G-Protein-coupled Estrogen Receptor 1 Agonist G-1 Perturbs Sunitinib Resistance-related Phosphoproteomic Signatures in Renal Cell Carcinoma

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Abstract. *Background: Metastatic renal cell carcinoma (RCC) often develops resistance to first-line targeted therapy such as sunitinib. G-Protein-coupled estrogen receptor 1 (GPER1) agonist G-1 was recently reported to regulate RCC physiology but the role of G-1 in RCC tumorigenesis and sunitinib resistance remains largely unknown. Materials and Methods: Parental and sunitinib-resistant 786-O cells were treated with GPER1 agonist G-1, and quantitative phosphoproteomics was performed. Bioinformatic analyses and validations, including immunoblotting, cell migration, and cell cycle distribution, were performed. Results: G-1 repressed cell proliferation and migration in both parental and sunitinib-resistant 786-O cells. Phosphoproteomic signatures, including phosphoinositide 3-kinase and protein kinase B (PI3K-AKT) as well as other pathways, were up-regulated in sunitinib-resistant cells but application of G-1 reversed this effect. Among phosphoprotein candidates, activating transcription factor 2 (ATF2) Thr69/71 phosphorylation was antagonistically regulated by sunitinib resistance and G-1. Conclusion: Our results open up the possibility for managing RCC and sunitinib resistance by GPER1 agonist G-1 and its regulated pathways.*

Renal cell carcinoma (RCC) is the leading cause of kidney malignancy, contributing to an estimated 4-5% of new cases in 2020 (1). In addition to radical or partial nephrectomy, sunitinib is the first-line standard therapy for low-risk recurrent or metastatic RCC (2, 3). Primary or secondary resistance, however, usually develops after sunitinib administration (4). Although the combination of other target therapeutics, such as immune checkpoint inhibitors, seems to be a promising treatment option (5), there is still a need to investigate alternative strategies for managing RCC, especially in cases of sunitinib resistance.

The incidence of RCC has a male-to-female ratio of 2:1 in young patients but becomes approximately 1:1 in patients over 70 years old (6), implying the inhibitory role of estrogen in RCC carcinogenesis. Estrogen can repress RCC growth by non-genomic signaling pathways *via* classical, nuclear estrogen receptors (7-9). In addition to nuclear estrogen receptors, G-protein-coupled estrogen receptor 1 (GPER1) was identified as a non-classical estrogen receptor, and its expression correlated with tumorigenesis and poor prognosis in several cancer types, including breast and prostate cancer (10). Treatment of *in vitro* cancer cell lines by the GPER1 agonist, G-1, led to variable results. For example, G-1 inhibited cell growth in prostate cancer (11) but promoted cell survival and migration in RCC cell lines (12). Recently, we demonstrated that estrogen inhibited RCC cell growth and alleviated sunitinib resistance *in vitro* through estrogen-triggered modulation of phosphorylation (9, 13). However, it remains unclear whether G-1 regulates phosphorylation dynamics in RCC tumorigenesis as well as and sunitinib resistance.

Quantitative phosphoproteomics has been used to study phosphorylation signaling in cancer development and drug resistance. Smith *et al.* found the novel phosphoproteomic

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signature in GPER1 agonist G-1-treated breast cancer cells (14). In addition, by combining immunoprecipitation with quantitative phosphoproteomics, a sunitinib-regulated tyrosine phosphorylation network was revealed (15). In the present study, we utilized a quantitative phosphoproteomics approach to survey G-1-regulated phosphorylation changes and their effect on sunitinib resistance with respect to cell proliferation, cell cycle, and cell migration. The identification of phosphoproteins that are regulated with G-1 may provide novel therapeutic targets for sunitinib-resistant RCC.

Materials and Methods

Chemicals. All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Generation of sunitinib-resistant RCC cells. The human RCC cell line 786-O was purchased from the Biological Collection and Research Center (Hsinchu, Taiwan) and maintained in RPMI 1640 medium (without phenol red) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA). Sunitinib-resistant 786-O cells were generated in-house by gradually increasing exposure up to 10 μ M sunitinib *in vitro* (16). Sunitinib resistance (SunR) was confirmed by cell growth and sunitinib accumulation in lysosomes (17).

Treatment with G-1 and determination of cell viability. Both parental (PAR) and SunR 786-O cells were seeded at 4×10^3 cells/well in 100 μ l culture medium in 96-well plates and cultured for 24 h. The cells were then treated with sunitinib (2.5 to 10 μ M), estrogen (1 to 5 μ M), GPER1 agonist G-1 (1 to 5 μ M), or GPER1 antagonist G-15 (1 to 5 μ M) for an additional 48 h. Cell viability and number were measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as previously described (18).

