RESEARCH

A unique model for SDH-deficient GIST: an endocrine-related cancer

James F Powers1, Brent Cochran2, James D Baleja2, Hadley D Sikes2, Xue Zhang2, Inna Lomakin1, Troy Langford3, Kassi Taylor Stein3 and Arthur S Tischler1

1Department of Pathology and Laboratory Medicine, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA
2Department of Developmental, Molecular and Chemical Biology, Tufts University School of Medicine, Boston, Massachusetts, USA
3Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Correspondence should be addressed to J F Powers: jpowers1@tuftsmedicalcenter.org

Abstract

We describe a unique patient-derived xenograft (PDX) and cell culture model of succinate dehydrogenase-deficient gastrointestinal stromal tumor (SDH-deficient GIST), a rare mesenchymal tumor that can occur in association with paragangliomas in hereditary and non-hereditary syndromes. This model is potentially important for what it might reveal specifically pertinent to this rare tumor type and, more broadly, to other types of SDH-deficient tumors. The primary tumor and xenografts show a very high proliferative fraction, and distinctive morphology characterized by tiny cells with marked autophagic activity. It is likely that these characteristics resulted from the combination of the germline SDHB mutation and a somatic KRAS G12D mutation. The most broadly relevant findings to date concern oxygen and oxidative stress. In paragangliomas harboring SDHx mutations, both hypoxic signaling and oxidative stress are putative drivers of tumor growth. However, there are no models for SDH-deficient paragangliomas. This related model is the first from a SDHB-mutated human tumor that can be experimentally manipulated to study mechanisms of oxygen effects and novel treatment strategies. Our data suggest that tumor growth and survival require a balance between protective effects of hypoxic signaling vs deleterious effects of oxidative stress. While reduced oxygen concentration promotes tumor cell survival, a further survival benefit is achieved with antioxidants. This suggests potential use of drugs that increase oxidative stress as novel therapies. In addition, autophagy, which has not been reported as a major finding in any type of SDH-deficient tumor, is a potential target of agents that might trigger autophagic cell death.

Introduction

SDH-deficient gastrointestinal stromal tumors (GISTs) are rare but sometimes lethal tumors that can occur in patients with paraganglioma (PGL) syndromes. These tumors do not harbor the KIT or PDGFRα mutations typical of conventional GISTs and are therefore not amenable to conventional GIST treatments with receptor tyrosine kinase inhibitors. They also metastasize to lymph nodes more frequently than conventional GISTs, and their clinical prognosis is not predictable by conventional risk stratification parameters (Boikos et al. 2016, Mullassery & Weldon 2016, Ricci 2016). Similar to PGLs, there is currently no cure for SDH-deficient GIST other
than complete surgical excision, and there is a paucity of experimental models.

This paper describes a unique PDX and cell culture model for basic and pre-clinical studies of SDH-deficient GIST. The model, which we have named ‘the Ian GIST model’, is derived from an aggressive gastric GIST that arose in a young man with a germline SDHB mutation and family history of multiple paragangliomas. Despite a remarkable multi-institutional translational research effort aimed at developing an effective treatment (‘the Ian GIST Project’, https://www.forian.org/), the entire clinical course before the patient died was approximately 2 years. In addition to loss of SDHB protein expression and SDH activity, the tumor was found to harbor a somatic KRAS G12D mutation, possibly contributing to its aggressive behavior.

This GIST model is important for two reasons. First, it is a unique human-derived model for a rare type of tumor that occurs in patients with hereditary SDHB mutations. Although the basic metabolic defect in all SDH-deficient tumors is similar, different types of tumor such as GISTs and paragangliomas are likely to be affected by that defect in different ways because of intrinsic differences in their cells of origin. It is well established that different types of cancer as well as individually distinct cancers have varied genetic and epigenetic characteristics that dictate different approaches to treatment (Burgess et al. 2017) and that even therapeutic outliers can lead to new markers of drug sensitivity (Burgess et al. 2017). Second, because SDH-deficient GIST does have similarities to other SDH-deficient tumors, information gained from studying this model might also lead to increased understanding of other tumor types and to improved treatments for them.

Materials and methods

Origin of the model

The tumor of origin was a gastric GIST that arose in a 17-year-old patient who had a germline SDHB mutation (423+1G>A) and family history of paragangliomas in two male relatives, but was himself previously healthy. Approximately 3 months after resection of the primary tumor, he presented with a 15 cm recurrent tumor in the residual portion of stomach, with extensive local invasion and abdominal dissemination.

