Possible role of estrogen receptor-alpha in the signaling mechanism of progesterone receptor membrane component-1 in human breast cancer cells

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ABBREVIATION

ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor 2
MHT	Menopausal hormone therapy
WHI	Women`s Health Initiative
WMS	The Million Women Study
PGRMC1	Progesterone receptor membrane component 1
Hpr6.6	Human membrane progesterone receptor
CK2	casein kinase 2 or II
TBCA	tetra-bromo-cinnamic acid
EGFR	epidermal growth factor receptor
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
EE	Ethinyl estradiol
Eq	Equilin
DHEq	17α-Dihydroequilin
P4	Progesterone
NET	Norethisterone acetate
MPA	Medroxyprogesterone acetate
LNG	Levonorgestrel
CMA	Chlormadinone acetate
DSG	Desogestrel

DNG	Dienogest
DRSP	Drospirenone
DYD	Dydrogesterone
NOM	Nomegestrolacetate
CYA	Cyproterone acetate

1 INTRODUCTION

1. 1 Breast cancer

1.1.1 Epidemiology of breast cancer in women

Breast cancer is one of the most common cancers worldwide. Women living in the US have a one in eight chance of being diagnosed with breast cancer. In 2013, an estimated 232,340 women were diagnosed with invasive breast cancer, 64,640 with carcinoma in situ and approximately 39,620 women died (American Cancer Society. 2013).

Breast cancer incidence and mortality increase with age. 79% of new cases and 88% of breast cancer deaths occurred in women at the age of 50 and older. During 2006-2010, the average age at diagnosis was 61 (American Cancer Society. 2013).

A large racial disparity remains in breast cancer. Non-Hispanic white women have the highest incidence rates of breast cancer. However, African-American women have a higher mortality rate for all age groups (American Cancer Society. 2013).

1.1.2 Classification of breast cancer

Greater than 95% of breast cancers are adenocarcinomas, which are divided into in situ carcinomas (15-30%) and invasive carcinomas (70-85%). Carcinoma in situ is limited to ducts and lobules by the basement membrane, and can be further divided into ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Invasive carcinoma refers to that the cells have the potential to invade into the surrounding adjacent normal tissue, reach regional lymph nodes and distant sites. No special type includes the majority of invasive carcinoma (79%), followed by lobular carcinoma (10%), tubular/cribriform carcinoma (6%), mucinous (colloid) carcinoma (2%), medullary carcinoma (2%), papillary carcinoma (1%) and metaplastic carcinoma (<1%).

Since the distinct biological features correlate with different responses to various treatments and affect the long-term prognosis, four subtypes of the most common breast cancer type (no special type) have been identified based on the status of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Clinically, various statuses of these receptors may lead to different prognostic features, which are represented by cell proliferation and tumor differentiation. These subtypes include luminal A, luminal B, basal-like and HER2 positive (Perou et al. 2000; Perou et al. 2011; Blows et al. 2010; Habashy et al. 2014; Yersal and Barutca. 2014; Carey et al. 2007; Carey et al. 2006).

Luminal A (50-60%): This is the largest group of breast cancer and is characterized by ER+ and / or PR+ / HER2-. The majority occurs in postmenopausal women, responds well to hormonal treatments and has good prognosis.

Luminal B (15-20%): This group is characterized by ER+ and / or PR+ / HER2+. This type of breast cancer is aggressive, more likely to have lymph node metastases and leads to low survival rates.

Basal-like (8-37%): This group is also referred to as triple-negative, because of the absence of ER, PR and HER2. Basal-like breast cancer is associated with high proliferation rate, frequent metastasis, poor prognosis and occurs more often in younger premenopausal women.

HER2-positive (15-20%): This group is distinguished by ER-, PR- and HER2+, which is usually poorly differentiated and with poor outcomes.

1.1.3 Risk factors of breast cancer

The assessment of breast cancer risk may help to determine whether an intervention is appropriate to prevent the development of diseases. Many risk factors are non-modifiable, such as age, race/ethnicity, family history, early

menarche and late menopause, while the other risk factors are modifiable, including obesity, alcohol consumption, smoking and use of combined estrogen and progestin menopausal hormones (American Cancer Society. 2013; Mahoney et al. 2008, Advani and Moreno-Aspitia. 2014). Several assessment models, such as Gail Model, Claus Model, BRCAPRO Model and Cuzick-Tyrer model etc., have been developed based on these risk factors and applied for breast cancer predication (Amir et al. 2003, Evans and Howell. 2007).

1.2 Menopausal hormone therapy and breast cancer

1.2.1 Menopausal hormone therapy induces breast cancer

Since the early 1940s, when estrogen was first introduced into clinical practice, the concern that menopausal hormone therapy (MHT) may cause breast cancer has existed. However, millions of women still use it for menopausal symptom relief, such as hot flushes and sweats (Rossouw et al. 2002). To understand if indeed MHT influence breast cancer, data was collected to provide more reliable evidence. Various methods were applied including case reports, case-control studies and recently some large prospective cohort studies. Consequently, the concepts about MHT and breast cancer have also changed over time.

In the 1990s, two studies provided important evidences. The Nurses' Health Study cohort collected information on the menopausal status of 121,700 women ages 30-55. After 725,550 person-years of follow-up, they found that estrogen alone and estrogen plus progestin were both associated with an increased risk of breast cancer (Colditz et al. 1995). The Collaborative Group on Hormonal Factors in Breast Cancer reanalyzed 51 studies on the relation between MHT and breast cancer risk in 21 countries, and suggested that the relative risk for women who had used MHT for 5 years or longer is statistically higher (Collaborative Group on Hormonal Factors in Breast Cancer. 1997).

In 1993, WHI started two large randomized placebo-controlled clinical trials to evaluate the overall health effects of estrogen alone and of estrogen plus progestin. In the estrogen plus progestin arms, 16,608 postmenopausal women aged 50-79 years with an intact uterus were enrolled, and received either 0.625mg/d of conjugated equine estrogen (CEE) plus 2.5mg/d of medroxyprogesterone acetate (MPA) or placebo. After 5.2 years of follow-up, the risk of breast cancer increased by 24% in women receiving estrogen plus progestin compared with placebo. And more breast cancers were diagnosed only after the first 4 years in the estrogen plus progestin arm compared with placebo (Rossouw et al. 2002) (Figure 1). In the estrogen alone arms, 10.739 postmenopausal women aged 50-79 years with prior hysterectomy were randomized to either 0.625mg/d of CEE or placebo. No increase or even a reduction in breast cancer risk in the estrogen alone arm compared to placebo was reported after an average of 7.1 years of follow-up (Anderson et al. 2004) (Figure 1). But the possible reduction missed statistical significance. In addition, subgroup analyses suggested that estrogen alone may be associated with more favorable outcomes in younger women compared with older women (Anderson et al. 2004; LaCroix et al. 2011).

After the publication of the Women`s Health Initiative (WHI) in 2002, the use of postmenopausal MHT in the United States dropped over one year from approximately 40% to 20% (Hersh et al. 2004), and continued to decline by 52% from 2001-2009 (Tsai et al. 2011). Subsequently, a significant decrease in breast cancer incidence was also observed in different countries, such as in the United States, Germany, Australia, France and so on (Clarke et al. 2006). And this corresponding decrease is not influenced by the changes in frequency of mammography (Chlebowski et al. 2009).

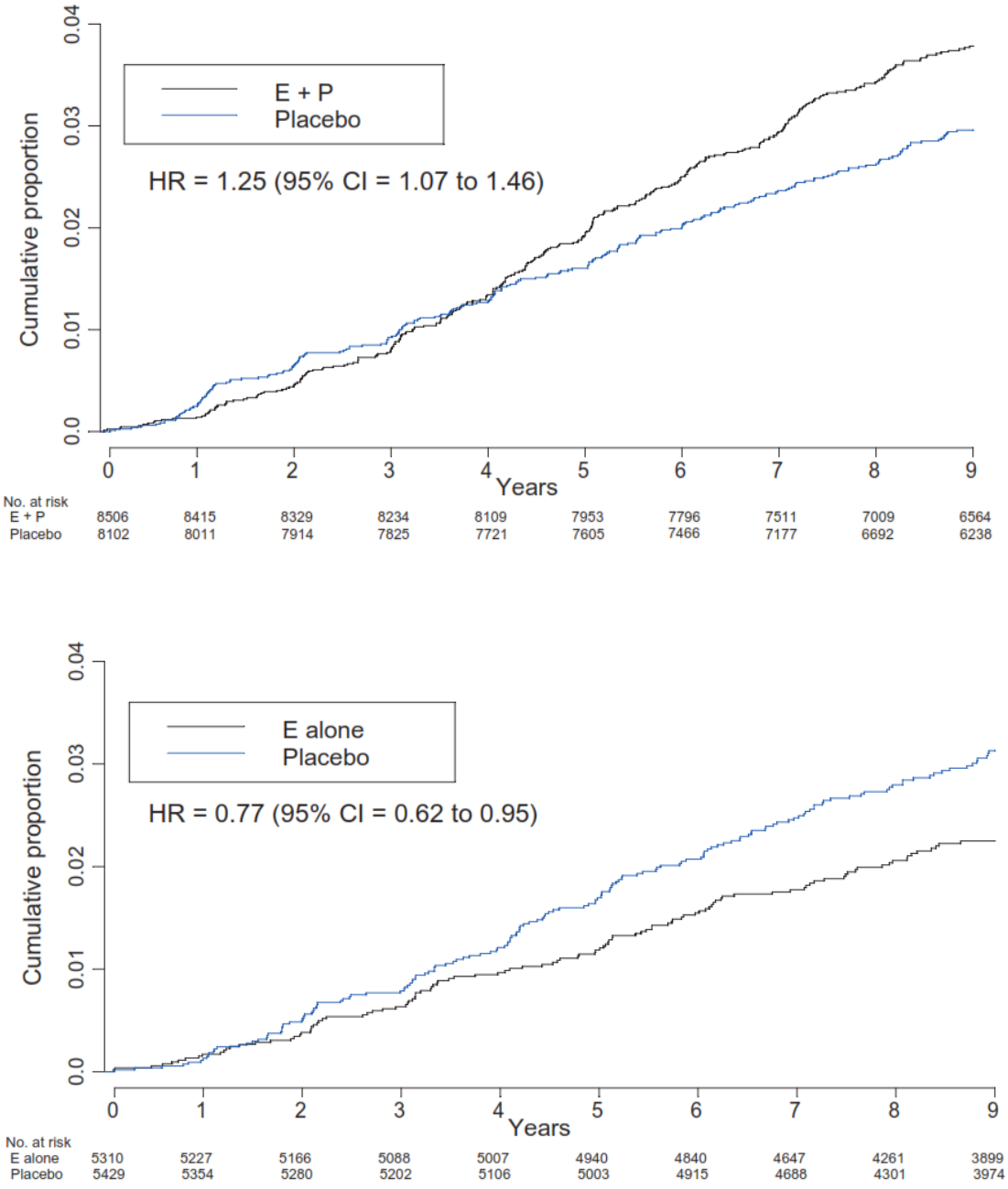

Figure 1. Women`s Health Initiative (WHI) Hormone Therapy Clinical Trials. Invasive breast cancer incidence (Chlebowski et al. 2012).

Another well-known cohort analysis is the Million Women Study (WMS) in UK, in which 1,084,110 women aged 50-64 were enrolled. Incidence of breast cancer was

found increased by a factor of 1.30 for current users of estrogen-only therapy, and by a factor of 2.00 for current users of estrogen-progestin preparations (Beral et al. 2003).

1.2.2 Different preparations of MHT and breast cancer risk

Based on all the data from observational studies and randomized controlled trials that was mentioned above, MHT is now established as carcinogenic to humans with respect to breast cancer. And it also shown a higher breast cancer risk following estrogen plus progestin therapy compared with estrogen alone. Moreover, some experimental and clinical data suggest that different types and regimens of MHT may have a different impact on the risk of breast cancer.

In the WHI hormone therapy trials, 0.625mg/d of CEE with or without 2.5mg/d of MPA were used, which represents the vast majority of MHT by far in the US (Rossouw et al. 2002). And in the UK, as reported in the MWS, the most commonly used estrogens are estradiol (E2) or CEE, and the progestins are mainly norethisterone acetate (NET), norgestrel and levonorgestrel (LNG) (Beral et al. 2003). In Europe, types and regimens of MHT vary in different countries. Instead of CEE, E2 is prescribed more frequently in most of European countries. The predominant progestins used in Denmark, Germany, Norway, and the Netherlands are the testosterone-derived progestins, mainly NET or LNG, whereas the progestin preferred in France, Italy and Spain is progesterone derivatives. Overall, the most frequently used regimen of combined MHT is progesterone plus NET in the sequential treatment mode (Table 1).

Stahlberg and collages (Stahlberg et al. 2004) analyzed data from the Danish Nurse Cohort, including information on MHT types and regimens, reproductive history and lifestyle-related factors of 10,874 women aged 45 years and above, who used estrogen (mainly E2) alone or estrogen combined with either progesterone-like progestins (MPA) or testosterone-like progestins (NET/LNG) in a sequential or continuous combined treatment mode. The result showed a 4-fold

increased risk of breast cancer with the continuous combined mode. And both progesterone-like progestins and testosterone-like progestins were associated with a statistically significant higher risk of breast cancer.

In the French E3N cohort study, data of 80,377 postmenopausal women aged between 45-65 years were assessed. Followed for an average of 8.1 postmenopausal years, 2,354 cases of invasive breast cancer occurred (Fournier et al. 2008). Among all the cases, different types of MHT were analyzed. Compared with the preparation of estrogen plus progesterone (P4) and estrogen plus dydrogesterone (DYD), the combinations of estrogen and other progestins, such as MPA, Nomegestrol acetate (NOM) or NET appears to be more harmful with regard to breast cancer. However, the association of these progestins with breast cancer risk, except for P4 and DYD, did not have significant difference from one another.

In another large prospective cohort, the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, 133,744 postmenopausal women from across Europe were enrolled (Bakken et al. 2011). The data regarding different preparations of MHT shows a 43% higher risk of continuous combined regimens compared with sequential regimens. And among women who used sequential regimens, the associations with breast cancer risk did not differ significantly between those who used testosterone-like or progesterone-like progestins. Among women who used estrogen-only regimens, risk did not vary significantly between those who used E2 or CEE.

	All (n=133,744)	Denmark (n=21,794)	France (n=33,125)	Germany (n=11,575)	Italy (n=14,074)	Norway (n=10,578)	Spain (n=9,360)	The Netherlands (n=10,935)	United Kingdom (n=22,303)
Type of estrogens (%)									
Conjugated equine estrogens	21.4	1.0	3.5	54.9	8.5	0.0	7.2	27.6	47.5
Estradiol	61.8	82.3	61.4	37.5	71.0	89.3	50.3	61.2	43.8
Low-potency estrogens	11.3	4.8	33.5	6.2	20.5	10.2	0.0	8.0	4.7
Other/unknown	5.5	11.9	1.6	1.4	0.0	0.5	42.4	3.2	3.9
Type of progestin (%)									
Micronized progesterone	9.5	0.0	24.4	0.1	2.2	0.0	1.1	1.0	0.0
Progesterone derivative	35.8	19.2	68.6	19.5	83.9	0.7	78.9	30.2	35.3
Testosterone derivative	53.2	80.8	4.3	80.0	13.9	99.3	0.0	64.6	64.7
Other/unknown	1.5	0.0	2.7	0.5	0.0	0.0	20.0	4.1	0.0

Regimen (%)									
Sequential	44.2	71.6	7.1	69.2	18.2	61.3	2.9	67.4	89.6
Continuous combined	15.3	24.4	2.2	24.5	0.7	38.1	0.0	5.8	8.5
Other/unknown	40.5	4.0	90.7	6.3	81.0	0.6	97.1	26.8	1.9

Table 1. Different types and regimens of MHT used among postmenopausal women in the cohort (the EPIC-study) (Bakken et al. 2011)

1.3 PGRMC1 and breast cancer

1.3.1 Introduction of PGRMC1

The WHI trial used the combination of CEE plus MPA. In contrast to the WHI combined arm, the estrogen only arm shows no increase but rather a reduction of breast cancer risk, which was significant for patients with more than 80% adherence to study medication. This result indicates a negative effect of progestins concerning breast cancer risk. However, the question remains open, in as far the combination of estrogens with synthetic progestins as well as with natural progesterone may elicit the same increased risk. Thus, there are still many questions on the extrapolation of the WHI results to all synthetic progestins and to natural progesterone.

Progestogens are conventionally thought to act via the activation of the intracellularly located progesterone receptors (PRs), PR-A and PR-B. Several in vitro studies indicate that progestogens can exert an anti-proliferative effect by the activation of these receptors in human breast cancer cells (Schoonen et al. 1995; Cappelletti et al. 1995; Krämer et al. 2006). Nevertheless, still other data suggested a proliferative effect of synthetic progestogens (Catherino et al. 1993; Franke and Vermes. 2003). Thus, the mechanisms by which progestogens act on human breast cells remain unclear. Moreover, many actions of progesterone are not be able to be explained by the classic genomic mechanism of steroid action involving activation of the intracellular transcription factors, PR-A and PR-B. On the other hand, evidence has accumulated that progesterone can also initiated rapid, cell surface-mediated actions by activating membrane receptors and their intracellular signal transduction pathways (Revelli et al. 1998; Norman et al. 2004; Thomas. 2012). Recent studies revealed that in addition to the intracellular-located receptors, progesterone receptor membrane component 1 (PGRMC1) is important to the activity of membrane-associated progesterone receptor (Cahill. 2007).

PGRMC1 was described as a putative progesterone-binding membrane receptor (Meyer et al. 1996). Thus, it was named Hpr6.6 (human membrane

progesterone receptor) before (Gerdes et al. 1998). PGRMC1 is a member of a multi-protein progesterone-binding complex. Because bacterially expressed PGRMC1 does not bind to progesterone (Min et al. 2005), it is now tentatively assumed that PGRMC1 does not bind natural progesterone (P4) by itself (Cahill. 2007), but requires an unknown protein that is associated only in partially purified PGRMC1 preparations (Peluso et al. 2008b).

1.3.2 PGRMC1 expression and function

In nonmalignant tissues, PGRMC1 is most highly expressed in the liver and kidney (Meyer et al. 1996). However, PGRMC1 is over-expressed in multiple types of cancer, including breast, thyroid, colon, ovary, and lung cancer (Rohe et al. 2009), and PGRMC1 levels correlate with tumor stage in ovarian cancer (Peluso et al. 2008a) and estrogen receptor status in breast cancer (Craven. 2008; Neubauer et al. 2008).

In breast cancer tissues, PGRMC1 is strongly expressed in about one-third of the investigated tissues. Crudden and colleagues (Crudden et al. 2005) showed that PGRMC1 is over-expressed in breast tumors compared with corresponding non-malignant tissue. Ji and colleagues (Ji et al. 2012) found that PGRMC1 does not exist in normal mammary gland, but its expression ranged from strong to minimal in breast cancer tissues according to immunohistochemistry. Furthermore, expression of PGRMC1 could be detected in 60 samples of breast cancer and was significantly correlated to lymph node metastasis, tumor size, TNM stage, overall survival rate, and tumor-free survival, but not to each patient's age or tumor differentiation. In a multivariate survival analysis, PGRMC1 was an independent prognostic factor of breast cancer (Ji et al. 2012). However, Causey and colleagues (Causey et al. 2011) found, in 28 frozen or paraffin-embedded breast cancer samples and ten control benign breast tissue samples by RelqPCR, which PGRMC1 mRNA levels decreased significantly with patient age. The different results may be due to different detection methods, as we know that the expression level of mRNA is not always fully translated into protein levels. Another possible reason may be that different ethnic groups have been investigated, one from China (Ji et al. 2012), while the other studies were

from the USA (Causey et al. 2011). Therefore, in order to harmonize the data, more studies among different countries using the same methods are necessary.

Little is known about how PGRMC1 functions. Since progesterone and the synthetic progestins used in MHT are able to activate PR-A/-B and PGRMC1 simultaneously, which suggests that in vivo the balance of the expression levels of both receptors might influence whether epithelial cells proliferate or not in the presence of progestogens.

Our laboratory has previously shown that the synthetic progestins bind to all progesterone receptors expressed by breast cancer cell line MCF-7. Binding to PR-A/-B in MCF-7 cells might transduce an anti-proliferative signal, countermanding the proliferative signal induced by low levels of PGRMC1. In contrast, the exogenously expressed PGRMC1, in MCF-7 cells that over expressed PGRMC1, might overrule the anti-proliferative effect of PR-A/-B. Therefore, it may be instructive to determine the expression ratio of PGRMC1 and PR-A/-B before MHT.

1.3.3 PGRMC1 in regulating breast cancer cell proliferation in vitro

Our laboratory compared the effect of the main synthetic progestins, which are used for MHT or natural progesterone alone, as well as combined with estrogens, on MCF-7 cells and PGRMC1 over-expressed MCF-7 cells (WT-12). As such, these cell lines mimic the PGRMC1 expression profile of breast cancer.

Interestingly, among all these natural progesterone (P4) and different synthetic progestins, including chlormadinone acetate (CMA), desogestrel (DSG), dienogest (DNG), drospirenone (DRSP), dydrogesterone (DYD), levonorgestrel (LNG), medroxy-progesterone acetate (MPA), nomegestrol (NOM) and norethisterone (NET), NET is most effective for the prolieration of all the cell lines, followed by DNG, DRSP, DSG, DYD, LNG, while no effect was found for CMA, NOM and P4. The proliferation effect on WT-12 cells is more significant than MCF-7 cells. These results demonstrated that progestogens act variant on

breast epithelial cells and different in their ability to induce proliferation. NET binds PGRMC1 with the highest affinity, which indicates MHT including NET might result in an increased risk for breast cancer development (Neubauer et al. 2009; Seeger et al. 2003).

Estrogen plus progestogens results showed estradiol (E2) can elicit a significant proliferation of WT-12 cells at 10^{-10} M. However, E2 alone at 10^{-12} M showed no significant effect. Addition of progesterone or the synthetic progestogens (MPA, NOM) in a sequentially or continuously combined manner did not significantly alter the estradiol-induced proliferation. However, NET showed a significant increased proliferation rate when combined with estradiol in a concentration of 10^{-12} M, and the increase being higher for the continuous combination. The E2- and NET-induced proliferation could be abrogated by the addition of an estrogen receptor antagonist. Women with breast cancer cells that over-express PGRMC1 may be more susceptible in developing breast cancer and thus may have a much higher breast cancer risk when treated with estrogen therapy alone and especially when treated with estrogen combined with certain synthetic progestins than those not expressing PGRMC1 (Ruan et al. 2012; Neubauer et al. 2013).

1.3.4 ER status and potential PGRMC1 functional model

Estrogen receptor (ER) status is a critical biomarker in breast cancer, not only because it is an indicator of prognosis of breast cancer, but also because ER is the target of tamoxifen and similar drugs. In our previous studies, Neubauer and colleagues suggested that PGRMC1 correlates with ER status (Neubauer et al. 2008). ER-negative tumors have elevated levels of PGRMC1, whereas PGRMC1 was phosphorylated in ER-positive tumors.

There are limited data concerning PGRMC1-activated signaling pathways. PGRMC1 has a role in regulating protein kinase-associated signaling in which PGRMC1 increases Akt activation and IkB phosphorylation leading to NFkB activation (Hand and Craven. 2003). PGRMC1 is a protein of 194 amino acids (21.5 kDa) with target sequences for two Src homology 2 (SH2), a Src

homology 3 (SH3) (Cahill. 2007), a tyrosine kinase site, and two acidophilic kinase sites, such as casein kinase 2 (CK2) (Table 2). This protein functions as a signaling adaptor molecule involved in membrane trafficking, and that the activity may be kinase- and/or ligand-regulated.

Unlike the traditional PRs, PGRMC1 does not bind directly to progesterone (Min et al. 2005), but rather binds to binding partners, such as P450. PGRMC1 shares key structural motifs with cytochrome b_5 (Mifsud and Bateman. 2002), a heme binding protein that activates cytochrome P450 proteins (Schenkman and Jansson. 2003). In ER-negative tumors, PGRMC1 binds and activates P450 proteins (Min et al. 2005), which metabolize drugs, hormones and lipids and produces a metabolite or by-product that triggers Akt phosphorylation. P450-mediated oxidative damage is a candidate mechanism for tumor formation (Min et al. 2005). And PGRMC1 promotes cell death in cancer cells after oxidative damage (Hand and Craven. 2003), possibly due to its activation of P450 proteins, activates the pro-survival protein kinase Akt. Akt is phosphorylated by the PDK1 protein kinase, and there is a putative PDK1 binding region on PGRMC1 (Cahill. 2007). On the other hand, PGRMC1 may also be able to bind directly to an Akt activator (Cahill. 2007). Because PGRMC1 has several potential binding sites for interacting proteins, it might act as a type of adaptor protein, providing docking sites for proteins that activate Akt, such as PDK1. Sites like S56 and S180 are required to activate Akt after oxidative damage in a PGRMC1-overexpressing cell line.

PGRMC1 contains several sites for phosphorylation and is phosphorylated in ER-positive tumors, suggesting that there is an ER-regulated kinase that phosphorylates PGRMC1. In the CK2-phosphorylated state, one or more of the N-terminal SH3 target motif and C-terminal SH2 target motif may be phosphorylated by an acidophilic kinase such as CK2, and do not interact with other proteins. However, the relevant kinase and the possible mechanism of ER regulating PGRMC1 to influence breast cancer progress remain unclear.

1.4 Aims of the following work

Aim of the first part of the present work is to determine the influence of modification of different PGRMC1 phosphorylation sites together with various preparations of estrogens and progestogens on the proliferation of breast cancer cell lines. Moreover, the main focus of the second part is on identifying the potential ER-regulated kinase that participates in the phosphorylation of PGRMC1. Furthermore, the transcription level of an ER reporter gene is evaluated, to prove the involvement of a cross-talk between PGRMC1 and ER in estrogen/progestogen-regulated breast cancers.

Table 2. Protein motifs predicted for PGRMC1 (SwissProt entry O00264) using the Scansite cell signaling interactions prediction "MotifScan" module (http://scansite.mit.edu/motifscan id.phtml) under medium stringency. (Cahill. 2007)

Position	Motif predicted	Consensus type	Sequence
S56[a]	Acidophilic S/T Kinase	CK2-P	QPAASGDSDDDEPPP
P62[a]	SH3 target sequence	Crk/Grb2/Abl/Src	DSDDDEPPPLPRLKR
P108	Kinase binding	ERK1 binding	KGRKFYGPEGPYGVF
Y112	Tyr-Kinase	Lck/Abl-p	FYGPEGPYGVFAGRD
Y138	SH2 target sequence	Shc	KEALKDEYDDLSDLT
T160	Kinase binding	PDK1 binding	SDWESQFTFKYHHVG
Y179[a]	SH2 target sequence	Fgr/SHIP	EGEEPTVYSDEEEPK
S180	Acidophilic S/T Kinase	CK2-P	GEEPTVYSDEEEPKD

Bold underlined amino acids represent those indicated in the 'Position' column, at the centre of each predicted motif.
[a]Motifs that were also predicted under high stringency "MotifScan" settings.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Table 3: Estrogens and Progestins

Name	Abbreviations	Company
Estrone	E1	Sigma (Munich, Germany)
Estradiol	E2	Sigma (Munich, Germany)
Estriol	E3	Sigma (Munich, Germany)
Estetrol	E4	Sigma (Munich, Germany)
Ethinyl estradiol	EE	Schering (Berlin, Germany)
Equilin	Eq	Steraloids (Newport, Island)
17α-Dihydroequilin	DHEq	Steraloids (Newport, Island)
Progesterone	P4	Sigma (Munich, Germany)
Norethisterone acetate	NET	Sigma (Munich, Germany)
Medroxyprogesterone acetate	MPA	Sigma (Munich, Germany)
Levonorgestrel	LNG	Sigma (Munich, Germany)
Chlormadinone acetate	CMA	Sigma (Munich, Germany)
Desogestrel	DSG	Sigma (Munich, Germany)

Dienogest	DNG	Schering (Berlin, Germany)
Drospirenone	DRSP	Schering (Berlin, Germany)
Dydrogesterone	DYD	LGM Pharma (Boca Raton, USA)
Nomegestrolacetate	NOM	LGM Pharma (Boca Raton, USA)
Cyproterone acetate	CYA	Schering (Berlin, Germany)

The compounds were dissolved in ethanol to give a concentration of 10^{-2} M and were stored as concentrated stock solutions at -20°C.

