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RESEARCH PAPER

## Progesterone receptor membrane component 1 promotes survival of human breast cancer cells and the growth of xenograft tumors

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### ABSTRACT

Triple negative breast cancers (TNBCs) are highly aggressive and grow in response to sex steroid hormones despite lacking expression of the classical estrogen (E2) and progesterone (P4) receptors. Since P4 receptor membrane component 1 (PGRMC1) is expressed in breast cancer tumors and is known to mediate P4-induced cell survival, this study was designed to determine the expression of PGRMC1 in TNBC tumors and the involvement of PGRMC1 in regulating proliferation and survival of TNBC cells *in vitro* and the growth of TNBC tumors *in vivo*. For the latter studies, the MDA-MB-231 (MDA) cell line derived from TNBC was used. These cells express PGRMC1 but lack expression of the classical P4 receptor. A lentiviral-based shRNA approach was used to generate a stably transfected PGRMC1-deplete MDA line for comparison to the PGRMC1-intact MDA line. The present studies demonstrate that PGRMC1: 1) is expressed in TNBC cells; 2) mediates the ability of P4 to suppress TNBC cell mitosis *in vitro*; 3) is required for P4 to reduce the apoptotic effects of doxorubicin *in vitro*; and 4) facilitates TNBC tumor formation and growth *in vivo*. Taken together, these findings indicate that PGRMC1 plays an important role in regulating the growth and survival of TNBC cells *in vitro* and ultimately in the formation and development of these tumors *in vivo*. Thus, PGRMC1 may be a therapeutic target for TNBCs.

**Abbreviations:** PGRMC1, progesterone receptor membrane component 1

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Breast cancer; endocrine; PGRMC1; progesterone; TNBC; xenograft

### Introduction

Breast cancer is diagnosed in over 230,000 women annually in the United States.<sup>1</sup> Breast cancer is among the most common invasive cancers, accounting for about 23% of invasive cancers in women worldwide.<sup>2</sup> Among these cases, approximately 12% are triple negative breast cancer (TNBC), characterized by absence of estrogen receptor (ESR1), progesterone receptor (PGR), and Her2-neu receptor (Her2).<sup>3</sup> Patients with TNBC have a poorer prognosis and more resistance to routine therapy than other forms of breast cancer. These cancers are also more aggressive and do not respond well to adjuvant endocrine treatments that target ESR1, PGR, or Her2.<sup>4</sup> Based on expression profiling, TNBCs often maintain a basal-like cell molecular phenotype and share both pathological and clinical features of BRCA1-related breast cancers. Being deficient in ESR1, PGR, and Her2, TNBCs represent a challenging form of breast cancer that is difficult to treat, and this is elevated further by the heterogeneity of the disease.

Estrogens have clearly been shown to stimulate breast cancer growth and progression, and ESR1 antagonists such as tamoxifen are one of the principal forms of adjuvant therapy.<sup>5,6</sup> In contrast, an understanding of the actions of

progestins in the development and progression of breast cancer is controversial.<sup>7,8</sup> In general, it is thought that progestins can both inhibit and stimulate proliferation of breast cancer cells.<sup>9</sup> The Women's Health Initiative Study firmly established that treatment with progestin in combination with estrogen (E2) for attenuating postmenopausal symptoms increases the risk of breast cancer, particularly TNBC.<sup>10</sup> This finding is corroborated to some extent in mice, in which progesterone (P4) promotes 7,12-dimethylbenz[ $\alpha$ ]anthracene-induced mammary tumors.<sup>11,12</sup> Breast cancer cells and tumors respond to progestins through an unknown mechanism, which generally act as pro-survival and proliferative factors.<sup>7,13–17</sup> Progestins may also promote angiogenesis and assist in immunoevasion, both of which contribute to tumor survival and growth.

Although TNBCs do not express the classical PGR, PGR-deficient cells still respond to P4, suggesting that a non-classical mechanism mediates the pro-survival and pro-growth effects of progestins in breast tumors. Two families of non-classical P4 receptors have been identified, including the progestin and adipo-Q receptor<sup>18</sup> and progesterone receptor membrane component (PGRMC) families.<sup>19</sup> PGRMC1 and PGRMC2 are 2 members of the PGRMC

family that were originally cloned as heme-1 domain proteins HPR6.6 and Dg6, respectively.<sup>20-22</sup> Based on binding affinity studies using PGRMC1 isolated from membrane fractions or generated as recombinant protein, several labs have now provided evidence that PGRMC1 binds P4 with moderate to high affinity.<sup>21,23,24</sup> More recently, PGRMC1 was shown through spectroscopic and mutagenesis studies to directly bind P4 at or near the heme binding domain.<sup>25</sup> The exact concentration of P4 in peripheral tissues is not known; however P4 concentration does vary from 1 ng/ml in serum to a 20  $\mu$ g/ml in ovarian peri-ovulatory intrafollicular fluid.

