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Original Research

Elevated sphingosine-1-phosphate lyase leads to increased metabolism and reduced survival in adrenocortical carcinoma

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Abstract

Objective: Adrenocortical carcinomas (ACCs) are invasive tumours arising in the adrenal cortex, and steroidogenic tumours are associated with worse prognostic outcomes. Loss-of-function mutations in sphingosine-1-phosphate lyase (SGPL1) cause primary adrenal insufficiency and as a key degradative enzyme in the sphingolipid pathway, SGPL1 also influences the balance of pro-proliferative and pro-apoptotic sphingolipids. We, therefore, hypothesized increased SGPL1 may be linked to increased disease severity in ACC.

Design: Analyse SGPL1 expression impact on patient survival and adrenal cancer cell phenotype. We analysed two ACC cohorts with survival and corresponding transcriptomic data, focusing on SGPL1 and sphingolipid pathway genes. *In vitro*, we generated SGPL1-knockout and overexpressing H295R adrenocortical cells to investigate the role of SGPL1 in cell signalling in ACCs.

Results: We found increased expression of several sphingolipid pathway receptors and enzymes, most notably *SGPL1* correlated with reduced patient survival in both cohorts. Overexpression of SGPL1 in the H295R cell line increased proliferation and migration while reducing apoptosis, while SGPL1 knockout had the opposite effect. RNA-seq revealed a global increase in the expression of genes in the electron transport chain in overexpressing cells, correlating with increased aerobic respiration and glycolysis. Furthermore, the opposite phenotype was seen in cells lacking SGPL1. We subsequently found the increased proliferation is linked to metabolic substrate availability and increased capacity to use different fuel sources, but particularly glucose, in overexpressing cells.

Conclusions: We, therefore, propose that SGPL1-overexpressing ACC tumours reduce patient survival by increasing fuel usage for anabolism and energy production to facilitate growth and invasion.

Keywords: SGPL1, sphingosine-1-phosphate lyase, sphingolipids, adrenocortical carcinoma

Significance

Adrenocortical carcinomas (ACCs) are associated with overall dismal patient survival, with limited treatment options available. Our studies incorporating analysis of public databases highlight several components of the sphingolipid pathway, most particularly Sphingosine-1-phosphate lyase (SGPL1) as potential therapeutic targets for ACCs. Our *in vitro* studies show overexpression of SGPL1 alters metabolic capacity and fuel usage in adrenocortical carcinoma cells, driving propensity for proliferation and migration, with SGPL1 knockout conferring a near opposite phenotype. The sphingolipid pathway may present the opportunity to provide bespoke patient treatment in ACC depending on the tumour expression of its key enzymes and receptors.

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Background

Adrenocortical carcinoma (ACC) has a global annual incidence of 0.7-2 individuals per million.[1,2] While rare, overall 5-year survival is poor at approximately 35% and the majority of patients develop associated metastases.[3,4] Outcomes remain dismal with conventional therapies including mitotane, a dicholorodiphenyltrichloroethane derived adrenolytic agent, particularly when associated with distant metastases where 5-year survival can be as low as 13%.[3,5] Identifying novel therapeutic targets, therefore, remains critical for this devastating disease.

Sphingolipids are defined by their 18-carbon amino alcohol backbone, modifications of which give rise to a variety of sphingolipid species playing an integral role in membrane biology and as bioactive signalling molecules.[6] Sphingolipid intermediates, particularly sphingosine/ceramide and sphingosine-1-phosphate (S1P), have opposing roles in regulating cancer cell death and proliferation, respectively.[7] The sphingolipid pathway is, importantly, amenable to therapeutic manipulation.[7] Sphingosine-1-phosphate lyase (SGPL1) carries out the final degradative step in the sphingolipid pathway, irreversible cleavage of S1P, governing the flux of the pathway away from ceramide to S1P (Figure 1A).

SGPL1 is implicated in adrenal disease, with total loss of enzyme activity and upstream accumulation of sphingolipid intermediates associated with a multisystemic syndrome incorporating primary adrenal insufficiency (PAI), first described in 2017.[8,9] *In vitro* modelling of the adrenal insufficiency disease using CRISPR-Cas9 engineered knockout of SGPL1 in the human adrenocortical tumour cell (H295R) line revealed significant reduction in steroidogenesis.[10] The disease mechanism has not yet been defined, however accumulation in upstream sphingolipids, in particular, sphingosine may directly attenuate the activity of steroidogenic factor 1 (SF-1).[11]

Sphingolipids play a role in regulating cellular bioenergetics[12] with a potential impact on cancer cell metabolism, with ceramides, in particular, regulating electron transport chain activity. As SGPL1 can also 'pull' the sphingolipid rheostat away from pro-apoptotic ceramide/sphingosine and towards anti-apoptotic S1P, we were prompted to consider the role SGPL1 may play in ACC.

In this study, we interrogate publicly available ACC transcriptomic data for the expression of sphingolipid pathway enzymes and receptors and find a number of associations with survival, most significantly with *SGPL1* expression. In determining sphingolipid intermediate flow (Figure 1A), expression of SGPL1 intrinsically influences adrenal tumour cell metabolism thereby impacting tumour progression. Using our original PAI *in vitro* model, together with a stable SGPL1-overexpressing H295R cell line, we investigate the role of SGPL1 in ACC metabolism, highlighting a novel regulatory mechanism for ACC tumour progression and a potential therapeutic target.

Methods

Cohort data

For mRNA abundance, 2 independent ACC cohorts were analysed; TCGA (The Cancer Genome Atlas) cohort[13] which included 79 ACC cases (https://portal.gdc.cancer.gov/projects/TCGA-ACC) and the cohort detailed by Assie and colleagues[14] that included 44 ACC cases (Gene Expression Omnibus data set GSE49280). For TCGA cohort, mRNA sequencing data were extracted from the GDC portal (https://portal.gdc.cancer.gov/; accessed October 7, 2020), and all calculations were performed on $\log_2$ values of FPKM-normalized read counts. Robust Multiarray Average algorithm normalized mRNA abundance was used for the Assie et al. Affymetrix data. The cohorts were divided into 2 equal-size groups according to the transcript abundance of the genes of interest. Overall survival was defined as time elapsed from the primary resection of ACC to death or last follow-up visit. Event-free survival was further analysed in the TCGA cohort (data available for 73 patients), with patients divided into their original groups as above for each gene of interest. One-way ANOVA testing was undertaken for comparison of COC subtyping with mRNA abundance ($\log_2$ values of FPKM normalized read counts). Data relating to hormone production for comparison with *SGPL1* abundance was available for 74 of the 79 patients in the TCGA cohort and all 44 patients in the cohort detailed by Assie and colleagues (grouped into hormone vs non-hormone producing). Survival curves were obtained by the Kaplan–Meier method and differences in survival were assessed with the log-rank test.

