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Ovarian tumor-initiating cells display a flexible metabolism

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Abstract

An altered metabolism during ovarian cancer progression allows for increased macromolecular synthesis and unrestrained growth. However, the metabolic phenotype of cancer stem or tumor-initiating cells, small tumor cell populations that are able to recapitulate the original tumor, has not been well characterized. In the present study, we compared the metabolic phenotype of the stem cell enriched cell variant, MOSE-L_{FFLV} (TIC), derived from mouse ovarian surface epithelial (MOSE) cells, to their parental (MOSE-L) and benign precursor (MOSE-E) cells. TICs exhibit a decrease in glucose and fatty acid oxidation with a concomitant increase in lactate secretion. In contrast to MOSE-L cells, TICs can increase their rate of glycolysis to overcome the inhibition of ATP synthase by oligomycin and can increase their oxygen consumption rate to maintain proton motive force when uncoupled, similar to the benign MOSE-E cells. TICs have an increased survival rate under limiting conditions as well as an increased survival rate when treated with AICAR, but exhibit a higher sensitivity to metformin than MOSE-E and MOSE-L cells. Together, our data show that TICs have a distinct metabolic profile that may render them flexible to adapt to the specific conditions of their microenvironment. By better understanding their metabolic phenotype and external environmental conditions that support their survival, treatment interventions can be designed to extend current therapy regimens to eradicate TICs.

Keywords

tumor-initiating cell; metabolism; glycolytic phenotype; AICAR; metformin; ovarian cancer microenvironment

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The authors have no conflict of interest to disclose.

Introduction

Ovarian cancer remains the 4th leading cause of cancer death among women[1]. Recent studies indicate that 75% of ovarian cancer cases are diagnosed in stage III or stage IV[2]. Standard treatment for advanced ovarian cancer includes cytoreductive surgery followed by platinum-based chemotherapy with a 50% to 70% relapse of disease within 18 months[3]. More recently, it has been reported that these platinum-resistant ovarian tumors are enriched with cancer stem cells, suggesting that these cells may be crucial to recurrent disease[4]. In fact, chemotherapy may enrich the cancer stem cell population as it targets and eradicates differentiated cancer cells[5].

Stem cells are a small group of cells able to phenotypically self-renew and produce differentiated daughter cells that have limited capacity for replication. In cancer, this long-lived stem cell population is referred to as tumor-initiating cell population or cancer stem cells (CSC/TICs)[6]. CSC/TICs within a tissue have a unique phenotype, distinctly different from their normal tissue counterparts[7]. Ovarian cancer CSC/TIC populations isolated from a human ascites were shown to have the ability for self-renewal, anchorage-independent growth *in vitro* and tumorigenicity, mimicking the original tumor *in vivo*[8]. It is unknown whether this distinct cancer cell population confers the same metabolic profile as other cancer cells exhibiting the well-documented Warburg effect[9, 10]. Human embryonic stem cells, adult hematopoietic stem cells and induced pluripotent stem cells exhibit a decrease in oxygen consumption, producing more ATP through glycolysis than through oxidative phosphorylation (OxPhos)[11, 12]. They also exhibit increased lactate secretion with a concomitant increase in acidification of their medium compared to their differentiated counterparts[11]. However, the metabolic phenotype of ovarian cancer CSC/TICs is not well described.

Metabolic changes are necessary to sustain unrestricted growth in cancer cells. As cancer cells rapidly divide, the need for the synthesis of lipids, nucleotides and proteins for cell division increases. A high carbon flux through glycolysis not only allows for pyruvate and ATP production, but also for carbons to be diverted for *de novo* macromolecule biosynthesis. This altered metabolism permits glucose-derived carbon intermediates to be used for macromolecular synthesis to sustain growth[13]. We have recently demonstrated that mouse ovarian surface epithelial (MOSE) cells, representing early (benign), intermediate, and late (aggressive and invasive) stages of ovarian cancer[14, 15] also exhibit an increasingly glycolytic phenotype[16]. As cells progress through distinct stages of the disease, they change their morphology and organization and increase their growth rate while acquiring the ability to grow as spheroids, invade collagen and form tumors *in vivo*[14, 15, 17]. By *in vivo* passaging, the late stage MOSE cells (MOSE-L) were enriched for tumor initiating cells - MOSE-L_{FFLV} (hereafter referred to as TICs)-that exhibit increased tumorigenicity[18]. This is consistent with other investigations demonstrating that ovarian CSC/TICs produce measureable tumors more rapidly than cancer cells[19, 20]. In the present studies, we investigated the metabolism of these highly aggressive cells. We hypothesized that due to the increased growth rate and the invasive nature of our TICs, their metabolism may be phenotypically distinct. These metabolic changes may be important to

drive the proliferation and tumorigenic potential of these tumor-initiating cells and, therefore, may present a target for the prevention of ovarian cancer metastatic outgrowths.

