THE ROLE OF PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 IN RECEPTOR TRAFFICKING AND DISEASE

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THE ROLE OF PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1
IN RECEPTOR TRAFFICKING AND DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Kaia Karli Hampton
Lexington, KY

Director: Dr. Rolf J. Craven,
Professor of Pharmacology and Nutritional Sciences
Lexington, KY
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The progesterone receptor membrane component 1 (PGRMC1) is a multifunctional protein with a heme-binding domain that promotes cellular signaling via receptor trafficking, and is essential for some elements of tumor growth and metastasis. PGRMC1 is upregulated in breast, colon, lung and thyroid tumors. We expanded the analysis of PGRMC1 in the clinical setting, and report the first analysis of PGRMC1 in human oral cavity and ovarian tumors and found PGRMC1 to correlate with lung and ovarian cancer patient survival. Furthermore, we discovered a specific role for PGRMC1 in cancer stem cell viability. PGRMC1 directly associates with the epidermal growth factor (EGFR) in cancer cells, and we reviewed multiple signaling-associated pathways that are important in trafficking wild-type and mutant EGFR. To better understand the potential of PGRMC1 in receptor tyrosine kinase trafficking, we extended our research to the insulin receptor (IR). Changes in insulin signaling have been linked to multiple diseases, because IR plays a key role in glucose metabolism, cellular survival and proliferation. We found PGRMC1 to co-precipitate with IR in cancer cells and in an adipose model system. PGRMC1 increased IR plasma membrane levels in multiple cancer cell lines, and was also found to increase plasma membrane levels of two glucose transporters. Treatment with a PGRMC1 ligand significantly increased IR levels in human adipocytes. Moreover, we demonstrate that both insulin binding and glucose uptake are dependent on PGRMC1.

KEYWORDS: PGRMC1, EGFR, IR, Receptor Trafficking, Cancer, Glucose

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October 27, 2017
THE ROLE OF PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 IN RECEPTOR TRAFFICKING AND DISEASE

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DEDICATION

This work is dedicated to my grandmother, Maija (Zarins) Grosc, and mother, Kristi (Grosc) Hampton: the strong and beautiful wives of LaVern Grosc, Ph.D., and Scott Hampton, Ph.D. Here’s to strong women. May we know them. May we be them. May we raise them.
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CHAPTER I: INTRODUCTION
Progesterone receptor membrane component 1 (PGRMC1) is a highly conserved ligand binding protein that is widely distributed throughout eukaryotic organisms (1). PGRMC1 belongs to the membrane-associated progesterone receptor (MAPR) family, which includes PGRMC1, PGRMC2, neudesin (NENF) and neuferricin (CYB5D2) (2). This family of proteins is involved in multifunctional physiological properties related to cholesterol and steroid biosynthesis, drug metabolism and response, autophagy, apoptosis, cell cycle regulation, cell migration, tumorigenesis and cancer progression, receptor trafficking and neural functions (2).

1.1 PGRMC1: Discovery and Nomenclature

PGRMC1 has been identified independently by various groups in multiple biological contexts. PGRMC1 was initially discovered and purified in 1996 by Meyer and colleagues, as a high-affinity progesterone-binding site derived from porcine liver membranes (3, 4). In the same year, the gene was isolated from the livers of dioxin treated rats, and named 25-Dx (5). In 2001, PGRMC1 was identified as an immunologically defined antigen and named inner zone antigen (IZA) after a monoclonal antibody specific for rat adrenocortical cells (6). The cDNA encoding IZA was later found to be identical to that encoding the previously reported putative membrane-associated progesterone receptor (MPR) and 25-Dx (7).

In 2003, Craven and colleagues at the University of North Carolina discovered and cloned the *Saccharomyces cerevisiae* yeast member of the
MAPR family, which they named Dap1 (Damage-associated response protein), and were the first to link the MAPR family member to sterol regulation and damage response (8). In *Caenorhabditis elegans*, the Dap1 homologue, termed VEM-1, was found to be involved in axon guidance in the developing roundworm (9).

The human PGRMC1 homologue was first cloned in 1998 and named human progesterone receptor 6.6 (Hpr6.6) (10). In the same paper, they identified another related human protein of a separate gene (Dg6) that corresponds to the present PGRMC2 nomenclature (10). The first published utilization of “PGRMC1” was reported in 2005 (11), as hmPR/PGRMC1, and later as PGRMC1 in 2006 (12). The current nomenclature approved by the HUGO Gene Nomenclature Committee is PGRMC1 (1).

1.2 PGRMC1: Structure and localization

PGRMC1 and the MAPR family proteins share a similar non-covalent heme-binding domain that is similar to cytochrome b5 (1, 13). The PGRMC1 protein consists of 195 amino acids and contains a short luminal peptide, a single N-terminal transmembrane segment and C-terminal cytochrome b5 like domain (13, 14). RT-PCR experiments detect a single transcript corresponding to a protein ~25kDa, however the protein may exist in different states of phosphorylation resulting in slight changes in the apparent molecular weight (MW) (5). The putative structure of PGRMC1 contains a prominent ligand binding cleft (Figure 1.1) and contains binding sites for Src homology 2 (SH2)
and Src homology 3 (SH3) domain-containing proteins and consensus phosphorylation sites for tyrosine kinases (1). Additionally, PGRMC1 contains several YXXϕ motifs (ϕ is a large hydrophobic amino acid) that are also known as immunoreceptor tyrosine-based activation motifs (ITAMS) (15). These motifs are predominantly involved in cellular protein trafficking and functions include: targeting membrane proteins to membrane compartments, internalization of receptors after ligand-binding, and interacting with vesicle coat protein adaptor protein complexes (1, 15).

In 2006, Craven and colleagues at the University of Kentucky were the first to demonstrate heme binding directly to PGRMC1, and that a mutation in the heme-1 domain at the Asp120 residue (D120G, Figure 1.1A) blocks heme-binding activity (16). Heme is composed of ferrous iron and protoporphyrin IX and is a cofactor in numerous reactions associated with energy production and metabolism. Indeed, the only known biochemical function of PGRMC1 is heme binding, and heme binding has been characterized through multiple spectroscopic and crystallographic studies (16-18). PGRMC1 binds to heme via a conserved tyrosine residue in a five-coordinate (5C) high-spin (HS) configuration and has been indicated in heme transport (18, 19). Recent crystallographic studies have revealed that PGRMC1 can form a stable dimer through stacking interactions of two protruding heme molecules, and that heme binding is required for dimer formation (Figure 1.1B-C) (20). The SH3 and SH2 target sequences in PGRMC1 lie adjacent to one another on the opposite surface to the heme-binding site, potentially providing a site for the recruitment of
proteins to the surface of PGRMC1 (15). Based on the structure published in *Nature Communications*, it has been suggested that phosphorylation of PGRMC1 at position Y113 may promote the membrane trafficking function of PGRMC1 (14). Furthermore, the membrane trafficking activity may switch from a dimer-dependent “receptor insertion” function to a monomer-dependent “receptor withdrawal” phenotype in the presence of carbon monoxide (CO) (Figure 1.1C) (14, 15).

A structure-based screen performed by Yokohama and colleagues identified four aromatic ligands for the *Arabidopsis thaliana* PGRMC1 homolog, AtMAPR2, which are highly conserved with the human PGRMC1 in the heme-1 domain (21, 22). One of the ligands with the highest binding affinity to the heme binding pocket of PGRMC1 is called AG205 (Figure 1.1D), and was found to bind PGRMC1 by the Craven group (15, 22, 23). Since then, the small molecule has been categorized as an inhibitor of PGRMC1, although in certain settings it may act as agonist (15). Although PGRMC1 does not share any apparent homology to nuclear or membrane-bound hormone receptors (13, 24), partially purified and recombinant progesterone have been found to bind PGRMC1 (19, 25). Recombinant PGRMC1 has only a moderate steroid specificity for progesterone, and a considerable body of evidence has moved away from PGRMC1 itself containing progesterone-binding activity (3, 15, 24). It has been proposed that PGRMC1 either forms a complex with other progesterone-binding proteins or that it is involved in the regulation of cell surface localization of other proteins that contain progesterone-binding activity (15, 26).
Since the initial discovery of PGRMC1 in rat liver membrane fractions (3), PGRMC1 has been found to be highly expressed in mammalian liver and kidney (10). Other tissues with PGRMC1 expression include the steroidogenic and reproductive tissues, brain, breast, heart, lung, skeletal muscle, pancreas, and other organs (2). In the cell, PGRMC1 is predominantly localized to endosomes and the endoplasmic reticulum (27, 28). PGRMC1 also localizes to the actin cytoskeleton and binds actin (29) and is found in the centromeric region of chromosomes during oocyte meiosis (30). PGRMC1 is also detected in the cytoplasm, plasma membrane, and nucleus in some cell types (18, 31).

1.3 Interactions and functional implications

1.3.1 Cytochrome P450 stimulation and cholesterol metabolism

Cytochrome P450 enzymes are heme-dependent monooxygenases that participate in the detoxification of xenobiotics and metabolism of pharmaceutical drugs (32). Although the number of P450 enzymes varies among species, the P450 catalytic cycle is well conserved (32). PGRMC1 has been shown to interact with several cytochromes P450 (P450s) in unicellular eukaryotes and multicellular organisms to activate enzymatic activity in sterol biosynthesis and protect cells from DNA damage (8, 33). The first evidence supporting a role for PGRMC1 in P450 activation was demonstrated in the PGRMC1 yeast homolog, Dap1. Dap1 was found to mediate resistance to the azole drugs, itraconazole and fluconazole (inhibitors of sterol synthesis), via activation of Erg11 (human
Cyp51) (8, 34) and regulate Erg5 (human Cyp61A1) in the cholesterol synthetic pathway (34). Dap1 increased the levels of Erg11 (human Cyp51) in a heme-dependent manner (35). Yeast cells lacking Dap1 are sensitive to DNA damage and demonstrate a partial arrest in sterol synthesis (34).

Importantly, Dap1 function is conserved in humans. In 2007, Esplanshade and colleagues provided evidence for the direct role of the human PGRMC1 in cytochrome P450 function. In their studies, RNAi-mediated knockdown of human PGRMC1 reduced the activity of Cyp51 and blocked cholesterol synthesis (32). Additional co-immunoprecipitation experiments demonstrated stable binding of PGRMC1 to Cyp51, Cyp3A4, Cyp7A1 and Cyp21A2, indicating a role for PGRMC1 in cholesterol synthesis, drug and hormone metabolism and bile acid synthesis (32, 36). PGRMC1 also binds to cytochrome P450 reductase (CPR) (33).

In addition to binding P450 proteins, PGRMC1 binds to the cholesterol sensing proteins Insig (insulin-induced gene) and Scap (SREBP cleavage activating protein) in Cos7 cells (37). Both Scap and Insig form a complex with the transcription factor SREBP (sterol regulatory element binding protein) and are involved in a feedback regulation loop. SREBP exists as a precursor form in the endoplasmic reticulum (ER) until the cholesterol synthesis process is initiated. Under low cholesterol conditions, the Insig/Scap/SREBP complex translocates to the Golgi apparatus where SREBP becomes proteolytically cleaved for entry into the nucleus to induce SRE-driven genes responsible for
synthesis (1). Scap escorts SREBP to the Golgi apparatus, and Insig can inhibit the process by binding to Scap (36).

The PGRMC1/Insig/Scap/SREBP complex regulates fatty acid lipogenesis and cholesterol synthesis, which leads to the downstream stimulation of isoprene and sterol synthesis (15). Depletion of either PGRMC1 or SCAP protein levels via pharmacological inhibition (anti-psychotic drugs) was shown to increase lipogenesis and cholesterogenesis in rat livers, and the effect was reversed with administration of the steroid antagonist mifepristone (MIF) (38). PGRMC1 also plays a central role in cholesterol homeostasis, as it has been shown to catalyze the enhanced ATP-independent incorporation of cholesterol into biological membranes (15).

This PGRMC1-containing complex has been suggested to modulate diabetes, autophagy and cancer lipogenesis in a cell-type specific manner (15). More specifically, it has recently been demonstrated that N-glycosylation under the high cytoplasmic glucose conditions of tumor cells is required for tumor growth, and PGRMC1 may play a role (15).

1.3.2 Damage response and regulation

Cells have developed a variety of pathways for responding to cellular damage, and the response to damage is critical for cellular survival. Cells respond to DNA damage and oxidative damage by removing the damage, delaying cell cycle progression, and activating or repressing gene transcription (8, 28). Unresolved damage can lead to proliferation, growth arrest, senescence,
apoptosis or necrosis. Therefore proteins that regulate responses to cellular damage in different cell types are of great importance.

Indeed, the PGRMC1 yeast homolog (Dap1), and more specifically the YPL170W gene, was discovered while conducting a genetic search for novel yeast genes involved in damage response regulation (8). The DNA damaging agent methyl methane sulfonate (MMS) is widely used in yeast damage repair studies, and yeast containing a deletion of the open reading frame of YPL170W resulted in arrested growth in the presence of MMS (8). Furthermore, a point mutation within the heme-1 domain in Dap1 also results in damage sensitivity in the presence of MMS (34). These results support the requirement of Dap1 for growth and cell cycle progression following cellular damage in the yeast model system (8, 34).

1.3.3 Chemoresistance

In 1996, the rodent PGRMC1 homolog (25-Dx) was found to be upregulated in dioxin-induced liver tumors, suggesting its first implication in cancer (5). In 2003, Difilippantonio and colleagues found PGRMC1 to be consistently about 2-4 fold more abundant in lung cancer cell lines compared with healthy lung cell lines, however the finding was not extended to primary clinical tissues (39). In 2005, the Craven group showed via western blot that PGRMC1 is significantly elevated in breast cancer tumors compared with matched nonmalignant tissue and demonstrated limited overexpression in colon and thyroid tumors (40). In cancer cell lines, PGRMC1 was found to be
overexpressed in cell lines originating from breast, thyroid and colon cancers, and PGRMC1 was localized to the perinuclear region of the cell (40). In the same year, three isoforms of PGRMC1 were identified in breast cancer tumors, two of which were differentially more abundant in tumors lacking the estrogen receptor (ER) (41). In ovarian tumors, PGRMC1 expression increases with tumor stage (42). PGRMC1 is also secreted by lung cancer cells, as PGRMC1 was significantly elevated in the plasma of lung cancer patients compared to noncancerous patients (43). Thus, PGRMC1 has been proposed as a potential biomarker for multiple cancers.

Because the yeast PGRMC1 homolog, Dap1, was found to mediate damage response to a DNA-alkylating agent, the Craven lab tested the role of PGRMC1 in resistance to oxidative damage (8). In Hand et al 2003, the human PGRMC1 protein (fused to an HA epitope) was expressed in the human breast cancer cell line, MCF-7 (24). PGRMC1 expressing cells became sensitized to cell death following oxidative damage and PGRMC1 was found to regulate cell death through a novel oxidative damage response pathway independent of Akt and IkB (24). These results are consistent with the previous findings in the yeast homolog, Dap1, and the model in which PGRMC1 regulates cell death (8).

The ability to respond to oxidative stress is a key feature of carcinogenesis, and one of the primary goals in cancer research is to develop new ways of inhibiting cancer specific growth. Doxorubicin (Adriamycin) is an anthracycline antibiotic that is a component of many treatment regimens used for chemotherapy, often used in the treatment of breast cancer. Doxorubicin acts as
a DNA replication inhibitor, specifically blocking the activity of the DNA unwinding protein, topoisomerase II, to initiate apoptosis or cell cycle arrest (44). Other chemotherapeutic agents include camptothecin analogs and nitrogen mustards, which work to inhibit topoisomerase I and alkylate DNA accordingly (40). The utility of doxorubicin, and many chemotherapies, is limited by its inability to kill all of the cells within a tumor. Many researchers utilize microarray-based screens to search for chemotherapy-regulated genes, and in 2005, the Craven lab observed PGRMC1 to be transcriptionally induced by doxorubicin in the breast cancer cell line, MDA-MB-231 (44).

To further investigate the role of PGRMC1 in chemotherapy susceptibility, PGRMC1 was inhibited via RNA inhibition (RNAi) in MDA-MB-231 cells (16). Treatment with doxorubicin and camptothecin was found to significantly increase cell death in cells containing the PGRMC1 RNAi. Furthermore, deletion of the heme-binding domain in PGRMC1 also increased susceptibility of breast cancer cells to chemotherapeutics (16). These results indicate PGRMC1 inhibition, and a mutation in the PGRMC1 heme-binding domain, increases the ability of chemotherapeutic drugs to kill cancer cells (16). Other groups have provided more evidence in support of this role, as PGRMC1 has been found to attenuate cisplatin-induced death in both MDA-MB-231 cells and ovarian cells, as well as death caused by erlotinib and doxorubicin in HCT116 colon cancer cells (15).
1.3.4 Tumorigenesis and EGFR

Tumor growth and invasion are critical steps in the spread of cancer, and the proliferation of many cancers are driven by receptor-tyrosine kinases. As mentioned earlier, PGRMC1 plays a significant role in oxidative damage, and is induced during dioxin-induced tumorigenesis (5, 28). Oxidative damage activates transmembrane receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR), which can lead to subsequent activation of the serine-threonine kinase Akt (28). PGRMC1 regulates the response to oxidative damage in the human breast cancer cell line, MCF-7, by sensitizing cells to cell death (28). Although PGRMC1 hyperactivates the apoptotic protein Akt and damage response protein IkB in MCF-7 cells, cell death was found to be independent of Akt and IkB (28). Some of the most significant findings in cancer research involve the identification and targeting of signaling pathways activated in tumors, and in 2010, the Craven lab found PGRMC1 to promote cancer progression and associate with EGFR (22, 23).

