transfected PGRMC1 did not illicit a suppression of PGRMC1 expression if there is any regulatory role of E_2. This indicates a possible loss of negative feedback control when PGRMC1 is overexpressed by E_2, and patients with overexpressed PGRMC1 might be at a higher risk of more complicated and uncontroable conditions. However, progestogens demonstrated diverse effects on PGRMC1 expression in different cell lines. Cells treated with P_4, NET and MPA demonstrated significant decreases of PGRMC1 expression in MCF-7EVC and T47DEVC cells; whereas PGRMC1 expression increased in MCF-7WT12 and T47DWT3 cells. Although the regulatory mechanism of PGRMC1 expression remains indefinite to warrant further investigations, P_4 showed consistent potency of downregulating PGRMC1 expression only in MCF-7EVC and T47DEVC cells. From the clinical aspects, given the observations of increased breast cancer risk with PGRMC1 overexpression, a steroid

type-dependent manner of breast cancer risk for HRT in postmenopausal women should cause sufficient attention. As indicated by an earlier study (233), usage of P_4 might be the optimal strategy for the HRT consumers. However, for those women with elevated PGRMC1 level, this risk should be taken into consideration when balancing between the benefit and the risk.

4.2. Effects of PGRMC1 overexpression on sexual hormone receptor expressions

Sexual hormones showed distinct impacts on ER-α and PR expressions in the four cell lines, mainly depending on the component of progestogen and the status of PGRMC1 expression. In the present study, all progestogns exhibited consistent downregulatory effects on ER-α expression in all cell lines, regardless of the status of PGRMC1 expression. Progestogen effects on ER-α expression have been reported divesely in the earlier studies. For example, in an early clinical investigation conducted by Vihko et al (234) which included 605 primary and 150 metastatic breast carcinoma lesions from both pre- and post-menopausal women, serum P_4 levels showed no correlation with tumor ER concentrations. In a similar subsequent clinical study (235), Teruel et al found no correlation between serum P_4 levels and the presence of ER in breast adenocarcinomas. Furthermore, in an *in vitro* study conducted by Hegde et al (236), MCF-7 cells treated with 150 nM P_4 for 48 hours showed a 2.2-fold increase in ER-α mRNA expression, but no significant increase was observed at the protein level analyzed by immunoblotting. Similarly, in the postmenopausal women with advanced breast cancer, Lundgren et al (237) found that high doses of MPA for one and eight weeks of treatment reduced ER levels by 26.9% and 20.0%, respectively. However, in one study conducted by Noguchi et al (238), postmenopausal women with ER and PR positive breast tumors administered with 400 mg oral MPA three times daily for seven days before surgery showed no significant post surgical changes of ER levels compared with control patients. These divergences may be atrributed to the differences of experimental conditions and treating durations among those experiments conducted, and reflect the heterogeneity and complexity of the characteristics of breast cancer cells among different investigational centers which might raise a great challenge of therapy decision making and tumor response predicting for individual patient in the clinical practice.

Additionally, in the present study, P_4 downregulated PRA and PRB expressions in MCF-

7EVC and T47DEVC cells; NET and MPA upregulated PRA expression and upregulated PRB expression in MCF-7EVC and T47DEVC cells. Based on the results of the pesent study, in MCF-7WT12 and T47DWT3 cells, significant regulations of progestogens were observed only for ER-α expression but not for PRA and PRB expressions. However, individual effects of PGRMC1 status on regulatory actions of progestogens, especially that of synthetic progestins, have not been largely studies. In another aspect, with extrinsic PGRMC1 transfection in the present study, expression levels of ER-α and PR decreased marketly in MCF-7WT12 and T47DWT3 cells compared to the corresponding MCF-7EVC and T47DEVC control cells, especially for PR, which was almost completely absent either before or after any hormone treatment. In the clinical treatments, the presence of ER-α and PR is a hallmark of better prognosis for breast cancer endocrine therapy. This is in consistency with the trend of worseness of tumors and unresponsiveness to endocrine therapies in patients haboring excessive PGRMC1.

