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Metabolic characterization of slowly cycling melanoma cells

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METABOLIC CHARACTERIZATION OF SLOWLY CYCLING MELANOMA CELLS

by

SOLOMON CHONG-ZHI TANG

THESIS

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MASTER OF SCIENCE

in

Biomedical Imaging

in the
Copyright 2015

By

Solomon Chong-Zhi Tang
ACKNOWLEDGEMENTS

My gratitude goes out towards a great cast of people who not only made this experience possible, but also meaningful. First and foremost, I would like to thank Dr. Sergey Magnitsky for providing an interesting and meaningful project for me to work on. I am grateful for his patience and guidance throughout the course of the project. Dr. John Kurhanewicz, Dr. Susan Noworolski, and Dr. Xiaojuan Li have also been instrumental as committee chair and members in providing advice and encouragement. I would like to give thanks to Sergio Wong, Charles Trulliet and Geetha Mohan for being my cell culture mentors. I am indebted to their effort in helping me when I needed it the most. Special thanks goes to Trey Jalbert, Renuka Sriram, and especially Jinny Sun for their NMR expertise, dedication, and drive which made this project possible. I would like to thank my classmates in the MSBI program for being amazing friends and making my time here at UCSF truly an experience of a lifetime. Lastly, I would like to express my gratitude to my family, the Loomer family, and Cleo for their everlasting support and encouragement in all my endeavors.
Metabolic characterization of slowly cycling melanoma cells

Solomon Tang

ABSTRACT

The low efficacy of existing methods of treatment for late-stage melanoma has been attributed to the development of drug resistant cells. Following conventional treatments, enrichment of slowly cycling melanoma cells has been reported. Recently, a unique biological marker histone 3 K4 demethylase JARID1B was used to characterize slow-cycling cells within a rapidly proliferating population of melanoma cells. High JARID1B expression is almost exclusive to slow-cycling melanoma cells. This phenotype of cells has been shown to be temporarily distinct, dynamic, and necessary for continuous tumor growth. This study aims to seek metabolic changes between JARID1Bhigh subpopulations and bulk melanoma tumor cells. Targeting this subpopulation of drug resistant cells may improve the efficacy of existing treatments. This project aims to identify and quantify metabolic differences by analyzing H NMR spectral data of cell extracts and culture media of slow-cycling JARID1Bhigh melanoma cells in comparison to highly proliferating bulk melanoma cells. We have demonstrated great improvement in acquiring consistent data for H NMR analysis over previous attempts. Our results show enhanced glucose and glutamine consumption, increased lactate/alanine ratios, and elevated concentrations of myo-inositol and choline derivatives in slow-cycling J/EGFPhigh subpopulations compared to highly proliferative tumor cells. These preliminary findings suggest heightened metabolic versatility in slow cycling melanoma cells in accordance with recent revisions of cancer metabolism and may help understand the dynamic stem-ness of these seemingly quiescent cells.
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INTRODUCTION

Mmelanoma is a cancer of melanocytes that is marked by irregular and discolored masses in the skin. Responsible for 90% of skin cancer mortalities, melanoma is the most dangerous form of skin cancer due to his high capability to invade and metastasize to other organs [1]. While these highly heterogeneous and aggressive tumors can be cured if excised at an early stage, median survival rates decreases to below 9 months if the tumor is allowed to metastasize [2].