Peptide dimethyl labeling and phosphopeptide enrichment. For quantitative phosphoproteomic analysis, both PAR and SunR 786-O cells were treated either with vehicle only (*i.e.* 0.1% dimethyl sulfoxide) or with 2 μ M G-1 for 48 h. Cellular proteins were then harvested in a lysis buffer containing 4% sodium dodecyl sulfate, 100 mM triethylammonium bicarbonate, as well as protease and phosphatase inhibitor cocktails, and sonicated using a Bioruptor Plus Sonicator (Diagenode, Denville, NJ, USA). After acetone precipitation, protein pellets were resuspended in 100 mM triethylammonium bicarbonate, and 8 M urea, followed by trypsin digestion and dimethyl labeling as previously described (9). Peptides from G-1-treated PAR 786-O cells, vehicle-treated SunR cells, and G-1-treated SunR cells were labeled with stable isotopic formaldehyde ($^{13}\text{CD}_2\text{O}$, or heavy labeled). For the vehicle-treated PAR 786-O cells, the peptides were labeled with formaldehyde (CH_2O , or light labeled). After dimethyl labeling, equal amounts (500 μ g) of the heavy- and light-labeled peptides were combined and subjected to sequential phosphopeptide enrichment by Ga- and Fe-immobilized metal affinity chromatography as previously described (19). The eluted phosphopeptides were further fractionated into six fractions using the high-pH reverse-phase StageTip method (20).

Nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) and statistical analysis of phosphosites.

The high-pH reverse phase-fractionated phosphopeptides were analyzed using an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled on-line to a Dionex Ultimate 3000 RSLC nano system (Thermo Fisher Scientific). Phosphopeptides were separated by in-house-prepared capillary column (100 μ m $\times$ 15 cm tip column) packed with 3- μ m ReproSil-Pur 120 C18-AQ reverse-phase beads (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany), and data-dependent acquisition in LTQ-Orbitrap XL were performed as described previously (9). In the present study, three independent biological batches with duplicate nanoLC-MS/MS analyses were performed. Phosphopeptide identification and dimethyl quantification were performed using MaxQuant software (version 1.5.3.8) (21) against a reviewed SWISS-PROT sequence database (version 2016_05, with 20,207 human canonical protein sequences) as previously described (9). The false discovery rate (FDR) at the peptide and protein levels was fixed at 1% in MaxQuant. The localization probability of each identified phosphorylation site (phosphosite) was also determined by MaxQuant. All nanoLC-MS/MS raw files as well as MaxQuant-generated result data were deposited onto the ProteomeXchange (22) Consortium via the Proteomics Identifications (PRIDE) partner repository with the dataset identifier PXD021254.

All phosphosite analyses were performed in Perseus platform (version 1.6.2.3) (23). Only high-confidence phosphosites (localization probability >0.75 or class I phosphosites) (24) were analyzed. For the SunR group, light-labeled intensity (PAR) and heavy-labeled intensity (SunR) were filtered to remove missing values. The phosphosite intensities were normalized by the internal reference scale (IRS) method (25), log₂-transformed, and subjected to permutation FDR testing with s0 setting of 0.1 to identify dysregulated phosphosites ($p < 0.01$) (23). The G-1-treated group was analyzed using the same approach. For comparing the G-1-treated and untreated SunR groups, differences in phosphosite levels were calculated as the 'ratio of ratios', according to a previous study (26). We first filtered for phosphosites with quantification information in the SunR (vs. PAR) and G-1-treated SunR (vs. PAR) groups. After IRS normalization across the two groups, the expression ratio for each group was calculated as SunR/PAR or (G-1-treated SunR)/PAR. Finally, the obtained ratio of G-1-treated SunR was divided by that of SunR. The resulting expression ratios were subjected to permutation FDR testing.

Bioinformatics of differentially regulated phosphosites. Gene Ontology network analysis was performed on the dysregulated phosphosites using Cytoscape (v3.8.0) (27) plug-in ClueGO (v2.5.7) (28, 29). Briefly, the regulated phosphosites were clustered (cut-off p -value=0.01) using ontologies/pathways information from the Gene Ontology biological pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) (30, 31), Comprehensive Resource of Mammalian Protein Complexes (32), and WikiPathway (33) databases. Enriched clusters were annotated by AutoAnnotate (v1.3.3) (34), a Cytoscape application for summarizing networks with semantic annotations. In addition, the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation module (35) with default settings was used to analyze the Gene Ontology information of the regulated phosphosites. The kinase-substrate relationship of the regulated phosphosites were analyzed using the post-translational modification-signature enrichment analysis (PTM-SEA) algorithm (36) with default settings and a cut-off FDR of 0.01. Finally, the network visualization of the regulated phosphosites was performed using the Cytoscape application PhosphoPath (v3.2) (37).