Crispr-Cas9 knockout of SGPL1

NCI-H295R cells were purchased from ATCC. Steroidogenic potential was monitored by regular cortisol assays and western blots for steroidogenic enzymes. Cells were discarded if they showed a significant drop in steroidogenesis, or at passage 30. H295R cells were cultured according to manufacturer's instructions and screened monthly for mycoplasma contamination.

We used the same 4 pairs of oligonucleotides previously used to create SGPL1 knockout HeLa lines,[15] as gRNAs for Crispr-Cas9 gene editing in H295R cells. We cloned each pair into pSpCas9(BB)-2A-GFP (PX458), a gift from Feng Zhang (Addgene plasmid #41838) according to the protocol described in.[16] Each of the 4 plasmids was then transfected individually into H295R cells using Lipofectamine™ 3000 according to manufacturer's instruction (Invitrogen). After 3 days, individual GFP-positive cells were isolated by fluorescence-activated cell sorting and seeded into separate wells in 96-well plates for each plasmid. Colonies were expanded and clonal knockout cell lines identified by Sanger sequencing. Knockout was confirmed by western blotting.

Generation of SGPL1-overexpressing cell lines

pCMV-SGPL1-C-GFPspark® (Origene) was packaged into lentiviral particles in HEK293T cells and concentrated in Vivapsin 20 columns (Sartorius). The concentrate was added to H295R cells and, after confirming successful transduction by fluorescence microscopy, 7 days later, the population of GFP-positive cells was isolated by fluorescence-activated cell sorting and expanded. SGPL1 overexpression was validated by western blotting. For the hygromycin cell line, we cloned a T2A-Hygromycin sequence in place of the C-terminal GFP linker and packaged lentiviral particles as above. Cells were then treated with 100 µg/ml Hygromycin B for 2 weeks, then returned to normal media to recover prior to validation and subsequent experiments.