The low efficacy of existing methods of treatment for late-stage melanoma has been attributed to the development of drug resistant cells after conventional cytotoxic treatments, as well as the existence of intrinsically heterogeneous drug resistant cells. These acquired resistance mechanisms include overexpression of anti-apoptotic proteins and drug efflux pumps in addition to increased DNA repair [3-4]. Vemurafenib, a recently FDA approved drug for the treatment of late-stage melanoma, has been shown to improve overall survival rates by selectively inhibiting the proliferation of cells with unregulated B-raf protein kinases. However, vemurafenib only functions in 60% of melanoma patients who have the V600E BRAF mutation and the median relapse time after initial tumor shrinkage is 5 months. Melanomas without the V600E BRAF mutation are not inhibited by vemurafenib, but may promote tumor growth instead [5-6]. In addition, various groups have discovered mechanisms of resistance to vemurafenib. Resistant melanomas that overexpress cell surface protein PDGFRB can create an alternative survival pathway. Activation of platelet-derived growth factor β, cRAF-1 kinase, and insulin growth factor 1 as well as a mutation of NRAS gene can reactivate
the normal BRAF, mitogen-activated protein kinase, and phosphoinositide 3-kinase survival pathways [7-10]. While the heterogeneity of melanoma tumors make the search for novel treatments increasingly diverse and challenging, common survival mechanisms for all types of melanoma exist. Conventional treatments such as chemotherapy, immunotherapy, and radiotherapy are effective against highly proliferative bulk tumor cells, but studies have reported enrichment of quiescent cancer cells after such treatments [11] Slow-cycling melanoma and glioblastoma cells survive cisplatin and temozolomide treatment respectively [3, 12]. If these surviving subpopulations of quiescent cells are responsible for tumor maintenance and relapses, combination treatments that target this phenotype could increase the efficacy of existing treatment strategies.

Recently, a unique biological marker histone 3 K4 demethylase JARID1B was used to characterize slow-cycling cells within a rapidly proliferating population of melanoma cells [13]. JARID1B is a member of the jumonji family of chromatin regulators. It is involved in tissue development, cancer, stem cell biology and marginally expressed in normal tissue [14-16]. High expression of JARID1B is almost

![Dynamic Stemness Model](image.jpg)

**Figure 5:** Dynamic stemness model of slow-cycling JARID1B\textsuperscript{high} melanoma cells. A small subpopulation of JARID1B\textsuperscript{high} cells are temporarily distinct and required for continuous tumor maintenance. Knockdown of JARID1B eventually leads to tumor exhaustion. Figure from [3].
exclusive to slow-cycling melanoma cells that have doubling times greater than four weeks. It has been shown that knockdown of JARID1B in JARID1B-positive melanomas using shRNA constructs initially lead to increased proliferation followed by tumor exhaustion which suggests that the JARID1B<sup>high</sup> subpopulation is necessary for continuous tumor growth. In addition, single isolated JARD1B<sup>high</sup> cells can give rise to highly proliferative progeny that are more resistant to treatment. JARID1B expression is dynamically regulated as JARD1B-negative cells can become positive (Figure 1) [13]. These JARID1B<sup>high</sup> cells account for approximately 5%-10% of bulk melanoma tumor cells [3]. Cisplatin and vemurafenib treatment results in the enrichment of a slow-cycling subpopulation that has high expression of JARID1B [13].

Considering JARID1B’s role in treatment resistance and continuous tumor maintenance, we asked if the slow-cycling JARID1B<sup>high</sup> subpopulation is metabolically different than highly proliferative JARID1B<sup>low</sup> cells. Metabolic characterization of slow-cycling JARID1B-positive cells is an important step toward understanding the dynamic nature of the JARID1B phenotype. The discovery of any real differences could be exploited to selectively kill or prevent the enrichment of slow-cycling melanoma cells following anticancer therapies. Targeting these slow-cycling cells may provide a promising new combination treatment strategy that eliminates both bulk tumor cells as well as drug resistant slow-cycling cells.

Proton nuclear magnetic resonance (H NMR) spectroscopy is a powerful method for the analysis of metabolic differences as a function of disease, gender, age, nutrition, genetic background, and the targeted analysis of biochemical pathways [17]. While NMR-based investigations have been done to study metabolic changes in melanoma
compared to melanocytes, little effort has been devoted to metabolic profiling of subpopulations within bulk melanoma cells [18-20]. This project aims to identify and quantify metabolic differences by analyzing NMR spectral data of cell extracts and culture media of slow-cycling JARID1B\textsuperscript{high} melanoma cells in comparison to highly proliferating bulk melanoma cell extracts.

**MATERIALS AND METHODS**

*Media Preparation*

Tu2\% media was created using the following ingredients: MCDB 153 medium (Sigma, M-7403; 4 parts), L-15 medium (Mediatech, MT 10-045-CV; 1 part), 2\% fetal bovine serum (ThermoFisher, 12662-011), 5 μg/ml bovine insulin (Sigma, I-5500), and 1.68 mM calcium chloride (Sigma, C-5670). FACS buffer solution consists of Dulbecco’s phosphate-buffered saline with 0.04\% EDTA (UCSF Cell Culture Facility) and 2\% fetal bovine serum.

