Research article

Breast cancer proteomics reveals correlation between estrogen receptor status and differential phosphorylation of PGRMC1

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Abstract

Introduction Breast tumors lacking the estrogen receptor-α (ER-α) have increased incidence of resistance to therapy and poorer clinical prognosis.

Methods Whole tissue sections from 16 cryopreserved breast cancer tumors that were either positive or negative for the ER (eight ER positive and eight ER negative) were differentially analyzed by multiplex imaging of two-dimensional PAGE gels using 54 cm isoelectric focusing. Differentially detected spots of Progesterone Receptor Membrane Component 1 (PGRMC1) were shown to differ in phosphorylation status by differential two-dimensional polyacrylamide gel electrophoresis of phosphatase-treated tumor proteins. Site directed mutagenesis was used to create putative phosphorylation site point mutants in PGRMC1. Stable transfectants of these mutants in MCF7 cells were assayed for their survival after oxidative stress, and for AKT kinase phosphorylation. Immune fluorescence using anti-PGRMC1 monoclonal antibody 5G7 was performed on breast cancer tissue microarrays.

Results Proteins significantly differentially abundant between estrogen receptor negative and estrogen receptor positive tumors at the 0.1% level were consistent with published profiles, suggesting an altered keratin pool, and increased inflammation and wound responses in estrogen receptor negative tumors. Two of three spots of PGRMC1 were more abundant in estrogen receptor negative tumors. Phosphatase treatment of breast tumor proteins indicated that the PGRMC1 isoforms differed in their phosphorylation status. Simultaneous mutation of PGRMC1 serine-56 and serine-181 fully abrogated the sensitivity of stably transfected MCF7 breast cancer cells to peroxide-induced cell death. Immune fluorescence revealed that PGRMC1 was primarily expressed in ER-negative basal epithelial cells of mammary ductules. Even in advanced tumors, high levels of ER or PGRMC1 were almost mutually exclusive in individual cells. In five out of five examined ductal in situ breast cancers of comedo type, PGRMC1 was expressed in glucose transporter 1 negative or positive poorly oxygenated cells surrounding the necrotic core, surrounded by a more distal halo of ER-positive cells.

Conclusions PGRMC1 phosphorylation may be involved in the clinical differences that underpin breast tumors of differing ER status.

CK: cytokeratin; DCIS: ductal carcinoma in situ; ER: estrogen receptor; FCS: fetal calf serum; GLUT: glucose transporter; HA: hemaglutinin; HIF: hypoxia-inducible factor; IEF: isoelectric focusing; IPG: immobilized pH gradient; PGRMC: progesterone receptor membrane component; PMF: peptide mass fingerprinting; RPMI: Roswell Park Memorial Institute; SAP: shrimp alkaline phosphatase.
Introduction
Breast cancer is among the most common forms of cancer observed in women, with approximately 185,000 new cases and 40,000 deaths estimated in the USA in 2008 [1]. Endogenous estrogens, which have effects on many organs, are thought to play a major role in the development of the breast, suggesting that increased sensitivity or longer exposures to estrogens is involved in greater risk for tumorigenesis [2-4].

The classical estrogen receptor (ER)-α is found in 50% to 80% of breast tumors and ER-α status is essential in making clinical decisions about endocrine therapy with anti-estrogens, which inhibit the mitogenic activity of estrogens in breast cancer. There are three classes of anti-estrogens currently in clinical use: selective estrogen receptor modulators (for example, tamoxifen); aromatase inhibitors; and 'pure' estrogen antagonists such as fulvestrant, which – like tamoxifen – binds to ERs competitively. However, in contrast to tamoxifen, fulvestrant's binding leads to rapid degradation and loss of the ER-α protein [5,6].

Clinically, a positive ER-α status correlates with favorable prognostic features, including a lower rate of cell proliferation and histologic evidence of tumor differentiation. ER-α status is also prognostic for the site of gross metastatic spread. For reasons unknown, ER-α-positive tumors are more likely to initially manifest clinically apparent metastases in bone, soft tissue, or the reproductive and genital tracts, whereas ER-α-negative tumors more commonly metastasize to brain and liver. Several studies have correlated ER-α expression with lower Matrigel invasiveness and reduced metastatic potential of breast cancer cell lines [7,8]. Moreover, when ER-α-positive cells are implanted in nude mice, tumors appear only in the presence of estrogens and are poorly metastatic as compared with those developed from ER-α-negative breast cancer cell lines [9,10]. This paradox suggests that ER-α expression could be associated with or involved in pathways that hinder cancer progression.

At the transcriptome level, gene expression analysis has revealed that different molecular subtypes exist within ER-α-positive and ER-α-negative breast cancers, and these are associated with different clinical outcomes. ER-α-positive tumors exist in at least two subtypes, luminal A and luminal B, which vary markedly in terms of gene expression and prognosis [11]. Conversely, hormone-receptor-negative breast cancer comprises two distinct subtypes, the Her2 (human epidermal growth factor receptor 2) subtype and the basal-like subtype [11,12], which differ in biology and behavior, and are both associated with a poor outcome.

Importantly, a very similar subdivision of breast cancers has been produced based upon immunohistochemistry, conducted to analyze patterns of protein expression in tumor sections, which suggests that a few protein biomarkers can be used to stratify breast cancers into different fundamental groups [13,14]. One set of biomarkers comprises the family of cytokeratins (CKs). They can be grouped into the 'luminal CKs' (CK-7/8, CK-18, and CK-19) and into the 'basal CKs' (CK-5/6 and CK-14) [13].

