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ARTICLE

Crosstalk between progesterone receptor membrane component 1 and estrogen receptor α promotes breast cancer cell proliferation

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Abstract

Progesterone (P4) and estradiol (E2) have been shown to stimulate and regulate breast cancer proliferation via classical nuclear receptor signaling through progesterone receptor (PR) and estrogen receptor α (ERα), respectively. However, the basis of communication between PR/ERα and membrane receptors remains largely unknown. Here, we aim to identify classical and nonclassical endocrine signaling mechanisms that can alter cell proliferation through a possible crosstalk between PR, ERα, and progesterone receptor membrane component 1 (PGRMC1), a membrane receptor frequently observed in breast cancer cells. While P4 and E2 treatment increased cell proliferation of ER+/PR+/PGRMC1 overexpressing breast cancer cells, silencing ERα and PR or treatment with selective estrogen receptor modulator (SERM) tamoxifen, or (PR-antagonist) RU-486 decreased cell proliferation. All four treatments rapidly altered PGRMC1 mRNA levels and protein expression. Furthermore, P4 and E2 treatments rapidly activated EGFR a known interacting partner of PGRMC1 and its downstream signaling. Interestingly, downregulation of ERα by tamoxifen and ERα silencing decreased the expression levels of PGRMC1 with no repercussions to PR expression. Strikingly PGRMC1 silencing decreased ERα expression irrespective of PR. METABRIC and TCGA datasets further demonstrated that PGRMC1 expression was comparable to that of ERα in Luminal A and B breast cancers. Targeting of PR, ERα, and PGRMC1 confirmed that a crosstalk between classical and nonclassical signaling mechanisms exists in ER+ breast cancer cells that could enhance the growth of ER+/PR+/PGRMC1 overexpressing tumors.

Introduction

It is estimated that in the U.S. breast cancers will account for 30% of all newly diagnosed cancers in women with 42,170 women expected to die from breast cancer alone in 2020 [1]. 17β-estradiol (E2) has long been considered a promoter of breast cancer [2–4]. However, both progesterone (P4) and E2 can promote the growth of breast cancers [5]. Although, the effect of E2 and estrogen receptor (ER) in breast cancers are well studied the role of P4 and progesterone receptor (PR) remains controversial.

Approximately, 70% of all breast cancers diagnosed in postmenopausal women are ER+/PR+ [6, 7]. ER+ breast cancers are readily treated with either the selective estrogen receptor modulator tamoxifen or selective estrogen receptor down-regulator fulvestrant [8]. Even with their clinical success, over 50% of patients develop de novo or acquired resistance along with undesired side effects [9, 10]. Therefore, other biomarkers that may associate with the receptors ER and PR should be explored for patients diagnosed with ER+/PR+ breast cancers. Novel membrane receptors with the ability to facilitate nonclassical signaling (non-genomic) of steroid hormones have been described [11–13]. Contrary to the well-established classical signaling (genomic) actions of E2 and P4, nonclassical signaling enables these hormones to bind receptors on cellular membranes that lack DNA binding domains but can formulate fast acting

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responses usually through the activation of secondary messengers [14–16].

The progesterone receptor membrane component 1 (PGRMC1) has been shown to be overexpressed in a spectrum of cancers [17–19]. Multiple studies show that PGRMC1 is overexpressed in both ER+ and triple-negative breast cancers and its overexpression correlates with higher lymph node status, tumor grade, and larger tumors [20, 21]. We recently demonstrated that PGRMC1 plays a major role in controlling the growth of breast cancer cells through the activation of the PI3K/AKT/mTOR and EGFR signaling pathways [22]. Both in vitro and in vivo studies have also shown that multiple progestins such as medroxyprogesterone acetate [23, 24], drospirenone [25], desogestrel [25], dydrogesterone [25], levonorgestrel [25], dienogest [26], and norethisterone [25, 27, 28] can promote breast cancer cell proliferation and growth of xenografts tumors that overexpress PGRMC1. Although, PGRMC1 is directly linked to progestogens the exact signaling mechanism behind this remains largely unknown. Recent studies, however, have demonstrated that multiple estrogens, including E2, equilin, and ethinylestradiol have the ability to increase cell proliferation of ER+/PGRMC1 overexpressing cells compared to ER+ only cells [29]. Therefore, giving rise to the possibility that a classical and nonclassical endocrine signaling crosstalk between ER/PR and PGRMC1 exists and that this crosstalk could promote the growth of breast cancers.

Here, we demonstrate that E2 and P4 can quickly increase PGRMC1 expression. Further, we observed that both hormones can quickly activate AKT/mTOR and EGFR signaling pathways while their respective inhibitors suppress these effects. Interestingly, silencing ERα resulted in decreased PGRMC1 expression while PGRMC1 silencing decreased ERα expression indicating a crosstalk between ERα and PGRMC1. On the other hand, silencing PR showed no effects to PGRMC1 expression and similarly PGRMC1 silencing displayed no effects to PR expression. Furthermore, analysis of both Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) datasets, suggest that *ESR1, PGR*, and *PGRMC1* gene levels are similarly elevated in invasive breast carcinoma tissues. Finally, Gene Ontology (GO) molecular function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified that *ESR1, PGR*, and *PGRMC1* only recognized steroid binding as a common feature between the three. Giving rise to the notion that PGRMC1 is clearly understudied. Therefore, we speculate that a crosstalk exists between classical and nonclassical endocrine signaling and thus show that ERα and PGRMC1 directly regulate each other.

Materials and methods

Cell culture

Breast cancer cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA) and cultured in phenol red-free RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml of streptomycin, and 100 units/ml of penicillin (Life Technologies, Grand Island, NY, USA). The cell lines were maintained in an atmosphere of 95% air, 5% CO_2 and incubated at 37 °C.

Hormone and drug treatments

For steroid hormone-free conditions, ZR-75-1 breast cancer cells were cultured in phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped FBS (Equitech-Bio, Kerrville, TX, USA) 48 h prior to treatment. Breast cancer cells were then treated with either 17β-estradiol (2, 4, 6, 8, and 10 nM), progesterone (0.1, 0.5, 1, 5, and 10 µM), tamoxifen (10, 20, 30, 40, and 50 µM), or RU-486 (1, 5, 10, 50, 100 µM) (Sigma Chemical Co. St. Louis, MO, USA).