Methods and Materials

Cell Culture

The MOSE cell model has been developed from C57BL/6 mice as described[14]. Classification into early-benign (MOSE-E), transitional intermediate and late-aggressive (MOSE-L) phenotypes was established based on their morphology, growth rate, anchorage-independent and spheroid growth capacity and tumorigenicity *in vivo*[14]. MOSE-L cells (1×10^6 cells) were injected intraperitoneally (IP) into syngeneic C57BL/6 mice; tumor cells were harvested from ascites 8 weeks later and propagated in cell culture for 4 passages, eliminating all other cell types. These cells were subsequently transduced with firefly luciferase lentiviral particles (GeneCopeia) to facilitate *in vivo* imaging and a high luciferase-expressing subclone (MOSE-L_{FFLV}) was isolated, referred to as TICs (tumor initiating cells) throughout this manuscript. This cell line represents a highly aggressive, tumor-initiating cell variant of the MOSE-L cell line[18] that is able to clonally expand and grow as spheroids in low attachment, serum-free conditions at high efficiency. As few as 100 cells injected in immune competent, syngeneic mice generates lethal peritoneal dissemination; IP injection of 1×10^4 TICs induces fatal disease after 21 days (compared to the parental MOSE-L: 1×10^6 cells are fatal after 120 days)[14, 18]. The specific characteristics of these cells will be reported elsewhere. For the purpose of this study, all MOSE cells were cultured in DMEM (Sigma) supplemented with 4% FBS (Atlanta Biologicals) and 100 ug/ml each of penicillin and streptomycin (Gibco) at 37°C in a humidified incubator with 5% CO₂; the TICs medium was supplemented with 4 µg/ml puromycin (Sigma) to maintain the expression of the firefly luciferase plasmid.

Animals

6 month-old C57BL/6 mice (Harlan Laboratories) were housed in a 12 h light and 12 h dark cycle with free access to water and standard rodent chow. 1×10^4 TICs in 100µl sterile PBS were injected IP. After 3 weeks, mice were euthanized by CO₂ asphyxiation and matched tumors from ascites and diaphragm were harvested from individual mice, digested as described[18, 21], and a single cell solution was plated into tissue culture dishes. Cells were cultured for 3 passages with puromycin to eliminate other tumor-associated cells before performing mitochondrial stress tests to assess oxygen consumption rate and extracellular acidification rate, using the Seahorse Biosciences XF24 Analyzer (see below). All animal studies were approved by the Virginia Tech Institutional Animal Care and Usage Committee.

Real-time PCR (qPCR)

Cells were seeded in 100 mm culture dishes and harvested 72 hours later. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed on 500ng of RNA using the ImProm-II Reverse Transcription System (Promega). Primer pairs were designed with NCB Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) or Beacon Design software (see primers in Supplemental Table 1). qPCR was performed using

50ng of cDNA with SensiMix Plus SYBR Mastermix (Quantace) in the ABI 7900HT (Applied Biosystems) with the following parameters: 42 cycles at 95°C for 10 minutes, 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 15 seconds, followed by a dissociation curve segment. Data was quantified using the CT method and expressed relative to RPL19 as the housekeeping gene as described previously[15, 17]. Data are expressed as mean $\pm$ SEM from three biological replicates performed in duplicate.

Glucose and Fatty Acid Oxidation using Dual-Labeled ^{14}C -Glucose and ^3H -Palmitate to Assess Substrate Choice for Oxidation

Cells were seeded at 5×10^5 cells/well in 6-well cell culture plates, incubated for 3 h, and washed with PBS. Following 2 h of incubation in serum-free medium, glucose and fatty acid oxidation were assessed via co-incubation with U- ^{14}C glucose and U- ^3H -palmitic acid. Glucose oxidation was measured via $^{14}\text{CO}_2$ production as previously described[22]. Incorporation of $^{14}\text{CO}_2$ derived from U- ^{14}C glucose into NaOH was measured using a LS 6500 scintillation counter (Beckman Coulter). Concomitantly, fatty acid oxidation was assessed by measuring $^3\text{H}_2\text{O}$ generation from 9,10-U- ^3H -palmitate (Perkin Elmer) as previously described[23]. Data presented as the mean $\pm$ SEM from three independent experiments performed in replicates of six, corrected for protein content.

Glucose Oxidation

Cells were seeded at 2.5×10^5 cells/ well in 6-well cell culture plates, incubated for 3 h, and washed with PBS. Following 2 h of incubation in serum-free medium, glucose oxidation was measured via $^{14}\text{CO}_2$ production essentially as previously described[22] except that glucose was substituted for BSA-bound palmitate. Incorporation of $^{14}\text{CO}_2$ derived from U- ^{14}C glucose into NaOH was measured using a LS 6500 scintillation counter (Beckman Coulter). Data are presented as the mean $\pm$ SEM from three independent experiments performed in replicates of three or six, corrected for protein content.

Fatty Acid Oxidation

Cells were seeded at 2.5×10^5 cells/ well in 6-well cell culture plates, incubated for 3 h, and washed with PBS. Following 2 h of incubation in serum-free medium, glucose and fatty acid oxidation were assessed via co-incubation with 1- ^{14}C palmitate. Fatty acid oxidation was measured via $^{14}\text{CO}_2$ production as previously described[22] and acid soluble metabolites were measured as the remaining label after acidification. Incorporation of $^{14}\text{CO}_2$ derived from 1- ^{14}C palmitate into NaOH was measured using a LS 6500 scintillation counter (Beckman Coulter). Data presented as the mean $\pm$ SEM from three independent experiments performed in replicates of three or six, corrected for protein content.

Lactate Assay

After the 3 h incubation with radioactive isotopes in the dual-labeled glucose and fatty acid experiments, 300 μl of medium was collected and assayed for lactate concentration using a colorimetric kit according to the manufacturer's instructions (BRSC, University of Buffalo). Data presented are the mean $\pm$ SEM from three independent experiments performed in six biological replicates, corrected for protein content.

De Novo Fatty Acid Synthesis

de novo fatty acid synthesis was assessed by measuring the quantity of U-¹⁴C-glucose (Perkin Elmer) from the dual-label glucose and fatty acid experiment that was partitioned into fatty acids as previously described[24, 25]. Data presented are the mean $\pm$ SEM from three independent experiments performed in six biological replicates, corrected for protein content.

