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## Somatostatin Receptor 2 signaling promotes growth and tumor survival in Small Cell Lung Cancer

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### Abstract

Somatostatin receptor 2 (SSTR2) is overexpressed in a majority of neuroendocrine neoplasms, including small cell lung carcinomas (SCLCs). SSTR2 was previously considered an inhibitory receptor on cell growth, but its agonists had poor clinical responses in multiple clinical trials. The role of this receptor as a potential therapeutic target in lung cancer merits further investigation. We evaluated the expression of SSTR2 in a cohort of 96 primary tumors from patients with SCLC and found 48% expressed SSTR2. Correlation analysis in both CCLE and an SCLC RNAseq cohort confirmed high level expression and identified an association between NEUROD 1 and SSTR2. There was a significant association with SSTR2 expression profile and poor clinical outcome. We tested whether SSTR2 expression might contribute to tumor progression through activation of downstream signaling pathways, using *in vitro* and *in vivo* systems and down regulated SSTR2 expression in lung cancer cells by shRNA. SSTR2 downregulation led to increased apoptosis and

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dramatically decreased tumor growth *in vitro* and *in vivo* in multiple cell lines with decreased AMPK $\alpha$  phosphorylation and increased oxidative metabolism. These results demonstrate a role for SSTR2 signaling in small cell lung carcinoma and suggest that SSTR2 is a poor prognostic biomarker in SCLC and potential future therapeutic signaling target.

### Keywords

Somatostatin; survival; cancer progression

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### Introduction:

Small cell lung cancer (SCLC) is a high grade poorly differentiated neuroendocrine carcinoma of the lung which represents approximately 15% of diagnosed lung cancers and up to 25% of lung cancer deaths<sup>1</sup>. SCLC is associated with early metastasis and poor patient survival<sup>2</sup>. Overall survival in SCLC is dismal with a five year survival of ~ 2% for extensive stage metastatic disease, which comprises 70% of cases at initial diagnosis<sup>3</sup>. Unfortunately, SCLC has not benefited from recent advances in targeted therapies<sup>1, 4</sup> and treatment has not significantly advanced in the last 20 years. Clearly, SCLC is in desperate need of efficacious targeted agents. One promising treatment avenue has focused on exploiting the neuroendocrine phenotype of SCLC. We previously established a link between neuroendocrine signaling and SCLC<sup>5</sup> and herein we expand those results and demonstrate a role for somatostatin signaling in the growth and pro tumor survival of SCLC.

Somatostatin receptors (SSTR) are 7 spanning trans-membrane G protein coupled receptors with multiple functions in both normal and tumor tissues. Somatostatin receptors have widespread but variable tissue expression in normal tissue. They are diversely expressed in multiple tumor types including a subset of breast, prostate, pancreatic, neuroendocrine, and hepatocellular carcinomas<sup>6, 7</sup>. Five subtypes of SSTRs have been isolated; however, research has focused on SSTR2, which is the best characterized member of the SSTR family. SSTR2 has multiple direct and indirect effects on cell cycling, angiogenesis, apoptosis, and growth factor signaling. It classically signals by inhibition of adenylate cyclase, inhibits calcium influx, increases p53 influx, and acts primarily through downstream mitogen activated protein kinase (MAPK) and protein kinase B (AKT) as well as other downstream kinases<sup>6-9</sup>. SSTR2 is canonically viewed as an inhibitory receptor on cell growth, but reports using SSTR2 agonists to activate this pathway to date have resulted in few significant anti-tumor responses with the exception of low grade carcinoid tumors as well as control of adrenocortical syndrome in small cell lung carcinoma<sup>6, 7, 10</sup>. Given the above findings, it is surprising that despite SSTR2's canonical role as an inhibitory receptor, trials have had poor clinical responses to SSTR2 agonists in high grade neuroendocrine and other malignancies<sup>6</sup>. Our investigations demonstrate that SSTR2 signaling is not inhibitory in high grade neuroendocrine malignancy, but rather serves as an important pro tumor survival signal in a subset of SCLC cell lines/tumor tissue *in vivo* and *in vitro* with loss of SSTR2 expression leading to profound effects on apoptosis with significant clinical implications.

## Materials and Methods:

### CCLE and RNAseq analysis:

Data was obtained from the CCLE database and analyzed using both R, heatmap2, pheatmap, and IGV analysis tools respectively, making use of the available resources for adenocarcinoma, squamous cell carcinoma of the lung, and we specifically analyzed and performed correlative analysis on 53 SCLC cell lines.<sup>11</sup> RNAseq analysis was performed using data released by George et al<sup>12</sup> on a cohort of 81 primary SCLC patient tumor samples. Spearman correlation was calculated and used for hierarchical clustering. Both raw and normalized (across the sampled human primary tumors) FPKM was used for clustering analysis and evaluation of gene expression for the RNAseq samples. mRNA expression from the CCLE SCLC cell lines was determined using the Affymetrix CEL file converted using Robust Multi-Array Average followed by quantile normalization (Supplemental Figure 2) followed by normalization across the 53 SCLC lines (Figure 1).

### Immunohistochemistry:

Immunohistochemical (IHC staining and antigen retrieval for somatostatin receptor 2A (SSTR2A, Biotrend, Schwabhausen, Germany) was performed on 5  $\mu$ M formalin-fixed paraffin-embedded sections as described<sup>13</sup> and interpreted visually by an experienced pathologist (CS). The tissues were scored for no (0), weak (1+), moderate (2+) or strong (3+) and % of immunoreactive tumor cells to obtain an IHC score (1–300). IHC score was obtained by multiplying the % of SSTR2+ (1–100%) cells by the staining intensity observed (1+ to 3+). SSTR2A immunoreactivity was evaluated in all cellular elements within the pathologic specimens, including tumor cells, inflammatory cells and the endothelium of small blood vessels. Immunoreactivity from neovasculature, plasma cells, or macrophages were excluded from the IHC scoring. The tumor microarrays (TMAs) used in this study were previously described<sup>5</sup>. TMAs were stained for SSTR2A as noted. Pathology blocks were retrieved from the archives of the Department of Pathology at Vanderbilt University Medical Center, Nashville VA Medical Center, and St. Thomas Hospital in Nashville, Tennessee. The summary of patient characteristics for the 98 tumors is in Supplemental Table 1. Images were captured using an Olympus BX41 (Olympus). The study was approved by the institutional review board at each medical center.

### Immunoblotting:

Western blotting was performed similarly to<sup>14</sup>. Briefly, cells were de-adhered, lysed in lysis buffer containing phosphatase and protease inhibitors at 4°C, centrifuged, and stored at –80°C. Protein concentration was quantified by BCA and 20–40  $\mu$ g of protein was loaded per well of 4–20% polyacrylamide Tris Glycine wells (Life Technologies, Grand Island, NY). Blots were visualized using ECL reagent (Perkin-Elmer, Waltham, MA). Human phospho kinase array analysis (R&D systems ARY003B) was performed per manufacturer recommendations. Phospho kinase array immunoblots were quantified using an inverse intensity method in Photoshop using mean pixel intensity and the manufacturer specified dot size<sup>15</sup>.