Silencing ERα, PR, and PGRMC1 in breast cancer cell lines

Silencing was performed by transfecting ZR-75-1 breast cancer cells with multiple siRNA's targeting ERα, PR, or PGRMC1 (Origene, Rockville, MD, USA) using Mirus bio TransIT siQUEST transfection reagent (Mirus Bio, Madison, WI, USA). Non-specific scrambled sequences were used as appropriate controls. Briefly, 5×10^5, ZR-75-1 breast cancer cells were seeded in 6-well plate and transfected with siRNA sequences (A, B, and C) with concentrations ranging from 20 to 60 nM for 48 h. The siRNA to transfection reagent ratio was 1:1, in accordance with the manufacture's protocol. Efficient silencing was confirmed by western blot analysis. ERα, PR, and PGRMC1 siRNA sequences utilized are listed below:

ERα

SR301461A-rArCrCrUrUrGrCrArGrArUrArUrGrUrUrUrArArCrCrArAGC

SR301461B-rArCrArCrCrArUrArGrUrArArUrGrUrCrUrArArUrArUrUCA

SR301461C-rGrGrCrArArArUrArGrArGrUrCrArUrArCrArGrUrArGrCTC

PR

SR303485A-rCrGrArGrUrGrGrArCrArUrArArGrArArArUrCrArGrArAGA

SR303485B-rUrGrUrGrGrArGrArGrUrUrArArUrArCrArArArCrArUrACC

SR303485C-rGrGrUrArArArCrArGrArUrArGrCrUrArCrArArUrArUrUGT
PGRMC1
SR323253A-rGrArUrCrArArCrUrUrUrUrArGrUrCrArUrGrArUrGrUrUCT
SR323253B-rCrArArUrUrGrArCrUrUrArArCrUrGrCrArUrGrArUrUrUCT
SR323253C-rUrCrArArCrUrUrUrUrUrArGrUrCrArUrGrArUrGrUrUrCrUGT

Cell proliferation assay

ZR-75-1 breast cancer cells were seeded at a density of 0.6×10^4 cells/well in 96-well plates for 24 h prior to treatment or transfection. Cell proliferation was measured 24, 48, 72, and 96 h following 2, 4, 6, 8, 10 nM of 17β-estradiol or 0.1, 0.5, 1, 5, 10 μM of progesterone, 10, 20, 30, 40, 50 μM of tamoxifen, or 1, 5, 10, 50, 100 μM of RU-486 treatment. Cell proliferation was also measured following silencing of ERα, PR, and PGRMC1. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was used to measure cell proliferation and the absorbance was read at 490 nm utilizing a microplate reader (CLARIOstar, BMG, LABTECH).

Western blot analysis

Protein was extracted from ZR-75-1 breast cancer cells using the mammalian Protein Extraction Reagent (mPER) according to the manufacture's protocol (Thermo Scientific, US) following 17β-estradiol, progesterone, tamoxifen or RU-486 treatment or transfection with siRNA targeting ERα, PR, or PGRMC1. Protein concentration was quantitated using the PierceTM BCA Protein Assay Kit (Thermo Scientific, US). Equal amounts of protein were loaded to Mini-Protean TGX polyacrylamide gels (Bio-Rad) and separated by SDS-PAGE. Proteins were then transferred onto PVDF membranes and blocked for 1 h with 5% BSA in 1x TBST. After blocking, primary antibodies were used to probe the respective proteins for 2 h followed by the appropriate secondary antibody and developed using the enhanced chemiluminescence kit (Thermo Scientific, US). Images were captured using the Fuji LAS 4000 imager (Fuji Systems, Japan). Primary antibodies used were as follows: PGRMC1 (13856S), AKT (14702S), p-AKT (4060S), p-EGFR (3777S), mTOR (2972S), p-mTOR (2971S), ERα (13258S), PR (8757S) all of which were obtained from Cell Signaling Technology; EGFR (ab-2430-1) purchased from Abcam and β-actin (A1978) from Sigma-Aldrich. ImageJ software was utilized to measure the band intensity.

Quantitative RT-PCR

Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) RNA was isolated and reverse transcribed using the RT2 first strand kit (Qiagen; Cat. No. 330401). The obtained cDNA was subsequently used for qRT-PCR, performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The results were analyzed by the comparative Ct method ($2^{-\Delta\Delta CT}$). The appropriate primers used were as follows:

PGRMC1 forward 5′-CGACGGCGTCCAGGACCC-3′
reverse 5′-TCTTCCTCATCTGAGTACACAG-3′
PRA forward 5′-CCTCGGACACCTTGCCTGAA-3′
reverse 5′-CGCCAACAGAGTGTCCAAGAC-3′
PRB forward 5′-TAGTGAGGGGGCAGTGGAAC-3′
reverse 5′-AGGAGGGGGTTTCGGGAATA-3′
ESR1 forward 5′-CCTAACTTGCTCTTGGACAGGA-3′
reverse 5′-GCCAGCAGCATGTCGAAGAT-3′
GAPDH forward 5′-CAGCCTCAAGATCATCAGCAATGC-3′
reverse 5′-AGACCACCTGGTGCTCAGTGTAG-3′

Immunohistochemistry (IHC)

Breast cancer tissues were acquired through the Texas Tech Physicians Breast Care Center at El Paso from patients who underwent surgery. Tissues were collected from patients after their informed consent and the study was approved by the Texas Tech University Health Sciences Center El Paso Institutional Review Board. IHC for PGRMC1 was done using human breast tissue and human breast lesions. Tissue samples were first incubated at 58 °C for 2 h then deparaffinized using xylene for 20 min. Tissues were gradually hydrated in a series of alcohol baths 100, 95, 70, 50, and 30% EtOH, followed by 5 min in distilled water bath. Epitope retrieval was processed with trilogy (Cell Marque, Rocklin, CA) followed by blocking in 1% fetal calf serum and 1% bovine serum albumin in TBS for 15 min. To avoid non-specific antibody binding, tissues were incubated for 10 min in perox-free blocking reagent (Cell Marque, Rocklin, CA). Tissues were then incubated with PGRMC1 antibody (Cell Signaling Technology, #13856S) (1:50) dilution for 3 h in a humidified chamber. Slides were further washed with PBS for 5 min and incubated with Ultra Marque polyscan HRP (Cell Marque, Rocklin, CA) for 1 h. Tissues were washed three times with PBS and stained with 3,3′-diaminobenzidine chromogen solution (Cell Marque, Rocklin, CA) for 20 min followed by hematoxylin to counterstain cell nuclei. The tissues were then dehydrated with a series of ethanol baths 30, 50, 70, 95, and 100% EtOH, and finished in a xylene bath. Using mounting media (Surgipath Medical Industries, Richmond, IL), tissues were cover-slipped and imaged using a Nikon ECLIPSE 50i

microscope. Both normal breast tissue and breast lesions were quantified using imageJ software.

In silico analysis

Genetic alterations including amplification, deep deletion, high and low mRNA expression of *ESR1*, *PGR*, and *PGRMC1* were analyzed by cBioPortal (www.cbioportal.org/) utilizing the METABRIC breast carcinoma and TCGA breast invasive carcinoma datasets [30]. OncoPrints demonstrating genetic amplification, deep deletion, high and low mRNA were obtained through cBioPortal following gene submission. Protein–protein interaction network was analyzed using the STRING 11.0 (http://string-db.org/) database to observe known and predicted interactions between the proteins of interest. Significantly enriched pathways including Molecular Function GO and KEGG pathway analysis were also obtained using STRING 11.0.

