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Alpha-Ketoglutarate Influences the Self-Renewal and Differentiation of Pluripotent Stem Cells

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Los Angeles

Alpha-Ketoglutarate Influences the
Self-Renewal and Differentiation of Pluripotent Stem Cells

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy
In Molecular Biology

by

Tara Ann TeSlaa

2017
ABSTRACT OF THE DISSERTATION

Alpha-Ketoglutarate Influences the
Self-Renewal and Differentiation of Pluripotent Stem Cells

by

Tara Ann TeSlaa
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2017
Professor Michael Alan Teitell, Chair

Human pluripotent stem cells (hPSCs) hold great potential for regenerative medicine due to their ability to self-renew indefinitely in *in vitro* culture and to differentiate into all three germ layers. However, the use of hPSCs in the clinic has been limited by the lack of differentiation protocols that produce fully mature and functional cell types. Development of more efficient differentiation strategies therefore will be key to fully realizing the therapeutic potential of hPSCs. Differentiation occurs through epigenetic changes that turn off genes important for self-renewal and activate genes required for cellular maturation and specialization. In addition, cellular metabolism shifts to address varying energetic and biosynthetic demands. Many metabolites, whose levels are influenced by the overall metabolic network, act as cofactors for enzymes that control the epigenetic state of the cell. α-Ketoglutarate (αKG), a TCA cycle metabolite, acts as a cofactor for αKG-dependent dioxygenases which included the JmjC-domain containing family of histone demethylases (JHDMs) and the Ten-eleven translocation (TET) methylcytosine oxidases. Succinate, another TCA cycle metabolite, acts as an inhibitor of the same αKG-dependent dioxygenases. Therefore, changes in the αKG-to-succinate ratio caused by changes in cellular metabolism impact the activity of αKG-dependent dioxygenases and therefore gene expression. Both JHDMs and TETs have known roles in
pluripotent stem cell self-renewal and differentiation. Recently, αKG has been reported to support self-renewal in mouse embryonic stem cells (mESCs). However, mESCs differ from traditionally maintained hPSCs in numerous ways including their stages of pluripotency. Both mESCs and human embryonic stem cells are derived from the inner cell mass, but mESCs are traditionally maintained in an earlier developmental state, called the naïve state, corresponding to the preimplantation embryo. hESCs are traditionally maintained in a more differentiated state, called the primed pluripotent state, corresponding to the post-implantation epiblast. Therefore, because the role of αKG in hPSCs has not previously been investigated, we examined the role of αKG in primed hPSC differentiation. We discovered that αKG can accelerate the differentiation of primed hPSCs likely through its action on αKG-dependent dioxygenases. Because αKG promotes self-renewal in mESCs, we investigated whether αKG promotes differentiation of primed mouse pluripotent stem cells derived from the post-implantation embryo called Epiblast stem cells (EpiSCs). αKG also promoted differentiation in mouse EpiSCs which suggests that the role of αKG is dependent on the stage of pluripotency and is not species dependent. To further confirm the role of αKG in hPSC differentiation, we decreased the αKG-to-succinate ratio by inhibition of αKG producing enzymes or succinate consuming enzymes during hPSC differentiation. Both manipulations led to a delay of hPSC differentiation. Finally, manipulation of the αKG-to-succinate ratio led to changes in DNA hydroxymethylation and histone methylation levels suggesting an epigenetic mechanism. Taken together, my data suggests that αKG plays a context specific, differentiation promoting role in primed pluripotent stem cells.
The dissertation of Tara Ann TeSlaa is approved.

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University of California, Los Angeles

2017
DEDICATION

This dissertation is dedicated to my parents Gaylon and Sara TeSlaa.
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PUBLICATIONS AND PRESENTATIONS


TeSlaa T., Zhang J., Iman S., Nuebel E., Miyata K., Koehler C., and Teitell M.A. The Role of Metabolism on Human Pluripotent Stem Cell Pluripotency and Differentiation. Poster, 9th Annual UCLA Broad Center of Regenerative Medicine and Stem Cell Research Meeting, Stem Cell Pathways to the Clinic, Los Angeles, CA, 2013.


TeSlaa T., Miyata K., Escobar S.L., Nuebl E., Nili M., Koehler C.M., and Teitell M.A. The Role of Mitochondrial Metabolism in Human Pluripotent Stem Cell Self Renewal and Differentiation. Poster, 10th Annual UCLA Broad Center of Regenerative Medicine and Stem Cell Research Meeting, Cancer, Stem Cells, and the Immune Response, Los Angeles, CA, 2014.


