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The Impact of the Mitochondrial Metabolism of Induced Pluripotent Stem Cells Upon Differentiation

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by

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Contents

List of Figures

List of Tables

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Stefanie Shahan

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ABSTRACT OF THE THESIS

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Research Advisor: Professor Jeffrey Millman

Induced pluripotent stem cells (iPSCs) can be differentiated into any cell type found in the body. The derivation of a stem cell derived β cell (SC-β) capable of responding to glucose by secreting insulin was hugely significant for diabetes research and opened up the possibility of cell replacement therapy to combat this widespread disease (Pagliuca et al. 2014). The optimization of differentiation procedures such as this could improve yield, function, cost, and efficiency of a stem cell-derived product. Current approaches to improve differentiation are primarily focused on signal transduction pathways, while the metabolic state of the cells has received little attention. Using a Seahorse XF24 extracellular flux analyzer, we found that iPSCs have heavy reliance on fatty acid oxidation (FAO), a trait not shared by many mature cell types. Manipulation of stem cells and their mitochondrial oxidation by manipulating cell culture composition or adding small molecules BPTES, etomoxir, and UK5099 was shown to be possible in both suspension and adherent differentiations. Expression of key mitochondrial genes changed drastically during differentiation of iPSCs to SC-β cells. Thus, this body of work has demonstrated the importance of and paved the way for future research on the importance of mitochondrial oxidation to differentiation.

Chapter 1

Introduction and Background

1.1 An Overview of Induced Pluripotent Stem Cell Differentiation

In 2007, the Yamanaka group generated induced pluripotent stem cells (iPSCs) from a human dermal fibroblast line; the iPSCs are capable of self-renewal and differentiating into a variety of cell types, and in addition share most morphological traits and behaviors of embryonic stem cells (ESCs). This landmark discovery has since prompted an influx of research on the characteristics and differentiation potential of iPSCs, as it offers a way to derive patient-specific cells of any type at high volumes from an easily obtainable tissue sample that can then be used for cell therapy (Takahashi et al. 2007).

1.1.1 Stem Cell Pluripotency and Differentiation

iPSCs and ESCs share many characteristics, pluripotency being the most critical to their use in medicine. Both types of stem cell are capable of converting to many somatic cell types through the process of differentiation. Notably, some iPSCs have slightly reduced pluripotency (Stadtfelt et al. 2010), but both murine iPSCs and ESCs are capable of generating viable mice progeny through tetraploid complementation, demonstrating their potential to develop into any somatic cell type (Zhao et al. 2009). For ethical reasons, pluripotency tests akin to these cannot be conducted within human iPSCs, but it is possible that they are similarly pluripotent. Through differentiation of pluripotent stem cells, large quantities of specific cell types can be generated and implanted back into a patient suffering from a disease, and in the future could be used to grow whole replacement organs.

Thus, a vital aspect of using stem cells for cell therapy is the optimization of this differentiation. Differentiation can be costly, plagued with cell death and lack of efficiency, and take extensive culture time to complete. Optimizing the process to reduce these issues is essential for the transition of stem cells from research subject to plausible, available medical therapy. Differentiation is often altered by changing factors and their concentrations, the duration spent in certain differentiation steps, alteration of cell cluster sizes, or oxygen availability; however, even the best modern differentiations have much room for improvement. As an example, even in a well characterized cardiac differentiation that has been partially optimized by research groups, generation of the desired cell type can take over a week, is affordable but still costly, and results in 64-89% of cells expressing appropriate cell markers (Burridge et al. 2011). Cardiac differentiation has existed for years and still struggles with these issues; other differentiations are relatively new, and researchers hope to similarly optimize them.

1.1.2 Diabetes and the Stem Cell-Derived β-Cell

A recent and significant advancement in the field of stem cells and differentiation is the derivation of stem cell-derived β-cells (SC-β), pancreatic cells that release insulin in response to a glucose challenge, which was only accomplished in 2014 (Pagliuca et al. 2014). These cells have great potential for cell therapy, as 29.1 million people in the United States alone suffer from diabetes ("CDC 2014 Diabetes Report" 2014), a disease where pancreatic β-cells either die or become nonfunctional, causing high blood glucose levels in the body. Aside from diabetes' immediate medical health issues, which include potentially fatal hyperglycemia, ketoacidosis, and coma, the disease can even be correlated with other health issues, like retinal or neurologic disorders (Diabetes Control and Complications Trial Research Groupa 1994). Replacement of nonfunctional β cells by implantation with alternative cells could solve this modern health crisis; one option for replacement is SC-β cells, which are patient-specific, can be generated in large quantities, and can be tested for functionality (ability to release insulin in response to a glucose challenge) prior to implantation. Therefore, it is vital that the differentiation of β-cells be optimized. Though altering factors, timing, and oxygen availability are all important to differentiation optimization, this research will explore characterization of stem cells, the starting block of any differentiation, and the possible influence of shifts in mitochondrial metabolism and its oxidation of certain metabolites.

1.1.3 Mitochondrial Metabolism and Differentiation

One distinct characteristic of human embryonic stem cells (hESCs) that differs from mature cells is their metabolism: they are heavily reliant on glycolysis, an anaerobic process that generates ATP using glucose, to generate their energy. Accordingly, their mitochondria differ from other cell types: hESCs possess scarce mitochondria that are localized to the edges of the cell, are underdeveloped with poor cristae formation, and appear to have restricted oxidative capacity (Varum et al. 2011). As hESCs differentiate, their mitochondrial networks increase in mass, branch out, and localize to other areas of the cell (St. John et al. 2005), and their mitochondria become more active as oxidative phosphorylation (OXPHOS) becomes the primary source of energy (Cho et al. 2006). Despite iPSCs being generated from adult cells with developed mitochondria and a reliance on OXPHOS, they seem to revert to a state similar to hESCs in terms of metabolism and mitochondria (Prigione et al. 2010) and, upon differentiation, share similar mitochondrial regulation mechanisms as hESCs (Armstrong et al. 2010, Varum et al. 2011). It has been shown that altering hESC mitochondrial biogenesis can influence commitment to a germ layer in differentiation (Prowse et al. 2012).

Thus, it has been acknowledged that certain mitochondrial behavior is related to stem cell identity and changes upon differentiation, but it is still debated as to whether this is a result of differentiation or a mediator of it (Rehman et al. 2010), and the exact method to influence the mitochondria in a beneficial way is unknown, aside from attempting to increase the mitochondrial mass in some mature cell types and the application of specific metabolites.

At the forefront of research on the topic of stem cell differentiation, application of specific metabolites and level of saturation of these metabolites have arisen as a way to influence differentiation (Yanes et al. 2010), and it is known that different media formulations can impact differentiation efficiency in hESC cultures transitioning to hematopoietic and neural cell types (Lee et al. 2015). Differences in iPSC and hESC behavior, as well as some of the effects of reprogramming a fibroblast into an iPSC, have been attributed to metabolome differences related to mitochondrial metabolism and levels of specific fatty acids (Panopoulos et al. 2012). Alteration of methionine metabolism has been shown to reduce stem cell pluripotency, and initiated unspecific differentiation (Shiraki et al. 2014).

Thus, recent literature has repeatedly been directed towards the subject of metabolites and their influence upon stem cell metabolism and differentiation potential, but the exact connection between metabolites and differentiation has not yet been elucidated. If the mechanisms underlying mitochondrial behavior that regulates differentiation exists and can be identified, optimization of differentiation through influencing the mitochondria may be possible.

1.2 OXPHOS and Mitochondrial Metabolism

Mitochondria are organelles that serve many essential functions within the cell: the generation of ATP for energy through OXPHOS, the mediation of apoptosis (Wang 2001) and the regulation of potentially cytotoxic reactive oxygen species (ROS), and more (Marchi et al. 2011). Manipulation of the mitochondria, and possible control of differentiation through the mitochondria, requires understanding of its function.

1.2.1 Mitochondrial Structure and Function

Mitochondria are organelles possessing two membranes, an inner and an outer membrane, and thus have two internal compartments: the matrix, which exists inside of the inner membrane, and the intermembrane space. The outer membrane is dense with transport proteins and thus is somewhat permeable, allowing a broad set of small molecules from the cytosol to filter into the intermembrane space. In contrast, the inner membrane is more impermeable, though it also contains transport proteins, and has invaginations called cristae that increase its area to facilitate exchange of molecules (Alberts et al. 2002). The mitochondria uptakes food molecules, such as pyruvate and fatty acids, which it then converts to acetyl CoA and passes into the citric acid cycle, which generates electrons. These electrons are used to fuel the electron transport chain, a series of protein complexes that takes in oxygen and electrons to move hydrogen ions across the inner membrane and establish a proton gradient. This proton gradient can be used to power the protein complex of ATP synthase, which uses the energy of the proton motive force generated by the gradient to convert ADP to ATP. This ATP is then exported to the cytosol, where it can be utilized by the rest of the cell as energy. Overall, one molecule of glucose can generate approximately 30 molecules of ATP, and this production requires consumption of oxygen. This entire process is summarized in Figure 1.1. One

final thing to note is that the inner membrane is not completely impermeable to protons; some protons can pass through, consuming oxygen to do so, without interaction with ATP synthase or the electron transport chain. This is called proton leak (Jastroch et al. 2010).

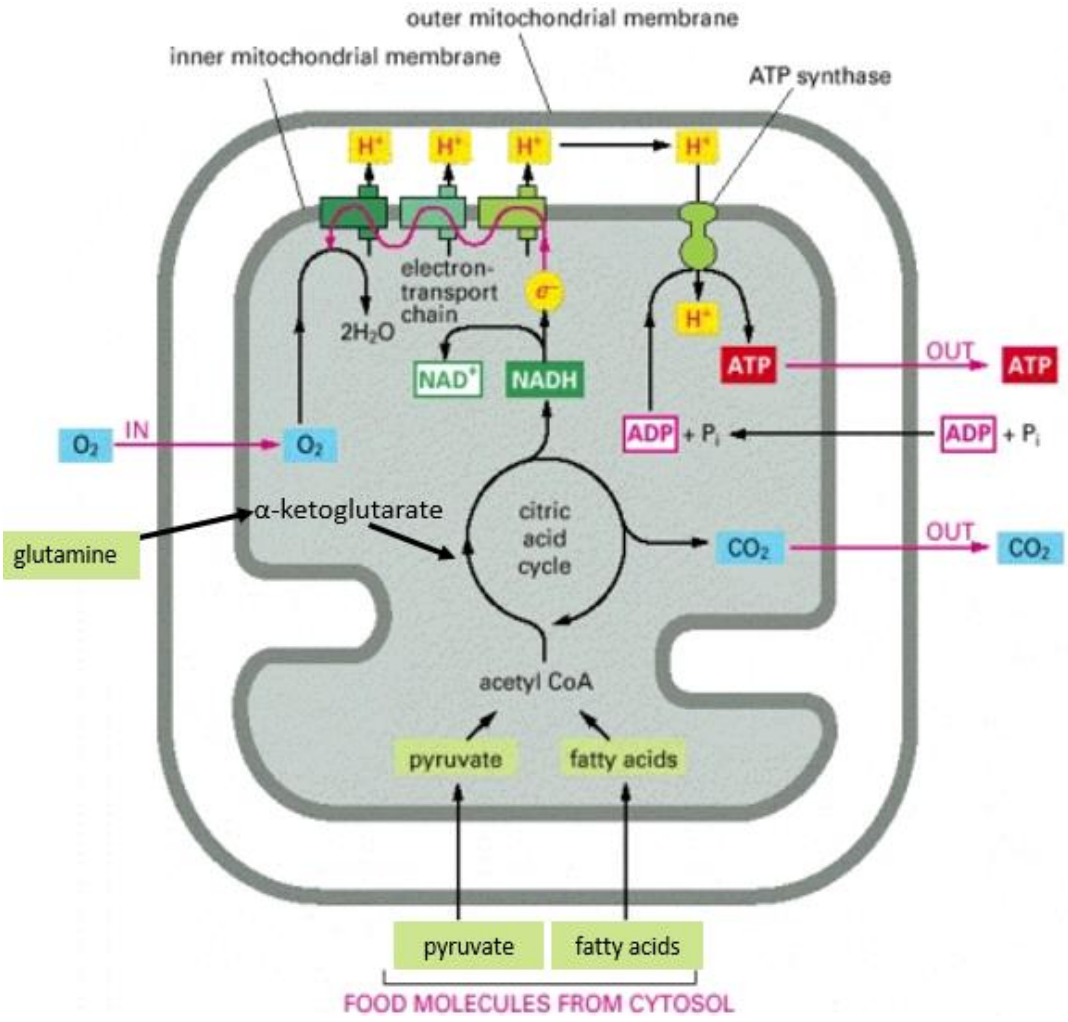

Figure 1.1 An Overview of Mitochondrial Structure. This figure was taken directly from Alberts et al., 2002. Food molecules will often be referred to as "mitochondrial fuels."

1.2.2 Generation of Pyruvate Through Glycolysis

Pyruvate, one of the food molecules commonly taken up by the mitochondria, is generated primarily through the process of glycolysis, an aerobic process which occurs in the cytosol. Glycolysis is a reaction chain that consumes a glucose molecule and NAD^+ to produce a net output of two pyruvate, two ATP molecules, and the electron carrier NADH. Pyruvate will then often be shuttled into the mitochondria to be used in OXPHOS, but it can also undergo the process of fermentation, which in human beings generates lactic acid and NAD^+, which must be replenished if glycolysis is to occur (Alberts et al. 2002). This process is summarized in Figure 1.2.

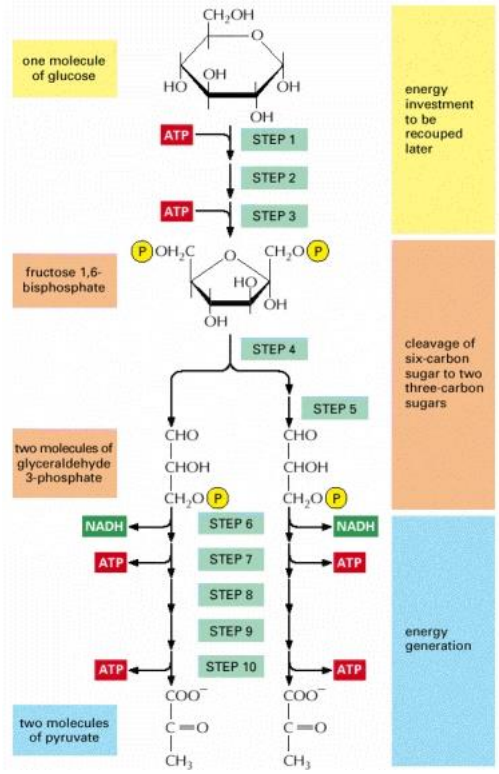

Figure 1.2 An Overview of Glycolysis. This figure was taken directly from Alberts et al., 2002.

Comparatively, glycolysis produces less ATP than OXPHOS. It is also important to note that glycolysis is an anaerobic process, while mitochondrial OXPHOS requires oxygen for the electron transport chain's function. Thus, in a hypoxic environment, cells will upregulate glycolysis enzymes and shift to glycolysis for ATP production (Robin et al. 1984), and in addition will regulate

OXPHOS (Taylor 2008). Shifts similar to this are not limited to solely hypoxic environments—cell types utilize glycolysis to different degrees. The upregulation of glycolysis and role as the primary generator of ATP is well documented in both cancer cells (Ganapathy-Kanniappan et al. 2013) and stem cells, which are known to have increased glycolysis and decreased but still present OXPHOS (Ito et al. 2014).

1.2.3 Healthy Mitochondrial Function Is Essential to β-Cell Function

Having outlined the processes of glycolysis and OXPHOS, and having described the mitochondria, it is now possible to understand how essential healthy mitochondrial function is to the function of a β-cell. Figure 1.3 gives a simple overview of how glucose stimulates insulin release. The process of insulin release as a response to glucose moves directly through the mitochondria, and relies on the mitochondria to process pyruvate and produce ATP. Once ATP has been produced, it inhibits the outward flow of potassium by blocking potassium channels and depolarizes the cell, causing an inward flow of calcium that then stimulates insulin granule exocytosis. Without proper functioning of the mitochondria, insulin release can be disrupted.

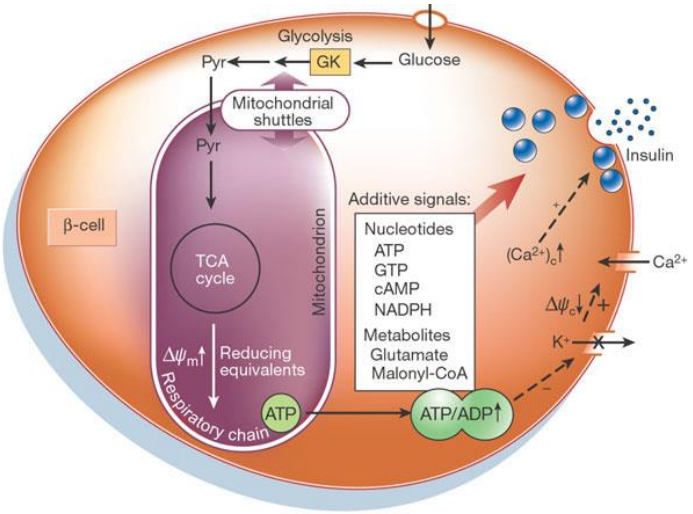

Figure 1.3 An Outline of Insulin Release Due to Glucose Uptake. This figure was taken directly from Maechler and Wollheim's 2001 article.

