# Secretion and expression regulation of progesterone receptor membrane component1 (PGRMC1) in breast cancer cells

**Inaugural-Dissertation**

**zur Erlangung des Doktorgrades**

**der Medizin**

**der Medizinischen Fakultät**

**der Eberhard Karls Universität**

**zu Tübingen**

**vorgelegt von**

**Bo Ma**

**aus**

**Zhejiang, China**

**2015**

**Dekan: Professor Dr. I. B. Autenrieth**

**1. Berichterstatter: Professor Dr. H. Seeger**

**2. Berichterstatter: Privatdozent Dr. E. Ruckhäberle**

# Summary

In women breast cancer still is the most prevalent cancer and one of the leading causes of death. Progesterone receptor membrane component-1 (PGRMC1) highly more expressed in cancerous breast tissue than in benign surrounding breast tissue may be involved in tumorigenesis and increase breast cancer risk. In recent investigations it could be shown that estrogens and certain synthetic progestogens can induce an increased proliferation rate in breast cancer cells via PGRMC1 suggesting a possible importance of the kind of estrogen and progestogen in terms of breast cancer risk when used as hormone therapy in the postmenopause. However, the detailed mechanisms through which PGMRC1 mediates proliferative effects elicited by progestogens and regulating its expression are still little known. It remained elusive whether PGRMC1 is secreted by breast cancer cells into plasma and thus might be used for cancer risk screening. Here we analyzed PGRMC1 expression and the proliferative effect of progestogens in various breast cancer cell lines. In BM cells (endogenous estrogen receptor (ER-α) and PGMRC1), medroxyprogesterone acetate (MPA), norethisterone (NET), levonorgestrel (LNG) and drospirenone (DRSP) significantly increased the proliferation. However, these progestogens didn't obviously alter the proliferation of SUM225CWN cells (only endogenous PGRMC1). In MCF-7 breast cancer cells, the presence of PGRMC1 can sensitize E2-induced PS2 mRNA levels, an estrogen response element. Interestingly it could be shown for the first time that certain progestogens such as NET also were able to induce an enhanced PS2 expression in MCF-7/PGRMC1 cells. No effect was found for progesterone. Addition of NET to E2 did not obviously alter the PS2 mRNA levels as compared with E2 only. This effect of E2 and NET could be blocked by antagonists of ER-α, PGRMC1 and CK2, a kinase which is involved in cell proliferation, the effect being highest for the ER-α-antagonist. In order to elucidate the possible involvement of RANK/RANKL, its expression was investigated in various breast cancer cell lines. However, the expression of RANK and RANKL couldn't be detected in these cell lines using western blot. Estradiol and progestogens didn't significantly change cell proliferation and RANKL expression in ER-α negative, RANKL overexpressing cells. In co-cultures of MDA-MB-231 and MCF-7/PGRMC1 cells with osteoblast-like cells no induction of RANKL expression was found in the breast cancer cells. Thus the possible involvement of RANK/RANKL in the PGRMC1 mediated progestogenic effect remains unclear. To elucidate probable mechanisms on the influence on endogenous PGRMC1 expression, MCF-7 cells were transfected with a mimic of miRNA let-7i, since let-7i is considered as a tumor suppressor to inhibit malignant growth of cancer cells. We demonstrated that

let-7i targets PGRMC1 and inhibited endogenous PGRMC1 mRNA expression. Further, an analysis of PGRMC1 expression in the supernatant of breast cancer cells by western blot revealed that PGRMC1 could be secreted by these cells. However, first investigations with plasma samples from women with and without breast cancer weren't successful in detecting PGRMC1.

In summary we conclude from these results that ER-α may play an important role in the signal transduction of PGRMC1 activated by estradiol and progestogens, and PGRMC1 may participate in the ER-α/PS2 pathway. PGRMC1 expression might be regulated by a complex mechanism that might involve the suppressive action of miRNAs, e.g. let-7i, which could become a therapeutic target. Moreover, PGRMC1 might be a potential predictive biomarker for breast cancer risk especially in postmenopausal women using hormone therapy.

# Contents

# List of figures

# List of tables

# Abbreviations

| | |
|---|---|
| 3'UTR | 3' untranslated region |
| ANOVA | Analysis of variance |
| ATCC | American type culture collection |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CDKs | Cyclin-dependent kinases |
| CKIs | Cyclin-dependent kinases inhibitors |
| CMA | Chlormadinone acetate |
| CYP | Cytochromes P450 |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphat |
| DRSP | Drospirenone |
| dsRBD | Double-stranded RNA binding domain |
| DY | Dydrogesterone |
| E2 | Estradiol |
| ER | Estrogen receptor |
| ERK | Extracellular regulated protein kinases |
| ER-α | Estrogen receptor-alpha |
| FSH | Follicular stimulating hormone |
| FUL | Fulvestrant |
| GAPDH | Glyceraldehyde-3-phosphate degydrogenase |
| HRP | Horseradish peroxidase |
| HRT | Hormone replacement therapy |
| IHC | Immunohistochemistry |
| Insig | Insulin-induced gene |
| I k B | Inhibitor of NF- k B |

| | |
|---|---|
| LNG | Levonorgestrel |
| MAPR | Membrane-associated progesterone receptor |
| MCF-7 | Michigan Cancer Foundation-7 |
| miRNA | Micro RNA |
| MPA | Medroxyproges-terone acetate |
| MWS | Million women study |
| NET | Norethisterone |
| P4 | Progesterone |
| PAGE | Polyacrylaraide gel electrophoresis |
| PAI1 | Plasminogen activator inhibitor 1 |
| PAIRBP1 | Plasminogen activator inhibitor RNA-binding protein 1 |
| PCR | Polymerase chain reaction |
| PGRMC | Progesterone receptor membrane component |
| PKB | Protein kinase B |
| PKC | Protein kinase C system |
| PR | Progesterone receptor |
| RANK | Receptor activator of nuclear factor κB |
| RANKL | Receptor activator for nuclear factor k B ligand |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RU486 | Mifepristone |
| SDS | Sodium dodecyl sulfate |
| SH2 | Src homology domain 2 |
| SH3 | Src homology domain 3 |
| SIGC | Spontaneously immortalized granuloser cells |
| SRE | Sterol regulatory element |
| SREBP | Sterol regulatory element binding protein |
| TEMED | Tetramethylenediamine |
| Tris | Tris (hydroxyraethyl) aminomethane |

# 1. Introduction

Progesterone receptor membrane component (PGRMC) is a member of membrane associated progesterone receptor (MAPR) family, which is widely distributed in eukaryotic organisms [1], it includes PGRMC1 and PGRMC2. PGRMC1 is a highly conserved ligand protein including a transmembrane region on N-terminal and a cytochrome b5 region.

## 1.1 PGRMC1: discovery and localization

In 1996, PGRMC1 was initially extracted from membrane fragments of mice hepatic [2], and was indicated that PGRMC1 distributed in the endoplasmic reticulum of hepatic extracts.

### 1.1.1 Discovery and nomenclature

The protein named PGRMC1 was purified in 1996 by Falkenstein and his colleagues [3]. In this year, Selmin and colleagues reported this gene sequence was aroused in 2,3,7,8-tetrachloro dibenzodioxine (TCDD) treated mice hepatocytes and named 25-Dx [4].

In 2001, Falkenstein and colleagues [5] found that this protein was decreased from 56 kDa to 28 kDa through reducing agent dithiothreitol (DTT). In 2005, Selmin and coauthors named the 25-Dx as mPR [6]. Runko et al. reported that the antigen bound to the monoclonal antibody, which recognized Ventral Midline Antigen (VEMA). This PGRMC1 protein of mice was termed VEMA in their results [7]. PGRMC1 was an immunodominant antigen and termed Inner Zone Antigen (IZA) [8].

In 2004, Runko and colleagues identified a lower eukaryotic PGRMC1 homolog, named VEM-1 in Caenorhabditis elegans nematode. By a transgenic null mutation it was demonstrated that VEM-1 was intervened in the conduction of neurons and neuritogenesis in the nematode ventral midline [9]. In unicellular eukaryotes as Saccharomices cerevisae yeast, PGRMC1 homolog, named Dap1 (damage-associated response protein), has been found to interfere with cytochrome P450 sterol synthesis and avoid DNA destroyed [10, 11]. The protein 'Putative steroid receptor' is also a PGRMC1 homolog [12, 13].

In conclusion, the construction Phylogenic Tree depended on the resemblance of PGRMC1-related members of the MAPR family shows a wide eukaryotic distribution, but doesn't distribute in prokaryotes [1].

### 1.1.2 Subcellular localization

PGRMC1 was first discovered to localize in the endoplasmic reticulum of hepatocytes [14]. In human MCF-7 cells, PGRMC1 was observed in perinuclear localization, consistent with endoplasmic reticulum [15]. Beausoleil and colleagues found PGRMC1 expression in nucleus of HeLa cells [16], the next year Sauer and co-workers observed phosphopeptides in PGRMC1 from spindles [17]. Raza and others identified 28 kDa form in microsomal and 58 kDa in cytoplasm fractions of mice adrenal gland [8]. Immunohistochemistry (IHC) studied on Pukinje cells recommended PGRMC1 was localized in endoplasmic reticulum and Golgi apparatus [18].

In SIGC cells, PGRMC1 was distributed upon the cells surface [19, 20]. PGRMC1 was located on the surface of sperm, since some antibodies may hold back progesterone initiated acrosome reaction [21, 22]. PGRMC1 expression was increased in spinal cord-injured rats after progesterone

treatment, and the protein was immunolocalized on the membrane of neuronal cells [23]. Leel and colleagues suggested that PGRMC1 could be converted from membrane bound to cytoplasm forms [24].

## 1.2 Structure and functional model of PGRMC1

The specific proteins, which PGRMC1 interacts with are closely related to their subcellular localization.

Some direct proteins that interact to PGRMC1 were observed in mammalians, while other proteins were demonstrated in lower organisms. PGRMC1 can interact with itself and compose dimer formation. Suchanek and colleagues detected that PGRMC1 was directed to constitute cross-linked homodimerics [25]. In 2005 Suchanek et al. demonstrated an interaction between co-transfected and affinity-tagged PGRMC1 and Plasminogen activator inhibitor RNA-binding protein 1 (PAIRBP1) [25]. PGRMC1 was participated in the progesterone-mediated inhibition of apoptosis in expression PAIRBP1 cells [19, 20]. The cytoplasmic progesterone receptor (PR) didn't present on granulosa/luteal cells after progestogen retreated. PR blocker RU486 could not prevent the action of progestogen anti-apoptosis. Nevertheless, PGRMC1 and PAIRBP1 antibodies could block the role of progestogen, indicating that PGRMC1 or PAIRBP1 involved in the effect of progesterone [26].

Suchanek and coworkers also demonstrated the interactions among PGRMC1 and SREBP cleavage-activating protein (SCAP), Insig-1 in COS7 cells [25]. Insig-1 and SCAP was participated in the feedback control process, which regulated cholesterol synthesis through increasing sterol regulatory element (SRE) and SRE-binding protein (SREBP) [27-30].

In mammals, PGRMC1 is involved in steroidogenesis. The repression of

does-dependence occurred the presence of the IZA (synonym of PGRMC1) in mice microsomal fraction [31]. In steroid tissues, IZAgs (PGRMC1) localization indicated that PGRMC1 was participated in the synthesis and metabolism of steroids [8].

Wright et al. observed that PGRMC1 bound to glucocorticoid site localized rat liver microsomes [32]. The result indicated that this site combined to the glucocorticoid and was contested by a variety of complexness such as progestogen and metyarapone.

### 1.2.1 Structural model

A pattern according to the homologous Arabidopsis 1J03 by Cahill, shows the composition of PGRMC1 [1]. N- and C-terminal areas of cytochrome b5 region were located in the identical side, which formed a relatively protein binding pocket. Accordingly the N-terminal of PGRMC1, CK2 and SH3 target motif were likely to overlap each other in associated PGRMC1 structure. Similarly, CK2 and SH2 target motifs were situated on the contrary sides of binding-sites for ligand. It indicates that PGRMC1 may interact with corresponding proteins via binding sites. Therefore, it was suggested that the binding of ligand might influence the assumed interacting motif of PGRMC1. (Fig. 1)

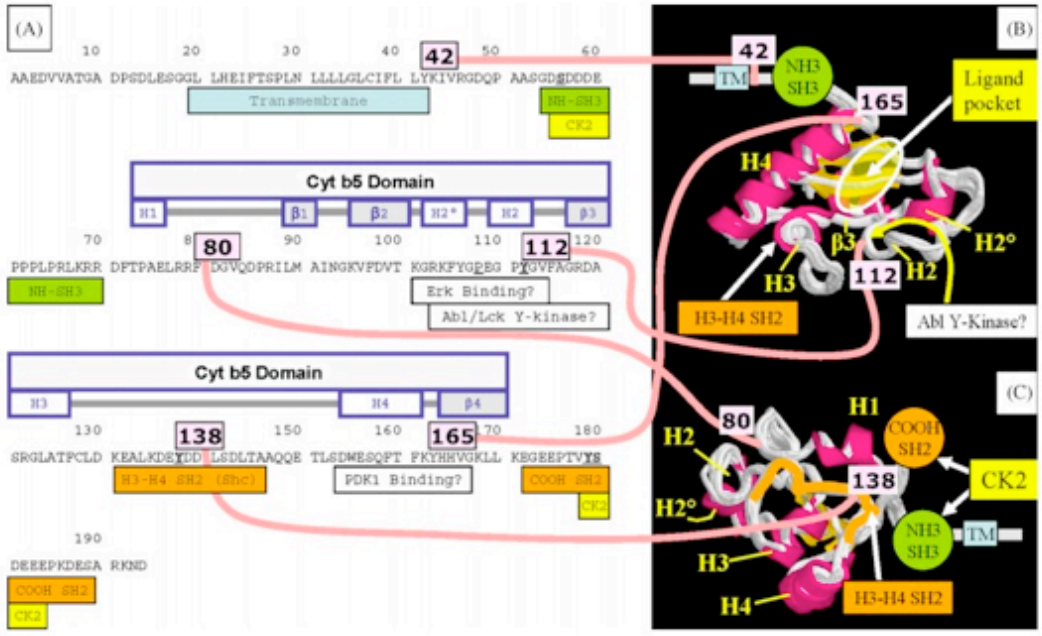


**Figure 1: PGRMC1 model structured by Arabidopsis 1J03 NMR.** (A) The amino acid sequence of PGRMC1 (SwissProt O00264). The cytochrome b5 domain is indicated above the sequence, with the positions of helices and beta sheets. The position of tyrosines of the putative ITAM/YXX motifs are shown in pink boxes above the amino acid sequence. (B and C) The structure of PGRMC1 as modelled with the Arabidopsis 1J03 coordinates (which were depicted with RasMol), showing the positions of the color-coded features from (A). (B) View from the side of the ligand binding pocket. (C) View from the side opposite to that containing the ligand-binding pocket. The positions of PGRMC1 features which are not present in the 1J03 structure are schematically portrayed by circles which are color-coded to correspond with (A). H2 was not shaded red as a helix by the RasMol software, although the helical arrangement of the peptide backbone is obvious (Adapted from Cahill, 2007).

### 1.2.2 Functional model

As previously mentioned, the motif of protein putatively connected with signaling is suggestive that PGRMC1 is participated in membrane transport and signal transduction functions. Noticeable, the motifs of SH3 and SH2 could cause protein centered on cell membrane and facilitate the activity of cells. It offered a model of PGRMC1 as a ligand signal transduction protein. In this hypothetical model of the CK2-phosphorylated state, N-terminus of SH3 domain and C-terminus of SH2 domain could be phosphorylated by CK2, but

didn't react with other proteins. Because the CK2 C-terminus was conservatived and overlapped with SH2 domain, CK2 may regulate these domains function. Moreover it was observed that the dephosphorylated CK2 domain may relocate PGRMC1. If the SH2 domain is participated, tyrosine phosphorylation could occur simultaneously. It is supposed that the interaction of the presumed domains are adjusted through tyrosine phosphorylated Y112 and ligand pocket [1]. (Fig. 2)

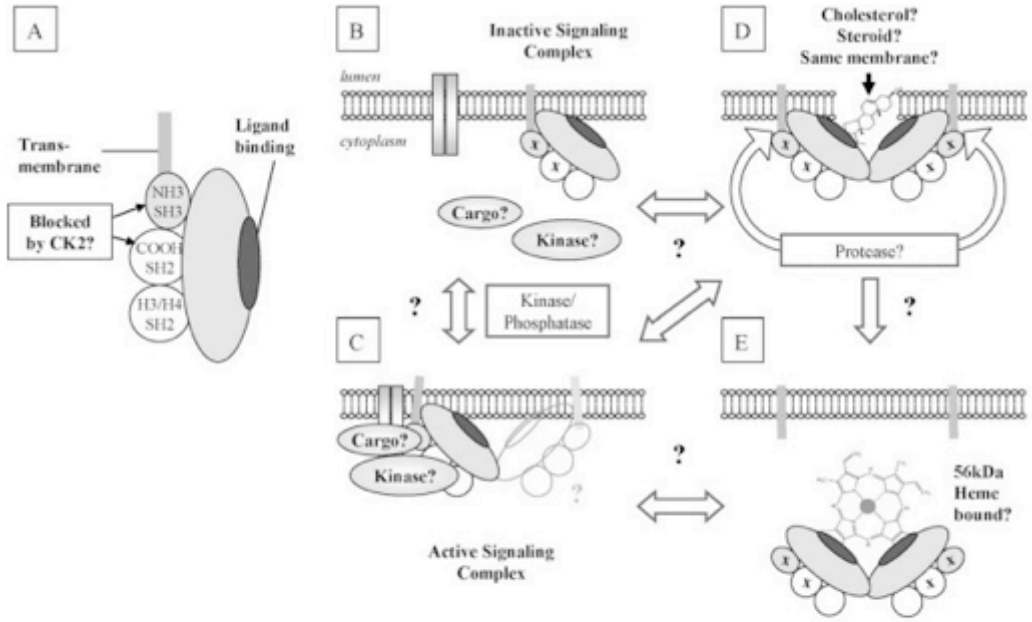


**Figure 2: PGRMC1 function model.** (A) Schematic model of PGRMC1, based upon the model structure of Fig. 1. (B) According to the hypothetical model, when PGRMC1 is phosphorylated by CK2 at S56 and S180 the putative SH3 and SH2 target sequences are unavailable to interact with SH3- and SH2-domain-containing proteins. (C) In the absence of S56 and/or S180 phosphorylation of these interaction motifs PGRMC1 possibly forms a functional protein complex. (D) Cholesterol and/or steroid binding may require dimerisation of PGRMC1 from a '28 kDa' monomer to a '56 kDa' dimer. It is conceivable that the subunits bind to membranes on different subcellular organelles. (E) Protease action, such as by the S1P or S2P proteases which cleave the cholesterol sensitive SREBP, may release a '56 kDa' dimer to the cytoplasm, where it can bind heme. These speculative scenarios are intended to be mechanistically illustrative, and neither mutually exclusive nor functionally comprehensive. Translocation(s) between sub-cellular locations may be involved in change between putative functional scenarios (arrows) (Adapted from Cahill, 2007).

## 1.3 PGRMC1 and disease

PGRMC1 was initially cloned as the dioxin induced gene, suggesting that it is involved in stress-alleviation or stress response. Progesterone has neuroprotective effects. Labombarda et al. have observed that PGRMC1 expression was increased when injured mice was cured with progestogen [23], and PGRMC1 expression was is detected in encephalon, which was increased after trauma [33]. Notwithstanding steroids did not combine to PGRMC1 directly, it bound to other protein compounds and affected steroid hormone synthesis through the metabolism of cholesterol. PGRMC1 was detected in the has been detected in the T-lymphocytes [34]. PGRMC1 may be involved in the disease of the immune system.

So far more research is focused on the relationship between PGRMC1 and cancer. PGRMC1 expression is higher in several cancers or tumor cells such as breast, thyroid, cervix, colon, and lung [35] than normal tissues. In 2005, Craven et al. suggested that PGRMC1 pertained to gene that regulate sensitivity of tumor cell to antineoplastic drugs [36]. Furthermore, it is suggested that PGRMC1 was involved in the regulation of chemo-sensitivity [37]. However PGRMC1 plays an effect of the connector proteins regardless of its combining targets. PGRMC1 is involved in the UNC-40/DCC receptor, which is in signal transduction. UNC-6/Netrin is related with the dependent receptor UNC-40/DCC In C. elegans. These cause apoptosis or survival response. The signal path of UNC-40/DCC, UNC-5 and Netrin are crucial to carcinogenesis in mammals. Netrin leads to apoptotic. Therefore UNC-5 and UNC-40/DCC are thought to be a cancer suppressor, which absence could cause tumor [38-41].

This could be involved in neoplastic status. Signal transduction demands the

accumulation of proteins in enriched cholesterol and insoluble detergent which named lipid rafts [42]. Nevertheless, every presumed influences of PGRMC1 to neoplasm are not certainly related with the raft and cholesterol. Therefore the pathway of mevalonate that prevents HMG-CoAR function was discovered for anti-tumor therapy [43-45].

### 1.3.1 PGRMC1 expression in breast cancer

Breast cancer is the most prevalent tumor worldwide. PGRMC1 is more highly expressed in breast tumor tissue than non-malignant organizations. Nevertheless, PGRMC1 expression is usually detected within stromal tissue [23]. Crudden and coauthors suggested that PGRMC1 expression is higher in is breast cancer than relevant non-tumor tissues [35]. But Ji and coworkers showed that the expression of PGRMC1 ranged from minimal to strong in breast tumor tissues according to IHC and didn't exist in normal mammary gland [46]. Multivariate analysis showed that PGRMC1 was a prognostic factor for breast tumor. PGRMC1 expression was strongly correlated with the progression of breast tumor, and may serve as a useful prognostic indicator of malignancy [46]. However, Causey and coworkers investigated 28 breast tumor specimen and 10 corresponding non-tumor tissues by RelqPCR and found that PGRMC1mRNA decreased observably with patient age [47]. The different results may be due to different detection methods, as mRNA levels are not always fully translated into protein levels. Another possible reason may be that different ethnic groups have been investigated, one from China [46], while the other was from the USA [47]. Therefore, it is necessary that more studies among different countries take the same methods. Neubauer and colleagues detected that the expression of PGRMC1 is elevated in ER (estrogen receptor)-negative breast cancer [48]. Neubauer and colleagues found that PGRMC1 expression colocqalizes with hypoxia in DCIS. Therefore, PGRMC1 levels may affect cell survival in response to stress conditions and

have a predictive value for prognosis [48, 49].

### 1.3.2 Proliferative effect of progestins on breast cancer via PGRMC1

We have been performing in vitro experiments with MCF-7 cells over-expressing PGRMC1. The results indicate that PGRMC1 mediates progestin dependent proliferative effect in these cells. The natural progesterone and progestogens applied to hormone therapy are distinct in proliferative effects on MCF-7 and MCF-7 WT12 (MCF7/PGRMC1) cells: progesterone, chlormadinone acetate (CMA) and nomegestrol act neutrally, whereas dienogest, drospirenone, dydrogesterone, MPA and norethisterone increase cells proliferation and may increase breast cancer risk. In WT-12 cells, the same progestins, detected a significant increase compared with MCF-7. The highest proliferation was observed in NET. Certain propestogens elevated the proliferation of PGRMC1 over-expressing cells [50]. The influence of progestins on proliferation might rely on their particular pharmacology. Then we investigated the influence of different concentrations of E2 as well as the present of the progesterone or progestins on MCF-7 cell lines over-expressing PGRMC1: MPA [51], P [51, 52], NET [52] and NOM [52]. E2 caused a significant does-dependent proliferation in WT-12 cells. Progesterone combined with E2 has no influence at any of E2 concentration. But, MPA indicated a pronounced proliferative effect at a low E2 concentration. NET significantly stimulated cell proliferation more pronounced in the continuous combined with E2 model than the sequential. No effect was seen for NOM, and P [52]. It may be concluded that NOM and P bound with E2 are neutral in the risk of mammary tumorigenesis at least in women over-expressing PGRMC1. In the mouse model, MCF-7/PGRMC1-inoculated cells were more sensitive towards E2 and shown a stronger proliferative response in the presence of norethisterone as compared to MCF-7 comprising carrier control. It suggested that over-expressing PGRMC1 cells could be easily formed into cancer in

women to receive NET/E2 treatment [53]. In the blocking experiment, only the presence of estrogen receptor antagonist fulvestrant was able to totally block the EP effect combination, i.e. E2 plus NET. Partial significant inhibitory effects could be found in the presence of AG205 (PGRMC1 antagonist), no effect was observed for PR A/B antagonist (RU486) [52]. Thus might make a significant effect in signal transduction of PGRMC1 activated via estrogen or progestins.