Statistical analysis

Hormone, drug treatments, and siRNA transfection studies were performed as three independent experiments with six experimental replicates and are represented as the mean ± SD. Four independent breast tissues and breast lesions were used for imaging and quantification. Difference between the control and experimental groups were analyzed by unpaired Student's *t* test, using GraphPad Prism 7 software, version 7.0 (GraphPad Prism Software, San Diego, CA, USA). p values <0.05 were considered statically significant.

Results

PGRMC1 is rapidly transcriptionally activated upon hormone treatment

P4 is a key ligand of both PR and PGRMC1, using ER+/PR+/PGRMC1 overexpressing, ZR-75-1 breast cancer cells, we demonstrate that it can increase cell proliferation while its antagonist RU-486 decreases cell proliferation (Supplementary Figs. 1 and 2). We wanted to study the effects of P4 on both classical and nonclassical receptors, therefore we measured the mRNA levels of PGRMC1 and PR isoforms, PRB and PRA following P4 and RU-486 treatment at multiple time points. We rationed that nonclassical signaling would occur rapidly mainly through the membrane receptor PGRMC1. Therefore, mRNA levels were measured starting at 5 min up to 24 h. Interestingly P4 rapidly increased PGRMC1, PRB, and PRA relative mRNA expression within 5 min and profoundly increased and sustained elevated mRNA levels of all three receptors at 30, 60, and 3 h (Fig. 1A). Further, antagonizing PR with RU-486 clearly suppressed PRB and PRA mRNA expression specifically at 30, 60, and 3 h, which were previously activated by P4 but had no clear effects on PGRMC1 mRNA levels (Fig. 1B). These data suggested that P4 has the ability to rapidly activate both nonclassical and classical signaling mechanisms through PGRMC1, PRB, and PRA. Further, we also wanted to determine if E2 induced the rapid activation of PGRMC1. For this, we first studied the effect of E2 and observed that E2 also increases the proliferation of ZR-75-1 cells and tamoxifen inhibits cell proliferation (Supplementary Figs. 3 and 4). We measured the mRNA levels of both PGRMC1 and ESR1 upon E2 and tamoxifen treatment from 5 min to 24 h. Interestingly, both E2 and tamoxifen alter PGRMC1 and ESR1 mRNA expression (Fig. 1C, D). These results indicated that both P4 and E2 can rapidly impact the transcriptional levels of PGRMC1.

PGRMC1 protein levels are altered upon hormone treatment

Although initial results indicate that P4 can transcriptionally activate PGRMC1 by increasing and sustaining its mRNA expression from 30 min up to 3 h. We measured the protein levels of PGRMC1, PRB, PRA, and ERα following P4, RU-486, E2 and tamoxifen treatments for 30, 60, and 180 min. The importance of analyzing protein levels became apparent as P4 treatment sustained PRB levels but caused PRA levels to significantly decrease which is inversely correlated with our mRNA data (Fig. 2A, C). PR isoforms PRB and PRA are known to be expressed at equal levels in normal mammary cells [31, 32]. Interestingly, an imbalance in PRB/PRA ratio is often observed in breast malignancies [33, 34]. Further, studies have also shown that PRA the truncated isoform is the dominant driver in PR-positive breast cancer cells [35]. Because steroid hormones are deemed to signal through nuclear hormone receptors and are known to exert their effects from hours to days. We speculate that at these time points, progesterone is primarily binding to PGRMC1 and exerting rapid nonclassical effects (Fig. 2A). However, progesterone could also be binding to PRA causing it to become internalized and priming the receptor to become elevated at a later time point. Interestingly, after initially decreasing PGRMC1 expression following P4 treatment for 30 min, its levels began to raise following 60 and 180 min of treatment (Fig. 2A, C). While RU-486 clearly suppressed PRB and PRA expression, similar to our mRNA data it had minimal effects on PGRMC1 expression (Fig. 2B, D). E2, on the other hand, sustained ERα expression but also mirrored our mRNA expression as it decreased PGRMC1 expression (Fig. 2E, G). Finally, treatment with tamoxifen