The disruption of insulin release due to an error in this process can lead to diabetes. Notably, mutations in mitochondrial DNA (mitochondria have their own set of DNA, separate from the nucleus of the cell they reside in) can initiate diabetes, with no other known mutation (Sivitz and Yorek 2010). This implies that a disruption in solely mitochondrial function is enough to cause diabetes, highlighting the importance of healthy mitochondria to β-cell function.

1.2.4 Mitochondrial Properties Essential to this Work

Fully exploring all mitochondrial structures and functions in detail would be impossible, but there are several key properties that are relevant to this work.

Many food molecules serve as fuel for the citric acid cycle, some of the most prominent being pyruvate, fatty acids, and amino-acids. This work will focus upon pyruvate oxidation, fatty acid oxidation (FAO), and glutamine oxidation, the amino acid that literature suggests is indispensable for stem cells (Tohyama et al. 2016).

Pyruvate and fatty acids are both converted into acetyl-CoA upon entering the mitochondrial matrix, which can be input into the citric acid cycle. Glutamine is converted to glutamate (catalyzed by the enzyme glutaminase) and then α-ketoglutarate, which is also a substrate in the citric acid cycle. Pyruvate and fatty acids are both moved into the mitochondrial matrix by transport proteins on the inner membrane: mitochondrial pyruvate transporters MPC1 and MPC2, and carnitine palmitoyltransferase I (CPT1) for long-chain fatty acids, among possible others. These transporters specifically can be inhibited by the small molecules UK5099 for the pyruvate transporters, and etomoxir for CPT1. Application of these inhibitors prevents pyruvate oxidation or FAO. Glutamine oxidation can be inhibited by the application of Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), which inhibits glutaminase, the enzyme that converts glutamine to glutamate.

Other mitochondrial processes can also be inhibited or disrupted, including the action of ATP synthase, certain complexes in the electron transport chain, and the proton gradient. Disruptions of these processes can reveal information about the mitochondria, and are implemented in this experimental work.

Oligomycin binds and inhibits ATP synthase, preventing the exchange of protons across the gradient for conversion of ADP to ATP. Antimycin A and rotenone disrupt and destroy the proton gradient, preventing any consumption of oxygen by the electron transport chain and use of the gradient by ATP synthase. Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) is an uncoupler, transporting protons across the inner cell membrane before they are used by ATP synthase, allowing for the continuous operation of the electron transport chain, and maximal use of oxygen by the mitochondria.

These chemicals and their effects can be seen summarized in Figure 1.4. These inhibitors and disrupted processes specifically will be utilized in this work.

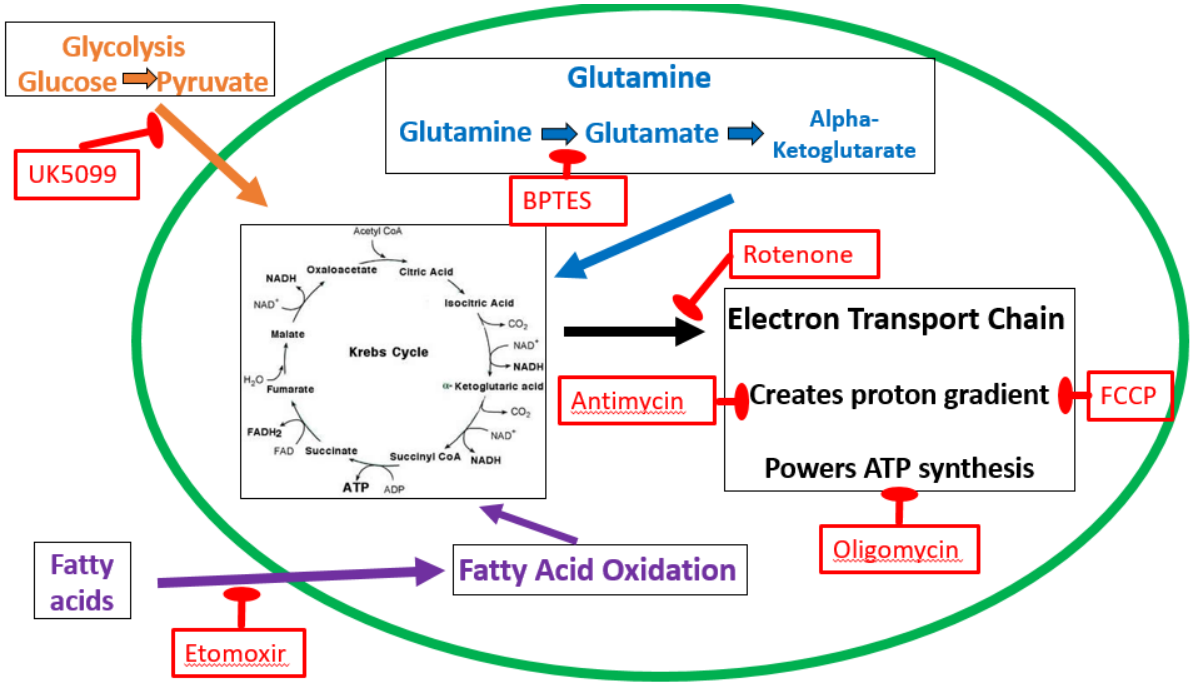

Figure 1.4 Inhibition and Disruption of Mitochondrial Processes in this Research. All substances listed in red are inhibitors, and arrows demonstrate where they affect mitochondrial respiration.

Chapter 2

Characterization of Stem Cell Mitochondrial Metabolism

2.1 Introduction

Following the creation of human induced pluripotent stem cells (iPSCs) in 2007 (Yamanaka 2007), research has focused on the optimization of differentiation protocols to produce large quantities of mature, functional cells. Ideally, cells could be collected from a patient, converted to an iPSC, differentiated into mature cells, and implanted back into the patient. Current differentiation methodology is effective, but plagued with cell death, low efficiency, and extensive culture time; thus, optimizing these processes is necessary to move forward. Often, optimization involves altering media formulations, applied factors, the sizing of clusters, or the time duration of certain differentiation steps. As stem cells are the starting point of any differentiation, and cell loss often occurs early in the process, characterization of stem cell behavior and biology is imperative to improving differentiation.

Human embryonic stem cells (hESCs) have been thoroughly studied in literature. As mentioned in chapter one, a distinct characteristic of hESCs that differs from mature cell types is their metabolism: they are heavily reliant on glycolysis, and according to some studies possess underdeveloped mitochondria (Varum et al. 2011). Despite iPSCs being generated from adult cells with developed mitochondria and a reliance on oxidative phosphorylation (OXPHOS), they appear to revert to a state similar to hESCs in terms of metabolism and mitochondria (Prigione et al. 2010). As they differentiate, stem cells grow to rely upon OXPHOS more than glycolysis, a shift possibly attributed to mitochondrial mass and network branching.

Due to the heavy reliance on glycolysis to generate ATP within a stem cell and the clear shift to OXPHOS in mature cells, most literature on stem cells has focused on glycolysis, rather than the

mitochondria and OXPHOS. However, it has been shown that the mitochondria of stem cells are carefully regulated, and that mitochondrial behavior or the metabolites taken up by the cell, specifically fatty acids, could possibly affect differentiation (Alakpa et al. 2016). It has also been shown that increased glucose concentration can suppress murine ESC neural differentiation (Yang et al. 2016). This emerging evidence that metabolites and mitochondrial consumption of available fuels within stem cells has prompted our investigation into the mitochondrial oxidation of these fuels. As the Millman lab specializes in endodermal differentiation to a stem cell-derived β cell, we chose to study this differentiation process specifically. Thus, this body of work sought to characterize the mitochondrial behavior of stem cells and several other mature cell types related to the β cell.

2.2 Abstract

Most research on stem cells has stressed their metabolic dependence on glycolysis for energy production, emphasizing a decrease in mitochondrial oxidative activity in immature cells. However, mitochondrial oxidation still occurs and its use of energy sources could be cell type dependent. Through analysis with a Seahorse XF24 extracellular analyzer, we were capable of quantifying the ATP production, maximal respiration, and reliance of cells on specific fuel sources in terms of their oxygen consumption. We demonstrated that stem cell mitochondrial metabolism relied heavily on fatty acid oxidation (FAO) as opposed to pyruvate or glutamine oxidation. This was observed within HUES8, an hESC line, and two iPSC lines that were reprogrammed from 1016 fibroblasts, one line a clone of the other. Stem cells were also shown to have the largest capacity for FAO by using analysis of their maximal respiration. Combined, these two findings indicate that stem cells require mitochondrial FAO, though at this point we can only hypothesize as to why this may be.

Comparatively, mature cell types such as fibroblasts and INS-1s were found to rely less upon FAO and fibroblasts had lower capacity for its use, indicating that they favored pyruvate or glutamine oxidation. Thus, we conclude that this reliance on FAO is characteristic of stem cells, and that shifts in oxidation pathway reliance may be linked to differentiation or cell identity.

2.3 Materials and Methods

2.3.1 The Seahorse XF24 Extracellular Flux Analyzer

To analyze mitochondrial OXPHOS, the Seahorse XF24 extracellular flux analyzer (henceforth referred to as the Seahorse) was implemented. The Seahorse is capable of measuring oxygen consumption rate (OCR) as well as rate of acid efflux (ECAR) in a 24 well plate. To quantify cellular respiration on a Seahorse, we injected solutions with oligomycin, FCCP, and then a solution of antimycin A and rotenone, which will be called a mitochondrial functionality test. The specific impact of each of these chemicals upon the mitochondria was detailed in Section 1.2.4, but is summarized in Table 2.1. We optimized concentrations of these chemicals for use with a 1016 SeVA stem cell line (a line used within Pagliuca et al. 2014 and Millman et al. 2016), an iPSC line reprogrammed from 1016 fibroblasts. We kept the applied concentrations consistent through all Seahorse runs, with injections resulting in final concentrations of 3μM oligomycin, 0.25μM FCCP, 2μM antimycin A, and 1μM rotenone.

Table 2.1 Mitochondrial Functionality Test Chemicals

Term	Function	Final Concentration
Oligomycin	ATP synthase inhibitor	3 μM
FCCP	Mitochondrial uncoupler of ATP synthesis and the electron transport chain	0.25 μM
Antimycin A	Binds cytochrome c reductase to inhibit electron transport, eliminating the proton gradient of the mitochondrial inner membrane (Ma 2012)	2 μM
Rotenone	Inhibitor of the electron transport chain through mitochondrial complex I.	1 μM

Analysis of cell behavior following injection of these chemicals allows quantification of cell behavior.

A general overview of cell properties as related to Seahorse measurement and injections can be viewed in Figure 2.1, allowing quantification of mitochondrial baseline respiration, ATP production, maximal mitochondrial respiration, proton leak, and non-mitochondrial respiration.

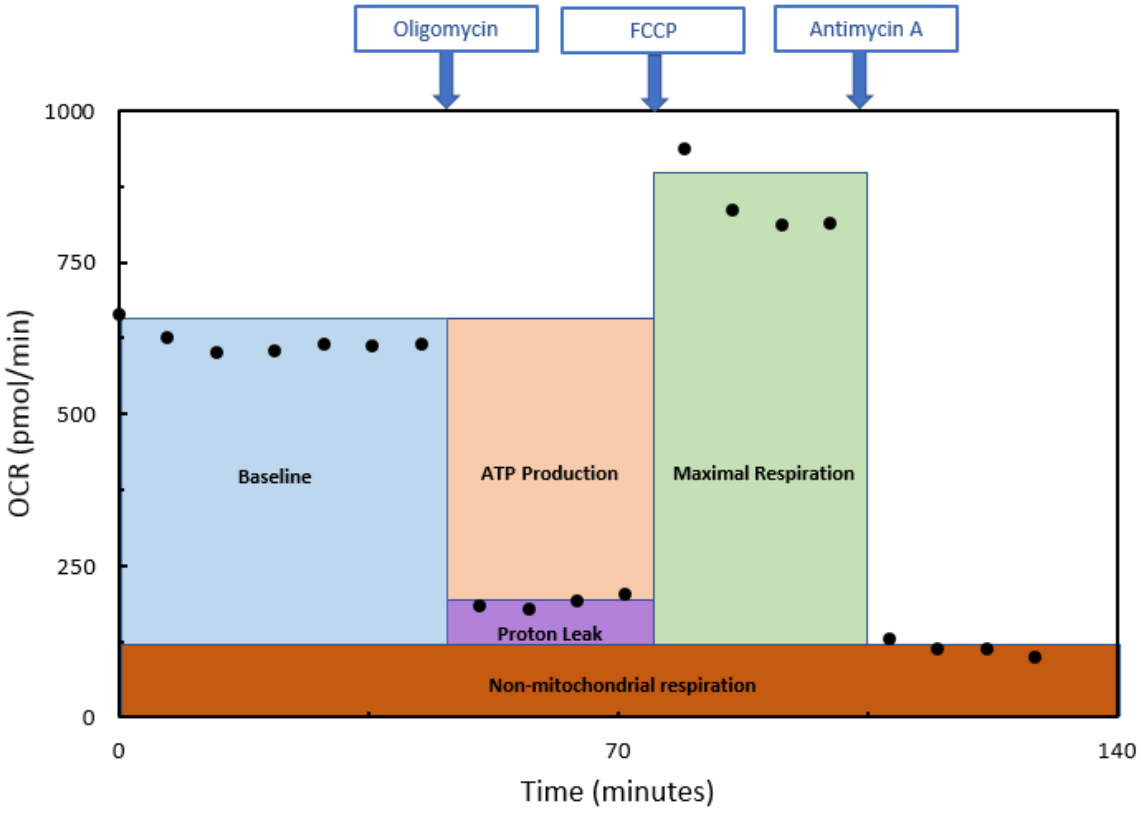

Figure 2.1 Seahorse XF24 Mitochondrial Functionality Test. A Seahorse mitochondrial functionality test involves injection of solutions with the chemicals oligomycin, FCCP, and antimycin A/rotenone to assess aspects of mitochondrial oxygen consumption.

Each of these mitochondrial properties and its definition as used in this body of work can be seen in Table 2.2.

Table 2.2 Mitochondrial Functionality Test Terminology

Term	Definition	Mathematical Definition
Baseline	The OCR of cells at a resting state.	The average of data collected prior to any injection.
ATP production	The OCR used for production of ATP by mitochondrial ATP synthase.	The difference between baseline and the average of data following the injection of oligomycin.
Proton leak	OCR related to protons crossing into the mitochondrial matrix without ATP synthase (Jastrovich 2010).	The difference between the average of data following the injection of oligomycin and the average of data following the injection of antimycin A/rotenone.
Maximal Respiration	The maximum OCR the cellular mitochondria are capable of consuming.	The maximum OCR following injection of FCCP, with non-mitochondrial respiration removed.
Non-mitochondrial respiration	OCR of processes occurring outside of the mitochondria.	The average of data following the injection of antimycin A/rotenone.

To allow for easily comprehensible data analysis, and to allow for simple comparison between Seahorse runs, the measured Seahorse OCR will be mathematically adjusted throughout this analysis. Two main adjustments will be performed: representing OCR as a fraction of ATP production, and representing OCR as a fraction of baseline. When representing OCR as a fraction of ATP production, the average baseline value is set to one, and the average value following oligomycin injection is set to zero. Similarly, when representing OCR as a fraction of baseline, the average baseline value is set to one, and the average value following antimycin A/rotenone injection is set to zero. Both eliminate non-mitochondrial respiration from the measurements. These two adjustments were performed upon Figure 2.1, and can be seen in Figure 2.2.

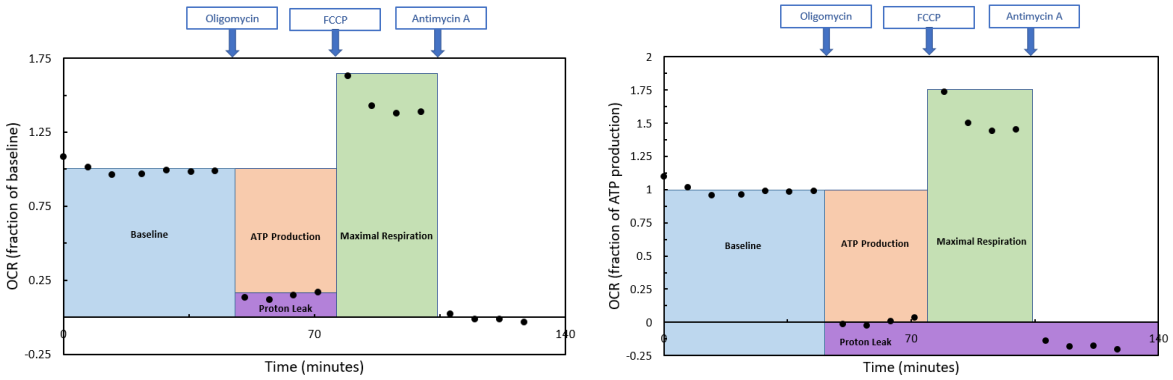

Figure 2.2 Seahorse XF24 Mitochondrial Test Mathematical Adjustments. A Seahorse mitochondrial functionality test with OCR values, adjusted to set either oligomycin injection at zero or the antimycin A injection to zero.