4.3. Effects of PGRMC1 overexpression on E_2 induced cell proliferation

ER-α signaling plays a critical role in cancer development and progression. In an *in vitro* study using MCF-7 and MCF-7WT12 cells conducted by Neubauer et al (239), E_2 was revealed to promote cell proliferation in a dose dependent manner, with a more pronounced effect observed in MCF-7WT12 cells. In another *in vitro* study conducted by Telang et al (240), MCF-7 cells treated with 5 nM, 10 nM and 20 nM E_2 exhibited an 83.3%, 120% and 140% increase in the surviving population. A *in vitro* study also demonstrated a 17.6% decrease in the population doubling time, a 91.9% increase in the saturation density and a 1.2-fold increase in the number of anchorage independent colonies with 20 nM E_2 treatment in MCF-7 cells (241). Similarly, E_2's promoting effect on cell proliferation in T47D cells has also been confirmed. In one *in vitro* study conducted by Belkaid A (242), promotion of cell proliferation was observed with the treatment of 2 nM E_2 for 5 days. An early *in vitro* study also showed that 10 nM E_2 could stimulated the proliferation over that of control in T47D cells although only slightly compared with the stimulation in MCF-7 cells (243). In another *in vitro* study conducted by Sotoca et al (244), T47D cells showed a clear E_2-dependent increase in cell proliferation with a 31% maximal increase at 100 pM E_2 treatment.

Plasma estradiol levels in postmenopausal women following one year's treatment of

commonly used 0.625 mg conjugated equine estrogen are 14.3-31.4 pg/ml, corresponding to 0.04-0.08×10^{-9} M E_2 (245), similar to the 13.0 pg/ml level in postmenopausal breast cancer patients (246). The present study used a higher level of 10^{-9} M E_2, and showed 100.7% and 122.9% increases in cell proliferation in MCF-7EVC and T47DEVC cells, which are in accordance with those previous results. In the presence of PGRMC1 overexpression, increases of 171.2% and 175.6% in cell proliferation with 10^{-9} M E_2 treatment were observed in MCF-7WT12 and T47DWT3 cells. Results in MCF-7WT12 and T47DWT3 cells are in accordance with one published research (247) conducted with MCF-7WT12 cells which showed a 2-fold increase of cell proliferation treated with 10^{-10} M E_2 for 6 days, whereas E_2 at a lower level of 10^{-12} M demonstrated no promoting effect. This implies a possible promoting effect of PGRMC1, directly or indirectly, on E_2 induced cell proliferation. However, few studies have been conducted in term of the impact of PGRMC1 overexpression on ER-α signaling pathway up till now. In an early *in vitro* study (170), E_2 has demonstrated no affinity to PGRMC1, and there is not supposed to be an effective E_2-PGRMC1 binding event to exert a direct promoting effect on cell proliferation. To further confirm the relation between the promoting regulation of PGRMC1 overexpression and the ER-α signaling pathway, all cell lines in the present study were treated with estrogen receptor antagonist, i.e. 10^{-6} M and 10^{-7} M TAM in SM medium to determine the cell proliferation in a condition of absolute invalid ER-α binding when possible intracellular ER binding substance production exists. The results showed that with the treatment of single agent TAM, no extra promoting effects on cell proliferation were observed in any cell lines. Based on these, the assumption that ER-α signaling involves in the promoting effect of PGRMC1 overexpression on cell prolifertion might be reasonable.

No research has been conducted to explore the direct impact of PGRMC1 overexpression on the regulation of ER-α and PR expressions in breast cancer up till now, although a positive correlation between PGRMC1 and ER-α expession but not PR expression was reflected in one immunohistochemical study conducted with human breast cancer samples (248). The present study used 10^{-9} M E_2 to evaluate the effect of E_2 on ER-α and PR expressions and to detect a possible role of PGRMC1 overexpression in these regulations. Results showed significant increase of ER-α expression in all cell lines. However, increased expressions of PRA and PRB were only observed in MCF-7EVC and T47DEVC cells, whereas both PRA and PRB expresstions

