Progesterone Receptor Membrane Component 1 (Pgrmc1): A Heme-1 Domain Protein That Promotes Tumorigenesis and Is Inhibited by a Small Molecule

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ABSTRACT

Tumorigenesis requires the concerted action of multiple pathways, including pathways that stimulate proliferation and increase metabolism. Progesterone receptor membrane component 1 (Pgrmc1) is related to cytochrome \( b_5 \), binds to heme, and is associated with DNA damage resistance and apoptotic suppression. Pgrmc1 is induced by carcinogens, including dioxin, and is up-regulated in multiple types of cancer. In the present study, we found that Pgrmc1 increased in vivo tumor growth, anchorage-independent growth, and migration. Pgrmc1 also promoted proliferation in the absence of serum in A549 non-small cell lung cancer cells but enhanced proliferation regardless of serum concentration in MDA-MB-468 breast cancer cells. Pgrmc1 promotes cholesterol synthesis and binds to Insig (insulin-induced gene), Scap (sterol regulatory element binding protein cleavage activating protein), and PAI1 proteins, but Pgrmc1 did not affect cholesterol synthesis in lung cancer cells. Pgrmc1 is also associated with progesterone signaling and plasminogen activator inhibitor (PAI1) RNA binding protein, but neither progesterone activity nor PAI1 transcript levels were altered in Pgrmc1-knockdown lung cancer cells. Pgrmc1 homologues bind to aryl ligands identified in an in silico screen, and we have found that a Pgrmc1 ligand induced cell death in a Pgrmc1-specific manner in multiple breast and lung tumor cell lines. Our data support a role for Pgrmc1 in promoting cancer-associated phenotypes and provide a therapeutic approach for targeting Pgrmc1 with a small molecule in lung and breast cancer.

For the last several decades, cancer has been treated with pharmacological compounds that kill proliferating cells nonspecifically, causing widespread side effects during cancer treatment. Some of the most significant findings in cancer research involve the identification and targeting of signaling pathways that are activated in tumors. As the targeting approaches have evolved, increasing the diversity of targets is a priority in cancer research.

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ABBREVIATIONS: Pgrmc1, progesterone receptor membrane component 1; AtMAPR2, Arabidopsis thaliana membrane-associated progesterone receptor 2; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; LDLR, low-density lipoprotein receptor; NENF, neuronal-derived neurotrophic factor; PAI1, plasminogen activator inhibitor; PAIR-BP1, plasminogen activator inhibitor RNA binding protein; Hpr, human membrane progesterone receptor; Insig, insulin-induced gene; Scap, sterol regulatory element binding protein cleavage activating protein; snRNA, short hairpin RNA; AG-205, 5-[(1-[4-(chlorophenyl)-1H-tetrazol-5-yl]sulfanyl)acetyl]-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole; RNAI, RNA interference; FACS, fluorescence-activated cell sorting; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle medium; GC-MS, gas chromatography/mass spectroscopy; hbd, heme binding deficient.
dependent survival (Rohe et al., 2009). One of the best-characterized binding partners for Pgrmc1 is the RNA binding protein PAIR-BP1 (plasminogen activator inhibitor 1 mRNA binding protein) (Peluso et al., 2005), although the role of Pgrmc1 in RNA metabolism is unclear.

Pgrmc1 promotes cell signaling in breast cancer cells (Hand and Craven, 2003; Neubauer et al., 2008), activating the serine-threonine kinase Akt after damage and the anti-apoptotic protein IκBα basally (Hand and Kaprielian, 2004). Pgrmc1 was originally named Hpr6.6 (human membrane progesterone receptor) or mPR (membrane progesterone receptor) and was identified as a component of an uncharacterized progesterone receptor complex (Cahill, 2007; Losel et al., 2007). Although Pgrmc1 copurifies with progesterone-binding proteins (Peluso et al., 2008b), it contains no apparent homology to hormone receptors, and recombinant Pgrmc1 does not bind directly to progesterone (Min et al., 2005).

In nonmalignant tissues, Pgrmc1 is mostly highly expressed in the liver and kidney (Gerdes et al., 1998). The primary Pgrmc1 binding partners in nonmalignant tissues likely include P450 proteins (Hughes et al., 2007), and both the yeast and human Pgrmc1 proteins contribute to cholesterol synthesis (Rohe et al., 2009). Pgrmc1 also binds to the cholesterol regulators Insig (insulin-induced gene) and Scap (sterol regulatory element binding protein cleavage activating protein) (Suchanek et al., 2005), although the biological function of this interaction is unclear. Furthermore, the role of Pgrmc1 in cholesterol synthesis has been tested only in embryonic kidney cells (Hughes et al., 2007) and not in cancer cells.