Mitochondrial Respiration

Mitochondrial respiration of the cells was determined using an XF24 extracellular flux analyzer (Seahorse Bioscience) as described[26] with some modifications. Cells were seeded into XF24 V7 cell culture microplates at a density of 50,000 cells per well and incubated for 48 h. The medium was changed and the experiments were conducted in serum-free, bicarbonate-free medium after 1 h incubation. Cells were loaded into the XF24 and experiments consisted of 3-minute mixing, 2-minute wait, and 3-minute measurement cycle. Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitors 0.5 μ M oligomycin (Calbiochem), which inhibits ATP synthase, or in the presence of 0.3 μ M carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, Sigma), the mitochondrial uncoupler, to assess maximal oxidative capacity[27]. All experiments were performed at 37°C. FCCP-stimulated oxygen consumption rate (OCR) and oligomycin-stimulated extracellular acidification rate (ECAR) were calculated by the oligomycin- or FCCP-induced change minus the basal rates. Data presented are the mean $\pm$ SEM from six independent experiments performed in replicates of six or seven and corrected for protein.

Determination of mitochondrial integrity

Cells were grown on sterile glass coverslips for 24h, incubated with 2 μ M JC-1 (Life Technologies) for 20 min or 3 μ M mitoxonTM (Molecular Probes) for 10 min, to visualize mitochondria membrane potential or superoxide presence, respectively. Cells were rinsed and imaged in warm HBSS buffer on a Nikon 80i epifluorescence microscope equipped with UV, FITC and TRITC filters, and a DS-U2 monochromatic camera and using the NIS Elements BR 4.0 software (Nikon Instruments, Inc.); images were assembled with Adobe Photoshop®. To label active mitochondria, cells were treated with 7nM tetramethylrhodamine ethyl ester (TMRE) and 1nM mitotracker Green (both from Molecular Probes) for 45 min, rinsed twice, trypsinized and collected in Krebs Ringer solution containing 7nM TMRE and propidium iodide for dead cell exclusion. Flow cytometric analyses were performed on a FACSaria (BD Biosciences) and data was analyzed using Flowjo (TreeStar) software. Controls included unstained or singly stained cells for compensation.

Determination of survival and proliferation

For the proliferation assay, cells were plated at 3×10^3 cells per well in 96-well plates and allowed to adhere overnight. Increasing concentrations of 5-aminoimidazole-4-carboxamide 1- β -D ribofuranoside (AICAR) (Tocris Biosciences) and metformin (Sigma-Aldrich) were administered and cells were allowed to grow for 48 and 72h, respectively. An MTT assay

was performed as described[28]. Data presented are the mean $\pm$ SEM from three to four independent experiments performed in replicates of six per drug concentration.

For the survival assay, cells (5×10^3) were plated in 96-well plates and allowed to grow for 48 h at 37°C. Plates were washed with PBS and media with the indicated substrates were added for 24 h. At 24 h, 5 l of alamar blue was added and plates were incubated for 3 h at 37°C. The levels of reduced alamar blue were measured by a fluorescence plate reader using an excitation wavelength at 560nm and an emission wavelength of 590nm. Substrate media were as follows: 1) full medium (high glucose (HG) DMEM with 4% FBS + 60 μ M bovine serum albumin (BSA, fatty acid-free fraction V, (Calbiochem); 2) full substrate medium (HG DMEM with 200 μ M 2:1 oleate:palmitate (Sigma) in 60 μ M BSA); 3) glucose only medium (no glucose, no glutamine DMEM + 60 μ M BSA and 25mM glucose; and 4) fatty acid only medium (no glucose, no glutamine DMEM + 200 μ M 2:1 oleate:palmitate 2:1 in 60 μ M BSA). The 2:1 oleate:palmitate ratio was chosen to reflect *in vivo* fatty acid concentrations, as palmitate alone has been shown to be highly toxic and pro-apoptotic to cells[29, 30] and does not represent physiological conditions. All media were supplemented with 4mM glutamine. Data presented are the mean fluorescence units $\pm$ SEM from three independent experiments performed in replicates of six.

Statistics

Data are presented as mean $\pm$ SEM. Results were analyzed with a one-way ANOVA followed by Tukey's post ad hoc test. When comparing different cell types grown in different media, results were analyzed with a two-way ANOVA followed by a Bonferroni post ad hoc test. All results were analyzed by Prism (GraphPad). Results were considered significant at $p < 0.05$.

Results

Our previous studies have shown that during progression, the late stage MOSE-L develop a glycolytic phenotype[16], concomitant with increased proliferation and tumorigenicity compared to the non-tumorigenic MOSE-E[14]. This suggests that these changes may be critical for the aggressive phenotype and as such targets for treatment strategies. However, it is unknown whether the metabolism of the highly aggressive TICs is different than their precursor cell population. Since this is critical to eliminate cells that can recapitulate fatal disease after conventional chemotherapy, we assessed changes in the metabolism of the TICs to identify potential targets for the design of intervention studies.

