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Role of PGRMC1 in breast cancer progression and increased breast cancer risk upon progestin-based hormone therapy

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1. Introduction

1.1. Cancer: definition and characteristics

Cancer cells are characterized by two essential attributes: they proliferate despite the usual limitations of cell division and are capable of invading adjacent tissues and spreading to distant sites in the body[1,2]. The combinations of these behaviors make cancer cells extremely harmful. An anomalous cell, which grows against the regular control mechanisms, will lead to a tumor or *neoplasm*. As long as the neoplastic cells stay conglobate and do not invade nearby tissue, the tumor is referred to as **benign** (non-cancerous). A tumor is referred to as **malignant** (cancerous) or as cancer, if the cells are able to invade to surrounding tissue or spread to other parts of the body (figure 1)[1,3].

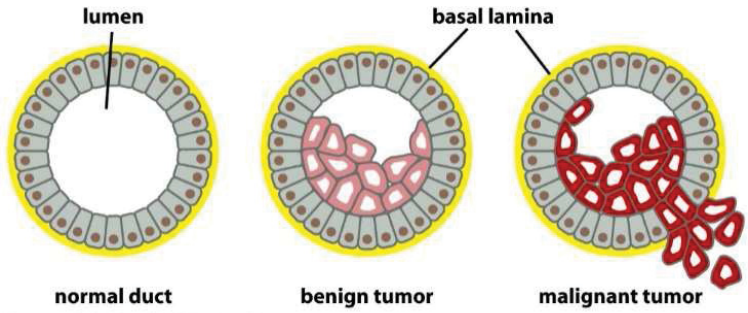


Figure 1. Benign and malignant tumors.
A benign tumor remains within the basal lamina, which marks the boundary of the normal structure. A malignant tumor destroys the integrity of the tube as illustrated. *Molecular Biology Of The Cell, Alberts B., 2011*[1]

This invasive growth, which is an essential characteristic of cancer cells, enables cells to detach from the primary tumor, reach the blood vessels or the lymph system and form secondary tumors or metastases in distant organs. The broader a tumor spreads, the harder it gets to eradicate it, which is why most deaths from cancer are due to metastatic disease[2,4]. Metastatic spread - the development of metastases - is the least understood aspect of carcinogenesis and is responsible for 90 % of cancer caused mortality[5].

Tumors are classified by tissues and cell types, they grew out of. With a share of 80 %, tumors from epithelial cells are the most common cancer type in humans and are referred to as **carcinomas**. Tumors, which develop from connective tissue or muscle cells, are termed **sarcomas**[1,2,6]. Besides, there are cancer types which cannot be categorized into these two groups, such as the different types of leukemia and lymphoma, which develop from hematopoietic cells, and tumors from cells of the nervous system. Cancer types, which

derive from different cell types often lead to very distinct disorders, which is why *cancer* is not one disease but rather a term for a large family of diseases[1–3].

The transformation of cells from benign to malignant behavior, characterized by uncontrolled growth of tumor cells and the ability to spread to distant regions of the body, is referred to as **malignant progression**. Although there are many causes for malignant progression, two major underlying commonalities of cancers are genetic mutations and altered epigenetic regulation[1,7]. Genetic mutations either result from germline mutations, i.e. a mutation that is passed on to descendants through their reproductive cells or by acquiring somatic mutations in the DNA over time, due to carcinogenic agents. Epigenetic changes are alterations in the gene expression pattern, which can be caused by variations in heterochromatin packaging or DNA methylation (figure 2)[1,7]. Cancer-critical genes can in general be divided into two groups - **tumor suppressor genes** and **oncogenes** - depending on whether their loss or gain of function contributes to cancer development[7]. A loss of function of tumor suppressor genes turns off cell inhibition mechanisms that under normal conditions prevent uncontrolled growth. In contrast, oncogenes stimulate cells to proliferate in case of gain of function mutations. However, a single genetic and/or epigenetic alteration is not sufficient to turn a normal cell into a malignant cell; it can only be achieved by several changes[1,7].

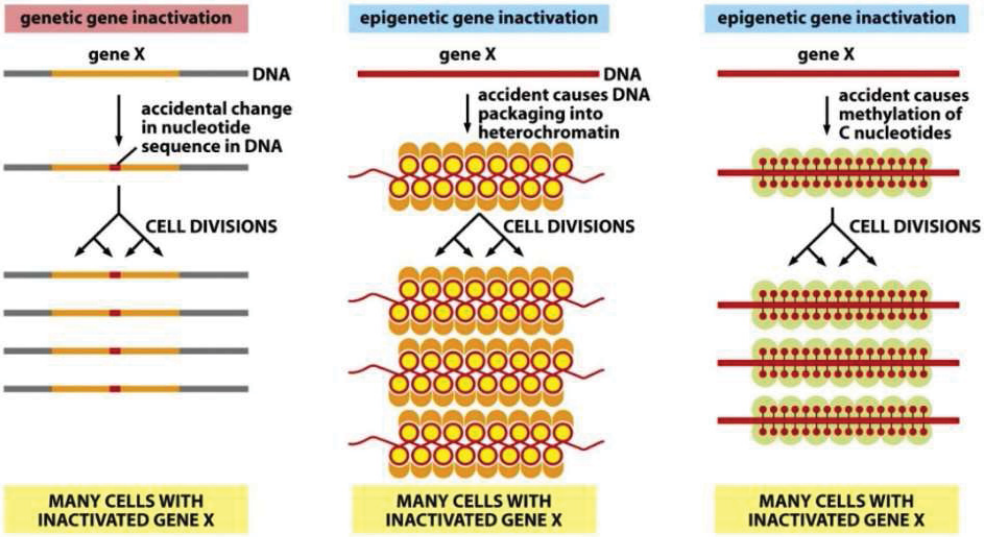


Figure 2. Comparison of genetic and epigenetic changes in tumors.
A mutation results from an irreversible change in the DNA sequence. In contrast, epigenetic changes are either caused by site-specific changes in histone modification or by site-specific DNA-(de)methylation. *Molecular Biology Of The Cell, Alberts B., 2011*[1]

The process of accumulation of alterations which changes normal cells into tumor cells is the first phase of **carcinogenesis** and is referred to as **tumor initiation**. During **tumor promotion**, the second step of cancer development, the descendants of a single initiated cell are stimulated to survive (resist apoptosis) and undergo clonal growth (expand in number). The third and last phase of carcinogenesis is the actual malignant transformation and is referred to as **tumor progression**[1,4]. The cells have undergone further mutations in tumor suppressor genes and oncogenes and thus acquire greater malignancy, characterized by abnormal growth speed and invasiveness. Cells, which have left the primary lesion and are disseminated to ectopic sites, are called disseminated tumor cells (DTCs). Various studies reason a parallel progression of primary tumors and metastases, suggesting that tumor cells leave the primary tumor before they acquire full malignancy and undergo malignant transformation at distant sites[8]. After numerous cell divisions, a tumor-mass has developed, displacing the healthy tissue. The cells become decreasingly differentiated, i.e. differ from the tissue of origin and exhibit different characteristics (figure 3)[7].

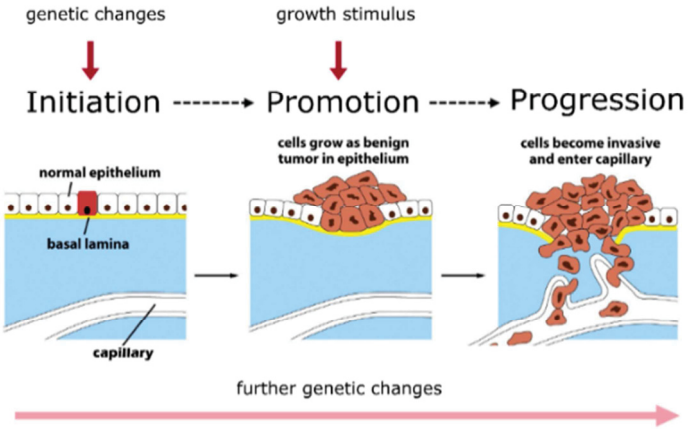


Figure 3. The three steps of carcinogenesis: tumor initiation, tumor promotion, tumor progression
Carcinogenesis is the process of initiating and promoting cancer, i.e. the process by which normal cells are transformed into cancer cells. Adapted from: Molecular Biology Of The Cell, Alberts B., 2011[1]

To be *successful* as a cancer cell, a cell must develop a whole range of abnormal capabilities during its development. Different tumors display different combinations of features. However, cancer cells exhibit basic behavioral characteristics obtained during their multistep development, albeit acquired through different mechanisms[9,10]. In important reviews from 2000 and 2011, Hanahan and Weinberg proposed a list of several functional properties, that most tumors have acquired and are involved in the pathogenesis of tumors, termed the **hallmarks of cancer**, which are (figure 4): (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) avoiding immune destruction, (4) enabling replicative immortality,

(5) tumor promoting inflammation, (6) activating invasion and metastasis, (7) inducing angiogenesis, (8) genome instability and mutation, (9) resisting cell death and (10) deregulating cellular energetics[9,10].

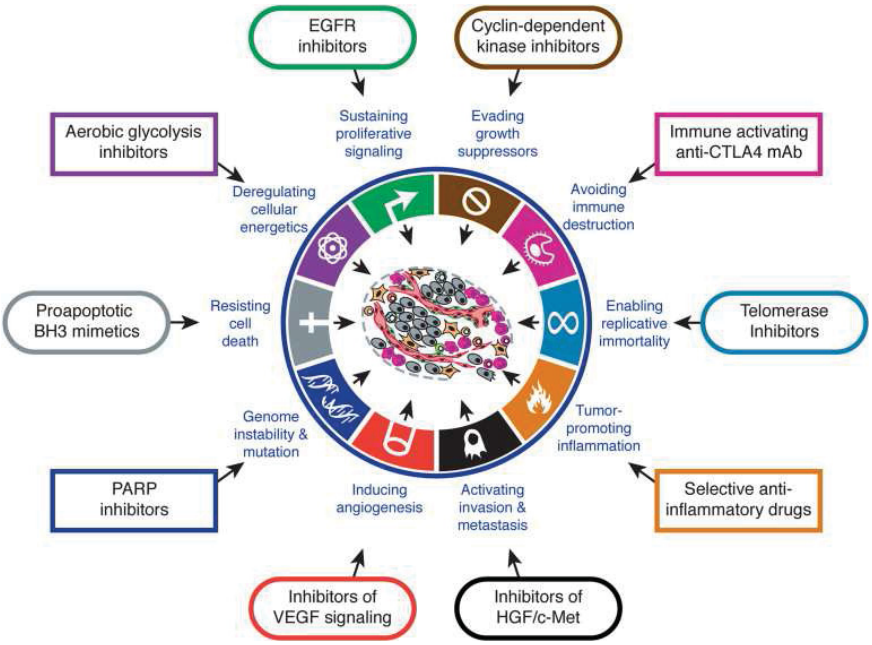


Figure 4. The hallmarks of cancer
The figure pictures the 10 hallmarks of cancer postulated by Hanahan and Weinberg and examples of therapeutic targeting of the hallmarks of cancer. Drugs are and were developed to interfere with and to target each of the acquired capabilities necessary for tumor growth and progression. Hallmarks of Cancer: The Next Generation, Hanahan D, Weinberg R. A., *Cell*, 2011[10].

Knowing this collection of subversive abilities and the various mechanisms of tumor cells to acquire those, are important to understand the pathogenesis of cancer and to fight tumors in a targeted way. Thus, over the last decades, a large number of mechanism-based anti-cancer drugs have been developed to treat cancers. Examples of therapies developed to target each of the hallmarks of cancer necessary for tumor growth and progression are depicted in figure 4[10].

1.2. Breast cancer

1.2.1. Epidemiology

In 140 of 184 countries, breast cancer is the most frequently diagnosed cancer among women and thus the most common cancer in women worldwide (figure 5)[11,12].

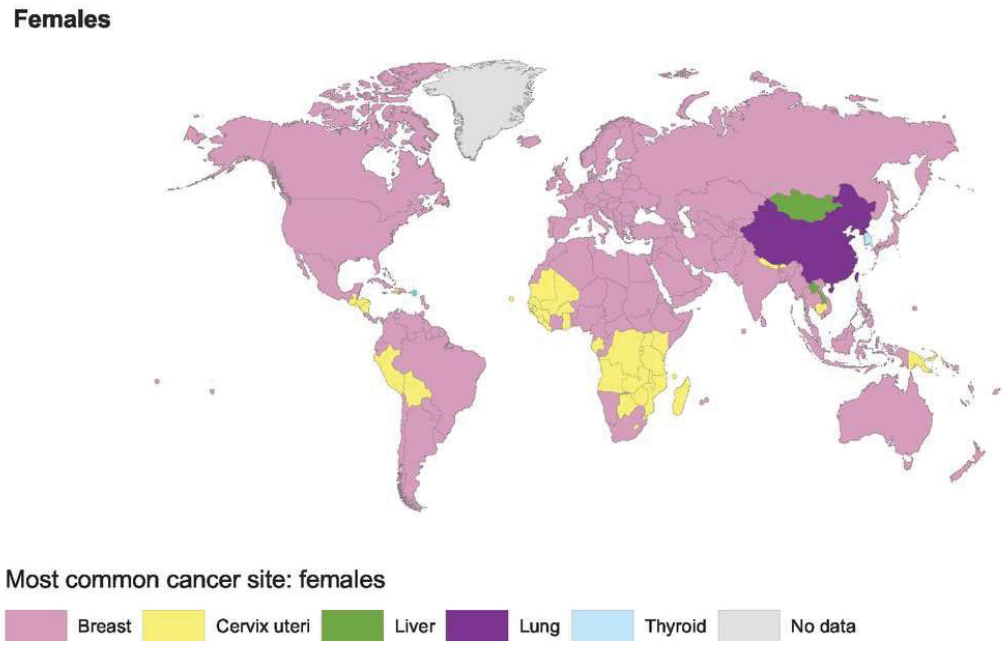


Figure 5. Top cancer for females per country
Estimated age-standardized rates of incident cases, worldwide in 2012. Data source: GLOBOCAN 2012, Graph production: Cancer Today, © International Agency for Research on Cancer 2016[12].

Due to screening programs and various therapy options, prognosis for breast cancer is generally good in developed countries. In contrast, survival in economically developing countries is relatively poor, because diagnosis is often made late during or even after progression of the disease and treatment options are limited[11,13].

Breast cancer accounts for 25 % of all new cancer cases in women every year, way ahead of other cancer types, such as colorectal cancer (9.2 %) and lung cancer (8.8 %) (figure 6)[11,12].

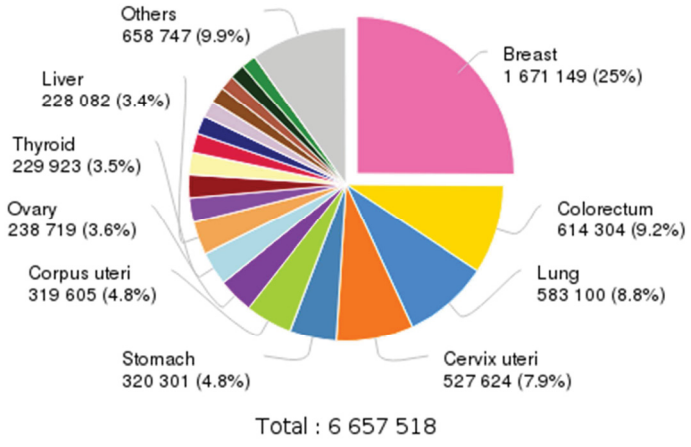


Figure 6. Top 20 cancer sites for females
Estimated number of incident cases, females, worldwide (top 20 cancer sites) in 2012.
Data source: GLOBOCAN 2012, Graph production: Cancer Today, © International Agency for Research on Cancer 2016[12].

In 2012 about 1.7 million new breast cancer cases were diagnosed worldwide and estimated 521,900 women died from breast cancer, making it the leading cause of cancer death among women (figure 7)[11]. Breast cancer incidence rates between different countries vary largely. Higher incidence rates are observed in Northern and Western Europe, Northern America and Australia, whereas lower rates are reported in Africa and Asia. 47 % of the newly diagnosed breast cancer incidences in 2012 occurred in economically developed countries, which however only represent 18 % of the world population. Migration studies suggest that these differences are caused less by genetic factors but due to environmental factors and differences in lifestyle[11–13].

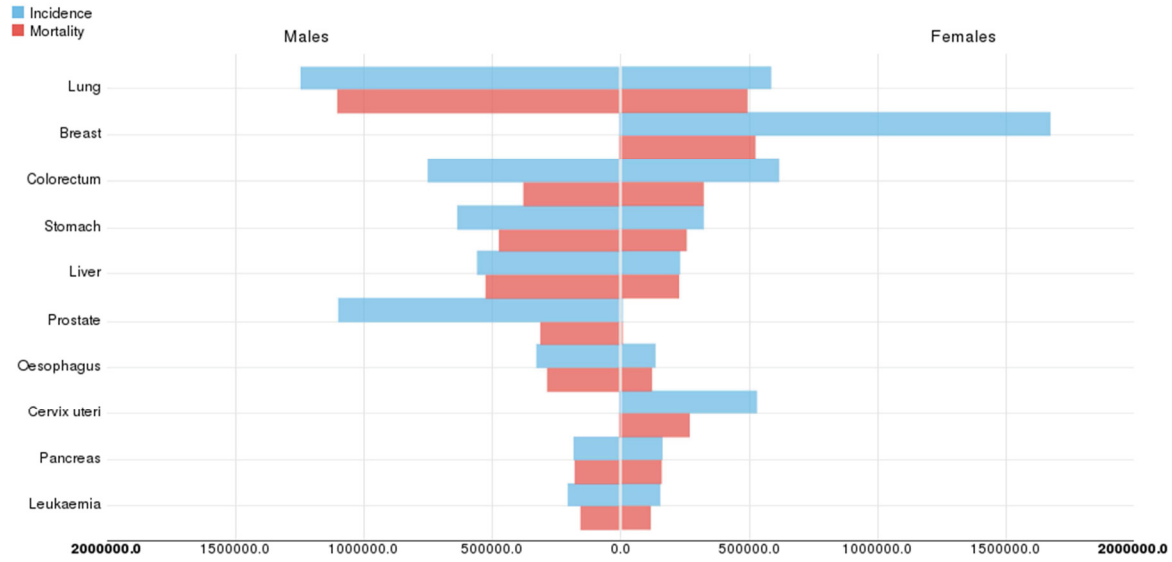


Figure 7. Estimated number of cancer cases for both sexes
Estimated number of cancer cases (incidence and mortality), both sexes, worldwide in 2012. Data source: GLOBOCAN 2012, Graph production: Cancer Today, © International Agency for Research on Cancer 2016[12].

1.2.2. Subtypes and treatment options

Breast cancer can be classified into molecular subtypes based on the expression of distinct receptors. The molecular classification is important for the design of individualized therapies, which significantly improved within the last decades and are among others a reason for decreased breast cancer mortality rates since 1990 in western countries[11,13].

Based on gene signature and expression profiling, four subtypes of breast cancer are defined: luminal A, luminal B, human epidermal growth factor receptor 2-positive (Her2+), and basal like (table 1)[14,15].

Table 1. Molecular subtypes of breast cancer[14]

Subtype	Receptor expression	Proliferation rate	Treatment option
Luminal A	ER+/PR+/Her2+/-	Ki-67 < 14 %	Endocrine therapy
Luminal B	ER+/PR-/Her2+/-	Ki-67 > 14 %	Endocrine therapy
Her2+	ER-/PR+/Her2+	-	HER2-targeted therapy
Basal-like	ER-/PR-/Her2-	Ki-67 50 – 60 %	Chemotherapy

Breast cancer classification by receptor expression. ER: Estrogen Receptor, PR: Progesterone Receptor, Her2: human epidermal growth factor receptor 2, Ki-67: Antigen Ki-67.

Luminal tumors exhibit expression of the hormone receptors estrogen receptor (ER) and/or progesterone receptor (PR), and are further subdivided into the **luminal A- and luminal B-type**, depending on the positivity for PR and the proliferation rate. Since the luminal subtypes grow hormone-dependent, they can effectively be treated with hormone-therapy, such as inhibition of estrogen-synthesis (e.g. aromatase-inhibitors), ER-agonists (tamoxifen) or selective ER degrading agents (fulvestrant)[14,16]. The **Her2+-type** is characterized by amplification and overexpression of the Her2 (ERBB2) oncogene, but absence of ER expression. Her2+ tumors can effectively be treated with anti-Her2 therapies, such as trastuzumab (Herceptin®), a monoclonal antibody, directed against Her2[14,16,17]. **Basal-like** tumors are defined by absence of the three main breast cancer markers ER, PR and Her2. Thus, the majority of these tumors are also referred to as triple-negative breast cancer (TNBC). This tumor subgroup is associated with poor outcome and features the greatest treatment challenge, due to lack of molecular-based targeted therapy[14,17,18]. Therefore, conventional chemotherapy is still the state of the art treatment for this subtype. Nevertheless, the molecular analysis of basal-like tumors has identified potential targets for directed therapy, including immune checkpoint inhibitors (e.g. PD-L1-inhibitors), androgen receptor inhibitors, angiogenesis-inhibitors (e.g. Bevacizumab) and substances that target DNA damage response (PARP-inhibitors)[14,18].

1.2.3. Factors associated with increased breast cancer risk

The breast cancer incidences worldwide increased considerably within the last 40 years (figure 8). In Germany, the rate of new breast cancer incidences per 100,000 citizens for example increased from 53.3 in 1970 to 90.0 in 2007[12].

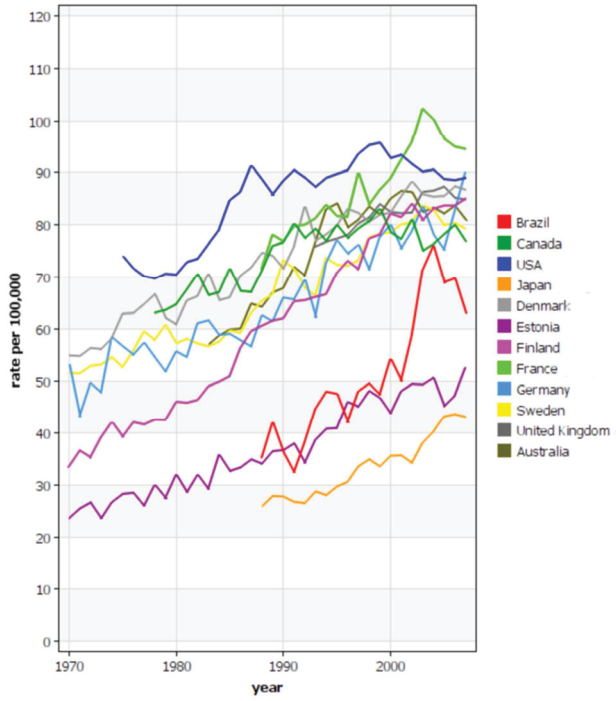


Figure 8. New diagnosed breast cancer incidences per year from 1970 – 2010
New diagnosed breast cancer incidences per year between 1970 and 2010 in developing- (Brazil) and developed (others) countries. Depicted is the breast cancer rate per 100,000. International Agency for Research on Cancer (IARC)[12].

This increase is linked to a set of extrinsic, i.e. lifestyle-related and environmental breast cancer risk factors[11]. However not only external factors, but also intrinsic, i.e. inherited factors play an important role in breast cancer risk. Important intrinsic and extrinsic factors associated with a higher breast cancer risk are: higher age, positive family anamnesis of breast cancer, obesity, lack of physical activity and certain lifestyle habits, such as smoking[11,19–24]. Additionally, hormonal factors play an important role in increased breast cancer risk. Studies indicated that a lower total number of menstruation cycles is associated with a lower risk of breast cancer[25]. Further, women who have had more menstruation cycles, because their menarche started early (< age 12) or went through menopause late (> age 55), have a slightly higher risk of breast cancer[11,26]. This indicates that a longer exposure to the hormones estrogen and progesterone is associated with a higher breast cancer risk. Further, childbirth and younger age at first full-term pregnancy (< age 30) are associated with a lower risk of breast cancer[25,27,28]. Studies revealed that the breast cancer risk decreases with the number of children born; nulliparity increases the risk by 30 %[25,29].
Besides endogenous estrogen and progesterone also exogenous hormones - used in hormone therapy and contraception - have been associated to higher breast cancer risk[30–32].

The increased breast cancer risk upon application of synthetic hormones is important for the studies of this work and will be discussed in more detail in paragraph 1.4.2..

1.3. Steroid hormones and steroid hormone receptors

As described above, accumulating research suggests that prolonged exposure to endogenous ovarian hormones and exogenous synthetic hormones is implicated in breast cancer development[25]. However, the underlying exact mechanism of how female hormones increase the breast cancer risk remains unknown. One out of four breast cancers is hormone dependent, meaning hormones are required for their growth[11,25]. Therefore, endocrine therapy targeting estrogen receptors and estrogen signaling in different ways are an effective tool to combat hormone dependent breast cancer[33–35]. Concluding, it can be said that steroid hormones and their receptors play an important role in breast cancer, and the research and molecular analysis of their interplay in the disease is of particular importance for a better understanding of the carcinogenesis of breast cancer.

1.3.1. Steroid hormones

Cholesterol is a precursor for the five main classes of steroid hormones: (pro)gestagens, glucocorticoids, mineralocorticoids, androgens and estrogens. These hormones are effective signaling molecules, regulating a range of body functions (figure 9)[1,36].

Progestogens, androgens and estrogens are summarized as sex hormones. One distinguishes between male- (androgens) and female sex hormones (estrogens, progestogens), however, female and male individuals, synthesize both groups physiologically[36]. **Androgens** (such as testosterone) regulate the expression of male secondary sexual characteristics. **Estrogens**, such as estrone (E1) or estradiol (E2), regulate the maturation and growth of the internal female sexual organs and the expression of secondary female sexual characteristics. Estrogens, together with progesterone (P4), a progestagen (or gestagen), are responsible for the regulation of the female cycle and pregnancy[36–38]. P4 further primes the uterus for the implantation of the fertilized egg and is responsible for the maintenance of pregnancy[37].

Glucocorticoids, such as cortisol, promote gluconeogenesis as well as glycogen synthesis and increase fat- and protein degradation. They also inhibit inflammatory reactions and enable animals to be responsive to stress[36,37].

The function of **mineralocorticoids** (mainly aldosterone) is the regulation of water and electrolyte balance. In this complex regulation, they play a role in the salt- and water

retention in the body by promoting the reabsorption of Na^+ and the excretion of K^+ and H^+ in the distal tubules of the kidney, leading to an increase in blood volume and blood pressure[37]. Steroid hormones are mainly synthesized in the following organs: gestagens in the corpus luteum, estrogens in the ovaries, androgens in the testes, glucocorticoids and mineralocorticoids in the adrenal cortex[1,37].

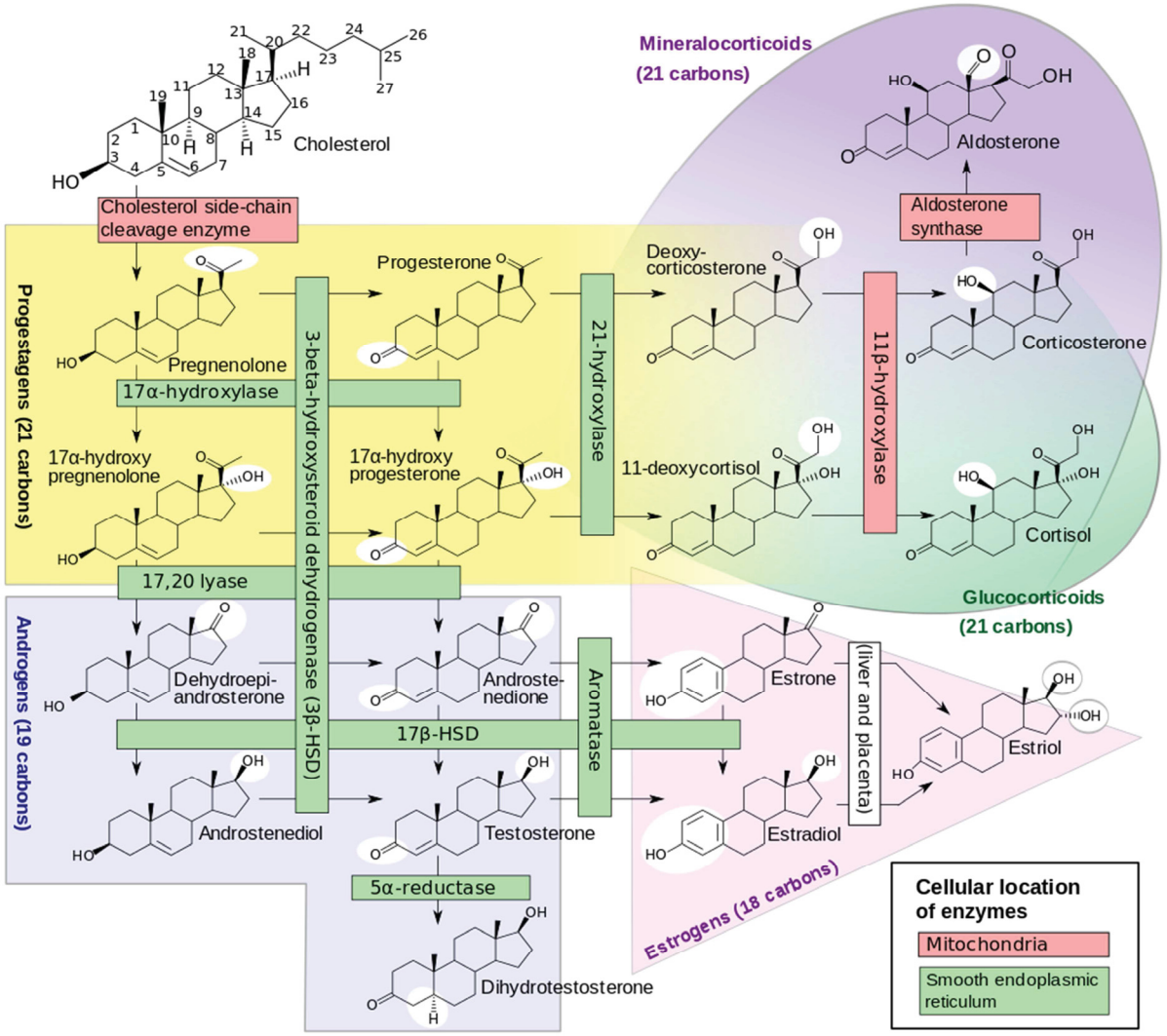


Figure 9. Diagram of the pathways of human steroidogenesis
The figure illustrates the synthesis of steroids from cholesterol and the enzymes involved in steroid biogenesis. Mitochondrial enzymes are colored pink and smooth endoplasmic reticulum enzymes green. This figure provides a schematic simplified topology. Diagram of the pathways of human steroidogenesis, Häggström M, Richfield D, *WJM,* 2014[39].

1.3.2. Steroid hormone receptors

Steroids are hydrophobic molecules and can therefore diffuse through the cell membrane[36]. Within the cell they bind to specific intracellular receptor proteins – the steroid hormone receptors, which can be divided into nuclear steroid hormone receptors and membrane steroid hormone receptors[40,41].

Nuclear steroid hormone receptors

The **estrogen receptors α, β, γ** (ERα, ERβ, ERγ), the **progesterone receptor** (PR), the **androgen receptor** (AR), the **glucocorticoid receptor** (GR) and the **mineralocorticoid receptor** (MR) belong to the nuclear receptor superfamily and are responsible for the signal transduction of steroid hormones inside the cell[40,42]. Like all nuclear receptors, steroid receptors are transcription factors, which are present in the cytoplasm and are translocated into the nucleus after activation. Although the nuclear hormone receptors have very distinct functions, they share a common structure and mode of action[42,43]. Using the **estrogen receptor α (ERα)** as an example, the structural organization and mechanism of action of steroid receptors will be illustrated (figure 10). Nuclear hormone receptors are evolutionarily conserved and are composed of five structural and functional domains: two transcriptional activation function domains (AF-1 and AF-2), a DNA-binding domain (DBD), a variable hinge-domain (H) and a ligand-binding domain (LBD) (figure 10 A)[43–45]. The inactive receptor molecule usually is bound to inhibitor proteins. Binding of the ligand (estrogens or synthetic compounds) to the LBD results in conformational changes of ERα, leading to dissociation of the inhibitor and dimerization of the receptor[45,46]. Different ligands can induce unique conformational changes of ERα and receptor dimerization. The receptor dimer is translocated into the nucleus, where the receptor-ligand complex binds to control elements in the DNA and specifically influences the expression of single genes. The DBD binds to specific positions in the DNA, referred to as estrogen-response-elements (EREs) (figure 10 B)[45].

The binding of a ligand and the associated conformational change causes binding of co-activators, which in turn change the binding of the receptor to the DNA. The co-activators are often enzymes catalyzing reactions that alter the chromatin structure[36]. In other cases, ligand binding can have the opposite effect and induces co-repressors to bind to the receptor and reduce transcription. Known co-activators of ERα are NcoA-1 (nuclear hormone receptor coactivator-1) and SRC-1 (steroid receptor coactivator-1)[45,47]. In contrast,

prohibitin-2 (PHB2) is a known mediator of transcriptional repression of ERα via recruitment of histone deacetylases[48–51]. It competes with NcoA-1 for modulation of ERα transcriptional activity[45,52].

ERα can also be activated in a ligand-independent signaling pathway by phosphorylation of ERα by second messenger signaling cascades in the cell[45,53]. Pathways involved in the ligand-independent activation of ERα are among others the MAPK-pathway, the PI3K pathway and the protein kinase A (PKA) pathway (figure 10 B)[45].

Membrane steroid hormone receptors

Besides the classical genomic pathway via nuclear steroid hormone receptors, steroid signals can also be mediated by a non-genomic pathway via membrane (m) steroid hormone receptors, including mERs, mPRs, mARs, mGRs and mMRs[40,41]. Membrane steroid hormone receptors are usually bound to the plasma membrane and are also referred to as extra-nuclear steroid receptors. The steroid-signal is integrated into intracellular signaling by interaction of the receptors with cytoplasmic signal transduction pathways[40,41,54]. In case of plasma membrane bound ERs (mERs), binding of estrogens e.g. results in activation of MAPK/ERK signaling pathway (figure 10 B)[45].

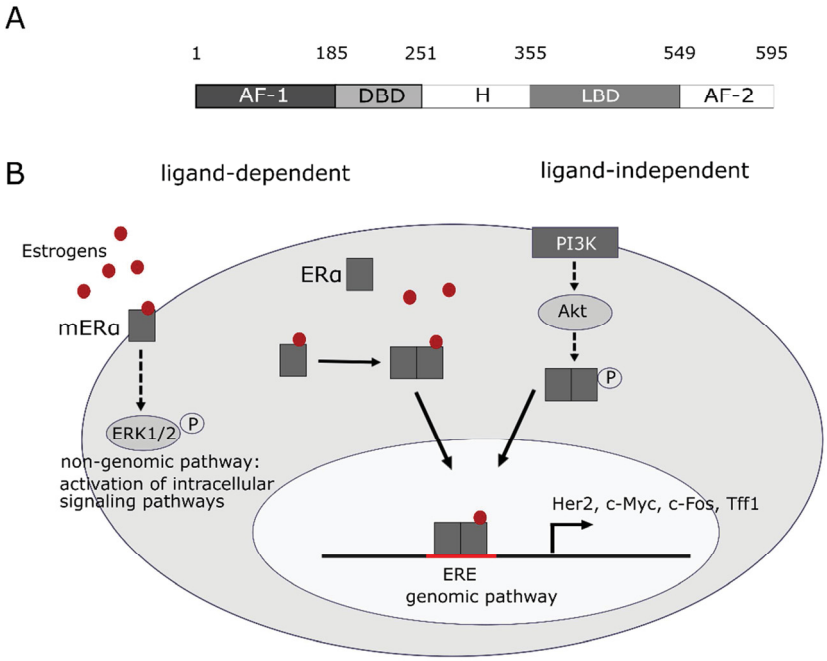


Figure 10. Structural description of human ERα and schematic representation of estrogen receptor ligand-dependent and ligand-independent signaling.
A: Schematic structure of human estrogen receptor α (595 aa). Hormone receptors are evolutionarily conserved and have five distinct structural and functional domains: DNA-binding domain (DBD), hinge domain (H), ligand-binding domain (LBD), and two transcriptional activation function domains AF-1 and AF-2. B: ER-mediated signaling occurs in a ligand-dependent and ligand-independent mechanism. Receptor dimerization (homodimers: ERα:ERα), leads to translocation into nucleus and

binding to specific EREs in the regulatory regions of estrogen responsive genes. In the ligand-dependent "*non-genomic*" pathway, ligand interacts with plasma membrane-bound ERs, which results in activation of cytoplasmic signaling pathways, such as ERK1/2. In *ligand-independent signaling* pathway, phosphorylation/activation of ERα by other active signaling cascades in a cell. Abbreviation: Akt: protein kinase B, ERE: estrogen-response element, ERα: estrogen receptor α, ERK1/2: extracellular signal-regulated kinase, Her2: Human epidermal growth factor receptor 2, PI3K: phosphatidylinositol-4, 5-bisphosphate 3-kinase, P: indicates phosphorylation, Tff1: Trefoil factor 1[45].

1.4. Hormonal contraception and hormone therapy

Besides the above described endogenous hormones, exogenous hormones were developed to be applied as agents e.g. in hormonal contraception and hormone therapy in menopause[55,56].

1.4.1. Hormonal contraception

Hormonal contraception, i.e. the use of hormones to prevent fertilization, is among the most reliable reversible methods of contraception and has revolutionized the reproductive life of women since the 1960s[57]. Hormonal contraceptives vary in their composition, dosage and application modes and therefore have different partial effects, side effects and risks[58–61]. They are available in various forms, including oral pills, vaginal ring, patches, injections, implants and intrauterine devices (IUDs)[56,62]. In Germany, 75 % of the 20- to 29-year old women and 44 % of the 30- to 44-year old women use hormonal contraception. Oral contraception (birth control pill) is the most commonly used hormonal contraception method; 72 % of the 20- to 29-year old and 16 % of the 30- to 44-year old use *the pill*[63].

Hormonal contraceptives are divided into two classes: **combined contraceptives** and **progestin-only contraceptives**. Combined contraceptives contain an estrogen (usually ethinylestradiol) and a component of synthetic analogs of gestagens, referred to as **progestins**[56,59,64]. Combined hormonal contraceptives lead to the inhibition of follicular maturation and ovulation. The contraceptive effect is primarily ensured by progestins[64,65]. Estrogen was originally included for stabilization of the endometrium and to reduce the incidence of breakthrough bleeding. However, it was also found to inhibit follicular development and to prevent ovulation[64]. Progestin-only contraceptives contain solely progestins. The mechanism of action of progestin-only contraceptives depends on the progestin activity and -dose. Hormonal contraceptives further increase the viscosity of the cervical mucus, making it less permeable for sperm[56].

1.4.2. Hormone therapy

Menopausal hormone therapy (HT) is administered in peri- and post-menopause for treatment of climacteric symptoms, such as hot flashes and attacks of sweating. HT is expected to reduce these symptoms and to improve quality of life. The aim of HT is not, to reconstitute physiological conditions, but to treat the symptoms. Therefore, the term hormone *replacement* therapy was substituted by hormone therapy[55,66,67].

The treatment can be carried out with use of different estrogens, such as estradiol (E2), estriol (E3) or conjugated equine estrogens (CEE). HT, which only includes the use of estrogens, is referred to as **estrogen-only hormone therapy (EHT)**[32,55]. However, in addition to estrogens, hormone therapy usually includes progestins, which are added to prevent the development of endometrial hyperplasia and an associated risk of endometrial cancer due to estrogen administration. This HT is referred to as **combined estrogen-progestin hormone therapy (CHT)**[55,68]. In peri- and early menopause, preferably a sequential combined HT, i.e. a daily dose of both estrogen and progestin, is applied. Continuous combined hormone therapy is usually prescribed to postmenopausal women[55]. Estrogens are taken daily, while progestins are given in the second half of the cycle, to mimic the normal menstruation cycle. For both HTs special combined estrogen-progestin drugs were developed, which are available in various administration forms (E2: oral, transdermal, intranasal, intramuscular, topical; E3: mainly topical; CEE: oral; progestins: oral, transdermal)[55].

1.4.3. Exogenous hormones

Exogenous hormones can either be bioidentical or synthetic analogs. Bioidentical hormones have the same chemical and molecular structure as endogenous hormones, while synthetic analogs are derivates of the natural hormones, with a similar mode of action[66,68–70].

Examples for bioidentical hormones are E2 and E3, which are used in HT as well as P4, which is also applied for HT sometimes[69,70].