In the present study, we demonstrate that Pgrmc1 promotes tumor growth, anchorage-independent growth, migration, and serum-independent proliferation in cancer cells. We also show that a Pgrmc1 ligand induces growth arrest in a similar manner to Pgrmc1 inhibition by short hairpin RNA (shRNA). The results suggest that Pgrmc1 promotes cancer progression and is a potential target for therapeutic development in breast and lung cancer.

Materials and Methods

Tissue Culture. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% serum supreme (Thermo Fisher Scientific, Waltham, MA) and antibiotics and grown at 37°C in 5% CO₂ in air. A549 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-468 cells were a kind gift from Dr. Rita Plattner (University of Kentucky, Lexington, KY). For progesterone treatments, cells were grown in phenol red-free media containing 10% charcoal-stripped fetal bovine serum for 1 week and then tested for viability in the same media or in phenol red-containing media at 37°C.

RNA Interference. Lentiviruses expressing shRNAs were prepared from the plasmids pGIPZ and V2LHS.90636 (Open Biosystems, Huntsville, AL) by the Viral Vector Core at the Translational Core Laboratories, Cincinnati Children’s Hospital Research Foundation, Cincinnati, OH. For lentiviral infection, 500,000 cells/100-cm² dishes were infected with 1 × 10⁶ lentiviral particles in 8 µl/ml polybrene (Millipore Bioscience Research Reagents, Temecula, CA) overnight, washed, and selected in 5 µg/ml puromycin for 5 days. The cells were then sorted by fluorescence-activated cell sorting (FACS) for the 20% most fluorescent fraction, which represents a nonclonal population. Gene inhibition with siRNA transfected was performed as described (Mallory et al., 2005b; Crudden et al., 2006). The Ad-LacZ and Ad-Pgr-hbd (previously called Ad-Hprhbd) (hbd = heme binding-deficient) adenoviruses have been described previously (Crudden et al., 2006) and were used at the same doses. To target Pgrmc1, we used an oligonucleotide duplex corresponding to the 21 base pairs after position 509 in the Pgrmc1 open reading frame (sPGR; Ambion, Austin, TX). The control was “control silencer 1” (Ambion).

Immunological and RNA Techniques. Protein levels were analyzed by Western blot as described (Mallory et al., 2005b). The antibodies used in the study were anti-κu70 (A-9; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), antiphospho-ERK1/2 (E-4; Santa Cruz Biotechnology, Inc.), anti-ERK1/2 (K-23; Santa Cruz Biotechnology, Inc.), and antiphosphotyrosine (PY-69; Santa Cruz Biotechnology, Inc.). Western blots for Pgrmc1 were performed with a polyclonal antibody called anti-PGR-UK1, which was raised in rabbits to an internal 10-amino acid peptide. In all cases, Western blots were performed at least in duplicate.

Transcript levels were assessed as described previously (Mallory et al., 2005b). PCRs contained primers to either Pgrmc1, LDLR, PAI1, Pgrmc2, or NENF in combination with primers to actin, which served as an internal control for cDNA loading. Pgrmc1 and actin were amplified with the primers HPR (Crudden et al., 2006), ACT-F, and ACT-R (Cance et al., 1992). The primer sequences for LDLR were LDLR (GCTCAAGACCG-GAAAAGCATC) and LDLR + 379R (CGAATCCGGAGAGATGG-CAC); the primers for PAI1 were PAI1 + 90F (GTGCCCACTGTCC-CTCAAGC) and PAI1 + 360R (GATCGCGTCTGTTGTGTCGTA); the Pgrmc2 primers were PGR2 + 500F (AGAGATGAATATGAT-GATCT) and PGR2 + 750R (GACTCCGAGACGTCTGTA); and the NENF primers were NENF + 150F (GAGAGGACCTGGCGGAGTA) and NENF + 400R (GGGTATTTGGGTTGTTGAC).

Proliferation, Anchorage-Independent Growth, and Migration Assays. For growth curves, cells were plated in 24-well dishes, harvested, and counted with a hemocytometer. In some cases, viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, where cells were treated in 96-well dishes and then incubated with 0.5 mg/ml MTT. The MTT solution was then removed, and crystals were dissolved in dimethyl sulfoxide (DMSO) and quantitated by measuring A560. FACS was performed as described previously (Mallory et al., 2005b). Cells were fixed in 70% ethanol, washed, and resuspended in phosphate-buffered saline (PBS) containing 20 µg/ml propidium iodide and 20 µg/ml DNase-free RNase. After an overnight incubation, the samples were analyzed at the University of Kentucky Flow Cytometry Facility. For soft agar assays, cells were resuspended in 0.4% agar in DMEM at a density of 200 cells/ml and overlaid on 1% agar, which was also dissolved in 1× DMEM. Cells were plated in triplicate, and the assay was performed at least two separate times. Cells were grown for 4 weeks and imaged with an Olympus (Tokyo, Japan) IX70 microscope. The parameters of 30 to 35 different colonies were calculated with Pictureframe 2.3 software (Optronics, Inc., Goleta, CA).

