THE ROLE OF SIRTUIN 3 IN OVARIAN CANCER METASTASIS

A Thesis in
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by
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ABSTRACT

Epithelial ovarian cancer (EOC) is highly metastatic with late-onset symptoms, making it the leading cause of death in women with gynecologic malignancies. The proposed mechanism of metastasis involves aggressive, free-floating spheroid-like cellular aggregates that shed from the primary tumor, survive for some time in anchorage independence and eventually invade the omentum of the peritoneal cavity and nearby organs. Preliminary data revealed an upregulation of the antioxidant superoxide dismutase (SOD2) in ovarian cancer spheroids and confirmed its recently emerging, dichotomous role as a tumor promoter. We hypothesized that sirtuin 3 (SIRT3), a key regulator of SOD2, would similarly be increased in spheroids and help to promote ovarian cancer metastasis. While we observed the SIRT3 protein increase in ovarian cancer spheroids, we unexpectedly saw – upon generating SIRT3 shRNA-mediated knockdown cells – an increase in metastatic functions such as migration and proliferation. These data point to a tumor suppressive function of SIRT3 in ovarian cancer, as previously established in breast cancer. Interestingly, bioenergetics analysis characterized SIRT3 knockdown cells as having increased rates of glycolysis and glucose-dependent mitochondrial respiration. Moreover, they displayed a dependency on glucose for cell survival, while those cells with SIRT3 expression were able to survive in glucose-depleted conditions. These results may hint at clues as to why ovarian cancer spheroids increase expression of SIRT3 during the anchorage-independent stage of metastasis in the ascites fluid-containing peritoneal cavity. Efforts are underway to establish if a transient increase in SIRT3 during this anchorage-independent spheroid stage enhances cellular metabolic flexibility to rely on alternate fuel sources in situations of glucose deprivation, as may be the case in the ascites fluid.
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Chapter 1

BACKGROUND

1.1 Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer-related death in American women, with an estimated 22,000 diagnoses and 14,000 deaths for 2016 alone (1). The five-year survival rate for all stages of ovarian cancer is 45%, but patients are often diagnosed at late stage with distant metastatic lesions, in which case the rate dismally drops to 28% (1). Approximately 25% of women are diagnosed with stage I or II cancer, whereas 58% of diagnoses are of metastatic stage III and 17% of patients are presented with stage IV cancer (2). The lifetime incidence of ovarian cancer is 1.39% (1 in 72 women), and the lifetime risk of death is 1.04% (1 in 92 women) (2). Presently, there are no reliable screening methodologies for prevention or diagnostic modalities for early detection. Therefore, all efforts in understanding the underlying pathogenesis of this deadly disease are essential towards finding early markers of the disease and better treatments.

Symptoms

Symptoms of ovarian cancer are non-specific and often correlate with retrospective identification of abdominal discomfort, lower back pain, bloating, nausea, unusual constipation or diarrhea, frequent urination and lack of appetite as common presenting signs (3). These symptoms are often overlooked as simple gastrointestinal issues, thus contributing to the difficulty in making ovarian cancer early diagnosis a challenge.
Risk factors

The most clearly associated risk factor for the development of ovarian cancer is family history (4); however, other common risk factors include age, with the median age of ovarian cancer at diagnosis being 63 years (2), and BRCA1 and BRCA2 genetic mutations, which cause defective homologous recombination repair mechanisms (5). Factors that suppress ovulation, such as full-term pregnancies, oral contraceptive use, and lactation are considered to be protective against the development of ovarian cancer (2).

Types

There are three major classifications of primary ovarian tumors: epithelial, sex-cord stromal, and germ cell. Epithelial ovarian carcinomas (EOCs) are the most common, accounting for 90% of ovarian cancers (6). Histological subtypes of EOCs, characterized by origin and utilization of pro-tumorigenic cell signaling pathways, include high-grade serous (the most common, representative of stages III and IV), low-grade serous, mucinous, endometrioid, clear cell, and transitional tumors (7, 8).

Origin

The origin of ovarian cancer has remained elusive over the years. It is not collectively agreed to arise from ovarian surface epithelial cells (OSE), as Sir Spencer Wells first proposed in 1872 (9). In 1999, Dubeau refuted Wells’ hypothesis and suggested an alternative cancerous origin: secondary Müllerian tract structures (10). At the beginning of the 21st century, Piek further supported Dubeau and postulated that the origin of serous ovarian adenocarcinomas likely arose from the fallopian tube (11). In Piek’s experience, flushing the fallopian tube for
culture purposes yielded easily-obtainable epithelial cells, which supported the idea that tubal cells may be transported and grafted onto the ovaries, to later present as cancer (11). There is also compelling genomic evidence linking early fallopian tube Serous Tubal Intraepithelial Carcinoma (STIC) lesions as a possible source of ovarian carcinoma: STIC lesions and ovarian carcinomas both share identical, DNA-damaging TP53 mutation signatures (12, 13). Thus, current research suggests that the primary ovarian cancers may be derived from the fallopian tube. This paradigm shift for ovarian carcinogenesis highlights the need for further clinical evaluation of the fallopian tube to establish characteristics of metaplastic changes and for experimental cell lines representing the fallopian tube. This information may also lead to direct efforts for prevention and treatment (i.e., tubal ligation or salpingectomy for the tying or removal of the fallopian tube).

Current treatment

The primary line of treatment for ovarian cancer at the time of initial presentation is optimal cytoreductive surgery (14). In those patients where surgical resection cannot reduce the burden of disease to less than 1 cm of visible disease, chemotherapy using paclitaxel and platinum-based drugs is the standard of care (14). Through mechanisms not fully understood, the majority of the 70% of ovarian cancer patients who initially respond to cisplatin therapy develop resistance and relapse (15). If the relapse occurs in greater than 6 months’ time, the cancer is challenged again with a platinum based regimen. If recurrence occurs in less than the 6-month time frame, there are limited, efficacious options. There are only two targeted therapies that have been FDA-approved: antiangiogenic agent Bevacizumab (for patients who do not respond to platinum chemotherapy) and poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) inhibitor Olaparib (for patients with BRCA mutations and who have been treated with at least three other chemotherapeutic drugs). Several agents are currently in clinical development phases
and additionally aim to target [human] epidermal growth factor receptor family members, pro-inflammatory cytokines, folate receptors, and the insulin-like growth factor system (16).