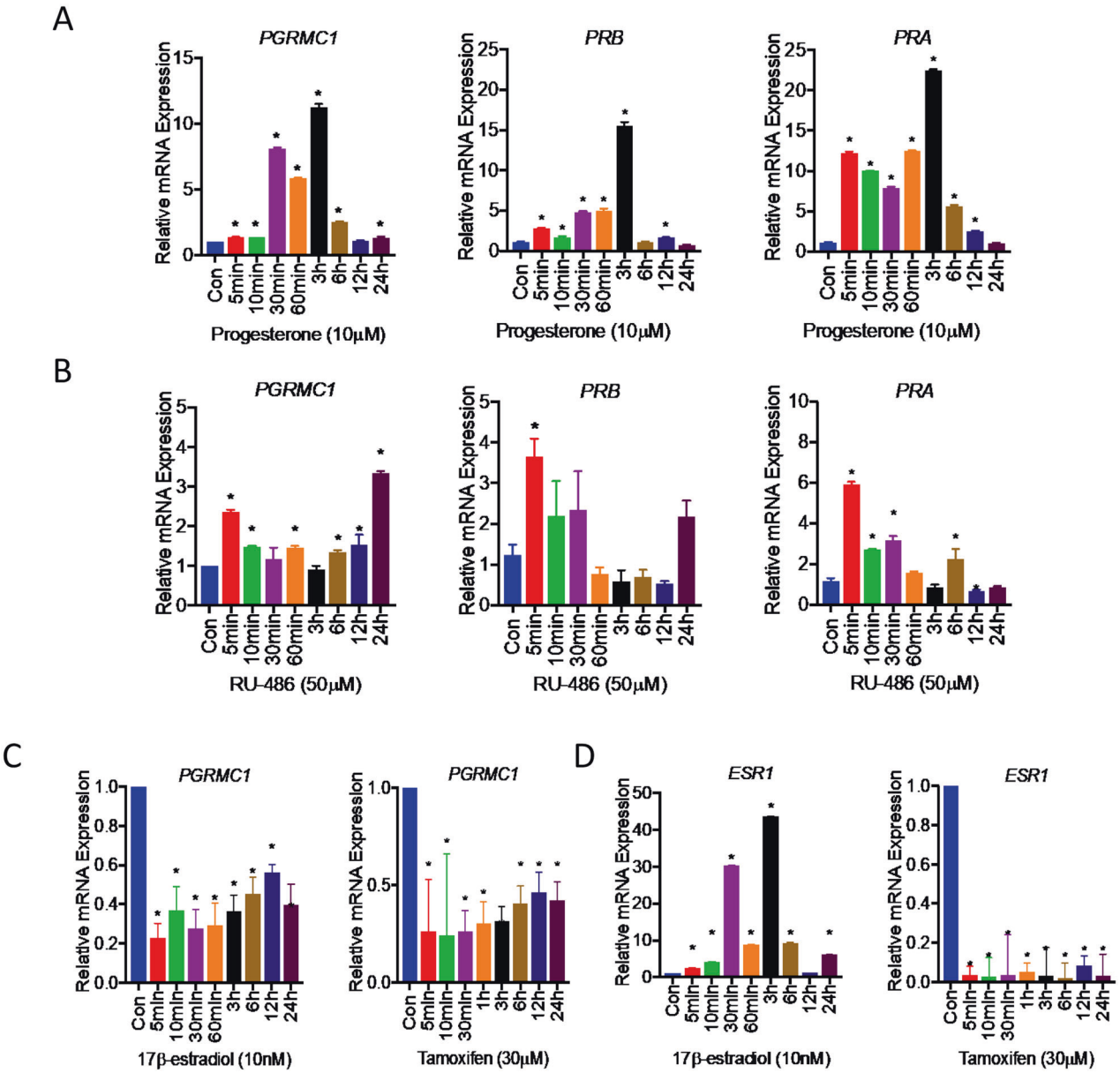

Fig. 1 P4, RU-486, E2, and tamoxifen alter PGRMC1 mRNA expression. A Relative mRNA expression of PGRMC1, PRB, and PRA in ZR-75-1 breast cancer cells following 10 μM P4 treatment after 5, 10, 30, and 60 min, and 3, 6, 12, and 24 h. **B** Relative mRNA expression of PGRMC1, PRB, and PRA in ZR-75-1 following 50 μM RU-486 treatment after 5, 10, 30, and 60 min, and 3, 6, 12, and 24 h. **C**, **D** Relative mRNA expression of PGRMC1 and ESR1 in ZR-75-1 cells following 10 nM 17β-estradiol and 30 μM tamoxifen treatment after 5, 10, 30, and 60 min, and 3, 6, 12, and 24 h. Statistical analysis is the mean ± SD. $*p < 0.05$ as compared with control (calculated using one-way ANOVA, multiple comparisons).

demonstrated that a decrease in ERα correlates with a decrease in PGRMC1 expression (Fig. 2F, H).

P4 and E2 can rapidly activate downstream targets of PGRMC1 and cell survival pathways

To determine if the rapid activation of nonclassical signaling via PGRMC1 impacts the proliferative activity of breast cancer cells, ZR-75-1 cells were treated with P4, RU-486, E2 and tamoxifen for 30, 60, and 180 min. PGRMC1 has been shown to interact with EGFR in cellular membranes [36]. Interestingly, P4 treatment rapidly increases the phosphorylation of EGFR, AKT, and mTOR (Fig. 3A, C). Although treatment with RU-486 had minimal impact on the phosphorylation of EGFR and actually increased the phosphorylation of AKT and mTOR (Fig. 3B, D). RU-486 has the potential to behave as both an agonist and antagonist for PR [37] and coupled with the fact that RU-486 seems to have minimal impact on PGRMC1 expression could indicate that RU-486 does not impact nonclassical signaling.

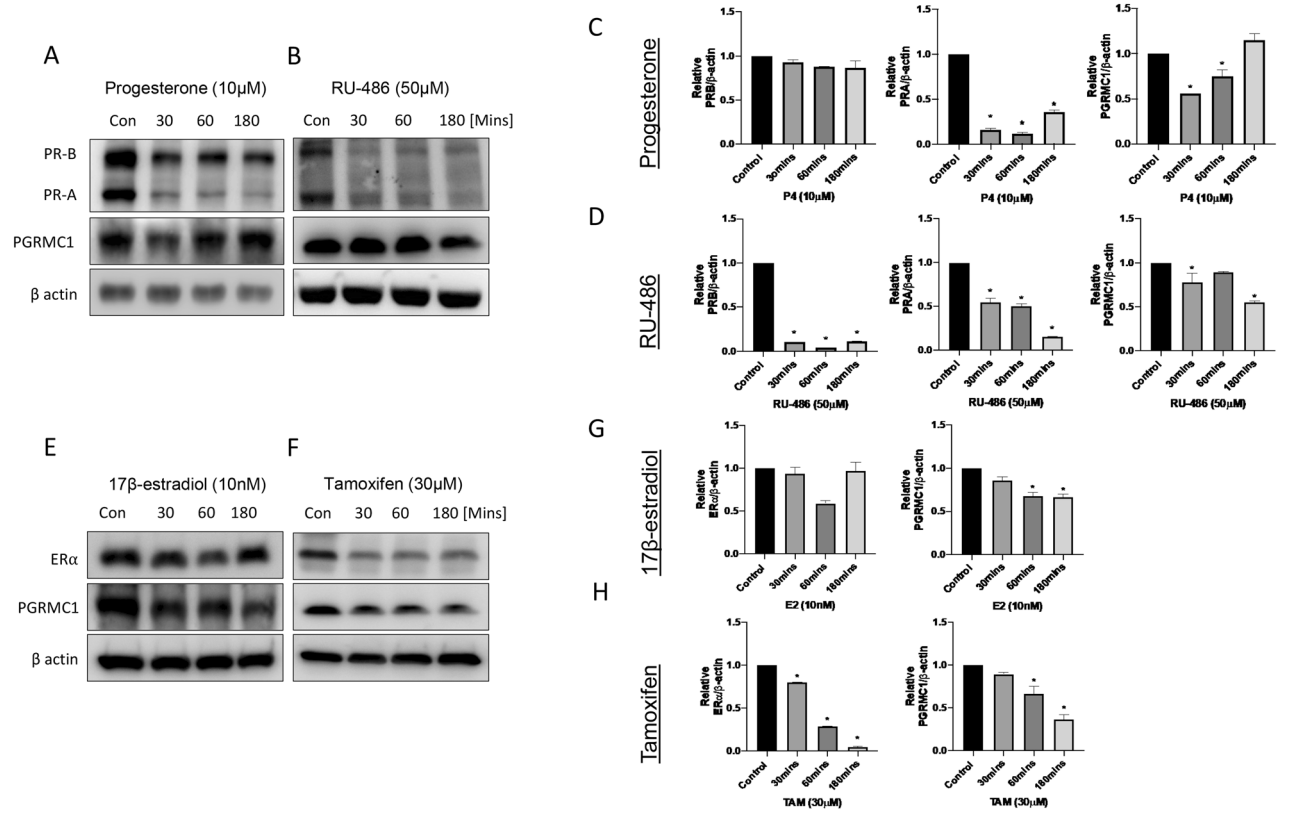

Fig. 2 PGRMC1 protein expression is quickly altered by P4, RU-486, E2, and tamoxifen. A, **B** Western blot analysis of PRB, PRA, and PGRMC1 expression in ZR-75-1 breast cancer cells following 10 μM P4 or 50 μM RU-486 treatment after 30, 60, and 180 min. **C**, **D** Densitometric analysis following 10 μM P4 or 50 μM RU-486 treatment after 30, 60, and 180 min of PRB, PRA, and PGRMC1 expression. **E**, **F** Expression of ERα and PGRMC1 by western blot following 10 nM 17β-estradiol or 30 μM tamoxifen treatment after 30, 60, and 180 min. **G**, **H** Densitometric analysis following 10 nM 17β-estradiol or 30 μM tamoxifen treatment after 30, 60, and 180 min of PRB, PRA, and PGRMC1 expression. Analysis of **C**, **D**, **G**, and **H** are the mean ± SD. $*p < 0.05$ as compared with control (calculated using one-way ANOVA, multiple comparisons).