2.3.2 Cell Culture in Preparation for a Seahorse Run

To prepare for a Seahorse run, the selected cells were single-cell dispersed within an appropriate media for their respective line, and plated upon a Seahorse XF24 Cell Culture 24 well microplate coated with fibronectin. Four wells out of the twenty-four served as background wells, and were left with only a fibronectin coating and media. Cell lines were typically maintained for approximately 48 hours within the microplate, feeding stem cell lines every twenty-four hours. Upon reaching the desired cell confluency, the media was aspirated and replaced with 500 μL of RPMI 1640 (Sigma-Aldritch) medium containing 20mM glucose and 1mM sodium pyruvate (note that we altered this formulation within select later experiments, and INS-1 cells require a RPMI 1640 media at 2mM glucose). The microplate was then placed within a 37 ºC non-CO_2 incubator for 90 minutes before testing using the Seahorse, to eliminate temperature variations. Meanwhile, a Seahorse sensor cartridge was prepared for solution injection, and placed within the 37 ºC non-CO_2 incubator for at least 60 minutes before insertion into the Seahorse for calibration. Cell lines, appropriate media formulations for their culture, typical seeding densities, and ideal final confluency for a Seahorse run can be seen in Table 2.3.

Table 2.3 Culturing Cell Lines Within Seahorse XF24 Microplates. Abbreviations include Dulbecco's modified Eagle's medium (DMEM), FBS (fetal bovine serum), penicillin-streptomycin (P/S), and non-essential amino acids (NEAA).

Cell Line	Media	Culture Duration within Microplate	Seeding Density (cells/well)	Ideal Final Confluency (%)
1016 SeVA iPS (adherent)	mTeSR1	48 hours	100,000	80-90
1016 Fibroblast	DMEM + 20% FBS + 1% P/S+1% NEAA +1% GlutaMax+ 20mM glucose	48 hours	50,000	100
HUES8 embryonic stem cell (suspension)	mTeSR1	48 hours	100,000	80-90
C9 SeVA iPS (adherent)	mTeSR1	48 hours	100,000	80-90
INS-1	RPMI 1640 + 10% FBS+ 1% P/S + 1% pyruvate	48 hours	90,000	80-90

2.3.3 Analysis of Specific Mitochondrial Oxidation Pathways with Seahorse

In addition to the data provided by the mitochondrial functionality test, additional inhibitory small molecules can be injected to determine the reliance of the cells upon specific types of mitochondrial oxidation, such as pyruvate oxidation. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), etomoxir, and UK5099 were injected with the Seahorse prior to the mitochondrial functionality test injections. BPTES, etomoxir, and UK5099 inhibit glutamine oxidation, fatty acid oxidation (FAO), and pyruvate oxidation, respectively. When injected by the Seahorse, each results in a loss of OCR, corresponding to how much the cell was dependent on the related mitochondrial fuel source for oxidation and oxygen consumption. We will thus call this loss of respiration "dependence," and its value in a variety of cell types can be compared.

A summary of relevant information for each chemical is listed in Table 2.4.

Table 2.4 Small Molecules Impacting the Mitochondria

	Final concentration	Target of Inhibition	Related method of OXPHOS
BPTES	30 μM	Glutaminase	Glutamine oxidation
Etomoxir	40 μM	Carnitine palmitoyltransferase	Fatty acid oxidation/β oxidation
UK5099	4 μM	Mitochondrial pyruvate carriers	Pyruvate oxidation

The effects of each of these chemicals was further characterized by calculating the concentration of NAD^+ with an NAD^+ kit (Cayman Chemical) following a 20 minute treatment with RPMI 1640, 10mM pyruvate, and 20mM glucose as well as a small molecule with the concentration listed in Table 2.4. There was an observed increase in NAD^+ concentration following etomoxir and UK5099 treatment.

To efficiently compare different Seahorse runs and dependencies, data was mathematically adjusted to be a fraction of ATP production, as detailed in section 2.3.1. This allows each dependence to be represented as a fraction of total ATP production, which is comprehensible.

The INS-1 cell line, a rat insulinoma line provided by the Urano lab at Washington University, varied drastically from other cell types in its behavior during a mitochondrial functionality test; this was likely due to its response to glucose. Thus, a Seahorse run with INS-1 cells was started with an RPMI 1640 solution with 2 mM glucose and 1mM sodium pyruvate, with all following injections resulting in a 20 mM glucose environment, and the first glucose injection being accompanied by one of the three small molecules mentioned above, at concentrations of 30 μM BPTES, 100 μM etomoxir, and 200 μM UK5099.

2.4 Results

2.4.1 Stem Cells Rely Primarily on FAO

To begin assessing the mitochondrial respiration of undifferentiated iPSCs, we measured total OCR and used injection of oligomycin, FCCP, antimycin A, and rotenone to understand what cellular processes contributed to OCR. We measured that mitochondrial respiration was 1.48 +/- 0.412 femtomole/minute/cell, maximal respiration was 2.82 +/- 0.36 femtomole/minute/cell, proton leak was 0.283 +/- 0.0768 femtomole/minute/cell, and non-mitochondrial respiration was 0.395 +/- 0.113 femtomole/minute/cell. This data confirmed our assumption that the majority of OCR was due to mitochondrial respiration. To explore this further, we used injection of BEU to determine what fuels the undifferentiated iPSC were using for mitochondrial respiration. we found the cells primarily relied on fatty acids for OXPHOS. This was surprising because undifferentiated iPSCs generate a lot of pyruvate due to the high level of glycolysis and, though it has been hypothesized that the low level of activity still occurring within the mitochondria of a stem cell might serve to support lipogenesis (Zhang et al. 2016), the degree to which this occurs and its comparison to other types of mitochondrial oxidation has not been shown.

Prior to a mitochondrial functionality test, the small molecules BPTES, etomoxir, and UK5099 were injected using the Seahorse onto 1016 SeVA iPSCs (Figure 2.3). Injection of etomoxir impacted FAO, and its addition to the cells prompted a loss of OCR. This loss accounted for nearly all the oxygen consumed by ATP production in the mitochondria, indicating that iPSCs rely heavily on FAO for oxygen consumption.

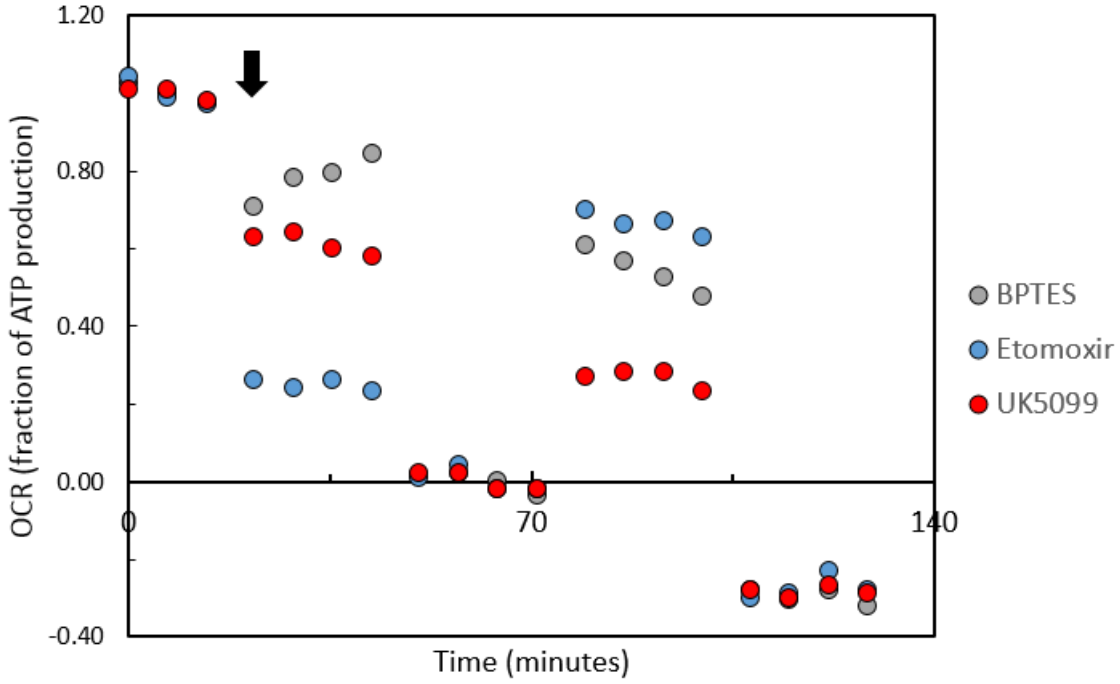

Figure 2.3 1016 SeVA iPSCs rely upon FAO over other fuel sources. A mitochondrial functionality test preceded by injection of a small molecule at the black arrow: BPTES, etomoxir, or UK5099.

To confirm that this reliance upon FAO was not a property only of 1016 SeVA iPSCs, two other stem cell lines were analyzed. HUES8 cells obtained from the Melton laboratory at Harvard, which are hESCs, were also analyzed with the Seahorse after being in suspension culture. C9 iPS cells, which are clones of the 1016 SeVA iPSC, were also tested and found to possess a similar mitochondrial dependence profile, with high FAO. All stem cell lines analyzed share a similarly large loss of respiration upon etomoxir injection, accounting for a large amount of the oxygen consumption involved in ATP production (Figure 2.4). The cells analyzed were from both suspension and adherent cultures, so this loss of respiration was not related to the cell culture system.

It is important to note, when comparing dependencies of cell types, that the fractional dependence of each cell type adds up to over 1. This is not an error, although it implies that the total oxygen consumption of each type of mitochondrial oxidation adds up to more than the respiration consumed by ATP production, which should not be possible. In reality, the application of each of the small molecules can influence respiration generated by each of the fuel sources studied. For example,

etomoxir's inhibition of fatty acid transport into the matrix of the mitochondria prevents FAO, reduces input to the TCA cycle, and then limits output to the electron transport chain, which consumes oxygen. However, as pyruvate and glutamine oxidation also provide substrates to the TCA cycle, their products' movement through the cycle and the following change in oxygen consumption are also impacted. Thus, the dependence of the cell on each fuel source measured here is not exact; it still demonstrates that in stem cells FAO contributes the most to cellular respiration and ATP production.

Additionally, it should be noted that HUES8 ES cells have a negative dependence upon glutamine oxidation. In these cells, the reaction to BPTES was for OCR to increase; possibly, this was due to some kind of oxygen consumption compensation from other fuel types or the mitochondria.

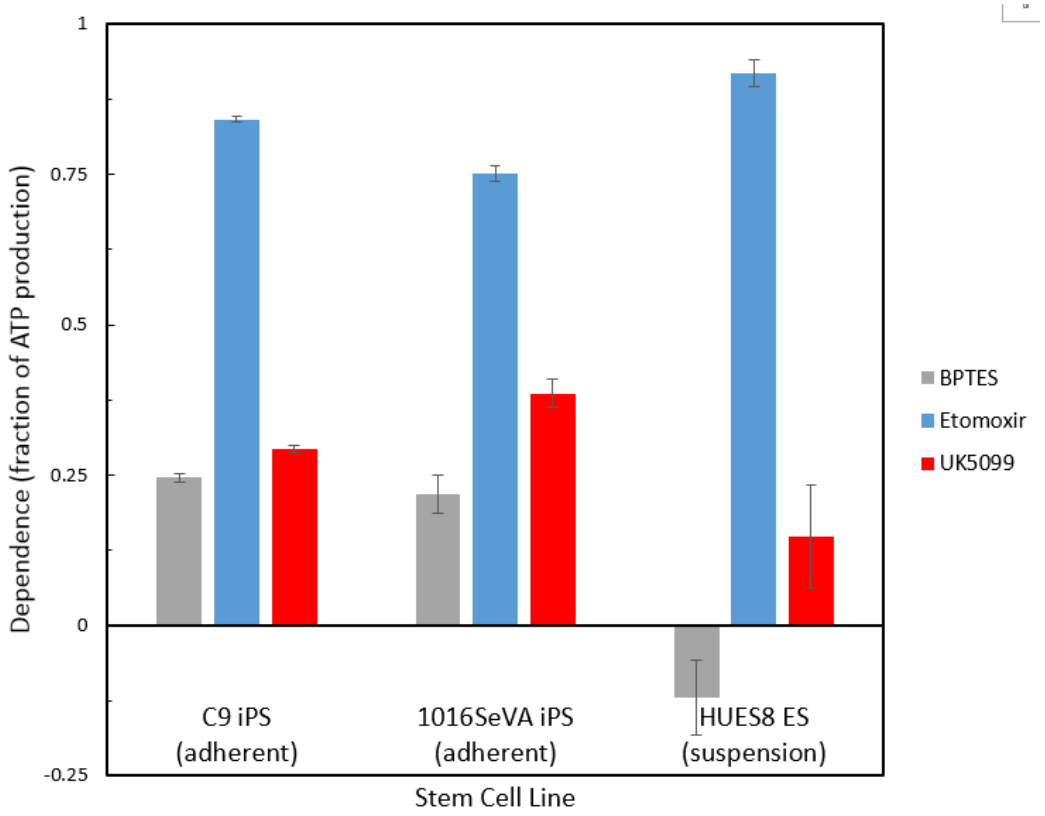

Figure 2.4 FAO reliance is characteristic of stem cells. Bar graph comparison of the dependence of various stem cell lines upon different types of mitochondrial oxidation. Dependence is defined as the loss of OCR following injection of a small molecule.

As a way to observe the effects of the small molecules upon the 1016 SeVA iPSCs, and better characterize the effects of the small molecules, the concentration of NAD^+ was quantified. BPTES saw a limited effect upon NAD^+ concentration, while both etomoxir and UK5099 appeared to cause a mild increase in NAD^+ concentration after 20 minutes of exposure. This exposure time was chosen because it mimics the Seahorse timing, and a drop in oxygen consumption due to the small molecules occurs far before 20 minutes have passed. Hypothetically, as the TCA cycle slows less NAD^+ consumption would occur, so it is plausible that there is an increased concentration of NAD^+ following application of these small molecules.

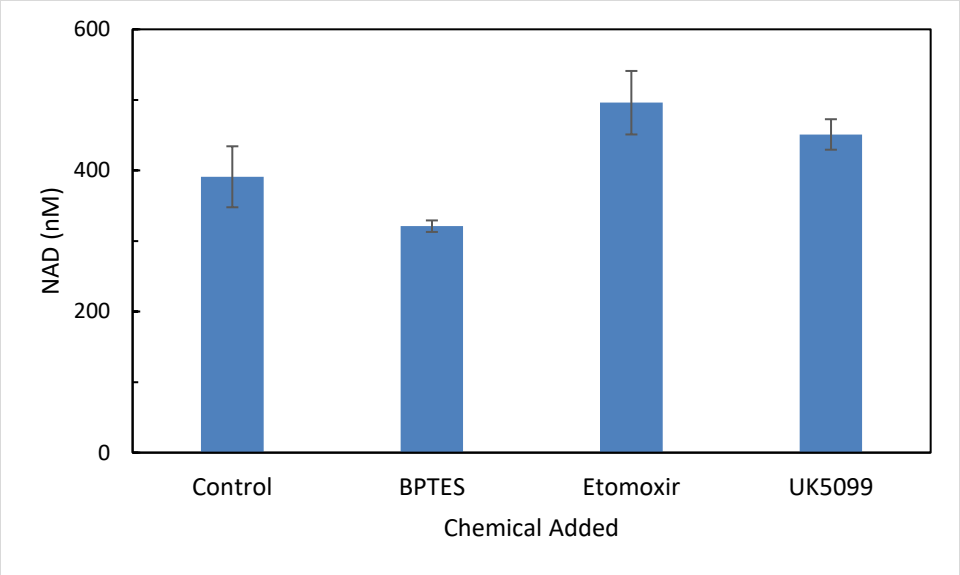

Figure 2.5 Application of small molecules impacts NAD concentration minimally. Bar graph comparison of the cellular NAD concentrations following application of the small molecules for twenty minutes of exposure.

2.4.2 Other Cell Types Do Not Rely Heavily on FAO Like Stem Cells

Having determined that stem cell lines are reliant on FAO, it is important to know if this is a property inherent to stem cells, or if it is seen in other cell types. We chose to study two cell types that are involved in the process of taking a patient sample to a β cell: 1016 fibroblasts and stage 1 definitive endoderm (DE) cells, derived from iPSCs. The relationship between these cell types can be seen in Figure 2.6.