**Metastasis**

Ovarian cancers frequently invade the omentum, a large fold of peritoneum which overlies the surface of the abdominal cavity and stores fat (17). This metastasis often accounts for the abdominal pain that patients experience. Epithelial ovarian cancers can metastasize via the hematogenous and lymphatic routes, but most commonly metastasis is thought to occur via the transcoelomic route of peritoneal seeding. In this latter means, illustrated by Shield and co-workers (18), primary tumor cells shed into the peritoneal cavity where they form spheroid-like cellular aggregates carried by ascites fluid (Fig. 1). Recent *in vitro* and *in vivo* studies have established that anchorage-independent ovarian cancer spheroids contribute to tumor generation and survival, metastasis and chemotherapy resistance (19-22). Again, understanding the biology of this aggressive population of free-floating cells is imperative for designing more targeted, better effective EOC therapies; for it is thought that these populations of cells are responsible for recurrent diseases.
Figure 1. **Ovarian cancer spheroids metastasize to the omentum.** Ovarian cancer spheroids are free-floating, aggressive bodies of cells that shed from primary tumors and metastasize commonly to the omentum. [Figure taken from (18).]

### 1.2 Metabolism, mitochondria, and cancer

An increasingly expanding field of research for general cancer therapy is the targeting of metabolism. In the 1920s, Nobel Laureate Otto Warburg famously hypothesized that cancer cells lower their mitochondrial respiration and predominately generate the cellular energy currencies adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) through high rates of glycolysis followed by lactic acid fermentation (23). In these cancer cells’ wasteful alteration of glucose metabolism, glycolysis is decoupled from the normal, complete oxidation of glycolytic end-product pyruvate to acetyl-CoA, which enters the Krebs cycle to ultimately undergo oxidative phosphorylation (24). Marie and Shinjo (25) have summarized the “Warburg Effect” in their comparative illustration of glucose metabolism in normal versus cancer cells (Fig. 2). Another alteration common in cancer is increased glutamine metabolism. Glutamine-derived α-
ketoglutarate feeds into the Krebs cycle and serves as a major contributor to citrate and also malic enzyme-dependent pyruvate production – both of which are major energy sources for cancer cells (26, 27). A third, common metabolic alteration in cancer is the reliance of cells on fatty acid metabolism. Fatty acids may be oxidized to carbon dioxide, incorporated into membranes or serve as storage for cancer cell fuels to drive growth (24). Interestingly, fatty acid oxidation (FAO) was recently shown to be a primary metabolic pathway for metastatic ovarian cancer cells located on the fat-rich omentum (28). In all these cases, metabolic reprogramming of metabolism often occurs at the level of mitochondria.

Figure 2. The Warburg effect in cancer cells. In the presence of oxygen, normal cells metabolize glucose and oxidize pyruvate to carbon dioxide through the Krebs cycle and Oxidative Phosphorylation (TCA/OXPHOS), whereas lactate is produced when oxygen is limited. The Warburg effect describes how cancer cells, regardless of oxygen availability, convert most glucose to lactate, which generates far less ATP. [Figure taken from (25).]

Mitochondria are double membrane-bound “powerhouse” organelles that generate a generous supply of ATP (Fig. 3). The outer membrane contains various enzymes that catalyze reactions such as fatty acid elongation and tryptophan degradation. The intermembrane space contains cytochrome C, an important protein whose release from mitochondria officially initiates and commits a cell to apoptosis. In the matrix of the mitochondrial inner membrane, a series of
chemical reactions occur within the Krebs cycle, where energy is generated from the oxidation of acetyl-CoA from carbohydrate, fat, and protein metabolism. The inner membrane cristae is a center for oxidative phosphorylation and more redox signaling, as electrons from NADH and flavin adenine dinucleotide (FADH2) are transported through the electron transport chain (ETC) complexes to the final acceptor, oxygen, which becomes reduced. The transfer of electrons through the ETC drives the pumping of protons across the inner mitochondrial membrane, and this gradient produces ATP. However, oxygen can be prematurely and incompletely reduced, yielding reactive oxygen species (ROS).

Figure 3. Mitochondrial metabolism of nutrients. The mitochondrion, the central, white organelle of the blue-colored cell, is a hub for ATP production. Various nutrients, including glucose, fatty acids, and amino acids, are metabolized to ATP through several enzymatic processes, including fatty acid β-oxidation; the TCA, or Krebs, cycle; and oxidative phosphorylation. [Figure taken from (29).]
A frequent hallmark of cancer cells is increased ROS production. ROS are implicated in cellular damage and signaling pathways involved in migration, invasion, tumor cell survival and proliferation, metastasis, angiogenesis and inflammation (reviewed in (30)). Exogenous ROS can result from various exogenous chemicals, including xenobiotics as well as ionizing radiation, while endogenous ROS can be produced in several ways within the cell. ROS are generated when NADPH oxidases transfer electrons across the plasma membranes of cells (31); when endoplasmic reticulum stress is enhanced by misfolded proteins (32); and in oxidative phosphorylation upon the premature and incomplete reduction of oxygen (33). In cases of mitochondrial dysfunction when oxygen is improperly reduced, the toxic superoxide radical, $O_2^{-}$, is produced. To neutralize superoxide and maintain a redox balance, cells employ an antioxidant system consisting of superoxide dismutases (SOD1,2,&3), catalase, glutathione and thioredoxin systems (34). Exogenous antioxidants such as vitamins C and E may also contribute to detoxification of ROS.