Ethinylestradiol (EE) is an E2 derivate and the most frequently used synthetic estrogen. EE is mainly used in contraception. It acts as an ER agonist and differs from E2 by an additional ethynyl group, which influences its pharmacokinetic properties: compared to E2, EE has a reduced first-pass effect in the liver, resulting in increased bioavailability[71–73].

Progestins are synthetic derivates of gestagens, and are most commonly used in hormonal contraception and HT[55,56,74,75]. Various progestins are available and are applied in different

forms. Within the last decades, progestins were refined and enhanced and can be grouped into different generations, depending on their market access[56,76]. Since in this work, progestins play an important role, in the following, the mechanism of action and the effects of progestins will be described in detail.

Progestins are structurally related to progesterone or nortestosterone (table 2). However, they differ from their molecule of origin and from each other in metabolism, pharmacokinetics, pharmacodynamics, potency and binding affinity to PR, causing different biological effects[75,77,78]. The impact of progestins on (breast) cells is broad and has not yet been fully elucidated. Progestins are primarily designed as PR agonists and act by binding to and activating the PR. Activation of PRs in the hypothalamus and pituitary gland by progestins inhibits the release of gonadotropin-releasing hormone (GnRH)[74,75,77]. Thereby the secretion of gonadotropins is suppressed, which leads to the inhibition of follicular maturation and ovulation. In the endometrium, they have anti-estrogenic effects and prevent endometric hyperplasia[55,56,75,79]. Some progestins also act as androgen receptor antagonists, which in some cases can be used for treatment of different androgen-dependent conditions[80,81]. In addition to these typical progestogenic anti-estrogenic and anti-androgenic activities, progestins exhibit agonistic or antagonistic off-target effects, by binding to androgen, glucocorticoid and mineralocorticoid receptors with varying binding affinities (table 2)[78,79,82–84].

Table 2. Most commonly used progestins and their target receptors

			Mode of action, receptor binding						
Progestin			Progesterone	Anti-estrogen	Anti-androgen	Androgen	Gluco-corticoid	Estrogen	Anti-mineralo-corticoid
Progesterone	Progesterone	**P4**	+	+	(+)	-	(+)	-	+
	Medroxyprogesterone (acetate)	**MPA**	+	+	-	(+)	+	-	-
	Cyproteron(acetate)	**CPA**	+	+	+	-	+	-	-
	Chlormadinonacetat	**CMA**	+	+	+	-	+	-	-
	Dydrogesterone	**DYD**	+	+	-	-	-	-	-
Nortestosterone	Norethisteron(acetate)	**NET**	+	+	-	+	-	+	-
	Levonorgestrel	**LGS**	+	+	-	+	-	-	-
	Nomegestrel(acetate)	**NOM**	+	+	(+)	-	-	-	-
	Dienogest	**DGN**	+	+	+	-	-	-	-
	Drospirenon	**DSP**	+	+	+	-	-	-	+

Shown are commonly used progestins and their abbreviations. Progestins can be divided into two groups; they are structurally related to either progesterone or nortestosterone. All progestins have anti-estrogenic effects and are used in HT to prevent endometric hyperplasia. Some progestins also exhibit anti-androgenic actions and are used for androgenic-dependent conditions. Besides, progestins can have off-target effects and cause receptor activation by binding to androgen receptors, glucocorticoid receptors and estrogen receptors and can act as anti-mineralocorticoids. +: effect, -: no effect, (+): weak effect[75,77–79,83,84,91]

The progestins NET, LGS and MPA are known to have androgenic activity, which can increase the tendency to greasy skin and body hair. Further, also estrogenic effects can be mediated, however probably only by NET, due to potential metabolization to estrogenic compounds[78,85–87].

To date, little is known regarding the effect of progestins on mPR, they might however be relevant for some biological effects[88]. Recent studies further indicate potential effects of progestins on Progesterone Receptor Membrane Component-1 (PGRMC1)[55,89,90].

1.4.4. Breast cancer risk

As endogenous estrogen and progesterone, also exogenous hormones, used in postmenopausal hormone therapy and in contraception, have been associated with increased breast cancer risk[11,74].

Hormonal contraception

An association between hormonal contraception and higher risk of breast cancer has been analyzed in various clinical studies. However, there are still many unanswered questions not least due to inconsistent outcomes[92–94]. A meta-analysis of 54 epidemiological studies between 1976 and 1992, reported a significant increased relative risk of having breast cancer for current users of combined oral contraceptives[30]. However, the composition of (oral) contraceptives has changed significantly within the last decades; new progestins have been designed and the hormone dosage has decreased. Since the year 2000 various other cohort studies have been performed, addressing an potential breast cancer risk upon hormonal contraception use[92–101]. The majority of these studies observed the effect of combined oral contraceptives on breast cancer risk, since this is the most commonly used hormonal contraception method. Figure 11 provides an overview of clinical studies published after the year 2000. Figure 11 A presents the relative breast cancer risk (RR) for women who used hormonal contraceptives in their lifetime (*ever use*), while figure 11 B represents the risk of current users of hormonal contraceptives (*current use*)[95–101].

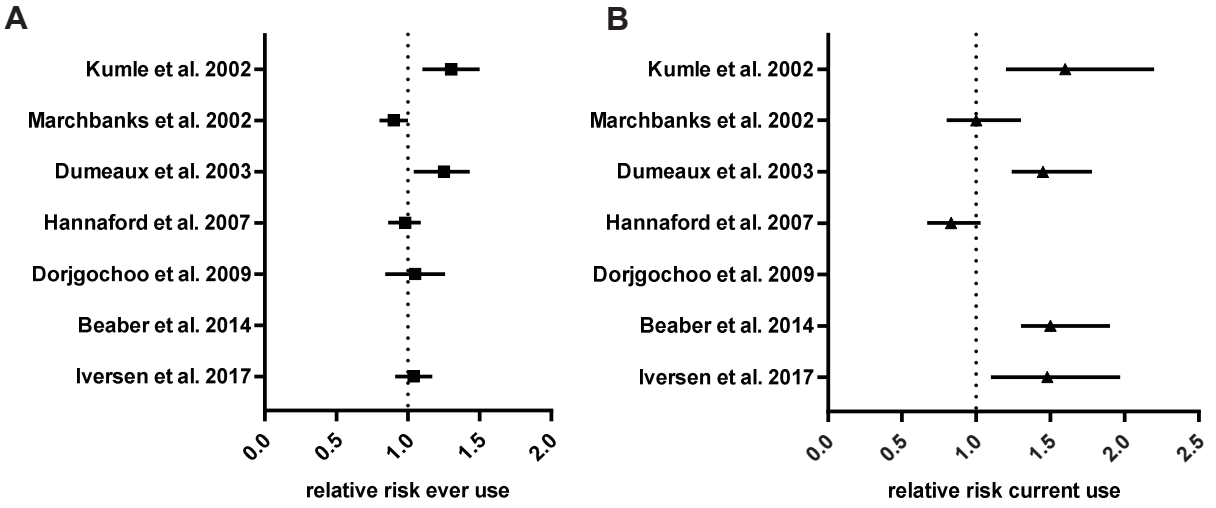


Figure 11. Effect of hormonal contraception on breast cancer risk, overview of recent clinical studies
A: Relative risk of ever users of hormonal contraception compared to never users (relative risk never users = 1.0, dotted line), confidence interval (CI): 95 %, B: Relative risk of current users of hormonal contraception compared to never users (relative risk never users = 1.0, dotted line), confidence interval (CI): 95 %[95–101].

The considered studies revealed inconsistent outcomes concerning a higher breast cancer risk for users, who used (oral) hormonal contraceptives in their lifetime. Dumeaux et al. and Kumle et al. report an 25 % and 30 % elevated breast cancer risk for women, who used hormonal contraceptives in their lifetime, while the other studies did not detect an

increased risk compared to women, who never used hormonal contraceptives (*never use*)[95–101]. For current users of hormonal contraceptives, four out of six studies evaluated a 45 – 60 % higher breast cancer risk compared to never users[95,97,100,101]. In contrast, two other studies did not observe an increased risk[96,98]. These variations might be effected by various factors, such as different compositions of the cohorts (age, ethnicity, reproductive factors) or implementation of the recruitment in different countries and associated compositions of the contraceptives. Most of the studies revealed distinct risks for breast cancer dependent on the duration of use of (oral) contraceptives (table 3); in most cases, women, who used hormonal contraception for 10 years or more had a higher risk for breast cancer compared to women, who had taken hormones for less than 10 years[95,97,98]. However, Kumle et al. observed that the relative risk of breast cancer for users of hormonal contraception decreases after discontinuation. 5 - 10 years after last use, the relative risk was again the same as for women, who never used oral contraceptives[95]. Kumle et al. further compared the relative breast cancer risk of progestin-only and estrogen/progestin combined oral contraceptives. For *ever users* they did not detect an elevated risk for progestin-only oral contraceptives (1.1, 95 % CI: 0.8 – 1.7), while for users of combined oral contraceptives, they found a 30 % increased risk (1.3, 95 % CI: 1.1 – 1.6). However, for *current users*, they assessed a 60 % and 50 % higher risk for progestin-only (1.6, 95 % CI: 1.0 – 2.4) and combined (1.5, 95 % CI: 1.0 – 2.0) oral contraceptives[95].

Table 3. Effect of hormonal contraception on breast cancer risk, overview of recent clinical studies

	N	never use	ever use	current use	duration of use RR (95 % CI)	
			RR (95 % CI)	RR (95 % CI)	≤ 10 years	> 10 years
Iversen et al. 2017	46,022	1.0	1.04 (0.9 – 1.2)	1.48 (1.1 – 2.0)	-	-
Beaber et al. 2014	23,054	1.0	-	1.50 (1.3 – 1.9)	-	-
Dorjgochoo et al. 2009	28,514	1.0	1.05 (0.8 – 1.3)	-	-	-
Hannaford et al. 2007	45,950	1.0	0.98 (0.9 – 1.1)	0.83 (0.7 – 1.0)	0.98 (0.8 – 1.2)	1.22 (1.0 – 1.5)
Dumeaux et al. 2003	96,362	1.0	1.25 (1.1 – 1.5)	1.45 (1.1 – 1.7)	1.22 (0.9 – 1.5)	1.40 (1.1 – 1.8)
Marchbanks et al. 2002	9,257	1.0	0.90 (0.8 – 1.0)	1.00 (0.8 – 1.3)	0.9 (0.8 – 1.1)	0.9 (0.7 – 1.3)
Kumle et al. 2002	103,027	1.0	1.30 (1.1 – 1.5)	1.60 (1.2 – 2.0)	1.20 (1.0 – 1.5)	1.35 (1.0 – 1.8)

N: number of women/size of cohort. Never use: women who never used hormonal contraceptives in their lifetime. Ever use: Relative risk of women who ever used hormonal contraception in their lifetime compared to never users confidence interval (CI): 95 %. Current use: Relative risk of current users of hormonal contraception compared to never users, confidence interval (CI): 95 %. Duration of use, < 10 years: women who used hormonal contraceptives for 10 years or less than 10 years. > 10 y: women who used hormonal contraceptives for more than 10 years [95–101].

(Post)menopausal hormone therapy

Initially, case reports and retrospective studies pointed towards an association of estrogen-only (EHT) and combined estrogen/progestin (CHT) hormone therapy, administered for climacteric symptoms, and a higher risk of breast cancer. In various prospective large cohort studies, such as the Million Women (MW) Study (1,084,110 women) and the Women's Health Initiative (WHI) (27,547 women), this hypothesis was further investigated[32,68,77,102–108]. The studies almost consistently suggest that CHT but not EHT increases the risk of breast cancer (figure 12), indicating a potential role of progestins in breast cancer carcinogenesis[102–108]. In the majority of the studies, current users of estrogen-only therapy were reported to reveal a similar relative breast cancer risk as women, who do not use HT (figure 12 A) (average relative risk from five cohort studies: 1.05 (95 % CI: 0.7 – 1.8))[102,104–108]. In contrast, women, who currently use combined estrogen/progestin hormone therapy, have a significant higher relative risk for breast cancer. Depending on the study, current users of CHT have a 22 % - 286 % increased relative breast cancer risk (figure 12 B) (average relative risk from five cohort studies: 1.88 (95 % CI: 0.9 – 8.9)[102,103,105–108].

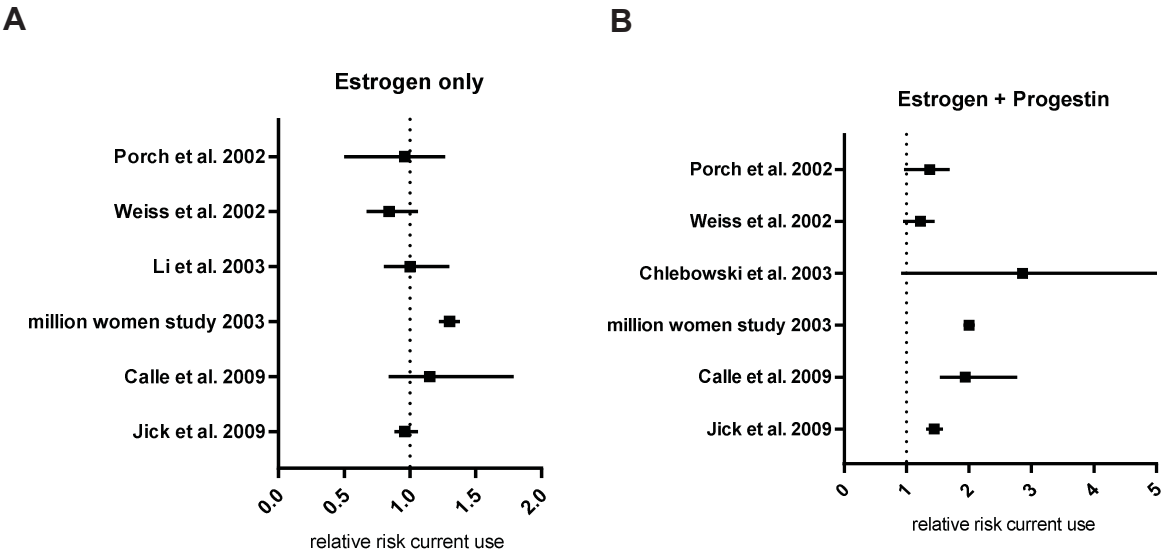


Figure 12. Effect of hormone therapy on breast cancer risk, overview of recent clinical studies
A: Relative risk of current users of estrogen-only hormone therapy compared to never users (relative risk never users = 1.0, dotted line), confidence interval (CI): 95 %, B: Relative risk of current users of combined estrogen/progestin hormonal contraception compared to never users (relative risk never users = 1.0, dotted line), confidence interval (CI): 95 %[102–108].

The same trend can be observed for users, who ever used EHT and CHT (table 4).

Table 4. Effect of hormone therapy on breast cancer risk, overview of recent clinical studies

	N	never use	Estrogen only RR (95 % CI)		Estrogen + Progstin RR (95 % CI)	
			ever use	current use	ever use	current use
Jick et al 2009	22,573	1.0	0.96 (0.9 – 1.1)	-	1.44 (1.3 – 1.6)	-
Calle et al. 2009	68,369	1.0	1.02 (0.7 – 1.8)	1.15 (0.9 – 1.8)	1.55 (0.8 – 2.8)	1.94 (1.5 – 2.8)
MW study 2003	1,084,110	1.0	-	1.30 (1.2 – 1.4)	-	2.00 (1.9 – 2.1)
WHI 2003	27,547	1.0	1.00 (0.8 – 1.3)	1.00 (0.7 – 1.3)	1.49 (1.1 – 2.0)	2.86 (0.9 – 8.9)
Weiss et al. 2002	3,823	1.0	-	0.84 (0.7 – 1.1)	-	1.22 (1.0 – 1.5)
Porch et al. 2002	17,835	1.0	-	0.96 (0.7 – 1.4)	-	1.37 (1.1 – 1.8)

N: number of women/size of cohort. Never use: women who never used hormone therapy in their lifetime. Ever use: Relative risk of women who ever used hormone therapy in their lifetime compared to never-users confidence interval (CI): 95 %. Current use: Relative risk of current users of hormone therapy compared to never users, confidence interval (CI): 95 %[102–108]. WHI: women's health initiative (Chlebowski et al. and Li et al.)[103,104]

As reported for hormonal contraception, various studies indicated that the relative risk of breast cancer increases with time of use of CHT[102,103,106]. Chlebowski et al. (WHI) observed that after 4 years of CHT, the breast cancer risk is significantly elevated compared to the placebo group (figure 13 A)[103,109]. In fact, the National Institute of Health stopped the WHI estrogen/progestin trial early, due to significantly elevated breast cancer cases in the CHT-cohort compared to the placebo group[109,110]. For EHT also with longer duration of intake, no increased breast cancer risk could be observed by various studies[102,104–106]. Li et al. (WHI) even found a decrease in breast cancer risk with longer duration of intake (figure 13 B), however this could not be confirmed by other studies[102,104,106,109].

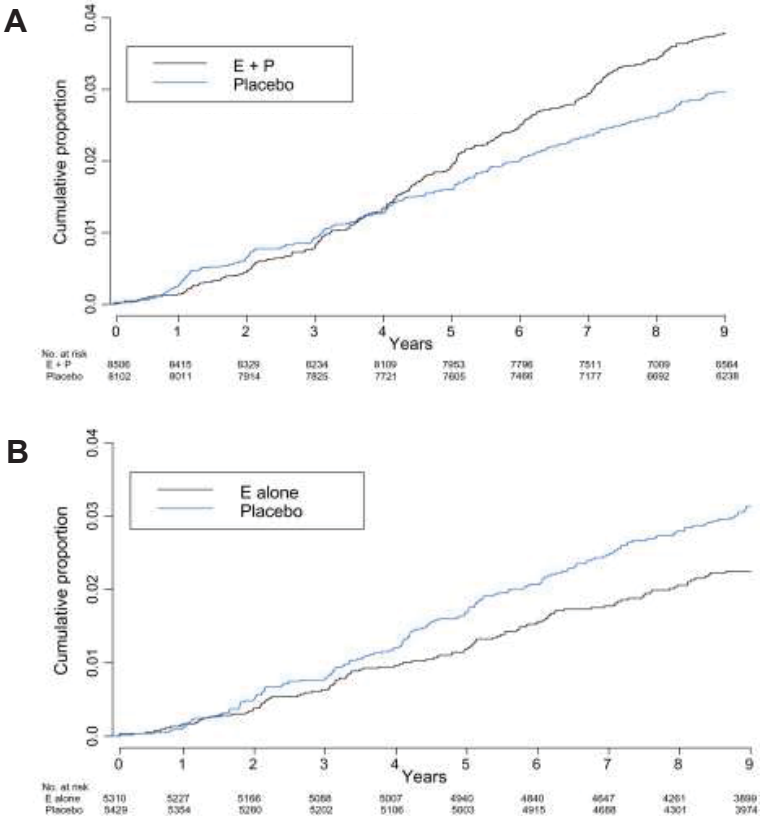


Figure 13. Women's Health Initiative (WHI) hormone therapy clinical trials for combined estrogen/progestin therapy and estrogen-only therapy.
A: combined estrogen/progestin hormone therapy. B: estrogen-only hormone therapy. Between 1993 and 1998, 10 739 women with prior hysterectomy were randomly assigned to estrogen alone (conjugated equine estrogen [CEE], 0.625 mg/d) or matching placebo and 16 808 women with an intact uterus were randomly assigned to estrogen plus progestin (conjugated equine estrogen, 0.625 mg/d plus medroxyprogesterone acetate [MPA], 2.5 mg/d) or a matching placebo. Depicted are the invasive breast cancer incidences by hormone therapy group. E + P = estrogen plus progestin; E alone = estrogen alone. Chlebowski R. T., Anderson G. L., J Natl Cancer Inst., 2012[109].

Further, Fournier et al. demonstrated that the risk of breast cancer differs, depending on the type of progestin used. With a relative risk of 2.74 (95 % CI: 1.42–5.29), 2.57 (95 % CI: 1.81–3.65) and 2.11 (95 % CI: 1.56–2.86) the progestins medrogestone, cyproterone acetate and norethisterone acetate were found to exhibit the highest breast cancer risk. In contrast to combined estrogen/progestin therapy, no increased breast cancer risk has been reported for the combined therapy of estrogens and progesterone (relative risk: 1.08 (95 % CI: 0.89–1.31)[111].

1.5. Progesterone Receptor Membrane Component 1 (PGRMC1)

Progesterone Receptor Membrane Component 1 (PGRMC1) is expressed in different cellular systems and contexts and has a wide range of cellular functions[89,112–118,118–123].

PGRMC1 is a member of the membrane-associated progesterone receptor (MAPR) family, which consists of four homologous proteins, including PGRMC1, the closely related PGRMC2,

Neudesin a protein with neurotrophic activity and Neuferricin (CYB5D2). They share a cytochrome b5 (cyt*b*5) related heme-binding domain and are known to interact with cytochrome P450 (CYP) enzymes[124,125].

PGRMC1 was independently discovered in 1996 by two groups. Selmin et al. purified upregulated PGRMC1 from the liver of dioxin treated rats and named the protein 25-Dx[126]. In the same year, Meyer et al. isolated PGRMC1 from porcine liver membranes as a high-affinity progesterone binding site[127]. In a review from 2001, the same group refers to it as progesterone membrane binding protein mPR[128]. However, it is important to note that PGRMC1 and the conventional mPRs or the nuclear PR do not exhibit a homologous amino-acid- or protein structure[112,124]. The human PGRMC1 homolog was cloned in 1998 by Gerdes et al. and initially referred to as Hpr6.6 (heme progesterone receptor 6.6), underlining its amino acid similarity to the cytochrome b5 heme binding domain[129]. In addition, homologous PGRMC1 proteins have also been found in other lower eukaryotes, indicating an evolutionary highly conserved structure. The nematode homolog of PGRMC1 - VEM-1 - was found in the genome of *C. elegans* and shares 37 % amino acid similarity with PGRMC1[130,131]. The yeast homologous protein is referred to as Dap1 and was found in 2003 in *S. cerevisiae*, while the *A. thaliana* homolog is termed AT2G24940[132,133].

PGRMC1 is expressed in various tissues. It is primarily found in the liver and kidney, but also in other organs, such as brain, breast, heart, lung, pancreas as well as reproductive tissues[124,129,134,135]. Further, PGRMC1 reveals increased expression in breast cancer and other tumor entities, where it contributes to carcinogenesis[89,112,114,117,119,122,136–141]. Its subcellular localization is not fully elucidated yet, but it was suggested to be dependent on various factors such as the cell system, dimerization/multimerization or posttranslational modifications[113,142–144]. It is often colocalized with CYP proteins in the smooth endoplasmic reticulum, but is also localized in the cytoplasm, the nucleus, the plasma membrane and mitochondria[127,142,144–147]. In a large-scale detection of nuclear phosphoproteins, Beausoleil et al. observed phosphopeptides of PGRMC1 in the nucleus of HeLa cells[142]. Further, biotinylated PGRMC1 could be detected in the extracellular compartment of cancer cells[148]. These distinct observed subcellular localizations of PGRMC1 imply that in order to understand its various biological functions, it is important to unravel the role of PGRMC1 in its subcellular context.

AG-205 is a small molecular inhibitor of PGRMC1, which has been designed in an in-silico screening project to find inhibitors against MAPRs[133]. AG-205 alters the spectroscopic properties of PGRMC1 and inhibits the viability of PGRMC1 expressing cancer cells[149–152].

1.5.1. Structure

The main splice variant of PGRMC1 consists of 195 amino acids (primary structure: figure 14 A), its calculated mass amounts 21.7 kDa, while the experimental mass is slightly higher and is about 25 kDa, probably due to various posttranslational modifications[135,153]. Its structure is different from other mPRs and nuclear PRs[112,115]. PGRMC1 exhibits an N-terminal extracellular domain (EC), a helical transmembrane domain (TM) and a cytoplasmic domain (C). As a member of the MAPR family, PGRMC1 contains a cytochrome b5-like domain (cyt*b*5), which includes a heme binding moiety (figure 14 B)[112]. PGRMC1 further possesses several peptide motifs, which are potentially involved in protein-protein interactions. These include a Src homology 3 (SH3) target sequence at P63, and two Src homology 2 (SH2) target sequences at Y139 and Y180 (figure 14 B), which require Tyr-phosphorylation for interaction with SH2 domains[112,113]. Recently, Kabe et al. published the crystallographic structure of the cytosolic domain of PGRMC1, which confirms previous predictions obtained from modeling studies and proves that PGRMC1 dimerization is mediated by heme-binding (figure 14 C)[117]. PGRMC1 dimerization or multimerization was reported by other groups before. Min et al. suggested that PGRMC1 might alter its subcellular distribution dependent on multimerization, which could change its function[144,154].

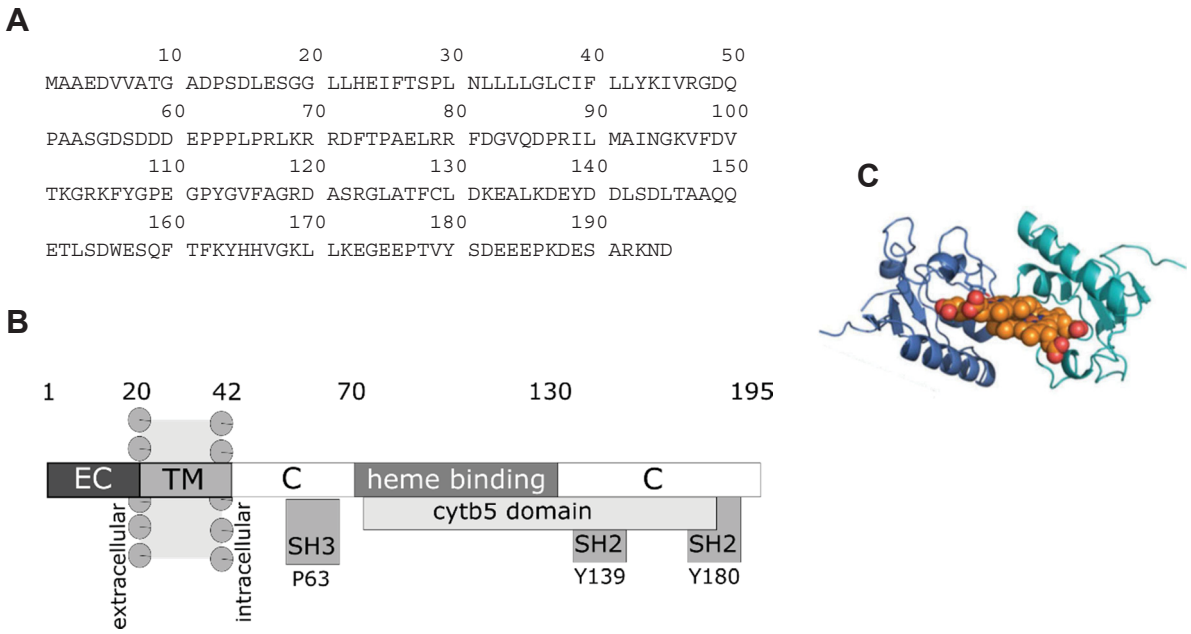


Figure 14. Structure pf PGRMC1
A: primary structure of PGRMC1, each letter represents one amino acid, depicted by the 1-letter code. The main splice variant of PGRMC1 consists of 195 amino acids[135,153]. B: secondary structure of PGRMC1. N-terminal extracellular domain (EC), helical transmembrane domain (TM), cytoplacmic domain (C), cytochrome b5-like domain (cytb5 domain), including a heme binding moiety (heme binding)[112]. Peptide motifs: Src homology 3 (SH3) target sequence at P63, Src homology 2 (SH2) target sequences at Y139 and Y180[112,113]. C: PGRMC1 dimerization mediated by heme-binding, crystallographic structure of the cytosolic domain of PGRMC1. Kabe Y. et al., *Nat Commun*, 2016[117].

1.5.2. Functions

PGRMC1 is a multifunctional protein and various cellular processes have been attributed to it, including P4 signal transduction and steroid response, heme-binding, binding and regulation of cytochrome P450 enzymes, cholesterol/steroid synthesis, vesicle trafficking as well as regulation of cell proliferation and -migration, apoptosis and cell cycle[112,116–118,121,134,146,147,152,155–159]. The most reported functions will be discussed further below.

Progesterone binding

PGRMC1 was discovered by Meyer et al., when searching for alternative membranous high affinity progesterone binding sites and was therefore suggested as a putative progesterone receptor[127]. Since then PGRMC1 has often been associated with activities dependent on P4 in various cell systems[114,118,119,152,155,159–162]. The Peluso group repeatedly detected PGRMC1 to induce P4-dependent anti-apoptotic action in different cell types[119,120,149,152]. Further, PGRMC1 was reported to mediate decreased susceptibility of cancer cells to various chemotherapeutic drugs, such as doxorubicin, dependent on P4[119,140]. In Purkinje cells of the cerebellum PGRMC1 is involved in P4 response as well as P4 biosynthesis[156,157].

Despite clear evidence of PGRMC1, to induce P4-dependent effects, so far, many studies with bacterially expressed PGRMC1 failed to prove interaction of PGRMC1 and P4[112,114]. Recently, Kaluka et al. assessed the P4-binding capacity of PGRMC1 by UV–vis and resonance Raman spectroscopy in the presence of heme. They provided evidence, supporting the P4-binding activity of PGRMC1 and observed that P4 binds to heme-bound PGRMC1 in a putative heme/ligand-binding pocket, resulting in induced conformational changes of the bound heme[162].

Other studies further suggest that posttranslational-modifications, such as phosphorylation, ubiquitinylation and SUMOylation are crucial for PGRMC1, to bind P4 with high-affinity, which is why studies with bacterially expressed PGRMC1 potentially failed to prove its P4-binding activity[114,118,162].

Heme binding and interaction with cytochrome P450 enzymes (CYPs)

As other members of the MAPR family, which share conserved heme binding capacity, PGRMC1 binds heme on its cyt*b*5 related domain[117,134,154]. Kabe et al. reported that PGRMC1 forms dimers via heme-heme stacking, a mechanism which has never been observed in eukaryotes before (figure 15)[117]. Increased heme levels are a risk factor of various cancer types and iron or heme deprivation can suppress carcinogenesis[117,124,163–167]. However, the underlying mechanism of how heme participates in cancer progression is not fully understood yet. The results of Kabe et al. provide further insight into the role of heme and PGRMC1 in cancer pathogenesis. They claim that the dimerization is heme dependent and essential for interaction with and activation of epidermal growth factor receptor (EGFR) and cytochrome P450 enzymes[117].

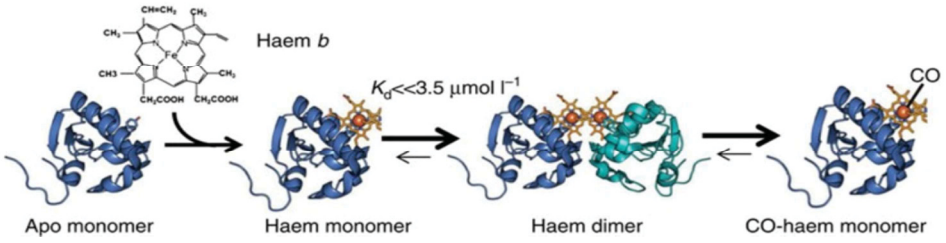


Figure 15. Heme dependent dimerization of PGRMC1
Structure of the stable PGRMC1 dimer formed through stacked heme. The heme iron is five-coordinated by Tyr113, and the open surface of the heme mediates dimerization. Carbon monoxide (CO) interferes with PGRMC1 dimerization by binding to the sixth coordination site of the heme. Kabe Y. et al., *Nat Commun*, 2016[117].

Interaction of PGRMC1 and its homologs with various members of the CYP family via its cyp*b*5 domain was detected before by other groups[121,124,168–170]. CYPs are heme proteins

(contain heme as a cofactor) with oxidoreductase activity, which exist in various but not all living organisms (animals, plants, fungi, bacteria, archaea, viruses)[171–173]. In humans, 57 different CYP proteins are known, which are found in all organs especially the liver. They play an important role in the metabolization of water-insoluble endogenous and exogenous substances and are the major enzymes involved in drug metabolism. CYPs can deactivate drugs directly through bioconversion or facilitating their excretion from the body. In addition, CYPs are involved in the synthesis of cholesterol, steroid hormones, prostaglandins, retinoids and vitamins and the metabolization of polyunsaturated fatty acids[116,124,171,173–175]. Because PGRMC1 is capable of binding heme and CYPs require heme as a cofactor, a potential role of PGRMC1 as heme-chaperone was suggested resulting in alterations of the enzymatic activity of CYPs. Thus, by interaction with CYPs, PGRMC1 is potentially involved in various functions associated with cytochrome P450 enzymes[115,176]. Kabe et al. provided evidence that interaction of PGRMC1 with CYPs mediates resistance to the chemotherapeutic agent doxorubicin, by facilitating its degradation[117]. This interaction may also explain the PGRMC1-mediated decreased susceptibility of cancer cells to cytostatic agents such as paclitaxel and cisplatin[120,168,177,178]. PGRMC1 is further involved in cholesterol- and steroid synthesis by interaction with CYPs, catalyzing the respective reactions[115,124,134,179]. Hughes et al. reported that PGRMC1 is involved in cholesterol biosynthesis by stably binding and activating CYP51A1 (Lanosterol 14α-demethylase), a protein involved in cholesterol- and ergosterol biosynthesis[116]. Knockdown of PGRMC1 lead to reduced activity of CYP51A1 and associated cholesterol synthesis[116]. Further, PGRMC1 is involved in the conversion of androgens (testosterone/ androstenedione) into estrogens (estradiol/estrone) by regulating the activity of CYP19A1 (aromatase)[179].

Vesicle trafficking

Internalization and vesicle trafficking of membrane proteins and - receptors are facilitated by conserved consensus motifs, located in their cytoplasmic domain[180]. The short-sequence signal motifs are recognized by adaptor proteins involved in the endocytic-, secretory- and sorting pathway, which ensure the proper trafficking and accurate distribution of membrane receptors to the cell surface and into cellular compartments of the endosomal/lysosomal system[1,180]. Runko et al. detected several YXXϕ signal motifs in the PGRMC1 primary structure and its homologs. YXXϕ signal motifs are well characterized short sequence motif, which are found in the C-terminus domains of different membrane receptors and are

involved in their internalization, trafficking and sorting process[130,181]. Various subsequent studies have investigated the role of PGRMC1 in vesicle trafficking[158,179,182,183]. Co-localization of PGRMC1 and caveolin and localization of PGRMC1 in cellular vesicles was demonstrated, suggesting a role of PGRMC1 in regulating vesicle trafficking (reviewed in Cahill, 2007)[112]. Various studies pointed towards a role of PGRMC1 in regulating and stabilizing insertion of receptors in the cell membrane in different cell types[158,179,182]. Ahmed et al. reported that PGRMC1 is required for the translocation of EGFR to the cell surface and its stable cell surface localization[158]. Zhang et al. demonstrated the same for GLP-1R (glucagon-like peptide-1 receptor), a receptor involved in insulin secretion in pancreatic beta cells[182]. Both groups showed that the PGRMC1 inhibitor AG-205 prevents translocation of the EGFR and GLP-1R to the surface, implying an important role of PGRMC1 in regulating cellular vesicle transport pathways[158,182].

1.5.3. Regulation by phosphorylation

PGRMC1 exhibits a variety of independent cellular functions. This diversity of functions was assumed to be regulated by a variety of posttranslational modifications, including phosphorylation, ubiquitination, acetylation and SUMOylation[113,114,142]. According to the phosphosite database, the phosphorylation sites pS57, pY113, pY180 and pS181 are the most commonly detected sites[113,184]. In a proteomics project, differential PGRMC1 phosphorylation in ER-positive and -negative breast cancer has been observed, indicating that not only the expression level but also the phosphorylation of PGRMC1 may play a role in breast cancer[141]. Therefore, investigation of the regulation of PGRMC1 by posttranslational modifications, foremost PGRMC1 phosphorylation status, will further our knowledge about its biological functions and downstream signaling. As described above, PGRMC1 exhibits two SH2-domain target sequences, which require Tyr phosphorylation (Y139, Y180) for SH2-domain containing proteins to bind and thereby inducing conformational changes of the receptor (figure 14 B)[112,113]. Recruitment of proteins might in turn activate further downstream signaling pathways, as known for other Tyr-phosphorylated steroid receptors. However, phosphorylation of S181 and T178 is predicted to sterically inhibit phosphorylation of Y180 and to attenuate protein interaction[113].

Likewise, binding of SH3 domains to SH3 target sequence P63 might be affected by phosphorylation of S57 (reviewed in Cahill et al., 2016)[113]. Further, phosphorylation of Y113 might be responsible for the membrane trafficking function of PGRMC1, but at the same

time prevent heme-binding due to steric interference, indicating a reciprocal regulation[113,117].

1.5.4. Role of PGRMC1 in cancer

Despite various treatment options, breast cancer is still the most common cause of cancer death worldwide for females[185]. Therefore, searching for new targets for treatment of breast cancer is of particular importance. PGRMC1 has been shown to be involved in cancer pathology and could therefore represent a target for cancer therapy (reviewed in Cahill, 2016)[114]. PGRMC1 protein and mRNA are upregulated in various tumors, including colon, lung, ovary, cervix and breast, suggesting a role of the receptor in carcinogenesis[122,136,137,139,141,151,158]. In breast cancer, the expression of PGRMC1 correlates with metastasis of lymph nodes, larger tumor size, poorer overall- and tumor-free survival[137,139,186]. Further, PGRMC1 is associated with resistance to the chemotherapeutic agent doxorubicin by promoting its degradation via CYP enzymes and resistance to treatment with cisplatin and paclitaxel[120,140,149,177,187]. Although the contribution of PGRMC1 to cancer progression was investigated in various studies, the role of PGRMC1 in cancer signaling pathways and its activation mechanism are not fully understood yet.

PGRMC1 participates in various cellular functional properties, which are involved in the pathogenesis of tumors and hallmarks of cancer. Interaction of PGRMC1 and EGFR was proven by various groups and suggested to thereby facilitate oncogenic signaling pathways, leading to increased proliferation, migration and invasion of cancer cells[158,166,179,187,188]. In fact, PGRMC1 was observed to be important for cancer cell proliferation in vitro and tumor growth and metastasis in vivo[119,187]. Knockdown of PGRMC1 in xenograft models led to reduction of tumor volume, tumor numbers, metastasis and enhanced response to chemotherapeutic agents[119,120,140,161,187]. Further, a role of PGRMC1 might play a role in cell cycle progression[121,159,189]. Ahmed et al. demonstrated that inhibition of PGRMC1 by AG-205 induces G1 cell cycle arrest, indicating a role of the receptor in this phase of the cell cycle[121].

1.5.5. Role of PGRMC1 in increased breast cancer risk associated with hormonal contraception and hormone therapy

In *in vitro* as well as in *in vivo* experiments revealed that treatment with certain synthetic progestins, used in contraception and post-menopausal hormone therapy, can cause a proliferative effect of breast cancer cells overexpressing PGRMC1[89,90,123,190–192]. This could

be of particular importance with regard to the detected elevated breast cancer rates, associated with hormonal contraception and HT, observed in various clinical studies (see 1.4.2.)[92,109].

In previous studies, our group demonstrated that PGRMC1 is involved in the mode of action of progestins on breast cancer cells. Overexpression of PGRMC1 in MCF7 breast cancer cells (MCF7/PGRMC1) resulted in increased proliferation upon progestin treatment, as compared to empty vector control cells (MCF7/EVC)[90,123,190,193–197]. As also observed in clinical studies, in our *in vitro* studies, various progestins exhibited different effects on proliferation of breast cancer cells. The progestins drospirenone (DSP), desogestrel, dydrogesterone (DYD), levonorgestrel (LGS), medroxyprogesterone acetate (MPA) and norethisterone (NET) significantly enhanced the proliferation rate of MCF7/PGRMC1 cells, as compared to the control cells, whereas the progestins chlormadinone acetate (CMA) and nomegestrel (NOM), as well as P4, did not enhance proliferation at concentrations of 1 µM[195]. In *in vivo* studies, a sequential combined treatment of E2 and NET significantly increased tumor growth of MCF7/PGRMC1 cells, compared to E2-only treatment, whereas MCF7/EVC cells did not respond to NET treatment[190]. Considering that PGRMC1 is expressed in breast tissue and overexpressed in breast cancer, further investigation of PGRMC1 activation and the resulting response of breast cancer cells is essential for the better understanding of the effects of progestins on breast cancer risk.

1.6. Aim of the study

This study is divided into two main parts: investigation of the role of PGRMC1 in breast cancer progression and investigation of its role in higher breast cancer risk upon progestin-based contraception and hormone therapy.