**Antibody Generation, affinity purification and testing:**

Anti-SSTR2 polyclonal antibody (VU430AP) was generated and purified by the Vanderbilt Antibody and Protein Resource (VAPR). Briefly, rabbits were immunized against peptide (WSTNTSNQTEPYDLTSN), sera was affinity purified and processed for use in immunoblotting (Supplemental Figure 1). New polyclonal antibody was generated for use in immunoblotting due to a lack of available precise SSTR2 immunoblot antibody at the start of these studies. This antibody was tested by IHC in FFPE samples, but did not stain the epitope to IHC quality criteria, likely due to formalin effects.

**Antibodies and Reagents:**

Anti SSTR2 for immunoblotting was generated as noted below. All antibodies were used per manufacturer recommendations and include  $\alpha$ -tubulin, BCL-xL, BIM, BID, BAX, Cleaved caspase 9, caspase 9, Cleaved Caspase 7, Caspase 7, Cleaved caspase 3, caspase 3, p-AKT, AKT, p-ERK, ERK (Cell signaling, Danvers, MA), BCL-2 (Santa Cruz, Dallas, TX), GAPDH (Genetex, Irvine, CA), and  $\beta$ -actin (Sigma, St. Louis, MO). Octreotide was obtained from SelleckChem. SSTR2A antibody for immunohistochemistry was obtained from Gramsch Laboratories.

**Cell Culture:**

All cells were cultured in RPMI-1640 medium or DMEM/F12 (Gibco by Life Technologies) containing 10% heat-inactivated FBS (Gibco by Life Technologies), at 37°C, 100% humidity, and 5% CO<sub>2</sub>, and IX Penicillin/Streptomycin (Gibco by Life Technologies). Cells were tested every 6 months for mycoplasma using the ELISA-based Myco Alert PLUS Mycoplasma Detection Kit (Lonza). Cell lines were purchased from ATCC and authenticated by STR validation by biosynthesis and matched at least 8 assessed STR alleles to database parental cell lines.

**shRNA:**

shRNA generation and lenti viral infection was performed as in<sup>5, 16</sup> using the GIPz system targeted to SSTR2. Knockdown of SSTR2 was confirmed by immunoblotting. Constructs were obtained from Sigma or Dharmacon with the following target sequences: SSTR2 KD#1: GCAACACACTTGTCATTTA, SSTR2 KD#2: TGTGTGTTTGTGTATTGAA, SSTR2 KD#3: GGCTCTGTGGTGTCAACCA, SSTR2 KD#4: GGCAACACACTTGTCATT, SSTR2 KD#5: TGAAGACCATCACCAACATTT, SSTR2 KD#6: CCCTTCTACATATTCAACGTT. For cells that had been infected with shRNA lentivirus, cells were maintained in additional selection media that contained 1–3  $\mu$ g/mL puromycin (Sigma). Transfected cells were of low-passage number (<15 passages).

**Cell Proliferation Assays:**

Cells were plated at 1–5 X10<sup>3</sup> per well in a 96 well plate were allowed to adhere up to 24 hours, then washed with IX PBS prior to addition of supplemented media. At the respective time points, CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) solution was added into each well and the experiment was carried out as per the manufacturer's instructions (Promega, Madison, WI). Briefly, cells were gently shaken, and the plates were

incubated at 37°C for 1–4 hours. The absorbance was detected at 490 nm and 630nm (reference) with a Microplate Reader (Epoch Microplate Spectrophotometer, Biotek). Reference absorbance was used to correct for background lid condensation as indicated.

**Metabolic Assay:**

Seahorse testing was performed as described in the Seahorse XF Cell Mito Stress test kit User Guide with 40,000 living cells loaded per well onto Poly-L-lysine coated plates. Wells were observed use phase contrast light microscopy after the washing steps and imaged using DAPI staining and automated and manual microscopy after the seahorse run to confirm that no significant number of cells were lost during the protocol or with washing. Testing was performed with at least 3 well replicates per sample and 3 independent experimental replicates with similar findings.

**Clonogenic Assay:**

Cells were plated in 6 well plates in duplicate at 100 cells per well. They were incubated for two weeks then washed and stained with Crystal Violet (Sigma) before imaging

**Mice:**

6–8 week old athymic outbred fox nl nu/nu (Charles River Labs) mice were injected with 2 million cells of the specified cell line along with 1:1 in matrigel (BD Biosciences) into bilateral mouse flank. For the experiments herein, 10 mice were observed for 4 weeks and tumors were flash frozen or fixed in 10% buffered neutral formalin. Aliquots from injected cells were lysed for western blotting at time of injection. All animals in this study were maintained in AAALAC accredited mouse facilities at Vanderbilt University Medical Center and in accordance with IACUC regulations and protocols at Vanderbilt University Medical Center.

**Flow Cytometry:**

Cells were fixed, washed and processed as described in<sup>17</sup> and stained with propidium iodide for cell cycle analysis. Flow cytometry was performed on a FACScaliber. Data analysis was performed using Modfit software and cell quest.

**Statistical Analysis:**

Statistical analysis for proliferation assays were performed using a 2 tailed Student's t test. Results with a P <0.05 were considered to be statistically significant. Growth Curves and volume analysis was performed using a Linear Mixed model and by Log transformation and Wilcoxon testing. Tumor microarray studies were evaluated using Pearson and Wilcoxon testing as appropriate and using a Cox proportional Hazards Model including age and smoking status. Kaplan Meier plots were produced for all patients, as well as limited and extensive disease subgroups. These statistical analyses were performed in R (<http://www.r-project.org>). Additional spearman clustering analysis was performed on both SCLC cell lines and SCLC primary tumors.

## Results:

### SSTR2 is highly expressed in SCLC cell lines and primary tumors:

We first examined the expression pattern of somatostatin (SST) and its associated receptors based on the CCLE databases and published tissue data<sup>18</sup>. Our analysis uncovered that somatostatin components are broadly expressed in many NSCLC, as well as in SCLC cell lines. Recent genomic analyses of primary SCLCs also confirm high level expression (FPKM (Fragments per kilobase per million mapped reads) >2) of SSTR2 in 47% of 81 primary SCLC tumors and several tumors have very high level expression (30–48 FPKM) with 23% of the tumors analyzed expressing high levels of both SST (somatostatin) and SSTR2 (conjugate receptor) (Fig. 1B).<sup>12, 19, 20</sup> Most cell lines also had high level expression in both the ligand somatostatin and at least one of the downstream receptors (Fig. 1B and supplemental Figure 2). Our laboratory has confirmed this upregulation of SSTR2 in multiple cell lines, primary SCLC, and patient derived xenografts using a novel purified polyclonal SSTR2 antibody with high specificity (Fig. 1 and Supplemental Fig. 1 and methods for validation). This antibody was utilized to assess SSTR2 expression in multiple neuroendocrine, squamous cell carcinoma, and adenocarcinoma cell lines (Fig. 1C). The highest expression was noted in the SCLC cell lines tested which are consistent with elevated expression in most SCLC cell lines in the CCLE database (Supplemental Fig. 2). There was low level expression in human bronchial epithelial cell lines compared to carcinoma cell lines (Fig. 1C).