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TeSlaa T., Chaikovsky A., Escobar S.L., Graeber T.G., Braas D., and Teitell M.A. α-Ketoglutarate accelerates the initial differentiation of primed human pluripotent stem cells. Poster, Keystone Symposium, Mitochondrial Communication, Taos, NM, 2017.
Chapter 1:

Introduction
Clinical Potential and Current Roadblocks of Human Pluripotent Stem Cells (hPSCs)

Human pluripotent stem cells (hPSCs) encompass both human embryonic stem cells (hESCs) which are derived from the inner cell mass (ICM) of the human blastocyst (Thomson et al., 1998) and human induced pluripotent stem cells (hIPSCs) which are reprogrammed from somatic cells to a pluripotent state through forced expression of a combination of transcription factors required for pluripotency (Boyer et al., 2005; Takahashi et al., 2007). hPSCs self-renew in culture and can differentiate into all three germ layers making them useful for regenerative medicine applications in which cell replacement is a viable therapy (Gearhart, 1998). However, some current differentiation protocols produce cell types that are immature and as a result exhibit only limited functionality (Später et al., 2014). Extended time in culture can induce maturation of hPSC derived cells (Lundy et al., 2013). However, time in culture increases the cost and labor required for production of hPSC-derived cells decreasing the feasibility of their use clinically. Furthermore, protracted time in culture of hPSCs can lead to chromosomal aberrations (Baker et al., 2007; Draper et al., 2004; Laurent et al., 2011; Mayshar et al., 2010).

Variability exists between individual hESC and hIPSC lines resulting in a disparity in the ability of individual lines to differentiate into each of the three germ layers. This problem, termed lineage bias, complicates efforts to improve differentiation protocols (Burrows et al., 2016; Cahan and Daley, 2013; Nazareth et al., 2013; Newman and Cooper, 2010; Osafune et al., 2008). Genetic diversity, epigenetic alterations, or genetic alterations contribute to lineage bias (Burrows et al., 2016; Cahan and Daley, 2013; Kim et al., 2011). In the case of hIPSCs, cell type of origin can also lead to an epigenetic memory that influences lineage bias (Kim et al., 2011). Strategies to overcome lineage bias are also important in enabling the use of hPSCs clinically (Wright et al., 2014). In addition, considerable heterogeneity exists
within single hPSC lines. Both lineage primed and self-renewing subpopulations exist within single hPSC lines (Hough et al., 2014).

**Developmental Stages of Pluripotency in Mouse and Human**

Pluripotent cells can be derived from two stages of development: the inner cell mass (ICM) of the preimplantation blastocyst or from the epiblast after implantation into the uterus (Tesar et al., 2007). In mice, cells derived from the developmentally immature ICM of the embryo, referred to as mouse embryonic stem cells (mESCs), are in maintained in this undifferentiated state that has been termed the naïve pluripotent state. Mouse cells derived from the slightly more mature postimplantation epiblast are termed epiblast stem cells (EpiSCs) and are maintained in this more differentiated state termed the primed pluripotent state (Brons et al., 2007; Martin, 1981; Tesar et al., 2007). Self-renewal of naïve mESCs is promoted *in vitro* by the presence of leukemia inhibitory factor (LIF). Naïve mESCs can also efficiently contribute to chimera production (Smith et al., 1988; Williams et al., 1988). Mouse EpiSCs, in contrast, self-renew in response to basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGFβ), and cannot contribute to chimera formation when transplanted into the early preimplantation gastrula (Greber et al., 2010; Mascetti and Pedersen, 2016).

Traditionally cultured hPSCs, despite being derived from the ICM, share several characteristics with EpiSCs including similar morphologies, metabolism and epigenetics and therefore are considered primed pluripotent stem cells (PSCs) (Nichols and Smith, 2009). While primed PSCs cannot produce a chimeric mouse when injected into the early blastocyst, when transplanted into the post-implantation gastrula primed hPSCs are able to colonize the gastrula and depending on the site of injection can differentiation into each germ layer in a predictable manner (Mascetti and Pedersen, 2016). This stage-matched
incorporation of PSCs into the developing embryo emphasizes the differences in developmental stages in these cell states and culture conditions despite shared their potential to differentiate into all germ layers.

Naïve pluripotent stem cells have some practical advantages over the primed pluripotent state. Naïve PSCs have low levels of both DNA methylation and trimethylation of histone H3 lysine 27 when compared to primed PSCs (Bernstein et al., 2006; Hayashi et al., 2008; Leitch et al., 2013; Marks et al., 2012; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). These epigenetic differences result in naïve mESC cell lines that are relatively homogeneous and do not exhibit lineage bias (Marks et al., 2012). Furthermore, primed PSCs generally undergo apoptosis in response to dissociation into single cells (Chen et al., 2010; Ohgushi et al., 2010; Watanabe et al., 2007). In contrast to primed pluripotent stem cells, mESCs can easily survive dissociation into single cells for clonal expansion making them easier to genetically modify. Therefore, much research has been aimed at identifying conditions in which hPSCs can be either reprogrammed from a primed to a naïve state or to derive hPSCs in a naïve pluripotent state. This goal has been achieved through a variety of different protocols that utilize many approaches to promote naïve pluripotency including the expression of naïve state transcription factors, the addition of growth factors, and treatment with small molecule inhibitors (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014). Gene expression analysis and transcriptional analysis of transposable elements determined that naïve hPSCs produced with protocols from Theunissen et al. and Takashima et al. best resembled the in vivo human preimplantation ICM (Huang et al., 2014; Theunissen et al., 2016). Interestingly, naïve hPSCs exhibit two active X chromosomes despite $XIST$ expression which is lost when naïve hPSCs transition into the primed pluripotent state. Further differentiation causes $XIST$ re-expression and X chromosome inactivation (XCI) which is maintained during differentiation (Sahakyan et al., 2017). This is an improvement from conventional primed hPSCs which do not undergo XCI during differentiation (Patel et al., 2017). While these naïve hPSCs exhibit many features of in vivo preimplantation pluripotency, incorporation into the mouse blastocyst remains inefficient. It is unclear
whether this is due to species differences or imperfect naïve hPSC conditions. In addition, naïve hPSCs produced with Theunissen et al. protocol have a scrambled pattern of DNA methylation and lose methylation at imprinted loci (Pastor et al., 2016). Furthermore, transition of naïve hPSCs to the primed pluripotent state, which is the first step of differentiation, is inefficient and slow. Therefore, much more work needs to be done to determine the differences between the naïve and primed pluripotent state and to improve PSCs differentiation for clinical application.