### 1.3.3 Probable mechanism of PGRMC1 proliferation

The mechanisms through which PGMRC1 mediates proliferative effects stimulated by progestins and may participate in tumorigenesis are still uncertain. Some studies suggested that PGRMC1 participates in signal transduction of protein kinases, which increased Akt activation through PGRMC1 and IkB phosphorylation and leads to NFkB activation [15]. PDK1 phosphorylates and PGRMC1 contains binding sites for PDK1 [1]. However, the mechanism is still unclear. PGRMC1 contains several potential docking sites for binding proteins such as PDK1, which could increase Akt activation. A membrane-impermeable progesterone conjugate results in an increased cellular $Ca^{2+}$ and the following phosphatidylinositol-3-kinase results in ERK1/2 and PKC phosphorylation [54, 55]. However, PGRMC1 could induce ERK2 phosphorylation. So ERK2 participates in the inhibitive feedback of signaling via the adjacent SH2 protein motif. To elucidate the probable mechanisms, the expression level of 15 downstream proteins of the signal transduction pathways were detected with reverse-phase protein array technology, and confirmed by western blot. The expression level of ERK1/2 in MCF-7 WT12 was lowered by about 40% than that of MCF-7. However, the expression of ERK1/2 did not alter by stimulation of cells with progestins [54]. In rat neural progenitor cells, Liu and coworkers suggested that progesterone induces a PGRMC1-dependent cell proliferation via the ERK signaling pathway [56]. PGRMC1 can bind to P450 proteins [11], Insig-1 [25]. In summary, different

amounts of phosphorylated PGRMC1 may influence the tumor physiology [1]. According to the result of receptor blocking experiment, ER-α might make a significant effect the signal transduction of PGRMC1 activated via progestins. It is still unknown how PGRMC1 acts. Therefore, more and further studies are needed.

## 1.4 Aims of the studies

PGRMC1 is highly expressed in several tumors, such as thyroid, ovary, colon, lung, and breast cancer. From the studies published so far, it became evident that PGRMC1 is required for tumor cell anti-apoptotic, proliferation, metastasis, and invasion. However, it remains elusive how PGRMC1 regulates the proliferation elicited by progestins and whether PGRMC1 is secreted by breast cancer cells. In spite of PGRMC1 plays a pronounced effect in tumorigenesis, the detailed mechanisms regulating its expression remain unclear so far.

The purpose of this work was to study PGRMC1 expression and the influence of estradiol and different progestins on various breast cancer cells. Moreover, the effect of PGRMC1 in E2-induced PS2 expression was analyzed. It is not sure, if the various cell lines constitutively express Receptor Activator for Nuclear Factor κB Ligand (RANKL) whereas Receptor Activator of Nuclear Factor κB (RANK) and Osteoprotegerin (OPG) are detected in various breast cancer cell lines. Therefore, we planned to screen various cell lines for RANK and RANKL expression such as MCF-7, T47D, HCC70, HCC202, AR, BM and SUM225CWN.

In addition, we further studied the regulatory mechanism that effects the expression of PGRMC1 in breast tumor cells, whether let-7i influences the mRNA level of PGRMC1. Furthermore, the presence of PGRMC1 protein (fragments) in cell culture supernatant was detected to develop a method to measure PGRMC1 expression from body fluids.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals and biological reagents

Actin antibody (I-19)-R (Santa cruz, US)

Agarose LE (Biozym, US)

Albumin Standard (Thermo, US)

Blue separopore CL-6B (Bio-world, Dublin)

BCA protein assay reagent kit (Thermo, US)

Chloroform (AppliChem, Germany)

Coomassie brilliant blue R250 (Sigma, US)

D-(+)-Glucose solution (Sigma, US)

DMEM medium (Gibco, US)

DMSO (AppliChem, Germany)

DMSO (Sigma, US)

Donkey anti-goat IgG-HRP (Santa cruz, US)

DPBS (Gibco, US)

ECL western blotting analysis system (Amersham, UK)

ER-α antibody (H-184) (Santa cruz, US)

Estradiol (Sigma, US)

Ethanol (Merck, Germany)

Fetal bovine serum (Gibco, US)

Formaldehyde (Roth, Germany)

GAPDH primer (Qiagen, Germany)

Goat anti-mouse IgG-HRP (Santa cruz, US)

Goat anti-rabbit IgG-HRP (Santa cruz, US)

G418 sulfate (Merck, Germany)

HEPES buffer solution (Gibco, US)

HA-probe antibody (Y-11) (Santa cruz, US)

Hyclone FBS (Thermo, US)

Hygromycin B (Invitrogen, Carlsbad, US)

Let-7i mimic (Qiagen, Germany)

Let-7i primer (Qiagen, Germany)

LightCycler 480 SYBR Green I Master kit (Roche, Germany)

Massruler low range DNA ladder (Thermo, US)

Methanol (Roth, Germany)

miRNeasy Mini Kit (cat. no. 217004) (Qiagen, Germany)

miScript II RT Kit (Qiagen, Germany)

miScript SYBR Green PCR Kit (Qiagen, Germany)

M-MLV Reverse Transcriptase (Promega, US)

Nonfat milk (Sucofin, Germany)

Opti-MEM® I medium (Gibco, US)

Page ruler prestained protein ladder (Fermentas, Germany)

PBS tablet (Gibco, US)

Penicillin/streptomycin (Sigma, US)

PDH primer (Invitrogen, Germany)

PGRMC1 antibody (#12444) (Cell signaling, US)

PGRMC1 antibody (G-21) (Santa cruz, US)

PGRMC1 primer (Qiagen, Germany)

Phosphatase inhibitor cocktail (Sigma, US)

Ponceau S solution 0.1% (Sigma, US)

Protease inhibitor cocktail kit (Thermo, US)

PVDF blot membrane (Amersham Lifescience, Sweden)

QIAzol Lysis reagent (Qiagen, Germany)

Random Primers, Oligo (dT) 15 Primer (Promega, USA)

RANK antibody (Amgen, Germany)

RANK antibody (Santa cruz, US)

RANKL antibody (Amgen, Germany)

Recombinant RNasin Ribonuclease Inhibitor (Promega, USA)

Rotiphorese® gel 30 (Roth, Germany)

RNeasy Mini Kit (cat. nos. 74104 and 74106) (Qiagen, Germany)

RPMI medium 1640 (Gibco, US)

SDS loading buffer (Roth, Germany)

Silencer negative control (Ambion, Germany)

siPORT™ NeoFX™ Transfection Agent (Invitrogen, Germany)

SNORA73A (Qiagen, Germany)

Sodium pyruvate (Gibco, US)

TEMED (Roth, Germany)

TFF1 primer (Qiagen, Germany)

Tris-HCl (Roth, Germany)

Triton X-100 (Roth, Germany)

Trypan blue stain (0.4%) (Gibco, US)

Trypsin-EDTA (Sigma, US)

TWEEN-20 (Roth, Karlsruhe, Germany)

### 2.1.2 Equipment

-20°C refrigerator (Bosch, Germany)

-80°C refrigerator (Heraeus, Germany)

Balance CP323S-OCE (Sartorius, Germany)

Biological safety cabinet (Heraeus, Germany)

Cell culture flask (Greiner Bio-One, Germany)

Cell freezing tube (Greiner Bio-One, Germany)

Cell incubator (Binder, Germany)

Centrifuge biofuge pico (Heraeus, Germany)

Centrifuge 5417 R (Eppendorf, Germany)

Electrophoresis cell (Biorad, US)

Electronic pipettes (Pipetus, Germany)

Electrophoresis power supply (Consort, Belgium)

Electrophoretic transfer cell (Biorad, US)

ELISA (Tecan sunrise, Germany)

Filter paper (Whatman, UK)

Fluovert FU microscope (Leica, Germany)

Folie bag sealer (Krups, Germany)

Gel electrophoreses and blotting equipment (BioRad, US)

Ice maker (Scotsman, US)

Incubator shaker (Heidolph Titramax, Germany)

LightCycler®480 Instrument (Roche, Germany)

Lumi-Imager (Roche, Germany)

Magnetic stirrer (Uniequip, Germany)

Microliter™Syringes (Hamilton, US)

MS1 Minishaker (IKA, Germany)

PH meter (Mettler Toledo MP225, Germany)

Photometer (Eppendorf, Germany)

Pipettes (Abimed, Germany)

PVDF blot membrane (Amersham Lifescience, Sweden)

Shaker (Roth, Germany)

Thermal Cycler (MJ Research PTC-220) (Roche, US)

Thermomixer compact (Eppendorf, Germany)

Transferpette (Eppendorf, Germany)

Transilluminator (Herolab, Germany)

Vacuum concentrator 5310 (Eppendorf, Germany)

Vortex (Heidolph, Germany)

Vortex-Genie (SI, US)

Waterbath (Vivo, Germany)

## 2.2 Methods

### 2.2.1 Methods for proliferation of breast cancer cell lines

2.2.1.1 Cell culture

Human breast cancer cells T47D, HCC70, HCC202, MCF-7, HBL100 and SUM225CWN were availabled from American Type Culture Collection (ATCC). BM and AR were isolated from Brigitte Gückel. All cells were planted in a humid 5% $CO_2$ atmosphere at 37°C. HCC70 and HCC202 breast cancer cell lines were grown at RPMI-1640 media having 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). BM and AR cell lines were planted in DMEM culture media containing 1% penicillin/streptomycin and 10% FBS. HBL100 was cultured in McCoy's 5A media having 1% penicillin/streptomycin and 10% FBS. SUM225CWN was cultured in Ham's F-12 media having containing 10% FBS, 0.1% Hydrocortisone, 0.05% Insulin and 1% penicillin/streptomycin. MCF-7 and T47D cells were routinely grown at RPMI-1640 media supplemented with 1% penicillin/streptomycin, 25 mmol/l HEPES and 10% FBS.

Cells were seeded into a 100-mm culture plate using culture media. Then, cells were passaged using 0.25% trypsin/EDTA reagent (sigima). Trypsinization was blocked through mixing normal growth medium. They were moved to a 15-ml pipe and centrifuged for 5 minutes at 1,000 rpm. Afterwards, cells were treated with 5 ml of growth media and planted.

2.2.1.2 Dissolution of estradiol, progesterone, and synthetic progestins

Estradiol (E2), progesterone (P4) and the synthetic progestins medroxyprogesterone acetate (MPA), norethisterone (NET), chlormadinone

acetate (CMA), levonorgestrel (LNG), and desogestrel (DSG), were availabled from Sigma (Munich, Germany). drospirenone (DRSP) was a gift from Schering (Berlin, Germany). dydrogesterone (DYD) and nomegestrol (NOM) were purchased from LGM Pharma (Boca Raton, USA). All drugs were diluted with alcohol and stored at -20°C as $10^{-2}$ mol/l solutions. During the experiment, the final concentration of ethanol was less than 0.01%. This ethanol concentration was used in controls.

2.2.1.3 Cell proliferation

Proliferation was measured by stripping medium, i.e. phenol red-free medium supplemented with charcoal/ dextran FBS (Thermo, USA). About 5,000 cells were planted in 96-well dishes with growth media. The second day, the media was replaced with stripping media, and cells were incubated for two days. Afterward, the progestins were mixed into the growth medium. After five days, proliferative effect was determined using MTT method. In the same conditions, cells were incubated with blocker for five days.

2.2.1.4 In vitro 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay

Cell proliferation was analyzed through determining the yellow tetrazolium salts MTT. MTT was diluted 1:4 in culture phenol red-free media.

Then, the media was poured from the culture dish. 100 µl MTT was poured to per well and cultured three hours. Incubator was centrifuged ten minutes, and then the supernatant was removed. The crystals were dissolved through adding 100 µl dimethyl sulfoxide (DMSO) to each well. It was analyzed by ELISA at 550 nm. The result was proportional with the number of cells.

In the previous study, MTT was better than BrdU-assay. In this experiment, 0.925 correlation coefficient was detected. In own in-house experiments, we have verified MTT against cytometric analysis, and 0.96 correlation coefficient was found. The proliferation assay was executed triplicates and repeated three times, with each assay producing the same outcomes.

2.2.1.5 Western blot analysis

Western blot analysis is a common method to detect proteins using specific antibodies. Cells were washed two times using phosphate buffered saline (PBS), then lysed in Protein Extraction Reagent (PER) in the light of the recommendations (Pierce, Rockford, IL, USA). The concentration of protein was detected through bicinchoninic acid (BCA). In total, 25 µg protein extracts were analyzed each lane on a polyacrylamide gelatin and separated by electrophoresis. The SDS-PAGE gel had 12% acrylamide. This gelatin was blotted onto a nitrocellulose membrane (Amersham, USA) through a blot system at 4°C over night. Afterwards these membranes were blocked for two hours using 5% dried low fat milk powder dissolved in TBST buffer at ordinary temperature. Then, the membrane was interacted with the first antibody at 4°C over night. After washing three times with TBST, the second antibody incubated the membrane for two hours at ordinary temperature. Chemiluminescence was produced through the ECL WB Analysis System (Amersham, Germany). The signal was detected and quantified by Lumi-Imager and Lumi-Analyst 3.1 software (Boehringer, Mannheim, Germany). Western blot analysis was used to measure PGRMC1 and ER-α expression. The first antibody of analyzing cell lysates were rabbit anti-PGRMC1 antibody (#12444, 1:500, Cell signaling) and rabbit anti-ER-α antibody (H-184, 1:500, Santa Cruz). The secondary antibody was Goat

anti-rabbit (IgG-HRP, 1:500, Santa Cruz). Blot was re-probed with actin (I-19, 1:1000, Santa Cruz) to provide protein loading controls.

2.2.1.6 Statistical analysis

The proliferation assay was performed triplicates, with each assay producing the same outcomes. Since the data wasn't normally distributed, these results were analyzed by ANOVA with the logarithmated value. The overall alpha level is set to 0.05.

### 2.2.2 Methods for PS2

2.2.2.1 Cell culture

MCF-7, ER-positive primary breast tumor cells, were availabled from ATCC. This cell line was grown in RPMI-1640 media having 1% penicillin/streptomycin, 25 mmol/l HEPES and 10% FBS in a humid 5% $CO_2$ atmosphere at 37°C. MCF-7 cell lines were seeded into a 100-mm culture plate useing RPMI-1640 growth medium and normally subcultured once a week.

2.2.2.2 Transfection of MCF-7 cell lines (EVC and WT-12)

MCF-7 cell lines were steady transfected through plasmid pcDNA3.1/Hygro having heme agglutinin-tagged PGRMC1 using lipofectamineTM 2000 (Invitrogen, Karlsruhe, Germany), according to the protocol. $5x10^5$ cells were transfected and incubated with RPMI-1640 media for one day. Afterwards, the media was replaced with media supplemented with 100 µg/ml hygromycin B. Transfection rates were measured by cotransfection of a GFP-expressing plasmid and immune fluorescence analysis. Cells were incubated for two

weeks, then limiting dilutions were executed three times to choose for stable cloned cells.

Successful transfection was validated through PCR via chromosomal DNA. The sequences of the primers were 5'-CTGCTGCATGAGATTTTCACG-3' hybridizing to nucleotides 71-91 of PGRMC1 open reading frame and 5'-GCATAGTCCGGGACGTCATA-3' hybridizing to the sequence coding for the HA tag. PCR products were sequenced. For experiments we used MCF-7/PGRMC1 clone WT-12. For control experiments MCF-7 transfected with 3HA were used (EVC-cells).

2.2.2.3 Total RNA isolation

RNA was obtained from cell pellets by the RNeasy Mini Kit (Qiagen) in accordance with the recommendations. The cell pellet was placed in -80°C for later experiments. Frozen pellet was lysed by adding appropriate volume of Buffer RLT Reagent. Then, the sample was pipeted into an RNeasy Mini column and centrifuged at room temperature. At the end, the RNeasy Mini spin column was added 50 µl RNase-free water to elute the RNA. Final RNA concentration and quality was measured with a spectrophotometer (Nano2Drop, Saveen Werner). The separated RNA samples were confirmed using 1.5% agarose gel electrophoresis.

2.2.2.4 cDNA synthesis from total RNA

Total RNA were used as the starting material in the reverse transcription reaction. We used miRNeasy Kits for purification of total RNA including miRNA. After RNA isolation, cDNA was synthesized using reverse transcriptase PCR with the RT cDNA Synthesis Kit (Promega). 1µg of total RNA was heated

together with Oligo (dT) 15 Primers and Random Primers for 10 minutes at 70ºC, and reduced temperature on the ice. Afterwards, this sample mixed with reverse-transcription components in the light of Table 1. The reverse-transcriptase mixture has all ingredients of cDNA synthesis.

The reaction was interacted for 60 minutes at 37ºC, and warmed at 95ºC for five minutes to inactivate components, then setted on the ice. Finally, 27.5 µl water of RNase-free was added to the synthesized cDNA, and 50 µl cDNA was placed in -20ºC freezer. The integrity of cDNA was checked by actin.

**Table 1. PS2 Reverse-transcription reaction components:**

| Component | Volume/reaction |
|---|---|
| 5x Buffer | 5 µl |
| M-MLV Reverse Transcriptase | 1 µl |
| dNTP | 5 µl |
| Recombinant RNasin Ribonuclease Inhibitor | 0.5 µl |
| Template RNA | 11 µl |
| Total volume | 22.5 µl |

2.2.2.5 Quantitative real-time PCR (qPCR)

For qPCR experiments, RNA was obtained from cell pellets by the RNeasy Mini Kit (Qiagen). cDNA for qPCR was synthesized using RT cDNA Synthesis Kit (Promega) as previously mentioned.

qPCR evaluated PS2 mRNA was executed on synthesized cDNA by the TFF1 primers (for sequences see Table 4). To analyze PS2, 2 µl cDNA was added into qPCR system using the LightCycler 480 SYBR Green I Master kit (Roche).

The qPCR reaction (see Table 2) was executed on LightCycler®480 Instrument (Roche, Germany). The following cycle parameters (see Table 3) were adopted. PDH RTPCR was used for normalization. Because PDH level of the sample was no difference under this condition, PDH was a good reference gene. For all primers, cDNA serial dilution was used as a standard curve. Water without cDNA was adopted for the negative control. The particularity of primer was validated using 2% agarose gel electrophoresis. The specificity of amplification was confirmed using melting curve of PCR product. The LightCycler® 480 1.5 Software was used to analyze the result and relative quantification by Ct value.

**Table 2. Reaction mix for detection of PS2 mRNA:**

| Component | Volume/reaction(384-well) |
|---|---|
| 2x LightCycler480 SYBR Green I Master | 5 µl |
| 10x Primer Assay | 1 µl |
| RNase-free water | 2 µl |
| Template cDNA | 2 µl |
| Total volume | 10 µl |

**Table 3. Cycling conditions of PS2 mRNA:**

| Step | Time | Temperature | Additional comments |
|---|---|---|---|
| **Initial activation step** | 5 min | 95° C | |
| **cycling:** | | | |
| Denaturation | 10 s | 95° C | |
| Annealing and extension | 30 s | 60° C | |
| Cycle number | 40 cycles | | Cycle number depends on the amount of template cDNA and abundance of the target. |

**Table 4. Primer sequences for PS2 qPCR:**

| Primer | Forward (Sequence 5'-3') | Reverse (Sequence 5'-3') |
|---|---|---|
| TFF1 | ATGGCCACCATGGAGAACAAGG | CTAAAATTCACACTCCTCTTCTGG |
| PDH | CAGCCTCAAGATCATCATCAGCAATGC | AGACCACCTGGTGCTCAGTGTAG |

2.2.2.6 Statistical analysis

All data were describe as means ± SD and data shown is the result of at least three replicates of three or more independent runs for each assay. Normal distributed data was further analyzed by ANOVA-test to compare variance of averages of several groups. For qPCR studies, Ct values from duplicates of each sample were normalized to those of PDH and analyzed using the ΔΔCt method. Value was regarded as statistical significance at $p<0.05$ for all statistical outcomes.

### 2.2.3 Methods for RANK/RANKL

2.2.3.1 Cell culture

Human breast tumor cells HCC70, HCC202, T47D, HBL100, MCF-7, BM, AR and SUM225CWN were maintained as mentioned above. L-cells RANKL, over-expressing RANKL was grown in DMEM media having 1% penicillin/streptomycin, 10% FBS, 1% NaCl, and 600 µg/ml of G418 sulfate.

2.2.3.2 Co-cultures of breast cancer cells with osteoblast-like cells

MDA-MB-231 cell lines were maintained using RPMI-1640 media containing 10% FBS and 1% penicillin/streptomycin. CAL72 osteoblast-like cell lines were

maintained in DMEM medium having 1% penicillin/streptomycin, 1% insulin-transferrin-sodium selenite, 2% HEPES and 10% FBS. SAOS2 cell lines were planted in McCoy's 5A media having 10% FBS and 1% penicillin/streptomycin. Cal72 and Saos2 cell lines ($2\times10^5$/60 mm plate) were co-cultured with tumor cell lines ($2\times10^5$/60 mm dish). MCF-7 WT12 and MDA-MB-231 cells were studied in co-culture systems. First, osteoblast-like cells were cultured. Then, before sowing osteoblast-like cells, $2\times10^5$ tumor cell lines were planted into 60 mm dish at two hours and co-cultured for the indicated periods (1 week or 2 weeks). The culture medium was changed every three days. All cell lines were grown at 37°C.

2.2.3.3 Western blot analysis

Western blot analysis was carried out as mentioned above. Cells were washed two times using PBS and lysed in PER kit. The concentration of protein was measured using the bicinchoninic acid. Each lysate (25 µg of protein) was analyzed each lane by SDS-PAGE. When we studied RANK expression, protein was separated through a 10% acrylamide and blotted onto nitrocellulose membrane. However, we use a 12% separating gel to detect RANKL. Immunoblotting was executed with the first antibody against RANK (1:1000; 1:500; 1:200), RANKL (1:1000; 1:500; 1:200) and Actin (1:1000), respectively. The gel was blotted onto a Hybond ECL nitrocellulose membrane at 4°C over night using a blot system. Then, membranes were blocked for 2 hours in TBST containing 5% dried low fat milk and immersed using the first antibody at 4°C over night. The membrane was immersed using the secondary antibody for two hours after washing thrice by TBST. Chemiluminescence was produced through the ECL WB Analysis System (Amersham, Germany). The signal was detected and quantified by Lumi-Imager and Lumi-Analyst 3.1 software (Boehringer, Mannheim, Germany).

2.2.3.4 Cell proliferation

Cell proliferations were analyzed through determining the yellow tetrazolium salt MTT. MTT was diluted 1:4 in DMEM phenol red-free media. It was analyzed by ELISA at 550 nm. The result was proportional with the number of cells.

2.2.3.5 Statistical analysis

The proliferation assay was performed triplicates, with each assay producing the same result. Since the data was not normally distributed, these results were analyzed by ANOVA with the logarithmated value. The overall alpha is set to 0.05.