Our model was developed from a PDX established by a commercial service (Champions Oncology, Hackensack, NJ, USA) in nude mice from a tumor deposit in the patient’s abdomen. With authorization from the deceased patient’s family, we received cryopreserved passage 3 xenograft tissue and expanded through passage 6 in nude and/or NSG mice. This project is approved by the Institutional Review Board at Tufts Medical Center.

Histology and immunohistochemistry

Histologic sections of the primary tumor, the recurrence and xenografts were examined by a pathologist (AST) to assess phenotype drift in consecutive passages. Immunohistochemical stains were performed following previously reported protocols with antibodies shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). All control tissues showed appropriate immunoreactivity.

Electron microscopy

Fragments of xenograft tissue were fixed in commercially prepared fixative (Electron Microscopy Sciences, Hatfield, PA, USA) containing 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer. They were then postfixed in 2% OsO₄ dehydrated in a graded ethanol series followed by propylene oxide and embedded in Epon. Ultrathin (90 nm) sections on copper grids were stained with 0.2% lead citrate and 1% uranyl acetate and examined with a Philips 208S electron microscope at 80kV.

Transcriptional profiling

RNA sequencing was performed by the Tufts genomics core with an Illumina HiSeq 2000. Paired-end RNA sequencing data were preprocessed using Trimmomatic (Bolger et al. 2014) to cut or filter poor quality reads. The preprocessed data were then aligned using TopHat2 pipeline (Kim et al. 2013). The featureCounts software (Liao et al. 2014) was used to map reads to genes, and edgeR (Robinson et al. 2010) was used to calculate RPKM (Reads Per Kilobase of transcript per Million mapped reads) values for genes.

Metabolomic analysis

In vivo metabolomic analyses were performed to test whether the major metabolites produced in the GIST xenografts are consistent with metabolic profiles reported in SDH-deficient paragangliomas and in an SDH-deficient line of fibroblast-like cells derived from mouse adrenal glands (Letouze et al. 2013, Lussey-Lepoutre et al. 2015). ¹H NMR was used for unbiased metabolite profiling and
13C-NMR was used to trace the metabolic fate of glucose and determine the contribution of glycolysis and/or TCA cycle/anaplerotic pathways to the metabolite profile (Bruntz et al. 2017). Four replicate subcutaneous tumor nodules (bilateral flank implants from two mice, each 1 cm in greatest dimension) were analyzed. A xenograft of a KIT-mutated, SDH-intact, GIST (GIST-T1) (Taguchi et al. 2002) in NSG mouse was used as an SDH-intact control. Thirty minutes before killing, the mice were injected intraperitoneally with a 3 mmol/kg dose of 100% uniformly 13C6-labeled glucose (the natural abundance of 13C is 1%) (Eloqayli et al. 2004). Tumor tissue was dissected cold and frozen in liquid nitrogen. Metabolites were extracted with chloroform/methanol. To each frozen tissue sample, 0.4 mL of ice-cold methanol and 0.085 mL water were added and the tissue was minced to a fine grain on ice. After sonication to homogeneity, 0.4 mL of cold chloroform was added, which was then processed as described by Wu et al. (2008).

The polar methanol/water layer was dried using a speed-vac and reconstituted in 50 mM phosphate D2O buffer pH 7.0 with 0.5 mM of NMR standard (DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid). 1H NMR spectra of each sample were collected at 25°C on a Bruker Avance 600 spectrometer using 256 scans and a NOE1D pulse sequence. 13C NMR spectra were also measured using 10,000–20,000 scans. The 1H data were processed and analyzed using CHENOMX NMR Suite 8.0 to identify and quantify compounds present and measure their concentrations. Identity of compounds in 13C data were based on published reference spectra (Wishart et al. 2009) (and on 2D 1H-13C correlation spectra using HSQC (heteronuclear single-quantum coherence).

**Immunoblots**

Protocols for routine protein extraction and immunoblotting were as previously described (Powers et al. 2000). Proteins were resolved on gradient 4–15% polyacrylamide gels to permit probing for a wide range of molecular weights. Blots were sequentially probed, stripped and re-probed with the same antibodies used for immunohistochemistry, at dilutions optimized for immunoblots.