Beyond the traditional naïve mESC culture, mouse PSCs can be grown in an even more homogeneous undifferentiated pluripotent state termed the ground state. Rather than being maintained on a feeder layer with serum and LIF (S/L), ground state mESCs are grown in feeder free conditions in the presence of LIF and two inhibitors (2i/L) that support self-renewal, a FGF/Mek/Erk inhibitor PD184352 and a Gsk-3 inhibitor CHIR99021 (Nichols et al., 2009; Ying et al., 2008). Ground state mESCs exhibit lower levels of both DNA and histone methylation when compared to mESCs cultured on feeders (Ficz et al., 2013; Habibi et al., 2013). Furthermore, the gene expression pattern of mESCs cultured in 2i/L confirms a more homogeneous population than when mESCs are cultured in S/L (Ficz et al., 2013).

**Metabolism in Pluripotency and Differentiation**

Previous work from our lab and others has shown that primed hPSCs rely on glycolysis for ATP production and shift toward oxidative phosphorylation (OXPHOS) during differentiation (Zhang et al., 2011; Zhou et al., 2012). Upon differentiation, changes in metabolism can be seen before the decrease in the expression of pluripotent markers NANOG and OCT4 (Moussaieff et al., 2015). One regulator of primed hPSC metabolism is uncoupling protein 2 (UCP2) which is highly expressed in primed hPSCs and decreases during retinoic acid induced differentiation (Zhang et al., 2011). UCP2 is thought to promote glycolysis in hPSCs by acting as a transporter of C4 metabolites out of the mitochondria suppressing
respiration (Vozza et al., 2014). Enhancement of glycolysis, by use of hypoxic environments for example, promotes pluripotency (Covello et al., 2006; Forristal et al., 2010). Culture in hypoxic conditions enhances reprogramming of fibroblasts to iPSCs by enhancing the shift to glycolytic metabolism that occurs during the acquisition of the pluripotent state (Mathieu et al., 2014; Yoshida et al., 2009).

While OXPHOS is low in primed hPSCs when compared to many differentiated cells, glutamine is critical for hPSC survival and self-renewal as it fuels the TCA cycle and is a precursor of glutathione (GSH), an important antioxidant in hPSCs (Marsboom et al., 2016; Tohyama et al., 2016; Zhang et al., 2016). High levels of GSH are maintained and used to combat oxidative stress and DNA damage by high expression of glutathione peroxidase 2 (GPX2) in primed hPSCs (Dannenmann et al., 2015). UCP2 expression in hPSCs also promotes the pentose phosphate pathway, which produces NADPH important for reducing glutathione disulfide (GSSG) back to GSH for antioxidant protection (Zhang et al., 2011). Low GSH levels induced by the withdrawal of glutamine from hPSC media results in oxidation and degradation of pluripotency transcription factor OCT4 leading to differentiation. In this way OCT4 acts as a sensor of the redox state of the cell coordinating growth factor signaling and oxidative stress with differentiation (Marsboom et al., 2016).

Feeder cells can greatly influence the metabolic state of primed hPSCs. Feeder free conditions promote greater dependence on glycolysis when compared to primed hPSCs co-cultured with mouse embryonic fibroblasts (MEFs). This feeder free induced shift in metabolism involves increase serine, nucleotide, and lipid biosynthesis (Gu et al., 2016; Zhang et al., 2016). Furthermore, feeder free conditions enhance use of glucose and glutamine into the TCA cycle while feeder MEF conditioned medium causes more respiration, probably due to an increase in fatty acid oxidation. Supplementation of feeder free medium
with lipid results in primed hPSC metabolism with increased respiration similar to MEF conditioned medium metabolism (Zhang et al., 2016).