Qualitative immunoblotting. Ten micrograms of cellular lysates were used for western blotting as previously described (9). The primary antibodies used in the present study and their dilution factors were as follows: anti-phospho (p)-activating transcription factor 2 (ATF2) (Thr69/71) (Cat. 9225) at 1:1000, anti-ATF2 (Cat. 9226) at 1:1500; anti-cyclin-dependent kinase 1 (CDK1) (Cat. 9116) at 1:1000 from Cell Signaling Technology (Danvers, MA, USA); anti-cyclin B (Cat. sc-245) at 1:1000 and anti-cyclin D (M20) (Cat. sc-718) from Santa Cruz Biotechnology (Dallas, TX, USA); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. 60004-1-Ig) at 1:40000 from Proteintech (Rosemont, IL, USA).

Cell migration assay. Migration of PAR and SunR 786-O cells following G-1 treatment was measured using the Oris™ Cell Migration Assay kit (Platypus Technologies, Madison, WI, USA) according to the manufacturer's protocol. Briefly, 5×10^4 cells were seeded onto each well of a 96-well plate and cultured for 24 h. After the stopper was removed from each well, fresh culture media with or without 2 μ M G-1 was added to the cell culture for 48 h. The cells were then stained with a cell-permeant derivative of calcein, calcein acetoxymethyl, and the number of migrated cells was determined by measuring fluorescence at 485/528 nm excitation/emission using an Infinite M200 PRO microplate reader (Tecan, Zürich, Switzerland).

Cell-cycle profiling by flow cytometry. Cell-cycle profiles were determined by propidium iodide (PI) DNA staining followed by flow cytometry (38). Briefly, 5×10^5 PAR or SunR 786-O cells were treated with or without 2 μ M G-1 for 48 h. The cells were harvested by trypsinization and fixed with 70% ethanol. For PI staining, ethanol-fixed cells were incubated in PI staining buffer containing 0.2 mg/ml RNase A, 0.1% Triton X-100, and 20 μ g/ml PI in phosphate-buffered saline, for 30 min in the dark at room temperature. Fluorescence emitted at 585 nm from the PI-stained DNA was detected using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The percentages of cells (from a total of 104 cells) in the different cell phases were determined using FlowJo software, version 8.7 (BD Life Sciences, Ashland, OR, USA).

Results

GPER1 agonist G-1 inhibited cell growth of both PAR and SunR RCC cells. We first generated SunR 786-O cells by incubating the cells with half-maximal inhibitory concentration of sunitinib, *i.e.* 10 μ M, for more than 6 months (16), and confirmed that SunR 786-O cells showed less growth inhibition by sunitinib than PAR cells ($p < 0.01$) (Figure 1A). In agreement with previous studies (8, 9, 13), estrogen repressed growth of both PAR and SunR 786-O cells at relatively high concentrations (1 to 5 μ M) (Figure 1B).

GPER1, a transmembrane estrogen receptor (39), was shown to participate in RCC cell physiology (12). In addition to endogenous estrogen, GPER1 can also be regulated by the synthetic agonist G-1 and antagonist G-15 (40, 41). We therefore investigated whether GPER1 agonist/antagonist is able to regulate growth of 786-O cells. The concentration ranges for GPER1 agonist/antagonist, *i.e.* 1 to 5 μ M, were chosen based on previous studies (12, 14). The GPER1

agonist G-1 significantly inhibited cell growth of PAR as well as SunR cells, and SunR cells were more susceptible to G-1, *i.e.* at 1 μ M, than PAR cells (Figure 1C). Although GPER1 antagonist G-15 also caused growth inhibition in both PAR and SunR cells (Figure 1D), G-1 showed greater growth inhibition than G-15 at the same concentrations, especially in SunR cells (Figure 1C and 1D). Collectively, these data suggest that the GPER1 agonist G-1 is a potential growth inhibitor in both PAR and SunR cells.