For Redox immunoblots (Cox et al. 2010), frozen GIST tissue samples were first placed in petri dishes on ice and soaked with 100μL of 200mM methyl methane thiosulfonate (MMTS, purchased from Sigma) in PBS (1×). The samples were then sliced with razor blades and placed in a 1.5 mL tube along with an additional 900μL of 200mM MMTS in PBS. The tissue samples were incubated on ice for 30 min. After incubation, the tissue sample was centrifuged at 250g for 5 min. The MMTS solution was removed from the tube and the cells were washed with 1 mL of PBS. The samples were then centrifuged and washed with PBS a second time. After centrifugation again, 100μL RIPA buffer with HALT protease inhibitor cocktail (1×, Thermo Fisher) was added to the samples. The samples were then homogenized with a manual tissue homogenizer. The lysed cells were then re-pelleted at 12,000g for 10 min. The supernatant of the solution was then collected and the protein content in the solution was assessed via the BCA assay. Twenty micrograms of protein were loaded into a Tris–tricine acrylamide gel and subjected to SDS-PAGE under non-reducing conditions (i.e. with no β-mercaptoethanol in the sample buffer). After the separated lysates were transferred to a PVDF membrane (Bio-Rad) (1 h at 100 V), the blot was blocked and incubated with either goat primary antibody for Prx2 (R&D Systems, Catalog # AF3489) (at a dilution of 1:800) or rabbit primary antibody for Prx3 (Abcam, Catalog #ab129206) (at a dilution of 1:10,000) at 4°C overnight and then with donkey anti-goat IR 688 (Licor) (at a dilution of 1:10,000) or donkey anti-rabbit IR 800 (Licor) (at a dilution of 1:10,000) for 1 h at approximately 22°C. β-Tubulin was used as a loading control for the blots. The tagged proteins were visualized using an Odyssey CLx Infrared Imaging System. In order to determine the fraction of oxidized protein for each tumor sample, the gel analyzer tool in ImageJ (https://imagej.nih.gov) was used. This tool calculates position-dependent intensities for the entirety of each band. The area under the intensity peak associated with the oxidized band was divided by the sum of the areas under the intensity peaks of both the oxidized and reduced bands.

**Cell cultures**

Culture medium consisted of RPMI-1640 with 15% fetal bovine serum, glutamine and penicillin/streptomycin, enriched with MEM non-essential amino acids (glycine, alanine, asparagine, aspartic acid, glutamic acid, proline, serine; each 0.1 mM, Life Technologies), fatty acid supplement (Sigma) and uridine (50μg/L, Sigma). Concentrations of aspartic acid and sodium pyruvate were further supplemented individually to final concentrations of 3 mM and 5 mM respectively (Supplementary Table 2).

Xenograft tissue at room temperature was minced into small fragments in complete culture medium, and then gently triturated in a 1 mL polystyrene pipet followed by
a 9° Pasteur pipet until approximately 80% of the tissue volume was dissociated. The resulting cell suspension was separated from the residual fragments, centrifuged at 500g and resuspended in fresh medium.

In order to test the influence of ambient O₂ concentration on cell growth and survival, we compared cultures routinely maintained in ~20% O₂ (‘normoxia’, 95% air/5% CO₂) vs 10% or 5% O₂ in Billups-Rothenberg modular incubator chambers. For the lowered O₂ concentrations, the gas mixtures contained either 80% or 85% N₂ plus 5% CO₂. Cells were plated in black 96-well assay plates (Costar) at a density of 5 × 10⁴ cells/well, and survival was quantitated at 3 days using a CyQuant NF Cell Proliferation Assay kit (Molecular Probes). Use of this DNA-based assay was chosen to avoid potential confounding effects of abnormal metabolism (Quent et al. 2010). All cultures were maintained at 37°C in a water-saturated atmosphere.

Growth factors and inhibitors

Stem cell factor (SCF, also known as KIT ligand) was obtained from Cell Signaling Technologies. Recombinant human insulin-like growth factor 1 (IGF1) was from Abcam. ERK inhibitors U0126 and PD98059 were from New England Biolabs and Promega, respectively.

Results

Histopathology

The primary tumor was a 4.5 cm ulcerated gastric mass. A few patchy areas of the tumor showed classic GIST patterns (Miettinen et al. 2010) consisting of mildly discohesive, polygonal epithelioid cells with slightly eosinophilic cytoplasm and pleomorphic nuclei or hypercellular spindle cells with relatively bland nuclei. In contrast, at least 90% of cells in the tumor showed an unusual gradation from epithelioid to round shape and progressive decrease in cell size, with many cells becoming smaller than neutrophils (~12 µm) and exhibiting almost no cytoplasm (Fig. 1A and Supplementary Fig. 1). Some areas showed prominent myxoid material between cells or in large pools, suggesting a possible collision tumor between GIST and adenocarcinoma (Supplementary Fig. 1B). This was ruled out by immunohistochemical stains for GIST markers KIT and DOG1 (Rammohan et al. 2013) showing transition between cell types (Fig. 1B and Supplementary Fig. 4), and by the absence of staining for cytokeratin markers indicative of carcinomas. Mitoses were very numerous in classic epithelioid areas (up to five mitoses per single high power field) (Supplementary Fig. 2) and sparse or absent in the smallest cells. Staining for...
proliferation marker Ki67 showed a comparable pattern, ranging from approximately 80% to 20% in different parts of the tumor (Fig. 1D). An abdominal tumor deposit sampled approximately 1 year after resection of the primary consisted almost entirely small cells with patches of myxoid material (Supplementary Fig. 3).