Further, because ZR-75-1 cells are ER+, it is not unexpected that E2 treatment significantly increased the phosphorylation of EGFR and AKT (Fig. 3E, G) and inhibiting ER with tamoxifen drastically decreased the phosphorylation of EGFR, AKT, and mTOR (Fig. 3F, H). These data indicate that P4 and E2 have the potential to signal through PGRMC1 via the activation of its binding partner EGFR and downstream targets AKT/mTOR.

ERα and PGRMC1 regulate each other independently of PR

ZR-75-1 cells were first transfected with multiple siRNAs targeting PR and ERα. As expected, silencing both receptors drastically decreased cell growth in a time-dependent manner (Fig. 4A, D). Although, impairing PR expression with RU-486 did not have significant effects to PGRMC1 expression, we evaluated the expression of PGRMC1 following PR silencing. Upon successful silencing of PR, it was intriguing to see no change to PGRMC1 expression (Fig. 4B, C). On the other hand, tamoxifen decreased both ERα and PGRMC1 expression, thus we proceeded to silence ERα, and study PGRMC1 expression. After successfully silencing ERα, it was compelling to see a significant decrease in PGRMC1 expression (Fig. 4E, F). To understand the connection between ERα, PR, and PGRMC1, we proceeded to silence PGRMC1. Upon PGRMC1 silencing we observed no change in PRB or PRA expression, however, we observed a significant decrease to the expression of ERα (Fig. 4G, H). A correlation between PGRMC1 and ERα expression in breast tumors has been annotated and may provide an insight into a novel mechanism that exists between PGRMC1 and ERα [38]. Our results show that by disrupting ERα signaling, we simultaneously decrease PGRMC1 gene expression, leading to a possible crosstalk between ERα and PGRMC1 in breast cancer cells.

Functional annotation analysis of *ESR1*, *PGR*, and *PGRMC1* in breast cancers

METABRIC and TCGA data allowed us to identify the genetic alterations of tumors from patients that were

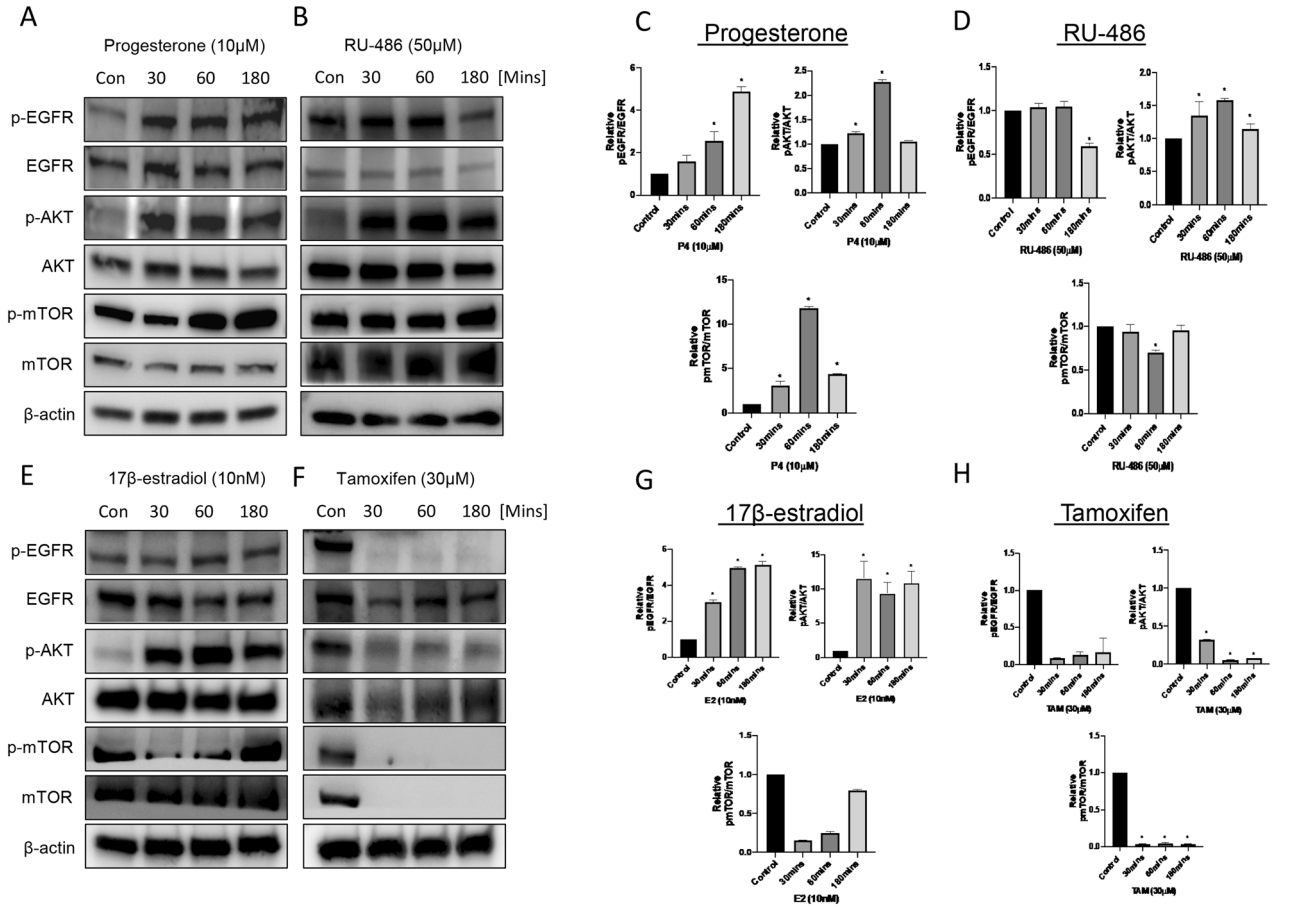

Fig. 3 Survival pathways associated with PGRMC1 are activated by P4 and E2. A, **B** Changes to the phosphorylated and total forms of EGFR, AKT, and mTOR were assessed by western blot after ZR-75-1 breast cancer cells were treated for 30, 60, and 180 min with 10 μM P4 or 50 μM RU-486. **C**, **D** Densitometric analysis of phosphorylated form of EGFR, AKT, and mTOR following 10 μM P4 or 50 μM RU-486 treatment after 30, 60, and 180 min. **E**, **F** Western blot analysis of phosphorylated and total forms of EGFR, AKT, and mTOR following 10 nM 17β-estradiol or 30 μM tamoxifen treatment after 30, 60, and 180 min. **G**, **H** Densitometric analysis of the phosphorylated form of EGFR, AKT, and mTOR following 30, 60, and 180 min following 10 nM 17β-estradiol or 30 μM tamoxifen treatment. Analysis of **C**, **D**, **G**, and **H** are the mean ± SD. $*p < 0.05$ as compared with control (calculated using one-way ANOVA, multiple comparisons).