### 2.2.4 Methods for let-7i

2.2.4.1 Cell transfection with miRNA

MCF-7 cell lines were transfected using siPORT™Neo FX™Transfection reagent, in accordance with the reverse transfection recommendations. Let-7i mimic was obtained from Qiagen (Germany) and used at 50 nM concentration. The negative control RNA (Silencer negative universal control) was obtained from Qiagen (Germany) too.

Before the transfection, cells were treated using trypsin and diluted with normal growth media. Then, 5 µl Transfection reagent siPORT™ NeoFX™ was added into Opti-MEM® I agent to 100 µl, and 5 nm miRNA or control was diluted in Opti-MEM® I media. The diluted miRNA was added to the diluted siPORT™

NeoFX™Transfection agent. The mix was incubated 10 minutes at room temperature and dispersed in a 6-well dish, and then $2x10^5$ cells were added into one well and mixed. Transfected cells were cultured for 48 hours at 37°C in normal cell culture conditions (Fig. 3).

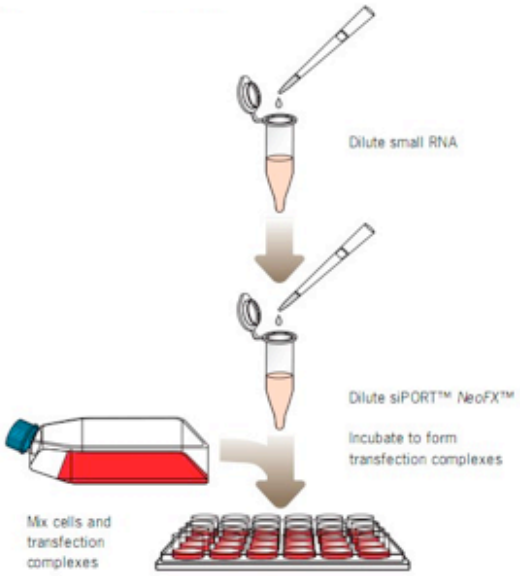


**Figure 3: Reverse Transfection Overview.** Reverse transfection involves simultaneously transfecting and plating cells, much like procedures used for transfecting suspension cells. siPORT™ NeoFX™ Transfection Agent and RNA are mixed, incubated, distributed to culture wells, and overlayed with cells (Adapted from siPORT™NeoFX™Transfection Agent User Guide).

2.2.4.2 Total RNA containing miRNA isolation

RNA containing miRNA were isolated from cell pellets by the miRNeasy Mini Kit (Qiagen) in the light of the manufacturer's recommendations. The cell pellet was placed in -80°C for later experiments. Frozen pellet was lysed by adding 700 µl QIAzol Lysis Reagent and cultured at 15-25°C for five minutes. 140 µl of chloroform was mixed into the lysate. The mixture was shaken vigorously for fifteen and separated at 12,000 x g at 4°C for fifteen minutes by centrifugation. The upper layer having RNA was moved to another collection pipe. Avoid transferring any interphase. Then, the sample was pipeted into an RNeasy®

Mini column and centrifuged at room temperature. At the end, the RNeasy Mini spin column was added 50 µl RNase-free water to elute the RNA. Final RNA concentration and quality was measured with a spectrophotometer (Nano2Drop, Saveen Werner). The separated RNA samples were confirmed using 1.5% agarose gel electrophoresis.

2.2.4.3 cDNA synthesis from total RNA containing miRNA

Total RNA containing miRNA were used as the starting material in the reverse transcription reaction. It is not necessary to enrich for small RNA. We used miRNeasy Kits for purification of total RNA including miRNA. After RNA isolation, cDNA was synthesized using reverse transcriptase PCR with the miScript II RT cDNA Synthesis Kit (Qiagen). 1µg of total RNA was used in each reaction and mixed with reverse-transcriptase mixture on ice in accordance with Table 5. The reverse-transcription mixture has all ingredients of cDNA synthesis.

The reaction was interacted for 60 minutes at 37ºC, and warmed at 95ºC for five minutes to inactivate components, then setted on ice. Then, 80 µl water of RNase free was added to the synthesized cDNA and 100 µl cDNA was placed in -20ºC freezer. The integrity of cDNA was checked by actin.

**Table 5. Let-7i Reverse-transcription reaction components:**

| Component | Volume/reaction |
|---|---|
| 5x miScript HiFlex Buffer | 4 µl |
| 10x Nucleics Mix | 2 µl |
| miScript Reverse Transcriptase Mix | 2 µl |
| RNase-free water | Variable |
| Template RNA | Variable |
| Total volume | 20 µl |

2.2.4.4 Polymerase chain reaction (PCR)

2.2.4.4.A Semi-quantitative PCR

DNA amplification was performed using PCR. PCR reaction (25 µl) was executed using Taq DNA polymerase (Promega) in the light of the protocol. The components listed below were mixed:

| | |
|---|---|
| Template | 1 µl (50-100 ng) |
| dNTP | 2.5 µl |
| 2.5mM $MgCl_2$ | 0.75 µl |
| 10×Buffer | 2.5 µl |
| forward primer | 1 µl |
| reverse primer | 1 µl |
| Taq | 0.2 µl(2.5U ) |
| H2O | 14.8µl |
| DMSO | 1.25 µl |
| Total volume | 25 µl |

The following cycle parameters were adopted: initial activation 94ºC for 3 minutes; denaturation 94ºC for 30 seconds; annealing 56.3ºC for 30 seconds and extension 72ºC for 18 seconds (repeated 32 times).

PCR reactions were performed on Thermal Cycler (MJ Research PTC-220) and the products were analyzed using 2% agarose gel electrophoresis.

2.2.4.4.B Quantitative real-time PCR (qPCR)

For qPCR experiments, RNA containing miRNA were isolated from cells transfected 5 nM miRNA or control (Silencer negative control; Ambion) after 48 hours by the miRNeasy Mini Kit (Qiagen). cDNA for qPCR was synthesized by the miScript II reverse transcription kit (Qiagen) as described above.

To analyze the let-7i, 1 µl cDNA was added in qPCR system using the miScript SYBR Green PCR kit (Qiagen). The qPCR reaction (see Table 9) was executed using the LightCycler®480 Instrument (Roche, Germany). The following cycle parameters (see Table 7) were used. Let-7i was quantified using let-7i primer (Qiagen) and normalized using SNORA73A primer (Qiagen). Because no differences in SNORA73A levels were detected under this condition, SNORA73A was a good reference gene (All tests were performed twice. For analysis the results, the LightCycler® 480 1.5 Software was used).

qPCR evaluated PGRMC1 mRNA (see Table 8) was executed on synthesized cDNA by the PGRMC1 primers (for sequences see Table 10) and LightCycler 480 SYBR Green I Master kit (Roche). The following cycle parameters (see Table 5) were used. Threshold cycle readings were used to calculate the relative amounts of PGRMC1 by normalizing them to the signal of GAPDH after validating that GAPDH mRNA levels did not differ among treatments.

For all primers, cDNA serial dilution was used as a standard curve. Water

without cDNA was adopted for the negative control. The particularity of primer was verified through 2% agarose gel electrophoresis to confirm single amplification. LightCycler® 480 1.5 Software was used to analyze the result and relative quantification by Ct value.

**Table 6. Reaction mix for detection of mature miRNA:**

| Component | Volume/reaction(384-well) |
|---|---|
| 2x QuantiTect SYBR Green PCR Master Mix | 5 µl |
| 10x miScript Universal Primer | 1 µl |
| 10x miScript Primer Assay | 1 µl |
| RNase-free water | Variable |
| Template cDNA | ⩽1 µl |
| Total volume | 10 µl |

**Table 7. Cycling conditions of mature miRNA:**

| Step | Time | Temperature | Additional comments |
|---|---|---|---|
| **Initial activation step** | 15 min | 95° C | |
| **3-step cycling:** | | | |
| Denaturation | 15 s | 94° C | |
| Annealing | 30 s | 55° C | |
| Extension | 30 s | 70° C | Perform fluorescence data collection. |
| Cycle number | 40 cycles | | Cycle number depends on the amount of template cDNA and abundance of the target. |

**Table 8. Reaction mix for detection of PGRMC1 mRNA:**

| Component | Volume/reaction(384-well) |
|---|---|
| 2x LightCycler480 SYBR Green I Master | 5 µl |
| 10x Primer Assay | 1 µl |
| RNase-free water | 2 µl |
| Template cDNA | 2 µl |
| Total volume | 10 µl |

**Table 9. Cycling conditions of PGRMC1 mRNA:**

| Step | Time | Temperature | Additional comments |
|---|---|---|---|
| **Initial activation step** | 15 min | 95° C | |
| **3-step cycling:** | | | |
| Denaturation | 15 s | 95° C | |
| Annealing | 5 s | 58° C | |
| Extension | 10 s | 72° C | Perform fluorescence data collection. |
| Cycle number | 40 cycles | | Cycle number depends on the amount of template cDNA and abundance of the target. |

**Table 10. Primer sequences for PGRMC1 qPCR:**

| Primer | Forward (Sequence 5'-3') | Reverse (Sequence 5'-3') |
|---|---|---|
| PGRMC1 | CGACGGCGTCCAGGACCC | TCTTCCTCATCTGAGTACACAG |
| GAPDH | CAGCCTCAAGATCATCATCAGCAATGC | AGACCACCTGGTGCTCAGTGTAG |

2.2.4.5 Statistical analysis

All data were described as means ± SD and data shown is the result of at least three replicates of three or more independent runs for each assay. Normal distributed data was further analyzed by ANOVA-test to compare variance of averages of several groups. For qPCR studies, Ct values from duplicates of each sample were normalized to those of SNORA73A or GAPDH and analyzed using the ΔΔCt method. Value was regarded as statistical significance at $p<0.05$ for all statistical outcomes.

### 2.2.5 Methods for supernatant

2.2.5.1 Cell types

MCF-7 and T47D were cultured. PGRMC1 was transfected into MCF-7 cell lines as mentioned above. The same methods were used to transfect T47D cell lines.

2.2.5.2 Western blot analysis

Western blot was excused as mentioned above. The concentration protein was determined using BCA. In total, 25 μg of protein was analyzed per lane and separated on 12% acrylamide gel via electrophoresis. Western blot was used to measure PGRMC1 expression. The first antibody of analyzing cell lysates was rabbit anti-PGRMC1 antibody (G21, 1:200, Santa Cruz). The secondary antibody was goat anti-rabbit (IgG-HRP, 1:500, Santa Cruz). Blot was re-probed with actin (I-19, 1:1000, Santa Cruz) to provide protein loading controls. For cell supernatant, the primary antibody was rabbit anti-PGRMC1

antibody (#12444, 1:200, Cell Signaling). Goat anti-rabbit (IgG-HRP, 1:500, Santa Cruz) was used as secondary antibody.

2.2.5.3 Cell supernatant samples

MCF-7 cell was cultured in RPMI-1640 media without serum for 24 hours. Then the medium was concentrated 10-fold using a filter tube Amicon Ultracel (Millipore, Billerica, MA) (Fig. 4). 0.25 ml slurry separopore blue CL-6B (Bio-world, Dublin) was added to the concentrated media for 30 minutes. The slurry was centrifuged at 1,000g for three minutes, then 20 µl was detected via western blot. The supernatant of T47D was obtained in the same way.

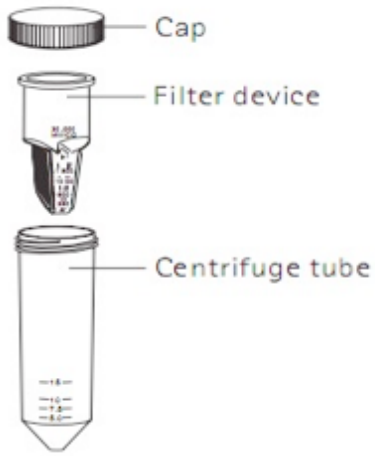


**Figure 4: The Amicon Ultracel device is supplied with a cap, a filter device, and a centrifuge tube.** (Adapted from Amicon Ultracel User Guide)

2.2.5.4 PGRMC1 detection in plasma from breast cancer patients

The specimens of plasma were obtained from the Women's Hospital of Tübingen University, and the agreement was ratified by the Institutional Review Board. Plasma samples were performed by centrifugation at 13,000g for ten minutes, and then the supernatants were dissolved into Tris solution and mixed with 200 µl slurry for 30 minutes. The slurry was centrifuged at

1,000g for three minutes and 5 μl was detected via western blot.

2.2.5.5 Statistical analysis

The experiment was performed triplicates, with each assay producing the same outcomes.

# 3. Results

## 3.1 Proliferation of breast cancer cell lines endogenously expressing PGRMC1

### 3.1.1 PGRMC1 expression in various breast cancer cell lines

PGRMC1 is a membrane of multi-protein progesterone binding complex and the molecular weight is 26 kDa. To analyze PGRMC1 expression in various breast tumor cell lines, lysates prepared from HCC202, HCC70, SUM225CWN, HCC1500, HBL100, BM and AR cell lines were analyzed by western blot. In Figure 5, the results of PGRMC1 levels in these cells are depicted. Endogenous PGRMC1 was detected in HCC70, SUM225CWN, HBL100, BM and AR cell lines. The highest expression was observed in SUM225CWN cell lines.

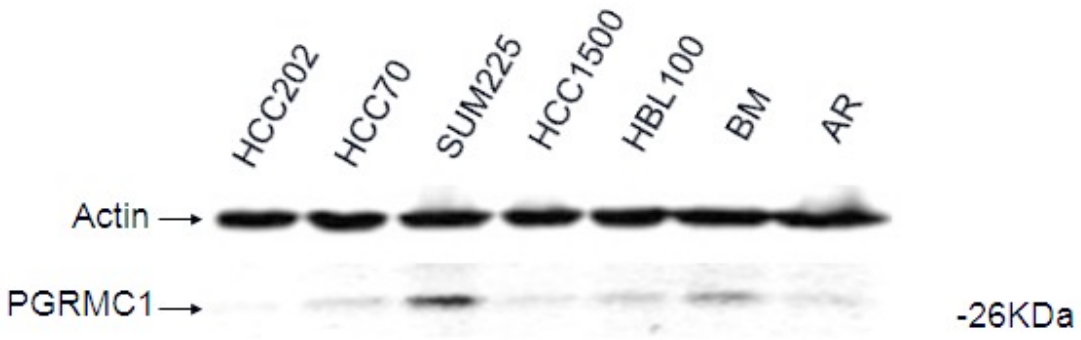


**Figure 5: PGRMC1 expression in various breast cancer cells.** Western blot analysis of lysates prepared from HCC202, HCC70, SUM225CWN, HCC1500, HBL100, BM and AR cell lines. PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody (#12444, Cell signaling) followed by anti-rabbit horseradish peroxidase antibody. Actin was used as a loading control (upper panel).

### 3.1.2 ER-α expression in various breast cancer cells

ER-α, also known as -NR3A1 is encoded by the-gene-ESR1 and its molecular weight is 66 kDa. To analyze the expression of ER-α in various breast tumor cells, lysates prepared from T47D, HCC70, HCC202, SUM225CWN, HBL100, BM, AR and MCF-7 cell lines were detected by western blot. ER-α was detected in MCF-7, T47D and BM cells. However, ER-α expression wasn't observed in other cell lines (Fig. 6).

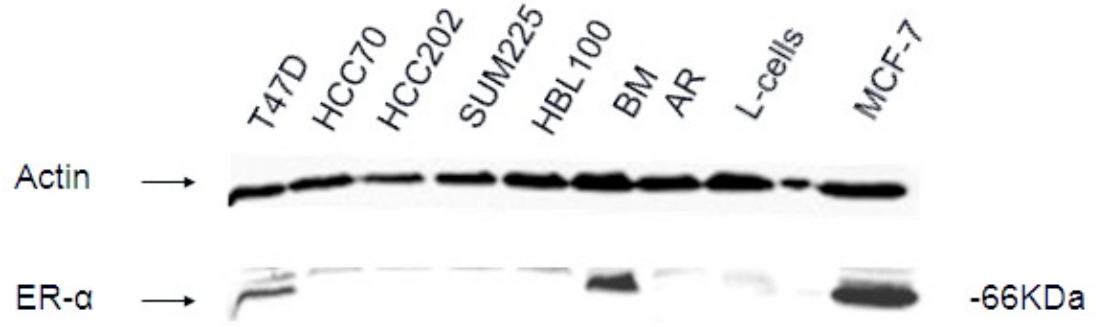


**Figure 6: ER-α expression in various breast cancer cells.** Western blot analysis of lysates prepared from T47D, HCC70, HCC202, SUM225CWN, HBL100, BM, AR and MCF-7 cell lines. ER-α is detected with a polyclonal rabbit anti-ER-α antibody (H-184, Santa cruz) followed by anti-rabbit horseradish peroxidase antibody. Actin was used as a loading control (upper panel).

### 3.1.3 Effect of estradiol and progestins on the proliferation of breast cancer cell lines expressing PGRMC1 with and without co-expressing endogenous ER-α

To analyze the influence of estradiol and progestogens on the proliferation of breast tumor cell lines, BM (endogenous ER-α and PGRMC1) and SUM225CWN cells (only endogenous PGRMC1) were stimulated with these hormones respectively. In Figure 7, the results of the proliferation assays in BM cells are depicted. MPA, NET, LNG, DSP and E2 significantly increased the proliferation. Incubation of DNG and DSG resulted in an increased

proliferation at the concentration of $10^{-6}$ M, no significant effect was seen at $10^{-7}$ M. For P4, NOM, DYD and CMA, no significant effect was found. In Figure 8, the results using SUM225CWN cells are shown. At the same condition, estradiol and progestins didn't obviously alter the proliferation.

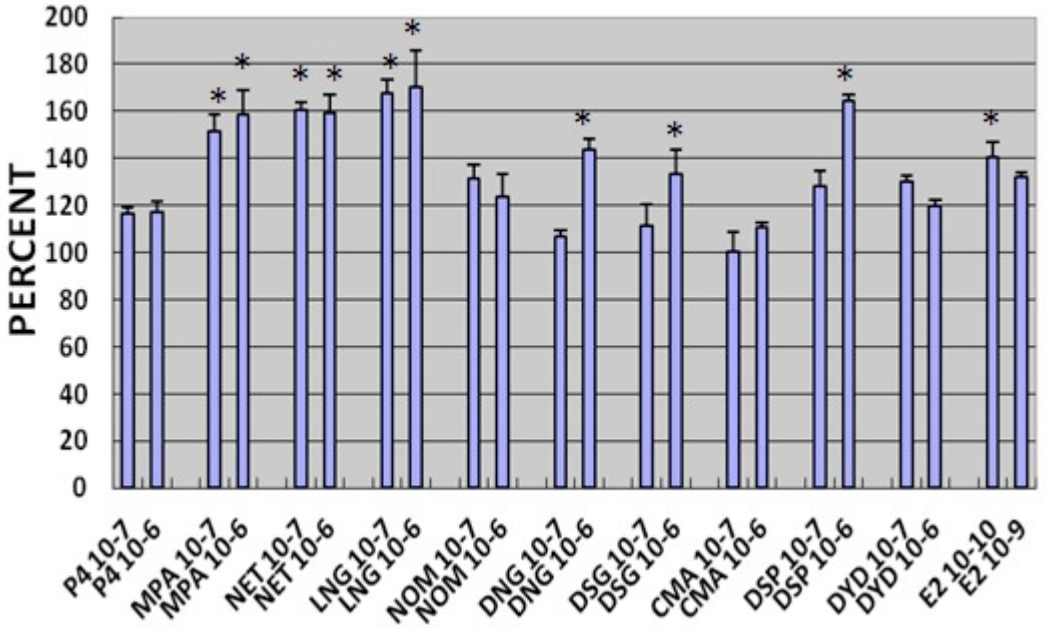


**Figure 7: Proliferation assays of BM with estradiol and all progestins.** BM cell lines were treated using estradiol (E2; $10^{-10}$ or $10^{-9}$ M) and progesterone (P4), chlormadinone acetate (CMA), drospirenone (DSP), dienogest (DNG), desogestrel (DSG), dydrogesterone (DYD), levonorgestrel (LNG), medroxyprogesterone acetate (MPA), nomegestrel (NOM), norethisterone (NET) (each at $10^{-7}$ or $10^{-6}$ M). After 5 days, the proliferation was detected. Data was standardized using untreated cells (means ± SD; *P<0.05 vs untreated control).

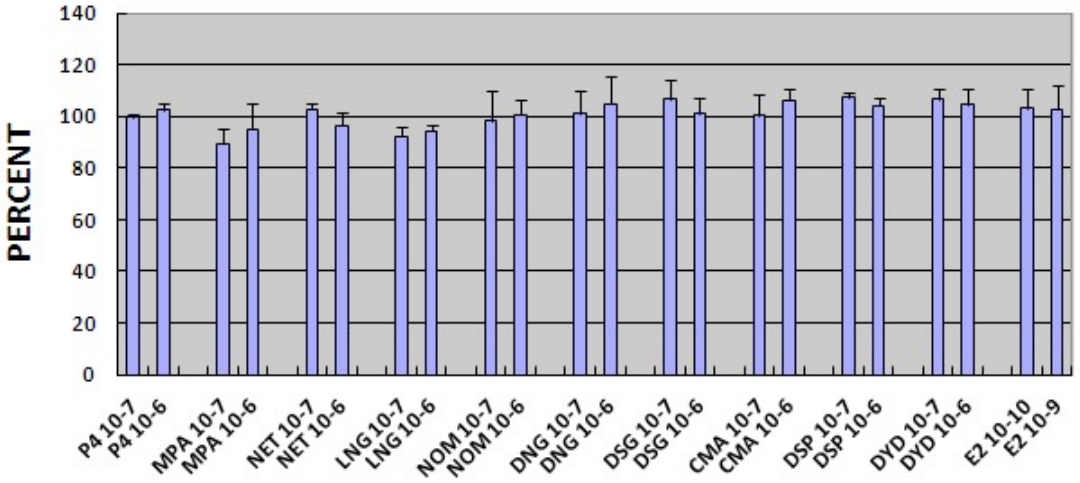


**Figure 8: Proliferation assays of SUM225CWN with estradiol and all progestins.** SUM225CWN cell lines were treated with estradiol (E2; $10^{-10}$ or $10^{-9}$ M) and progesterone (P4), chlormadinone acetate (CMA), drospirenone (DSP), dienogest (DNG), desogestrel (DSG), dydrogesterone (DYD), levonorgestrel (LNG), medroxyprogesterone acetate (MPA), nomegestrel (NOM), norethisterone (NET) (each at $10^{-7}$ or $10^{-6}$ M). After 5 days, the proliferation was detected. Data was standardized using untreated cells.

## 3.2 PGRMC1 sensitizes PS2 expression by E2-induced

### 3.2.1 Effect of E2 on PS2 expression in MCF-7 cells

In this experiment, different E2 ($10^{-10}$, $10^{-9}$ and $10^{-8}$ M) concentrations were treated to MCF-7 cell lines, and PS2 mRNA levels were measured by qRT-PCR after one hour. E2 increased PS2 expression. Treatment of MCF-7 cells with $10^{-9}$ M E2 significantly increased native PS2 mRNA about 40% of control value ($P<0.05$; Fig. 1). In this cell lines, this effect was increased with the increasing dose (Fig. 9). Therefore, $10^{-9}$ M E2 was utilized in further experiments.