To assay migration, serotonin-stained shRNA-expressing cells were suspended in DMEM containing 1% bovine serum albumin and placed in the top well of invasion chambers (BD Biosciences, San Jose, CA). Medium containing 10% serum was placed in the lower chamber as a chemoattractant. Cells were allowed to invade for 16 h at 37°C. Cells on the upper surface of the membrane were removed, and cells on the underside were fixed, stained with 0.5% crystal violet in 20% methanol, and counted.

Tumor Xenografts. Athymic nude female mice (5–6 weeks old) were purchased from Harlan (Indianapolis, IN). The mice were randomly divided into two groups (n = 8 per group) and injected subcutaneously with either A549/con or A549/RNA interference (RNAi) cells (3 × 10⁶ in 100 µl of PBS) into the upper flank. The mice were
monitored daily for tumor growth for 3 weeks. Tumor volume (mm$^3$) was calculated by using the following formula $V = (W^2 \times L)/2$, where $W$ is width (small diameter) and $L$ is length (long diameter). The tumor was excised and weighed (g), and proteins were extracted with a Polytron PT-1300 homogenizer (Kinematica, Inc., Bohemia, NY). For orthotopic xenografts, 2 x 10$^6$ cells were washed and suspended in PBS, then injected into a mouse tail vein with a 30-gauge needle. After a 42-day incubation, mice were euthanized, and their lungs were removed surgically, washed in PBS, and fixed in paraformaldehyde. Tumor cells were fluorescent because of the expression of the green fluorescent protein, and tumors were visualized with an Olympus MVX10 dissecting microscope and quantitated with Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). All animal protocols were approved by the Institutional Animal Care and Use Committee before the experiment.

**Statistics.** All data are expressed as mean ± standard deviation and analyzed by using Student’s $t$ test to assess the significance between groups. Lung metastasis data were presented as a median and range and analyzed by the Mann–Whitney test using software from Minitab Inc. (State College, PA). All measurements were taken of triplicate Pgrmc1 preparations.

**Heme Spectroscopy.** The GST-Pgrmc1 fusion protein (previously called GST-Hpr6), consisting of amino acids 43 to 195 of the Pgrmc1 coding sequence, has been described (Cruden et al., 2006). The purification of GST-Pgrmc1 was identical to the earlier description, except that 50 $\mu$M 6-aminolevulinic acid was included in the culture medium after induction with isopropyl $\beta$-D-thiogalactoside. The purified protein was assayed by bicinchroninic acid assay (Thermo Fisher Scientific) and verified by SDS-polyacrylamide gel electrophoresis. For spectroscopy, 17 $\mu$M eluted Pgrmc1 was incubated with either 0.1% DMSO (final concentration) or 0.1% DMSO and 10 to 50 $\mu$M AG-205 at 37°C for 10 min. The respective samples were then measured in the reference or sample cuvettes of a Varian Inc. (Palo Alto, CA) Cary 100 spectrophotometer, and the absorbance spectrum from 370 to 550 nm was scanned. Measurements were taken of 30 colonies from each cell population were measured, and the results model that Pgrmc1 promotes tumor formation and tumor cell migration in A549 cells.

**Results**

**Pgrmc1 Contributes to Tumorigenesis In Vivo and Tumor-Associated Phenotypes In Vitro.** Because Pgrmc1 has elevated expression in tumors (Cruden et al., 2005; Peluso et al., 2008a) and is associated with tumorigenesis (Selmin et al., 1996; Nie et al., 2006), we determined the extent to which Pgrmc1 regulates tumor growth. A549 human nonsmall cell lung cancer cells were infected with a lentivirus derived from the pGIPZ plasmid (A549/con) or a lentivirus containing pGIPZ expressing a short hairpin RNA targeting Pgrmc1 (A549/RNAi). Pgrmc1 was efficiently inhibited (Fig. 1A) in A549/RNAi cells. Ku70 is shown throughout as a control for sample loading. Athymic nude mice were injected with A549/con or A549/RNAi cells, and the excised tumor weight of A549/con was 2.9-fold more than that of A549/RNAi tumors (Fig. 1B; $p = 9 \times 10^{-6}$, $t$ test). In addition, the tumor volume was 8.1-fold larger for A549/con compared with A549/RNAi ($p = 0.0007$; $t$ test, data not shown). Next, we introduced tumors to the lung via tail vein injection. A549/con cells efficiently formed tumors in the lung (Fig. 1C, top), whereas no fluorescent tumor nodules were detected in mice injected with A549/RNAi cells (Fig. 1C, bottom). The results are shown graphically in Fig. 1D. The results indicate that Pgrmc1 promotes in vivo tumor growth.