The major morphological differences between xenografts and the patient’s tumors were a more compact architecture, increasingly monomorphic population of intermediate-to-small cells, loss or greatly decreased amounts of myxoid material and uniform expression of Ki67 in 80–90% of the tumor cells. Immunohistochemical staining for KIT was maintained through at least in vivo passage 6, tending to be most intense in the largest cells and often undetectable in the smallest (Fig. 1). In contrast, staining for DOG1, which was sparse in the primary tumor, was almost lost by in vivo passage 4.

The most distinctive ultrastructural features were degenerating, malformed mitochondria and extensive autophagic vacuoles containing mitochondria and other cytoplasmic components. Many cells contained numerous cytoplasmic vacuoles and loss of most identifiable cytoplasmic structures (Fig. 2).

In both the primary tumor and xenografts, immunohistochemical staining for SDHB was lost in all tumor cells and retained in endothelial cells (Fig. 1C and G) consistent with loss of the WT SDHB allele (Dahia et al. 2005, van Nederveen et al. 2009), while staining for SDHA (Korpershoek et al. 2011, Oudijk et al. 2013, Papathomas et al. 2015) was retained.

![Ultrastructural features of the PDX cells.](image)
Metabolite profile

Of 55 readily identifiable metabolites in the Ian GIST xenografts, lactate comprised approximately 24% and succinate 11% of the total (Table 1). Fumarate was almost undetectable, with a succinate/fumarate ratio of 679±151 (n=4 tumors), consistent with loss of SDH activity. Four other metabolites that each comprised >5% of the total were taurine, glycine, myo-inositol and alanine. All others accounted for <3%. The eight most abundant metabolites comprised >75% of the total (Table 1). In contrast, our analysis of a control xenograft of a KIT-mutated, SDHB-intact GIST model GIST-TI showed 0.54% succinate, consistent with reported values of less than 1% succinate in other non-SDH-deficient tumors (Euceda et al. 2017).

The 13C-glucose label ended up as a small amount of glucose, and abundant lactate, alanine and glutamate. The labeled glutamate contained nearly equal amounts of [13C2-4,5]Glu and [13C2-2,3]Glu isomers (Supplementary Fig. 5A), indicating nearly equal activity of pyruvate dehydrogenase (PDH) and the anaplerotic pathway catalyzed by pyruvate carboxylase (PC) respectively (Lussey-Lepoutre et al. 2015, Bruntz et al. 2017). There was also robust incorporation of glucose into succinate. In contrast, the ratio of [13C2-4,5]Glu and [13C2-2,3]Glu isomers in the GIST-TI xenograft was approximately 6:1 (Supplementary Fig. 5B). These findings are consistent with increased activity of PC previously reported in SDH-deficient cells (Lussey-Lepoutre et al. 2015).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% of total metabolites* (mean, n=4)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>23.85</td>
<td>21.78–26.95</td>
</tr>
<tr>
<td>Taurine</td>
<td>11.49</td>
<td>10.82–12.54</td>
</tr>
<tr>
<td>Succinate</td>
<td>11.03</td>
<td>10.76–11.40</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.63</td>
<td>8.18–9.30</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>8.04</td>
<td>6.65–8.63</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.38</td>
<td>5.04–5.72</td>
</tr>
<tr>
<td>O-Phosphoethanolamine</td>
<td>4.10</td>
<td>3.78–4.37</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3.3</td>
<td>2.15–4.32</td>
</tr>
<tr>
<td>Sum</td>
<td>75.82</td>
<td></td>
</tr>
</tbody>
</table>

*The table lists the eight most abundant metabolites expressed as percentage of total metabolites, showing the average of percentages in four individual tumors.