In addition to these molecular portraits, it has been shown that expression patterns present in primary breast cancers are also observed in their respective metastases [15]. Other gene expression profiles have distinguished breast cancers according to the differential expression of a wound response signature. More than 20 years ago, based on histologic similarities between tumors and wound healing, Dvorak [16] proposed that the tumor stroma is 'normal wound healing gone awry'. Since then it has been discovered that genes induced in a fibroblast serum-response program are expressed in tumors by the tumor cells themselves, by tumor-associated fibroblasts, or both [17]. The molecular features that define this wound-like phenotype are evident at an early clinical stage, persist during treatment, and predict increased risk for metastasis and death in breast, lung, and gastric carcinomas.

We previously published a system for proteomic analysis involving differential radioactive labeling of samples and separation using 54 cm immobilized pH gradient (IPG)-isoelectric focusing (IEF) [18,19]. In the present study we used this system to identify protein species with pronounced and consistent differential abundance between sample categories. We employed large homogenous invasive ductal breast carcinomas, which are well suited for conventional proteomics analysis, but are becoming increasingly rare because of improved screening programs. Differential proteomic analysis of pooled tumors that were selected on the basis of being either ER-α-positive or ER-α-negative unexpectedly revealed differentially abundant phosphorylated isoforms of the cytochrome b5-domain protein progesterone receptor membrane component (PGRMC)1 (reviewed in [20]) between these tumors.

Materials and methods
Quantitative population multiplex proteomics
The tumors used in the present study were from a tissue archive previously described [19]. Tumors with large homogenous lesion regions were selected, assayed for RNA integrity, and classified as being either positive or negative for ER-α, as described previously [19]. Frozen tumor sections of 10 μm were lysed directly into SDS buffer, separately iodinated in inverse replicates with each of 125I and 131I, and separated by 54 cm daisy chain IPG-IEF after sample pooling, as described previously [18]. Radioimaging, image processing with the Pic/GREG software (Fraunhofer Institute for Applied Information Technology, 53754 Sankt Augustin, Germany), statistical analysis of the gels, and identification of proteins by mass spectrometry were performed as described previously [19]. There were no identifications of multiple proteins from single spots. The peptide mass fingerprinting (PMF) MASCOT
scores [21] of all identifications are provided, indicating the reliability of individual probability-based protein identification assignments.

Shrimp alkaline phosphatase analysis
Cryogenic slices from six patients (30 slices T433, 40 slices T443, 40 slices T469, 40 slices T470, 35 slices T623, and 30 slices T640) were each extracted with 200 μl aliquots of shrimp alkaline phosphatase (SAP)-dephosphorylation buffer (50 mmol/l Tris [pH 8.5], 5 mmol/l MgCl₂, 0.25% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, supplemented with 1× EDTA-free complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, Switzerland)). This precooled buffer was added directly on ice to the frozen slices in Eppendorf tubes and the tissue was mechanically homogenized using a plastic pellet pestle. Tubes were vortexed and incubated for 30 minutes at 4°C, followed by centrifugation for 15 minutes at 14,000 g at 4°C. Supernatants were collected and pooled together, and the protein concentration was assayed using the BCA method, as described previously [19]. The yield was approximately 4 mg protein. Thirty units of SAP and containing the following phosphatase inhibitors: activated vanadate, sodium fluoride, and sodium fluoride were added into 800 μl of protein in 400 μl in SAP-dephosphorylation buffer, followed by mixing and incubation for 16 hours at 37°C. In parallel, a mock incubation control was performed on 800 μg protein in the same buffer without addition of SAP and containing the following phosphatase inhibitors: activated vanadate, sodium fluoride, and sodium glycero phosphate at final concentrations of 1 mmol/l, 5 mmol/l, and 5 mmol/l, respectively. The incubation was performed in parallel at 37°C for 16 hours. After incubation the proteins were frozen at -80°C. A nonincubated raw lysate control containing 800 μg protein in 400 μl SAP buffer was frozen at -80°C without additions or incubation. Frozen protein mixtures were thawed, precipitated, and resuspended at 1 μg/μl in boiling 0.1 M Tris, 2% SDS (pH8.5). Sixty micrograms of protein were then used for iodination with each of 125I or 131I. Differential inverse replicate ProteoTope analysis was as described above for 54 cm daisy chain IPG-IEF after rehydration loading overnight to pH 5 to 6 IPG (ProteoSys AG, Mainz, Germany).

Cell culture
MCF-7 cells stably transfected with PGRMC1 and mutants, respectively, were established. Five micrograms of expression vector pcDNA3.1 containing hemaglutinin (HA)-tagged mPR (PGRMC1) wild-type or HA-tagged mutants S56A, S180A, S56A/S180A, S66A/C128S/S180A, Y138F, Y179F, or Y179F/S180A were transfected into MCF-7 breast cancer cells. For transfections, a transfection device and kits from AMAXA Biosystems (Gaithersburg, MD, USA) were used, in accordance with the manufacturer’s recommendation. A total of 2 × 10⁶ cells were transfected with circular plasmids and plated with Roswell Park Memorial Institute (RPMI)-medium for 24 hours. Then medium was changed to RPMI medium complete medium containing 60 μg/ml hygromycin B and cells were cultured for 2 weeks for selection of stable integration events. After 2 weeks single colonies had formed and limiting dilution assays were performed to select for colonies grown from a single cell. Colonies were trypsinized, counted, and diluted in twofold dilutions to obtain clones.

For functional assays MCF-7 cells and derivatives were maintained in RPMI medium containing 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, and penicillin and streptomycin. PGRMC1 expression in all cell lines was confirmed by Western blot using an HA-specific monoclonal antibody [22]. Charcoal/dextran-treated FCS was obtained from Hyclone (Acros Organics N.V., Geel, 2440 Belgium; Catalogue Nr SH30068).