kept netagive in MCF-7WT12 and T47DWT3 cells. Sexual hormones have been reported to regulate the expression of their receptors in breast cancer cells in previous studies. In one *in vitro* study conducted with MCF-7 cells (249), 1 nM and 10 nM E_2 alone were reported to significantly upregulated ER-α expression by 1-2 and 4-5 folds respectively, whereas in the same study, PRA and PRB expressions demonstrated no significant changes, which is contrary to the results from the present study for MCF-7EVC and T47DEVC cells. However, the current results are in accordance with another study in which PR levels significantly increased with 10^{-10} M E_2 treatment in MCF-7 cells (250). The regulation of PR expression is a complex and mutifactorial prosess. Except for ER regulation, signaling events involving of EGFR family and IGF-1R, and crosstalk between ER and these signaling pathways have been reported to downregulate PR (251). Recent studies have revealed that some MicroRNAs are regulators for PR expression. One recent study conducted by Gilam et al (252) using surgical samples from ER positive, HER2 negative breast cancer pitients to identify MicroRNAs that regulate PR expression by *in vitro* Luciferase binding assays revealed that miR-181a, miR-23a and miR-26b are negative regulators of PR expression in ER-positive breast cancers. An earlier study conducted by Maillot et al (253) using MicorRNA microarrays and RT-PCR experiments in breast cancer cell lines showed that E_2 treatment repressed the expression of a broad set of MicroRNAs in an ER-dependent manner, and transcriptome analysis also demonstrated that E_2-repressed miR-26a and miR-181 are negative regulators of PR gene. In addition, diversity of microenvironment including cultural media and cultural conditions may also influence cell responses to treatments. All these may at least partly explain the result variability among different investigational centers. In another aspect, PR gene is a downstream gene of ERE, usually induced by activation of ER-α signaling, so PR can be regarded as a marker for functional ER signaling. The present study showed negative expressions both before and after 10^{-10} M E_2 treatment in MCF-7WT12 and T47DWT3 cells, indicating a possible loss of ER-α signaling in PGRMC1 overexpressing breast cancer cells. Given the observation of increased cell proliferations in PGRMC1 overexpressing breast cells, mechanisms besides ER-α signaling may be involved in the promoting action. Further investigations are warranted to explore the mechanism of ER-α signaling inactivation by PGRMC1 overexpression.

4.4. Effects of PGRMC1 overexpression on progestogen induced cell

proliferation

The Women's Health Initiative (WHI) randomized trial raised the concern of breast cancer risk with combined HRT regiments in postmenopausal women by a 1.26-fold higher hazard ratio after mean intervention of MPA plus conjugated equine estrogens for 5.6 years and in total mean follow-up of 7.9 years (151). Subsequent epidemiological evidence revealed distinct risks of carcinogenesis and progression in breast tissues with different progestogen treatments. P_4 has no association with increased breast cancer risk in normal postmenopausal women, either alone or in combination with E_2. Synthetic progestins exhibit greater risks for breast cancer compared with natural progesterone, with various risks depending on the progestin contained within the regimen. In the French E3N study, in the total mean follow-up of 7.9 years, estrogen plus P_4 demonstrates no significant relative risk for invasive breast cancer compared with HRT never-use, while regimens of E_2 plus NET and MPA demonstrated 2.11- and 1.48-fold higher risks, respectively (150). In another large scaled randomized controlled trial, postmenopausal women receiving treatment of conjugated equine estrogen plus MPA demonstrated 1.25- and 1.96-fold higher risks in term of occurrence of invasive breast cancer and death directly attributed to breast cancer, respectively compared with those receiving placebo pills (254).

However, *in vitro* studies have reported controversial cell responses to P_4 treatment. In two *in vitro* studies conducted by Hegde et al (236) and Zhao et al (255), P_4 promotes cell proliferation by more than 35% tested by MTT assay in MCF-7 cells, with much higher increase observed in T47D cells (255). Similarly, in the study conducted by Fjelldal et al (256), P_4 at physiological levels of up to approximately 10^{-6} M slightly elevated cell densities, whereas higher levels of P_4 caused a steep fall in cell densities; in another *in vitro* study with T47D cells conducted by Chen et al (257), P_4 at the concentration of 100 nM for 24 hours inhibited cell proliferation via introducing transcription of its target gene mitogen-activated protein kinase phosphatase 1; and in the study with PR-transfected MDA-MB-231 cells conducted by Lin et al (258), 10^{-12} M to 10^{-6} M P_4 markedly inhibited DNA synthesis and cell growth in a dose dependent manner. This inconsistency is partly explained by the diversities of physiological state within the cells and the incubation conditions among different investigational centers. On the contrary, synthetic protestins are consistently reported to promote breast cancer cell proliferation. In the recent *in vitro* study conducted by Sweeney et al (259),

consistant promoting effects of 10^{-6} M NET and 10^{-6} M MPA on breast cancer cell proliferation was reported.