Molecular indicators of elevated H$_2$O$_2$ in the mitochondria and cytosol

In light of recent studies that have linked complex II of the mitochondrial electron transport chain with increased levels of hydrogen peroxide (Quinlan et al. 2012, Kluckova et al. 2015), we hypothesized that SDH deficiency could result in increased steady-state fluxes of H$_2$O$_2$ in the mitochondria and perhaps in the cytosol. In order to measure relative H$_2$O$_2$ levels within the different GIST cells, we employed a previously described methodology (Cox et al. 2010) to measure the oxidation products of molecular targets of H$_2$O$_2$. Unlike free radical species, such as hydroxyl radical, which react readily with multiple targets inside cells, H$_2$O$_2$ reacts much more selectively within cells (Winterbourn 2008). In particular, studies have shown that virtually all of the H$_2$O$_2$ inside cells reacts with peroxiredoxins (Winterbourn 2008, Lim et al. 2015), a family of cysteine-based antioxidant proteins that form disulfide-linked dimers upon reaction with H$_2$O$_2$, on the basis of the high second-order rate coefficient for their reaction with H$_2$O$_2$ and the intracellular abundance of the these proteins (on the order of 100μM in many types of cells) (Peskin et al. 2007, Selvaggio et al. 2018). Since these enzymes act as the primary sink for intracellular H$_2$O$_2$, several studies have used the oxidation status of peroxiredoxins (i.e. oxidized dimers vs reduced monomers) as proxies for the intracellular H$_2$O$_2$ level (Low et al. 2007, Kumar et al. 2009, Sobotta et al. 2013, Poynton & Hampton 2014). This approach combines both the H$_2$O$_2$ specificity of peroxiredoxins as well as the H$_2$O$_2$ sensitivity of cells’ natural peroxide sensors.

In order to assay for oxidized peroxiredoxin dimers in both the mitochondria and cytosol in the two different types of GIST, lysates from GIST cells both with and without functional SDH were subjected to non-reducing SDS-PAGE (in which the proteins were denatured but the disulfide bonds were not reduced), transferred to a PVDF membrane, and stained for both peroxiredoxin 3 (PRDX3; located in the mitochondria) and peroxiredoxin 2 (PRDX2; located in the cytosol). The fraction of oxidized PRDX3 and PRDX2 dimers compared to reduced PRX3 and PRX2 monomers for each tumor indicated high levels of H$_2$O$_2$ in the mitochondria and cytosol of tumor cells in the xenograft tissue (Fig. 3). Densitometry showed the ratio of oxidized PRDX3 to total PRDX3 protein to be consistently higher than the corresponding ratio for PRDX2 (0.983±0.002 for PRDX3 compared to 0.792±0.032 for PRDX2, P=0.0095 two-tailed Student’s t-test), consistent with a greater degree of mitochondrial compromise. Comparison of the fraction
of oxidative stress already present in vivo, as implied by the Prx blots, we therefore tested the effects of decreased O₂ concentrations, both alone and in combination with antioxidants. Over a 3-day period in culture, there was a marked, concentration-dependent increase in tumor cell survival in the presence of 10% or 5% O₂, and survival was further enhanced by addition of either N-acetylcysteine or a proprietary antioxidant mix. However, despite this beneficial effect, there was a sharp decline in Ki67 expression compared to the tumor cells in vivo (Fig. 4). By 2 weeks in culture almost all tumor cells had died or been overgrown by mouse cells, which grew vigorously under hypoxic conditions. The rapid switch between populations of human and mouse cells was confirmed by immunoblots that showed almost undetectable SDHB during the initial 3-day culture period, consistent with the overwhelming predominance of SDH-deficient human tumor cells, and its re-emergence by 2 weeks. In addition, immunoreactivity for Ki67 using a human-selective antibody disappeared over the same time course.

In order to get clues to understand the basis for loss of tumor cell proliferation in culture, RNA sequencing analysis was performed on the xenograft and the cultured cells to identify changes in mRNA expression. We found 226 genes upregulated by >ten-fold and 120 genes downregulated by the same amount in cells cultured in 10% O₂ as compared to the xenograft. The complete list is given in Supplementary Table 3. Analysis of pathways with Ingenuity Pathway Analysis software indicated that genes and pathways most affected in upregulated genes are matrix metalloproteases, cytokines and WNT5A and WNT5B. The cytokine induction could be an indicator of a senescence-associated secretory phenotype (Lasry & Ben-Neriah 2015). The downregulated genes were most involved in connective tissue and growth regulation pathways. Figure 5 shows the changes in expression of genes of known interest to GIST. While there was little change in the GIST marker DOG1, there was a >30-fold decrease in IGF1 expression when cells were put into culture. However, addition of IGF1 to the culture of these cells was not sufficient to rescue the growth defect (data not shown). There was an almost ten-fold increase in HIF2 expression consistent with an increase in hypoxic stress in culture. There was a four-fold increase in KIT ligand expression, but a two-fold decrease in KIT receptor expression. Consistent with this, there was nearly a three-fold increase in CBL expression, which can downregulate the KIT receptor through the ubiquitin pathway (Zeng et al. 2005). Thus, below we examined the expression of KIT protein in the cultures.