Table 4: Inhibitors

Name	**Function**	**Company**
TBCA	Casein Kinase II Inhibitor III	Calbiochem (Darmstadt, Germany)
Fulvestrant	Estrogen receptor antagonist	Sigma (Munich, Germany)
Tamoxifen	Selective estrogen receptor modulator	Sigma (Munich, Germany)
AG205	PGRMC1 inhibitor	Timtec, Inc. (Newark, USA)

The compounds were dissolved in DMSO to give a concentration of 10^{-2} M and were stored as concentrated stock solutions at -20°C.

The growth factors Epidermal Growth Factor (EGF), Fibroblast Growth Factor-Basic (bFGF) and Insulin-like Growth Factor (IGF-I) were purchased from Sigma Chemicals. The compounds were reconstituted according to the manufacturer's instructions stated on the package insert and were stored in aliquots at -20°C.

2.1.2 Cells and cell culture

MCF-7

MCF-7, an ER+ and PR+ invasive breast ductal carcinoma cell line isolated in 1970 from a 69-year-old Caucasian woman, was purchased from American Type Culture Collection (ATCC). MCF-7 cells stably transfected with PGRMC1 and mutants, respectively, were established. Expression plasmid pcDNA3.1 containing hemagglutinin (HA)-tagged mPR (PGRMC1) wild-type or HA-tagged mutants S56A, S180A, S56A/S180A, were transfected into MCF-7 breast cancer cells (Table5). Stable transfection was verified by PCR using chromosomal DNA and primers spanning intron 1 to distinguish integrated PGRMC1 cDNA from the chromosomal sequence. The sequences of the primers were 5'-CTGCTGCATGAGATTTTCACG-3' hybridizing to nucleotides 71–91 of PGRMC1 open reading frame and 5'-GCATAGTCCGGGACGTCATA-3' hybridizing to the sequence coding for the HA tag. PCR products were sequenced.

Untransfected cells were routinely cultured in RPMI-1640 medium containing 10% (v/v) heat inactivated fetal calf serum (FCS), 25mM HEPES and 1% penicillin/streptomycin at 37°C in a humid 5% CO_2 atmosphere. Transfected cells were also maintained in the same condition but with an additional 100 µg/mL hygromycin B, purchased from Invitrogen, Karlsruhe, Germany. Cells were fed every three to four days. Cultures were split weekly at a ratio of 1:3 to 1:4 after treatment with trypsin (0.04%)-EDTA (0.03%) for 5 minutes followed by trypsin neutralisation with medium, both purchased from Gibco, and centrifugation at 1200rpm for 5 minutes. The cell pellet was resuspended in the appropriate growth

medium for subculture or assay work.

Table 5: MCF-7 and mutant cell lines

Name	Mutant	Sequence
MCF-7	Untransfected	
Mut A	S56A	serine-56 to alanine: AGC → GCC
Mut B	S180A	serine-180 to alanine: TCA → GCA
Mut C	S56A/S180A	serine-56 to alanine: AGC → GCC serine-180 to alanine: TCA → GCA
WT-12	PGRMC1 wild-type	
EVC	Empty vector control	

T47D

T47D, a human ER+ primary breast cancer cell line, was purchased from American Type Culture Collection (ATCC). T47d cells were stably transfected with expression vector pc-DNA3.1 containing hemagglutinin-tagged (3HA) PGRMC1 as described below.

Untransfected cells were routinely cultured in RPMI-1640 medium containing 10% (v/v) heat inactivated fetal calf serum (FCS), 25mM HEPES, 1mM sodium pyruvate, and 1% penicillin /streptomycin at 37°C in a humid 5% CO_2 atmosphere. Transfected cells were also maintained in the same condition but with additional 40µg/mL hygromycin B, purchased from Invitrogen, Karlsruhe, Germany. Cells were fed every three to four days. Cultures were split weekly at a ratio of 1:3 to 1:4

after treatment with trypsin (0.04%)-EDTA (0.03%) for 5 minutes followed by trypsin neutralization with medium, both purchased from Gibco, and centrifugation at 1200rpm for 5 minutes. The cell pellet was resuspended in the appropriate growth medium for subculture or assay work.

Table 6: T47D and mutant cell lines

Name	Mutant
T47D	Untransfected
WT-3	Wild-type
EVC	Empty vector control

2.2 Methods

2.2.1 Transfection of T47D cells

T47D cells were stably transfected with expression vector pc-DNA3.1 containing hemagglutinin-tagged (3HA) PGRMC1 using lipofectamine^{TM} 2000 (Invitrogen, Karlsruhe, Germany), in accordance with the manufacturer's recommendation. A total of 5×10^5 cells were transfected and plated with RPMI medium for 24h. Then, the medium was changed to RPMI complete medium containing hygromycin B. Cells were cultured for 2 weeks for selection of stable integration events. Transfection rates were measured by cotransfection of a GFP-expressing plasmid and immune fluorescence analysis. After 2 weeks single colonies had formed and limiting dilutions were performed three times to select for colonies grown from a single cell.

Stable transfection was verified by PCR using chromosomal DNA and primers spanning intron 1 to distinguish integrated PGRMC1 cDNA from the chromosomal

sequence. The sequences of the primers were 5'-CTGCTGCATGAGATTTTCACG-3' hybridizing to nucleotides 71–91 of PGRMC1 open reading frame and 5'-GCATAGTCCGGGACGTCATA-3' hybridizing to the sequence coding for the HA tag. PCR products were sequenced. And the efficiency of the transfection was determined using the Immune fluorescence analysis as described below.

2.2.2 Immune fluorescence analysis

The immune cytochemical fluorescence analysis of cytospins was performed using a humidified chamber. For preparation of cytospins, 5×10^4 cells were resuspended in 500mL PBS spun onto slides using Cytospin 2 centrifuge (Shandon, Waltham, MA, USA) at 1000 rpm for 5 min. Then, the cytospins were dried overnight at room temperature and stored at -20°C until further use.

Table 7: Antibodies used for Immune fluorescence analysis

	Name	Dilution	Company
Primary antibody	Anti-Cytokeratin Pan FITC conjugated (C11)	1:400	Santa Cruz, CA, USA
Primary antibody	Rabbit anti-HA (Y11)	1:100	Santa Cruz, CA, USA
Secondary antibody	Goat anti-mouse AlexFluor 594	1:100	Invitrogen, Karlsruhe, Germany

Cytospins were investigated by fixing the cells in 50ml 0.05% formalin in PBS for 90s at room temperature, followed by a wash step for 3min in PBS. Then the slides were placed on ice and permeabilized using 50ml 0.1% Triton X-100 in PBS for 15min. Afterwards, the cytospins were washed three times for 3min with PBS. Blocking of unspecific antibody binding sites was performed with serum of the

species, which was the source of the secondary antibody – goat in our case. To that aim, goat serum was diluted 1: 10 in Antibody Diluent (Dako Cytomation, Glostrup, Denmark) and 50ml of this diluted serum was pipetted onto the slides and incubated for 30min at room temperature. Then the block was removed and incubated with the first antibody for 60min at room temperature, followed by washing the cytospins in PBS for 3min. Secondary antibodies were incubated for 30min at room temperature. Finally, the cytospins were washed twice for 3min with PBS and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, USA).

2.2.3 MTT assays

Proliferation of treated cells was also determined by measuring the enzymatic cleavage of the tetrazolium ring of the yellow tetrazolium salt 3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) by mitochondrial dehydrogenase, resulting in a blue, water-insoluble formazane salt. The MTT salt was dissolved in RPMI 1640 medium without phenol red to a concentration of 1mg/ml filtered sterile and further diluted 1: 4 with RPMI 1640 medium without phenol red.

Cells were trypsinized and counted using a Neubauer Cell Counter (Hemocytometer). 90μL of the marker ‘tryphan blue’ and 10μL of cell suspension were mixed thoroughly in a well in a 96-well plate, kept specifically for this purpose. 10μL of the marker/cell suspension mixture was placed on either side of a 0.1mm-deep chamber in the Hemocytometer and observed under a microscope. The number of cells in a defined area was counted and the cell concentration derived from the count.

Cells were seeded with 5000 cells per well in a 96-well plate. After 24h of incubation, the culture medium was changed to medium with stripped FCS. Estrogens alone or in combination with different progestins in a continuous

combined of sequential regimen were then added in to the medium. After 6 days of stimulation, medium was decanted from the 96-well plate used to incubate the cells. Then 100ml MTT solution was added to each well and incubated for 3h at 37°C. Crystallized formazane salt was centrifuged for 10 min with 400g and supernatant was discarded. The resulting salt was solubilized in 0.1ml sterile DMSO added into each well and the plate was shaken for 7min at room temperature. Analysis was performed using an ELISA-reader at a wavelength of 550nm. The resulting extinction is proportional to the amount of cells present in the well. (Diary plans in table 8, 9, and 10).

Table 8: Normal stimulation

Day 0	Cell seeding
Day 1	Change to medium with stripped FCS
Day 3	Change to estrogens or progestins in Medium with stripped FCS
Day 5	Change to estrogens or progestins in Medium with stripped FCS
Day 7	Change to estrogens or progestins in Medium with stripped FCS
Day 9	MTT assay

Table 9: Continuous combined stimulation

Day 0	Cell seeding
Day 1	Change to medium with stripped FCS
Day 3	Change to estrogens plus progestins in Medium with stripped FCS
Day 6	Change to estrogens plus progestins in Medium with stripped FCS
Day 9	MTT assay

Table 10: Sequential stimulation

Day 0	Cell seeding
Day 1	Change to medium with stripped FCS
Day 3	Change to estrogens in Medium with stripped FCS
Day 6	Change to estrogens plus progestins in Medium with stripped FCS
Day 9	MTT assay

2.2.4 Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in M-PER mammalian protein extraction reagent containing Halt Protease Inhibitor Cocktail according to the manufacturer's protocol (both from Pierce, Rockford, IL, USA). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). In total, 25µg of protein extract was loaded per lane onto a 10% polyacrylamide gel and separated by electrophoresis. The gel was blotted onto a

Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) at 15V for 90min using a semi-dry blot system. The membrane was blocked for 2h at room temperature using 5% dried low fat milk powder dissolved in TBST buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20). Then, the first antibody was incubated overnight at 48°C. After washing three times with TBST, the second antibody was incubated for 2.5h at room temperature. Chemiluminescence was generated using the ECL Western Blotting Analysis System (Amersham). The signals were measured and quantified with Lumi-Imager and LumiAnalyst 3.1 software (Boehringer, Mannheim, Germany).

Table 11: Antibodies used for western blot analysis

	Name	Dilution	Company
Primary antibody	Phospho-PGRMC1-specific mouse monoclonal antibody 3G11A2	1:200	
Primary antibody	Anti-Actin antibody produced in rabbit	1:1000	Santa Cruz, CA, USA
Secondary antibody	Goat anti-mouse IgG-HRP	1:1000	Santa Cruz, CA, USA
Secondary antibody	Goat anti-rabbit IgG-HRP	1:1000	Santa Cruz, CA, USA

2.2.5 Extraction of RNA and cDNA synthesis

Expression of TFF1 was determined with real-time polymerase chain reaction (RT-PCR). Messenger RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -20°C until cDNA synthesis using a standard protocol. Briefly, 1µg total RNA was incubated for

30min with 2µl DNase I (Amplification Grade DNase I Kit; Sigma, St. Louis, MO, USA) at room temperature. After thermal inactivation of DNase I at 70°C for 10min, cDNA synthesis was done using random and oligo(dT) primers and Superscript II RNase H reverse transcriptase (Invitrogen). Reverse transcriptase reaction was performed at 42°C for 50 min and 72°C for 15min.To check cDNA quality control, RT-PCR was performed for the pyruvate dehydrogenase β- subunit (PDH) to verify absence of genomic DNA.

2.2.6 Quantitative real-time PCR

Quantitative RT-PCR (qRT-PCR) was performed with the LightCycler System (Roche) and SYBR Green incorporation according to the manufacturer's instructions. At the end of the PCR, the reaction melting curve was determined to check for the purity of the PCR reaction.

For efficiency-corrected relative quantification of gene expression, triplicate reactions were set up. Expression of PDH was used to normalize the relative regulation of candidate genes employing the efficiency corrected equation. Efficiency of every single PCR reaction was determined by using Rest-384 software.

Table 12: Primers used for qRT-PCR

Name	**Company**
Hs_TFF1_1_SG	Qiagen, (Hilden, Germany)
PDH classic	Invitrogen (Karlsruhe, Germany)

2.3 Statistics

All proliferation experiments were done in triplicates and were repeated at least three times, with each experiment yielding essentially identical results. Statistical analysis was done by ANOVA with the logarithmated values followed by Dunnett's procedure from triplicates of at least three independent experiments. The overall alpha level was set at 0.05.

Most of the qRT-PCR experiments were done in triplicate once, due to the lack of time, while some of them were repeated twice. From these values, means were calculated alone with standard deviation (S.D.). Statistical analysis was performed using Rest-384 software.

3. RESULTS

3.1 Transfection of T47D cells with PGRMC1 coding expression plasmids

Transfection rates measured by cotransfection of a GFP expressing plasmid were around 40-50%. After limiting dilution PGRMC1-3HA transfected T47D cells were prepared for cytospins and immune fluorescence analysis was performed using anti-HA antibody indicating that the transfected cell line was almost devoid of untransfected MCF-7 cells (Fig. 2).

Western Blot analysis for the PGRMC1-3HA fusion protein resulted in a single band at approximately 30 kDa, which is the predicted size of 28kDa for PGRMC1 plus approx. 3kDa for the three HA tags. The untransfected T47D produced only a very faint signal at 28 kDa indicating a weak intrinsic PGRMC1 expression. Similar signal intensities for the housekeeping protein actin (approx. 42kDa) indicate loading of equal amounts of total protein.

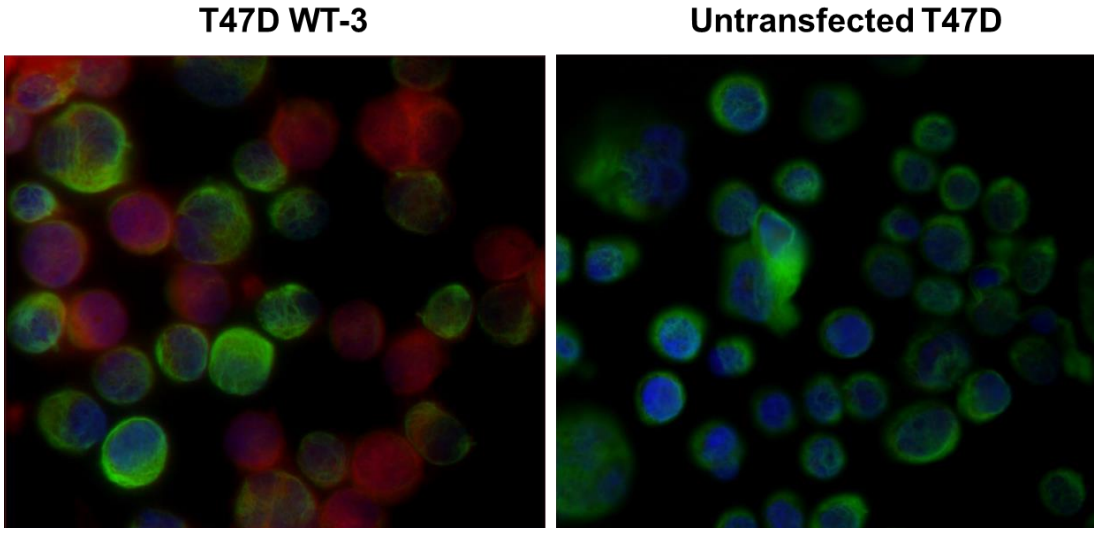

Figure 2. Over-expression of PGRMC1 in T47D cells. Immunofluorescence analysis of cytospin preparations from T47D (left) and T47D/PGRMC1-3HA (right) cells. PGRMC1-3HA is detected with anti-HA antibody conjugated with Alexa 594. Cell nuclei are stained with DAPI. Magnification: 40×.

PCR products from chromosomal DNA of the transfected cell lines provided the expected signal in the agarose gel. Sequencing of PCR products confirmed the PGRMC1 wild type sequence. At the end of the experiments the high purity of the T47D/PGRMC1-3HA cells was validated by immune fluorescence analysis of cytospins and by sequencing PCR products. These results indicated that T47D/ PGRMC1-3HA cells are highly pure, overexpress PGRMC1 wild type protein and can be used for functional assays.

MTT assay was performed to in investigated potential differences in the basal proliferation of T47D and T47D/PGRMC1-3HA (WT-3) cells. The latter showed a significant higher proliferation after day 6.

3.2 MTT assay

3.2.1 Proliferative effect of progestogens or estrogens alone on MCF-7 and T47D cells

First, a fixed concentration of progesterone (P4) or different synthetic progestins (10^{-6} M, 10^{-7} M) was incubated with both cell lines. The proliferation rate was measured after 6 days (Fig. 3, 4).

All MCF-7 cell lines except MutC were able to significantly respond to different progestins (Fig. 3). P4 and NOM triggered only the proliferation of MutA and EVC, but in a relatively low level. In comparison to all other synthetic progestins tested, NET had the strongest proliferative effect especially on MutB. MutC did not respond to any progestins. And the proliferation effect of EVC was also weaker than MutA, MutB and WT-12.

P4 and all progestins were able to significantly stimulate the proliferation of T47D EVC and untransfected T47D cells (Fig. 4). WT-3 did not respond to P4 and NOM at the concentrations of either 10^{-6} M or 10^{-7} M. Also, at the concentration of 10^{-7}M, MPA, LNG, CYP did not have any effect on WT-3 cells. EVC and T47D had similar

reactions on different progestins, while the proliferative rate of WT-3 was obviously higher than the other T47D cell lines.

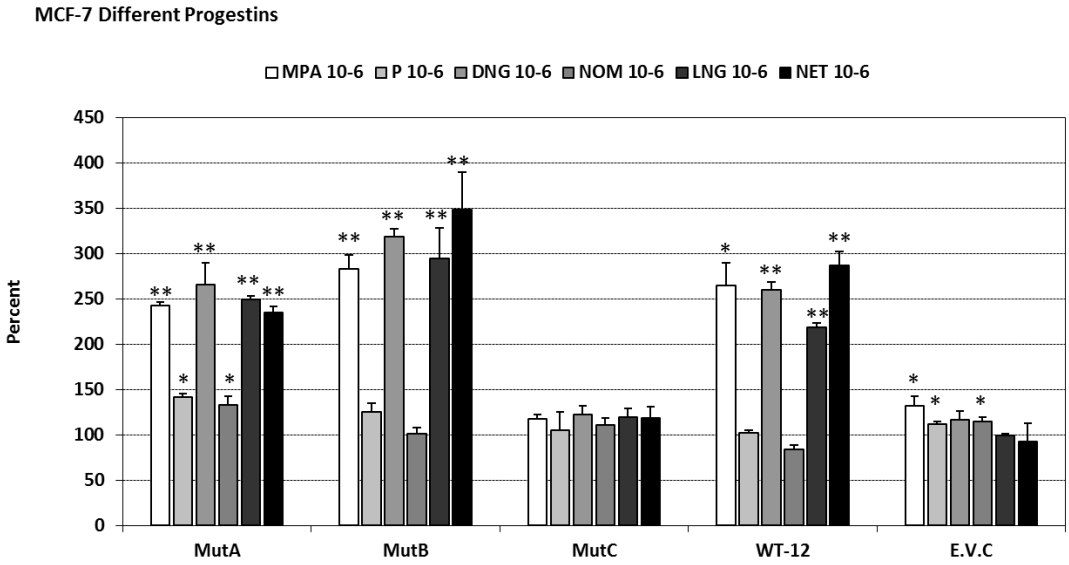

Figure 3. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with MPA, P4, DNG, NOM, LNG, and NET each at concentration of 10^{-6}M. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. controls).

With regard to the proliferative effect triggered by different estrogens, MCF-7 and T47D cell lines were incubated with E1, E2, E3, E4, Eq, DHEq, and EE at concentrations of 10^{-12}M, 10^{-11}M and 10^{-10}M, respectively. The proliferation rate was measured after 6 days (Fig. 5, 6).

Dose-dependent effects on cell proliferation of estrogens except DHEq were observed (Fig. 5): for all the estrogens except DHEq at concentrations from of 10^{-12}M to 10^{-10}M with a maximal effect at 10^{-10}M. Almost no effects were observed in EVC and untransfected MCF-7 cells within the investigated concentration ranges

for all the estrogens used in this experiment. EE showed the strongest effect compared to all the other estrogens at all three concentrations.

The similar dose-dependent proliferative effects of estrogens on T47D cells were also detected (Fig. 6). However, unlike MCF-7 cells, T47D EVC and untransfected T47D cells were able to react to different estrogens. All the estrogens except DHEq increased the proliferation of T47D cells significantly, and would reach their maximal effect at 10^{-10} M, the highest concentration that we tested.

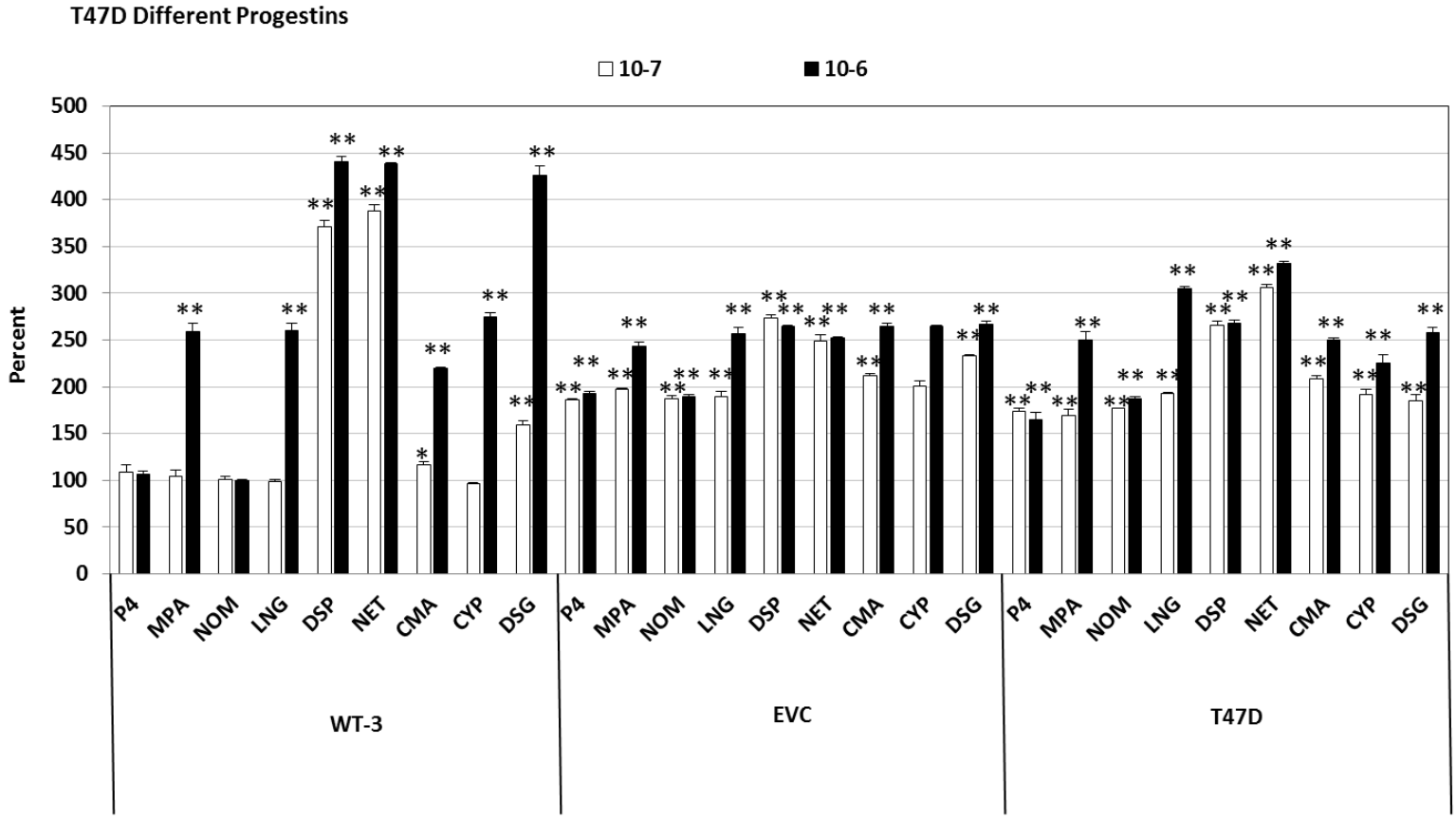

Figure 4. T47D WT-3, EVC, and untransfected T47D cells were incubated with P4, MPA, DNG, NOM, LNG, DSG, CMA, CYP and NET each at concentrations of 10^{-7}M and 10^{-6}M. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. controls).

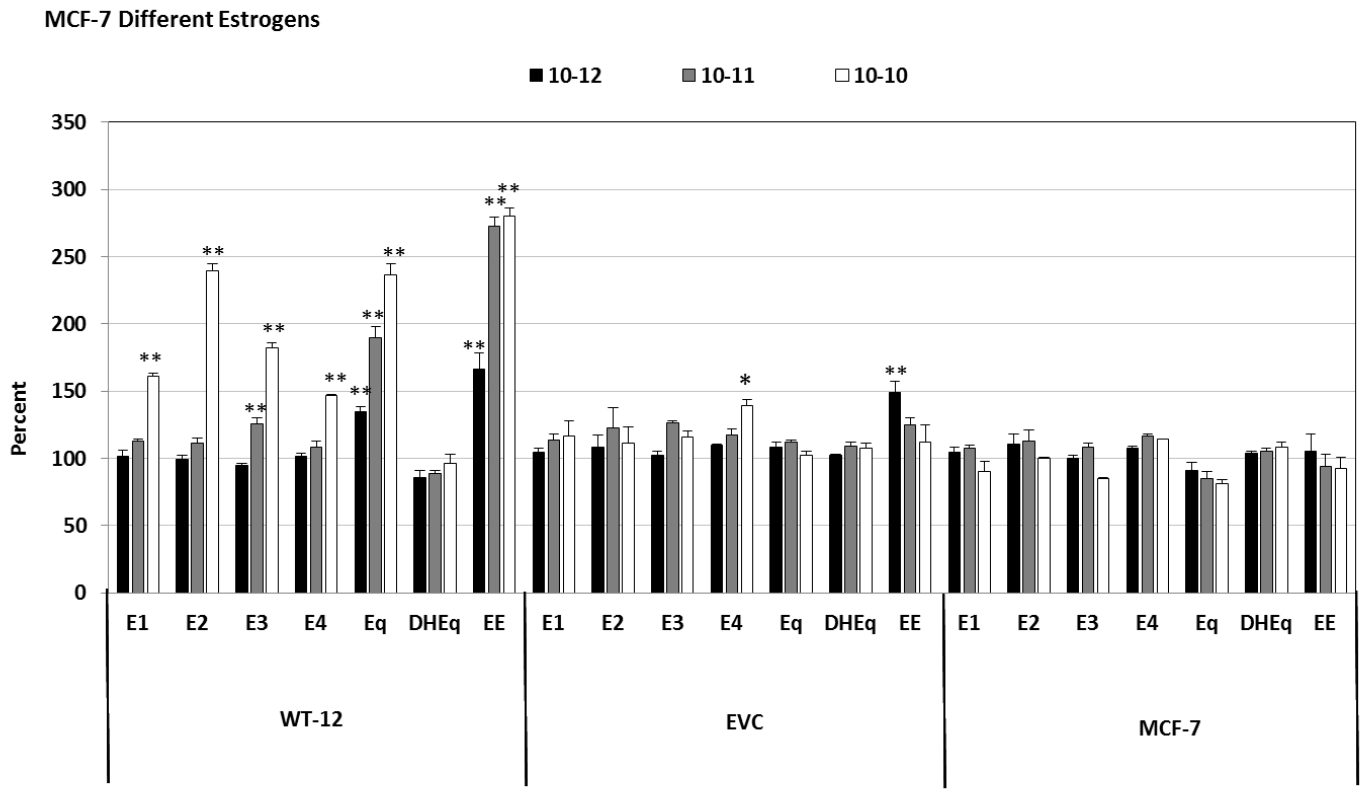

Figure 5. MCF-7 WT-12, EVC and untransfected MCF-7 cells were incubated with E1, E2, E3, E4, Eq, DHEq and EE each at concentration of 10^{-12}M, 10^{-11}M and 10^{-10}M. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. controls).