**SOD2**

Mitochondrial manganese-containing superoxide dismutase (SOD2) is an antioxidant enzyme that detoxifies ROS by catalyzing the dismutation of superoxide ($O_2^{-}$) to oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$). For this reason, SOD2, for many years, was solely appreciated as a tumor suppressor (35). However, the increase in $H_2O_2$ production (a stable ROS) by elevated SOD2 has been shown to enhance the invasion and migration of tumor cells (36). Particularly in ovarian clear cell carcinoma, redox signaling in $H_2O_2$-mediated tumorigenesis was found to be through the oxidation and inactivation of phosphatases that regulate Akt and focal adhesion kinase (FAK) signaling pathways (37). Preliminary data from our laboratory show increased SOD2 activity in patient ascites-derived EOC spheroids (Fig. 4A) and SOD2 protein
levels in numerous ovarian cancer cell lines when cultured as anchorage-independent 3D spheroids compared to 2D attached monolayer grown cultures (Fig. 4B). Preliminary data (not shown) also show a decrease in spheroid cell size and viability upon SOD2 knockdown, suggesting a causative, tumorigenic role of SOD2. Therefore, we looked into upstream regulators of SOD2 activity that might also have a pro-tumorigenic role in ovarian cancer metastasis. We honed in on the mitochondria-localized deacetylase enzyme sirtuin 3 (SIRT3).

**Figure 4. SOD2 activity and protein is increased in ovarian cancer spheroids.** A) In patient ascites-derived EOC samples, SOD zymography reveals a marked increase in SOD activity in anchorage-independent grown spheroids (3D) compared to attached 2D conditions. B) Immunoblot of several wild-type ovarian cancer cell lines shows a similar increase in SOD2 protein in 3D spheroids compared to 2D monolayer cultures.

**SIRT3**

In this past decade alone, the biomedical sciences field has witnessed a striking rise in sirtuin research (38). There are seven mammalian homologs of silent information regulator 2 proteins (sirtuins 1-7) localized in either the nucleus, cytoplasm or mitochondria and having either deacetylase and/or ADP-ribosyltransferase activity. Full length SIRT3 is a 44-kDa nuclear protein that is proteolytically processed in the mitochondria to become a mature, enzymatically active, 28-kDa nicotinamide adenine dinucleotide (NAD+) dependent lysine deacetylase (39). Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) powerfully stimulates SIRT3 protein and RNA levels (40). SIRT3 then deacetylates, or activates, a broad range of well-defined substrates including long-chain-acyl-CoA dehydrogenase (LCAD)
(to induce fatty acid oxidation), SOD2 (to maintain ROS homeostasis), isocitrate dehydrogenase 2 (to facilitate the Krebs cycle), and acetyl-CoA synthetase 2 and glutamate dehydrogenase (to generate acetyl-CoA and amino acids to fuel the Krebs cycle) (39).

At lysine position 42 (K42), SIRT3 deacetylates and activates LCAD, which is one of several enzymes to catalyze FAO (41). FAO provides an essential source of acetyl-CoA, which then enters Krebs cycle for ATP energy output. In an elegant study by Nieman and co-workers., adipocyte-ovarian cancer cell co-culture revealed a direct transfer of fluorescently-labeled lipids to the cancer cells, which provided the energy for their proliferative growth (28). The authors also demonstrated that metastatic ovarian cancer cells rely primarily on FAO as a major metabolic pathway. Therefore, adipocytes favorably contribute to the ovarian cancer tumor microenvironment in the omentum by enhancing tumor growth (42).

SIRT3 has been shown to deacetylate and activate SOD2 at K122 and K68. When K122 was mutated to arginine, mimicking the deacetylated state, SOD2 activity was increased and mitochondrial superoxide levels were decreased (43). To further connect this to SIRT3, SOD2 activity was increased in SOD2−/− mouse embryonic fibroblasts (MEFs) co-expressing both wild-type SOD2 and wild-type gene Sirt3 (43). Supporting K68 regulation, overexpression of SIRT3 in HEK293T cells decreased the percentage of K68-acetylated SOD2 from approximately 54% to 36%, whereas knockdown of SIRT3 increased the percentage from 54% to 88% (44).

Similar to SOD2, SIRT3 has been characterized as both a tumor suppressor and tumor promoter. In the SIRT3 tumor suppressor-supporting literature, a murine study found that seven of twenty female SIRT3−/− mice spontaneously developed mammary tumors, while no
SIRT3**+** mice did so (45). Histological examination identified these mouse tumors as estrogen receptor- and progesterone receptor positive, which parallels the common breast cancer subtype in humans. Further, SIRT3**−** MEFs from the same study portrayed increased superoxide levels, an indication that the activating role of SIRT3 on SOD2 activity has been lost. Also in breast cancer, a study showed 74% of human invasive lesions to be immunohistochemically negative for SIRT3 compared to the 23% decreased SIRT3 expression in normal breast tissue (46). This low SIRT3 expression correlated with multiple subtypes of breast cancers, making it an attractive, potential biomarker. Other cancers in which SIRT3 is downregulated and acts as a tumor suppressor are hepatocellular carcinoma (47), lung adenocarcinoma (48), and gastric carcinomas (49). SIRT3 commonly acts as a tumor suppressor by suppressing ROS, most likely through the activation of SOD2, and by subsequently destabilizing ROS-responsive transcription factor hypoxia-inducible factor-1α (HIF-1α) (49-51). ROS emanating from mitochondria have been shown to cause HIF stabilization (52, 53). Under normoxia (21% oxygen), prolyl hydroxylases (PHDs) hydroxylate the proline residues of HIF-1α, resulting in the direct stabilization and degradation of HIF-1α protein levels. While hypoxia (0.3-3% oxygen) can directly decrease PHD activity, it can also cause increased ROS production from complex III of the mitochondrial ETC, which results in diminished PHD activity (54).