Because PGRMC1 is upregulated in tumors, the Craven lab sought to determine the extent to which PGRMC1 regulates tumor growth. Human lung cancer cells, A549, were infected with lentiviruses containing either a control plasmid (A549/con) or a plasmid containing a short hairpin RNA targeting the PGRMC1 gene (A549/RNAi). RNA inhibition successfully established a PGRMC1-knockdown model system (Figure 1.2A) (22). In *in vitro* studies, PGRMC1-knockdown cells failed to proliferate in the absence of exogenous serum and generate growth in soft agar, supporting a role for PGRMC1 in cell
proliferation and anchorage-independent growth (22). When cells were injected into athymic nude mice, only the mice who received control cells (A549/con) efficiently formed tumors (Figure 1.2B-D), indicating PGRMC1 promotes tumor growth \textit{in vivo}. (22). Additionally, the PGRMC1 ligand, AG205, induced cell death in multiple breast and tumor cell lines (22).

A variety of tumors have EGFR overexpression, and activation of the EGFR-tyrosine kinase has been linked to increased proliferation, metastasis and decreased apoptosis (23). There are a growing number of drugs that inhibit EGFR, including cetuximab, erlotinib, and gefitinib, however patient response varies (23). After establishing the promotion of cancer proliferation in A549 non-small cell lung cancer cells (22), the Craven lab tested the model that PGRMC1 elevates growth factor receptor function. Indeed, PGRMC1 was found to increase susceptibility to the EGFR inhibitors AG-1478 and erlotinib in the lung cancer cell line, A549, and the breast cancer cell line, MDA-MB-231 (23). More specifically, PGRMC1 co-precipitated with EGFR and increased EGFR plasma membrane levels (Figure 1.3A, lane 3) (23). The PGRMC1-EGFR association was not altered after EGF stimulation, indicative to a constitutive interaction (23). To determine EGFR plasma membrane regulation, PGRMC1 was inhibited by both shRNA and siRNA, and underwent cell surface labeling and immunofluorescence techniques. EGFR plasma membrane levels were significantly lower in both RNAi PGRMC1-knockdowns via western blot analysis (Figure 1.3A, lane 4) (23). Immunofluorescence revealed an increase in the intracellular pool of EGFR in PGRMC1-knockdown cells (Figure 1.3C) and
reduction of EGFR at the plasma membrane (23). Additionally, inhibition of PGRMC1 function by the small molecule inhibitor, AG205, also prevented EGFR translocation to the cell surface (23). The cellular region in which PGRMC1 and EGFR co-localize was mapped to the microsomal lumen (Figure 1.4), as both proteins were found to co-fractionate in the high density fraction in a density gradient centrifugation (23). Taken together, these data demonstrate a model in which PGRMC1 associates with EGFR to stabilize EGFR levels at the plasma membrane (Figure 1.4). It is important to note that PGRMC1 did not regulate the receptor kinase HER2, suggesting a trafficking specificity (23).

In a separate study published in *Nature Communications*, crystallographic and protein analyses further characterized the EGFR-PGRMC1 interaction. Kabe and colleagues reported that heme is required for PGRMC1 dimerization, interactions with EGFR, and cancer proliferation and chemoresistance (20). EGFR and PGRMC1 co-precipitated in human colon cancer cells, and treatment with succinylacetone (SA), an inhibitor of heme biosynthesis, disrupted the interaction (20). The same pattern of PGRMC1 dependence for the trafficking of EGFR was reported in zebrafish oocyte cell membranes by Thomas and colleagues (45). Furthermore, treatment with AG205 resulted in a decrease in EGFR-dependent signaling and EGFR expression (45). These results suggest a widespread vertebrate generality for EGFR trafficking.

Tumor invasion is a critical step in the spread of cancer, and the PGRMC1-EGFR complex has been linked to the NGAL-MMP9 complex involved in tumorigenesis. Neutrophil gelatinase-associated lipocalin (NGAL) is an iron-
binding protein that forms a complex with and stabilizes the matrix metalloproteinase MMP9 to promote tumor invasion and survival (46). PGRMC1 was found to increase the transcription and protein levels of NGAL in lung cancer cells (46). Additionally, PGRMC1 elevated MMP-9 activity and the NGAL-MMP9 complex activity (46). NGAL expression was required for tumor formation, and the EGFR inhibitors erlotinib and AG1478 suppressed NGAL activity (46). Together these results support the model in which PGRMC1 increases the NGAL-MMP-9 complex by activating EGFR to drive tumor growth (46).

1.3.5 PGRMC1 and membrane trafficking

In addition to the PGRMC1-EGFR interaction and presence of multiple immunoreceptor tyrosine-based activation motifs (ITAMs), subsequent studies have substantiated a role for PGRMC1 in membrane trafficking. PGRMC1 has been found to associate with membrane progesterone receptor α (mPRα) on cell membranes (26). Membrane progesterone receptors (mPRs) are seven transmembrane receptors that belong to the progesterone and adipocytokine receptor family (PAQR), comprising of 5 subtypes. These proteins are expressed on cellular surfaces, are coupled to G proteins, and initiate a variety of intracellular signaling pathways associated with G proteins (26). In a study conducted by Thomas et al., PGRMC1 was found to enhance mPRα expression and function in both cancerous and noncancerous cell lines, specifically increasing the availability of mPRα to the plasma membrane (26).
Analogously to the case with EGFR surface localization, PGRMC1 associates with the glucagon-like peptide-1 receptor (GLP-1R) and mediates downstream signaling (47). Glucagon-like peptide-1 (GLP-1) is a gastrointestinal hormone that is secreted by intestinal L cells upon food intake and enhances glucose-stimulated insulin secretion (GSIS) from pancreatic beta cells (47, 48). The glucagon-like peptide-1 receptor (GLP-1R) is a member of the large G protein-coupled receptor family and is involved in glucose homeostasis. Affinity purification and mass-spectrometry (AP-MS) techniques identified PGRMC1 as an interactor with GLP-1R, and the direct interaction was confirmed by co-immunoprecipitation and co-immunofluorescence (47). Furthermore, the cell surface localization of GLP-1R in pancreatic beta cells was found to be dependent upon interactions with PGRMC1 (47). Overexpression of PGRMC1 enhanced GLP-1-induced insulin secretion (GIIS) whereas inhibition of PGRMC1, via RNAi and ligand treatment with AG205, significantly reduced GIIS (47).

GLP-1R can signal through c-Src to trans-activate EGFR (Figure 1.5), and an increase in EGFR activity has been linked to increased insulin secretion (47, 49). Zhang et al. found treatment with the epidermal growth factor (EGF) increased GIIS, and inhibition of EGFR or (downstream) PI3K blocked PGRMC1’s capability of GIIS (47). The EGFR/PI3K pathway was not found to mediate GLP-1-induced insulin secretion, therefore suggesting a novel role for PGRMC1 to participate in PGRMC1-mediated GIIS through EGFR/PI3K to potentiate insulin exocytosis (Figure 1.5) (47). These results suggest a role for
PGRMC1 in the maintenance of glucose homeostasis, and more importantly the involvement in type 2 diabetes, as the insulinotropic properties of GLP-1 are maintained in patients with type 2 diabetes (47).

1.4 Summary

Over the past two decades, research has compellingly demonstrated roles for PGRMC1 in heme-binding, steroidogenesis, p450 regulation, tumorigenesis and membrane trafficking. It has been postulated that PGRMC1 is a signaling hub protein that may be relevant to multiple disease states and biological processes (14). In normal tissues, PGRMC1 increases lipid synthesis via cytochrome p450 proteins. In cancer cell lines, PGRMC1 increases proliferation, invasion, tumor growth, and renders cells susceptible to oxidative damage.

PGRMC1 is overexpressed in multiple cancers, and associates with tumor stage and patient survival in ovarian and lung cancer accordingly (43). In this dissertation, we expand the analysis of PGRMC1 in the clinical setting to include over 600 tumor sections and lung-tumor derived stem cells. We report the first clinical analysis of PGRMC1 levels in human oral cavity and ovarian tumors, and found PGRMC1 expression to correlate with patient survival. Additionally, PGRMC1 was detected in lung-tumor derived stem cells, and drug treatment with the PGRMC1 ligand, AG205, triggered stem cell death whereas other treatments did not.

One of the most note-worthy binding partners of PGRMC1 is the epidermal growth factor receptor (EGFR), and this association is dependent on
PGRMC1 binding to heme. PGRMC1 colocalizes with EGFR in the endoplasmic reticulum (ER) in microsomal vesicles, and is responsible for trafficking the receptor tyrosine to the plasma membrane (23). Although there are many drugs that inhibit EGFR, the degree to which patients respond to these drugs varies markedly between those expressing wild-type EGFR vs mutants of EGFR. My work investigated multiple signaling-associated pathways that are important in trafficking wild-type and mutant EGFR with the goal being stimulation of new approaches to targeting the distinct forms of the receptor.

More recently, PGRMC1 has been linked to GLP-1-induced insulin secretion via the GLP-1R (glucagon-like peptide-1 receptor). PGRMC1 associates with and activates GLP-1R to enhance the insulinotropic actions of GLP-1 (49). Furthermore, a global gene expression profile revealed decreased PGRMC1 RNA levels in insulin-resistant, high BMI patients compared to matched insulin-sensitive subjects (50). In addition to its highly conserved role in membrane trafficking, these data support a role for PGRMC1 in the regulation of glucose metabolism, and my work sought to explore the potential role of PGRMC1 in trafficking another receptor tyrosine kinase, the insulin receptor (IR).

We report here that PGRMC1 co-precipitates with the insulin receptor (IR) in multiple cancer cell lines and in an adipose model system. More specifically, PGRMC1 increases the plasma membrane levels of IR and two glucose transporters, GLUT-4 and GLUT-1 in cancer cells, with that latter being key downstream targets of the IR pathway and cellular uptake of glucose. Insulin binding to IR initiates the translocation of glucose transporters to the plasma
membrane for glucose entry, and we demonstrate that both insulin binding and glucose uptake are dependent on PGRMC1. Furthermore, the PGRMC1 ligand, AG205, was shown to destabilize plasma membrane IR levels in cancer cells, with no effect on total IR protein levels. Alternatively, treatment with PGRMC1 ligands significantly increased total IR protein levels in fully differentiated human subcutaneous adipocytes.

The number of pathways affected by PGRMC1 can be expected to include other functions at various sub-cellular pathways, and the uncharacterized work should almost certainly provide new future therapeutics for the treatment of cancer and other diseases (15).
Figure 1.1 PGRMC1 structure, heme binding and the small molecule AG205.

(Figure legend on following page)
Figure 1.1 PGRMC1 structure, heme binding and the small molecule AG205.

(A) A model for the human structure of PGRMC1 adapted from Rohe et al., 2009 (See Appendix A for copyright information) (36). Asterisks represent identical residues between PGRMC1 and Dap1 (yeast homolog), while pound signs indicate key non-conserved residues. The D120 residue is critical for heme binding. (B) Close-up view of the binding pocket of PGRMC1 and heme stacking (adapted from Kabe et al., 2016, see Appendix B for copyright information (20)).

(C) Transition model for structural regulation of PGRMC1 in response to heme and CO. When heme binds to PGRMC1, a dimer formation can occur between two PGRMC1 proteins. The presence of CO disrupts the dimer formation (adapted from Kabe et al., 2016 (20)).  (D) The PGRMC1 ligand AG205 chemical structure and binding to the binding pocket of PGRMC1 (adapted from Yoshitani et al., 2005, see Appendix C for copyright information (21)).
**Figure 1.2 PGRMC1 promotes tumorigenesis *in vivo*.** A549 cells were infected with lentiviruses derived from the pGIPZ plasmid (A549/con) or pGIPZ expressing a short hairpin RNA targeting PGRMC1 (A549/RNAi). Athymic nude mice were injected with A549/con or A549/RNAi cells. (A) PGRMC1 expression was inhibited in the excised A549/RNAi tumors (top, lane 2), whereas ku70 was unchanged. (B) Excised tumor weight of A549/con was 2.9-fold more than A549/RNAi. (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) Graphical representation of panel C. *, ≤ 0.05; **, ≤ 0.01; ***, ≤ 0.005. (Adapted from Ahmed et al., 2010a, see Appendix D for copyright information (22)).
Figure 1.3 PGRMC1 increases EGFR levels at the plasma membrane. (A)
The cell surface proteins of A549/con and A549/RNAi cells were biotin-labeled with sulfo-
succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate and purified using avidin-
agarose columns. Lanes 1 and 2 and Western blots of the proteins that failed to bind to the avidin-
agarose columns (unbtn), whereas lanes 3 and 4 are Western blots of the avidin-bound pro-
teins (biotin). There was a 7.4-fold decrease in EGFR at the plasma membrane in A549/RNAi cells (p=0.01) (B-C) EGFR was stained by immunofluorescence (IF) in A549/con and A549/RNAi cells, respectively, and indicate an increased intracellular pool of the receptor (adapted from Ahmed et al., 2010b, see Appendix E for copyright information (23)).
Figure 1.4 Membrane trafficking of EGFR directed by PGRMC1. A schematic representation of PGRMC1 binding to EGFR intracellulary, transporting EGFR to the plasma membrane for stabilization of the receptor, and the complex becoming endocytosed and associating together in an endosome.
Figure 1.5 GLP-1 induced insulin secretion mechanism with PGRMC1 and EGFR. The diagram shows GLP-1 involved in regulating insulin secretion in pancreatic beta cells. The blue lines represent the classical way GLP-1R regulates insulin exocytosis, mainly through cAMP and protein kinase A (PKA) signaling. The purple lines represent PGRMC1 and its role in increasing GLP-1 induced insulin secretion (GIIS) through activating EGFR and its downstream PI3K activity (adapted from Zhang et al., 2014, see Appendix E for copyright information (47)).
CHAPTER II: PATHWAYS DRIVING THE ENDOCYTOSIS OF MUTANT AND WILD-TYPE EGFR IN CANCER

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2.1 Introduction

2.1.1 Regulation of wild-type EGFR trafficking

EGFR is over-expressed in a large number of tumors and is one of the best characterized oncogenic targets. EGFR binds to multiple extracellular growth factors, triggering conformational changes, dimerization of the receptor and phosphorylation of numerous residues in its cytoplasmic domain (51, 52). Some of the phosphorylated sites serve as docking points for downstream signaling molecules, while others are bound by negative regulatory proteins that drive endocytosis of the receptor. Specifically, Cbl (Casitas B-lineage lymphoma), an E3 ubiquitin ligase (53), is recruited to tyrosine 1045 phosphorylated EGFR by the adaptor protein GRB2, which promotes EGFR ubiquitylation and entrance into clathrin coated pits (Figure 2.1) (54, 55). There are a number of recent reviews on EGFR signaling and trafficking (56), so we will focus on several areas acting upstream on wild-type and mutant EGFR that have not been reviewed recently. MIG6/RALT (mitogen-inducible gene/receptor-associated late transducer) inhibits EGFR (57-61) by associating with the receptor through a carboxy-terminal ERB-binding region (EBR). MIG6-EGFR binding physically obstructs EGFR asymmetric dimer formation (58, 61) and suppresses EGFR activity by stabilizing an inactive conformation of the receptor (62). Many of these findings were reviewed in 2011 (63). MIG6 decreases proliferation and migration in a variety of cell types in vitro (63). In MIG6/Errfi1 knockout mice, endogenous EGFR is hyper-activated, causing hyperplasia of
epidermal keratinocytes and extreme sensitivity to chemical carcinogenesis (64) that is reversed by gefitinib.

More recently, Hopkins, et al., showed that mammary gland terminal end buds in Errf1-null mice had increased luminal filling (65). This hyper-proliferation was not due to EGFR hyper-activation but decreased ABL activity, suppressing apoptosis in this setting (65). ABL is a cytoplasmic tyrosine kinase associated with tumor cell survival and metastasis in cancer cells but also with stress-associated apoptosis through p73 in normal tissues (66-68). MIG6 binds to ABL via its conserved ERB domain in the absence of epidermal growth factor, suggesting a mechanism in which MIG-6 senses EGF deprivation to induce apoptosis (65).

In addition to inhibiting EGFR catalytic activity, MIG6 increases EGFR internalization and trafficking to the lysosome (69), even for EGFR mutants that are not trafficked by CBL-mediated endocytosis (70). Indeed, computational modeling suggests that MIG6 and CBL contribute equally to EGFR endocytosis (71), although this model is based on a limited number of cell lines. MIG6-dependent EGFR endocytosis is thought to be clathrin-dependent, involving binding between MIG6 and the SH3 domains of the intersectins ITSN1 and ITSN2 (Figure 2.1) (70). Moreover, MIG6 associates with the SNARE protein syntaxin 8 (STX8, Figure 2.1), elevating levels of the STX8-EGFR complex, which is essential for EGFR endosomal trafficking (69). The findings of Frosi, et al. suggests that clathrin-dependent EGFR endocytosis results in lysosomal degradation (70). However, this model contrasts with a previous study indicating
that clathrin-dependent EGFR endocytosis is associated with receptor recycling and sustained activation (72). The latter study employed high levels of ligand, and the two studies were performed in very different cell types, HeLa (72) and mouse NR6 cells (70), implying that some aspects of EGFR endocytosis and signaling may be organism – or cell type-specific. Furthermore, there is recent biochemical evidence that MIG6 tyrosine phosphorylation weakens its ability to inhibit EGFR, even though the proteins remain associated (73). Thus, the activation of various signaling pathways may have a profound effect on CBL function.