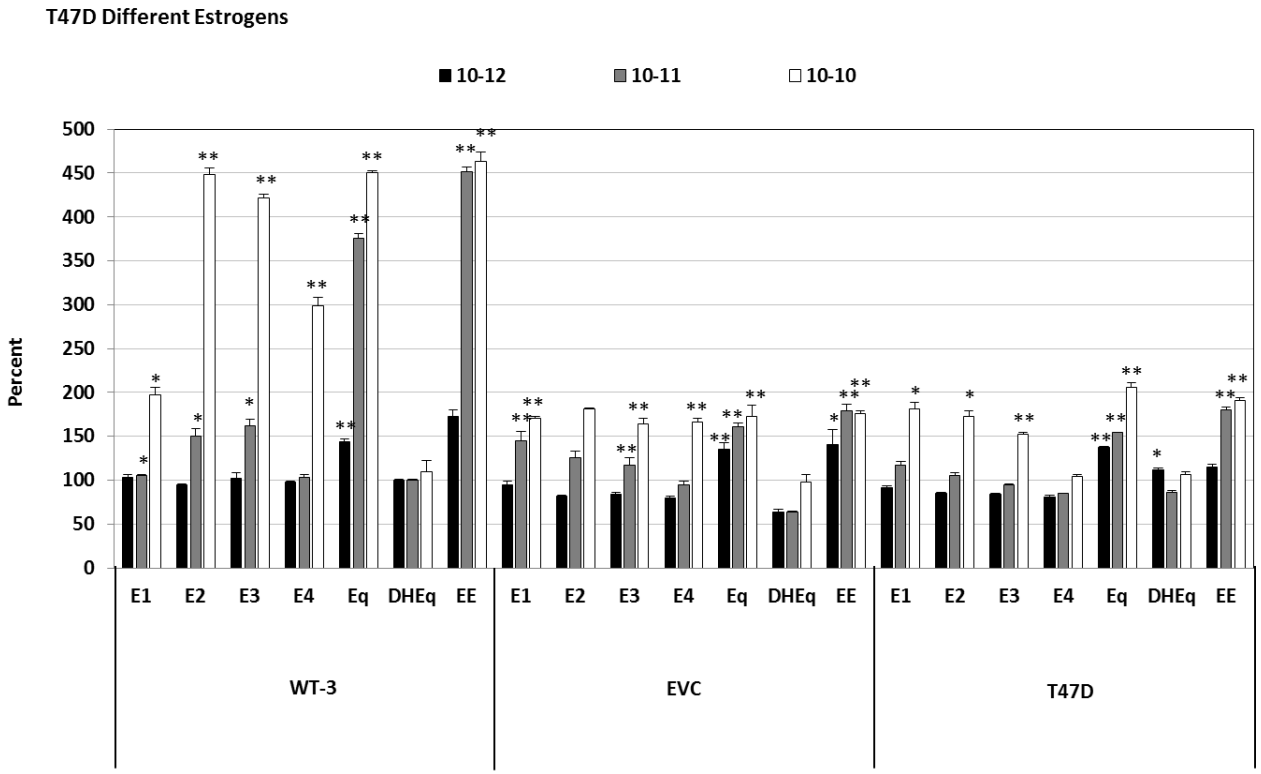

Figure 6. T47D WT-3, EVC, and untransfected T47D cells were incubated with E1, E2, E3, E4, Eq, DHEq and EE each at concentrations of 10^{-12}M, 10^{-11}M and 10^{-10}M. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. controls).

3.2.2 Proliferative effect of estrogens in combination with progestins on MCF-7 and T47D cells

To investigate the proliferative effect of MCF-7 and T47D cells after stimulation of estrogens in combination with progestins. Cells were incubated with estrogens alone as well as estrogens in addition with progestins in either a continuous combined or a sequential manner. By continuous combined manner, it means that cells were incubated 6 days with the combination of estrogens and progestins. And by sequential manner, cells were stimulated 3 days by estrogens alone, and by the combination of estrogens and progestins for the next 3 days. The proliferation rate was measured after 6 days (Fig. 7-12).

For MCF-7 cells and mutants, as can be seen in Figure 7, 8, NET alone at the concentration of 10^{-7} M elicited significant proliferation of MutA, MutB and WT-12, which is consistent with the results mentioned above. E2 alone at the concentrations of 10^{-12} M and 10^{-10} M elicited also a significant proliferative increase of MutA, MutB and WT-12 cells. And the effect was clearly dose-dependent. However, the increased proliferation of EVC was triggered only by E2 alone at a lower concentration range, 10^{-14} M and 10^{-12} M. Addition of NET (10^{-7} M) to E2 in both continuous combined and sequential manner altered the E2-induced proliferation significantly: for MutA at the E2 concentration of 10^{-14} M and 10^{-12} M continuously and at the concentration of 10^{-14} M sequentially, for MutB at the E2 concentration of 10^{-14} M continuously, and for WT-12 at the E2 concentration of 10^{-14} M and 10^{-12} M continuously and at the concentration of 10^{-14} M sequentially.

In a further experiment with MCF-7 WT-12 cells EE and E2 (10^{-10} M and 10^{-9} M) was continuously or sequentially added with CMA, CYP, DSP, LNG, and NET at the concentration of 10^{-6} M. As can be seen in Figure 9, 10, CMA and CYP alone had no significant effect on WT-12 cells, while DSP and NET had obviously strong

effect. EE and E2 in both concentrations elicited significant proliferation of WT-12, but the addition of some progestins increased these proliferative effects compared with estrogens alone: for CMA added in EE at the concentration of 10^{-9} M continuously, for CYP added in EE at the concentration of 10^{-9} M continuously and sequentially, for DSP added in EE at the concentration of 10^{-9} M continuously and at EE10^{-10} M, 10^{-9} M and E2 10^{-10} M sequentially, and for NET in all the combination that we tested.

For T47D cells, the proliferative effects of estrogen and progestins were also investigated. As shown in Figure 11, 12, the effects of 10^{-6} M NOM, 10^{-7} M DSP, 10^{-7} M and 10^{-6} M DSG, 10^{-7} M and 10^{-6} M NET, 10^{-7} M and 10^{-6} M CMA, and 10^{-7} M CYP on T47D WT-3 cells in the presence of E2 at the concentration of 10^{-10} M in the continuous combined manner significantly increased. Moreover, the proliferation of EVC was also significantly enhanced by the continuous combined E2 and NOM (10^{-7} M and 10^{-6} M), as well as by E2 and NET (10^{-7} M and 10^{-6} M). However, no significant difference of the effect of estrogen and progestin combination compared with estrogen alone could be found for T47D WT-3 and EVC cells in the sequential manner.

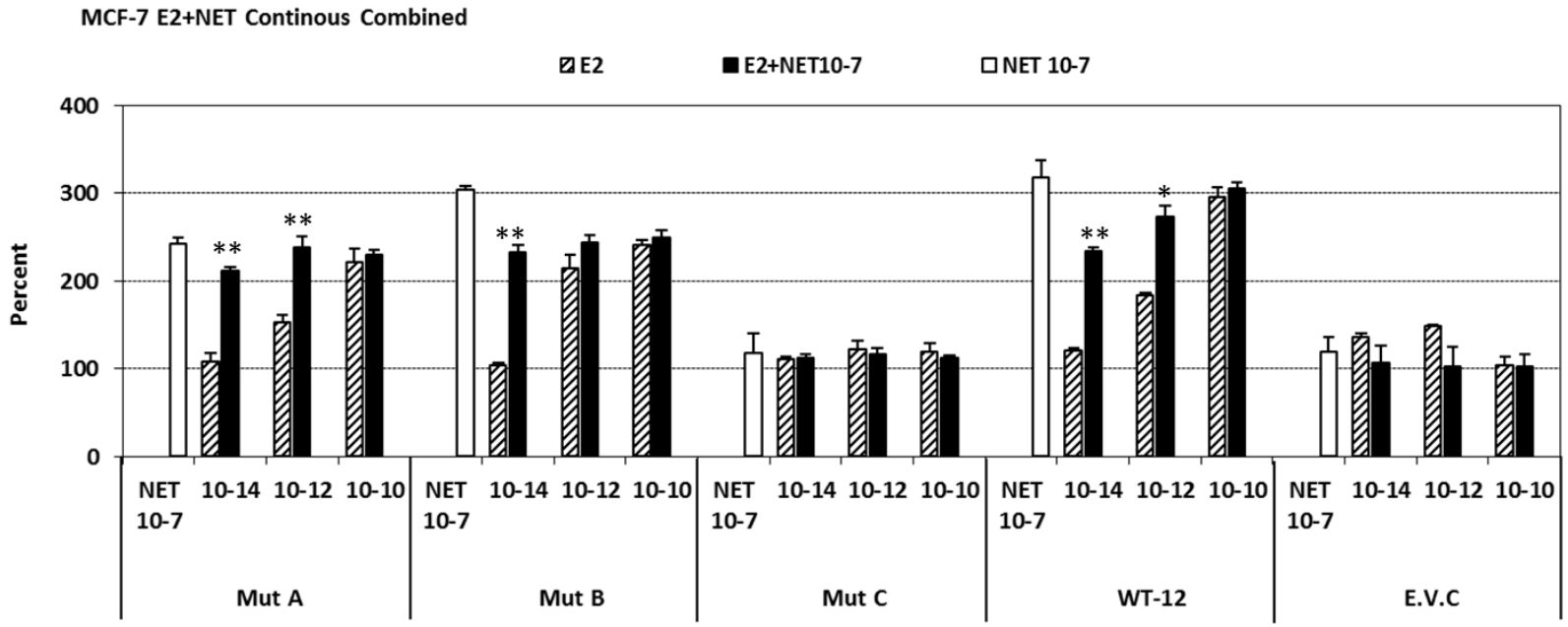

Figure 7. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with E2 (10^{-14}M, 10^{-12}M and 10^{-10}M) alone and in combination with NET (10^{-7}M) continuously. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

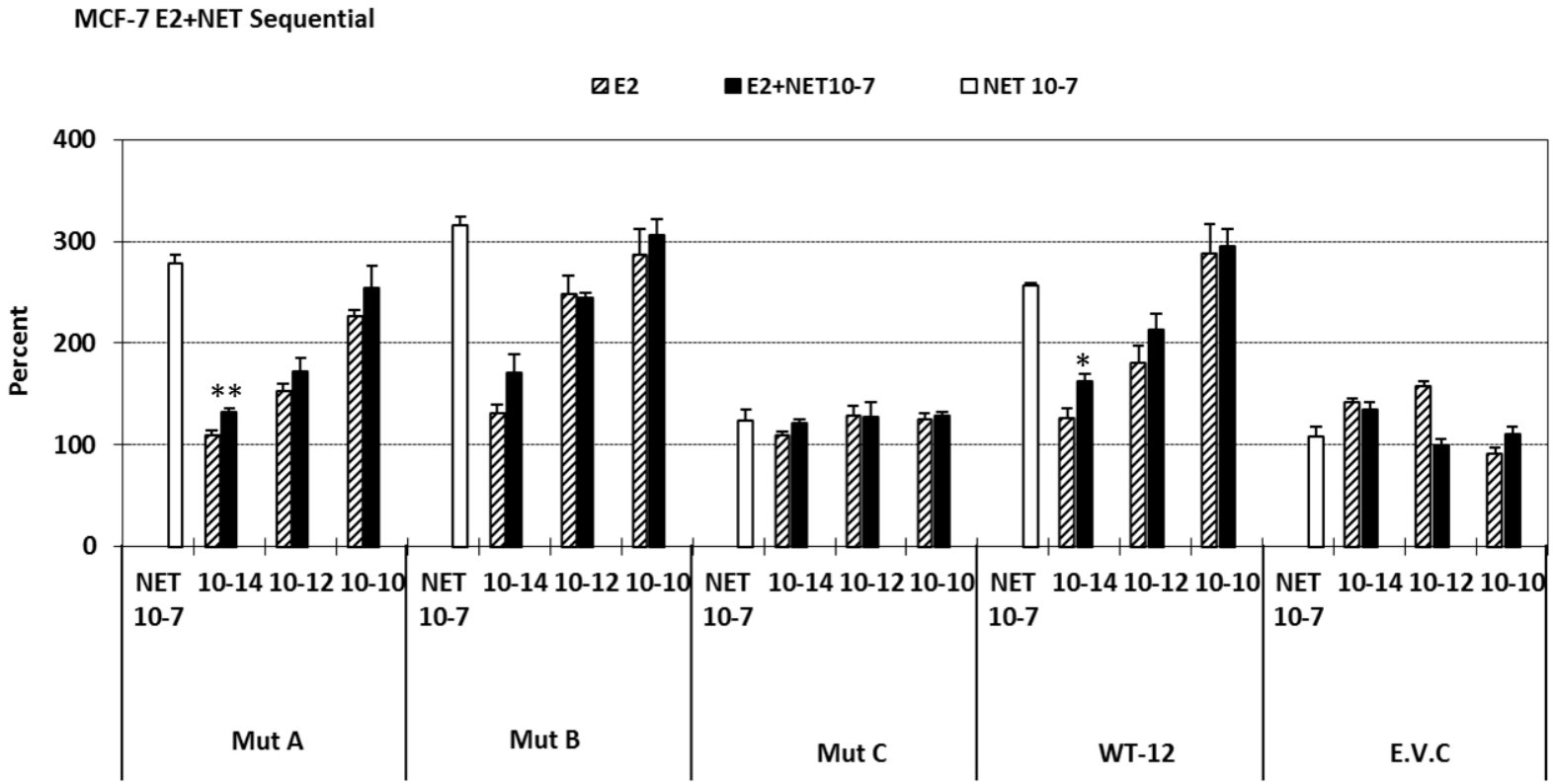

Figure 8. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with E2 (10^{-14}M, 10^{-12}M and 10^{-10}M) alone and in combination with NET (10^{-7}M) sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

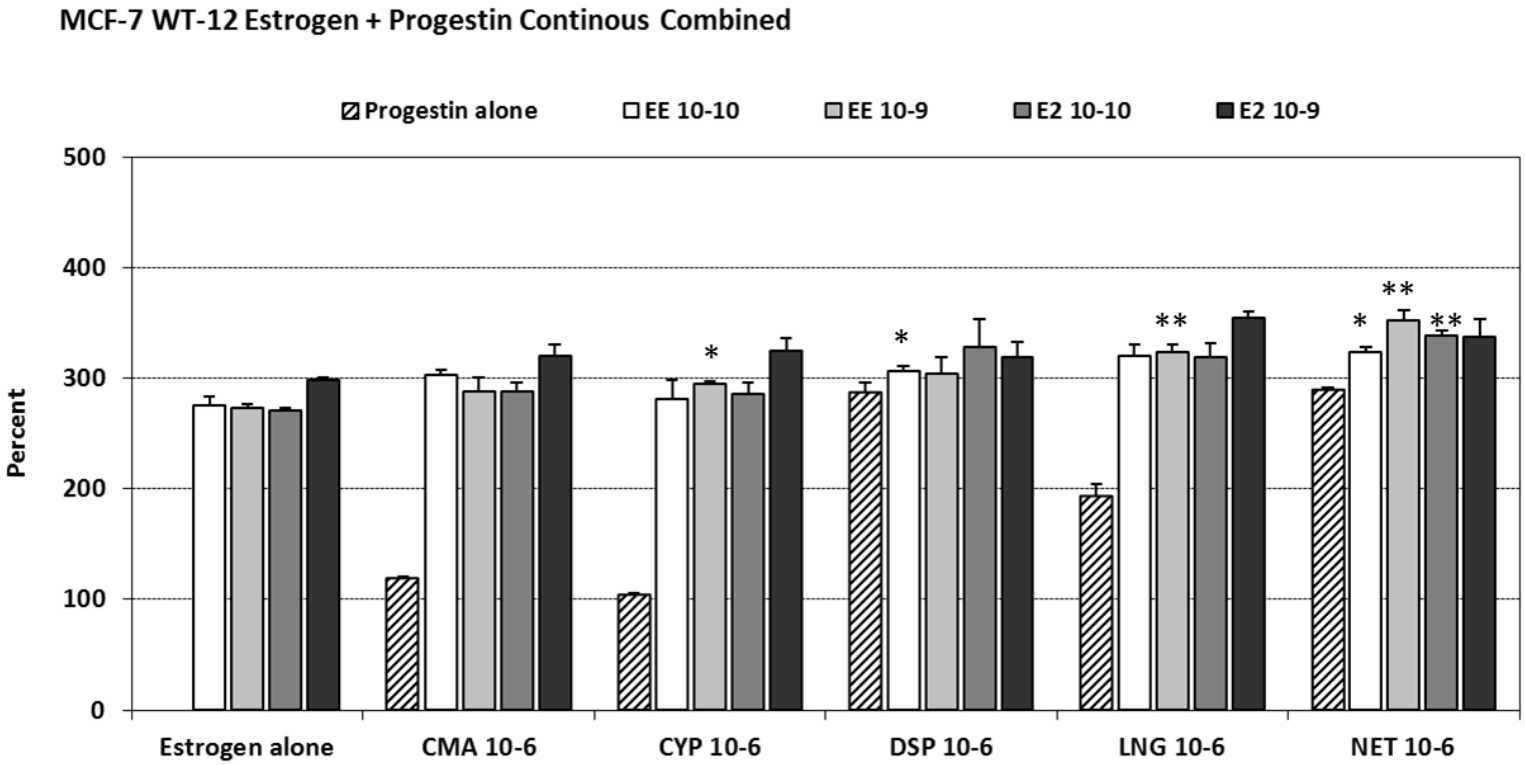

Figure 9. MCF-7 WT-12 cells were incubated with EE and E2 (10^{-10}M and 10^{-9}M) alone and in combination with various progestins (10^{-6}M) continuously. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

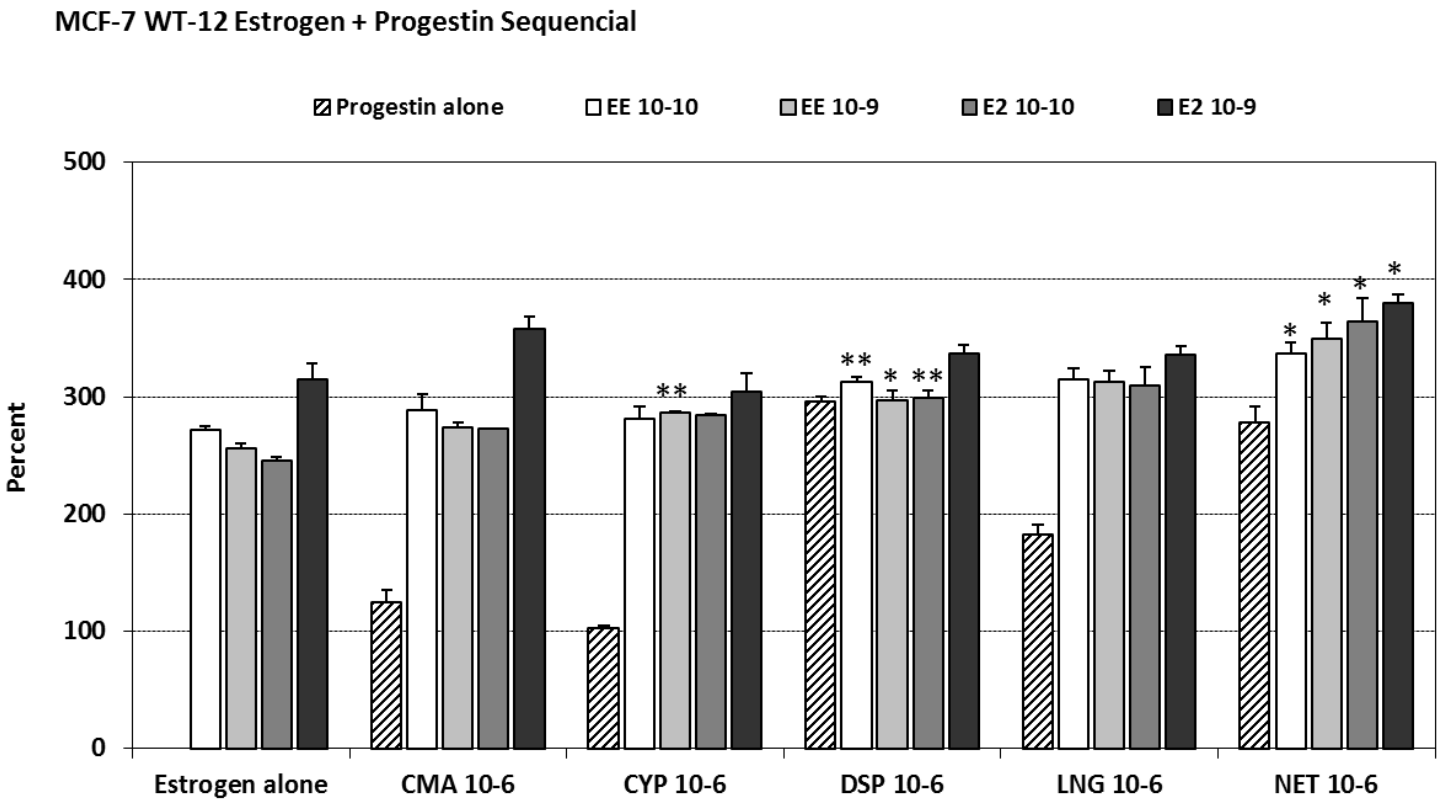

Figure 10. MCF-7 WT-12 cells were incubated with EE and E2 (10^{-10}M and 10^{-9}M) alone and in combination with various progestins (10^{-6}M) sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

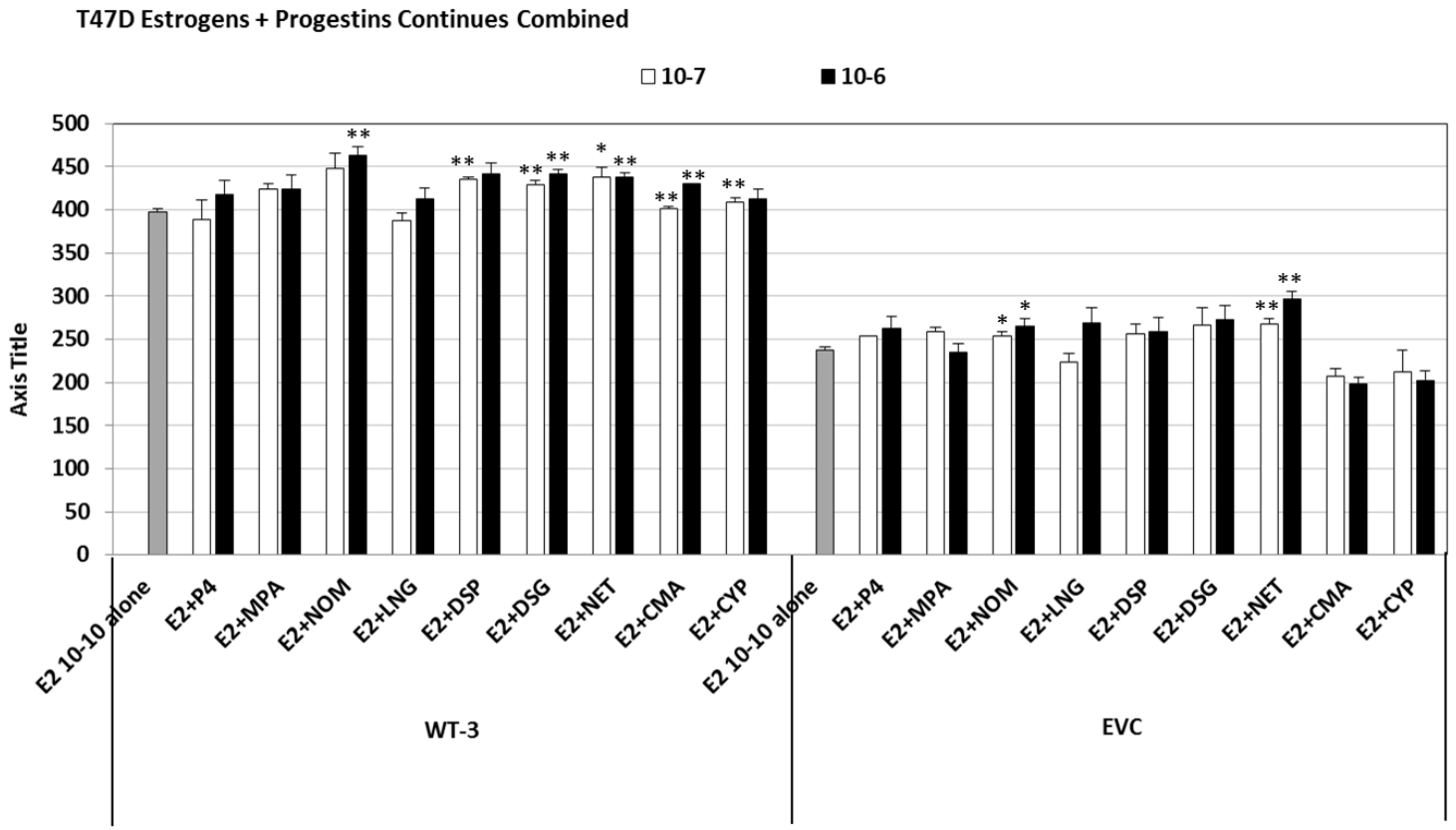

Figure 11. T47D WT-3 and EVC cells were incubated with E2 (10^{-10}M) alone and in combination with various progestins (10^{-7}M and 10^{-6}M) continuously. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

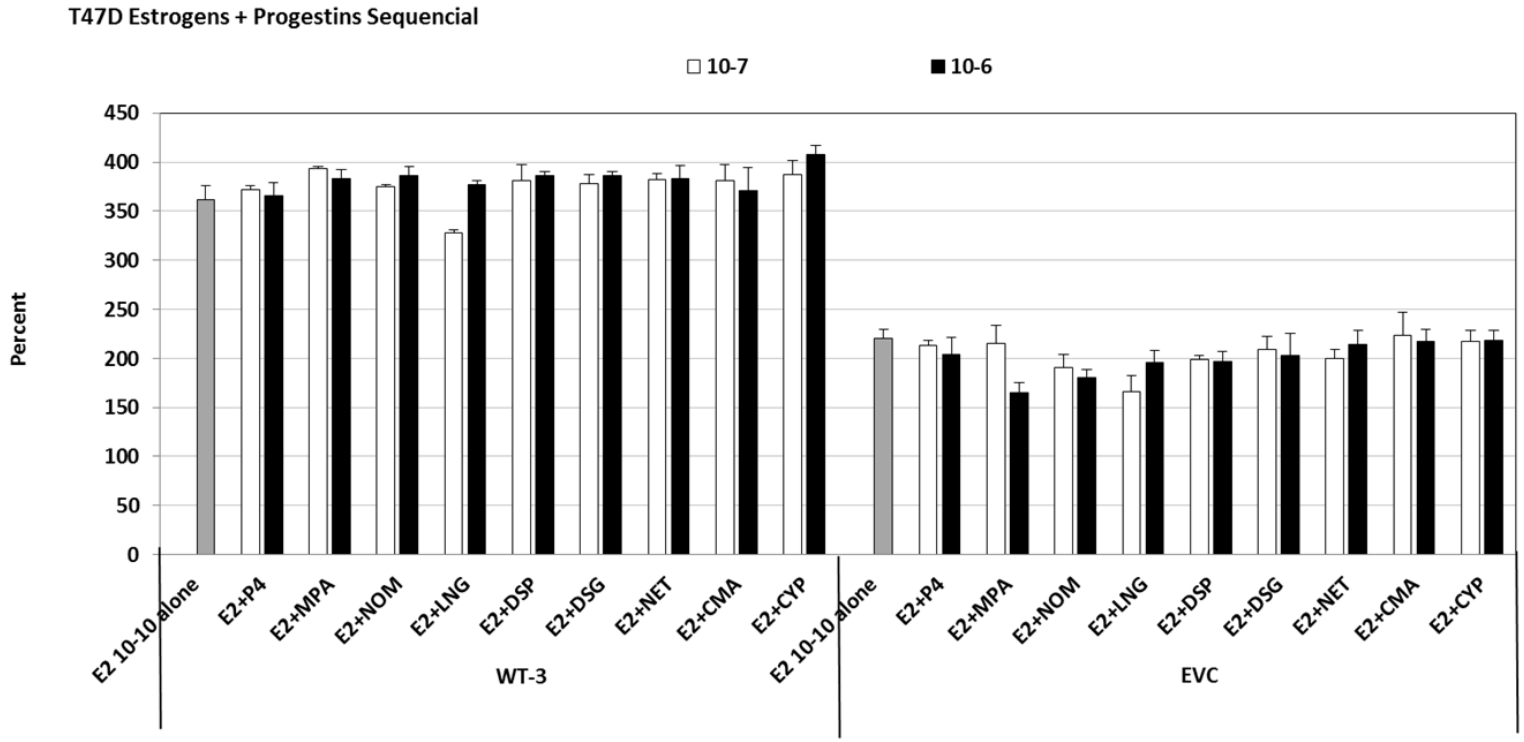

Figure 12. T47D WT-3 and EVC cells were incubated with E2 (10^{-10}M) alone and in combination with various progestins (10^{-7}M and 10^{-6}M) sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

3.2.3 Blocking effect of TBCA on the prolieferation of MCF-7 cells

First, NET or TBCA was used alone and in combination continuously or sequentially on MCF-7 WT-12 cells to determine the best manner. By continuous combined manner, it means that cells were incubated 6 days with NET and TBCA combination. And by sequential manner, cells were treated 3 days by TBCA alone, and by NET and TBCA combination for the next 3 days. The proliferation rate was measured after 6 days (Fig. 13).