*Cell Culture*

For this experiment, we obtained WM3734 and WM3734\textsuperscript{JARID1Bprom-EGFPBlast} cells from Dr. Herlyn of the Wistar Institute. WM3734\textsuperscript{JARID1Bprom-EGFPBlast} is a line of human melanoma cells that was used to isolate live JARID1B-positive cells. It was created via [3].
lentiviral infection of a pLU-JARID1Bprom-EGFP construct which drives cytoplasmic EGFP expression controlled by the JARID1B promoter (J/EGFP) [3]. All cells were suspended in Tu2% with 10% DMSO (Sigma, D2438) as a cryopreservant and stored in a liquid nitrogen cryo-dewar at a density of 1.5x10^6 cells/mL until seeded. Quickly thawed in a 37°C water bath, the cells were reconstituted in L-15 + 10% FBS and centrifuged at 200 rcf for 3 minutes. This was repeated twice before seeding to remove any trace of DMSO which is toxic to the cells after being thawed. WM3734\textsuperscript{JARDID1Bprom-EGFPblast} cells were seeded at a concentration of 4 x 10^4/cm^2 in Tu2% media. To maintain logarithmic growth, cells were passaged at a 1:3 ratio upon reaching 70% confluence. Adherent cells were washed with D-PBS and incubated with 0.05% trypsin (ThermoFisher, 25300-054) for 2 minutes at 37°C. Cells were harvested, reconstituted in L-15 + 10% FBS and centrifuged at 200 rcf for 3 minutes before finally being transferred to their new flask in Tu2% media. All cells were incubated at 37°C under a gas phase of 95% air/5%CO².

**FACS of J/EGFP Signals**

Adherent WM3734\textsuperscript{JARDID1Bprom-EGFPblast} cells were harvested after being washed with D-PBS, incubated with 0.05% trypsin at 37°C and suspended in FACS buffer solution. All cells were kept on ice during transportation to the sorting facility. For detection of JARID1B promoter controlled EGFP expression, WM3734\textsuperscript{JARDID1Bprom-EGFPblast} cells were sorted using a FACSARia III instrument (BD Biosciences) at the Gladstone Flow Cytometry Core. A non-transformed line of WM3734 cells was used as a negative control for autofluorescent signals. Cells were sorted using a 100 micron
nozzle under at approximately 4500 events/sec with 75% efficiency using the Purity sorting mode. Each population aliquot was approximately $1.5 \times 10^6$ cells and kept on ice. To allow cells to recover from the sorting process, aliquots were seeded in 100 mm cell culture plates (Corning) with 5 mL Tu2% media for 24 hours before metabolite extraction. Unsorted cells were counted using a TC20 Automated Cell Counter (Bio-Rad) and plated at an equal density at the same time.

Cell sorting for previous data sets followed a similar method, but aliquots were instead seeded in T75 flasks for 3 days before extraction. Previous cell counts were obtained manually using a hemocytometer.

**Cell Extraction**

The extraction process for newly acquired data followed a dual-phase extraction method as previously described [21]. Initially, media from each plate was transferred to respective 50 mL conical centrifuge tubes (Falcon). Cells were kept on ice and then washed with ice-cold D-PBS three times to remove traces of media left behind. 3 mL of ice-cold methanol was added to each plate for 5 minutes. To prevent methanol evaporation during manipulation, plates were kept covered for the duration. Then, with a small cell scraper (Corning), cells were scraped and transferred to their respective 50 mL glass centrifuge tubes. Following the addition of 3 mL of ice-cold chloroform to each tube, cells were vigorously vortexed before adding 3 mL of ice-cold water. Cells were vortexed again before being placed at 4°C overnight for phase separation. The final methanol:chloroform:water ratio was 1:1:1 (v/v/v). The upper methanol-water phase containing water-soluble metabolites was carefully separated from the lower chloroform
lipid phase without disturbing the protein interface. The aqueous phase was stored at -80°C until lyophilized at -100°C and 50 mTorr for 24 hours. The remaining chloroform phase and protein interface were allowed to evaporate before being stored at -80°C.