Although some studies suggest that MIG6 and CBL act through separate mechanisms, other findings suggest interplay between their EGFR endocytic pathways. Notably, intersectin 1 (ITSN1) forms complexes with both MIG6 and CBL, mediated by intersectin SH3 domains binding to the proline-rich carboxy-terminus of CBL (74) or proline-rich sequences located in the RED (RALT Endocytic Domain) of MIG6 (70), increasing repression of EGFR signaling (70) and EGFR ubiquitylation (74). ITSN1, which has been reviewed recently (75), recruits other proteins downstream, particularly SHP2, SRC homology-2 containing phosphotyrosine phosphatase (Figure 1, (76, 77)).

SPRY2 is a CBL-binding protein (78) that can be tyrosine phosphorylated (79), driving its association with CBL and inhibiting CBL-RTK binding (Figure 2.1) (54). SPRY2 is de-phosphorylated by SHP2, releasing CBL (80). ITSN1 recruits SHP2 to SPRY2, disrupting the inhibitory effect of SPRY2 on CBL, promoting EGFR ubiquitylation and endocytosis (Figure 2.1) (76). SPRY2 has been
extensively reviewed elsewhere (81). However, the role of the MIG6-ITSN complex in regulating CBL-SPRY complex formation is presently unknown. Thus, the interactions between EGFR, CBL, ITSN and MIG6 may be overlapping, perhaps to fine tune the temporal signaling through the receptor and to provide redundancy in the system.

2.1.2 Sigma-2 receptor associates with EGFR, increases plasma membrane EGFR levels and promotes invasion

PGRMC1 (progesterone receptor membrane component 1) is a cytochrome b5-related protein that binds heme and is implicated in cellular trafficking (36). There are compelling data that PGRMC1 is identical to the sigma-2 receptor (S2R). A highly selective S2R probe cross-linked directly to PGRMC1; S2R ligand binding decreased with PGRMC1-knockdown and increased with PGRMC1 over-expression; the apoptotic activity of an S2R ligand decreased with PGRMC1 knockdown; the PGRMC1 ligand AG205 displaced S2R ligand binding (22, 82). Notably, PGRMC1 was proposed to be a sigma receptor more than a decade earlier based on the ability of some sigma ligands to displace microsomal progesterone binding (83). It is still formally possible that PGRMC1 is not itself the S2R but is part of a complex that is tightly associated with S2R, and numerous experiments are under way to further dissect this possibility. S2R\textsuperscript{PGRMC1} also plays a key role in membrane-associated progesterone signaling (12, 84, 85), but S2R\textsuperscript{PGRMC1} is not homologous to known steroid receptors and direct binding of progesterone to recombinant PGRMC1
has not been demonstrated. However, progesterone binding was detected to partially purified PGRMC1 (85), and RNAi inhibition of PGRMC1 decreased progesterone binding activity (85) suggesting that \( \text{S2R}^{\text{PGRMC1}} \) may influence progesterone signaling through a binding partner. Indeed, Thomas, et al. demonstrated that PGRMC1 forms a complex with mPR\( \alpha \) and recruits the receptor to the plasma membrane (26). Together, these proteins may be part of a larger membrane progesterone receptor complex.

In many peripheral tissues and in tumors, numerous groups have localized \( \text{S2R}^{\text{PGRMC1}} \) to the endoplasmic reticulum, endosomes, intracellular puncta and microsomal fractions (3, 23, 27). Interestingly, \( \text{S2R}^{\text{PGRMC1}} \) localizes to a significant extent to the plasma membrane (86-88) and nucleus (89) in neuronal cells, and its interactions with receptors may occur at the plasma membrane in those tissues. Indeed, it is intriguing to speculate that \( \text{S2R}^{\text{PGRMC1}} \) might bind to a membrane progesterone receptor in neuronal tissues, where \( \text{S2R}^{\text{PGRMC1}} \) is co-expressed with membrane progesterone receptors (87), although this model is currently untested.

A number of groups have found that \( \text{S2R}^{\text{PGRMC1}} \) plays a profound role in regulating cellular signaling, particularly the Akt and ERK pathways (22, 23, 28, 90), and in searching for the mechanism underlying this effect, we found that \( \text{S2R}^{\text{PGRMC1}} \) associates with EGFR and co-localizes with EGFR within endosomes (23). Furthermore, \( \text{S2R}^{\text{PGRMC1}} \) inhibition decreased plasma membrane levels of EGFR (Figure 2.1), and EGFR was de-stabilized by \( \text{S2R}^{\text{PGRMC1}} \) inhibition in some— but not all — cell types (23). Thus, we propose a model that \( \text{S2R}^{\text{PGRMC1}} \)
contributes to the trafficking of EGFR to the plasma membrane. An alternate model is that S2R^{PGRMC1} inhibits the endocytosis of EGFR, but S2R^{PGRMC1} was not detected at the plasma membrane in lung cancer cells (23), suggesting that any inhibition would be indirect.

Because S2R^{PGRMC1} associates with EGFR, we searched for downstream events regulated by this interaction and found that S2R^{PGRMC1} has a profound impact on protease activation in lung cancer cells (46). Specifically, the S2R^{PGRMC1}-EGFR complex increases the Lys310 acetylation and Ser535 phosphorylation of the NF-κB transcription factor, which in turn drives the expression of NGAL/LCN2 (46), a binding protein for matrix metalloproteinases such as MMP9 (91). Indeed, MMP9 activity was largely dependent on S2R^{PGRMC1} expression in lung cancer cells. These activities required EGFR and were elevated by exogenous EGFR expression (46). We note that other proteases, including MMP-2 and cathepsin D, were also activated in an S2R^{PGRMC1}-dependent manner (46), and cathepsin D plays a key role in tumor invasion and metastasis (92).

S2R^{PGRMC1} is appealing as a cancer target because recent events suggest that it can be efficiently inhibited both by “PGRMC1” ligands, such as AG205 (21, 22), and by a number of small molecule “sigma-2 receptor” ligands, including siramesine, PB28, SV119, CB-64D, SM-21 and others (93-98). Some of these ligands have been extensively tested in vitro, in vivo and in clinical trials and had relatively minimal side effects. However, it is unclear whether any of these
ligands alter EGFR trafficking. The interactions between these ligands and progesterone will likely reveal new elements of the $S2R^{PGRMC1}$ mechanism.

### 2.1.3 RAK/FRK increases EGFR trafficking

Our efforts in studying EGFR led to a second heretofore unknown pathway regulating EGFR trafficking. SRC family intracellular tyrosine kinases associate with growth factor receptors, including EGFR, and are important in mitogenic signaling through these receptors (99). Indeed, SRC was the prototypical oncogene, being mutated in transforming avian viruses. There are eight SRC-related tyrosine kinases with a common SH2 (SRC homology)-SH3 domain structure and a myristoylation site at the amino terminus (100). The BRK/RAK/SRC42A/ SRM kinases form a subgroup of proteins called the BRK family, that are related to SRC structurally but differ in the amino terminal sequences and multiple other sites (101). In addition, the BRK/RAK/SRC42A/SRM proteins have widely divergent roles in cell proliferation.

While the majority of SRC-related kinases have a positive role on cell proliferation and survival, the RAK/ FRK (FYN-related kinase (102, 103) inhibits growth when expressed in a number of cancer cell types (104, 105). RAK/FRK phosphorylates and binds to the PTEN tumor suppressor, stabilizing PTEN and promoting growth arrest, both in vitro and in vivo (106). In addition, RAK/FRK associates with the RB (retinoblastoma) tumor suppressor (104) and phosphorylates a negative regulatory site on SRC (102). Thus, there are multiple potential mechanisms through which RAK/FRK can inhibit cell growth.
We found that RAK phosphorylated tyrosine 1173 of EGFR and co-precipitated with EGFR (107). The RAK-EGFR interaction required both the SH2 and SH3 domains of RAK and increased after EGF stimulation. As a result, RAK decreased the levels of EGFR at the plasma membrane (Figure 2.1) (107), although it is unclear whether this was due to increased EGFR endocytosis, decreased EGFR transport to the plasma membrane, or some other factor.

Interestingly, BRK/PTK6 (breast tumor kinase/protein tyrosine kinase 6) also binds to EGFR and phosphorylates the receptor (108). In addition, BRK phosphorylates CBL and promotes its degradation (109), potentially decreasing EGFR endocytosis (Figure 2.1). BRK/PTK6 drives breast tumor formation in vivo in mouse models (110, 111) and xenografts (111). BRK is also a key effector of the MET receptor tyrosine kinase (111-113), and its stability is elevated by HER2 (114, 115).

Thus, two closely related RAK-BRK family members associate with EGFR, although their functions in the complex are opposite. The third family member, SRC42A, inhibits tyrosine kinase activity in Drosophila (116) in addition to playing a key role in development. SRM (SRC related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites) is induced in tumors and phosphorylates docking protein 11 (117) but does not perform an essential role in development (118), and any association with EGFR is unknown. It is intriguing to speculate that the EGFR-BRK and EGFR-RAK complexes (and possibly SRM or SRC42A complexes in Drosophila) may form in very different environments and with different dynamics during signaling.
2.1.4 EGFR mutants drive tumor growth and have altered intracellular trafficking

Mutant forms of EGFR are associated with cancer development, including lung cancer in non-smokers, and with elevated sensitivity to EGFR inhibitors (119-123), such as erlotinib and gefitinib. It has become increasingly clear that distinct EGFR mutants have different patterns of regulation and trafficking (Table 1). For example, Furukawa, et al. reported that EGFR-Δ746-750 has sustained activation of downstream effectors and is not phosphorylated on Y1045, the CBL binding site, resulting in impaired endocytosis (124). In the EGFR-L858R mutant, Y1045 is phosphorylated (121-123). However, in human cancer cells, EGFR-L858R is down-regulated (125, 126), but their assessment of ubiquitylation and CBL binding were markedly different (125, 126). In contrast, Furukawa, et al., found normal EGFR-L858R-CBL binding with unaffected downstream signaling (124). However, the latter studies were in mouse fibroblasts and simian COS-7 cells and may not reflect the signaling environment of cancer cells. The EGFR-L858R mutant has impaired nuclear EGFR localization resulting in decreased DNA repair activity (127). Interestingly, the EGFRvIII mutant is also trafficked atypically, with the majority of the receptor being recycled to the plasma membrane rather than being degraded, even though the EGFRvIII mutation is on the extracellular surface of the protein (128).

MIG6 expression is elevated in cells expressing EGFR-L858R (129), and MIG6 is required for the endocytosis of wild-type and mutant EGFR (129, 130). However, MIG6 tyrosine phosphorylation is elevated in cells expressing EGFR-
L858R and EGFR-Δ746-750 (131), suggesting a weaker ability to inhibit the receptor (Table 1). SPRY2 prevents endocytosis of both wild-type and Δ746-749/A750 mutant EGFR (130). Thus, in a limited number of cell lines, MIG6 and SPRY2 do not discriminate between wild-type and mutant EGFR in their endocytic functions (130). However, a recent study suggests that, although MIG6 is more efficient against the wild-type EGFR, it has an increased role in the endocytosis of EGFR Δ746-A750 compared to wild-type EGFR, because CBL is less active against the mutant (71). The EGFR-vIII mutant does not undergo ligand-induced endocytosis due to low levels of phosphorylation (132), and MIG6 is inactive against it (69).

In contrast to MIG6 and SPRY, S2R<sup>PGRMC1</sup> does not act equally on wild-type and mutant EGFR. An S2R<sup>PGRMC1</sup> inhibitor was active against cells expressing wild-type EGFR, but had no activity against lung cancer cells expressing EGFR mutant (23). However, the cell lines had different genetic backgrounds, and factors other than EGFR could have affected S2R<sup>PGRMC1</sup> inhibitor sensitivity. For that reason, we expressed wild-type EGFR and the EGFR-Δ747-749/A750P mutant in MDA-MB-435 cells, which do not express EGFR, and found that S2R<sup>PGRMC1</sup> co-precipitated with wild-type EGFR-2.4-fold more than the mutant (Figure 2.2). The mechanism underlying this specificity is unclear. However, S2R<sup>PGRMC1</sup> is enriched in endosomes in lung cancer cells (23), and the decreased endocytosis of mutant EGFR may limit the access of the two receptors to each other. Because S2R<sup>PGRMC1</sup> binds mutant EGFR poorly (and
inhibitors are inactive against cells expressing it), we do not expect that S2R functions through a SPRY pathway for EGFR regulation.

In stark contrast to S2R\textsuperscript{PGRMC1}, we found that RAK/ FRK bound preferentially to the EGFR $\Delta$746-749/A750P mutant compared to the wild-type protein (107). This may be due to the increased activity of the mutant EGFR, because RAK binding to wild-type EGFR increased after ligand stimulation (107). The results suggest that RAK may have elevated tumor suppressive activity in tissues expressing mutant EGFR. It is intriguing to speculate that tumors expressing mutant EGFR may have decreased RAK expression, but this concept has not been tested. Because RAK and MIG6 are both active against EGFR mutants, it is intriguing to speculate that they may be mechanistically related. However, this remains to be tested.

2.2 Perspectives: Current and Future Work

Mutant forms of EGFR are associated with some types of cancer and have differential trafficking compared to the wild-type receptor. Furthermore, specific trafficking proteins are distinct in their regulation of wild-type and mutant EGFR. In spite of the dramatic advances in the field, there are numerous questions remaining about MIG6, such as the conditions under which its tyrosine phosphorylation changes and the key players directing these alterations. In addition, new targets for MIG6 play key roles in proliferation and apoptosis. In normal tissues lacking MIG6, breast cells proliferated due to loss of ABL (65), but it is unclear how the MIG6-ABL interaction changes in different non-malignant
cell types and during cancer progression. In normal tissues, ABL is thought to be pro-apoptotic, while in cancer cells, ABL drives proliferation, survival and metastasis. But it is unknown whether ABL no longer binds MIG6 in cancer cells or whether binding changes in the presence of apoptotic stimuli.

The relatively poor binding of S2R$^{PGRMC1}$ to the EGFR-Δ746-749/A750P mutant (Figure 2.2) may offer important clues to its interaction with EGFR. If the mutant is sustained at the plasma membrane, it is likely that S2R$^{PGRMC1}$ is prevented from binding to mutant EGFR because EGFR is not internalized, consistent with the endosomal localization of S2R$^{PGRMC1}$ in lung cancer cells (23). For wild-type EGFR, EGF stimulation did not affect EGFR-S2R$^{PGRMC1}$ binding (23), suggesting that the activated state of EGFR-Δ746-749/A750P is not responsible for the change in binding to S2R$^{PGRMC1}$. Future work will include a broader analysis of S2R$^{PGRMC1}$ binding to additional EGFR mutants, particularly EGFR-L858R.

The RAK/FRK tyrosine kinase decreases the plasma membrane pools of EGFR, and it is intriguing to speculate that RAK/FRK might influence the phosphorylation of EGFR trafficking proteins, including SPRY2, MIG6 and ITSN. We predict that RAK/FRK would increase MIG6 activity, possibly by decreasing MIG6 tyrosine phosphorylation. Conversely, we speculate that RAK/FRK might decrease the tyrosine phosphorylation of SPRY2, because SPRY2 phosphorylation is associated with decreased EGFR ubiquitylation (Figure 2.1). Current research is focusing on the role of RAK/FRK-PTEN binding in regulating EGFR.
2.3 Conclusions

EGFR levels at the plasma membrane are balanced by competing positive and negative mediators. The impact of these pathways changes for the mutant forms of the receptor, and some of the regulatory proteins have altered expression in cancer. While kinase inhibitors are active against tumors expressing mutant EGFR, their activity is limited against those expressing wild-type EGFR, and some activities of EGFR may be kinase-independent. S2R\textsuperscript{PGRMC1} inhibitors are attractive in this setting because they inhibit EGFR-dependent cancer cell proliferation and are most active against the wild-type form of the protein (23).
**Table 1** EGFR-L858R and EGFR-Δ746-750 mutants differ from wild-type

**EGFR in regulation and trafficking.** Question marks indicate unknown, and a negative sign implies no effect.

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<th>Wild-type</th>
<th>L858R</th>
<th>Δ746-750</th>
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<tbody>
<tr>
<td>Endocytosis by MIG6</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Endocytosis by RAK</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Prevents endocytosis by SPRY2</td>
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<td>S2R association</td>
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<td>Sensitivity to S2R inhibitor</td>
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<td>MIG6 Expression</td>
<td>+</td>
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<td>MIG6 tyrosine phosphorylation</td>
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Figure 2.1 Selected pathways regulating EGFR endocytosis and degradation. In the top panel, EGFR levels at the plasma membrane are increased by S2R\textsuperscript{PGRMC1}. The diagrams, from left to right, below, show different binding partners for EGFR. GRB2 recruits CBL to EGFR resulting in lysosomal degradation. SPRY2 phosphorylation drives its association with CBL, inhibiting CBL binding to EGFR. ITSN1 can recruit SHP2 to dephosphorylate SPRY2, releasing CBL to bind EGFR. MIG6 physically obstructs EGFR dimerization and binds to STX8 and ITSN1/2 to promote lysosomal degradation of EGFR. BRK phosphorylates EGFR to inhibit EGFR internalization, while RAK/FRK has the opposite activity.
Figure 2.2 S2RPGRMC1 preferentially associates with wild-type EGFR.