Conversely, SIRT3 functioned as a tumor promoter in the following cancers where it was found to be overexpressed. In several oral squamous cell carcinoma cell lines, experimental downregulation of SIRT3 promoted apoptosis and inhibited cell growth and proliferation in vitro and reduced tumor burden in vivo (55). In esophageal squamous cell carcinoma, increased SIRT3 expression correlated with worse prognoses (56). Downregulation of SIRT3 by RNAi in colon cancer resulted in decreased cell proliferation, migration and invasion, and increased ROS formation and HIF-1α expression levels (57). Recent reports have
demonstrated the pro-proliferative role of SIRT3 in melanoma (58) and renal cell carcinoma (59), in which SIRT3 knockdown induced senescence, cell cycle arrest, and inhibited tumorigenesis in vivo (58), and also impaired cell proliferation by inhibiting glutamate dehydrogenase and glutamine utilization in pyruvate-compromised cells (59).

These studies suggest a dichotomous role of SIRT3 in cancer, as previously demonstrated for one of its target proteins, SOD2. SIRT3 likely acts as a tumor suppressor by activating SOD2, thereby decreasing mitochondrial superoxide levels and destabilizing HIF-1α, a major regulator of tumor cell aerobic glycolysis and cell survival under hypoxia. Conversely, SIRT3 may act as an oncoprotein in tumor cells where it is highly expressed. Here, SIRT3 may help protect cells from ROS buildup in mitochondria by enhancing SOD2 activity and regulating metabolism, allowing tumor cells to enhance their flexibility to utilize different fuel sources in times of glucose deprivation. More so, the function of SIRT3 may depend on the stage of ovarian cancer. Opposite roles for SIRT3 have previously been documented in breast cancer. SIRT3 has been shown to be overexpressed in lymph-node positive breast cancer compared to normal breast tissue, implicating a pro-survival role of SIRT3 in advanced-stage breast cancer (60). In contrast, SIRT3 expression was decreased in stage IIA, IIB, and III malignancy breast samples compared to normal breast and stage I malignancy samples, suggesting a tumor suppressive role of SIRT3 (45). The impact of SIRT3 in various stages of ovarian tumorigenesis is yet to be determined.

1.3. Specific aims

The role of SIRT3 in ovarian cancer – as a tumor suppressor or promoter – has not been established. Due to increased SOD2 protein and activity in ovarian cancer spheroids (Fig. 4), we
hypothesize that SIRT3 is also increased in spheroids, thereby regulating ovarian metastasis through its role in enhancing cellular superoxide scavenging and metabolic reprogramming.

Aim 1 was to assess SIRT3 protein and RNA levels in serous ovarian cancer cell lines and human samples cultured in anchorage-independent spheroids versus attached monolayer-grown controls. Genetic manipulation of SIRT3 expression and characterization of the effects of SIRT3 knockdown on spheroid morphology, migration, and growth of ovarian cancer cells were studied.

Aim 2 was to understand the mechanistic role of SIRT3 in ovarian cancer metastasis. While understanding the SIRT3-SOD2 crosstalk in ovarian metastatic cells was of interest, this study also focused on investigating the metabolic pathways in which SIRT3 could have an important role. In Aim 2, we examined the hypothesis that SIRT3 increase in spheroid cells serves to prime ovarian cancer cells for metastasis to the omentum by inducing FAO through LCAD activation.

We measured the cells’ usage and dependency on long-chain fatty acid-, glutamine-, and glucose-oxidation pathways using a Seahorse metabolic analyzer.
Chapter 2

MATERIALS AND METHODS

2.1. Cell lines and culture conditions

Ascites fluid from a patient with high-grade serous ovarian adenocarcinoma (sample EOC7), that contained suspended ovarian cancer cells, was provided by our clinical collaborator, Dr. Rebecca Phaeton (Penn State Hershey). The OVCA433 cell line, derived from a patient with late-stage serous ovarian adenocarcinoma, was a generous gift from Dr. Susan K. Murphy (Duke University, Durham, NC). The OVCAR3 cell line, derived from the malignant ascites of a patient with progressive ovarian adenocarcinoma, was purchased from American Type Culture Collection (ATCC, Manassas, VA). OVCA433 and OVCAR3 cells were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma) and maintained in 5% CO₂ at 37°C. EOC7 was maintained in complete MCDB105/M199 medium supplemented with 10% FBS and 1% of 100x penicillin-streptomycin antibiotic (Corning).

2.2. Immunoblotting

Cells were grown in monolayer conditions to 70-80% confluency before being lysed in RIPA buffer (+ protease/phosphatase inhibitors). Cells grown as spheroids using 6-well, ultra-low attachment (ULA) dishes (Cornell) (seeding of 30,000 cells per well) were harvested on Day 5. Electrophoresis was carried out on 4-12% SDS-PAGE precast gels (BioRad), followed by western transfer to PVDF membranes and 1-hour blocking with 5% non-fat milk/TBS with 0.1% Tween20. Primary antibodies used were anti-SIRT3 (Cell Signaling Technology, #5490) at a 1:1,000 dilution, and GAPDH (Thermo Fisher Scientific, #4300) and β-Actin (Thermo Fisher
Scientific, #4302) at 1:10,000 dilutions (all diluted in 5% non-fat milk/TBS with 0.1% Tween20). Immunoreactive protein bands were visualized using SuperSignal West Femto chemiluminescence substrate (Thermo Fisher Scientific) and the ChemiDoc MP system (BioRad). Protein bands were normalized to GAPDH and Actin loading controls.