Differences in metabolism also exist between the naïve and primed pluripotent states. When compared to primed hPSCs, naïve hPSCs have a higher flux of overall glucose metabolism with both glycolysis and OXPHOS reported to be higher in the naïve state when compared to the primed pluripotent state. In contrast to the human system, mouse naïve PSCs are less glycolytic and more oxidative than their primed counterparts, mouse EpiSCs (Zhou et al., 2012). This difference may reflect a developmental difference that exists between human and mouse species. In human embryos and naïve hPSCs, high levels of nuclear N-Myc may promote increased glucose metabolism and a greater localization of N-Myc is seen in the nucleus of the ICM when compared to trophoblast cells. Furthermore an N-Myc inhibitor decreases the proliferation of naïve hPSCs while having no effect on primed hPSCs (Gu et al., 2016).

**Interconnections Between Metabolism and Epigenetics**

Epigenetic alterations are chemical modifications to histones or DNA that result in changes in gene expression. Cofactors and co-substrates of many of these reactions are intermediates of metabolic pathways, and therefore serve as a link between the metabolic state of the cell and gene expression (Kaelin Jr and McKnight, 2013). Naïve mESCs are dependent on threonine catabolism for their survival as threonine can fuel one carbon metabolism that is required for nucleotide biosynthesis and s-adenosyl methionine (SAM) production, which acts a methyl donor for DNA and histone methylation (Wang et al., 2009). Inhibition of threonine dehydrogenase (TDH), which converts threonine to serine, in naïve mESCs decreases trimethylation of histone H3 lysine 4 (H3K4me3) and promotes differentiation (Shyh-Chang et al., 2013). In humans, however, the TDH gene is no longer functional due to three inactivating mutations (Wang et al., 2009). In primed hPSCs, methionine acts as the methyl donor for DNA and histone
methylation and withdrawal of methionine from the medium potentiates differentiation into all three germ layers (Shiraki et al., 2014). Acetyl-CoA acts as an acetyl group donor for histone acetylation and addition of acetate to culture media promotes self-renewal in both naïve mESCs and primed hPSCs (Moussaieff et al., 2015).

Several enzymes involved in demethylation of DNA and histones require a TCA cycle metabolite, α-ketoglutarate (αKG), to act as a cofactor. This group of enzymes, called αKG dependent dioxygenases include the JmjC-domain containing family of histone demethylases (JHDMs) and the ten-eleven translocation (TET) methylcytosine dioxygenases (Loenarz and Schofield, 2011). JHDMs hydroxylate methylated lysines forming an unstable hydroxymethyl group that is spontaneously lost as formate effectively removing the methyl group (Klose and Zhang, 2007). TET enzymes function by hydroxylating 5-methylcytosine (5mc) in DNA to form 5-hydroxymethylcytosine (5hmc), which can either exist as a stable epigenetic mark or be further converted back to an unmethylated cytosine by the same TET enzymes (Ito et al., 2010; Tan and Shi, 2012). When maintained in 2i/L medium in the ground state, mESCs use αKG to support self-renewal and maintain the demethylation of DNA and histones (Carey et al., 2014).

However, many studies support the role of αKG and αKG-dependent enzymes in promoting differentiation. Knockout of the ten eleven translocation (Tet) enzymes, members of the αKG-dependent dioxygenase family, impairs differentiation of mESCs through deregulation of developmental promoters (Dawlaty et al., 2014). Knockout of only Tet2 leads to delayed gene induction during differentiation (Hon et al., 2014). Several JmjC domain containing histone demethylases (JHDMs), also members of the αKG-dependent dioxygenase family, have been shown to be important in pluripotent stem cell (PSC) differentiation. Jumonji and AT-Rich Interaction Domain Containing 2 (JARID2), also known as Jumonji, knockout mESCs display delayed expression of differentiation markers due to delayed
demethylation of H3K27 (Shen et al., 2009). UTX (ubiquitously transcribed tetratricopeptide repeat X), a H3K27 demethylase, is important for the activation of HOX genes in early differentiation, while another H3K27 demethylase, JMJD3, is involved in gonad development (Agger et al., 2007; Hong et al., 2007). Furthermore, an oncometabolite produced by mutations in isocitrate dehydrogenase (IDH), 2-hydroxyglutarate (2HG), acts as a competitive inhibitor of αKG-dependent dioxygenases resulting in a block in differentiation in tumors harboring these mutations (Losman et al., 2013; Lu et al., 2012; Xu et al., 2011). The role of αKG in primed hPSC differentiation has previously been unexplored despite these numerous connections. Addition of αKG could serve as a simple way to promote epigenetic remodeling, which is required for hPSC differentiation. In addition, because epigenetics play a role in primed hPSC line to line variability and lineage bias, αKG-promoted differentiation could be a strategy to address these problems and enable efficient differentiation of hPSCs for clinical applications (Burrows et al., 2016; Kim et al., 2011; Nazareth et al., 2013).
References


Zhou, W., Choi, M., Margineantu, D., Margaretha, L., Hesson, J., Cavanaugh, C., Blau, C.A., Horwitz, M.S., Hockenbery, D., Ware, C., et al. (2012). HIF1α induced switch from bivalent to
exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. The EMBO journal 31, 2103-2116.
Chapter 2:

Pluripotent stem cell energy metabolism: an update
Pluripotent stem cell energy metabolism: an update