Glucose and fatty acid oxidation

Three methods were employed to assess glucose and fatty acid oxidation as a function of substrate availability. First, we measured glucose oxidation in a low-glucose medium with ^{14}C glucose to assess oxidation of glucose alone in this condition. The glucose oxidation was lower in MOSE-L than in the benign MOSE-E, confirming our previous results; this, however, did not reach statistical significance due to the lower than expected glucose oxidation rate in the MOSE-E, a result of the transitional nature of our MOSE model. There were no statistically significant differences in glucose oxidation between the

MOSE-L and the TICs (Figure 1A). Next, we measured fatty acid oxidation in a glucose-free medium, forcing the cells to rely on this substrate for oxidation. MOSE-L and TICs showed a significant lower CO₂ production derived from ¹⁴C-palmitate than MOSE-E, (p<0.001) (Figure 1B) and a decrease in acid soluble metabolites, a product of incomplete β-oxidation (MOSE-L p<0.05; TIC p<0.01) (Figure 1C). As shown in Figure 1D, this resulted in the total decrease of fatty acid oxidation (CO₂ plus ASMs, p<0.001) in glucose-free medium in both the MOSE-L and TICs. Since there was no statistically significant difference between MOSE-L and TICs, these results demonstrate that the TICs show the same preference for glucose utilization as the MOSE-L under these conditions.

To assess preferential substrate selection, the cells were incubated with both ¹⁴C-glucose and ³H-palmitate in serum-free HG DMEM. As shown in Figure 2A, glucose oxidation in TICs was significant lower than in MOSE-E (p<0.001) and MOSE-L (p<0.01). Furthermore, TICs exhibited a significantly lower fatty acid oxidation than MOSE-E (p<0.001) and MOSE-L (p<0.05) (Figure 2B). When glucose and fatty acid oxidation were combined, there was a significant decrease in total oxidation of these substrates in the TICs compared to MOSE-E (p<0.001) and MOSE-L (P<0.01) (Figure 2C). Taken together, TICs had a greater percentage of their oxidation attributed to glucose rather than fatty acids as compared to the MOSE-E cells (67.8% versus 51.2%, p<0.01) (Figure 2D). The oxidation attributed to glucose in TICs (67.8%) was also higher than in the MOSE-L (57.4%), albeit not statistically significant. Overall, these results indicate that in the presence of both substrates, TICs appear to be more glycolytic in a glucose-rich environment. The contribution of additional substrates for energy was not assessed and, therefore, this decrease in oxidation is not indicative of a damaged OxPhos system, but rather a decreased preference for glucose and fatty acid oxidation through oxidative phosphorylation.

Substrate synthesis and expression of transporters

Based on the results of the dual label experiments, we next explored *de novo* fatty acid synthesis and lactate excretion. There were no differences in fatty acid synthesis from ¹⁴C-glucose between MOSE-L cells and TICs (Figure 3A) but an increase in TICs compared to MOSE-E (p=0.067). This was expected since cancer cells can activate lipolysis in adjacent white adipose tissue[31] and glutamine production in adjacent muscle tissue[32] and utilize these substrates rather than enhance their *de novo* synthesis; this correlates well with changes in the expression of transporters (see below). With the decrease in total oxidation seen in TICs, we next assessed the lactate levels in the medium after the 3 h incubation period with the labeled isotopes. As expected, the lactate levels were higher in the TICs compared to MOSE-E (p<0.001) and MOSE-L (p<0.001) cells (Figure 3B), indicating an increased rate of glycolysis.

Given the changes in substrate usage between the cell lines, we assessed the expression of a panel of select genes involved in substrate transport and metabolism. Figure 3C illustrates the changes in mRNA of the five expressed glucose and fatty acid transporters (*Fat/Cd36* was not expressed as indicated by CT values >35). TICs showed significant increased mRNA levels of five transporters over MOSE-E (*Glut1* p<0.01; *Glut2* p<0.05; *Glut4*, *Fatp1*, *Fabp4* p<0.001) and an increase in mRNA level in transporters over MOSE-L (*Glut4*

$p < 0.05$; *Fatp1* $p < 0.001$; *Fabp4* $p < 0.001$). Additionally, there was a decrease in mRNA expression of *Glut3* ($p < 0.01$ compared to MOSE-E). Other transporters were not differentially expressed (Table 1). Together, this suggests an overall increased capacity of TICs for substrate uptake, both for glucose and fatty acids.

TICs expressed higher levels of metabolic genes commonly seen elevated in cancer (Table 1). There was a significant increase in the mRNA levels of *Pdhh* (master regulator of aerobic versus anaerobic metabolism) ($p < 0.05$ vs MOSE-E), *Pdk1* (the regulator of PDH; $p < 0.01$ vs MOSE-E, $p < 0.01$ vs MOSE-L), and *Ucp2* ($p < 0.001$ vs MOSE-E, $p < 0.001$ vs MOSE-L) (Figure 3C). The increase in *Pdk1* in TICs, which decreases PDH activity, is consistent with the glycolytic phenotype observed. Additionally, the increase in *Pdhh*, one subunit of the pyruvate dehydrogenase complex, may be indicative of the TIC's ability to switch between glycolysis and OxPhos when needed. We also observed increases in the mRNA levels of *Ucp3* in MOSE-L and TICs, and reduced levels of *Pkm2* and *HkII*, but these were very highly variable and, therefore, not statistically significant. There were no changes detected in the expression of *CrAT*.

Impact of limited substrates and metabolism modulators on cell survival

Next, we subjected the cells to extreme nutrient deficiencies to assess survival under extreme conditions. All media were supplemented with glutamine since earlier studies demonstrated that none of the cell lines could survive without glutamine (data not shown). HG DMEM was supplemented with 4% FBS and vehicle (0.4% BSA, control), 200 μ M oleate:palmitate (2:1), or only with vehicle; the last treatment group received glucose-free DMEM with 200 μ M oleate:palmitate only. As shown in Figure 4 A-B, all cells survived when cultured in glucose-containing medium, irrespective of the presence of fatty acids. However, significantly more TICs were able to survive in medium containing the 2:1 oleate:palmitate only (26.2% of DMEM ctrl with 4% FBS, $p < 0.001$) than MOSE-E (13.5%); MOSE-L were not able to adapt to the extreme nutrient deficiency (survival 0.58% of control) (Figure 4B).