Cell viability assays
Cell viability was measured using an assay that utilized the fluorometric determination of ATP levels as an indicator of cell viability, namely the ATP-TCA kit (TCA-100; DCS Innovative Diagnostik Systeme, Hamburg, Germany), as follows. Cells were incubated with 50 μl tumor cell extraction reagent and 50 μl RPMI medium for 30 minutes at room temperature. Then, 50 μl supernatant was transferred into a 96-well plate and 50 μl chemoluminescence reagent was added. Chemoluminescence was quantified using a luminescence reader (Berthold, Bad Wildbad, Germany). The fluorometric readout is presented in relative light units (higher relative light unit values are associated with higher ATP levels and therefore higher levels of cell viability).

Phosphorylation of Akt
A total of 1 × 10⁶ cells were seeded in a six-well plate and cultured overnight at 37°C and 5% carbon dioxide in RPMI medium and 10% FCS. Cells were treated with 1 mmol/l H₂O₂ in RPMI for 30 minutes at 37°C and 5% carbon dioxide. Subsequently, cell culture dishes were transferred immediately to ice and lysed in M-PER mammalian protein extraction reagent containing protease inhibitors. Protein concentration was determined using the BCA Protein Assay Kit (both from Pierce, Rockford, IL, USA). Twenty micrograms of protein was loaded per lane onto a 10% polyacrylamide gel and separated by electrophoresis.

The gel was blotted on Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) and blocked overnight at 4°C using 5% milk in TBST buffer. Western blot for P-Akt was performed using rabbit anti-P-Akt 1/2/3 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature. The following antibodies were incubated with a single membrane: mouse anti-Akt-1 to detect total Akt (1:200; Santa Cruz Biotechnology), rabbit anti-HA-probe to detect HA-tagged exogenous PGRMC1 (Y-11; 1:200; Santa Cruz Biotechnology), and rabbit anti-actin (I-19; 1:500; Santa Cruz Biotechnology). As secondary antibodies, either goat anti-rabbit IgG-horse radish peroxidase (1:2,500; Santa Cruz Biotechnology), or biotinylated anti-mouse IgG (H+L; 1:2,000; Vector laboratories, Burlingame, CA, USA) followed by

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streptavidin/HRP (1:1,000; DakoCytomation, Hamburg, Germany) were applied. Chemoluminescence was generated using ECL Western Blotting Analysis System (Amersham). The signals were measured with a Lumi-Imager (Boehringer, Mannheim, Germany).

Immune fluorescence analysis
Monoclonal anti-PGRMC1 antibody 5G7 was generated in mice inoculated with the cytoplasmic domain (Δ43hpr6) of bacterially expressed PGRMC1, representing a 25 kDa protein of which the amino-terminal 43 amino acids of hpr6 were deleted. For immune fluorescence analysis, paraffin-embedded breast cancer tissue was used. Paraffin-embedded sections (5 μm) of a tissue microarray were prepared and labeled with antibodies, in accordance with standard protocols. Sections were deparaffinized and boiled in 10 mmol/l citrate buffer for 1 minute each. The secondary antibody was then applied to the slide for 60 minutes. Sections were washed with phosphate-buffered saline for 1 minute each. The secondary antibody was then rinsed with antibodies, in accordance with standard protocols. Sections were deparaffinized and boiled in 10 mmol/l citrate buffer for 4 minutes in a pressure cooker to expose the antigen. Blocking of unspecific antibody binding sites was performed with goat normal serum (10% dilution in antibody diluent [DakoCytomation; Dako Denmark A/S, 2600 Glostrup, Denmark]). Slides were incubated for 60 minutes with the first antibody and rinsed in three changes of phosphate-buffered saline for 1 minute each. The secondary antibody was then applied to the slide for 60 minutes. Sections were washed again to remove the unbound antibody. For counter staining, Vectashield (Vector Laboratories) was used to embed labeled tissue. Labeling reactions were performed in a humified chamber. The following primary antibodies were used: 5G7 monoclonal antibody (1:10), ER-α (1: 200; DCS, Hamburg, Germany) and glucose transporter (GLUT)1 (1:100; Dianova, Hamburg, Germany). Goat anti-mouse AlexaFluor 594 and goat anti-rabbit AlexaFluor488 (both 1:1,000; Invitrogen, Karlsruhe, Germany) were used as secondary antibodies. Preincubation of the 5G7 with recombinant PGRMC1 protein was performed overnight at 4°C at a protein concentration of 0.5 μg/ml. Control was treated in the same way but without recombinant protein.

Results
Quantitative multiplex proteomic analysis of pooled tumor samples
We applied a sample pooling strategy to the analysis of clinical protein samples, which permitted generation of effective results from limited amounts of sample [19]. Eight ER-α-positive and eight ER-α-negative tumors were randomly assigned to the subpools summarized in Table 1, each sub-pool containing normalized equal amounts of protein from two tumors. Pooled samples were differentially quantified according to the regimen summarized in Table 1 (subpool ER+1 [containing T378 and T392] was differentially compared with subpool ER-1 [containing T433 and T443], ER+2 was compared with ER-2, ER+3 was compared with ER-3, and ER+4 was compared with ER-4). Thus, there were four differential comparisons, each performed in inverse replicate 54 cm serial IEF-IPG to generate eight IEF samples and 24× two-dimen-
sional PAGE gels, which were differentially quantified by ProteoTope imaging. Additionally, proteins from all eight tumors from each category were pooled into two master pools, which were also compared by ProteoTope (as presented in Figure 1). Similar gel sets were performed for the paired tumor pools in Table 1 and quantified. Spots were matched across gels, and their intensities were analyzed relative to ER-α status. Synthetic average gel images of the comparisons of pools from Table 1 were constructed by computer, as shown in Figure 2. The two-dimensional PAGE positions of the statistically most significant differential protein spots identified by mass spectrometry are indicated in Figure 2, and their identities are shown in Figure 3. In total, proteins from 325 spots were identified by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) PMF with MASCOT scores greater than 70, of which 72 spots represented 16 proteins that were identified in more than one protein spot (Additional file 1 [Table S1]).