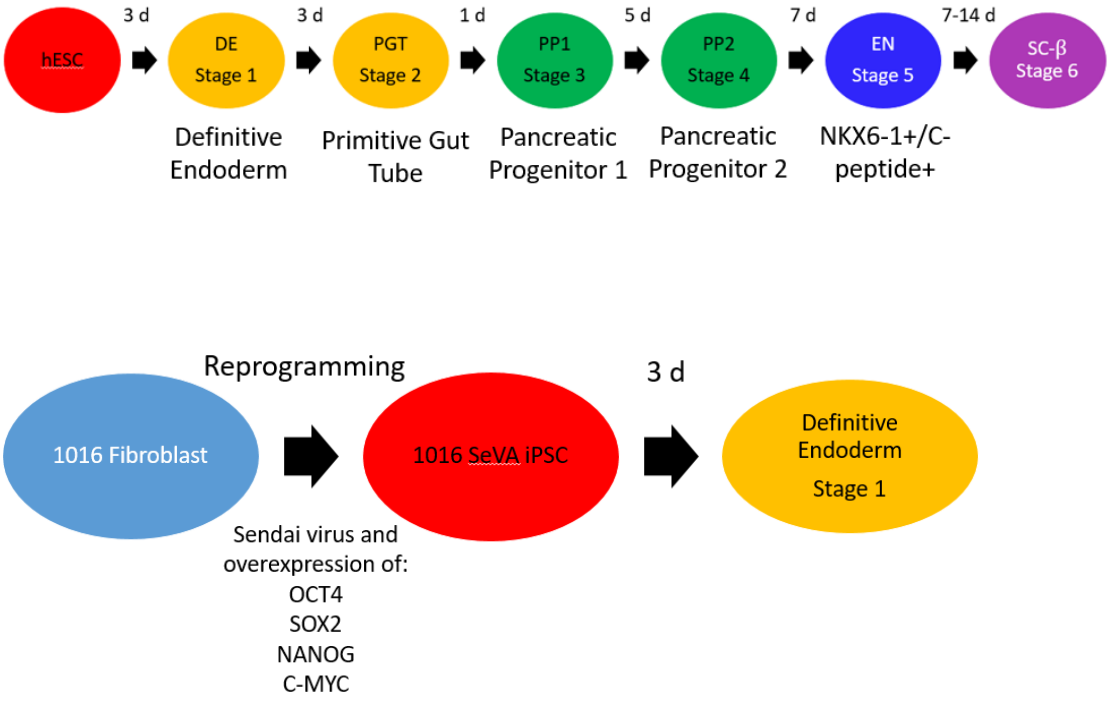

Figure 2.6 The protocol of stem cell-derived β-cell differentiation. The general outline of a SC-β cell differentiation from a human embryonic stem cell, which will be elaborated on in Chapter 3 (upper). The early portion of this process, using an iPSC rather than an hESC, is also shown and will be implemented in this chapter (lower).

Though all of the stem cell lines are heavily dependent on FAO, this trait is not shared by 1016 fibroblasts, which 1016 SeVA iPS cells were reprogrammed from. The FAO dependence of the 1016 fibroblasts is significantly reduced, according to a students' t-test with p=0.05 (Figure 2.7). Thus, reprogramming alters the reliance on specific mitochondrial oxidation fuels. To study if dependence also shifts during differentiation, 1016SeVA cells were cultured and differentiated into stage 1 DE using the protocol outlined in Figure 2.6, which is part of a longer protocol to generate β cells from stem cells (Figure 4.1). Stage 1 DE cells had significantly altered dependence upon glutamine oxidation as determined by a students' t-test, with p=0.05, differing from the 1016 SeVA cells even after only one stage of differentiation (Figure 2.7). Thus, the dependence of stem cells upon FAO is not a trait shared by all cell types, and may be a characteristic of stem cells; this dependence also shows a shift even in early stages of endodermal differentiation.

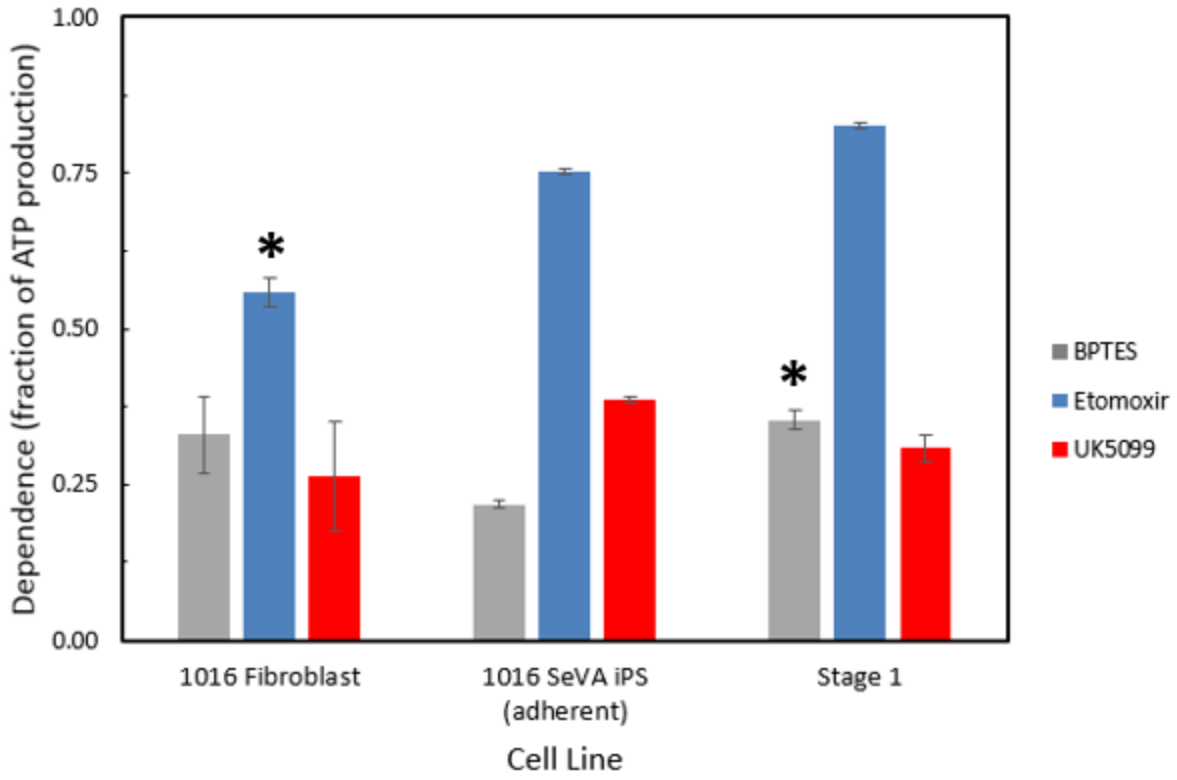

Figure 2.7 Reliance on fuel sources alters throughout early endodermal differentiation. Bar graph comparison of the dependence of various cell lines upon different types of mitochondrial oxidation. Dependence is defined as the loss of OCR following injection of a small molecule.

In order to provide a comparison of iPSC mitochondrial behavior to another mature cell type, we used a Seahorse on INS-1 cells, a rat insulinoma cell line. This cell line was also chosen because, as a β cell line, it provides insight into the appropriate mitochondrial behavior of a β cell. However, it is cancerous as well as non-human, and cancer cells tend to possess higher rates of glycolysis and lower rates of OXPHOS (Ganapathy-Kanniappan et al. 2013), so it may not be a representation of a healthy human β cell.

INS-1 cells have a unique response to injection in a Seahorse run, likely due to glucose response upon injections, and thus cannot have a calculated dependence akin to other cell lines within this body of

research. However, their response to the small molecules can be quantified and compared (Figure 2.8), and UK5099 injection causes the greatest loss in OCR. This indicates that INS-1 cells primarily implement pyruvate oxidation, rather than FAO. This agrees with literature (Patterson et al. 2014).

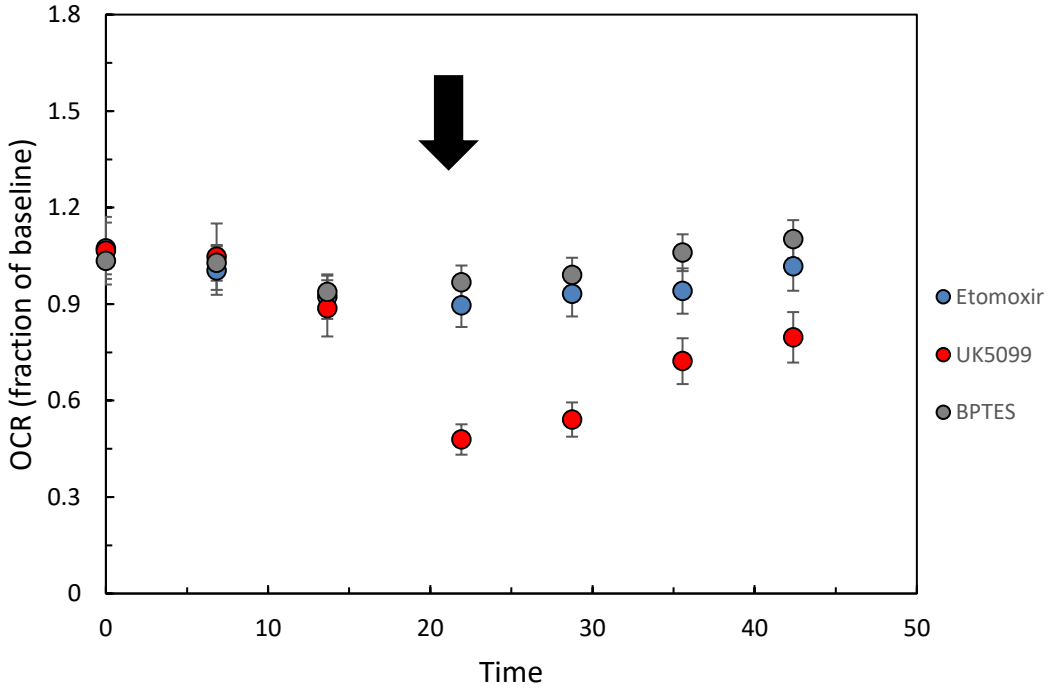

Figure 2.8 INS-1 cells rely upon pyruvate oxidation. INS-1 cells within within 2mM glucose media were injected with BPTES, etomoxir, or UK5099, along with glucose, resulting in a 20mM final concentration of glucose at the black arrow. As INS-1 cells have a glucose response, they cannot be analyzed similarly to the iPS cells, but relative response to etomoxir or UK5099 may be observed.

Thus, two cell types, INS-1 and fibroblasts, were observed to have reduced dependence on FAO in comparison to stem cells, and there is an evident change in mitochondrial behavior besides a mere increase in OXPHOS and reduction in glycolysis. Additionally, as a stage 1 DE cell also possessed a different dependence profile, this research indicates that dependencies alter as a population differentiates, and that dependency may be related to cell maturity.

Having established the mitochondrial metabolic behavior of several cell types related to SC-β cell differentiation, we decided to run a Seahorse with small molecules and a functionality test on SC-β

cells derived using the protocol listed in Figure 2.6. The only significant change in reliance (established with a student's t-test with p=0.05) on the different fuel types was a decrease in pyruvate oxidation in the SC-β cells. As we have not analyzed human islets, it is difficult to know if this replicates human β cell behavior, though it is definitely a contrast from INS-1 cells, as it has a low reliance on pyruvate oxidation.

This could be the true mitochondrial behavior of human β-cells, but it is also possible that the SC-β cells are instead behaving metabolically in an immature way. In that situation, this Seahorse data has provided a target for further research on how to improve SC-β cell differentiation, implying that increasing reliance on pyruvate oxidation at baseline could improve functionality. Additionally, this interpretation would indicate that running a Seahorse mitochondrial functionality test with small molecules could also serve as a method to assess if a SC-β cell has matured.

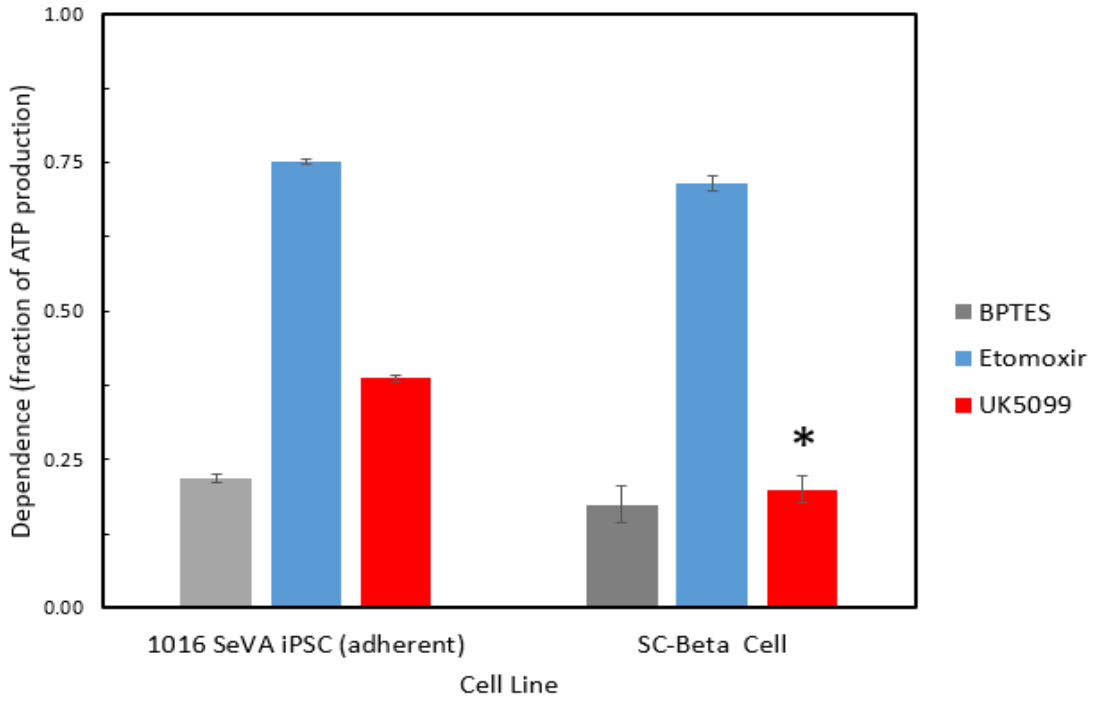

Figure 2.9 Stem-cell derived β-cells lower reliance on pyruvate oxidation in comparison to iPSCs. Bar graph comparison of the dependence of SC-β cells and 1016 SeVA iPSCs upon different types of mitochondrial oxidation. Dependence is defined as the loss of OCR following injection of a small molecule.

2.4.3 Analyzing the Mitochondrial Capacity of Stem Cells

Another approach to analyzing the effect of the small molecules upon the mitochondria is quantifying the change in maximal respiration, the definition of which can be seen in Figure 2.2, quantifying maximal respiration as a fraction of the baseline OCR. Maximal respiration can indicate the capacity for cells to utilize certain fuel sources, which is separate from the actual usage of these fuels at baseline (dependence). The experimental method is identical to that implemented in Figure 2.3. To allow for comparison, maximal respiration is represented as a fraction of the baseline OCR (Figure 2.10). 1016 SeVA iPS cells showed the greatest loss in maximal respiration when presented with etomoxir, demonstrating the cells' capacity for FAO above other types of oxidation, a trend similar to the dependence of stem cells on FAO. Again, 1016 fibroblasts and stage 1 DE cells do not demonstrate similar capacities for the three different fuel sources, with stage 1 cells exhibiting greater capacity for pyruvate oxidation at maximal respiration.

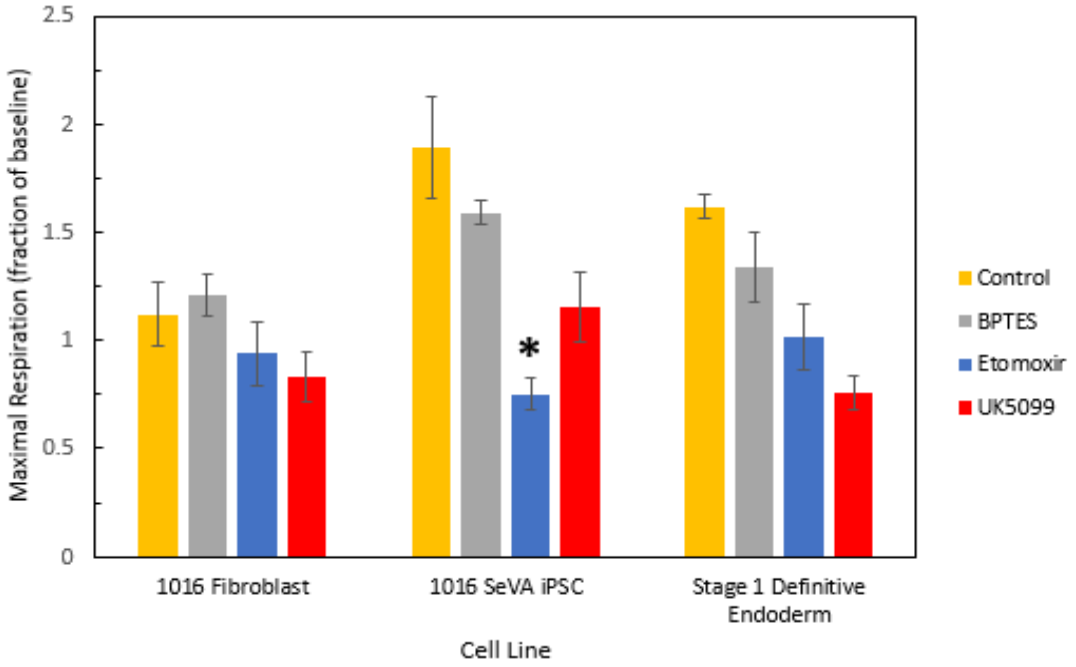

Figure 2.10 1016SeVA iPSCs have a greater capacity for FAO than other fuel sources. Maximal respiration was calculated as a fraction of the baseline OCR, with a value of one being equivalent to baseline, and compared between three cell lines at varying stages of maturity. Control here represents an injection of DMSO and the resultant maximal respiration. A statistically significant difference exists between the maximal respiration with etomoxir and with BPTES or the control, DMSO.