Metabolic modulators have gained much attention in recent years due to their successful application in diabetes and metabolic syndrome but also show promise in cancer treatment. We next examined the differential impact of metabolic modulators on cell viability using the monophosphate kinase (AMPK) agonists AICAR and the FDA-approved anti-diabetic drug metformin- both positively modulate glucose utilization and insulin sensitivity via AMPK-dependent and independent mechanisms[33]. As shown in Figure 4C, TICs were significantly more resistant to AICAR treatment than either MOSE-E or MOSE-L as indicated by a significantly higher EC_{50} (EC_{50} 254.4 μ M, 135.7 μ M and 103.9 μ M, respectively; $p < 0.05$). In contrast, TICs were more sensitive to metformin treatment than MOSE-E or MOSE-L (Figure 4C, right panel) as reflected in their lower EC_{50} (11.0 mM) than MOSE-E (EC_{50} 19.0 mM) and MOSE-L (EC_{50} 30.3 mM).

TICs have an increased FCCP stimulated-OCR and oligomycin stimulated-ECAR

To further investigate the ability of TICs to adapt their metabolism to varying extracellular conditions, we used the Seahorse Extracellular Flux Analyzer to measure OCR and

glycolysis rate indirectly via ECAR. FCCP, a mitochondrial uncoupler was used to stimulate maximal OCR due to the FCCP-induced proton leak through the inner mitochondrial membrane, decreasing the proton motive force[27]. To offset the proton leak, cells ramp up OCR presenting an indicator of a cell's maximum respiration. As shown in Figure 5, MOSE-L had a significantly decreased FCCP-stimulated OCR compared to the MOSE-E ($p<0.001$), measured as the difference in FCCP-stimulated OCR minus basal OCR, confirming our previous studies[16]. TICs increased their FCCP stimulated-OCR to the same level as the benign MOSE-E cells (different from MOSE-L $p<0.001$), indicating that TICs have the capacity to up-regulate OxPhos when necessary after uncoupling, exhibiting an increased OCR reserve.

The cells were also challenged with oligomycin, an ATP Synthase inhibitor to assess ECAR. When ATP Synthase is inhibited, ATP production in the electron transport chain is blocked, forcing the cells to switch to glycolysis for ATP production. When challenged with oligomycin, TICs had a significantly higher oligomycin-stimulated ECAR than the MOSE-E ($p<0.01$) and MOSE-L ($p<0.05$) (Figure 6 A-B), measured as the difference in oligomycin-stimulated ECAR minus basal ECAR. This increase in oligomycin-stimulated ECAR suggests that TICs have a higher glycolytic reserve and are able to increase the flux through this pathway for ATP production when OxPhos is slowed.

Mitochondrial organization and function

The mitochondrial membrane potential (Ψ_m) is associated with the capacity of the cells to generate ATP. To determine if the differences in cellular metabolism are the result of defective mitochondria as proposed by Otto Warburg, we visualized the Ψ_m using fluorescent dye staining of live cells. As shown in figure 7, JC-1 stained MOSE-E cells exhibit a more organized mitochondrial network throughout the cell; in the MOSE-L and TICs, the mitochondria are less organized and appear more round (left panels, green). Hyperpolarized mitochondrial membranes accumulate the dye and shift its emission spectrum into the TRITC range; as shown in Figure 7 (second column, red), hyperpolarized mitochondria are visible in all MOSE-E cells but in only about 10% of the MOSE-L and none in the TICs. This suggests that the mitochondria of the TICs may not be hyperpolarized and inactive. However, hydrogen peroxide can quench JC-1 red emission[34]. We used mitosoxTM staining to visualize superoxides in living cells; while the staining was very photo labile and its quantitation rather unreliable, the highest staining intensity was observed in the TICs while most of the MOSE-E exhibited little staining (data not shown). This suggests that the lack of red JC-1 emission may not be the result of a depolarized mitochondrial membrane but a quenching of the dye by reactive oxygen species. To confirm this, we stained the cells with mitotracker Green and TMRE and quantitated intracellular fluorescence by flow cytometry; both dyes label active mitochondria regardless of their membrane potential but are not sensitive the hydrogen peroxide quenching. As shown in figure 7B, the uptake of mitotracker Green in MOSE-L was significantly higher than in MOSE-E ($p<0.01$) but lower than in the TICs ($p<0.001$). The mean geometric fluorescence of TMRE in the MOSE-L was significantly higher than in MOSE-E ($p<0.001$); TICs showed also a higher TMRE fluorescence than the MOSE-E ($p<0.001$) but significantly less

than MOSE-L ($p < 0.01$). This indicates that both the MOSE-L and the TICs have active, highly polarized mitochondria.

Comparison of tumors from the diaphragm and ascites

TICs were originally isolated from the ascites of mice injected IP with MOSE-L, suggesting an enrichment for aggressive, tumor-initiating cells in the peritoneal cavity. For an initial assessment of the impact of the tumor microenvironment on tumor cell mitochondrial function and metabolism, tumors were harvested from the diaphragm and from the ascites of C57BL/6 mice three weeks after cell implant and subsequently underwent a mitochondrial stress test (Figure 8 A-D). No significant differences in OCR were observed, with a trend towards a lower FCCP-stimulated OCR in the ascites tumor cells than in those harvested from the diaphragm ($p = 0.09$). There was a significant higher basal ECAR in the ascites tumor cells ($p < 0.01$), suggesting a decrease in glycolytic rate after tumor cell attachment and outgrowth (Figure 8C). No differences in the oligomycin-stimulated ECAR rate were observed.