For further methodology, please see the supplemental data

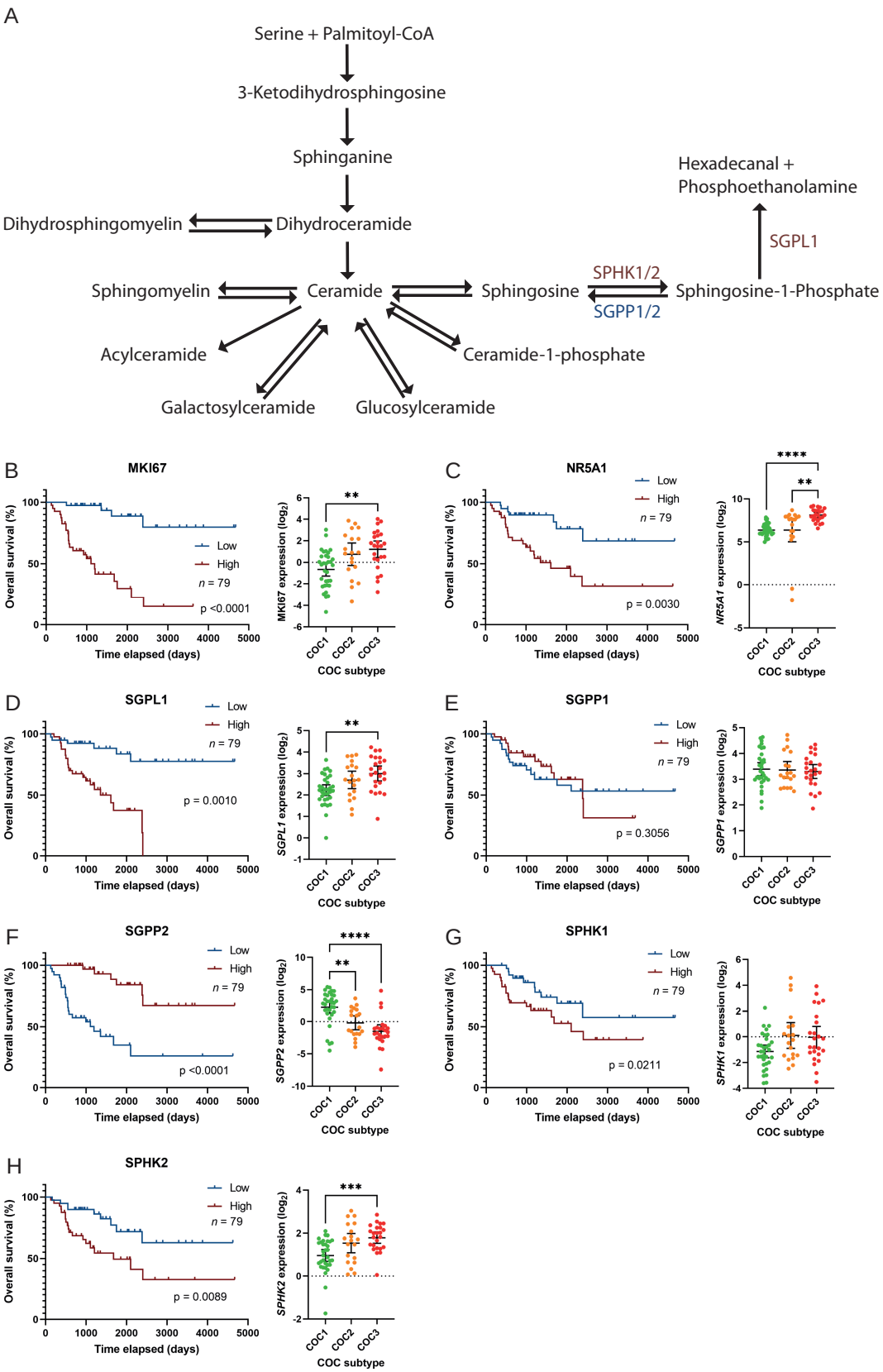

Figure 1. High SGPL1 expression confers worse patient survival prognosis. (A) Sphingolipid synthesis pathway. SGPL1 sits at the end of the sphingolipid pathway, acting as a 'rheostat' together with SPHK1/2 and SGPP1/2 controlling the flow of sphingolipids between anti-apoptotic sphingosine-1-phosphate and pro-apoptotic ceramide and sphingosine. Enzymes in red push the pathway towards the anti-apoptotic state, those in blue do the opposite. (B–H) Patient survival and gene expression data for current ACC biomarkers MKI67 (B) and NR5A1 (C) as well as SGPL1 (D), SGPP1 (E), SGPP2 (F), SPHK1 (G), and SPHK2 (H). Data taken from TCGA ACC cohort. For each gene, we present Kaplan–Meier survival curves for high expressing (median and above) and low expressing (below median) groups and cluster-of-cluster (COC)-based expression analysis. Tick marks indicate right-censored data. $n = 79$ patients. Data displayed are mean ± 95% CI. * = adj. $P < 0.05$, ** = adj. $P < 0.01$, *** = adj. $P < 0.001$, **** = adj. $P < 0.0001$. Kaplan–Meier (log-rank) test for survival, Kruskall–Wallis test with Dunn's multiple comparisons between each pairing. Non-significant comparisons are not displayed for figure clarity.

Results

High SGPL1 expression is associated with poor survival prognosis in ACC

We sought to examine how the expression of sphingolipid enzymes and particularly SGPL1 correlates with survival probability in ACCs. Within the TCGA database, there were 79 patients with ACCs and corresponding RNA-seq expression data. Sorting the patients into high ($n = 40$) and low ($n = 39$) expression for each gene, we found decreased survival associated with high expression of established prognostic/diagnostic biomarkers *MKI67* and *NR5A1* (SF-1)[17–20] (Figure 1B and C). Seventy-six of the 79 patients with RNA-seq data also had cluster of cluster (COC) scoring, incorporating DNA copy number, DNA methylation, RNA expression, and microRNA expression data to classify patients into 1 of 3 clusters. Cluster of cluster group I (COCI) have the least aggressive tumours, with only 7% disease progression rate while COCIII are the most aggressive and disease progression rate is 96%.[13] MKI67 and NR5A1 expression were both significantly higher in COCIII than COCI (Figure 1B and C). Strikingly, high SGPL1 expression is significantly correlated with lower survival, and expression is increased in the COCIII subgroup (Figure 1D). Sphingosine-1-phoshate phosphatase 1 (*SGPP1*) expression does not correlate with reduced survival or COC subtype (Figure 1E), while high *SGPP2* expression is associated with *increased* survival and *SGPP2* expression is significantly higher in COCI than COCII and COCIII (Figure 1F). In contrast, high *SPHK1* and *SPHK2* expression correlate with reduced survival, and *SPHK2* is expressed significantly higher in COCIII (Figure 1G and H).

We subsequently analysed SGPL1 expression in a small cohort of ACC tissues, using nuclear Ki67 staining intensity as a marker of increased proliferation and a proxy for a more aggressive tumour (Figure S1A). We found a significant correlation of H-scores for SGPL1 and nuclear Ki67 in our patient dataset (Figure S1B). There was no significant difference in SGPL1 expression between cortisol-producing and non-producing tumours (Figure S1C). SGPL1 expression in adrenocortical adenoma was not analysed in this small series nor has it been described in the literature.

Extracellular S1P signals through a family of 5 g-protein-coupled receptors, sphingosine-1-phosphate receptors 1-5 (S1PR1-5), each coupled to a different subset of G-alpha subunits with distinct downstream signalling. S1PR1 and S1PR3 are considered pro-tumourigenic, S1PR2 can have both pro- and anti-tumourigenic effects, while the picture is less clear for S1PR4 and S1PR5.[21] We found increased *S1PR2* and *S1PR3* expression correlated with reduced survival in the TCGA ACC cohort (Figure S2B and C). No correlations were found for the other S1P receptors (Figure S2A, D and E).

COCII-III are largely similar to the transcriptomic subtype C1A defined by Assie and colleagues,[14,22] while C1B is similar to COCI. We stratified our cohort by C1A/C1B subclass, finding similar distribution of data as found with COC analyses (Figure S3A–L). The subclass of C1A patients had increased expression of *SPGL1*, *SPHK1*, *SPHK2*, *S1PR3*, *and S1PR5* and reduced expression of *SGPP2* and *S1PR4*.

Event-free survival analysis of TCGA ACC patients recapitulated results for overall survival analysis (Figure S4A–L). Given the loss of SGPL1 reduces steroidogenesis, we hypothesized increased SGPL1 may be associated with increased steroidogenesis.[8,9] Steroid hormone-producing ACCs have worse clinical outcomes than hormone non-producing ACCs, and we found increased *SGPL1* expression in steroid-producing tumours (Figure S4M).

We then analysed the dataset from Assie and colleagues,[14] which include 44 ACC patients and corresponding survival and Affymetrix array data. Similar to the TCGA data, we found reduced survival in patients with high *SGPL1* and increased survival in patients with high *SGPP2* (Figure S5). Both datasets seem to indicate enzymes that reduce the pool of ceramide and sphingosine and increase the flow of metabolites towards S1P (*SGPL1*, *SPHK1/2*) reduce patient survival while those that increase sphingosine and ceramide levels (*SGPP2*) increase patient survival. We also found reduced survival in patients with higher *S1PR2* expression (Figure S5H). *S1PR3* was not included in this Affymetrix array data. As with the TCGA cohort, we found increased *SGPL1* expression in steroidogenic compared with steroid non-producing ACCs (Figure S5K).

SGPL1 overexpression correlates with increased proliferation and migration in H295R cells

To understand how increased SGPL1 might reduce patient survival, we generated an *SGPL1* overexpression cell line using NCI-H295R cells. Wild-type H295R cells were transduced with lentiviral particles facilitating the integration of a gene encoding SGPL1 fused to GFP at the C-terminus, followed by cell sorting for GFP-positive cells. GFP fusion at the C-terminus of SGPL1 does not affect lyase function.[23] We also generated a knockout line via Crispr-Cas9 to examine the effect of reduced SGPL1 expression (Figure 2A).