**Anchorage-independent growth is a hallmark of cancer cells, and we determined the extent to which Pgrmc1 regulates soft agar growth. A549/con cells readily formed colonies in soft agar, whereas A549/RNAi cells formed small microcolonies that failed to proliferate (Fig. 2, A and B). The radii of 30 colonies from each cell population were measured, and A549/con colonies were 2.9-fold larger, a difference that was highly significant ($p = 6 \times 10^{-13}$, $t$ test). A box plot (Fig. 2A) shows the range (vertical line) and confidence intervals (boxes) of the colony sizes. In addition, migration in A549/con cells was 3.4-fold higher than that of A549/RNAi cells, an effect that was highly significant (Fig. 2C; $p = 0.002$, $t$ test). The results model that Pgrmc1 promotes tumor formation and tumor cell migration in A549 cells.

**Pgrmc1 Promotes Tumor Cell Proliferation.** One of the hallmarks of cancer cells is their ability to survive in the absence of exogenous growth factor stimulation, and A549/con cells continued to divide for 7 days after serum deprivation (Fig. 3A, solid line). In contrast, Pgrmc1-knockdown cells failed to proliferate (Fig. 3A, dashed line) and underwent a modest, but significant, increase in G1-phase cells and a decrease in S- and G2/M-phase cells in A549/RNAi cells (Fig. 3B). The percentage of G1-phase cells increased from 87 ± 1.2 for A549/con to 77 ± 1.1 ($p = 0.0004$, $t$ test) for A549/RNAi

![Fig. 1. Pgrmc1 increases tumor growth in mouse xenografts. A549 cells were infected with a lentivirus derived from the pGIPZ plasmid (A549/con) or a lentivirus containing pGIPZ expressing a short hairpin RNA targeting Pgrmc1 (A549/RNAi). Athymic nude mice were injected with A549/con or A549/RNAi cells. A, Western blot analysis revealed that Pgrmc1 expression was inhibited in the excised A549/RNAi tumors (top, lane 2), whereas ku70 was unchanged. B, the excised tumor weight of A549/con was 2.9-fold more than A549/RNAi. The results support a model in which Pgrmc1 promotes in vivo tumor growth. C, A549/con cells efficiently colonized the lungs after tail vein injection (top, fluorescent image on left and bright field on right), whereas A549/RNAi cells were deficient in lung colonization (bottom). D, the results from C were quantitated and are presented graphically. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.](image-url)
The S-phase percentage decreased from 22/0.2 to 17/0.9 (p < 0.0005), and the G2/M-phase percentage decreased from 11/1.1 to 6/0.2 (p < 0.002) after 24 h. The change in cell number was minor at this time point because the initial doubling time for the control cells was 3.5 days under these conditions. In media containing 10% serum, A549/con and A549/RNAi cells grew at equivalent rates (data not shown), suggesting that Pgrmc1 loss can be supplemented by a component of serum.

The Pgrmc1-D120G mutant fails to bind to heme and sensitizes breast cancer cells to chemotherapy (Crudden et al., 2006). We expressed Pgr-hbd in A549 cells (Fig. 3C) using an adenovirus called Ad-Pgr-hbd, with an adenovirus called Ad-LacZ as a control. In media lacking serum, cells expressing Pgr-hbd failed to proliferate (Fig. 3D, dashed line), whereas cells infected with the same dose of the control vector (Ad-LacZ), continued to proliferate for 6 days. The difference in viability between the two populations was significant at days 3 (p = 0.008), 6 (p = 0.0004), and 8 (p = 0.005). Pgr-hbd had no effect on viability in complete media with 10% serum (data not shown).

In MDA-MB-468 breast cancer cells transfected with an siRNA duplex targeting Pgrmc1, Pgrmc1 expression was efficiently inhibited (Fig. 3E), and we detected a 36% decrease in proliferation (Fig. 3F). The decrease in proliferation was highly significant (p = 0.0015 at day 5 and 0.0017 at day 8), and there was a similar growth defect in cells with and without serum. Transfection with siRNA inhibited cell growth nonspecifically in A549 cells maintained without serum (data not shown), preventing an analogous experiment in A549 cells.