diagnosed with invasive breast carcinoma. METABRIC data samples demonstrated a correlation in the percentage between *ESR1* and *PGRMC1*, further both were mainly overexpressed due to amplification and retained high mRNA levels within different samples (Fig. 5A). TCGA data mirrored those of METABRIC, as a strong correlation was observed between *ESR1*, and *PGRMC1* in the percentage of samples that demonstrated genetic alteration (Fig. 5B). Genetic alterations to *PGR* on the other hand were lower in METABRIC and equal to that of *ESR1*, in TCGA datasets, however the majority of the genetic alterations from TCGA observed in *PGR* are from deep deletion, which could signify that *ESR1* is more closely involved with *PGRMC1* in breast tumor samples. Intriguingly, GO analysis demonstrated that *ESR1, PGR*, and *PGRMC1* only have steroid binding in common from known annotated molecular functions (Fig. 5C). Further, KEGG pathway analysis did not identify *PGRMC1* in either estrogen signaling pathway or breast cancer (Fig. 5D). Finally, we looked at the mRNA levels of *ESR1, PGR*, and *PGRMC1* in Luminal A and B breast cancer tissue from METABRIC and TCGA datasets. Interestingly, while the mRNA levels of *ESR1* and *PGRMC1* are highly comparable in METABRIC samples, *ESR1, PGR*, and *PGRMC1* have similar expression in TCGA samples (Fig. 5E, F). These data demonstrate that *ESR1* and *PGRMC1* are similarly expressed in breast cancer tissue and could be more closely related than previously thought in breast cancers.

PGRMC1 is overexpressed in breast cancer tissue

To assess the expression of PGRMC1 in human breast cancers, we examined its expression by immunohistochemistry in normal and breast lesions from non-tumor and tumor tissue. Immunohistochemical analysis of normal breast tissue demonstrated minimal PGRMC1 expression in

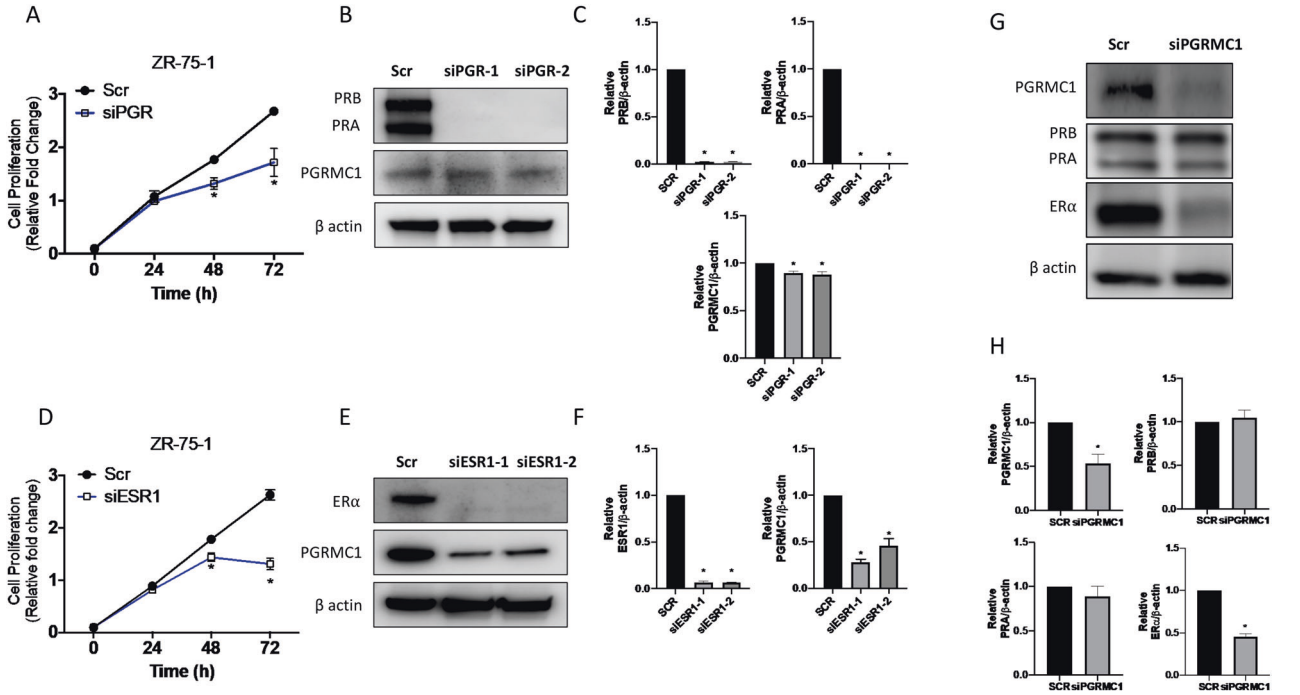

Fig. 4 PGRMC1 and ERα regulate each other irrespective of PR. **A** Cell proliferation was assessed via MTS assay following PR silencing after 24, 48, and 72 h in ZR-75-1 breast cancer cells. **B** Western blot analysis of PRB, PRA, and PGRMC1 following PR silencing using multiple siRNAs. **C** Densitometric analysis of PRB, PRA, and PGRMC1 following PR silencing. **D** MTS assay was used to assess cell proliferation following ERα silencing after 24, 48, and 72 h. **E** Protein expression by western blot analysis of ERα and PGRMC1 following ERα silencing using multiple siRNAs. **F** Densitometric analysis of ERα and PGRMC1 following ERα silencing. **G** Western blot analysis of PGRMC1, PRB, PRA, and ERα following PR silencing. **H** Densitometric analysis of PGRMC1, PRB, PRA, and ERα after PGRMC1 silencing. Analysis of **C**, **H**, and **F** are the mean ± SD. $^{*}p < 0.05$ as compared with control (calculated using one-way ANOVA, multiple comparisons).

normal tissue samples (Fig. 6A). Further, strong staining for PGRMC1 was observed in the breast lesions, particularly in the cellular membranes of cells (Fig. 6B). Quantification of normal breast tissue versus breast lesions solidified our findings (Fig. 6C). Taken together, our results support our claim that PGRMC1 is overexpressed in breast cancers and its expression can be altered by P4 and E2. Both hormones can rapidly activate PGRMC1 downstream signaling pathways EGFR/AKT/mTOR and a crosstalk between PGRMC1/ERα can promote breast cancer cell proliferation (Fig. 6D).

Discussion

Multiple studies have demonstrated that P4 can have an effect on membrane receptors and activate nonclassical signaling pathways. Interestingly, PGRMC1 a member of the MAPR family of membrane proteins, shares no homology with the nuclear steroid hormone receptors, but actually contains a cytochrome b5-like domain capable of binding P450 enzymes via a heme-dependent manner [39–41]. In multiple cancers, PGRMC1 is capable of promoting cancer cell survival and is involved in chemotherapeutic resistance [42, 43]. Here, we demonstrate that there is a crosstalk between PGRMC1 and ERα irrespective of PR in ER+/PR+/PGRMC1 overexpressing breast cancer cells.