Role of PGRMC1 in breast cancer progression

PGRMC1 expression is upregulated in breast cancer and elevated expression of PGRMC1 is associated with increased tumor growth and poorer outcome, suggesting a contribution of PGRMC1 on breast carcinogenesis. However, the role of PGRMC1 in breast cancer, its activation mechanism and involved signaling pathways and are not fully understood yet. Therefore, the aim of the present study was to further **investigate the contribution of PGRMC1 on breast cancer progression**, the last step of carcinogenesis. For this purpose, the effect of PGRMC1 overexpression and –knockdown on cell proliferation was examined. With

the aim to **gain deeper insight into PGRMC1 signaling in breast cancer**, we further searched for potential PGRMC1-interaction partners as well as proteins and signaling pathways which might be regulated by PGRMC1. A special focus was placed on PGRMC1-dependent expression and activity of ERα.

Various studies suggest regulation of PGRMC1 functions by phosphorylation, indicating a role of the receptor not only at expression level. Based on this background, we aimed to **investigate the phosphorylation status of PGRMC1** and the role of PGRMC1 phosphorylation in breast cancer progression and in activation of signaling cascades. To further **investigate the previously described role of PGRMC1 in resistance to anti-cancer treatment**, we correlated PGRMC1 expression to therapy response, examined potential changes in PGRMC1 expression levels in matching pre- and post-therapy surgical specimens and investigated the effect of PGRMC1 overexpression on resistance to anti-cancer therapy.

Role of PGRMC1 in increased breast cancer risk upon progestin-based contraception and hormone therapy

In previous studies, we gave evidence that PGRMC1 is involved in the mode of action of progestins on breast cancer cells. PGRMC1 was demonstrated to forward progestin signals, resulting in enhanced proliferation of MCF7 breast cancer cells, indicating a potential role of PGRMC1 in the often-reported increased breast cancer risk upon progestin-based hormone therapy. To further **study the role of PGRMC1 in higher breast cancer risk upon progestin-based contraception and hormone therapy**, we investigated cell proliferation of different PGRMC1-overexpressing breast cancer cells upon treatment with various progestins used in hormonal contraception and HRT. To **investigate the biological activity of progestins associated with regulation of PGRMC1 activity**, we further examined the effect of progestin-treatment on phosphorylation of PGRMC1 and investigated the significance of PGRMC1 phosphorylation for proliferation. To **gain deeper insight into PGRMC1 signaling upon progestin-treatment**, we searched for potential PGRMC1-interaction partners as well as PGRMC1-regulated proteins and signaling pathways in cells treated with the progestin NET.

2. Material and Methods

2.1. Material

2.1.1. Chemicals

Chemical	Source
1% Nonidet P 40 Substitute (NP-40)	Sigma-Aldrich, St. Louis, USA
β-Estradiol (E2)	Sigma-Aldrich, St. Louis, USA
AG205	Sigma-Aldrich, St. Louis, USA
Agarose	Sigma-Aldrich, St. Louis, USA
Cyproterone acetate (CPA)	Sigma-Aldrich, St. Louis, USA
4',6-Diamidin-2-phenylindol (DAPI)	Thermo Fisher Scientific, Waltham, USA
1,4-Dithiothreitol	Sigma-Aldrich, St. Louis, USA
DMSO	Sigma-Aldrich, St. Louis, USA
DMSO Hybri-Max™	Sigma-Aldrich, St. Louis, USA
Doxorubicin (dox)	Sigma-Aldrich, St. Louis, USA
Drosperinone (DSP)	Sigma-Aldrich, St. Louis, USA
Dydrogesterone (DYD)	Sigma-Aldrich, St. Louis, USA
Epidermal growth factor (EGF)	Cell Signaling Technology, Danvers, USA
Epirubicin (epi)	Sigma-Aldrich, St. Louis, USA
Estradiol (E2)	Sigma-Aldrich, St. Louis, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, USA
Formaldehyde solution (4.5 %)	Merck KGaA, Darmstadt, Germany
Glycine	Sigma-Aldrich, St. Louis, USA
Hematoxylin Solution, Mayer's	Sigma-Aldrich, St. Louis, USA
Iodocetamide	Sigma-Aldrich, St. Louis, USA
Medroxyprogesterone acetate (MPA)	Sigma-Aldrich, St. Louis, USA
Methanol	Sigma-Aldrich, St. Louis, USA
NaCl	Sigma-Aldrich, St. Louis, USA
Nomegestrol (NOM)	Sigma-Aldrich, St. Louis, USA
Norethisterone (NET)	Sigma-Aldrich, St. Louis, USA
Papanicolaou Stain, EA 50	Sigma-Aldrich, St. Louis, USA
Paraformaldehyde (4 %)	Merck KGaA, Darmstadt, Germany

PhosSTOP™ Phosphatase Inhibitor	Roche, Basel, Switzerland
Progesterone (P4)	Sigma-Aldrich, St. Louis, USA
cOmplete™ Protease Inhibitor Cocktail	Roche, Basel, Switzerland
Quinalizarin	Sigma-Aldrich, St. Louis, USA
Skim milk powder	Sigma-Aldrich, St. Louis, USA
Sodium deoxycholat	Sigma-Aldrich, St. Louis, USA
Sodium dodecyl sulfate	Sigma-Aldrich, St. Louis, USA
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich, St. Louis, USA
TRIS	Sigma-Aldrich, St. Louis, USA
Triton X-100	Merck KGaA, Darmstadt, Germany
Trypan blue	Sigma-Aldrich, St. Louis, USA
Tween20	Sigma-Aldrich, St. Louis, USA

2.1.2. Cell lines

Purchased cell lines:

Name of cell line	Source
MCF7	ATCC, Manassas, USA
T47D	ATCC, Manassas, USA
MDA-MB-231	ATCC, Manassas, USA

Stably transfected cell lines:

Name of cell line	Characteristics	Mutation
MCF7/PGRMC1	MCF7 cells overexpressing 3 x HA-PGRMC1	Wild type
MCF7/PGRMC1-GFP	MCF7 cells overexpressing GFP-PGRMC1	Wild type
MCF7/EVC	MCF7 cells transfected with empty vector	-
MCF7/PGRMC1-S57A	MCF7 cells overexpressing 3 x HA-PGRMC1	S57 → A
MCF7/PGRMC1-S181A	MCF7 cells overexpressing 3 x HA-PGRMC1	S181 → A
MCF7/PGRMC1-S57A/S181A	MCF7 cells overexpressing 3 x HA-PGRMC1	S57 → A S181 → A
T47D/PGRMC1	T47D cells overexpressing 3 x HA-PGRMC1	Wild type
T47D/EVC	T47D cells transfected with empty vector	-
MDA-MB-231/PGRMC1	MDA-MB-231 cells overexpressing 3 x HA-PGRMC1	Wild type
MDA-MB-231/EVC	MDA-MB-231 cells transfected with empty vector	-

2.1.3. Cell culture media, additives and reagents

Medium/additive/reagent	Source
RPMI 1640 medium	Gibco by Thermo Fisher Scientific, Waltham, USA
Phenol-red free RPMI 1640 medium	Gibco by Thermo Fisher Scientific, Waltham, USA
Fetal bovine serum (FBS)	Gibco by Thermo Fisher Scientific, Waltham, USA
Charcoal stripped fetal bovine serum	Gibco by Thermo Fisher Scientific, Waltham, USA
Penicillin/streptomycin	Gibco by Thermo Fisher Scientific, Waltham, USA
HEPES	Gibco by Thermo Fisher Scientific, Waltham, USA
Trypsin	Gibco by Thermo Fisher Scientific, Waltham, USA
PBS	Gibco by Thermo Fisher Scientific, Waltham, USA
Opti-MEM® Reduced Serum Medium	Gibco by Thermo Fisher Scientific, Waltham, USA
Hygromycin B	Gibco by Thermo Fisher Scientific, Waltham, USA

2.1.4. Antibodies

Primary antibodies:

Ab against	species	Application	Product no.	Source
β-actin	rabbit	Western blot	Sc-1616	Santa Cruz Biotech., Dallas, USA
Akt	mouse	Western blot	5G3	Cell signaling, Cambridge, UK
p-Akt	rabbit	Western blot	D9E	Cell signaling, Cambridge, UK
CYP51A1	rabbit	Western blot, ICC	ab210792	Abcam, Cambridge, UK
EGFR	rabbit	Western blot	D38B1	Cell signaling, Cambridge, UK
p-EGFR	rabbit	Western blot	D7A5	Cell signaling, Cambridge, UK
ERα	rabbit	Western blot	D8H8	Cell signaling, Cambridge, UK
ERK1/2	rabbit	Western blot	137F5	Cell signaling, Cambridge, UK
p-ERK1/2	rabbit	Western blot	9101	Cell signaling, Cambridge, UK
Her2	rabbit	Western blot	29D8	Cell signaling, Cambridge, UK
p-Her2	rabbit	Western blot	6B12	Cell signaling, Cambridge, UK
MEK 1	mouse	Western blot	61B12	Cell signaling, Cambridge, UK
p-MEK1/2	rabbit	Western blot	41G9	Cell signaling, Cambridge, UK
PGRMC1	rabbit	Western blot	D6M5M	Cell signaling, Cambridge, UK
PGRMC1	goat	ICC	ab48012	Abcam, Cambridge, UK
PGRMC1	mouse	IHC	5G7	EMBL, Monterotondo, Italy
pPGRMC1	mouse	Western blot, IHC	3G11A2	EMBL, Monterotondo, Italy
PHB1	rabbit	Western blot, ICC	ab75766	Abcam, Cambridge, UK
PHB2	rabbit	Western blot, ICC	E1Z5A	Cell signaling, Cambridge, UK

Secondary antibodies:

Secondary ab	Application	Prod. no.	Source
Goat-anti-rabbit-HRP	Western blot	Sc-2004	Santa Cruz Biotechnology, Dallas, USA
Goat-anti-mouse-HRP	Western blot	Sc-2005	Santa Cruz Biotechnology, Dallas, USA
Donkey-anti-goat Alexa fluor 488	ICC	A-11055	Thermo Fisher Scientific, Waltham, USA

2.1.5. Blocking peptides

Protein	Peptide sequence	Source
PGRMC1	CGEEPTVY**S**DEEEPK	PANATecs, Heilbronn, Germany
pPGRMC1	CGEEPTVY**pS**DEEEPK	PANATecs, Heilbronn, Germany

2.1.6. Primers

Primers for PCR/sequencing:

Primer	Sequence	Source
BGHrev	5' TAGAAGGCACAGTCGAGG 3'	Biomers GmbH, Ulm, Germany
T7	5´ TAATACGACTCACTATAGGG 3´	Biomers GmbH, Ulm, Germany

Primers for qRT-PCR:

Primer	Source
RT² qPCR Primer Assay for Human TFF1	Qiagen, Hilden, Germany
RT² qPCR Primer Assay for Human ESR1	Qiagen, Hilden, Germany
RT^2 qPCR Primer Assay for Human PDH	Qiagen, Hilden, Germany
Random Primers	Promega, Mannheim, Germany

2.1.7. siRNAs

siRNA	Source
FlexiTube GeneSolution GS10857 for PGRMC1	Qiagen, Hilden, Germany
FlexiTube GeneSolution GS2099 for ESR1	Qiagen, Hilden, Germany
Negative Control siRNA	Qiagen, Hilden, Germany

2.1.8. Commercially available kits

Kit	Source
DNeasy Tissue kit	Qiagen, Hilden, Germany

RNeasy Mini kit	Qiagen, Hilden, Germany
Omniscript RT kit	Qiagen, Hilden, Germany
QuantiFast SYBR Green PCR kit	Qiagen, Hilden, Germany
Pierce™ HA-Tag IP/Co-IP Kit	Thermo Fisher Scientific, Waltham, USA
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, USA
Duolink® In Situ Red Starter Kit Goat/Rabbit	Sigma-Aldrich, St. Louis, USA
ZytoChem-Plus HRP kit	Zytomed Systems GmbH, Berlin, Germany
SilverQuest™ Silver Staining Kit	Thermo Fisher Scientific, Waltham, USA
Pierce™ Coomassie Plus Bradford Assay Kit	Thermo Scientific, Waltham, USA

2.1.9. Reagents

Reagent	Source
lipofectamineTM 2000	Thermo Fisher Scientific, Waltham, USA
RNasin®	Promega GmbH, Mannheim, Germany
HiPerFEct Transfection Reagent	Qiagen
DreamTaq DNA Polymerase (5 U/µL)	Thermo Fisher Scientific, Waltham, USA
Amersham ECL Western Blotting Detection Reagent	GE Healthcare, Little Chalfont, UK
cell lysis buffer CLB1	Bayer AG, Leverkusen, Germany
RPPA spotting buffer CSBL1	Bayer AG, Leverkusen, Germany

2.1.10. Consumables

Consumable	Source
CryoTubes®	Greiner Bio-One, Solingen, Germany
Chamber slides	Nunc Lab-Tek, Thermo Fisher Scientific, Waltham, USA
E-Plate 96 VIEW	OLS OMNI Life Science Bremen
CELLSTAR® well plates (6, 24, 96)	Greiner Bio-One, Solingen, Germany
CELLSTAR® Cell Culture Flasks	Greiner Bio-One, Solingen, Germany
CELLSTAR® Falcon tube (15 ml, 50 ml)	Greiner Bio-One, Solingen, Germany
TipOne® Filter Tips	STARLAB International, Hamburg, Gemany
Costar® Stripette®	Corning, New York, USA
FrameStar® 384-well plate	4titude® Limited, Surrey, United Kingdom

Cell lifter	Corning, New York, USA
Eppendorf® Safe-Lock microcentrifuge tubes	Eppendorf AG, Hamburg, Germany
Microscope slides	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Coverslips	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Mini-PROTEAN® TGX™ precast gels	Bio-Rad Laboratories, Inc., Hercules, USA
Immun-Blot® PVDF Membranes	Bio-Rad Laboratories, Inc., Hercules, USA
DAKO Protein Blocking Solution	Dako, Glostrup, Denmark
DAKO antibody diluent	Dako, Glostrup, Denmark
DAKO Fluorescent Mounting Medium	Dako, Glostrup, Denmark
Superfrost® Plus Micro Slide	VWR Interantional, Radnor, USA
SuperBlock Blocking Buffer	Thermo Fisher Scientific, Waltham, USA

2.1.11. Devices and instruments

Device	Name	Source
Inkubator	HERACELL 150i	Thermo Fisher Scientific, Waltham, USA
Centrifuge	Megafuge 1.0	Heraeus Instruments, Hanau, Germany
Microplate reader	Anthos Reader HT2	Anthos Mikrosysteme GmbH, Krefeld, Germany
	xCELLigence SP	OLS OMNI Life Science, Bremen, Germany
Thermocycler	T3000	Biometra GmbH, Göttingen
Thermoshaker	Peqlab Thriller	VWR International GmbH, Darmstadt
LightCycler	LightCycler® 480 System	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Electrophoresis Cell	Mini-PROTEAN®	Bio-Rad Laboratories, Inc., Hercules, USA
Power Supply	PowerPac™ Universal	Bio-Rad Laboratories, Inc., Hercules, USA
Imaging System	ChemiDoc™ MP-System	Bio-Rad Laboratories, Inc., Hercules, USA
Fluorescence microscope	Axioplan 2 Imaging	Carl Zeiss Microscopy GmbH, Jena, Germany
Chromatigraphy System	Ultimate 3000 Rapid Separation Liquid	RSLC, Thermo Fisher, Dreieich, Germany
Trap column	Acclaim PepMap100	Thermo Scientific, Dreieich, Germany

analytical column	Acclaim PepMapRSLC	Thermo Scientific, Dreieich, Germany
Mass spectrometer	Orbitrap Elite	Thermo Scientific, Dreieich, Germany
Distal coated Silica Tip emitters		New Objective, Woburn, USA
Zeptosens technology		Bayer AG, Leverkusen, Germany
Zeptosens hydrophobic chips		Bayer AG, Leverkusen, Germany
	NanoPlotter 2	GeSim, Grosserkmannsdorf, Germany
	ZeptoREADER	Bayer AG, Leverkusen, Germany

2.1.12. Software

Software	Source
GraphPad PRISM	Graphpad Software Inc., La Jolla, USA
ImageJ 1.51d	NIH, Bethesda, USA
MaxQuant environment	Max Planck Institute of Biochemistry, Planegg, Germany
Mocrosoft Excel 2016	Microsoft Corporation, Redmond, USA
ZeptoVIEW Pro 3.1 array analysis software	Bayer AG, Leverkusen, Germany
SPSS 20.0	IBM, Armonk, USA

2.2. Methods

2.2.1. Tissue samples

2.2.1.1. Patient's samples

The selection of the patient's cohort and evaluation of regressive changes was carried out by Dr. Martin Wurster; the patient's cohort, immunohistochemical status, therapy and response to therapy were reported before by Wurster et al.[198].

Tissue samples from 69 breast cancer patients with non-metastatic invasive primary breast carcinoma have been included. Neo-adjuvant therapy and surgery has been reported before[198]. The therapeutic response was assessed by means of histopathological sections of the removed tumors. The regressive changes in the tumor were evaluated from 0 to 4 using a semi quantitative evaluation system (0 = no effect, 1 = resorption and tumor sclerosis, 2 = minimally present invasive residual tumor [< 0.5 centimeter], 3 = non-invasive tumor, 4 = no

tumor can be detected) according to the tumor regression grading described by Sinn et al.[199]. Samples were sorted into two groups using this evaluation: patients which responded to treatment (responder: R) and patients which did not respond to treatment (non-responder: NR).

2.2.1.2. Immunohistochemistry and labeling

Immunohistochemical staining and labeling of PGRMC1 and pPGRMC1 was carried out by Dr. Isabel Wurster and was reported before by Willibald et al. and Wurster I.[122,200].

The immunohistochemical analysis was performed on tissue microarrays (TMA) produced from cut core biopsies and surgical resection specimens. Tissue samples were fixed in 4 % buffered formalin (pH 7.0) and embedded in paraffin. IHC was performed on TMA sections (4 µm), mounted onto *Superfrost* glass slides. In total four TMAs have been produced with 150 cores each. For IHC ZytoChem-Plus HRP kit, was used. Briefly, before incubation with primary antibody, unspecific binding was blocked with SuperBlock Blocking Buffer for 5 min. After washing once, primary antibodies against PGRMC1 (5G7) and phosphorylated PGRMC1 at S181 (pPGRMC1) (3G11A2) were diluted 1:50 in antibody diluent and applied. 3,3'-diaminobenzidine (DAB) was used as chromogen. Finally, the slides were counterstained with Mayer's haematoxylin solution for 10 sec and mounted for examination. For each antibody, a positive tissue sample was used as positive control. For negative control, the same section was incubated without the primary antibody. Incubation steps were performed in a humified chamber. Counter staining was done with Papanicolaou Stain for 30 sec.

PGRMC1 labelling was scored according to the *immune reactive score* (IRS) established by Remmele and Stegner[201]. This score calculates the percentage of positive nuclei (0 = 0 %; 1 < 10 %; 2 = 10 – 50 %; 3 = 51 – 80 % and 4 > 80 % of positive cells) and the staining intensity (0 = “negative”, 1 = „weak“, 2 = „moderate“, and 3 = „strong” staining). The final IRS itself is calculated by multiplying staining intensity and percentage of positive nuclei providing scores between 0 and 12. A consultant pathologist blinded to clinical outcome reviewed all paired biopsies and surgical specimens containing tumor tissue as well as connective non-tumor tissue. Labelled sections were investigated blinded without knowing the kind of treatment regimen. All sections were digitally documented and labelling was semi quantitatively scored.

2.2.2. Cell biological methods

2.2.2.1. Cultivation of breast cancer cell lines

MCF7, T47D and MDA-MB-231 cells were maintained in RPMI 1640 medium, supplemented with 10 % fetal bovine serum, 100 units/ml penicillin/streptomycin and 25 mM HEPES in a humidified incubator at 37 °C in the presence of 5 % CO_2. At 80 % confluency, cells were washed with PBS and incubated for 5 min at 37°C with 0.05 % trypsin to detach the cells from the surface of the flask. Subsequently, cells were resuspended in culture medium and centrifuged for 5 min at 1100 rpm. The cell pellet was resuspended, splitted and transferred into new cell culture flasks.
MCF7, T47D and MDA-MB-231 cells were authenticated regularly by Microsynth AG (Balgach, Swiss); the last authentication was performed on July 14 2017.

2.2.2.2. Cryoconservation

For preparation of cryopreserved cell suspensions, cells were detached from the flask surface with trypsin as described above. After centrifugation, the cell pellet was resuspended in 1 ml culture medium containing 5 % DMSO Hybri-Max™, transferred into CryoTubes® and slowly frozen at -80°C in a freezing container. On the next day, the cells were transferred to liquid nitrogen for long-term storage.

2.2.2.3. Determination of cell number

For determination of cell number, an aliquot of the cell suspension was stained with 0.4 % trypan blue. The cell count was determined using a hemocytometer.

2.2.2.4. Cytospins

For preparation of cytospins, cells were resuspended in PBS (50,000 cells/ml). 200 µl cell suspension was loaded into a cytospin chamber and centrifuged at 800 rpm for 2 min. Remaining PBS was removed and the slides were dried overnight, encircled with a liquid blocker pen and stored at -20°C until further use.

2.2.2.5. Cell harvest

For harvesting, cells were washed with ice-cold PBS twice, harvested using a cell lifter, transferred in 1 ml PBS into a pre-cooled Eppendorf tube and centrifuged at 1100 rpm for 5 min. After removing the supernatant, the pellet was washed again in 1 ml PBS, the cells were centrifuged at 1100 rpm for 5 min and PBS was removed to receive a *dry* pellet.

2.2.2.6. Stable transfection

MCF-7, T47D and MDA-MB-231 cells were stably transfected as reported before[141]. MDA-MB-231/PGRMC1 and MDA-MB-231/EVC cells were a kind gift from Dr. Michael Cahill (Charles Sturt University, Wagga Wagga, Australia). Cells were transfected with the expression vector pcDNA3.1/Hygro(+) containing 3 x HA-tagged (3x human influenza hemeagglutinin-tagged) PGRMC1 using lipofectamine^{TM} 2000 transfection reagent, according to the manufacturer's recommendation. MCF7 cells were additionally transfected with GFP-tagged PGRMC1 wild-type and HA-tagged PGRMC1 phosphorylation-site mutants S57A, S181A, S57A/S181A. For this purpose, putative phosphorylation sites at Serine 57 and Serine 181 were removed by site directed mutagenesis and replaced with Alanine (S57A, S181A). Prior to transfection, cells were plated in 6-well plates in complete RPMI medium without antibiotics for 24 h to receive 70 - 90% confluency at the time of transfection. For transfection, 5 µg of DNA and 5 µL of Lipofectamine 2000^{TM} were incubated separately in 250 µL Opti-MEM® medium. After 5 min of incubation at room temperature, the diluted plasmids and Lipofectamine 2000^{TM} were combined and incubated for 20 min at room temperature. The DNA-Lipofectamine complexes were then added to each well, and the cells were incubated for a defined time at 37°C in a CO_2 incubator. 48 h after transfection, 5 x 10^5 cells transfected with expression vector pcDNA3.1/Hygro(+), containing a hygromycin resistance gene, were transferred to 10 cm dishes and complete RPMI medium, containing 100 µg/ml hygromycin B was added. Cells were cultured for selection of stable integration events. After two 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, western blot and immunofluorescence staining, to isolate PGRMC1-overexpressing clones.

2.2.2.7. Treatment

2.2.2.7.1. Hormones

For progestin treatment, cells were seeded in complete medium. After 48 h, the medium was changed to steroid-free medium (phenol-red free RPMI 1640 medium, 10 % charcoal stripped fetal bovine serum, 100 units/ml penicillin/streptomycin and 25 mM HEPES) and incubated for 48 h. Treatment was performed with NET, DYD, DSP, MPA, CPA, NOM and P4 at concentrations of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M or the respective amount of DMSO as a

control, for a defined time period (72 h for MTT assay, 24 h for mass spectrometry analysis, phosphorylation and RPPA)in steroid-free medium. Treatment with E2 was performed with 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M or the respective amount of DMSO as a control.

2.2.2.7.2. EGF

For EGF treatment, MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S181A cells were seeded in complete medium. After 24 h the medium was removed and replaced with medium containing epidermal growth factor (EGF). EGF was used at 10 ng/ml and incubated with cells for 10 min at 37°C.

2.2.2.7.3. Quinalizarin

To investigate signaling of PGRMC1 phosphorylation, simultaneous to treatment with NET (10^{-6} M), MCF7/PGRMC1 cells were treated with the CK2 inhibitor quinalizarin in concentrations of 10, 50, 100 and 500 nM or the respective DMSO volume as a control for 24 h.

2.2.2.7.4. AG205

To investigate effects of PGRMC1 inhibition on cell proliferation, MCF7 cells were treated with the PGRMC1 inhibitor AG205. Cells were seeded in complete medium. After 24 h, the medium was removed and cells were treated with 3.125, 6.25, 12.5, 25, 31.25, 50, 100 and 200 µM AG205 in complete medium for 72 h.

2.2.2.7.5. Epirubicin and Doxorubicin

For anthracycline-treatment, MCF7/EVC and MCF7/PGRMC1 cells were seeded in complete medium. After 48 hours, the medium was changed to serum-free medium. Treatment was performed with 10^{-6} M P4 or vehicle (0.001% DMSO) and Epirubicin (epi) (Doxorubicin (dox) in various concentrations (2000 nM, 1000 nM, 500 nM, 250 nM, 125 nM, 62,5 nM, 31,25 nM, 15,62 nM) in steroid-free medium for 48 h.

2.2.2.7.6. Fulvestrant

To investigate effects of ERα downregulation on cell proliferation, MCF7/PGRMC1 cells were treated with the selective ER downregulator fulvestrant. Cells were seeded in complete medium containing 100 nM fulvestrant. After 24 h, the cells were harvested, seeded in fulvestrant-containing medium into 96-well plates and cultured for 24 h, 48 h and 72 h.

2.2.2.8. MTT assay

Cells (5 x 10^4 cells per well) were seeded in triplicates in 96-well plates in complete medium and treated as described above. For cell viability assay, the medium was aspirated and cells were washed with PBS. 100 µl of steroid-free medium, supplemented with 0.25 mg/ml MTT, was added per well. After 3 h of incubation at 37 °C, MTT solution was aspirated and 100 µl DMSO was added. Following 1 h of incubation at 37 °C, absorbance was measured at 540 nm, using a microplate reader.

2.2.2.9. xCELLigence

Cells (5 x 10^4 cells per well) were seeded in 96-well plates (E-plate 96 VIEW) and treated with NET and DMSO as described above. The xCELLigence system was used for real-time monitoring of cell viability according to manufacturer's instruction [202].

2.2.3. Molecular biological methods

2.2.3.1. PCR

Stable transfection of the PGRMC1 gene or the empty vector was verified by polymerase chain reaction (PCR) using BGHrev and T7 primers. Genomic DNA was extracted using DNeasy Tissue kit according to the manufacturer's recommendation. 24 µl master mix (12.5 µl Dream Taq Polymerase, 9.5 µl H_2O, 1 µl T7 primer (200 nM), 1 µl BGHrev primer (200 nM)) was added to 1 µl template.

The PCR was carried out in a thermocyler according to the following temperature program (40 cycles) (Table 5). PCR products were visualized by gel electrophoresis in a 2 % agarose gel.

Table 5. Temperature program used for PCR

PCR-step	Temperature	Time
Initial denaturation	95°C	3 min
Denaturation	95°C	30 sec
Hybridization	55°C	30 sec
Elongation	72°C	1 min
Final Elongation	72°C	5 min
Cooling	4°C	∞

2.2.3.2. siRNA knockdown of endogenous PGRMC1

For knockdown of endogenous PGRMC1 in MCF7, T47D and MDA-MB-231 cancer cell lines, FlexiTube GeneSolution for PGRMC1 was used, containing 4 siRNAs that specifically target human PGRMC1 mRNA. Cells were transfected with the PGRMC1 siRNAs or negative control siRNA using HiPerFect Transfection Reagent according to the manufacturer's instructions. For MCF7 and T47D cells 5 nM of siRNA was used, for MDA-MB-231 cells 10 nM siRNA was used. Cells were harvested after 24 h, 48 h and 72 h to verify knockdown of PGRMC1 by Western blot analysis.

For MTT-assay, cells were pre-incubated with siRNA against PGRMC1 for 24 h in in cell culture flasks to fully knock-down the endogenous protein. Afterwards the cells were seeded in 96-well plates and again treated with siRNA. Cell proliferation was measured after 24 h, 48 h and 72 h of incubation.

2.2.3.3. siRNA knockdown of endogenous ERα

For knockdown of ERα in MCF7/PGRMC1, MCF7/EVC, T47D/PGRMC1 and T47D/EVC cell lines, FlexiTube GeneSolution for ESR1 was used. Cells were transfected with the ESR1 siRNAs or negative control siRNA as described above. For knock-down of ERα, 5 nM of siRNA was used.

For MTT-assay, cells were pre-incubated with siRNA against ESR1 for 24 h in in cell culture flasks, afterwards seeded in 96-well plates and again treated with siRNA. Cell proliferation was measured after 24 h, 48 h and 72 h of incubation.

2.2.3.4. qRT-PCR

RNA was isolated from a cell pellet of 0.5×10^6 cells using the RNeasy Mini Kit according to the manufacturer's specifications.

Reverse transcription of RNA into cDNA was performed with the Omniscript RT kit. 500 ng RNA were diluted in RNase-free water in a volume of 12.75 µl and incubated for 5 min at 65 °C. Then, 7.25 µl master-mix (2.0 µl 10x Buffer, 2.0 µl dNTP mix, 2.0 µl Random Primer (DNA hexamers of randomized sequence), 1.0 µl Omniscript RTase, 0.25 µl RNasin® (Ribonuclease Inhibitor) was added, the samples were vortexed and incubated for 1 h at 37 °C and afterwards for 5 min at 93 °C for denaturation of RTase. For qPCR, QuantiFast SYBR Green PCR kit and RT² qPCR Primer assays for ESR1, TFF1 and PDH were used. Primers were

diluted 1:10 and added to the master mix (5.0 µl SYBR Green mix, 3.0 µl RNase-free water, 1.0 µl Primer). 1 µl cDNA and 9 µl master mix were added to wells of a 384-well plate. The plate was sealed and centrifuged for 1 min at 1000 rpm before starting the qPCR program (5 min at 95 °C, 55 cycles: 10 sec at 95°C, 30 sec at 58°C). qPCR was performed using the LightCycler® 480 System.

2.2.4. Protein chemical methods

2.2.4.1. Immunoprecipitation and co-immunoprecipitation

For immunoprecipitation, cells were harvested as described above. The cell pellet (5 x 10^6 cells) was lysed using 500 µl mild-lysis buffer (20 mM TRIS, 137 mM NaCl, 1% Nonidet P 40 Substitute (NP-40), 2 mM EDTA) containing protease- and phosphatase inhibitors. The cell pellet was resuspended in lysis buffer, vortexed and incubated on a thermoshaker at 750 rpm and 4 °C for 30 min. Immunoprecipitation was performed using Pierce™ HA-Tag IP/Co-IP Kit following the manufacturer's instructions.

2.2.4.2. Western blot

For western blot analysis, cells were harvested as described above. Cells were lysed in RIPA lysis buffer (50 mM TRIS, 150 mM NaCl, 1 % NP-40, 0.5 % Sodium deoxycholate, 0.1 % SDS), containing protease- and phosphatase inhibitors. Protein concentration was determined using Pierce™ BCA Protein Assay Kit. 25 µg of total protein was loaded onto Mini-PROTEAN® TGX™ precast gels and protein samples were separated in a electrophoresis cell by polyacrylamide gel electrophoresis using SDS-running buffer (25 mM TRIS, 192 mM Glycine, 0,1 % SDS). Protein was transferred to Immun-Blot® PVDF Membranes over night at 4 °C, using western blotting-buffer (25 mM TRIS, 200 mM Glycine, 20 % Methanol). Membranes were blocked with 5 % skim milk powder in TRIS-buffered saline (TBS) (20 mM TRIS, 150 mM NaCl) containing 0.1% Tween20 (TBS-T) for 60 minutes at room temperature and incubated with the respective primary antibodies over night at 4 °C. After washing membranes with TBS-T, secondary antibodies were applied in 1 % skim milk powder/TBS-T and incubated at room temperature for one hour. Membranes were again washed with TBS-T and ECL-reagent was applied prior to chemiluminescent imaging using ChemiDoc™ MP-System.

Densitometric analysis of PGRMC1 and pPGRMC1 immunoblots was performed on scanned immunoblot images, using the ImageJ gel analysis tool[203]. The gel analysis tool was used to obtain the absolute intensity for each band and corresponding actin band. The relative

intensity of each band representing total protein and phosphorylated protein was calculated by normalizing the absolute intensity of the band to the corresponding control band.

2.2.4.3. Immunocytochemistry

For immunocytochemistry (ICC) cells were prepared on cytospins, fixed with 4 % PFA for 15 min at room temperature and washed three times for 5 min with TBS-T. Afterwards, cells were permeabilized with 0.1 % Triton X-100 in PBS for 10 min at room temperature and washed three times for 5 min with TBS-T. DAKO Protein Blocking Solution was added and incubated for 1 h at RT. Cells were subjected to immunofluorescence staining with primary antibodies diluted in antibody diluent overnight at 4 °C. Afterwards, the slides were washed three times for 5 min with TBS-T and respective fluorophore labelled secondary antibody were added to the samples and incubated for 1 h at RT in the dark. The slides were washed three times for 5 min with TBS-T and incubated with 2 µg/ml 4',6-Diamidin-2-phenylindol (DAPI) for 5 min at room temperature. Antibody incubation steps were performed in a humified chamber. The slides were washed with distilled water, mounted with Fluorescent Mounting Medium and dried overnight. The cells were examined by fluorescence microscopy using the Axioplan 2 Imaging fluorescence microscope.

2.2.4.4. Proximity ligation assay

For proximity ligation assay (PLA), MCF7 cells were prepared on cytospins as described above and fixed and permeabilized as described above for ICC. After permeabilization the Duolink® In Situ Red Starter Kit Goat/Rabbit was used according to the manufacturer's recommendation. The cells were examined by fluorescence microscopy using the Axioplan 2 Imaging fluorescence microscope. Dots per cell were quantified using imageJ.

2.2.4.5. Mass spectrometry

Mass spectrometry was performed in cooperation with Dr. Gereon Poschmann and Prof. Dr. Kai Stühler (Molecular Proteomics Laboratory, BMFZ, Heinrich Heine University, Düsseldorf, Germany).

PGRMC1 was immunoprecipitiated from four individual replicates of MCF7/PGRMC1-HA cells as described in 2.2.4.1.. As a negative control, GFP-labelled MCF7/PGRMC1 cells (MCF7/PGRMC1-GFP) were used. Resulting protein preparations were shortly separated in a 4-12% polyacrylamide gel (about 4 mm running distance), silver stained and processed as

previously described[204]. Briefly, samples were destained, reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin peptides extracted from the gel and finally resuspended in 0.1 % trifluoroacetic acid. Subsequently, the samples were analyzed on a liquid chromatography coupled electrospray ionization Orbitrap mass spectrometer. An Ultimate 3000 Rapid Separation Liquid Chromatography System was used for peptide separation: peptides were initially pre-concentrated on a trap column (Acclaim PepMap100, 3 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 2 cm length) at a flow rate of 6 µl/min for ten minutes using 0.1 % TFA as mobile phase and thereafter separated on an analytical column (Acclaim PepMapRSLC, 2 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 25 cm length) at a flow rate of 300 nl/min at 60°C using a 2 h gradient from 4 to 40 % solvent B (0.1 % (v/v) formic acid, 84 % (v/v) acetonitrile in water) in solvent A (0.1 % (v/v) formic acid in water). The liquid chromatography system was online coupled to a Orbitrap Elite mass spectrometer via a nano electrospray ionization source and peptides injected by distal coated Silica Tip emitters using a spray voltage of 1.45 kV. The mass spectrometer was operated in positive, data-dependent mode with capillary temperature set to 225 °C. First, full scans (350-1700 m/z, resolution 60,000) were recorded in the Orbitrap analyzer of the instrument with a maximal ion time of 200 ms and the target value for automatic gain control set to 1,000,000. In the linear ion trap part of the instrument subsequently up to twenty double- and triple-charged precursors with a minimal signal of 500 were isolated (isolation window 2 m/z), fragmented by collision induced dissociation (CID) and analyzed with a maximal ion time of 50 ms and the target value for automatic gain control set to 3000 (available mass range 50-2000 m/z, resolution 5400). Already analyzed precursors were excluded from further isolation and fragmentation for 45 sec.

For data analysis, the MaxQuant environment (version 1.5.3.8, was used with standard parameters if not otherwise stated. Spectra were searched against 20187 Swiss-Prot entries from the Homo sapiens proteome (UP000005640, downloaded on 18th November 2015 from UniProt KB). Label-free quantification was enabled as well as the *match between runs* option. Tryptic cleavage specificity was chosen, as well as carbamidomethyl at cysteines as fixed and methionine oxidation, phosphorylation (threonine, serine and tyrosine), acetylation at protein n-termini and ubiquitination at lysine (GlyGly, +114.0429) as variable modifications. Mass tolerances were 20 ppm (first search) and 4.5 ppm (second search after recalibration) for precursor masses and 0.5 Da for fragment masses. Phosphorylation sites

were reported showing the highest probability calculated form an MS/MS spectrum peak matches. Peptides and proteins were accepted at a false discovery rate of 1 %. For relative quantification of phosphorylated peptides, peptide intensities were normalized to progesterone receptor amounts by dividing them by the total progesterone receptor intensity.

2.2.4.6. RPPA

Reverse Phase Protein Arrays (RPPA) was conducted in cooperation with Berthold Gierke and Dr. Michael Pawlak, Natural and Medical Sciences Institute (NMI) at the University of Tuebingen, Reutlingen, Germany.

RPPA using Zeptosens technology were used for analysis of signaling protein expression and activity profiling as described earlier[205–208]. For the analysis, flash frozen cell pellets were lysed by incubation with 100 µl cell lysis buffer CLB1 for 30 minutes at room temperature. Total protein concentrations of the lysate supernatants were determined by Bradford Assay. Cell lysate samples were adjusted to uniform protein concentration in CLB1, diluted 10-fold in RPPA spotting buffer CSBL1 and subsequently printed as series of four dilutions (starting concentration at 0.3 µg/µl plus 1.6-fold dilutions) and in two replicates each. All samples were printed as replicate microarrays onto Zeptosens hydrophobic chips using a NanoPlotter 2 applying single droplet depositions (0.4 nl volume per spot). After printing, the microarrays were blocked with 3 % w/v albumin, washed thoroughly with double distilled H_2O, dried in a stream of nitrogen and stored in the dark at 4 °C until further use. Protein expression and activity levels were measured using a direct two-step sequential immunoassay and sensitive, quantitative fluorescence read-out. A single array was probed for each protein. Highly specific and upfront validated primary antibodies were incubated at the respective dilution in Zeptosens assay buffer overnight (15 h) at room temperature. Arrays were washed once in assay buffer and incubated for 45 min with Alexa647-labeled anti-species secondary antibody (Invitrogen, Paisley, UK). Arrays were then washed as before and imaged using a ZeptoREADER instrument in the red laser channel. Typically, six fluorescence images were recorded for each array at exposure times of between 0.5 and 16 sec. Negative control assays incubated in the absence of primary antibody (blank assays) were performed to measure the non-specific signal contributions of the secondary antibody. In addition, one chip out of the print series was stained to measure the relative amount of

immobilized protein per spot (protein stain assay). The following primary antibodies were used:

Protein	modification	Product no.	Source
Akt		4685	Cell signaling, Cambridge, UK
Akt - phospho	Ser473	4060	Cell signaling, Cambridge, UK
Akt - phospho	Thr308	13038	Cell signaling, Cambridge, UK
BRCA1 - phospho	Ser1524	9009	Cell signaling, Cambridge, UK
c-Fos		2250	Cell signaling, Cambridge, UK
c-Myc		5605	Cell signaling, Cambridge, UK
c-Myc - phospho	Thr58/Ser62	9401	Cell signaling, Cambridge, UK
EGFR		4405	Cell signaling, Cambridge, UK
EGFR - phospho	Tyr1068	2234	Cell signaling, Cambridge, UK
ER		8644	Cell signaling, Cambridge, UK
ERK1/2		6495	Cell signaling, Cambridge, UK
ERK1/2 - phospho	Thr202/Tyr204	4370	Cell signaling, Cambridge, UK
GSK3 beta		9315	Cell signaling, Cambridge, UK
GSK3 beta - phospho	Ser9	9336	Cell signaling, Cambridge, UK
HDAC1		34589	Cell signaling, Cambridge, UK
Her2		A0485	Dako, Glostrup, Denmark
Her2 - phospho	Tyr1221/1222	2247	Cell signaling, Cambridge, UK
Histone H3		4499	Cell signaling, Cambridge, UK
Histone H3 - acetyl	Lys14	7627	Cell signaling, Cambridge, UK
Histone H3 - monomethyl	Lys4	9723	Cell signaling, Cambridge, UK
MEK1		12671	Cell signaling, Cambridge, UK
MEK1/2 - phospho	Ser217/221	9154	Cell signaling, Cambridge, UK
MEK2		9147	Cell signaling, Cambridge, UK
PR		3127	Cell signaling, Cambridge, UK
Rb		9309	Cell signaling, Cambridge, UK
Rb - phospho	Ser807/811	8516	Cell signaling, Cambridge, UK
S6		2217	Cell signaling, Cambridge, UK
S6 - phospho	Ser235/236	2211	Cell signaling, Cambridge, UK
S6 - phospho	Ser240/244	2215	Cell signaling, Cambridge, UK

RPPA assay images were analyzed using ZeptoVIEW Pro 3.1 array analysis software. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample).