In the present study, P_4 alone demonstrated no significant promoting effect on cell proliferation in MCF-7ECV and T47DEVC cells, whereas in PGRMC1 overexpressing cells, P_4 elicited significant promotion of cell proliferation in MCF-7WT12 and T47DWT3 cells. On the contrary, synthetic progestins NET and MPA promoted cell proliferation in all cell lines, irrespective of cell line types and PGRMC1 abundance. Those results are in accordance with the clinical observations described above and one published observation that PGRMC1 is associated with aggressive phenotype and poor prognosis in both ER positive and negative breast cancers (230). Distinctions of progestogen effect on cell proliferation may be explained by their differences in binding affinity and structure. Synthetic progestins generally have higher relative biding affinites to human PR than natural P_4 (260-267). In one *in vitro* study using both PR positive and negative T47D cells, a high degree of similarity of transcriptional responses was found between synthetic progestins and natural P_4, with each progestin regulating 77% to 91% of the genes regulated by P_4 (268). Compared with P_4, synthetic progestins, whether structurally related to P_4 or to testosterone, have additional partial binding affinities to androgen, glucocorticoid and mineralocorticoid receptors, hence allowing the activation of other receptor signalings (269). For example, MPA can demonstrate androgenic properties by binding to AR to induce AR signaling specific transcriptional activities and promote cell proliferation in both absence or presence of ER and PR (149). In addition, MPA can also function through glucocorticoid receptor (259). Overall, for the female population requiring HRT, P_4 is preferable to other synthetic progestins in terms of breast cancer risk.

In another aspect, ER-α and PR expressions with treatments of different progestogens were determinded in the present study. All progestogens demonstrated consonant downregulating effects on ER-α expression in the four cell lines, whereas opposite impacts on PR expression were observed between natural P_4 and synthetic progestins. P_4 significantly downregulated PRA and PRB in MCF-7EVC and T47DEVC cells. However, NET and MPA significantly upregulated both PRA and PRB in MCF-7EVC and T47DEVC cells. By contrast, PRA and PRB expressions were both negative before and after any progestogen treatment in MCF-7WT12 and T47DWT3 cells. These observations further support the assumption of inactivation of ER-α signaling and loss

of sexual hormonal regulation of their receptors in MCF-7WT12 and T47DWT3 cells.

According to the results of the present study, overexpression of PGRMC1 appears to be an promoting factor for progestogen's regulation of cell proliferation. This is in accordance with the previous results to some extent. One study conducted by Zhou et al (270) demonstrated that cell proliferation increased by 40% and 97% in MCF-7WT12 cells with the treatments of 10^{-6} M MPA and 10^{-7} M NET. In another study conducted by Neubauer et al (271), both NET and MPA, but not progesterone, elicited a significant promotion of cellular proliferation in MCF-7WT12, however a lower response was observed in MCF-7EVC cells. A recently published research (272) used a mouse xenograft model to compared the effect of E_2 combined with P_4 or NET or placebo on tumor volumes. The results revealed that after 12 days of E_2 implantation combined with following 6-7 weeks of progestogen pellet implantation, NET but not P_4 or placebo significantly increased tumor growth. Despite the various extents of promoting effect of each progestin and different experimental conditions displayed in these studies, a consistency of elevated breast cancer risk in the usage of synthetic progestins compared with natural P_4 and the promoting effect exerted by PGRMC1 are comfirmed. This implies a relatively higher safety in the P_4 containing HRT regiments compared with synthetic progestin containing HRT regiments in terms of both carcinogenesis and a possible progression in an underlying cancer condition, especially in those with PGRMC1 overexpression which might increase susceptibility.