Quantitative phosphoproteomics revealed sunitinib resistance- and G-1-regulated phosphosites. Although G-1 is believed to be a specific GPER1 agonist (41), G-1 may affect both GPER1-dependent and -independent signaling pathways. To further dissect the possible phosphorylation signaling pathways regulated by G-1 treatment, chronic sunitinib treatment, and G-1 treatment in SunR cells, quantitative phosphoproteomics revealed 2893 and 3098 quantifiable phosphosites in the SunR and G-1 treatment groups, respectively. After IRS normalization (25), followed by permutation FDR testing ($p < 0.01$), we identified 615 up-regulated and 886 down-regulated phosphosites in SunR 786-O cells (Figure 2A) as well as 629 up-regulated and 690 down-regulated phosphosites in G-1-treated PAR 786-O cells (vs. control) (Figure 2B).

We further analyzed the dysregulated phosphosites using ClueGO (28, 29) to explore the functions of phosphosites in SunR and G-1-treated PAR 786-O cells. For SunR 786-O cells, two clusters were up-regulated in response to chronic sunitinib treatment, including the adherens junction organization as well as vascular endothelial growth factor receptor (VEGFR) pathway and focal adhesion (Figure 2C). This agrees with previous observations, where sunitinib-resistance up-regulated VEGF-related pathways (4, 17). In contrast, phosphosites involved in transcription factor binding and mRNA splicing were down-regulated, implying global repression of gene transcription in SunR cells (Figure 2A). Microtubule organization, negative regulation of organelle organization, as well as nuclear division and segregation were down-regulated in SunR cells (Figure 2A).

On the other hand, G-1 up-regulated phosphosites involved in protein sumoylation and ubiquitinylation, nucleocytoplasmic transport, DNA integrity checkpoints, and actin filament bundles (Figure 2B). Phosphosites involved in the receptor-signaling pathway, adhesion/tight junctions, and spliceosome were down-regulated in G-1-treated PAR cells (Figure 2B).

G-1 antagonized sunitinib resistance-related phosphorylation pathways. To explore the potential effect of G-1 on the sunitinib resistance-regulated phosphosites, the phosphosite intensities in G-1-treated SunR cells were compared to those of the untreated SunR cells. We identified 24 up-regulated (Log_2 ratio > 0.5) and 10 down-regulated (Log_2 ratio < -0.5)

Figure 1. *G-Protein-coupled estrogen receptor 1 (GPER1) agonist G-1 suppressed cell growth in both parental (PAR) and sunitinib-resistant (SunR) 786-O cells. SunR 786-O cells were first generated by chronic exposure to 10 μM sunitinib for over 6 months. Both PAR and SunR 786-O cells were incubated with sunitinib (2.5 or 10 μM) (A), estrogen (1 or 5 μM) (B), GPER1 agonist G-1 (1 or 5 μM) (C), or GPER1 antagonist G-15 (1 or 5 μM) (D) for 48 h, and then viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All treatments were repeated in triplicate. Significantly different at $p < 0.05$ from *vehicle control, and #indicated group.*

phosphosites ($p < 0.01$) (Figure 3A). Enrichment for protein functions and pathway information of the differentially expressed phosphosites was determined by Fisher's exact test ($p < 0.01$) using gene annotation databases (Table I). For example, arrest defective 1 and *N*-acetyltransferase human (ARD1–NATH) complex, Ku-antigen and *N*-methyl-D-aspartic acid receptor-regulated protein 1 (Ku-antigen–NARG1) complex, as well as activating transcription factor 2, hexokinase-1 and voltage-dependent anion channel-1 (ATF2–HK1–VDAC1) complex were enriched. In addition, mitogen-activated protein kinase (MAPK) signaling pathway and nucleotide-binding domain (NOD)-like receptor signaling

pathway were enriched. Finally, proteins related to DNA-damage checkpoint and repair were enriched.

To address the potential kinase motif(s) for the regulated phosphosites, the differentially expressed phosphosites were subjected to PTM-SEA analysis (36). Kinase motifs related to the cyclin dependent kinase (CDK) family were down-regulated in both SunR and PAR 786-O cells (Figure 3B). These data indicate that sunitinib resistance reduced cell-cycle/division activity, and G-1 further reduced CDK activities, such as of CDK1, CDK2, CDK4, and CDK6. On the other hand, several kinases and pathways, including C-C chemokine receptor type 7 (CCR7), phosphoinositide 3-kinase

Figure 2. Quantitative phosphoproteomics revealed sunitinib resistance- and G-protein-coupled estrogen receptor 1 agonist G-1-regulated phosphosites in sunitinib-resistant (SunR) (A) and G-1-treated (B) 786-O cells compared with the parental cells (PAR). Upper panel: Volcano plots of differentially regulated phosphosites (false discovery rate < 0.01 and $s_0 = 0.1$). Lower panel: The ClueGO enriched protein functions of the regulated phosphosites. Red and green colors denote up-regulated and down-regulated phosphosites, respectively.