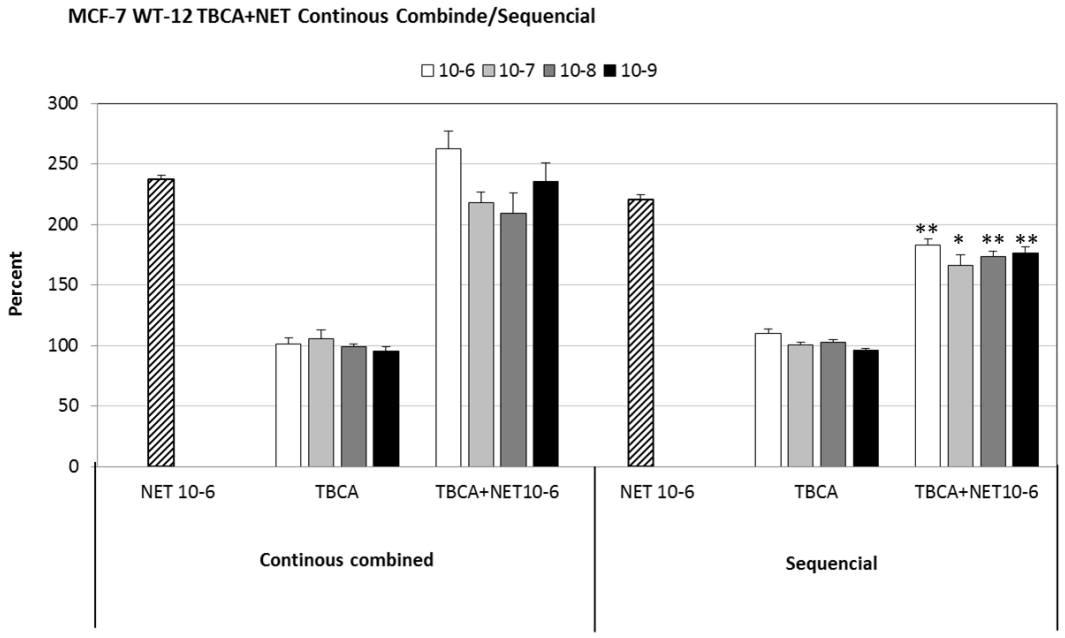

Figure 13. MCF-7 WT-12 cells were incubated with NET (10^{-6}M) and TBCA (10^{-6}M, 10^{-7}M, 10^{-8}M and 10^{-9}M) alone respectively, and in combination continuously or sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. NET alone).

TBCA alone showed no effect on the proliferation of WT-12 cells. And NET alone showed strong effect of increased cell proliferation. In the sequential manner, the proliferative effect of NET was significantly reduced compared with NET alone.

However, the reduction seemed not dose-dependent. The similar blocking effect was not observed in the continuous combined manner. Therefore, the sequential manner was chosen for further kinetic experiments.

To determine the best blocking concentration of TBCA, titration experiments were then performed. TBCA alone showed no effect on the proliferation of all MCF-7 cells. And NET and E2 alone showed consistently intensify of cell proliferation. As can be seen in Figure 14,15, the NET-induced effect could be reversed by the addition of TBCA: for Mut A at the concentration of TBCA 10^{-6} M and 10^{-4} M, for MutB at the concentration of TBCA 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M, for WT-12 at the concentration of TBCA 10^{-7} M, 10^{-6} M and 10^{-5} M, and for EVC at the concentration of TBCA 10^{-4} M. Also, the E2-induced effect could be reversed. Especially, the proliferation of WT-12 caused by E2 was significantly blocked by TBCA at all the concentrations that we tested. However, these blocking effects did not seem to be dose-dependent. Moreover, MutC still not reacted to any stimulation. For further experiments, 10^{-6} M was chosen.

Three parallel experiments were then performed to investigate the ability of TBCA on blocking the effect of NET and E2 at various concentrations. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were stimulated by NET, E2 and GF (EGF, FGF and IGF-I) at different concentrations. As shown in Figure 16, NET alone elicited the proliferation of MutA, MutB, WT-12, and EVC cells significantly, while TBCA alone had no effect, which is consistent with the results mentioned above. The combination of TBCA 10^{-6} M and NET 10^{-8} M, 10^{-6} M reduced significantly the proliferation rate of MutB and WT-12. As shown before, E2 at various concentrations enhanced the proliferation of MutA, MutB and WT-12 cell (Fig. 17). However, MutC and EVC seemed not sensitive to E2. Addition of TBCA (10^{-6} M) could reverse the effects of E2 on MutA at the E2 concentration 10^{-10} M, on MutB at 10^{-12} M and 10^{-10} M, and on WT-12 at all E2 concentrations that we tested. Figure

18 showed the results regarding GF. This experiment was designed to exclude the potential universal effect of TBCA. The results showed that GF could elicit increased cell proliferation of all MCF-7 cells, even for MutC cells. And the induction was not reversed by TBCA.

Finally, the combination of TBCA and three different progestins was tested. As shown in Figure 19-21, MPA, DNG and LNG were all able to significantly trigger the cell proliferation of MutA, MutB and WT-12 at a higher concentration range. No effect of TBCA alone was observed. TBCA reversed the effect of MPA as well as LNG at the concentration of 10^{-6}M on MutB cells significantly. And it also reversed the effect of DNG at the concentration of 10^{-6}M on MutA, MutB and WT-12 cells significantly.

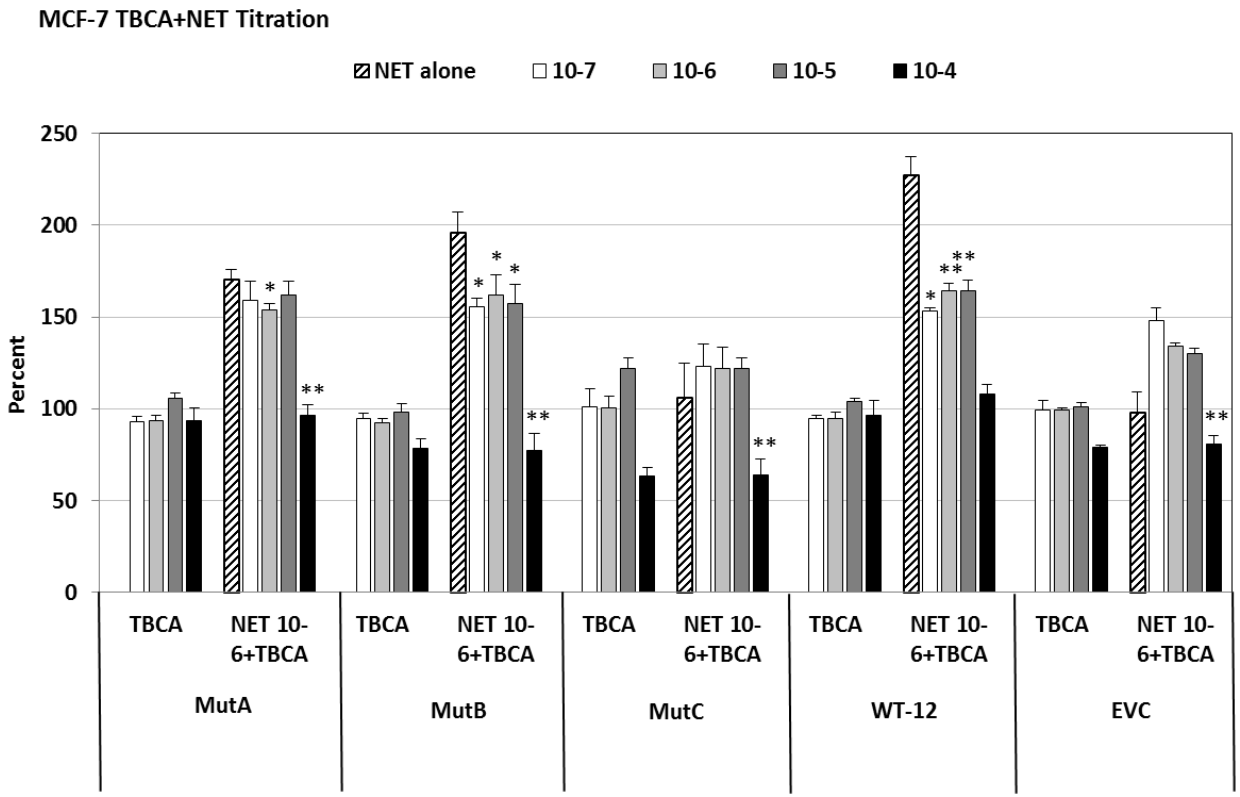

Figure 14. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with NET (10^{-6}M) and TBCA (10^{-7}M, 10^{-6}M, 10^{-5}M and 10^{-4}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. NET alone).

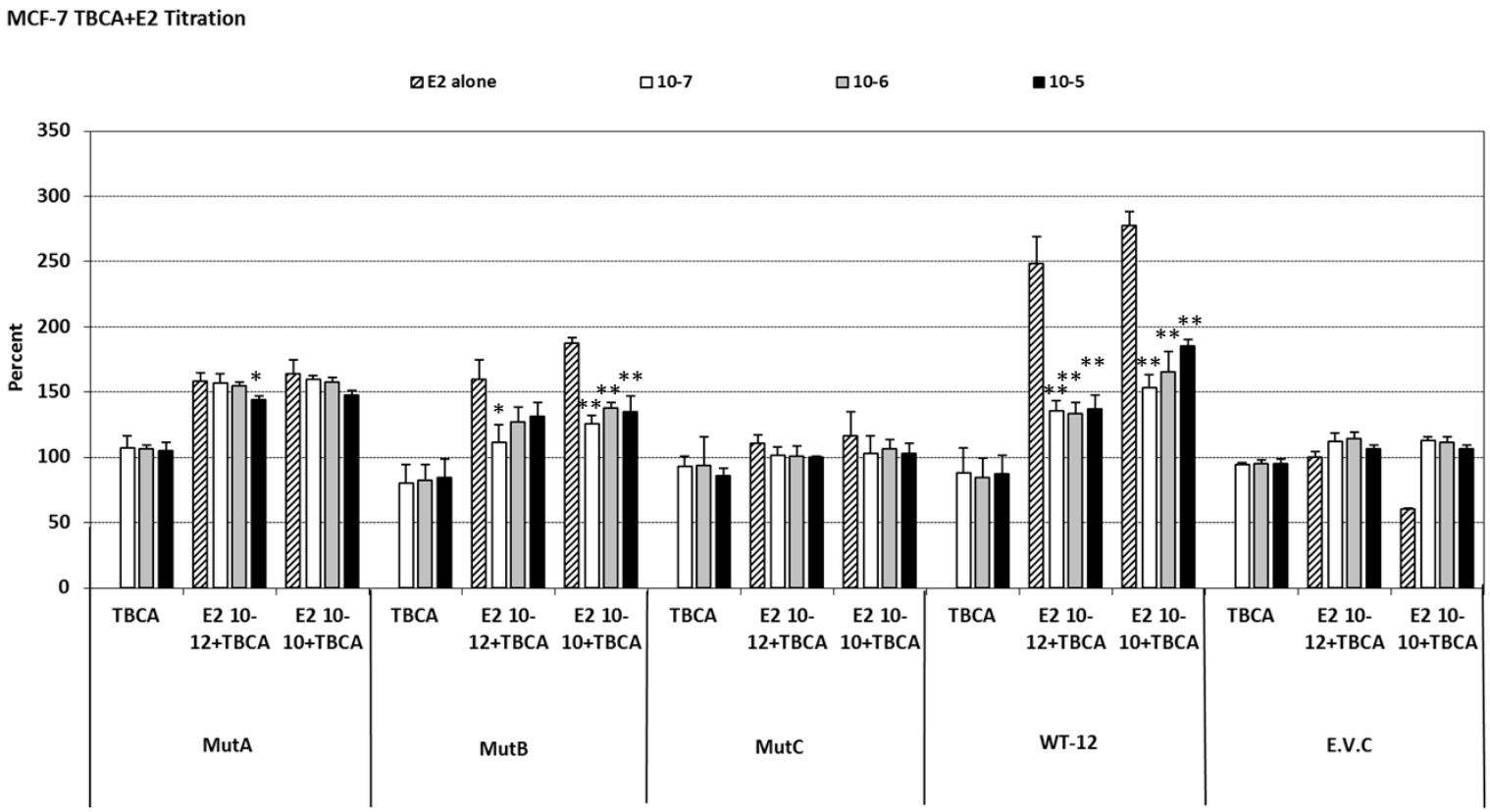

Figure 15. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with E2 (10^{-12}M, 10^{-10}M) and TBCA (10^{-7}M, 10^{-6}M and 10^{-5}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

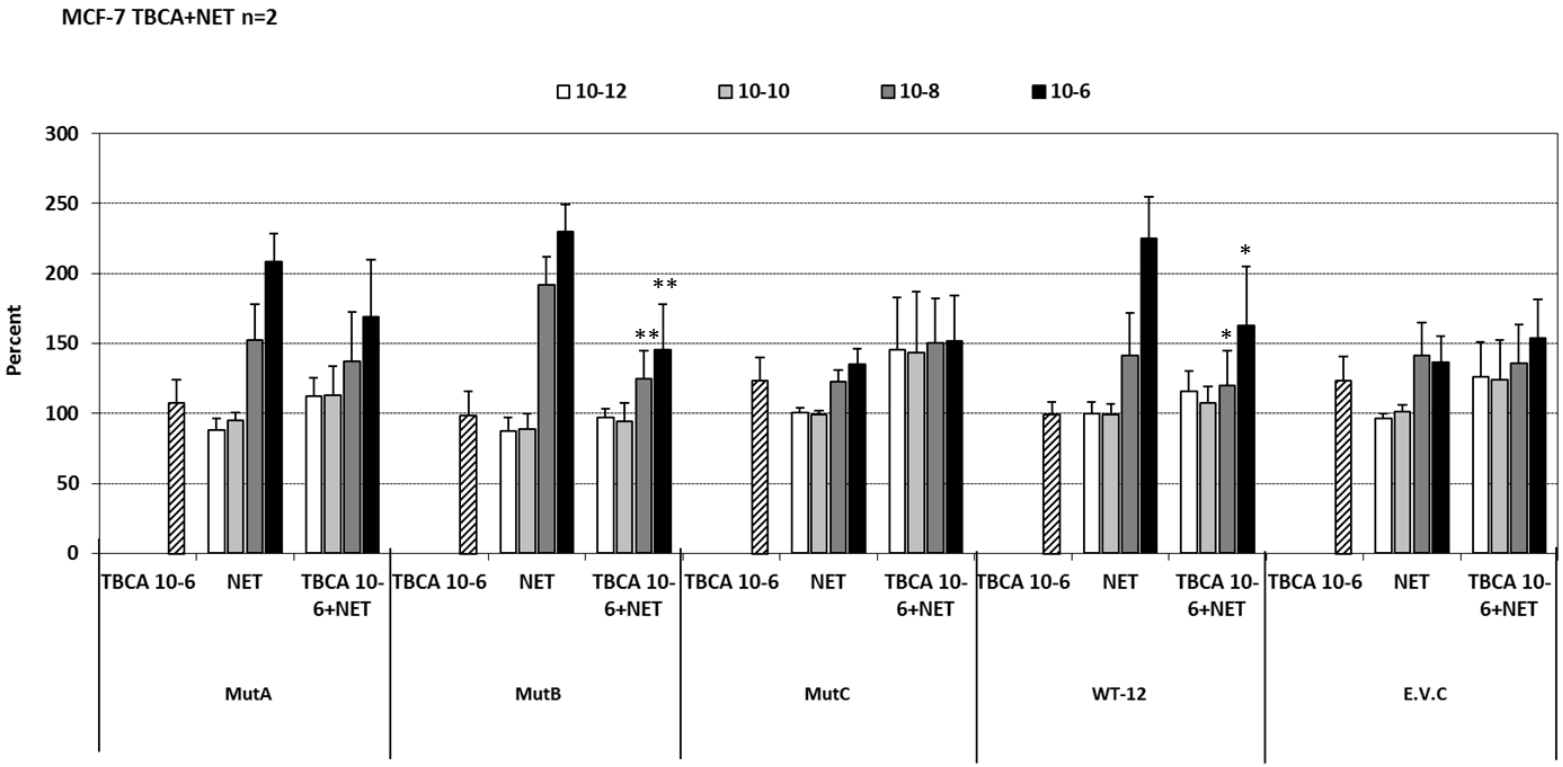

Figure 16. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10^{-6}M) and NET (10^{-12}M, 10^{-10}M, 10^{-8}M and 10^{-6}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. NET alone).

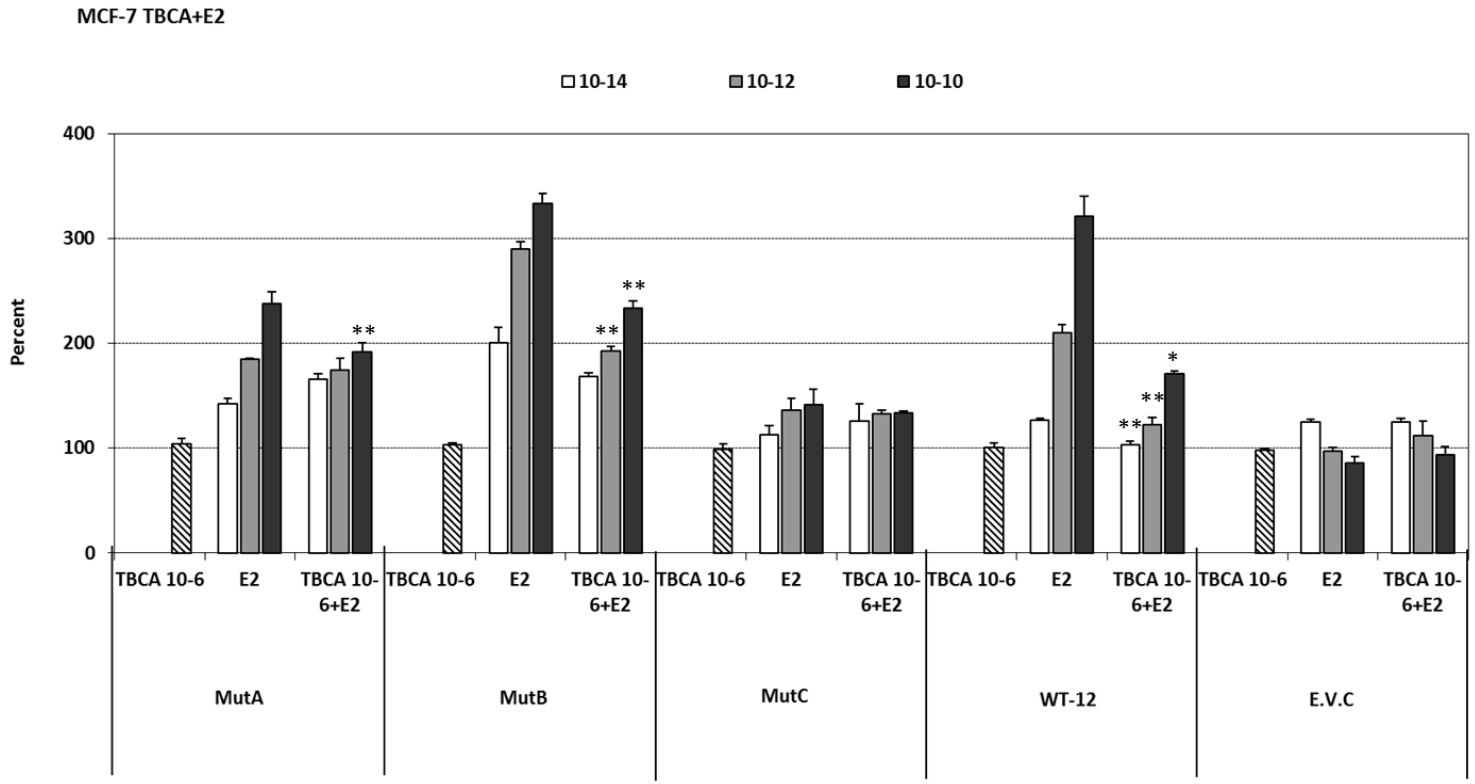

Figure 17. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10-6M) and E2 (10^{-14}M, 10^{-12}M and 10^{-10}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

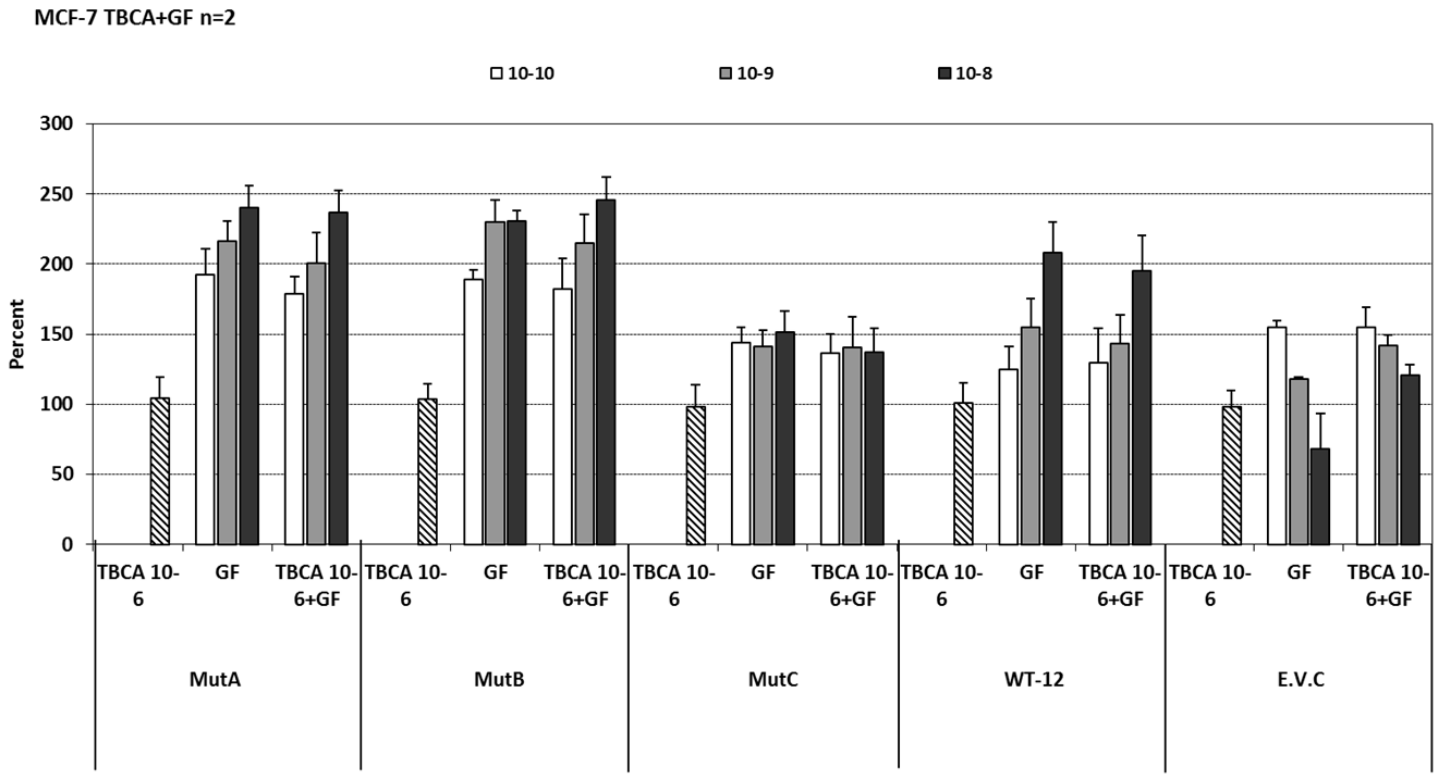

Figure 18. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10^{-6}M) and growth factors EGF, FGF and IGF-I 10^{-10}M, 10^{-9}M and 10^{-8}M (GF 10^{-10}M, 10^{-9}M and 10^{-8}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. GF alone).

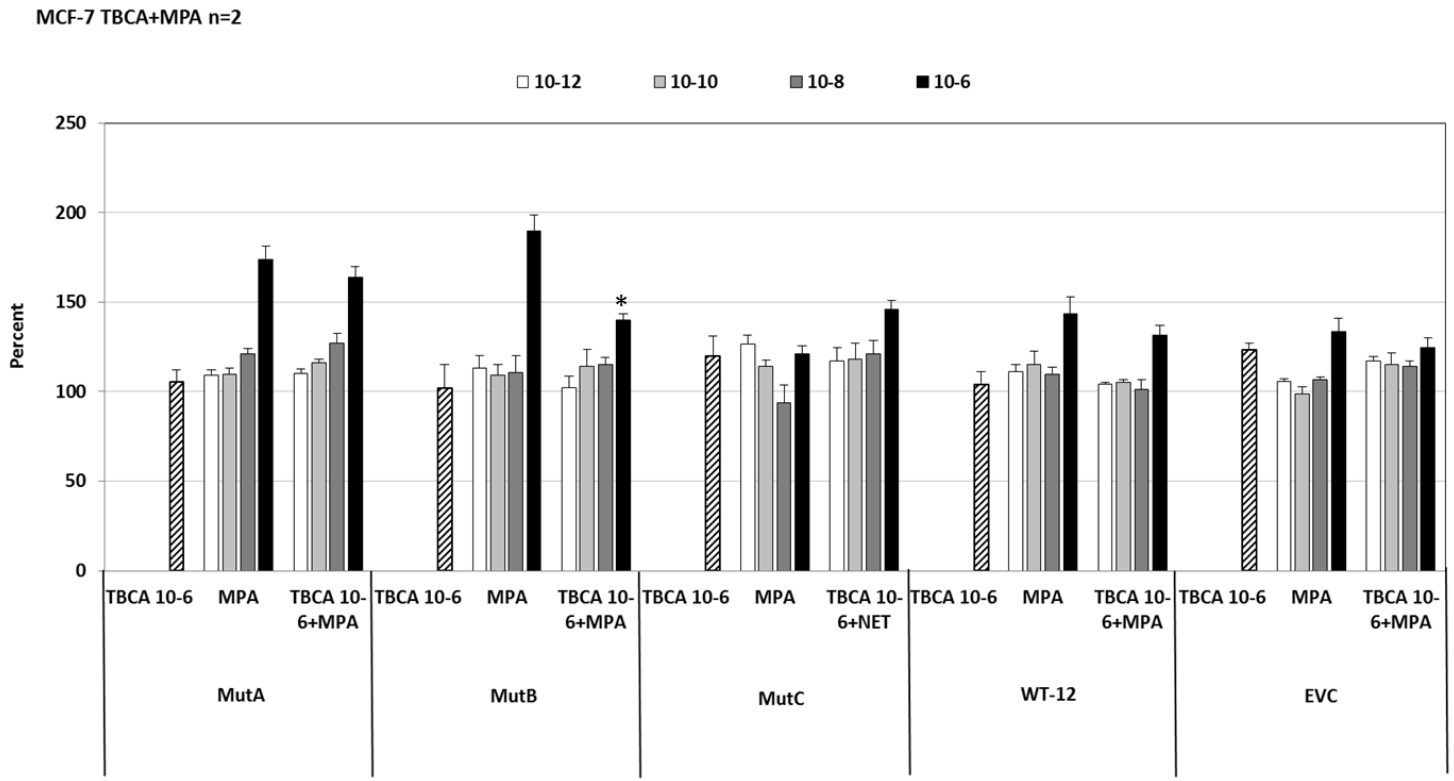

Figure 19. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10^{-6}M) and MPA (10^{-12}M, 10^{-10}M, 10^{-8}M and 10^{-6}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. MPA alone).