The differential results observed for this study (Figure 3) provided a protein profile that was consistent with published studies on this clinical system [23-25]. The profile of differentially abundant proteins detected between ER-α-positive and ER-α-negative tumors (Figure 3) shows similarity with the recently reported gene expression profile identified as being specific for the wound response reported for ER-α-negative tumors [17,26]. In the wound response, a genetic program is activated when cells within a tissue are exposed to serum proteins, indicative of permeabilization of the vascular endothelium and local injury. This expression profile provides an index for the extent of wound healing activity in cancers, and this correlates negatively with overall survival and positively with the incidence of metastasis [27,28]. Our results are consistent with the previously reported activated wound response in ER-α-negative tumors. In particular, CK-8, cathepsin B, heat shock protein 27, and ferritin light chain were less abundant in ER-α-negative tumors than in ER-α-positive tumors, whereas vimentin, apolipoprotein A1, cyclophrin A, transferrin, carbonic anhydrase, and PGRMC1 were more abundant. This is reminiscent of the wound response signature reported by Velardo and coworkers [26], in which PGRMC1 was upregulated late in the response (presented in Supplementary Table 1 of the report by Velardo and coworkers; Al010357 EST [VEMA] ventral midline antigen = PGRMC1). The serum proteins apolipoprotein A-I and albumin were recently found to be more abundant in a proteomic analysis of injured spinal cord tissue, whereas heat shock protein 27 was found to be downregulated in this wound response relevant system [29]. Taken together, these data indicate that the number of patients analyzed suitably identified differences in protein abundances that were strongly correlated with the presence of the ER-α, indicating that our experimental system yielded results that reflected the biology underlying the ER-α-positive versus ER-α-negative test design.
Figure 1

Fifty-four centimeter differential ProteoTope analysis. The panels show actual images from the inverse replicate labeled ProteoTope comparison of all tumors pooled. (a) Inverse ProteoTope comparison of all ER-positive samples pooled into one sample (ER+1 to ER+4 from Table 1) co-electrophoresed with pooled all ER-negative samples (ER-1 to ER-4 from Table 1). Proteins from ER-positive tumors are labeled with I-125, differentially compared with proteins from pooled ER-negative labeled tumors with I-131. The upper panels show the individual signal detected for each isotope, depicted in false spectral color. The signals for each isotope were normalized against each other for total relative intensity in the lower dual channel images, where the signal for I-125 is blue, the signal for I-131 is orange, and equal amounts of both signals produces gray or black signal. Two pure sources each of I-131 and I-125, as well as a 50% mixture of both isotopes, are measured on 2 mm sources pipetted next to each dried gel as imaging controls. The pH ranges of the 18 cm IPGs used for serial IEF are indicated above the panels, and the radioactive iodine isotope signals depicted in each panel are indicated on the right. In this experiment all iodination reactions were performed on 60 μg protein. In the examples shown, the I-125 signal is systematically stronger in all gels (compare lower panels for individual isotopes). Arrows in the enlarged lower panel show three spots identified as PGRMC1. (b) The top panels show the inverse replicate experiment of panel a, where sample ER+1 is labeled with I-131, and sample ER-1 is labeled with I-125. The panel presentation otherwise follows that given above for panel a. Similar gels were produced for all corresponding differential analyses depicted in Table 1. ER, estrogen receptor; IEF, isoelectric focusing; IPG, immobilized pH gradient; PGRMC, progesterone receptor membrane component.
PGRMC1 is more abundant in ER-α-negative tumors

PGRMC1, which had not previously been directly associated with ER-α status in breast cancer, was detected in three separate spots. Two of these were significantly more abundant in ER-α-negative tumors (Figure 3), and these were the more basic two spots (Figure 2).

Two-dimensional PAGE isoforms of PGRMC1 differ in phosphorylation status

To assess whether differences in distinct two-dimensional PAGE spot isoforms were due to distinctly phosphorylated species of PGRMC1, we treated proteins from primary breast tumors with SAP and quantified differences in protein isoform abundances using inverse-replicate ProteoTope (Figure 4). Importantly, SAP-dependent differences in relative signal intensity were reproducibly detected in inverse replicate labeled experiments, in which the intensity of spot 1–24 was reduced upon SAP treatment, and the intensity of spot 1–22 increased after SAP treatment. The middle spot 1–23 exhibited variable abundance changes, perhaps because of experimental variation. By contrast, when the mock treatment was compared with the raw extract, the ratios between both samples approximated 1:1. Qualitatively similar overall results were observed in an independent replicate (data not shown). Thus, the differences in intensity of respective spots between samples were not due to the incubation, but rather were due to the presence of phosphatase activity in the incubation mixture. This result demonstrates that the most acidic PGRMC1 spots can be dephosphorylated, whereupon they migrate to one of the more basic spots in two-dimensional PAGE. Taken together with the results presented in Figure 3 for these three protein spots, this provides evidence that PGRMC1 is more highly phosphorylated in ER-α-positive than in ER-α-negative tumors. Because phosphatase treatment did not totally eliminate any PGRMC1 spots, it is possible that protein species within these two-dimensional PAGE spots may also differ by modifications other than phosphorylation.

Phosphorylation site mutants of PGRMC1 can affect cell survival

Based upon some of the predicted and observed phosphorylation sites for PGRMC1 [20,30,31], we constructed a panel of HA-tagged PGRMC1 expression plasmids based upon pcDNA3_MPR_3HA [32], with amino acid substitutions at the positions of serine-56, serine-180, tyrosine-138, and tyrosine-179 (Figure 5). Because of the proposed role of disulfide bridging to form a 56 kDa dimeric form of PGRMC1, one of the mutants also involved substitution of the conserved cysteine-128 to serine. This residue is the only cysteine in the human PGRMC1 cytochrome b5 domain and is the only phylogenetically conserved cysteine in the protein. On the surface of the protein at the carboxyl-terminal end of helix 3, this cysteine is ideally situated on the protein to physically interact with other proteins (Figure 5) [20].