and protein kinase B (PI3K-AKT), epidermal growth factor receptor (EGFR), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2), tyrosine-protein kinase KIT (KIT) receptor, and interleukin 33 (IL33) signaling pathways, as well as glycogen synthase kinase 3 beta (GSK3B) and MAPK1 kinases were notably up-regulated in SunR 786-O cells. EGFR and PI3K-AKT pathways are involved in sunitinib resistance (42, 43). CCR7, GSKB, and KIT pathways have been correlated with prognostic and therapeutic resistance in RCC cells (44-46). G-1 treatment also alleviated these sunitinib resistance-activated kinases/pathways. Finally, motifs related to mammalian target of rapamycin (mTOR) and polo-like kinase 1 (PLK1) were up-regulated in G-1-treated SunR 786-O cells compared to that in untreated cells, indicating that G-1 may activate the mTOR and PLK1 pathways in SunR 786-O cells.

We also performed hierarchical clustering of the G-1-regulated phosphosites in SunR 786-O cells (Figure 3C). In

general, SunR 786-O and G-1-treated SunR 786-O cells showed opposite expression profiles. For example, phosphosites in clusters 1 and 2 were up-regulated in SunR 786-O cells but down-regulated in G-1-treated SunR 786-O cells. In clusters 3-5, phosphosite expression was down-regulated in SunR 786-O but up-regulated in G-1-treated SunR 786-O cells (Figure 3C). These data suggest that G-1 can antagonize changes in phosphorylation during chronic sunitinib treatment. In addition, PhosphoPath analysis (37) showed that 20 out of 34 regulated phosphosites showed functional connections centered on ATF2, filamin A (FLNA), p21 activated kinase 1 (PAK1), and SH3 domain-containing kinase-binding protein 1 (SH3KBP1) (Figure 3D). Finally, we used the DAVID functional annotation module with UniProtKB keywords (UP_KEYWORDS), KEGG pathway (KEGG_PATHWAY), and Gene Ontology (GOTERM_MF_DIRECT) to identify enrichment in phosphosite clusters (35). Functions in the MAPK signaling

Figure 3. G-Protein-coupled estrogen receptor 1 agonist G-1-regulated phosphoproteomic signatures in sunitinib-resistant (SunR) 786-O cells. A: Volcano plot of G-1-regulated phosphosites ($p < 0.01$, Log_2 ratio > 0.5 or < -0.5) in G-1-treated SunR 786-O cells compared with untreated SunR cells. B: Heatmap of phosphoproteomic signatures using the normalized enrichment scores by post-translational modification–signature enrichment analysis. The significantly enriched or depleted signatures at false discovery rate < 0.05 are marked by asterisks. C: Hierarchical clustering of G-1-regulated phosphosites in SunR cells. The comparative expression profiles of the selected phosphosites are also shown. D: The network of G-1-regulated phosphosites in SunR cells depicted by PhosphoPath analysis. The relative expression of specific phosphosites is indicated by small colored boxes from left to right in: SunR, G-1-treated parental, G-1-treated SunR vs. untreated SunR (SunR&G-1 vs. SunR), and G-1-treated SunR (SunR&G-1) 786-O cells, with red and green colors denoting up-regulation and down-regulation, respectively.

Table I. Protein functions of phosphosites significantly regulated by G-protein-coupled estrogen receptor 1 agonist G-1 in sunitinib-resistant 786-O cells.

Source	Enriched protein functions
CORUM	ARD1–NATH complex
CORUM	Ku antigen–NARG1 complex
CORUM	ATF2–HK1–VDAC1 complex
CORUM	TP53BP1-containing complex
CORUM	PTIP–DNA damage-response complex
CORUM	MDC1–TP53BP1–SMC1 complex
CORUM	MDC1–H2AX–TP53BP1 complex
CORUM	Prohibitin 2 complex, mitochondrial
KEGG brite	MAPK signaling pathway
KEGG brite	NOD-like receptor signaling pathway
KEGG brite	Cortisol synthesis and secretion
KEGG brite	Insulin secretion
KEGG brite	Cocaine addiction
KEGG brite	Viral carcinogenesis
KEGG brite	Aldosterone synthesis and secretion
KEGG brite	Thyroid hormone synthesis
KEGG brite	Amphetamine addiction
Reactome	G ₂ /M DNA damage checkpoint
Reactome	Nonhomologous end-joining
Reactome	Recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks
Reactome	SUMOylation of transcription factors
Reactome	Processing of ends of DNA double-strand breaks
Reactome	Activation of the AP-1 family of transcription factors