Figure 3
Redox blot sequentially incubated with antibodies against human peroxiredoxin-2 (PRDX2) and peroxiredoxin-3 (PRDX3). The bands at approximately 22 kDa represent the reduced monomer PRDX2 or PRDX3 proteins, while bands at about 44 kDa represent the respective oxidized dimers. The amount of oxidized dimer relative to the total reduced monomer plus oxidized dimer in each lane is an indication of the relative amount of H₂O₂-induced stress (Cox et al. 2010). PRDX2 acts as a sensor for H₂O₂ stress in the cytosol of the cells, while PRDX3 acts in the mitochondria. Beta-tubulin is a loading control. Lanes 1-3 are from separate xenografts of SDH-deficient GIST. Lane 4, which shows the highest amounts of both oxidized dimers and reduced monomers, is from a primary conventional GIST with intact SDHB.

Cell culture studies
Cell culture experiments were undertaken with two complementary objectives. The first was to study short-term primary cultures in order to identify critical factors regulating tumor cell survival, growth and differentiation independently of potentially confounding changes that might occur in cell lines. The second was to use data gained from those studies to guide efforts to establish cell lines, which would be needed for more extensive mechanistic investigations.

A striking and almost immediate finding was that the cells are averse to oxygen. Primary cultures were initially plated in a routinely employed atmosphere of 95% air/5% CO₂. This resulted in rapid cell death, often obvious within hours and leading to loss of almost all tumor cells within two weeks. In light of the high levels of oxidative stress already present in vivo, as implied by the Prx blots, we therefore tested the effects of decreased O₂ concentrations, both alone and in combination with antioxidants. Over a 3-day period in culture, there was a marked, concentration-dependent increase in tumor cell survival in the presence of 10% or 5% O₂, and survival was further enhanced by addition of either N-acetylcysteine or a proprietary antioxidant mix. However, despite this beneficial effect, there was a sharp decline in Ki67 expression compared to the tumor cells in vivo (Fig. 4). By 2 weeks in culture almost all tumor cells had died or been overgrown by mouse cells, which grew vigorously under hypoxic conditions. The rapid switch between populations of human and mouse cells was confirmed by immunoblots that showed almost undetectable SDHB during the initial 3-day culture period, consistent with the overwhelming predominance of SDH-deficient human tumor cells, and its re-emergence by 2 weeks. In addition, immunoreactivity for Ki67 using a human-selective antibody disappeared over the same time course.

In order to get clues to understand the basis for loss of tumor cell proliferation in culture, RNA sequencing analysis was performed on the xenograft and the cultured cells to identify changes in mRNA expression. We found 226 genes upregulated by >ten-fold and 120 genes downregulated by the same amount in cells cultured in 10% O₂ as compared to the xenograft. The complete list is given in Supplementary Table 3. Analysis of pathways with Ingenuity Pathway Analysis software indicated that genes and pathways most affected in upregulated genes are matrix metalloproteases, cytokines and WNT5A and WNT5B. The cytokine induction could be an indicator of a senescence-associated secretory phenotype (Lasry & Ben-Neriah 2015). The downregulated genes were most involved in connective tissue and growth regulation pathways. Figure 5 shows the changes in expression of genes of known interest to GIST. While there was little change in the GIST marker DOG1, there was a >30-fold decrease in IGF1 expression when cells were put into culture. However, addition of IGF1 to the culture of these cells was not sufficient to rescue the growth defect (data not shown). There was an almost ten-fold increase in HIF2 expression consistent with an increase in hypoxic stress in culture. There was a four-fold increase in KIT ligand expression, but a two-fold decrease in KIT receptor expression. Consistent with this, there was nearly a three-fold increase in CBL expression, which can downregulate the KIT receptor through the ubiquitin pathway (Zeng et al. 2005). Thus, below we examined the expression of KIT protein in the cultures.