From mechanical aspect, the role of PGRMC1 in the proliferative effect of progestogens in breast cancer remains an open and complicated issue. Currently, it is believed that the involvement of PGRMC1 on cell proliferation is mainly associated with the rapid non-genomic actions instead of nuclear PR signaling. Several *in vitro* experiments have been conducted that might at least partly help to understand this issue. In one *in vitro* experiment conducted by Neubauer et al (273), by stimulating with membrane-impermeable bovine serum albumin-fluorescein isothiocyanate conjugated P_4 at 10^{-6} M, proliferation in PGRMC1 (phosphorylated at serine 180) transfected MCF-7 cells increased by approximately 35% compared with MCF-7 control cells. This effect was independent of PR functional status. However, unconjugated P_4 showed an antiproliferative effect in both PGRMC1 transfected MCF-7 cells and control cells. In addition, in the same experiment, vascular endothelial growth factor A expression was roughly three-fold higher in PGRMC1 transpected MCF-7 cells compared to MCF-7

control cells. These findings suggest that PGRMC1 functions outside the nuclear PR signialing pathway. In addition, plasminogen activator inhibitor 1 RNA-binding protein (PAIRBP1) is a widely accepted protein involved in the action of PGRMC1 on P_4 activity. PAIRBP1 resides at the plasma membrane and in the cytoplasm (274,275). It has no binding site for P_4, but has shown to interact with PGRMC1 to transduct P_4's antiapoptotic action (188,276). Amino acids 70 to 130 in PGRMC1 sequence is the essential portion for PAIRBP1 binding (276). *In vitro* studies conducted in ovary cell lines by Peluso et al have comfirmed the increased binding capacity and cell responsiveness to P_4 with forced PAIRBP1 expression (274) as well as a significant attenuation of P_4's antiapoptotic actions with PAIRBP1 blockage (275). However, these findings are obtained from ovary cell lines, further investigations in breast cancer cell lines are warranted. It is worth noting that previous investigations have also found the conformational or structural basis of PGRMC1, such as dimerization and phosphorylation, is warranted for the functional exertion. In this case, a quantitative change may not parallel to a functional change. However, to the author's knowledge, no study has been conducted to characterize progestogen binding profiles or to compare the progestogen impact on PGRMC1 dimer or polymer up till now.

4.5. Effect of PGRMC1 overexpression on TAM resistance

Drug resistance is a major problem limiting efficacy in the cancer treatment. In brest cancers, the close relationship between the ER status and the response to hormonal treatment has been widely reported. Patients with high receptor values show better clinical outcomes than those with lower values. In one clinical study with 156 advanced breast cancer patients conducted by Stewart et al (277), responses to first TAM endocrine therapy were observed in 50% and 27% of ER+PR+ and ER+PR- patients, compared with 27% and 6% of ER-PR+ and ER-PR- patients. In another clinical study conducted by Lippman et al (278), out of 85 metastatic breast cancer patients, 65% of ER+ patients versus 9% of ER- patients achieved an objective response to endocrine therapy. In addition, the likelihood of an objective response to endocrine therapy appeared to increase as the ER amount increased. However, about 50% ER positive breast cancers acquire resistance to endocrine therapies that block ER-α actions through different mechanisms (279-281). In the treatment-naive patients with ER+ metastatic breast cancer, up to 50% do not respond to first line treatment with TAM (*de novo* resistance) (282); and almost all patients with metastatic disease and as many as 40% of

patients receiving adjuvant TAM eventually relapse and die from their diseases, despite an initial positive drug response (acquired resistance) (283). As far as now, numerous *in vitro* and *in vivo* studies have been conducted and several mechanisms responsible for resistance to endocrine therapies have been hypothesized, such as loss of ERs, altered signaling pathways, change of microRNA expressions, TAM metabolism, and crosstalk between the ER signaling cascades and growth factors (280,284-287).