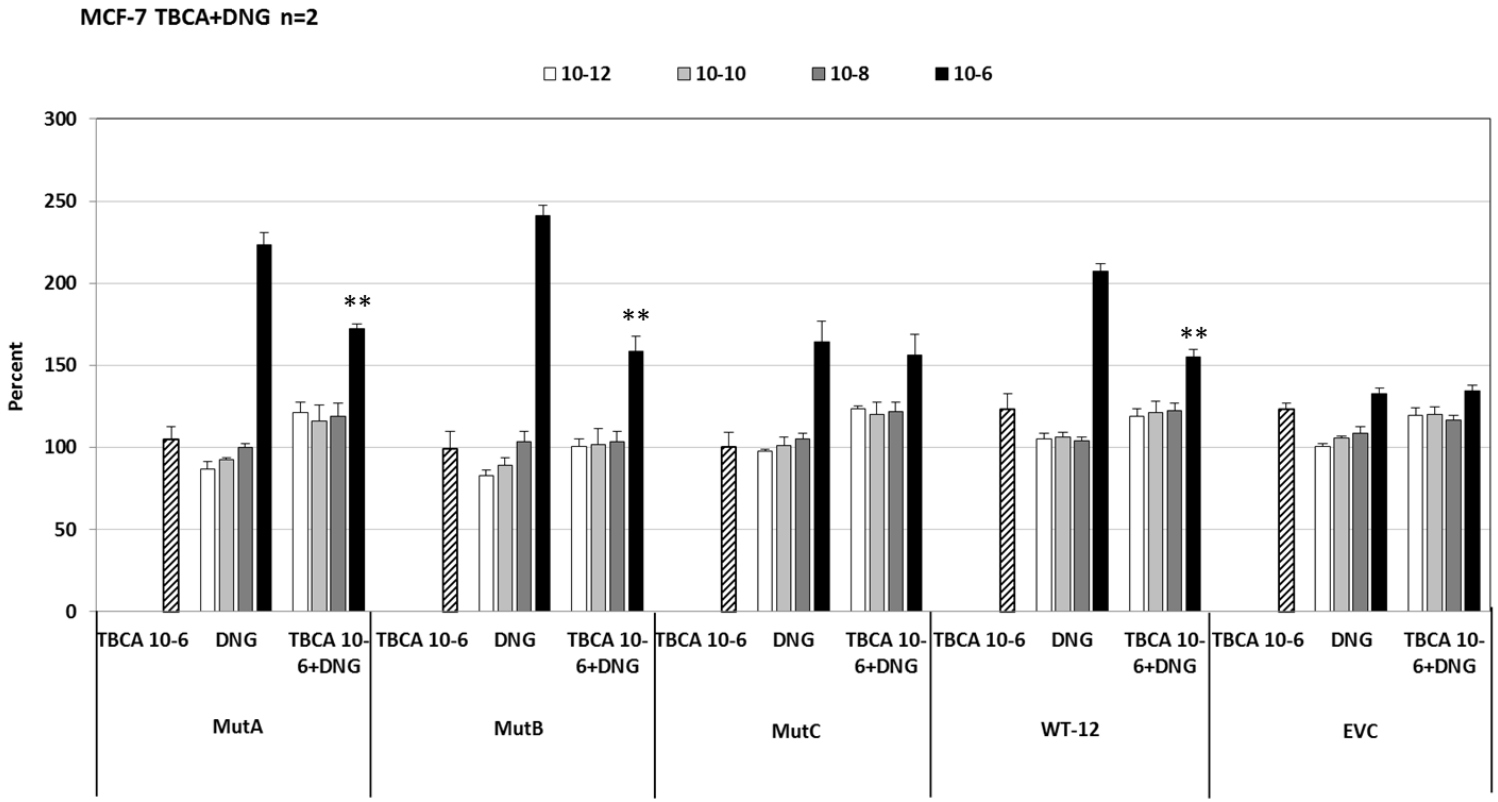

Figure 20. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10^{-6}M) and DNG (10^{-12}M, 10^{-10}M, 10^{-8}M and 10^{-6}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. DNG alone).

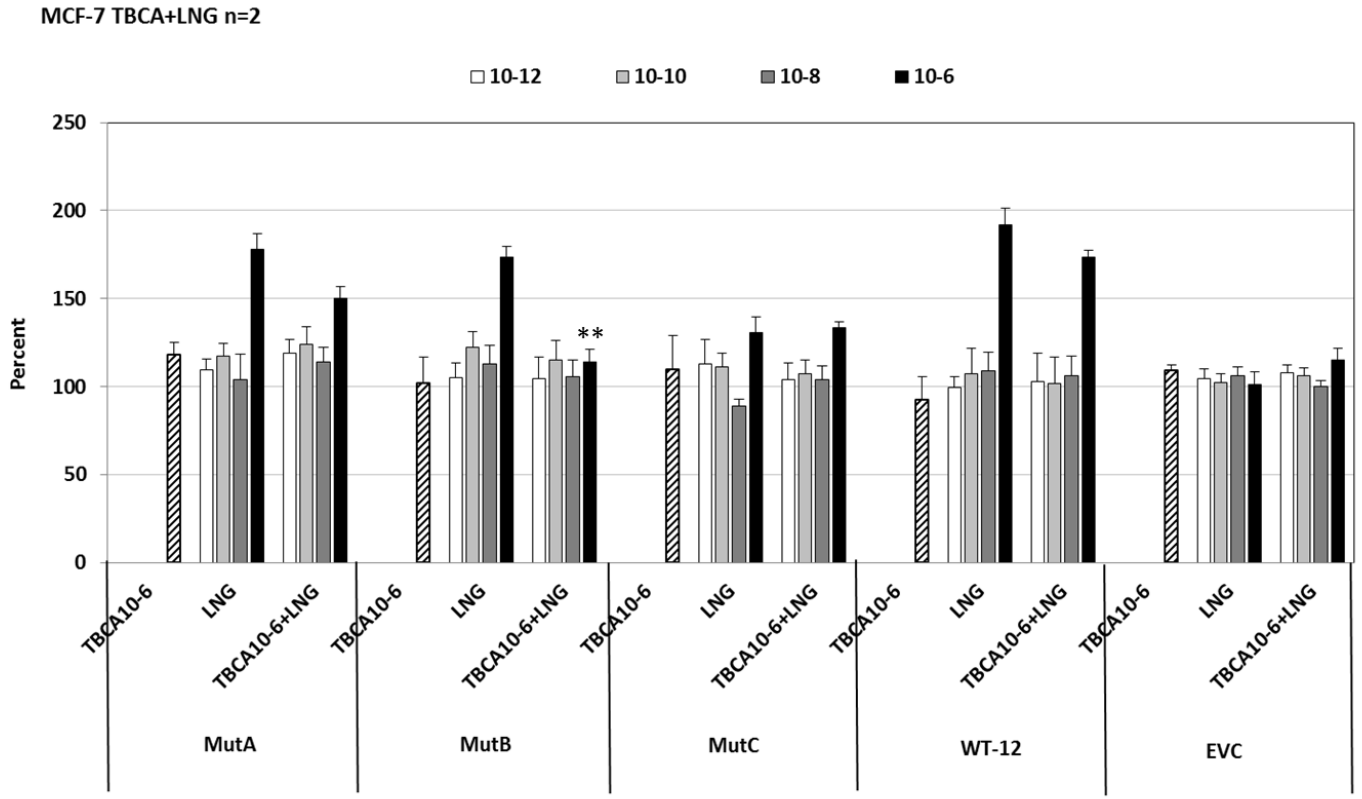

Figure 21. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with TBCA (10^{-6}M) and LNG (10^{-12}M, 10^{-10}M, 10^{-8}M and 10^{-6}M) alone respectively, and in combination sequentially. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. LNG alone).

3.2.4 Blocking effect of tamoxifen on the prolieferation of MCF-7 cells

E2 and tamoxifen were used alone and in combination continuously or sequentially on MCF-7 MutA, MutB, MutC, WT-12 and EVC cells to determine the best manner. By continuous combined manner, it means that cells were incubated 6 days with E2 and tamoxifen combination. And by sequential manner, cells were treated 3 days first by either E2 alone or tamoxifen alone, and then by E2 and tamoxifen combination for the next 3 days. The proliferation rate was measured after 6 days.

As shown in Figure 22-24, tamoxifen alone at all concentrations did not trigger any increase of MCF-7 MutA, MutC and EVC proliferation, but enhanced slightly proliferation of MCF-7 MutB and WT-12 cells. When MCF-7 cells were stimulated with E2 plus tamoxifen continuously (Fig. 22), the proliferation of the proliferative effect caused by E2 on MutA, MutB and WT-12 could be significantly reduced. When the cells were previously treated by tamoxifen (Fig. 23), and then add E2 in the system, the reduction of E2-induced proliferation of MutA, MutB and WT-12 cells was more obvious. However, when E2 was added first, no blocking effect was found (Fig. 24).

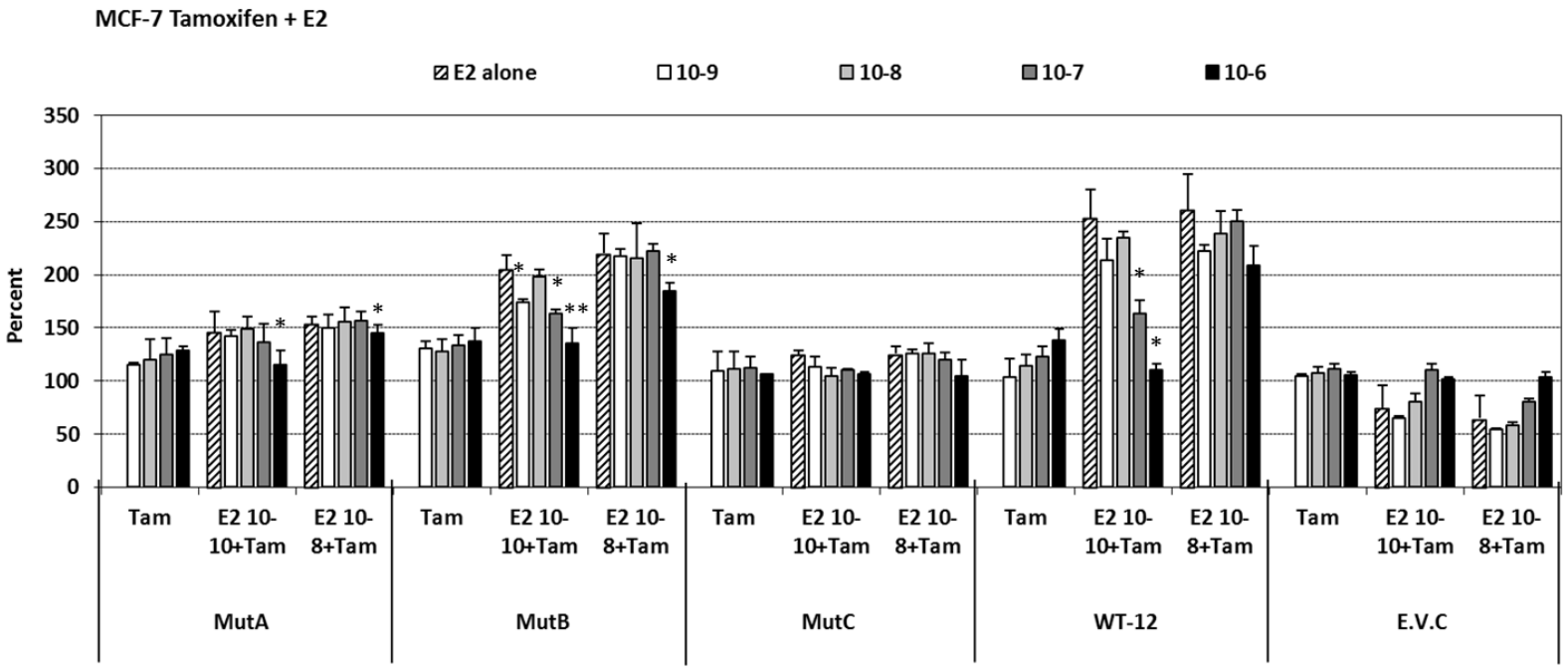

Figure 22. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with tamoxifen (10-9M, 10-8M, 10^{-7}M and 10^{-6}M) and E2 (10^{-10}M and 10^{-8}M) alone respectively, and in combination continuously. Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

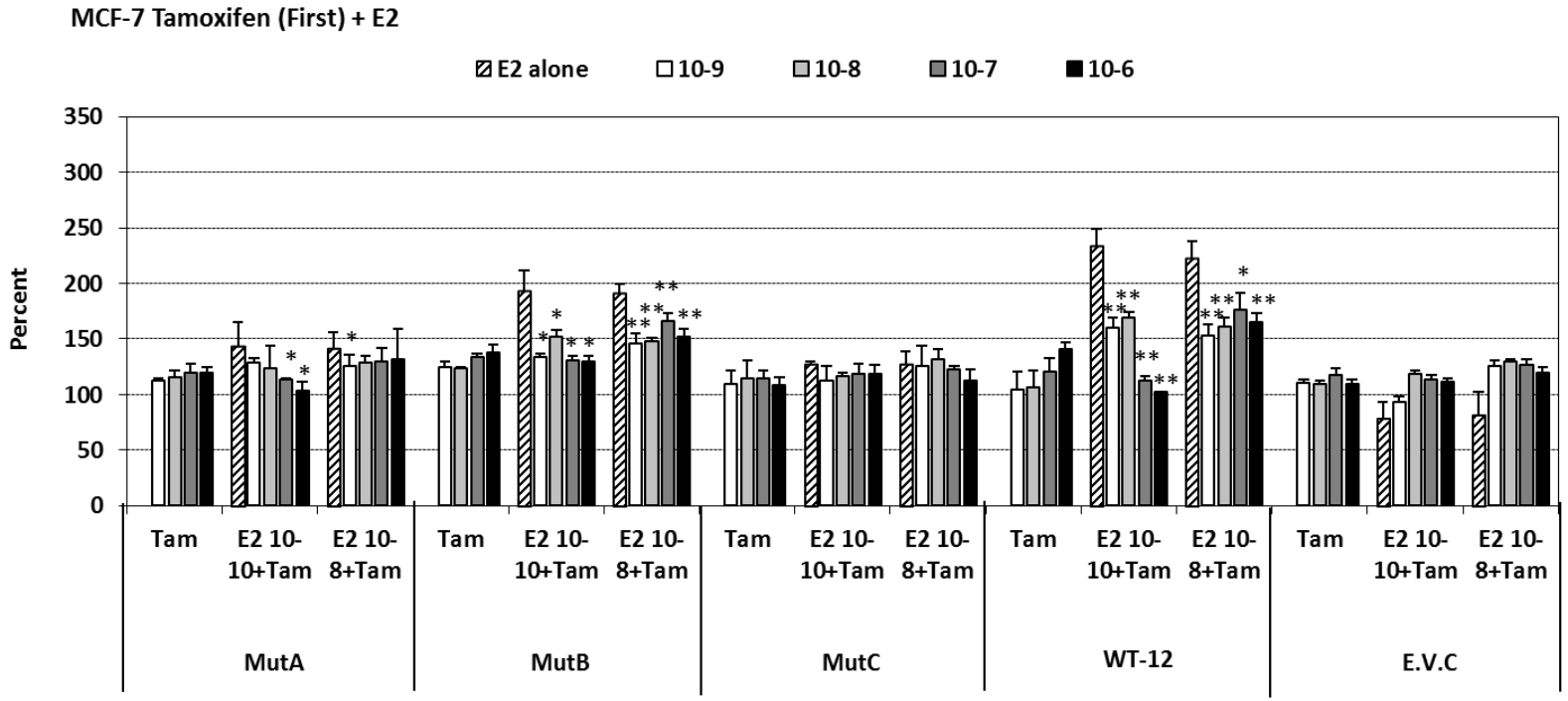

Figure 23. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with tamoxifen (10-9M, 10-8M, 10^{-7}M and 10^{-6}M) and E2 (10^{-10}M and 10^{-8}M) alone respectively, and in combination sequentially (tamoxifen alone for the first 3 days). Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

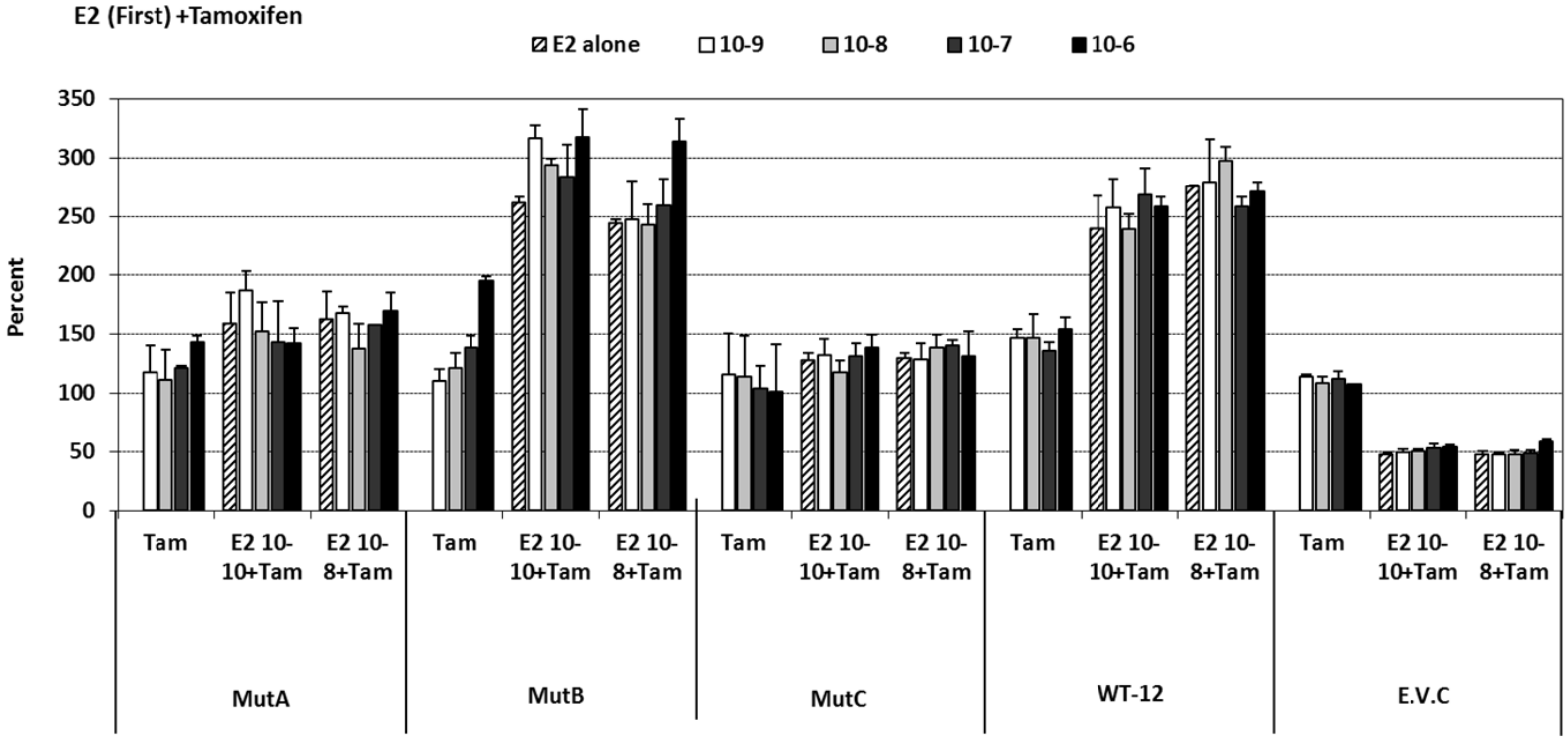

Figure 24. MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with tamoxifen (10-9M, 10-8M, 10^{-7}M and 10^{-6}M) and E2 (10^{-10}M and 10^{-8}M) alone respectively, and in combination sequentially (E2 alone for the first 3 days). Cell proliferation was measured after 6 days. Data were normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. E2 alone).

3.3 Western blot analysis

MCF-7 MutA, MutB, MutC, WT-12, and EVC cells were incubated with NET (10^{-6} M) alone and in combination with TBCA (10^{-6} M) sequentially. After 120min, protein was extracted from cells. A western blot of equal total protein amounts (25µg/lane) was then performed.

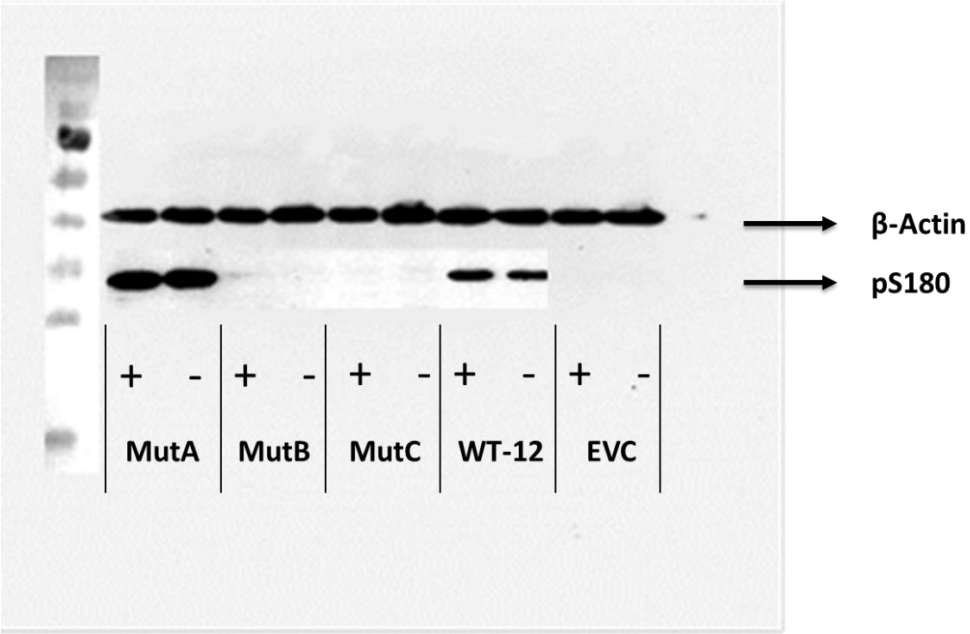

Figure 25. Western Blot analysis with phospho-PGRMC1-specific mouse monoclonal antibody 3G11A2. Upper panels depict loading controls for actin. Lower panels represent the expression of phosphorylated protein pS180. (+: TBCA 10^{-6}M + NET 10^{-6}M; -: NET 10^{-6}M alone).

As can be seen in Figure 25, because MutB, MutC were MCF-7 cells which was stably transfected with plasmid encoded by PGRMC1 mutation at the site S180A, no expression of pS180 was detected in both cell lines. Moreover, EVC is an empty vector control that was transfected with pcDNA3.1-3HA plasmid, so no expression of pS180 was found either. For MutA and WT-12, the expression of pS180 was detected. However, no significant difference regarding pS180

expression between NET alone and blocking with TBCA was found. This western blot experiment was also performed using E2 as the stimulation. And exact same result was observed (data not shown).

3.4 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to measure expression of the endogenous estrogen responsive gene TFF1 in MCF-7 cells and T47D cells stably transfected with wild-type PGRMC1 or mutant PGRMC1 expression plasmids, after stimulation of either estrogen of progestins.

3.4.1 Effect of E2 alone on transcription of TFF1 in MCF-7 and T47D cells

TFF1 has been reported as an estrogen-responsive gene, whose mRNA expression can be induced by estrogen. Based on this theory, the first experiment was performed, using mRNA extracted from MCF-7 and T47D cells, which were incubated with E2 at various concentrations.

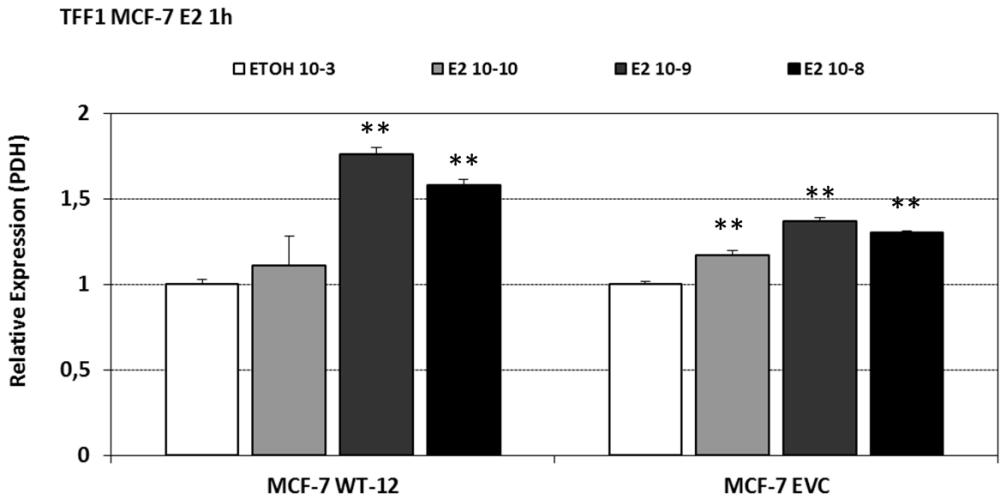

Figure 26. MCF-7 WT-12, and EVC cells were incubated with E2 (10^{-10}M, 10^{-9}M and 10^{-8}M) for 1h. After mRNA isolation and reverse transcriptase PCR, the

quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

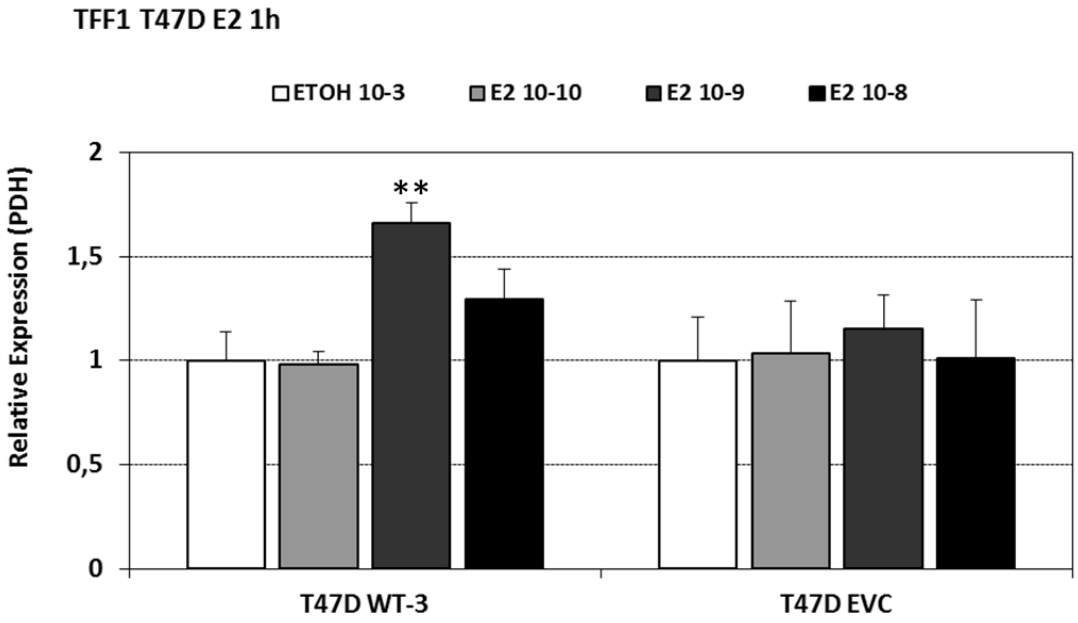

Figure 27. T47D WT-3 and EVC cells were incubated with E2 (10^{-10}M, 10^{-9}M and 10^{-8}M) for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

As can be seen in Figure 26, 27, the TFF1 expression of both MCF-7 and T47D cells was indeed significantly up-regulated: for MCF-7 WT-12 cells at the concentration of 10^{-9} M and 10^{-8} M, for MCF-7 EVC at all the concentrations that we

tested, and for T47D WT-3 at the concentration of 10^{-9}M, whereas T47D did not react to E2 stimulation.

3.4.2 Time-dependent effect of E2 and NET on transcription of TFF1 in MCF-7 and T47D cells

Subsequently, a series of time-dependent experiments were performed. We treated MCF-7 and T47D cells with E2 (10^{-8}M, 10^{-9}M) and NET (10^{-7}M) alone, respectively, for 0.5h, 1h, 2h and 4h. The different time periods allowed us to assess rapid, direct transcriptional effects as well as more delayed, potentially secondary effects.

Both E2 and NET triggered a significant increase of TFF1 expression in MCF-7 WT-12 cells after an incubation time of 0.5h, but not in EVC cells (Fig. 28, 29). After 1h, the stimulation for WT-12 and also EVC resulted in significantly elevated activation of TFF1. A similar effect was observed in MCF-7 WT-12 and EVC cells after 2h incubation. However, EVC cells did not react to NET 10^{-7}M. After 4h, NET was able to significantly elevate the expression of TFF1 in WT-12 cells, but not EVC cells. And E2 elicited significantly the expression of TFF1 in WT-12 cells only at the concentration of 10^{-9}M.