2.3. Statistical analysis

Statistical analysis of PGRMC1 and pPGRMC1 abundance patient's specimen was performed in cooperation with a biomathematician. For calculations SPSS 20.0 software was used. Since the data were not normally distributed Wilcoxon- and Friedman-test were applied. To find correlations between two parameters correlation coefficient given as Spearmans' rho (r_s) was used. To show differences between abundance intensity the sign-test was applied.

Unless otherwise stated, statistical analyses were performed using GraphPad Prism 5.0 Software (GraphPad Software, Inc.). The unpaired t-test was used to determine significant differences between the samples/groups.

P-values < 0.05 were regarded as significant (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, ****: $p < 0.001$).

All experiments were repeated a minimum of three times.

3. Results

3.1. Role of PGRMC1 in breast cancer progression

3.1.1. High expression of PGRMC1 in breast cancer correlates with poor outcome

An integrative data analysis tool (Kaplan-Meier-Plotter: kmplot.com/analysis) was used to assess the relevance of PGRMC1 expression levels on the clinical outcome of breast cancer patients[209]. Using microarray gene expression data of breast cancer patients, the Kaplan Meier plotter is capable to assess the effect of 54,675 genes on survival, using samples of 5,143 breast cancer patients. Significant correlation of high PGRMC1 expression with poorer overall survival (figure 16), relapse free survival (RFS) (figure 17) and distant metastasis-free survival (DMFS) (figure 18) was evaluated.

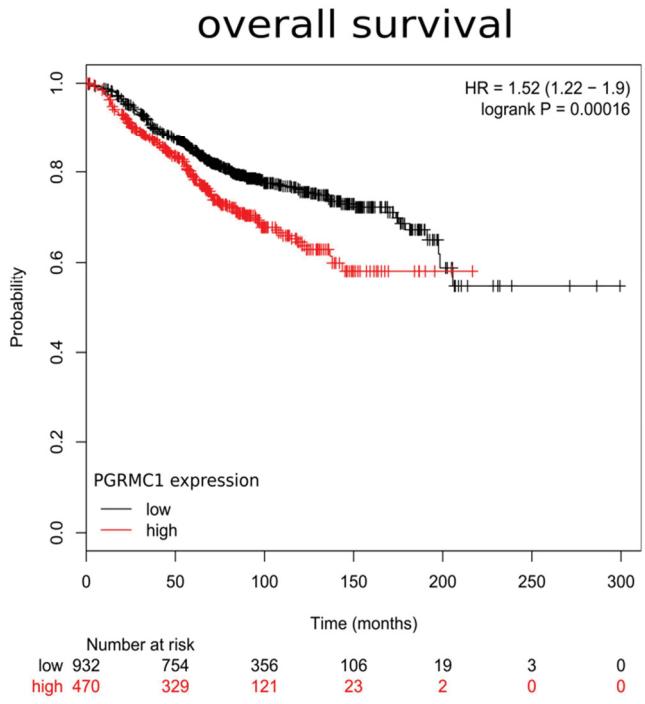


Figure 16. Overall survival rate of patients with high (red) and low (black) expression of PGRMC1.
Data from 1402 patients were included in the analysis. Analysis was done using *http://kmplot.com/*. p = 0.00015

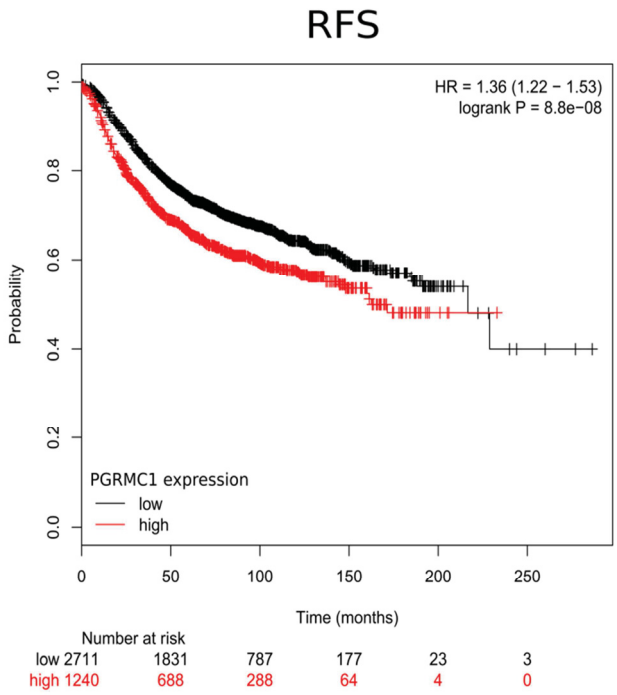


Figure 17. RFS rate of patients with high (red) and low (black) expression of PGRMC1.
Data from 3951 patients were included in the analysis. Analysis was done using *http://kmplot.com/*. p < 0.00001

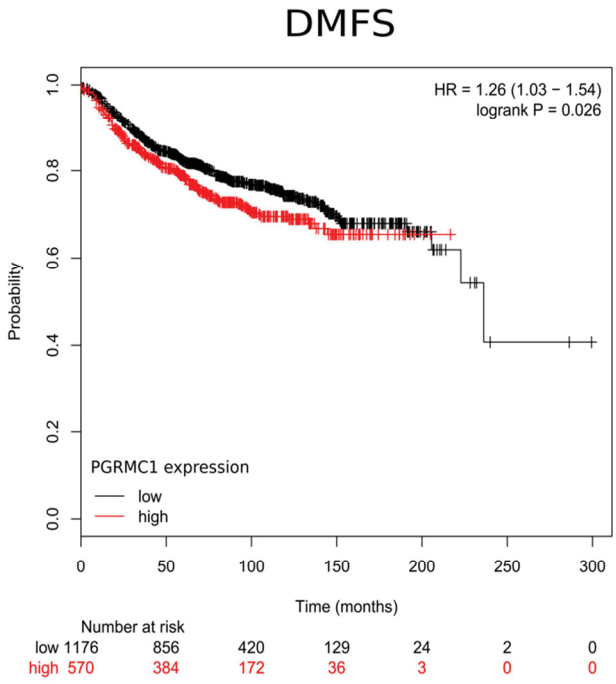


Figure 18. DMFS rate of patients with high (red) and low (black) expression of PGRMC1.
Data from 1746 patients were included in the analysis. Analysis was done using *http://kmplot.com/*. p = 0.026

3.1.2. PGRMC1 promotes proliferation of breast cancer cells

The poorer outcome for breast cancer patients with high PGRMC1 expression levels, indicate a potential role of the receptor in breast cancer carcinogenesis. Therefore, to further elucidate the role of PGRMC1 in cancer progression, PGRMC1 overexpressing breast cancer cell lines MCF7/PGRMC1, T47D/PGRMC1 and MDA-MB-231/PGRMC1 were generated[141]. Cells transfected with the empty vector (MCF7/EVC, T47D/EVC, MDA-MB-231/EVC) were respectively used as a control.

PCR was carried out to prove integration of the PGRMC1 gene or the empty vector into the genome of the cell lines (figure 19).

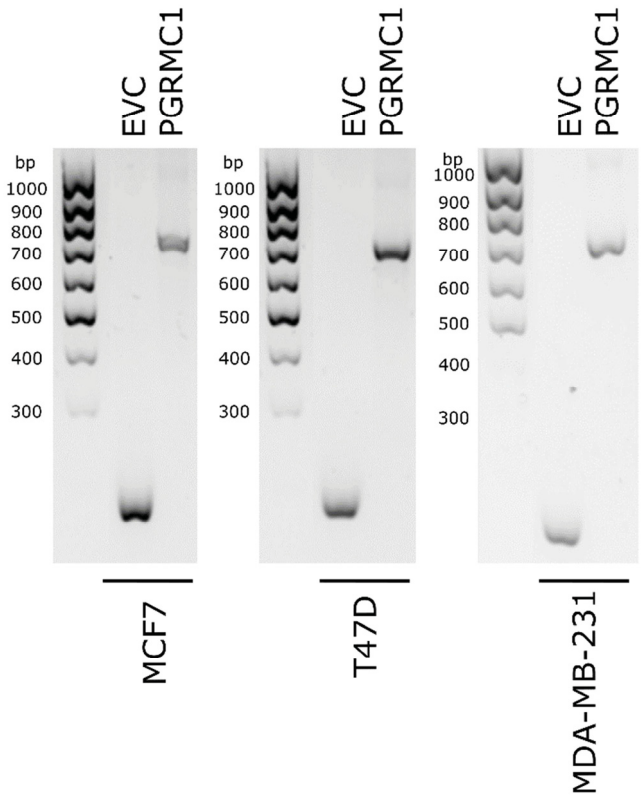


Figure 19. PCR analysis for examination of integration of the PGRMC1 gene in MCF-7, T47D and MDA-MB-231 cells. PCR products were separated with a 2% agarose gel. The PCR products are derived from MCF-7/EVC, T47D/EVC, MDA-MB-231/EVC cells and MCF-7/PGRMC1, T47D/PGRMC1, MDA-MB-231/PGRMC1 cells after amplification with the primer pair T7 forward/ BGH reverse.

Overexpression of the PGRMC1 protein in MCF7/PGRMC1, T47D/PGRMC1 and MDA-MB-231/PGRMC1 cells in comparison to the respective EVC controls was further verified by Western blot analysis (figure 20) and immunocytochemistry (MCF7: figure 21, T47D: figure 22, MDA-MB-231: figure 23).

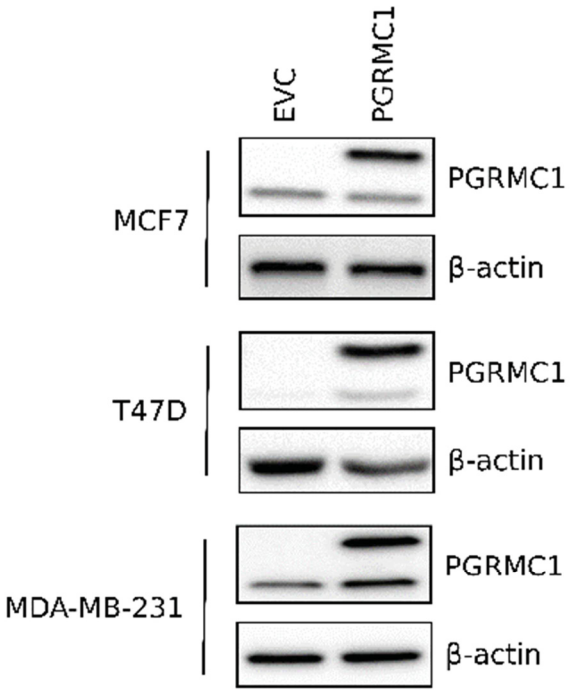


Figure 20. Western blot analysis of MCF7/EVC, T47D/EVC, MDA-MB-231/EVC cells and MCF7/PGRMC1, T47D/PGRMC1, MDA-MB-231/PGRMC1 cells.
EVC cells reveal expression of endogenous PGRMC1, PGRMC1 overexpressing cells reveal expression of endogenous (lower band, 23 kDa) and exogenous 3xHA-tagged PGRMC1 (upper band, 28 kDa).

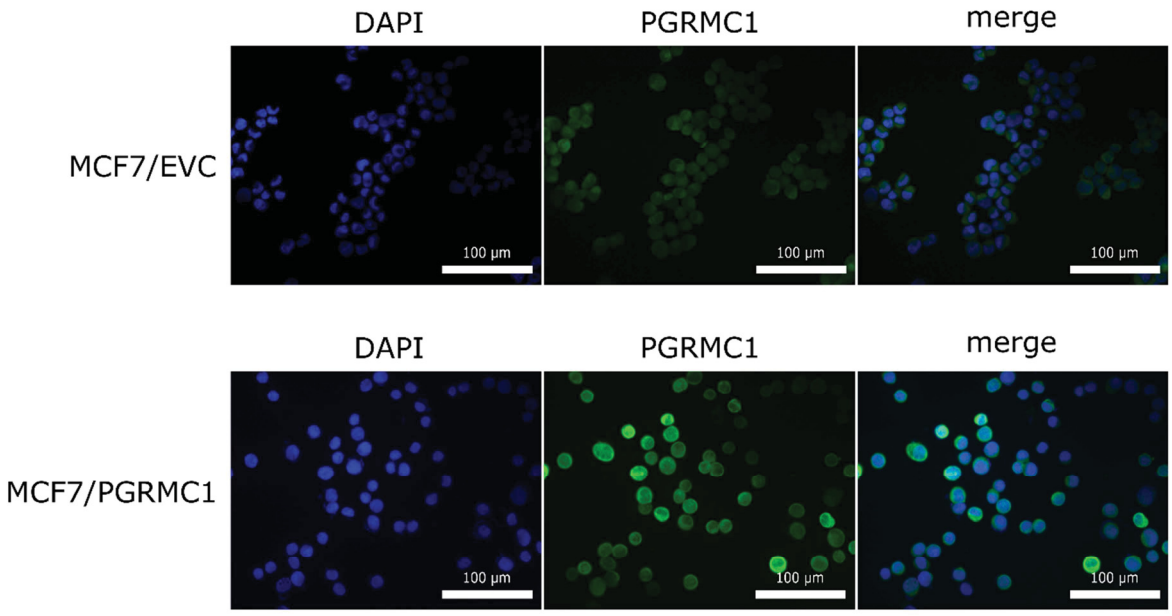


Figure 21. Fluorescent immunocytochemistry of PGRMC1 protein in MCF7/EVC and MCF7/PGRMC1 cell lines.
Cells were stained for cellular PGRMC1 with goat-anti human PGRMC1 antibody and AF488-conjugated donkey anti-goat secondary antibody. Nuclear stain: DAPI. Magnification 40x. Cells were grown in culture medium and then prepared on cytospins.

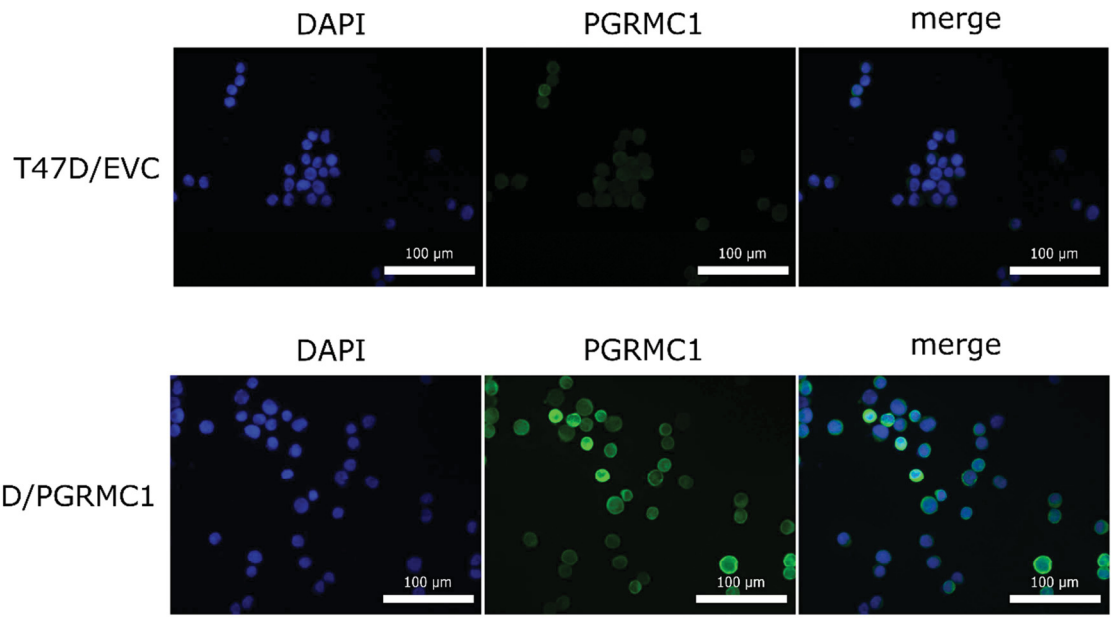


Figure 22. Fluorescent immunocytochemistry of PGRMC1 protein in T47D/EVC and T47D/PGRMC1 cell lines.
Cells were stained for cellular PGRMC1 with goat-anti human PGRMC1 antibody and AF488-conjugated donkey anti-goat secondary antibody. Nuclear stain: DAPI. Magnification 40x. Cells were grown in culture medium and then prepared on cytospins.

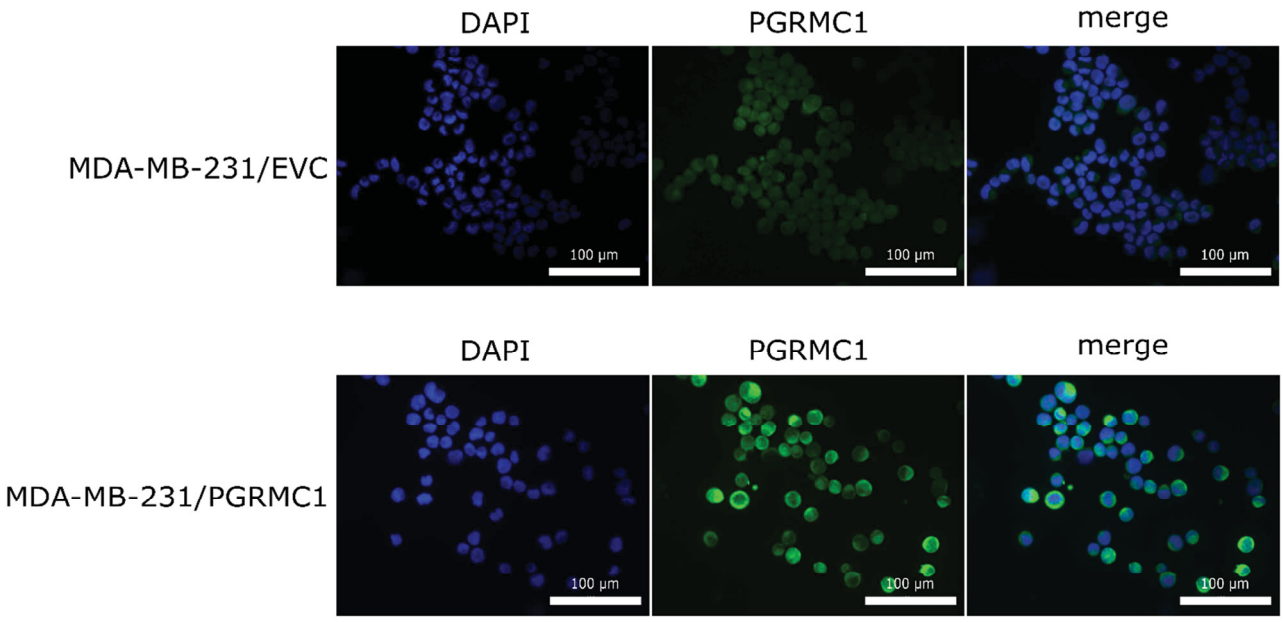


Figure 23. Fluorescent immunocytochemistry of PGRMC1 protein in MDA-MB-231/EVC and MDA-MB-231/PGRMC1 cell lines.
Cells were stained for cellular PGRMC1 with goat-anti human PGRMC1 antibody and AF488-conjugated donkey anti-goat secondary antibody. Nuclear stain: DAPI. Magnification 40x. Cells were grown in culture medium and then prepared on cytospins.

With the purpose to study the role of high PGRMC1 expression in breast cancer on tumor progression, we investigated the impact of PGRMC1 overexpression on proliferation of the transfected breast cancer cell lines. Overexpression of PGRMC1 in the cell lines MCF7 and T47D, representing the luminal A breast cancer subtype, resulted in significant enhanced proliferation compared to the respective EVC cell line (figure 24, figure 25). In contrast, overexpression of PGRMC1 in MDA-MB-231 cells, representing the basal breast cancer subtype, did not lead to increased proliferation; the overexpressing- and the EVC cell line exhibited an identical proliferation rate (figure 26).

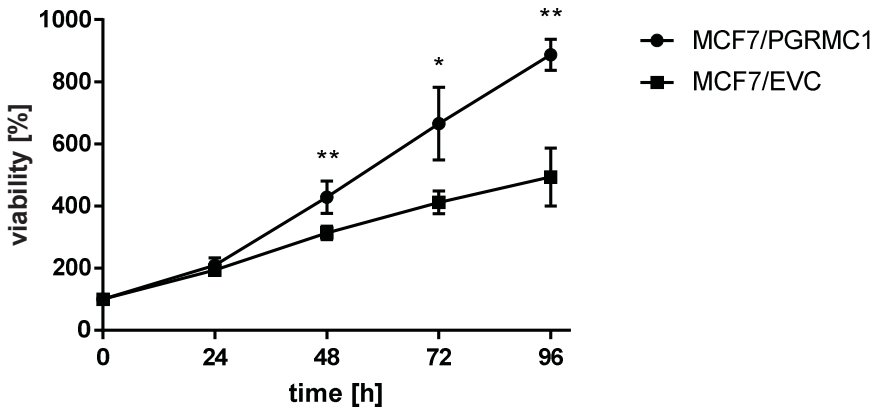


Figure 24. Cell viability of MCF7/EVC and MCF7/PGRMC1 cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$.

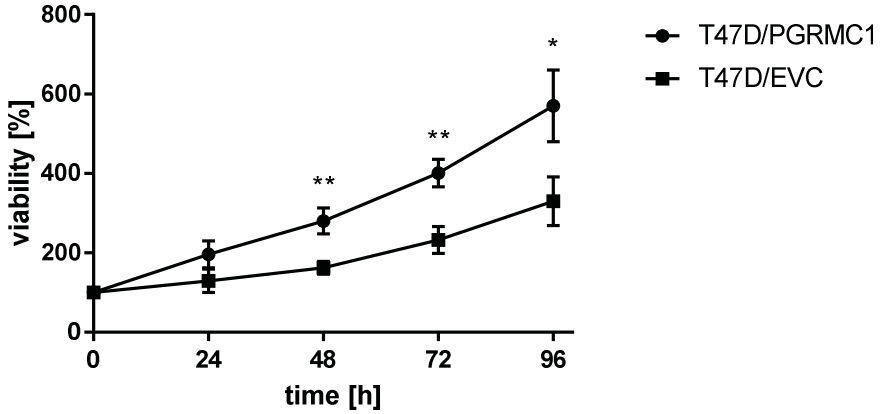


Figure 25. Cell viability of T47D/EVC and T47D/PGRMC1 cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$.

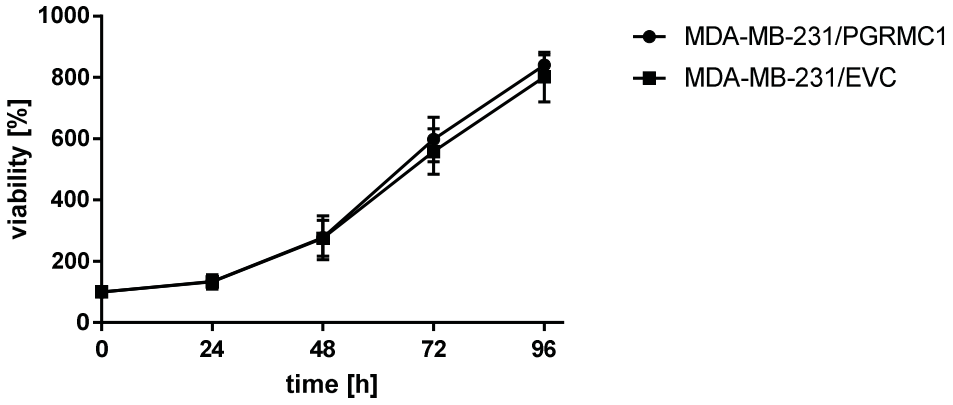


Figure 26. Cell viability of MDA-MB-231/EVC and MDA-MB-231/PGRMC1 cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %).

3.1.3. PGRMC1 inhibition and downregulation reduces proliferation of breast cancer cells

Next, we used the selective PGRMC1 inhibitor AG-205, which was shown to interact with the cytochrome b5/heme-binding domain of PGRMC1 and to alter the subcellular localization of the receptor, to investigate the effect of PGRMC1 inhibition on tumor cell proliferation. AG-205 was applied in various concentrations to MCF7 cells and its effect on cell viability was assessed. With increasing concentrations of AG-205, we observed a decrease in cell proliferation of the used cell line (figure 27).

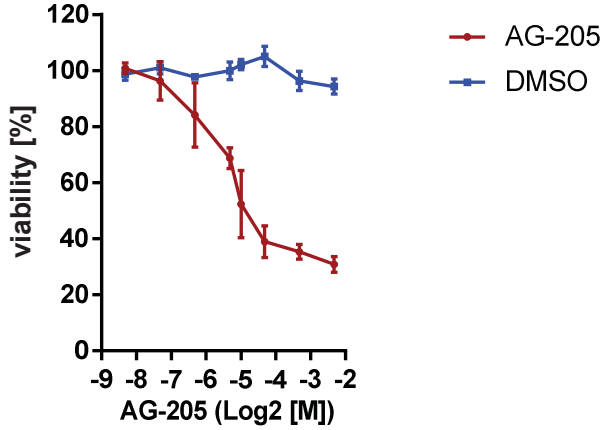


Figure 27. Dose-response curves from MCF7 cells treated with AG205.
Cells were treated with AG205 (3.125, 6.25, 12.5, 25, 31.25, 50, 100 and 200 µM) and respective volume of DMSO over 72 h. Cell viability was analyzed by MTT assay. Values were normalized to untreated cells (100 %).

Since inhibition of endogenous PGRMC1 in MCF7 cells resulted in a significant decrease in proliferation, we further aimed to investigate the effect of PGRMC1 knockdown on proliferation of breast cancer cells. Four different siRNAs targeting endogenous PGRMC1

were used for knockdown of PGRMC1 in MCF7, T47D and MDA-MB-231 cells (figure 28). Treatment of MCF7 and T47D cells with siRNA directed against PGRMC1, resulted in significant decreased cell proliferation compared to cells treated with control siRNA (figure 29, figure 30). However, in MDA-MB-231 cells, knockdown of PGRMC1 did not show any effect (figure 31).

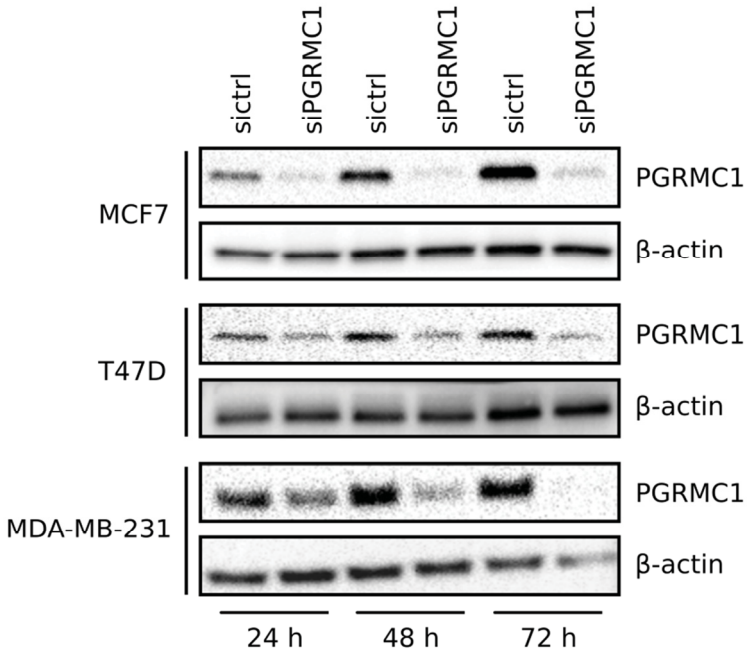


Figure 28. Western blot analysis of knockdown of PGRMC1 in MCF7, T47D and MDA-MB-231 cells.
Cells were treated with siRNA against PGRMC1 (siPGRMC1) and scrambled siRNA (sictrl). Cells were harvested and lysed after 24 h, 48 h and 72 h.

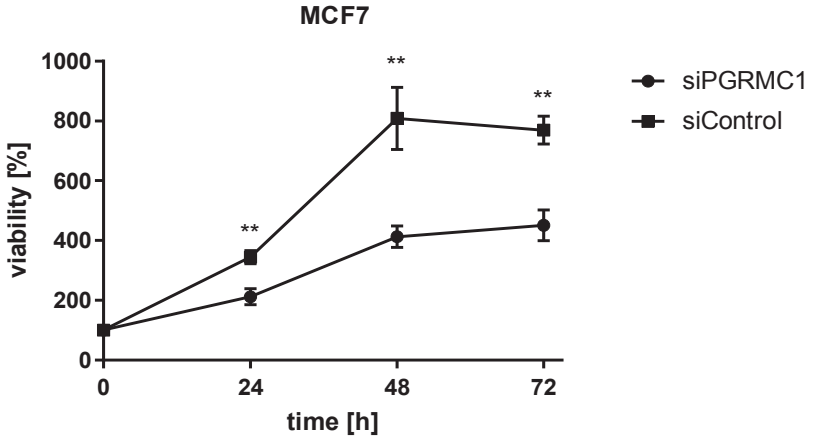


Figure 29. Cell viability of MCF7 cells, treated with siRNA against PGRMC1 (siPGRMC1) and scrambled siRNA (siControl).
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h and 72 h. Values were normalized to t = 0 (100 %). **: $p < 0.01$.

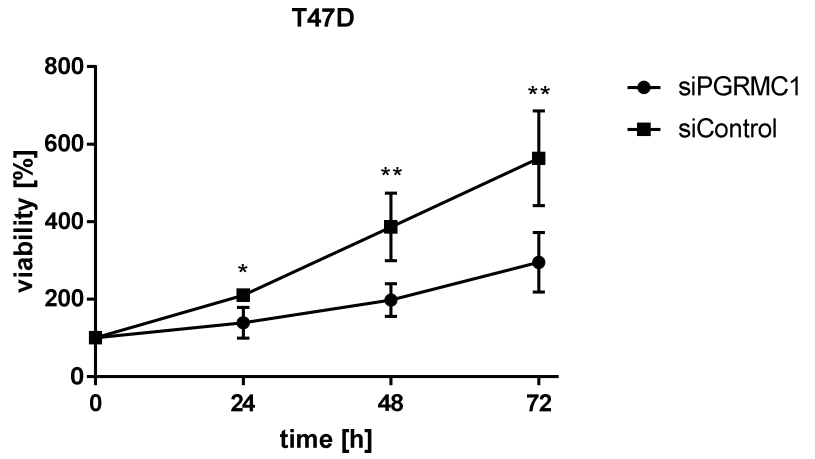


Figure 30. Cell viability of T47D cells, treated with siRNA against PGRMC1 (siPGRMC1) and scrambled siRNA (siControl). Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h and 72 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$.

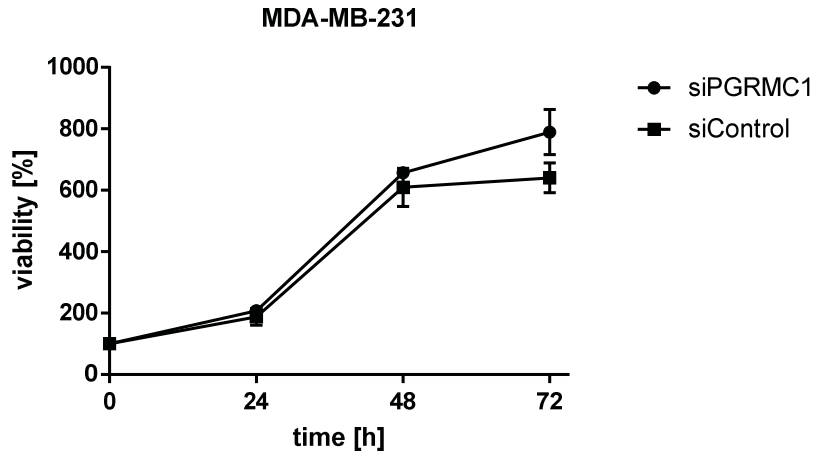


Figure 31. Cell viability of MDA-MB-231 cells, treated with siRNA against PGRMC1 (siPGRMC1) and scrambled siRNA (siControl).
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h and 72 h. Values were normalized to t = 0 (100 %).

3.1.4. Detection of PGRMC1 phosphorylation sites

PGRMC1 exhibits various functions, which are potentially regulated by differential phosphorylation, indicating a role of the receptor not only at expression level but also at the level of phosphorylation in breast cancer[113]. We investigated PGRMC1 phosphorylation in MCF7/PGRMC1 cells by mass spectrometry after immunoprecipitation of PGRMC1 from whole-cell lysates. Peptides containing phosphorylation of PGRMC1 at the sites Ser54, Ser57, Ser181, Thr178 and Tyr180 could be identified (supplemental table 1). Using the scansite tool (http://scansite.mit.edu/), potential protein kinases, responsible for phosphorylation of the respective amino acid and potential binding domains were identified (table 6)[210].

Table 6. PGRMC1 phosphorylation sites identified by mass spectrometry in MCF7/PGRMC1 cells.

Amino acid	Position	Sequence (localization probability)	Motif	Motif Group	Stringency
S	S54	IVRGDQPAA**S**(0.863)GD**S**DDDEPPPLPR	DNA PK	DNA damage kinase group	low
S	S57	GDQPAA**S**GD**S**(0.999)DDDEPPPLPR	Casein Kinase 2	Acidophilic serine/threonine kinase group	high
Y	Y180	EGEEP**T**V**Y**(0.851**)S**DEEEPKDESAR	SHIP SH2	Src homology 2 group (SH2)	medium
S	S181	EGEEPTVY**S**(1)DEEEPKDESARK	Casein Kinase 2	Acidophilic serine/threonine kinase group	medium
T	T178	LLKEGEEP**T**(0.9)V**YS**DEEEPKDESAR	Casein Kinase 2	Acidophilic serine/threonine kinase group	low

Localization probability indicates the probability of which the phosphorylation is the stated amino acid. Protein motifs as predicted from scansite website (http://scansite3.mit.edu/)[210]. (DNA PK: DNA-dependent serine/threonine protein kinase, SH2: Src homology 2, SHIP: SH2-containing inositol phosphatase).

Results reported in paragraph 3.1.4. were published in Willibald et al.[123]

3.1.5. PGRMC1 and pPGRMC1 are upregulated in breast cancer

Previous studies imply regulation of PGRMC1 functions by phosphorylation. In MCF7 breast cancer cell lines, PGRMC1 exhibited various phosphorylation sites, including S181A. Based on this background, we aimed to describe the abundance status of PGRMC1 and its phosphorylated version at serine 181 (pPGRMC1) in breast cancer and surrounding tissue by immunohistochemistry.

Abundance of PGRMC1- and pPGRMC1 was investigated in tissue samples of 69 breast cancer patients. Every analyzed tumor sample was positive for PGRMC1 and pPGRMC1, with a majority of the specimen revealing strong positive staining for PGRMC1 (85.5%) and pPGRMC1 (92.7%) (table 7).

Table 7. Expression of PGRMC1 and pPGRMC1 in tissue biopsies of 69 breast cancer patients.

	PGRMC1 N (%)	pPGRMC1 N (%)
negative	0	0
positive	67 (97.1)	67 (97.1)
weak	1 (1.5)	2 (2.9)
moderate	7 (10.1)	1 (1.4)
strong	59 (85.5)	64 (92.7)
Total	69 (100.0)	69 (100.0)

N = number of specimen, bracketed percentage of total.

For PGRMC1 as well as for pPGRMC1 a median IRS of 12 was detected in tumor tissue (PGRMC1: range 4 – 12, pPGRMC1: range 3 – 12). Compared to tumor tissue, significant lower abundances of PGRMC1 (median IRS of 2, range 0 – 6) and pPGRMC1 (median IRS of 2, range 0 – 6) were detected in connective tissue (figure 32).

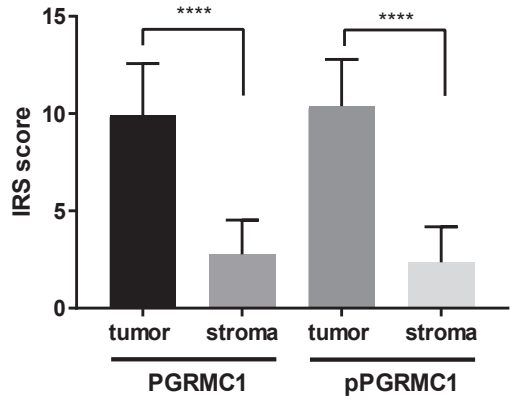
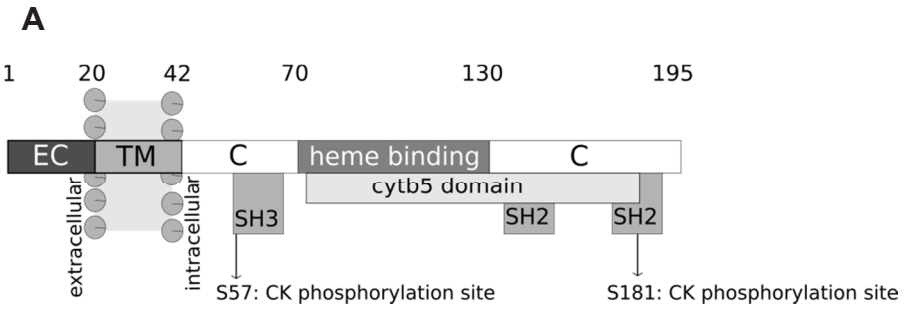


Figure 32. Mean IRS score of PGRMC1 and pPGRMC1 abundance in tumor tissue and tumor stroma
Mean IRS score of PGRMC1 in tumor tissue (9.92±2.7) and tumor stroma (2.92±1.7) and pPGRMC1 in tumor tissue (10.45±2.4) and tumor stroma (2.27±1.8) prior to neoadjuvant therapy (****: $p < 0.0001$).

Results reported in paragraph 3.1.5. were published in Willibald et al.[122] *and were reported before by Wurster I.*[200].

3.1.6. PGRMC1 phosphorylation is essential for increased proliferation

PGRMC1 phosphorylation was presumed to play an important role in regulating its function. To analyze if PGRMC1 phosphorylation at certain phosphosites is important for the enhanced cell proliferation observed for MCF7/PGRMC1 cells, we stably transfected MCF7 cells with PGRMC1, exhibiting point mutations at the CK2-phosphorylation sites S57 (MCF7/PGRMC1-S57A) and S181 (MCF7/PGRMC1-S181A) and a double-mutation at S57 and S181 (MCF7/PGRMC1-S57A/S181A) (figure 33).



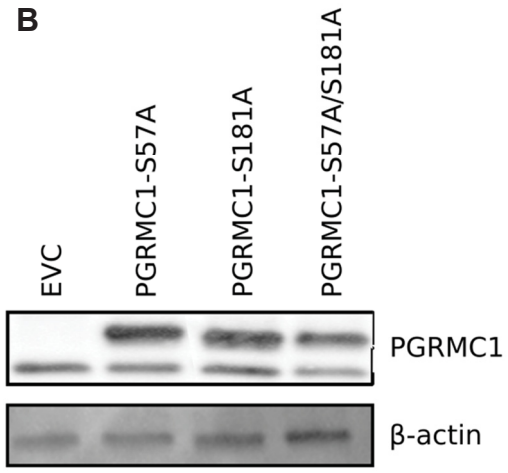


Figure 33. Overexpression of PGRMC1 mutants in MCF7 cells.
A: Schematic structure of PGRMC1 indicating the point-mutated phosphorylation sites S57A, S181A. B: Western blot analysis of MCF7/EVC, MCF7/PGRMC1-S57A, MCF7/PGRMC1-S181A and MCF7/PGRMC1-S57A/S181A cells. EVC cells show expression of endogenous PGRMC1, PGRMC1 overexpressing cells show expression of endogenous (lower band, 23 kDa) and exogenous 3xHA-tagged PGRMC1 (upper band, 28 kDa).