Discussion

Tumor initiating cells are, per definition, capable of recapitulating the original tumor and are therefore important targets for chemotherapeutic and preventive efforts. We have reported previously that the metabolism of ovarian cancer cells change to a more glycolytic phenotype as the cells progress from a benign to a highly aggressive phenotype[16]. The present studies provide an initial characterization of the metabolic phenotype of highly aggressive ovarian TICs. We demonstrate that TICs were more glycolytic than benign or late-stage MOSE cells, showing a decrease in glucose and fatty acid oxidation, and an increased lactate secretion. However, the contribution of glutamine to fatty acid synthesis (which can account for up to 25% of total fatty acid carbons in glioblastoma tumors[35]) was not assessed and, therefore, the utilization of ^{14}C -glucose may not reflect the actual number of carbons being incorporated into fatty acids. Nonetheless, TICs were metabolically more flexible and increased their OCR when their mitochondria were uncoupled by FCCP and increased their ECAR to overcome an ATP Synthase inhibition by oligomycin. While the organization of the mitochondria appeared to be altered, the activity as measured by fluorescence dyes was highly upregulated in MOSE-L but less in TICs. This metabolic phenotype was accompanied by an increase in expression of *Pdk1*, *Pdhb*, *Glut1*, *Glut2*, *Glut4*, *Ucp2*, *Fatp1* and *Fabp4*, indicating an increased capacity for substrate uptake and utilization. Together, these changes result in a highly adaptable metabolic phenotype that allows for an increased survival in extreme substrate deficiency and reduced response to the metabolic modulator AICAR. Furthermore, this phenotype appears to be partially maintained in ascites tumors *in vivo* but after tumor cell attachment and secondary outgrowth, cells resemble a more differentiated phenotype, comparable to the MOSE-L.

The TICs have demonstrated an ability to increase OxPhos when uncoupled by FCCP and increase glycolysis when ATP synthase in the ETC was slowed; whereas the MOSE-L cells exhibit the classic glycolytic Warburg effect and were neither able to increase glycolysis or OxPhos, nor survive in the presence of AICAR to the same extent as the TICs despite their

increase in mitotracker Green and TMRE fluorescence. This flexibility may account for the increased survival of TICs in glucose deprivation when cells would have to rely on OxPhos for ATP demand. Flow cytometric analysis of mitochondrial dye uptake indicated that both the MOSE-L and TICs have viable polarized mitochondria, although the more differentiated MOSE-L do not seem able to switch solely to OxPhos. This could be the result of a disorganized network of mitochondria, and future studies will focus on high resolution mapping to define the organization and dynamics of the mitochondria between cell types.

The TIC's preference for substrates is largely unknown. One could hypothesize that when deprived of glucose, the TICs have an increased flexibility to utilize other substrates. Specifically, glutamine has been implicated as an important substrate, as it readily converts to α -ketoglutarate, feeding into pools of cellular citrate through reductive carboxylation via isocitrate dehydrogenase (see recent review[36]). Glutamine can thus supply carbons for de novo fatty acid synthesis. In addition to citrate, glutamine has also been shown to produce the TCA cycle intermediates fumarate and malate, as well as aspartate in glucose-depleted medium[37].

The observed increasingly glycolytic phenotype of TICs is similar to the glycolytic nature of normal tissue stem cells that reside within the body[11, 12, 38] and seems to be required to maintain their "stemness" (see recent review[39]) or promote the expression of genes associated with a stem-like genotype[40]. Stem cells are characterized by a higher glycolytic flux[41], and a similar[38] or reduced oxygen consumption[42] that is reversed upon differentiation. Other studies have shown that induced pluripotent or embryonic stem cells (hESCs) are highly glycolytic but have a lower FCCP-stimulated OCR[38, 43] and oligomycin-stimulated ECAR[38] than their somatic counterparts. The authors suggest that the hESCs may be near their maximal glycolytic capacity and therefore have a reduced reserve for increased glycolysis[38]. The metabolic phenotype of CSC/TICs is also controversially discussed; glioblastoma stem cells have been reported to be less[44] or more glycolytic than their daughter cells[45-47], or exhibit either phenotype in the same tumor[48]. Colon cancer or osteoblastoma CSC/TICs express higher levels of glycolytic genes[49] and are more glycolytic[50] than their differentiated counterparts. Our results indicate a more glycolytic and flexible metabolic function in the TICs.

It is possible that CSC/TICs perform similarly to hESCs, but due to the reduced glycolytic reserve capacity of more differentiated cancer cells[16], the CSC/TICs' glycolytic reserve capacity is greater, closer to the capacity of benign cells. However, a recent study demonstrated the glycolytic nature of an osteosarcoma stem-like cell line with minimal contribution of OxPhos to cellular ATP production but a reduced mitochondrial function with a low spare respiratory capacity and coupling efficiency, and the lack of adaptive response to glucose deprivation[50]. It is unclear at this point if species or tissue differences, the choice of cells serving as comparisons and the choice of stem-cell markers for stem cell isolation impact these contradictory results, or if the generation of our TICs via IP enrichment from a primary cell line may also select for a specific phenotype.