Additionally, SSTR2 expression was assessed for correlation with a thirteen gene neuroendocrine panel. The highest and most significant correlation in primary SCLC was observed for a correlation between NEUROD1 expression and SSTR2 expression. Known SCLC and neuroendocrine associated genes including the ligand somatostatin, c-myc and synaptophysin were also correlated with SSTR2 expression in primary tumors. Normalized cell line data also noted a high correlation with neuroendocrine markers NEUROD 1 and synaptophysin. Other notable neuroendocrine subset markers such as DLL3 or ASCL1 were not significantly correlated with SSTR2 expression. Reactome pathway analysis (Supplemental Table 3 and Supplemental Figure 3) of highly upregulated genes from both CCLE and primary tumor data showed enrichment in neuroendocrine pathways with primary tumors enriched in developmental signaling pathways. The full correlation analysis is described in Supplemental Table 2.

The above work showed that SSTR2 is highly expressed in multiple SCLC cell lines and in both SCLC and NSCLC primary tissues. We evaluated SSTR2 protein expression by IHC staining and intensity scoring of a tumor microarray (TMA) collected from 98 archival primary SCLCs. We utilized a commercially available SSTR2 antibody which demonstrated significant expression (1+ or greater observed expression intensity) in 47 out of 98 (48%) of SCLC patient tumors with both membranous and cytoplasmic staining appreciated by IHC (Fig. 1D). This correlates well with the observed RNAseq expression (47%) in a separate SCLC cohort (Fig. 1B). SSTR2 expression was scored and separated into low and high SSTR2 expression patterns in TMA tumors based on observed positive tumor cell staining after antigen retrieval and IHC staining. The majority of SSTR2 high samples had an IHC

score of 10–100 out of 300. There were no statistically significant differences observed in patient characteristics between the low and high SSTR2 scoring expression patterns including age, gender, race, stage, or smoking status (Supplemental Table 1).

### **SSTR2 expression in SCLC confers poor prognosis:**

Analysis of SSTR2 expression patterns in this 98 patient cohort revealed a survival advantage in SCLC patients with low expression of SSTR2 with a 2 year survival of 39% in the low SSTR2 expressing patients compared to 17% in the high SSTR2 expressing arm (defined as SSTR2 expression in tumor tissue 1+ or greater or IHC score  $\geq 1$ ) (Supplemental Fig. 4).

This difference in long term survival was driven by survival differences in patients with limited stage disease (SCLC confined to one radiation field). Based on Cox proportional hazards analysis we noted a link between the effect of SSTR2 expression on risk of death (hazard) and stage. We therefore continued our analysis by stage specific survival analysis. Of note, 2 patients in cohort without stage information were excluded from this stage survival analysis. In limited stage disease (n=52), the average OS was 12 months in the SSTR2 high expressing group and 36 months in the low expressing group (Fig. 2A) ( $p=0.001$ ). There was no significant difference in overall survival between the high or low SSTR2 expression tumors in the extensive stage (widely metastatic) population (Fig. 2B). Cox proportional hazards modeling on 52 limited stage patients with either low or high SSTR2 expression showed a hazard ratio of 0.23–0.87 (95% confidence interval) favoring low SSTR2 expression independent of age or smoking status (Supplemental Table 4). These results call into question the prevailing dogma that signal transduction via SSTR2 activation leads to a reduction in proliferation and increased apoptosis and suggests SSTR2 signaling may support tumor growth and maintenance. We sought to further investigate the molecular basis of these clinical findings via multiple *in vitro* and *in vivo* experiments to determine why high SSTR2 expression may convey increased risk.

### **Loss of SSTR2 expression decreases tumor growth and induces apoptosis:**

To further evaluate the role of SSTR2 signaling we created multiple stable short hairpin RNA (shRNA) knockdown cell lines with reduced SSTR2 expression including a bronchiolar atypical carcinoid cell line (H727), and two adherent SCLC lines (H1048, H841) to assess the effects of SSTR2 knockdown on proliferation, cell signaling and cell death (Fig. 3 A). Down regulation of SSTR2 by shRNA significantly decreased proliferation *in vitro* in most cell lines with successful SSTR2 transcriptional knockdown compared to scrambled controls. The anti-proliferative effect of SSTR2 knockdown was consistent across multiple cell lines, multiple constructs, and 3 different cell types of lung carcinoma with the exception of one subclone KD4.1 (Fig. 3B). Clonogenic assays confirmed that these cells have reduced proliferative potential by colony formation assays (Fig. 3C). Altogether, we observed reduced proliferative potential in high grade neuroendocrine cell lines with decreased expression of SSTR2.

To better characterize these findings, we performed western blotting assessing apoptotic and proliferative/pro-tumor survival pathways (Fig. 4A–B). Cleaved effector caspases 3, 7, and 9

were increased in H1048 cells with SSTR2 knockdown (Fig. 4A and supplemental Fig. 5). This increased apoptotic milieu was not observed in the atypical carcinoid cell line H727 (Fig. 4B). Global changes in BCL family expression were also observed with loss of SSTR2 (supplemental Fig. 5). Flow cytometry confirmed expected changes in apoptosis with H1048 SSTR2 knockdown cells demonstrating increased apoptosis and changes in cell cycling with a significantly increased pre G1 phase (Fig. 4C). H1048 cells had increased pAKT levels and stable pERK levels in response to knockdown of SSTR2. Overall, H1048 shRNA scrambled controls and SSTR2 knockdown cell lines had notable differences in downstream signaling events and differences in apoptosis coinciding with reduced cell viability with loss of SSTR2 function.

### **Loss of SSTR2 expression decreases tumor growth and induces apoptosis in mouse xenografts.**

The next step involved the extension of these *in vitro* results into an *in vivo* model. The H1048 SCLC line was injected into a nude mouse xenograft model. The mouse xenograft model showed significant qualitative and quantitative differences between H1048 SSTR2 knockdown and scrambled construct xenografts. SSTR2 knockdown was unstable off selection with initial knockdown of SSTR2 only maintained in 10% of tumors *in vivo* after 32 days in mouse without selection (Fig. 5B). SSTR2 KD tumors demonstrated initial growth delay. Tumor volumes were initially significantly different between knockdown and control constructs during the first 2 weeks of tumor growth (by Wilcoxon testing  $p < 0.05$ ), but both volumes and growth rates converged (Fig. 5A) leading to no significant differences at day 32 in most samples (Fig. 5A). However, the tumor xenografts which maintained knockdown of SSTR2 had reduced size compared to control flank injections (Fig. 5B–C). Ki67 staining was similar between groups, but cleaved caspase 3 staining was overall increased in the 10% of xenografts with maintained knockdown, a result consistent with increased apoptosis (Fig. 5C). Overall, the combination of *in vivo* and *in vitro* data suggests that SSTR2 signaling has a heretofore-undocumented role as a pro-tumor survival signal in small cell lung carcinoma. Xenograft studies recapitulate the observed *in vitro* phenotypes with tumor growth delay, increased apoptosis *in vivo* with SSTR2 KD, and strong selective pressure for SSTR2 expression.