Proliferation of these cell lines was analyzed and compared to the proliferation of cells overexpressing the non-mutated PGRMC1 wild-type. The S57A mutant (figure 34), the S181A mutant (figure 35) as well as the S57A/S181A double mutant (figure 36) revealed significant lower proliferation rates compared to MCF7/PGRMC1 cells, pointing towards an important role of PGRMC1 phosphorylation at S57 and S181 in PGRMC1-activation inducing cell proliferation (figure 33 A).

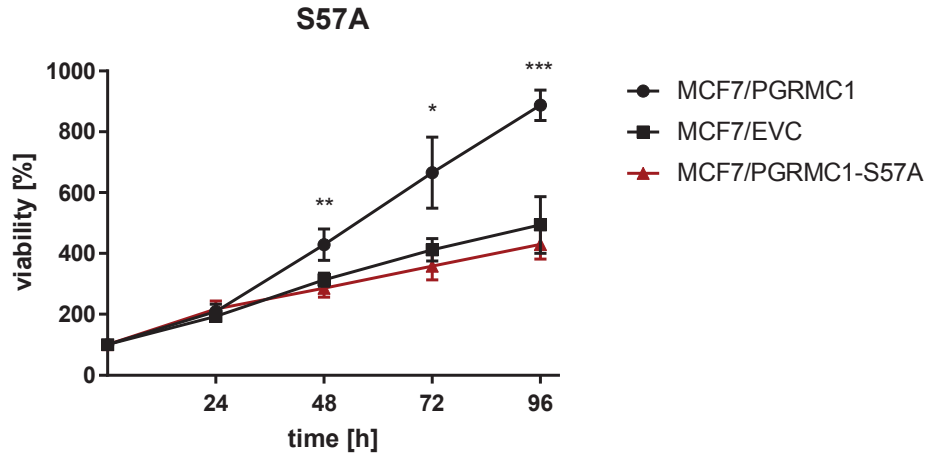


Figure 34. Cell viability of MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S57A cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

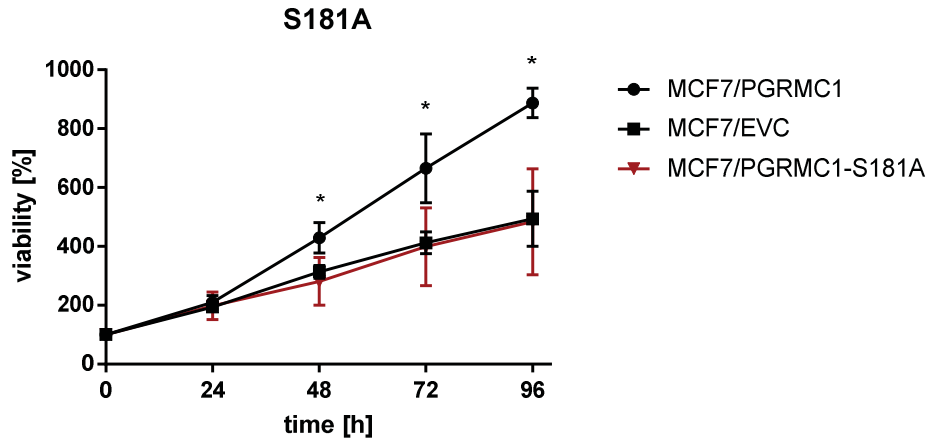


Figure 35. Cell viability of MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S181A cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$.

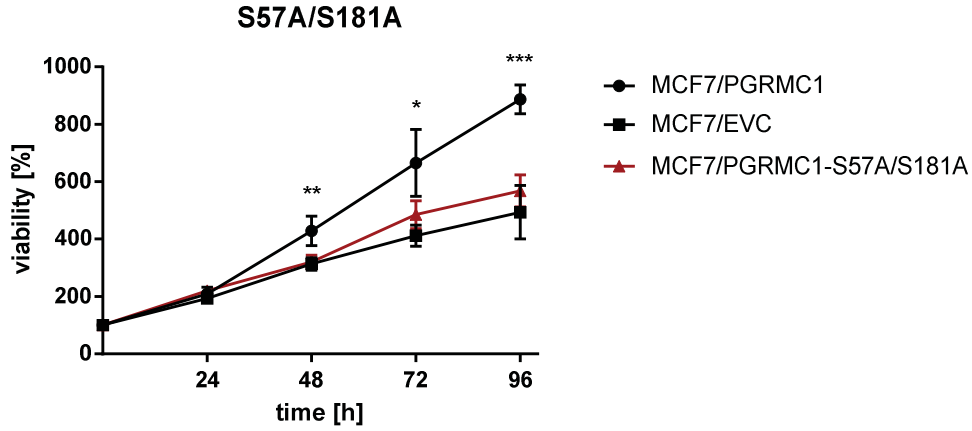


Figure 36. Cell viability of MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S57A/S181A cells.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h, 72 h and 96 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

3.1.7. PGRMC1 mediates upregulation of ERα and ERα downstream targets and activation of EGFR signaling

Since we found PGRMC1 overexpression and -knockdown to only have proliferation-enhancing or -diminishing effects on cells representing the luminal A breast cancer subtype, while on cells representing the basal breast cancer subtype no effect of neither PGRMC1 overexpression nor PGRMC1 knockdown could be observed, we hypothesized that PGRMC1 signaling resulting in increased cancer cell proliferation might be dependent on factors which affect proliferation of cells of the luminal A subtype but not the basal subtype, as for example ERα. Additionally, the results using PGRMC1 phosphorylation-site mutants indicate an important role of PGRMC1 phosphorylation at S57 and S181 in activating the receptor

and further downstream targets. To identify factors, which are involved in PGRMC1 signaling, leading to enhanced proliferation of breast cancer cells, we analyzed the expression and activation (by phosphorylation) of various proteins, which are known to play a role in important signaling cascades in breast cancer. For this purpose, the effect of PGRMC1 overexpression on protein expression and -activation was analyzed in PGRMC1-overexpressing MCF7 and -MDA-MB-231 cells by reverse phase protein array technology (RPPA) and was compared to the respective EVC cells. To further investigate the role of PGRMC1 phosphorylation, we additionally used the PGRMC1 phosphorylation-site mutant cell line MCF7/PGMC1-S181A.

RPPA analysis revealed significant elevated expression of ERα in MCF7/PGRMC1 cells compared to EVC cells. Interestingly, only a tendency but no significant effect for elevated ERα expression could be observed for MCF7/PGRMC1-S181A cells. Additionally, significant higher abundance of Her2 and c-Myc was detected in MCF7/PGRMC1 cells, proteins which are expressed dependent on ERα. However, expression of c-Fos, another ERα target gene, was the same for all 3 cell lines (figure 37). As expected, the triple-negative cell lines MDA-MB-231/EVC and MDA-MB-231/PGRMC1 did not express ERα, Her2 and PR. Besides, MDA-MB-231/PGRMC1 did not show increased abundance of c-Myc, as observed for PGRMC1/MCF7 cells (figure 38).

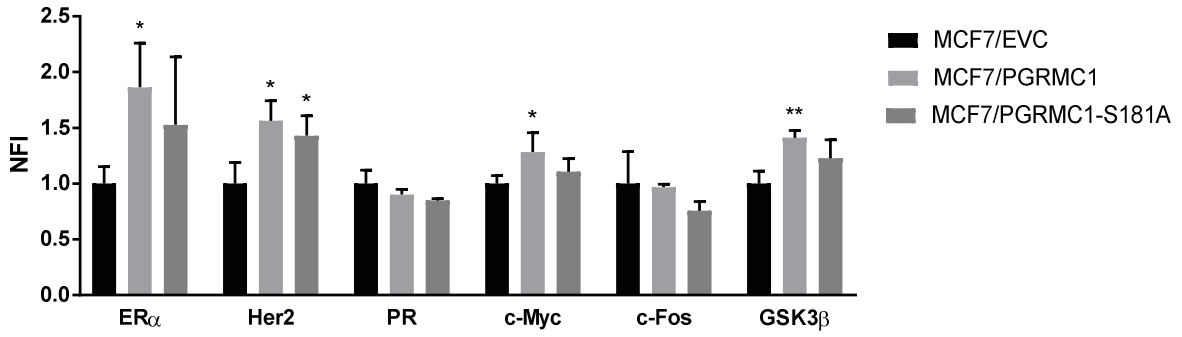


Figure 37. RPPA analysis of protein expression.
Protein expression of ERα, Her2, PR, c-Myc, c-Fos and GSK3β analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). Protein expression was normalized to MCF7/EVC and protein expression measured in MCF7/EVC cells was set to 1. Up-/downregulation of protein expression in MCF7/PGRMC1 and MCF7/PGRMC1-S181A were calculated. *: $p < 0.05$, **: $p < 0.01$.

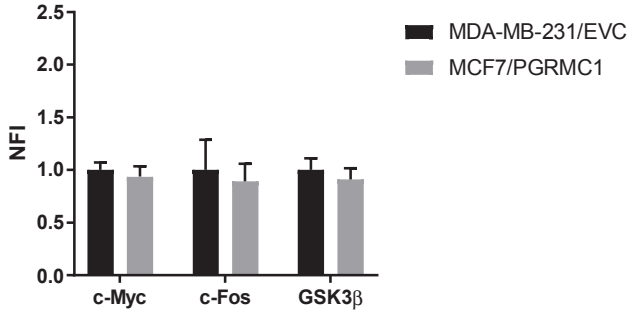


Figure 38. RPPA analysis of protein expression.
Protein expression of c-Myc, c-Fos and GSK3β analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). Protein expression was normalized to MDA-MB-231/EVC and protein expression measured in MDA-MB-231/EVC cells was set to 1. Up-/downregulation of protein expression in MCF7/PGRMC1 were calculated.

To verify the results obtained by RPPA, western blot analysis was performed and expression of ERα in PGRMC1 overexpressing MCF7 as well as -T47D cells was analyzed. As shown by RPPA, western blot analysis revealed increased expression of ERα in PGRMC1 overexpressing MCF7 and -T47D cell lines compared to the respective EVC cells (figure 39).

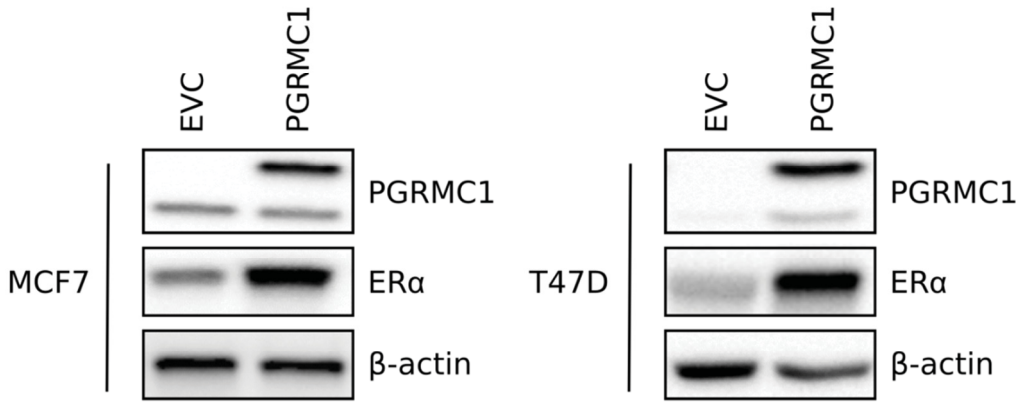


Figure 39. Western blot analysis of ERα expression in MCF7/EVC, T47D/EVC cells and MCF7/PGRMC1, T47D/PGRMC1 cells. PGRMC1 overexpressing cells (MCF7/PGRMC1, T47D/PGRMC1) show increased expression of ERα compared to EVC cells (MCF7/EVC, T47D/EVC).

Analysis of ERα mRNA (ESR1) expression in MCF7/PGRMC1 cells further revealed significant elevated ESR1 mRNA expression in PGRMC1 overexpressing cells compared to EVC cells (figure 40).

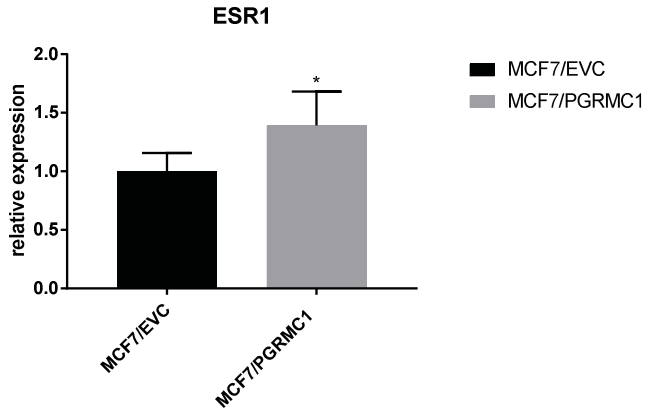


Figure 40. Analysis of ESR1 mRNA expression in MCF7/EVC and MCF7/PGRMC1 cells.
PGRMC1 overexpressing cells (MCF7/PGRMC1) show increased expression of ESR1 mRNA compared to EVC cells (MCF7/EVC). *: $p < 0.05$

The RPPA results indicate that increased expression of ERα also results in upregulated expression of ERα dependent genes, such as HER2 and c-Myc. To further investigate this hint, expression of TFF1 (trefoil factor 1), an ERα target gene, was analyzed. As expected, quantification of mRNA expression of TFF1 in MCF7/PGRMC1 cells revealed significant increased expression of TFF1 in PGRMC1 overexpressing MCF7 cells compared to EVC cells (figure 41).

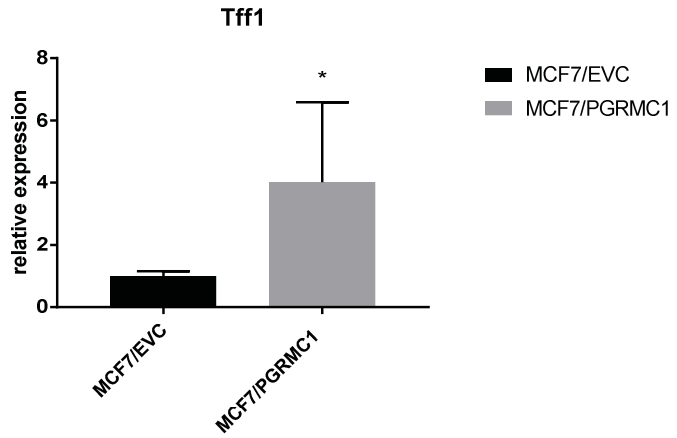


Figure 41. Analysis of TFF1 mRNA expression in MCF7/EVC and MCF7/PGRMC1 cells.
PGRMC1 overexpressing cells (MCF7/PGRMC1) show increased expression of TFF1 mRNA compared to EVC cells (MCF7/EVC). *: $p < 0.05$

Analysis of altered protein phosphorylation and respectively activation exhibited significant elevated phosphorylation of EGFR (p-Tyr1068), Akt (p-Ser473 and p-Thr308), MEK1/2 (p-Ser217/Ser221), ERK1/2 (p-Thr202/Tyr204) and S6 (p-Ser240/Ser244) in PGRMC1 overexpressing MCF7 cells compared to the EVC cell. In contrast, in MCF7/PGRMC1-S181A cells, no enhanced phosphorylation of the mentioned proteins could be detected (figure 42).

Furthermore, also in MDA-MB-231/PGRMC1 cells no upregulation of phosphorylation compared to the EVC cell was observed (figure 43). These results strongly suggest an association of PGRMC1 expression and activation on important pathways, known to be upregulated in cancer cells and to increase their malignancy.

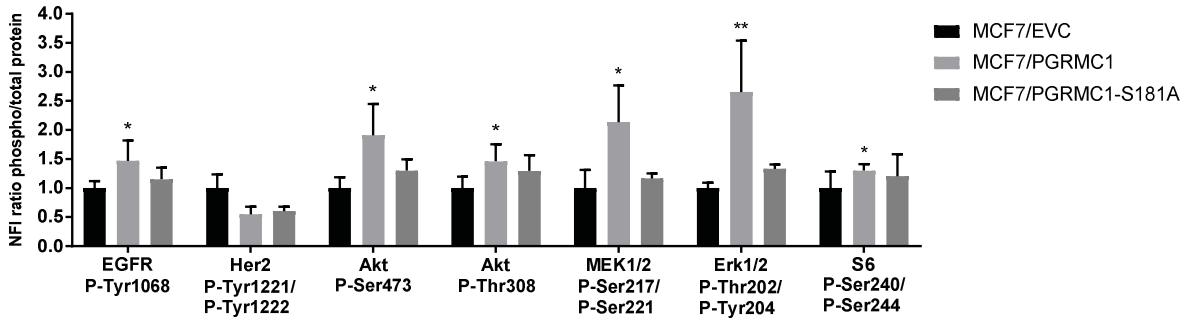


Figure 42. RPPA analysis of protein phosphorylation.
Protein phosphorylation of EGFR P-Tyr1068, Her2 P-Tyr1221/Tyr1222, Akt P-Ser473, Akt P-Thr308, MEK1/2 P-Ser217/Ser221, Erk1/2 P-Thr202/Tyr204 and S6 P-Ser240/Ser244 analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots, applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). NFI ratio of phospho-protein/total protein was calculated, normalized to MCF7/EVC and ratio in MCF7/EVC cells was set to 1. Up-/downregulation of protein phosphorylation in MCF7/PGRMC1 and MCF7/PGRMC1-S181A were calculated. *: $p < 0.05$, **: $p < 0.01$.

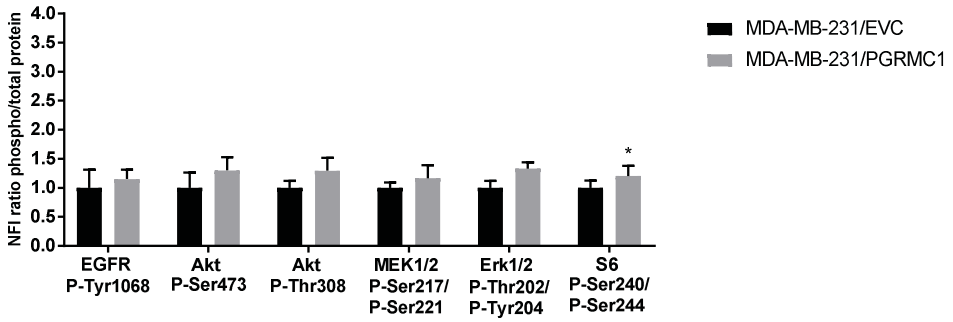


Figure 43. RPPA analysis of protein phosphorylation.
Protein phosphorylation of EGFR P-Tyr1068, Akt P-Ser308, Akt P-Thr308, MEK1/2 P-Ser217/Ser221, Erk1/2 P-Thr202/Tyr204 and S6 P-Ser240/Ser244 analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). NFI ratio of phospho-protein/total protein was calculated, normalized to MDA-MB-231/EVC and ratio in MDA-MB-231/EVC cells was set to 1. Up-/downregulation of protein phosphorylation in MDA-MB-231/PGRMC1 were calculated. *: $p < 0.05$, **: $p < 0.01$.

The RPPA results were additionally verified by western blot analysis. To see a fast effect, EGFR signaling was induced with the EGFR ligand EGF and phosphorylation of EGFR, Her2, Akt, MEK1/2 and ERK1/2 was analyzed by Western blot. As seen in RPPA analysis, an increased phosphorylation of EGFR (p-Tyr1068), Akt (p-Ser473), MEK1/2 (p-Ser217/Ser221) and ERK1/2 (p-Thr202/Tyr204) was observed in MCF7/PGRMC1 cells compared to MCF7/EVC cells upon treatment with EGF (figure 44 A), while all 3 cell lines showed a similar expression

level of the respective total protein; however, as seen in RPPA, Her2 was upregulated in MCF7/PGRMC1 cells compared to MCF7/EVC cells (figure 44 B).

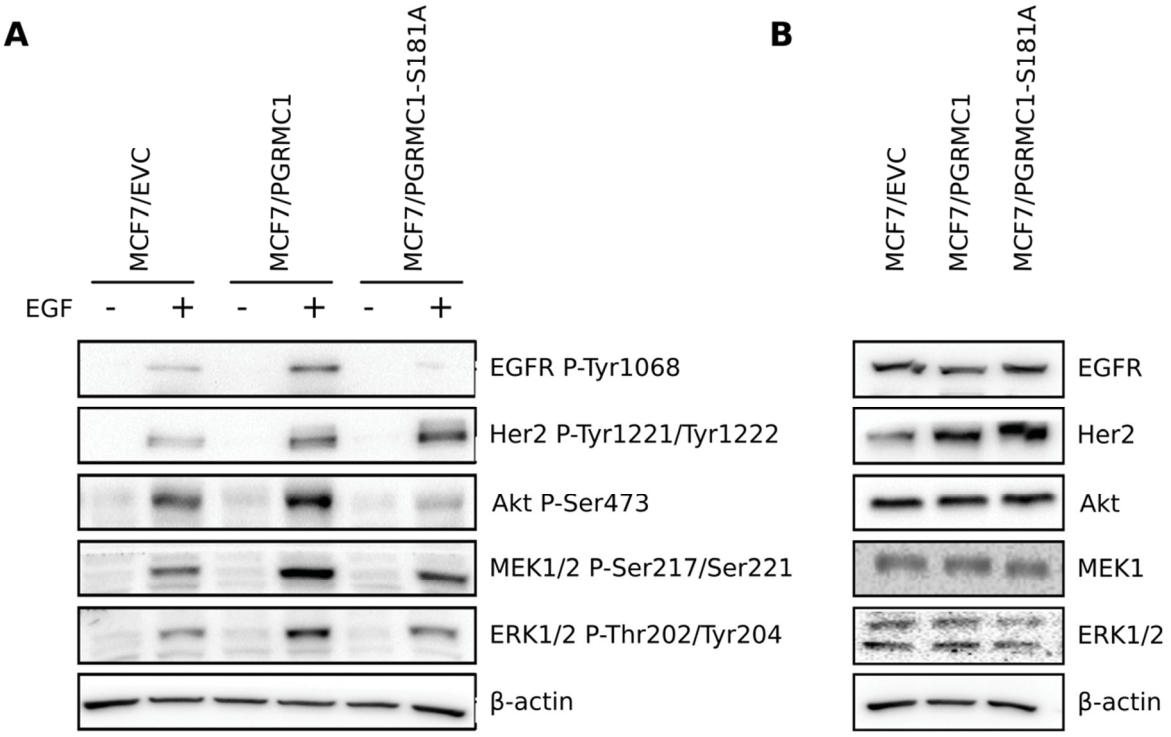


Figure 44. Western blot analysis of protein phosphorylation.
A: Protein phosphorylation of EGFR P-Tyr1068, Her2 P-Tyr1221/Tyr1222, Akt P-Ser473, MEK1/2 P-Ser217/Ser221 and Erk1/2 P-Thr202/Tyr204 verified by Western blot analysis. Cells were treated with EGF [10 ng/ml] for 10 min. B-actin was used as a housekeeper. B: Total protein expression of EGFR, Her2, Akt, MEK1/2 and Erk1/2 verified by western blot analysis. B-actin was used as a housekeeper.

3.1.8. Downregulation of ERα diminishes proliferation of MCF7/PGRMC1 cells

Increased proliferation of PGRMC1 overexpressing breast cancer cells was exclusively detected for ERα-positive cells and PGRMC1-dependent upregulation of ERα and ERα-dependent genes was observed. Therefore, we reasoned that PGRMC1 induces enhanced proliferation via upregulating ERα expression. We aimed to confirm this hypothesis by downregulation of ERα in PGRMC1-overexpressing cells and to determine the effect on cell proliferation. ERα was downregulated by the selective ERα downregulator fulvestrant in MCF7/PGRMC1 cells, leading to significant decreased proliferation compared to cells treated with DMSO as a control (figure 45).

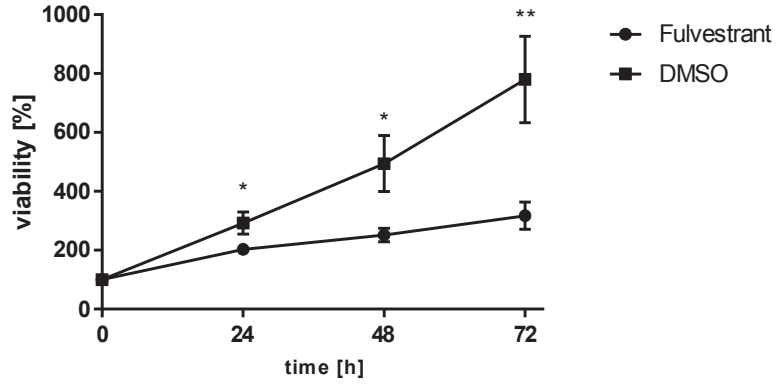


Figure 45. Cell viability of MCF7/PGRMC1 cells, treated with Fulvestrant.
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h and 72 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$.

To confirm the role of ERα in PGRMC1 signaling, ERα was downregulated in MCF7/PGRMC1 cells by using four different siRNAs targeting ESR1 mRNA (figure 46).

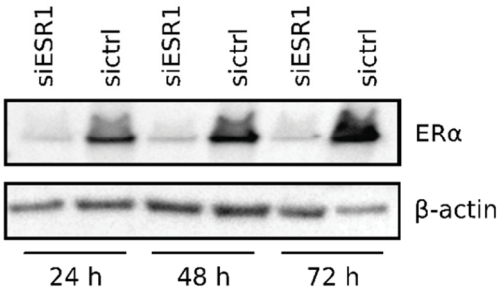


Figure 46. Western blot analysis of knockdown of ERα in MCF7/PGRMC1 cells.
Cells were treated with siRNA against ESR1 (siESR1) and scrambled siRNA (sictrl). Cells were harvested and lysed after 24 h, 48 h and 72 h.

As shown for fulvestrant, downregulation of ERα by siRNA also resulted in significant decreased proliferation of MCF7/PGRMC1 cells compared to siControl cells (figure 47).

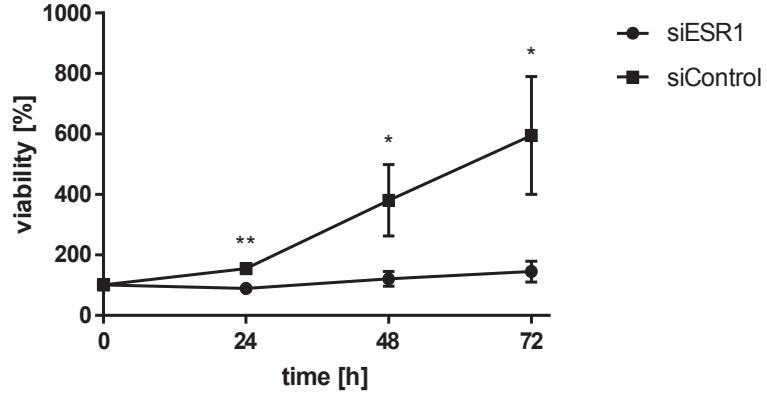


Figure 47. Cell viability of MCF7/PGRMC1 cells, treated with siRNA against ESR1 (siESR1) and scrambled siRNA (siControl).
Cells were grown in normal culture medium and proliferation was analyzed by MTT assay at t = 0 h, 24 h, 48 h and 72 h. Values were normalized to t = 0 (100 %). *: $p < 0.05$, **: $p < 0.01$.

3.1.9. PGRMC1 overexpression sensitizes breast cancer cells to Estradiol

PGRMC1 overexpressing cells exhibit elevated expression of ERα, as well as higher expression of ERα target genes, suggesting increased transcriptional activity of ERα in MCF7/PGRMC1 cells. Based on these observations, we hypothesized that PGRMC1 overexpressing cells are more sensitive to the ERα ligand E2 and should react with enhanced proliferation. As expected, treatment of MCF7/PGRMC1 and MCF7/EVC cells with increasing concentrations of E2 resulted in elevated proliferation of both cell types. However, MCF7/PGRMC1 cells exhibited significant enhanced proliferation compared to EVC cells especially at very low E2 concentrations. The PGRMC1 overexpressing cell line reacted with increased proliferation already at an E2 concentration of 10^{-12} M, while MCF7/EVC cells started with elevated proliferation at a E2 concentration of 10^{-11} M (figure 48), indicating enhanced susceptibility of MCF7/PGRMC1 cells to E2 treatment, potentially due to higher expression of its receptor ERα.

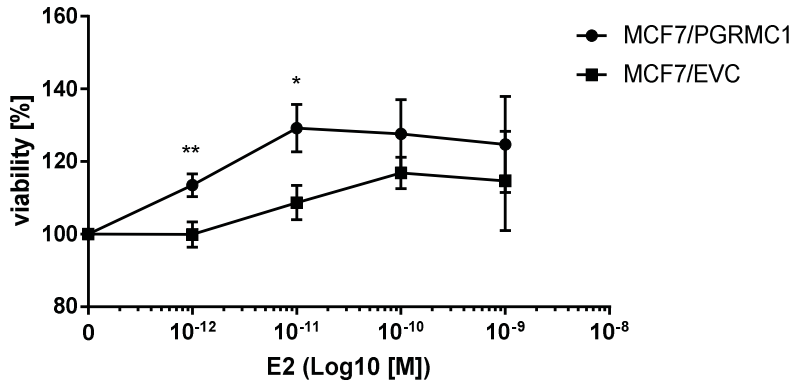


Figure 48. Dose-response curves from MCF7/PGRMC1 and MCF7/EVC cells treated with E2.
MCF7/PGRMC1 and MCF7/EVC cells were treated with E2 (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} M) for 72 h. Cell viability was analyzed by MTT assay. Values were normalized to untreated cells (100 %). *: $p < 0.05$, **: $p < 0.01$.

3.1.10. PGRMC1 overexpression decreases susceptibility of breast cancer cells to anthracycline treatment

PGRMC1 was reported to interact with cytochrome P450 enzymes and could thereby potentially play a role in degradation of drugs, including chemotherapeutics[117,189]. Aiming to further investigate the role of PGRMC1 in resistance to cytostatic agents, used in breast cancer, we treated MCF7/PGRMC1 cells and MCF7/EVC cells with the anthracyclines Epirubicin (Epi) and Doxorubicin (Dox). Additionally to treatment with anthracyclines, the cells were treated with the PGRMC1 ligand P4. MCF7/PGRMC1 cells revealed significant

decreased susceptibility to Epirubicin and Doxorubicin treatment as compared to the EVC cells, however only for simultaneous treatment with P4 (figure 49 A, C). Treatment of MCF7/PGRMC1 cells with various concentrations of anthracyclines revealed significant decreased susceptibility of the cells to anthracyclines when simultaneously treated with P4 (figure 49 B, D).

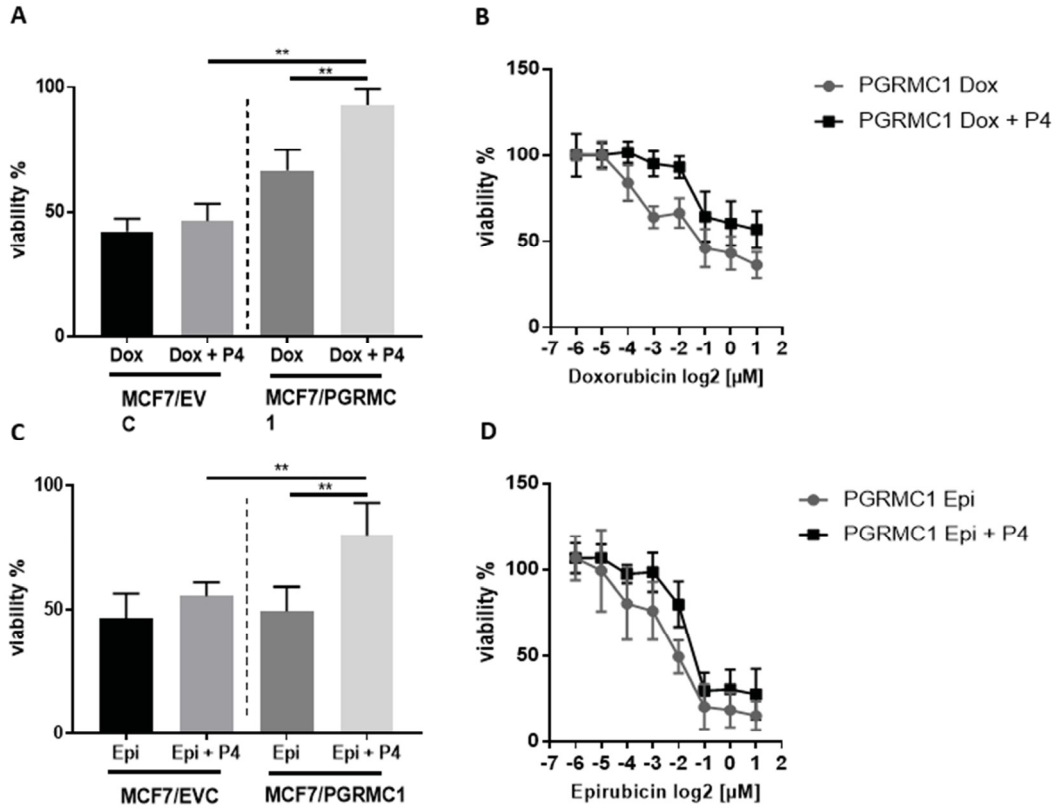


Figure 49. Treatment of MCF7/EVC and MCF7/PGRMC1 cells with Dox and Epi
A, C: Treatment of MCF7/EVC and MCF7/PGRMC1 cells with Dox [250 nM] or Epi [250 nM]. When simultaneously treated with P4 [1 µM] MCF7/PGRMC1 cells are significantly less susceptible to anthracycline treatment as compared to MCF7/EVC cells ($p < 0.01$). B, D: Dose-response curves from MCF7/PGRMC1 cells treated with Doxorubicin and Epirubicin. Cells are less susceptible to anthracycline treatment, when simultaneously treated with P4 [1 µM]. Cell viability was analyzed by MTT assay. Values were normalized to untreated cells (100 %). **: $p < 0.01$.

Results reported in paragraph 3.1.10. were published in Willibald et al.[122]

3.1.11. High PGRMC1 expression correlates with resistance to anthracycline treatment

To further investigate the potential role of PGRMC1 in resistance to cancer therapy, we determined the PGRMC1 expression in tumors of 68 breast cancer patients prior to neoadjuvant therapy and correlated it to therapy response. The patients received neoadjuvant therapy either based on four to six cycles (21-day interval) of anthracyclines (n = 17) or taxanes (n = 30) or a daily endocrine therapy with Letrozol or Exemestane (n = 22)

for six months. After neoadjuvant therapy, 49.4 % of the patients showed response to treatment (partial remission: **R**), while 50.7 % (34 patients) did not respond to therapy (stable disease or progress: **NR**). For each treatment, the patients were stratified for therapy response and correlation between PGRMC1 abundance and response to therapy was determined. Stratifying the patients for therapy response, we observed a significant higher IRS of PGRMC1 in the anthracycline-treated patient cohort, which did not respond to neoadjuvant therapy compared to patients, which responded to anthracycline-based therapy. For the taxane-based- as well as the Aromatase-inhibitor treated cohort, no differences in PGRMC1 abundance between responders and non-responders could be detected (figure 50).

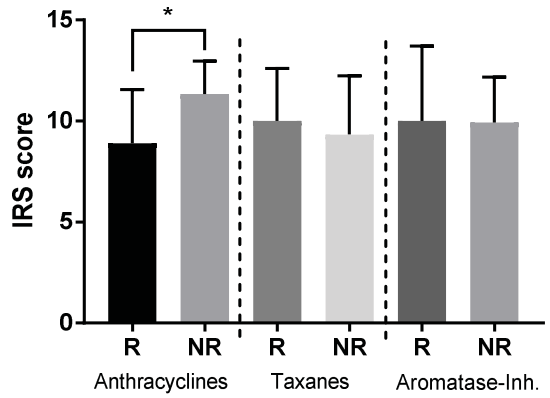


Figure 50. IRS score of PGRMC1 tumor tissue prior to neoadjuvant therapy.
Patients were divided into treatment groups and stratified for therapy response (R: responders, NR: non-responders). For patients treated with anthracyclines, a significant higher IRS score was detected for patients who did not respond to neoadjuvant therapy as compared to patients which responded to anthracycline-based neoadjuvant therapy *: $p < 0.05$.

Results reported in paragraph 3.1.10. were published in Willibald et al.[122]

3.1.12. PGRMC1 interaction partners

To date, little is known about PGRMC1 signaling in breast cancer in general and its interaction partners in particular. With the aim to gain deeper insight into the PGRMC1 signaling cascade in breast cancer, we searched for potential PGRMC1-interaction partners. PGRMC1-HA was immunoprecipitated from whole cell lysates of MCF7/PGRMC1-HA cells using an antibody directed against the HA-tag. As a control, MCF7 cells overexpressing GFP-tagged PGRMC1 (MCF7/PGRMC1-GFP) were used. Potential co-immunoprecipitated PGRMC1 interaction partners were detected by mass spectrometry. An overview of the detected interaction partners with significant higher intensities for PGRMC1-HA samples is shown in figure 51. The most significant proteins are proteins with a very high value for

student t-test difference HA_GFP and –log students t-test p-value HA_GFP, meaning the most significant proteins can be found in the upper right quarter.

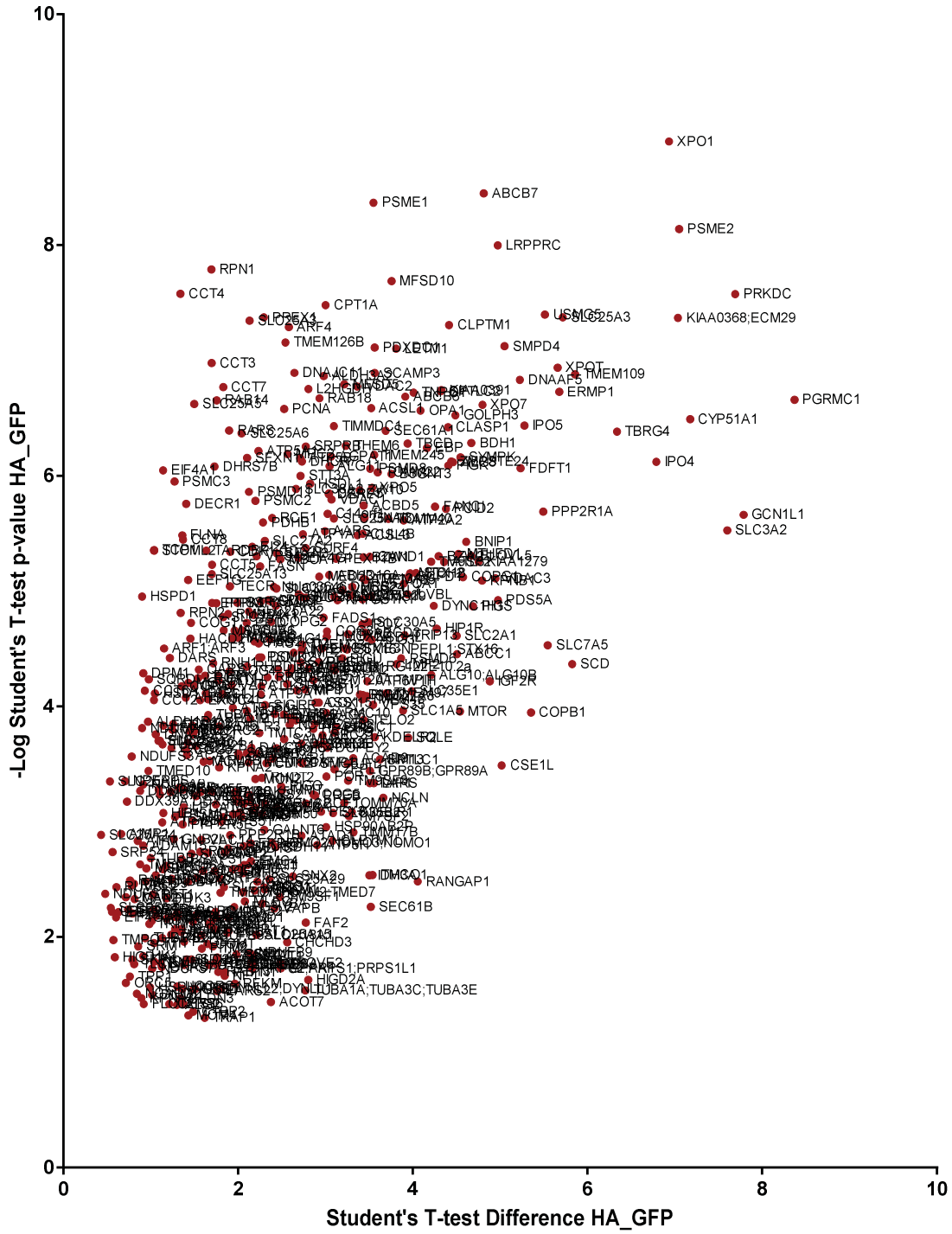


Figure 51. PGRMC1 interaction partners detected by mass spectrometry.
Scatter plot of proteins revealing significant higher intensities in PGRMC1-HA samples compared to PGRMC1-GFP samples identified by mass spectrometry. Plotted is the fold change (x-axis) versus the significance (y-axis).