MDA-MB-435 human breast cancer cells, which do not express EGFR (lower panel, lane 1), were transfected with a control plasmid (lane 1), the plasmid pcDNA3.1-EGFR (lane 2, a gift from Drs. Penni Black, University of Kentucky, and William Pao, Vanderbilt University) or the plasmid pBabe-EGFR-Δ746-749/A750P Addgene, Cambridge, MA). In the top two panels, lysates were immuno-precipitated using previously described conditions (23) with the anti-EGFR antibody IMC-C225 (Erbitux, ImClone Systems, Branchburg, NJ). Precipitates were then analyzed by western blot with (top panel) the anti-S2RPGRMC1 antibody PGR-UK1 (22) or (middle panel) an anti-EGFR polyclonal antibody (1005, Santa Cruz Biotechnology). Because of the very different molecular weights of the proteins, the blot was cut in half before probing. The bottom panels show the same unprecipitated cell lysates that were used for the precipitation reactions analyzed by western blot using EGFR and GAPDH polyclonal antibodies, the latter as a control for protein loading. The result shown
is representative of three independent experiments. We have previously shown that the EGFR-Δ746-749/A750P mutant is highly tyrosine phosphorylated in this system compared to wild-type EGFR (107).
CHAPTER III: PGRMC1 ELEVATION IN MULTIPLE CANCERS AND ESSENTIAL ROLE IN STEM CELL SURVIVAL

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3.1 Introduction

PGRMC1 is induced in a number of cancer types (36), including breast, ovarian and lung cancers, and a small study indicated that PGRMC1 is associated with poor survival in lung adenocarcinoma (43). PGRMC1 is also expressed in sebaceous carcinomas (133). PGRMC1 plays a causative role in cancer progression, because in vitro, PGRMC1 increases tumor cell proliferation, chemotherapy resistance and invasion, and in vivo, PGRMC1 increases tumor growth, angiogenesis and metastasis (12, 22, 134-137). There are a number of potential mechanisms through which PGRMC1 might promote tumor growth. PGRMC1 associates with the epidermal growth factor receptor (EGFR) and regulates susceptibility to the EGFR inhibitor erlotinib by increasing plasma membrane pools of EGFR (23). PGRMC1 also increases EGFR levels in Zebrafish (45). In lung cancer cells, the EGFR- PGRMC1 complex drives invasion, at least in part, by activating matrix metalloproteinases (46).

PGRMC1 is also detected in the nucleus in some cell types, where it regulates transcription (31) and in the centromeric region of chromosomes during oocyte meiosis (29, 30). PGRMC1 also localizes to the actin cytoskeleton and binds actin. In lung cancer cells, the prominent localization for PGRMC1 is cytoplasmic puncta, including early endosomes, and numerous groups have reported similar findings in other cell types. Finally, PGRMC1 is secreted by lung cancer cells, where it has a pro-proliferative function, and is detected in the plasma of lung cancer patients (138).
There is a growing consensus that PGRMC1 is critical for the transport of specific receptors to the plasma membrane. The receptors include EGFR, GLP1R, glucagon-like peptide 1 receptor (47), and mPR1α, membrane progesterone receptor α (26). PGRMC1 binds to mPR1α and transports it to the plasma membrane (26). Indeed, PGRMC1 was originally identified as a putative hormone receptor or “receptor membrane component” (3, 4, 10). Partially purified PGRMC1 binds to progesterone (25, 85), and recently, progesterone binding by recombinant PGRMC1 was reported (19), suggesting a direct role for PGRMC1 in progesterone function. PGRMC1 has an established role in progesterone signaling (12, 84, 134), and in some diseases, such as breast cancer, this contributes to hormonal growth and anti-apoptotic signaling (139). However, PGRMC1 shares no homology with hormone receptors (13) but has motifs that are structurally related to cytochrome b5, and PGRMC1 binds heme (7, 13, 16, 24, 32, 34, 140, 141), an evolutionarily conserved function (32, 34, 142) that is distinct from progesterone binding (19).

According to the cancer stem cell theory, tumors contain a sub-population of cells with extended replicative potential that contribute to drug resistance (143, 144). Cancer stem cells are thought to arise from mutations to either normal stem cells or transit amplifying cells, with key signaling contributions from the tumor microenvironment. PGRMC1 is detectable in amniotic-derived mesenchymal cells (145) and has been identified as an important hormonal signaling intermediate in neuronal stem cells (84), but its expression and function in cancer-derived stem cells have not been determined.
We demonstrate here that PGRMC1 is elevated in multiple tumor types, including head and neck cancer and in oral cancer. Using immunohistochemistry of paraffin-embedded tissue, we also confirm previous findings from western blots of frozen tissue that PGRMC1 staining correlated with survival in lung cancer patients. According to the stem cell theory, cancer stem cells are critical for the long-term survival of a tumor population and its therapeutic resistance. We report here that PGRMC1 is abundant in lung cancer-derived stem cells from patients, and PGRMC1 inhibition triggered cell death in lung cancer stem cells where other therapeutic classes failed.

3.2 Materials and Methods

3.2.1 Tissue arrays

The tissue arrays used were as follows. For head and neck cancers: HN483, HN242a and HN802 (US Biomax, Inc. Rockville, MD). For oral cancer, OR802 (US Biomax, Inc.). For lung cancer, BC04011, BC041114, BS04011, LC991 (US Biomax), IMH-305 and IMH-340 (Imgenex, Inc., San Diego, CA). For ovarian cancer, BC11115 and OV951 (US Biomax).

3.2.2 Immunological reagents and techniques

Tissue arrays were stained with an anti-PGRMC1 antibody raised to a recombinant protein composed of amino acids 43-195 of PGRMC1 (ProteinTech Group, Inc., Chicago IL). Immunohistochemistry was performed by the
University of Kentucky Histology Laboratory using the Dako Envision kit (Carpinteria, CA) and following the manufacturer's instructions. Staining was analyzed and scored by a board-certified pathologist (Dr. Stewart), as well as other authors, and analyzed statistically using Microsoft Excel. Kaplan-Meier curves were analyzed and prepared using Graphpad Prism software. Blocking was performed with an equimolar concentration of purified, recombinant PGRMC1-glutathione S-transferase fusion protein spanning the antigenic region, and the protein has been described (16).

Protein levels were analyzed by western blot as previously described (40), blotting electrophoresed proteins to Immobilon-P membranes and developing using the West Pico chemiluminescent substrate (Pierce). Blots were performed at least in duplicate. The antibodies used were the following: anti-GAPDH (Santa Cruz, FL-335), anti-LCB (Cell Signaling, D11), anti-SQSTM1/p62 (Cell Signaling, 5114), anti-caspase 3 (Santa Cruz, sc-7138), anti-PARP (Santa Cruz, sc-7150) and anti-PGRMC1 (22).

3.2.3 Lung cancer stem cells

Single-cell suspensions were isolated from patients at the Edwards Cancer Center using a gentleMACS Dissociator (Miltenyi, Auburn, CA), and C Tubes using a standardized, semi-automated protocol based on a combination of mechanical tissue disruption and incubation with a 50% solution 0.025% trypsin and Accutase (Innovative Cell Technologies, San Diego, CA). Cells were serially plated in 12-well, 6-well, 10-cm treated dishes and cultured to subconfluence in
RPMI-1640 medium supplemented with 5% irradiated, heat inactivated, defined fetal bovine serum (Thermofisher/Hyclone), and 50 U of penicillin and 5 mg of streptomycin/mL of medium (Thermofisher/Mediatech). Cancer stem cells were selected and proliferated using a hydrodynamic focusing bioreactor as previously described (146). After seven days of growth in the bioreactor, cells were then removed and counted using a CelloMeter automated counter and trypan blue exclusion to determine cellular viability and cell number. Cells were then immunophenotyped using fluoresceinated antibodies to CD133, CD44, CD24 and CXCR4 with an Accuri C-6 flow cytometer. For live cell assays, lung tumor-derived stem cells (positive for CD133, CD43, SSEA3/4, Oct4, alkaline phosphatase, aldehyde dehydrogenase and telomerase) were purchased from Celprogen, Inc. and were maintained in Human Lung Cancer Stem Cell Complete Growth Media with serum (Celprogen, Inc., Torrance, CA).

Cell viability was determined by Cell Titer Blue Cell Viability Assay (Promega). Erlotinib was from LC Laboratories (Woburn, MA), and other treatments were LY294002 (Sigma), PD98059 (Sigma) and AG205 (Tocris). For drug treatments, cells were maintained in complete media and switched to serum free media (DMEM containing antibiotics) and various drugs for 24 hours. For microscopy, cells were treated with vehicle or 10 μM AG205 for an additional 24 hours. Bright-field images were captured on a Nikon Eclipse TE200 microscope at 20x magnification. For viability assays, cells were treated for 72 hours in complete media, and viability was assayed according to the manufacturer’s instructions. Absorbance was measured using a Spectra Max M2
spectrophotometer (Molecular Devices). For western blot analysis, cells were lysed in radioimmunoprecipitation buffer containing protease inhibitors and phosphatase inhibitors.

For dye exclusion, cells were trypsinized and treated with 5 μg/ml Hoechst 33342 (Sigma) at 37°C for 45 minutes. Cells were then washed with PBS (Gibco) and incubated in fresh media for 45 minutes at 37°C to allow for dye efflux before being centrifuged and analyzed again by FACS.

3.3 Results

3.3.1 PGRMC1 in airway cancers

PGRMC1 is essential for lung tumor formation and metastasis, so we determined PGRMC1 levels by immunohistochemistry in 330 lung cancer samples using tissue microarrays, which included 58 patients with survival data. Tissues were stained for PGRMC1 and scored by a board-certified Pathologist. The antibody- generated to amino acids 43-195 of PGRMC1- was a commercial product from Proteintech Group, and to our knowledge, no controls for staining have been performed with this product. In staining of a tissue from an earlier study, we found that recombinant PGRMC1 fusion protein blocked staining with the antibody, suggesting that it is specific (Figure 3.1 A-B).

Consistent with earlier western blotting studies (43), PGRMC1 was significantly higher in lung tumors than normal tissue (p=2 X 10-7, 2-sided t-test) and was higher in stage I tumors compared to stage II (p=3X10-5, 2-sided t-test)
but was not associated with age at diagnosis or gender. High PGRMC1-expressing tumors had a significantly worse overall survival (p=0.02 by Gehan-Breslow-Wilcoxon test), with median survival times of 25 months for high expressers versus 108 for low expressers (Figure 3.2 A).

PGRMC1 staining was analyzed in 173 tissues from the head and neck, including the oral cavity, in tissue microarrays. Tumors from multiple sites within the head and neck region stained strongly for PGRMC1 (Figure 3.1 C-F), including tumors of the submaxilla, cheek, parotid gland, gingiva and larynx (Figure 3.4). PGRMC1 was significantly elevated in all head and neck tumors (n=173) compared to corresponding normal tissues (n=23, p=0.004, 2-sided t-test). There was no association with stage or gender, but PGRMC1 staining was higher in tumors from patients younger than 60 (p=0.03, n=113). For example, the highest levels of staining were located in the tongue, and PGRMC1 was elevated in tongue tumors (n=16, Figure 3.1 C-D) relative to normal tongue tissue (n=18, p=0.009).

3.3.2 PGRMC1 in ovarian cancer

In ovarian tissues, PGRMC1 staining was not significantly different in normal ovarian tissue compared to tumor adjacent tissue, clear cell carcinomas or mucinous papillary adenocarcinomas (Figure 3.1G). In contrast, PGRMC1 was significantly elevated in serous papillary adenocarcinoma (p=4 X 10^{-5}), papillary serous cystadenocarcinoma (Figure 1.H, p=0.006) and endometroid carcinoma (p=0.02, Figure 3.5). PGRMC1 was also elevated in metastatic
serous papillary adenocarcinoma compared to normal tissue (p=0.003), but was not significantly different from the primary serous papillary adenocarcinoma. This difference was highly significant (Figure 3.2B, p=0.0011 by Mantel-Cox test and 0.0014 by Gehan-Breslow-Wilcoxon test).

3.3.3 PGRMC1 in cancer-derived stem cells

The association between PGRMC1 levels and tumors suggested a potential role for PGRMC1 in stem cell maintenance. Stem cells were isolated from surgical specimens at Marshall University using a PBRX bioreactor, and western blots revealed abundant PGRMC1 in isolated stem cells as well as in the bulk of the tumor (Figure 3.3A). The isolated stem cells were pre-screened for expression of CD133, CD44, CD24 and CXCR4 as described (146).

To assess the role of PGRMC1 in stem cell viability, we treated lung tumor-derived stem cells with the PGRMC1 ligand AG205 (22), which had an IC50 of 95 μM (Figure 3.3B, solid lines), and induced cell rounding (Figure 3.3C). In contrast, stem cells were highly resistant to the EGFR inhibitor erlotinib, a widely used drug for lung cancer, and the ERK inhibitor PD98059, which is active against a variety of cancer cell lines (Figure 3.3B, dashed lines). Because the cells rounded and detached, apoptosis is a potential cell death mechanism, but AG205 treatment did not cause changes in markers of apoptosis, including cleavage of PARP and caspase-3 (Figure 3.6). The ability to efflux dyes is a key marker of cancer stem cells, and we found that lung cancer stem cells had the
properties typical of a “side scatter” population (Figure 3.7). However, drug efflux did not change with AG205 treatment.

In some cases, autophagy can be a cell death mechanism that acts as an alternative to apoptosis (147), and PGRMC1 has been implicated in autophagy (138), a metabolic process in which aged proteins and organelles are degraded in the lysosome (148). LC3B is both a key mediator of autophagy and an autophagy substrate that is cleaved and lipidated to the mature LC3B-II form. We detected increased levels of LC3B-II upon PGRMC1 ligand treatment, suggesting that autophagy was induced (Figure 3.3D, top panel). PGRMC1 ligands induce autophagy in lung cancer but also arrest the process, so that autophagy substrates are not degraded. In stem cells, PGRMC1 ligand treatment increased LC3B-II levels but did not decrease levels of the autophagy substrate p62. The autophagy inhibitor chloroquine behaved similarly (data not shown), consistent with an autophagy arrest in cancer stem cells treated with PGRMC1 ligands. However, it is unlikely that autophagy arrest caused cell death, because chloroquine had no effect on cell viability. Notably, AG205 caused an increase in PGRMC1 levels (Figure 3.3D, third panel), suggesting that AG205 alters PGRMC1 stability.

3.4 Discussion

PGRMC1 is a membrane-associated protein implicated in the transport of multiple receptors to the plasma membrane, and multiple studies have proposed PGRMC1 ligands as therapeutics or diagnostic agents for cancer. In the present
study, we demonstrate that PGRMC1 was elevated in a large cohort of lung tumors, which included multiple lung cancer pathologies, and where it was associated with poor survival. To our knowledge, this is also the first report of PGRMC1 expression in the oral cavity, and we detected strong PGRMC1 expression in multiple organs, particularly in the tongue. In some tissues, PGRMC1 was enhanced on the outer surface of the tissue (Figure 3.1C), suggesting a role in secretion or trafficking. Indeed, PGRMC1 is secreted in lung tumors (43).

This is, to our knowledge, the first report of PGRMC1 expression in a relatively large cohort of ovarian cancers. PGRMC1 has been previously shown to play a key role in ovarian tumor growth, apoptosis resistance, invasion, angiogenesis, drug resistance and metastasis (25, 134, 149-152). There are also numerous reports linking PGRMC1 to normal ovarian function, including follicle development (153, 154), and is aberrant in premature ovarian failure (155). Thus, the finding that high PGRMC1-expressing tumors correlate with poor overall survival emphasizes the importance of PGRMC1 as a therapeutic target in ovarian cancer.

The current study is, to our knowledge, the first report of PGRMC1 expression in cancer stem cells. PGRMC1 was detected in stem cells isolated using two different approaches, and the second commercial source was verified by measuring dye exclusion. The ability of the PGRMC1 ligand AG205 to induce rounding and inhibit viability in the stem cells differed markedly from known inhibitors. The mechanism through which PGRMC1 functions in the cells is
enigmatic, because EGFR inhibition had no effect on tumor stem cell growth, and
the cells did not die from apoptosis. We were also unable to detect changes in
signaling (i.e. global tyrosine phosphorylation), protein degradation (ubiquinated
protein levels) and PGRMC1-sensitive metabolism (INSIG-1 and CYP51 levels).
AG205 treatment induced the early event of autophagy, LC3-II accumulation, but
autophagy inhibition with chloroquine did not affect viability, suggesting that other
mechanisms are at work. We are currently investigating candidate mechanisms
in signaling and metabolism.

PGRMC1 was identified as the sigma-2 receptor in 2011, and there is an
ongoing, vigorous debate as to whether the proteins are identical. The sigma-2
receptor (S2R) is a membrane-associated protein that binds a number of
pharmacological compounds, including signature ligands SV-119, siramesine,
SM-21 and PB-28 (156-159), among others, as well as multiple anti-depressants
and stimulants. S2R and PGRMC1 have very similar patterns of being induced in
cancer, and S2R is detectable in stem cells. Xu, et al. identified PGRMC1 based
on its ability to bind and cross-link to the ligand WC-21 (82). The sigma-2
receptor probe RMH-4 was competed by classical S2R ligands, such as DTG,
siramesine, SV119 and WC-26, and equally by the PGRMC1 ligand AG205 (82).
Furthermore, RNAi knockdown of PGRMC1 in HeLa cells decreased binding of
labeled RMH-4, while PGRMC1 over-expression increased binding (82).

In contrast to the reports of the Mach group and co-workers, Abate, et al.
demonstrated that PGRMC1 knockdown in MCF-7 breast cancer cells did not
affect DTG (1,3-di-o-tolyguanidine) binding in membrane fractions (160). At the
moment, it is difficult to reconcile these findings, except that they were performed with different cell lines and ligands. In addition, Chu, et al. recently showed that PGRMC1 knockout in NSC34 cells did not affect binding of DTG or photolabeling of the 18 kDa “S2R” with [125I]-lodo-azido-fenropriomorph. Based on these findings, the authors stated in the title of the paper that “The sigma-2 receptor and the progesterone receptor membrane component 1 are different binding sites derived from different genes”. There are some caveats. NSC34 cells are a hybrid of mouse embryonic spinal cord cells and mouse neuroblastoma cells and represent a very specialized example of cell biology, as do HeLa and PC12 cells.