### **Loss of SSTR2 expression inhibits pAMPK $\alpha$ signaling.**

To better understand the signaling implications of loss of SSTR2 function, we tested the expression of phospho proteins downstream of SSTR2 by phospho protein array experiments. We found that down regulation of SSTR2 in SCLC lines induced the upregulation of pAKT and STAT6 as was seen in immunoblotting experiments (Figs. 4 and Supplemental Figure 6). Decreases in pAMPK $\alpha$ 1, and pAMPK $\alpha$ 2 were also observed (Supplemental Figure 6).

Because pAMPK regulates aerobic metabolism, we tested whether oxygen consumption rate (OCR) (measured using the Seahorse XFe 96 instrument) changed with SSTR2 knockdown. H1048 cells with and without SSTR2 knockdown were tested via a mitochondrial stress test kit<sup>21, 22</sup>. SSTR2 KD cells had significant increased basal respiratory rate, as well as maximal respiration as evaluated by carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP)



treatment (Figure 6 A–C). The change in maximal respiration was greater than 60% higher in the SSTR2 knockdown group compared to the control cell with scrambled shRNA construct (Figure 6). The extracellular acidification rate (ECAR) was also elevated suggesting increased overall metabolic activity. These results demonstrate that SSTR2 KD is associated with decreased pAMPK $\alpha$ , 2, increased O<sub>2</sub> consumption, and increased overall mitochondrial activity.

## Discussion:

Somatostatin signaling has been proposed as an inhibitory signaling pathway serving to inhibit cAMP, reduce intracellular Ca influx, reduce proliferation, and induce apoptosis<sup>7, 23–25</sup> in multiple cell types and carcinomas including well differentiated neuroendocrine carcinoid tumors. However, these findings do not match the clinical experience with high grade neuroendocrine carcinomas such as SCLC with multiple unsuccessful SSTR2 agonist trials<sup>26, 27</sup>. Herein, we have demonstrated that SSTR2 is highly expressed in multiple SCLC cell lines, as well as in SCLC patient tumors and contributes to tumor progression. Notably, there was a strong correlation between NEUROD1 expression and SSTR2 expression in both cell lines and primary SCLC tumors. NEUROD1-predominant SCLC cell lines have been associated with variant pathology and NEUROD1 high mouse models have associations with cMyc expression and a more aggressive phenotype<sup>28, 29</sup>. Our pathway analysis of genes whose expression is correlated with SSTR2 expression shows enrichment of neuronal pathways and developmental biology pathways including EPH signaling which has also been associated with poor prognosis and drug resistance. The data presented herein argues that SSTR2 may contribute to this aggressive phenotype. Furthermore, our data demonstrates that SSTR2 expression does not inhibit cancer cell survival in SCLC. Our *in vivo* and *in vitro* studies show decreased growth with loss of SSTR2 in multiple small cell lung carcinoma and related contexts. Our results suggests that SSTR2 signaling supports cellular survival in small cell lung carcinoma and potentially in high grade neuroendocrine carcinomas. This data also helps explain the lack of clinical efficacy of SSTR2 agonists despite widespread high level expression of SSTR2. This makes solid evolutionary sense as SCLC carcinomas show high somatic mutation rates<sup>12</sup> with very high level Ki67 staining and mitotic rates. It would make very little evolutionary sense for such mutationally, mitotically, and metabolically active carcinomas to maintain high levels of somatostatin receptor expression unless there is some survival advantage for these cancer cells. This survived signaling is further supported by the very high levels of expression in many tumors and that some tumors overexpress both ligand and receptor suggesting autocrine/paracrine signaling in this pathway.

Furthermore, our shRNA knockdown data shows there is strong selective pressure towards SSTR2 expression as most tumor xenografts evade knockdown of this receptor off selection and there is rescue of the growth phenotype with re-expression. Our human *ex vivo* data confirm that this high level of expression may have clinical utility and that tumors with high levels of SSTR2 expression have worse prognosis. Associating this data with the lack of ASCL1 expression in SSTR2 high tumors and positive NEUROD1 association with SSTR2 expression and the worse outcomes associated with SSTR2 may contribute to a variant neuroendocrine differentiation with chemotherapy/chemoradiation resistance or EMT like

phenotype with worse outcomes with concurrent chemoradiation<sup>30</sup>. Accordingly, our data suggests that SSTR2 signaling may have a hereto unexpected pro survival/oncogenic role in SCLC.

The existing knowledge about the signaling of SSTR2 notes initial activation of the pathway by ligand (SST or other agonist) binding to SSTR2, which is associated with inhibition of Ca<sup>++</sup> release, and/or inhibition of adenylate cyclase leading to inhibition of secretion of neuropeptides, and with cell type dependent activation of the downstream phospho tyrosine phosphatases (PTPs) SHP1 and SHP2<sup>7</sup>. SHP1 signaling has known roles in inhibiting AKT phosphorylation<sup>31</sup> and pro tumor survival signals. SSTR2 signaling activation has been previously characterized as leading to cell cycle arrest or apoptosis in low grade neuroendocrine tumors and is approved for use in low grade carcinoid tumors<sup>8</sup>. This ligand receptor pathway activation has been previously characterized as arresting the cell cycle, leading to apoptosis, and reducing hormone secretin and has served as rationale to treat carcinoid syndrome and associated neuroendocrine tumors<sup>23</sup>. In contrast, our results offer new and very different insights in the function of this receptor activation in high grade neuroendocrine malignancy such as SCLC. Namely, that SSTR2 receptor expression supports SCLC survival and tumor growth.

The concept of SSTR2 activation leading to p27 activation and cell cycle arrest is intriguing given that both p53 and Rb (mutated in most SCLC) interact cooperatively with p27<sup>32, 33</sup>. One potential explanation is that defects in p53 and Rb may lead to paradoxical signaling with SSTR2 activation leading to increased cell survival. The underlying signaling responsible for the increased apoptosis with loss of SSTR2 in our SCLC models is unclear, though the changes in oxygen consumption and increased mitochondrial activity consistent with decreased pAMPK suggest changes in oxidative metabolism and mitochondrial function may be associated with the increased observed apoptosis<sup>34</sup>. This increased pAKT may be a compensatory survival mechanism in response to cellular stress.

The data presented herein supports an anti-apoptotic function of SSTR2 signaling in SCLC. Loss of SSTR2 function led to changes in viability and cell cycle in H841 and HI 048 cell lines and increased apoptosis in SCLC cell lines *in vitro* and *in vivo*. *In vivo* data showed dramatic reduction in tumor growth which resolved with re-expression of SSTR2. Negative controls did not have any growth degradation and multiple shRNA knockdown constructs in multiple cell lines showed reduced cell viability suggesting loss of SSTR2 leads to increased apoptosis. The atypical carcinoid cell line H727 did not demonstrate the same level of apoptosis as the HI048 SCLC line, suggesting this phenomenon may be cell type specific and related to basal cellular apoptosis/cell stress. We examined Bcl2 family gene expression and found downregulation of multiple Bcl family genes with loss of SSTR2 expression (Supplemental Fig 5). SSTR2 has been linked to an inverse Bcl family regulation previously, but this data suggests that this inverse relationship does not exist in SCLC<sup>35, 36</sup>. This suggests that somatostatin receptor downstream signaling is cell type specific.