Many pathways affected by S1P signalling through the S1P receptors converge on proliferation (Ras, PI3K). We found increased viability in SGPL1-OE cells compared with wild-type, and significantly reduced viability in SGPL1-KO cells (Figure 2B). Differences in viability may be partly due to changes in the balance of pro- and anti-apoptotic sphingolipids and ceramides. Mass spectrometry for a panel of sphingolipids from cellular lipid fractions revealed increased levels of all sphingolipid intermediates including and upstream of S1P in SGPL1-KO cells (Figure 2C). In contrast, SGPL1-OE cells had reduced ceramide and sphingomyelins compared with wild-type. There was no difference in the level of sphingosine compared with wild-type, however, there was a reduction in S1P, confirming the SGPL1-GFP fusion protein is catalytically active. We found increased phosphoethanolamine (PE) in the SGPL1-KO cells, however, it is worth noting the breakdown of S1P by SGPL1 is not the only method of PE synthesis. There was conversely a lower level of PE in the OE cells, though this may also be due to a rapid turnover of PE to other lipid metabolites such as CDP-ethanolamine, phosphatidylethanolamine or phosphatidylcholine. Studies have shown reduced autophagic flux in response to the loss of SGPL1, partly due to the loss of PE.[24] Unlike the previous report in neuronal cells, we found no difference in autophagic flux in SGPL1-KO or OE H295R cells (Figure S6A–J), indicating differences seen in PE (Figure 2C) do not translate to differences in autophagy.

Lowered ceramide levels may reduce apoptotic drive in OE cells. We assessed apoptosis by treating cells with 0, 25, 50, and 100 µg/ml cycloheximide (CHX) for 24 h and performed western blotting for cleaved caspase-3 and cleaved poly-ADP ribose polymerase (PARP). We found a significant reduction in cleaved PARP and cleaved-caspase 3 expressions in the

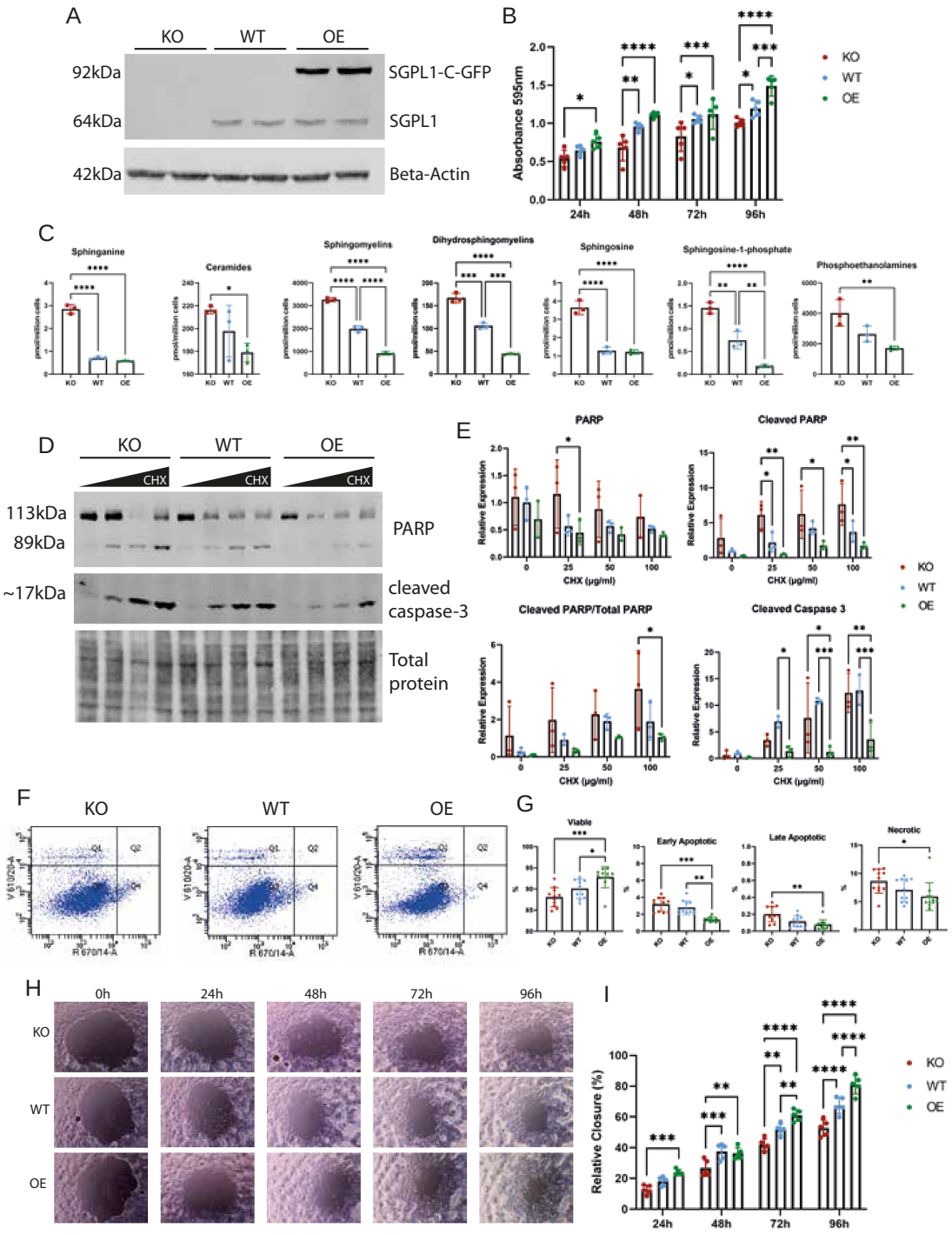

Figure 2. Increased SGPL1 expression correlates with increased proliferation and migration, reduced apoptosis in H295R cells. (A) Loss of SGPL1 expression in KO cells and increase in SGPL1-GFP in OE cells. (B) MTT assay revealed increased proliferation at 96 h in SGPL1-OE cells, decreased in KO. (C) Cellular levels of sphingolipid species measured by LC-MS. (D) Reduced expression of cleaved-caspase 3 and PARP in OE cells following cycloheximide treatment. Representative western blot, $n = 3$ experimental repeats. (E) Densitometric quantification of 3 experimental repeats of (D). (F) FACS plot showing viable cells (Q3), Early apoptotic cells (Q4), late apoptotic cells (Q2), and dead cells (Q1) as determined by Annexin V/PI staining. (G) Quantification of cell populations in (F), $n = 11$ experimental repeats. (H) Representative circle scratch wounds at from 0 to 96 h in SGPL1-KO, WT, and OE H295R cells. (I) Quantification of (H), $n = 5$ experimental repeats. Data displayed are mean ± SD. * = adj. $P < 0.05$, ** = adj. $P < 0.01$, *** = adj. $P < 0.001$, **** = adj. $P < 0.0001$. One-way ANOVA with Tukey's multiple comparisons between each pairing. Non-significant comparisons are not displayed for figure clarity.