Previously acquired extracts followed a similar extraction protocol. However, key differences include using trypsin at 37°C for 3 minutes to remove adherent cells with an additional PBS wash before adding equal parts methanol, chloroform, and water at ice-cold temperatures. During the trypsinization process, cell counts were acquired manually with a hemocytometer.

A total of two completed experiments were conducted (n = 2) in addition to existing extract samples (n = 4).

Sample Preparation for H NMR Analysis

Media samples were prepared by adding 60 μL of D₂O + 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid (TSP) as an internal chemical shift indicator and concentration reference to 540 μL of media in a microcentrifuge tube and mixing thoroughly.

Extract samples were prepared by reconstituting lyophilized extracts with 600 μL of 1mM TSP in D₂O and vortexed vigorously to ensure the complete dissolution of lyophilized samples along the tube walls. Samples were centrifuged at 2000 RCF for 3 minutes and then transferred to standard 5 mm NMR test tubes (Wilmad).

Previously prepared NMR samples utilized a coaxial insert containing 20 μM TSP in D₂O as an external reference. All samples were stored at 4°C until analyzed.

H NMR Spectroscopy
New 1D $^1$H NMR spectra were acquired on an 800 MHz Bruker Avance spectrometer with a Bruker Automatic Sample Changer. All measurements were performed at 298 K without sample rotation. 64 scans of 32k points were acquired with a spectral width of 12.33 ppm, and acquisition time of 1.03 s. 90° pulses were used with a relaxation time of 12 s, dwell time of 41.6 μs, and receiver gain of 57 dB.

Previously acquired magnetic resonance spectra were acquired using 600 MHz Bruker AvanceIII instrument using similar settings as described above, but with 1536 scans instead.

**H NMR Data Processing**

Spectra were manually phased, baseline corrected and calibrated using the Processor module of ChenoMx (NMR Suite 8.1, Evaluation) to the 0.0 ppm TSP peak. Concentrations of each metabolite were manually fitted using the Profiler module of ChenoMx and normalized by the number of cells per sample.

**RESULTS**

**FACS of J/EGFP Signals**

To discriminate between slow-cycling melanoma cancer cells and highly proliferating bulk tumor cells, a JARID1B-promoter-EGFP-reporter construct was used as previously described [3]. This model allowed us to isolate a population of slow-cycling melanoma cells based on JARID1B promoter-induced EGFP expression. High, medium and low J/EGFP population were set as the highest, median, and lowest 10%
of fluorescent signal using fluorescent-automated cell sorting (FACS; Figure 3A). While prior in vitro studies have confirmed a strong correlation between JARID1B and JARID1B promoter-induced EGFP expression using a threshold set to the maximum 5% of fluorescent signal [3], our threshold bins for each population aliquot were set to 10% fluorescent signal windows in order to increase population sizes within a limited 3 hour sorting period. Due to the desired purity of the sort, collection of cellular debris was avoided by rejecting events with low side scatter area (SSC-A) and forward scatter area (FSC-A). In addition, only FSC and SSC singlets were accepted to avoid the collection of adherent doublet cells (Figure 3B). Gating for each of the distinct populations was set manually using event dot plots. Minimal overlap of non-transformed WM3734 cells within each gated aliquot of true GFP-positive cells was achieved (Figure 3C). Histogram data of J/EGFP intensity shows that the transformed WM3734JARDID1Bprom-EGFP cells have a distinct signal shift away from non-transformed WM3734 cells. Successful segmentation of each subpopulation within WM3734JARDID1Bprom-EGFP cells using gated dot plots is also reflected in histogram form (Figure 3D).
Figure 7: FACS results of J/EGFP signals from WM3734\textsuperscript{JARDID1Bprom-EGFP} cells. A) Distribution of cell populations. B) Dot plots of forward and side scatter area, width, and height with gates to maintain cell purity. C) Dot plots of J/EGFP signal in non-transformed and transformed cells with subpopulation aliquot gates. D) Histogram plots of J/EGFP intensity. Low, medium, and high subpopulation aliquots are shown in purple, green and red groups respectively.
Table 1: Peak assignments and average concentrations of metabolites identified from hydrophilic cell extracts of previously acquired spectra. *s* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet, *dd* doublet of doublet. *PC* phosphocholine, *GPC* sn-glycero-3-phosphocholine.