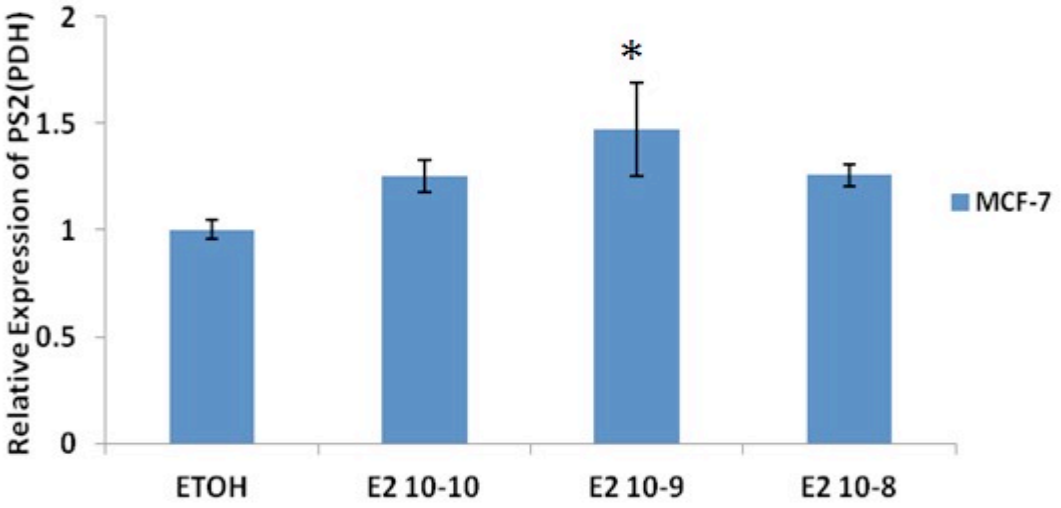


**Figure 9: The role of E2 on PS2 in MCF-7 cell lines.** Cells were stimulated with $10^{-10}$, $10^{-9}$, and $10^{-8}$ M E2, respectively and endogenous PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

### 3.2.2 PGRMC1-expressing cells show increased E2-induced level of PS2

In this experiment, a fixed E2 concentration ($10^{-9}$ M) was incubated with MCF-7 EVC and MCF-7 WT12 cell lines. PS2 mRNA levels were measured after one hour. In these cell lines, E2 could elicit an increase of PS2 mRNA (Fig. 10). In WT-12, which was transfected with PGRMC1, E2 induced a significant increase of about 60% (Fig. 10).

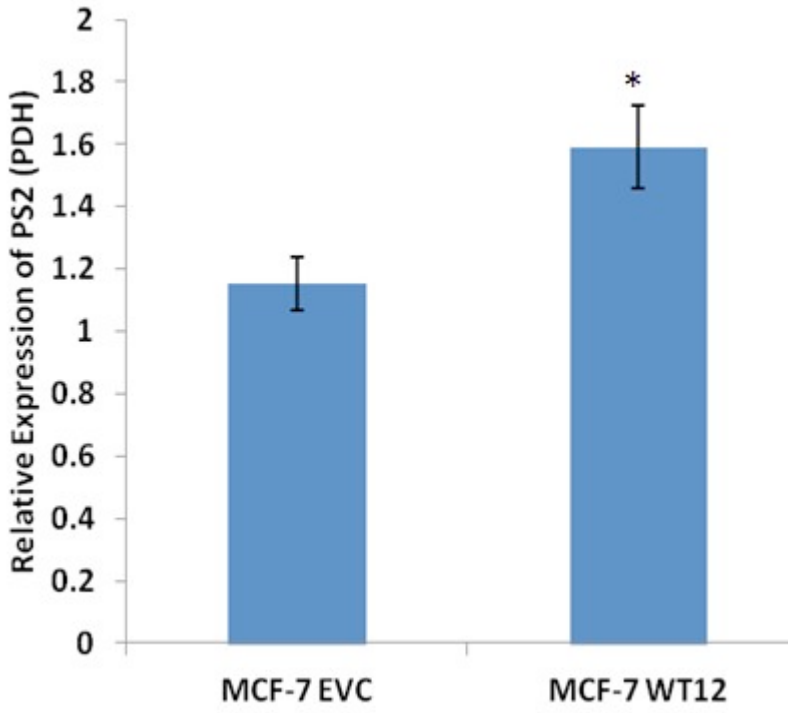


**Figure 10: PGRMC1 shows elevated E2-induced level of PS2.** MCF-7 with a plasmid control (EVC) and MCF-7 cells over-expressing PGRMC1 (WT-12) were stimulated with estradiol ($10^{-9}$ M). PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

### 3.2.3 PS2 blocking experiment (fulvestrant, TBCA, AG205)

The influence of receptor inhibitors to PS2 mRNA levels was examined. In blocking experiments, fulvestrant (ER-α inhibitor), TBCA (CK2 inhibitor) and AG205 (PGRMC1 inhibitor) were used. As can be seen in Figure 11, E2-induced effect could be significantly blocked when fulvestrant was added to the incubation. Addition of CK2 II Inhibitor TBCA to E2, however, partial abrogated the E2-induced PS2 mRNA levels in WT-12 cell (Fig. 12). Furthermore, inhibitory effects occurred in the presence of AG 205 (Fig. 13).

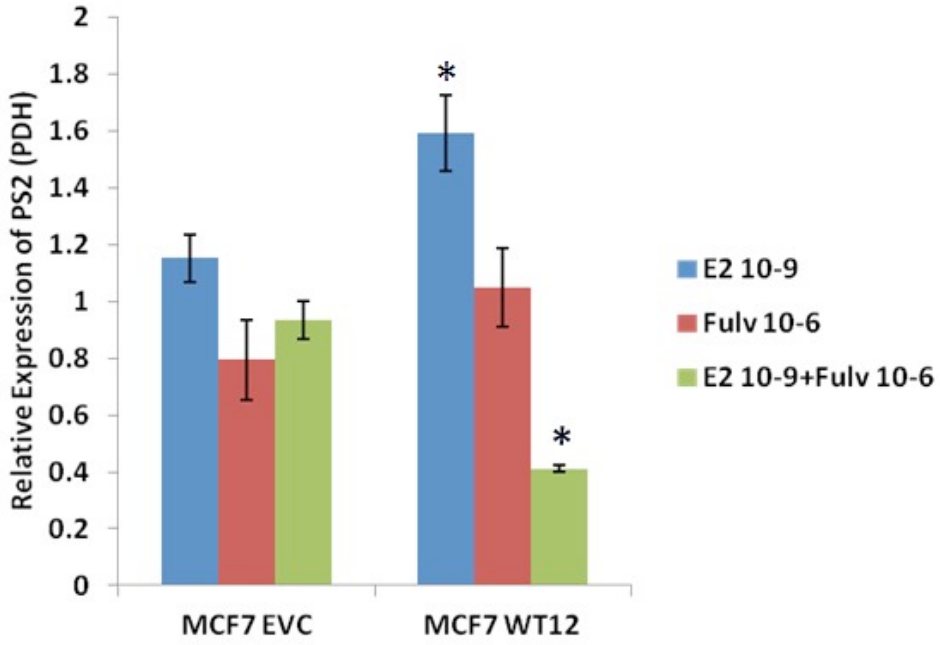


**Figure 11: PS2 blocking experiment (Fulvestrant).** MCF-7 EVC and MCF-7 WT12 were incubated with estradiol (E2; $10^{-9}$ M) and fulvestrant (Ful; $10^{-6}$ M) alone and in combination. PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

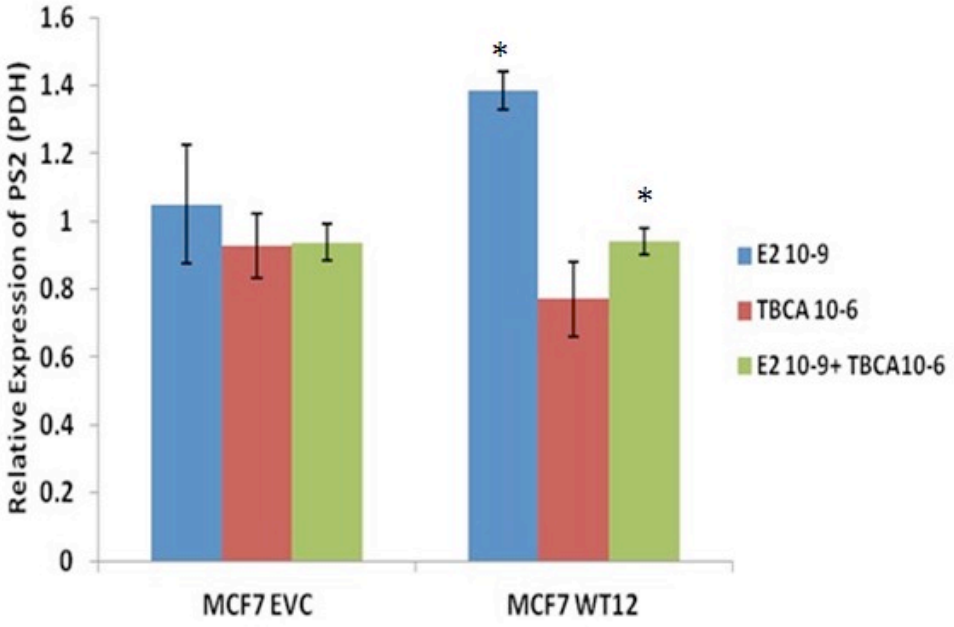


**Figure 12: PS2 blocking experiment (TBCA).** MCF-7 EVC and MCF-7 WT12 were incubated with estradiol (E2; $10^{-9}$ M) and TBCA (TBCA; $10^{-6}$ M) alone and in combination. PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

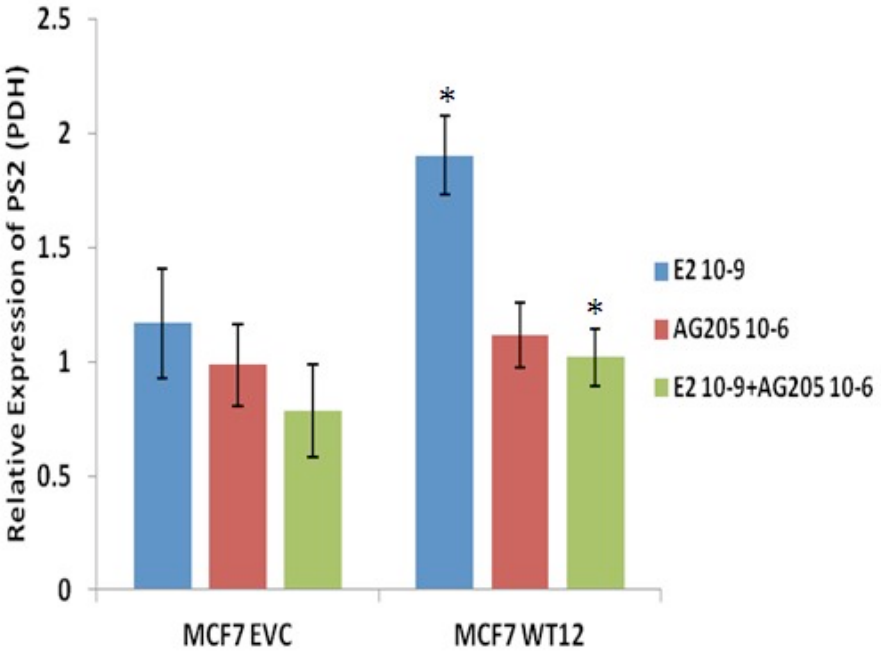


**Figure 13: PS2 blocking experiment (AG205).** MCF-7 EVC and MCF-7 WT12 were incubated with estradiol (E2; $10^{-9}$ M) and AG205 (AG205; $10^{-6}$ M) alone and in combination. PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

### 3.2.4 Effect of E2, P4, NET and E2 combined with P4 or NET on PS2 expression

In Figure 14, the results of the PS2 mRNA levels in MCF-7 EVC and MCF-7 WT12 cells are depicted. NET (60%) and E2 (40%) significantly increased the expression of PS2 mRNA in WT-12, which was transfected with PGRMC1. For P4, no effect was found. Addition of the NET ($10^{-6}$ M) in E2 combined manner did not obviously alter the PS2 mRNA levels as compared with the effect of E2 only (Fig. 14).

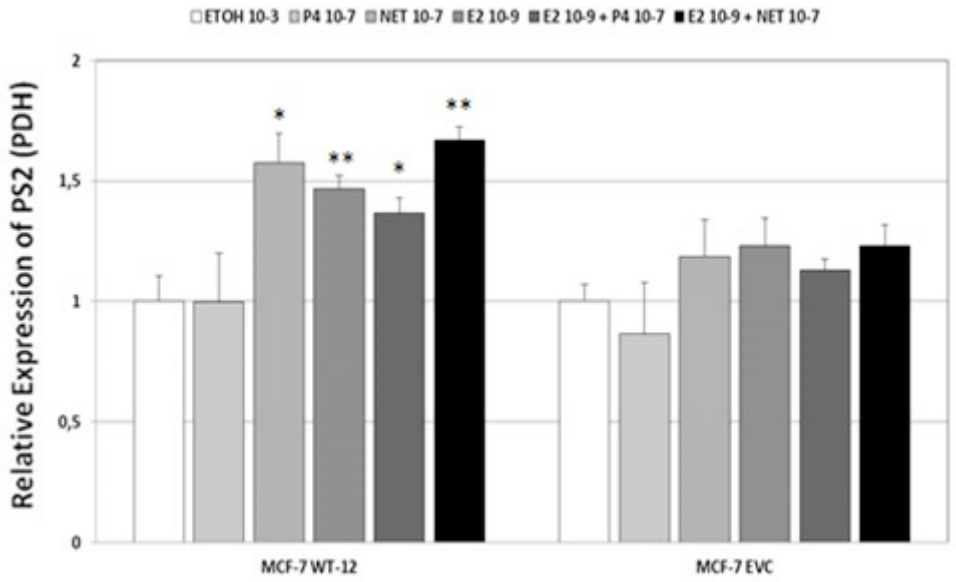


**Figure 14: Effect of E2, P4, NET and E2 combined with P4 or NET on PS2 expression.** MCF-7 EVC and MCF-7 WT12 were incubated with estradiol (E2; $10^{-9}$ M), progesterone (P4; $10^{-6}$ M) and norethisterone (NET; $10^{-6}$ M) alone or E2 in combination with P4 and NET. PS2 expression was detected using qPCR, relative to PDH. Data was standardized using untreated cells (means ± SD; *, **P different from control).

## 3.3 RANKL and RANK expression in various breast cancer cell lines

### 3.3.1 RANK expression in the various cell lines

RANK is a type I membrane protein and the molecular weight of RANK is 90 kDa. To test RANK expression in various breast tumor cell lines, lysates prepared from T47D, HCC70, HCC202, SUM225CWN, HBL100, BM, AR and HCC1500 cell lines were detected by western blot. However, the polyclonal mouse anti-RANK antibody (N1H8, Amgen, Inc) (Fig. 16) and the polyclonal goat anti-RANK antibody (SC-7625, Santa Cruz) (Fig. 15) didn't detect this protein in these cell lines.

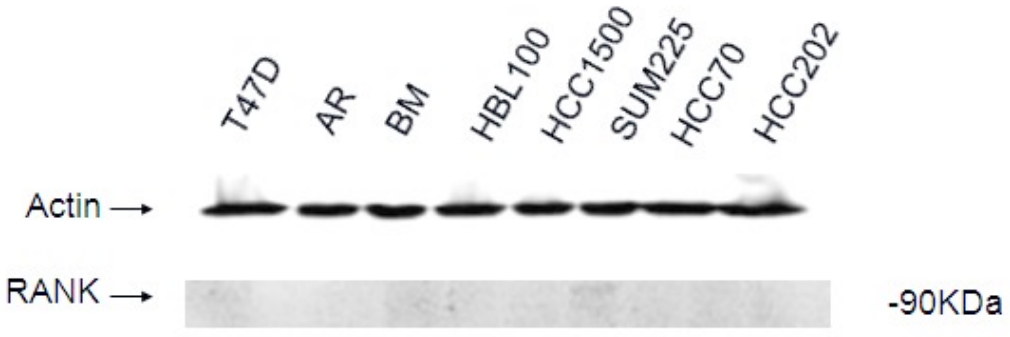


**Figure 15: RANK expression in the various cell lines (SC-7625).** Western blot analysis of lysates prepared from T47D, AR, BM, HBL100, HCC1500, SUM225CWN, HCC70 and HCC202 cell lines. RANK is detected with a polyclonal goat anti-RANK antibody (SC-7625, Santa Cruz) followed by anti-goat horseradish peroxidase antibody. Actin provided a loading control (upper panel).

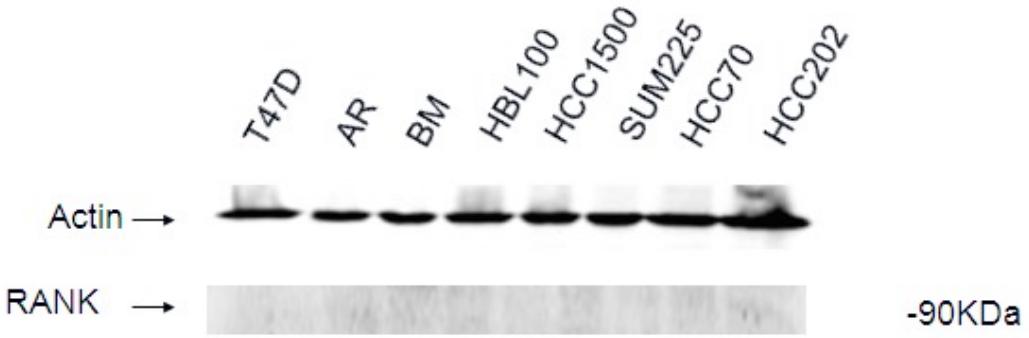


**Figure 16: RANK expression in the various cell lines (N1H8).** Western blot analysis of lysates prepared from T47D, AR, BM, HBL100, HCC1500, SUM225CWN, HCC70 and HCC202 cell lines. RANK is detected with a polyclonal mouse anti-RANK antibody (N1H8, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

### 3.3.2 RANKL expression in the various cell lines

RANKL is a type II transmembrane protein and the molecular weight of it is 35-40 kDa. In this experiment, we used a polyclonal mouse anti-RANKL antibody (m366, Amgen, Inc) to evaluate RANKL expression in T47D, HCC70, HCC202, SUM225CWN, HBL100, BM, AR, and MCF-7 cell lines. As positive

control for RANKL L-cells were used which are transfected with RANKL expression plasmid. RANKL expression was verified by western blot only in the L-cells, while under the same experimental conditions in the breast cancer cell lines this protein was not detectable (Fig. 17).

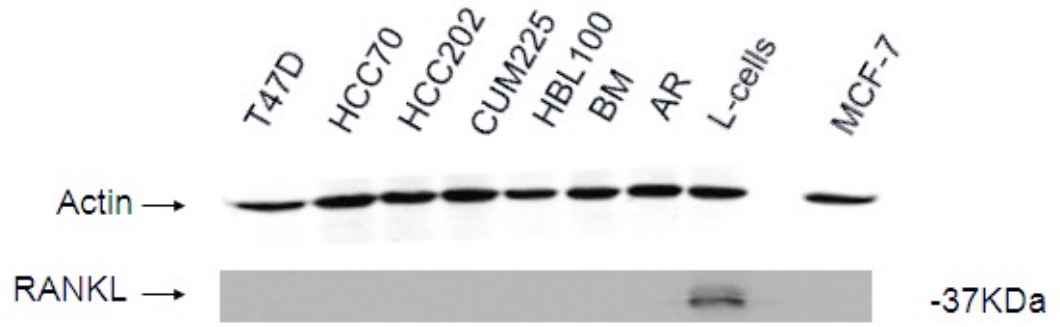


**Figure 17: RANKL expression in various cell lines.** Western blot analysis of lysates prepared from T47D, HCC70, HCC202, SUM225CWN, HBL100, BM, AR, L-cells RANKL and MCF-7 cell lines. RANKL is detected with a polyclonal mouse anti-RANKL antibody (m366, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

### 3.3.3 Co-culture of breast cancer cells with osteoblast-like cells

Through a co-culture system, we analyzed the expression of RANKL in breast tumor cells with or without osteoblast-like cells by western blot. Cal72 and Saos2 are the human osteoblast-like cells. RANKL expression was verified by western blot only in the control L-cells, while under the same experimental conditions co-cultures of MCF-7 WT12 and MDA-MB-231 cell lines with Cal72 and Saos2 cells did not detect RANKL expression (Fig. 18 and 19).

Then, we analyzed RANK expression in breast tumor cells with or without osteoblast-like cells by western blot. RANK expression was verified using western blot in Cal72 and MDA-MB-231 cell lines, while under the same experimental conditions MCF-7 WT12 and Saos2 cells did not express RANK (Fig. 20 and 21).

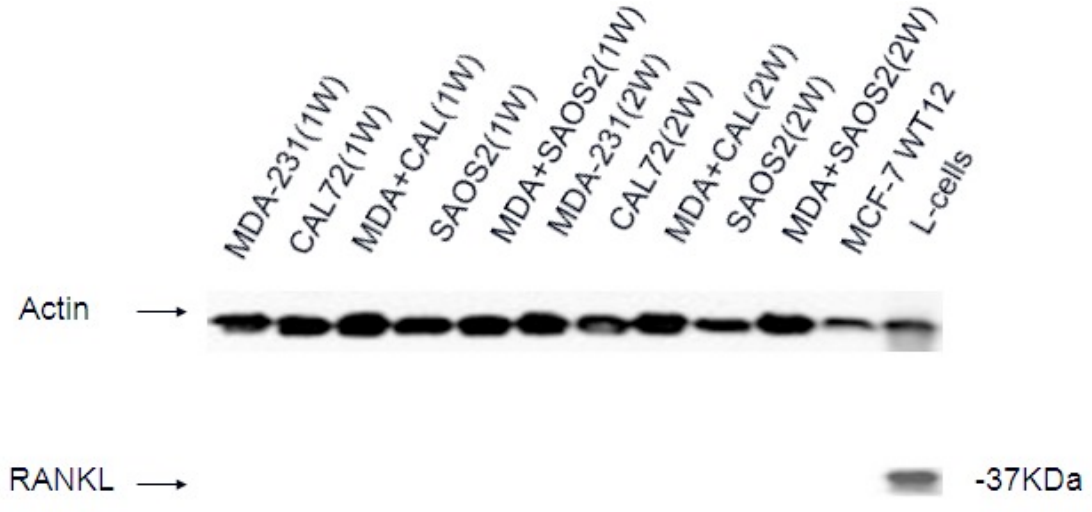


**Figure 18: RANKL expression in MDA-MB-231 co-culture systems.** Co-culture of MDA-MB-231 with Cal72 and Saos2 cells for 1 week or 2 weeks. Expression of RANKL is assessed by western blot. RANKL is detected with a polyclonal mouse anti-RANKL antibody (m366, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

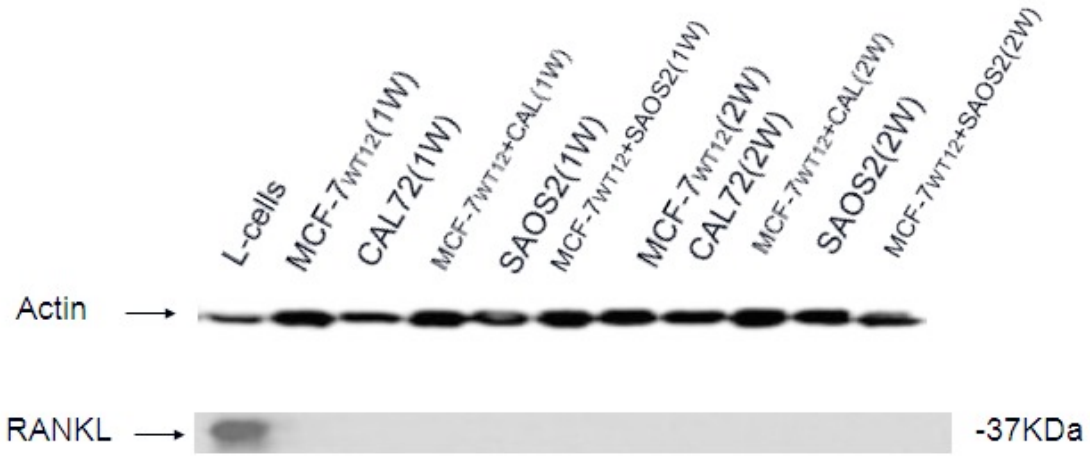


**Figure 19: RANKL expression in MCF-7 WT12 co-culture systems.** Co-culture of MCF-7 WT12 with Cal72 and Saos2 cells for 1 week or 2 weeks. Expression of RANKL is assessed by western blot. RANKL is detected with a polyclonal mouse anti-RANKL antibody (m366, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