It is also significant to note that cells with different identities use different amounts of their maximal capacity while sitting at baseline. Fibroblasts at baseline operate close to their maximal capacity for respiration, demonstrated in Figure 2.10 with the control's maximal respiration as a fraction of baseline being close to one. Meanwhile, 1016 SeVA iPS cells have much unused capacity for respiration, with the control's maximal respiration being approximately two times the baseline. Inducing a cell to tap into this unrealized respiratory capacity could also be related to differentiation.

2.5 Conclusions

Despite the tendency of a stem cell to rely upon glycolysis for ATP production, mitochondria in stem cells are still active, producing ATP, and appear to undergo dynamic shifts in behavior and fuel usage when differentiating. Using the Seahorse XF24 Extracellular Flux Analyzer, which measures OCR, we were able to quantify mitochondrial properties such as ATP production and maximal respiration. Additionally, we were able to utilize the small molecules BPTES, etomoxir, and UK5099, each of which resulted in a loss of OCR upon their application. Etomoxir and UK5099 inhibit the uptake of fatty acids and pyruvate into the cell, respectively, which inhibits FAO and pyruvate oxidation. BPTES inhibits glutaminase, an enzyme involved in glutamine oxidation. The loss of OCR measured upon each small molecule's application is approximately equal to the OCR of the related fuel's oxidation; we deemed this loss of OCR upon small molecule application to be called "dependence," or the reliance of a cell upon a certain type of mitochondrial fuel oxidation.

1016 SeVA iPSCs were found to rely heavily upon FAO, with it accounting for close to 75% of ATP production, followed by a mild reliance on pyruvate oxidation and glutamine oxidation. To confirm that this applied to all stem cells, and not just one line, this conclusion was confirmed in hESCs and a clone of the 1016 SeVA iPSCs, C9 iPSCs.

As another method of analyzing the relationship of stem cell mitochondria and FAO, maximal respiration was quantified following application of the small molecules. Any decrease in mitochondrial respiration following small molecule injection, as compared to a control, represents the contribution of the related mitochondrial fuel oxidation. We call the maximal mitochondrial respiration that can

come from a certain type of fuel oxidation the "capacity." The 1016 SeVA iPSCs demonstrated the greatest capacity for FAO, larger than their capacity for glutamine or pyruvate oxidation. This, combined with the dependence, indicates that stem cell mitochondria engage in FAO primarily when they're at a resting state, and that if challenged to produce more ATP or process more fatty acids, they would be capable of doing so using FAO.

Though this research cannot confirm exactly why this would be true, we can hypothesize. As glycolysis is the primary source of ATP in stem cells, it can be assumed that a large amount of pyruvate is produced. Some of this pyruvate is being routed to cellular respiration in the mitochondria, as application of the mitochondrial pyruvate transporter inhibitor UK5099 causes a drop in cellular respiration, but is not the most prominent source of ATP production in the cell. FAO in stem cell mitochondria could exist to create acetyl-CoA, which can be used as a building block in lipogenesis. Lipogenesis is a cytosolic process which synthesizes fats, its first step being acetyl-CoA carboxylation. As both pyruvate and fatty acids are converted to acetyl-CoA within the mitochondria, both can impact lipogenesis (Kersten 2001). A stem cell could break down fatty acids with FAO to generate different fats that it requires, and this has been shown to occur at a high rate in hPSCs and to be important for stem cell renewal and pluripotency (Zhang et al. 2016). Alternatively, the mitochondria could simply favor FAO over pyruvate oxidation for energy synthesis, so that pyruvate could be routed into fermentation and fuel further glycolysis with low oxygen consumption. The heavy dependence on and capacity for FAO in a stem cell could occur due to many factors, and further studies might reveal the precise reason for this mitochondrial behavior.

Regardless of why stem cells possess a dependence on and capacity for FAO above other types of oxidation, this is a trait not shared to the same degree by other cell types analyzed with Seahorse. To prepare for characterization of endodermal differentiation to an SC-β cell, we chose to study cell types within this differentiation, focusing upon cells at the beginning of the process, when fibroblasts are reprogrammed to stem cells and those stem cells are committed to an endodermal differentiation (DE, stage 1 cells).

In fibroblasts, though FAO still accounts for the majority of ATP production, it is significantly lower than in iPSCs. In Stage 1 DE cells, FAO accounts for a statistically similar amount of ATP production, but also decreases its dependence on glutamine oxidation.

To compare stem cells to an actual β-cell line, we conducted a Seahorse run on INS-1 cells, a rat insulinoma line. Unfortunately, these cells responded aberrantly to the mitochondrial functionality test we had conducted on other cell types, and to the injection of small molecules, increasing in OCR upon injection of many of the chemicals. We concluded that this behavior was due to a glucose response of the cells, which resulted in an increase in OCR. Thus, analysis of the INS-1 line was limited to injection of small molecules at higher concentrations, which could cancel out the glucose response and demonstrate dependencies upon specific fuel sources. We found that application of UK5099 resulted in the largest loss of OCR, indicating that a mature β-cell line relied on pyruvate oxidation over other types. Of course, the INS-1 line is inhuman and cancerous, which could affect its mitochondrial behavior and make it a flawed representation of a true human β, but overall its behavior fits with the narrative that is developing for the behavior of stem cells as opposed to their differentiated counterparts.

Overall, the quantification of other cell types indicates that high reliance on and capacity for FAO, and low reliance on and low capacity for other types of oxidation, is characteristic of a stem cell, as shown by analysis with a Seahorse XF24 Extracellular Flux Analyzer. 1016 SeVA iPSC mitochondrial behavior and high FAO contrasts with the parent cell line they were reprogrammed from (1016 fibroblasts), as well as mature rat insulinoma INS-1 cells and stage 1 DE cells derived from 1016 SeVA iPSCs. Thus, as a cell differentiates, its dependence on and capacity for certain types of oxidation will shift. This property could allow for control of differentiation by manipulating dependence and capacity for certain mitochondrial fuel types. From here on we will call the dependencies upon specific fuel sources, and each cell type's capacity for use of different fuel sources, the oxidative metabolic profile of the cell.

Chapter 3

Manipulation of Mitochondrial Metabolism

3.1 Abstract

As we have shown that the oxidative metabolic profile may be related to cell identity and differentiation, it is vital to know if a researcher could easily influence this profile and thereby possibly influence differentiation. We show that the oxidative metabolic profile of an induced pluripotent stem cell can be influenced by media composition: specifically, the presence of glucose, the use of TeSR E8, or the small molecule etomoxir. The use of TeSR E8 and glucose starvation demonstrated the potential for a researcher to easily manipulate mitochondrial metabolism with media formulation, specifically showing that the dependence of both media alterations changed dependence, with TeSR E8 appearing more reliant on FAO and glucose starvation resulting in a decreased dependence on the three studied forms of fuel oxidation.

Additionally, we demonstrated that etomoxir treated cells are capable of maintaining stem cell resting baseline behavior (dependence on fuel types) while undergoing changes in capacity for mitochondrial fuels, specifically an increase in capacity for pyruvate oxidation. To take advantage of this property and apply small molecules to differentiations, it is important to determine if long-term iPSC culture with small molecules is possible. We cultured iPSCs for multiple passages in the presence of small molecules impacting mitochondrial oxidation without loss of stem cell identity. This indicates that researchers could easily manipulate the oxidative metabolic profile of a stem cell immediately prior to differentiation, opening possibilities for exploring the impact of different fuel sources upon differentiation.

3.2 Materials and Methods

The Seahorse mitochondrial functionality test and small molecule injection were implemented, as detailed in section 2.2.3 of this thesis, for study of cells in modified media. To determine if media formulation had an effect upon mitochondrial behavior, culture was conducted in a variety of media prior to Seahorse quantification; details can be seen in Table 3.1.

Table 3.1 Modified Media Formulations

Media	Description
TeSR E8	Simplified mTeSR1 media from STEMCELL Technologies, claims to use only 8 essential factors for maintaining stem cells
Glucose-starved	In the Seahorse run, the RPMI 1640 media was created without glucose. Cells sat within this media for 90 minutes prior to the Seahorse run.
Etomoxir treated	iPSCs were seeded in a Seahorse microplate for 48 hours, and were exposed to 40 μM etomoxir in mTeSR1 media during the final 24 hours before the media was replaced with RPMI and the Seahorse was run.

For analysis of long-term culture with the small molecules BPTES, etomoxir, and UK5099, 1016 SeVA iPSCs were single-cell dispersed and plated in 16 wells of a 24 well culture plate treated with fibronectin. The iPSCs were fed approximately every twenty-four hours, and after feeding the wells with fresh media, small molecules were added to match the final concentrations used in the Seahorse experiments, with 30 μM BPTES, 40 μM etomoxir, and 4 μM UK5099 at n=4 wells. Control wells with DMSO were also plated and treated similarly. The iPSCs were passaged by single-cell dispersal with TrpLE, and viability and cell count were taken with a ViCELL Cell Counter and Cell Viability Analyzer. During passaging, samples were saved in RNAlater from every well for later qPCR analysis. A pictorial depiction of this experiment is shown in Figure 3.1.

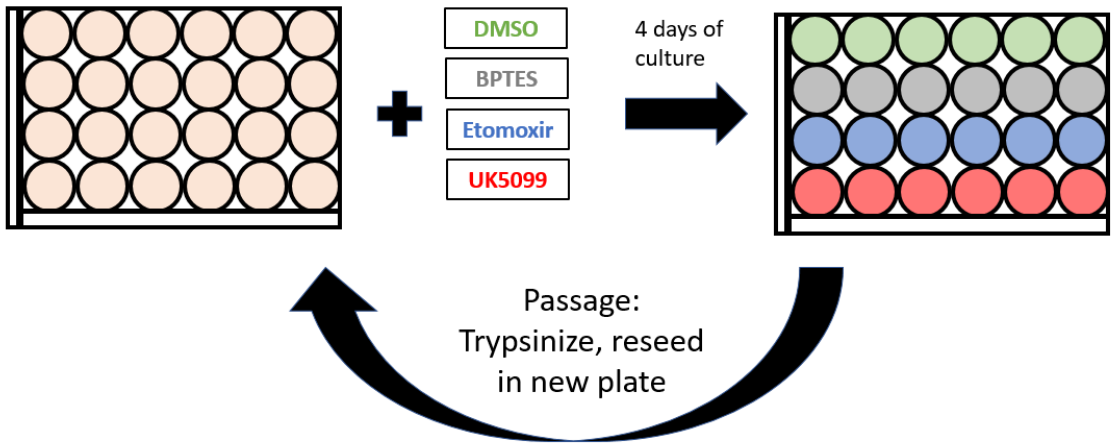

Figure 3.1 Protocol for long-term treatment with small molecules. 1016 SeVA stem cells were raised in mTeSR1 in a 24 well plate, with a small molecule added (DMSO served as control), then passaged and seeded into a new 24 well plate. This circular protocol was repeated up to 5 times.

3.3 Results

To determine if varying media conditions could affect the oxidative metabolic profile of a iPSC, and thus allow a researcher to control the oxidative metabolic profile easily, 1016 SeVA iPSCs were cultured within a variety of media and then analyzed using the Seahorse.

A mitochondrial functionality test upon the Seahorse with small molecule injection was used to analyze 1016 SeVA iPSCs under varying media conditions. Differences in dependence upon all three studied fuel sources were observed; thus, differences in media formulation may impact the mitochondrial dependence profile of a cell line. All iPSCs remained heavily dependent upon FAO, however, which is understandable, as stem cell identity is maintained in TeSR E8 (Chen et al. 2011) and glucose-starvation and etomoxir treatment are transient treatments. Even if glucose starvation and etomoxir treatment could induce differentiation, there would likely not be enough time to change cell identity during this experiment.

3.3.1 Media Formulation Can Alter Mitochondrial Metabolism

1016 SeVA iPSCs raised in TeSR E8 were analyzed with a Seahorse run (Figure 3.2) and shown to have significantly reduced pyruvate dependence, while it had an increased dependency on FAO, according to a student's t-test with p=0.05. TeSR E8 has fewer supplements than mTeSR1, and potentially the cells may increase FAO to support increased turnover of their fatty acid population, allowing generation of fats they do not have easy access to. Another explanation may be that TeSR E8 supports a higher degree of "stemness," promoting higher FAO and lowering other forms of oxidation.

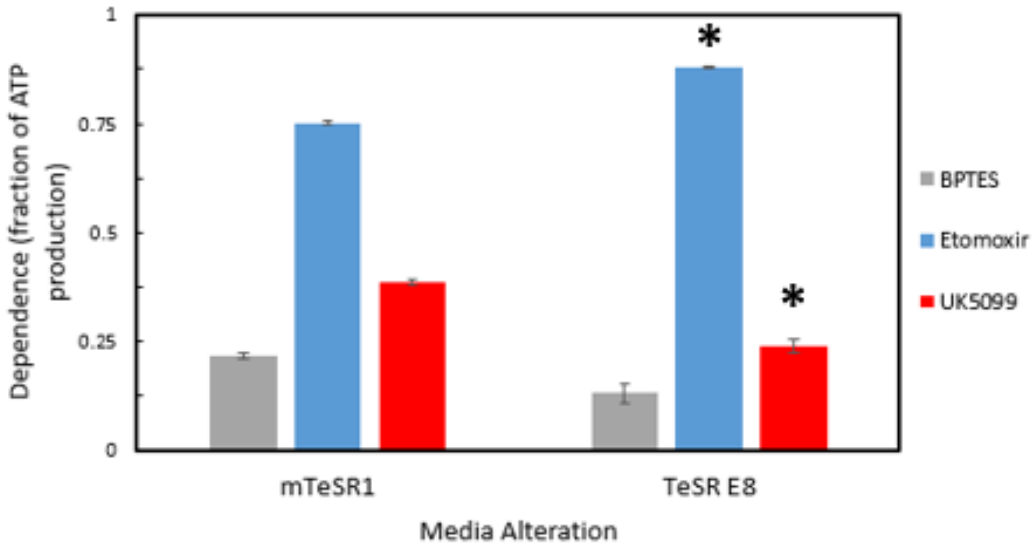

Figure 3.2 TeSR E8 media can influence mitochondrial fuel dependencies. 1016 SeVA stem cells were raised within TeSR E8 media, then analyzed by injecting small molecules prior to a typical Seahorse mitochondrial functionality test to determine dependencies. Statistically significant difference from the mTeSR1 control is indicated by (*).

1016 SeVA iPSCs raised in mTeSR1 were glucose starved for 90 minutes within RPMI 1640 media, with pyruvate added, immediately prior to a Seahorse run. We found their dependence on FAO to be significantly lowered, as calculated by a student's t-test with p=0.05, indicating that other fuels were being oxidized (the mitochondria also oxidizes amino acids besides glutamine, for example). This effect could also be a sign of cell distress or death; as stem cells are reliant upon glycolysis to provide their ATP production, an absence of it could significantly stress iPSCs.

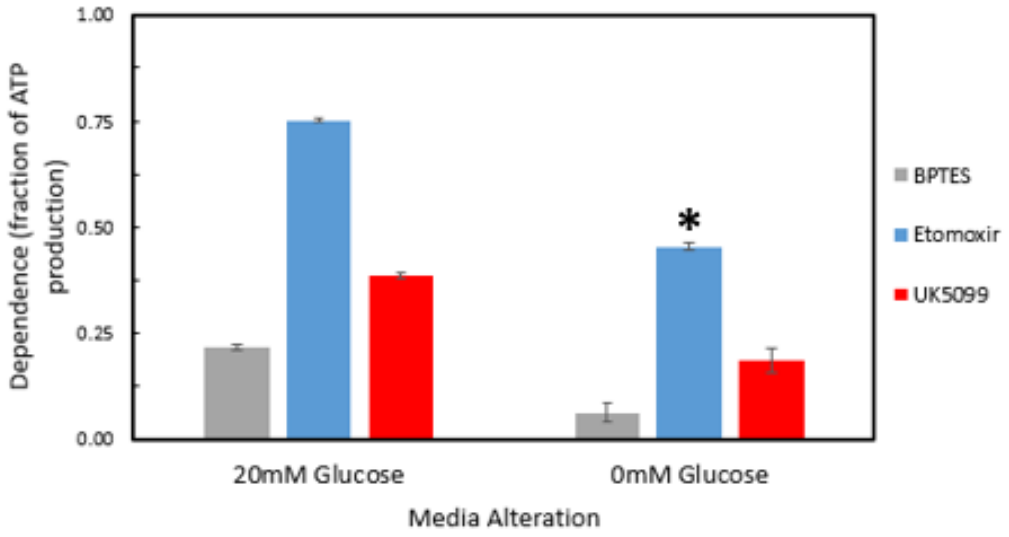

Figure 3.3 Glucose starvation can influence mitochondrial dependence on FAO. 1016 SeVA stem cells were raised within mTeSR1 media, and in the 90 minute period where we applied RPMI 1640 and pyruvate were exposed to a glucose-free environment. Then, the cells were analyzed by injecting small molecules prior to a typical Seahorse mitochondrial functionality test to determine dependencies. Statistically significant difference from the 20mM control is indicated by (*).