<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>PPM AND MULTIPLICITY</th>
<th>CONCENTRATION (PMOL/CELL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>J/EGFP EXPRESSION</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
</tr>
<tr>
<td>LEUCINE</td>
<td>0.96 (d), 1.67(m), 1.71(m), 1.73(m), 3.73(t)</td>
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<tr>
<td>VALINE</td>
<td>0.99 (d), 1.04 (d), 2.26 (m), 3.60 (d)</td>
<td>1.85</td>
</tr>
<tr>
<td>ETHANOL</td>
<td>1.18 (d), 3.65 (q)</td>
<td>48.17</td>
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<tr>
<td>THREONINE</td>
<td>1.33 (d), 3.56 (d), 4.24 (m)</td>
<td>9.22</td>
</tr>
<tr>
<td>LACTATE</td>
<td>1.33 (d), 4.11(q)</td>
<td>20.77</td>
</tr>
<tr>
<td>ALANINE</td>
<td>1.48 (d), 3.78 (q)</td>
<td>8.76</td>
</tr>
<tr>
<td>ACETATE</td>
<td>1.91 (s)</td>
<td>59.03</td>
</tr>
<tr>
<td>GLUTAMATE</td>
<td>2.05 (m), 2.14 (m), 2.34 (m), 2.37 (m), 3.75 (m)</td>
<td>23.04</td>
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<tr>
<td>GLUTAMINE</td>
<td>2.13 (m), 2.16 (m), 2.42 (m), 2.47(m), 3.77 (t), 6.87 (s)</td>
<td>9.63</td>
</tr>
<tr>
<td>GLUTATHIONE</td>
<td>2.16 (q), 2.18(q), 2.55 (m), 2.58 (m), 2.92 (dd), 2.97 (dd), 3.75 (d), 3.78 (t), 3.79 (d), 4.55 (t), 8.2 (s), 8.5 (s)</td>
<td>9.97</td>
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<tr>
<td>SUCCINATE</td>
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<td>ASPARTATE</td>
<td>2.67 (dd), 2.79 (dd), 3.78 (dd)</td>
<td>2.26</td>
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<tr>
<td>CREATINE</td>
<td>3.01 (s), 3.94 (s)</td>
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<tr>
<td>PHOSPHATE</td>
<td>3.04 (dd), 3.18 (dd), 3.93 (q), 6.89 (d), 7.19 (d)</td>
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<td>TYROSINE</td>
<td>3.04 (s), 3.93(s)</td>
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<td>CREATINE</td>
<td>3.20(s), 3.52(m), 4.06(m)</td>
<td>0.77</td>
</tr>
<tr>
<td>CHOLINE</td>
<td>3.22(s), 3.59(m), 4.16 (m)</td>
<td>6.58</td>
</tr>
<tr>
<td>PC</td>
<td>3.23 (s), 3.63(dd), 3.68 (m), 3.69 (dd), 3.88 (m), 3.92 (m), 3.96 (m), 4.33 (m)</td>
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<td>GPC</td>
<td>3.24 (t), 3.42 (t)</td>
<td>4.31</td>
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<td>TAURINE</td>
<td>3.28 (t), 3.53 (dd), 3.62 (d), 4.06 (t)</td>
<td>17.59</td>
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<td>MYO-</td>
<td>3.35 (s)</td>
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<td>INOSITOL</td>
<td>3.55 (s)</td>
<td>12.55</td>
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<td>METHANOL</td>
<td>3.56 (dd), 3.65 (dd), 3.77 (m)</td>
<td>6.43</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>3.82 (dd), 3.94(dd)</td>
<td>9.63</td>
</tr>
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Previously acquired spectral data of cell extracts were analyzed for differences in metabolite concentrations. Trends such as higher concentrations of lactate, glutamine, and glycerophosphocholine (GPC) with higher J/EGFP expression were observed. However, the error within and between groups of each metabolite was incredibly large and suggested that the quality of the acquired data was insufficient for any statistical conclusions to be relevant (Table 1; Figure 4).

**Figure 8:** Metabolite concentrations in different subpopulations of J/EGFP expressing melanoma cells from prior experiments. There exist large discrepancies in concentration levels between many metabolites which are involved in cellular respiration. However, the error within each metabolite concentration was incredibly large and suggests that the quality of the acquired data is insufficient for any conclusions to be made.