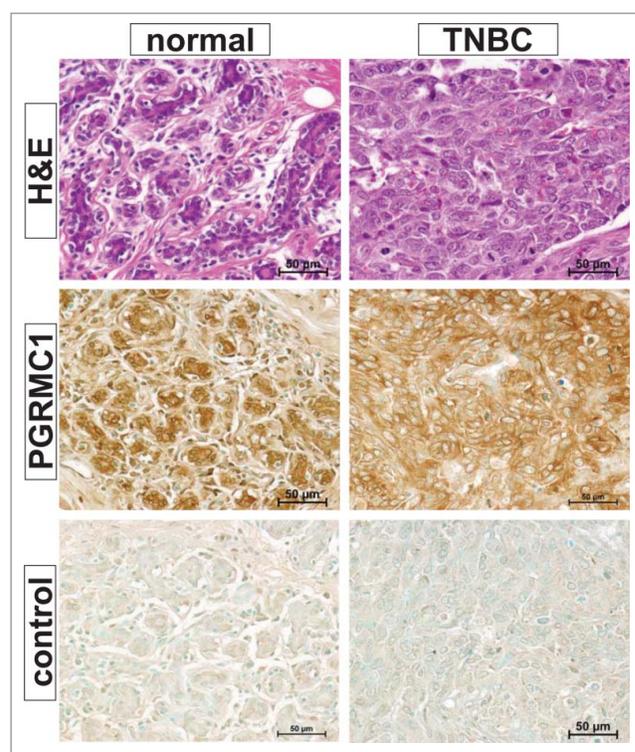
PGRMC1 is expressed in normal and malignant breast tissue, but a clear relationship between the level of expression and different types of breast cancer has not been established. Therefore examining PGRMC1 expression and function in TNBCs is important given that PGRMC1 regulates tumor growth and chemoresistance in ovarian<sup>26-28</sup> and endometrial cancers<sup>29</sup> and promotes proliferation and migration in breast cancer cells.<sup>30</sup> Moreover, PGRMC1 is up-regulated by carcinogens such as dioxin<sup>31</sup> and is overexpressed in a number of other cancers including lung, colon, and thyroid.<sup>32,33</sup> In this study, we initially assessed the expression of PGRMC1 in TNBCs and then assessed its function by developing TNBC cell lines in which PGRMC1 remained intact or was constitutively depleted using shRNA technology. These cell lines were used to assess the role of PGRMC1 in regulating proliferation and cell survival *in vitro* and tumor formation and progression in xenograft tumors in mouse models.

## Results

### **PGRMC1 expression in matched non-malignant mammary and TNBC tissues**

PGRMC1 protein expression was assessed in matched normal and grade III invasive TNBC samples obtained from University of Connecticut Health Center Research Tissue Repository Core Facility by immunohistochemistry (IHC). The absence of PGR, ESR1, and HER2 expression was demonstrated by IHC as part of the pathological analysis of the breast tumors used in this study. Based on IHC, TNBC tumors expressed PGRMC1 at levels comparable to normal mammary tissue (Fig. 1). Subcellular localization of PGRMC1 was highest in the perinuclear space in both tissues. Whereas PGRMC1 localized to the nuclei of normal breast epithelial cells, it was essentially absent from the nuclei of triple negative breast cancer cells.

To expand this analysis, Oncomine (www.oncomine.org) was used to search The Cancer Genome Atlas for expression of *PGRMC1* mRNA in patient matched normal mammary and TNBC tissues. Among the 593 matched breast cancer samples, 49 (*i.e.*, 8.3%) displayed the TNBC phenotype. After converting the log<sub>2</sub> of the median-centered ratio values to fold change values, it was revealed that differential expression between normal and TNBC matched tissues ranged from 0.64–3.64-fold. Of the 49 samples, 45 (92%) showed elevated *PGRMC1* in TNBC samples compared with normal tissues. However, only 12 matched samples (24.5%) showed increased *PGRMC1* expression in TNBC tissue greater than 2-fold. Collectively, the IHC and



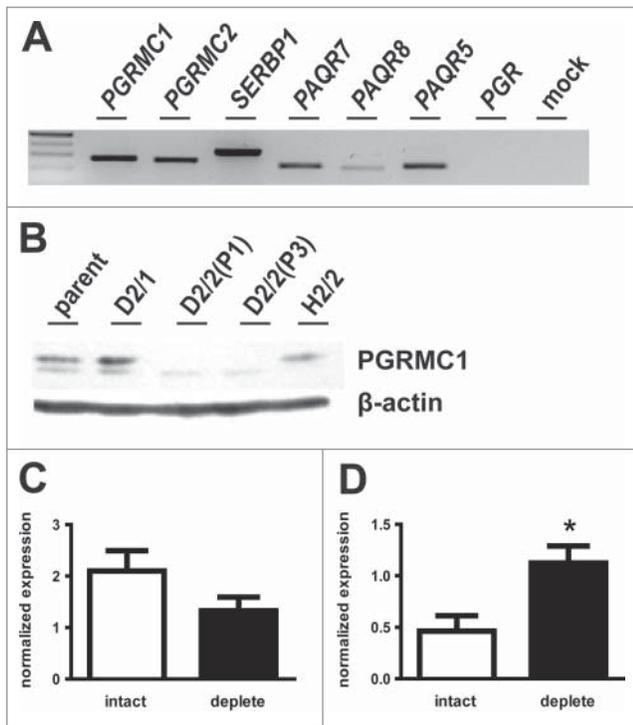
**Figure 1.** Representative images of hematoxylin and eosin stained sections and PGRMC1 expression in non-malignant breast tissue and triple negative breast cancer (TNBC) from the same patients. Samples of normal mammary tissue (left panels) and TNBC (right panels) were hematoxylin and eosin stained or stained for PGRMC1 expression by immunohistochemistry ( $n = 3$  individual patients). Some sections (bottom panels) were used as negative controls in which primary antibody was omitted.

Oncomine data indicate that that PGRMC1 is only minimally increased in TNBC compared with non-malignant mammary tissue.