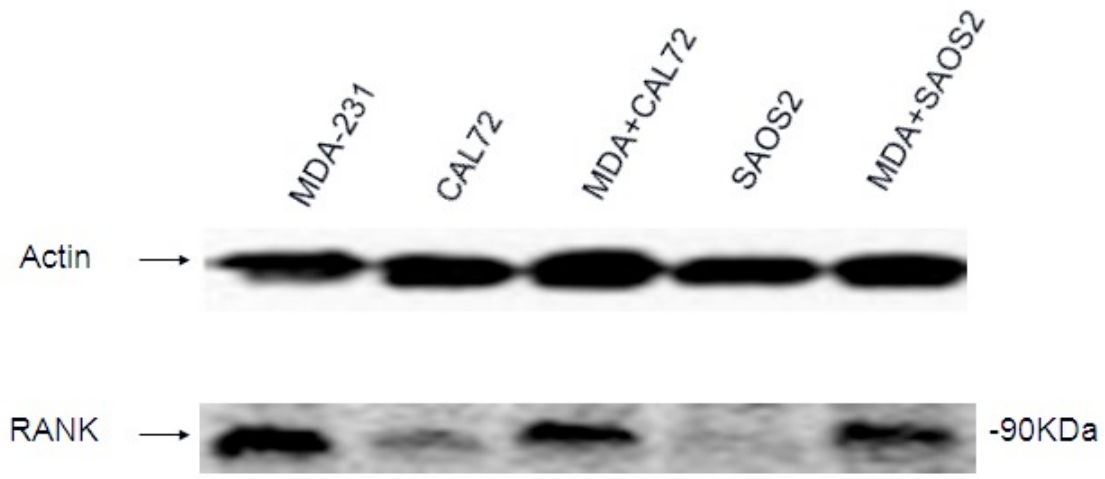


**Figure 20: RANK expression in MDA-MB-231 co-culture systems.** Co-culture of MDA-MB-231 with Cal72 and Saos2 cells. Expression of RANK is assessed by western blot. RANK is detected with a polyclonal mouse anti-RANK antibody (N1H8, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

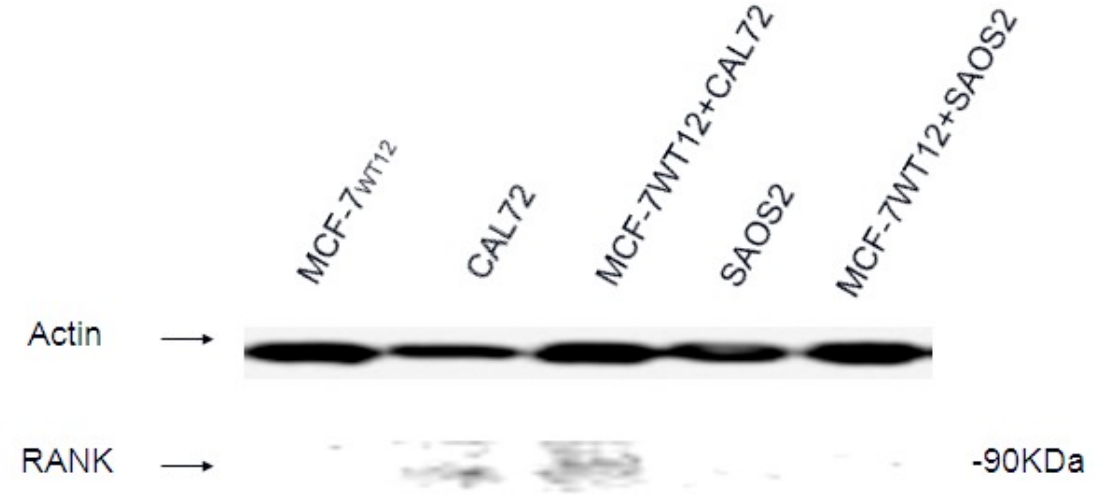


**Figure 21: RANK expression in MCF-7 WT12 co-culture systems.** Co-culture of MCF-7 WT12 with Cal72 and Saos2 cells. Expression of RANK is assessed by western blot. RANK is detected with a polyclonal mouse anti-RANK antibody (N1H8, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

### 3.3.4 Effect of estradiol and all progestins on L-cells RANKL cells in vitro

In order to analyze the influence of estradiol and progestins on the proliferation of over-expressing RANKL cells, L-cells were incubated with these hormones. In Figure 22, the results of the proliferation assays in L-cells RANKL are depicted. Incubation of estradiol for 6 days didn't significantly increase the proliferation at $10^{-10}$or $10^{-9}$ M. At the same condition, addition of progesterone or the synthetic progestogens didn't obviously alter the proliferation of L-cells.

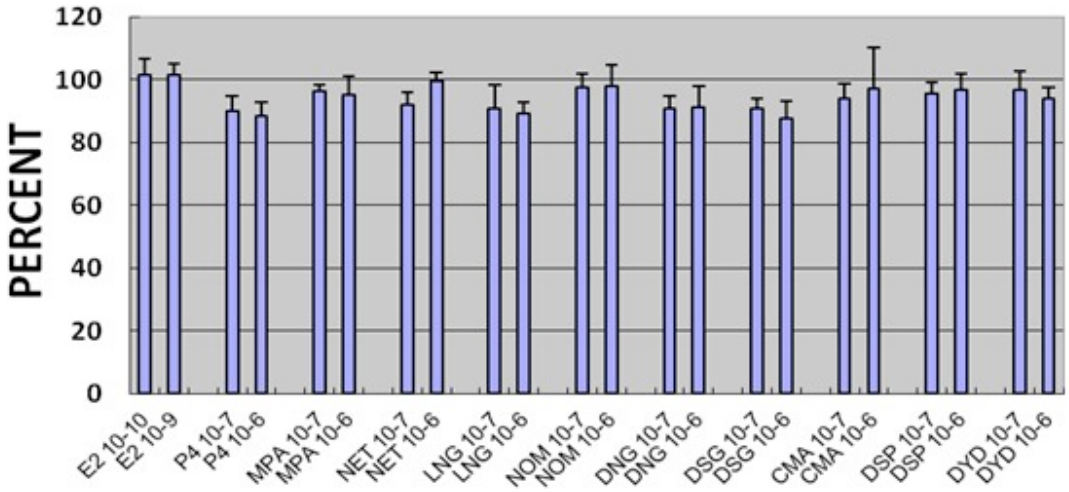


**Figure 22: Proliferation assays of L-cells RANKL with estradiol and all progestins.** L-cells were stimulated with estradiol (E2; $10^{-10}$ or $10^{-9}$ M) and progesterone (P4), chlormadinone acetate (CMA), drospirenone (DSP), dienogest (DNG), desogestrel (DSG), dydrogesterone (DYD), levonorgestrel (LNG), medroxyprogesterone acetate (MPA), nomegestrel (NOM), norethisterone (NET) (each at $10^{-7}$ or $10^{-6}$ M). After 5 days, the proliferation was detected. Data was standardized using untreated cells.

In the next experiment, we analyze the influence of estradiol and progestins on RANKL expression. L-cells were stimulated with these drugs respectively and RANKL levels were determined by western blot. At this treatment, E2 didn't

significantly change the expression of RANKL in L-cells. Furthermore, addition of progesterone or the synthetic progestogens didn't obviously alter RANKL levels (Fig. 23).

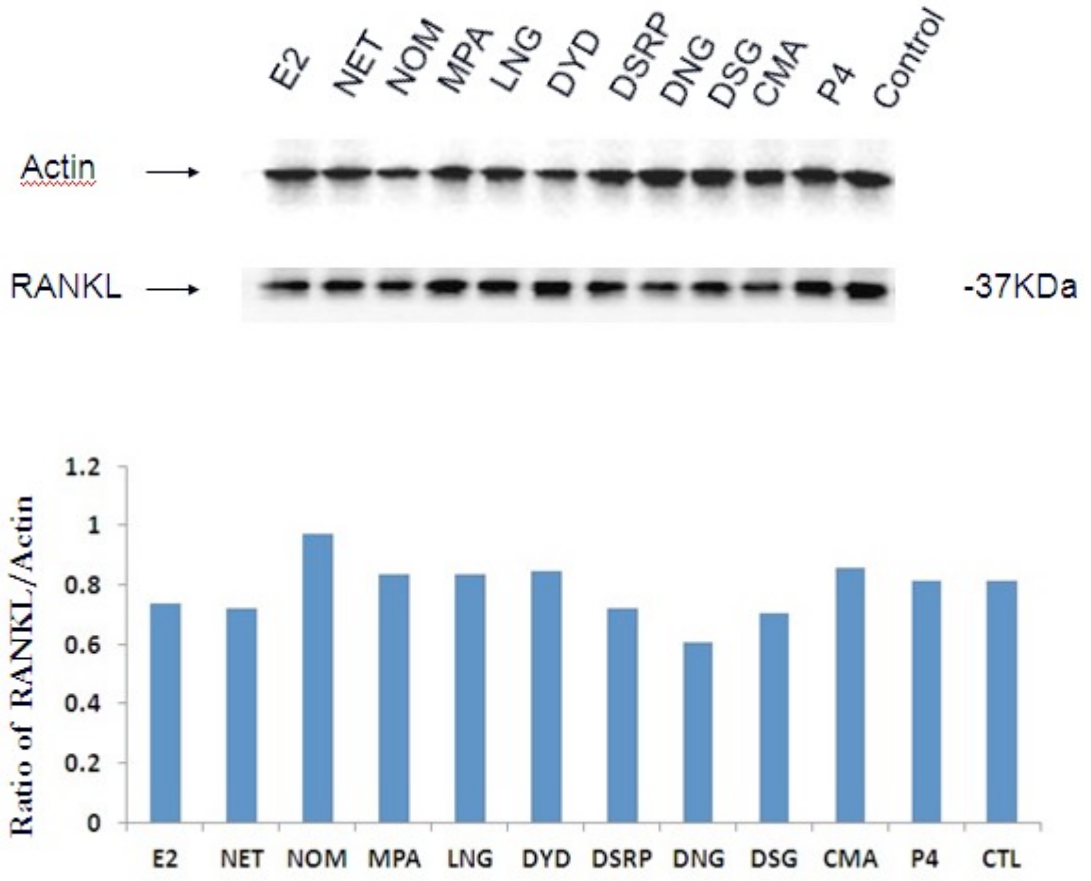


**Figure 23: Effect of estradiol and progestins on RANKL expression in L-cells.** Western blot analyzes RANKL expression in L-cells. Cells were incubated with estradiol (E2; $10^{-9}$ M) and norethisterone (NET), progesterone (P4), nomegestrel (NOM), medroxyprogesterone acetate (MPA), levonorgestrel (LNG), dydrogesterone (DYD), drospirenone (DSRP), dienogest (DNG), desogestrel (DSG), chlormadinone acetate (CMA) (each at $10^{-6}$ M). RANKL is detected with a polyclonal mouse anti-RANKL antibody (m366, Amgen, Inc) followed by anti-mouse horseradish peroxidase antibody. Actin provided a loading control (upper panel).

## 3.4 Let-7i regulates the expression of PGRMC1 mRNA

For the purpose of test the impact of let-7i on endogenous PGRMC1 expression, we transfected let-7i mimic into MCF-7 cells. After 2 days, PGRMC1 mRNA levels were measured.

In order to identify the result of transfection, we detected the level of let-7i. We found let-7i (Fig. 24 lane 2) was significantly increased compared to control (Fig. 24 lane 1). Thus, this proved that the transfection was successful.

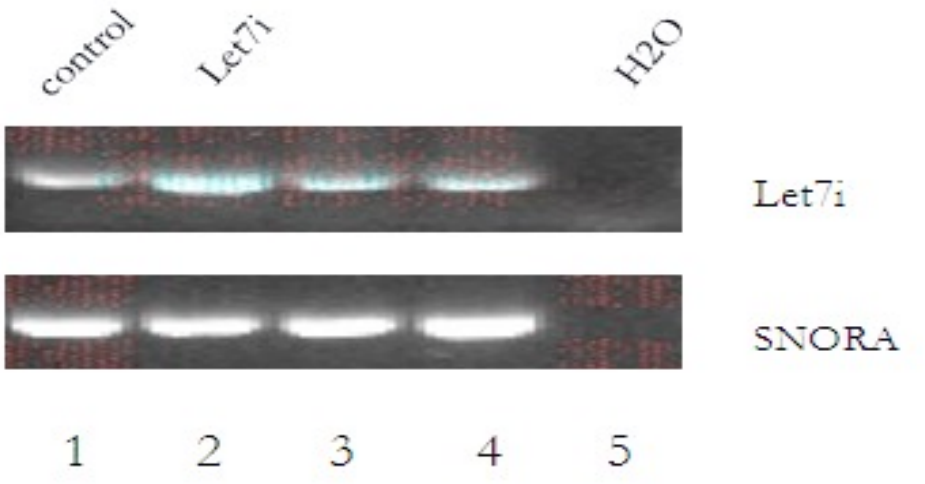


**Figure 24: Semi-quantitative RT-PCR analysis PGRMC1 mRNA and let-7i in MCF7 cell lines transfected with the let-7i mimic and silencer.** cDNA samples were made from MCF-7 cell lines transfected with let-7i (Lane 2), Control is shown in Lane 1. cDNA integrity was monitored using primers specific. Gene names are given on the right. All amplifications were performed in triplicate, representative results were presented.

The effect on let-7i expression by the transfection reagent was confirmed by amplification plot of real-time PCR. After 48 hours, let-7i was obviously higher than control (Fig. 25A).

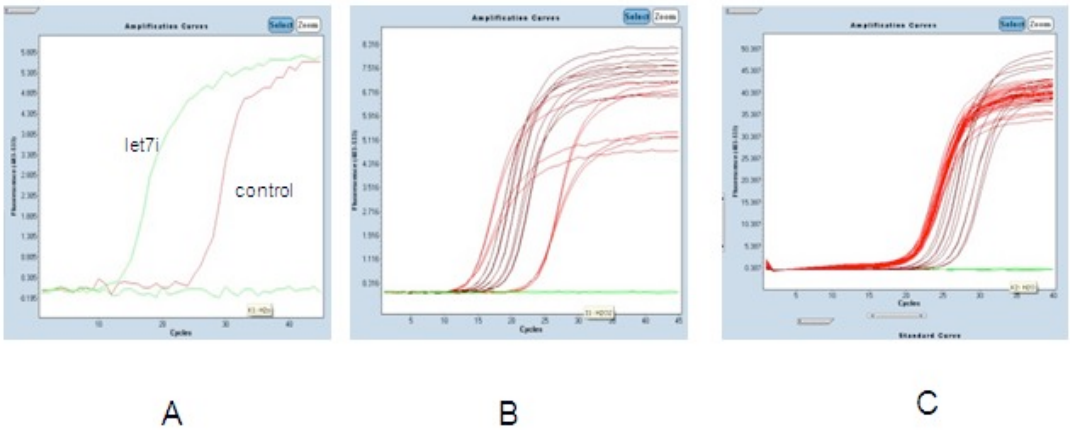


**Figure 25: Amplification plot of let-7i and PGRMC1.** (A) Real-time reverse transcription-PCR showed let-7i was obviously increased by transfection reagent let-7i mimic. (B) Amplification plots of let-7i. (C) Amplification plots of PGRMC1.

A dissociation curve analysis may be optionally performed to aid in verifying the character and status of PCR product (s). Dissociation curve was the qPCR software analysis step. Dissociation curve analysis of let-7i and PGRMC1 shows that each sample yielded only one peak, which generated by the products of amplification (Fig. 26).

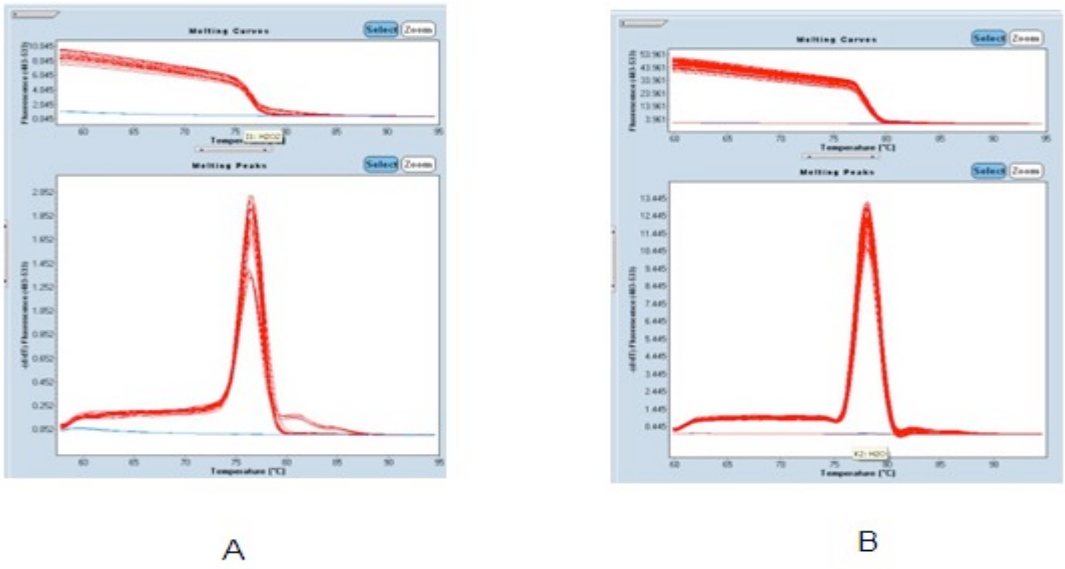


**Figure 26: Dissociation curve analysis of let-7i and PGRMC1.** (A) Dissociation curve analysis of mature miRNA let-7i PCR products showing single peaks from the specific amplification products. (B) Dissociation curve analysis of PGRMC1 PCR products showing single peaks from the specific amplification products.

Let-7i mimic reduced the PGRMC1 expression at mRNA level contrast to the control ($P<0.05$; Fig. 27A). Cells transfected with let-7i were increased 30 times than the control group ($P<0.05$; Fig. 27B). Therefore, it demonstrated that let-7i targets PGRMC1 and negatively affects the level of PGRMC1 in MCF-7 cells.

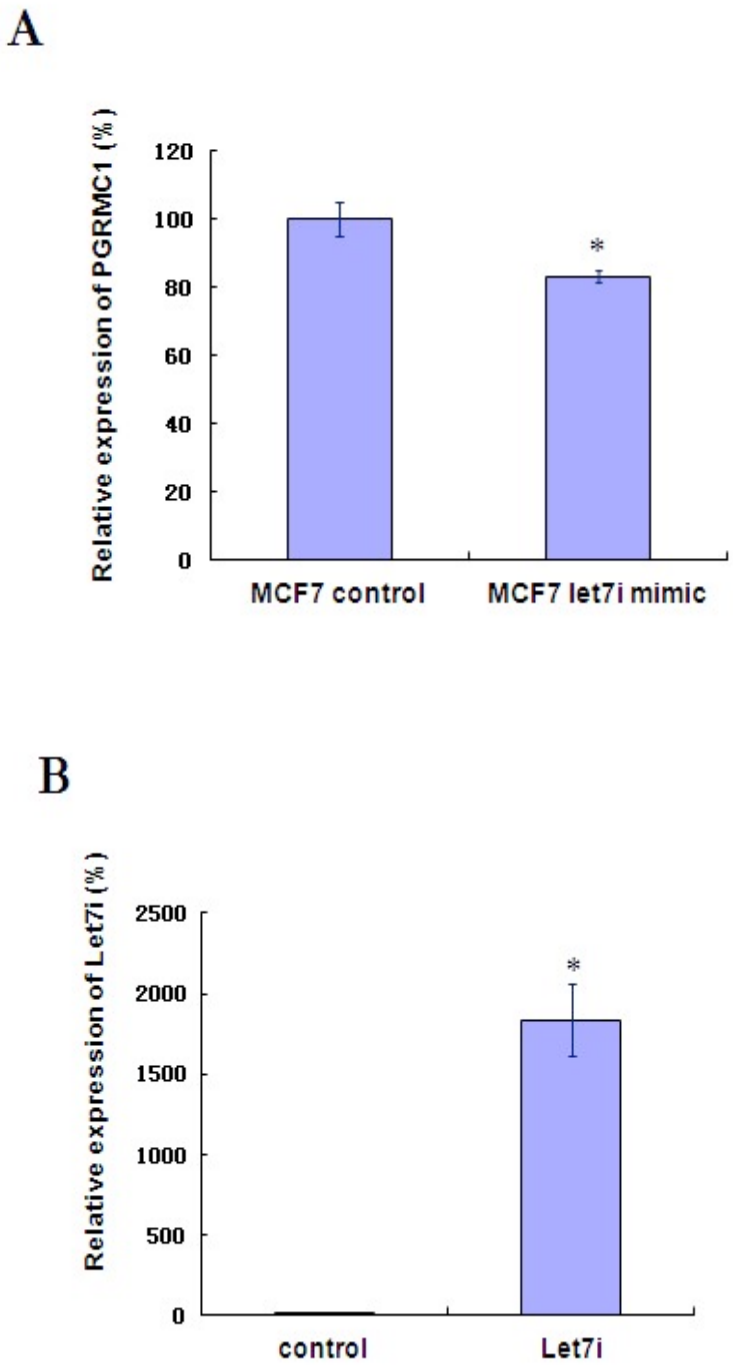


**Figure 27: Analysis of the effect of let-7i on mRNA expression of PGRMC1 in MCF-7 transfected with let-7i mimic or silencer control.** For qPCR, RNA containing miRNAs were isolated from cells transfected using 5 nM miRNA or control after 48 hours. (A) The mRNA of PGRMC1 after transfection was determined via qPCR. Normalization was quantified by GAPDH. (B) The let-7i level after transfection was determined via qPCR. Normalization was quantified by SNORA73A. Data was standardized using untreated cells (means ± SD; *P<0.05 vs control).

## 3.5 Secreted PGRMC1 by breast cancer cell lines

### 3.5.1 PGRMC1 expression in breast cancer cell lines

Western blot analysis for the MCF-7 WT12 protein produced an about 30 kDa band. This band was the predicted 28 kDa for PGRMC1 plus approximately 3 kDa for the three HA tags (Fig. 28a). The endogenously PGRMC1 provides only a very faint signal at 28 kDa indicating a weak intrinsic PGRMC1 expression (Fig. 28a). Nevertheless, merely the rabbit anti-PGRMC1 antibody (#12444, Cell Signaling) could detect this band. The polyclonal antibody (G-21, Santa Cruz) did not under the identical situation (Fig. 28b). This band could be disappeared through incubating of PGRMC1 antibody and recombinant peptide (Fig. 30). PGRMC1 antibody (#12444, Cell Signaling) could analyzed endogenously and exogenously PGRMC1 expression by Western Blot, which suggested that they were phosphorylated (Fig. 30a). The band of PGRMC1 was blocked by recombinant peptide (Fig. 30b). In MCF-7/PGRMC1 cell lines, no signal was observed. To provide protein loading controls, actin (approximately 42 kDa) was measured with rabbit anti-actin antibody.

The expression of PGRMC1 was analyzed by western blot in MCF-7/PGRMC1-3HA cells (MCF-7 WT12) and T47D/PGRMC1-3HA cells (Fig. 28 and 29). The rabbit anti-PGRMC1 antibody (#12444, Cell Signaling) exhibited highly sensitivity because it could detect PGRMC1 in the MCF-7 and T47D cells, whereas the G-21 antibody (G-21, Santa Cruz) showed a faint band at the corresponding condition (Fig. 28 and 29 lower panel). Maybe G-21 antibody is not high sensitivity. Therefore, the rabbit anti-PGRMC1 (#12444, Cell Signaling) was used to detect PGRMC1 expression in cell supernatant.