**H NMR Spectral Analysis**

Previously acquired spectral data of cell extracts were analyzed for differences in metabolite concentrations. Trends such as higher concentrations of lactate, glutamine, and glycerophosphocholine (GPC) with higher J/EGFP expression were observed. However, the error within and between groups of each metabolite was incredibly large and suggested that the quality of the acquired data was insufficient for any statistical conclusions to be relevant (Table 1; Figure 4).
Inspection of previously acquired spectra revealed large water peaks with inadequate suppression, poor shimming, and incomplete separation of lipophilic and protein layers that significantly altered the true baseline of pure metabolite peaks (Figure 5, 6). In addition, cell counts acquired for each population exhibited wild variation within and between data sets that artificially skewed metabolite concentrations during normalization (Table 2). As such, a push to acquire new, clean data was issued. While a total of 5 new individual experiments were conducted, only 2 were successful.

Table 2: Cell count data for each experiment conducted. Large variation of counts between subpopulations and across individually conducted experiments can be observed. Consistency of cell counts is greatly improved for new experiments.

<table>
<thead>
<tr>
<th>J/EGFP SUBPOPULATION</th>
<th>PREVIOUS</th>
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<th>NEW</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
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<tr>
<td>UNSORTED</td>
<td>1.05 x 10⁷</td>
<td>7.13 x 10⁶</td>
<td>1.29 x 10⁶</td>
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<td>LOW</td>
<td>2.85 x 10⁶</td>
<td>1.48 x 10⁶</td>
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<tr>
<td>MEDIUM</td>
<td>5.40 x 10⁶</td>
<td>1.60 x 10⁶</td>
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<td>HIGH</td>
<td>1.83 x 10⁶</td>
<td>1.05 x 10⁶</td>
<td>6.90 x 10⁵</td>
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</table>

In addition to acquiring new H NMR spectra of variable expressing J/EGFP subpopulation cell extracts, H NMR spectra of media samples from each subpopulation taken 24 hours after incubation was recorded and analyzed as well. While trends in metabolite concentrations between each J/EGFP subpopulation can be observed in both media and cell extract experiments, the sample size of each new experiment is extremely limited (n = 2). While no statistical inferences can be made, observed trends and concentrations of metabolites of each subpopulation are stable between experiments (Table 3, 4).
Figure 5: Comparison of previously acquired H NMR spectra (top) to newly acquired spectra (bottom).

Figure 6: Comparison of 1.33 ppm lactate doublets from previous (left) and new (right) cell extract spectra. Orange, blue, and black curves respectively represent low, medium, and high J/EGFP expression from the same data set. Note the poor shim and obstructive lipid bump that greatly broadens and skews the line shape of the old acquisition.
Table 3: Concentrations of metabolites identified from each hydrophilic cell extract experiments of newly acquired spectra.