To further investigate pathways and cellular functions, which involve PGRMC1 protein-protein interactions, the dataset was analyzed using Gene Ontology (GO;

geneontology.org)[211,212]. The database assigns the proteins to respective GO-annotations, i.e. biological functions. Figure 52 shows the number of proteins detected for particular GO-biological processes.

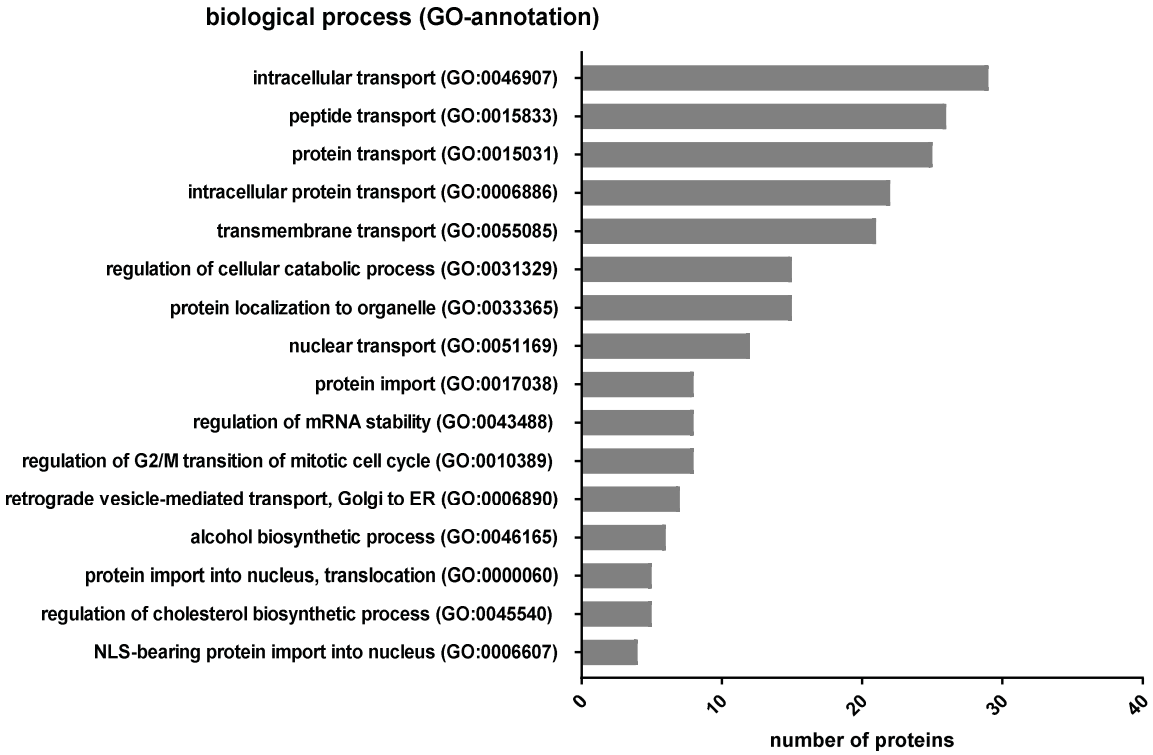


Figure 52. PGRMC1 interaction partners associated with GO-annotations for biological functions.
Number of proteins assigned to the respective biological function. The dataset was analyzed using Gene Ontology (geneontology.org). Total number of proteins in the dataset: 100.

Among the detected interaction partners, various proteins involved in cellular (protein) transport processes were found, including proteins participating in vesicle trafficking, such as COPB1 (Coatomer subunit beta) and COPG1 (Coatomer subunit gamma-1), which mediate the biosynthetic protein transport from the endoplasmic reticulum via the Golgi up to the trans Golgi network[213–216].

Interestingly, also various proteins, involved in nuclear export and import processes, such as Exportin-1, -2, -5, -7 and -T as well as Importin-4 and -5 were detected. Proteins which are imported from the cytoplasm into the nucleus carry nuclear localization signals (NLS). Their nuclear import is mediated by interaction of NLS with importin transport receptors. To investigate if PGRMC1 might be translocated into the nucleus by importins, the nls-mapper (nls-mapper.iab.keio.ac.jp) was used for prediction of importin dependent nuclear localization signals[217]. Using the nsl-mapper, we found a nsl-signal within the PGRMC1

primary structure (figure 53), strongly suggesting nuclear transport and therefore nuclear function of PGRMC1.

```
         10         20         30         40         50
MAAEDVVATG ADPSDLESGG LLHEIFTSPL NLLLLGLCIF LLYKIVRGDQ
         60         70         80         90        100
PAASGDSDDD EPPPLPRLKR RDFTPAELRR FDGVQDPRIL MAINGKVFDV
        110        120        130        140        150
TKGRKFYGPE GPYGVFAGRD ASRGLATFCL DKEALKDEYD DLSDLTAAQQ
        160        170        180        190
ETLSDWESQF TFKYHHVGKL LKEGEEPTVY SDEEEPKDES ARKND
```

Figure 53. Primary structure pf PGRMC1 exhibits a NLS.
Main splice variant of PGRMC1. Red marked amino acids: predicted importin dependent nuclear localization signals (NLS) identified by NSL-mapper (nls-mapper.iab.keio.ac.jp).

Further, proteins involved in cholesterol biosynthesis were detected, including Acyl-CoA desaturase (SCD), Squalene synthase (FDFT1), Squalene monooxygenase (SQLE) and Lanosterol 14-alpha demethylase (CYP51A1).

3.1.13. PGRMC1 interacts with CYP51A1

In the present study, CYP51A1 was within the five interaction partners with the highest significance. CYP51A1 is a cytochrome P450 enzyme, which is involved in cholesterol biosynthesis. Cytochrome P450 enzymes (CYPs) are known to be key players in the biosynthesis and breakdown of steroids (including estrogen and testosterone), lipids, and vitamins. Further, CYPs are important enzymes involved in metabolization of toxic compounds, including drugs and endogenous metabolism products. To further verify the potential interaction of PGRMC1 and CYP51A1, western blot analysis and proximity ligation assay were performed. As already observed in mass spectrometry (figure 54 A), interaction of PGRMC1 and CYP51A1 could be detected after co-immunoprecipitation of PGRMC1-HA followed by Western blot analysis detecting CYP51A1 (figure 54 B). To validate this result with a method independent of immunoprecipitation, interaction of PGRMC1 and CYP51A1 was investigated by proximity ligation assay (PLA) in MCF7 cells. PLA revealed interaction of PGRMC1 and CYP51A1 (figure 55), supporting the results from mass spectrometry.

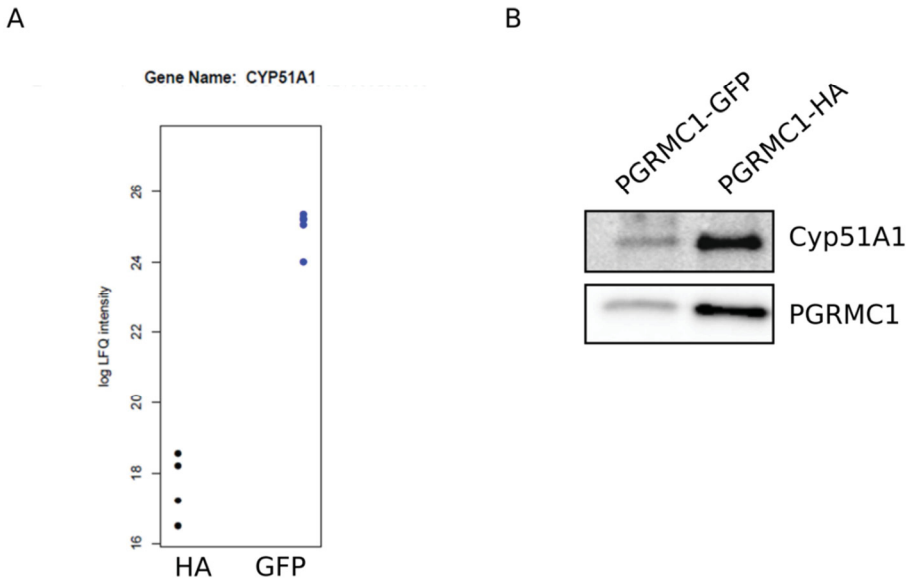


Figure 54. Interaction of CYP51A1 with PGRMC1.
A: Mass spectrometry results of immunopurified PGRMC1-GFP (GFP) and PGRMC1-HA (HA) samples for CYP51A1, log2 normalized intensity. B: Western blot analysis of immunopurified PGRMC1-GFP and PGRMC1-HA samples. CYP51A1: rabbit-anti-CYP51A1 antibody, PGRMC1: rabbit-anti-human PGRMC1 antibody.

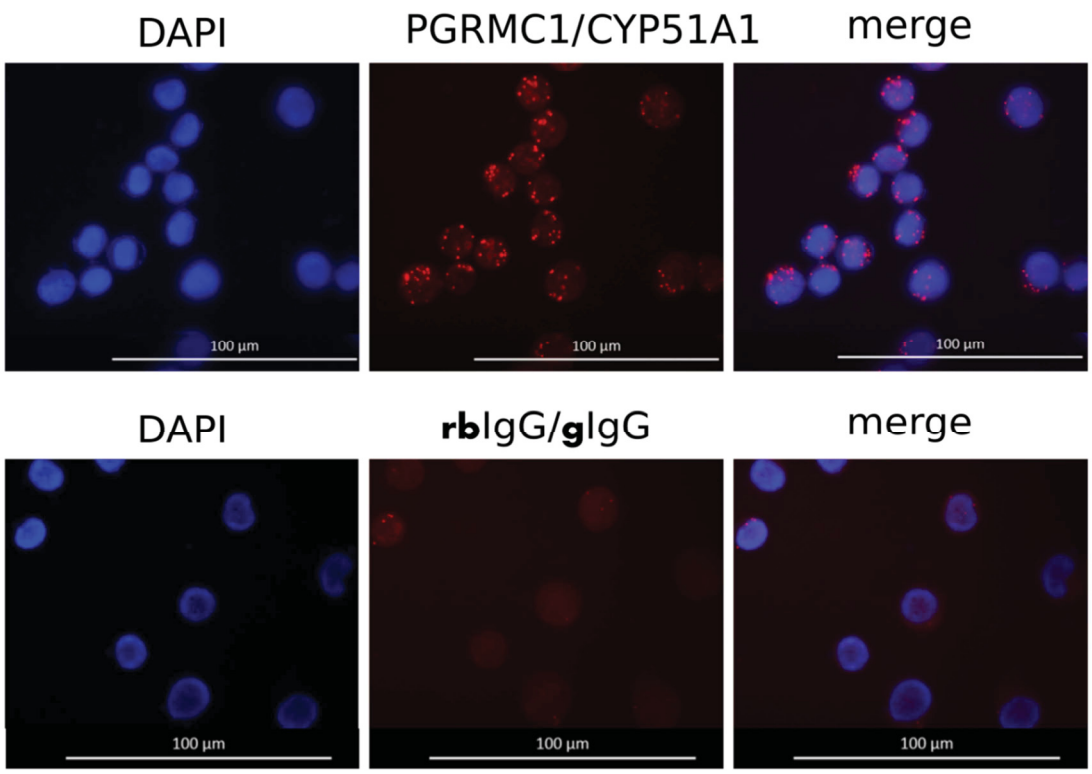


Figure 55. Proximity ligation assay for protein interactions between PGRMC1 and CYP51A1 in MCF7 cells.
Each red spot represents a single interaction. Nuclear stain: DAPI. Magnification 40x. Cells were grown in culture medium and then prepared on cytospins. Negative control: rabbit IgG, goat IgG isotype controls.

3.2. Role of PGRMC1 in increased breast cancer risk upon progestin-based contraception and hormone therapy

3.2.1. PGRMC1 promotes proliferation breast cancer cells upon progestin treatment

In previous studies, PGRMC1 was shown to forward progestin signals, resulting in enhanced proliferation of breast cancer cells, indicating a potential role of PGRMC1 in the often-reported higher breast cancer risk upon progestin-based hormone therapy.

To further study this effect, we used the above described PGRMC1-overexpressing breast cancer cell lines MCF7/PGRMC1, T47D/PGRMC1 and MDA-MB-231/PGRMC1 and investigated cell proliferation upon treatment with various progestins used in hormone therapy [10^{-6} M]. Treatment of MCF7/PGRMC1 and T47D/PGRMC1 revealed significant increased proliferation upon treatment with the progestins NET, DYD and DSP compared to the respective EVC cells. For MCF7/PGRMC1, significant higher proliferation was also observed for treatment with MPA. Treatment of MCF7/PGRMC1 and T47D/PGRMC1 cells with CPA, NOM and P4 did not lead to enhanced proliferation compared to the respective EVC cells (figure 56, figure 57). In contrast, treatment of PGRMC1 overexpressing MDA-MB-231 cells with any progestin or P4 did not induce increased proliferation compared to MDA-MB-231/EVC cells (figure 58).

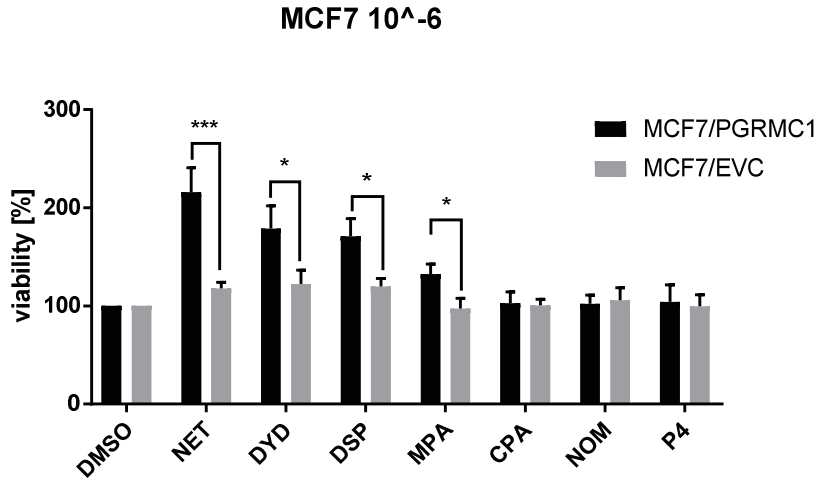


Figure 56. Cell viability of MCF7/EVC and MCF7/PGRMC1 cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

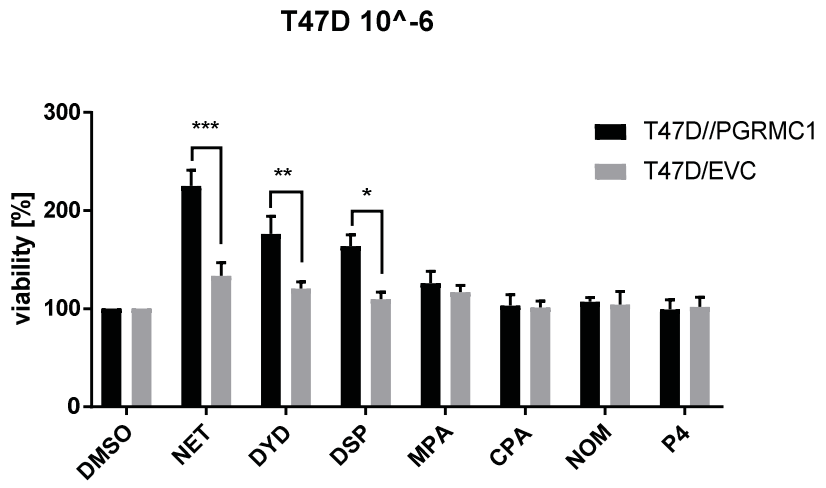


Figure 57. Cell viability of T47D/EVC and T47D/PGRMC1 cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

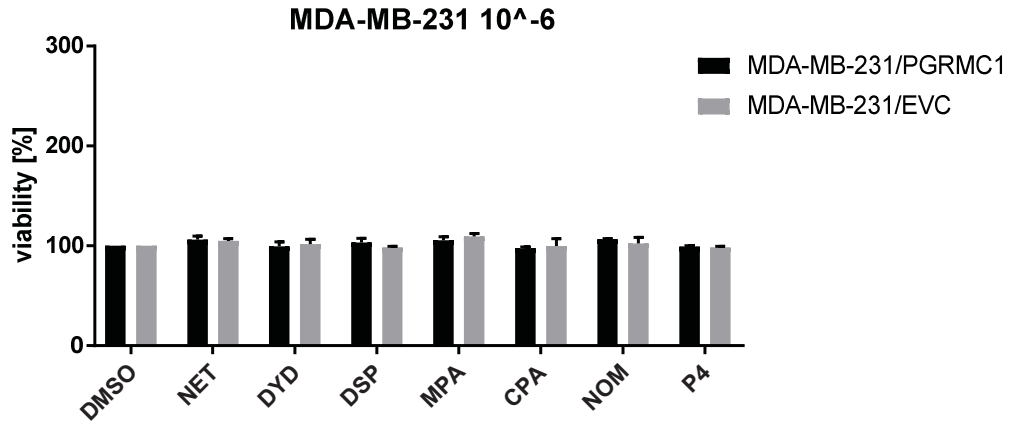


Figure 58. Cell viability of MDA-MB-231/EVC and MDA-MB-231/PGRMC1 cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

To analyze if effects of progestins on MCF7/PGRMC1 and T47D/PGRMC1 cells can also be observed at very low concentrations, PGRMC1 overexpressing and the respective EVC cells were treated with progestins in concentrations ranging from 10^{-6} M to 10^{-9} M. For the progestin NET, we detected significant higher proliferation for PGRMC1-overexpressing cells even at 10^{-9} M compared to DMSO treated cells. For the progestins DYD and DSP significant elevated proliferation compared to the DMSO control was observed at concentrations down to 10^{-8} M, while for MPA only the highest concentration (10^{-6} M) resulted in significant increased proliferation. CMA and NOM as well as P4 did not enhance proliferation of PGRMC1-overexpressing MCF7 and T47D cells compared to the DMSO control

(MCF7/PGRMC1: figure 59, T47D/PGRMC1: figure 61). For MCF7/EVC cells, we detected significant increased proliferation for 10^{-6} M and 10^{-7} M NET, 10^{-6} M DYD and 10^{-6} M DSP (figure 60). For T47D/PGRMC1 significant elevated proliferation was detected for 10^{-6} M and 10^{-7} M NET and DYD compared to the DMSO control (figure 62). However, proliferation of PGRMC1 overexpressing cells was always significantly higher compared to the respective EVC cell line.

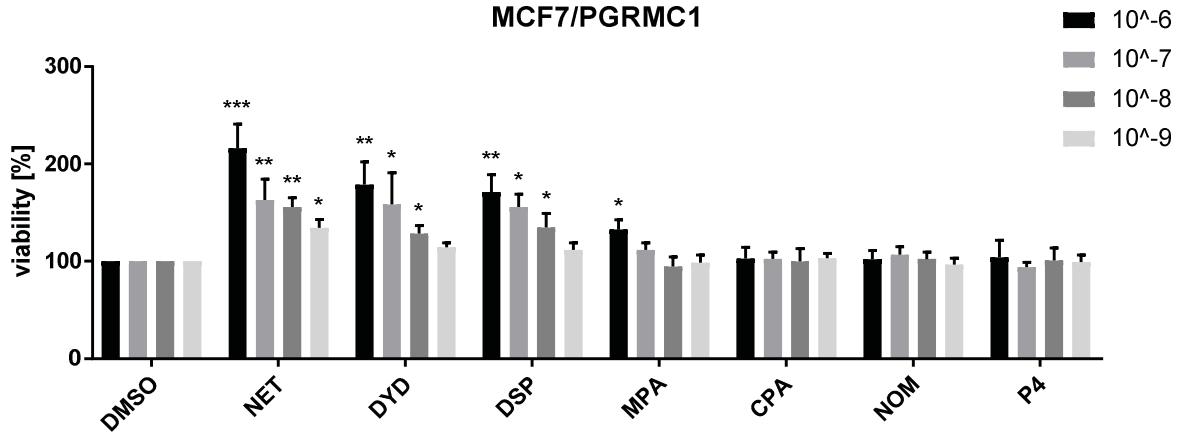


Figure 59. Cell viability of MCF7/PGRMC1 cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

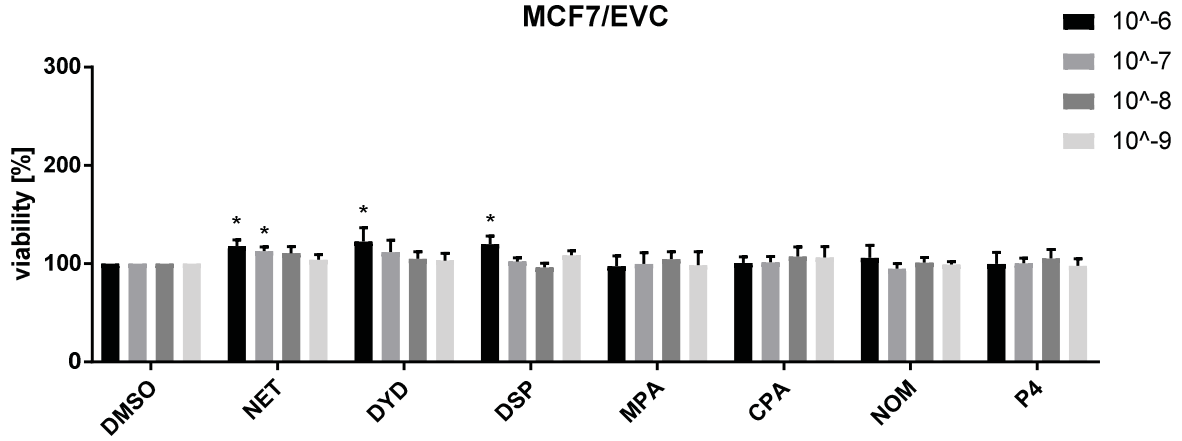


Figure 60. Cell viability of MCF7/EVC cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$.

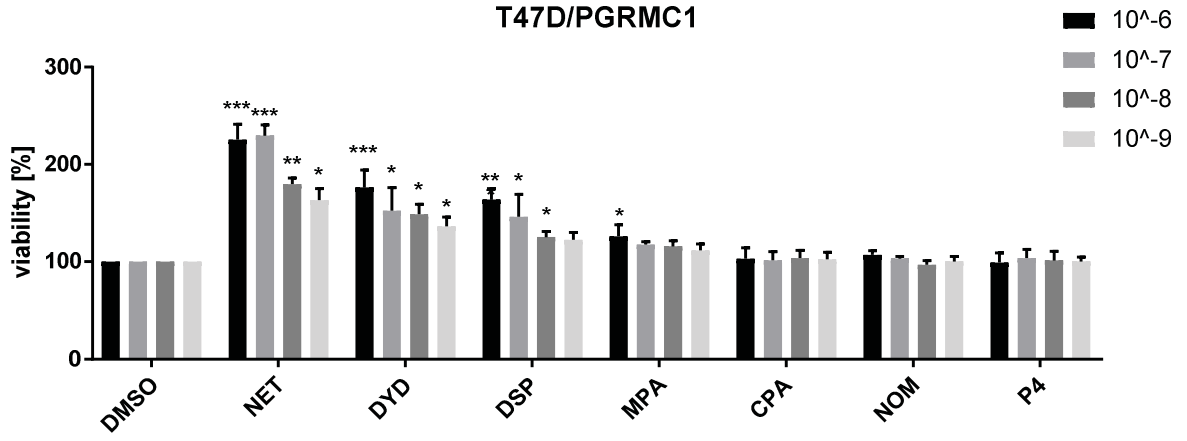


Figure 61. Cell viability of T47D/PGRMC1 cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

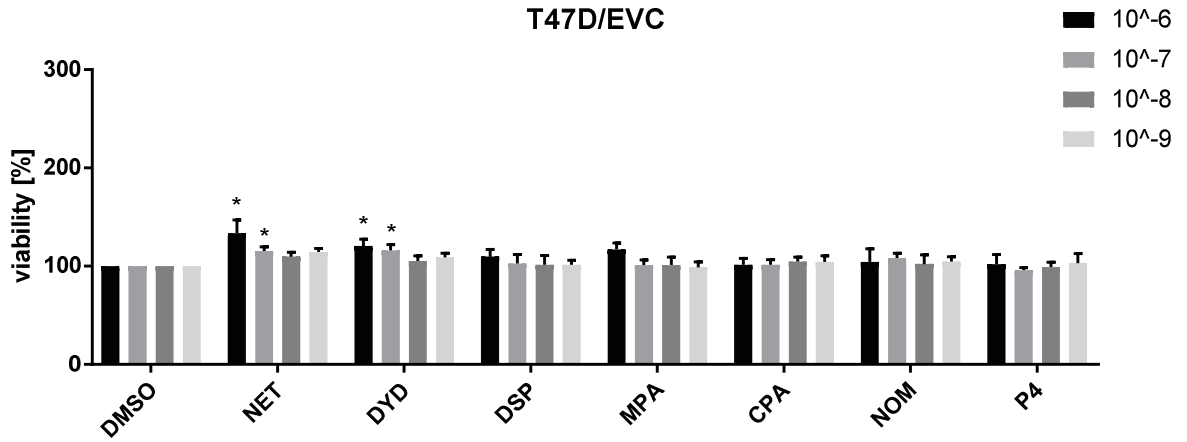


Figure 62. Cell viability of T47D/EVC cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET, DYD, DSP, MPA, CPA, NOM, P4 [10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to DMSO treated cells. *: $p < 0.05$.

Results reported in paragraph 3.2.1. were partly published in Willibald et al.[123]

3.2.2. PGRMC1 is phosphorylated at Ser181 upon progestin treatment

The above results indicate a role of PGRMC1 in the signaling cascade after binding of progestins to hormone receptors, however to date nothing is known regarding a potential activation of the receptor by progestin signals. To investigate, whether treatment with NET has an influence on PGRMC1 activation by induction of posttranslational modifications, the phosphorylation status of PGRMC1, immunopurified from DMSO- and NET-treated breast cancer cells, was determined by mass spectrometry and Western blot analysis. Comparison

of NET- and DMSO-treated samples by mass spectrometry revealed a significant higher relative signal intensity of the phosphopeptide EGEEPTVY**pS**DEEEPKDESARK (Ser181 phosphorylation site) for NET-treated samples, compared to DMSO-treated samples (figure 63 C, supplemental figure 1). No significant differences between NET- and DMSO-treated samples for the phosphosites pS54, pS57 and pT178 (figure 63 A, B, D) was observed.

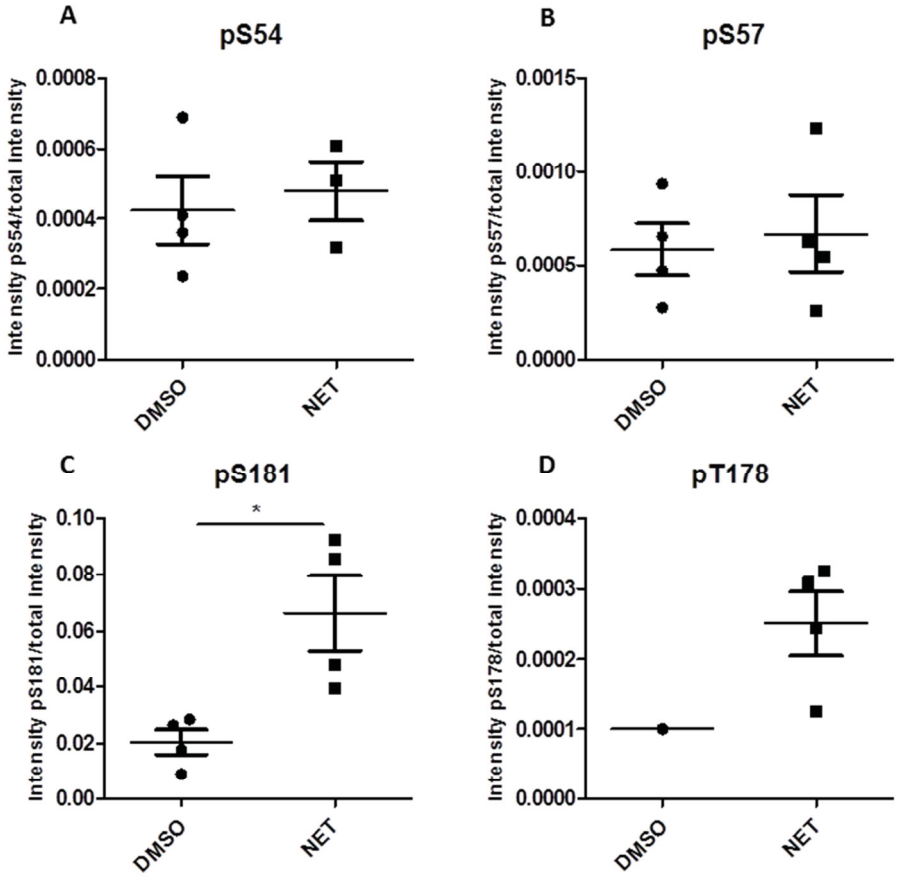


Figure 63. PGRMC1 phosphorylation upon treatment with NET.
Intensity of phosphorylation in NET- and DMSO-treated MCF7/PGRMC1 cells after 72 h of treatment identified by mass spectrometry. A: S54 phosphorylation (DMSO: n = 4, NET: n = 4), B: S57 phosphorylation (DMSO: n = 4, NET: n = 4), C: S181 phosphorylation (DMSO: n = 4, NET: n = 4), (*: $p < 0.05$), D: T178 phosphorylation (DMSO: n = 1, NET: n = 4).

Using an anti-phospho Ser181-PGRMC1 (anti-pPGRMC1) antibody, mass spectrometry results were validated by Western blot analysis using NET- and DMSO-treated PGRMC1-overexpressing MCF7 and T47D cells (figure 64 A) as well as endogenously PGRMC1-expressing MCF7 and T47D cells (figure 65 A). As observed by mass spectrometry analysis, significant higher PGRMC1 phosphorylation at Ser181 in NET-treated samples compared to DMSO-treated samples could be identified. Expression levels of total PGRMC1 did not differ significantly in NET- and DMSO-treated MCF7/PGRMC1 (figure 64 B) and T47D/PGRMC1 (figure 64 C) cells as well as MCF7 (figure 65 B) and T47D cells (figure 65 C).

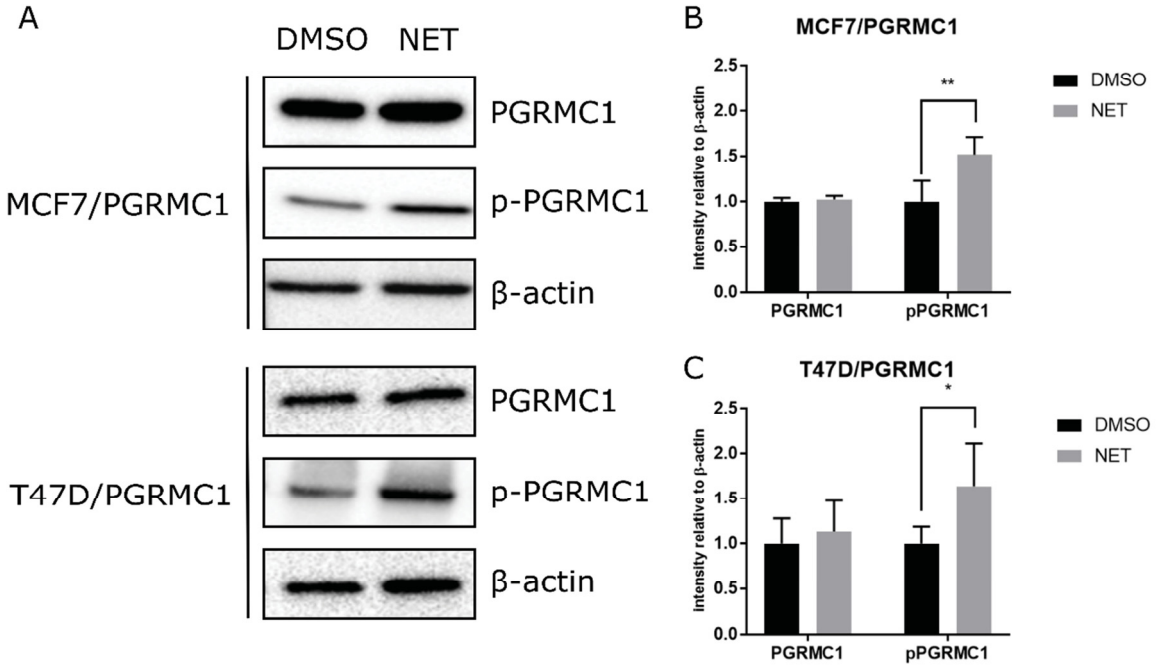
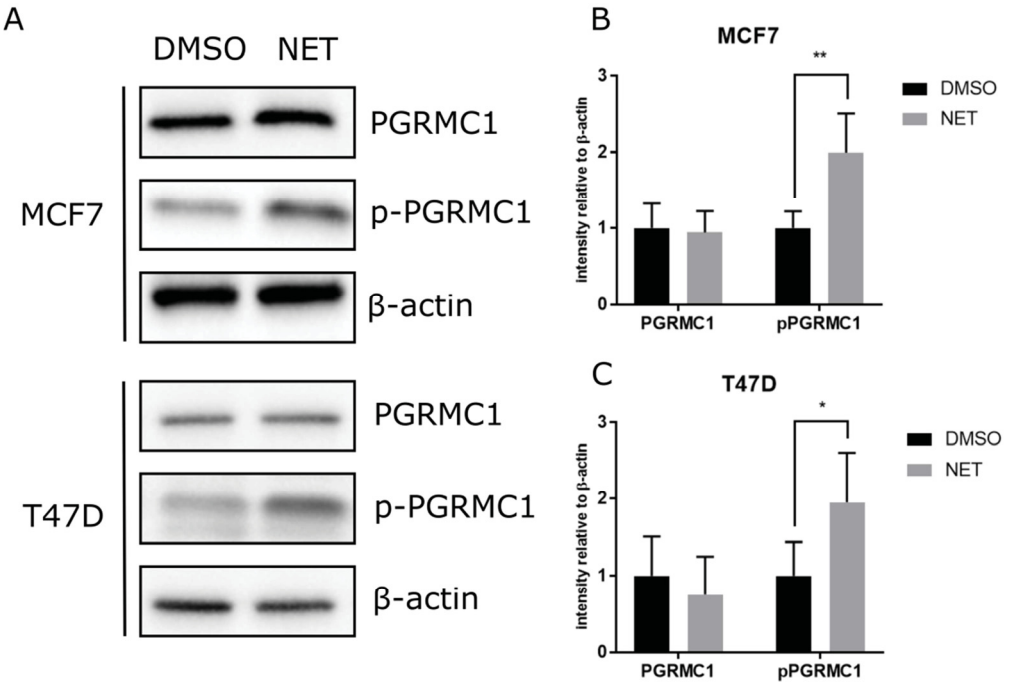


Figure 64. Phosphorylation of PGRMC1 at S181 upon NET treatment.
A: Western blot analysis of PGRMC1 and pPGRMC1 in MCF7/PGRMC1 and T47D/PGRMC1 cells after treatment with NET and DMSO. B-actin was used as loading control. B: Densitometric analysis of Western blot results of MCF7/PGRMC1 cells treated with NET and DMSO (n = 3). Intensity was normalized to corresponding DMSO control. **: $p < 0.01$. C: Densitometric analysis of Western blot results of T47D/PGRMC1 cells (n = 3). Intensity was normalized to corresponding DMSO control. *: $p < 0.05$.

Figure 65.



Phosphorylation of PGRMC1 at S181 upon NET treatment.
A: Western blot analysis of PGRMC1 and pPGRMC1 in MCF7 and T47D cells after treatment with NET and DMSO. B-actin was used as loading control. B: Densitometric analysis of Western blot results of MCF7 cells treated with NET and DMSO (n = 3). Intensity was normalized to corresponding DMSO control. **: $p < 0.01$. C: Densitometric analysis of Western blot results of T47D cells (n = 3). Intensity was normalized to corresponding DMSO control. *: $p < 0.05$.

Results reported in paragraph 3.2.2. were published in Willibald et al.[123]

3.2.3. PGRMC1 is phosphorylated by CK2 upon progestin treatment

Scansite analysis revealed that the Ser181 PGRMC1 phosphorylation site is likely to be phosphorylated by CK2 protein kinase (scansite score 0.498)[210]. Therefore, to examine the role of CK2 in PGRMC1 phosphorylation, the highly selective CK2 inhibitor quinalizarin (K_i = 520 nM) was used and MCF7/PGRMC1- and T47D/PGRMC1 cells were treated with NET and increasing concentrations of the inhibitor[218,219]. In Western blot analysis with lysates of the treated cells, decreasing signals for pPGRMC1 with increasing quinalizarin concentrations could be observed. Significant lower signals could be detected for quinalizarin concentrations 100 nM and 500 nM. While the phosphorylation of PGRMC1 decreased with increasing quinalizarin concentrations, the amount of total protein was the same for every concentration used (figure 66 B, C).

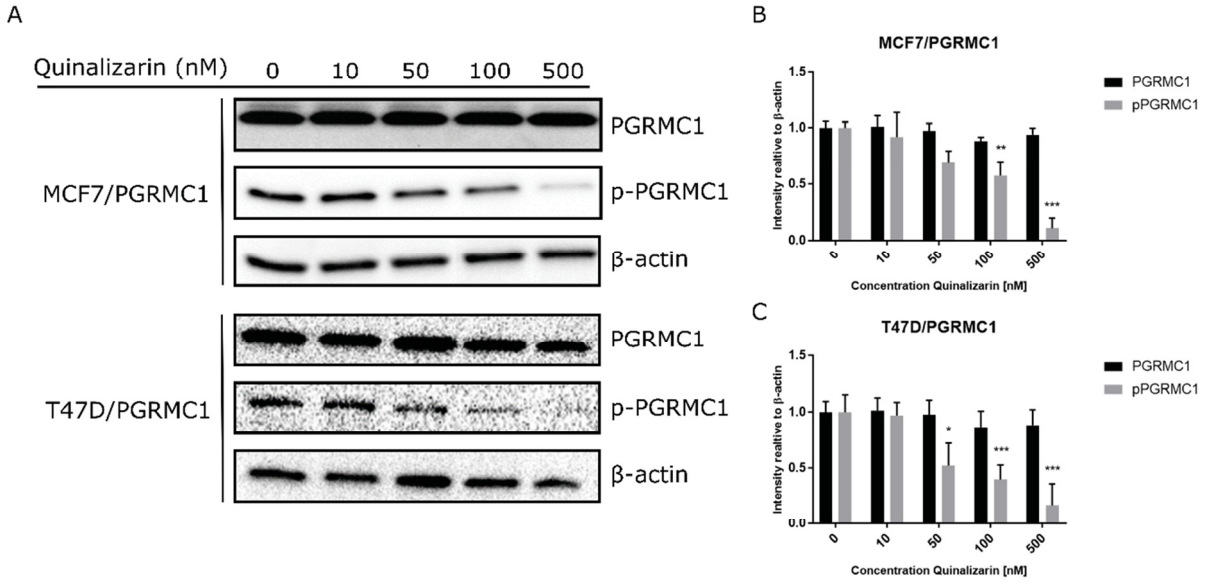


Figure 66. Phosphorylation of PGRMC1 at S181 upon treatment with NET and quinalizarin.
A: Western blot analysis of PGRMC1 and pPGRMC1 after 24 h of treatment. MCF7/PGRMC1 and T47D/PGRMC1 cells were treated with NET and 0, 10, 50, 100 and 500 nM quinalizarin for 24 h. β-actin was used as loading control. B: Densitometric analysis of Western blot results of MCF7/PGRMC1 cells (n = 3). Intensity was normalized to 0 nM quinalizarin. C: Densitometric analysis of Western blot results of T47D/PGRMC1 cells. (n = 3). Intensity was normalized to 0 nM quinalizarin. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$

Results reported in paragraph 3.2.3. were published in Willibald et al.[123]

3.2.4. PGRMC1 phosphorylation is essential for increased proliferation upon progestin treatment

To further investigate the impact of PGRMC1 phosphorylation on cell proliferation, we used the above described transfected MCF7/PGRMC1 cells, exhibiting point mutations at the CK2

phosphorylation sites S57 (MCF7/PGRMC1-S57A) and S181 (MCF7/PGRMC1-S181A) and the double-mutant with point-mutations at S57 and S181 (MCF7/PGRMC1-S57A/S181A). Cells were treated with NET for 72 h and cell viability was examined by MTT assay. Mutation at the S181 phosphorylation site, as well as double mutations at S181 and S57 phosphorylation sites resulted in significant lower proliferation rates after 72 h of stimulation, compared to MCF7 cells transfected with wild-type PGRMC1. No significant different proliferation rate could be detected for MCF7/PGRMC1-S57A cells, compared to MCF7/PGRMC1 cells (figure 67).