1016 SeVA iPSCs raised in mTeSR1 were treated with etomoxir for 24 hours prior to the start of a Seahorse run, which revealed a significant increase in FAO dependency following a brief recovery period (Figure 3.4). This recovery period occurred when etomoxir was removed from the cells for 1.5 hours prior to the start of the Seahorse run. As the dependency on FAO increased following this treatment, it is possible that the iPSCs were attempting to recover, following culture where they had reduced fatty acid transport into the mitochondria.

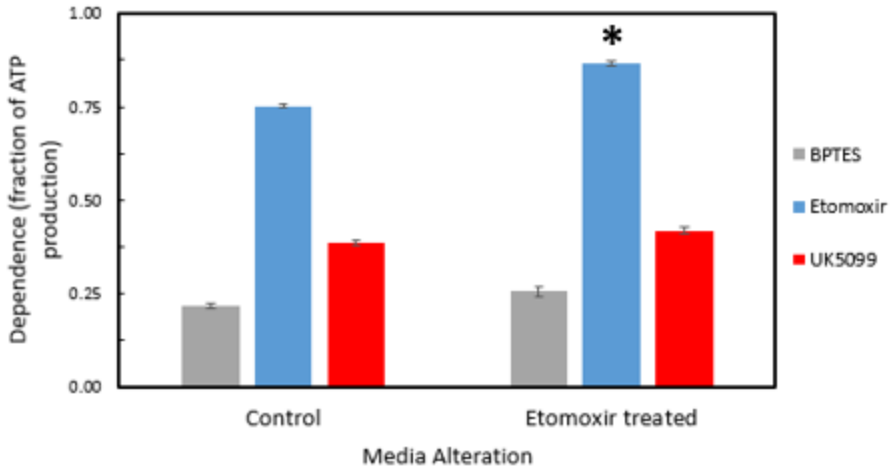

Figure 3.4 Etomoxir treatment increases reliance on FAO after brief recovery period. 1016 SeVA stem cells were raised within mTeSR1 media, and in the 24 hours before the Seahorse run were exposed to etomoxir. Then, the cells were analyzed by injecting small molecules prior to a typical Seahorse mitochondrial functionality test to determine dependencies. Statistically significant difference from the 20mM control is indicated by (*).

As can be seen, etomoxir's effect on the resting behavior of a stem cell seems to alter only FAO. However, maximal respiration can also be analyzed as a fraction of the baseline OCR (Figure 3.5). We found that the impact of UK5099 upon maximal respiration increased significantly following 24 hours of etomoxir treatment, as determined by a student t-test between the two conditions with $p=0.05$. This indicates that although baseline behavior remains unchanged, it is possible to alter stem cell capacity for a specific fuel source. It is possible that this somewhat consistent baseline behavior, coupled with a change in capacity, could indicate that the stem cells are still behaving similarly to stem cells at baseline, with high FAO, but could be 'primed' to behave differently, especially if they were to undergo differentiation.

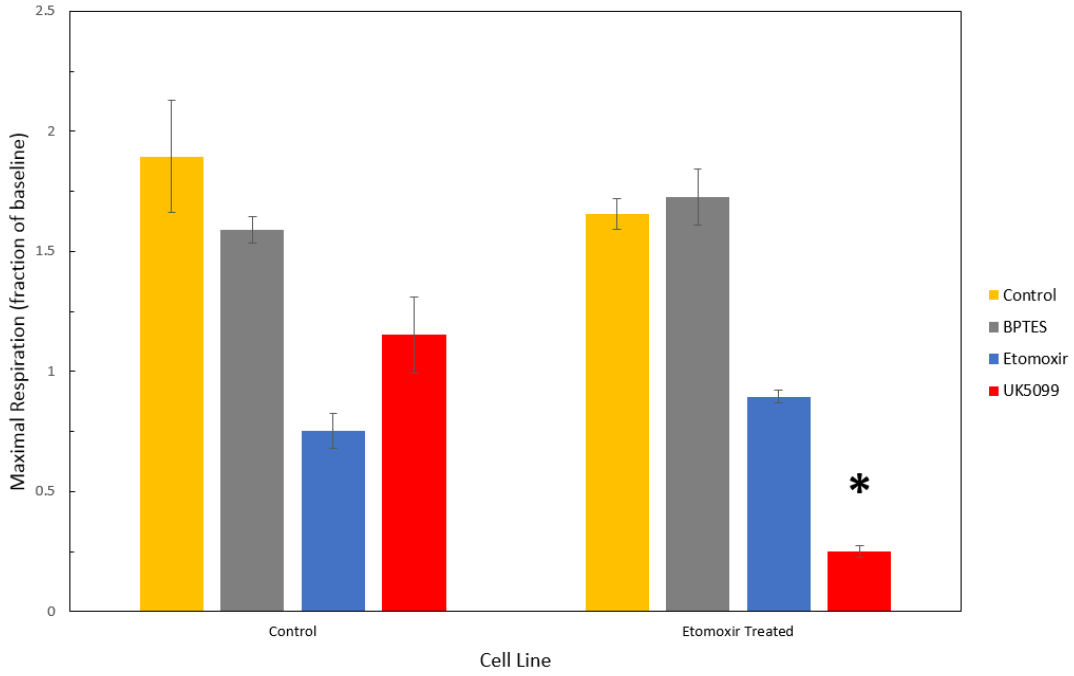

Figure 3.5 Etomoxir treatment increases capacity for pyruvate oxidation. 1016 SeVA stem cells were raised within mTeSR1 media, and in the 24 hours before the Seahorse run were exposed to etomoxir. Then, the cells were analyzed by injecting small molecules prior to a typical Seahorse mitochondrial functionality test to determine capacity.

3.3.2 Long-Term Treatment with Small Molecules Is Plausible

Having established that treatment with small molecules can impact the mitochondrial metabolism of stem cells, it is important to discern if this treatment can be applied to differentiation. Applying small molecules to iPSC culture over a period of days prior to a differentiation, long-term, might allow for alteration of their mitochondrial metabolism, and in extension their differentiation. To ascertain the plausibility of long-term culture with the three small molecules impacting mitochondrial fuel usage—BPTES, etomoxir, and UK5099— 1016 SeVA iPSCs were raised in mTeSR1 with addition of the small molecules. These stem cells were capable of retaining essential stem cell gene expression for up to five passages with the small molecules, as shown through the maintenance of the OCT4 and NANOG genes (Figure 3.6), which are related to stem cell self-renewal and proliferation, respectively. Statistically, there was no significant difference between the expression of these genes between the

conditions. UCP2, which encodes uncoupling protein 2 and serves to separate ATP synthase and the electron transport chain, was also measured and found to be maintained throughout five passages.

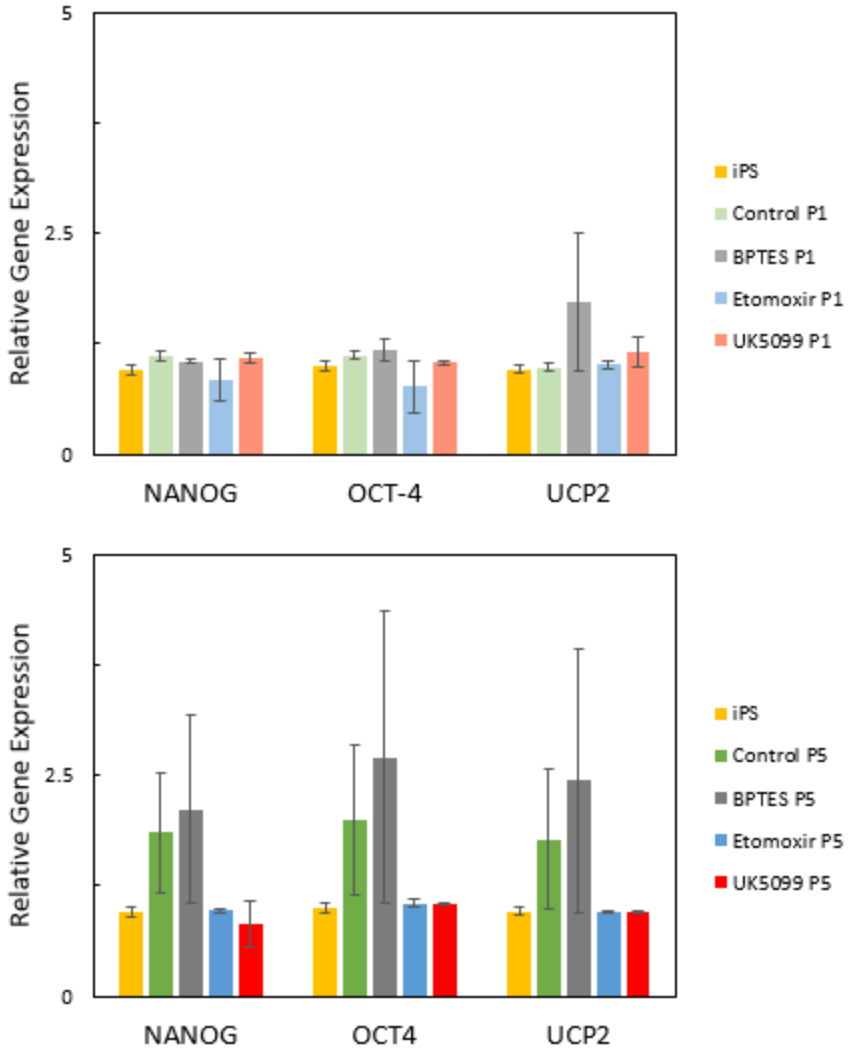

Figure 3.6 Small molecule treated iPSCs retain stem cell relative gene expression. 1016 SeVA iPSCs were maintained within a 24 well plate with 30 μM BPTES, 40 μM etomoxir, or 4 μM UK5099 for up to 5 passages. Samples were collected upon passaging and analyzed with qPCR for relevant stem cell genes.

While expression of important stem cell genes were maintained, morphology was also retained. Small molecule treated cells were capable of reaching confluency (Figure 3.7) with minimal visible differences between their cell morphologies and that of the control wells. Retaining the ability to grow

to confluency and keep growth rate serves not just as an indicator of stemness, but also as an essential component of being able to proceed effectively through differentiation, producing a large enough quantity of treated cells to proceed through the differentiation process and be implanted. Notably, brown debris is visible in the BPTES wells; possibly hydrophobic BPTES falling out of solution.

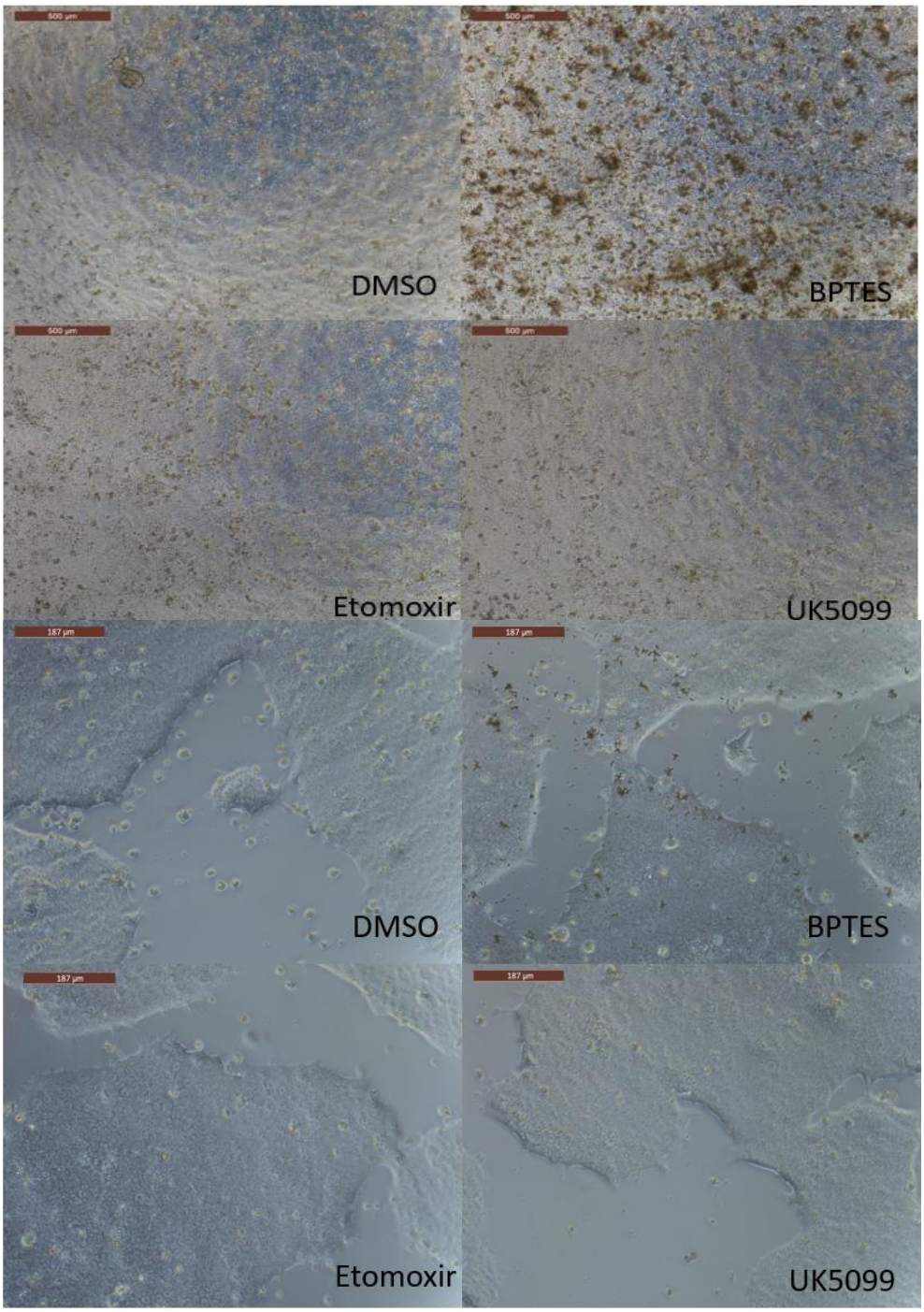

Figure 3.7 Culture with small molecules does not alter cell morphology or confluency. 1016 SeVA iPSCs were maintained within a 24 well plate with 30 μM BPTES, 40 μM etomoxir, or 4 μM UK5099 for up to 5 passages, and pictures at two different magnitudes (5X above, 10X below) were taken, demonstrating no apparent change in morphology or ability to reach confluency.

Despite retaining the ability to reach confluency and maintain cell morphology, a loss of cell viability was observed in later passages, and growth rate was slightly altered (Figure 3.8). Cell death appears to peak during passage 3, and cell count was lowest during passage 2 and 3, but both issues began to resolve as cells approached passage 5. These negative effects would not be enough to prevent long-term use of these small molecules upon an iPSC population. This raises the possibility that iPSCs could be treated with BPTES, etomoxir, or UK5099 prior to a differentiation in the long-term.

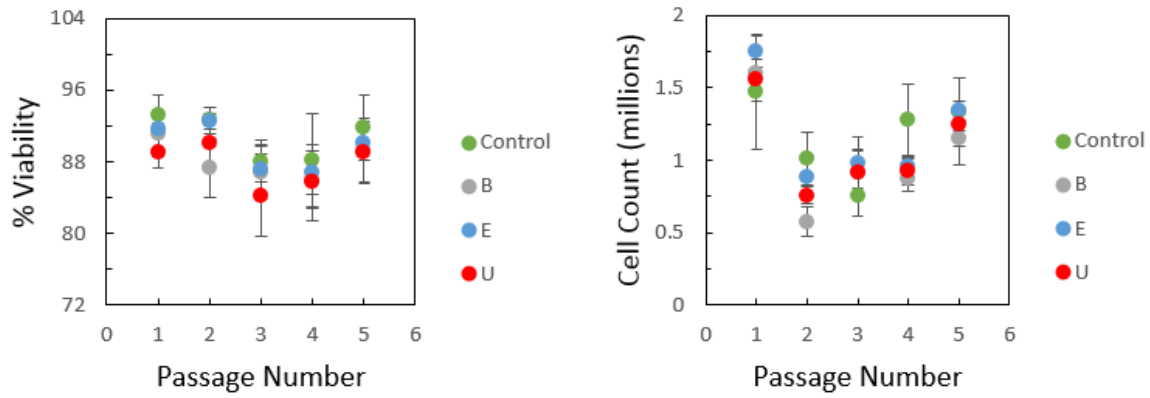

Figure 3.8 iPSCs treated with small molecules retain acceptable viability and growth rate. 1016 stem cells were raised under small molecules impacting mitochondrial function, demonstrating minor loss in viability and growth rate.