Stable expression of PGRMC1 (Hpr6.6) enhances the susceptibility of MCF-7 breast cancer cells to lethality caused by H₂O₂ exposure [22]. We transfected MCF-7 cells with each of the PGRMC1 expression plasmids from Figure 5 and established stable transfectant cell lines. Expression of exogenous PGRMC1 was confirmed in all cell lines using an anti-HA tag antibody. The different cell lines exhibited grossly similar cell
survival in medium containing 10% FCS (Figure 6a). Upon treatment with 50 μmol/l H2O2, the viability of untransfected control cells was reduced, and the expression of wild-type PGRMC1 greatly sensitized cells to H2O2 stress, resulting in lower cell viability (Figure 6b), as expected. Expression of many of the mutant PGRMC1 proteins produced viability comparable to that of nontransfected MCF-7 control cells. However, two of the cell lines (S56A/S180A and Y179F/S180A) exhibited higher viability levels than native MCF-7 cells. The S56A/S180A-expressing cells appeared impervious to the effects of H2O2, whereas the response of Y179F/S180A-expressing cells varied from marginal survival rates (Figure 6b) to no enhanced survival in other experiments (Figure 7; data not shown).

The above experiment was performed with replicate design but using charcoal/dextran-treated 10% FCS. Charcoal-stripping removes steroid hormones and other hydrophobic components such as cholesterol from the FCS. All cell lines were able to grow in this medium (Figure 6c); however, the degree of viability was greatly impaired in the presence of H2O2 for all cell lines except the S56A/S180A double mutant (Figure 6d). Therefore, charcoal pretreatment of FCS possibly removes some component that is necessary for the marginal survival of PGRMC1 mutant Y179F/S180A but not for S56A/S180A.
Hand and Craven \[22\] also observed phosphorylation of the kinase Akt upon H\(_2\)O\(_2\)-induced death of MCF-7 cells; we therefore assayed the degree of Akt phosphorylation in these cells by Western blot. As expected, based on the work reported by Hand and Craven \[22\], control MCF-7 cells or cells stably expressing exogenous wild-type PGRMC1 exhibited an increase in Akt phosphorylation that correlated with reduced viability after H\(_2\)O\(_2\) exposure. However, the S56A/S180A mutant that was able to survive peroxide treatment also did not exhibit marked phosphorylation of Akt (Figure 7). In some experiments the Y179F/S180A mutant also exhibited partially reduced levels of Akt phosphorylation (data not shown). Taken together, these data are consistent with differences in the phosphorylation status of PGRMC1 observed in breast cancers, potentially being able to influence the clinically relevant survival phenotype of those cancers. However, further experiments will be necessary to confirm this hypothesis and to elucidate the mechanisms involved.

### Table 1

| Experimental variable designation | Pools | Tumor number | RNA quality | Tumor status | Lymph node status | Grade | ER status | PR status | Her2/neu status | Age of patient (years) |
|----------------------------------|-------|--------------|-------------|--------------|------------------|-------|-----------|-----------|-------------------|------------------------|
| ER positive                      |       | T378         | ok          | 2            | 0                | 2     | 12        | 4         | 0                 | 75                     |
| ER+1                             |       | T392         | ok          | 2            | 0                | 2     | 12        | 2         | 2\(^a\)           | 61                     |
| ER+2                             |       | T460         | ok          | 2            | 0                | 2     | 4         | 8         | 3                 | 79                     |
| ER+3                             |       | T464         | ok          | 2            | 0                | 2     | 12        | 8         | 0                 | 50                     |
| ER+4                             |       | T288         | ok          | 2            | 0                | 2     | 12        | 4         | 1                 | 76                     |
| ER negative                      |       | T711         | ok          | 4            | 2                | 3     | 8         | 6         | 0                 | 65                     |
| ER+1                             |       | T712         | ok          | 2            | 1                | 2     | 9         | 4         | 0                 | 58                     |
| ER+2                             |       | T425         | ok          | 2            | 1                | 2     | 12        | 0         | 0                 | 78                     |

Individual tumors are designated by their tumor bank T registration numbers. Experimental, clinical, and histopathological parameters are listed. Eight ER-positive and eight ER-negative tumors are grouped into four pools of two tumors each, as indicated. Clinical data comprise the following: tumor status ranging from pT1 (tumor 2 cm or smaller in greatest dimension) to pT3 (tumor >5 cm); lymph node status from pN0 (no regional lymph node metastasis) to pN3 (metastasis to ipsilateral internal mammary lymph nodes \[s\]) and pNx (regional lymph node cannot be assessed); tumor grade from 2 (moderately differentiated) to 3 (poorly differentiated); histopathologic data for ER and PR (0 = undetectable; 1 to 3 = weakly positive; 4 to 7 = moderately positive; 8–12 = highly positive); and Her2/neu-status (0 = negative, positive +1 to +3). Ages for each patient are given in years. \(^a\)No gene amplification detected. \(x\), pNx regional lymph nodes cannot be assessed. ER, estrogen receptor; HER, human epidermal growth factor receptor; PR, progesterone receptor.
PGRMC1 location in tumors