Figure 3
Redox blot sequentially incubated with antibodies against human peroxiredoxin-2 (PRDX2) and peroxiredoxin-3 (PRDX3). The bands at approximately 22 kDa represent the reduced monomer PRDX2 or PRDX3 proteins, while bands at about 44 kDa represent the respective oxidized dimers. The amount of oxidized dimer relative to the total reduced monomer plus oxidized dimer in each lane is an indication of the relative amount of H₂O₂-induced stress (Cox et al. 2010). PRDX2 acts as a sensor for H₂O₂ stress in the cytosol of the cells, while PRDX3 acts in the mitochondria. Beta-tubulin is a loading control. Lanes 1-3 are from separate xenografts of SDH-deficient GIST. Lane 4, which shows the highest amounts of both oxidized dimers and reduced monomers, is from a primary conventional GIST with intact SDHB.
In addition to its SDHB mutation and consequent loss of SDH activity, this GIST was unusual in also harboring a somatic KRAS mutation. This suggested that the unusual morphology, extremely high proliferative fraction, partial loss of the characteristic marker KIT and extreme fragility of the tumor cells in vitro might result from aberrant RAS/MAPK signaling. To test the effects of inhibiting this signaling cascade, we used two structurally different inhibitors of MAP kinase signaling, U0126 and PD98059 (Dokladda et al. 2005). Low concentrations of U0126, sufficient to cause approximately a 50% decrease in MAPK phosphorylation, caused a 2- to 2.5-fold increase in KIT expression (n=4 independent experiments). This effect was not seen with PD98059. Paradoxically, this modest inhibition of ERK also maintained short-term proliferative activity, as indicated by Ki67 expression, at a higher level than in control cultures (Fig. 6). However, the percentage of labeled cells in culture remained markedly lower than that in xenograft tissue. Long-term maintenance with either inhibitor did not result in sustained proliferation or establishment of cell lines.

Discussion

The Ian GIST model is a unique human-derived model for SDH-deficient GIST, a rare mesenchymal tumor that can occur in patients with SDHB mutations.

GISTs comprise <1% of all gastrointestinal tumors (Oudijk et al. 2013). SDH-deficient GISTs, account for ~3% of all GISTs and ~5% of gastric GISTs in an unselected adult population (Gill et al. 2011). Approximately 50% arise in the context of Carney Triad, a non-hereditary syndrome caused by post-zygotic promoter hypermethylation of SDHC and consisting of SDH-deficient PGL, SDH-deficient GIST and pulmonary chondroma. Approximately 30% are associated with germline mutations of SDHA (Oudijk et al. 2013).
In a 2016 NIH study, 34 of 63 SDH-mutant GISTs harbored mutations in SDHA, 16 in SDHB, 12 in SDHC and 1 in SDHD (Boikos et al. 2016). All developed in the stomach, consistent with earlier reports.

SDH-deficient GISTs may occur in patients with or without a history of PGLs, and the lifetime risk of developing SDH-deficient GIST in patients with SDHB mutations is uncertain. It is also unclear whether SDHB confers a worse prognosis than other mutated SDHx genes, as is the case with paraganglioma (Gimenez-Roqueplo et al. 2008). However, most SDH-deficient GISTs follow an indolent course even after they disseminate (Boikos et al. 2016). The tumor represented by the Ian GIST model was unusual for its morphology, high proliferative fraction, decreased expression of the GIST markers KIT and DOG1 and rapid clinical course. It is likely that these characteristics resulted from the combination of germline SDHB and somatic KRAS mutations.

The most distinctive morphological feature of this tumor model is the presence of tiny cells with marked autophagic activity. Comparable findings have been reported in a model of nutrient starvation caused by growth factor deprivation (Lum et al. 2005). In that model, decreased cell size was caused by autophagic consumption of cytoplasmic constituents, with progressive loss of ribosomes and the ER/Golgi network. The latter would also result in reduced expression of markers associated with differentiated function. We hypothesized that in the present model conditions comparable to exogenous nutrient starvation might be caused by superimposing metabolic demands caused by KRAS mutation (Eser et al. 2014) on loss of SDH activity, which alone results in decreased function of TCA cycle anaplerotic pathways (Lussey-Lepoutre et al. 2015) and loss of ATP production by oxidative phosphorylation.

As a preliminary test of this hypothesis, we used two MEK inhibitors, U0126 and PD98059, which have previously been employed to study the effects of kinome remodeling on cellular function and differentiation (Duncan et al. 2012). Consistent with this hypothesis, concentrations of U0126 causing approximately a 50% decrease in MAPK phosphorylation in cell culture produced a 2–2.5-fold increase in KIT expression and also maintained short-term proliferative activity as indicated by Ki67 expression. PD9059 did not affect KIT but had a comparable effect on Ki67 (data not shown). While neither inhibitor is entirely specific for MEK and both might have targets outside the RAS/MAPK cascade (Dang & Lowik 2004, Dokladda et al. 2005), these results suggest that use of the Ian GIST model to study kinome remodeling (Duncan et al. 2012) might be a fruitful area of investigation. They are of particular interest in view of...
recent studies showing that treatment of cancer patients with kinase inhibitors can select for survival of subsets of tumor cells known as drug-tolerant persister (DTP) populations (Terai et al. 2018).