2.3. Real-time semi-quantitative RT-PCR

Total RNA was isolated from monolayers and spheroids (RNeasy Mini Kit; Qiagen) and converted to cDNA via reverse transcription (iScript cDNA synthesis; BioRad), according to manufacturer’s instructions. Real-time semi-quantitative RT-PCR was performed using an Applied Biosystems 7500 Real Time PCR cycler and iTaq Universal SYBR Green Supermix (BioRad). The following primer pairs were used: SIRT3 forward: 5’-GGACCGCGCTTTGC-3’; SIRT3 reverse: 5’-GGACCGCGCTTTGC-3’; 18S rRNA forward: 5’-GGACCGCGCTTTGC-3’; and 18S rRNA reverse: 5’-GGACCGCGCTTTGC-3’. Data were analyzed using the comparative threshold cycle (CT) method, and values were normalized to the ribosomal subunit 18S rRNA levels.

2.4. Generation of SIRT3 stable knockdown cell lines

For Lipofectamine 2000 shRNA transfection (Invitrogen, Carlsbad, CA), OVCA433 and OVCAR3 cells were plated at respective densities of 200,000 and 400,000 cells per well in 6-well plates and allowed to come to 70-80% confluency. The following SIRT3 shRNA purified plasmids ( Origene TR309432 ) were transfected at final concentrations of 4 µg/µL (OVCA433) and 1 µg/µL (OVCAR3):

#1: [5’- GTACAGCAACCTCCAGCA GTACGATCTCC-3’]
Scrambled non-targeting shRNA (SCR) was used as a transfection control. Transfection media was changed to normal RPMI media 5 hours post-transfection, and 10 µg/mL puromycin-supplemented media was used to select transfected cells at 72 hours post-transfection. Cells were maintained in a stable selection RPMI media containing 10% FBS, 0.5 µg/mL puromycin, and 1% of 100x penicillin-streptomycin antibiotic (Corning).

2.5. Spheroid morphology assay

1000 cells per well were seeded under normal culture conditions in 96-well ULA plates (Cornell). The morphology of spheroids was manually captured at selected time points (12, 24, 48, 72, 96, 120 hours; 10x magnification) using a Keyence BZ-X700 microscope.

2.6. Spheroid migration assay

Migration was assessed by transferring spheroids from the 96-well ULA plate to a normal 96-well cell culture plate, using a sterile 200 µl pipette with a cut tip. Images of outgrowth were captured immediately after transfer and up to 2 days afterwards. The surface areas covered by the migrating cells were measured using the line tool in Image J software.
2.7. Clonogenicity assay

This survival assay measures the ability of single cells to survive, attach and proliferate to form individual colonies. SCR and shSIRT3-stably transfected Ovca433 cells monolayer cells were plated in triplicate in 6-well cell culture dishes at a density of 3000 cells per well and monitored for up to 12-14 days. Upon colony formation (≥50 cells/colony), colonies were stained and visualized with 0.05% crystal violet dye.

2.8. Seahorse XF96 extracellular flux analysis to assess metabolic phenotype

The Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience; Billerica, MA) simultaneously measures the oxygen consumption rate (OCR), as a measure of cellular respiration and the extracellular acidification rate (ECAR), as an indication of lactic acid production and glycolytic flux, in live cells. Several pharmacological inhibitors can be used throughout the assay to determine which metabolic pathways are preferentially utilized by the cells. The metabolic Phenotype Test simultaneously measures the use of mitochondrial respiration and glycolysis in cells under baseline and energy-demanding stressed conditions. Stress conditions are induced by adding Oligomycin A, which inhibits ATP synthase and drives cells to increase glycolysis to meet energy demands. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), an ionophore that uncouples mitochondria, is added to increase OCR as the mitochondria try to recover the mitochondrial membrane potential. For the Phenotype Test, cells were plated in normal growth media at a density of 10,000 cells per well. The media was replaced with Seahorse XF media (25 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, no FBS and sodium bicarbonate) one hour prior to the assay. OCR and ECAR
measurements were taken at baseline and after simultaneous injection of 1 µM Oligomycin A and 0.75 µM FCCP.

The Fuel Flex Test can measure the dependency (the requirement for a fuel to meet metabolic demand), capacity (the ability for a fuel to meet metabolic demand), and flexibility (capacity minus dependency) of live cells to oxidize and utilize glucose, glutamine, and/or long chain fatty acids. For the Fuel Flex Test, cells were plated in normal growth media at a density of 10,000 cells per well. The media was changed to Seahorse XF media one hour prior to the assay. OCR and ECAR measurements were taken at baseline and after injection of the following pathway inhibitors: 2 µM UK5099 (of glucose oxidation), 3 µM BPTES (of glutamine oxidation), and 4 µM Etomoxir (of long-chain fatty acid oxidation).

2.9. Glucose-deprivation stress test

SCR and shSIRT3 cells were plated in a 6-well cell culture plate at a density of 150,000 cells per well in RPMI media substituted with 10% FBS. The following day, the wells were washed with PBS and the media was changed to FBS-depleted RPMI or FBS-and-glucose-depleted RPMI. FBS contains 94 mg/dL glucose, and it was essential to remove all traces of glucose. At 72 hours, the percentage of apoptotic, dead cells were measured using the Muse™ Annexin V and Dead Cell Kit (Millipore, MCH100105).