Tara Teslak1 & Michael A Teitel1,2,3,4,5,6,7*

Abstract

Recent studies link changes in energy metabolism with the fate of pluripotent stem cells (PSCs). Safe use of PSC derivatives in regenerative medicine requires an enhanced understanding and control of factors that optimize in vitro reprogramming and differentiation protocols. Relative shifts in metabolism from naïve through "primed" pluripotent states to lineage-directed differentiation place variable demands on mitochondrial biogenesis and function for cell types with distinct energetic and biosynthetic requirements. In this context, mitochondrial respiration, network dynamics, TCA cycle function, and turnover all have the potential to influence reprogramming and differentiation outcomes. Shifts in cellular metabolism affect enzymes that control epigenetic configuration, which impacts chromatin reorganization and gene expression changes during reprogramming and differentiation. Induced PSCs (iPSCs) may have utility for modeling metabolic diseases caused by mutations in mitochondrial DNA, for which few disease models exist. Here, we explore key features of PSC energy metabolism research in mice and man and the impact this work is starting to have on our understanding of early development, disease modeling, and potential therapeutic applications.

Keywords: differentiation; epigenetics; metabolism; mitochondria; pluripotency

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See the Glossary for abbreviations used in this article.

Introduction

Energy production in early mammalian development depends upon many factors, including substrate availability, uptake, and O2 tension. All mammalian cells produce ATP by differing proportions of glycolysis and oxidative phosphorylation (OXPHOS), with the balance between these processes at specific developmental stages or states of cellular activation controlled by multiple intra- and extracellular factors. Glycolysis is the enzymatic conversion of glucose to pyruvate, which generates 2 net ATP molecules per mole of glucose. Cells that depend mainly on glycolysis for ATP production further convert pyruvate to lactate, which is excrated. By contrast, cells in oxygen-rich environments may prefer OXPHOS for more efficient ATP production, which on average nets 34 additional ATP molecules per glucose by oxidizing pyruvate to acetyl-CoA in the mitochondrial tricarboxylic acid (TCA) cycle. During pre-implantation development of early mouse embryos, ATP is produced mainly by OXPHOS from uptake of pyruvate, lactate, amino acids, and triglyceride-derived fatty acids (Brinster & Toddy, 1979; Martin & Loese, 1995; Janssen et al., 2008; Loese, 2012). This is followed by a shift to a more balanced mixture of glycolysis and OXPHOS with increasing glucose uptake in the low O2 microenvironment of an implanting blastocyst (Loese & Barton, 1984; Houghton et al., 1994; Zhou et al., 2012). In vitro studies report a similar increase in glucose uptake in early human embryos advancing to the blastocyst stage in a dish (Gardner et al., 2001). Pyruvate and glucose uptake and amino acid turnover are predictors of human blastocyst quality and enhanced viability for in vitro fertilization protocols (Houghton et al., 2002; Biston et al., 2009). In concept, in vitro differences in early mammalian embryo energy metabolism should be replicated in vitro by cells obtained from distinct stages of embryonic development that are maintained in similar culture conditions.

Human embryonic stem cells (hESCs) originate from the blastocyst inner cell mass and hold great clinical potential for cell replacement therapies because of their high proliferative capacity and their ability to differentiate into any cell type in the body (Thomson et al., 1998). However, the clinical use of differentiated hESCs is limited by ethical concerns regarding the method of hESC acquisition and by potential alloimmune rejection (Zhao et al., 2011). To help circumvent these issues, mammalian somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) through ectopic expression of different combinations of transcription factors, such as the "Yamanaka cocktail" of POUSF1, SOX2, RLF4, and MYC (Takahashi & Yamanaka, 2006; Takahashi et al., 2007) or by other
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<td>ShcA</td>
<td>5-hydroxytryptophan synthetase</td>
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<td>SmC</td>
<td>5-hydroxytryptamine synthetase</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<td>Drp1</td>
<td>dynamin-related protein 1</td>
</tr>
<tr>
<td>EBs</td>
<td>embryonic bodies</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAO</td>
<td>fatty acid oxidation</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
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<tr>
<td>NAGl</td>
<td>human monosaccharide transporter</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFIC</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>NIF</td>
<td>nuclear factor I</td>
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<tr>
<td>P53</td>
<td>p53 tumor suppressor protein</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PKA</td>
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<td>PKB</td>
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<td>protein kinase C</td>
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<tr>
<td>PKD</td>
<td>protein kinase D</td>
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<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAF</td>
<td>Raf proto-oncogene</td>
</tr>
<tr>
<td>RKIP</td>
<td>oncoprotein regulator of kinase</td>
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</table>
However, iPSCs metabolically resemble developmentally more mature, glycolytic mouse embryo stem cells (mESCs), obtained from the post-implantation embryo, instead of mESCs, which show a bivalent metabolism that can switch between glycolysis and OXPHOS on demand (Zhou et al., 2012). This metabolic comparison is consistent with biomarker and functional features of standard laboratory iPSCs that are "primed", or more mature, than naïve, or ground state iPSCs. Naïve iPSCs, similar to mESCs that represent the least mature pluripotent stage, have recently been obtained by iPSC exposure to chemical inhibitor and growth factor cocktails or by transient expression of two transcription factors combined with two chemical inhibitors and human leukemia inhibitory factor (Fig 1) (Gallon et al., 2018; Takaishina et al., 2014; Theunissen Thoiroud et al., 2014; Warren et al., 2014). iPSCs reset to a naïve state through transient ectopic expression of NANOG and KLF4 require a higher level than "primed" iPSCs, similar to pre-implantation mouse embryos and naïve mESCs (Fig 1) (Takaishina et al., 2014). The regulation of energy metabolism therefore appears intertwined with genetic and epigenetic mechanisms that control PSC maturation state through pathways that require further elucidation.