Endocrine resistance and the lack of novel targets is a persistent problem in luminal-like breast cancers. Over a third of women diagnosed with early-stage breast cancer treated with endocrine therapy in the form of tamoxifen may become untreatable within 2–5 years if resistance develops [44, 45]. Furthermore, prolonged tamoxifen use could increase the risk of endometrial cancers in postmenopausal women [46]. The majority of patients diagnosed with ER+ tumors also express PR [46]. This has a severe impact on treatments as patients with ER+/PR+ tumors respond favorably to endocrine therapy such as tamoxifen, while ER+/PR− tumors have also been shown to respond poorly to endocrine therapy [47]. Studies by Mohammed et al. describe that under estrogenic conditions, in ER+ tumors, PR is capable of re-directing ER chromatin [48]. Based on the evidence, we can argue that this dogma has since carried over to breast cancer etiology and P4 is perceived as a good hormone that can inhibit breast cancer cell proliferation. However, multiple studies have demonstrated that progestogens can actually increase the risk of developing breast cancer [49–51] and in vivo studies further demonstrated that

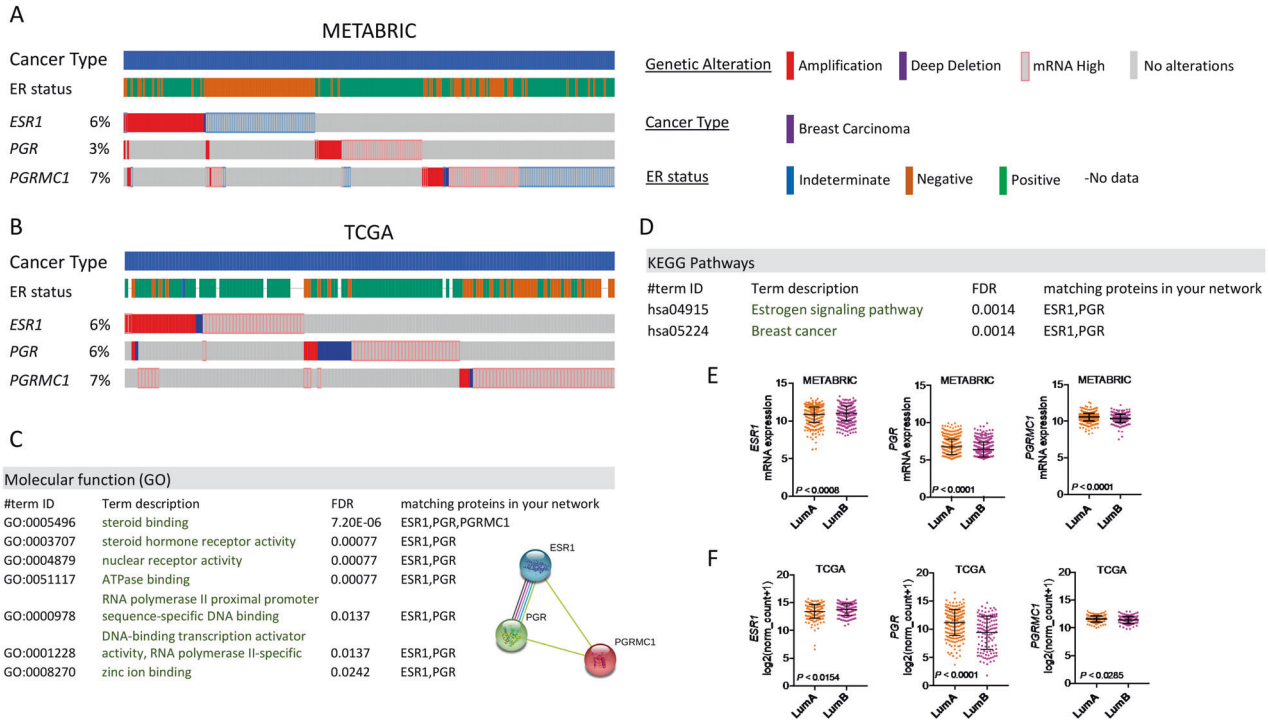

Fig. 5 METABRIC and TCGA dataset analysis of *ESR1, PGR*, and *PGRMC1* in breast cancer patients. A, **B** Oncoprint diagram illustrating amplification, deep deletion, and high mRNA gene expression of ESR1, PGR, and PGRMC1 gene expression in breast carcinoma patients/samples obtained from METABRIC ($n = 1904$) and TCGA ($n = 817$) based on ER status. **C**, **D** Protein–protein interactions by STRING analysis of protein networks between *ESR1, PGR*, and *PGRMC1*. Proteins were categorized by terms based on gene ontology (GO): molecular function and KEGG pathways which identified matching proteins based on the significance levels defined by false discovery rate (FDR). **E** mRNA expression of *ESR1, PGR*, and *PGRMC1* in luminal A and B breast carcinoma samples from METABRIC database. **F** Log2 (normalized_count + 1) gene expression of *ESR1, PGR*, and *PGRMC1* in luminal A and B breast carcinoma samples from TCGA database.

administration of both E2 and P4 are required for mammary carcinogenesis compared to E2 alone which failed to induce mammary carcinogenesis [52]. These results are in line with the WHI estrogen-alone trial, which demonstrated that estrogen alone does not increase the risk of breast cancer but actually decreases it, while the combination of estrogen plus progestin is associated with an increased risk [53, 54].

Our study demonstrated that P4 treatment can independently increase breast cancer cell proliferation, while silencing PR impaired cell growth of ER+/PR+/PGRMC1 overexpressing breast cancer cells. This is particularly interesting because P4 may be impacting patients that present with breast tumors that overexpress PGRMC1. Historically, steroid hormones have been deemed to undergo classical signaling, whereby hormones bind exclusively to hormone receptors with DNA binding capabilities that will exert their actions from hours to days. However, non-classical signaling has emerged as another way for steroid hormones to bind to receptors anchored on cellular membranes exerting their effects within minutes [55–57]. Further, evidence of direct P4-binding to PGRMC1 has been demonstrated [58] and our results showed that both P4 and E2 can rapidly impact PGRMC1 levels within minutes. Previously we demonstrated that PGRMC1 mainly signals through the activation of EGFR and PI3K/AKT signaling pathways [22]. Therefore, it was of major interest to study if P4 and E2 would have any effect on theses signaling pathways. Rapid activation of the PI3K pathway was observed as both P4 and E2 increased the phosphorylation of AKT and mTOR, while tamoxifen decreased phosphorylation of AKT and mTOR. All treatments, however, altered the phosphorylation of EGFR, this is particularly interesting because PGRMC1 and EGFR are known to interact in cellular membranes [36]. At these time points no increased expression of PGRMC1 was observed following E2 treatment, perhaps because ERα prioritized PR expression over PGRMC1. Since P4 binds to both PR and PGRMC1, we hypothesized that PGRMC1 may be under the control PR. To test this hypothesis we silenced PR, ERα, and PGRMC1. To our surprise, PR silencing had no effect on PGRMC1 expression, rather ERα silencing significantly suppressed PGRMC1 expression. Further, PGRMC1 silencing had no effect on PRB or PRA expression, strikingly though ERα expression was significantly decreased. These results closely resembled our observations from the RU-486 treated group, which had no effect on