<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>EXPERIMENT 1</th>
<th>EXPERIMENT 2</th>
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<td>Concentration per cell (pM/cell)</td>
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<tr>
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<td>J/EGFP Expression</td>
<td>J/EGFP Expression</td>
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<tr>
<td></td>
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<td>Unsorted Low Medium High</td>
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<td>ACETATE</td>
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<td>11.50 7.22 14.61 20.13</td>
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<td>ALANINE</td>
<td>53.8 31.6 42.6 41.9</td>
<td>22.42 16.90 23.57 26.69</td>
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<td>ARGinine</td>
<td>16.7 14.4 14.5 12.8</td>
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<td>4.2 2.5 3.1 3.5</td>
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<td>8.3 5.5 7.7 7.9</td>
<td>3.67 2.99 4.28 5.03</td>
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<td>CREATINE PHOSPHATE</td>
<td>13.2 9 11.1 11.1</td>
<td>5.50 4.81 6.17 7.07</td>
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<td>43.17 35.51 46.00 53.57</td>
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<td>19.29 12.09 17.94 18.85</td>
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<td>GLYCEROL</td>
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<td>14.38 14.76 16.44 20.51</td>
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<td>86.9 46.1 64.7 63.1</td>
<td>36.21 24.55 35.94 40.19</td>
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<td>25.9 15 19.6 22.3</td>
<td>10.79 8.02 10.89 14.20</td>
</tr>
<tr>
<td>LACTATE</td>
<td>133.3 59.6 80.5 96.9</td>
<td>55.54 31.87 44.72 61.72</td>
</tr>
<tr>
<td>LEUCINE</td>
<td>13.7 10.2 11.8 12.4</td>
<td>5.71 5.45 6.56 7.90</td>
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<tr>
<td>MYO-INOSITOL</td>
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<td>8.92 6.58 9.61 12.23</td>
</tr>
<tr>
<td>PC</td>
<td>25.4 15.1 20.6 19.2</td>
<td>10.58 8.07 11.44 12.23</td>
</tr>
<tr>
<td>PYRUVATE</td>
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<td>THREONINE</td>
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<td>14.17 11.23 15.22 17.26</td>
</tr>
<tr>
<td>TYROSINE</td>
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<td>3.83 3.58 4.11 5.54</td>
</tr>
<tr>
<td>VALINE</td>
<td>8.5 6.5 6.5 8</td>
<td>3.54 3.48 3.67 3.70</td>
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Figure 7: Average concentration of metabolites in J/EGFP expressing melanoma cell extracts from new. Values were normalized to subpopulation cell counts.
Figure 8: Relative concentration change of metabolites between J/EGFP expressing melanoma subpopulations. J/EGFP<sup>low</sup> population was used as the reference.
Table 3: Metabolite consumption and production of different J/EGFP expressing melanoma subpopulations in Tu2% media after 24 hours. Concentrations have been normalized to subpopulation cell count.

<table>
<thead>
<tr>
<th>METABOLITE</th>
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<th>EXPERIMENT 2</th>
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<tr>
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<td>Difference from Standard (pM/cell)</td>
<td>Difference from Standard (pM/cell)</td>
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<td></td>
<td>Unsorted</td>
<td>Low</td>
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<td>ALANINE</td>
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<td>36.19</td>
</tr>
<tr>
<td>PYRUVA TE</td>
<td>-188.56</td>
<td>-121.13</td>
</tr>
</tbody>
</table>

![Glucose Consumption](chart1)

![Pyruvate Consumption](chart2)

![Glutamine Consumption](chart3)

![Lactate Production](chart4)
DISCUSSION

The Warburg effect cites an increase in glucose consumption and glycolysis rates ending in the production and excretion of lactate even under normoxic conditions for most cancers including melanoma [22-24]. Some have hypothesized that the preference for inefficient fermentation of glucose to ATP in cancer cells is due to defects in mitochondrial oxidative metabolism [25]. Other interpretations of the Warburg hypothesis postulate that cancer cells depend more heavily on rapid aerobic glycolysis instead of mitochondrial respiration because glycolysis is more efficient in terms of the required solvent capacity and speed of reaction despite being less efficient in terms of ATP yield per glucose uptake [26]. These interpretations are counter to the original inference of damaged mitochondria in cancer cells. In fact, a growing body of evidence shows that oxidative phosphorylation is persevered in many cancer cells including melanoma [27]. Glutamine has been implicated in melanoma to be a major alternative
source of carbon input to the tricarboxylic acid cycle (TCA) that allows the decoupling of mitochondrial TCA from glycolysis [28]. Not only can glutaminolysis maintain oxidative phosphorylation through the TCA, it can also provide carbon to support fatty acid synthesis [29]. This has led to a recent revision of the Warburg hypothesis that suggests the massive consumption of glucose by cancer cells could allow them to meet the needs of other biosynthetic pathways by diverting carbon flux from glycolysis while still contributing to energy production [30].

Analysis of media samples have shown that J/EGFP\textsuperscript{high} cells have increased glucose consumption as well as increased lactate production in comparison to J/EGFP\textsuperscript{low} cells (Figure 9). These results are consistent with previous experiments in the lab have demonstrated increased FDG uptake in slow-cycling J/EGFP\textsuperscript{high} cells compared to J/EGFP\textsuperscript{low} cells [31]. The lactate/alanine ratio has been correlated with oxidative stress as the conversion of pyruvate to lactate and alanine is coupled with NAD\textsuperscript{+} and NADH reactions. The increased lactate/alanine ratio and pyruvate consumption in the J/EGFP\textsuperscript{high} subpopulation (Figure 9) provide further evidence of functioning mitochondrial respiration. Coupled with the marked enhancement of glutamine consumption in the J/EGFP\textsuperscript{high} subpopulation (Figure 9), our findings are not only in alignment with the emerging revision of the Warburg hypothesis and cancer metabolism paradigm, but also illustrates metabolic versatility that may contribute to the dynamic stemness of slow-cycling melanoma cells and their intrinsic drug resistance.