Metabolic regulation of self-renewal, reprogramming, and differentiation

Reprogrammed iPSCs maintain an "epigenetic memory" or chromatin signature of the cells from which they were generated that can impact their re-differentiation potential and function (Kim et al., 2010, 2011; Bar-Nur et al., 2011). Changes in cellular metabolism can impact the activity of epigenome-modifying enzymes, as discussed below (Kerstin Williams & McKnight Steven, 2013). Therefore, manipulation of culture conditions could erase or generate new epigenetic marks during iPSC reprogramming, PSC differentiation, or steady-state growth that will affect the functional potential of the end resulting cell. For therapeutic utility, identifying specific, reproducible, and chemically defined culture conditions to produce safe and functional differentiated cells from iPSCs, or by transdifferentiation protocols, will be required. Several key cell types targeted for cell replacement therapies have high energy demands, such as cardiomyocytes and neurons. Therapeutic applications will therefore require re-establishment of a cell type-specific, fully functional mitochondrial network to support the energy and other mitochondrial-supplied factors for these replacement cell types. Importantly, mitochondrial dysfunction due to impaired nucleus and mitochondrial encoded genes has been linked to >400 named human diseases, including multiple neurodegenerative disorders and cancer (Nunnari & Saarma, 2012).

The discovery that hypoxia maintains self-renewal and increases the efficiency of reprogramming to pluripotency has stimulated studies to determine the role of oxygen tension in cell fate determination. A striking difference in mitochondrial morphology between PSCs and their differentiated derivatives has similarly spurred studies to decipher the mechanisms that control cell state-specific mitochondrial structure and function. Adding to this complexity are state-specific levels of cellular metabolites, such as the AMP/ATP ratio and amino acid availabilities, which can impact PSC gene expression and cell function. In the first part of this review, the effect that these components of cellular metabolism have on self-renewal and differentiation is explored.

Oxygen tension and hypoxia-inducible factors (HIFs)

Reduced O₂ (1-5%) can be used for iPSC tissue culture to mimic the hypoxic early embryonic microenvironment in vivo. Transcription factors such as hypoxia-inducible factor 1α (HIF1α) and 2α (HIF2α) control the genomic response to low O₂ tension by promoting the expression of genes such as pyruvate dehydrogenase kinase 1

Figure 1. Influence of energy metabolism on pluripotent status.

Naïve human pluripotent stem cells (iPSCs) show an increase in ATP production through oxidative phosphorylation (OXPHOS) compared to more mature, "primed" iPSCs. Primed iPSCs can be converted to the naïve state through ectopic expression of NANOG and KLF4, inhibition of the ERK pathway by two inhibitors (D), and stimulation with human leukemia inhibitory factor (L) (Takaishina et al., 2014). Alternatively, the naïve state can be induced with a cocktail of five inhibitors and growth factors (D) (Theunissen Thoiroud et al., 2014). Stem cells can be reprogrammed with OCT4, SOX2, KLF4, and c-MYC (OCT4, SOX2, KLF4, c-MYC). Fibroblasts are more resistant than primed iPSCs. Factors that activate glycolysis and inhibit OXPHOS promote induced PSC (iPSC) reprogramming. Vitamin C enhances iPSC reprogramming as an antioxidant and as a cofactor for epigenetic enzymes. Rapamycin, an inhibitor of the mTOR pathway, also increases the efficiency of iPSC reprogramming. Withdrawal of methionine from iPSC culture, which is required to maintain DNA and histone methylation, promotes differentiation.
(PDH), lactate dehydrogenase A (LDHA), and glycogen phosphorylase (GP)). These enzymes help regulate energy metabolism in hypoxia. In hypoxic conditions, HIF1α and HIF2α are degraded in the presence of oxygen, while under normoxic conditions, they are stabilized and activated. HIF1α also regulates the expression of genes involved in glycolysis and glucose metabolism, leading to increased glucose uptake and metabolism in hypoxic conditions. HIF1α activation can also promote angiogenesis by increasing the expression of angiogenic factors such as VEGF.

Hypoxia-denovo replication (HDDR) is another mechanism that contributes to genomic instability in hypoxic cells. DDR involves the replication of DNA in the absence of active oxygen, leading to increased DNA damage and mutagenesis. DDR is induced by hypoxia and can promote the survival of hypoxic cells by allowing them to escape the cell cycle arrest induced by hypoxia.