OE cells compared at all 3 doses (Figure 2D and E). To assess baseline apoptotis, we performed flow cytometry with annexin V and propidium iodide (PI). Even in unstimulated cells, there were fewer early and late apoptotic cells in the OE group. These data collectively indicate there is increased viability in SGPL1 overexpressing cells, which may in part be due to reduced apoptosis. The reduced viability in the KO cells does not appear to be due to differences in apoptosis.

Among the other pro-tumourigenic pathways downstream of S1PR signalling is migration. Signalling through S1PR3,

in particular, induces alterations in Rac and Rho signalling, both of which may contribute to increased invasiveness.[21] To assess differences in migration, we seeded cells at full confluency and then produced a circular wound via a vacuum pump, as described in[25] and monitored wound closure. Cells were pre-treated with 5μg/ml mitomycin C to inhibit proliferation. SGPL1-OE cells filled the wound significantly faster than WT cells, while SGPL1-KO cells migrated significantly slower (Figure 2H and I). Crucially, there were no differences in viability in cells treated with mitomycin C in a parallel experiment, indicating the differences are likely due to differences in migratory capacity (Figure 2J).

Transcriptomic analysis highlights the enrichment of calcium handling and oxidative phosphorylation genes in SGPL1-KO cells

Prolonged gain or loss of SGPL1 causes cell- and tissue-type specific changes in the levels of sphingolipids.[23,24,26–28] We hypothesized this may be due to the transcriptional reprogramming of the different cell types in response to SGPL1 levels and consequent lipid imbalance. RNAseq was performed on all three cell lines and principal component analysis revealed the transcriptomic signatures of the biological replicates clustered together and in distinct groups (Figure 3A). Using a cut-off of an adjusted *P*-value less than 0.05, there were 2711 significantly differently expressed genes between SGPL1-WT and -KO, 2568 between SGPL1-WT and –OE, and 5330 between SGPL1-KO and -OE (Figure 3B and C). Interestingly, we did see some differences in the expression of enzymes in the sphingolipid pathway (Figure S7A). Ceramide synthase 4 and 5 (*CERS4/5*) were upregulated in OE cells while *CERS6* was downregulated. Different ceramide synthase family members are selective for different acyl-CoA substrates and consequently generate different ceramide chain lengths. Differences in expression may reflect differences in substrate availability caused by lipid dysgenesis or alterations in other metabolic pathways.

We analysed significantly different genes by ingenuity pathway analysis (IPA) to determine whether particular pathways were functionally enriched in our comparisons. While there were no obvious candidate pathways that were significantly enriched in both WT vs. OE and KO vs. OE, calcium signalling was functionally enriched in the KO group in comparison to both OE and WT (Figure 3D), potentially indicating altered calcium storage in the KO cells. Examining the significant genes further, we found increased expression of calcium pumps (*ATP2A2, ATP2B1*) and decreased calcium channels (*RYR1, RYR3*) in KO cells, all of which regulate the flow of calcium ions between the ER into the cytoplasm (Figure S7B). We assessed calcium ER storage by Fura-2-AM assay and stimulating with 1 μM thapsigargin. While there was a significantly smaller calcium release from the ER in the SGPL1-KO compared with the SGPL1-OE cells, neither was different with respect to the wild-type (Figure S7C and D). Equally, there were no differences in the baseline calcium levels (Figure S7E), potentially indicating the cytoplasmic calcium levels were similar.

SGPL1 expression drives increased oxidative phosphorylation gene expression and subsequent metabolic rate in H295R SGPL1-OE cells

Gene set enrichment analysis (GSEA) of all genes revealed altered expression of genes in the Steroid Hormone Biosynthesis pathway in KO cells compared with WT (Figure 3E), mimicking what we found in a recent parallel study where we found that SGPL1-KO H295R cells are less steroidogenic. There were no differences in steroidogenic gene expression or cortisol production between WT and OE cells (data not shown), similar to what we showed in Figure S1C.

Instead, we found a striking enrichment in oxidative phosphorylation genes in the OE group compared with the WT and KO (Figure 3E, red box). Intriguingly, nuclear-encoded ETC genes (e.g. *NDUFB10*, *UQRCQ*, and *ATP5F1D*) were downregulated in the KO but mitochondrial encoded genes (e.g. *MT-ND1, MT-ATP6*) were upregulated relative to WT, potentially indicating changes in mitochondrial organization, as we have shown in SGPL1-KO HeLa cells.[27] Western blot using an OXPHOS antibody cocktail (Figure 3F) showed increased protein expression of NDUFB8, SDHB, and UQCRC2 (Figure 3G), mirroring the RNA-seq data (Figure S8A–C).

This led us to hypothesize there may be increased respiration in the OE cells which could account for the increased proliferative capacity. We used the Seahorse MitoStress Test kit to measure oxidative respiration (Figure 4A). SGPL1-OE cells have higher rates of basal respiration, higher maximal respiration, and ATP production (Figure 4B), perhaps due to the increased expression of the proteins relied on for these processes (Figure 3G). We also find increased basal and maximal glycolysis in OE cells (Figure 4C and D).

We sought to determine whether increases in metabolic rate and proliferation were due to an increase in fuel usage rate or a modified fuel preference. We grew cells in nutrient-poor media and supplemented with either 17.4 μM D-glucose, 2.5 mM L-glutamine, 100 μM palmitic acid, or a combination of 2 or all 3, and observed the effects on proliferation. Growing cells in minimal nutrient media abrogated all growth differences between WT and OE, while OE cells grew significantly faster in media replete with all 3 fuel sources (Figure 4E and F). KO cells grew marginally but significantly slower even in blank media. Supplementing with either L-glutamine, palmitic acid or both together did not produce differences in growth rate between WT and OE (Figure S9A–C). Only in the presence of D-glucose did we reproduce the differences in growth rate (Figure 4G, Figure S9D and E).