2.10. Statistical analysis

Statistical analysis has either been performed on experiments with at least three independent trials or technical replicates within a single trial (method disclosed in legend keys). Data are expressed
as mean ± SEM. Statistical data analysis (Student t-test using the Holm-Sidak method) was performed using GraphPad Prism Software v6 (*p < 0.05 deemed significant). Experiments with less than three trial sets are therefore representative and currently being repeated for confirmation.
Chapter 3

RESULTS

3.1. SIRT3 is overexpressed in ovarian cancer spheroids.

Because we have previously observed increased SOD2 activity, protein (Fig. 4) and RNA (not shown) levels in 3D anchorage-independent spheroids of numerous ovarian cancer cell lines and human EOC samples compared to attached 2D monolayers, we measured the protein and RNA levels of SIRT3, which is a SOD2 regulator. In our patient sample, EOC7, and in two selected ovarian cancer cell lines, we witnessed an increase in SIRT3 protein when cultured as 3D spheroids versus as 2D monolayers (Fig. 5A-C). SIRT3 RNA trends were inconclusive and varied depending on the cell line (Fig. 5D). However, since anchorage-independent spheroids mimic a phase of the ovarian cancer spread, we hypothesized that the increase in SIRT3 expression gives cells an advantage during metastatic progression and that SIRT3 may act as a tumor promoter in ovarian cancer.
Figure 5. **SIRT3 is increased in ovarian cancer spheroids.** Representative immunoblot of SIRT3 in spheroid and monolayer-cultured cells from A) patient-derived ascites (n=1) and B) ovarian cancer cell lines (n=3). Quantitation of three independent trials of both C) protein and D) RNA analysis of SIRT3 in spheroids and monolayers. *p<0.05; t-test analysis, statistical comparison between 2D and 3D of each cell line.

3.2. **SIRT3 knockdown promotes epithelial phenotype in spheroids, enhances migration, and increases proliferative growth.**

To study the role of SIRT3 in ovarian cancer metastasis, we employed lentiviral shRNA to generate stable SIRT3-knockdown (shSIRT3) ovarian cancer cell lines. OVCAR3 shSIRT3 cells are currently being generated, so the remaining data are of OVCA433 shSIRT3 cells. Of the four shRNA constructs, #2 and #3 showed the greatest suppression of SIRT3 protein compared to the non-specific targeting scramble control (SCR), which was comparable to wildtype (WT) OVCA433 (Fig. 6). For this reason, these knockdown cell lines (SCR, #2, and #3) were used for further studies.
Figure 6. **Generation of stable shSIRT3 OVCA433 cell lines.** Immunoblot analysis of SIRT3 shRNA transfected into the OVCA433 cell line. Constructs #2 and #3 yielded the best shRNA-mediated knockdown of SIRT3 compared to the WT and SCR controls.

Next, we grew OVCA433 knockdown cells as spheroids, using a 96 well ULA plate for microscopy and 6-well ULA plates for immunoblot purposes. Since we saw an increase in SIRT3 expression in wildtype cells under anchorage-independent spheroid growth, we hypothesized that a decrease in SIRT3 expression may have deleterious effects on spheroid formation or cell survival under anchorage independence. However, SIRT3 knockdown cells appeared to grow larger sized spheroids compared to scrambled controls (Fig. 7). In addition, beginning as early as 24 hours, we noticed morphological differences, with shRNA SIRT3 spheroids displaying a more epithelial phenotype (Fig. 7). We describe this phenotype as closely resembling a ‘squamous’ epithelial cell. Upon investigation of E-Cadherin and vimentin levels, we did not observe significant SIRT3-dependent differences in expression of these epithelial or mesenchymal markers, respectively. In the future, we will assess if SIRT3 knockdown increases mucin production, a common phenotype of ovarian cancer.
Figure 7. **SIRT3 knockdown spheroids are large and epithelial-like.** A) Representative time course experiment (n=3 trials) (10x magnification) in OVCA433 cells. B) 20x magnification at 48 hours from one trial. All images in same scale.
To further characterize the effects of SIRT3 knockdown in ovarian cancer cells, we assessed the migratory and colony-forming abilities of these cell lines. Upon transfer of viable spheroids to normal cell culture plates, we observed that cells from the SIRT3 knockdown spheroids migrated efficiently onto the culture dish following spheroid adherence, whereas the scrambled cells did not (Fig. 8).

Figure 8. SIRT3 knockdown cells are migratory. A) Representative time course of one well of one trial (n=6 wells per experimental group). Areas measured and outlined in yellow in ImageJ software. B) Quantitation of the change in migratory increase in area from t=0 to 48 hours after transfer from ULA to normal 96 well plates. Data as mean ± SEM.

In clonogenicity assays, SIRT3 knockdown cells formed larger colonies than scrambled counterparts (Fig. 9A). However, in counting the colonies, there was no significant difference in the number of colonies formed between SIRT3 scrambled and knockdown cells. This indicated that the cells were seeded with equal density and that cell survival may not be significantly affected, although SIRT3 knockdown increased the proliferative ability of cells to induce larger colony size (Fig. 9B).
3.3. SIRT3 knockdown increases rates of glycolysis and mitochondrial respiration in cells.

Aim 2 of our proposal sought to understand the mechanism of SIRT3 action in ovarian cancer. We first wanted to determine the role of SIRT3 in general bioenergetics of cancer cells, and performed a Seahorse Cell Energy Phenotype test. Using the Seahorse Report Generator tool, we plotted our data on an energy map: the utilization of each pathway - mitochondrial respiration (Oxygen consumption rate – OCR) and glycolysis (Extracellular Acidification Rate – ECAR) - at baseline and upon injection of the FCCP/Oligomycin A stressor mix. We discovered that our
SIRT3 knockdown cells shifted towards a more glycolytic energetic state (utilizing both metabolic pathways, but predominately glycolysis) compared to the more quiescent, scrambled cells (not energetic for either metabolic pathway) (Fig.10). SIRT3 knockdown cells were also the most sensitive to the FCCP and Oligomycin A inhibitors, becoming even more energetic in response to these stressors which cause increased respiration and glycolysis.

![Graph showing XF Cell Energy Phenotype](image)

**Figure 10. SIRT3 knockdown cells are energetic.** At baseline levels (open box) and after FCCP and Oligomycin A stressors (closed box), OVCA433 shSIRT3 #2 and #3 cells are shifted toward a more glycolytic and energetic state compared to SCR cells. n=1 trial.