The ion transport chain

Mitochondria and the electron transport chain

Mitochondria play a crucial role in cellular energy metabolism, generating a significant portion of the ATP required for cell function. In hypoxic conditions, mitochondria can be more susceptible to damage and dysfunction, leading to reduced ATP production and increased free radical accumulation. This can contribute to the hypoxia-induced genomic instability and genomic dysfunction observed in hypoxic cells. Understanding the mechanisms by which hypoxia affects mitochondrial function and DNA stability can provide insights into the regulation of hypoxic response and the development of strategies to mitigate the effects of hypoxia on cell survival and function.
although vitamin C may also impact reprogramming efficiency through epigenetic mechanisms described below.

ATP and ROS production by OXPHOS is further limited by several mechanisms in iPSNs, such as by the expression of uncoupling protein 2 (UCP2) (Zhang et al., 2011). UCP2 transports four carbon TCA cycle intermediates out of the mitochondria, effectively reducing carbon substrate for use in OXPHOS (Vozza et al., 2014). Also, nuclear genes encoding multiple subunits of cytochrome C oxidase (complex IV of the ETC), which donate electrons to O2, are expressed at a lower level in mIPSNs compared to nESCs (Zhou et al., 2012). DMEO-induced differentiation of mIPSNs increases ETC complex I and complex IV activities along with mitochondrial biogenesis to support an increase in mitochondrial ATP production (Han et al., 2013). The ETC maintains the mitochondrial inner membrane electrochemical potential, Δψ, which is required to prevent mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic intermembrane space (IMS) proteins, such as cytochrome c, that induce apoptosis (Green & Kroemer, 2004). When ETC activity is low, Δψ can be additionally supported by the hydrolysis of ATP in the complex V ATP synthase, which results in the translocation of protons from the mitochondrial matrix to the IMS to increase Δψ (Hatefi, 1985). iPSNs have relatively low respiration and ETC activity; therefore, ATP hydrolysis activity of the ATP synthase helps to maintain Δψ and sustain cell viability (Zhang et al., 2011). Interestingly, iPSNs maintain a higher Δψ than their differentiated derivatives (Chung et al., 2007; Armstrong et al., 2010; Privone et al., 2011). This has been proposed to enable rapid metabolic changes during differentiation (Folmes Clifford et al., 2012b; Folmes et al., 2012a) and possibly to maintain a fragmented mitochondrial network (Mattner et al., 2005).

iPS reprogramming of mouse embryonic fibroblasts (MEFs) causes major changes in the expressed proteome in two stages. ETC complex I and complex IV proteins are reduced early during reprogramming, in contrast to components of ETC complexes II, III, and V, which are transiently induced during a second, intermediate reprogramming phase (Hansson et al., 2012). The efficiency and speed of iPS reprogramming is enhanced when OXPHOS is decreased by inhibition of any of the ETC respiratory complexes, consistent with a required shift toward glycolysis (Fig 1) (Son et al., 2013b).

An increase in OXPHOS capacity is required for proper cardiomyocyte lineage-directed differentiation from iPSNs. Cardiomyocyte differentiation induces the expression of nuclear-encoded genes for mtDNA transcription factors, mtDNA replication factors, components of the fatty acid oxidation (FAO) machinery, enzymes of the TCA cycle, and ETC subunits (St John et al., 2005; Chung et al., 2007; Tolyhama et al., 2013). Cardiomyocyte-directed differentiation is enhanced by the generation of ROS by NADPH oxidase-like enzymes (Sauer et al., 2000; Crespo et al., 2010). Agonists of peroxisome proliferator-activated receptor α (PPARα), a highly expressed nuclear hormone receptor in the heart associated with FAO, promote cardiomyogenesis of mESCs through increasing ROS production (Shantiparan et al., 2008).

Differences in carbon substrate types can be used to purify metabolically mature mouse cardiomyocytes following differentiation from mIPSNs because of key differences in metabolite handling capacity between mouse cardiomyocytes and mIPSNs. Fetal cardiomyocytes preferentially consume lactate for the production of ATP (Fisher et al., 1981; Werner & Szecsey, 1987). Therefore, cardiomyocytes derived in vitro from PSCs can utilize lactate in the absence of glucose to produce ATP, whereas nESCs and iPES are unable to use lactate for ATP production. When cultured in glucose-free media supplemented with lactate, functional mouse cardiomyocytes can be recovered at ~99% purity (Tobiyama et al., 2013).