To determine whether OE cells have an increased usage and reliance on D-glucose, we performed substrate oxidation assays, measuring basal respiration before and after the addition of an inhibitor of glucose, glutamine or free fatty acid metabolism, and thereafter maximal respiration (Figure 4H–K). We saw significantly higher basal respiration in the OE cells before treatment, as previously (Figure 4L). Treating with BPTES, a glutaminase inhibitor, produced only a modest decrease in respiration, while Etomoxir, an inhibitor of carnitine palmitoyltransferase-1, reduced respiration significantly more in the KO cells (Figure 4M). UK5099, a mitochondrial pyruvate carrier inhibitor, produced the greatest decrease in respiration in all 3 groups. OE cells were still capable of higher maximal respiration following treatment with BPTES and Etomoxir, but not UK5099 (Figure 4N). These data indicated the difference in proliferation may be due to the increased ability to use glucose to either produce ATP or for anabolism. We performed Seahorse Fuel Flux assays to measure each cell types dependence (how much it relies on that fuel source normally) and capacity (ability to use that fuel source if no others are available) for glucose, fatty acids and glutamine. We found

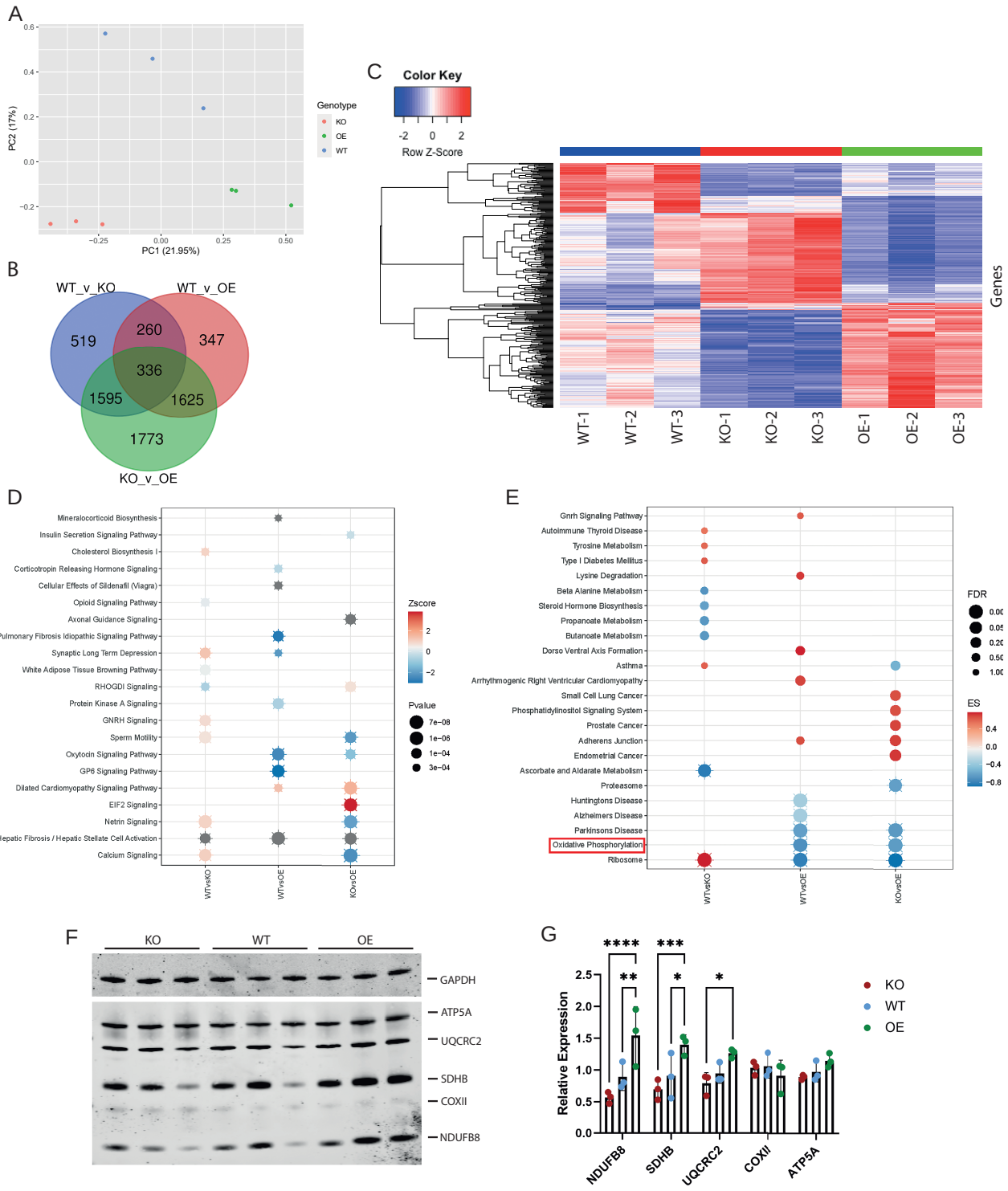

Figure 3. RNA-seq analysis identifies enrichment of differentially expressed genes regulating calcium handling in SGPL1-KO cells, oxidative phosphorylation in OE. (A) Principal component analysis (PCA) plot of the 9 samples analysed by RNA-seq, showing the clustering of samples by genotype. (B) Venn diagram of differentially expressed genes from each comparison showing large amount of overlap between WT vs KO and KO vs OE analysis, as well as WT vs OE and KO vs OE, but less of an overlap between WT vs KO and WT vs OE. (C) Heatmap of differentially expressed genes. Row clustering by unsupervised hierarchical clustering. (D) Dot-plot of top 10 functionally enriched pathways from DE genes from each comparison by ingenuity pathway analysis (IPA, QIAGEN). Colour indicates the direction of functional enrichment by *z*-score and size of dot indicates significance. Grey dots indicate low confidence in predicting the direction of functional enrichment, despite enrichment for genes in that pathway. 'Spikes' indicate significant enrichment. Analysis indicates likely increased calcium signalling in KO compared with WT and in KO compared with WT (colours flipped due to analysis being conducted as OE vs KO rather than KO vs OE). (E) Gene Set Enrichment Analysis reveals increased expression of genes involved in oxidative phosphorylation in SGPL1-OE cells compared with WT and SGPL1-KO, and reduced expression in KO compared with WT. The figure shows top 10 enriched pathways in each comparison. Positive enrichment scores indicate increased expression in the first group (i.e. WT in 'WT vs KO') compared with the second, while a negative enrichment score indicates increased expression in the second group compared with the first. (F, G) Western blot with OXPHOS cocktail antibody in untreated H295R SGPL1-KO, WT, and SGPL1-OE lysates. Representative image of experimental triplicates. Increased expression of NDUFB8, SDHB, and UQCRC2 in OE cells. Data displayed are mean ± SD. * = adj. $p < 0.05$, ** = adj. $P < 0.01$, *** = adj. $P < 0.001$, **** = adj. $P < 0.0001$. One-way ANOVA with Tukey's multiple comparisons between each pairing. Non-significant comparisons are not displayed for figure clarity.