As a compromise, it could be argued that the sigma-2 receptor is a separate entity from PGRMC1, but S2R-ligand binding requires PGRMC1, perhaps due to a direct interaction between S2R and PGRMC1. However, this is not consistent with the ability of IAF to photolabel S2R equally in control and PGRMC1-knockout cells. Another possibility is that the original probe, WC-21, has non-S2R binding activity, including binding to a PGRMC1 complex. However, the ability of multiple well characterized S2R ligands to efficiently compete for WC-21 binding argues against this possibility (82). Clearly, there are many questions to be addressed in this field, including the identity of the 18 kDa “S2R” protein, and we have used the nomenclature “PGRMC1” in this study until the situation is resolved.

In summary, the present findings implicate elevated PGRMC1 expression in a broad range of tumor types. Tumors of the upper airways are intriguing for therapeutics, because topical delivery systems can allow greater penetrance of
chemicals and biologicals targeting PGRMC1. In addition, we provide evidence linking PGRMC1 expression to cancer stem cells, which are notorious for their resistance to therapeutics, and the cancer stem cells in this study were highly resistant to powerful agents such as erlotinib and PD98059. In contrast, PGRMC1 inhibitors had activity against cancer stem cells, suggesting a role for PGRMC1 in maintaining cancer stem cell viability. In contrast, PGRMC1 inhibitors had activity against cancer stem cells, suggesting a role for PGRMC1 in cancer stem cell maintenance and the importance for inhibitors such as AG205 for future therapeutics targeting cancer stem cells.

3.5 Conclusion

PGRMC1 is broadly expressed in a variety of tumors, where its expression is elevated in comparison to corresponding normal tissues. In some diseases, PGRMC1 expression correlates with poor patient survival, while in breast cancer, the correlation between PGRMC1 and survival is more complex and may depend on the patient population or epitopes being analyzed. PGRMC1 was expressed in two different patient-derived tumor stem cell populations and was required for viability in those cells. The results support PGRMC1 as a tumor biomarker and therapeutic target for multiple types of cancer.
Figure 3.1 Immunohistochemistry of PGRMC1 in multiple tissues.

Immunohistochemistry of (A) a lung tumor section using the anti-PGRMC1 antibody 12990 from Proteintech Group, Inc. (B) The same section stained with the same antibody plus recombinant PGRMC1 fusion protein. (C-H) PGRMC1 staining in multiple tissues, as indicated in the figure.
Figure 3.2 PGRMC1 correlates with patient survival. (A) Kaplan-Meier plot in samples from patients with lung cancer, comparing high PGRMC1 staining tumors (solid line) with low PGRMC1 staining tumors (dashed line). (B) A similar analysis to panel a, for ovarian cancers.
Figure 3.3 Treatment with AG205 reduces stem cell viability. (Figure legend on following page)
Figure 3.3 Treatment with AG205 reduces stem cell viability. (A) Bulk tumor cells and tumor stem cells were isolated from a patient with lung cancer and analyzed by western blot for PGRMC1 (upper panel) and GAPDH (lower panel, loading control). PGRMC1 was abundant in both the bulk tumor cells and the stem cells. (B) Cancer-derived stem cells were treated with various ligands and assayed for viability. Neither the EGFR inhibitor erlotinib nor the ERK inhibitor PD98059 were active against the cells, while the PGRMC1 ligand AG205 inhibited viability of the cells. (C) Bright field imaging of cancer-derived stem cells treated with AG205 revealed cell rounding in the majority of the cells. (D) Western blot analysis of stem cells treated with vehicle (lane 1) or AG205 (lane 2) revealed increased levels of the autophagy initiating protein LC3B-II (top panel, lower band), while the levels of the autophagy substrate p62 were unchanged (second panel). PGRMC1 levels increased following treatment (third panel), and GAPDH served as a loading control.
Figure 3.4 Immunohistochemistry of tumors and tissue from the head and neck region. (A) Squamous cell carcinoma of right submaxilla, stage II. (B) Squamous cell carcinoma of left cheek, stage II. (C) Squamous cell carcinoma with necrosis of right parotid gland, stage II. (D) Mucoepidermoid carcinoma of gingiva, stage I. (E) Squamous cell carcinoma of larynx, stage IV. (F) Normal larynx tissue.
Figure 3.5 Immunohistochemistry of ovarian tumors and tissue. (A) Clear cell carcinoma, stage IIIB. (B) Endometrioid carcinoma, stage IIIB. (C) Papillary serous cystadenocarcinoma, stage IIIB. (D) Papillary serous cystadenocarcinoma, stage IIIC. (E) Serous surface papillary carcinoma. (F) Normal ovary tissue.
Figure 3.6 Treatment with AG205 did not induce apoptosis. Western blot analysis of stem cells treated with vehicle (lane 1) or 10 μM AG205 (lane 2), as described in the methods section. Treatment with PGRMC1 ligand AG205 did not induce cleavage of PARP (top panel) or caspase-3 (middle panel), which are common apoptosis markers. GAPDH (bottom panel) served as a loading control.
Figure 3.7 Measurements of drug efflux in lung cancer stem cells. Drug efflux pattern of lung cancer stem cells follows typical “side scatter” population. Lung cancer-derived stem cells were treated with vehicle control (A) or Hoechst 33342 (B-D). In panel B, there were no further treatments, whereas cells were treated with 50 μM AG205 for 24 hours (C) or 48 hours (D). In triplicate measurements, drug treatment did not affect dye exclusion to a significant extent.
CHAPTER IV: INSULIN RECEPTOR PLASMA MEMBRANE FUNCTION
INCREASED BY THE PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1

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4.1 Introduction

Changes in insulin signaling have been linked to multiple diseases, most typically diabetes (161) but also loss of cognitive function (162) and therapeutic resistance in cancer (163, 164). More recently, a growing body of evidence has demonstrated the importance of IR expression in cancer cell proliferation and tumor development (165). IR is a tetramer comprised of 2 alpha (ligand binding) and 2 beta (kinase domain) chains that is expressed in numerous tissues. The human IR encodes two isoforms, IR-A (lacking exon 11) and IR-B, with the latter having a predominant role in metabolic insulin action (166).

Signaling from the IR through the IRS-1/PI3K/AKT pathway causes a rapid translocation of the GLUT-4 glucose transporter from intracellular vesicles to the plasma membrane (167), increasing the cellular uptake of glucose (168), a step that is crucial to preventing complications of multiple diseases.

IR is actively transported in a cycle of plasma membrane export, activation and internalization (169-172) and disruptions to this cycle are a candidate mechanism for insulin resistance. IR is internalized after ligand binding through clathrin-dependent (173, 174) or caveolin-dependent (175) mechanisms that govern the degradation and recycling of the receptor complex (176, 177). While the signaling pathways downstream of insulin binding are quite well characterized, the proteins driving the plasma membrane localization of IR are less well understood.

PGRMC1 (progesterone receptor membrane component 1 (1) plays an important role in signaling by transporting transmembrane receptors to the
plasma membrane (26, 178), and these receptors include tyrosine kinases (26, 47). PGRMC1 localizes to endosomes and the endoplasmic reticulum (27, 36), as well as the plasma membrane (86), consistent with its highly conserved role in trafficking. In cancer, the best characterized trafficking target for PGRMC1 is the EGFR (epidermal growth factor receptor) tyrosine kinase (20, 23, 45, 46), and PGRMC1 increases plasma membrane levels of EGFR (23), increasing cellular signaling (22, 28, 90). In contrast, the EGFR-related protein HER2/neu was not PGRMC1-dependent (23), suggesting a trafficking specificity.

In addition to tyrosine kinases, PGRMC1 also increases plasma membrane pools of GLP-1R and MPRα1, a plasma membrane progesterone receptor (26, 47). The latter likely contributes to progesterone binding activity by the PGRMC1 complex, which was how PGRMC1 was originally identified (3, 4, 10, 83). Some types of progesterone signaling are dependent on PGRMC1 in diverse regions of the body, including the brain, ovaries and the pancreas (179-182). Binding to the liganded GLP-1R complex likely contributes to glucose homeostasis, as PGRMC1 has been found to modulate glucose-induced insulin stimulation in beta cells (47).

PGRMC1 has numerous other binding partners, including cytochrome P450 proteins (32, 33, 183), PAIR-BP1 (plasminogen activator inhibitor RNA binding protein 1 (182, 184)) and α-tubulin (30). PGRMC1 is attractive as a therapeutic target because it has a small molecule ligand, called AG205 (22) that was identified by our group and has been verified as a PGRMC1-targeting ligand by others (18, 22, 45, 47, 82).
In the present study, we have investigated the role of PGRMC1 in trafficking IR and promoting glucose uptake. There is a precedent for PGRMC1 being associated with insulin signaling, because a clinical study of insulin-resistant, high BMI subjects demonstrated decreased PGRMC1 RNA levels compared to insulin-sensitive subjects (185). However, this study did not provide a direct link between IR and PGRMC1. Based on previous findings by our group that PGRMC1 regulated receptor tyrosine kinase trafficking, we posited that down-regulated PGRMC1 could disrupt normal IR function. In the results, we show that PGRMC1 has a direct role in regulating IR trafficking and glucose transport.

4.2 Methods

4.2.1 Tissue Culture

A549 cells were obtained from ATCC (Manassas, VA) and verified by Genetica LLC (Cincinnati, OH). HUH7 cells were generously provided by Dr. Brett Spear (University of Kentucky College of Medicine). Cells were maintained in DMEM (Corning, Manassas, VA) containing 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO) and antibiotics and were maintained at 37°C in 5% CO2 and air. The A549 derivatives infected with lentiviruses expressing short hairpin RNAs were prepared from the plasmids pGIPZ (control) and V2LHS_90636 (PGRMC1-knockdown) and have been previously described (23).
4.2.2 Reagents and Treatments

AG205 was purchased from Timtec, Inc., (Newark, NJ). Dose response and time courses were performed previously (data not shown) to establish the most effective concentrations and times. A549 and HUH7 cells were treated with AG205 (20 μM) for 90 minutes and underwent protein analysis or cell surface labeling. A549 control and PGRMC1-knockdown were plated on glass bottom microwell dishes (MatTek Corporation, Ashland, MA) for imaging. A549 control and PGRMC1-knockdown were treated with recombinant human insulin, Cy5 labeled (Nanocs Inc., New York, NY) at a concentration of 100 nM for 5 minutes and 15 minutes. Cells were visualized using a Nikon A1R+ resonant scanning confocal system at the University of Kentucky Imaging Facility and analyzed with NIS-Elements C imaging software.

4.2.3 Cell Surface Labeling Assays

For a single experiment, four dishes of 90-95% confluent cells were used. After the removal of media, cells were washed twice with ice-cold phosphate-buffered saline (PBS) (VWR, Radnar, PA) and labeled with sulfo-NHS-SS-biotin (sulfosuccinimidyl-2(biotinamido)-ethyl-1,3-dithipropionate) for 30 minutes at 4°C on a rocking platform. The labeled proteins were purified with avidin agarose using the Cell Surface Protein Isolation Kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. For comparison, the intracellular protein pool that did not bind avidin-agarose was also collected and stored as the “unbound” or “cytoplasmic” fraction. Cell surface labeling reactions were
performed at least in triplicate and fraction levels were confirmed via SDS-PAGE gel separation and staining with Coomassie Blue as previously described (23). Western blots of biotin-labeled eluates and unbound fractions were performed at least in triplicate.

4.2.4 Immunological Techniques

For western blot analysis, cell lysates were prepared by incubating cells in RIPA buffer (50mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) for 10 minutes at 4°C. Lysates were cleared by centrifugation at 14,000 x g for 10 minutes at 4°C, and proteins were separated by gel electrophoresis. The antibodies used in this study were anti-insulin receptor beta (Novus Biologicals, Littleton, CO) anti-insulin receptor β (Cell Signaling, Danvers, MA), PGRMC1 (Abcam, Cambridge, MA), anti-proliferating cell nuclear antigen (PCNA; PC-10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (Santa Cruz), anti-GLUT4 (Santa Cruz), anti-GLUT1 (Santa Cruz). Western Blots for PGRMC1 were performed with the PGR-UK1 polyclonal antibody directed to the sequence QPAASGDSDDDE of the PGRMC1 coding sequence (22). Western blots were performed at least in triplicate. For immunoprecipitations, cells were gently scraped off dishes and lysed in Nonidet P-40 buffer (1% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) for 10 minutes at 4°C. Lysates were cleared by centrifugation at 14,000 x g for 10 minutes at 4°C, and bound to Protein A/G-
agarose beads (Santa Cruz) containing antibody. Nonspecific antibodies matching the host species of the primary antibodies were included as negative controls. The reactions were rotated end over end at 4°C for 1.5 hours, centrifuged to collect precipitates, and washed 3 times in lysis buffer. The beads were resuspended in 1x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and analyzed via western blot.

4.2.5 2-Deoxyglucose Uptake

For radioactive glucose uptake measurements, A549 control and PGRMC1-knockdown cells (5 x 10^5/well) were plated in separate 12-well plates in serum containing medium (DMEM) overnight. Cells were then washed twice in PBS, and incubated in RPMI-1640 media (VWR, Radnor, PA) containing 1% BSA (VWR, Radnor, PA) for 2 hours before glucose uptake studies. Cells were then washed twice with PBS and incubated in 1 ml PBS containing 0.1 mM 2-deoxyglucose and 1 μCi/ml 2-deoxy-D-[3H] glucose (Perkin Elmer, Boston, MA) for 5 minutes at 37°C. Cytochalasin B (20 μM; Sigma Aldrich, St. Louis, MO) was added to the relevant wells with the deoxyglucose mixture to serve as a negative control. Then, cells were washed three times in ice-cold PBS, solubilized in 0.4 ml of 1% SDS for 10 minutes at room temperature and was counted in 4 mL of Biosafe II scintillation fluid (Research Products International Corp., Mount Prospect, IL) for 1 minute on a Beckman LS6500 scintillation counter.
4.2.6 Statistics

Figures show representative blots and each experiment was performed at least three times. Quantitation of bands was performed using Adobe Photoshop software (Adobe Systems, San Jose, CA). Data are expressed as mean ± standard deviation and were analyzed by using Student’s paired, two sided t test to assess significance. All measurements were considered significant if $P \leq 0.05$ (*); $P \leq 0.01$ (**); $P \leq 0.001$ (***)

4.3 Results

4.3.1 PGRMC1-mediates elevation of IR plasma membrane levels

To test the role of PGRMC1 in plasma membrane stability of IR, we compared membrane levels of IR in control and PGRMC1-knockdown A549 human lung cancer cells. Extracellular proteins were biotinylated and purified with avidin column chromatography and then analyzed by western blot using an antibody to the IR β sub-unit. We will refer to IRβ as IR. IR levels decreased in KD cells relative to controls (Figure 4.1A, compare lanes 3 and 4). IR was not detectable in the cytoplasmic fraction (Figure 4.1A, compare lanes 1 and 2), which was diluted relative to the membrane fraction. A coomassie brilliant blue-stained gel of the fractions revealed few changes in band intensity (Figure 4.1B). Verification of this protocol for precipitation of the cytoplasmic and plasma membrane fractions in A549 cells has been previously reported (23). In multiple
experiments, IR plasma membrane levels decreased 2.3-fold in PGRMC1-knockdown cells relative to controls (p<0.005, t-test Figure 4.1C). In contrast, depletion of PGRMC1 did not affect the total protein levels of IR (Figure 4.1D).

In lung cancer cells, PGRMC1 is inhibited by the ligand AG205 (23). To examine pharmacological inhibition of PGRMC1 on IR plasma membrane stability, we treated HUH7 human liver cancer cells, in addition to A549 human lung cancer cells, with AG205. Cell surface proteins were labeled, purified and analyzed by western blot as described above. Plasma membrane levels of IR were profoundly reduced after treatment with AG205 in both cell lines (Figure 4.2A-B, compare lanes 3 and 4). The nuclear protein PCNA (proliferating cellular nuclear antigen) served as a control for intracellular proteins (Figure 4.2A-B, compare lanes 1 and 2) and Coomassie Blue-stained gel of the fractions showed no variability in band intensity (Figure 4.2C-D). In multiple experiments, AG205 treatment decreased plasma membrane IR levels in both A549 and HUH7 cells by 57.5-fold and 6.8-fold respectively (Figure 4.2G-H, p<0.005 and p<0.01 respectively). As before, total cellular protein levels of IR were not affected after treatment with AG205 (Figure 4.2E-F). Together, these results suggest that PGRMC1 inhibition via AG205 treatment also decreases plasma membrane levels of IR. In these experiments, IR was detected in the unlabeled “cytoplasmic” fraction. This differed from the control and PGRMC1-knockdown cells in Figure 4.1. We speculate that the infection and selection process of the cells in Figure 4.1 may have increased the fractions of IR present at the plasma membrane.
4.3.2 IR and PGRMC1 co-precipitate

PGRMC1 interacts directly with the EGFR receptor tyrosine kinase and the [8 membrane spanning] receptor [MAPR1a], suggesting that the regulation of IR by PGRMC1 could be direct. Both PGRMC1 and IR were immuno-precipitated from A549 lung cancer cells and HUH7 liver cancer cells and analyzed by western blot. IR was efficiently precipitated (Figure 4.3A, upper panel), and PGRMC1 co-precipitated with IR (Figure 4.3A, lower panel) in both cell lines (lanes 2 and 4). Similarly, PGRMC1 was efficiently precipitated (Figure 4.3B, upper panel), and IR co-precipitated with PGRMC1 (Figure 4.3B, lower panel) in both cell lines (lanes 2 and 4). The same lysates were precipitated with a host specific antibody that matched to the antibodies for IR and PGRMC1 (Figure 4.3A-B, lanes 1 and 3). As an additional control, IR and PGRMC1 were precipitated from A549 control and PGRMC1-knockdown cells, and PGRMC1 was only detected in control cells (Figure 4.3C, lane 2).