Pgrmc1 Did Not Alter Sterol Intermediates in Cancer Cells. Pgrmc1 increases cholesterol synthesis in embryonic kidney cells (Hughes et al., 2007), but only when cells are grown in medium containing high levels (40–50 mM) of a cholesterol precursor, mevalonic acid. These concentrations were toxic to A549 cells, but when the cells were grown in medium containing lipoprotein-deficient serum with 10 mM mevalonic acid we did not detect any change in cholesterol levels in A549/RNAi cells relative to control cells (Fig. 4, A and B). Low cholesterol levels stimulate the synthesis of the low-density lipoprotein receptor (LDLR), but we did not detect any changes in LDLR expression (Fig. 4E) in Pgrmc1-knockdown cells (Fig. 4D).

In ovarian cancer cells, Pgrmc1 binds to PAIR-BP1, a PAI1 mRNA binding protein (Peluso et al., 2006, 2008b), and the target of PAIR-BP1 is the PAI1 transcript. However, we did not detect any changes in PAI1 mRNA levels in Pgrmc1-knockdown cells (Fig. 4F). Pgrmc1 is part of a family of three related proteins that includes Pgrmc2 and NENF/Neudesin/Spuf (secreted protein of unknown function; Fig. 4C). In Pgrmc1-knockdown cells, Pgrmc2 transcription increased (Fig. 4G), whereas Neudesin levels were unchanged (Fig. 4H). Pgrmc1 has also been implicated in progesterone signaling. In A549 cells, progesterone inhibits growth (Ishibashi et al., 2005), and we found that Pgrmc1 knockdown cells were slightly more sensitive to progesterone than control cells (Fig. 4I). Thus, the results do not support a model in which Pgrmc1 drives proliferation and migration by increasing the levels of PAI1, Pgrmc2, or NENF transcripts or suppressing progesterone signaling.

Pgrmc1 Aromatic Ligands Inhibit Cancer Cell Growth. There are four aromatic ligands for the Arabidopsis thaliana Pgrmc1 homolog, AtMAPR2 (Yoshitani et al., 2005), which is highly conserved with human Pgrmc1 in the heme-1 domain (Fig. 5A, underlined residues). AtMAPR2 and Pgrmc1 also share Tyr106 and Tyr112, which are required for heme binding in Pgrmc1/IZA (Min et al., 2005) and are boxed in black in Fig. 5A. One of the AtMAPR2 ligands with the highest bind-
ing affinity is called AG-205, an aromatic compound (Fig. 5B). The addition of AG-205 to purified Pgrmc1 caused a shift in absorbance at approximately 400 nm, with a smaller shift at approximately 530 nm (Fig. 5C). The results indicate that AG-205 alters the spectroscopic properties of the Pgrmc1–heme complex. We tested the activity of AG-205 toward cancer cells and found that a 20 μM dose of AG-205 inhibited cancer cell viability 72 h after treatment in A549 (Fig. 5D), MDA-MB-231 (Fig. 5E) and MDA-MB-468 cells (Supplemental Fig. 2A). The growth inhibitory activity depended on the culture conditions, with AG-205 inhibiting proliferation in low serum conditions (Fig. 5D, solid line) but not in 10% serum (Fig. 5D, dashed line) in A549 cells (Fig. 5D) and MDA-MB-468 cells (Supplemental Fig. 1A). In MDA-MB-231 cells, the dependence on serum was less pronounced but was still significant (Fig. 5E).

AG-205 inhibited cell cycle progression. When A549 cells were maintained in 0.1% serum, AG-205 increased the fraction of G1-phase cells (93% ± 2 versus 78% ± 0.6 for control cells; \( p = 9 \times 10^{-5} \) by t test) and sub-G1 cells (8% ± 2 versus 0.07% ± 0.2 for control cells; \( p = 2 \times 10^{-3} \) by t test). In contrast, S-phase cells (4% ± 0.6 versus 16% ± 1 for control cells; \( p = 1 \times 10^{-4} \) by t test) and G2/M-phase cells (3% ± 0.6 versus 6% ± 0.6 for control cells; \( p = 3 \times 10^{-3} \) by t test) were depleted from the AG-205-treated population. These results are shown graphically in Fig. 6A.