This data also suggests a link between SSTR2 signaling, pAMPKa, and metabolic regulation in mammalian cells. Direct effects of SSTR2 signaling in SCLC are under-characterized. We found changes in pAMPKa and 2 with loss of SSTR2 signaling which correspond well to

the increased oxidative respiration observed with loss of SSTR2 in SCLC. This opens up the potential for a role of SSTR2 in metabolic regulation in SCLC.

Existing literature has noted the potential for somatostatin signaling to have a pro-growth/pro-survival impact on developmental signaling and retinal hypoxia<sup>37, 38</sup>. This is in accord with existing clinical data which has not shown a consistent patient survival advantage for octreotide or SSTR2 agonist treatment in high grade neuroendocrine carcinomas<sup>6, 39, 40</sup>. Multiple clinical trials involving somatostatin agonists in cancer treatments have failed to demonstrate a clinical benefit, with the notable exception of well differentiated low grade neuroendocrine tumors<sup>26, 41</sup>. It is noteworthy that these trials were unsuccessful despite high level expression of SSTR2 and other targets in poorly differentiated neuroendocrine carcinomas. However, while agonists have been explored in clinical trials, there has been little therapeutic exploration of SSTR2 antagonist signaling to date in SCLC.

The high level of SSTR2 expression noted in these studies is of particular interest due to new higher affinity pharmacological agents which use the SSTR2 receptor as a target for delivery of cytotoxic drug or radio isotope. These agents may have best activity in more aggressive disease and SSTR2 expression would serve as a natural biomarker for response. SSTR2 antagonist radiolabeled peptides may be particularly effective given our data as well as improved binding efficiency and are in pre-clinical testing now<sup>42</sup>. Our work both confirms the high level of expression of SSTR2 in most SCLCs including primary tumor and cell lines and draws a link between level of expression and disease prognosis in limited stage patients.

A limitation of this work is that our existing data is unable to distinguish between differential downstream signaling effects due to an altered distribution of GPCRs from effects solely due to reduction in SSTR2 expression. In the setting of differential signaling and heterodimerization, it is possible that reducing SSTR2 expression could lead to differential signaling through alternative GPCRs or somatostatin receptor heterodimers. This phenomenon is a topic of active investigation. Our studies also focus on SSTR2 knockdown in cancer cells and do not assess systemic effects on angiogenesis or hormone secretion as would be seen with pharmacologic antagonism<sup>43</sup>. To our knowledge, there are also no current published pharmacologic signaling studies with SSTR2 antagonists in SCLC. The initial pharmacologic studies for these “antagonists” were performed in the stimulated and overexpressed environment of HEK293 cells or CHO cells with significant contextual and cell type specific differences from high grade neuroendocrine tumors<sup>44, 45</sup>. Confirming and comparing these results with new SSTR2 antagonists under development will be vital to better understand clinical SSTR2 signaling in SCLC. It is also notable that we did not observe a difference in OS in extensive stage disease human samples, but this may reflect known differences in treatment regimen in limited stage disease with the addition of radiation and the extremely poor baseline prognosis of extensive stage SCLC. This raises questions of a role for SSTR2 in radiation resistance which is a topic of active inquiry in our laboratory.

The data presented above demonstrates that somatostatin receptors are widely expressed in both SCLC and selected non-small cell lung cancers, that this signaling is not anti-proliferative in SCLC, but has pro-survival/anti-apoptotic effects in both SCLC and high

grade neuroendocrine carcinomas. This is potentially paradigm changing because somatostatin agonists are currently used to treat multiple hyper-secretory syndromes associated with SCLC or well-differentiated neuroendocrine tumors. They are also used theoretically in symptomatic higher grade carcinomas and are still in actively recruiting clinical trials for the treatment of high grade SCLC (NCT01417806). Our data supports that SSTR2 signaling in these highly lethal neuroendocrine carcinomas confers a cancer cell survival advantage and that loss of SSTR2 function leads to metabolic changes in SCLC suggesting new potential therapeutic targets for this devastating disease, particularly in limited stage disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Novelty and Impact:**

Small cell lung cancer (SCLC) is a highly aggressive and metastatic neuroendocrine carcinoma of the lung with a dismal survival and no improvement in decades. Somatostatin receptor 2 (SSTR2) is a G protein coupled receptor which canonically reduces growth. This work shows high levels of SSTR2 in SCLC cell lines and primary tumors, high SSTR2 expression is correlated with worse patient survival in SCLC, and *in vivo* SSTR2 signaling plays a pro tumor survival role in SCLC.