### **Development of PGRMC1-intact and PGRMC1-deplete breast cancer cell lines**

The MDA-MB-231 (MDA) breast cancer cell line, which is deficient in PGR, ESR1, and HER2, was used to assess the function of PGRMC1.<sup>34</sup> Conventional RT-PCR confirmed that these cells do not express PGR, but they do express members of the progesterone membrane receptor component (PGRMC) family *PGRMC1* and *PGRMC2*, as well as the PGRMC1-interacting protein *serpine 1 mRNA binding protein (SERBP1)*. MDA cells also express 2 members of the progestin and adipoQ receptor (PAQR) family, *PAQR5* and *PAQR7*, at moderate levels, and *PAQR8* at low levels (Fig. 2A).

Having demonstrated the expression of PGRMC1 in these cells, a lentiviral-based approach was used to constitutively knockdown *PGRMC1*. While parental MDA cells and those transformed with a *PGRMC1* shRNA that was ineffective at knocking down PGRMC1 (D2/1 clone) expressed PGRMC1 protein, MDA cells transformed with a second *PGRMC1* shRNA showed >90 % knockdown efficiency (Fig. 2B, D2/2 clone). Knockdown of *PGRMC1* was maintained through multiple passages [compare passage 1 (D2/2 (P1)) with passage 3 (D2/2(P3))] (Fig. 2B).



**Figure 2.** Expression of progesterone receptors in PGRMC1-intact and PGRMC1-deplete MDA breast cancer cell lines. (A) As shown by RT-PCR, parental MDA-MB-231 breast cancer cells do not express the classical PGR. MDA cells do express several putative non-classical progesterone receptors, including PGRMC1, PGRMC1, the PGRMC1 interacting protein serine 1 mRNA binding protein (SERBP1), and members of the progestin and adipoQ receptor (PAQR) family, PAQR5, PAQR7, and PAQR8. (B) Western blot showing expression of PGRMC1 in parental MDA cells and MDA cells treated with the pLKO-1 empty vector (D2/1), shRNA against *PGRMC1* (D2/2, PGRMC1-deplete cells) or shRNA against *PGRMC2* that was ineffective at knocking down *PGRMC2* (H2/2, PGRMC1-intact cells). (C) *PAQR7* expression in PGRMC1-intact and PGRMC1-deplete cells normalized to  $\beta$ -actin ( $n = 3$ ). (D) *PGRMC2* expression in PGRMC1-intact and PGRMC1-deplete cells normalized to  $\beta$ -actin ( $n = 3$ ).

The H2/2 clone, derived from parental MDA cells transformed with a shRNA that was ineffective at knocking down *PGRMC2* mRNA, yielded expression of PGRMC1 consistent with the parental MDA cell line. An expression analysis of *PGRMC2* and *PAQR7* in both cell types revealed that while *PAQR7* expression did not differ ( $P = 0.14$ , Fig. 2C), a compensatory up-regulation in *PGRMC2* expression ( $P = 0.04$ , Fig. 2D) was observed in PGRMC1-deplete cells.

### **PGRMC1 mediates the anti-proliferative and anti-apoptotic effects of P4**

P4 suppressed mitosis in PGRMC1-intact MDA cells in a dose-dependent manner with a maximum suppression of approximately 50% ( $p \leq 0.05$ , Fig. 3A). The anti-proliferative effect of P4 was lost in PGRMC1-deplete cells cultured for 24 and 48 h, suggesting that PGRMC1 mediates the actions of P4 (Fig. 3B, C). It was also of interest to determine if P4 suppressed stress-induced apoptosis of breast cancer cells and whether or not PGRMC1 mediates the anti-apoptotic actions of P4 as has been shown in granulosa cells,<sup>35</sup> as well as ovarian<sup>26,36</sup> and endometrial cancer cells.<sup>29</sup> While P4 treatment did not change basal

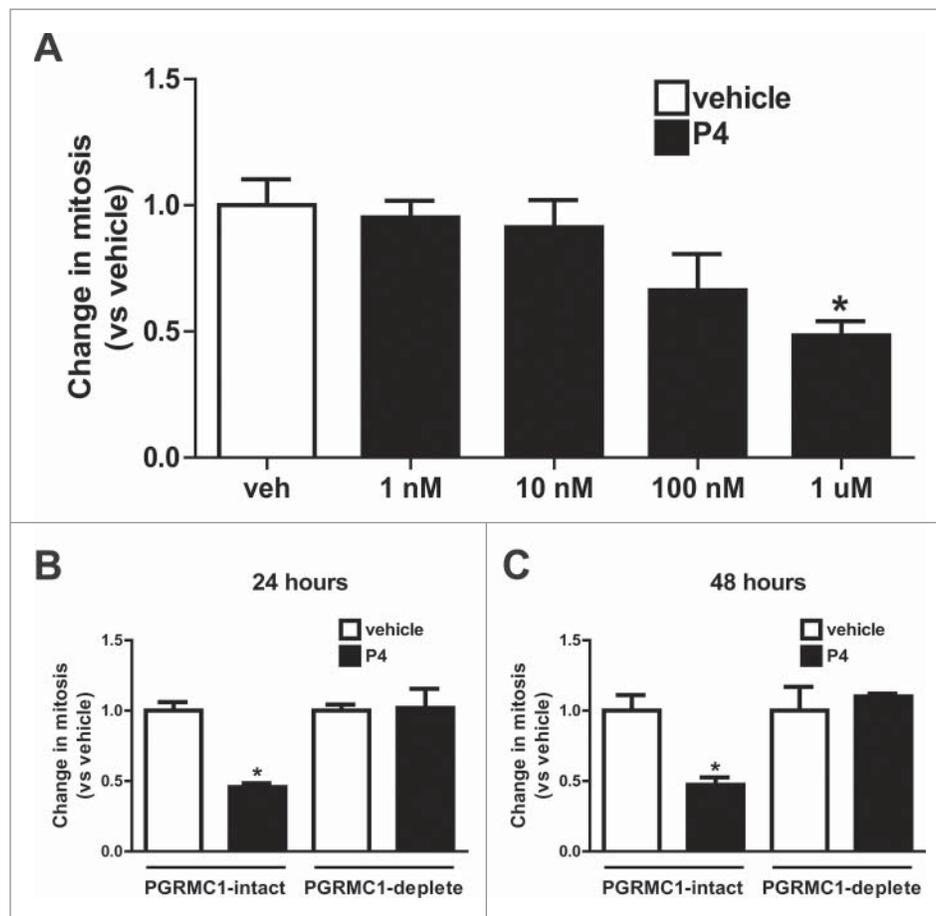
apoptosis (~5%) of PGRMC1-intact MDA cells, the chemotherapeutic agent doxorubicin (Dox) increased apoptosis to 32% after 48 h of treatment ( $p \leq 0.05$ , Fig. 4A). Dox-induced apoptosis was reduced by approximately 50% when PGRMC1-intact cells were pretreated with P4 for 30 min. The survival action of P4 was lost in PGRMC1-deplete cells (Fig. 4B).