A Pgrmc1 Ligand Increases Pgrmc1 Levels and Alters Signaling. In A549 cells, AG-205 increased Pgrmc1 levels (Fig. 6B, top) by 4.6 ± 0.8-fold (\( p = 0.03 \), t test). In contrast, ku70 levels (Fig. 6B, bottom panel) were unaffected by AG-205. RNA levels, estimated by semiquantitative reverse transcription-polymerase chain reaction, were unchanged (Supplemental Fig. 1B), suggesting that the increased Pgrmc1 protein levels occur posttranscriptionally.

ERK1/2 activation is associated with increased invasion (Simon et al., 1996), and Pgrmc1 inhibition decreased ERK phosphorylation in A549 cells (Fig. 6C, top, lanes 1 and 2), while having little effect on total ERK expression (Fig. 6C, bottom, lanes 1 and 2). We then tested the same endpoint with a separate approach and found that A549 cells infected with a control adenovirus (Ad-LacZ) maintained high basal levels of ERK phosphorylation (Fig. 6C, top, lane 3), whereas the same cells expressing a heme-binding-deficient Pgrmc1 mutant (Ad-Pgr-hbd) had reduced levels of ERK phosphorylation (Fig. 6C, top, lane 4). ERK levels were unaffected by the expressed genes (Fig. 6C, bottom, lanes 3 and 4). Because Pgrmc1 expression corresponded with increased ERK phosphorylation levels, we tested the effect of AG-205 by using

Fig. 3. Pgrmc1 promotes proliferation in the absence of serum in cancer cells. A, viability in A549/con and A549/RNAi cells was measured by cell counting from days 1 to 7 after serum withdrawal. Solid lines represent control cells, and Pgrmc1-inhibited cells are indicated by a dashed line. Measurements were in triplicate, and the results are representative of three separate experiments. B, the cell cycle profiles A549/con and A549/RNAi cells were analyzed by FACS 24 h after serum deprivation, and there was a small, but significant, increase in the G1 population in A549/RNAi cells and a corresponding decrease in S and G2/M populations. C, Western blot showing the expression of the inactive mutant Pgr-hbd in cells infected with the Ad-Pgr-hbd adenovirus (lane 2), but not in cells infected with the same dose of control Ad-LacZ adenovirus (lane 1). D, viability in Ad-LacZ (solid line) and Ad-Pgr-hbd-infected cells (dashed line), measured by cell counting 1 to 7 days after infection in media lacking serum. E, Western blot demonstrating Pgrmc1 inhibition by siRNA transfection in MDA-MB-468 cells. F, viability of MDA-MB-468 cells transfected with a control siRNA (solid line) or Pgrmc1-targeting siRNA (dashed line). Measurements were in triplicate, and the results are representative of triplicate repeats.
the same approach. AG-205-treated cells had markedly lower levels of phosphorylated ERK relative to vehicle-treated cells (Fig. 6D, top, lanes 1 and 2), whereas ERK levels were unaffected (Fig. 6D, bottom). The results suggest that Pgrmc1 has a role in regulating cell signaling and AG-205 treatment has a similar endpoint to Pgrmc1 inhibition.

Finally, we assessed the specificity of AG-205 for Pgrmc1 and found that Pgrmc1-inhibited A549 cells exhibited decreased toxicity after AG-205 treatment (Fig. 6E, dashed line) compared with control cells at doses of 10, 20, and 40 μM (p = 0.04, 0.0008, and 0.009, respectively, t test). Using an independent technique, we found that AG-205 partially reversed the loss of viability caused by Pgrmc1-hbd expression compared with control cells expressing LacZ (Fig. 6F, dashed and solid lines, respectively) at doses of 3, 6, 12, and 25 μM (p = 1 × 10^{-5}, 3 × 10^{-8}, 6 × 10^{-9}, and 5 × 10^{-6}). We note that AG-205 was less toxic in cells infected with adenovirus (Fig. 6F). The results indicate that Pgrmc1 contributes significantly to AG-205 toxicity.

Discussion

The findings presented here support a model linking Pgrmc1 to cancer progression. Pgrmc1 is overexpressed in breast tumors (Crudden et al., 2005), is induced by chemotherapy (Mallory et al., 2005b), and promotes chemotherapy resistance in breast cancer cells (Crudden et al., 2006). In separate papers, Peluso et al. subsequently showed that Pgrmc1 is overexpressed in ovarian cancer, where it contributes to chemotherapy resistance (Peluso et al., 2008a) and suppresses apoptosis (Peluso et al., 2008b). However, many tumors express Pgrmc1 before chemotherapy, suggesting a role in the early stages of tumorigenesis (Selmin et al., 1996). In the present study, we present evidence that Pgrmc1 promotes tumor growth in vivo. We inhibited Pgrmc1 expression by using a sequence-specific shRNA that had been previously characterized in human kidney cells (Hughes et al., 2007)
and used two separate in vivo assays for tumor growth. Pgrmc1 was identified previously as an up-regulated gene in lung cancer (Difilippantonio et al., 2003), and this is the first phenotypic characterization of Pgrmc1 in lung cancer. We have also identified a small-molecule ligand for Pgrmc1 that inhibits tumor cell growth.