We generated a new specific monoclonal antibody (5G7) against the cytoplasmic domain of PGRMC1 lacking the amino-terminal first 46 amino acids. This antibody recognized endogenous PGRMC1 in breast cancer tissues (Figure 8). The PGRMC1 signal (red in Figure 8) could effectively be abrogated by competitive pre-incubation of the antibody with recombinant PGRMC1 protein (Figure 8a). Co-incubation of anti-ER-α antibody (green signal) and anti-PGRMC1 antibody (red) revealed that these proteins were predominantly expressed in different cells, even in ER-α-positive tumors. Remarkably, very few individual cells were observed that exhibited abundant levels of both ER-α and PGRMC1 (yellow in Figure 8b). In ductal in situ breast cancers of comedo-type, PGRMC1 was present in cells surrounding the necrotic centre of the tumor, whereas ER-α was expressed in cells more distal to the necrotic centre (Figure 8c, i to 8c, v). The green fluorescence in the comedo necrotic zone was due to autofluorescence of necrotic cellular material (Figure 8c, iv). The cells expressing PGRMC1 were presumably in the hypoxic zone, and so we performed co-immunofluorescence labeling with 5G7 anti-PGRMC1 and anti-GLUT-1, a hypoxia-inducible factor (HIF)-1 inducible marker for hypoxic cells [33]. Although not all PGRMC1-expressing cells expressed GLUT-1, the vast majority of GLUT-1-positive cells co-expressed PGRMC1. Where PGRMC1 and GLUT-1 proteins were expressed in the same cells, PGRMC1 exhibited a perinuclear location that contrasted markedly with the cytoplasmic membrane localization of GLUT-1 (Figure 8c, vi to 8c, vii). This result provides confirmation of the cellular location of PGRMC1 that was observed for over-expressed HA-tagged PGRMC1/Hpr6 [22].

Figure 4

PGRMC1 isoforms in breast tissue differ in phosphorylation status. Shown are differential inverse replicate ProteoTope quantification of PGRMC1 spots 1–22, 1–23, and 1–24 from Figure 2 and Figure 3 from SAP-treated and control samples. The original sample represents proteins pooled from several primary breast tumors for experimental treatment. The treatment nomenclature is as follows: phosphatase treated sample (+SAP), mock incubation control with phosphatase inhibitors and without addition of SAP (-SAP), and original sample frozen before incubation (Raw). Spot numbers are indicated. Quantification of the differential ratio of signal intensities from two samples per two-dimensional PAGE gel for each of the spots from the indicated inverse replicate gel pairs +SAP versus -SAP, +SAP versus Raw, and -SAP versus Raw. The ratio of signal for control and treated samples increases in a phosphatase-dependent manner, consistent with at least some fraction of spots 1–24 and 1–23 in the original sample representing phosphorylated isoforms of PGRMC1 present in spot 1–22. PGRMC, progesterone receptor membrane component; SAP, shrimp alkaline phosphatase.

Figure 5

PGRMC1 expression plasmids expressing wild-type PGRMC1 and various amino acid mutants. Mutations were introduced by polymerase chain reaction, after which the entire open reading frame of product plasmids was confirmed correct by DNA sequencing. Individual codon mutations were encoded by the following sequences: serine-56 to alanine (S56A) AGC → GCC; cysteine-128 to serine (C128S) TGC → AGC; tyrosine-138 to phenylalanine (Y138F) TAC → TTC; tyrosine-179 to phenylalanine (Y179F) TAC → TTC; and serine-180 to alanine (S180A) TCA → GCA. Amino acid numbering is according to human PGRMC1 Uniprot O00264, which does not include the initiator methionine. Uniprot Q6IB11 corresponds to the same sequence with initiator methionine included, in which mutated human PGRMC1 amino acids would be numbered as S57, C129, Y139, Y180, and S181. The position of three tandem influenza virus hemaglutinin epitope sequences (HA) is indicated at the carboxyl-terminus of the open reading frame. PGMRC, progesterone receptor membrane component.
Discussion
Validation of the differential abundance profile

Despite the small number of well characterized tumors employed in this analysis, the sample size is sufficient to detect marked and consistent differences between the test classes with reliable significance. A discussion of the protein abundance profile obtained is provided in Additional file 1. Taken together, these results suggest that our comparison of just eight patients from each group of ER-α-positive or ER-α-negative tumors (Table 1 and Figure 3) has provided useful results that grossly reflect the known differences in biology between these cell types. Therefore, previously unreported protein differences were of extreme interest.

PGRMC1 and cancer implications

We demonstrate a higher abundance of hypophosphorylated PGRMC1 isoforms in the specific subpopulation of clinically relevant ER-α-negative cancers. Further studies in a larger patient collective will be necessary to correlate specific PGRMC1 isoforms with other tumor markers in addition to ER-α.

We identified three two-dimensional spots corresponding to PGRMC1 (Additional data file 1 [Table S1] and Figure 3), two of which were significantly more abundant in ER-α-negative tumors (spots 1–22 and 1–23 in Figure 3). Phosphatase treatment of primary breast cancer proteins demonstrated that these different isoforms of PGRMC1 differed at least partly in their phosphorylation status (Figure 4).

PGRMC1 was previously reported to be more abundant in a variety of cancers, including breast cancer (although differential ER-α status was not reported), and a perinuclear localization was suggested to implicate it in a role involving cytochrome P450 activation and steroid metabolism [34]. The differential abundance of PGRMC1 protein between breast cancers of different ER-α status is notable because we previously identified the distantly related cytochrome b5-domain feudesin/SPUF protein and cytochrome b5 itself to have been slightly yet significantly differentially abundant between breast tumors that were all positive for the ER-α but which differed in the expression level of the cytoplasmic progesterone receptor [19]. Indeed, cytochrome b5 was also marginally yet signifi-
cantly more abundant in the ER-α-positive tumors in our present study (1.2-fold [P = 0.03]; spot 1–32 in Figure 3). Hughes and colleagues [35] recently reported that PGRMC1 and a fungal homolog are present in evolutionarily conserved protein complexes with respective members of the cytochrome P450 class of enzymes, including the Cyp51A1 protein, which is involved in the production of cholesterol from lanosterol. Furthermore, they demonstrated that reduction in the level of PGRMC1 mRNA and protein produced an elevation in lanosterol levels. A variety of experiments suggest a role of cholesterol in the biology of PGRMC1, as reviewed by Cahill [20]. The rate-limiting enzyme of the mevalonate pathway leading to cholesterol synthesis is hydroxymethylglutaryl-coenzyme A reductase, and this enzyme is both regulated by cholesterol levels [36,37] and is diagnostic of a recently identified class of poor prognosis apocrine breast cancers that were both ER-α and progesterone receptor negative [38].