4.3.3 Cellular binding of insulin dependent on PGRMC1

Insulin is the most potent physiological agent known, and insulin’s biological actions are direct results of the interaction with IR (186). To determine the effect of PGRMC1 on insulin binding, A549 control and PGRMC1-knockdown cells were incubated with insulin labeled with the fluorophore cyanine-5 (we will refer to this as “Cy5-insulin”). Cy5-insulin bound readily to control A549 cells (Figure 4.4C), while binding was largely undetectable in PGRMC1-knockdown
cells (Figure 4.4D). In the absence of Cy5-insulin, no fluorescence was observed (Figure 4.4A-B). To better understand the pharmacodynamics of insulin binding, A549 control and PGRMC1-knockdown cells were incubated with Cy5-labeled insulin for a longer period of time (15 minutes) and real-time data was collected via video recording on a Nikon A1R+ confocal microscope (video not attached). At the end of the time-lapse, insulin binding was 10-fold higher in control cells compared to PGRMC1-knockdown cells (Figure 4.4E). Thus, the results support the model that PGRMC1 mediates the interaction of IR with its ligand, insulin.

4.3.4 PGRMC1 mediates Glucose Transporter Plasma Membrane Levels and facilitates Glucose Uptake

IR stimulation causes the glucose transporter GLUT-4 to translocate from intracellular vesicles to the plasma membrane (167, 187). Because PGRMC1 elevated IR plasma membrane levels, we posited that it would also increase plasma membrane GLUT-4. Indeed, plasma membrane GLUT-4 levels declined in PGRMC1-knockdown cells (Figure 4.5A, upper panel). Interestingly, the levels of the constitutive glucose transporter, GLUT-1, also declined in PGRMC1-knockdown cells, although to a lesser extent (Figure 4.5A, lower panel). In triplicate experiments, the decreases in GLUT-4 and GLUT-1 in A549/RNAi cells were 2.6-fold (p<0.01) and 1.5-fold (p=0.02), respectively (Figure 4.5B). The total protein levels of GLUT-4 and GLUT-1 did not change between control and PGRMC1-knockdown cells (Figure 4.5C).
Because PGRMC1 increased the levels of glucose transporters at the plasma membrane, we tested whether glucose transport was also affected. A549 control and PGRMC1-knockdown cells were incubated with 3H-glucose and washed extensively. PGRMC1-knockdown cells exhibited a 1.5-fold decrease in basal uptake of 3H-glucose compared to control cells (Figure 4.5D, p=0.0007, t-test). The actin polymerization inhibitor cytochalasin B, which arrests the transport of glucose transporters to the plasma membrane, served as a negative control.

4.4 Discussion

IR plays a crucial role in metabolism and performs key functions in the muscle, fat and brain (188). IR is also over-expressed in cancer (189), where it has a ligand-dependent transforming activity in fibroblasts (190). Cancer cells typically express the IR-A form (191), which is also expressed in embryonic tissues and in the brain, and differs from the IR-B isoform, which has an additional 12 amino acids due to a splice variant in exon 11 (192, 193). IR-A acts as a receptor for IGF-II, which is induced in cancer, with equal affinity to IGF-1R (191), suggesting that IR directs tumor-specific signaling that promotes metabolism.

The current work demonstrates a key role for PGRMC1 in maintaining IR at the plasma membrane. We primarily used A549 cells because we have a verified and well characterized RNAi-mediated knockdown model system in A549 cells (22, 23, 46). A549 cells express IR, and IR increases proliferation and
therapeutic resistance in this non-small cell lung cancer cell line (163, 194, 195). Our group previously showed that PGRMC1 is essential for trafficking the EGFR receptor tyrosine kinase in cancer cells and is associated with EGFR (23). This finding was elegantly extended by Kabe, et al., who showed that PGRMC1 forms heme-dependent dimers that associate with EGFR, driving downstream signaling, cell transformation and tumor metastasis (20). We report here that PGRMC1 also associates with IR and increases IR plasma membrane levels. Future research will determine whether PGRMC1 complexes with IR are also heme-dependent.

PGRMC1 elevates the plasma membrane levels of two glucose transporters and increases glucose transport (Figure 4.6). The most likely mechanism of activated GLUT-4 plasma membrane levels is increased signaling through IR, while extracellular GLUT-1 levels were elevated by PGRMC1 to a lesser extent. Mammalian glucose transport is mediated by a family of membrane glycoproteins, including GLUT-1 and GLUT-4 (196). The GLUT-1 isoform is ubiquitously expressed and facilitates basal glucose uptake and transport across blood tissue barriers, while the GLUT-4 isoform is predominately found in the muscle, fat and heart tissues and mediates the rate-limiting step in regulated transport in these tissues (197). There is evidence supporting an interaction between GLUT-1 and GLUT-4 in insulin sensitive tissues, where GLUT-1 is localized to the plasma membrane and GLUT-4 is distributed in intracellular compartments until insulin signaling initiates the translocation of GLUT-4 to the plasma membrane (198). The concentration of GLUT-4 at the cell surface and
duration for which the protein is maintained at the surface governs the rate of
glucose transport into fat and muscle cells (199). We note that EGFR has been
reported to associate with the sodium/glucose co-transporter SGLT-1 in a kinase-
independent manner (200), and glucose transport could be affected via PGRMC1
through this interaction. There is also evidence for cross-talk between IR and
EGFR in the A549 cell line (163). Finally, we cannot exclude the possibility that
PGRMC1 interacts directly with the glucose transporters, particularly GLUT-1.

The findings are consistent with our earlier observation that PGRMC1
decreases AMPK threonine 174 phosphorylation (138), an activating
phosphorylation event. AMPK activation increases glucose transport and
decreases competing pathways in energy metabolism (201, 202). Thus, AMPK
activation in PGRMC1-depleted cells may be a consequence of the depleted
 glucose transport detected in the present study. However, AMPK is also capable
of directing GLUT-4 trafficking to the plasma membrane (202), suggesting that, in
addition to stabilizing IR plasma membrane pools, PGRMC1 may also play a
more direct role in regulating glucose transporters.

The pathways controlling IR plasma membrane trafficking are less well
characterized than some other receptors, such as EGFR. However, key proteins
include LMBD1/limb region domain containing 1 (203), PKCε/protein kinase Cε
(204) and the adaptor protein GRB10/growth factor receptor bound protein 10
(177, 205). IR is ubiquitinated and associates with the ubiquitin ligase NEDD4
via GRB10 and with the E3 ubiquitin ligase mitsugumin 53 (206) and
APS/adaptor protein with pleckstrin homology and Src homology 2
domains/SH2B adaptor protein 2 (207). This group of proteins affect the internalization and degradation of IR, likely with variations between the two IR isoforms, in different tissues and with different types of ligand stimulation (177). An important future direction of the research will be to identify PGRMC1-interacting partners in regulating IR trafficking.

In these experiments, the PGRMC1 ligand AG205 decreased the levels of IR at the plasma membrane without affecting overall IR protein concentrations. AG205 appears to act as an inhibitor of PGRMC1 in cancer cell lines, because AG205 and PGRMC1 inhibition by RNA interference had a similar effect on proliferation (22), NGAL/neutrophil gelatinase-associated lipocalin secretion (46) and autophagy induction (138). AG205 acts to a significant extent through PGRMC1, because PGRMC1-knockdown cells fail to arrest in response to AG205 (22). The current findings indicate that AG205 treatment causes distinct effects on protein stability in cancer cells, because EGFR levels were decreased at the doses used here (23), while IR was unaffected. The underlying mechanism through which AG205 and PGRMC1 affect protein stability is unclear.

These results presented here indicate a novel mechanism through which IR is trafficked by PGRMC1 (Figure 4.6). Several studies have shown that the IR pathway is directly involved in cancer progression and development, suggesting that this is an important new target for cancer prevention and therapy. We have found that PGRMC1 directly associates with IR, maintains IR at the plasma membrane and increases insulin binding and glucose uptake in cancer cells.
(Figure 4.6). Both genetic manipulation and the PGRMC1 inhibitor AG205 altered IR at the plasma membrane in cell lines from different tissue types. A future direction of the research will be to determine the systemic activity of AG205 and other potential PGRMC1 ligands. In prior in vivo experiments in our laboratory, effects on glucose levels were not determined and studies of efficacy were limited by the quantity of the drug that could be obtained. However, the present studies suggest that PGRMC1 may be an important metabolic regulator with the potential to target signaling in cancer.
Figure 4.1 PGRMC1 increases IR plasma membrane levels. (A) Western blot analysis of plasma membrane (PM) protein levels from control (“con”, lanes 1-2) and PGRMC1-knockdown cells (“KD”, lanes 3-4) labeled with biotin and purified by avidin-agarose. Proteins that were not detected at the plasma membrane are indicative of cytoplasmic (cyto) proteins (lanes 1-2). The experiment was performed five separate times. (B) Coomassie-stained gel of the samples represent total protein levels. (C) Graphical representation of IR plasma membrane levels reduced in knockdown (KD) vs control (con) cells (p<0.001). (D) Western blot example of total IR protein levels in control and knockdown cells. ***, p<0.001, compared with the vehicle-treated group.
Figure 4.2 Treatment with PGRMC1 ligand AG205 decreases IR plasma membrane levels. (Figure legend on following page)
Figure 4.2 Treatment with PGRMC1 ligand AG205 decreases IR plasma membrane levels. Western blot analysis of plasma membrane (PM) protein levels in A549 (A) and HUH7 (B) cell lines from control (lanes 1-2) and AG205 (20 μM) treated (lanes 3-4 respectively) labeled with biotin and purified by avidin agarose. Proteins that were not detected at the plasma membrane are indicative of cytoplasmic (cyto) proteins (lanes 1-2). PCNA serves as a control for intracellular proteins. Coomassie Blue-stained gel represents total protein levels in A549 (C) and HUH7 (D) cell lines respectively. (E-F) Western blot analysis of total IR protein levels in A549 cells and HUH7 cells -/+ AG205 treatment. IR plasma membrane levels were significantly reduced after treatment with AG205 in both A549 (G) and HUH7 (H) cells (p<0.001 and p<0.01 respectively). **, p<0.01; ***, p<0.001 compared with the vehicle-treated group.
Figure 4.3 IR co-precipitates with PGRMC1. (A) IR and (B) PGRMC1 were immunoprecipitated from A549 cells (lanes 1 and 2) and HUH7 cells (lanes 3 and 4) and probed (western blot analysis) for IR (top) or PGRMC1 (bottom). (C) IR (top panel) and PGMC1 (bottom panel) were immunoprecipitated from control (lane 1) or PGRMC1-knockdown (“KD”, lane 2) cells. Immunoprecipitation reactions were probed for PGRMC1 (top and bottom panels).
Figure 4.4 Cellular binding of insulin is dependent on PGRMC1. (Figure legend on following page)
Figure 4.4 Cellular binding of insulin dependent on PGRMC1. To visualize insulin binding, control ("con") and PGRMC1-knockdown ("KD") A549 cells were incubated with Cy5-labeled insulin (100 nM) and imaged. (A-B) Control and PGRMC1-knockdown cells before insulin treatment ("untr") and (C-D) 5 minutes after the addition of Cy5-labeled insulin. Fluorescence revealed a reduction of insulin binding in PGRMC1-knockdown cells. Images are representative of experiments performed in triplicate. (E) In a separate experiment, control and PGRMC1-knockdown cells were incubated with Cy5-labeled insulin (100 nM) for 15 minutes. The insulin binding was recorded live (videos in supplemental figure 1) and the NIS-Elements C imaging software data was exported to excel. Insulin binding was significantly lower in PGRMC1-knockdown cells (p<0.001, t-test).
**Figure 4.5 PGRMC1 increases glucose uptake.**

(A) Western blot analysis of plasma membrane (PM) protein levels from control (“con”) and PGRMC1-knockdown cells (“KD”, lanes 2 and 4) labeled with biotin and purified by avidin agarose.  

(B) Graphical representation of reduced GLUT-4 and GLUT-1 levels at the plasma membrane in PGRMC1-knockdown cells (p<0.01 and p<0.05).  

(C) Western blot analysis of total GLUT-1 and GLUT-4 levels did not change in control vs knockdown cells.  

(D) Radioactive glucose uptake assay in control and
PGRMC1-knockdown cells. Knockdown of PGRMC1 significantly reduced the uptake of 3H-glucose in A549 cells (p<0.001). Cytochalasin B served as a negative control (inhibits glucose transport). *, p<0.05; **, p<0.01; ***, p<0.001 compared with the vehicle-treated group.
Figure 4.6 PGRMC1 trafficks IR to the plasma membrane and promotes glucose entry. Schematic representation for the model in which PGRMC1 associates with IR to increase IR levels at the plasma membrane. IR at the plasma membrane is capable of initiating the signaling cascade to translocate glucose transporters from GLUT containing vesicles to the plasma membrane for glucose entry. Red arrows represent events mediated by PGRMC1.
CHAPTER V: A NOVEL REGULATION OF INSULIN RECEPTOR LEVELS IN HUMAN AND RODENT ADIPOCYTES

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5.1 Introduction

Rates of diabetes are expected to increase due to rampant obesity and will become a worldwide health crisis in the future (208). The manifestations include hypertension, hyperlipidemia, hyperglucocorticoidemia and type 2 diabetes. The latter is associated with insulin resistance, a heterogeneous disease characterized by defects in insulin signaling (209). The insulin receptor (IR) is a receptor tyrosine kinase that initiates the process, and IR signaling is coordinated through multiple cascades (210). Binding of insulin to its receptor (IR) triggers downstream signaling that includes IRS1/2 (insulin receptor substrate), AKT and ERK (174). IR is a tetramer comprised of 2 alpha (ligand binding) and 2 beta (kinase domain) chains that is expressed in numerous tissues. Signaling from the IR through the IRS-1/PI3K/AKT pathway results in a rapid translocation of the GLUT-4 glucose transporter from intracellular vesicles to the plasma membrane, increasing the cellular uptake of glucose (175), a step that is critical in preventing diabetes complications.

Adipose tissue performs a key function in glucose homeostasis, because it acts as a reservoir for circulating glucose, converting the sugar into lipid for storage (211). When there is an excess of adipose tissue, such as in high BMI individuals, the glucose catabolism is disrupted, and diminished insulin receptor function is thought to contribute (212). Indeed, obese individuals show decreased insulin binding in skeletal muscle, adipose tissue and liver (50). Mouse models of skeletal-muscle specific vs fat-specific IR-knockout (MIRKO and FIRKO accordingly) suggest that glucose uptake by muscle is more
significant for diabetes, which was anticipated since the muscle is believed to account for 80% of glucose disposal (210, 213). However, other studies suggest that adipose is a critical site for glucose metabolism and the development of obesity and its associated abnormalities (214).

PGRMC1 (progesterone receptor membrane component 1) plays an important role in signaling by transporting transmembrane receptors to the plasma membrane, including receptor tyrosine kinases (1, 26, 47, 178). PGRMC1 localizes to endosomes, the endoplasmic reticulum and the plasma membrane, the latter being consistent with its highly conserved role in trafficking (23, 27, 86). In cancer, PGRMC1 associates with the EGFR (epidermal growth factor receptor) tyrosine kinase (20, 23, 45, 46), increases plasma levels of EGFR, and increases cellular signaling (22, 23, 28, 90). In contrast, the EGFR-related protein HER2/neu was not PGRMC1-dependent (23), suggesting a trafficking specificity.

In addition to tyrosine kinases, PGRMC1 also increases plasma membrane pools of GLP-1R and the plasma membrane progesterone receptor, MPRα1 (26, 47). The latter likely contributes to progesterone binding activity by the PGRMC1 complex, which was how PGRMC1 was originally identified (3, 4, 10, 83). Binding to the liganded GLP-1R complex likely contributes to glucose homeostasis, as PGRMC1 has been found to modulate glucose-induced insulin stimulation in beta cells (47). PGRMC1 has numerous other binding partners, including cytochrome P450 proteins, PAIR-BP1 (plasminogen activator inhibitor RNA binding protein 1) and α-tubulin (30). PGRMC1 is an appealing therapeutic
target because it has a small molecule ligand, called AG205 (22), that was identified by our group and has been verified by others (22, 82). Based on photoaffinity cross-linking studies with a labelled sigma-2 receptor ligand called WC-21, PGRMC1 was identified as the sigma-2 receptor (82). Sigma receptors are low molecular weight receptors for multiple endogenous and exogenous ligands (96). It is notable that PGRMC1 was originally thought to be a sigma receptor (83).

In the present study, we have investigated the role of PGRMC1 in adipose specific IR regulation. There is a precedent for PGRMC1 being associated with insulin signaling, because a clinical study of insulin-resistant, high BMI subjects demonstrated decreased PGRMC1 RNA levels compared to insulin-sensitive subjects (185). However, this study did not provide a direct link between IR and PGRMC1. Based on previous findings by our group that PGRMC1 regulated receptor tyrosine kinase trafficking via direct binding, we posited that PGRMC1 binds to IR. Furthermore, in adipose tissue from high BMI patients, a small molecule PGRMC1-directed therapeutic increased levels of IR. This is the first report of increased IR protein levels in high BMI patients through pharmacological intervention. The findings suggest that PGRMC1 is important for signaling though the IR and may play an important role in diabetes.
5.2 Material and methods

5.2.1 Human samples

Human tissue samples were from the lab of Dr. Phil Kern. Biopsies from abdominal subcutaneous adipose tissue were obtained from nondiabetic subjects that were deidentified and only paired with their respective BMI. Tissue samples were immediately placed on ice and stored at -80°C until analysis. After thawing, tissue samples were lysed in 1mL cold RIPA buffer (50mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) via pipetting and sonication (30 seconds). After a 5 minute incubation at 4°C, samples were cleared at 1,000 x g. The infranatant was collected and cleared at 1,000 x g for an additional 5 minutes at 4°C. Protein quantification was performed using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA).