Increased expression of substrate transporters in TICs allow for the utilization of substrates such as lactate, glutamine, ketones and fatty acids from the host. This is a normal process in

muscle, brain and, importantly, ovaries. In the ovaries, this energy transfer from the granulosa cells to the oocytes supports their maturation in early oogenesis[51]. Cancer cells utilize an aberration of this normal energy transfer between cells, termed “parasitic metabolism” or “reversed Warburg effect”[52]. Ovarian cancer cells that had metastasized to the omentum increased the expression of FABP4 at the cancer-adipocyte boundary and increased the uptake of fatty acids and subsequent ATP production through β -oxidation[31]. While there is little information on the expression of substrate transporters in CSC/TICs, the elevated expression of these transporters in addition to metabolism enzymes and regulators of mitochondrial function (*Pdhhb*, *HkII*, *Pkm2*, *Pdk1*, *Ucp2,3*) suggest that our TICs are able to control and adapt their metabolism, substrate uptake and utilization of available substrates for energy production. Interestingly, *Ucp2* which controls electron transport chain flux and subsequent rates of mitochondrial oxidation is highly elevated in our TICs similar to pluripotent human stem cells, but is reduced in differentiated cells[38]. Based on this finding and the mitoxoxTM staining, future studies will investigate contribution of *Ucp2* in modulating mitochondrial membrane potential and ROS levels, critical for CSC/TICs survival.

We further investigated the metabolic phenotype of the TICs via the modulation of their metabolism by activating AMPK, an energy-sensing enzyme that is activated in response to increases in the AMP:ATP ratio[53] to shift the cellular metabolism from energy-consuming pathways to energy-producing pathways[54]. In the present study, we investigated the impact of the AMPK agonists AICAR and metformin. AICAR has the ability to induce ovarian cancer cell death through AMPK modulation and inhibition of the mTOR pathway[55]. Metformin is thought to work by blocking complex I in the electron transport chain, thereby raising the AMP:ATP ratio, resulting in indirect AMPK activation[56]. AICAR concentrations above 128 μ M induced toxicity in MOSE cells but TICs were more resistant. In contrast, metformin was more toxic for TICs, comparable to the reported more effective killing of breast cancer stem cells[57, 58] or ALDH-expressing ovarian CSC/TICs[59] than their parental non-stem cancer cell populations. It is not known if the effective *in vitro* dosages are of physiological relevance; however, since metformin toxicity towards CSC/TICs is drastically enhanced by low glucose concentrations[60], the effective *in vivo* concentrations may vary dependent on the CSC/TICs location within the peritoneal cavity. Together, our results suggest that Metformin but not AICAR could be used in an ovarian cancer treatment regimen to specifically eliminate CSC/TICs.

While still preliminary, our results also show that the tumor microenvironment affects our TICs metabolic phenotype and that metastatic tumor cells can adapt to a secondary environment and thrive. This is important since especially visceral obesity has been associated with a higher risk of developing and dying of ovarian cancer[61, 62]. We have shown previously that the individual fat pads of the peritoneal cavity are distinct entities regarding their immune and progenitor cell profile[21]; both are altered by parity and the presence of peritoneal cancer cells[18], and a high-fat diet (unpublished data, manuscript in preparation). This can decrease (parity) or increase (high fat) the tumor burden. Our data suggests that the TICs metabolic flexibility or plasticity allows for their survival under minimalist conditions but also for an adaptation to the variable conditions found within the

peritoneal cavity and the utilization of available substrates from the host to satisfy their energy requirements[39]. Given the preferred localization of TICs to the omentum, diaphragm and other intraperitoneal fatpads[18] and their expression of transporters (also seen in omental metastases at the adipocyte-tumor cell interface[31]), it is likely that this flexibility makes the TICs more resilient to microenvironmental changes and allows for their adaption and survival in these altered microenvironments. This requires the development of drugs that specifically target these changes in the metabolic phenotype of tumor-initiating cells to prevent recurrent CSC/TICs outgrowth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ovarian cancer TICs exhibit a decreased glucose and fatty acid oxidation
- TICs are more glycolytic and have highly active mitochondria
- TICs are more resistant to AICAR but not metformin
- A flexible metabolism allows TICs to adapt to their microenvironment
- This flexibility requires development of specific drugs targeting TIC-specific changes to prevent recurrent TIC outgrowth

Figure 1. TICs display a decrease in fatty acid oxidation compared to the MOSE-E cells
 Changes in (A) ¹⁴C- glucose oxidation in low-glucose DMEM, (B) ¹⁴C- fatty acid oxidation into CO₂ in glucose-free DMEM, (C) fatty acid oxidation of incomplete beta-oxidation into acid soluble metabolites (ASMs) in glucose-free DMEM, and (D) total fatty acid oxidation as CO₂ plus ASMs were determined. Data are presented as mean ± SEM. Different from MOSE-E, *p 0.05, **p 0.01, ***p 0.001.

Figure 2. OxPhos and substrate preference

Changes in (A) glucose oxidation from dual-label experiments with ^{14}C -glucose and ^3H -palmitate as competing substrates, (B) fatty acid oxidation from dual-label experiments with ^{14}C - glucose and ^3H -palmitate as competing substrates, (C) total oxidation from either ^{14}C - glucose and ^3H -palmitate , and (D) percentage of glucose and fatty acids used for oxidation, where the total oxidation are the values from figures E and F, adjusted to 100%.

Data are presented as mean $\pm$ SEM. Different from MOSE-E, **p 0.01, ***p 0.001.

Different from MOSE-L, bar with *p 0.05, **p 0.01.