### **PGRMC1 promotes tumor initiation and growth**

Because PGRMC1 promotes cell survival, we hypothesized that PGRMC1 would facilitate the establishment and growth of xenograft breast tumors in immunocompromised mice. It was of interest to determine whether or not PGRMC1 promoted the growth of tumors when established subcutaneously. PGRMC1-intact or PGRMC1-deplete MDA cells were injected subcutaneously into the flank of NOD/SCID mice. Tumors were measured with calipers every third day on days 17–29 post-injection. By 29 days, flank tumors derived from PGRMC1-intact MDA cells were 2.5-fold larger than tumors derived from PGRMC1-deplete MDA cells ( $p < 0.05$ , Fig. 5A). It is well-established that MDA cells do not establish intraperitoneal tumors at a high percentage in nude mice. We therefore used this line of immunocompromised mice to evaluate establishment of PGRMC1-intact and PGRMC1-deplete tumors. Intraperitoneal inoculation of nude mice with  $5 \times 10^6$  PGRMC1-intact cells resulted in the establishment of tumors in 40% of the mice. Interestingly, only 10% of nude mice inoculated with PGRMC1-deplete cells generated intraperitoneal tumors, suggesting that PGRMC1 expression confers a selective advantage in establishing xenograft tumors (Fig. 5B).

### **Discussion**

Triple negative breast cancer has a poor prognosis and few treatment options exist for women with this aggressive form of breast cancer. Understanding how mammary cells that lack PGR remain responsive to P4 is critical for understanding the etiology and progression of this disease, as well as for developing new treatment options. With regard to the level of PGRMC1 expression in breast cancer, it was previously reported that PGRMC1 expression was higher in approximately 50% of breast tumors that were not distinguished by type or grade. PGRMC1 protein and mRNA are likewise elevated in several other cancers compared with corresponding normal tissues.<sup>32,37,38</sup> In this study, PGRMC1 protein was found to be expressed in TNBC at a level consistent with matched non-malignant breast tissue. Further analysis of *PGRMC1* expression using The Cancer Genome Atlas database indicated that *PGRMC1* mRNA expression was increased by 2–3.64-fold in 25% of the available TNBC samples compared with matched normal breast tissue. Overall, PGRMC1 expression is only marginally increased in TNBC compared with normal tissue or is not differentially expressed at all depending upon the sample. This finding highlights heterogeneity even within the TNBC category of breast cancer. As in other cell types, PGRMC1 expression was most evident in the cytoplasm of both normal breast tissue and TNBC. PGRMC1 expression was also observed in the nuclei of many cells of normal breast tissue, in contrast to TNBC in which PGRMC1 nuclear staining

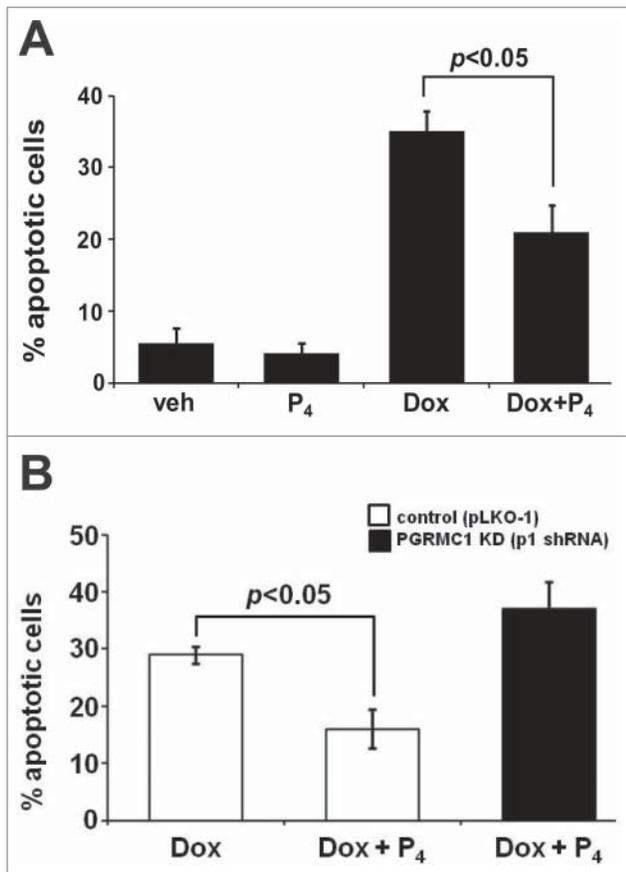


**Figure 3.** PGRMC1 mediates the anti-proliferative effect that progesterone (P4) exerts in MDA cells. (A) Dose response curve showing change in cells undergoing mitosis following 24 hours of treatment with vehicle or the indicated concentrations of P4. (B, C) After 24–48 hours of treatment, PGRMC1-intact cells display a reduction in mitosis in response to P4 (1  $\mu$ M). PGRMC1-deplete cells do not display this reduction in mitosis in response to P4. \* $p < 0.05$  compared with vehicle control,  $n = 3$ .