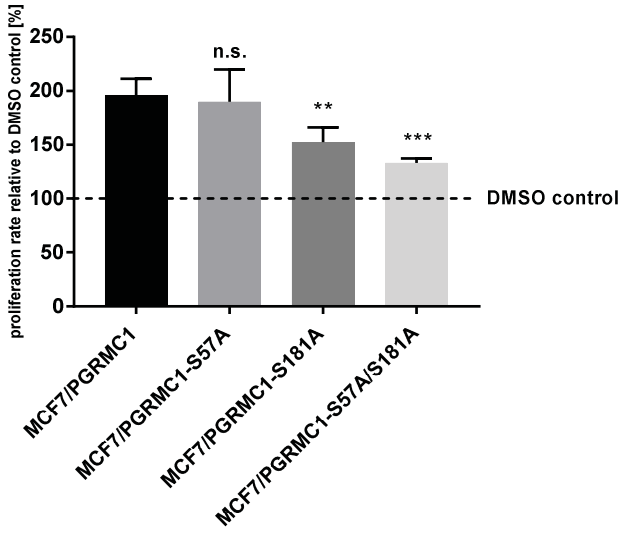


Figure 67. Cell viability of MCF7/PGRMC1 MCF7/PGRMC1-S57A, MCF7/PGRMC1-S181A and MCF7/PGRMC1-S57A/S181A cells upon treatment with progestins.
Cells were grown in stripped medium supplemented with NET [10^{-6} M] or DMSO [0.01 %]. Proliferation was analyzed by MTT assay after 72 h. Values were normalized to respective DMSO treated cells. **: $p < 0.01$, ***: $p < 0.005$.

Results reported in paragraph 3.2.4. were published in Willibald et al.[123]

3.2.5. PGRMC1 mediates upregulation of ERα downstream targets and activation of EGFR signaling upon progestin treatment

Increased proliferation upon progestin treatment was only detected in ERα-positive PGRMC1-overexpressing cell lines. Therefore, we reasoned, that the PGRMC1-dependent upregulation of ERα might also play a role in enhanced proliferation of PGRMC1 overexpressing cells upon progestin treatment. To further investigate activation of cellular functions and signaling pathways dependent on PGRMC1 activation by progestins, regulation of protein expression and -activation upon treatment with NET was analyzed. MCF7/EVC and MCF7/PGRMC1 cells were treated with NET and vehicle control and expression and activation of proteins important in breast cancer signaling with special regard to ERα

expression/activation were analyzed. Since PGRMC1 phosphorylation was shown to be important for inducing increased proliferation upon NET treatment, further MCF7/PGRMC1-S181A cells were analyzed. Expression was normalized to the regarding DMSO control, which was set to 1 in each case.

Interestingly, upon treatment with NET, decreased expression of ERα in all three cell lines was observed. In NET treated MCF7/PGRMC1 cells, ERα expression was significantly lower compared to MCF7/EVC cells. Further, upregulation of the ERα dependently expressed protein c-Myc was found upon NET treatment, which was significantly higher in MCF7/PGRMC1 cells. However, this could not be confirmed for MCF7/PGRMC1-S181A cells (figure 68).

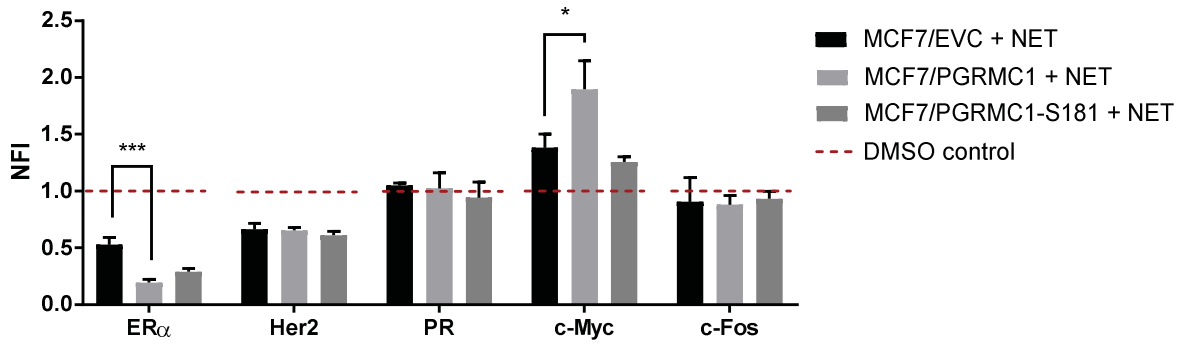


Figure 68. RPPA analysis of protein expression upon NET treatment.
Protein expression of ERα, Her2, PR, c-Myc and c-Fos analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). Protein expression was normalized to the respective DMSO control and protein expression measured in the respective DMSO control cells was set to 1 (dashed line). Up-/downregulation of protein expression in NET-treated MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S181A cells was calculated. *: $p < 0.05$, ***: $p < 0.005$.

To further investigate the activity of ERα as a transcription factor in dependence of PGRMC1 and NET treatment, as readout, we analyzed the expression of the ERα-dependent gene TFF1. TFF1 expression was investigated in NET- and DMSO-treated MCF7/PGRMC1 and MCF7/EVC cells. In both cell lines, treatment with NET resulted in significant increased expression of TFF1 compared to the respective DMSO control. However, the expression of TFF1 upon treatment with NET was significantly elevated in MCF7/PGRMC1 cells compared to MCF7/EVC cells (figure 69).

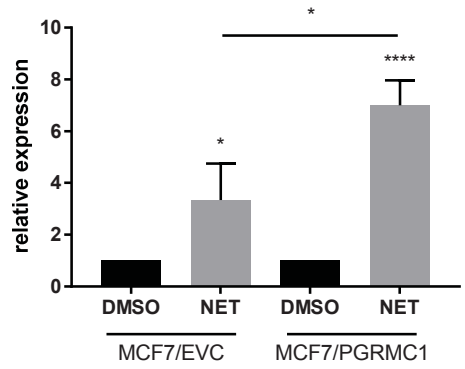


Figure 69. Analysis of TFF1 mRNA expression in MCF7/EVC and MCF7/PGRMC1 cells.
Cells were treated with DMSO [0.01 %] or NET [1 µM] for 24 h. Intensity was normalized to respective DMSO control. *: $p < 0.05$, ****: $p < 0.001$.

Since in progestin-independent studies, an effect of PGRMC1 on activation of the EGFR signaling cascade was observed, we further analyzed the PGRMC1-dependent activation of EGFR and its downstream targets upon activation by NET. As for analysis of ERα expression and -activation, MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S181A cells were used. Analysis of protein activation by phosphorylation dependent on NET, revealed a significant upregulation of Her2 phosphorylation (P-Tyr1221/Tyr1222) in all three cell lines. However, phosphorylation of Her2 was significantly higher in NET-treatedMCF7/PGRMC1 cells compared to MCF7/EVC and MCF7/PGRMC1-S181A. Further, significant upregulation of pAkt (P-Ser472 and P-Thr308) and pMEK1/2 (P-Ser217/Ser221) was detected in NET-treated MCF7/PGRMC1 cells, which could not be confirmed for EVC cells and MCF7/PGRMC1-S181A cells (figure 70).

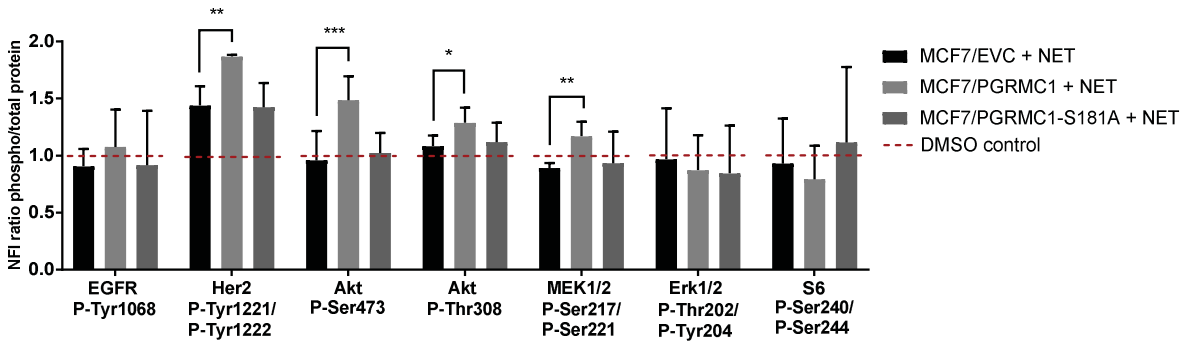


Figure 70. RPPA analysis of protein phosphorylation upon NET treatment.
Protein phosphorylation of EGFR P-Tyr1068, Her2 P-Tyr1221/Tyr1222, Akt P-Ser473, Akt P-Thr308, MEK1/2 P-Ser217/Ser221, Erk1/2 P-Thr202/Tyr204 and S6 P-Ser240/Ser244 analyzed by RPPA. Sample signals were quantified as protein–normalized, blank-corrected mean fluorescence intensities (NFI) of the single spots applying linear fits and interpolating to the mean of the four printed sample dilutions (eight spots per sample). NFI ratio of phospho-protein/total protein was calculated, normalized to the respective DMSO control and ratio in of the respective DMSO control cell was set to 1 (dashed line). Up-/downregulation of protein phosphorylation in MCF7/EVC, MCF7/PGRMC1 and MCF7/PGRMC1-S181A was calculated. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

3.2.6. PGRMC1 interaction partners upon progestin treatment

With the aim to further analyze the PGRMC1 signaling cascade upon activation of PGRMC1 by progestin signals, we searched for PGRMC1 interaction partners upon progestin treatment. MCF7/PGRMC1-HA cells and the control cell line MCF7/PGRMC1-GFP were treated with NET and DMSO as a control. MCF7/PGRMC1-HA and potential interaction partners were subsequently (co-)immunopurified from the cell lysates and detected by mass spectrometry. The volcano plot (figure 71) stratifies proteins, exhibiting significant increased signals in the PGRMC1-HA NET treated samples compared to the corresponding DMSO treated samples which might therefore be potential PGRMC1 interaction partners upon progestin treatment.

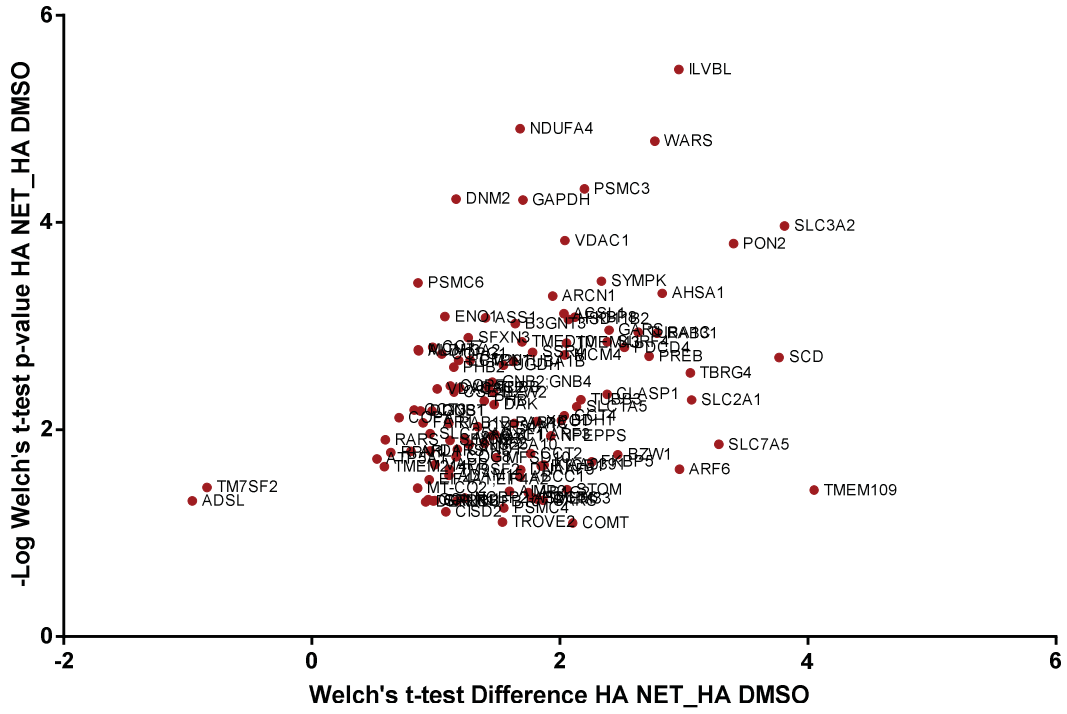


Figure 71. PGRMC1 interaction partners upon NET treatment.
Scatter plot of proteins revealing significant higher intensities in PGRMC1-HA/NET samples compared to PGRMC1-HA/DMSO samples identified by mass spectrometry. Plotted is the fold change (x-axis) versus the significance (y-axis).

In the NET and the DMSO treated PGRMC1-HA samples, PGRMC1 revealed similar signal intensities; those were significantly different to the intensities in the DMSO and NET treated PGRMC1-GFP controls (figure 72).

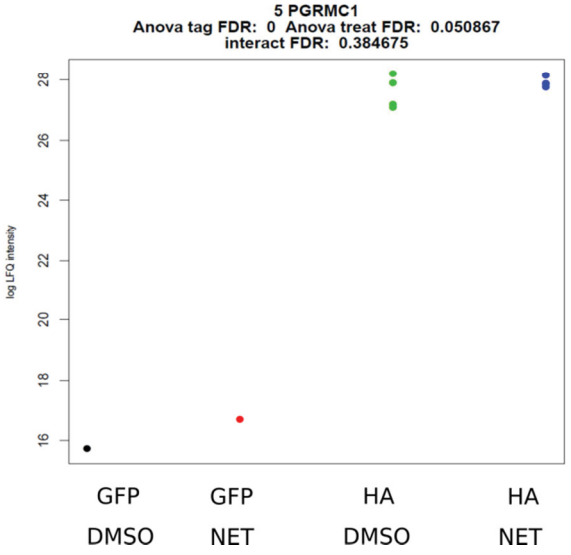


Figure 72. Intensity for PGRMC1 detected by mass spectrometry.
Mass spectrometry results for PGRMC1 of PGRMC1-GPP (GFP) and PGRMC1-HA (HA) samples immunopurified from DMSO or NET treated cells.

3.2.7. PGRMC1 interacts with PHB1 and PHB2 upon progestin treatment

Within the proteins revealing higher intensities for NET treated samples compared to DMSO treated samples, i.e. potential interaction partners of PGRMC1 upon progestin treatment, we found Prohibitin 1 (PHB or PHB1) and Prohibitin 2 (PHB2) (figure 73).

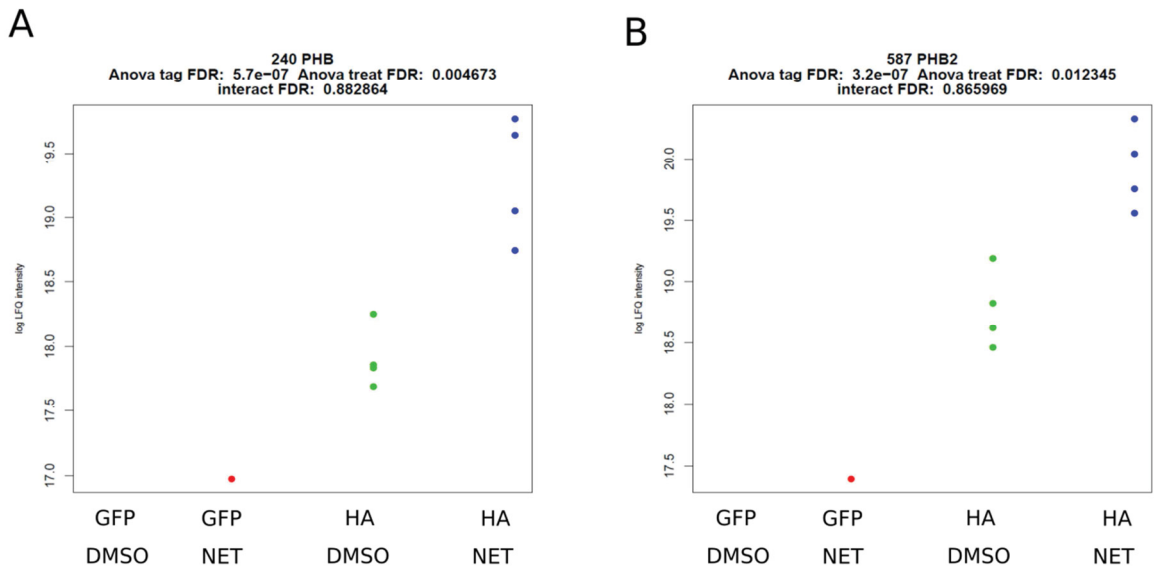


Figure 73. Interaction of PHB1 (PHB) and PHB2 with PGRMC1.
A: Mass spectrometry results of immunopurified PGRMC1-GFP/DMSO (GFP DMSO), PGRMC1-GFP/NET (GFP NET), PGRMC1-HA/DMSO (HA DMSO) and PGRMC1-HA/NET (HA NET) samples for PHB1, log2 normalized intensity. B: Mass spectrometry results of immunopurified PGRMC1-GFP/DMSO (GFP DMSO), PGRMC1-GFP/NET (GFP NET), PGRMC1-HA/DMSO (HA DMSO) and PGRMC1-HA/NET (HA NET) samples for PHB2, log2 normalized intensity.

Both prohibitins are suggested to modulate transcriptional activity by directly or indirectly interacting with transcription factors, including ERα. In fact, PHB2 is known as a ERα

coregulator that potentiates the inhibitory activities of antiestrogens and represses the activity of estrogens[48,220]. Because of their role as transcription factor modulators, we were interested in the interaction of PGRMC1 with PHB1 and PHB2. The results from mass spectrometry were verified by immunoprecipitation followed by Western blot analysis using antibodies against PHB1 and PHB2 respectively. Western blot analysis supported the results from mass spectrometry. Both, PHB1 (figure 74) and PHB2 (figure 75) revealed significant higher signals in Western blot analysis.

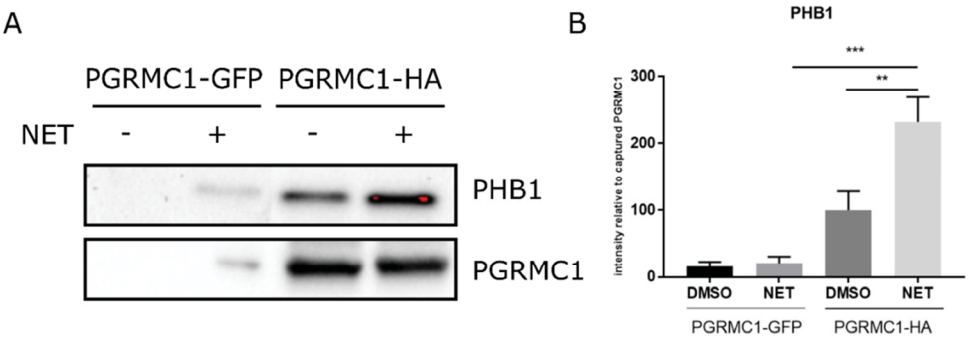


Figure 74. Interaction of PGRMC1 and PHB1 upon NET treatment verified by western blot.
A: Western blot analysis of immunopurified PGRMC1-GFP/DMSO, PGRMC1-GFP/NET, PGRMC1-HA/DMSO and PGRMC1-HA/NET samples. B: Densitometric analysis of PHB1 expression. Expression is normalized to PGRMC1-HA/DMSO (100 %). **: $p < 0.01$, ***: $p < 0.005$.

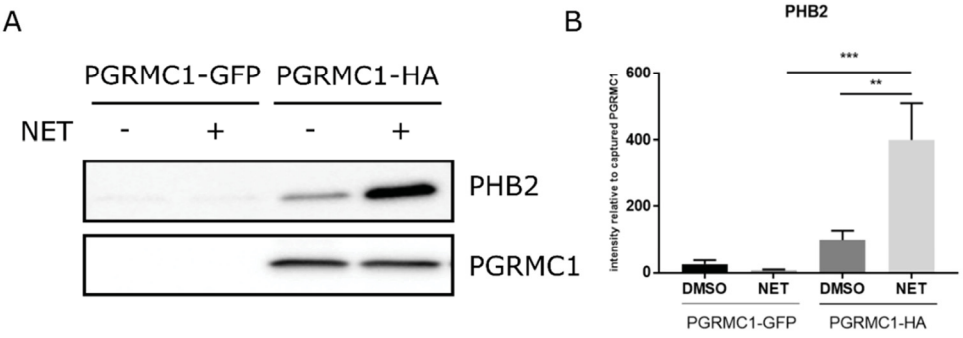


Figure 75. Interaction of PGRMC1 and PHB1 upon NET treatment verified by western blot.
A: Western blot analysis of immunopurified PGRMC1-GFP/DMSO, PGRMC1-GFP/NET, PGRMC1-HA/DMSO and PGRMC1-HA/NET samples. B: Densitometric analysis of PHB2 expression. Expression is normalized to PGRMC1-HA/DMSO (100 %). **: $p < 0.01$, ***: $p < 0.005$.

To verify the result independently of immunopurification and PGRMC1 overexpression, we further validated the result by PLA. Endogenously PGRMC1-expressing MCF7 cells were treated with NET and DMSO and interaction of PGRMC1 with PHB1 (figure 76) and PHB2 (figure 78) was analyzed. For PHB1/PGRMC1 as well as for PHB2/PGRMC1 interaction, we found significant more signals (dots per cell) when MCF7 cells were treated with NET compared to DMSO treated cells (figure 77, figure 79).

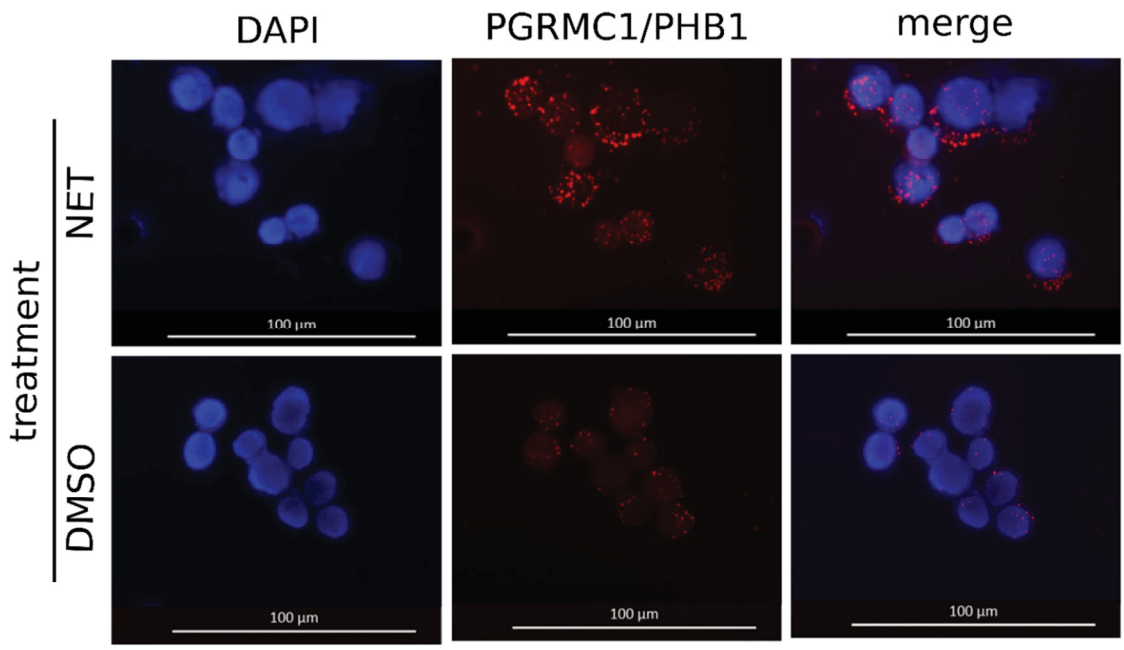


Figure 76. Proximity ligation assay for protein interactions between PGRMC1 and PHB1 upon NET treatment.
MCF7 cells were treated with DMSO or NET for 72 h and then prepared on cytospins. Each red spot represents a single interaction. Nuclear stain: DAPI. Magnification 40x.

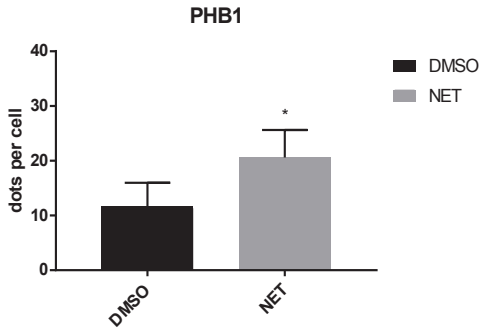


Figure 77. Analysis of PLA for interaction of PGRMC1 with PHB1 upon treatment with NET.
Five images were taken per slide. Cell number and PLA signals were quantified using imageJ. (n = 3). *: $p < 0.05$

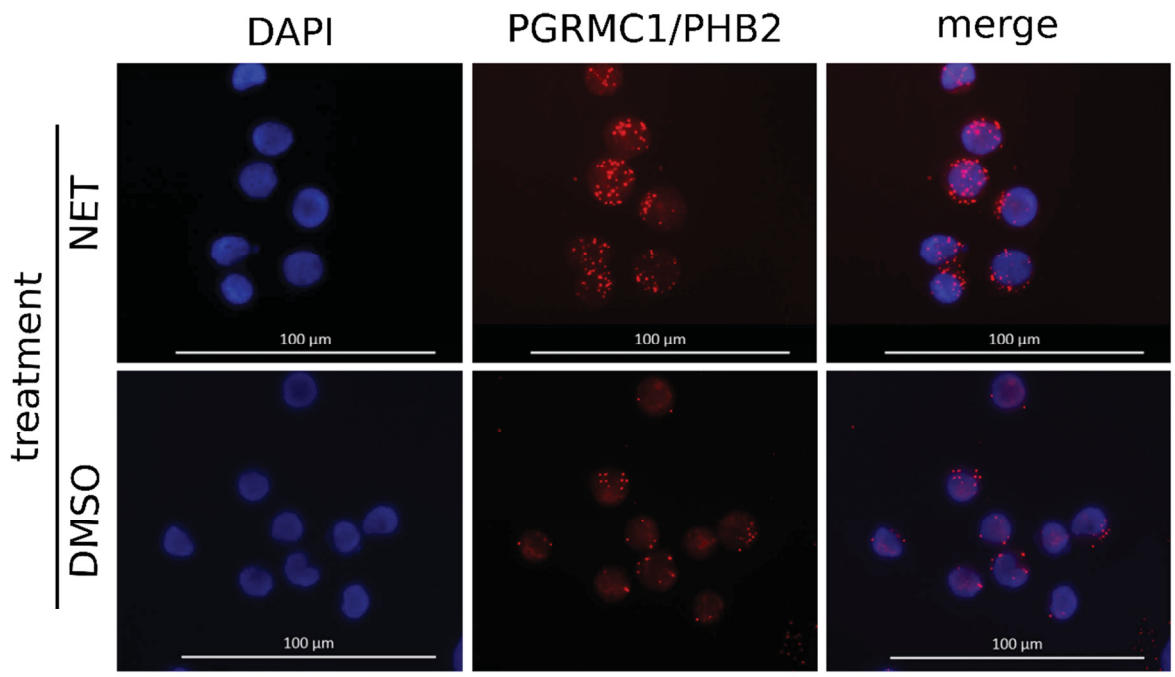


Figure 78. Proximity ligation assay for protein interactions between PGRMC1 and PHB2 upon NET treatment.
MCF7 cells were treated with DMSO or NET for 72 h and then prepared on cytospins. Each red spot represents a single interaction. Nuclear stain: DAPI. Magnification 40x.

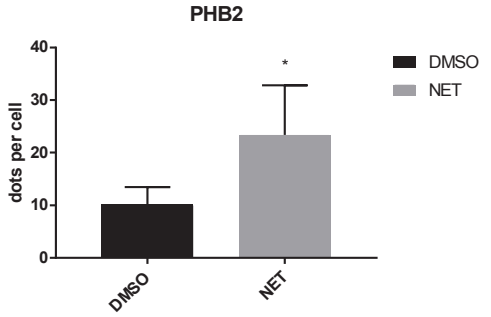


Figure 79. Analysis of PLA for interaction of PGRMC1 with PHB2 upon treatment with NET.
Five images were taken per slide. Cell number and PLA signals were quantified using imageJ. (n = 3). *: $p < 0.05$

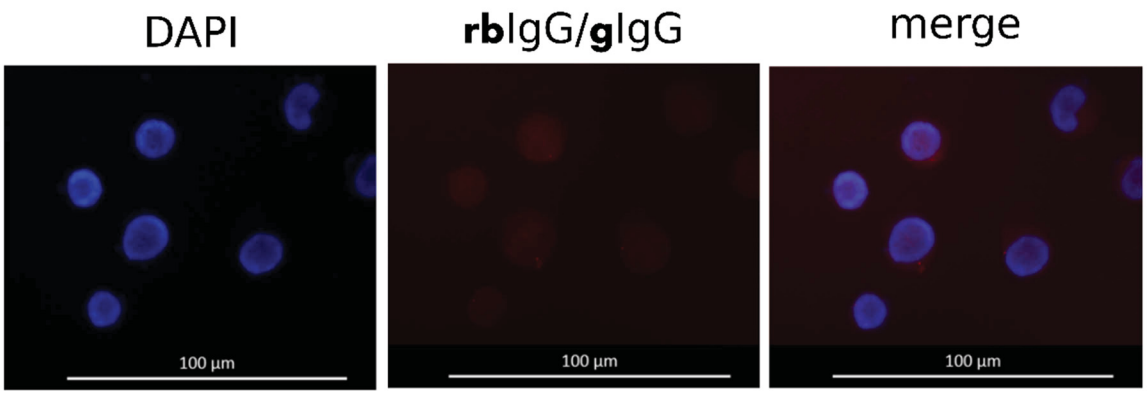


Figure 80. PLA negative control: rabbit IgG, goat IgG isotype controls.
MCF7 cells were treated NET for 72 h and then prepared on cytospins. Nuclear stain: DAPI. Magnification 40x.

4. Discussion

4.1. Role of PGRMC1 in breast cancer

Various studies indicated PGRMC1 to promote a more aggressive phenotype and to participate in carcinogenesis of breast cancer. However, to date little is known regarding the role of PGRMC1 signaling in breast cancer cells. Therefore, in the present study, we aimed to further investigate the contribution of PGRMC1 to breast cancer progression and to signaling cascades important in breast cancer.

4.1.1. PGRMC1 expression is associated with poor prognosis and promotes progression of ERα-positive breast cancer cells

We used an integrative data analysis tool to assess the impact of high PGRMC1 expression on the clinical outcome of breast cancer patients. The analysis revealed correlation of elevated PGRMC1 levels in tissue of breast cancer with poorer outcome. These results are in accordance with other studies, reporting worse survival rates for patients with elevated PGRMC1 expression in breast-, lung-, ovarian- and kidney cancer[137–139,221]. However, all of these studies only analyzed a small patient's cohort, while we used data of more than 5000 breast cancer patients.

In the present study, we detected significant elevated expression of PGRMC1 in tumor compared to connective tissue of the breast. We mainly observed strong PGRMC1 abundance in tumor tissue: 85.5 % of the investigated specimen revealed a high PGRMC1 expression status. These results are supported by data from other research groups, who detected significant elevated expression of PGRMC1 in breast cancer compared to non-malignant breast tissue[119,137,221]. Using immunohistochemical analysis, Ji et al. detected PGRMC1 expression in breast cancer tissues ranging from strong to minimal. Further, elevated PGRMC1 mRNA or protein levels in tumor tissue and a significant lower expression in the surrounding non-tumorous tissue were found for other cancer types before[151,160,222]. These data again point towards a potential role of PGRMC1 in (breast) cancer carcinogenesis.

To further investigate the impact of PGRMC1 expression on breast cancer progression, the last step of carcinogenesis, we analyzed the effect of PGRMC1 overexpression and -downregulation on cancer cell proliferation. Overexpression of PGRMC1 in the hormone-receptor positive cell lines MCF7 and T47D resulted in significant increased proliferation,

while knockdown of endogenous PGRMC1 caused significant decreased proliferation. Cell proliferation was further diminished when endogenously PGRMC1-expressing cells were treated with the PGRMC1 inhibitor AG-205. The same effect was reported before for uterine sarcoma cells (Lin et al.) and renal cell carcinoma cells (Zhang et al.); both groups observed decreased cell proliferation upon PGRMC1 knockdown and elevated cell proliferation upon PGRMC1 overexpression[140,178]. Additionally, our results are in accordance with other in vivo and in vitro studies: Downregulation of PGRMC1 resulted in decreased cell proliferation of lung-, breast-, endometrial- and kidney cancer cells in vitro and lower tumor numbers and reduced tumor volume in mouse xenograft studies[119–121,138,140,190]. PGRMC1 could participate in cancer cell progression and thereby promote an aggressive cancer phenotype in multiple ways: PGRMC1 promotes cellular processes which are important hallmarks of cancer, such as cell-cycle progression, anti-apoptosis and autophagy[150,159,178,189,223,224]. Further, strong evidence exists that PGRMC1 interacts with EGFR a protein which is involved in activation of oncogenic signaling pathways. Interaction of PGRMC1 and EGFR was reported to induce EGFR phosphorylation and activation of EGFR downstream targets, such as Akt and ERK[139,158,187,188]. Besides, it has been supposed that the contribution of PGRMC1 in cholesterol- and steroid synthesis and steroid response might promote carcinogenesis[112]. Interestingly, in the basal-like cell line MDA-MB-231 cells we could not observe any effect of PGRMC1 overexpression or -downregulation on cell proliferation. While most studies found an effect of PGRMC1 up- or downregulation on cell proliferation also Kabe et al. did not detect and effect of PGRMC1 knockdown on proliferation of HCT116 cells[187]. This strongly indicates a cell-system dependent function of PGRMC1. As MDA-MB-231 cells, HCT116 cells lack expression of ERα[225].

4.1.2. PGRMC1 phosphorylation is essential for increased proliferation

The diverse functions of PGRMC1 might be regulated by a variety of posttranslational modifications, foremost by its phosphorylation[113]. Further, as recently reviewed by Cahill et al., PGRMC1 phosphorylation might play a crucial role not only in terms of its function, but also in its protein-protein interaction and subcellular localization[112–114,117,118]. Investigation of PGRMC1 function and its regulation by phosphorylation status is therefore important for a better understanding of its function. In the present study, we observed PGRMC1 phosphorylation at S54, S57, S181, T178 and Y180 in MCF7 breast cancer cells. According to the phosphosite database, pS181, pY113, pS57 and pY180 are the most commonly detected

sites[113,184]. Phosphorylation site Y180 is a potential SH2-target sequence, requiring tyrosine phosphorylation for SH2 protein domains to bind and thereby inducing conformational changes of the receptor. However, phosphorylation of S181 and T178 by CK2 is predicted to sterically inhibit phosphorylation of Y180 and to attenuate protein interaction, which might explain the low signals for the pY180 phosphosite in the present study[113,114]. Likewise, binding of SH3 domains to the SH3 target sequence at P63 might be affected by phosphorylation of S57 by CK2[112,113]. Although phosphorylation of Y113 is reported to be among the most common PGRMC1 phosphorylation sites, in the present study phosphorylation of Y113 could not be detected by mass spectrometry[184]. Phosphorylation of Y113 might be responsible for the membrane trafficking function of PGRMC1, but at the same time prevent heme-binding due to steric interference, suggesting a reciprocal regulation[113,117]. Phosphorylation at S181 is among the most commonly detected PGRMC1 phosphorylations and is expected to be constitutively phosphorylated[113]. In the present study, significant elevated levels of phosphorylated PGRMC1 at S181 in breast cancer tissue compared to the surrounding tissue were found, indicating a potential role of PGRMC1 phosphorylation at S181 in breast cancer progression. Interestingly, PGRMC1 expression in tumor tissue was observed close to the nucleus as well as in the cytoplasm, whereas phosphorylated PGRMC1 was only found in the cytoplasm (data not shown), supporting the assumption that functions of PGRMC1 vary, depending on its phosphorylation status. In previous studies, we observed that PGRMC1 mutation at S57 and S181 results in a modified function of PGRMC1 in MCF7 cells. Upon H_2O_2 exposure, susceptibility to cell death of stably PGRMC1-overexpressing MCF7 cell lines carrying point mutations at S57 and S181 was altered, as well as phosphorylation of Akt[141]. Here we could further show that mutation of S57, S181 and double mutation of S57 and S181 significantly diminishes proliferation of MCF7/PGRMC1 cells. While proliferation of MCF7/PGRMC1 cells was significantly higher compared to MCF7/EVC cells, the proliferation rate of MCF7/PGRMC1-S57A, MCF7/PGRMC1-S181A and MCF7/PGRMC1-S57A/S181A cells was comparable to EVC cells. This indicates that PGRMC1 phosphorylation at S57 and S181 is crucial for PGRMC1 downstream signaling, resulting in increased proliferation. Various attempts to explain this effect are conceivable. As discussed above, phosphorylation of S57 and S181 might induce protein-protein interactions between the receptor and downstream signaling proteins. Mutation of the amino acid and thus deletion of the respective phosphorylation site might

therefore prevent downstream signaling. As discussed elsewhere, another approach could be that deletion of S57 and S181 phosphorylation sites enable phosphorylation of P63 and Y180, which is otherwise sterically inhibited by pS57 and pS181[112–114]. Phosphorylation of P63 and Y180 could then in turn lead to recruitment of SH3- and SH2-target proteins, resulting in altered downstream signaling. Further, a lack of S57 and S181 phosphorylation and thus a gain of P63 and Y180 phosphorylation might induce conformational changes of PGRMC1, causing altered biological functions of the receptor.

4.1.3. PGRMC1 mediates upregulation of ERα expression and EGFR signaling

Since we found an effect of PGRMC1 expression on cell proliferation only in cells representing the luminal A breast cancer subtype, while in a cell line, representing the basal like breast cancer subtype, we hypothesized that PGRMC1 signaling, resulting in enhanced proliferation, might be dependent on factors, present in luminal A and absent in basal like cells. Further, RPPA and qPCR analysis revealed upregulation of ERα protein- and mRNA expression in PGRMC1 overexpressing cells and higher abundance of proteins and mRNA, which are expressed dependent on the transcription factor ERα (cMyc, Her2, Tff1). Downregulation of ERα in MCF7/PGRMC1 cells resulted in significant decreased proliferation. We therefore conclude that PGRMC1 signaling leading to elevated proliferation is dependent on ERα. The results imply a mechanism, by which PGRMC1 mediates ERα expression, which induces tumor progression. To date, no mechanism by which PGRMC1 might induce upregulation of ERα expression is known. In breast cancer several mechanisms for modulation of ERα expression are reported, contributing to the pathogenesis of tumors[226,227]. ERα expression can be upregulated at the transcriptional level by increased promoter activity and expression of the ESR1 gene, by ESR1 gene amplification or by diminished degradation of ERα protein through proteasomal pathways[227]. The 8 ERα coding regions on chromosome 6 are transcribed from at least seven promoters, which is why the ESR1 promoter activity is affected by various factors[226]. Since we detected upregulation of both, ERα protein and ERα mRNA, we assume a mechanism of upregulated transcription of the ESR1 gene. Like other genes, transcription of the ESR1 gene can be regulated by epigenetic mechanisms, including methylation and acetylation. Transcription can be activated by histone H3 and H4 acetylation and H3 methylation, which induces chromatin decondensation. Here, we could no detect significant up- or downregulation of

H3 acetylation and methylation as well as expression of Histone deacetylase 1 (HDAC1) (data not shown), suggesting that altered ERα expression is not due to epigenetic changes.

We also detected upregulation of GSK3β, which is involved in protection of ERα from proteasomal degradation upon ubiquitylation and plays an important role in ERα protein stabilization and turnover[227,228]. GSK3β is further involved in E2-induced ERα phosphorylation and thus its transcriptional activity, which could also explain the elevated expression of ERα target genes[228].