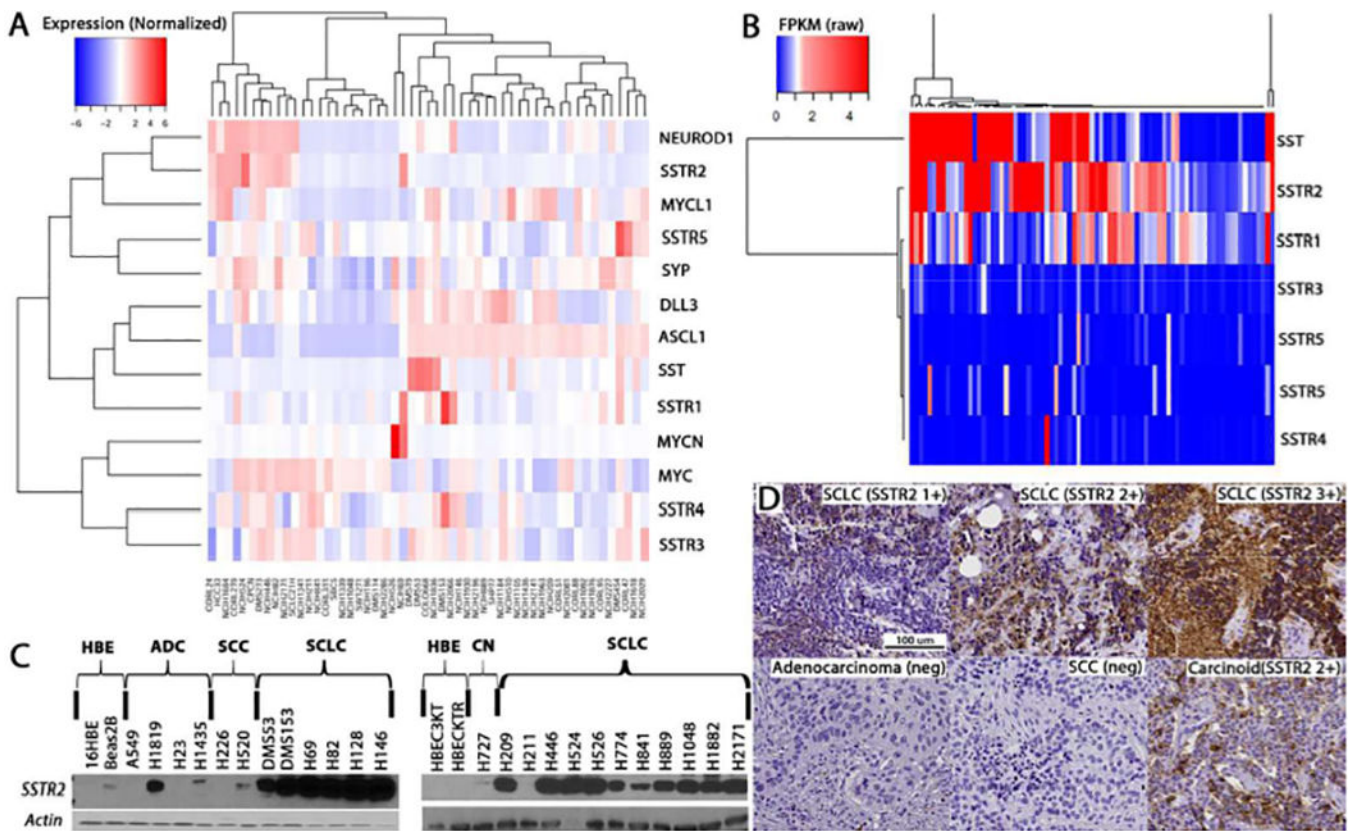
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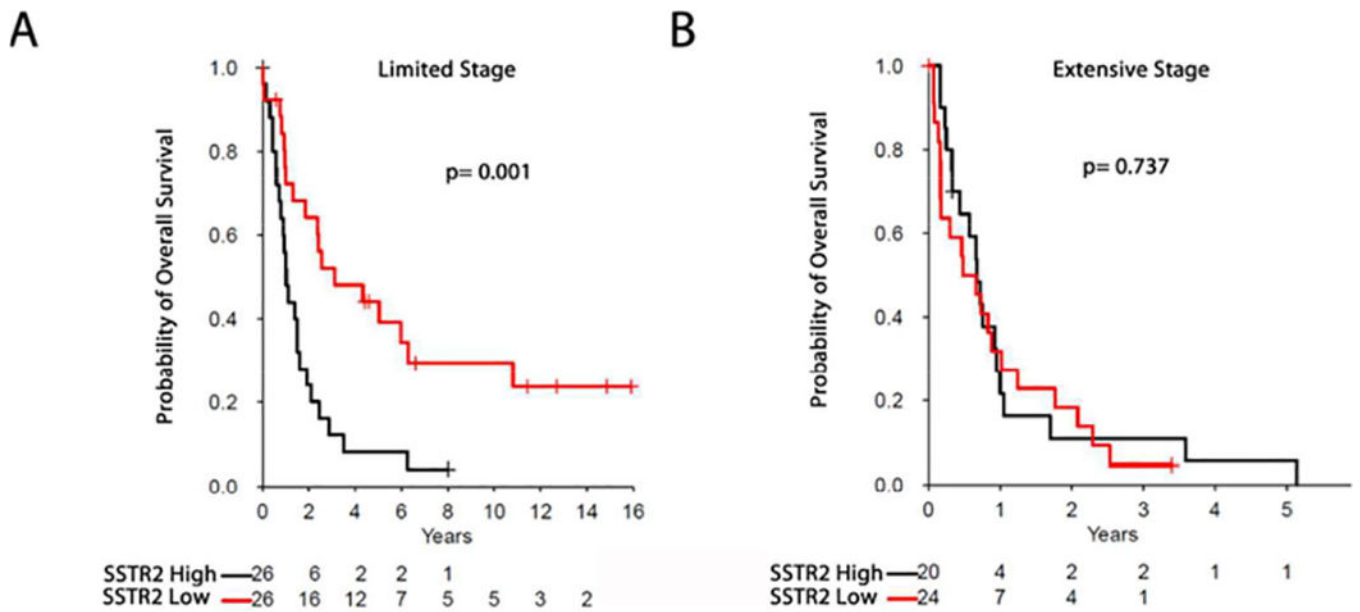
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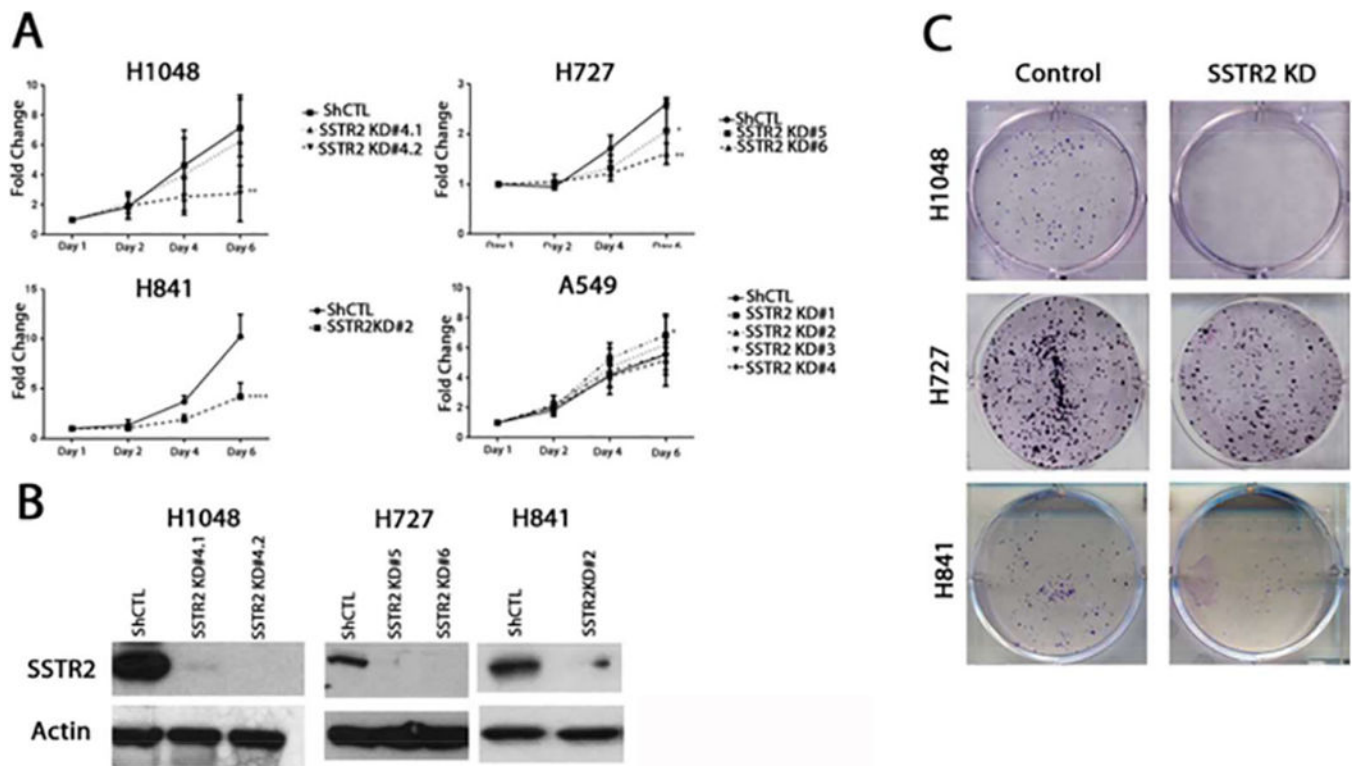
**Figure 1. SSTR2 expression in lung Carcinoma tumors and cell lines.**

A) Normalized expression data on 53 SCLC cell lines from CCLE<sup>12</sup> on selected Neuroendocrine and SST components with overexpression of SSTR2 in most cell lines. B) RNA seq data and FPKM (fragments per kilobase per million mapped reads) showing SST pathway expression in 81 primary SCLC, data released by<sup>12</sup> C) Immunoblotting for SSTR2 and loading control beta actin in cell lines from CCLE with overexpression of SSTR2 in most cell lines and low level expression in SCC or adenocarcinoma lines. D) SSTR2A staining of primary Lung Carcinoma including small cell lung carcinoma (SCLC) tumors, adenocarcinoma, squamous cell carcinoma, and carcinoid with staining intensity used in IHC scoring.