was not observed. This finding differs from PGRMC1 expression in ovarian cancer, where cancer progression from stage IIIC grade 2 to stage IIIC grade 3 ovarian tumors correlated with increased nuclear PGRMC1 expression.<sup>26</sup> An interesting inverse expression pattern exists between PGRMC1 and the classical progesterone receptor (PGR) in ovarian tumors in which a high level of PGRMC1 and concomitant low level of PGR are observed.<sup>28</sup> Loss of PGR in TNBC samples did not correlate with a major increase in PGRMC1 expression over non-malignant breast tissue as seen in ovarian cancer. This suggests that at least in TNBC, PGR and PGRMC1 are not transcriptionally coupled as is suggested in ovarian cancer.

While in TNBC the difference in PGRMC1 expression between normal and TNBC tissues was not great, changes in the subcellular localization (*i.e.*, cytoplasmic versus nuclear) of PGRMC1 likely contributes to the unique properties of different cancer types within the same tissue, as well as tissue-specific cancers. The nuclear localization of PGRMC1 was previously observed in highly mitotic rat granulosa cells of preantral and antral follicles *in vivo* and becomes localized to the cytoplasm and plasma membrane of granulosa cells of preovulatory follicles when the frequency of mitosis is reduced.<sup>24</sup> Similarly, nuclear PGRMC1 expression was reduced in granulosa cells in which mitosis is reduced by contact inhibition.<sup>39</sup>

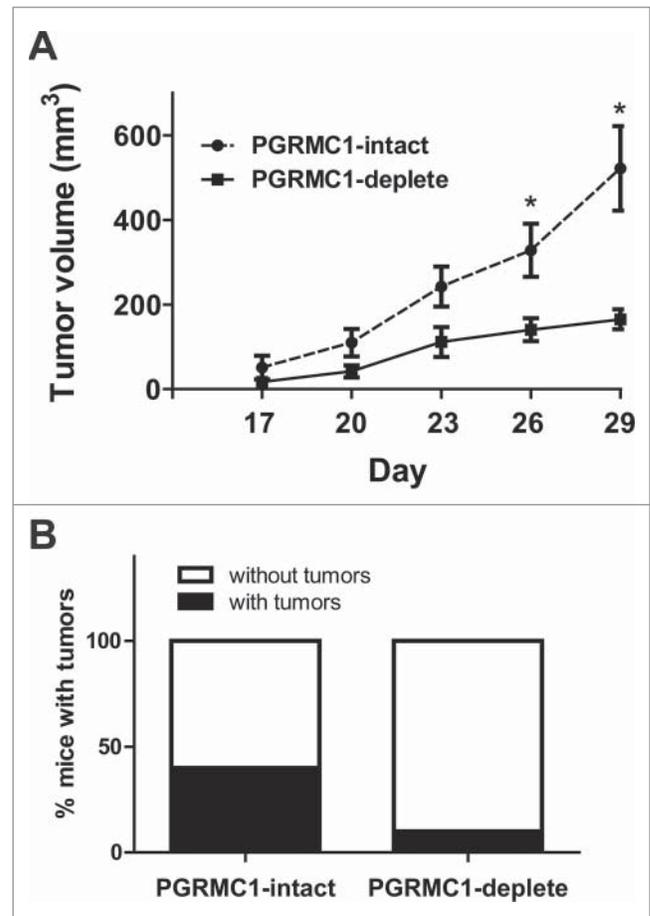
The ability of P4 to regulate mitosis has been demonstrated by numerous *in vitro* studies. In granulosa cells from various sources a role for PGRMC1 in mediating the anti-proliferative actions of P4 has been clearly established. Specifically P4 slows mitogen-induced proliferation of rat granulosa cells isolated from both immature and mature preovulatory rat follicles.<sup>24</sup> Similar findings have been established in human granulosa/luteal cells and spontaneously immortalized granulosa cells (SIGCs).<sup>40</sup> Depletion of PGRMC1 using siRNA eliminates the anti-mitotic actions of P4, suggesting that P4 activation of PGRMC1 is necessary for blocking progression through the cell cycle in granulosa cells.<sup>41</sup> More recently, PGRMC1 was shown to interact with PGRMC2 to suppress entry of SIGCs into the cell cycle.<sup>42</sup> During early gestation, PGRMC1 localizes to the nuclei of mitotic cells within the uterus at the interface between the undifferentiated and terminally differentiated stroma during decidualization.<sup>43</sup> A role for PGRMC1 in enhancing microtubule stability during mitosis has also been proposed, wherein PGRMC1 was found to directly interact with  $\beta$ -tubulin.<sup>44</sup> In addition to its role in metaphase, PGRMC1, along with its binding partner PGRMC2, is involved in regulating entry into the G1 stage of the granulosa cell cycle.<sup>42,45</sup> PGRMC1 forms a physical interaction with aurora kinase B on metaphase II chromosomes in bovine oocytes, and failure to do so is suggested to play a role in increased aneuploidy in cows with reduced antral



**Figure 4.** PGRMC1 mediates the anti-apoptotic effect that progesterone exerts in MDA cells. (A) PGRMC1-intact MDA cells undergo apoptosis in response to the chemotherapeutic agent doxorubicin (Dox, 2  $\mu\text{g}/\text{ml}$ ). The percentage of apoptotic cells is reduced by 50% when cells are pretreated with P4 (1  $\mu\text{M}$ ). (B). The anti-apoptotic actions of P4 are lost upon constitutive depletion of PGRMC1 using lentiviral-based shRNA knockdown ( $n = 3$ ).

follicle counts.<sup>46</sup> These studies in reproductive tissues provide foundational information about the mitotic/meiotic functions of PGRMC1 as a mediator of the actions of P4 in ovarian somatic and germ cells.