The role of Pgrmc1 in tumor growth in vivo is likely related to its function in anchorage-independent growth, an activity that is shared with multiple signaling pathways (Reddig and Juliano, 2005). Pgrmc1 also increased migration, although the molecular target of Pgrmc1 in the migration pathway is unknown. Tumor growth, anchorage-independent growth, and migration are determined in three-dimensional culture conditions in the presence of serum. In two-dimensional culture, Pgrmc1 promoted the proliferation of lung and breast cancer cells in distinct patterns. In A549 lung cancer cells, Pgrmc1 promoted proliferation primarily in the absence of serum, suggesting that serum components were capable of providing a proliferative signal that was absent in Pgrmc1-knockdown cells. One potential model to explain the serum-dependent growth phenotype of Pgrmc1 in A549 cells is that Pgrmc1 controls the secretion or action of a serum component. Indeed, A549 cells harbor the K-ras mutation, and this leads to constitutive EGFR ligand secretion (Toulany et al., 2007), forming an autocrine loop that promotes cell survival.

In support of a model linking Pgrmc1 to signaling, Pgrmc1 expression corresponded with increased phosphorylation of ERK1/2, a key intermediate in many signaling cascades. Furthermore, we previously showed that Pgrmc1 (formerly named Hpr6.6) increases damage-associated kinase signaling in breast cancer cells (Hand and Craven, 2003). To further test the relationship between Pgrmc1 and kinase signaling, we analyzed tumors from Pgrmc1-expressing A549/con and Pgrmc1-knockdown A549/RNAi cells by Western blot with antibodies to phosphotyrosine. The pattern of tyrosine phosphorylation differed markedly between the A549/con and A549/RNAi xenografts. Specifically, proteins of molecular mass 50, 34, and 26 kDa increased in A549/RNAi cells (Supplemental Fig. 2, pp50, pp34, and pp26), whereas a protein of 82 kDa decreased (pp82). Tumors were ranked by weight, and the phosphorylation pattern of the median-sized tumors is shown. Findings from model organisms also support a role for Pgrmc1 in signaling, because the C. elegans Pgrmc1 homolog, VEM-1 (ventral midline), is associated with membrane signaling via the UNC-40/DCC (deleted in colorectal cancer) pathway during C. elegans development.

Fig. 5. The AG-205 compound binds to Pgrmc1 and inhibits tumor cell viability. A, Pgrmc1 shares homology with AtMAPR2 (underlined residues), which was the target for a screen for small-molecule ligands. The putative heme-binding Tyr106 and Tyr112 are indicated by black boxes. B, the molecular structure of AG-205 includes multiple aromatic components. C, spectroscopic scan of Pgrmc1 with vehicle control compared with vehicle plus 50 μM AG-205. The decrease in relative absorbance at 400 nm is consistent with an AG-205-induced change in the spectral properties of Pgrmc1-heme. The scan is representative of four separate scans using different Pgrmc1 protein preparations. The lower scan is a comparison of purified Pgrmc1 without and with the vehicle control (DMSO). D and E, A549 cells (D) and MDA-MB-231 cells (E) are shown in viability assays of cells maintained in 0.1% serum (solid line) or 10% serum (dashed line) and treated with increasing doses of AG-205. Percentage of viability was determined by cell counting, and % viability refers to the cell number relative to the untreated control. The results show a highly significant loss of viability after AG-205 treatment when cells are grown in the absence of serum growth factors.
development (Runko and Kaprielian, 2004) and is important for axon guidance during development, a function that may be related to the invasive phenotype in Pgrmc1-knockdown cells. The Pgrmc1-related protein Neudesin/NENF is secreted and triggers activation of the ERK and Akt pathways in neurons (Kimura et al., 2008). Although we have no evidence that Pgrmc1 is secreted, we are currently testing the effect of Pgrmc1 on multiple signaling pathways. In MDA-MB-468 cells, Pgrmc1 promoted growth in the absence and presence of serum, unlike A549 cells. MDA-MB-468 cells have elevated levels of growth factor receptors (Goldenberg et al., 1989), and the relationship between Pgrmc1 and these pathways is the subject of an ongoing investigation.