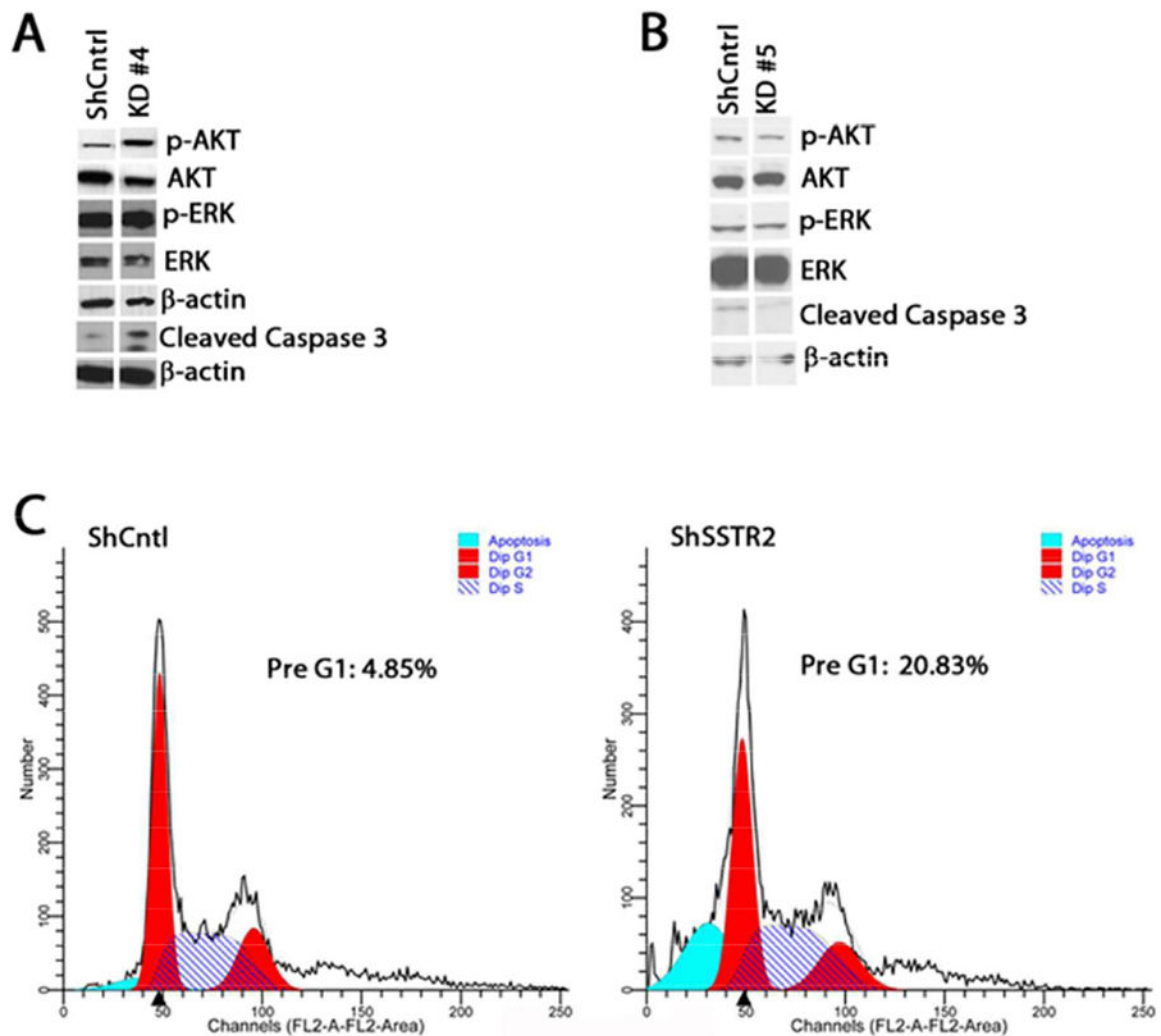
**Figure 2: Improved Median Survival in Limited Stage SCLC patients with low SSTR2 expression.**

Results from a 96 SCLC patient cohort assembled in a TMA and stained for SSTR2. Panel (A) demonstrates prolonged survival with low/no SSTR2 expression compared to high/observed SSTR2 expression in limited stage disease n=52, (HR: 0.23–0.87 (95% confidence) based on multivariate Cox Proportional hazards analysis (adjusted for age, smoking status, and race). Panel (B) represents the same analysis in patients with extensive stage disease n=44, and fails to demonstrate any survival advantage in this population.



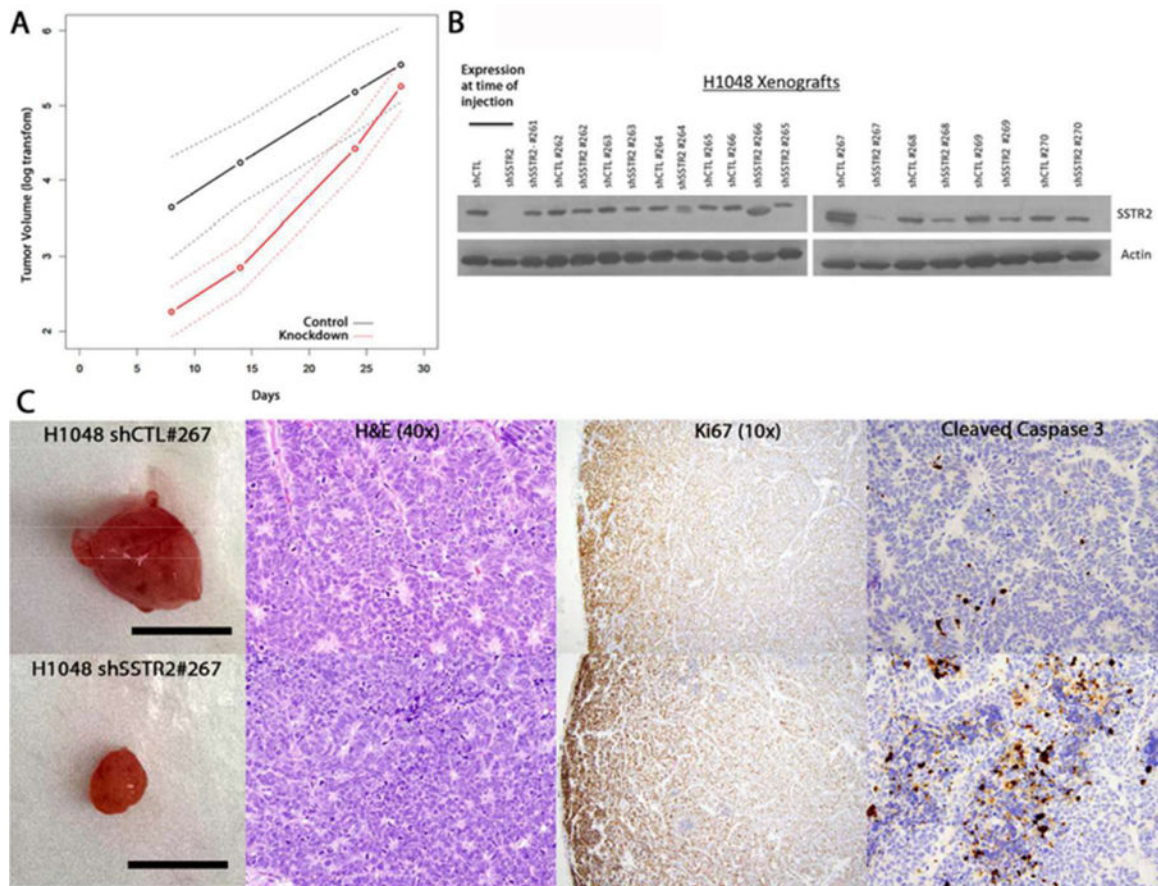
**Figure 3: Loss of SSTR2 expression leads to decreased viability in multiple high grade neuroendocrine cell lines.**