For T47D cells, the exact same experiment was performed as it for MCF-7 cells. As can be seen in Figure 30 and 31, T47D WT-3 and EVC cells did not result in any significant up-regulation of TFF1 expression after 0.5h incubation. However, after a longer incubation time, namely 1h, 2h and 4h, both E2 and NET were able to trigger a significant increase of TFF1 expression in WT-3. As for T47D EVC cells, except E2 at the concentration of 10^{-9}M, no significant increase of TFF1 expression was found.

Obviously, with regard to estrogen or progestins triggering the up-regulation of TFF1 expression, the incubation time of 1h was more sensitive for both MCF-7 and T47D cells. Hence, this time period was chosen for further experiments.

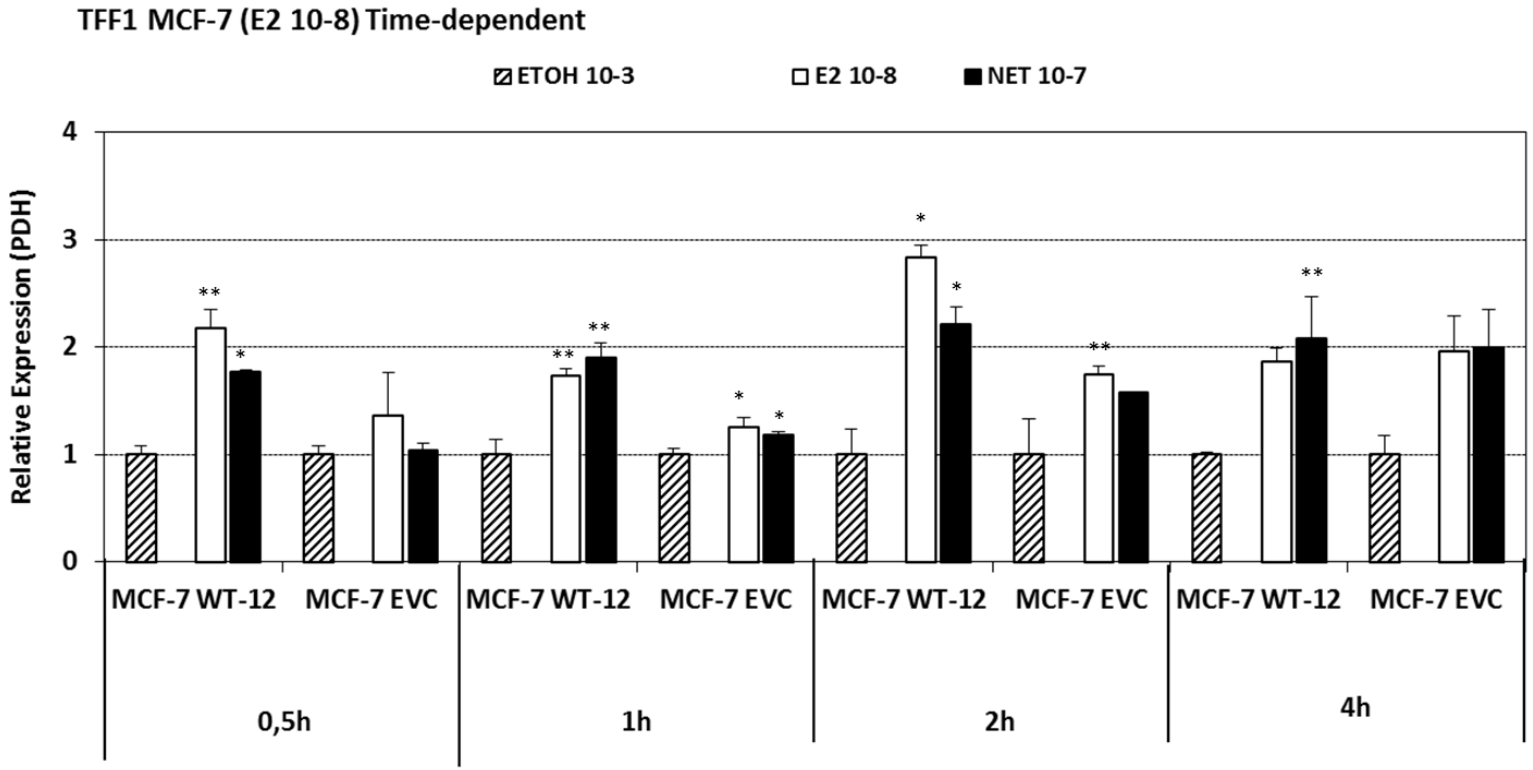

Figure 28. MCF-7 WT-12 and EVC cells were incubated with E2 (10^{-8}M) and NET (10^{-7}M) alone respectively for 0.5h, 1h, 2h and 4h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

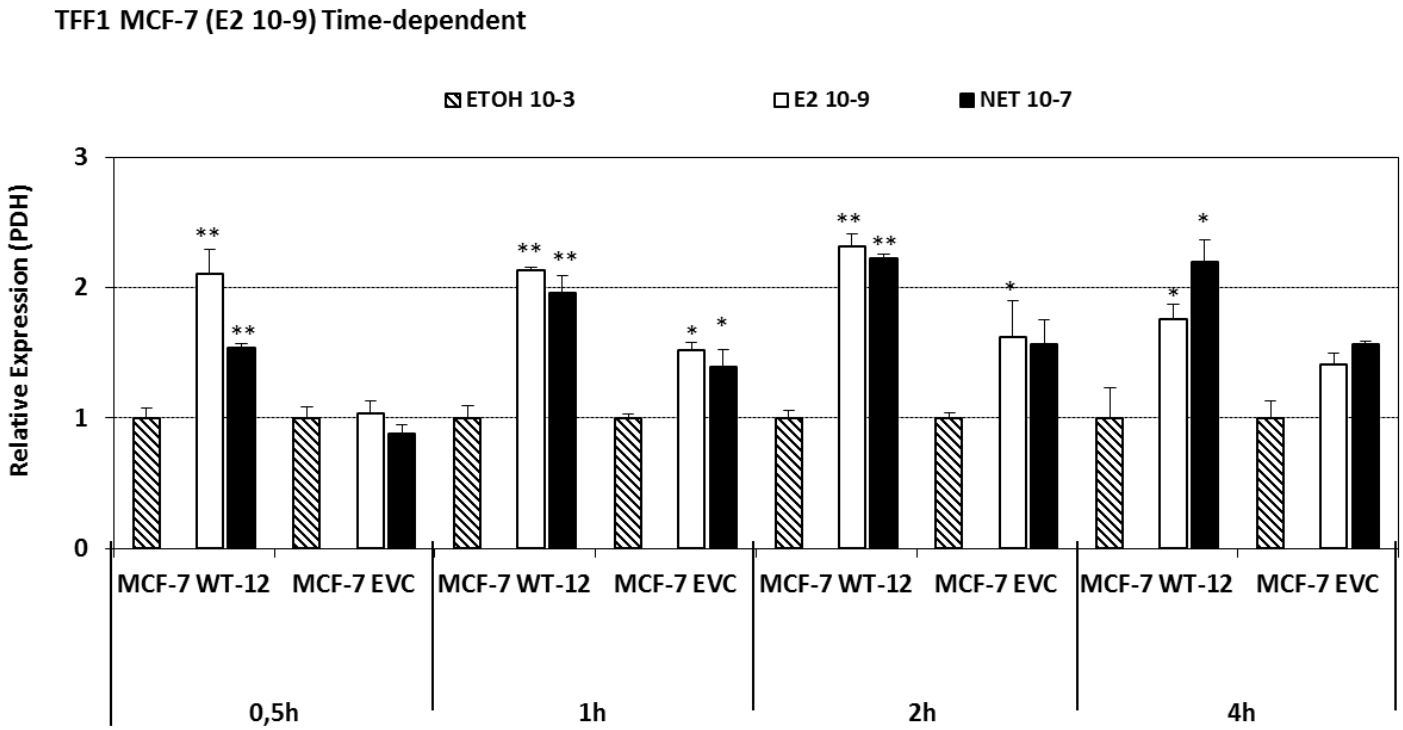

Figure 29. MCF-7 WT-12 and EVC cells were incubated with E2 (10^{-9}M) and NET (10^{-7}M) alone respectively for 0.5h, 1h, 2h and 4h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

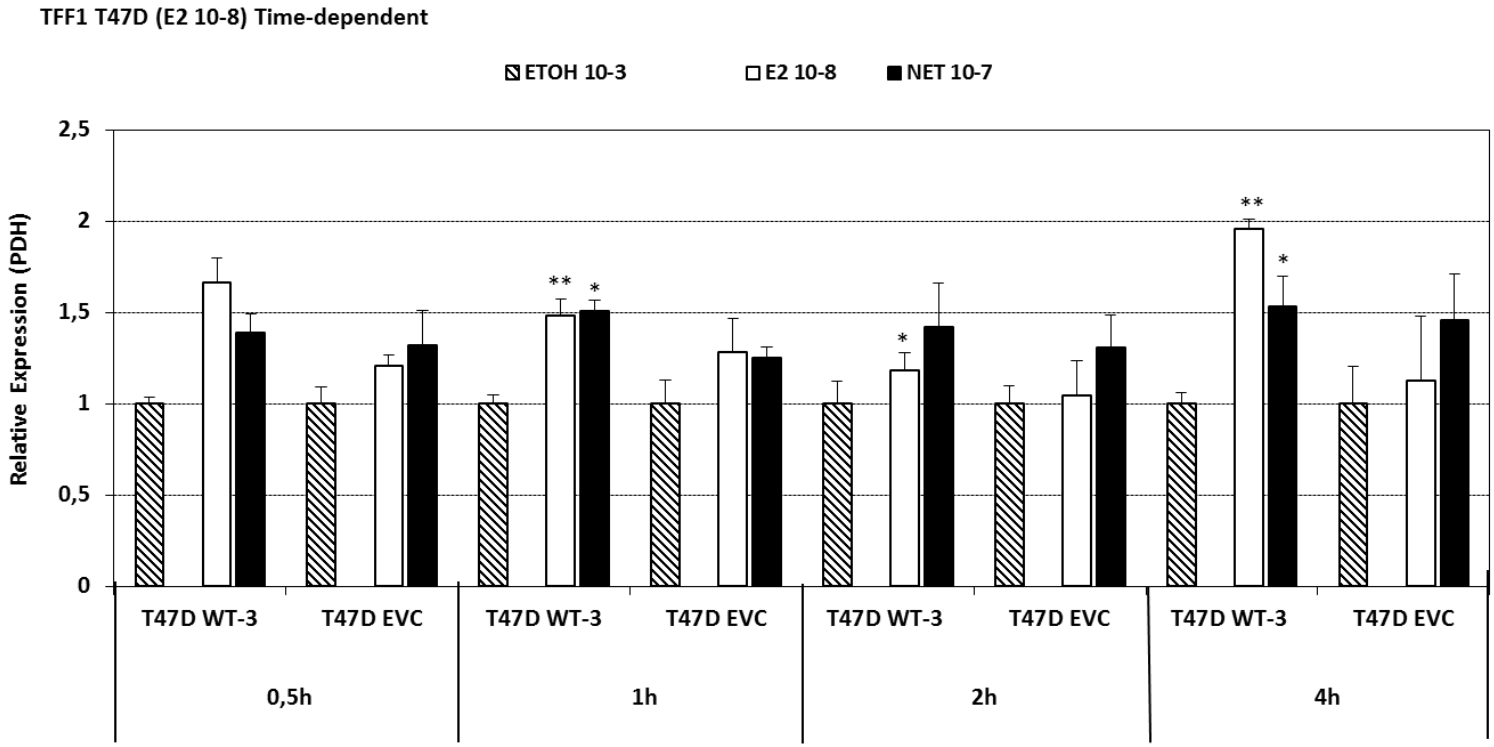

Figure 30. T47D WT-3 and EVC cells were incubated with E2 (10^{-8}M) and NET (10^{-7}M) alone respectively for 0.5h, 1h, 2h and 4h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

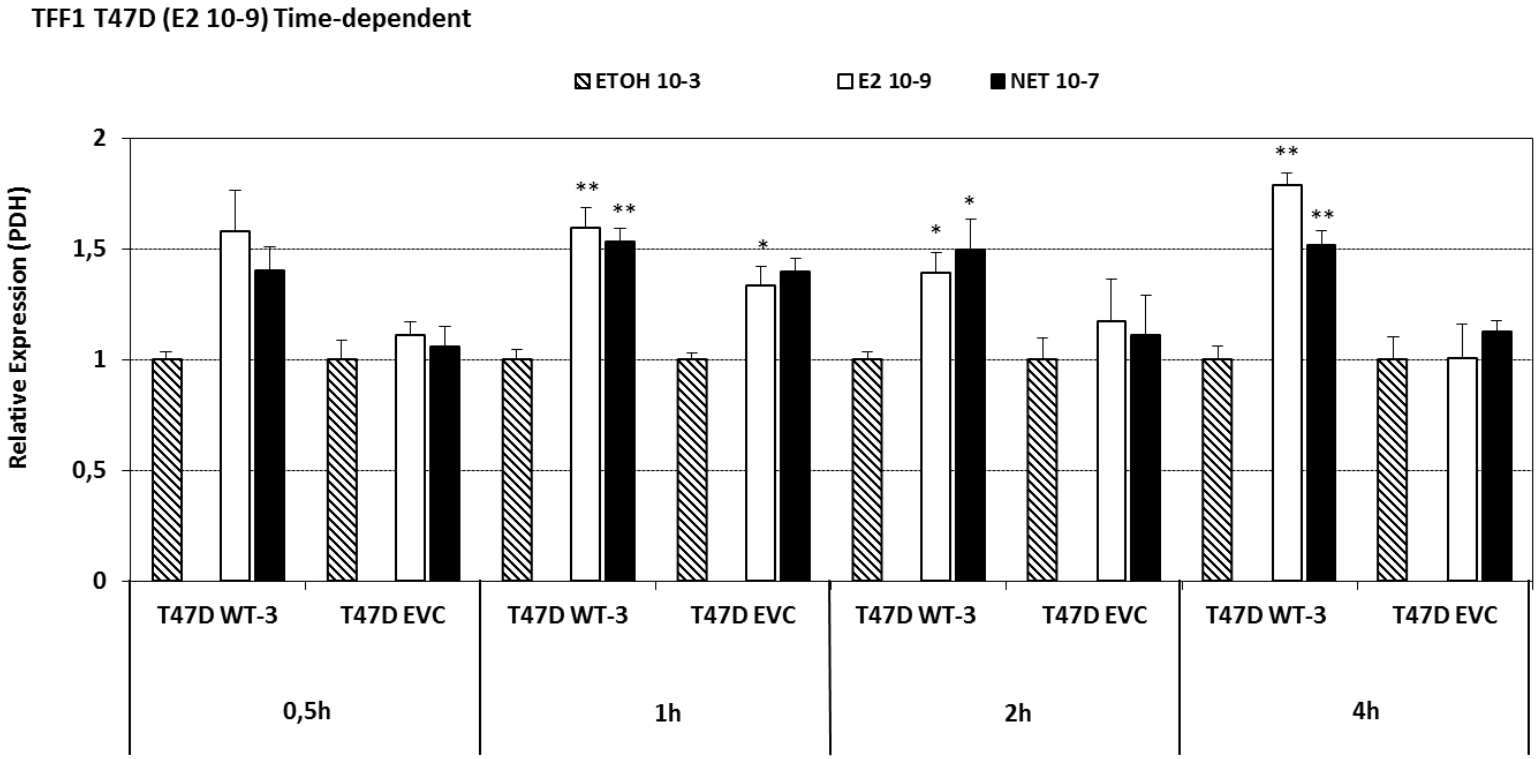

Figure 31. T47D WT-3 and EVC cells were incubated with E2 (10^{-9}M) and NET (10^{-7}M) alone respectively for 0.5h, 1h, 2h and 4h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

3.4.3 Effect of progestogens alone on transcription of TFF1 in MCF-7 and T47D cells

Since the elevated activation of TFF1 triggered by NET was detected, it would be really interesting for us to test the possible effects of other synthetic progestins. Therefore, we investigated first NET at various concentrations.

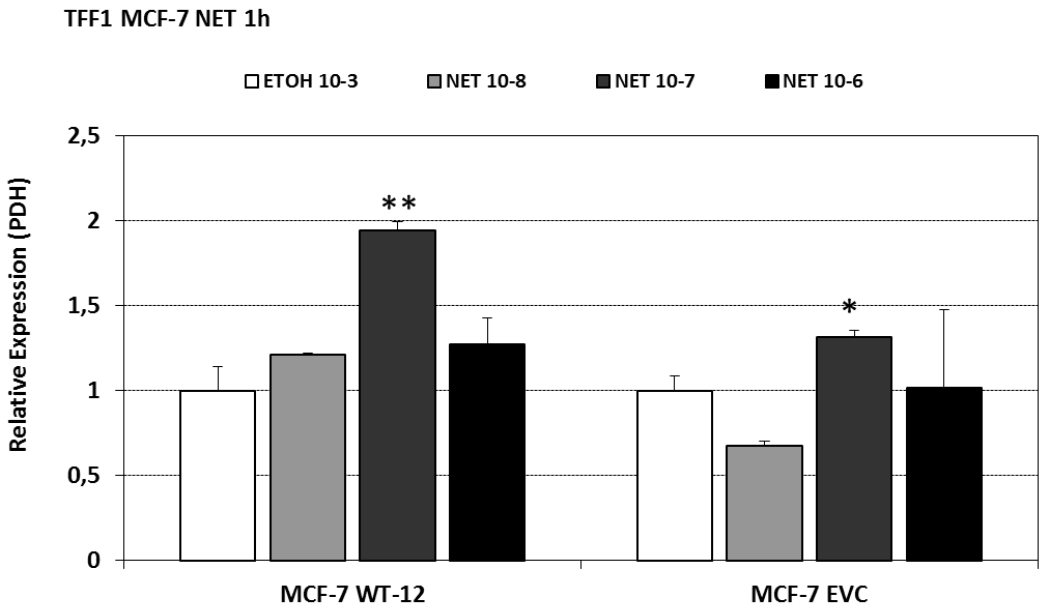

Figure 32. MCF-7 WT-12 and EVC cells were incubated with NET (10-8M, 10^{-7}M and 10^{-6}M) alone for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

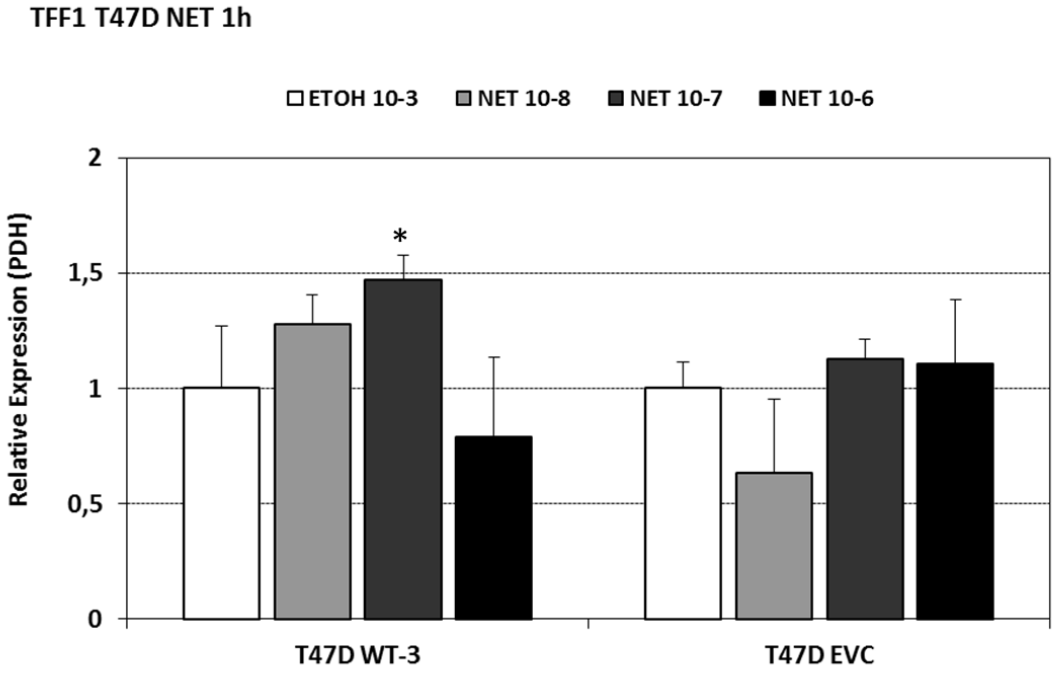

Figure 33. T47D WT-3 and EVC cells were incubated with NET (10^{-8}M, 10^{-7}M and 10^{-6}M) alone for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

NET at the concentration of 10^{-7}M triggered significantly the increase of TFF1 transcription level in MCF-7 WT-12, EVC, and T47D WT-3 cells (Fig. 32, 33). However, the transcription of TFF1 in T47D EVC cells did not alter significantly alter the NET stimulation compared with ethanol (10^{-3}M) stimulated control cells. Moreover, other concentrations of NET, even if it is higher than 10^{-7}M, could not cause any elevation of TFF1 expression.

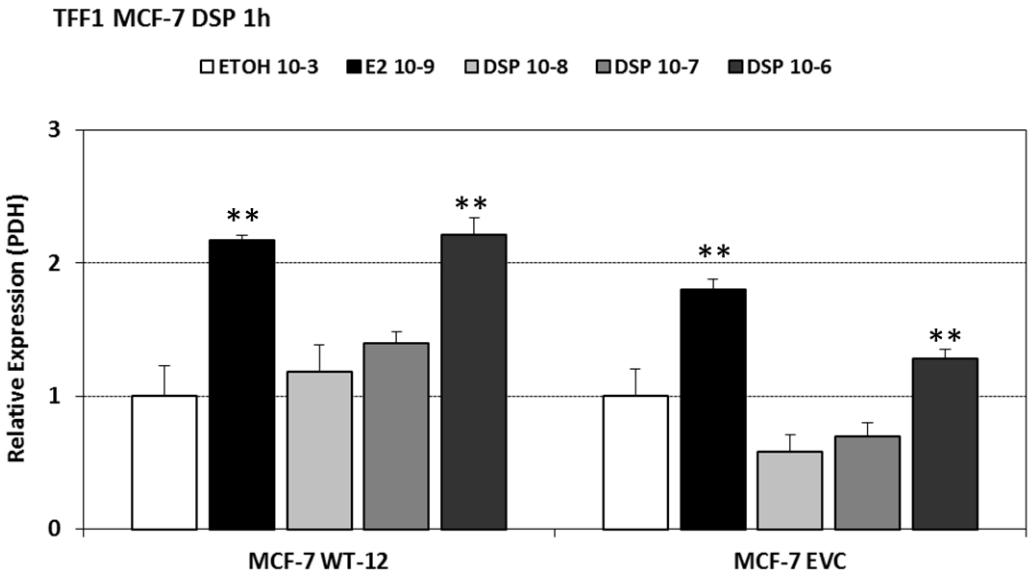

Figure 34. MCF-7 WT-12 and EVC cells were incubated with DSP (10^{-8}M, 10^{-7}M and 10^{-6}M) alone for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

Another synthetic progestin, DSP, which were commonly used clinically, was then tested. Additionally, E2 10^{-9}M was also used as a quality control of the experiment.

Consistently, the stimulation of E2 alone on MCF-7 WT-12, EVC and T47D WT-3 cells triggered significant increase of TFF1 transcription. It could represent the promising results of this experiment. As can be seen in Figure 34, 35, DSP elicited increased TFF1 expression in MCF-7 WT-12, EVC and T47D WT-3 cells only at the highest concentration that we tested. And T47D EVC cell reacted to neither E2 nor DSP stimulation.

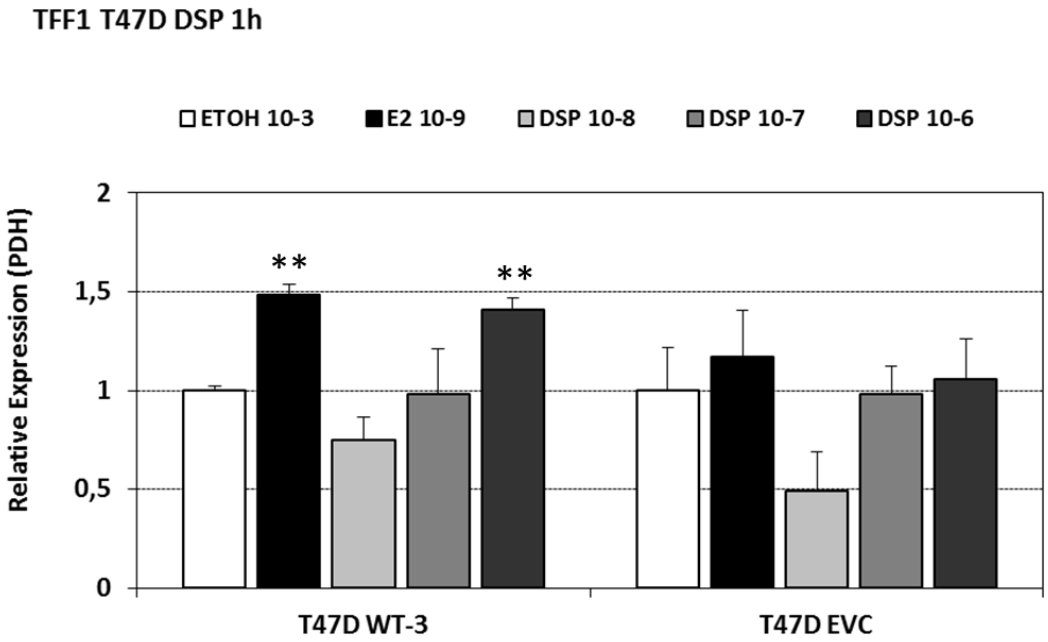

Figure 35. T47D WT-3 and EVC cells were incubated with DSP (10^{-8}M, 10^{-7}M and 10^{-6}M) alone for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

Furthermore, P4 and other progesins were tested, including MPA, NOM and CMA. As can be seen in Figure 36, P4, MPA and CMA at the concentration of 10^{-7}M were not able to result in any elevated activation of TFF1. Among all the progesting that we tested, only NOM at the concentration of 10^{-7}M enhanced the transcription of TFF1 in both MCF-7 WT-12 and T47D WT-3 cells. This effect of NOM is similar to the effect of NET (Fig. 37), except that MCF-7 EVC cells react to NET but not NOM.