Figure 3. TICs excrete more lactate and have an increased expression of substrate transporters (A) *De novo* fatty acid synthesis from ^{14}C - glucose and (B) non-labeled lactate excretion into the medium from the dual-label substrate experiments; (C) qPCR determination of glucose transporters 1, 2, 3, and 4 (*Glut1*, *Glut2*, *Glut3*, and *Glut4*), fatty acid transporter isoform 1 (*Fatp1*), fatty acid binding protein isoform 4 (*Fabp4*), pyruvate dehydrogenase subunit b (*Pdhb*), pyruvate dehydrogenase kinase 1 (*Pdk1*), and uncoupling protein 2 (*Ucp2*). Data are presented as mean $\pm$ SEM. Different from MOSE-E, *p 0.05, **p 0.01, ***p 0.001. Different from MOSE-L, bar with *p 0.05, **p 0.01, ***p 0.001.

Figure 4. Survival under limiting conditions

(A) Survivability in media containing the indicated substrates, measured via alamar blue; (B) percent survivability of the cells in DMEM without glucose, supplemented with 200 μ M oleate:palmitate 2:1 (bar 4) divided by the control high glucose DMEM supplemented with 4% FBS and 0.04% BSA (bar 1); Cytotoxicity of increasing concentrations of (C) AICAR and (D) metformin. Data are presented as mean $\pm$ SEM. *different from MOSE-E $p < 0.05$; ^different from MOSE-E $p < 0.01$; **different from MOSE-E $p < 0.001$; ++different from MOSE-L $p < 0.05$; #different from MOSE-L $p < 0.01$; +different from MOSE-L $p < 0.001$.

A.

B.

Figure 5. FCCP-stimulated oxygen consumption rate is increased in the TICs

Maximal respiration was measured following administration of carbonylcyanide-p-trifluoromethoxyphenyl hydrazine (FCCP), a mitochondrial uncoupler. (A) Image of a representative experiment measured over 2 h; (B) Change over baseline in OCR after FCCP treatment. Data are presented as mean $\pm$ SEM. Different from MOSE-E, ***p 0.001. Different from MOSE-L, bar with ***p 0.001.

Figure 6. Oligomycin-stimulated ECAR shows an increased glycolytic capacity in the TICs Extracellular acidification rate (ECAR), an indication of the rate of glycolysis was modified by oligomycin, an ATP synthase inhibitor (see Fig. 5). (A) Image of representative experiment measured over 2 h; (B) Change in ECAR over baseline after oligomycin treatment. Data are presented as mean $\pm$ SEM. Different from MOSE-E**p 0.01. Different from MOSE-L, bar with *p 0.05.

A

B

Figure 7. Changes in mitochondria organization and membrane potential

(A) Live-cell staining of cells with fluorescent mitochondria-specific dyes showing mitochondria organization and membrane hyperpolarization. (B) Changes in geometric mean fluorescence after mitotracker green and TMRE staining. Different from MOSE-E, *p 0.01, **p 0.001; different from MOSE-L, ^p 0.001, ^^p 0.01.

Figure 8. Cells harvested from the ascites are more glycolytic than those harvested from solid tumors

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were modified by oligomycin, carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), and rotenone on TICs that were harvested from the ascites of the mouse compared to TICs that were harvested from solid diaphragm tumors. (A) Basal OCR levels, measured over 2 h; (B) Change in OCR over baseline after FCCP treatment; (C) Basal ECAR levels, measured at 2 h; (D) Change in ECAR over baseline after oligomycin treatment. Data are presented as mean $\pm$ SEM, **p 0.01.

Table 1

Fold-change in mRNA levels for MOSE-L and TICs compared to MOSE-E.

Gene Name	MOSE-E	MOSE-L	TIC
Transport genes			
Glut 1	1.015 ± 0.12 ^a	1.477 ± 0.06 ^{a,b}	1.796 ± 0.14 ^b
Glut 2	1.111 ± 0.38 ^a	0.384 ± 0.29 ^{a,b}	11.430 ± 3.20 ^b
Glut 3	1.045 ± 0.22 ^a	0.008 ± 0.00 ^b	0.097 ± 0.01 ^b
Glut 4	1.024 ± 0.15 ^a	4.175 ± 0.34 ^b	7.182 ± 0.76 ^c
FATP1	1.079 ± 0.13 ^a	1.332 ± 0.03 ^a	4.095 ± 0.03 ^b
FABP4	1.004 ± 0.06 ^a	0.179 ± 0.01 ^b	2.155 ± 0.17 ^c
Glycolysis genes			
Pyruvate Kinase M2 (PKM2)	1.005 ± 0.07	0.714 ± 0.06	0.857 ± 0.10
Hexokinase II (HKII)	1.056 ± 0.26	0.910 ± 0.03	0.630 ± 0.08
Pyruvate Oxidation genes			
Pyruvate Dehydrogenase b (PDHb)	1.004 ± 0.06 ^a	1.367 ± 0.04 ^b	1.345 ± 0.07 ^b
Pyruvate Kinase 1 (PDK1)	1.002 ± 0.04 ^a	1.284 ± 0.11 ^{a,b}	2.149 ± 0.16 ^b
Mitochondrial genes			
Uncoupling protein 2 (UCP2)	1.000 ± 0.02 ^a	1.684 ± 0.08 ^b	3.853 ± 0.33 ^c
Uncoupling protein 3 (UCP3)	1.066 ± 0.26	4.083 ± 0.41	16.66 ± 8.10
Carnitine Acetyl Transferase (CrAT)	1.007 ± 0.08	0.786 ± 0.03	1.134 ± 0.13

qPCR data analyzed using the AACT method indicating fold-change over the MOSE-E cells, where different letters denote statistical significance ($p \leq 0.05$) and the same letters are not statistically different for the MOSE-E, MOSE-L and TIC cells. Data is presented as mean ± SEM.