5.2.2 Cell Culture

Fully differentiated human subcutaneous adipocytes from multiple donors were purchased from Zenbio, Inc., (Research Triangle Park, NC) and maintained in proprietary media according to the manufacturer's instructions. Lot#SL0055 contained differentiated human subcutaneous adipocytes derived from 10 female donors with a median age of 46.7 and median BMI of 26.8. Lot #L100610B contained differentiated human subcutaneous adipocytes from a female
Caucasian with a BMI of 23.3 and age of 40. Lot#L072709 contained differentiated human subcutaneous adipocytes from a female of unknown ethnicity with a BMI of 38.0 and age of 39. All donors were listed as non-smokers.

3T3-L1 cells were obtained from ATCC (Manassas, VA), verified by Genetica LLC (Cincinnati, OH) and cultured under the suggested conditions according to ATCC. 3T3-L1 cells were maintained in media purchased from Zenbio, Inc., (Research Triangle Park, NC) and differentiated according to manufacturer's protocol. Medium included: Preadipocyte Medium (cat# PM-1-L1), Differentiation Medium (cat# DM-2-1) and Adipocyte Medium (cat# AM-1-L1).

The collection of stromal vascular fraction was obtained from the visceral fat of male, Sprague-Dawley rats (Taconic Biosciences, Hudson, NY) after CO2 asphyxiation. Harvested fat was washed in ice-cold HBSS (VWR, Radnar, PA), finely minced, and digested in 1 mg/ml type II collagenase solution prepared in HBSS (Sigma Aldrich, St. Louis, MO) for 45 min at 37°C with vigorous shaking. The mixture was then filtered through 250 μM gauze mesh into a 50 mL tube and let stand 2-3 minutes. Infranatant containing the collagenase solution was removed, and the floating layer of adipocytes were washed 3x with 10 mL phosphate-buffered saline (VWR, Radnar, PA). The adipocytes were incubated with collagenase II solution (2mL), vortexed and left shaking for 10 minutes at 37°C. After incubation, 2 mL of high-glucose DMEM (Corning, Manassas, VA) containing 10% BCS (Sigma Aldrich, St. Louis, MO) was added, mixed and the
solution was filtered through 70 μM nylon mesh cell strainer (VWR, Radnor, PA) and plated for cell culture. After cells were approximately 100% confluent, cells were differentiated according to the Zenbio, Inc., manufacturer’s protocol (Research Triangle Park, NC).

5.2.3 Reagents and Treatments

AG205 was purchased from Timtec, Inc., (Newark, NJ) and PB28 was purchased from Sigma Aldrich (St. Louis, MO). Differentiated human subcutaneous adipocytes were treated with the PGRMC1 ligands, AG205 (10μM) and PB28 (1μM), for 24 hours and analyzed via western blot. Dose response and time courses were performed previously (data not shown) to establish the most effective concentrations and times. Differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells were incubated with AG205 (20uM) for 90 minutes and underwent protein analysis.

5.2.4 Immunological Techniques

For western blot analysis, cell lysates were prepared by incubating cells in RIPA buffer (50mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) for 10 minutes at 4°C. Lysates were cleared by centrifugation at 14,000 x g for 10 minutes at 4°C, and proteins were separated by gel electrophoresis. The antibodies used in this study were anti-insulin receptor beta (Novus Biologicals, Littleton, CO) anti-insulin receptor β (Cell
Signaling, Danvers, MA), PGRMC1 (Abcam, Cambridge, MA), and anti-GAPDH (Santa Cruz). Western Blots for PGRMC1 were performed with the PGR-UK1 polyclonal anti-body directed to the sequence QPAASGDSDDDE of the PGRMC1 coding sequence (22). Western blots were performed at least in triplicate.

For immunoprecipitations, cells were gently scraped off dishes and lysed in Nonidet P-40 buffer (1% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) for 10 minutes at 4°C. Lysates were cleared by centrifugation at 14,000 x g for 10 minutes at 4°C, and bound to Protein A/G-agarose beads (Santa Cruz) containing antibody. Nonspecific antibodies matching the host species of the primary antibodies were included as negative controls. The reactions were rotated end over end at 4°C for 1.5 hours, centrifuged to collect precipitates, and washed 3 times in lysis buffer. The beads were resuspended in 1x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and analyzed via western blot. The antibodies used in this study were anti-insulin receptor (Novus Biologicals, Littleton, CO), anti-insulin receptor β (Cell Signaling, Danvers, MA), PGRMC1 (Abcam, Cambridge, MA), anti-GAPDH (Santa Cruz).
5.3 Results

5.3.1 IR regulation in human differentiated adipocytes

The down-regulation of PGRMC1 in insulin-resistant diabetes suggest a role for PGRMC1 in regulating IR in adipocytes. To test this model, human tissue-derived adipocytes were treated with PGRMC1 ligand AG205 (22). PGRMC1 has been identified as the sigma-2 receptor in binding studies (82), so we also tested the activity of the sigma-2 receptor ligand PB28 (215). The cellular morphology of the adipocytes was unchanged upon treatment with either ligand. AG205 significantly increased IRβ protein levels in adipocytes derived from pooled BMI (body-mass index, median 26.8, range of [25.3-28.6]) donors (Figure 5.1A-B, p=0.004, t-test) and the high BMI donor (BMI 38, p=0.013, t-test). Treatment with PB28 increased IRβ protein levels in adipocytes derived from both pooled BMI donors and the high BMI donor, but only adipocytes derived from pooled BMI donors achieved statistical significance (Figure 5.1A-B, p=0.027, t-test). There was no significant change in IRβ protein levels after ligand treatments in adipocytes derived from the low BMI 23 donor. Protein analysis was performed by western blot using an antibody to the IR β sub-unit. Total basal IRβ protein levels were lower in adipocytes derived from pooled BMI donors and the high BMI donor (Figure 5.1A-B, p=0.001 and 0.006, respectively, t-test), indicative of reduced insulin sensitivity. In adipose tissue from subjects with varying BMI, both IR and PGRMC1 protein levels decreased in subjects with high BMI (Figure 5.2), consistent with RNA levels detected by microarray.
5.3.2 IR regulation in rodent model systems

Although human tissue-derived adipocytes are a highly accurate model system for studying human metabolic processes, they vary between individuals and populations. In order to further characterize the interaction of PGRMC1 with IR, we utilized two rodent model systems—the 3T3-L1 murine cell line and cells isolated from stromal vascular fractions derived from the subcutaneous adipose of Sprague Dawley rats. To determine the effect of the PGRMC1 ligands on IR levels in these rodent model systems, we treated differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells with AG205. Interestingly, the drug treatment significantly reduced total IR protein levels in both rodent model systems (Figure 5.3A-B, lanes 2. p<0.05 and p<0.01 respectively). These results were opposite of the pharmacological effects seen in human adipocytes.

Next, we investigated the potential interaction of PGRMC1 with IR. PGRMC1 interacts directly with the EGFR receptor tyrosine kinase and the [8 membrane spanning] receptor mPRα. Furthermore, we have detected a direct interaction between PGRMC1 and IR in human cancer cells (data in press). Both PGRMC1 and IR were immuno-precipitated from differentiated 3T3-L1 cells and analyzed by western blot. IRβ was efficiently precipitated (Figure 5.3C, upper panel lane 2), and PGRMC1 co-precipitated with IRβ (Figure 5.3C, lower panel lane 2). Similarly, PGRMC1 was efficiently precipitated (Figure 5.3D, upper panel lane 2), and IRβ co-precipitated with PGRMC1 (Figure 5.3D, lower panel lane 2). The same lysates were precipitated with a host specific antibody that matched to
the antibodies for IRβ and PGRMC1 (Figure 5.3C-D, lanes 1). These results suggest a direct interaction of PGRMC1 with IR.

5.4 Discussion

IR signaling is key in the progression of metabolic disease, and we have shown that IR levels are elevated in adipocytes treated with two different ligands for the PGRMC1 protein. This stands in stark contrast to the behavior of the PGRMC1 ligand AG205 in cancer cells, where it has no effect on overall IR levels (Hampton and Craven, in review) and decreases EGFR levels (23). Despite multiple sources confirming the identity between PGRMC1 and sigma-2 receptor (82, 216, 217), the co-identity of the two proteins is controversial (160, 218, 219). In a set of pooled adipocytes from 10 donors, AG205 and PB28 behaved similarly, but the trend of IR elevation by PB28 in a high BMI donor did not achieve significance.

Notably, the finding that AG205 increased IR levels in human adipocytes was not extended to two different widely used model systems for obesity- mouse 3T3-L1 cells and adipocytes from Sprague-Dawley rats. The results suggest an intriguing species-specific function for PGRMC1 in fat. Nagy and colleagues recently reviewed the physiological and metabolic profiles of rodent white adipose fat pads with white adipose fat depots in humans and urged researchers to carefully consider experimental designs given some of the stark differences in adipose tissue location and function among species (220). Furthermore, Barnard and colleagues argued dietary modification in rodent models has limited
translatable benefit for understanding the pathogenesis of human obesity and diabetes based on the constraints of HFD (high-fat diet) (221).

Finally, Elbein et al, showed that PGRMC1 RNA levels decrease in adipose tissue from insulin-resistant subjects compared to insulin-sensitive subjects (185). Our findings extend the observation in a small cohort of adipose tissues, revealing diminished PGRMC1 protein levels in two high BMI subjects. Thus, there is potentially a strong translational impact because the research will develop new small molecule therapeutics that will potentially enhance the treatment of insulin-sensitive diabetes and reverse the course of insulin-resistant diabetes. The overall impact of the study could be profound. With millions of people worldwide expecting to develop diabetes in the future, PGRMC1-directed therapeutics could become an important approach to treating the disease. There are a number of diabetes treatments currently available, including drugs that lower blood sugar (222), activate IR and alter the production of insulin. Because of the long-term course of the disease and the vast numbers of people becoming diabetic, however, new therapeutics are needed for diabetes.
Figure 5.1 PGRMC1 ligand treatment in human adipocytes. Insulin Receptor β (IRβ) levels increase significantly after 24 hour treatment with 10μM AG205 and 1μM PB28 in human subcutaneous cultured adipocytes. (A) Western blot analysis of human subcutaneous adipocytes derived from 10 separate donors. GAPDH served as a loading control. (B) IRβ protein levels were determined by western blot in fully differentiated human adipocytes sourced from multiple donors with a median BMI of 26.8 (Pooled), a donor with a 23 BMI (Low BMI),
and donor with a 38 BMI (High BMI). IRβ protein levels were significantly elevated after treatment with AG205 and PB28 (p=0.004 and p=0.027 respectively) in pooled BMI adipocytes. Treatment with AG205 significantly increased IRβ levels in adipocytes derived from a high BMI donor (p=0.013).
Figure 5.2 PGRMC1 decreases with glucose insensitivity in human adipose tissue. Adipose tissue samples biopsied from human patients matched with BMI. Protein analysis via western blot show a reduction of both PGRMC1 and IRβ levels with increasing BMI.
Figure 5.3 PGRMC1 ligand treatment and co-immunoprecipitation with IR in rodent model systems. Western blot analysis of total IRβ protein levels in differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells after AG205 (20μM) treatment. Total IRβ protein levels were significantly reduced after treatment with AG205 (lane 2) in both differentiated 3T3-L1 (A) and rat SVF-isolated (B) cells (p<0.05 and p<0.01 respectively). (C) IRβ and (D) PGRMC1 were immunoprecipitated (IP) from differentiated 3T3-L1 cells. Immunoprecipitation with an irrelevant antibody (con Ab, lane 1) is a control. (C) IRβ was immunoprecipitated (lane 2) and probed (western-blot) for IRβ (top panel) and PGRMC1 (bottom panel). (D) PGRMC1 was immunoprecipitated (lane 2) and probed for IRβ (top panel) and PGRMC1 (bottom panel).
CHAPTER VI: DISCUSSION
6.1 Summary of findings

Cancer is one of the leading causes of death, and the number of worldwide cases are expected to increase approximately 50% over the next 20 years, with the numbers rising from 14 million cases in 2012 to 21 million cases in 2030 (World Health Organization). PGRMC1 is a cytochrome bs related protein that is induced in a number of cancer types and is involved in tumor formation, invasion and metastasis. Prior research in the field has primarily focused on the role of PGRMC1 in breast, ovarian and lung cancer, and have correlated PGRMC1 expression with tumor progression and stage. Typical analyses include the use of cancer cell lines and clinical tumor samples. Certainly, such studies provide valuable information, however, there remains a need in the field to incorporate additional clinical samples, not limited to tumor samples.

Herein, we have analyzed PGRMC1 levels in over 600 tumor sections, including a larger cohort of lung tumors than in previous studies, and we report the first clinical analysis of PGRMC1 levels in human oral cavity and ovarian tumors compared to nonmalignant tissues. In lung and ovarian cancers, PGRMC1 was highly elevated and correlated with patient survival. In addition to clinical tumor samples, we report the first implication for PGRMC1 in cancer stem cells. PGRMC1 was detected in lung-tumor derived stem cells isolated from in the Appalachian population. More importantly, we report a essential role for PGRMC1 in cancer stem cell viability. Drug treatment with the PGRMC1 small
molecule, AG205, triggered stem cell death, while a chemotherapeutic and ERK inhibitor, erlotinib and PD98059, did not.

There are a number of potential mechanisms in which PGRMC1 might promote tumor growth, including its association with the epidermal growth factor receptor (EGFR). EGFR is over-expressed in a large number of tumors and is activated through changes in expression or mutations. Multiple drugs have been developed to inhibit EGFR, including chimeric antibodies targeting the extracellular domain and small molecule kinase inhibitors. The active kinase domain inhibitors are particularly active against mutant forms of the receptor, and subsequent mutations drive resistance to the inhibitors. This dissertation includes a review focusing on the trafficking of wild-type and mutant EGFR, and in doing so, found PGRMC1 inhibitors to be most active against the wild-type form in EGFR-dependent cancer cell proliferation.

Although the number of cancer cases can be appreciated, the number of diabetes cases worldwide overwhelmingly surpass cancer. In 2014, the World Health Organization reported 422 million people in the world living with diabetes. The widespread epidemic of obesity and type 2 diabetes has raised great concern for the impact of these disorders in cancer development, with a major concern being circulating insulin (223). Multiple studies have shown that the insulin receptor (IR) pathway is directly involved in cancer development and progression, and insulin is suggested to affect cancer growth (164). Like EGFR, IR is a member of the receptor tyrosine kinase family.
Analogous to our extensive data on the regulation of EGFR trafficking, we demonstrate a role for PGRMC1 in the trafficking of IR. PGRMC1 co-precipitates with IR and increases plasma membrane IR levels in multiple cancer cell lines. Insulin binding to IR initiates a rapid translocation of glucose transporters to the plasma membrane for glucose entry. Indeed, PGRMC1 also increases the plasma membrane levels of two glucose transporters, GLUT-4 and GLUT-1, and is essential for both glucose uptake and insulin binding. Taken together, our data support a role for PGRMC1 in promoting insulin binding and glucose uptake at least in part by binding IR and maintaining plasma membrane pools of the receptor.

Insulin signaling regulates many crucial aspects of cellular physiology, and the significance of our findings extend beyond the scope of cancer. Our goal is to explore the interaction of PGRMC1 and IR in multiple physiological settings, including the main organ linked with many disease states: adipose. Impaired insulin signaling is central in the development of type 2 diabetes, and obese individuals demonstrate decreased insulin binding due to a reduction of IR levels, without an alteration in ligand-receptor affinity (50). We obtained fully differentiated human subcutaneous adipocytes from donors ranging from low to high BMIs and treated them with PGRMC1 ligands. We show treatment with PGRMC1 ligands significantly increase IR protein levels in adipocytes derived from high BMI patients. Protein levels appear to be affected through the direct interaction of PGRMC1 and IR, as we demonstrate their co-precipitation in another adipose model system, differentiated 3T3-L1 cells. These findings are
remarkable; pharmacological treatment with PGRMC1 ligands restored IR levels derived from high BMI adipocytes to mimic IR levels derived from low BMI adipocytes.

6.2 Significance

This dissertation represents a significant advancement in understanding the implication for PGRMC1 in disease states. Previous research has focused on the role of PGRMC1 in membrane trafficking and cancer pathology, with an emphasis on EGFR. However, as new research provides alternative mechanisms and insights into disease pathology, the field of PGRMC1 must evolve accordingly.

6.2.1 The relationship between PGRMC1 and wild-type EGFR

Mutations in the EGFR tyrosine kinase domain often result in increased malignant cell survival, proliferation and metastasis (224). The discovery of mutations in EGFR has resulted in the development of many anticancer drugs that have provided a successful avenue for the treatment of certain cancers. However, subsequent mutations in the receptor can lead to drug resistance and only a subset of cancers contain EGFR mutations. Therefore tumors expressing wild-type EGFR are often harder to treat.