3.4 Conclusions

As established in Chapter 1, stem cells start with a reliance on and capacity for FAO and appear to undergo shifts in mitochondrial behavior throughout differentiation, resulting in alteration of dependence and capacity for certain types of mitochondrial oxidation. Control or manipulation of this shift in oxidation could allow a researcher to influence differentiation. We showed that alterations in media formulation were capable of altering dependence upon fuel sources.

Specifically, cells raised in TeSR E8 relied even more heavily upon FAO, indicating a possible increase in stemness or requirement for lipogenesis. Stem cells that were glucose starved demonstrated a decrease in oxidation of FAO, which could be the result of an increase in usage of other fuels or a

result of cell stress. Stem cells cultured with etomoxir demonstrated an increased dependence on FAO after they had 90 minutes to recover, but overall fairly unchanged dependences upon the different types of mitochondrial oxidation. However, there was a drastic increase in capacity for pyruvate oxidation, indicating that while resting cell behavior remained unaltered the mitochondria changed their ability to utilize pyruvate oxidation and FAO. This change in capacity could possibly influence future differentiation of a stem cell while still maintaining the baseline behavior characteristic of a stem cell, allowing a researcher to impact differentiation but not induce premature, uncontrolled differentiation.

Thus, a change in overall media formulation, a deprivation of one of the three studied fuels, and treatment with a small molecule were all possible and altered the mitochondrial metabolic profile of 1016 SeVA iPSCs. This opens up the possibility for future research into media formulations, fuel deprivation, or small molecule treatment. Knowing that manipulation of the oxidative metabolic profile is possible with the studied small molecules, it was imperative to determine if long-term treatment with etomoxir and the other small molecules was possible. Long-term treatment would allow manipulation of the stem cell population in preparation for a differentiation.

To study whether mitochondrial metabolism manipulation with small molecules prior to a differentiation would be possible, we conducted long-term culture with small molecules impacting three types of mitochondrial oxidation. We showed that 1016 SeVA stem cells could be cultured long-term with the small molecules BPTES, etomoxir, and UK5099, which inhibit glutamine, fatty acid, and pyruvate oxidation, respectively. This extended culture did result in lower cell viability and cell count, but not to a degree which made it difficult to culture them, or where there was massive cell death (typically viability remained above 85%). Their morphologies and ability to reach confluency also did not change, in total making it plausible for a researcher to use the small molecules in long-term culture. Ultimately, the treatment did not affect gene expression of stem cell markers from the control, indicating that the iPSCs had retained their pluripotency and self-renewal and were capable of differentiation. This implies that iPSCs can be cultured with small molecules in the long-term, while maintaining their identity as a stem cell, and allowing for possible influence on differentiation.

Thus, this body of work provides a researcher with easy, accessible tools to influence mitochondrial behavior in a stem cell by altering media components, which could potentially be incorporated with differentiation protocols to help optimize them.

Chapter 4

Mitochondrial Metabolism and SC-β Cell Differentiation

4.1 Abstract

While it is known that mitochondrial respiration, network formation, and possibly biomass change as an iPSC differentiates, it is vital to this research to know if mitochondrial uptake and usage of fuels alters as an iPSC differentiates. We discovered that mitochondrial uptake and likely mitochondrial metabolism shifts drastically but in a consistent pattern during endodermal differentiation to an SC-β cell, as tracked by gene expression of relevant mitochondrial oxidation genes. This indicates that alteration of mitochondrial oxidation and oxidation fuel sources could impact differentiation. We also discovered, in agreement with the literature established conclusion that mature cells tend to require more OXPHOS and mitochondrial activity than a stem cell, that relative gene expression of mitochondrial fuel transporters increased within SC-β cells.

To utilize this discovery that mitochondrial uptake of certain fuels alters through differentiation, we applied the small molecules BPTES, etomoxir, and UK5099 to an SC-β cell differentiation during transition to stage 1 DE cells. We conducted several differentiations (both adherent and in suspension) implementing these small molecules during this transition, taking samples at stage 1 and assessing definitive endoderm markers, and stopping the differentiation at stage 4 (pancreatic progenitors) to predict the potential for a full SC-β differentiation (Pagliuca et al. 2014). The stage 1 DE cells showed increases in DE markers with small molecule treatment, indicating improved differentiation. However, in stage 4 cells, the small molecule treatment resulted in no change from the control in relative gene expression at stage 4 for any of the small molecule treatments, indicating that addition of small molecules in those conditions did not improve the differentiation process. However,

this experimentation demonstrated the possibility of adding the small molecules BPTES, etomoxir, and UK5099 without halting or damaging endodermal differentiation in both adherent and suspension culture.

Thus, we've established that mitochondrial genes alter with differentiation and likely influence it, as well as indicating that a researcher could use the studied small molecules and implement them in differentiation to try and optimize the process, a useful tool.

4.2 Materials and Methods

A human embryonic stem cell line (HUES8) underwent endodermal differentiation in suspension culture, designed to generate glucose responsive, insulin producing stem-cell derived β-cells (Pagliuca et al., 2014) in six stages (Figure 4.1). This procedure has been modified since its initial design in 2014 within the Millman lab to increase glucose responsiveness, insulin production, and β-cell markers of the generated cells.

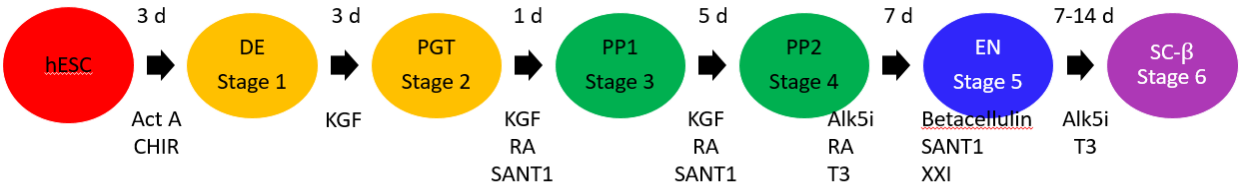

Figure 4.1 Differentiation protocol for a stem-cell derived β-cell. The listed factors and duration of the time steps can read about in detail in Pagliuca et al., 2014.

At every conclusion of a differentiation stage— barring stage 2, primitive gut tube (PGT)—samples were collected and stored in RNAlater. RNA was extracted, cDNA synthesized, and qPCR conducted upon the samples. Three separate differentiations were analyzed. To confirm a successful differentiation, stage 1, stage 4, and stage 6 gene markers were studied (Figure 4.2).

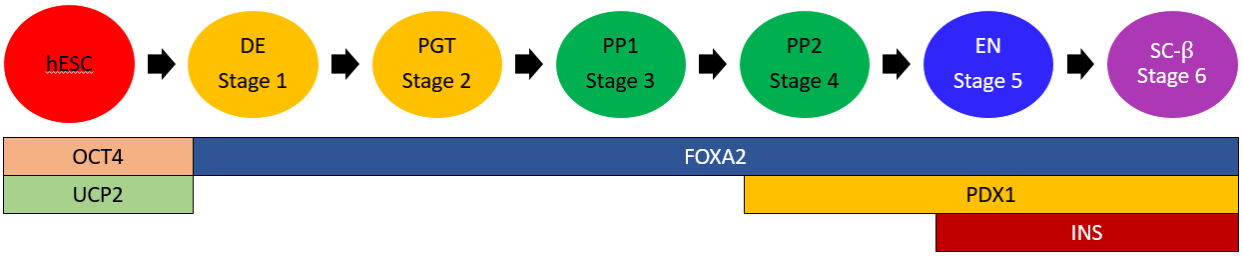

Figure 4.2 Expected appearance of differentiation markers for a stem-cell derived β-cell.

The related proteins and functions of these differentiation markers can be seen in Table 4.1. Two other differentiation markers, which will only be used to assess stage 1, are also shown.

Table 4.1 Differentiation marker gene summary

Gene	Protein and function
OCT4	Involved in embryonic stem cell self-renewal.
FOXA2	Encodes a transcription factor essential for β cell development (Lee 2005).
PDX1	Encodes a transcription factor essential for β cell development
INS	Encodes the hormone insulin, which is produced by β cells
GSC	Encodes a transcription factor, a definitive endoderm marker (McLean et al. 2007).
CER1	Encodes a transcription factor, a definitive endoderm marker (McLean et al. 2007).

Other genes included in the qPCR run were related to transport of fuel into the mitochondria, as detailed in Table 4.2.

Table 4.2 Mitochondrial metabolism gene summary

Gene	Protein and function	Related Oxidation Pathway
GLS	Glutaminase, an enzyme that catalyzes the conversion of glutamine to glutamate	Glutamine oxidation
CPT1A	Carnitine palmitoyltransferase I, transports long-chain fatty acids into the mitochondria	FAO
MPC1	Mitochondrial pyruvate carrier 1, transports pyruvate into the mitochondria	Pyruvate oxidation
MPC2	Mitochondrial pyruvate carrier 2, transports pyruvate into the mitochondria	Pyruvate oxidation

To test the potential for use of the small molecules during differentiation, an endodermal differentiation to SC-β cells was conducted with small molecules applied during the first phase of treatment to reach DE, or stage 1. This differentiation was conducted within 6 well plates with both adherent and suspension ESCs. The small molecules were not applied at any other time during the differentiation other than prior to reaching stage 1. The differentiation was halted once the cell population reached stage 4, and samples were collected and stored in RNAlater. RNA was extracted, cDNA synthesized, and qPCR was conducted to test for the endodermal stage 4 markers, PDX-1 and NKX6-1.

4.3 Results

4.3.1 Relative Gene Expression of Mitochondrial Transport Genes Displayed Trends Throughout SC-β Differentiation

In order to characterize the mitochondrial uptake of fuel sources throughout a differentiation, we analyzed RNA samples taken at each stage of an SC-β cell differentiation with qPCR, studying the relative gene expression of mitochondrial transport proteins, genes involved in glutamine oxidation, and genes involved in SC-β cell differentiation.

The RNA samples of each differentiation stage were analyzed by qPCR, studying genes related to transport of pyruvate and fatty acids into the mitochondria, or the gene related to glutaminase, an essential enzyme in glutamine oxidation. The temporal relative gene expression showed trends

throughout differentiation for all genes analyzed, with significant differences from iPS cells and, for many, from Stage 4 pancreatic progenitor cells, showing fluctuations (Figure 4.3). All significances were determined by a student's t-test with a $p=0.05$. The mitochondrial transporter genes GLS, MPC1, MPC2, and CPT1A all showed a significant increase in expression from iPSCs, indicating a likelihood that mitochondrial fuel uptake increases in SC-β cells. This indicates that a variation in expression of these transport proteins or enzymes, and possibly a change in uptake of their respective fuel sources, is likely related to successful differentiation. UCP2, which has previously been implicated in stunting human embryonic stem cell mitochondrial oxidation (Zhang et al. 2011) and decreasing upon differentiation, stayed relatively constant, increasing significantly in the final stages of differentiation.

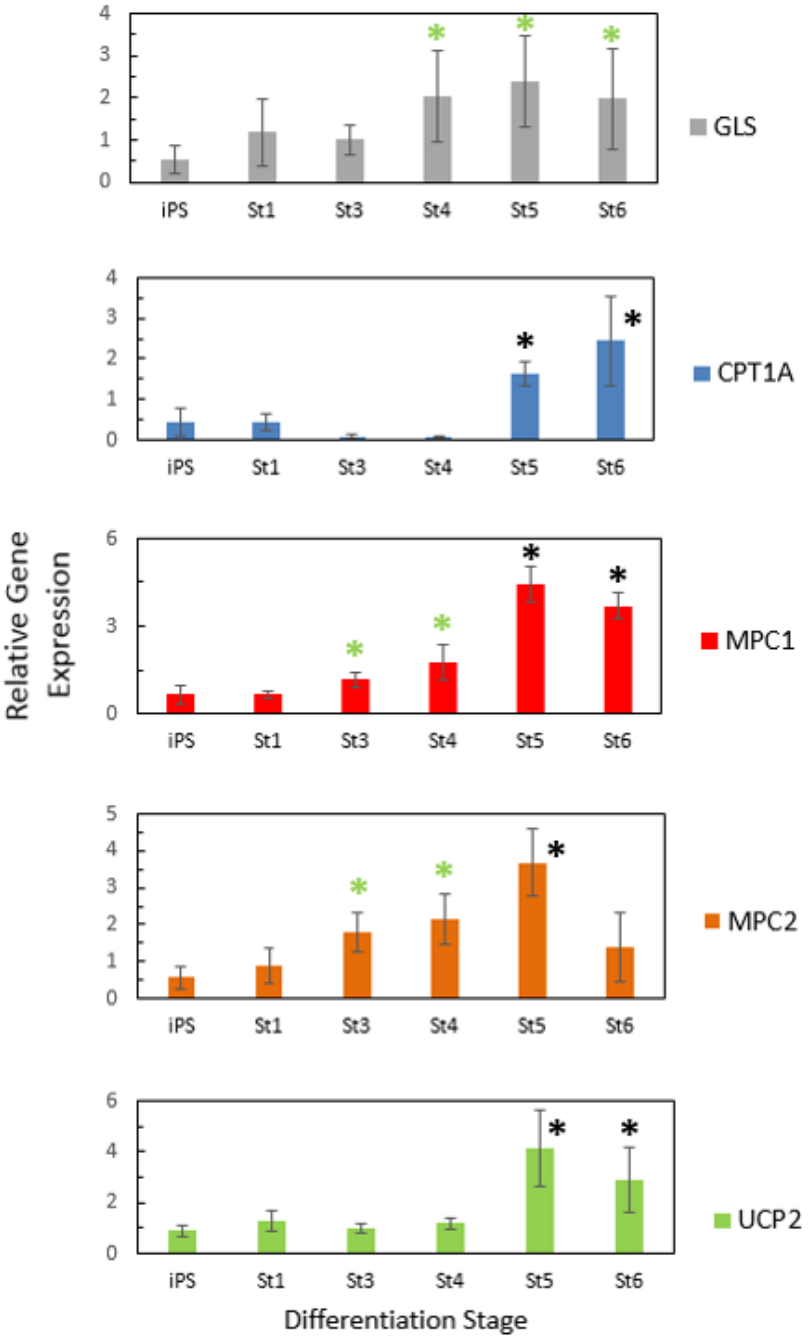

Figure 4.3 Mitochondrial gene expression correlates to differentiation stage. Average gene expression throughout several endodermal differentiations to an SC-β cell, with n=3 or 4. Those marked with a black (*) represent relative gene expression significantly different from iPS cells and from stage 4 cells, while those marked with a green (*) are significantly different from stage 4 cells.

To confirm that these findings were made with successful differentiations, several differentiation marker genes were analyzed using qPCR (Figure 4.4). These markers showed gene expression correlating to the expected gene expression shown in Figure 4.2. OCT4, a stem cell marker, expressed only prior to stage 1. FOXA2, which is intended to express initially in stage 1 of an endodermal differentiation, showed high expression in stage 1 and onwards. PDX1, which is known to begin expressing in stage 3 or 4, appeared as expected in the differentiation. Finally, INS was generated in all of the experiments, expressing in stage 5, a strong indicator of whether an SC-β cell has been generated. Thus, the differentiations analyzed for mitochondrial transport genes were valid differentiations that resulted in pancreatic progenitor cells, expressing appropriate pancreatic lineage genes.