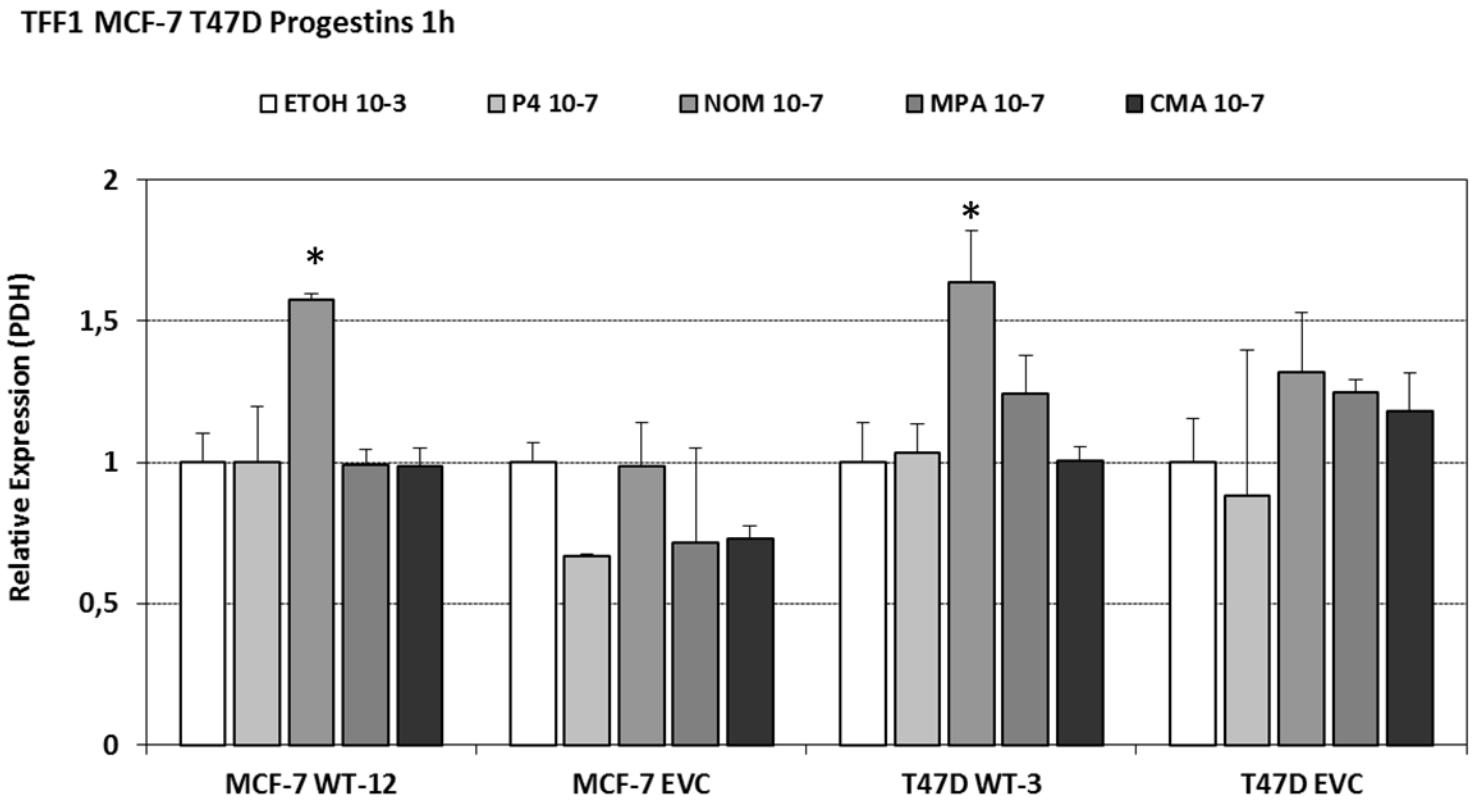

Figure 36. MCF-7 and T47D cells were incubated with P4, NOM, MPA and CMA at the concentration of 10^{-7}M for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

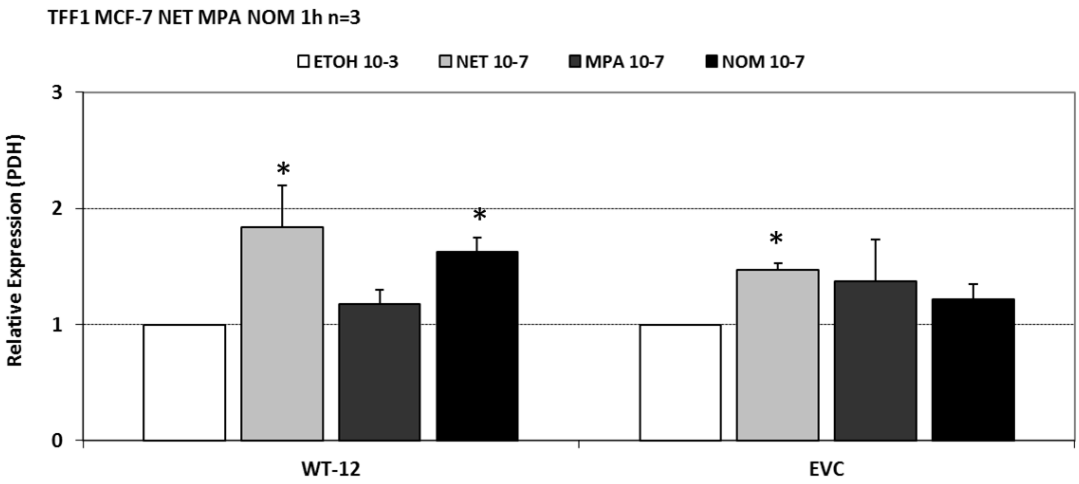

Figure 37. MCF-7 WT-12 cells were incubated with NET, NOM and MPA at the concentration of 10^{-7}M for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

3.4.4 Effect of estrogen plus progestogens on transcription of TFF1 in MCF-7 cells

In this study, we have already proved that estrogens as well as progestogens alone can trigger statistically significant induction of TFF1 transcription. Therefore, for further investigation, the effect of the combination of estrogen and different progestogens was detected. As can be seen in Figure 38, the transcriptional level of TFF1 gene was significantly elevated not only by E2 and NET alone respectively, but the combination of E2 and NET elicited a similar up-regulation. Moreover, although P4, as a neutral progestogen, would not trigger any increase of TFF1 expression, with combination of E2 enhanced significantly the transcription of TFF1. However, no significant differences of the proliferation rate was detected between NET/E2 alone and NET plus E2.

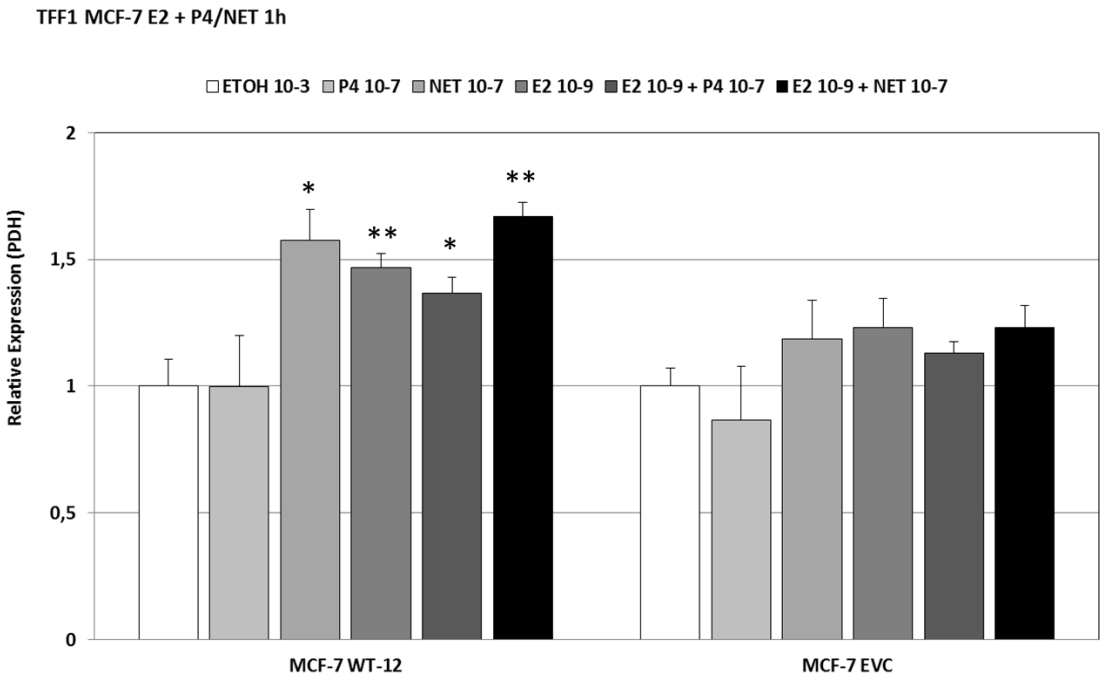

Figure 38. MCF-7 WT-12 and EVC cells were incubated with P4 (10^{-7}), NET (10^{-7}M), E2 (10^{-9}) alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

3.4.5 Blocking effect of fulvestrant on transcription of TFF1 in MCF-7 and T47D cells

A selective estrogen receptor modulator was then investigated in the blocking experiment. Unlike NET, fulvestrant was dissolved in DMSO, therefore, three different controls were applied in this experiment: ethanol (10-3M) as the control of NET alone, DMSO (10-3M) as the control of fulvestrant alone, and ethanol (10^{-3}M) plus DMSO (10^{-3}M) as the control of the combination of NET and fulvestrant.

Figure 39 and 40 exhibited the up-regulation of TFF1 expression triggered by NET, as well as the suppression of it caused by fulvestrant in MCF-7 WT-12, EVC and T47D WT-3 cells. However, T47D EVC cells did not show the similar down-regulation in the presence of fulvestrant. Again, MCF-7 MutC cells did not react to any stimulation.

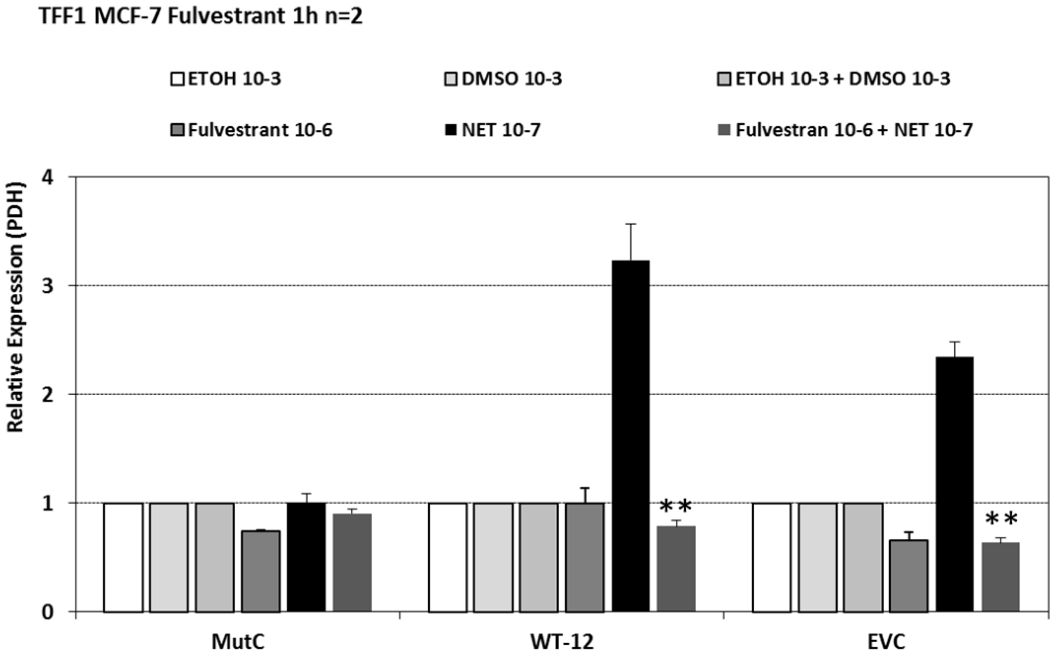

Figure 39. MCF-7 MutC, WT-12 and EVC cells were incubated with fulvestrant 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M), DMSO (10^{-3}M) or ethanol plus DMSO (10^{-3}M) stimulated control cells respectively. (Means±SD; *p < 0.05; **p < 0.01 vs.NET alone).

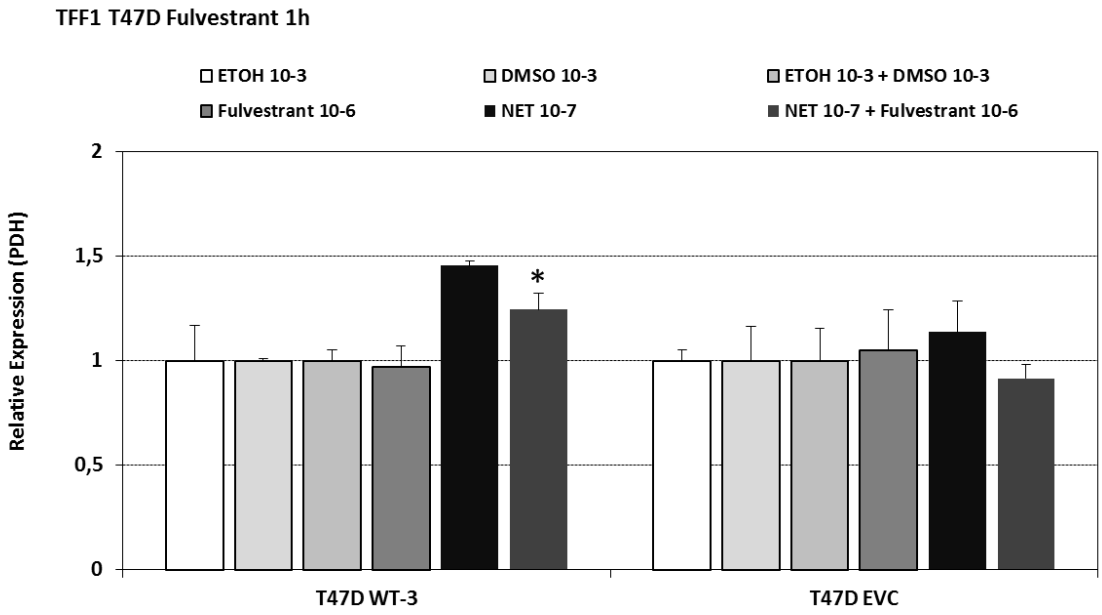

Figure 40. T47D WT-3 and EVC cells were incubated with fulvestrant 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M), DMSO (10^{-3}M) or ethanol plus DMSO (10^{-3}M) stimulated control cells respectively. (Means±SD; *p < 0.05; **p < 0.01 vs.NET alone).

3.4.6 Blocking effect of AG205 on transcription of TFF1 in MCF-7 and T47D cells

Furthermore, another blocking experiment regarding TFF1 transcription was performed on MCF-7 and T47D cells. Unlike NET, AG205 was dissolved in DMSO, therefore, three different controls were applied in this experiment: ethanol (10^{-3}M) as the control of NET alone, DMSO (10^{-3}M) as the control of AG205 alone, and ethanol (10^{-3}M) plus DMSO (10^{-3}M) as the control of the combination of NET and AG205.

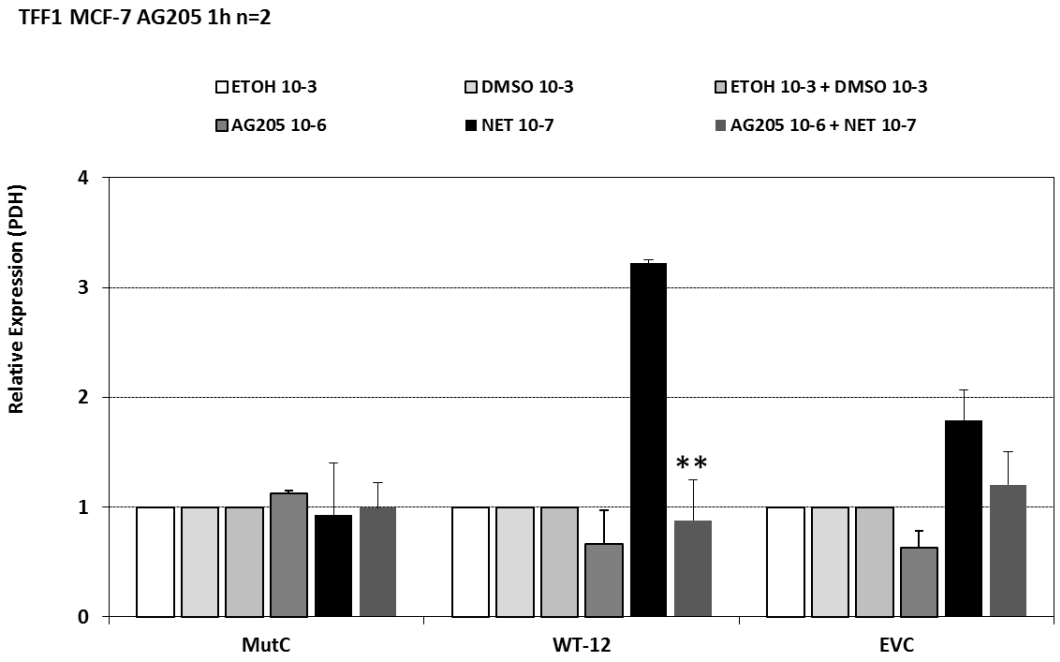

Figure 41. MCF-7 MutC, WT-12 and EVC cells were incubated with AG205 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M), DMSO (10^{-3}M) or ethanol plus DMSO (10^{-3}M) stimulated control cells respectively. (Means±SD; *p < 0.05; **p < 0.01 vs.NET alone).

Figure 41 and 42 demonstrated that in both PGRMC1 wild-type plasmid transfected cell line, MCF-7 WT-12 and T47D WT-3, the NET-regulated increase of TFF1 transcription was fully reversed in the presence of AG205. However, no significant down-regulation was observed in EVC transfected MCF-7 and T47D cells. Again, MCF-7 MutC cells did not react to any stimulation.

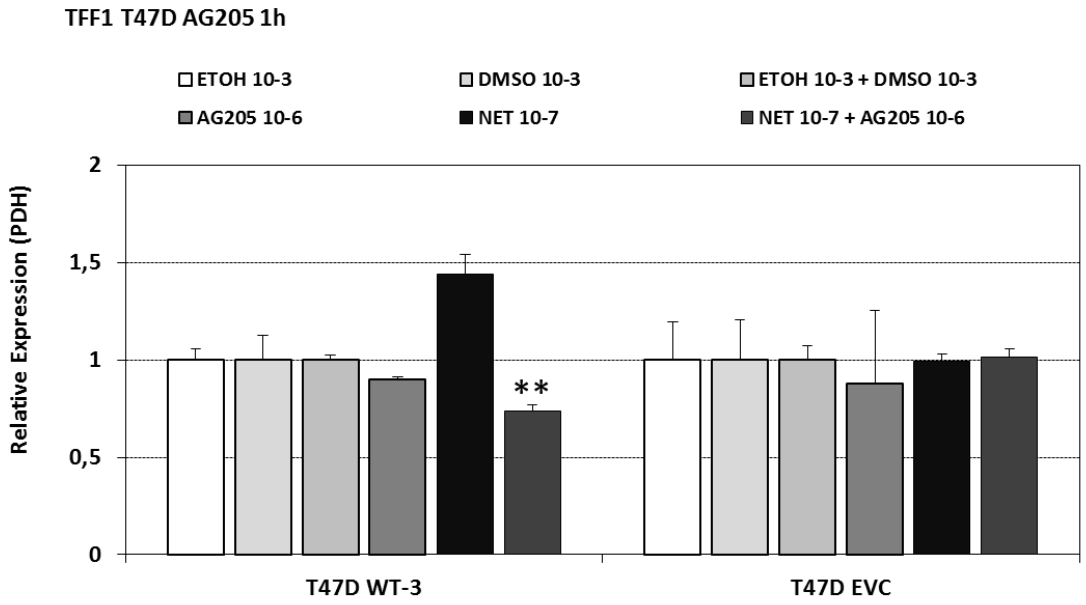

Figure 42. T47D WT-3 and EVC cells were incubated with AG205 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M), DMSO (10^{-3}M) or ethanol plus DMSO (10^{-3}M) stimulated control cells respectively. (Means±SD; *p < 0.05; **p < 0.01 vs.NET alone).

3.4.7 Blocking effect of TBCA on transcription of TFF1 in MCF-7 cells

As mentioned above, TBCA was used as an inhibitor to block the estrogens/progestins-triggered proliferative effect. In the next step of our experiment, the same blocking experiment was also used to measure the variation of the transcription level of TFF1 in MCF-7 cells.

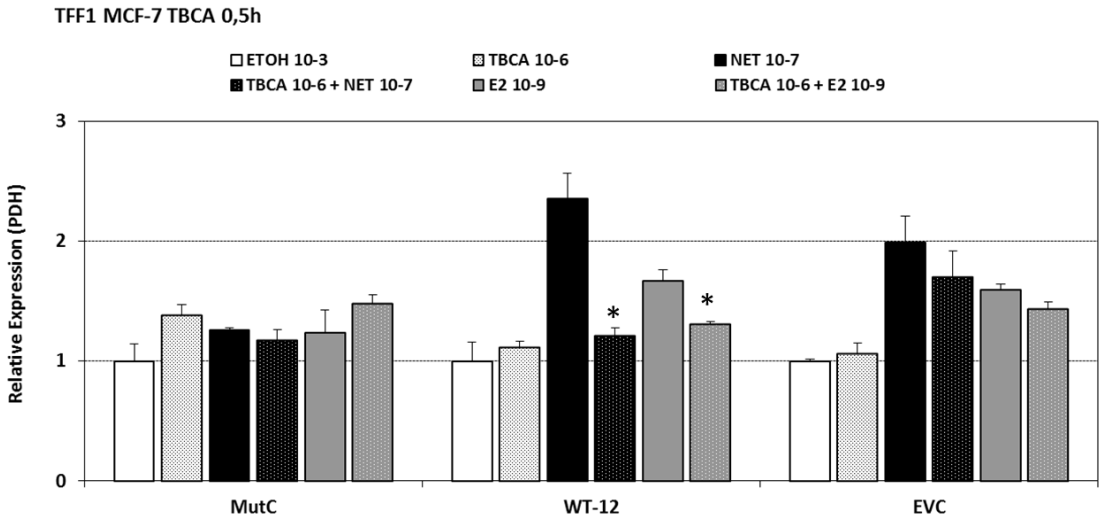

Figure 43. MCF-7 MutC, WT-12 and EVC cells were incubated with TBCA 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 0.5h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs. ethanol).

As can be seen in Figure 43, 44, TBCA did not induce or reduce the transcription of TFF1 in all the MCF-7 cell lines, whereas NET and E2 triggered consistently the elevation of TFF1 expression. Compared with NET or E2 alone respectively, the combination of NET plus TBCA and E2 plus TBCA reversed the up-regulation of TFF1 transcription in MCF-7 WT-12 cells completely, but not in EVC cells. We also included MCF-7 MutC cells in this experiment. However, no significant up-or down-regulation of TFF1 expression was detected.

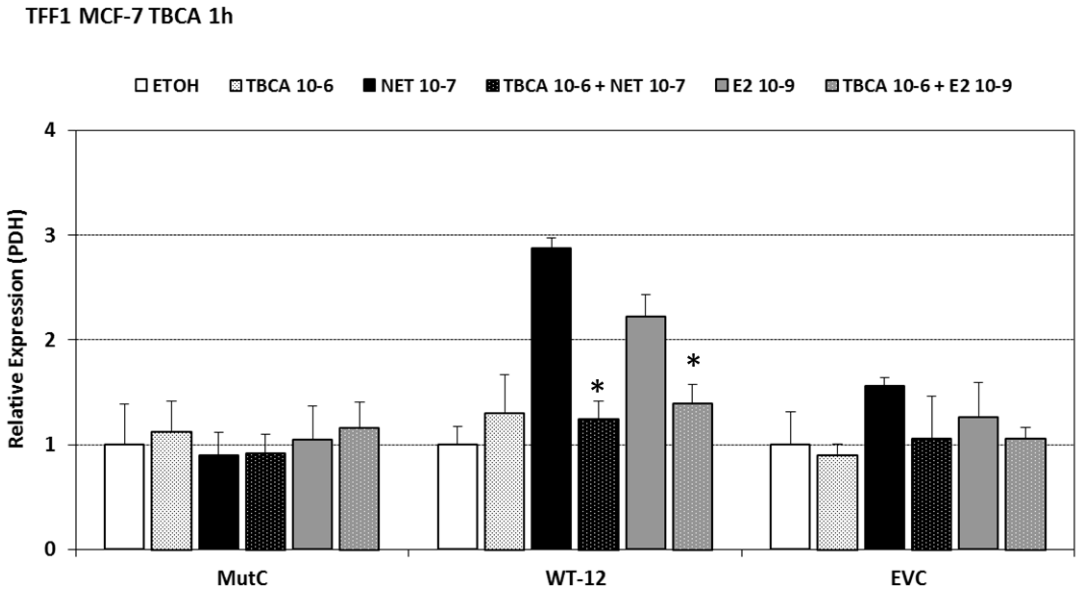

Figure 44. MCF-7 MutC, WT-12 and EVC cells were incubated with TBCA 10^{-6}M and NET 10^{-7}M alone respectively, or in combination for 1h. After mRNA isolation and reverse transcriptase PCR, the quantity of TFF1 cDNA in relation to the PDH cDNA was measured in the LightCycler System. The quantity of TFF1 cDNA in stimulated cells was normalized to ethanol (10^{-3}M) stimulated control cells. (Means±SD; *p < 0.05; **p < 0.01 vs.NET/E2 alone).

4 DISCUSSION

4.1 The effect of estrogen/progesterone on breast cancer cell proliferation

4.1.1 Proliferative effect of progestogens on breast cancer cells

For the past few years we have been performing in vitro experiments with MCF-7 cells overexpressing PGRMC1. At first, we showed in functional studies (Neubauer et al. 2009) that progesterone conjugated with BSA-FITC, which is membrane-impermeable, was able to increase the proliferation of MCF-7/PGRMC1 cells by 36% independent of the classical progesterone receptor. Unconjugated progesterone did not show any effect. In 2008, this work was awarded with a special prize for breast cancer research at the World Congress on Menopause in Madrid. In addition, this progesterone complex was able to increase the mRNA level of vascular endothelial growth factor A (VEGF-A) three-fold as compared to control cells (Neubauer et al. 2009).

These in vitro results correlate with in vivo studies by Peluso and colleagues (Peluso et al. 2009) using a mouse model with PGRMC1 expressing SKOV-3 ovarian cancer cells and PGRMC1-depleted SKOV-3. The microvasculature of tumors established by transplanted PGRMC1-depleted cells was only 14% of that of tumors derived from parental SKOV3-cells. In glia cells of the retina, progesterone also stimulated the VEGF expression (Swiatek-De et al. 2007).

In the present study, we tested the main progestins used for hormone therapy or contraception such as the synthetic progestins chlormadinone acetate (CMA), desogestrel (DSG), dienogest (DNG), drospirenone (DSP), dydrogesterone (DYD), levonorgestrel (LNG), medroxyprogesterone acetate (MPA) used in the Women's Health Initiative (WHI) study (Rossouw et al. 2002), nomegestrol (NOM) and norethisterone acetate (NET) reported in the Million Women Study (WMS) in UK (Beral et al. 2003) as well as the natural progesterone (P4) applied in the French E3N cohort study (Fournier et al. 2008). Since small modifications of their chemical

structure could change their property to proliferate breast cancer cells. In addition, we applied for the first time a new cell model, T47D, which was also stably transfected with the same PGRMC1-WT and empty vector plasmids.

As shown above, for the MCF-7 cell model, P4 and NOM act neutrally, whereas DNG, MPA, LNG and NET increase cell proliferation and thus may increase breast cancer risk. The actual results confirm our previous ones that NET is the progestin resulting in the strongest proliferative effects on WT-12 cells, and DNG, DYD and MPA were active only at the highest concentration tested (10^{-6}M), whereas P, CMA and NOM had no significant effect.(Ruan et al. 2012).

These differences can probably be aligned with the two different structural deviations: progestins are structurally related to testosterone (DNG and NET) as well as MPA, a pregnane derivative with androgenic properties, and acting proliferative, whereas progesterone and progesterone-related progestins (CMA and NOM) were neutral with the exception of DYD. The reason for this discrepancy is not known so far. Perhaps the effective metabolite dihydrodydrogeterone may be informative. DSP, a derivative of spirolactone, also displays a strong proliferative effect.

For the first time, another cell model T47D was included in this study. The proliferative effect of progestogens on T47D wild-type cells (WT-3) was similar to the results of MCF-7 wild-type cells (WT-12). However, in this experiment, CMA also triggered a relatively low, yet significant proliferative effect at both concentrations tested (10^{-7}M, 10^{-6}M). Interestingly, as control, T47D EVC and untransfected T47D cells appeared to be more sensitive to various progestins compared with MCF-7 control cells. Even P4 and NOM, which was total neutral in the MCF-7 model, had significant effect on T47D EVC and transfected T47D cells. Due to the exact same condition of these two experiment (including incubation time period, dissolution of progestogens, detective method, etc.), the distinction may arise only from the individual differences of MCF-7 and T47D.

T47D and MCF7 are two human hormone-dependent breast cancer cell lines which are widely used as experimental models for breast cancer studies. They were both originally derived from a metastatic site of pleural effusion (ATCC, www.atcc.org) and express estrogen receptors. Aka and colleges (Aka et al. 2012) reported that more than 164 proteins are differentially expressed between them. Specifically, Proteins involved in cell growth stimulation, anti-apoptosis mechanisms and cancerogenesis are more strongly expressed in T47D than in MCF7. These proteins include G1/S-specific cyclin-D3 and prohibitin. Proteins implicated in transcription repression and apoptosis regulation, including transcriptional repressor NF-X1, nitrilase homolog 2 and interleukin-10, are, on the contrary, more strongly expressed in MCF7 as compared to T47D.

4.1.2 Proliferative effect of estrogens on breast cancer cells

Although estrone (E1), estradiol (E2), estrol (E3) and estetrol (E4) are all endogenous estrogens, the functions of them are disparate: Estradiol (E2) is the predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. When a woman is pregnant, estriol (E3) is the predominant estrogen, which replaces estradiol (E2). Estetrol (E4) is only produced only during pregnancy by the fetal liver. When a woman reaches menopause, estrone (E1), a weaker form of estrogen, becomes predominant. Estradiol (E2) is the strongest of estrogens and available before menopause or pregnancy (Coelingh et al. 2008; Holinka et al. 2008; Ingle et al. 2014). E2 is still the most frequently used estrogen in menopausal hormone therapy (MHT) for postmenopausal women, as reported by many observational studies and randomized controlled trials, including in the Million Women Study (WMS) (Beral et al. 2003), whereas conjugated equine estrogens (CEE) was used in the WHI hormone therapy trials (Rossouw et al. 2002). CEE is a mixture of estrogens isolated from horse urine. Two of the major components of CEE are E1 (52.5-61.5%) and equilin (22.5-30.5%). E1 can be normally found in women; However Equilin is not, so there has been interest in the effects of equilin on the human

body (Sawicki et al. 1999). There also other Concomitants in CEE, such as 17α-Dihydroequilin (13.5-19.5%) and 17β-Dihydroequilin. Ethinyl estradiol (EE) is a derivative of E2, which is an orally bioactive estrogen used in many formulations of combined oral contraceptive pills. It is one of the most commonly used medications for this purpose.