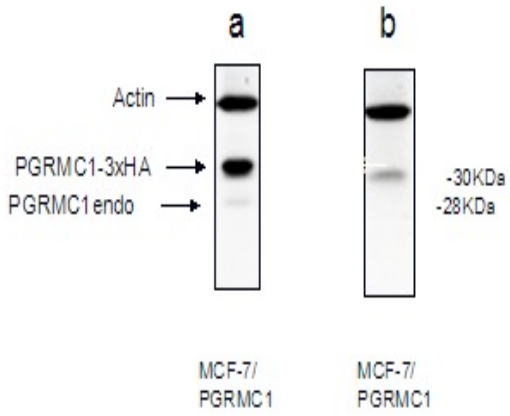


**Figure 28: PGRMC1 expression in MCF-7 WT12.** Western blot analysis for lysates prepared from MCF-7 WT12 cell lines. (a) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody (#12444, Cell Signaling) followed by anti-rabbit horseradish peroxidase antibody. Signals are visualized with chemiluminescence, and actin provided a loading control (upper panel). The exogenously and endogenously PGRMC1 signal was detected. (b) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody (G-21, Santa Cruz) followed by anti-rabbit horseradish peroxidase antibody. The exogenously PGRMC1 signal was detected only.

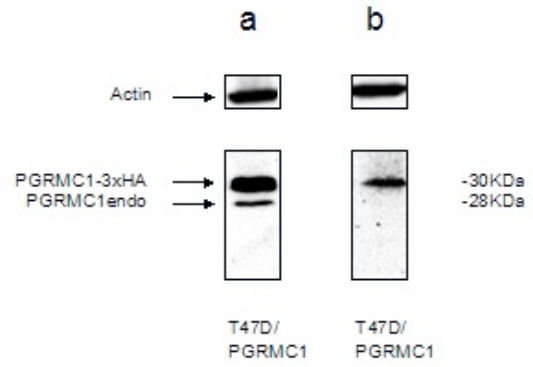


**Figure 29: PGRMC1 expression in T47D/PGRMC1-3HA cells.** Western blot analysis of lysates prepared from T47D/PGRMC1-3HA cells. (a) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody (#12444, Cell Signaling) followed by anti-rabbit horseradish peroxidase antibody. Signals are visualized with chemiluminescence, and actin provided a loading control (upper panel). The exogenously and endogenously PGRMC1 signal was detected. (b) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody (G-21, Santa Cruz) followed by anti-rabbit horseradish peroxidase antibody. The exogenously PGRMC1 signal was detected only.

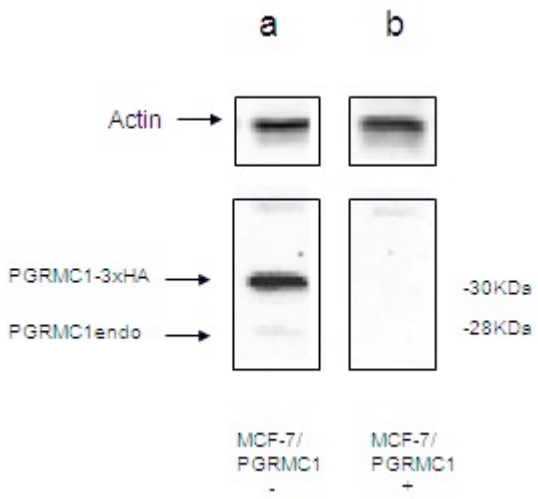


**Figure 30: Recombinant PGRMC1 of MCF-7 WT12 cells.** Western blot analysis for lysates prepared from MCF-7 WT12 cell lines. (a) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody followed by anti-rabbit horseradish peroxidase antibody. Signals are visualized with chemiluminescence. PGRMC1-3HA is detected at approximately 30 kDa. (b) No signals are detected. Here, the primary antibody was preincubated with recombinant PGRMC1. (Upper panels) The same lysates were incubated with an actin specific antibody for loading control.

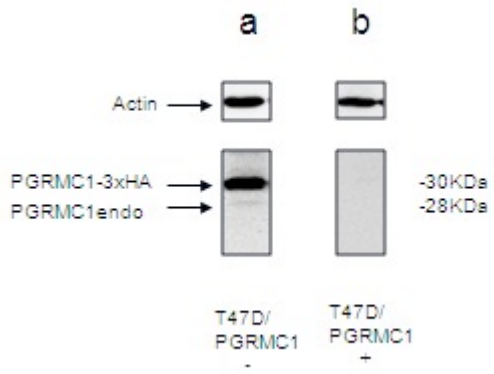


**Figure 31: Recombinant PGRMC1 of T47D/PGRMC1-3HA cells.** Western blot analysis of lysates prepared from T47D/PGRMC1-3HA cells. (a) PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 antibody followed by anti-rabbit horseradish peroxidase antibody. Signals are visualized with chemiluminescence. PGRMC1-3HA is detected at approximately 30 kDa. (b) No signals are detected. Here, the primary antibody was preincubated with recombinant PGRMC1. (Upper panels) The same lysates were incubated with an actin specific antibody for loading control.

### 3.5.2 PGRMC1 detection in supernatant from breast cancer cell lines

PGRMC1 is associated with secreted protein. We collected supernatant from the conditioned media without serum of breast cancer cell lines after 24 hours,

and then concentrated ten times prior to western blot. Supernatant was depurated through a cibacron blue column. Then PGRMC1 levels were detected by western blot. Theoretically, dead cells may release PGRMC1. Nevertheless, the cells used for the experiment were grown in cell cycle G1 and no cells were dead under this condition (24 hours without serum) [57]. We used slurry Separopore blue CL-6B to treat the samples. Separopore blue CL-6B is an agarose conjugate in saline suspension used to purify protein. PGRMC1 was analyzed through western blot in the supernatant from MCF-7/PGRMC1 (MCF-7 WT12) (Fig. 32A) or T47D/PGRMC1 cell lines (Fig. 32B). But, merely the rabbit anti-PGRMC1 antibody (#12444, Cell Signaling) could detect this band, under the identical situation the polyclonal antibody (G-21, Santa Cruz) did not. Therefore, the sensitivity of the antibody is important in this experiment.

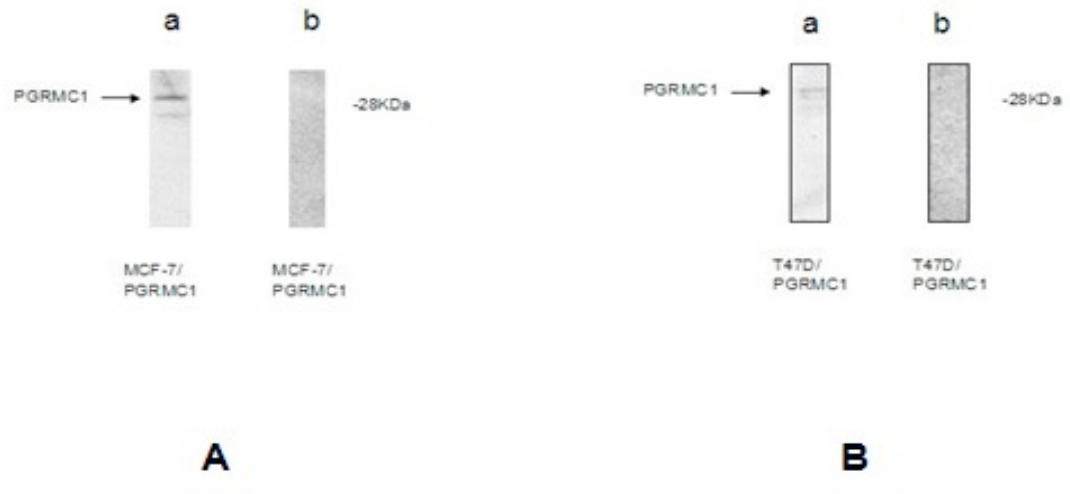


**Figure 32: Detection of PGRMC1 in supernatant from breast cancer cells by western blot analysis.** A) Conditioned media from MCF-7/PGRMC1 (MCF-7 WT12) were depurated through cibacron blue column and detected by western blot. The analysis shown the expression of PGRMC1 in the supernatant from MCF-7 WT12 cells used the polyclonal rabbit anti-PGRMC1 (Cell Signaling) (a) or the G-21 antibody (Santa Cruz) (b). The rabbit anti-PGRMC1 (Cell Signaling) measured the band, while the G-21antibody did not under the identical situation. B) Conditioned media from T47D/PGRMC1 cells were depurated through cibacron blue column and detected by western blot. The analysis shown the expression of PGRMC1 in the supernatant from T47D/PGRMC1 cells used the polyclonal rabbit

anti-PGRMC1 (Cell Signaling) (a) or the G-21 antibody (Santa Cruz) (b). The rabbit anti-PGRMC1 (Cell Signaling) measured the band, while the G-21antibody did not under the identical situation. For A and B, the supernatant was obtained and concentrated ten times prior to western blot.

### 3.5.3 PGRMC1 detection in plasma from breast cancer patients

We purified plasma from premenopausal and postmenopausal breast cancer patients, then PGRMC1 was analyzed by western blot. MCF-7/PGRMC1 (MCF-7 WT12) cell lines were utilized as positive control. PGRMC1 expression was determined only in MCF-7 WT12 cell line, while under the same experimental conditions this protein was not detectable in the plasma (Fig. 33).

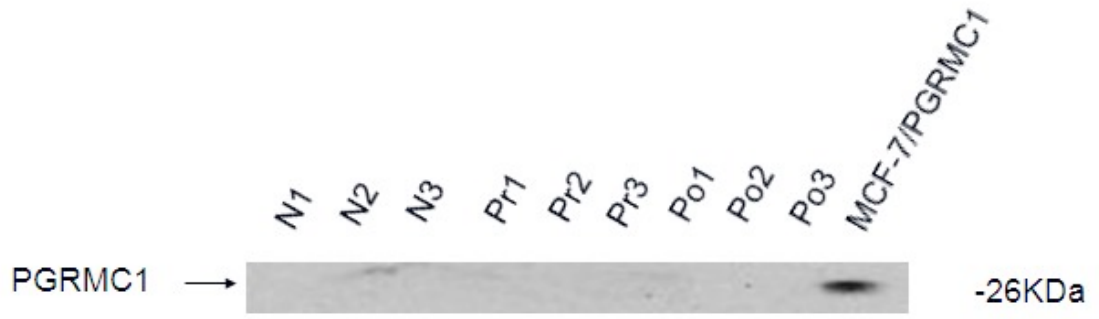


**Figure 33: PGRMC1 expression in plasma from breast cancer patients.** Plasma specimen was depurated through a cibacron blue column. ‘‘N’’ refers to normal control, and ‘‘Pr’’ means premenopausal breast tumor patients and ‘‘Po’’ refers to postmenopausal breast tumor patients. PGRMC1 expression was analyzed by western blot. PGRMC1 is detected with a polyclonal rabbit anti-PGRMC1 (Cell Signaling) followed by anti-rabbit horseradish peroxidase antibody.

# 4. Discussion

Breast cancer is the most prevalent tumor in women. According to statistics, women diagnosed with breast cancer is more than 1,000,000 per year and over 410,000 die of this disease [58]. PGRMC1 has been involved in breast tumor for a long time. Some studies have shown that PGRMC1 would be significant in carcinogenesis and elevate the risk of breast cancer.

PGRMC1 includes a cytochrome b5 region and has protein and phosphorylation binding sites for SH2 and SH3 regions of tyrosine kinases [1], which is a member of membrane-associated progesterone receptor (MAPR) family and is widely distributed in eukaryotic organisms [1]. PGRMC1 combined with some proteins participated in xenobiotic (Cyp3A4) [11, 59], hormone (Cyp21) [11], and sterol metabolisms (Cyp51) [11, 59, 60] and RNA binding (PAIR-BP1) [20, 61] in kidney tissue. Furthermore, PGRMC1 combines P450 proteins and plays activation in metabolism of cholesterol and hormone [11, 59, 62]. PGRMC1 has been detected in several tumors, such as including colon, lung, breast, thyroid, and ovary cancer [35, 63-67]. In addition, PGRMC1 is required for cancer cell apoptosis resistance, anchorage independent growth, cancer growth, and metastasis [57, 61, 68].

In this study, we demonstrated that ER-α plays a significant effect in the signal transduction of PGRMC1 activated by progestins and the presence of PGRMC1 can sensitize E2-induced PS2 mRNA levels. Furthermore, we evaluated the level of RANKL and RANK in various breast tumor cell lines and effect of estradiol and progestins on over-expressing RANKL cells. This was extended by findings showing that let-7i targets PGRMC1 and negatively affects PGRMC1 expression in breast tumor cells MCF-7. Moreover, PGRMC1

was present in breast cancer cell lines and could be secreted by these cells.

## 4.1 Proliferation of breast cancer cell lines endogenously expressing PGRMC1 with and without co-expressing ER-alpha

The interventional WHI (Women's Health Initiative) trial and Million Women trial demonstrated a probable relationship between progestin treatment and improved risk for breast tumor in women after menopause [69, 70]. In contrast to this, in French E3N-EPIC study which enrolled about 80,000 women after menopause, hormone therapy that included progestins MPA or NET induced an increased breast cancer risk [71, 72]. Nevertheless, in the WHI study, estrogen did not increase but to reduce the risk of breast cancer [73]. Progestogens are conventionally thought to perform their influences by the position of progesterone receptors (PR) in cells. Some research indicated that progestogen might play an anti-proliferative effect via PR in breast cancer cell lines [74-76]. These results are contrasted with the above-mentioned observation. Additional results suggested the proliferation of synthetic progestogens [77, 78]. Therefore, the exact mechanism of progestogens effects on human breast cancer is unknown. This may be depend on the specific conditions of cells.

PGRMC1 was found to be interrelated with the membrane-associated progesterone receptor activity. Furthermore, PGRMC1 is also highly expressed in many tumors and in tumor cells such as breast cancer. ER-α has a wide extent of physiological activation as a ligand transcription factors and overexpressed in approximately 75% of breast tumors. In humans, ER-α is encoded by the gene ESR1 [79]. ER-α over-expression is a major factor in human breast cancer prognosis. ER-α plays a significant effect in breast tumor

progression as well as outcome of patients [80].

We have surveyed the proliferation of progesterone and various synthetic progestins that are applied for hormone therapy on MCF-7 and MCF-7 WT12 (MCF7/PGRMC1) cells in vitro. In MCF-7 cell lines, DYD, DSG, DRSP, NET and LNG raised the proliferative effect, the influence is maximum for NET. In MCF-7 WT-12, these hormone detected an obviously improved than in MCF-7. However, it was found no effect in NOM, P and CMA [50]. The presence of PGRMC1 sensitizes some synthetic progestins induced proliferative effect in MCF-7 cells.

Our study has shown that some breast cancer cells, for example BM and SUM225CWN express PGRMC1. Interestingly ER-α was expressed in BM but not in SUM225CWN cells. The results of their proliferation assays with estradiol and all progestins are different. In BM cells, MPA, NET, LNG DSP significantly increased the proliferation and no significant effect was found for P4, NOM, DYD and CMA. The role of progestins on BM cells might rely on the particular pharmacology. However, these hormones didn’t obviously alter the proliferation of SUM225CWN. Thus ER-α plays a significant effect in the signal transduction of PGRMC1 activated by progestins. However, the exact mechanism is still unclear. Therefore, in order to verify this hypothesis and make it even more conclusive evidence, knockout or overexpression experiments for ER-α should be performed in the future and observe the proliferative effect of estradiol and progestins.

## 4.2 PGRMC1 sensitizes PS2 expression by E2-induced

PS2 (also called Trefoil factor 1) is a cysteine-rich acidic secreted protein [81, 82] and over-expressed in several tumors, including kidney, colon [83-85] and breast cancer [86, 87]. PS2 is a classical estrogen-regulated gene through an

ERE (estrogen response element) in its promoter [82]. Masiakowski and colleagues found that expression of the pS2 gene is controlled by estrogen in MCF-7 cells [88]. Our study confirms that estradiol can increase the mRNA levels of PS2 in the MCF-7 cell line. The promoter PS2 includes activator protein 1 (AP-1) and ERE site. Therefore, AP-1 site with ERE intermediate estradiol-induced the expression of PS2 [89, 90]. Furthermore, our investigations showed that the E2-induced PS2 expression is more pronounced in PGRMC1-expressing cells, such as in WT-12 cells. As shown in earlier experiments breast cancer cells over-expressing PGRMC1 are more sensitive towards the E2-induced proliferative effect. According to our results this proliferative effect might be mediated via an enhanced expression of PS2.

To explore which possible receptor-mediated mechanisms are included in the E2-induced PS2 expression in MCF-7 cell lines, antagonists of the ER-α receptor (fulvestrant), CK2 receptor (TBCA) or PGRMC1 (AG 205) were used. The presence of fulvestrant was able to obviously block the effect of E2. We have known that ER-α could induce several downstream genes level including PS2 [91]. Therefore, PGRMC1 may participate in the signal transduction of ER-α and PS2 activation by estrogen. The mechanism of interaction is still unclear.

CK2 (Casein kinase Ⅱ) is a conserved kinase protein that is constituted of catalysis and regulatory domains [92]. With a ubiquitous activity this enzyme has nearly 450 known substrates [93] and targets about 20% of the phosphoproteome [94]. CK2 was found to have both cytosolic and nuclear localization in eukaryotes cells and participated in important physiological procedures such as cellular proliferation, apoptosis and tumorigenesis [95, 96]. CK2 is a “lateral” (non-hierarchical) kinase [97] and has multiple effects on the signaling landscape in cancer [98]. CK2 might be a potential interaction sites

with PGRMC1. Therefore, we studied the effect of a serine/threonine kinase CK2 in PS2 expression through breast cancer cells over-expressing PGRMC1. We used the selective CK2 inhibitor, tetrabromocinnamic acid (TBCA), which acts as a potent and ATP-competitive inhibitor of Casein kinase Ⅱ. Surprisingly, treatment with TBCA decreased the E2-induced PS2 mRNA levels in PGRMC1 over-expressing cells. Therefore, CK2 is also intimately intertwined with PS2 and PGRMC1.

## 4.3 RANKL and RANK expression in various breast cancer cells

RANK belongs to the transmembrane protein family [99]. The gene resides on the human chromosome 18q22 [100, 101]. RANK is detected in osteoclasts and is involved in their differentiation and activation. RANK protein is also expressed on T cells, B cells, dendritic cells, and fibroblasts [102, 103]. Human RANK is constituted of 383 amino acids which participated in NF-κB function [104]. The binding of RANK could cause the activation of several signal transduction pathways [105, 106].

RANKL, a ligand for RANK, is a type II transmembrane protein and is found on the stromal cells, osteoblasts, and T-cells [107-109]. RANKL gene is found on 13q14 and includes about 6 exons [102]. RANKL is a tumor necrosis factor (TNF)-related cytokine coded for by a single gene. However, the RANKL protein exists in three isoforms [110] : two of which possess a transmembrane domain of either 317 or 270 aa and a third isoform of 243 aa that acts as a soluble ligand (sRANKL). The expression of RANKL is influenced in stromal and osteoblast cells by several elements. It was detected in fewer organizations, yet at a high level in lung and thymus, as well as low level in other organs such as spleen [111]. Some studies shown that RANKL was still

detected in breast epithelium only at pregnancy or lactation period [112]. It was also expressed by some cancer cells, so it could stimulate cancer cells proliferation [113] through autocrine and paracrine mode.

RANK and RANKL are involved in bone remodeling. Bone remodeling cycle is closely adjusted through RANK, RANKL, and osteoprotegerin (OPG). RANK mRNA expression is at a high levels through osteoclasts [114], and RANKL is expressed by most bone osteoblasts [115]. RANKL combines to RANK of osteoclasts, which results in osteoclast maturation [116, 117], increased osteoclast activity [117], and inhibits apoptosis of osteoclast [118]. OPG can inhibit RANK-RANKL interaction and osteoclast activity through combining to RANKL.

Mouse presented invalid for RANK and RANKL by gene melting exhibited disturbed lobulo-alveolar structures during pregnancy, eliminating lactation [112, 115]. Deletion of the construction was owing to decreased differentiation and increased apoptosis of alveolar bud in functional breast during lactation [112]. Other studies found that RANKL [119] and RANK were also involved in ductal side-branching, alveolar differentiation and lumen formation in breast [120].

RANK/RANKL were originally shown to make a significant effect in physiological processes of T-cells, dendritic, and osteoclasts cells [121]. Recent research has demonstrated that RANK/RANKL play an essential effect in breast carcinogenesis and bone metastases [122-125]. Some studies reported that RANK/RANKL protects human breast cancer cells and avoids apoptosis [124] and increases in motility in this cell lines [123]. Other studies shown that RANK and RANKL signal transduction blocked could slow bone metastases [126].

In 1999, Thomas and coworkers [127] first detected that breast tumor cells MCF-7, MDA-MB-231, T47D and breast tumor tissues display RANK, but didn't display RANKL. However, other studies lately demonstrated that RANKL was also detected in MDA-MB-231and MCF-7 cell lines [122, 125, 128, 129]. The expression level of RANKL mRNA and protein were detected and up-regulated under hypoxic conditions in MCF-7 cells [130]. In our experiments, RANK expression was verified in MDA-231 and Cal72 cell lines, while under the same experimental conditions MCF-7 and T47D did not detect RANK expression by western blot. Furthermore, we didn't find any constitutively expressed RANKL in various cancerous breast epithelial cell lines using western blot. The different results might owing to the basis level of RANKL expression is relatively low in these cell lines. Another possible reason may be the detection methods, as it is known that the expression level of mRNA is not always fully translated into protein. Therefore, we could use some methods to up-regulate the expression of RANKL, for example hypoxia inducible factor 1 (HIF-1). Since hypoxia can elevate RANK and RANKL expression in human breast cancer cell lines [130]. Another way, sensitive antibody should be recommended for detection of RANK and RANKL by western blot. Recent research suggested that the expression of RANKL in breast tumor cell lines was enhanced after co-cultured with primary osteoblasts like cells. Schubert and colleagues reported that co-cultured HCC70 cell lines with human primary osteoblasts increased RANKL expression[131]. Consistently, other studies also found that MDA-MB-231 cell lines were co-cultured with stromal cells and osteoblasts caused RANKL mRNA expression [132]. Therefore, using a co-culture system, we analyzed the RANKL level in breast tumor cells. However, co-cultured MCF-7/PGRMC1 and MDA-MB-231 cells with osteoblast-like cells didn't induced RANKL expression. The possible reason may be the detection methods, as it is known

that the expression level of mRNA is not always fully translated into protein. Different culture conditions and heterogeneity of cell lines may be another possible reason.

According to results of animal experiments RANK/RANKL also appear to be essential in mediating the progesterone effects on breast development via progesterone receptor B [112, 133]. MPA therapy, which is applied for progesterone contraceptives and HRP, is related to an improved breast cancer risk. MPA leads to an affluent RANKL-induced of RANKL in breast epithelium and the absence of RANKL reduced the occurrence of breast tumor induced by MPA. RANK-defect mouse was a significantly retarded in hormone and oncogene induced carcinogenesis. RANKL-RANK signal transduction was needed to the survival and proliferation for breast cancer cells [124]. Thus, these studies imply that progesterone-RANKL-RANK axes make a critical effect in breast cancer initiation. Our study showed that estradiol and progestins didn't significantly alter the proliferation of L-cells, over-expressing RANKL cell lines, and change the expression of RANKL. These observations may be due to the character of L-cells being different from tumor cells. L-cells are derived from a common totipotent stem cell. Furthermore, L-cells are estrogen receptor- negative cell lines. It may be the reason that progestins didn't alter the proliferation of L-cells.