A) Loss of SSTR2 leads to reduced cell viability in multiple cell lines and constructs (error bars are 95% CI  $*=p<0.05$ ,  $**p<0.01$ ). A549 does not express significant SSTR2 and does not show similar reduction in cell viability after shRNA treatment including multiple constructs. B) Immunoblots show effective knockdown of SSTR2 expression in multiple cell lines. C) Clonogenic assays confirm qualitatively reduced viability and colony formation in SSTR2 knockdown cell lines.



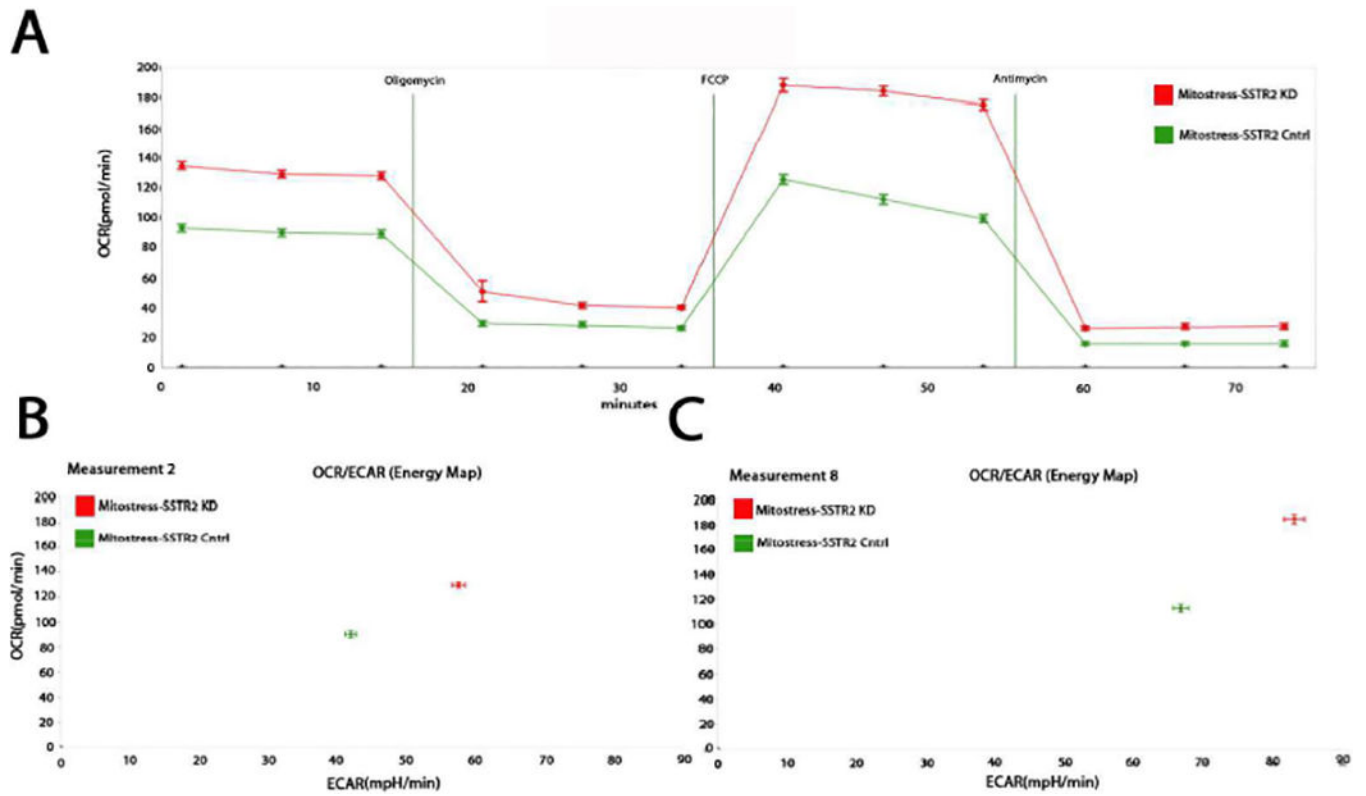
**Figure 4. Loss of SSTR2 leads to increased cleaved caspase and apoptosis.**

A) Immunoblotting of HI 048 *in vitro* cells with increased p-AKT, stable p-ERK, and increased caspase activity consistent with increased apoptosis with loss of SSTR2 (note SSTR2 knockdown for these clones is shown in Fig. 3). B) H727 immunoblots with stable p-AKT and mildly increased cleaved caspase. C) Propidium Iodide based flow cytometry with an increased pre G1 phase noted in HI048 SSTR2 KD cells (right) compared to shRNA controls (left) consistent with increased apoptosis.



**Figure 5. Loss of SSTR2 slows *in vivo* SCLC growth.**

A) Log transform and nonlinear modeling with REML fit (bar connotes 95% confidence) for 9 HI 048 control shRNA xenografts and matched 10 SSTR2 shRNA KD xenografts. Note the initial growth delay in the shRNA KD xenografts until day 20 post implantation. B) Immunoblotting of the 19 tumors and initial SSTR2 expression in control and KD cell lines C) Tumor imaging of representative shRNA CTL (top) and shSSTR2 xenografts (bottom) with maintained knockdown (1 cm scalebar) with Hematoxylin and Eosin, Ki67, and Cleaved Caspase 3 staining for tumor #267 both shRNA control xenograft (top), and SSTR2 shRNA knockdown xenograft (bottom) show similar Ki67 and increased cleaved caspase 3 in the SSTR2 KD xenograft.



**Figure 6. Loss of SSTR2 expression leads to changes in oxygen metabolism in SCLC lines.**  
 A) Mitochondrial Stress Testing of SSTR2 shRNA Ctrl and SSTR2 knockdown (KD) HI 048 cell lines show increased basal O<sub>2</sub> consumption (B) in the SSTR2 KD lines as well as significantly increased maximal respiration with FCCP Treatment (C). ECAR/OCR ratios show increased overall mitochondrial activity at baseline (B) as well as with FCCP treatment (C).