In the present study, PGRMC1 was shown to be necessary for mediating the anti-proliferative actions of P4 in MDA TNBC cells. This finding now parallels similar results in endometrial and ovarian cancer cells.<sup>29,57</sup> However, a role for progestins in general in regulating breast cancer cell proliferation is not at all clear. For example, MCF7 breast cancer cells showed varying levels of proliferative stimulation in response to different progestins.<sup>47</sup> Many women receive hormone replacement therapy (HRT) to alleviate common side effects associated with the menopausal transition. In this physiological setting, progestins may actually promote the development of breast cancer. Perhaps the most compelling evidence that progestins promote breast cancer comes from large scale clinical trials in which postmenopausal women are treated with different forms of HRT. At least 4 such studies, collectively involving 62,149 women, indicate that women receiving progestin in combination with estrogen are at a greater risk of developing breast cancer than women administered estrogen-alone therapy.<sup>48-51</sup> A large cohort study involving 46,355 postmenopausal women concluded



**Figure 5.** PGRMC1 enhances breast cancer cell tumorigenesis and growth of xenograft tumors *in vivo*. (A) Subcutaneous flank injection of PGRMC1-intact MDA cells into NOD/SCID mice resulted in the formation of larger tumors than does injection of PGRMC1-deplete MDA cells. Based on a 2-way ANOVA, \* $p < 0.05$  compared with PGRMC1-deplete tumors at corresponding time,  $n = 8$ . (B) Intraperitoneal injection of GFP-labeled PGRMC1-intact MDA cells into athymic nude mice resulted in the generation of tumors in a greater percentage of mice than injection of PGRMC1-deplete MDA cells ( $n = 10$ ).

that estrogen + progestin treatment resulted in an 8% increase in breast cancer risk as opposed to a 1% increased risk in women receiving estrogen-only therapy.<sup>50</sup> A portion of the well-publicized Women's Health Initiative Study involving 16,608 postmenopausal women was terminated in part because the risk of breast cancer to women receiving combined therapy was elevated beyond that for women receiving placebo.<sup>52</sup> Clearly, more research is needed to determine the molecular mechanisms whereby progestins and PGRMC1 interact to promote mammary tumor formation and growth.

It was also demonstrated here that PGRMC1 expression contributed to breast cancer cell survival *in vitro* and tumor initiation and growth *in vivo*. These data using the MDA TNBC cell line parallel our prior studies on PGRMC1 function in endometrial and ovarian cancers.<sup>29,53</sup> Physiologically, progestins have been known for years to block apoptosis in normal ovarian<sup>54</sup> and breast tissues.<sup>55,56</sup> P4 also blocks apoptosis in lactating mammary glands<sup>57</sup> and supplementation of P4 following weaning can prevent regression of mammary tissue by attenuating epithelial cell death.<sup>56</sup> A number of studies involving both primary cells<sup>58</sup> and transformed

cell lines<sup>13</sup> have demonstrated that P4 prevents cells from undergoing apoptosis during stress regardless of whether or not the cells express the classical PGR. Our findings here place PGRMC1 squarely in the middle of the pathway by which progestins promote survival of cancer cells derived from female reproductive tissues. We provided the first evidence that PGRMC1 confers resistance of endometrial cancer to chemotherapy. More specifically, endometrial tumors derived from cells expressing PGRMC1 grew much faster and were more resistant to the combined chemotherapeutic treatment of Carboplatin and Paclitaxel than PGRMC1-deplete endometrial tumors.<sup>29</sup> Likewise, PGRMC1 promotes the development, growth and Cisplatin insensitivity of human ovarian tumors.<sup>53</sup> Thus, the present studies are consistent with the concept that PGRMC1 plays a role in regulating cell survival and chemoresistance in MDA TNBC cells.

Exactly how progestins influence breast cancer cells remains unclear since some breast cancers such as TNBC lack the PGR. Several labs have now proposed that progestins signal through novel membrane progestin receptors. Members of the PAQR family, and PAQR7 in particular, have been proposed as mediators of P4 in breast cancer.<sup>59</sup> Interestingly, 2 labs have now demonstrated that PGRMC1 and PAQR7 interact to form a complete P4 binding and signaling apparatus. Indeed, the actions of P4 on entry into the cell cycle and apoptosis are dependent on both PGRMC1 and PAQR7.<sup>60,61</sup> While the exact mechanism has yet to be sorted out, our findings unequivocally demonstrate that PGRMC1 is necessary for mediating the actions of P4 *in vitro* and for TNBC growth *in vivo*. The next obvious step will be to determine PGRMC1 mechanism of action and how it might function in concert with PAQR family members to regulate P4 responses in breast cancer. PGRMC1 has been implicated in many cellular processes that may promote tumor cell survival and growth including sterol metabolism,<sup>62,63</sup> chemical detoxification,<sup>31</sup> chemoresistance,<sup>29,64,65</sup> gene transcription,<sup>39,66</sup> cell stress response<sup>67</sup> and survival,<sup>35</sup> mitosis,<sup>44,45,66</sup> and immune regulation.<sup>68</sup> A recent study identified several treatment targets that have been analyzed experimentally and in clinical trials for triple negative breast cancer.<sup>69</sup> These targets include epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and tyrosine kinases. Notably, PGRMC1 has been shown to interact with EGFR and increase plasma membrane EGFR levels.<sup>70</sup> Accordingly, PGRMC1 expression increases *in vitro* breast cancer cell proliferation in the presence of growth factors and medroxyprogesterone acetate.<sup>17</sup> A link between PGRMC1 and EGFR has also been identified in zebrafish oocytes as a component of estrogen-induced meiotic arrest.<sup>71</sup> In addition, PGRMC1 increases expression of VEGF.<sup>72,73</sup> Thus, PGRMC1 expression in TNBC may lead to enhanced functions of EGF and VEGF signaling pathways resulting to aggressive tumor growth and chemoresistance. It is perhaps not surprising that EGF and VEGF pathways are successful treatment targets for TNBC. As suggested by this and other studies,<sup>13,29,52,67</sup> inhibiting PGRMC1 could have multifaceted anti-cancer effects and thereby represent a potential candidate to target for chemotherapeutic agents.