Upregulation of ERα and its target gene c-Myc could not be detected in MCF7/PGRMC1-S181A cells, strongly indicating that PGRMC1 phosphorylation and -activation is important for activation of downstream signaling pathways facilitating increased ERα expression.

High levels of E2 have been reported to induce rapid proteasomal degradation of ERα, by a feedback-mechanism linked to ERα-mediated transcriptional response[226]. However, various other studies observed upregulation of steady-state ERα levels by long term exposure to E2[226,229–231]. The fact that many ERα promoter regions contain estrogen responsive elements supports this observation[226,232]. PGRMC1 might be involved in cholesterol synthesis and upregulation of cholesterol levels[112,114,115,233]. Since cholesterol is the precursor for the synthesis of steroid hormones, we hypothesize that PGRMC1 might also mediate synthesis of steroid hormones including E2, which then in turn could result in increased ERα expression.

We further found upregulation of phosphorylation of EGFR and its downstream targets in PGRMC1 overexpressing breast cancer cells. EGFR signaling pathways, including MAPK- and PI3K signaling cascades, essentially contribute to pathogenesis of breast cancer and are therefore important targets of anti-cancer agents[234–236]. Our results are supported by other studies, which proposed a role of PGRMC1 in promoting EGFR phosphorylation and activation[139,158,187,188]. Kabe et al. demonstrated that heme-dependent PGRMC1-dimerization mediates EGFR phosphorylation[187]. Besides, various groups found that knockdown of PGRMC1 results in decreased phosphorylation of EGFR and its downstream targets, such as Akt and ERK1/2, suggesting that PGRMC1 is involved in activation of EGFR and signaling cascades proceeding from EGFR[139,158,187,188]. However, an upregulation of EGFR signaling upon PGRMC1 overexpression has never been shown before. We assume that the PGRMC1-induced activation of EGFR considerably contributes to pathogenesis of breast cancer. As observed for upregulation of ERα, the phosphorylation-site mutant MCF7/PGRMC1-S181A

did not reveal increased activation of EGFR signaling, indicating that PGRMC1 phosphorylation at S181A is essential for its activation and capability to interact with EGFR. Since Kabe et al. demonstrated that PGRMC1-dimerization is required for interaction with EGFR, phosphorylation at S181A might also be important for receptor-dimerization.

Interestingly, we could not detect upregulation of EGFR signaling in PGRMC1 overexpressing MDA-MB-231 cells, which is why we reason that PGRMC1-dependent EGFR phosphorylation might be dependent on the cell system and factors differing between the two cell lines, such as the expression of hormone receptors. A crosstalk of the ERα- and EGFR signaling cascades has often been reported. Upon E2 activation, ERα can induce activation of the EGFR signaling pathway, e.g. resulting in phosphorylation of Akt (P-Ser473), via the non-genomic pathway[45,237,238]. Vice versa ERα activation can be accomplished independently of estrogens by members of the MAPK-signaling pathway and the PI3K pathway, which we have demonstrated to be upregulated in PGRMC1 overexpressing cells. Activation of EGFR by EGF for example leads to Ras-Raf-MEK-ERK1/2-mediated phosphorylation of ERα at Ser118[239–241]. Interestingly, Akt activation has been demonstrated to regulate ERα expression, which might be another approach to explain the upregulation of ERα in PGRMC1-overexpressing cells[237]. In summary, ERα is capable of phosphorylating and activating EGFR downstream pathways and vice versa members of the EGFR downstream pathways can phosphorylate and activate ERα, resulting in cancer cell progression. A PGRMC1-dependent bidirectional crosstalk of ERα expression/activation and EGFR signaling is conceivable and should be further investigated (figure 81).

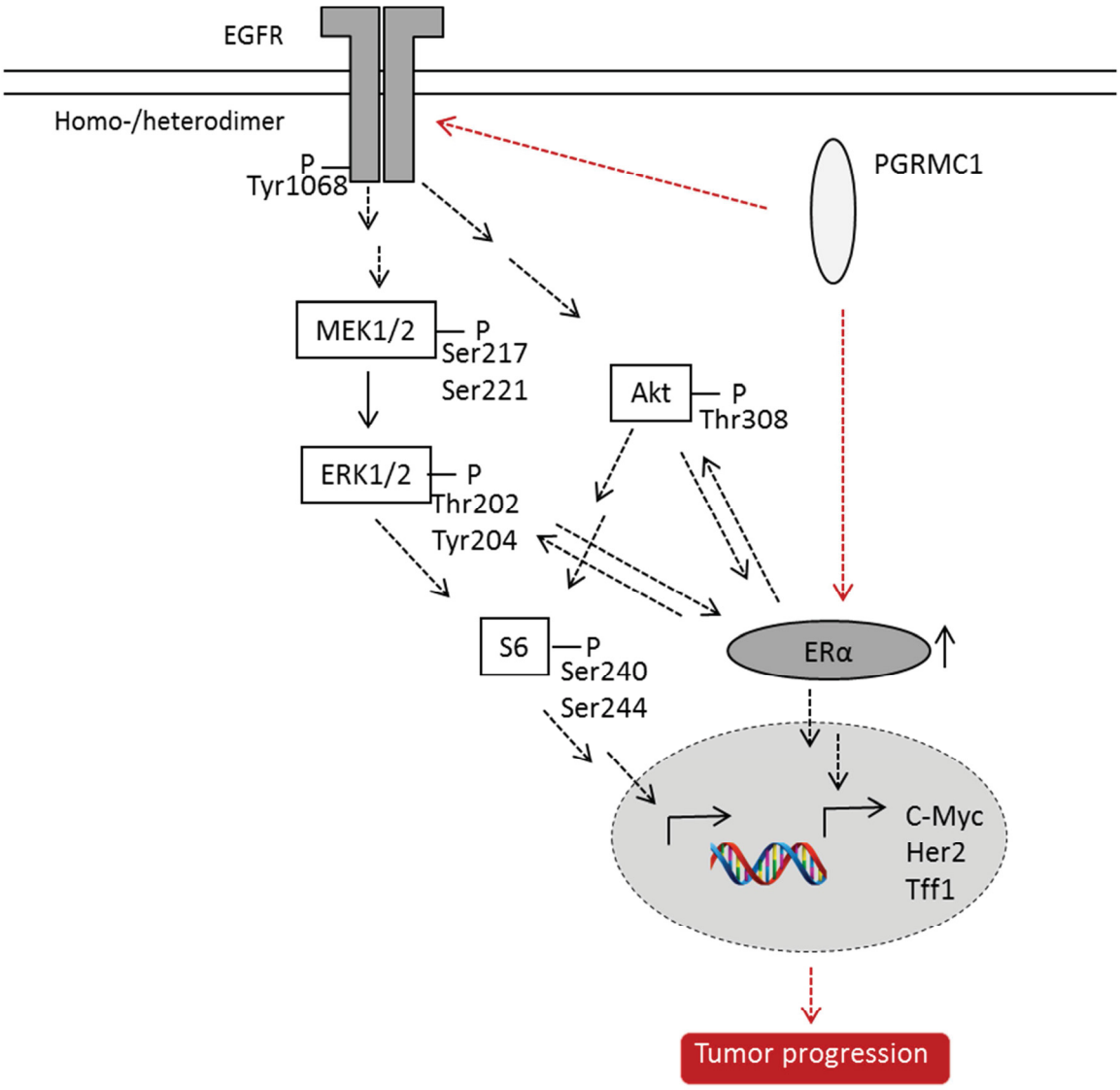


Figure 81. Potential crosstalk of PGRMC1/EGFR/ERα signaling cascades.
PGRMC1 mediates phosphorylation of EGFR and its downstream targets and upregulation of ERα and ERα-target genes. EGFR phosphorylation activates MAPK signaling cascade (including MEK1/2- , ERK1/2- and S6-phosphorylation) and PI3K signaling cascade (including Akt- and S6-phosphorylation). Phosphorylation of S6 induces transcription of genes, involved in the regulation of cell cycle progression, cell proliferation and glucose homeostasis. Upon ligand-dependent or –independent activation, ERα translocates into the nucleus and acts as a transcription factor and transcribes genes involved in tumor progression. Crosstalk of EGFR and ERα signaling cascades: ERα can be phosphorylated ligand-independently by members of the MAPK- and PI3K pathway. Vice versa, ERα signaling can activate Erk1/2 and Akt.

4.1.4. High PGRMC1 expression correlates with resistance to anthracycline treatment

In previous studies, PGRMC1 was shown to be associated with chemoresistance in cancer cells[120,140]. Recently, Kabe et al. proved that haem-mediated PGRMC1 dimerization is required for interaction with cytochrome P450 enzymes, facilitating the degradation of doxorubicin[187]. In cell line experiments in the present study, we further identified PGRMC1 to mediate resistance to the chemotherapeutic agents doxorubicin and epirubicin, however only with simultaneous treatment with P4. This supports the finding of Clark et al., who observed that PGRMC1 is responsible for the anti-apoptotic action of P4 in MDA-MB-231

breast cancer cells, which were treated with doxorubicin[119]. This result was further verified by correlation of PGRMC1 expression in breast cancer specimen before neoadjuvant therapy with therapy response (anthracyclines, taxanes or endocrine therapy). Significant higher levels of PGRMC1 were detected in patients who did not respond to anthracycline-based therapy as compared to patients who achieved partial remission, giving evidence for a coherence of PGRMC1 expression and response to cancer therapy.

Our findings from tissue of breast cancer patients along with the studies associating PGRMC1 expression with chemoresistance in breast cancer cells clearly indicate that PGRMC1 plays an important role in worse response to anti-cancer treatment, probably by interaction of the PGRMC1 dimer with cytochrome P450 enzymes, which are responsible for the degradation of various anti-cancer drugs. The fact that this effect is not detectable in a P4-free system indicates that the activation of PGRMC1 by its ligand P4 is required for its anti-apoptotic action.

4.1.5. PGRMC1 interacts with proteins, important for cellular transport and cholesterol biosynthesis

We detected various potential PGRMC1 interaction partners by mass spectrometry. Among the 100 most significant interaction partners, we found numerous proteins, which are involved in cellular transport processes, including vesicular transport. Since the function of proteins is usually dependent on its cellular localization, directed transport and sorting of proteins and lipids between cellular compartments is essential for an impeccable cellular function. Therefore, mediators of vesicle trafficking are important regulators of cellular physiologies e.g. by determining the cell surface presentation of receptors[214,216,242]. Various studies suggested a role of PGRMC1 vesicle trafficking before, however the precise role of PGRMC1 in vesicular transport is still under investigation[158,179,182,183]. Among the interaction partners, involved in vesicular transport, we detected interaction of PGRMC1 with COPB1 and COPG1, proteins which are involved in protein transport from the endoplasmic reticulum to the trans-Golgi network[213–216].

Interestingly, among the detected PGRMC1 interaction partners, we also found various proteins involved in nuclear import and -export and detected a NLS within the PGRMC1 primary structure. The fact that PGRMC1 exhibits a NLS and interacts with proteins involved in nuclear import and –export, strongly suggests a nuclear function of the protein. The nuclear function of proteins, which shuttle between the cytoplasm and the nucleus, is

regulated by controlled nuclear import and export, dependent on physiological conditions. Nuclear localization of PGRMC1 has been observed before. Beausoleil et al. detected PGRMC1 phospho-peptides in nuclear extracts of HeLa cells[142]. Min et al. suggested that intracellular distribution of PGRMC1 might depend on multimer formation of PGRMC1 after ligand binding[154]. Further, Peluso et al. detected that PGRMC1 is posttranslationally modified by small ubiquitin-like modifier protein-1 (SUMO), which leads to nuclear localization and the ability to mediate progesterone-dependent attenuation of the activity of transcription factor T-cell factor/lymphoid enhancer factor (Tcf/Lef)[118,243]. These findings suggest that PGRMC1 might alter its sub-cellular localization depending on posttranslational modification or activation status and thereby regulating its function. However, the nuclear function of PGRMC1 still requires further investigation and might be important to gain deeper insight into its biological function and its role in cancer.

Besides, we detected 8 proteins, involved in regulation of G2/M transition of mitotic cell cycle. A role of PGRMC1 in cell cycle progression has previously been reported[121,159,189]. Ahmed et al. demonstrated that inhibition of PGRMC1 by AG-205 induces G1 cell cycle arrest, indicating a role of the receptor in this phase of the cell cycle[121].

Additionally, 5 proteins, involved in cholesterol metabolism were detected. PGRMC1 was proposed before to be involved in cholesterol synthesis, potentially by interacting with proteins of the cholesterol synthesis pathway. Our results support this assumption. CYP51A1, also known as Lanosterol 14α-demethylase was among the proteins involved in cholesterol metabolism. CYP51A1 catalyzes lanosterol demethylation, resulting in an intermediate, which is the precursor for steroid synthesis. Interaction of PGRMC1 and CYP51A1 was detected before by Hughes at al. in human embryonic kidney cells (HEK293). In fact, they showed that PGRMC1 is required for cholesterol synthesis in mammals; knockdown of PGRMC1 resulted in reduced activity of CYP51A1 and thus accumulation of lanosterol in the cells[116]. We could confirm this interaction in the present study, strongly pointing towards an important role of PGRMC1 in synthesis of cholesterol and steroids, potentially resulting in altered functional properties of cells. Cholesterol metabolism is important for cancer cell proliferation as well as resistance to anti-cancer therapy and cholesterol pathway genes have been demonstrated to be upregulated in various cancers[244]. Elevated cholesterol metabolism induced by interaction of PGRMC1 with enzymes of the cholesterol biosynthesis pathway might contribute to malignant transformation in various

ways. Enhanced synthesis of sterols and lipids is essential for the rapid proliferation of cancer cells, since cholesterol is an important component of the cell membrane and important for its function[245]. In addition, cholesterol is the precursor for the synthesis of steroid hormones, which are known to promote the development and growth of various human cancers[233]. Besides, cholesterol metabolism could contribute to carcinogenesis by activating and augmenting major signaling pathways. Early steps of the cholesterol biosynthesis pathway provide intermediates which are important signal molecules for pathways upregulated in cancer, such as PI3K- and GTPase signaling[244,246]. Cholesterol is further a main component of lipid rafts, small domains in cell membranes which are distinguished by high contents of sphingomyelins, glycosphingolipids and cholesterol[247]. They serve as signaling platforms by recruiting and accumulating various proteins and signaling molecules. A wide range of signal transduction pathways have been found to occur in and to be dependent on lipid rafts, including pathways associated with cell survival and –proliferation (EGFR-signaling, insulin receptor signaling, etc.)[244,248].

4.2. Role of PGRMC1 in increased breast cancer risk upon progestin-based contraception and hormone therapy

In previous studies, we demonstrated PGRMC1 to forward progestin signals in MCF7 cells and therefore suggested a potential role of PGRMC1 in the higher breast cancer risk upon progestin-based contraception and hormone therapy. In the present study, we extended our previous studies to gain deeper insight into PGRMC1 signaling upon progestin treatment.

4.2.1. PGRMC1 promotes proliferation of ERα positive breast cancer cells upon progestin treatment

In previous *in vitro* and *in vivo* studies, we investigated the effect of various progestins on PGRMC1-overexpressing MCF7 cells. These studies revealed that upon treatment with selected progestins the proliferation rate and tumor volume is significantly higher compared to MCF7/EVC cells[90,192–194,196,197]. In the present study, besides elevated proliferation of MCF7/PGRMC1 cells, we also detected enhanced proliferation of T47D/PGRMC1 breast cancer cells upon treatment with selected progestins compared to T47D/EVC cells, again supporting our previous results. We observed significant higher proliferation of PGRMC1 overexpressing MCF7 and -T47D cells compared to EVC cells upon treatment with the progestins NET, DYD, DSP and MPA, while treatment with CPA, NOM and P4 did not increase

proliferation significantly. These results are in accordance with the results of Ruan et al., who also detected proliferation-enhancing effects of NET, DYD, DSP and MPA, whereas no effect could be observed for NOM and P4. They are further supported by a recently published xenograft study of Zhao et al., who found higher tumor volumes of PGRMC1 overexpressing MCF7 and T47D cells in NET treated mice compared to tumor volumes of the respective EVC cells[249]. As shown before by our group, tumors of E2/NET treated mice exhibited increased growth compared to tumors of E2-only treated mice[190]. These results indicate potential off target activities of the progestins NET, DYD, DSP and MPA on PGRMC1, as reported for other steroid receptors, resulting in enhanced proliferation. Binding of progestins might induce phosphorylation and conformational changes of PGRMC1 resulting in altered biological function of the receptor[114]. The effects of the various progestins on PGRMC1 overexpressing cells varied depending on progestin used. While NET increased cell proliferation even at nanomolar concentrations, MPA only showed effects when used at 1 µM. In the highest concentration used, NET and DYD also enhanced proliferation of MCF7 and T47D EVC cells, which is potentially attributed to the endogenous expression of PGRMC1 in those cells.

As shown for proliferation of cells without progestin-treatment, no effect could be observed of progestin-treatment on MDA-MB-231/PGRMC1 and MDA-MB-231/EVC cells, suggesting that also the progestin-mediated PGRMC1-signaling is dependent on factors, present in MCF7 and T47D cells but not in MDA-MB-231 cells, such as ERα.

In T47D cells, NET was shown before to be bioconverted into the a-ring reduced metabolites 3α,5α-norethisterone and 5α-norethisterone potentially by 5 alpha-steroid reductase and aldo-keto reductases. While NET is a PR-agonist, 3α,5α-norethisterone and 5α-norethisterone are ER-agonists[86,87]. 5 alpha-steroid reductase is abundant MCF7 cells, as well[250]. Therefore, it is also conceivable that instead of NET its metabolites might induce the described effects by binding to PGRMC1.

4.2.2. PGRMC1 phosphorylation at S181 is essential for increased proliferation upon progestin treatment

To gain deeper insights into the mechanism of action of progestins on PGRMC1, we aimed to further investigate PGRMC1 signaling upon NET treatment in the present study. For the first time, we could show that PGRMC1 is phosphorylated at S181 and thus probably activated by the progestin NET. As known for other steroid receptors, in the presence of NET or its metabolites PGRMC1 might potentially undergo conformational changes, which exposes the

phosphorylation site or induces dissociation from chaperones, enhancing phosphorylation of the receptor by kinases[53,251]. Serine 181 is the most commonly observed phosphosite of PGRMC1 and is predicted to be phosphorylated by CK2, a constitutively active kinase responsible for phosphorylation of a large proportion of proteins[184,252]. Cahill et al. suggested that the Y180 and the S181 phosphorylation sites are accessible for protein interactions, since they are located in unstructured regions of the protein, which were unstable under NMR conditions[113,114,117]. Thus, increased phosphorylation of PGRMC1 at S181 upon NET treatment might lead to augmented recruitment of enzymes or protein-protein interactions, resulting in altered signal transduction[113].

Besides characterization of PGRMC1 phosphorylation status, also identification of kinases and phosphatases responsible for phosphorylation and dephosphorylation is important to better understand PGRMC1 signaling. Simultaneous treatment of MCF7/PGRMC1- and T47D/PGRMC1 cells with NET and quinalizarin, a cell-permeable highly specific CK2 inhibitor, resulted in a decrease of S181 phosphorylation. This result confirms the assumption that CK2 is responsible for, or involved in, phosphorylation of PGRMC1 at S181. Our results are supported by a recently published quantitative phosphoproteome analysis using quinalizarin to detect CK2 target proteins. In this study, for the first time PGRMC1 was shown to be a target of CK2: immunoprecipitated PGRMC1 was phosphorylated in the presence of CK2, while in the absence of CK2 no PGRMC1 phosphorylation could be detected. Treatment with 1 µM quinalizarin revealed decreased phosphorylation[253].

Here we could prove that mutation of S181 and double mutation of S57 and S181 diminished susceptibility of MCF7/PGRMC1 cells to NET treatment. The proliferation rate of these cells upon NET treatment was similar to the proliferation rate of EVC cells, whereas sole mutation of S57 did not diminish cell proliferation. This indicates that PGRMC1 phosphorylation at S181 phosphorylation site is crucial for NET-induced downstream signaling of PGRMC1. As discussed above, phosphorylation of PGRMC1 at S181 could alter biological functions of the receptor in various ways and the underlying mechanism still has to be further investigated.

4.2.3. PGRMC1 mediates progestin-dependent upregulation of ERα dependent genes and activation of EGFR signaling

As demonstrated for progestin-independent PGRMC1 signaling, significant enhanced upregulation of ERα target genes c-Myc and TFF1 was detected in PGRMC1 overexpressing

cells upon progestin treatment. Further, upregulation of phosphorylation of Her2 and Her2/EGFR downstream targets was observed. These effects could not be shown for MCF7/PGRMC1-S181A cells. We therefore suppose that the progestin-induced phosphorylation at S181 activates PGRMC1, which in turn activates downstream signaling facilitating ERα expression and EGFR phosphorylation in the same way as discussed in 4.1.3.. The fact that NET mediated the strongest effect on PGRMC1 overexpressing cells could be due to the bioconversion of NET to the ER-agonists 3α,5α-norethisterone and 5α-norethisterone[86,87]. Besides activation of PGRMC1 and associated downstream targets, bioconverted NET might also directly bind to ERα, facilitating ligand-dependent ERα signaling. This is supported by the observation that ERα target genes were upregulated and ERα was downregulated in all three cell lines upon NET-treatment. The downregulation of ERα might be attributed to ligand-induced degradation of ERα mediated by ubiquitin ligase 26S proteasome[226]. This turnover is linked to ER-mediated transcriptional response, which explains the fact that the degradation of ERα was significantly stronger in MCF7/PGRMC1 cells compared to MCF7/EVC and MCF7/PGRMC1-S181A cells.

4.2.3. PGRMC1 interacts with PHB1 and PHB2 upon progestin treatment

Besides other interaction partners, we detected increased interaction PHB1 and PHB2 with PGRMC1 upon treatment with NET in PGRMC1-overexpressing cells as well as in endogenously PGRMC1-expressing MCF7 cells. Both prohibitins are known to be localized in the nucleus, where they act as transcription factor modulators. Particularly PHB2 was found to be a repressor of ERα activity[220,254–257]. Therefore, we hypothesized that the NET-mediated interaction of PGRMC1 with PHB1 and PHB2 might have an effect on the function and activity of certain transcription factors, including ERα. In a similar mechanism as known for interaction of PHB2 with nucleotide-exchange protein 3 (BIG3), interaction of PGRMC1 with PHB1 and PHB2 might inhibit the repressory effect of prohibitins on transcription factors, enhancing their activity[258]. This model might be a further explanation of elevated expression of ERα target genes in PGRMC1-overexpressing cells, and should be further investigated.

4.3. Conclusions and outlook

In the present study, we were able to gain further insights into the mechanisms of PGRMC1 activation and involvement in important breast cancer signaling pathways independent and

dependent on progestins. PGRMC1 promotes progression of cancer cells, potentially by activating signaling pathways facilitating ERα expression and activation as well as EGFR signaling, however the underlying mechanism not known yet. Therefore, future studies should address the mechanism of elevated ERα expression mediated by PGRMC1 and the role of PGRMC1 in facilitating EGFR signaling. The interaction partners detected in the present study will be an important starting point to further elucidate the PGRMC1 signaling cascade.

We detected various PGRMC1 phosphorylation sites and demonstrated that phosphorylation of PGRMC1 at Ser181 is important for its activation and function. Characterization of PGRMC1 phosphorylation and associated function requires further investigation and is important for a better understanding of the involvement of PGRMC1 in breast cancer progression and increased breast cancer risk in progestin-based hormone therapy. Future studies should address the role of the various PGRMC1 phosphorylation sites in activation and dimerization of PGRMC1 and downstream signaling cascades.

Further, the present study again demonstrates a potential role of PGRMC1 in cholesterol biosynthesis. Upregulation of cholesterol and its precursors contributes to pathogenesis of cancer in various ways. Future studies should therefore further investigate the capability of PGRMC1 in increasing cellular cholesterol- and steroid levels and the interaction of PGRMC1 with proteins of the cholesterol biosynthesis pathway also in regard to its phosphorylation.

Concluding, upregulation of PGRMC1 facilitates cancer progression and contributes to pathogenesis cancer. Therefore, PGRMC1 could be a potential anti-cancer therapeutic agent and its suitability as such should be considered.

5. Summary

PGRMC1 is upregulated in breast cancer and elevated expression of PGRMC1 is associated with increased tumor growth and poorer outcome, indicating a contribution of PGRMC1 in carcinogenesis of breast cancer. Further, PGRMC1 might be involved in transducing membrane-initiated progestin signals, suggesting a role of PGRMC1 in higher breast cancer risk upon progestin-based contraception and hormone therapy. However, the role of PGRMC1 in breast cancer, its activation mechanism and involved signaling pathways are not fully understood yet. Therefore, the aim of the present study was to investigate the role of PGRMC1 in breast cancer progression, the last step of carcinogenesis, and the role of PGRMC1 in increased breast cancer risk upon intake of progestins for contraception and hormone therapy.

For this purpose, the effect of PGRMC1 overexpression and –knockdown on cell proliferation was examined. Since the function of PGRMC1 was suggested to be regulated by differential phosphorylation, the PGRMC1 phosphorylation-status and the significance of PGRMC1 phosphorylation on breast cancer progression was investigated. With the aim to further elucidate PGRMC1 signaling in breast cancer, we searched for signaling pathways which might be regulated by PGRMC1. To gain deeper insight into the activation mechanism of PGRMC1 by progestins, all those studies were additionally conducted with treatment of breast cancer cells with progestins.

Overexpression of PGRMC1 in the hormone-receptor positive cell lines MCF7 and T47D resulted in significant enhanced proliferation, while knockdown of PGRMC1 caused significant decreased proliferation. Analysis of PGRMC1-dependent expression and activation of proteins revealed upregulation of ERα and ERα-dependent genes, as well as activation of EGFR signaling cascade. PGRMC1 phosphorylation at S56 and S181 was identified to be essential for enhanced proliferation and induction of enhanced ERα expression.

Treatment of PGRMC1-overexpressing breast cancer cells with various progestins resulted in significant increased proliferation compared to EVC cells. Further, treatment of breast cancer cell lines with the progestin norethisterone (NET) induced phosphorylation of PGRMC1 at the Casein Kinase 2 (CK2) phosphorylation site Ser181, which could be decreased by treatment with CK2 inhibitor quinalizarin.

These results emphasize an important role of PGRMC1 in breast cancer progression and display a potential embedding of PGRMC1 in signaling pathways, upregulated in breast cancer. PGRMC1 might therefore be an interesting target for anti-cancer therapy. Additionally, this study gives insights into the mechanism of differential phosphorylation of the receptor and its activation by progestins. It also confirms expression and activation of PGRMC1 as a model to investigate tumorigenesis and progression of breast cancer in progestin-based hormone therapy.

6. Zusammenfassung

Der hormonrezeptor PGRMC1 weist im Mammakarzinom eine erhöhte Expression auf. Außerdem korreliert eine hohe Expression von PGRMC1 im Mammakarzinom mit stärkerem Tumorwachstum und schlechterer Prognose, was eine Beteiligung von PGRMC1 an der Karzinogenese des Mammakarzinoms vermuten lässt. PGRMC1 scheint außerdem bei der Weiterleitung membran-initiierter Progestin-Signale beteiligt zu sein, wodurch der Rezeptor eine Rolle bei dem erhöhtem Brustkrebsrisiko bei progestin-basierter Kontrazeption und Hormontherapie spielen könnte. Die Rolle von PGRMC1 im Mammakarzinom, dessen Aktivierungs-mechanismus und beteiligte Signalwege sind jedoch noch nicht vollständig aufgeklärt. Daher war das Ziel der vorliegenden Arbeit, die Rolle von PGRMC1 bei der Progression des Mammakarzinoms, dem letzten Schritt der Karzinogenese, aufzuklären und dessen Rolle bei einem erhöhten Brustkrebsrisiko bei Einnahme von exogenen Hormonen zu untersuchen.

Zu diesem Zweck wurde die Auswirkung einer PGRMC1-Überexpression und eines -Knockdowns auf die Progression des Mammakarzinoms untersucht. Da diverse Studien auf die Regulation der Funktion von PGRMC1 durch differenzielle Phosphorylierung hinweisen, wurden außerdem der Phosphorylierungs-Status von PGRMC1 und die Bedeutung der Phosphorylierung für die Progresion des Mammakarzinoms analysiert. Mit dem Ziel, den PGRMC1 Signalweg weiter aufzuklären, wurde nach Signalwegen gesucht, die durch PGRMC1 reguliert werden. Um einen tieferen Einblick in die Aktivierung von PGRMC1 durch Progestine zu bekommen, wurden diese Experimente zusätzlich unter Behandlung von Brustkrebszellen mit Progestinen durchgeführt.

Die Überexpression von PGRMC1 in den hormonrezeptor-positiven Zelllinien MCF7 und T47D führte zu einer signifikant erhöhten Proliferation, während der Knockdown eine signifikant niedrigere Proliferation zur Folge hatte. Die Analyse einer PGRMC1-abhängigen Expression oder Aktivierung von Proteinen zeigte sowohl eine Überexpression von ERα und ERα-Zielgenen als auch eine erhöhte Aktivierung der EGFR Signalkaskade. Es konnte außerdem gezeigt werden, dass die Phosphorylierung von PGRMC1 essentiell für die erhöhte Proliferation und die Induktion einer erhöhten ERα Expression ist. Die Behandlung von PGRMC1-überexprimierenden Brustkrebszellen mit verschiedenen Progestinen resultierte in einer signifikant erhöhten Proliferation im Vergleich zu Leervektor-Kontrollzellen. Die Behandlung von Brustkrebszellen mit dem Progestin NET induzierte die Phosphorylierung

von PGRMC1 an der CK2-Phsophorylierungsstelle S181, welche durch Behandlung mit dem CK2 Inhibitor Quinalizarin reduziert werden konnte.

Diese Ergebnisse demonstrieren eine wichtige Rolle von PGRMC1 bei der Progression des Mammakarzinoms und lassen auf eine Einbettung von PGRMC1 in beim Mammakarzinom hochregulierte Signalwege schließen. PGRMC1 könnte daher ein wichtiges Target für die anti-Krebs Therapie darstellen. Zusätzlich bietet diese Studie Einblick in den Mechanismus der differenziellen Phosphorylierung des Rezeptors und dessen Aktivierung durch Progestine. Sie bestätigt außerdem die Expression und Aktivierung von PGRMC1 als Modell zur Untersuchung der Tumorentstehung und der Progression von Brustkrebs bei der Progestin-basierten Hormontherapie.

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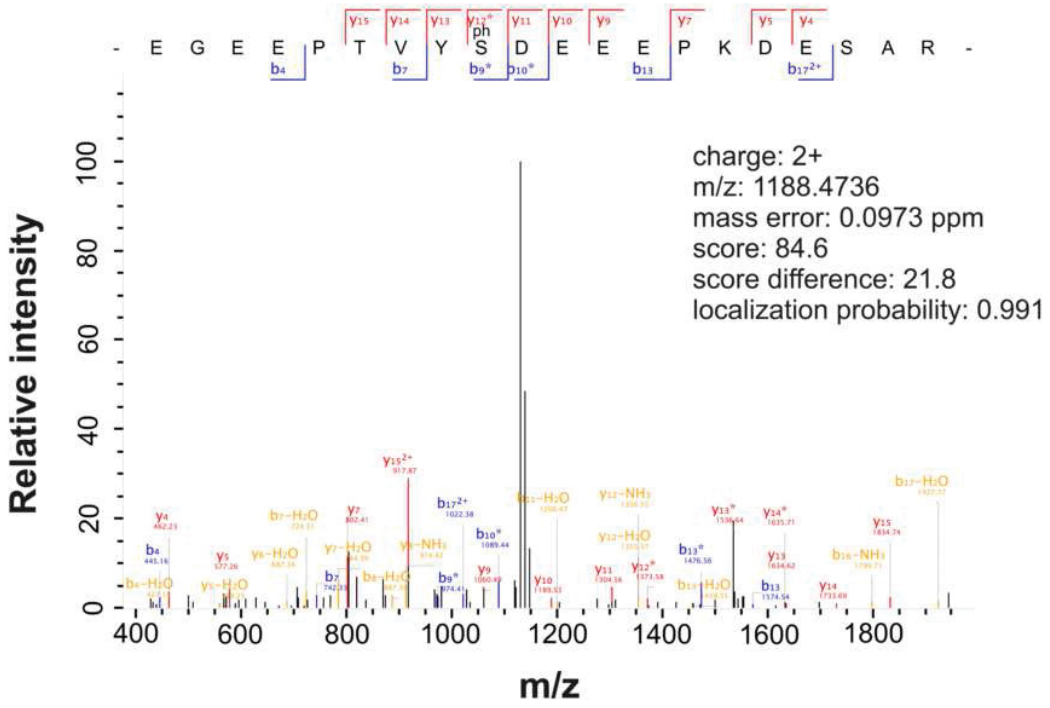
8. Supplemental figures and tables



Supplemental Figure 1. MS/MS spektrum
Annotated MS/MS spectrum of a 1188.4736 m/z two-fold charged precursor, fragmented by CID which can be attributed to the sequence EGEEPTVYSDEEEPKDESARK including one phosphorylation. A phosphorylation at S181 shows highest score resulting in a localisation probability of 99%.

Supplemental Table 1: PGRMC1 phosphopeptides detected by Mass spectrometry.

Phospho (STY) Probabilities	Mass error [ppm]	Score diff	Posterior error probability	Score	Score for localization	Delta score	Localization probability
EGEEPTVYS(1)DEEEPKDESARK	-0.569	38.8	2.75E-36	136.2	121.9	96.6	0.9998
IVRGDQPAAS(0.863)GDS(0.137)DDDEPPPLPR	0.397	8.0	2.91E-19	111.4	74.9	77.6	0.8626
GDQPAAS(0.001)GDS(0.999)DDDEPPPLPR	-0.085	32.0	2.76E-27	120.2	114.3	75.7	0.9994
LLKEGEEPT(0.9)VY(0.042)S(0.058)DEEEPKDESAR	1.112	11.9	3.85E-09	93.2	54.3	65.1	0.9001
EGEEPT(0.031)VY(0.851)S(0.117)DEEEPKDES(0.002)AR	0.176	8.6	0.0046791	50.1	50.1	28.1	0.8511

Phosphopeptides were detected using liquid chromatography coupled mass spectrometry. Sites were localized according to the fragment spectra matching pattern. Scores and probabilities are given for peptide and phospho site identification and localisation.

9. Appendix

9.1. List of figures

9.2. List of tables

9.3. Abbreviations

µ	Micro (10^{-6})
µg	microgram
µl	Microliter
µM	Micromolar
ab	Antibody
AF-1	Transcriptional activation function domain-1
AF-2	Transcriptional activation function domain-2
Akt	Protein kinase B
AP-1	Activator protein 1
AR	Androgen receptor
BCA	Bicinchoninic acid
BIG3	Nucleotide-exchange protein 3
BRCA1	Breast cancer type 1 susceptibility protein
BRCA1	Breast cancer type 2 susceptibility protein
C	Cytoplasmic domain
C. elegans	*Caenorhabditis elegans*
CEE	Conjugated equine estrogen
CHT	Combined estrogen-progestin hormone therapy
CK2	Casein Kinase 2
CI	Confidence interval
cm	Centimetre
CMA	Chlormadinonacetat
CO	Carbon monoxide
COPB1	Coatomer subunit beta

COPG1	Coatomer subunit gamma-1
CPA	Cyproteron(acetate)
CYB5D2	Neuferricin
CYP	Cytochrome P450
CYP19A1	Aromatase or estrogen synthetase
CYP51A1	Lanosterol 14α-demethylase
cytb5	Cytochrome b5
d	Day(s)
DAB	3,3'-diaminobenzidine
DAPI	4’,6-Diamidin-2-phenylindol
DBD	DNA-binding domain
DCIS	Ductal carcinoma in-situ
DGN	Dienogest
DMFS	Distant metastasis-free survival
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA PK	DNA-dependent serine/threonine protein kinase
dNTP	Deoxynucleotide
dox	Doxorubicin
DSP	Drospirenon
DTC	Disseminated tumor cell
DYD	Dydrogesterone
E	Estrogen-alone
E + P	Estrogen plus progestin
E1	Estrone
E2	Estradiol
E3	Estriol
EC	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHT	Estrogen-only hormone therapy

epi	Epirubicin
ER	Estrogen receptor
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
ERγ	Estrogen receptor-γ
ERE	Estrogen-response-elements
ERK1/2	Extracellular signal–regulated kinases 1/2
ESR1	Estrogen receptor 1 (gene)
et al.	et alii/et aliae/et alia
EVC	Empty vector control
FBS	Fetal bovine serum
FDFT1	Squalene synthase
FoxP3	fork box 3
g	Gram
GFP	Green fluorescent protein
GLP-1R	Glucagon-like peptide-1 receptor
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GR	Glucocorticoid receptor
GRP30	Orphan G-protein coupled receptor 30
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine-5'-triphosphate
h	Hour(s)
H	Hinge-domain
HA	Human influenza hemeagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her2 (also: ERBB2)	Human epidermal growth factor receptor 2
Hpr6.6	Heme progesterone receptor 6.6
HRP	Horseradish peroxidase
HT	Hormone therapy
ICC	Immunocytochemistry
IDC	Invasive ductal carcinoma

i.e.	id est
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
iNOS	Inducible nitric oxide synthase
(Co-)IP	(Co-)immunoprecipitation
IRS	Immune reactive score
IUD	Intrauterine device
kDa	Kilodalton
Ki-67	Antigen Ki-67
LBD	Ligand-binding domain
LGS	Levonorgestrel
m	milli
M	molar
ml	milliliter
mM	millimolar
MAPK	Mitogen-activated protein kinase
MAPR	Membrane-associated progesterone receptor
mAR	Membrane androgen receptor
MEK1/2	Mitogen-activated protein kinase kinase 1/2
mER	Membrane estrogen receptor
mg	Milligram
mGR	Membrane glucocorticoid receptor
min	Minutes
mm	millimetre
mMR	Membrane mineralocorticoid receptor
MPA	Medroxyprogesterone (acetate)
mPR	Membrane progesterone receptor
MR	Mineralocorticoid receptor
mRNA	messenger RNA
MTT	Thiazolyl Blue Tetrazolium Bromide
MW	Million Women (study)
n	nano

N	Number/size of cohort
NcoA-1	Nuclear hormone receptor coactivator-1
NET	Norethisteron(acetate)
ng	nanogram
NLS	Nuclear localization signals
nm	nanometre
nM	nanomolar
NOM	Nomegestrel(acetate)
NP-40	Nonidet P 40 Substitute
NR	Non-responder
p	p-value/ probability value
P/Pro	Proline
Ⓟ	Indicates phosphorylation
P4	Progesterone
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PD-L1	Programmed death-ligand 1
PGRMC1	Progesterone Receptor Membrane Component-1
PGRMC2	Progesterone Receptor Membrane Component-2
PHB1	Prohibitin-1
PHB2	Prohibitin-2
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PKA	Protein kinase A
PLA	Proximity ligation assay
pPGRMC1	Phospho-S181-PGRMC1
PR	Progesterone receptor
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
R	Responder
RFS	Relapse free survival
RNA	Ribonucleic acid

rpm	Revolutions per minute
RPPA	Reverse phase protein array
RR	Relative (breast cancer) risk
S6	S6 Ribosomal Protein
SDS	Sodium dodecyl sulfate
S/Ser	Serine
SCD	Acyl-CoA desaturase
S. cerevisiae	*Saccharomyces cerevisiae*
sec	Second(s)
SH-2	Src homology 2
SH-3	Src homology 3
SHIP	SH2-containing inositol phosphatase
Sictrl/siControl	scrambled siRNA control
siESR1	siRNA knockdown of ERα
siPGRMC1	siRNA knockdown of PGRMC1
siRNA	Small interfering RNA
SQLE	Squalene monooxygenase
SRC-1	Steroid receptor coactivator-1
SUMO	Small Ubiquitin-like Modifier
t	Time/time point
T/Thr	Threonine
Tcf/Lef	T-cell factor/lymphoid enhancer factor
TBS	TRIS-buffered saline
TBS-T	TBS containing Tween20
TFF1	Trefoil factor 1
TM	Transmembrane domain
TMA	Tissue microarrays
TNBC	Triple-negative breast cancer
UV/vis spectroscopy	Ultraviolet–visible spectroscopy
WHI	Women's Health Initiative
Y/Tyr	Tyrosine

9.5. Declaration

Ich, Marina Willibald, versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in vorgelegter oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolgreichen oder erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 26.09.2017

_____

Marina Willibald

10. Aknowledgement

An dieser Stelle möchte ich meinen besonderen Dank nachstehenden Personen entgegenbringen, ohne deren Mithilfe die Anfertigung dieser Dissertation niemals zustande gekommen wäre:

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