Regarding the proliferative effect of different estrogens on MCF-7 cells, the following interesting results were observed in our study. Estrone (E1), estradiol (E2), estrol (E3), estetrol (E4), ethinyl estradiol (EE) and equilin (Eq) are able to increase cell proliferation in a concentration-dependent fashion. Only 17α-Dihydroequilin was inactive. At the lowest concentration of 10^{-12}M, E1, E2, E3 and E4 had no effect whereas EE and Eq significantly increased the proliferation rate. At the highest concentration 10^{-9} M, all these estrogens acted proliferatively. As for control cells, MCF-7 EVC and untransfected MCF-7 cells did not demonstrated significant elevation. And again, for T47D WT-3 cells, a similar increase of proliferation after stimulation of various estrogens was observed. Even T47D EVC and untransfected T47D cells exhibited a low yet significant dose-dependent enhanced proliferation.

These results showed that the estrogenic effect on MCF-7 WT as well as T47D WT cells is clearly dose-dependent. E1, E2, E3, E4, EE and Eq elicited a significant increase in cell proliferation only at higher concentration (10^{-10}M). Thus choosing a low-dose oral or transdermal administration may be of importance in the presence of PGRMC1. However, the other publication demonstrated the opposite. Gérard and colleges revealed that E4 was 100 times less potent than E2 to stimulate the proliferation of human breast epithelial (HBE) cells. In addition, E4 exhibits antagonistic properties towards the proliferative effect of E2 on breast epithelial cells (Gérard et al. 2014).

Furthermore, in comparison to breast cancer cell lines overexpressing PGRMC1, the stimulation of estrogens did not or did only induce low proliferation rate of the

empty vector control cell lines, which may suggest that PGRMC1 enhances the sensitivity of breast cancer cells towards a strong estrogenic proliferative effect via an interaction of PGRMC1 and ERα.

4.1.3 Proliferative effect of progestogens/estrogens combination on breast cancer cells

The role of progestogen addition to estrogen therapy in the postmenopause has come under scrutiny since the results of the Women's Health Initiative (WHI) estrogen-only arm were published as compared to the WHI combined arm (Rossouw et al. 2002; Anderson et al. 2004). In comparison to the estrogen-only arm, in which a reduction of breast cancer risk was observed, in the combined arm increase of breast cancer risk was found. The French E3N cohort study has reported, using micronized progesterone in combination with estrogens, no increase in breast cancer risk when combining transdermal (patches) or percutaneous (gels) estradiol therapy with progesterone (Fournier et al. 2008). In 80377 women, breast cancer risk was increase with oral synthetic progestins, but not with progesterone and dydogesterone.

Consistent with our previous studies, we found in the present study that the addition of progestins to high concentration of E2 (10^{-10}M) does not influence the estradiol-induced MCF-7 proliferative. However, when adding the progestins to lower concentrations of E2 (10^{-14}M, 10^{-12}M), all progestins were effective in eliciting a proliferative effect as a mono substance also increased the proliferation, and NET showed the greatest effect. However, when we include another estrogen, EE, in this experiment and raise the concentration to 10^{-10}M and 10^{-9}M, the combination of EE and CYP, DSP, LNG and NET can still elicit significantly proliferation of MCF-7 WT-12 cells, which proved again that various estrogens and progestogens function differently.

Moreover, for the newly transfected T47D cells, the sensitivity was obviously higher than the MCF-7 cells. Significant increase of proliferation was found even in the

control cells T47D EVC. Interestingly, for the MCF-7 cells, no proliferative effect was elicited by CMA, NOM and P4, which were proved to be neutral in breast cancer risk when combined with E2, at least in women overexpressin PGRMC1. However, except P4, the combination of E2 with both CMA and NOM elicited significant increased proliferation of T47D WT-3 cells.

These effects was more pronounced in the continuous combined treatment than in the sequential therapy, i.e. 6 days combined treatment than the sequential therapy versus 3 days estrogens alone plus 3 days estrogen plus progestin. Collectively, results from this study provided evidence at the molecular level that differing regimens of menopausal hormone therapy (MHT) can cause disparate consequences. It would imply that the common regimen of continuous combined MHT may have adverse consequences whereas a sequential regimen, which is more physiological, could be an effective strategy to maintain health and function throughout menopausal aging.

At least for a sequential E2/NET combination, these data have been validated in the meantime in in vitro experiments by transplanting MCF-7/PGRMC1 cells in a mouse model developed by Dr S. Hyder (Liang et al. 2007). In this mouse model, MCF-7/PGRMC1-inoculated cells were more sensitive towards estradiol and elicited a stronger proliferative response in the presence of NET as compared to MCF-7 cells containing the vector control (Neubauer et al. 2013)

In summary, the data presented are very important in terms of the positive results of MHT and breast cancer risk in clinical studies so far. They provided an in vitro model that is able to explain the positive effect of MHT on breast cancer tumorigenesis. Furthermore, this effect may depend on the specific chemical structure and various preparations of MHT.

4.1.4 Phosphorylation site mutants of PGRMC1 affect cell proliferation

Protein motifs prediction for PGRMC1 revealed that each phosphorylation site was contained within known kinase recognition motifs (Table 2). P180 and T160 are present within consensus binding sites for ERK1 and PDK1, respectively. S56 and S180 are present within a consensus sequence for acidophilic kinase CK2 phosphorylation. And it also includes two SH2 target sequences (Y138, Y179), one SH3 target sequence (P62), and a tyrosine kinase site (Y112).

Since PGRMC1 contains several sites for phosphorylation, we transfected MCF-7 cells each of the PGRMC1 expression plasmids (Table 5), in which specific position is modified, namely MutA (S56A), MutB (S180A), MutC (S56A/S180A), WT-12 (wild-type) and EVC (empty vector) and established stable transfected cell lines. Expression of exogenous PGRMC1 was confirmed in all cell lines using an anti-HA tag antibody.

Unequal effects of estrogen/progestrogens on proliferation of various MCF-7 cells were detected in the present study. Cells with mutants on both N-terminal putative CK2 site (S56) and C-terminal CK2 site (S180), i.e. MutC, did not response to any stimulation. Because in MutC cells, the amino acid serine at position 56 and 180 is modified so that PGRMC1 cannot be phosphorylated by a kinase, which correlates with the assumption that phosphorylation of these sites may activate PGRMC1. By contrast, MutA and MutB cells showed practically the same increase of proliferation, compared with WT-12 cells, after stimulation of estrogens or progestrogens, which may suggest phosphorylation at only one of the site, i.e. S56 or S180, inactivates the receptor.

Similar result was detected in one previous study. Neubauer and colleges (Neubauer et al. 2008) found, using the same cell model, that the degree of viability was greatly impaired in the presence of the H_2O_2 for all cell lines except the S56A/S180A double mutant. The expression of PGRMC1 double mutant substantially reduced the sensitivity of cells to H_2O_2 stress, resulting in higher cell viability. The possible mechanism of survival of the S56A/S180 mutant deserves

some consideration. Phosphorylation of S56 presumably blocks phosphorylation of the interaction of PGRMC1 with another protein(s) through the predicted proline-rich SH3 target domain centered on P62, whereas phosphorylation of S181 presumably blocks phosphorylation of the adjacent Y179, which would be necessary for interaction with one or more presumed SH2-domain proteins (Cahill. 2008) Phosphorylation at certain site of may play a critical role in tumorigenesis mediated by hormone and may alter breast cancer risk in postmenopause women.

4.2 A potential kinase correlated with PGRMC1 phosphorylation, CK2

Protein kinase CK2 (official acronym for casein kinase 2 or II) is involved in cell proliferation and survival, and found overexpressed in virtually all types of human cancer, including breast cancer. Results from Neubauer and colleges (Neubauer et al. 2008) revealed that PGRMC1 is more abundant in ERα-negative tumors, but is more highly phosphorylated in ERα-positive tumors. Both acceptor serines of the predicted CK2 sites have been detected as phosphoserine peptides in PGRMC1 from HeLa cell nuclear extracts (Beausoleil et al. 2004). S180 was also detected in PGRMC1 from human mitotic spindle preparations (Sauer et al. 2005), and from a trypsin digest of total mouse liver protein extract (Jin et al. 2004). Because phosphorylated peptides are notoriously difficult to detect, it is possible that S56 was phosphorylated in all those samples. Moreover, it has also recently been demonstrated that several tamoxifen resistant breast cancer cell lines showed greater susceptibility to apoptosis upon inhibition of CK2 compared to tamoxifen sensitive MCF-7 cells (Yde et al. 2007).

Several lines of evidence suggested that CK2 is a potent suppressor of apoptosis in response to diverse apoptotic stimuli-thus its molecular down-regulation or activity inhibition results in potent induction of cell death (Qaiser et al. 2014). CK2 inhibition in cells causes rapid early decrease in mitochondrial membrane potential ($\Delta\psi m$), which may be a primary trigger for apoptotic signaling and cell death. Yao and colleges (Yao et al. 2007) also observed that a synthetic highly selective CK2

inhibitor, tetra-bromo-cinnamic acid (TBCA), could reduce prostate cancer cell proliferation in a dose-dependent manner resulting from CK2 inhibition. However, in our study, TBCA alone appeared to have no effect on MCF-7 cell lines which did not receive any hormone stimulation. Although TBCA reversed, not fully yet significantly, the proliferation of several cell lines triggered by E2 and NET, no dose-dependent effect in the titration experiment was detected. To ensure these results, the possible universal effect of TBCA was also excluded by using GF as the stimulation. In summary, all our data strongly suggest that CK2 was correlated with the hormone-induced proliferation of breast cancer cells, and the inhibition of CK2 greatly undermined this effect.

Interestingly, the reductive effect of TBCA appeared unequally on different PGRMC1 mutants: the proliferative effect of NET cannot be blocked in MutA cells, but can be reversed in MutB and WT-12 cells when NET was at a higher concentration range (10^{-8}M, 10^{-6}M), and with respect to E2-induced proliferation, the reductive effect of TBCA was clearly stronger. A study from Williams and colleges (Williams et al. 2009) revealed that estrogen receptor α (ERα) contains also several putative phosphorylation sites, and identified protein kinase CK2 as a kinase that phosphorylated two of them, i.e. S282 and S559 using motif analysis, in vitro kinase assays, and incubation of cells with CK2 kinase inhibitor. Therefore we conjecture that, if CK2 phosphorylated PGRMC1 and ERα simultaneously, the inhibition of phosphorylation on ERα may enhance the reduction of cell proliferation caused by inhibition of phosphorylation on PGRMC1, resulting in an unequal suppression of TBCA. However, E2 and NET are actually two different hormone components, thus, direct comparisons are inappropriate. Supposedly, this unequal effect could also be caused by the unpaired concentrations of E2 and NET.

To further demonstrate the involvement of CK2 in the phosphorylation of PGRMC1 and whether the phosphorylation of PGRMC1 was regulated by hormones, MCF-7 WT-12 and mutants were pretreated with TBCA, followed with E2 or NET, cell lysates were then subjected to western blot using a phosphor-specific PGRMC1

antibody (pS180). This antibody provides a tool to validate the phosphorylation site S180 and to begin to assess the functional significance of this site.

PGRMC1 in MutA and WT-12 cells was intact at the site of S180A. Thus, a strong phosphorylation at S180 was observed in these cells. The result suggests that the stimulation of NET did indeed lead to phosphorylation of PGRMC1 at the site S180. Additionally, the stimulation of E2 also phosphorylated PGRMC1 at the same site, which may extend the knowledge of the mechanism of E2-regulated breast cancer cell proliferation. However, TBCA did not suppress phosphorylation of s180a in MutA and WT-12 cells indicating that CK2 may not be responsible for the phosphorylation of S180 of PGRMC1. The western blot data was unexpected and completely contrary to our assumption. It is possible that other acidophilic kinase was involved, so identifying the right kinase for the phosphorylation of PGRMC1 will be an interesting future direction of the research. However, the experiment was performed only once, thus, the inaccuracy should also be taken under consideration.

4.3 Cross-talk between PGRMC1 and ER

4.3.1 Tamoxifen blocks the proliferative effect of E2

Recently, an increasing number of studies have found that estrogen can exert its action through not only the traditional genomic but also an extranuclear estrogen receptor (ER) pathway (Irsik et al. 2013; Levin. 2009). And also for many actions of progesterone, besides the intracellular transcription factors, i.e. PR-A and PR-B, a rapid, cell surface-mediated action is also involved (Revelli et al. 1998; Norman et al. 2004; Thomas. 2012). The extranuclear estrogen receptor includes membrane-associated receptors and cytoplasmic receptor (Lappano et al. 2013). Because estrogen receptor (ER) has no intrinsic transmembrane domain and/or kinase domain, the cytoplasmic ER requires association-proteins to translocate it to the plasma membrane and trigger the cytoplasmic pathway. The extranuclear ER pathway is involved in several crucial cellular functions such as cell proliferation,

migration, secretion, and apoptosis (Bolli et al 2011; Cortez et al. 2010). Knowledge on these novel estrogen actions is now significantly broadening our understanding of breast carcinogenesis, particularly regarding metastasis and drug resistance (Chakravarty et al. 2010; Williams et al. 2013). However, mechanisms underlying rapid extranuclear responses of estrogen signal are not yet fully understood (Williams et al. 2013; Acconcia and Marino. 2011).

Data from the present study demonstrated that E2 induced a rise in the proliferation rate of PGRMC1-overexpressing MCF-7 cell line. And this E2 – regulated proliferative effect could be fully reversed in PGRMC1-overexpressing MCF-7 cell line, but not EVC control cells, when the cells were pretreated by tamoxifen, an antagonist of ER in breast tissue, at a higher concentration. This may suggest an involvement of PGRMC1 in the estrogen-regulated ER signaling pathway. Our previous studies also showed that the presence of ERα is mandatory for the observed proliferative effect, since only fulvestrant was able to totally block the proliferative effect of an estradiol/norethisterone combination (Ruan et al. 2012). Thus, the effect of progestogens appears to be mediated not only via the classical genomic pathway involving nuclear receptors (PRs) but also including non-nuclear receptors such as PGRMC1 which may act via a cross-talk with the nuclear or the extranuclear ER receptor. In addition, the similar blocking effect of tamoxifen was also detected in PGRMC1 mutants, namely, MutA and MutB, but not in MutC. This may indicate that not only PGRMC1 mediates the intracellular estrogenic effects though ERα, but the integrity and phosphorylation of PGRMC1 play also a crucial role.

4.3.2 Estrogen and progetogens affect the transcription level of TFF1

Trefoil factors 1 (TFF1, formerly pS2), a small cysteine-rich acidic peptide consisting of 60 amino acids (Thim. 1989), is regarded as an indicator of the intact ER signaling pathway (Henry et al. 1990; Corte et al. 2006). The TFF1 gene contains an estrogen response element (ERE) and its expression can be regulated

by estrogen. To be specific, estrogen-bound ER is recruited to estrogen response elements (ERE) within the TFF1 promoter, resulting in an induction of TFF1 transcription (Espino et al. 2006). Estrogen stimulation of an estrogen-dependent breast cancer cell line induce significant (up to 100 fold) increase of TFF1 mRNA as well as an increase of protein level (Masiakowski et al. 1982). Moreover, some studies suggested that TFF1 expression might be useful in identifying the subgroup of ER-positive breast cancer patients being more responsive to aromatase inhibitors (AI) than to Tamoxifen (Zhou et al. 2011).

Similar result was observed in the present study: E2 stimulation could significantly induce the TFF1 transcription in both MCF-7 and T47D PGRMC1-overexpressing cell lines, as well as MCF-7 control cells. However, the rise of TFF1 expression appeared not to be dose-dependent, and the most efficient concentration for E2 was 10^{-9}M. Additionally, for the first time, we discovered in the present study that, like estrogen, progestegen stimulation can also induce TFF1 transcription. Because when the same experiment was performed using NET as the stimulation, the effect of increased TFF1 expression was again detected at the concentration of 10^{-7}M. Moreover, compared with the elevated effect of E2 and NET alone, the combination of E2 and NET did not significantly further induce the transcriptional level of TFF1. Taken together, these findings would support our assumption that PGRMC1 responding to stimulation of estrogen and progestogen and then transduced the signal, via a cross-talk with ER, to induce ER related gene transcription. Interestingly, although T47D presented a higher sensitivity to stimulations in the proliferation experiment compared with MCF-7 cells, the rise of TFF1 transcription in T47D cells was not as strong as it in MCF-7 cells.

In comparison to the classic genomic steroid action involving activation of the intracellular transcription factors, which typically occurs over a time scale of hours, the novel membrane-associated extranuclear signal transduction pathways is rapid and can be initiated within minutes (Revelli et al. 1998; Norman et al. 2004; Thomas. 2012). Accordingly, in the present study, we performed a series of

experiments to determine the correct time point, when E2 and NET would most effectively activate the membrane-associated signaling pathway. However, within different time periods of 0.5h, 1h, 2h and 4h, no obvious time-dependent effect was found. Due to the narrow range of the time period tested, our data do not clearly demonstrate that time is irrelevant in the membrane-associated signaling pathway. Therefore, a wider range of time period was required in future studies.

With regard to other progestins, at the concentration of 10^{-7}M, P4, MPA and CMA did not trigger any significant up-regulation of TFF1 transcription, whereas DSP and, surprisingly, NOM, which was proved to be neutrally in the proliferation experiment, enhanced the expression of TFF1. So far, we do not have explanation for this result, but further investigation was required.

Although PGRMC1 is clearly a component of membrane-associated progesterone signaling, its exact role and the mechanisms by which PGRMC1 mediates intracellular signaling are unclear, and limited data on signaling pathways through PGRMC1 suggest that its action is indirect, and it may act as an adaptor protein for a wide variety of proteins, such as multiple P450 proteins, including steroid regulatory element-binding protein cleavage activating protein (Scap), insulin-induced gene and epidermal growth factor receptor (EGFR) (Ahmed et al. 2010). And some of these downstream proteins may be keys of the cross-talk with the extranuclear ER signaling pathways. For instance, EGFR has been previously designated as a mediator of the non-genomic effects of E2. And initial evidence has been obtained that cell surface expression of EGFR is associated with PGRMC1. A recent publication demonstrated that estrogens and tamoxifen do not only exert their effects at the genomic level, but also function at the cell membrane activating downstream signaling pathways, since blocking EGFR would reverse the inhibitory effect of tamoxifen on breast cancer cells (Raffo et al. 2014).

4.3.2 Inhibitors block the transcription level of TFF1

In the last part of the present study, three inhibitors was applied, fulvestrant, AG205 and TBCA. Fulvestrant is widely used as a selective estrogen receptor down-regulator for ER positive breast cancer in postmenopausal women, which can effectively inhibit estrogen signaling in breast cancer. And as a PGRMC1 inhibitor, AG205 alters the spectroscopic properties of the PGRMC1-heme complex. And finally, TBCA is a CK2 inhibitor as described above. As expected, Inhibition of both ER and PGRMC1 signaling with fulvestrant and AG-205, respectively, causes a statistically significant decrease in TFF1 expression in MCF-7 and T47D breast cancer cells overexpressing PGRMC1, but not in empty vector control cells. And a reduction of proliferation rate in breast cancer cells was previously observed in our laboratory, when MCF-7 cells were treated with these inhibitors (Ruan et al. 2012). Taken together, these results demonstrate not only that maintenance of PGRMC1 signaling is required for TFF1 expression, but also the involvement of both PGRMC1 and ER during the estrogen/progestogen-induced TFF1 transcription.

Although in terms of protein levels, we failed to prove that CK2 was the right kinase which led to PGRMC1 phosphorylation, cell proliferation rate in the MTT experiment was dramatically reduced after the incubation of TBCA. So far, we did not have a better explanation for these conflict results, but the suppression of TFF1 transcription in MCF-7 and T47D breast cancer cells overexpressing PGRMC1 caused by TBCA may again indicate a correlation between PGRMC1 phosphorylation and ER status in the intracellular signaling pathway. Furthermore, pharmacological targeting of the cell signaling pathways that regulate PGRMC1 phosphorylation at certain sites may represent therapeutic targets for modulating ER function.

5 SUMMARY

As a treatment, menopausal hormone therapy (MHT) is commonly recommended clinically to relieve postmenopausal symptoms, such as hot flushes and sweats, and to address long-term biological changes, such as bone loss, that result from declining levels of the natural hormones in postmenopausal women. However, since the early 1940s, when estrogen was first introduced into clinical practice, the concern that MHT may cause breast cancer has existed. Therefore, various methods were applied, including case reports, case-control studies and recently some large prospective cohort studies, to provide more reliable evidence. Consequently, the concepts about MHT and breast cancer have also changed over time. According to previous research data from our laboratory, we believe that except for the traditional intracellular-located hormone receptors, membrane-associated signaling pathway which may be activated via progesterone receptor membrane component 1 (PGRMC1) is also important in terms of MHT related breast cancer risk.

To further understand the possible breast cancer risk and the mechanisms related, MCF-7 and T47D cells were stably transfected with PGRMC1 and mutants, respectively. In the present work, the influence of modification of different PGRMC1 phosphorylation sites together with various preparations of estrogens and progestogens on the proliferation of breast cancer cell lines was determined. Moreover, the potential estrogen receptor (ER)-regulated kinase, CK2 (official acronym for casein kinase 2 or II), that may participate in the phosphorylation of PGRMC1 was detected. Furthermore, the transcription of an ER reporter gene, Trefoil factors 1 (TFF1, formerly pS2), in these two breast cancer cell models, was evaluated, to prove the involvement of a cross-talk between PGRMC1 and estrogen receptor (ER) in estrogen/progestogen-regulated breast cancers.

Results from the present study clearly demonstrated that different progestogens as well as estrogens can induce, in varying degrees, the proliferation of PGRMC1

over-expressing breast cancer cells. And breast cancer cells containing various PGRMC1 mutans responded differently to stimulations, due to certain PGRMC1 phosphorylation sites. Moreover, both ER and CK2 inhibitor can significantly reverse the proliferative effect of progestogens and estrogens on PGRMC1 over-expressing breast cancer cells, which may indicate the vital role of ER status and CK2 in the membrane-associated PGRMC1 signaling pathway. However, western blot did not provide any strong evidence that CK2 was mandatory in PGRMC1 phosphorylation, although PGRMC1 was indeed phosphorylated after the stimulation of progestogens as well as estrogens. Therefore, an improvement of the current methods of proving the kinase or a thorough search of the right kinase which may cause the phosphorylation of PGRMC1 was required in future study. Furthermore, the data has also exhibited that the transcription of the estrogen-induced ER reporter gene, TFF1, in PGRMC1-overexpressing breast cancer cells, was significantly amplified by the stimulation of various progestogens and estrogens, which may again implicate the importance of ER in the membrane-associated PGRMC1 signaling pathway. Finally, a significant suppression of TFF1 transcription in breast cancer cells overexpressing PGRMC1, caused by three different inhibitors, was observed, which revealed the correlation between PGRMC1, CK2 and ER status. Thus blocking of ER activity by endocrine therapies seems also to be an effective mechanism to reduce breast cancer risk in patients overexpressing PGRMC1 whereby additional blocking by PGRMC1 and CK2-inhibitors may add some benefit in the development of resistance to endocrine therapy.

All the estrogens and progestogens applied in this study are also commonly used as MHT in postmenopausal women. Our results suggest that in terms of breast cancer risk in women overexpressing PGRMC1 kind and dosage of the estrogen and progestogen used should be considered.

6 ZUSAMMENFASSUNG

Menopause Hormontherapie (MHT) ist eine Behandlung, die häufig postmenopausalen Frauen empfohlen wird, um postmenopausale Symptome wie Hitzewallungen und Schweißausbrüche zu lindern, und um langfristige biologische Veränderungen wie Knochenschwund zu behandeln. Doch seit den frühen 1940er Jahren, als Östrogene erstmalig in der klinischen Praxis eingeführt wurden, existierte die Sorge, dass MHT Brustkrebs verursachen konnte. Daher wurden verschiedene Methoden angewandt, einschließlich Fallberichten, Fallkontrollstudien und einigen großen prospektiven Kohortenstudien, um den Zusammenhang zwischen MHT und einem erhöhten Brustkrebsrisiko zu untersuchen. Nach den bisherigen Forschungsergebnissen aus unserem Labor, vermuten wir, dass außer den traditionellen intrazellulären Hormonrezeptoren auch membranassoziierte Signalwege, die über Progesteronrezeptor-Membrankomponente 1 (PGRMC1) aktiviert werden können, in Bezug auf das mit MHT verbundene mögliche Brustkrebsrisiko wichtig sein können. Um das mögliche Brustkrebsrisiko und die zusammenhängenden Mechanismen weiter zu verstehen, wurden MCF-7 und T47D Zellen jeweils mit PGRMC1 und Mutanten stabil transfiziert. In der vorliegenden Arbeit wurde die Wirkung verschiedener Östrogene und Gestagene auf die Proliferation dieser PGRMC1-Varianten untersucht. Als Vertreter für mögliche Signalwege wurde die Caseinkinase 2 (CK2) herangezogen, die vermutlich an der Phosphorylierung von PGRMC1 beteiligt ist. Weiterhin wurde die Transkription eines ER-Reportergens, Kleeblatt-Factor 1 (TFF1, ehemals PS2), gemessen, um eine Interaktion zwischen PGRMC1 und Östrogenrezeptor (ER) am Östrogen/Gestagen-vermittelten Brustkrebs zu untersuchen.

Die Ergebnisse der vorliegenden Studie zeigen deutlich, dass verschiedene Gestagene sowie Östrogene die Proliferation beider PGRMC1-überexprimierenden Mammakarzinomzell-Linien steigern kann. Aufgrund bestimmter PGRMC1 Phosphorylierungsstellen reagierten die verschiedenen PGRMC1 Mutanten unterschiedlich auf die Steroidhormone. Wir konnten beobachten, dass sowohl ER-

als auch CK2-Inhibitoren die proliferativen Effekte von Östrogenen und Gestagenen signifikant eliminierten, was darauf hindeutet, dass der ER-Status und CK2 eine Schlüsselrolle in den membranassoziierten PGRMC1 Signalwegen spielen könnte. Obgleich PGRMC1 durch Stimulationen von Östrogenen und Gestagenen phosphoryliert wird, können die im Western Blot erhaltenen Daten nicht zwingend darauf hinweisen, dass CK2 bei der PGRMC1 Phosphorylierung notwendig ist. Darum wurde entweder eine Verbesserung der derzeitigen Methoden oder eine gründliche Suche nach der richtigen Kinase, die die Phosphorylierung von PGRMC1 verursachen kann, in weiterer Studie erforderlich. Außerdem können wir zeigen, dass sich die Transkription des Östrogen-induzierten ER Reportergens, TFF1, von PGRMC1-überexprimierenden Mammakarzinomzell-Linien durch Stimulation mit Östrogenen und Gestagenen signifikant verstärkt wurde, was die Bedeutung von ER an membranassoziierten PGRMC1 Signalwegen unterstreicht. Wir konnten hierbei beobachten, dass die Zunahme der TFF1-Expression von PGRMC1-überexprimierenden Mamma-karzinomzell-Linien durch drei Inhibitoren (ER-, PGRMC1 und CK2-Inhibitor) signifikant reduziert werden konnte, was auf eine Korrelation zwischen PGRMC1, CK2 und ER-Status hindeutet. Somit ist eine ER-Blockade zur Reduzierung für die beobachtete proliferative Effekt von Östrogenen und Gestagenen erfordlich wäre, würde die Stilllegung von ER die PGRMC1- vermittelte Proliferation auf jeden Fall verringern, was eine interessante Richtung für unsere zukünftige Studien wäre.

Alle Östrogene und Gestagene, die in dieser Studie untersucht wurden, sind üblicherweise als MHT bei postmenopausalen Frauen in Anwendung. Unsere Untersuchungen deuten daraufhin, dass das Brustkrebsrisiko bei Frauen mit erhöhter PGRMC1-Expression durch Art und Dosierung der verwendeten Östrogene und Gestagene beeinflusst werden könnte.

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8 DECLARATION OF AUTHORSHIP

I hereby certify that this thesis has been composed by me in its entirety and is based on my original work, unless stated otherwise. No other person's work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

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