We performed a Fuel Flex Test to 1) confirm the general, phenotypic characterization of SIRT3 knockdown cells and 2) attempt to identify the fuels most responsible for the enhanced basal rates of mitochondrial respiration and glycolysis in SIRT3 knockdown cells. As seen previously in the Phenotype Test, our Fuel Flex Test results showed that shSIRT3 ovarian cancer cells had high basal OCR, but even higher basal ECAR, compared to scrambled controls (Fig. 11). This verified that SIRT3 knockdown likely shift cells towards a more glycolytic phenotype. Although our first Fuel Flex test suggested that shRNA SIRT3 cells rely primarily on glucose as a fuel source, the
results of a replicate assay were inconclusive at the writing of this thesis. This was likely due to several technical issues, including solubilization of certain compounds such as Etomoxir and strong well-to-well differences in replicate assays after addition of glucose, fatty acid, and glutamine oxidation pathway inhibitors (UK5099, Etomoxir, and BPTES, respectively).

Figure 11. **SIRT3 knockdown cells have increased OCR and ECAR.** Fuel Flex Assay: shSIRT3 cells have higher basal OCR and ECAR values, respectively indicative of mitochondrial respiration and glycolysis rates. n=1 trial. Values taken at baseline (measurement 3) before the addition of fuel inhibitors.

3.4. Glucose deprivation sensitizes SIRT3 knockdown cells to apoptosis.

Finally, until we get an in-depth metabolic outlook using the fuel inhibitors in the Fuel Flex Test, we assessed the reliance upon the glycolytic pathway in SIRT3 knockdown cells in culture. In previously characterizing these cells as highly glycolytic, we hypothesized that when subjecting them to glucose-stress, cell death would be subsequently increased. Annexin V staining detected the apoptotic cells through the binding of phosphatidylserine, which translocates to the extracellular membrane upon initiation of apoptotic programmed cell death. When culturing cells in glucose-depleted RPMI media, we observed an increased population of apoptotic cells in SIRT3 knockdown cells as compared to scrambled cells (Fig. 12). This confirmed that SIRT3 knockdown cells rely on glucose oxidation as a major source of cellular energy.
Figure 12. SIRT3 knockdown cells utilize glucose for energy. Muse™ Annexin V staining at 72 hours reveals an increase in apoptotic cells upon SIRT3 knockdown and glucose deprivation. n=1 trial, no technical replicates.
Chapter 4
DISCUSSION AND CONCLUSION

In recent years, the sirtuin protein family has gained widespread interest in studies of metabolism, oxidative stress, neurodegenerative and cardiovascular diseases, and cancer. The role of SIRT3 in ovarian cancer had not been studied until recently, during the writing phase of this thesis. One sole report has found SIRT3 to be significantly downregulated in metastatic ovarian tissues and cell lines and function as a tumor suppressor; knockdown of SIRT3 enhanced migration and invasion in vitro and liver metastasis in vivo (61).

In the present study, we are the first to study the role of SIRT3 in ovarian cancer spheroids, which far better mimic and represent the metastatic population of ovarian cancer cells. We hypothesized that SIRT3 would act as a tumor promoter of ovarian cancer metastasis, given that one of its antioxidant targets, SOD2, is increased in spheroids and has been shown to mediate H$_2$O$_2$-driven metastasis in ovarian clear cell carcinoma (37). We are not sure why SIRT3 RNA levels were not consistently upregulated in spheroids between ovarian cancer cell lines, but we realize that the mRNA of a gene is not necessarily depictive of its protein levels. We simply interpret these conflicting data by proposing that SIRT3 may be regulated at the translational, protein level or that there may be increased SIRT3 protein degradation in spheroids. In future studies, we will assess whether manipulating translation alters SIRT3 levels. However, in contrast to RNA, we observed consistently increased SIRT3 in spheroids at the protein level, which suggested a possible role for SIRT3 in ovarian cancer cell metastasis. Upon observation of this increase, we hypothesized that SIRT3 expression may be increased to aid in cellular survival during anchorage independence, which is a critical step during transcoelomic metastasis. In this metastatic event, cells detach from the primary tumor on the ovary and spend time as cellular aggregates in
intraperitoneal cavity ascites fluid, prior to attachment to the omentum. We then proceeded to characterize the role of SIRT3 by generating stable SIRT3 knockdown cells. Upon SIRT3 knockdown we expected to see an inhibition of metastatic potential but instead witnessed an interesting increase in 1) spheroid size, 2) epithelial-like spheroid character, 3) proliferation, 4) migration, 5) rates of glycolysis and mitochondrial respiration, and 6) dependence on glucose. This encouraged us to rethink the role of SIRT3 in ovarian cancer metastasis. Why is SIRT3 increased in ovarian cancer spheroids, yet functions as a tumor suppressor?

Because we observed an enhanced epithelial phenotype upon SIRT3 knockdown, we initially thought it may be indicative of a reversal of an epithelial-to-mesenchymal (EMT) phenotype, previously described for aggressive ovarian cancer spheroids (62). Furthermore, in an *in vivo* ovarian cancer metastatic mouse model, forced epithelial-cadherin (E-cadherin) expression inhibited ovarian metastasis (63). However, in our study, SIRT3 knockdown did not prevent spheroid outgrowth and migration (Fig. 6), suggesting that this observed epithelial phenotype does not prevent metastatic progression in shSIRT3 spheroids.