The results presented in Figure 8 indicate that PGRMC1 is abundantly expressed in a population of ER-α-negative and GLUT-1-positive cells in the hypoxic zone surrounding necrotic tumor tissue. GLUT-1 is a membrane glucose transporter that is important in the enhanced rates of anaerobic metabolism of tumors, known as the Warburg effect [33]. Intriguingly, because not all PGRMC1-positive cells expressed GLUT-1 (Figure 8, vi), the population of PGRMC1-expressing cells may have given rise to those expressing GLUT-1, suggesting avenues for future experimentation.

The GLUT-1 and HIF-1 positive cells occupying the hypoxic tumor microenvironment adjacent to necrotic zones are resilient to chemotherapy and frequently give rise to metastases. Although a literature search revealed no directly reported association between the mevalonate pathway and hypoxia, the Wilm’s tumor suppressor protein WT1 is thought to suppress growth by downregulating the mevalonate pathway [39], and the hypoxic expression of WT1 is regulated by HIF-1 [40]. Hypoxic conditions have been shown to promote phenotypic de-differentiation in ductal breast carcinoma in situ. In mammary ductal in situ breast cancer of comedo-type, ductal carcinoma in situ (DCIS) cells surrounding the central necrosis exhibited high HIF-1α protein levels, down-regulated ER-α, and increased expression of the epithelial breast stem cell marker CK-19 [41]. These cells lost their polarization and acquired an increased nucleus/cytoplasm ratio, which are hallmarks of poor architectural and cellular differentiation. CK-19 is one marker for a cell population that contains mammary multipotent progenitor cells [42]. Therefore, hypoxia might induce dedifferentiation of epithelial cells, thereby promoting an aggressive phenotype in breast cancer. The hypoxia-induced downregulation of ER-α expression in DCIS has potential clinical relevance and suggests a reason that some ER-α-positive tumors become resistant to anti-estrogen treatment. Because PGRMC1 is upregulated in the cells close to the necrotic area, it conceivably plays a role in this phenomenon.
Location of endogenous PGRMC1 in breast cancer tissue. (a) Validation of PGRMC1-specific mouse monoclonal antibody 5G7. Breast cancer tissue was labeled with 5G7 with (left) or without (right) prior incubation of the antibody with recombinant PGRMC1 protein. Preincubation of the 5G7 with Δ43hpr6, the cytoplasmic domain of PGRMC1 protein, which served as the immunogen for 5G7, blocks specific detection of PGRMC1 (red). DAPI is used to detect cell nuclei (blue). Magnification: 63×. (b) Differential expression of PGRMC1 in ER-α-positive and ER-α-negative tumor cells. Depicted are 10 different breast cancer tissue samples (in subpanels i to xii) from a tissue microarray labelled for ERα (green) and PGRMC1 using monoclonal antibody 5G7 (red). (i) An ER-α-positive invasive ductalobular breast cancer (magnification: 10×). (ii) Higher magnification of upper boxed area in subpanel i, showing an ER-α-positive duct. (iii) Higher magnification of lower boxed area of subpanel i, showing an ER-α-negative duct. Subpanels iv to xii show nine different breast cancer tissues; on the right is shown the red signal without the green signal from the same image. Magnification: 20×. (iv) ER-α-positive invasive ductal carcinoma. (v) ER-α-negative invasive lobular breast cancer. (vi) ER-α-positive invasive ductalobular breast cancer. (vii) ER-α-negative invasive ductal carcinoma. (viii) ER-α-positive invasive ductal carcinoma. (ix) ER-α-positive invasive ductal carcinoma. (x) ER-α-negative invasive ductal carcinoma in situ. (xi) ER-α-positive invasive ductal carcinoma. (xii) ER-α-negative invasive ductal breast cancer. The figures indicate that PGRMC1 is differentially expressed in ER-α-positive and ER-α-negative tumor cells. DAPI (blue) is used to detect cell nuclei. (c) Differential expression of PGRMC1 in ER-α-positive invasive ductalobular breast cancer sample with a DCIS (comedo type). (i) The tissue is labeled for ER-α (green) and PGRMC1 using ant-PGRMC1 monoclonal antibody 5G7 (red). PGRMC1 is expressed in areas in which ER-α is not expressed. Magnification: 20×. (ii and iii) Depicted is the area of subpanel i with reduced green (ii) or red signal (iii). (iv) Shown is the negative control for subpanels i to iii, using an unlabeled consecutive section, indicating autofluorescence especially in the necrotic center. (v) the boxed area of subpanel i is enlarged. (vi) Depicted is the same ductal carcinoma in situ in a consecutive section labeled for glucose transporter (GLUT)-1 (green) and PGRMC1 using 5G7 monoclonal antibody (red). (vii) the boxed area of subpanel vi is enlarged. PGRMC1 is expressed in GLUT-1-positive, hypoxic cells in a perinuclear fashion. GLUT-1 protein is localized at the cytoplasmic membrane. This sample was taken from an ER-α-positive invasive ductalobular breast cancer with in situ carcinoma components of comedo-type, and the results depicted were typical for 5/5 comedo type tumors in the stained tissue array. Clinical patient data for the tumors depicted in panels b and c are provided in Additional file 1 (Table S2). DCIS, ductal in situ carcinoma; ER, estrogen receptor; PGRMC, progesterone receptor membrane component.
HIF-1 also induces the angiogenic growth factor vascular endothelial growth factor [33]. Swiatek-De Lange and colleagues [43] implicated PGRMC1 in the activation of vascular endothelial growth factor gene expression in retinal glial cells. Interestingly, PGRMC1 (AI010357 EST [VEMA] ventral midline antigen) was observed to be one of a number of genes upregulated in the late phase of a wound healing model involving injured spinal cord [26], at a time when vascular morphogenesis occurs in the healing tissue.