H NMR spectra of cell extracts also reflect the results found in the analysis of media samples (Figure 7, 8). The slow-cycling J/EGFP\textsuperscript{high} subpopulation of cells have elevated concentrations of glutamine and products of glutaminolysis such as glutamate,
pyruvate, lactate, and alanine. In addition, almost all identified amino acids were found in higher concentrations in the J/EGFP\textsuperscript{high} subpopulation compared to the J/EGFP\textsuperscript{low} and J/EGFP\textsuperscript{medium} subpopulations that represent highly proliferating bulk tumor cells. The exception to this trend is arginine. However, this is expected as melanoma has been known to be auxotrophic to arginine due to a lack of the urea cycle enzyme arginosuccinate synthetase [32]. Interestingly, the J/EGFP\textsuperscript{high} subpopulation also has a noticeably higher concentration of myo-inositol which has been shown to be chemo-preventative and blocks proliferation in other cancers such as lung cancer and breast cancer [33, 34]. As such, myo-inositol production may be one mechanism that contributes to the slow-cycling nature of JARID1B\textsuperscript{high} cells.

While unusually high glucose uptake is often considered a biomarker for cancer and is reflective of the high energetics required for the rapid proliferation characteristic of cancer cells J/EGFP\textsuperscript{high} cells do not proliferate rapidly despite their enhanced consumption of glucose [35]. One new hypothesis that may explain increased bioenergetics in non-proliferating cells is that slow-cycling cells may have increased cell motility and invasion. A new study that examined the role of choline and phosphocholine in the phospholipid metabolism pathway in prostate cancer cells showed that inhibition of phospholipase C, which converts phosphatidylcholine to phosphocholine, results in a decrease in cell migration [36]. Our results are in line with this hypothesis by showing an increase in choline and other choline derivatives in slow-cycling J/EGFP\textsuperscript{high} cells compared to J/EGFP\textsuperscript{low} cells in addition to increased flexibility and versatility in bioenergetics pathways.
The chief limitation to this project was the limited number of successful experiments. While a total of 5 experiments were conducted, each spanning the length of two weeks from initial seeding of cells to H NMR acquisition of media and cell extract, the current data set only consists of 2 samples (n = 2). Failures were due to purchasing non-cell culture plates that did not promote cells to adhere, sudden malfunction of the lyophilizer condenser overnight, and culture contamination. Even though stark differences in metabolite concentrations were found between J/EGFP expressing subpopulations after blind analysis of spectral data, the statistical power remains insufficient to achieve any semblance of statistical significance. However, the results from the two experiments are similar in both metabolite concentration values and overall trends between groups before and after normalization to cell count (Table 2, 3, 4).

CONCLUSION AND LIMITATIONS

Identifying metabolic differences in melanoma subpopulations is an important step toward understanding the dynamic stemness of slow-cycling JARID1B$^{\text{high}}$ melanoma cells. Our findings have shown that slow-cycling J/EGFP$^{\text{high}}$ melanoma cells have higher concentrations of intracellular metabolites such as glutamine, glutamate, lactate, alanine, phosphocholine and glycerophosphocholine compared to highly proliferative bulk tumor cells with lower J/EGFP expression. Evidence of functional mitochondrial respiration pathways such as glutamineolysis are consistent with recent revisions of the Warburg hypothesis that suggest the increase in glucose consumption could be used to fuel alternate biosynthetic pathways. The metabolic versatility and
flexibility of J/EGFP\textsuperscript{high} expressing cells to better utilize alternate sources of carbon could be a contributing factor towards the dynamic stemness and intrinsically resistant properties of slow-cycling melanoma cells. These preliminary findings show a promising future for this type of melanoma research.
REFERENCES


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