Previously, when hypothesizing SIRT3 to be a tumor promoter, we proposed that SIRT3 primes ovarian cancer cells for metastasis to the omentum by inducing FAO through LCAD activation. Initially, we sought to determine the direct effects of SIRT3 loss on LCAD acetylation, anticipating that 1) spheroids display a decrease in LCAD acetylation and hence activation of the FAO pathway; and that 2) SIRT3 knockdown would reverse these observations. Unfortunately after several attempts to use anti-acetyllysine and LCAD antibodies in immunoprecipitation assays, we were unsuccessful in demonstrating acetylation of LCAD under the conditions investigated.
Although we had technical difficulties in verifying that SIRT3 regulates FAO, we did observe that SIRT3 knockdown results in higher glycolytic rates and a potential dependency on glucose. SIRT3 has been shown to repress glycolysis through HIF-1α destabilization and the subsequent downregulation of target glycolytic genes in breast cancer (50, 64). Perhaps in ovarian cancer, the increase in SIRT3 during anchorage independence is a transient phenotype that better allows cells to flexibly utilize different fuel sources of the varying tumor environments (i.e. ascites fluid). It is feasible that cells are deprived of fuels, in particular glucose, and that the existence of SIRT3 allows cells to slow metabolism and/or switch dependence to other carbon sources. Our observations that SIRT3 knockdown cells are more susceptible to glucose deprivation, is one clue that SIRT3 may provide ovarian cancer cells with an advantage in nutrient stress situations.

Compared to parent monolayer-attached cells, increased glycolytic rates have been observed in human colon carcinoma- (65), glioma- (66), and transformed embryonic kidney tumor- (67) spheroids, with postulated causes pointing to the intrinsic hypoxic environment and poor vascularization of spheroids. However, unpublished evidence from our laboratory demonstrates a decrease in glycolytic flux in ovarian cancer spheroids compared to attached monolayers (Fig. 13). This supports our hypothesis that SIRT3 may be upregulated in spheroids to suppress tumorigenesis via repression of glycolysis and the subsequent shift to alternate fuel sources. To further elucidate the role of SIRT3 in nutrient stress, we plan to repeat the clonogenicity, migration, and spheroid morphology assays under glucose-stress conditions to determine if the tumor-suppressive function of SIRT3 is glucose-dependent. In ovarian cancer patients, spheroids are found in suspension in ascites fluid, which could potentially be a nutrient-stressed environment. We plan to measure glucose levels in human ascites compared to serum levels, and also use ascites fluid as conditioned media for cell culture, compared to low or high glucose media. We will assess whether SIRT3 advantageously protects spheroids in these potentially nutrient-stressed environments and conditions.
Figure 13. **Ovarian cancer spheroids exhibit decreased glycolytic flux.** Extracellular flux analysis (Seahorse XF24) reveals that ES-2 ovarian cancer spheroids (S) have a significantly lower ECAR/OCR ratio, indicating a decrease in glycolysis. Student T-test, ****p<0.0001. [Data provided by Usawadee Dier.]

In future Fuel Flex Test studies, we look forward to determining the main fuels oxidized upon SIRT3 knockdown within attached and anchorage-independent conditions. In addition, we plan to perform glucose-media depletion stress tests under anchorage-independent conditions. We anticipate that spheroids increase SIRT3 expression to deal with these nutrient stress situations, while SIRT3 knockdown spheroids will not be able to survive, due to their dependence on glycolysis.

We must not downplay the potential SOD2-SIRT3 axis when discussing ovarian cancer metastasis. SIRT3 deacetylates and activates SOD2, and possibly these two proteins must work in concert to promote metastasis. Loss of extracellular matrix (ECM) attachment strongly induces ROS (68), which can cause damage to DNA and even induce apoptosis (69). The detoxification of ROS is essential for anchorage-independent spheroid survival and tumor development (70). Studies have shown SIRT3 to greatly enhance the ability of SOD2 to reduce cellular ROS through K53 and K89 SOD2 deacetylation (71), and scavenge ROS through K68 SOD2.
deacetylation (44). Collectively, SIRT3 and SOD2 may provide a protective mechanism for decreasing oxidative stress within cellular spheroids and thus allowing survival. We will employ a mitochondrial superoxide fluorescent dye (MitoSOX) and SOD2 zymography studies to monitor antioxidant activity in SIRT3 knockdown ovarian cancer cells. We will consider generating a double-knockdown of SOD2 and SIRT3 in ovarian cancer cells and perhaps may demonstrate that spheroids require both enzymes to survive in anchorage independence.

Finally, it could be that SIRT3 expression is transiently regulated, only occurring during anchorage independence and before cells begin to proliferate. In future studies, we are interested in the regulation of SIRT3, as PGC-1α has been shown suppress ROS in a SIRT3-dependent manner (72). We plan to measure PGC-1α levels in various stages of ovarian cancer, and the subsequent effects on SIRT3 levels upon PGC-1α genetic manipulation.

This current study is one of the first to characterize the role of SIRT3 in ovarian cancer, and we are beginning to unravel its relationship to tumor metabolism and pro-survival pathways. We have identified a mechanism in which SIRT3 upregulation in spheroids likely provides a pro-survival, protective advantage, but we still need to determine at what stages of tumor progression SIRT3 is altered and how these changes affect spheroid survival in metastatic progression. This study already suggests promising, future translational potential. Once we better understand the function of SIRT3 in ovarian cancer progression, we can think of targeting SIRT3 based on the cancer stage and cell type. The only reported pharmacological activator of SIRT3 is honokiol (HKL), a natural compound derived from magnolia tree bark. HKL has been shown to block and reverse cardiac hypertrophy in mice (73). This compound may have similar effects on human-derived ovarian cancer cells, by slowing the migration, proliferation, and spheroid formation of cells. A novel SIRT3 inhibitor, LC-0296, has recently been developed and shown to inhibit head
and neck cancer cell survival and proliferation, and promote apoptosis (74). Aside from these agents, most pharmacological inhibitors and activators are broad-spectrum and non-specific across the sirtuin family. However, SIRT3, specifically, remains a valuable protein target of interest as we consider designing new therapeutic treatments for ovarian cancer patients.
REFERENCES


64. Schumacker PT. 2011. SIRT3 Controls Cancer Metabolic Reprogramming by Regulating ROS and HIF. Cancer Cell 19:299-300.