PGRMC1 protein affects the response to oxidative damage in the MCF-7 breast cancer cell line, influencing their susceptibility to oxidative cell death [22]. It is unclear whether this reflects a normal function of PGRMC1 or is a function of the conditions of over-expression. However, under these conditions, some of our phosphorylation site PGRMC1 mutants exhibited enhanced survival (Figure 6). Both survival and failure to induce Akt phosphorylation were associated with somewhat higher levels of the exogenous S56A/S180A mutant PGRMC1 protein detected by Western blot (Figure 7), but our data do not demonstrate that this higher level is reproducible, and similar levels of the other mutants did not protect against cell death, suggesting that elevated exogenous PGRMC1 protein abundance levels per se were not responsible for enhanced survival of MCF-7 cells expressing the S56A/S180A mutant. Indeed, over-expression of PGRMC1 above endogenous levels increased susceptibility to peroxide-induced death [22] (Figure 7). It is possible that the failure of the S56A/S180A mutant to be phosphorylated on those residues leads to accumulation of some biologically active species that is/are perhaps inappropriately cleared. For instance, sterol levels regulate the ubiquitination and degradation of both Insig-1 and hydroxymethylglutarate-coenzyme A reductase to downregulate the mevalonate pathway [44,45], and PGRMC1 interacts directly with Insig-1 [32].

The possible mechanism of survival of the S56A/S180A mutant deserves some consideration. Phosphorylation of S56 presumably blocks the interaction of PGRMC1 with another protein(s) through the predicted proline-rich SH3 target domain centered on P62, whereas phosphorylation of S181 presumably blocks phosphorylation of the adjacent Y179, which would be necessary for interaction with one or more presumed SH2-domain proteins [20]. Phosphorylation of Y179 probably requires the prior regulatory dephosphorylation of S180. C128 was essential for the vital function of the S56A/S180A mutant, and it is quite possible that dimerization via a cystine-mediated disulfide bond [20] is required for the rescuing function. Mutation of cysteine to serine is unlikely to have greatly affected protein structure. Furthermore, the inability of phosphorylated Y179 to interact with one or more unidentified SH2 domain-containing proteins may be responsible for the sus-

Figure 9

Hypothetical functions of PGRMC1 in cancer biology. PGRMC1 potentially occupies a regulatory nexus; presented is a schematic model for some hypothetical functions of PGRMC1 in cancer biology, many of which require future experimental validation. See also the supplementary discussion in Additional data file 1.
ceptibility of the Y179F/S180A to growth in charcoal-treated FCS.

Candidate PGRMC1-interacting proteins
It is reasonable to speculate that differences in the phosphorylation status of PGRMC1 can affect the proteins with which it interacts, and thereby affect cellular biology. The possible breast cancer relevance of known or suspected interactions of PGRMC1 with PAI1BP1/CGI-55, neogenin and DCC (deleted in colon cancer) are considered in the supplementary discussion included in Additional file 1. Future research should address what role, if any, these proposed interactions of PGRMC1 with those candidate interaction partners may play in breast cancer.

Conclusions
Taken together, this emerging picture strongly suggests that PGRMC1 is potentially able to impinge upon the regulation of cell biology that is centrally important for the clinical consequences of tumors, potentially maintaining not only cell migration and tissue morphogenesis but also tissue homeostasis. There are therefore a variety of theoretically possible mechanisms whereby differential PGRMC1 abundance and phosphorylation could affect tumor biology, perhaps with a central nexus functionality (Figure 9). This work suggests directions for further experiments that will be necessary to address the explicit role(s) of PGRMC1 in cancer.

We detected an expected wound response signature in ER-α-neg tumors that was associated for the first time with differential abundance, and phosphorylation of PGRMC1 between different tumor types. Furthermore, our data suggest that the phosphorylation status of PGRMC1 can affect cell survival in response to life-threatening conditions. Determination of the thus far poorly defined role of PGRMC1 in cancer biology could prove to be of great relevance to clinical cancer therapists. Indeed, relevance to a broad range of tissues and pathologies is quite probable.

Competing interests
In line with the policies of the German Human Genome Project (DHGP), patent applications (WO2006029836, WO2007039189, and EP1938103) have been filed regarding these results, of which SC, MN, DW, RK, and MAC are inventors. If commercial profits are generated therefrom, then ProteoSys AG, the University of Tübingen, and SC will be stockholders of ProteoSys AG during all or some of the period.

Authors' contributions
HN, SEC, and RK were involved in conception and design, acquisition of data (clinical), and analysis and interpretation of data. WW, SP, and WS were involved in the acquisition of data (proteomics). GSP was involved in involved in conception, design, and acquisition of data (proteomics). KS and UV were involved in acquisition of data (histology). TF and DW were involved in conception and design (clinical tumor samples). MAC was involved in conception and design (proteomics and some clinical), analysis and interpretation of data, and was also the main author of the manuscript.

Additional files

The following Additional files are available online:

Additional file 1
Supplementary materials. Presented are Supplementary discussions entitled 'Validation of the differential abundance profile' and 'Candidate PGRMC1 interacting proteins'. It additionally contains two supplementary tables: Table S1 ('Protein spots that contained multiple identifications of individual proteins as gene products') and Table S2 ('Clinical patient data for the tumours in Figure 8B and 8C').

See http://www.biomedcentral.com/content/ supplementary/bcr2155-S1.pdf

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