## Materials and methods

### Immunohistochemistry using human breast tissues

Matched human breast samples harboring normal or invasive triple negative tumor tissues (grade III) were obtained from the University of Connecticut Health Center Research Tissue Repository Core Facility (<http://biobank.uhc.edu>, n = 3). Paraffin embedded sections were cut to 5  $\mu$ m. Sections were deparaffinized, rehydrated in a graded series of ethanol washes, and exposed to hydrogen peroxide to quench endogenous peroxidase activity. Antigen retrieval was completed by boiling sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Non-specific binding was blocked by a 30 min incubation with BSA and donkey serum. Sections were subsequently incubated overnight at 4°C with anti-PGRMC1 (1:350; Sigma Aldrich, St. Louis, MO). Sections were then washed in PBS, incubated in biotinylated secondary antibody, and washed again. Sections were then exposed to horseradish peroxidase-conjugated streptavidin for 45 min at room temperature (Vector Laboratories, Burlingame, CA), washed in PBS and incubated with 3,3'-diaminobenzidine substrate. PGRMC1 was revealed as a brown precipitate and sections were then counterstained with hematoxylin. As a negative control, mammary sections were incubated using the same protocol, but with omission of primary antibody.

### Development of PGRMC1-intact and PGRMC1-deplete MDA cell lines

MDA-MB-231 breast cancer cells (MDA, ATCC® HTB-26™) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 292 mg/ml L-glutamine, 100  $\mu$ g/ml streptomycin, and 2.5  $\mu$ g/ml amphotericin B at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The pLKO-1 vector harboring a hairpin sequence for targeted knockdown of human *PGRMC1* and a hairpin sequence for targeted knockdown of *PGRMC2* were individually packaged into lentiviruses at the MGH Center for Cancer Research in association with the RNAi Consortium of the Broad Institute as described in detail.<sup>29,74,75</sup> Infection titers were first established by infecting HEK293T cells grown on 96-well microtiter plates with 25  $\mu$ l of diluted transfected supernatants containing lentiviral particles and 25  $\mu$ l polybrene (Sigma; 48 mg/kg). The estimated multiplicity of infection (MOI) for each virus was 1–2, which resulted in most transduced cells containing no more than one virus integrant.<sup>34</sup> The MDA cells were then infected using conditions as described for HEK293T cells. Here, MDA cells were treated with the pLKO-1 empty vector (D2/1 cells), *PGRMC1* shRNA (D2/2 cells, PGRMC1-deplete) or *PGRMC2* shRNA (H2/2 cells, PGRMC1-intact) that was ineffective at knocking down PGRMC2. After 24 h, DMEM-F12 culture medium containing viral particles was removed and cells demonstrating stable integration of the respective plasmids were selected by culturing cells for 48–72 h in puromycin (2  $\mu$ g/ml). PGRMC1 levels were determined by RT-PCR and Western blot analysis upon expansion of selected clones. Because H2/2 cells had comparable levels of PGRMC1 and PGRMC2 to parental MDA cells and had been exposed to lentivirus and puromycin

selection consistent with D2/2 cells, H2/2 cells were used as the control cell line (*i.e.*, PGRMC1-intact).

### Cell culture and treatments

PGRMC1-intact and PGRMC1-deplete MDA cells were seeded in triplicate at equal densities ( $1 \times 10^5$  cells/well) in 24 well culture plates. One day prior to each experiment, cells were rinsed with and converted to serum-free medium. For proliferation analysis, cells were treated with vehicle (0.03% ethanol in culture medium) or P4 (1  $\mu$ M) for 24 or 48 hours. At the end of each time point, mitotic cells were identified by Hoechst nuclear staining. Cells were first washed with PBS and fixed on ice for 10 min in 4% paraformaldehyde. Nuclei were stained with Hoechst 33258 (2  $\mu$ g/ml, Sigma Chemical Co.) in 80% PBS buffered glycerol.<sup>76</sup> The number of mitotic figures was determined from 4 fields of view per replicate, and data were presented as a change in mitosis versus the vehicle treatment. For evaluating the survival function of PGRMC1, MDA cells were treated with vehicle, doxorubicin (Dox; 2  $\mu$ g/ml; Alexis Biochemicals, San Diego, CA), P4 (1  $\mu$ M), or P4 for 30 minutes followed by Dox. After 48 h of treatment, cells were fixed and processed for Hoechst staining as described above. The number of cells showing evidence of nuclear condensation or fragmentation was recorded as a percent of the total cells counted in at least 3 fields of view per well.