There is relatively little known about the molecular mechanism of Pgrmc1, except that it binds to heme and coprecipitates with P450 proteins, Insig-1, Scap, and PAIR-BP1 (Rohe et al., 2009). However, we were unable to detect Insig-1 expression in A549 cells, and sterol regulatory element-binding protein processing was unaffected by Pgrmc1 deletion under our conditions (data not shown). Furthermore, we were unable to detect any changes in cholesterol intermediates in Pgrmc1-knockdown cells when assayed by GC-MS (Fig. 4) or by incubating cells with 14C-acetate and separating labeled lipids by thin-layer chromatography (data not shown), as described previously (Mokashi and Porter, 2005). Together, the results do not support a requirement for Insig and Scap binding or Pgrmc1-mediated cholesterol synthesis as a mechanism for driving cancer cell proliferation, although cholesterol synthesis may be an important feature of Pgrmc1 in nonmalignant tissues.

Pgrmc1 has been proposed to act as a progesterone receptor or progesterone signaling intermediate in multiple cell types (Losel et al., 2007), and Pgrmc1 is required for the antiapoptotic activity of progesterone in rat granulosa cells (Peluso et al., 2008b). In A549 lung cancer cells, progesterone inhibits proliferation (Ishibashi et al., 2005), and we found that this inhibitory activity was slightly elevated in Pgrmc1-knockdown cells (Fig. 4I). This result is not consistent with Pgrmc1 acting to promote progesterone signaling in lung...
cancer, but suggests that one potential mechanism through which Pgrmc1 promotes proliferation may be to antagonize progesterone-mediated growth inhibition. In rat granulosa cells, Pgrmc1 binds to PAIR-BP1, a PAI1 mRNA binding protein (Peluso et al., 2006, 2008b), and a target of PAIR-BP1 is the PAI1 transcript. Although we did not detect any change in PAI1 transcript levels in Pgrmc1-knockdown cells (Fig. 4F), we cannot exclude the model that a Pgrmc1–PAIR-BP1 complex regulates proliferation via an intermediate other than PAI1.

Pgrmc1 is related to Pgrmc2 (Gerdes et al., 1998) and NENF/Neudesin/Spfu secreted protein of unknown function (Fig. 4C) (Kimura et al., 2005, 2008). In Pgrmc1-knockdown cells, Pgrmc2 transcription increased (Fig. 4G), whereas Neudesin levels were unchanged (Fig. 4H), raising the possibility that Pgrmc1 suppresses Pgrmc2 transcription. To test for functional redundance between Pgrmc1 and Pgrmc2, we inhibited Pgrmc2 by RNAi in control and Pgrmc1-knockdown cells. Pgrmc2-knockdown cells proliferated normally, and Pgrmc1/Pgrmc2-knockdown cells proliferated in a similar manner to Pgrmc1-knockdown cells (data not shown), suggesting that the two proteins are not functionally redundant.

Pgrmc1 homologues are related to cytochrome b5, bind heme, and have reducing activity (Ghosh et al., 2005), and Pgrmc1 and its homologues regulate P450-mediated redox reactions (Mallory et al., 2005a; Min et al., 2005; Hughes et al., 2007). Pgrmc1 is also important in oxidative damage-induced signaling (Hand and Craven, 2003; Neubauer et al., 2008). Thus, one appealing model is that Pgrmc1-regulated redox activity drives a cellular process associated with proliferation. In support of this model, tyrosine phosphorylation increased in Pgrmc1-knockdown cells, including tumors from Pgrmc1-knockdown-injected mice, and protein phosphatases are regulated by a redox mechanism (Leslie et al., 2003; Chen et al., 2006). Their activation or inactivation by Pgrmc1 could contribute to the changes in tyrosine phosphorylation that we have observed. However, we emphasize that this model is largely speculative and is under investigation.

One appealing feature of Pgrmc1 inhibitors, such as AG-205, is that they potentially have a measure of specificity for tumors. Furthermore, AG-205 acted preferentially on Pgrmc1-expressing cells and was capable of reversing the effects of a dominant-negative Pgrmc1 mutant. The primary activity of AG-205 was in the absence of serum. The absence of serum actually resembles in vivo conditions, which Pgrmc1 promotes proliferation may be to antagonize progesterone-mediated growth inhibition. In vivo experiments with AG-205 are ongoing, and pilot experiments indicate that AG-205 is stable and deliverable in vivo. The results presented here indicate the existence of a novel pathway that promotes tumor proliferation, suggesting potential new avenues for research and the development of antineoplastic therapeutics.

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References


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