AP-1: Activator protein 1; ARD1: arrest defective 1; ATF2: activating transcription factor 2; ATM: ataxia telangiectasia mutated; CORUM: Comprehensive Resource of Mammalian Protein Complexes; H2AX: histone H2AX; HK1: hexokinase-1; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinase; MDC1: mediator of DNA damage-checkpoint protein 1; NARG1: *N*-methyl-D-aspartic acid receptor-regulated protein 1; NATH: *N*-acetyltransferase human; NOD: nucleotide-binding domain; PTIP: PAX2 transactivation domain-interacting protein; SMC1: structural maintenance of chromosomes protein 1; TP53BP1: p53-binding protein 1; VDAC1: voltage-dependent anion channel-1.

pathway, acetylation, DNA-binding, and alternative splicing, were enriched in clusters 1 and 2 (Table II). On the other hand, functions in the SH3 domain, cell–cell adhesion, transcription regulation, and methylation were enriched in clusters 3–5.

Integral network analysis highlighted the crosstalk of phosphorylation dynamics between G-1-treated PAR 786-O cells, SunR 786-O cells, and G-1-treated SunR 786-O cells. We dissected the possible crosstalk between G-1 and sunitinib in RCC cells by performing integral network analysis. We used the phosphosites with significant changes in at least one of the following conditions: G-1-treated PAR *vs.* PAR 786-O cells, SunR 786-O *vs.* PAR 786-O, and G-1-treated SunR *vs.* untreated SunR 786-O cells. A total of 2,286 phosphosites were analyzed by the PhosphoPath analysis. In agreement with PTM-SEA analysis (Figure 3B), PhosphoPath analysis indicated enrichment of the VEGFA-VEGFR2 signaling pathway ($p < 0.0001$), focal adhesion-PIK3-AKT-mTOR signaling pathway ($p < 0.0001$), and cell cycle checkpoint ($p < 0.0001$) (Figure 4).

G-1 inhibited ATF2 phosphorylation and cell-cycle progression. According to our phosphoproteomics data, ATF2 phosphorylation at Thr69 and Thr71 was differentially regulated in G-1-treated and untreated SunR 786-O cells (Figure 3C and 3D). In addition, ATF2 was also found in the VEGFA-VEGFR2 signaling pathway (Figure 4A) and focal adhesion-PIK3-AKT-mTOR signaling pathway (Figure 4B), suggesting that ATF2 may be affected by G-1-mediated and sunitinib resistance-related signaling pathways. In agreement with the phosphoproteomics data, we confirmed that Thr69/71 phosphorylation in ATF2 was up-regulated in SunR 786-O cells, and that G-1 treatment inhibited Thr69/71 phosphorylation in both PAR and SunR 786-O cells (Figure 5A). In contrast, ATF2 expression did not significantly change by treatment, demonstrating that G-1 and sunitinib resistance affect ATF2 activity by protein phosphorylation.

Recently, Hasegawa *et al.* reported that CDK1 and cyclin B complex promote cell-cycle progression into M phase by phosphorylating Thr69/71 in ATF2 (47). In addition, our phosphoproteomics data suggested that G-1 regulates the

Table II. Functional annotation of G-1-regulated phosphosite clusters in sunitinib-resistant 786-O cells by the Database for Annotation, Visualization and Integrated Discovery.

Category	Term	Genes	Fold enrichment	Enrichment score
Cluster 1-2				
KEGG_PATHWAY	MAPK signaling pathway	<i>PAK1, STMN1, ATF2</i>	17.2	0.74
UP_KEYWORDS	Acetylation	<i>PAK1, STMN1, DOCK7, ATF2</i>	1.2	0.74
UP_KEYWORDS	DNA-binding	<i>CENPA, ATF2, HOXA3</i>	3.6	0.27
UP_KEYWORDS	Alternative splicing	<i>PAK1, STMN1, TNS3, DOCK7, CENPA, ATF2</i>	1.2	0.20
Cluster 3-5				
UP_KEYWORDS	SH3 domain	<i>SH3K1, LASP1, BIN1</i>	4.1	0.83
GOTERM_MF_DIRECT	Cadherin binding involved in cell-cell adhesion	<i>KLC2, PDLI5, FLNA, LASP1, HNRNPK</i>	2.2	0.69
UP_KEYWORDS	Transcription regulation	<i>FLI1, TP53BP1, NAA15, RBL1, UIMC1, HNRNPK</i>	1.5	0.31
UP_KEYWORDS	Methylation	<i>HIST1H1E, COPA, TP53BP1, RCC1, LASP1, HNRNPK</i>	1.5	0.30