## 4.4 Let-7i regulates mRNA of PGRMC1

In spite of PGRMC1 express in several tumors and advance tumorigenesis, the detailed mechanisms regulating its expression are still little known. Some research showed that miRNAs could be involved in some genes expression and might regulate the expression of PGRMC1. MiRNA is 18-25 nucleotide (nt) small RNAs, which usually bind in the 3'-untranslated region of mRNA, resulting in degradation or translational repression of mRNAs, and potentially

lower levels of protein expression [134-138]. MiRNA makes a significant effect in almost every biological functions, such as development time, cell proliferation, and differentiation [139]. Deregulation of miRNA may related with human malignancies, occurrence, development, and outcomes [140]. Moreover, increasing results indicate that miRNA regulates cellular proliferation via various mechanisms. There are some miRNAs up-regulated in breast cancer, such as let-7, miRNA-125b, and miRNA-145 [141-143].

Let-7 is the second to be discovered miRNA [144]. Some research indicated that let-7 sequence of C. elegans is more conserved than mankind, which offered a role to miRNA as the genetic fundamental moderators in variety of living creatures [145]. Let-7 family miRNAs are important for carcinogenesis [146]. In mammals, let-7 is able to adjust a variety of oncogenes, for example RAS [147], MYC [147, 148], and HMGA2 [149]. Therefore, aberrant level of let-7 could lead to tumorigenesis and metastasis of several tumors, including breast, lung, colon, and ovarian cancer [142, 143, 150, 151]. While some genes targeted by let-7 miRNA were discovered, our study found another gene, PGRMC1. This research suggested that overexpression of let-7i negatively affects PGRMC1 expression in MCF7 cells, which probably offered an alternative interpretation for breast tumorigenesis.

The length of PGRMC1 3'-UTR is 1212bp. Current research indicated that let-7i inhibits the expression of PGRMC1. A study of silicon suggested that let-7 bound to the 3'-UTR of PGRMC1 [152]. The 3'-UTR of PGRMC1 was presumed binding the let-7 subtypes [152]. Next, bioinformatics revealed that let-7 accordant with the sequence of PGRMC1 3'-UTR, suggesting PGRMC1 may be a target of let-7, which was further confirmed by the cell transfection with miRNA [153].

To analysis the impact of let-7 on the mRNA level of PGRMC1, we transfected let-7i mimic into MCF-7 cells. After 2 days, Let-7i mimic down-regulated the expression of PGRMC1. qPCR detected that let-7i repressed PGRMC1 expression at the mRNA level. Over-expression of let-7 in MCF-7 cells inhibited the expression of PGRMC1 mRNA. To date, over 1,000 miRNAs have been found in humans (http://www.sanger.ac.uk/Software /). Whereas the number of mRNAs is about 30,000. Thus, a single miRNA may regulate many kinds of mRNA between the miRNA and mRNA [154]. Wendler and colleagues found that let-7i regulated PGRMC1 expression in ovarian cancer cells [152]. For this research, we found that let-7 miRNAs target PGRMC1 and negatively affects endogenous PGRMC1 mRNA expression in breast cancer MCF7 cells.

Some binging sites for miRNAs were found in the PGRMC1 3'-UTR, such as let-7i [152]. Additionally, we have shown that let-7 miRNA targets PGRMC1 and reduces expression of PGRMC1 in MCF-7 cells. In breast cancer, PGRMC1 expression and let-7 miRNA appears negative correlation. Furthermore, PGRMC1 promotes cellular proliferation and tumorigenesis [1]. Therefore our finding indicates a new regulatory mechanism in PGRMC1 intermediated proliferation of breast tumor cells via miRNAs. Since let-7i makes a significant effect in the inhibition of some mRNAs participated in tumor advances, such as PGRMC1, which could be the candidate for drug therapies and the interaction has to be investigated in the future.

## 4.5 Secreted PGRMC1 by breast cancer cell lines

PGRMC1 was initially purified in endoplasmatic reticulum from swine liver cells [2]. PGRMC1 includes several interplay regions for phosphorylation [155]. PGRMC1 is mainly situated in cellular membranes and colocalizes with Golgi apparatus or endoplasmic reticulum [57]. Some research also introduced

nucleus and chromosomal location [20, 156, 157]. PGRMC1 could be discovered in nuclear, microsomal, mitochondrial, cytosolic and membrane, indicating that it could change its subcellular localization in different cell lines. It was confirmed that PGRMC1 was exist in mammary tumors. Furthermore, Crudden et al. suggested that PGRMC1 was significantly higher in breast cancer than appropriate normal tissues [35]. Our results implicated that PGRMC1 was detected in mammary tumor cells and could be secreted by this cells. Thus, PGRMC1 is related to secreted proteins. It indicated the potential source of plasma PGRMC1.

PGRMC1 was expressed in breast tumor cells, while PGRMC1 was also expressed in the supernatant of the culture medium. Some studies implicated that PGRMC1 might conduce to the biological environment of tumorigenesis and could be used as biomarker or therapeutic target [158, 159]. PGRMC1 could be converted from membrane bound to cytoplasm form [24], and could positioned in the tumor cell secretory vacuoles [160]. Furthermore, breast tumor cells secrete PGRMC1. The research will demand confirmation of plasma in breast cancer patients compared to noncancer women in the next trials.

PGRMC1 belongs to secreted proteins which is an only basic composition and neurotrophic function [161], and combines to unconscious neuron receptors and promotes Akt signal [162]. PGRMC1 was secreted by tumor cells and was discovered in the supernatant from breast cancer cell lines by western blot. PGRMC1 promotes breast tumor cells growth and could lead to tumor formation. We first reported that PGRMC1 level was increased in supernatant of breast cancer cells. Nevertheless, the method demanded a refining procedure, which was not an easy step in clinical application. The sensitivity of antibody is important in this assay. Furthermore, we purified plasma of breast

tumor women. Then PGRMC1 was detected via western blot. However, PGRMC1 expression could not be detectable in plasma. The detection should use fewer purification procedures and a more sensitive antibody.

PGRMC1 plays a significant effect in cancer development and metastatic through resistant to apoptosis and be up-regulated in multiple cancers. Shakeel and colleagues showed that PGRMC1 could be discovered in patient plasmas with lung tumor and matched healthy patients. We described that PGRMC1 was detected in breast tumors. Furthermore, PGRMC1 is increased in ER-negative breast cancers [48]. This study demonstrated that PGRMC1 could be secreted by the breast tumor cell lines. The measurement of PGRMC1 levels could provide a method of determination who display a higher expression level of PGRMC1, and who may be predisposed to develop breast tumor. Therefore, PGRMC1 might be a predictor for cancer progression and a target for breast tumor treatment.

# 5. Conclusions

PGRMC1 is elevated in tissues of breast cancer patients and is important in tumorigenesis. Certain synthetic progestins may increase the proliferation of PGRMC1-expressing breast tumor cells, while progesterone and other synthetic progestins such as nomegestrol (NOM) or chlormadinone acetate (CMA) reacted neutrally. Our findings point out an important role of ER-α in the proliferative effect induced by progestins.

We confirm that the expression of the ER responsive gene pS2 is increased by estrogen in MCF-7 cells. Furthermore, we can demonstrate the new finding that the presence of PGRMC1 can sensitize E2-induced PS2 mRNA levels indicating for the first time that PGRMC1 may participate in the transcriptional regulation of PS2 by ER. In blocking experiments, antagonists of CK2 (TBCA), ER-α (fulvestrant) and PGRMC1 (AG 205) were able to block the effect of E2 corroborating our new finding and suggesting that CKII may be involved. Fittingly, PGRMC1 contains two putative CKII phosphorylation sites.

Since mice experiments published elsewhere [124] suggested an association of progesterone action and RANK/RANKL system we tried to investigate if PGRMAC1 may be involved in vitro. We were not able to detect RANKL endogenous expression in different breast cancer cells using western blot. Besides that progestins didn't significantly up-regulate RANKL expression in normal and PGRMC1 overexpressing cells. From that we conclude that in vitro a different unknown factor is missing, which may be present in vivo and may be responsible for establishing the progesterone-RANK/RANKL axis.

Regulation of PGRMC1 is quite unresolved, yet. Our findings confirmed

regulation of PGRMC1 expression by miRNA let-7i, in breast cancer cell lines, which has previously been shown in ovarian cancer cells [152], overexpression of let-7i negatively affects the expression of PGRMC1 in MCF7 breast cancer cells. Since let-7i is considered to be a suppressor inhibiting the growth of malignant tumor cells by acting RAS [147], HMGA2 [149, 163], and c-Myc [164] including PGRMC1, this miRNA may be a candidate target for future drug therapy.

PGRMC1 is expressed in breast tumor cells as well as has been suggested to have a prognostic effect in HRT. Therefore, an easy test of its expression in health women would be very helpful. The survey of PGRMC1 levels could provide a method of determination who display an increased expression level of PGRMC1 and who is likely to develop breast tumor treatment with HRT. It has previously suggested that PGRMC1 can be detected in plasma [160]. PGRMC1 might be a predictor for cancer progression and a target for breast tumor treatment. We were able to detect PGRMAC1 in supernatants from PGRMC1 overexpressing cells but not in the plasma of a small cohort of healthy persons and breast cancer patients. Further experiments are now needed to address the source of plasma PGRMC1 from breast tumor patients.

In conclusion, our findings shown that ER-α and PGRMC1 signaling seem to be linked. PGRMC1 expression was adjusted through complex mechanism that relates the repression action of let-7i, which may be a potential therapeutic target for breast cancer. Furthermore, PGRMC1 is related to secreted proteins and that the level of secreted PGRMC1 is potential biomarker for breast cancer risk.

# Zusammenfassung

Nach wie vor steht die Prävalenz von Brustkrebs bei Frauen an erster Stelle vergesellschaftet mit einer relativ hohen Todesursache. Die Progesteronrezeptor-membrankomponente-1 (PGRMC1), die in Mammakarzinomgewebe stärker exprimiert wird als in gesundem Stromagewebe, könnte eine wichtige Rolle in der Genese des Mammakarzinoms spielen. In früheren Untersuchungen konnte gezeigt werden, dass Estrogene und einige synthetische Gestagen über PGRMC1 eine erhöhte Proliferation in Mammakarzinomzellen induzieren können, ein Hinweis darauf, dass die Art des Estrogens und des Gestagens wichtig sein dürfte in Hinblick auf das Brustkrebsrisiko unter einer Hormontherapie in der Postmenopause. Allerdings ist noch unklar über welche Mechanismen dieser Effekt generiert wird und wie die endogene Expression von PGRMC1 reguliert werden kann. Offen ist ebenfalls die Frage, ob PGRMC1 von Brustkrebszellen in das Blut abgegeben wird und somit für ein Screening hinsichtlich eines möglichen erhöhten Brustkrebsrisikos verwendet werden könnte. In der vorliegenden Arbeit wurden die Proliferationsstimulierung via PGRMC1, Beeinflussung der Expression von PGRMC1 sowie mögliche Mechanismen der Signaltransduktion in verschiedenen Mammakarzinomzell-Linien sowie die Sekretion von PGRMC1 durch Karzinomzellen untersucht. In BM Zellen (endogene Expression von Estrogenrezeptor (ER-α) und PGMRC1), erhöhten Medroxyprogesteronacetat (MPA), Norethisteron (NET), Levonorgestrel (LNG) und Drospirenon (DRSP) signifikant die Proliferation. Allerdings konnten diese Hormone die Proliferation von SUM225CWN Zellen (nur endogenes PGRMC1) nicht stimulieren. In MCF-7 Zellen war die Anwesenheit von PGRMC1 mit einer Sensitivierung gegenüber dem Estradiol(E2)-induzierten PS2 mRNA Level, einem estrogenem Zielgen, vergesellschaftet. Interessanterweise konnte zum erstenmal nachgewiesen werden, dass auch bestimmte Gestagene wie NET in der Lage waren die PS2 Expression in MCF-7/PGRMC1 Zellen zu erhöhen, wohingegen kein Effekt für das natürliche Progesteron gefunden wurde. Diese Wirkung von E2 und NET konnte durch die Zugabe von Antagonisten gegenüber ER-α, PGRMC1 und CK2, einer Kinase, die in die Tumorzellproliferation involviert ist, blockiert werden, wobei der Effekt des ER-α-Antagonisten am stärksten war. Um eine mögliche Rolle von RANK/RANKL zu untersuchen, wurde zunächst die Expression dieser Komponenten in verschiedenen Mammakarzinomzell-Linien bestimmt. Mittels Westernblot konnte allerdings in keiner der untersuchten Zelllinien eine Expression von RANK oder RANKL nachgewiesen werden. In RANKL überexprimierenden, ER-α-negativen und PGRMC1-negativen Zellen konnte weder durch Estradiol noch durch Gestagene eine Erhöhung der Zellproliferation noch der RANKL-Expression bewirkt werden. In Kokulturen

von Osteoblasten mit Mammakarzinomzell-Linien mit und ohne Überexpression von PGRMC1 wurde versucht eine Induktion von RANK bzw. RANKL in den Mammakarzinomzell-Linien zu generieren. Dies gelang leider nicht, so dass die Rolle von RANK/RANKL als Mediator von Hormoninduzierten Proliferationen von PGRMC1 überexprimierenden Zellen noch offen ist. Als möglicher Mechanismus der Beeinflussung der PGRMC1-Expression wurden MCF-7 Zellen mit einem miRNA Let-7i Analogon transfiziert, da Let-7i als Tumorsuppressor fungiert. In der vorliegenden Arbeit konnte gezeigt werden, dass Let-7i die endogene PGRMC1-Expression inhibieren kann. PGRMC1 konnte im Überstand von verschiedenen Mammakarzinomzell-Linien mittels Westernblot nachgewiesen werden. Allerdings waren die ersten Versuche PGRMC1 in Plasmaproben von Patientinnen mit und ohne Mammakarzinom nachzuweisen nicht erfolgreich.

Zusammenfassend ist festzustellen, dass ER-α eine wichtige Rolle in der Signaltransduktion von PGRMC1 nach Aktivierung durch Estradiol und synthetische Gestagene spielen dürfte und dass eine Interaktion von PGRMC1 mit ER-α den PS2 Signalweg stimulieren kann. Die PGRMC1-Expression kann möglicherweise durch verschiedene miRNAs wie z.B. Let-7i beeinflusst werden und bietet somit ein mögliches therapeutisches Ziel. Des Weiteren könnte PGRMC1 ein prädiktiver Biomarker für das Brustkrebsrisiko in Frauen unter Hormontherapie darstellen.

# Curriculum Vitae

Personal Information:

Family Name: Ma

Given Name: Bo

Date of Birth: 26. 12. 1978

Nationality: Chinese

Gender: Male

## Education

| Institution/Location | Years | Degree | Field of Study |
|---|---|---|---|
| Zhejiang University, China | 1997.09-2002.07 | Bachelor | Clinical Medicine |
| Soochow University, China | 2007.09-2012.06 | Master | Medicine（Surgery） |
| University of Tübingen, Germany | 2013.01-Present | Dr. Med Candidate | Oncology (Breast cancer) |

## Professional Experience

| Institution/Location | Years | Title |
|---|---|---|
| The First Affiliated Hospital of Zhejiang University, China | 2001.06-2002.06 | Intern |
| Huzhou Center Hospital, China | 2002.06-2007.06 | Resident |
| Huzhou Center Hospital, China | 2007.06-2013.12 | Attending physician |
| Fudan University Cancer Hospital, Fudan University Cancer Institute, China | 2010.04-2011.06 | Visiting scholar |
| Huzhou Center Hospital, China | 2014.01-Present | Associate chief physician |

# Acknowledgments

It is my pleasure to have the opportunity to study and do research in the Center for tumor progression, University of Tübingen, Germany. I would like to express my thanks to everyone who has given me any help and support during my stay here.

First of all, I wanted to extent my sincerest appreciation to my supervisor, Prof. Dr. Harald Seeger, for his assistance, support and guidance, valuable advice during the progress of research. I would also like to thank for his valuable input and suggestions for this dissertation. The knowledge and wisdom he has imparted upon me will, without any doubt, forever remain a major contributor behind my every single step forward in the future. Dr. Harald Seeger provided an ideal environment for me to learn and grow as a scientist.

I would also like to thank Dr. Hans Neubauer for his constructive advice, sharing his expertise throughout my research.

I would like to thank my colleague Qianqian Ma, for her assistance and technical supports in organizing the lab work. Her friendliness, patience made our lab an exciting place to work. I am also thankful to all other colleagues of the Institute of Tumor progression for making the institute a good place to work.

My heartfelt thanks to my colleagues and friends, past and present: Silke, Christina, Helen, Beate, Cathlin, Wenling Ji, for their great friendship and the happy time sharing with me in Tübingen.

Family support served as the foundation upon which I stood during my study. I am indebted to my parents for their caring, support and love throughout my life. I am also thanks to my parents-in-law for support and understanding.

Last but not least, I want to express my profound gratitude to my wife and my sons for their always unconditional support, encouragement, patience and love while I returned to school to obtain this degree. I hope one day I will be able to pay back all the time that I took from them. Without their patient love and stimulating support, I believe this work can’t have been completed.

# Declaration of authorship

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

Some of the quantitative real-time PCR results were obtained with the help of a college in our lab, Qianqian Ma, from China.

# Reference


1. Cahill, M.A., *Progesterone receptor membrane component 1: an integrative review.* The Journal of steroid biochemistry and molecular biology, 2007. **105**(1): p. 16-36.
2. Meyer, C., et al., *Purification and Partial Sequencing of High-Affinity Progesterone-Binding Site (s) from Porcine Liver Membranes.* European Journal of Biochemistry, 1996. **239**(3): p. 726-731.
3. Falkenstein, E., et al., *Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells.* Biochemical and biophysical research communications, 1996. **229**(1): p. 86-89.
4. Selmin, O., et al., *Isolation and characterization of a novel gene induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in rat liver.* Carcinogenesis, 1996. **17**(12): p. 2609-2615.
5. Falkenstein, E., et al., *Chemical modification and structural analysis of the progesterone membrane binding protein from porcine liver membranes.* Molecular and cellular biochemistry, 2001. **218**(1-2): p. 71-79.
6. Selmin, O., et al., *Transcriptional activation of the membrane-bound progesterone receptor (mPR) by dioxin, in endocrine-responsive tissues.* Molecular reproduction and development, 2005. **70**(2): p. 166-174.
7. Runko, E., C. Wideman, and Z. Kaprielian, *Cloning and expression of VEMA: a novel ventral midline antigen in the rat CNS.* Molecular and Cellular Neuroscience, 1999. **14**(6): p. 428-443.
8. Raza, F.S., et al., *Identification of the rat adrenal zona fasciculata/reticularis specific protein, inner zone antigen (IZAg), as the putative membrane progesterone receptor.* European Journal of Biochemistry, 2001. **268**(7): p. 2141-2147.
9. Runko, E. and Z. Kaprielian, *Caenorhabditis elegans VEM-1, a novel membrane protein, regulates the guidance of ventral nerve cord-associated axons.* The Journal of neuroscience, 2004. **24**(41): p. 9015-9026.
10. Hand, R.A., et al., *Saccharomyces cerevisiae Dap1p, a novel DNA damage response protein related to the mammalian membrane-associated progesterone receptor.* Eukaryotic cell, 2003. **2**(2): p. 306-317.
11. Hughes, A.L., et al., *Dap1/PGRMC1 binds and regulates cytochrome P450 enzymes.* Cell metabolism, 2007. **5**(2): p. 143-149.
12. Yoshitani, N., et al., *A structure-based strategy for discovery of small ligands binding to functionally unknown proteins: Combination of in silico screening and surface plasmon resonance measurements.* Proteomics, 2005. **5**(6): p. 1472-1480.
13. Song, J., et al., *Letter to the Editor: Hypothetical protein At2g24940. 1 from Arabidopsis thaliana has a cytochrome b5 like fold*.* Journal of biomolecular NMR, 2004. **30**(2): p. 215-218.
14. Nölte, I., et al., *Localization and topology of ratp28, a member of a novel family of putative steroid-binding proteins.* Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 2000. **1543**(1): p. 123-130.

15. Hand, R.A. and R.J. Craven, *Hpr6. 6 protein mediates cell death from oxidative damage in MCF - 7 human breast cancer cells.* Journal of cellular biochemistry, 2003. **90**(3): p. 534-547.
16. Beausoleil, S.A., et al., *Large-scale characterization of HeLa cell nuclear phosphoproteins.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(33): p. 12130-12135.
17. Sauer, G., et al., *Proteome analysis of the human mitotic spindle.* Molecular & cellular proteomics, 2005. **4**(1): p. 35-43.
18. Sakamoto, H., et al., *Expression and localization of 25-Dx, a membrane-associated putative progesterone-binding protein, in the developing Purkinje cell.* Neuroscience, 2004. **126**(2): p. 325-334.
19. Peluso, J.J., et al., *Expression and function of PAIRBP1 within gonadotropin-primed immature rat ovaries: PAIRBP1 regulation of granulosa and luteal cell viability.* Biology of reproduction, 2005. **73**(2): p. 261-270.
20. Peluso, J.J., et al., *Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action.* Endocrinology, 2006. **147**(6): p. 3133-3140.
21. Falkenstein, E., et al., *Specific progesterone binding to a membrane protein and related nongenomic effects on Ca2+-fluxes in sperm.* Endocrinology, 1999. **140**(12): p. 5999-6002.
22. Buddhikot, M., et al., *Recognition of a human sperm surface protein involved in the progesterone-initiated acrosome reaction by antisera against an endomembrane progesterone binding protein from porcine liver.* Molecular and cellular endocrinology, 1999. **158**(1): p. 187-193.
23. Labombarda, F., et al., *Effects of injury and progesterone treatment on progesterone receptor and progesterone binding protein 25 - Dx expression in the rat spinal cord.* Journal of neurochemistry, 2003. **87**(4): p. 902-913.
24. Leel, V., et al., *Identification of a truncated ratp28-related protein expressed in kidney.* Biochemical and biophysical research communications, 2004. **316**(3): p. 872-877.
25. Suchanek, M., A. Radzikowska, and C. Thiele, *Photo-leucine and photo-methionine allow identification of protein-protein interactions in living cells.* Nature methods, 2005. **2**(4): p. 261-268.
26. Engmann, L., et al., *Progesterone regulation of human granulosa/luteal cell viability by an RU486-independent mechanism.* The Journal of Clinical Endocrinology & Metabolism, 2006. **91**(12): p. 4962-4968.
27. Sun, L.-P., et al., *Insig required for sterol-mediated inhibition of Scap/SREBP binding to COPII proteins in vitro.* Journal of Biological Chemistry, 2005. **280**(28): p. 26483-26490.
28. Yang, T., et al., *Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER.* Cell, 2002. **110**(4): p. 489-500.
29. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* The Journal of clinical investigation, 2002. **109**(9): p. 1125-1131.
30. Goldstein, J.L., R.B. Rawson, and M.S. Brown, *Mutant mammalian cells as tools to delineate*

*the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis.* Archives of biochemistry and biophysics, 2002. **397**(2): p. 139-148.