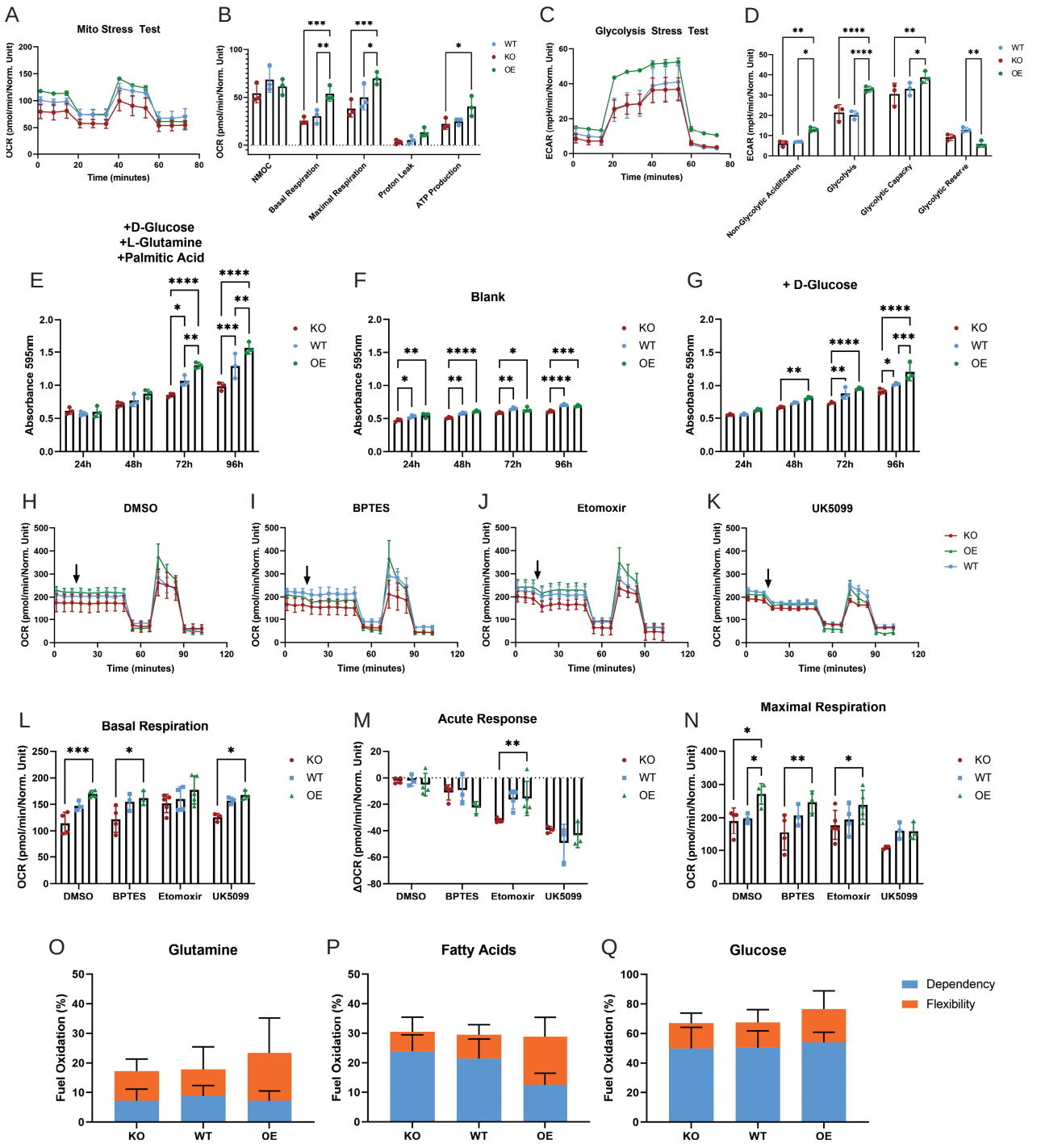

Figure 4. SGPL1 overexpression increases basal and maximal mitochondrial respiration, driving increased proliferation. (A) Representative oxygen consumption rate (OCR) experiment in H295R SGPL1-KO, WT, and SGPL1-OE cells following Mito Stress Test injection paradigm. $n = 8$ technical replicates. $n = 3$ experimental replicates. (B) Non-mitochondrial oxygen consumption (NMOC), basal respiration, maximal respiration, proton leak, and ATP production in each cell type. (C) Representative extracellular acidification rate (ECAR) experiment in H295R SGPL1-KO, WT, and SGPL1-OE cells following Glycolytic Stress Test injection paradigm. $n = 10$ technical replicates, $n = 3$ experimental replicates. (D) Non-glycolytic acidification, basal glycolysis, maximal glycolytic capacity, and non-glycolytic acidification in each cell type. (E, F, G) MTT assays of cells grown in basal media supplemented with glucose, glutamine, and palmitate (E), nothing (F), or glucose alone (G). (H, I, J, K) Representative OCR traces for Substrate Oxidation Stress Test paradigms for cells treated with DMSO (H), BPTES (I), Etomoxir (J), or UK5099 (K). (L) Basal respiration prior to drug addition. (M) OCR response to drug treatment. (N) Maximal respiration following drug treatment. (O, P, Q) Dependency and flexibility of each cell type to metabolize glutamine (O), fatty acids (P), and glucose (Q) for respiration. $n = 8$ technical replicates, $n = 3$ experimental replicates. Data displayed are mean ± SD. * = adj. $P < 0.05$, ** = adj. $P < 0.01$, *** = adj. $P < 0.001$, **** = adj. $P < 0.0001$. One-way ANOVA with Tukey's multiple comparisons between each pairing. Non-significant comparisons are not displayed for figure clarity.

that the OE cells have a greater capacity (dependency + flexibility) for glucose utilization, but not for fatty acids or glutamine. This could explain why in the presence of only glutamine or palmitate, the OE cells grow at the same rate as the WT cells, while in the presence of glucose they are able to grow faster.