To address these concerns, we summarized multiple signaling associated pathways that are important in trafficking wild-type and mutant EGFR. Furthermore, we found PGRMC1 to associate with wild-type EGFR 2.4-fold more
than a mutant EGFR, and a PGRMC1 inhibitor was active against cells expressing wild-type EGFR. These data indicate PGRMC1 as an attractive target in the inhibition of wild-type EGFR-dependent cancer cell proliferation.

6.2.2 PGRMC1 as a potential biomarker and therapeutic target for cancer stem cells

In the field of cancer research and care, the development of valid biomarkers can provide useful information in guiding clinical decision making and is becoming increasingly more important in the clinical management of cancer patients (225). Accordingly, The Precision Medicine Initiative pledged $70 million to the National Cancer Institute (NCI) in 2015, to “scale up efforts to identify genomic drivers in cancer and apply that knowledge in the development of more effective approaches to cancer treatment” (225).

In Chapter III, we include an analysis of PGRMC1 levels in over 600 tumor sections. PGRMC1 was significantly elevated in a large cohort of tumors derived from cancers of the airway, including the head and neck region. In lung cancer samples, PGRMC1 was significantly higher in stage I tumors compared to stage II tumors and correlated with patient survival. Furthermore, we report the first analysis of PGRMC1 expression in a large cohort of ovarian cancer and found high PGRMC1-expressing tumors to correlate with poor overall survival. These data implicate PGRMC1 as a potential biomarker in a broad range of tumor types.
PGRMC1 has been previously associated with drug resistance, a characteristic of cancer stem cells. The stem cell theory proposes that a subset of cancerous stem cells contribute to drug resistance and tumor maintenance. Therefore, in order to develop efficient treatments that can induce a long-lasting clinical response, cancerous stem cells must be considered in drug development. The association between PGRMC1 levels and tumors suggested a potential role for PGRMC1 in stem cell maintenance, and we report the first evidence detecting PGRMC1 expression in cancer stem cells. Remarkably, the PGRMC1 small molecule, AG205, also induced stem cell death, whereas the powerful agents (erlotinib and PD98059) did not.

6.2.3 The insulin receptor is proposed as a new target for cancer therapy

The identification of the mechanism(s) of insulin signaling in cancer development and progression may represent an important factor in the resistance to various anticancer drugs, particularly surrounding the dysregulation of the insulin receptor (IR) (164). Malignant cells have been reported to overexpress IRs that may reach or exceed expression levels physiologically observed in insulin target organs, such as adipose tissue or the liver (223). The insulin receptor consists of two isoforms, IR-A and IR-B, with the latter being predominant in most adult and differentiated tissues (164). Furthermore, IR-B is associated with metabolic and differentiating signaling, whereas IR-A mainly favors cell growth, proliferation and survival. Indeed, the IR-A isoform is predominantly expressed in cancer cells, and some studies suggest IR-A to be
involved in cancer stem cell biology (164). The characterization of insulin receptor trafficking has the potential to enhance our understanding of cancer development and progression while simultaneously generating new therapeutic targets.

Our data demonstrate that PGRMC1 associates with IR and trafficks IR to the plasma membrane in multiple cancer cell lines. Furthermore, insulin binding and glucose uptake were dependent on PGRMC1. Insulin has been known to have mitogenic properties; circulating insulin affects cancer growth and hyperinsulinemia is a major cancer risk factor for obese and diabetic patients (223, 226). Additionally, elevated blood glucose levels have been hypothesized to increase cancer risk and death in diabetic patients (226). Based on our findings, PGRMC1 may be an important regulator in insulin driven cancer development. Future studies involving the different IR isoforms may identify mechanisms and therapeutics to target the IR-A pathway in order to inhibit the tumor promoting effect of IR without impairing its metabolic effects.

6.2.4 Regulation of insulin receptor levels in human adipocytes derived from a high BMI donor

According to the Centers for Disease Control and Prevention (CDC), more than 1/3rd of the U.S. population is obese (36.5%). Adipocytes are one of the most highly insulin-responsive cell types in the body, and insulin is a critical regulator of adipocyte biology (227). Insulin signaling initiates at one central point, the insulin receptor (IR), to stimulate glucose transport, promotion of
adipocyte triglycerides storage, and uptake of fatty acids (210, 227). Chronic exposure to insulin leads to a down-regulation of insulin signaling via reduced insulin receptor expression at the plasma membrane, and obese individuals demonstrate a reduction in IR abundance in adipose tissue (50, 228).

We report a novel regulator for IR levels in human and rodent adipocytes. We show treatment with PGRMC1 ligands significantly increased total IR protein levels in fully differentiated human subcutaneous adipocytes. More specifically, IR levels in adipocytes derived from a donor with a high BMI were restored to levels comparable to adipocytes derived from a donor with a low BMI. Protein levels appear to be affected through the direct interaction of PGRMC1 and IR, as we demonstrate their co-immunoprecipitation in differentiated 3T3-L1 cells. This further validates our data in cancer cells demonstrating the role of PGRMC1 in IR signaling. However, pharmacological treatment did not affect total IR protein levels in cancer cells, as it did in adipocytes, indicating some form of cellular specificity. These data elegantly extend the findings of Kitamura and colleagues. In their study, they found overexpression of human IR in db/db mice restores blood glucose levels and improves the obesity phenotype (229). Thus, PGRMC1-directed therapeutics have the potential to provide an important approach in restoring IR function in obese individuals.

6.3 Limitations

This dissertation suffers from the universal limitations surrounding in vitro model systems. Due to ethical and practical concerns associated with human
experimentation, initial research is often conducted in animal model systems to examine preliminary efficacy, toxicity and pharmacokinetics. Indeed, human cancer cell lines and rodent model systems have driven critical advancements in understanding the mechanisms associated with disease states. However, the success rate of clinical translation is limited; the average rate of successful translation from animal model to clinical cancer trials is less than 8% (230). The biological differences between human and rodent models was elegantly demonstrated in our studies using adipocytes. The PGRMC1 ligand, AG205, increased insulin receptor levels in human samples, but decreased insulin receptor levels seen in rodent samples. Nevertheless, the FDA requires animal testing before any human is exposed to new molecular entities (230).

The complexity of the insulin signaling pathway also places limitations on the scope of this dissertation. The insulin receptor utilizes several substrates to facilitate multiple functions that include metabolism, growth and aging (210). Furthermore, insulin signaling varies amongst tissue type and cellular state. In two cancer cell lines, AG205 treatment affected glucose uptake and insulin binding, but had no effect on total IR protein levels, an effect seen in human derived adipocytes. It should also be noted that both the radioactive glucose uptake and insulin binding assays are downstream functions of insulin, and insulin can also bind to the IGF-1 receptor (IGF-1R). To address these issues, further experiments are necessary.

PGRMC1 has only one commercially available ligand, AG205, which has been used extensively in our research. However, recent studies challenge the
identification of PGRMC1 to be synonymous to the sigma-2 receptor (S2R) (82, 217). The sigma-2 receptor is a protein associated with tumor cell proliferation and binds to multiple ligands, including PB28, siramesine and haloperidol (82, 160). The structure of S2R is not known, and the gene remains to be cloned (82). We have discussed the studies in greater detail surrounding the identification of PGRMC1 as S2R in earlier chapters. The possibility that PGRMC1 is indeed the S2R would expand the pharmacological library for PGRMC1, making it an even more attractive therapeutic. However, until the field comes to a definitive consensus, there will be limitations on the use of ligand terms.

6.4 Future directions

This dissertation has provided novel evidence for the role of PGRMC1 in cancer stem cells, adipose metabolism and the regulation of two receptor tyrosine kinases, EGFR and IR. Despite advances in research, treatments and education, cancer and obesity remain worldwide epidemics. Multiple studies have established obesity as a predominant risk factor for numerous health conditions, including cardiovascular disease, type 2 diabetes and cancer. It is our hope that the work presented here will encourage future research into the biology of such disease states. Perhaps the greatest unanswered questions involve: (1) IR endocytosis and trafficking, (2) IR isoforms, IGF-1R and specificity and (3) glucose regulation and (4) the potential interactions between IR and EGFR.
6.4.1 IR endocytosis and trafficking

Ligand-dependent endocytosis and sorting for degradation of receptor-tyrosine kinases (RTKs) are critical steps in modulating the duration and intensity of receptor function (177). Many studies have investigated the pathways involved in EGFR trafficking, but very little is known about the endocytosis of IR. Similar to other RTKs, ligand stimulation at the cell surface promotes receptor internalization (177). IR endocytosis has been proposed to occur through both clathrin-dependent and caveolae-mediated pathways, with the latter experiments performed in primary adipocytes (177). In clathrin-mediated endocytosis, clathrin relies on adapter proteins to be recruited to the plasma membrane because clathrin does not bind directly to the plasma membrane or cargo receptors (231). Following endocytosis, complexes merge with early endosomes and either become recycled or proceed to the lysosome for degradation (231).

In a previous experiment performed by our lab, density gradient centrifugation divulged the co-localization of the PGRMC1-EGFR complex to intracellular microsomes (23). PGRMC1 was found in fractions associated with the endoplasmic reticulum (calnexin) and secretory vesicles (Rab5), which increases the likelihood for the PGRMC1-IR co-localization to occur in such (23). Numerous studies have proposed PGRMC1 to act as an adaptor protein, based on its modulation of cell surface expression of EGFR, mPRα and ERβ (23, 26). Taken together, these data would suggest PGRMC1 to associate with IR and internalize via clathrin-mediated endocytosis. Additionally, PGRMC1 may initiate
recycling of the PGRMC1-IR complex, because in the absence of PGRMC1, there are lower levels of IR at the plasma membrane.

Alternatively, PGRMC1 may inhibit the endocytosis of IR, which may explain why insulin binding was greater in control cells compared to PGRMC1-knockdown cells. Furthermore, clathrin-mediated endocytosis may be specific to cancer cells, as studies reported IR to become endocytosed via caveolae in adipose (177). Such data further demonstrate the complexity involved in insulin receptor function and signaling and require additional experiments to dissect the details.

6.4.2 IR isoforms, IGF-1R and specificity

This dissertation has demonstrated an interaction between PGRMC1 and IR and identified a role for PGRMC1 in insulin binding. However, it should be noted that insulin can also bind to another receptor, the IGF-1 receptor (IGF-1R), and the insulin receptor itself consists of two isoforms. The insulin receptor is composed of an extracellular α subunit and a transmembrane-spanning β subunit that are linked via disulfide bonds, and extracellular α subunits bind ligand (210). Alternative splicing at exon 11 encodes two isoforms of the receptor, isoform A and B, with the latter isoform containing 12 additional amino acids that are absent from the extracellular domain of IR-A (210). Insulin-sensitive tissues predominantly express the IR-B isoform, while IR-A is predominantly found in cancer cells and is expressed in the brain (164).
The IR isoforms can therefore provide specificity for pharmacological targets. Future research will need to identify the IR isoform in specific cell types and experimental models in order to provide specificity without impairing the total metabolic effect of insulin. In our data, drug treatment with a PGRMC1 ligand altered total IR protein levels in human-derived adipocytes, and not cancer cells. This is a significant finding because the highest expression of IR is found in adipocytes, and individuals possessing excess adipose have a greater risk for impaired insulin signaling (210).

Furthermore, intracellular targets may also play a role. The transcription factor SREBP-1c (sterol regulatory element–binding protein-1c) may play a critical role in the actions of insulin to regulate adipocyte gene expression, and PGRMC1 forms a PGRMC1/Insig/Scap/SREBP complex to regulate fatty acid lipogenesis and cholesterol synthesis (227). PGRMC1 may be working on a transcriptional level to alter IR levels and mediate adipocyte metabolism through the cholesterol/steroid pathway.

We also demonstrate a role for PGRMC1 to maintain IR levels at the plasma membrane in cancer cells. As mentioned earlier, the IR-A form is predominantly expressed in cancer cells and the brain, and future research should investigate the PGRMC1-IR interaction and mechanisms in both physiological settings. Indeed, insulin in the brain has been found to contribute to the control of nutrient homeostasis, cognition, memory and neuroprotective effects (232). Alterations in brain insulin metabolism have been suggested as a pathophysiological factor in neurodegenerative disorders, and Alzheimer’s
disease patients show a reduction in insulin receptor sensitivity and attenuated IR expression (233). Our lab is collaborating with the Thibault lab at the University of Kentucky to investigate the role of IR in cognition. The Thibault lab specializes in behavioral, electrophysiological, and calcium and glucose imaging to detect alternations in neuronal functions. Future research will investigate the role of PGRMC1 in insulin signaling.

IR and IGF-1R are both protein tyrosine kinases that belong to the IGF system that share a high degree of homology despite being products of two distinct genes (164). Because of the high degree of homology, the two receptors can heterodimerize to form hybrid receptors (164). Numerous studies have investigated the IGF axis in cancer, with an emphasis on IGF-1R and its involvement in cancer growth and transformation (223). Preliminary studies in our lab reveal PGRMC1 to regulate IGF-1R in an opposite manner from IR. We performed a membrane biotinylation experiment in A549 control and A549 PGRMC1-knockdown cells and found IGF-1R plasma membrane levels to increase in A549 PGRMC1-knockdown cells. IGF-1R is also overexpressed in cancer, and many clinical trials have investigated anti-IGF-1R blocking antibodies. IGF-1R blockade has shown only a moderate response in the clinic (223). Additionally, cells can develop resistance to IGF-1R inhibitors, and IGF-1R blockade results in enhanced IR-A homodimer formation and increases IR-mediated activity (223). In a study performed by Novosiadly and colleagues, expression of total IR levels inversely correlated with cixutumumab, a humanized monoclonal antibody against IGF-1R, efficacy in pediatric solid tumor models in
vivo (forest et al). Future research is therefore necessary to differentiate the mechanisms affected by PGRMC1 in both IR and IGF-1R mediated pathways.

6.4.3 Glucose regulation

Glucose homeostasis is coordinated through insulin release from the pancreatic islet beta cell in response to elevated blood glucose levels, and peripheral insulin responsive tissues to clear the excess glucose (210). Insulin binding to the IR initiates the mobilization of glucose transporters from intracellular vesicles to the plasma membrane. Insulin increases glucose uptake mainly through enriching concentrations of glucose transporters at the plasma membrane, and the rate of glucose transport into cells is thus governed by the concentration of glucose transporters at the cell surface and the duration for which the protein is remained there (199). The trafficking pathways of vesicles containing glucose transporters remain unclear, an area in which PGRMC1 is associated with.

Our data show PGRMC1 to be dependent for glucose transporters, GLUT-4 and GLUT-1, expression at the cell surface. Glucose transporter type 4 (GLUT-4) plays a central role in controlling whole body glucose homeostasis and glucose transporter type 1 (GLUT-1) is frequently upregulated during oncogenesis (234). We did not investigate the direct interaction between PGRMC1 and the glucose transporters, therefore future experiments will be carried out to answer the proposed hypothesis.
This dissertation also shows glucose uptake to be dependent upon PGRMC1. As previously mentioned, the concentration of glucose transporters at the cell surface directly affects the amount of glucose transport into the cell. Therefore it is possible that glucose uptake is dependent upon PGRMC1 maintaining glucose transporter levels at the plasma membrane. However, whether this function is dependent on IR signaling or PGRMC1 affecting the glucose transporter trafficking remains unclear. IR can signal through the phosphatidylinositol 3-kinase (PI3K) pathway, which is an essential mediator in glucose uptake (210). Inhibition of the PI3K enzyme completely blocks the stimulation of glucose uptake by insulin, introducing another area in which PGRMC1 can modulate the glucose uptake process (199). Furthermore, PGRMC1 associates with the glucagon-like peptide-1 receptor (GLP-1R) to enhance GLP-1 induced insulin secretion by modulating the EGFR-PI3K pathway to potentiate insulin exocytosis (49). We will discuss the potential cross talk between IR and EGFR in the next section. Future research on the signaling cascades involved in glucose uptake is necessary to determine the exact role PGRMC1 plays in the process.

6.4.4 The potential interaction between IR and EGFR

Our lab has established a role for PGRMC1 in regulating both EGFR and IR at the plasma membrane. Both proteins are intricately involved in a network of pathophysiological functions across numerous tissue types and disease states. Understanding the crosstalk between signaling molecules is fundamental
for designing molecular targeted therapeutics. The EGFR signaling pathway is one of the best understood receptor signaling pathways and have been used as main targets in cancer therapy. However, some cancers have been resistant to EGFR inhibition, suggesting a crosstalk between EGF and other pro-survival pathways (235).

Both EGFR and IR signal through the PI3K pathway, promote cellular growth and are involved in tumorigenesis. Furthermore, Capala and colleagues performed computational and experimental analyses investigating the crosstalk between EGF, IGF and insulin cell signaling pathways and found Erk1/2, Ak11, Jnk and p70S6k to be important for crosstalk (235). PGRMC1 has been shown to directly bind to the G-protein coupled receptor, GLP-1R (glucagon-like peptide-1 receptor), and initiate EGFR signaling in pancreatic β cells (23, 47). Several studies have shown crosstalk between G-protein coupled receptors and IR, and IR signaling is critical for the protective effect GLP-1 has on pancreatic β cell function and survival (236).

We have shown that PGRMC1 inhibition in A549 cells causes a reduction in both EGFR and IR plasma membrane levels. We have not investigated whether knockdown of PGRMC1 produces a similar cytoplasmic buildup of IR, as was shown with EGFR. We cannot exclude the possibility that EGFR signaling affects insulin binding and glucose uptake in the A549 cell line. Activation of EGFR has been reported to transiently increase glucose uptake, and is a stabilizer of an active glucose transporter, SGLT1 (200). Future studies should
carefully consider the role of PGRMC1 in modulating both EGFR and IR, and develop ways to target each accordingly.
APPENDIX A
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