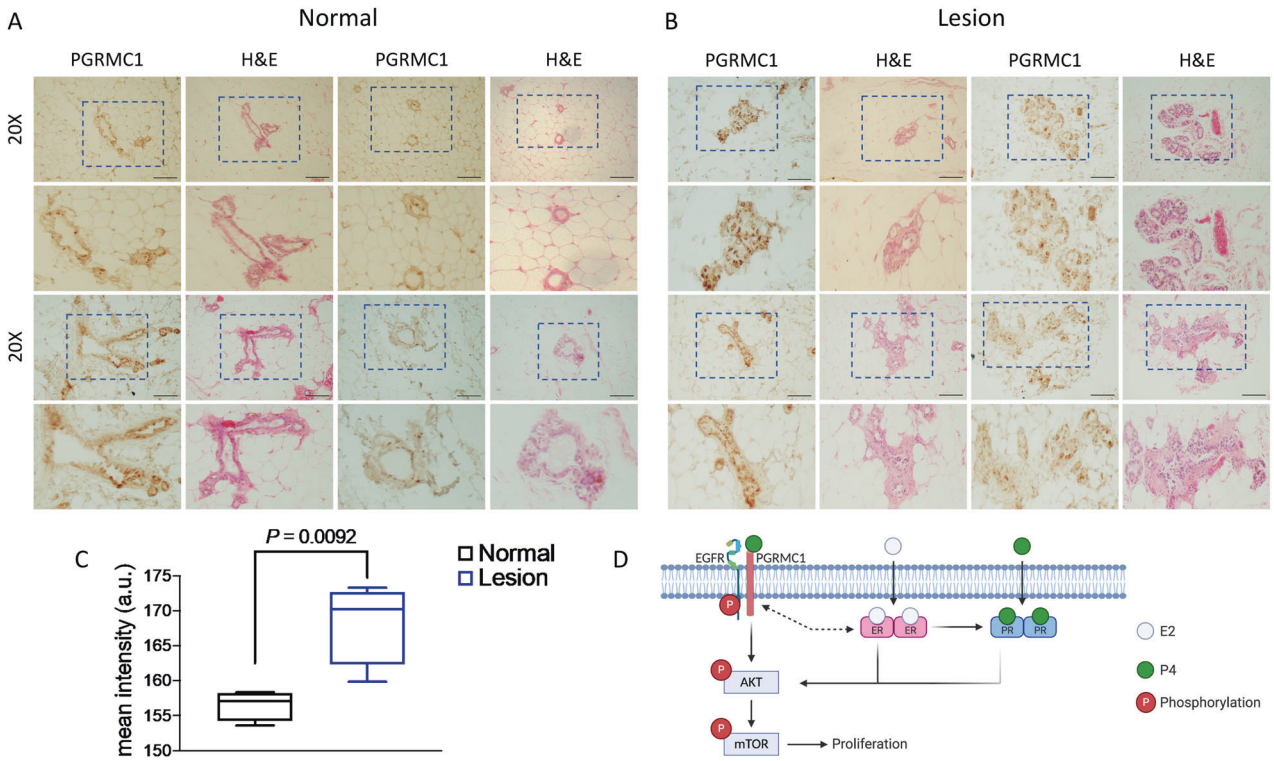

Fig. 6 PGRMC1 expression in human breast cancers and crosstalk model between PGRMC1/ERα. A, **B** Representative immunohistochemical and hematoxylin and eosin analysis of PGRMC1 expression in human non-tumor tissues and human breast lesions. Images were captured at ×20 magnification. **C** PGRMC1 IHC mean intensity of normal breast tissues ($n = 4$) and breast lesions ($n = 4$). **D** Schematic of downstream activation of EGFR and AKT/mTOR signaling pathway following P4 and E2 treatment. Relative PGRMC1 levels were quantified using ImageJ software and p values were calculated by Student's t test.

PGRMC1 expression even though PRB and PRA were significantly decreased. Tamoxifen, on the other hand, decreased both ERα and PGRMC1 expression. Recent studies by Asperger et al. demonstrated that overexpressing PGRMC1 in MCF7 cells increased mRNA levels of *ESR1*, while decreased *PGR* mRNA levels were observed [59]. One possible explanation for an existing crosstalk between PGRMC1 and ERα is through the regulation of cytochrome p450 (CYP) enzymes. CYPs play a major role in cholesterol/steroid hormone synthesis and evidence for a physical interaction between PGRMC1 and CYPs is strongly supported [60]. More specifically, PGRMC1 is capable of regulating CYP19/aromatase [61]. CYP19 is responsible for the conversion of androgens, testosterone, and androstenedione to estrogens, estradiol, and estrone, respectively [62]. Therefore, elevated levels of PGRMC1 particularly in tissue with high adipose concentration like the breast could be prone to more frequent activation of CYP19 leading to E2 production.

Undoubtedly, PGRMC1 seems to play a major role in ER+/PR+/PGRMC1 overexpressing cells. However, its role in tumorigenesis is less known. Here, we observed that PGRMC1 is mainly overexpressed due to amplification and both METABRIC and TCGA datasets demonstrated that *PGRMC1* mRNA is highly expressed and that this expression is comparable to *ESR1*, in human breast tumor tissue. Intriguingly, STRING protein–protein interaction network analysis of *ESR1, PGR*, and *PGRMC1* only recognized one similarity between the three receptors which was steroid binding. While KEGG analysis did not recognize *PGRMC1* in either ER signaling or breast cancer. These results clearly demonstrate that PGRMC1 remains understudied particularly in luminal-like breast cancers which express ERα, PR, and PGRMC1. Our current findings correlate with our previous results, we observed low mRNA and protein expression of PGRMC1 in normal-like breast epithelial cells compared to breast cancer cells [22]. Interestingly, in a cohort of breast cancer patients, 53.6% expressed ERα, 43.5% expressed PR, and 69.6% expressed PGRMC1, further PGRMC1 expression was minimally detected in normal tissues [20].

In summary, we show that E2 and P4 can rapidly activate AKT/mTOR and EGFR signaling pathways, while blocking ERα an PR inhibits these processes. In addition, we report a direct regulation between ERα and PGRMC1 as ERα signal disruption by tamoxifen or ERα, silencing decreased PGRMC1 expression and PGRMC1 silencing similarly decreased ERα expression irrespective of PR. Finally,

PGRMC1 possess the capabilities of promoting E2 and progestogen induced breast cancer cell proliferation and lends itself as a viable molecular target in ER+/PR+/PGRMC1 overexpressing tumors.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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