ATF2: Activating transcription factor 2; *BIN1*: myc box-dependent-interacting protein 1; *CENPA*: centromere protein A; *COPA*: coatamer subunit alpha; *DOCK7*: dedicator of cytokinesis 7; *FLI1*: Protein flightless-1 homolog; *FLNA*: filamin A; *HIST1H1E*: histone H1.4; *HNRNPK*: heterogeneous nuclear ribonucleoprotein K; *HOXA3*: homeobox A3; *KLC2*: kinesin light chain 2; *LASP1*: LIM and SH3 domain protein 1; *MAPK*: mitogen-activated protein kinase; *NAA15*: N-alpha-acetyltransferase 15; *PAK1*: p21 activated kinase 1; *PDLI5*: PDZ and LIM domain protein 5; *RBL1*: retinoblastoma-like protein 1; *RCC1*: regulator of chromosome condensation; *SH3K1*: SH3 domain-containing kinase-binding protein 1; *STMN1*: stathmin 1; *TNS1*: tensin 1; *UIMC1*: ubiquitin interaction motif containing 1.

cell-cycle checkpoint (Figure 4C). Therefore, we performed flow cytometry to examine the cell-cycle progression in cells. Firstly, G-1 treatment promoted S-phase entry in PAR 786-O cells (Figure 5B). The entry of S-phase after G-1 treatment may be explained by the increased expression of G1/S-phase cyclin D1 in response to G-1 treatment in PAR 786-O cells (Figure 5A). On the other hand, G-1 treatment blocked the G2/M phase transition in PAR 786-O cells (Figure 5B). This G2/M arrest coincided with reduced CDK1 and cyclin B expression by G-1 treatment, as well as the down-regulation of ATF2 Thr69/71 phosphorylation (Figure 5A). In contrast, G-1 treatment did not lead to any significant changes in cell-cycle progression in SunR 786-O cells (Figure 5B), although ATF2 phosphorylation as well as cell-cycle regulators, such as CDK1, cyclin B and cyclin D were inhibited by G-1 treatment in SunR cells.

Effect of G-1 on RCC cell migration. According to our phosphoproteomics data, both G-1 treatment and sunitinib resistance may affect cell migration and cell adhesion. We therefore monitored cell migration using Oris™ cell migration assays (48). Sunitinib resistance reduced cell migration by ~50% in 786-O cells, and G-1 inhibited cell migration by nearly 50% in both PAR and SunR 786-O cells (Figure 5C and D). Taken together, these data show G-1 can repress cell migration in both PAR and SunR 786-O cells.

Discussion

It has been shown that estrogen represses RCC cell growth *via* classical estrogen receptors (8, 9), and perturbs sunitinib resistance in *in vitro* RCC cell culture (13). The role of non-classical estrogen receptor GPER1 in RCC physiology, however, is still unclear. In the present study, we showed that GPER1 agonist G-1 repressed cell growth in both PAR and SunR 786-O RCC cells. Using quantitative phosphoproteomics, we further demonstrated that G-1 perturbs phosphoproteomic signatures involved in VEGFR pathway, cell-cycle progression, as well cell migration. Although the exact role of GPER1 remains to be further addressed, our observations imply the importance of the GPER1 pathway in regulating RCC growth. Because estrogen may induce genomic changes and cause delirious side-effects *via* classical estrogen receptors (49), we anticipate that the G-1-regulated extranuclear-initiated ('nongenomic') pathway, either GPER1-dependent or -independent, provides an alternative approach for managing RCC.

Our phosphoproteomics data also highlighted the potential roles of ATF2 in sunitinib resistance and G-1-regulated signaling pathways. Elevated ATF2 expression contributes to resistance to sorafenib, another first-line tyrosine kinase inhibitor, in liver cancer (50). Silencing of ATF2 inhibited the growth of pancreatic cancer cells and enhanced

Figure 4. *Continued*

sensitivity to chemotherapy (51). In addition, up-regulation of ATF2 expression is also correlated with poor prognosis of RCC (52). Together, these observations demonstrated that ATF2 might participate in RCC carcinogenesis and tyrosine kinase inhibitor resistance, although regulation of ATF2 phosphorylation adds another level of regulation to this system. In the present study, we observed the up-regulation

of Thr69/71 phosphorylation of ATF2 in sunitinib resistance, and treatment with G-1 reversed this. ATF2 Thr69/71 phosphorylation is required for its association with activator protein 1 (AP-1), nuclear localization, and transcriptional activation of downstream genes related to DNA repair and apoptotic signaling (53), which may promote resistance to therapeutic agents. The down-regulation of Thr69/71