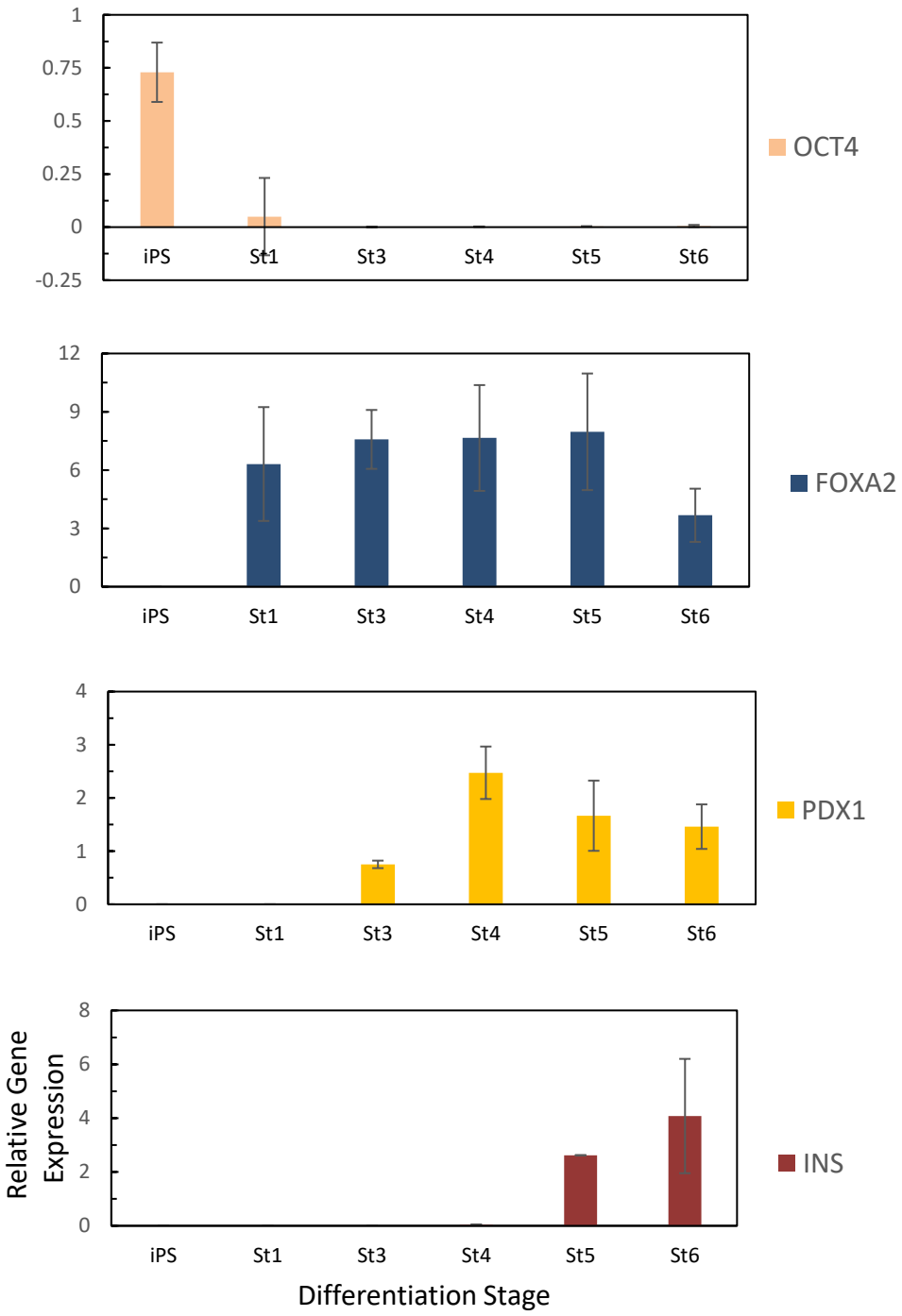

Figure 4.4 Differentiations successfully generated pancreatic progenitors, with expected temporal gene expression. Gene expression analysis to confirm a successful endodermal differentiation, showing high relative gene expression of appropriate markers.

4.3.2 Small Molecule Treated Cells Demonstrate Potential Improvement of SC-β Cell Differentiation

Having characterized relative gene expression of mitochondrial oxidation related genes, it was important to attempt to apply the results to an actual differentiation and manipulate it. The small molecules BPTES, etomoxir, and UK5099 were applied during the transition to Stage 1 DE cells, but otherwise differentiation was conducted with the same SC-β cell protocol as used in 4.3.1.

An endodermal differentiation to SC-β cells was conducted in both suspension and adherent cell populations, and samples were taken at stage 1 (DE) for qPCR analysis to determine how stage 1 marker gene expression was affected (Figure 4.5). Expression of certain genes involved in embryonic development significantly increased in small molecule treated cells, indicating possible improvement of SC-β cell differentiation.

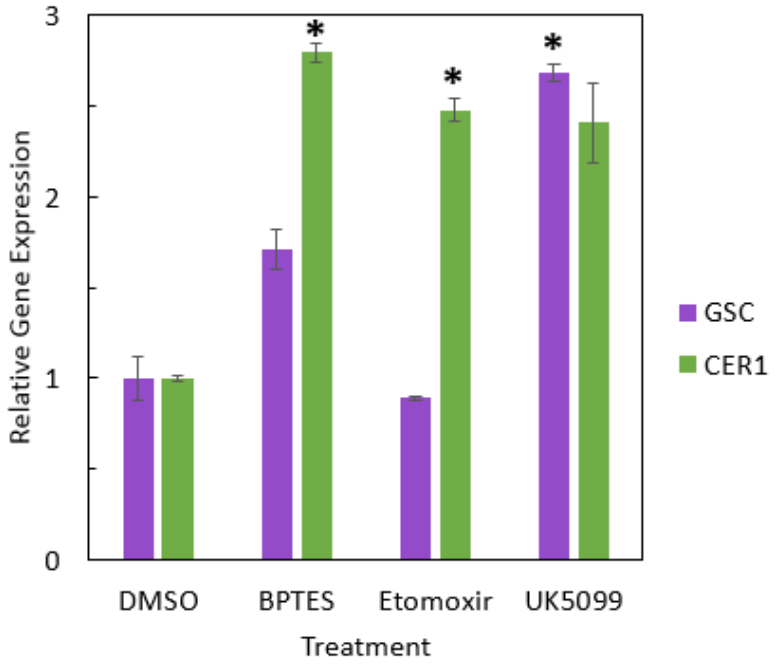

Figure 4.5 Small molecule treatment improves expression of embryonic development genes in SC-β differentiation. Gene expression analysis confirmed significantly increased expression of GSC and CER1 in cells having undergone SC-β cell differentiation to stage 1. (*) indicates significant difference from DMSO relative gene expression by a student's t-test.

The same endodermal differentiation to SC-β cells was conducted in both suspension and adherent cell populations, and halted at stage 4 to assess pancreatic progenitor potential to become a stem cell derived β cell; qPCR was used to analyze samples taken from the stage 4 cells to assess for successful differentiation by expression of stage 4 markers. The stage 4 markers were present, and similar to a DMSO control within suspension and some adherent cells, although UK5099 application appeared to cause lowering of the stage 4 markers within the adherent cells. This indicates that the inclusion of small molecules in media during differentiation for both suspension and adherent culture is plausible (Figure 4.6). Notably, BPTES was not included within adherent culture; this was for reducing cost, as early differentiations with BPTES did not show much promise for endodermal differentiation.

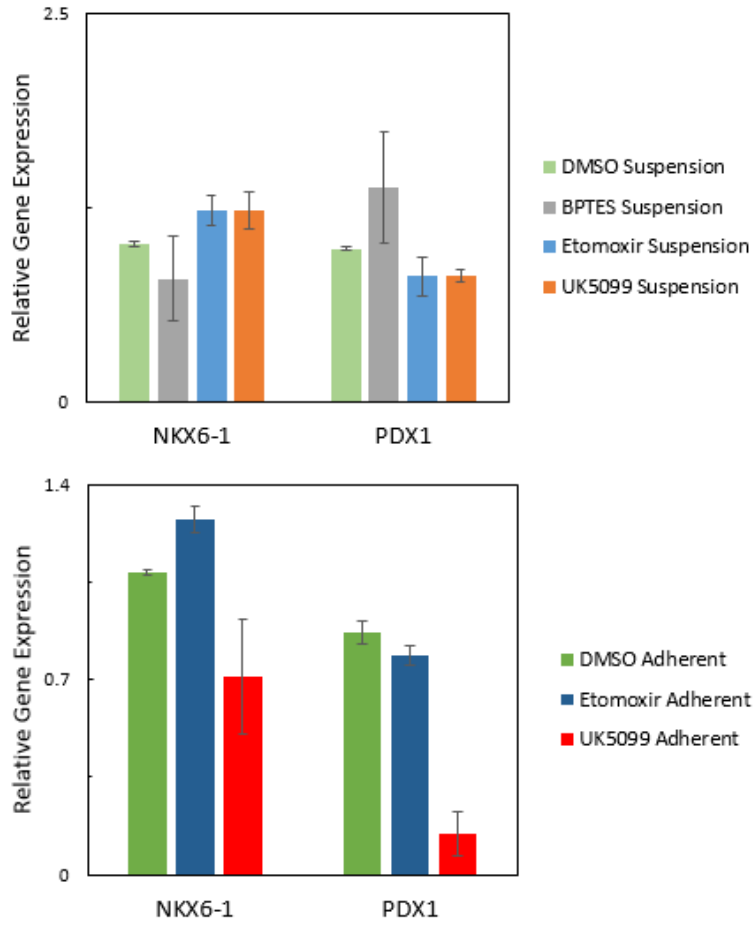

Figure 4.6 Endodermal differentiation is possible in the presence of small molecules. Gene expression analysis confirmed expression of NKX6-1 and PDX1 in cells having undergone SC-β cell differentiation to stage 4.

4.4 Conclusions

The previous chapters demonstrated that the mitochondrial metabolism and reliance of the mitochondria upon different fuel sources altered with cell identity, particularly within an endodermal differentiation, and that this mitochondrial metabolism could be controlled by a researcher using media formulations. Following these discoveries, it was important to characterize and potentially manipulate an endodermal differentiation. As the Millman lab specializes in the differentiation of SC-β cells, this differentiation protocol was chosen (Pagliuca et al., 2014).

Several differentiations were conducted, RNA was collected, and qPCR was used to analyze relative gene expressions. Notably, all differentiations studied and sampled demonstrated appropriate relative gene expression of stem cell markers, pancreatic progenitor markers, and SC-β cell markers, like INS. This serves as confirmation that the differentiations studied were valid SC-β cell differentiations, and their results are representative of the differentiation process.

We showed that relative gene expression of mitochondrial transport proteins related to oxidative phosphorylation followed trends throughout differentiation to a SC-β cell. In particular, the genes CPT1A, MPC1, and MPC2, all encoding mitochondrial fuel transporters, appeared to follow a pattern within differentiation. The relative gene expression of these transporters increased during the final stage of differentiation to an SC-β cell, implying that they might support greater quantities of FAO and pyruvate oxidation by allowing the mitochondria to uptake larger amounts of food molecules. We would expect cells which are more mature and rely on mitochondrial energy production, rather than glycolysis, to utilize pyruvate oxidation and FAO to a greater degree, allowing the relative gene expression results to be reasonable.

Despite transport genes' relative expression increasing during the final stage of differentiation (SC-β cells), their expression throughout the process of differentiation did not show a steady upward trend. Instead, relative gene expression lowered and rose throughout the differentiation protocol. This was not random; the fluctuations were consistent between several different experiments. These results

indicate that uptake of mitochondrial fuel sources could impact differentiation, or are at least closely related to the process.

The gene GLS, which encodes the enzyme glutaminase involved in glutamine oxidation, did not demonstrate any patterns throughout differentiation. It is possible that glutamine oxidation is not as vital to differentiation as FAO or pyruvate oxidation, or that the enzyme's expression alone is not representative of glutamine oxidation. Further research into glutamine oxidation could be important, but this study has revealed little of interest.

Having characterized several genes directly related to the three studied types of mitochondrial oxidation, and having established the importance of mitochondrial fuel uptake for endodermal differentiation to a SC-β cell, we decided to attempt to disrupt mitochondrial fuel uptake within an SC-β cell differentiation by using the small molecules BPTES, etomoxir, and UK5099.

SC-β cell differentiation was conducted in both suspension and planar culture, with the small molecules BPTES, etomoxir, and UK5099 applied during the first step of differentiation to a DE (stage 1) cell. We took samples upon the cells reaching stage 1 (DE) and conducted qPCR, finding that the relative gene expression of embryonic development genes CER1 and GSC were increased significantly in small molecule treated cells in comparison to the DMSO control. This indicates that treatment with small molecules could possibly improve differentiation; this is especially significant because the differentiation of DEs is already effective, and thus any improvement is especially interesting.

We halted the differentiation once the cells reached stage 4, pancreatic progenitor 2 (PP2) cells, as differentiation to stages 5 and 6 can be expensive and result in a more inhomogeneous cell population, which could skew results (Pagliuca et al., 2014). During this stage, it's possible to assess the potential of the cells to become functional SC-β cells by analyzing relative gene expression of PDX-1 and NKX6-1, both of which are endodermal lineage markers that should express during stage 4, PP2. In our differentiations, those treated by small molecules and those treated as a control (DMSO) showed

appropriate gene expression of PDX-1 and NKX6-1, and were assessed to have good potential for further differentiation to a functional SC-β cell.

Though this reserach did not demonstrate a benefit from application of small molecules during transition to stage 1 (definitive endoderm), it did demonstrate that SC-β cell differentiation with small molecules applied was possible, and allowed differentiation. This is vital information to any researcher hoping to continue this work and apply any of the studied small molecules to a differentiation.

As a side note, UCP2 relative gene expression was also studied and characterized within SC-β cell differentiation. Previous work with hPSCs has implicated UCP2 as a metabolism regulator, ensuring glycolysis produces much of the cell's ATP, and indicating that it should reduce in expression upon differentiation (Zhang et al. 2011). These conclusions were contradicted directly by this work, as UCP2 levels were maintained or increased. This could lead to three possible conclusions: the literature findings do not apply to endodermal differentiation to an SC-β cell (this is plausible, as cardiomyocytes and fibroblasts were tested), the assertion that UCP2 is high in stem cells but lowers upon differentiation is false, or the SC-β cells produced in our lab are flawed and have the potential for improvement if UCP2 is altered. While we're not certain which of these conclusions is true, there has been work done with SC-β cell differentiation that implies that lowering UCP2 could lead to better functionality and maturity of an SC-β cell (Pezzolla et al. 2015), which supports our results and serves as a lead for our lab in further optimization of SC-β cell differentiation. Regardless, UCP2 is a mitochondrial membrane protein, which emphasizes the importance of mitochondrial function during differentiation regardless of the type of fuel uptake.

In conclusion, mitochondrial uptake of specific fuels was shown to be related to the process of SC-β cell differentiation, we showed that small molecule treatment could improve differentiation to stage 1 DE cells, and we demonstrated that it was possible to run a differentiation with small molecules that impacted fuel uptake.

4.5 Future Directions and Final Conclusions

Though much literature on stem cell behavior and metabolism has focused upon the relationship between glycolysis and the mitochondria as the source of ATP synthesis, we assert that the mitochondria is still partially active in stem cells, and that its behavior can impact differentiation. Though the high rate of glycolysis generates much pyruvate, we found that stem cell mitochondria mainly processes fatty acids. Stem cells were found to rely heavily on mitochondrial FAO, and to have a great capacity for it, a trait that differed from other cell types and from cells that have committed to a lineage. Though we could not confirm the underlying reason for this trait, we can reasonably hypothesize that the oxidative mitochondrial metabolism favoring FAO over pyruvate oxidation could be the result of pyruvate undergoing fermentation to fuel further glycolysis rather than undergoing OXPHOS, or hypothesize that stem cells require extensive lipogenesis for self-renewal (Zhang et al. 2016) and pluripotency and thus conduct FAO for its support.

Having established that oxidative mitochondrial metabolism alters with differentiation, we moved to providing a toolset for a future researcher to use in study of this phenomenon. This work has set the foundation for future research into manipulation of differentiation, particularly SC-β cell endodermal differentiation, through manipulation of the oxidative metabolic profile, which we have defined as reliance upon and capacity for certain types of oxidation. Alteration of media composition, availability of food molecules like glucose, and application of small molecules were all shown to be simple, easily accessible, and effective methods of altering the oxidative metabolic profile.

From establishing the possibility of easy manipulation of oxidative metabolic profile, we moved on to quantifying and influencing actual SC-β cell differentiation. We determined that, in effective differentiations, the relative gene expression of specific mitochondrial transporter genes followed patterns throughout the endodermal differentiation process. This promoted our idea that pyruvate oxidation and FAO are heavily correlated with the process of differentiation. Unfortunately, our preliminary attempts to manipulate the oxidative metabolic profile with small molecules did not result in improvement of differentiation, as measured by relative gene expression of PDX-1 and NKX6-1 within pancreatic progenitor stage 4 cells. These attempts did prove the possibility of a successful

differentiation even with small molecule treatment within both adherent and suspension culture systems.

While literature has established that mitochondrial behavior alters with differentiation and could possibly control it, a characterizable change in mitochondrial behavior aside from morphology, general activity levels, and biomass (which is debatable) has not been presented. We believe this research supports the idea that mitochondrial uptake and oxidation of specific fuel types serves as the mechanism of mitochondrial change with differentiation.

If the work conducted here could serve as a basis for further research on SC-β cell differentiation, and result in an improvement or optimization of the process, that would fulfill the goal of this research. This would presumably be accomplished by manipulation of cell dependence and capacity throughout the differentiation process, encouraging the mitochondrial behavior to alter to that of a mature cell type and thus encouraging successful, efficient differentiation. Ideally, in continuation of the study of SC-β cell endodermal differentiation, cadaveric islets would undergo a Seahorse test. As it is, cadaveric β-cells are expensive, and as the glucose response of the INS-1 insulinoma cell line has prevented proper study of it, our group hesitated to invest the resources in a cadaveric β-cell Seahorse run at this time. However, further study of differentiation and the relative gene expression of various genes related to mitochondrial metabolism could be significant, and creating a relative expression profile for many mitochondrial genes across a differentiation would be beneficial.

Future research on this topic would investigate oxidative metabolic profile and gene expression throughout differentiation to other germ layers, and to other cell types, to determine the importance of mitochondrial OXPHOS and fuel sources in other differentiation protocols. This would establish the importance of mitochondrial metabolism and oxidation in the stem cell and its differentiation to any somatic cell.

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