Discussion

In recent years pan-genomic characterization has highlighted novel biomarkers for ACC,[17,22,29] some of which pose interesting therapeutic targets. However, as mitotane is still the only FDA and EMA-approved drug for the treatment of ACC, the search for new therapeutic targets remains critical. A recent study found a network of lipid metabolism genes upregulated in ACCs, including SGPL1.[30] We found a similar result in our studies, while we also found alterations in the expression of other sphingolipid pathway genes (SPHKs/SGPPs/S1PRs) also correlated with significant differences in survival, as was seen for SPHK1 in a previous study.[31] Therefore, the pathway as a whole may represent a possible therapeutic target and crucially, one that has been targeted in cancer and other diseases and for which drugs are already available.[31]

The sphingolipid pathway is gathering interest in cancer biology, particularly in view of the purported actions of pro-proliferative S1P.[30,32–35] High expression of *SGPL1* is uniquely associated with poor survival prognosis in ACC when compared with other cancers in the TCGA data set, indeed in some cancers, it confers an *improved* prognosis.[36–38] Upregulation of SGPL1 expression is however also described in ovarian cancer[39] and chemotherapeutic-resistant ovarian tumours.[40] SGPL1 plays an integral role in governing the dynamic balance of sphingolipid flux. While high SGPL1 leads to breakdown of S1P, it also in turn promotes catabolism and reduced accumulation of pro-apoptotic ceramides, as is seen in our *in vitro* ACC model.

SGPL1 may represent a viable therapeutic target in ACC. While total loss of lyase activity is associated with multisystemic disease, importantly, incomplete pharmacological SGPL1 inhibition has few side effects, as seen in clinical trials for the treatment of rheumatoid arthritis.[41] Fingolimod (FTY720), which is currently approved for the management of MS and inhibits S1PR1, 3, & 5, has been shown to have anti-tumour efficacy *in vitro* and *in vivo* in several cancer cell models. It is a pro-drug that is phosphorylated primarily by SPHK2 and was recently shown to be efficacious, inhibiting H295R growth *in vitro* and *in vivo*.[31] Interestingly, it has also been demonstrated as an SGPL1 inhibitor both *in vitro* and *in vivo* murine studies.[42] SPHK inhibition, including with isoform selective inhibitors, is being targeted in clinical trials for several cancers.[43,44] In a clinical study, the use of intravenous Safingol, a putative SPHK inhibitor, in patients with advanced solid tumours stabilized disease in 6 individuals, notably 2 with ACC, one of whom also had a significant reduction in metastases.[45] Collectively, these studies and ours suggest there is plenty of scope for developing and using drugs targeting various parts of the sphingolipid pathway in patient tumours.

Our data suggest the increased pro-tumourigenic features of SGPL1^{high} ACC tumours may be due, in part, to an increase in cellular respiration rates partly due to an increased capacity to metabolize fuel faster. Cancer cells are known to alter their metabolism, most famously via the Warburg effect, which serves to increase the anabolic capacity of cells in order to increase proliferation.[46] SGPL1 overexpressing cells had increased glycolysis, however, this did not come at the expense of reduced oxidative phosphorylation, rather, it was increased. We hypothesize this reflects an increase in the rate of utilization of sugars, for oxidative phosphorylation but potentially also for increasing anabolism. An increase in oxidative phosphorylation, even in the face of active glycolysis, has been found in other tumours, including pancreatic ductal adenocarcinoma, leukaemia, lymphomas and endometrial carcinoma, and is an emerging target for cancer therapy.[47] Increased mitochondrial DNA content and expression of electron transport chain proteins are amongst the mechanisms described in tumours with ongoing high OXPHOS activity.[47]

SGPL1 plays a critical role in Acetyl CoA regulation, which is used for *de novo* synthesis of ceramides, while the downstream conversion of ceramide to sphingosine releases free fatty acids[48] The increased glycolysis in OE cells may reflect the increased drive for Acetyl CoA generation from glucose to fuel the sphingolipid pathway, as the flux in this pathway is increased by high SGPL1 levels. The sphingolipid pathway can also impact OXPHOS where ceramides can influence the function of the mitochondria, for instance regulating electron transport chain activity and mitochondrial fission.[12] *In vitro* treatment with C16 ceramides, sphingosine or sphinganine inhibits complex IV,[49] with C16 ceramides purported to additionally inhibit complex II.[50] In these studies, the accumulation of ceramides results in a decrease in ATP production. In our OE cells, we found decreases in ceramides, which may allow for increased complex IV activity and metabolic capacity. It would be of interest to see whether increased cellular metabolic activity and sphingolipid profiling of patient-derived ACC mirror our *in vitro* model, with scope to test the impact of inhibitors of these pathways.

In this study, we find *SGPL1* expression is significantly higher in steroidogenic compared with non-steroid producing ACCs (Figure S4M and S5K), where steroidogenic ACC typically confer worse prognosis.[13] While we do not see a difference between steroidogenesis between WT and SGPL1 OE H295R cells, we have previously demonstrated that SGPL1 KO H295R cells have significantly reduced steroidogenic capacity, and the impact of the balance of accumulated sphingolipids on steroidogenesis is yet to be fully determined in this model.[10] Sphingolipids, such as ceramide and sphingosine, diminish steroidogenesis with sphingosine directly attenuating steroidogenic factor-1 activity.[11,51,52] Mitochondrial dysfunction associated with reduced steroidogenesis is also described in SGPL1 deficient patient cells.[27] Beyond its impact on cancer cell metabolism, proliferation, and migration, SGPL1 inhibition as a therapeutic strategy may also bear the added advantage of impeding steroidogenesis in ACC.

In conclusion, we have identified a potential novel regulator of ACCs, whereby increased *SGPL1* expression correlates with reduced overall patient survival. This may be due to increases in the expression/activity of metabolic enzymes, particularly those required for glucose metabolism. Increased ATP production and anabolisis could facilitate faster cell division which, paired with reduced apoptotic signalling, increases proliferation in cells with elevated *SGPL1* expression. Future work will aim to validate SGPL1 as a novel prognostic marker in a large independent series of ACCs at protein level and test the ability of inhibitors of the sphingolipid pathway to reduce the pro-tumourigenic phenotype of SGPL1^{high} cells and tumours, which we hope will lead to the elucidation of novel insights into the pathogenesis of ACCs and the generation of new therapeutics.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Conflicts of interests

The authors declare no competing interests.

Ethics approval and consent to participate

ACC specimens were collected from patients undergoing surgery at each of St Bartholomew's, University College and Hammersmith Hospitals, London, after written consent obtained from participants and under the study protocol Genetics of endocrine tumours (REC: 06/Q0104/133). This study protocol was approved by Huntingdon Research Ethics Committee. The study was performed in accordance with the Declaration of Helsinki. No individual patient data is presented in this manuscript.

Data availability

All fast q files for this research have been deposited in the NCBI Gene Expression Omnibus (GEO) repository and can be accessed via GSE190177.

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