### RNA isolation and RT-PCR

Total RNA was isolated from parental MDA cells using TriReagent (Sigma Chemical Co., St. Louis, MO) to evaluate the expression of P4 receptors and SERBP1. Total cellular RNA was subjected to DNase I digestion (RQ1 RNase-free DNase; Promega, Madison, WI) to eliminate potential genomic DNA contamination. cDNA was synthesized using SuperScript II reverse transcriptase and oligo-dT primer (Life Technologies, Carlsbad, CA). Expression of various known and purported P4 receptors was assessed by conventional RT-PCR using primer sets shown in Table 1. Each PCR product was sequenced to confirm specific amplification of the target gene. RT-PCR was also used to assess *PGRMC1* mRNA expression in PGRMC1-deplete cells infected with lentivirus to knock down *PGRMC1* expression.

### Western blot analysis

The efficiency of *PGRMC1* knockdown was evaluated at the protein level using Western blotting. Protein lysates were

collected from parental MDA cells, as well as MDA cells transformed with specified shRNA-containing pLKO-1 vector. After electrophoretic separation of 25 mg protein from each sample using the NuPage system (Life Technologies, Carlsbad, CA), proteins were transferred (30 V, 1 h) onto polyvinylidene difluoride membranes. Nonspecific binding was blocked with 5% fat-free milk in PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature. PGRMC1 antibody (1:1000 dilution; Sigma Aldrich, St. Louis, MO) was diluted in PBST with 5% fat-free milk and applied to membranes for overnight incubation at 4°C. Membranes were then washed (3  $\times$  10 min each) in PBST buffer and incubated with biotin-conjugated secondary antibodies (1:2500 dilution; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. Membranes were washed in PBST as before, and bound antibody was detected using enhanced chemiluminescent reagents based on the manufacturer's recommendations (Amersham, Piscataway, NJ). Antibody specificity was confirmed in a control experiment in which primary antibody was omitted. To verify equal protein loading, membranes were then stripped [1 M glycine (pH 2.5), 1 h, 37°C] and re probed with  $\beta$ -actin antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA).

### Development of human breast xenograft tumors in athymic nude and NOD/SCID mice

All animal studies were approved by Institutional Animal Care and Use Committees at Washington State University or Massachusetts General Hospital. For generating subcutaneous tumors,  $2 \times 10^6$  PGRMC1-intact or PGRMC1-deplete MDA cells were suspended 1:1 in PBS/Matrigel® (BD Biosciences) and subcutaneously injected into the right and left dorsal flank of 6–8 week old female NOD/SCID mice ( $n = 8$  each group). Tumor growth was measured externally every 3 d with calipers. Tumor growth was calculated using an ellipsoidal equation for determining tumor volume (V):  $V = [\text{length} \times (\text{width}^2)] / 2$ .<sup>77</sup> Tumors were excised following euthanasia by carbon dioxide asphyxiation and cervical dislocation and then weighed, fixed in 4% paraformaldehyde overnight, and paraffin embedded. To confirm observed differences in xenograft tumor growth by subcutaneous injection, tumor growth was also assessed in athymic nude mice following intraperitoneal inoculation of PGRMC1-intact and PGRMC1-deplete cells. To accomplish this, PGRMC1-intact and PGRMC1-deplete cells were first transformed with GFP using a lentiviral system according to manufacturer's recommendations (GenTarget; San Diego, CA). Next,  $5 \times 10^6$  GFP-labeled PGRMC1-intact or PGRMC1-deplete cells were injected intraperitoneally into female nude mice (6–10 weeks of age, The Jackson Laboratories, Bar Harbor, ME;  $n = 10$  per treatment group). Tumor growth was determined at 10–12 weeks post-inoculation. GFP-labeled tumors from individual mice were counted, weighed and paraffin embedded as before. Histological sections from flank and intraperitoneal tumors were generated and stained with hematoxylin and eosin (ScyTek Laboratories, Logan, UT) using manufacturer's recommendations.

Table 1. PCR Primers.

Gene name	Primer sequences
PGR	ACAGCTTCGAGTCATTACTCTCAG ACCTCCAAGGACCATGCCAG
PGRMC1	ACCTGCTGCTGCTGGCCTCTG CCTGGATGCATCTCTCCAGC
PGRMC2 PAQR5	AGAAGCGGGACTTCAGCTTG TCCCATTTCTCGAACACTCTCC ACTATGGTGCCGTCAACCTC TCCCAGGTGACGGATAAGC
PAQR7	CGGATGATCCAGCTTCTCT CGTGTGCAGAGGCTCATAGA
PAQR8	TACCTCACCTGCAGCCTTCT GCAACAGCCAGCACAGAATA
SERBP1 ACTNB	ATCGGACCCTTCGAGGTGC TCTTCGTTACAGAGGTGGTCG GGACTTCGAGCAAGAGATGG AGCACTGTGTTGGCTACAG

## Statistical analyses

All *in vitro* experiments were replicated at least 3 times and analyzed using a one-way ANOVA followed by Tukey's *post-hoc* test. Each *in vivo* experiment was independently replicated 6–10 times with different mice being used in each experimental replicate. Tumor volume data were analyzed with a 2-way ANOVA. Data in all graphs represent the mean  $\pm$  SEM from replicated experiments as analyzed with GraphPad PRISM software (version 4.0). Assignment of mice to each experiment was made randomly. Mean values were considered statistically different when  $p \leq 0.05$  regardless of the statistical test used.

## Disclosure of potential conflicts of interest

JJ Peluso was awarded a patent on the non-genomic regulators of P4 action. The remaining authors have no relevant financial or non-financial relationships to disclose.

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