31. Laird, S., G. Vinson, and B. Whitehouse, *Monoclonal antibodies against rat adrenocortical cell antigens.* Acta endocrinologica, 1988. **119**(3): p. 420-426.
32. Wright, M.C., G. Allenby, and A.J. Paine, *Effect of vitamin A deficiency on the expression of low affinity glucocorticoid binding site activity and glucocorticoid-dependent induction of CYP3A2 in rat liver.* Biochemical and biophysical research communications, 1997. **237**(2): p. 211-216.
33. Meffre, D., et al., *The membrane‐associated progesterone‐binding protein 25‐Dx is expressed in brain regions involved in water homeostasis and is up‐regulated after traumatic brain injury.* Journal of neurochemistry, 2005. **93**(5): p. 1314-1326.
34. Ulziibat, S., et al., *Identification of estrogen receptor β-positive intraepithelial lymphocytes and their possible roles in normal and tubal pregnancy oviducts.* Human Reproduction, 2006. **21**(9): p. 2281-2289.
35. Crudden, G., R. Loesel, and R.J. Craven, *Overexpression of the cytochrome p450 activator hpr6 (heme-1 domain protein/human progesterone receptor) in tumors.* Tumor Biology, 2005. **26**(3): p. 142-146.
36. Mallory, J.C., et al., *A novel group of genes regulates susceptibility to antineoplastic drugs in highly tumorigenic breast cancer cells.* Molecular pharmacology, 2005. **68**(6): p. 1747-1756.
37. Crudden, G., R.E. Chitti, and R.J. Craven, *Hpr6 (heme-1 domain protein) regulates the susceptibility of cancer cells to chemotherapeutic drugs.* Journal of Pharmacology and Experimental Therapeutics, 2006. **316**(1): p. 448-455.
38. Thiébault, K., et al., *The netrin-1 receptors UNC5H are putative tumor suppressors controlling cell death commitment.* Proceedings of the National Academy of Sciences, 2003. **100**(7): p. 4173-4178.
39. Arakawa, H., *Netrin-1 and its receptors in tumorigenesis.* Nature Reviews Cancer, 2004. **4**(12): p. 978-987.
40. Mehlen, P. and L. Mazelin, *The dependence receptors DCC and UNC5H as a link between neuronal guidance and survival.* Biology of the Cell, 2003. **95**(7): p. 425-436.
41. Llambi, F., et al., *Netrin‐1 acts as a survival factor via its receptors UNC5H and DCC.* The EMBO journal, 2001. **20**(11): p. 2715-2722.
42. Hope, H. and L. Pike, *Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains.* Molecular biology of the cell, 1996. **7**(6): p. 843-851.
43. Swanson, K.M. and R.J. Hohl, *Anti-cancer therapy: targeting the mevalonate pathway.* Current cancer drug targets, 2006. **6**(1): p. 15-37.
44. Duncan, R.E., A. El-Sohemy, and M.C. Archer, *Statins and cancer development.* Cancer Epidemiology Biomarkers & Prevention, 2005. **14**(8): p. 1897-1898.
45. Demierre, M.-F., et al., *Statins and cancer prevention.* Nature Reviews Cancer, 2005. **5**(12): p. 930-942.
46. Ji, S., A. Wu, and H. Yang, *Expression of progesterone receptor membrane component-1 is associated with the malignant phenotypes of breast cancer.* Journal of Southern Medical University, 2012. **32**(5): p. 635-638.
47. Causey, M.W., et al., *Transcriptional analysis of novel hormone receptors PGRMC1 and*

*PGRMC2 as potential biomarkers of breast adenocarcinoma staging.* Journal of Surgical Research, 2011. **171**(2): p. 615-622.

48. Neubauer, H., et al., *Breast cancer proteomics reveals correlation between estrogen receptor status and differential phosphorylation of PGRMC1.* Breast Cancer Res, 2008. **10**(5): p. R85.
49. Lundgren, K., C. Holm, and G. Landberg, *Hypoxia and breast cancer: prognostic and therapeutic implications.* Cellular and molecular life sciences: CMLS, 2007. **64**(24): p. 3233-3247.
50. Ruan, X., et al., *Progestogens and membrane-initiated effects on the proliferation of human breast cancer cells.* Climacteric, 2012. **15**(5): p. 467-472.
51. Neubauer, H., et al., *The presence of a membrane-bound progesterone receptor sensitizes the estradiol-induced effect on the proliferation of human breast cancer cells.* Menopause, 2011. **18**(8): p. 845-850.
52. Ruan, X., et al., *Nomegestrol acetate sequentially or continuously combined to estradiol did not negatively affect membrane-receptor associated progestogenic effects in human breast cancer cells.* Gynecological Endocrinology, 2012. **28**(11): p. 863-866.
53. Neubauer, H., et al., *Overexpression of progesterone receptor membrane component 1: possible mechanism for increased breast cancer risk with norethisterone in hormone therapy.* Menopause, 2013. **20**(5): p. 504-510.
54. Neubauer, H., et al., *New insight on a possible mechanism of progestogens in terms of breast cancer risk.* Hormone Molecular Biology and Clinical Investigation, 2011. **6**(1): p. 185-192.
55. Lange, S.D., et al., *Membrane‐initiated effects of progesterone on calcium dependent signaling and activation of VEGF gene expression in retinal glial cells.* Glia, 2007. **55**(10): p. 1061-1073.
56. Liu, L., et al., *Progesterone increases rat neural progenitor cell cycle gene expression and proliferation via extracellularly regulated kinase and progesterone receptor membrane components 1 and 2.* Endocrinology, 2009. **150**(7): p. 3186-3196.
57. Ahmed, I.S., et al., *Progesterone receptor membrane component 1 (Pgrmc1): a heme-1 domain protein that promotes tumorigenesis and is inhibited by a small molecule.* Journal of Pharmacology and Experimental Therapeutics, 2010. **333**(2): p. 564-573.
58. Coughlin, S.S. and D.U. Ekwueme, *Breast cancer as a global health concern.* Cancer epidemiology, 2009. **33**(5): p. 315-318.
59. Szczesna-Skorupa, E. and B. Kemper, *Progesterone receptor membrane component 1 inhibits the activity of drug-metabolizing cytochromes P450 and binds to cytochrome P450 reductase.* Molecular pharmacology, 2011. **79**(3): p. 340-350.
60. Mansouri, M.R., et al., *Alterations in the expression, structure and function of progesterone receptor membrane component-1 (PGRMC1) in premature ovarian failure.* Human molecular genetics, 2008. **17**(23): p. 3776-3783.
61. Peluso, J.J., J. Romak, and X. Liu, *Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations.* Endocrinology, 2008. **149**(2): p. 534-543.
62. Min, L., et al., *Molecular identification of adrenal inner zone antigen as a heme‐binding*

*protein.* FEBS Journal, 2005. **272**(22): p. 5832-5843.

63. Peluso, J.J., et al., *Regulation of ovarian cancer cell viability and sensitivity to cisplatin by progesterone receptor membrane component-1.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(5): p. 1592-1599.
64. Dressman, H.K., et al., *Gene expression profiles of multiple breast cancer phenotypes and response to neoadjuvant chemotherapy.* Clinical Cancer Research, 2006. **12**(3): p. 819-826.
65. Irby, R.B., et al., *Iterative Microarray and RNA Interference-Based Interrogation of the Src-Induced Invasive Phenotype.* Cancer research, 2005. **65**(5): p. 1814-1821.
66. Difilippantonio, S., et al., *Gene expression profiles in human non-small and small-cell lung cancers.* European Journal of Cancer, 2003. **39**(13): p. 1936-1947.
67. Rohe, H.J., et al., *PGRMC1 (progesterone receptor membrane component 1): a targetable protein with multiple functions in steroid signaling, P450 activation and drug binding.* Pharmacology & therapeutics, 2009. **121**(1): p. 14-19.
68. Peluso, J.J., et al., *Progesterone receptor membrane component-1 regulates the development and Cisplatin sensitivity of human ovarian tumors in athymic nude mice.* Endocrinology, 2009. **150**(11): p. 4846-4854.
69. Jones, P., *Risk and benefits of estrogen plus progestin in healthy postmenopausal women.* JAMA, 2002. **288**: p. 321-3.
70. Beral, V., et al., *Breast cancer and hormone-replacement therapy: the Million Women Study.* The Lancet, 2003. **362**(9392): p. 1330-1331.
71. Fournier, A., F. Berrino, and F. Clavel-Chapelon, *Unequal risks for breast cancer associated with different hormone replacement therapies: results from the E3N cohort study.* Breast cancer research and treatment, 2008. **107**(1): p. 103-111.
72. Fournier, A., et al., *Estrogen-progestagen menopausal hormone therapy and breast cancer: does delay from menopause onset to treatment initiation influence risks?* Journal of Clinical Oncology, 2009. **27**(31): p. 5138-5143.
73. Initiative, W.G.f.t.W.s.H., *Effects of conjugated equine estrogen in postmenopausal women with hysterectomy.* JAMA, 2004. **291**: p. 1701-1712.
74. Schoonen, W., J. Joosten, and H. Kloosterboer, *Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines.* The Journal of steroid biochemistry and molecular biology, 1995. **55**(3): p. 423-437.
75. Cappelletti, V., et al., *Effect of progestin treatment on estradiol-and growth factor-stimulated breast cancer cell lines.* Anticancer research, 1994. **15**(6B): p. 2551-2555.
76. Krämer, E.A., et al., *The effect of progesterone, testosterone and synthetic progestogens on growth factor-and estradiol-treated human cancerous and benign breast cells.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2006. **129**(1): p. 77-83.
77. Catherino, W., M. Jeng, and V. Jordan, *Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism.* British journal of cancer, 1993. **67**(5): p. 945.
78. Franke, H.R. and I. Vermes, *Differential effects of progestogens on breast cancer cell lines.* Maturitas, 2003. **46**: p. 55-58.

79. Greene, G.L., et al., *Sequence and expression of human estrogen receptor complementary DNA.* Science, 1986. **231**(4742): p. 1150-1154.
80. Fishman, J., M.P. Osborne, and N.T. Telang, *The Role of Estrogen in Mammary Carcinogenesisa.* Annals of the New York Academy of Sciences, 1995. **768**(1): p. 91-100.
81. Thim, L., *Trefoil peptides: from structure to function.* Cellular and Molecular Life Sciences CMLS, 1997. **53**(11-12): p. 888-903.
82. Ribieras, S., C. Tomasetto, and M.-C. Rio, *The pS2/TFF1 trefoil factor, from basic research to clinical applications.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1998. **1378**(1): p. F61-F77.
83. EMAMI, S., et al., *Induction of scattering and cellular invasion by trefoil peptides in src-and RhoA-transformed kidney and colonic epithelial cells.* The FASEB Journal, 2001. **15**(2): p. 351-361.
84. RODRIGUES, S., et al., *Trefoil peptides as proangiogenic factors in vivo and in vitro: implication of cyclooxygenase-2 and EGF receptor signaling.* The FASEB journal, 2003. **17**(1): p. 7-16.
85. Rodrigues, S., et al., *Induction of the adenoma-carcinoma progression and Cdc25A-B phosphatases by the trefoil factor TFF1 in human colon epithelial cells.* Oncogene, 2006. **25**(50): p. 6628-6636.
86. Madsen, J., et al., *Tissue localization of human trefoil factors 1, 2, and 3.* Journal of Histochemistry & Cytochemistry, 2007. **55**(5): p. 505-513.
87. Vestergaard, E.M., et al., *Trefoil factors in human milk.* Early human development, 2008. **84**(10): p. 631-635.
88. Masiakowski, P., et al., *Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line.* Nucleic Acids Research, 1982. **10**(24): p. 7895-7903.
89. Barkhem, T., et al., *pS2 Gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor α, an estrogen-responsive element, and the activator protein 1 response element.* Molecular pharmacology, 2002. **61**(6): p. 1273-1283.
90. Barkhem, T., et al., *Transcriptional synergism on the pS2 gene promoter between a p160 coactivator and estrogen receptor-α depends on the coactivator subtype, the type of estrogen response element, and the promoter context.* Molecular Endocrinology, 2002. **16**(11): p. 2571-2581.
91. Oxelmark, E., et al., *The cochaperone p23 differentially regulates estrogen receptor target genes and promotes tumor cell adhesion and invasion.* Molecular and cellular biology, 2006. **26**(14): p. 5205-5213.
92. Boldyreff, B., U. Mietens, and O.-G. Issinger, *Structure of protein kinase CK2: Dimerization of the human β-subunit.* FEBS letters, 1996. **379**(2): p. 153-156.
93. Bian, Y., et al., *Global screening of CK2 kinase substrates by an integrated phosphoproteomics workflow.* Scientific reports, 2013. **3**.
94. Salvi, M., et al., *Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2009. **1793**(5): p. 847-859.
95. Montenarh, M., *Cellular regulators of protein kinase CK2.* Cell and tissue research, 2010. **342**(2): p. 139-146.
96. Cozza, G., L. A Pinna, and S. Moro, *Kinase CK2 inhibition: An update.* Current medicinal

chemistry, 2013. **20**(5): p. 671-693.

97. Meggio, F. and L.A. PINNA, *One-thousand-and-one substrates of protein kinase CK2?* The FASEB Journal, 2003. **17**(3): p. 349-368.

98. Duncan, J.S. and D.W. Litchfield, *Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2.* Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 2008. **1784**(1): p. 33-47.

99. Wei, S., et al., *Receptor Activator of Nuclear Factor-κB Ligand Activates Nuclear Factor-κB in Osteoclast Precursors 1.* Endocrinology, 2001. **142**(3): p. 1290-1295.

100. Whyte, M.P., *Paget's Disease of Bone and Genetic Disorders of RANKL/OPG/RANK/NF-κB Signaling.* Annals of the New York Academy of Sciences, 2006. **1068**(1): p. 143-164.

101. Hughes, A.E., et al., *Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis.* Nature genetics, 2000. **24**(1): p. 45-48.

102. Anderson, D.M., et al., *A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function.* Nature, 1997. **390**(6656): p. 175-179.

103. Hsu, H., et al., *Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand.* Proceedings of the National Academy of Sciences, 1999. **96**(7): p. 3540-3545.

104. Darnay, B.G., et al., *Characterization of the intracellular domain of receptor activator of NF-κB (RANK) Interaction with tumor necrosis factor receptor-associated factors and activation of NF-κB and c-Jun N-terminal kinase.* Journal of Biological Chemistry, 1998. **273**(32): p. 20551-20555.

105. Schneeweis, L.A., D. Willard, and M.E. Milla, *Functional dissection of osteoprotegerin and its interaction with receptor activator of NF-κB ligand.* Journal of Biological Chemistry, 2005. **280**(50): p. 41155-41164.

106. Hofbauer, L.C. and A.E. Heufelder, *Role of receptor activator of nuclear factor-κB ligand and osteoprotegerin in bone cell biology.* Journal of Molecular Medicine, 2001. **79**(5-6): p. 243-253.

107. Suda, T., et al., *Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families.* Endocrine reviews, 1999. **20**(3): p. 345-357.

108. Wong, B.R., R. Josien, and Y. Choi, *TRANCE is a TNF family member that regulates dendritic cell and osteoclast function.* Journal of Leukocyte Biology, 1999. **65**(6): p. 715-724.

109. Theill, L.E., W.J. Boyle, and J.M. Penninger, *RANK-L and RANK: T cells, bone loss, and mammalian evolution.* Annual review of immunology, 2002. **20**(1): p. 795-823.

110. Ikeda, T., et al., *Determination of Three Isoforms of the Receptor Activator of Nuclear Factor-κB Ligand and Their Differential Expression in Bone and Thymus 1.* Endocrinology, 2001. **142**(4): p. 1419-1426.

111. Wada, T., et al., *RANKL-RANK signaling in osteoclastogenesis and bone disease.* Trends in molecular medicine, 2006. **12**(1): p. 17-25.

112. Fata, J.E., et al., *The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development.* Cell, 2000. **103**(1): p. 41-50.

113. Kim, N.-S., et al., *Receptor activator of NF-κB ligand regulates the proliferation of mammary epithelial cells via Id2.* Molecular and cellular biology, 2006. **26**(3): p. 1002-1013.

114. Burgess, T.L., et al., *The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts.* The Journal of cell biology, 1999. **145**(3): p. 527-538.
115. Simonet, W., et al., *Osteoprotegerin: a novel secreted protein involved in the regulation of bone density.* Cell, 1997. **89**(2): p. 309-319.
116. Malyankar, U.M., et al., *Osteoprotegerin is an αvβ3-induced, NF-κB-dependent survival factor for endothelial cells.* Journal of Biological Chemistry, 2000. **275**(28): p. 20959-20962.
117. Lacey, D., et al., *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* cell, 1998. **93**(2): p. 165-176.
118. Fuller, K., et al., *TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts.* The Journal of experimental medicine, 1998. **188**(5): p. 997.
119. Fernandez-Valdivia, R., et al., *The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse.* Developmental biology, 2009. **328**(1): p. 127-139.
120. Gonzalez-Suarez, E., et al., *RANK overexpression in transgenic mice with mouse mammary tumor virus promoter-controlled RANK increases proliferation and impairs alveolar differentiation in the mammary epithelia and disrupts lumen formation in cultured epithelial acini.* Molecular and cellular biology, 2007. **27**(4): p. 1442-1454.
121. Narducci, P., R. Bareggi, and V. Nicolin, *Receptor Activator for Nuclear Factor kappa B Ligand (RANKL) as an osteoimmune key regulator in bone physiology and pathology.* Acta histochemica, 2011. **113**(2): p. 73-81.
122. Tan, W., et al., *Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling.* Nature, 2011. **470**(7335): p. 548-553.
123. Jones, D.H., et al., *Regulation of cancer cell migration and bone metastasis by RANKL.* Nature, 2006. **440**(7084): p. 692-696.
124. Schramek, D., et al., *Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer.* Nature, 2010. **468**(7320): p. 98-102.
125. Gonzalez-Suarez, E., et al., *RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis.* Nature, 2010. **468**(7320): p. 103-107.
126. Canon, J.R., et al., *Inhibition of RANKL blocks skeletal tumor progression and improves survival in a mouse model of breast cancer bone metastasis.* Clinical & experimental metastasis, 2008. **25**(2): p. 119-129.
127. Thomas, R.J., et al., *Breast Cancer Cells Interact with Osteoblasts to Support Osteoclast Formation 1.* Endocrinology, 1999. **140**(10): p. 4451-4458.
128. Cross, S.S., et al., *Expression of receptor activator of nuclear factor κ β ligand (RANKL) and tumour necrosis factor related, apoptosis inducing ligand (TRAIL) in breast cancer, and their relations with osteoprotegerin, oestrogen receptor, and clinicopathological variables.* Journal of clinical pathology, 2006. **59**(7): p. 716-720.
129. Nicolin, V. and P. Narducci, *Soluble TRAIL could enhance bone destruction acting on Rank-ligand in estrogen-independent human breast cancer cell line MDA-MB-231.* Acta histochemica, 2010. **112**(2): p. 189-192.
130. Tang, Z.-N., et al., *Hypoxia induces RANK and RANKL expression by activating HIF-1α in breast cancer cells.* Biochemical and biophysical research communications, 2011. **408**(3):

p. 411-416.

131. Schubert, A., et al., *Expression of osteoprotegerin and receptor activator of nuclear factor-κB ligand (RANKL) in HCC70 breast cancer cells and effects of treatment with gonadotropin-releasing hormone on RANKL expression.* Gynecological Endocrinology, 2008. **24**(6): p. 331-338.

132. Park, H.-R., et al., *Expression of osteoprotegerin and RANK ligand in breast cancer bone metastasis.* Journal of Korean medical science, 2003. **18**(4): p. 541.

133. Mulac-Jericevic, B., et al., *Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform.* Proceedings of the National Academy of Sciences, 2003. **100**(17): p. 9744-9749.

134. Büssing, I., F.J. Slack, and H. Großhans, *let-7 microRNAs in development, stem cells and cancer.* Trends in molecular medicine, 2008. **14**(9): p. 400-409.

135. Chatterjee, S., et al., *Target-Mediated Protection of Endogenous MicroRNAs in C.elegans.* Developmental cell, 2011. **20**(3): p. 388-396.

136. Fabian, M.R., N. Sonenberg, and W. Filipowicz, *Regulation of mRNA translation and stability by microRNAs.* Annual review of biochemistry, 2010. **79**: p. 351-379.

137. Huntzinger, E. and E. Izaurralde, *Gene silencing by microRNAs: contributions of translational repression and mRNA decay.* Nature Reviews Genetics, 2011. **12**(2): p. 99-110.

138. Shruti, K., K. Shrey, and R. Vibha, *Micro RNAs: tiny sequences with enormous potential.* Biochemical and biophysical research communications, 2011. **407**(3): p. 445-449.

139. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* cell, 2004. **116**(2): p. 281-297.

140. Esquela-Kerscher, A. and F.J. Slack, *Oncomirs—microRNAs with a role in cancer.* Nature Reviews Cancer, 2006. **6**(4): p. 259-269.

141. Scott, G.K., et al., *Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b.* Journal of Biological Chemistry, 2007. **282**(2): p. 1479-1486.

142. Yu, F., et al., *let-7 Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells.* Cell, 2007. **131**(6): p. 1109-1123.

143. Takamizawa, J., et al., *Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival.* Cancer research, 2004. **64**(11): p. 3753-3756.

144. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.* nature, 2000. **403**(6772): p. 901-906.

145. Pasquinelli, A.E., et al., *Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA.* Nature, 2000. **408**(6808): p. 86-89.

146. Medina, P.P. and F.J. Slack, *microRNAs and cancer.* Cell cycle, 2008. **7**(16): p. 2485-2492.

147. Johnson, S.M., et al., *RAS Is Regulated by the let-7 MicroRNA Family.* Cell, 2005. **120**(5): p. 635-647.

148. Sampson, V.B., et al., *MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells.* Cancer research, 2007. **67**(20): p. 9762-9770.

149. Lee, Y.S. and A. Dutta, *The tumor suppressor microRNA let-7 represses the HMGA2 oncogene.* Genes & development, 2007. **21**(9): p. 1025-1030.

150. Michael, M.Z., et al., *Reduced Accumulation of Specific MicroRNAs in Colorectal*

*Neoplasia11Note: Susan M. O'Connor and Nicholas G. van Holst Pellekaan contributed equally to this work.* Molecular Cancer Research, 2003. **1**(12): p. 882-891.

151. Dahiya, N., et al., *MicroRNA expression and identification of putative miRNA targets in ovarian cancer.* PloS one, 2008. **3**(6): p. e2436.

152. Wendler, A., et al., *Involvement of let-7/miR-98 microRNAs in the regulation of progesterone receptor membrane component 1 expression in ovarian cancer cells.* Oncology reports, 2011. **25**(1): p. 273-279.

153. Zhao, Y., et al., *Let-7 family miRNAs regulate estrogen receptor alpha signaling in estrogen receptor positive breast cancer.* Breast cancer research and treatment, 2011. **127**(1): p. 69-80.

154. Pritchard, C.C., H.H. Cheng, and M. Tewari, *MicroRNA profiling: approaches and considerations.* Nature Reviews Genetics, 2012. **13**(5): p. 358-369.

155. Munton, R.P., et al., *Qualitative and quantitative analyses of protein phosphorylation in naive and stimulated mouse synaptosomal preparations.* Molecular & Cellular Proteomics, 2007. **6**(2): p. 283-293.

156. Peluso, J.J., et al., *Progesterone inhibits apoptosis in part by PGRMC1-regulated gene expression.* Molecular and cellular endocrinology, 2010. **320**(1): p. 153-161.

157. Krebs, C.J., et al., *A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors.* Proceedings of the National Academy of Sciences, 2000. **97**(23): p. 12816-12821.

158. Gribben, J.G., *Implications of the tumor microenvironment on survival and disease response in follicular lymphoma.* Current opinion in oncology, 2010. **22**(5): p. 424-430.

159. Ogino, S., et al., *Cancer immunology—analysis of host and tumor factors for personalized medicine.* Nature reviews Clinical oncology, 2011. **8**(12): p. 711-719.

160. Mir, S.U., et al., *Elevated progesterone receptor membrane component 1/sigma - 2 receptor levels in lung tumors and plasma from lung cancer patients.* International Journal of Cancer, 2012. **131**(2): p. E1-E9.

161. Kimura, I., et al., *Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity.* Journal of neuroscience research, 2005. **79**(3): p. 287-294.

162. Kimura, I., et al., *Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain.* Journal of Biological Chemistry, 2008. **283**(7): p. 4323-4331.

163. Mayr, C., M.T. Hemann, and D.P. Bartel, *Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation.* Science, 2007. **315**(5818): p. 1576-1579.

164. Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes & development, 2009. **23**(15): p. 1743-1748.