Considering its unusual features, it might be asked how relevant the Ian GIST model is likely to be either to other SDH-deficient GISTs or to paragangliomas and other SDH-deficient tumors. Our most relevant findings to date concern oxygen and antioxidants. Paragangliomas are known to occur with increased frequency at high altitudes, and hypoxic signaling is a putative phenotypic modifier and driver of tumor growth in patients harboring SDHx mutations (Her et al. 2015). Based largely on murine knockdown models, hyperoxia has been proposed as a potential treatment (Her et al. 2015). In addition, SDHx-mutated paragangliomas are reported to show high levels of markers associated with oxidative stress (Fiedler et al. 2012), which has been proposed as both a driver and inhibitor of tumor progression in other tumor types (Pani et al. 2004). Because of the latter role, superoxide dismutase has been proposed as a therapeutic target (Pani et al. 2004). The Ian GIST model is the first from an actual human tumor of a type known to be associated with SDHB mutations that can be experimentally manipulated and utilized to study mechanisms of oxygen effects and novel treatment strategies. The evidence thus far suggests that tumor growth and survival require a balance between protective effects of hypoxic signaling vs deleterious effects of oxidative stress. While reduced oxygen concentration clearly promotes tumor cell survival, a further survival benefit for tumor cells is achieved with N-acetylcysteine or other antioxidants. This suggests use of drugs that increase rather than decrease oxidative stress as novel therapies (Raj et al. 2011, Huang et al. 2016). Another major finding is the role of autophagy in maintaining tumor cell survival in xenografts. Autophagy is largely uninvestigated in any type of SDH-deficient tumor. However, autophagy pathways are potential targets of agents that may cooperate with kinase inhibitors and other drugs as triggers of apoptotic cell death (Terai et al. 2018). It is of interest that taurine, a sulfur containing amino acid present at very high levels in our xenografts, is both an antioxidant (Schuller-Levis & Park 2003) and a negative regulator of autophagy (Zhang et al. 2017).

The deleterious effects of oxygen observed in cell culture are not entirely unexpected because it has been recognized for some time that routine culture conditions in 95% air/5% CO₂ are not physiological either for normal tissues or tumors (Carreau et al. 2011). While the pO₂ in inspired air (~20% O₂) is 150mmHg, the value drops dramatically in normal solid tissues. For example, in liver and kidney, pO₂ has been measured at ~41 and 72mmHg (equivalent to ~5% and 10% O₂ respectively). In tumors of different histologic types, pO₂ ranged from 0 to 54mmHg (Carreau et al. 2011). The most important consequence of the present study might therefore be the way the dramatic response to O₂ may inform our approaches to other SDH-deficient tumors, both in terms of model development and pre-clinical drug testing. For more than 40 years, attempts to establish human paraganglioma and pheochromocytoma cell lines in cultures maintained at 95% air/5% CO₂ have been unsuccessful. Most pre-clinical drug testing of substitute cell lines has also been conducted under these conditions, which can exaggerate the efficacy of many chemotherapeutic agents (Carreau et al. 2011). Revisiting these studies may yield interesting results.

In summary, the Ian GIST model is potentially important both for what it might reveal specifically pertinent to this tumor type and more broadly to other types of SDH-deficient tumors, especially regarding metabolic and signaling characteristics shared across the spectrum of SDH-deficient tumor types. The presence of the KRAS mutation could make the model challenging as a tool for development of therapies.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0115.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research was supported principally by grants to AST from the SDHB Pheo Para Coalition, the Pheo Para Alliance, and the Paradifference Foundation. The work utilized NMR instrumentation that was purchased with funding from a National Institutes of Health SIG grant (S10OD020073). K T S acknowledges support from the NSF GRFP.

Acknowledgements
The authors thank Dr Andrey Gritzman for providing pathology slides of the patient’s primary tumor and recurrence, and Dr Andrea Califano for sharing transcriptome data not shown in this paper.
References


Huang BK, Langford TF & Sikes HD 2016 Using sensors and generators of H2O2 to elucidate the toxicity mechanism of piperlongumine and phenethyl isothiocyanate. Antioxidants and Redox Signalling 24 924–938. (https://doi.org/10.1089/ars.2015.6482)


Taguchi T, Sonobe H, Toyonaga S, Yamasaki I, Shuin T, Takano A, Araki K, Akimaru K & Yuri K 2002 Conventional and molecular cytogenetic characterization of a new human cell line, GIST-T1, established from gastrointestinal stromal tumor. Laboratory Investigation 82 663–665. (https://doi.org/10.1038/labinvest.3780461)


Winterbourn CC 2008 Reconciling the chemistry and biology of reactive oxygen species. Nature Chemical Biology 4 278–286. (https://doi.org/10.1038/nchembio.85)