In the present study, TAM response was tested in both empty vector and PGRMC1-transfected MCF-7 and T47D cells. It is worth mentioning that in the TAM response experiments, estrogen simulating environment was not selected as control because inhibiting the “crazy” growth of tumor cells to some extent is not the ultimate goal of therapy, but killing or stopping growth of tumor cells is the ideal outcome. Therefore using the growth resting experenmental cells under null hormone effect as control is clinically more meaningful. Results showed that TAM exerted suppressive effects on cell proliferation only in empty vector transfected breast cancer cells. Earlier studies have demonstrated a broad range of drug resistance involved by PGRMC1. In the budding yeast Dap1p (damage associated protein 1, a homologue of PGRMC1), deletion of DAP1 gene leads to sensitivity to the methylating agent methyl methanesulfonate (213). Dap1p also directs resistance to itraconazole and fluconazole, which are inhibitors of sterol synthesis. Elevation of PGRMC1 has been demonstrated to contribute to treatment resistance in numerous cancers (174), including endometrial cancer (182), uterine sacorma (222) and uterine cancer (288), ovarian cancer (289), lung cancer (289,290), head and neck cancer (289). However, PGRMC1's involvement in endocrine resistance is seldom investigated and still remains an open question. In one *in vitro* study conducted by Blassl et al (291). MCF-7EVC and MCF-7WT12 cells were treated with 10^{-10} M and 10^{-12} M E_2 plus 10^{-9} M to 10^{-6} M TAM in different sequences of "E_2-first", "TAM-first" and "continuous" regimens for 5 days. E_2 alone promoted cell proliferation by 250% and 150% respectively in MCF-7EVC and MCF-7WT12 cells. In MCF-7WT12 cells, "E_2-first" regimen resulted in an agonistic TAM effect compared with normal TAM effect in "TAM-first" and "continuous" regimens. In contrast, no agonistic TAM effect was observed in MCF-7EVC cells with any regimens. The author attributed the role of PGRMC1 overexpression in TAM reisistance to its crosstalk with ER-α. Unfortunately, no further investigation was conducted.

Due to the exclusive consuming population of menopausal women of TAM, the present

study only evaluated ER-α and PR expressions with treatments of TAM plus E_2. The results revealed significant upregulation of ER-α and downregulation of PRA and PRB with treatment of 10^{-6} M or 10^{-7} M TAM plus 10^{-9} M E_2. As for MCF-7WT12 and T47DWT3 cells, ER-α expressions were markedly lower but showed no significant alteration with drug treatments and PRA and PRB expressions kept negative before and after treatments. It is worth noting that TAM resistance occurred in accordance with downregulation of ER-α expression and loss of PR in MCF-7WT12 and T47DWT3 cells, which is in accordance with the findings observed in the clinical settings that endocrine response of TAM treatment in terms of both objective response and response duration increases with the increase of ER amount within the tumor (278,282), and that loss of PR is associated with a lower TAM response (277)(292). Based on the above observations, although how PGRMC1 overexpression contributes to inactivation of ER-α signaling is still unkown, yet determination of PGRMC1 overexpression so as to identify the breast cancer population at higher risk of TAM resistance is of great value. P_4 might be a safer option than synthetic progestins, however, in the condition of PGRMC1 overexpression, cautions should be taken when priscribing sexual hormones.

In the current study, in the context that addition of massive extrinsic transfected PGRMC1 surpressed both ER and PR expression, PGRMC1 appeared to shrink the regulation of ER and PR expressions with antiestrogenic treatments in MCF-7WT12 and T47DWT3 cells. Combined with the concurrent observations of significant increases of cell proliferation with both NET and MPA containing treatments in all cell lines, blockage of ER-α signaling with antiestrogenic agents is not sufficient to achieve a satisfactory suppression in the presence of NET and MPA, even though ER-α expression remains a relative higher level in MCF-7EVC and T47DEVC cells. These imply that PGRMC1 might promote cell proliferation and influence TAM response by changing sexual hormone recepter status, and that progestogens might be an important regulatory factor involved in those actions.

In conclusion, PGRMC1 overexpression adds to the promoting effects of cell proliferation by E_2 and synthetic progestins in breast cancer. TAM and P_4 lose antiproliferative effect in the status of PGRMC1 overexpression. E_2 and progestogens have diverse regulatory actions on PGRMC1 expression, whereas in PGRMC1 overexpression status progestogens lose suppressing action of PGRMC1 expresson. In addition, PGRMC1 overexpression seems to impel loss of ER-α and PR, which

indicates the potential inactivation of ER-α signaling pathway and might be one of the main mechanisms of TAM resistance. Thus, in the clinical aspect, determination of PGRMC1 overexpression so as to identify the breast cancer population at higher risk of disease progression and TAM resistance is of great value. P_4 might be a safer option than synthetic progestins, however, in the condition of PGRMC1 overexpression, cautions should be taken when prescribing sexual hormones.

5. Summary

Breast cancer remains the leading malignancy in women around the world. Since over two thirds of breast cancers are estrogen receptor-α (ER-α) positive and higher incidence is found in postmenopausal women, endocrine therapy is the mainstay of adjunctive therapy for this population. However, resistance with longer treatment has aroused wide concern and is not yet fully understood. Progesterone receptor membrane component 1 (PGRMC1) as an upregulated membrane bound protein in tumorigenesis and progression has been reported to correlate with higher incidence of endocrine resistance. Thus, a better understanding of how PGRMC1 is involved in this process could help to stratify patients and guide decision making.