Figure 4. *G*-Protein-coupled estrogen receptor 1 agonist *G*-1 perturbed phosphorylation networks. Significantly altered selected phosphosites were analyzed by PhosphoPath. Enriched phosphorylation networks from vascular endothelial growth factor A-vascular endothelial growth factor receptor 2 (VEGFA-VEGFR2) pathway (A), focal adhesion-phosphoinositide 3-kinase-protein kinase B-mammalian target of rapamycin (PIK3-AKT-mTOR) signaling pathway (B), and cell-cycle checkpoint (C) are shown. The relative expression of each phosphosite is indicated by small colored boxes from left to right in: Sunitinib-resistant (SunR), *G*-1-treated parental, *G*-1-treated SunR vs. untreated SunR, and *G*-1-treated SunR 786-O cells, with red and green colors denoting up-regulation and down-regulation, respectively.

phosphorylation, as shown by *G*-1 treatment, may restrict ATF2 to the cytoplasm, and possibly be correlated with mitochondrial death (54). According to our results, ATF2 phosphorylation participated in the focal adhesion pathway, which affects cell migration. Therefore, the exact role of ATF2 phosphorylation in the GPER1 pathway warrants future studies.

Sunitinib resistance is correlated with cell survival and migration. However, there is controversy over how sunitinib resistance contributes to cell migration; in some studies, sunitinib resistance increased the cell migration rate (55, 56), while others have reported its reduction (57). In the present study, we demonstrated that sunitinib resistance in 786-O cells reduced cell migration. These results may be dependent on the sunitinib concentration, as low-dose sunitinib (1 μ M) seemed to promote cell migration, while high concentrations, such as 10 μ M, inhibited cell migration. As the half-maximal inhibitory concentration of sunitinib is $\sim$ 10 μ M in the 786-O cell lines (Figure 1A), we anticipate that high-dose sunitinib would provide a better model mimicking acquired resistance, as shown in previous studies (16).

Although *G*-1 inhibits cell growth and antagonizes sunitinib-resistant pathways, our phosphoproteomics analysis demonstrated that mTOR kinase was up-regulated in SunR 786-O cells, but inhibited in *G*-1-treated SunR 786-O cells. When compared to SunR cells, however, *G*-1 treatment of

SunR cells further up-regulated mTOR kinase activity. Similar results were also observed for PLK activity. RCC is known to activate the mTOR pathway during the development of resistance to therapies (4). In a recent study, activation of the PLK pathway was shown to promote proliferation and suppress apoptosis in RCC (58). Taken together, the significant up-regulation of mTOR kinase and PLK kinase in *G*-1-treated SunR 786-O cells raises concerns about the potential side-effects of *G*-1 on sunitinib-resistant cells.

Some limitations are present in this study. Although *G*-1 has been recognized as a specific GPER1 agonist, it may regulate RCC growth and phosphorylation dynamics in a GPER1-independent manner. Therefore, further studies are required to dissect the exact role of *G*-1 by using RCC cells with different GPER1 expression levels, such as *GPER1* knock-down or overexpression.

Conclusion

In conclusion, we demonstrated that the GPER1 agonist *G*-1 can regulate phosphorylation pathways related to RCC cell growth and migration, and antagonize the phosphorylation pathways activated by sunitinib resistance. Our results suggest a promising strategy for managing RCC and sunitinib resistance by GPER1 agonist *G*-1 and its regulated pathways.

Figure 5. G-Protein-coupled estrogen receptor 1 agonist G-1 down-regulated cell-cycle progression and cell migration. A: Parental (PAR) and sunitinib-resistant (SunR) 786-O cells were treated with dimethyl sulfoxide (DMSO) or 2 μ M G-1 for 48 h, and subjected to the designated analyses. A: Immunoblotting of cell-cycle-related proteins phospho-activating transcription factor 2 (P-ATF2) at Thr69 and 71 (T69/71), ATF2, cyclin-dependent kinase 1 (CDK1), cyclin B, cyclin D, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). B: Quantitation of cells in each cell-cycle phase (average $\pm$ SD) from flow cytometry. C: Typical photograph of migrated cells stained by calcein-AM. D: Quantification (average $\pm$ SD) of migrated cells shown in (C). All treatments were repeated in triplicate. Significantly different at $p < 0.05$ from *vehicle control, and #indicated group.

Conflicts of Interest

The Authors declare that they have no competing interests in regard to this study.

Authors' Contributions

SKC, YCW, and WCK designed the experiments. TYL and HJW performed experiments. CJH, and WCK analyzed the data. SKC, CJH, and WCK wrote the article.

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