The present study was undertaken to evaluate the impact of PGRMC1 overexpression (1) on the regulation of cell proliferation by 17-β estradiol (E_2) and progestogens; (2) on the response to tamoxifen (TAM) treatment; (3) on the regulation of ER-α, progesterone receptor (PR) and intrinsic PGRMC1 expression by E_2 and progestogens so as to discover the possible correlation between PGRMC1 and sexual hormone signaling pathways in breast cancer, as well as to investigate a tentative role of PGRMC1 in endocrine resistance.

The present study was undertaken in empty vector or PGRMC1 transfected estrogen receptor (ER) positive breast cancer cells, ie. MCF-7ECV, T47DEVC, MCF-7WT12, and T47DWT3 cells. Cells were incubated with single E_2 or progestogens, and with TAM plus E_2 for 5 days. Progestogens used in the present study included progesterone (P_4), medroxyprogesterone acetate (MPA) and norethisterone (NET). E_2 was used at 10^{-9} M, and other agents were used at 10^{-7} M and 10^{-6} M. Cell proliferation was measured by Methyl thiazoletetrazolium (MTT) test. Expressions of ER-α, PR and PGRMC1 were measured by western blot. SPSS statistical software 23.0 was used for quantitative analysis. Multi-group comparison was carried out using ANVOA followed by Bonferroni test. $P<0.05$ was considered statistically significant.

Results showed that

1) E_2 as well as MPA and NET significantly promoted proliferation in all cells, whereas P_4 demonstrated promoting effect only in MCF-7WT12 and T47DWT3 cells. When treated with TAM plus E_2, significant promoted cell proliferations were only observed

in MCF-7WT12 and T47DWT3 cells.

2) In MCF-7WT12 and T47DWT3 cells, no significant signals were observed for progesterone receptor A (PRA) and progesterone receptor B (PRB) either before or after any treatment.

3) E_2 significantly upregulated ER-α and intrinsic PGRMC1 expressions in all cells and upregulated PRA and PRB expressions in MCF-7EVC and T47DEVC cells.

4) All progestogens downregulated ER-α expression in all the cells. P_4, NET and MPA significantly downregulated intrinsic PGRMC1 expression in MCF-7EVC and T47DEVC cells, whereas significantly upregulated intrinsic PGRMC1 expression in MCF-7WT12 and T47DWT3 cells. P_4 significantly downregulated, whereas NET and MPA significantly upregulated PRA and PRB expressions in MCF-7EVC and T47DEVC cells.

5) E_2 plus TAM significantly upregulated ER-α and downregulated PGRMC1 expressions in MCF-7EVC and T47DEVC cells, whereas no significant changes were observed in MCF-7WT12 and T47DWT3 cells; PRA and PRB expressions were significantly downregulated in MCF-7EVC and T47DEVC cells.

In conclusion, PGRMC1 overexpression adds to the promoting effects of cell proliferation by E_2 and synthetic progestins in breast cancer. TAM and P_4 lose antiproliferative effect in the status of PGRMC1 overexpression. E_2 and progestogens have diverse regulatory actions on PGRMC1 expression, whereas in PGRMC1 overexpression status progestogens lose suppressing action of PGRMC1 expresson. In addition, PGRMC1 overexpression seems to impel loss of ER-α and PR, which indicates the potential inactivation of ER-α signaling pathway and might be one of the main mechanisms of TAM resistance. Thus, in the clinical aspect, determination of PGRMC1 overexpression so as to identify the breast cancer population at higher risk of disease progression and TAM resistance is of great value. P_4 might be a safer option than synthetic progestins, however in the condition of PGRMC1 overexpression, cautions should be taken when prescribing sexual hormones.

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7. Declaration of contributions

The work was carried out in the women's hospital under the supervision of Professor Harald Seeger. The study was designed by Professor Harald Seeger.

All tests were carried out by me independently. The statistical evaluation was carried out independently by me.

I certify that I wrote the manuscript independently and that I did not use any sources other than those I have indicated.

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Place, Date

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Signature

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