Mitochondrial dynamics

The dynamic fusion and fission/fragmentation of an interlacing mitochondrial network enables mixing of mitochondrial contents and the degradation of damaged mitochondria to maintain robust energy and metabolite production (Tiwg et al., 2008; Westermann, 2012). Mitochondrial network fusion status is a determinant of maximal respiratory capacity (Chen et al., 2005; Yu et al., 2006). PSCs show a punctate, fragmented mitochondrial network that progressively fuses during differentiation, which increases respiratory capacity (Zhang et al., 2011). The GTPase dynamin-related protein 1 (DRP1), which causes mitochondrial fission, can be inhibited to induce a fused mitochondrial network. Pharmacological inhibition of Drp1 to maintain a fused mitochondrial network inhibits iPS reprogramming (Vazquez-Martín et al., 2012a), although shRNA knockdown of Drp1, also resulting in mitochondrial fusion, did not impair iPS reprogramming of MEFs (Wang et al., 2014). These paradoxical results could be reconciled by off target effects of the Drp1 inhibitor, insufficient Drp1 shRNA knockdown, or a combination of these or other confounders. Interestingly, reduced expression 1 (REX1) zinc finger-containing protein that is required to maintain PSC self-renewal and is expressed during lineage non-specific retinoic acid-induced differentiation. REX1 expression also increases the expression of cyclin B1, which leads to the phosphorylation and activation of DRP1. Fusion of the mitochondrial network, and increased glycolytic metabolism that is characteristic of PSCs (Son et al., 2013a). The expression pattern of REX1 is concordant with DRP1 activation and mitochondrial fusion associated with pluripotency.

Mitochondrial network fusion requires fusion of the outer mitochondrial membrane, mediated by mitofusin-1 and -2 (MFN1 and MFN2), and fission of the inner mitochondrial membrane, mediated by optic atrophy 1 (OPA1) (Westermann, 2010). Mfn1, Mfn2, and Opa1 are all required for viable embryonic mouse development (Chen et al., 2005; Alisi et al., 2007). Opa1 also helps to remodel cristae folds of the inner mitochondrial membrane to help mitochondria adapt to changing metabolic demands (Patten et al., 2014). At least five different isoforms of the Opa1 protein exist due to differential splicing and proteolytic cleavage. Prohibitin 2 (Phb2), a nucleo- encoded mitochondrial protein, is expressed at high levels in mESCs and promotes expression of the long isoforms of Opa1. Ecotypic expression of Phb2 in mESCs inhibits lineage-directed differentiation toward neurons and endoderm and causes mitochondrial swelling (Kowro et al., 2014). Mfn2 and Opa1 are required for the differentiation of mESCs into beating cardiomyocytes (Kasahara et al., 2013), suggesting that shifting to OXPHOS during cardiomyogenesis also requires mitochondrial network fusion. Mfn2 also tether mitochondria to the sarcoplasmic reticulum, which is required for Ca2+ signaling and...
energy metabolism in cardiomyocytes (Chen et al., 2012a). Juncoto-
phin 2 (Jpx2), which is also part of the junctional membrane
complexes that physically link mitochondria with the sarcoplas-
mic reticulum, is required for proper mitochondria function, Ca\textsuperscript{2+}
homeostasis, and the differentiation of mESCs into cardiomyocytes
(Liang et al., 2012). Opening of the mitochondrial permeability tran-
sition pore (PTP) enables macromolecular diffusion across the mi-
 tochondrial inner membrane, which inhibits ATP production by
OXPHOS (Hunter et al., 1976; Kim et al., 2005). Inhibition of the PTP
promotes cardiomyocyte differentiation of mESCs through increas-
ing mitochondrial function (Horn Jenifer et al., 2011; Cho et al.,
2014). Interestingly, antioxidant exposure during PTP inhibition
synergistically enhances cardiomyogenesis by an unknown mecha-
nism(s) (Cho et al., 2014).

AMPK, mTOR, and autophagy

Adenosine monophosphate (AMP)-activated protein kinase (AMPK)
is a sensor of the AMP/ATP and ADP/ATP energy charge ratio
in cells and coordinates the cellular response to changes in energy
status. In response to increasing AMP/ATP and ADP/ATP ratios,
AMPK becomes phosphorylated by liver kinase B1 (LKB1) to ac-
ivate catabolic pathways that generate ATP and inhibit anabolic
pathways that consume ATP (Hardie et al., 2011). Phosphorylated
AMPK inhibits protein translation by inactivating the mammalian
target of rapamycin (mTOR) signaling pathway. mTOR exists as
two distinct protein complexes, complex 1 (mTORC1) and complex
2 (mTORC2). mTORC1 is an amino acid sensor that regulates protein
translation and autophagy. Autophagy is a process that degrades
cytoplasmic macromolecules and organelles to provide substrates
for energy production, or to remodel cellular functions with changes
in differentiation or activation state, and provides a rapid cellular
response to changing environmental conditions (Mizushima &
Levine, 2010). Chemical activation of AMPK in mouse and human
fibroblasts decreases iPSC reprogramming efficiency, potentially
from a failure to fully induce Oct4 gene expression (Vazquez-Martín
et al., 2012b). mTOR activity decreases during iPSC reprogramming
whereas rapamycin, an inhibitor of the mTOR pathway, enhances
iPSC reprogramming of mouse fibroblasts (Fig 1) (Chen et al., 2011;
He et al., 2012; Morita et al., 2013). Concomitantly, hyperactivation of
mTORC1 by knockdown of tuberous sclerosis 2 (Tsc2), an upstream
kinase inhibitor of mTOR, suppresses iPSC reprogramming (He
et al., 2012). Inhibition of the mTOR pathway leads to activation of
autophagy and enhances iPSC reprogramming efficiency, possibly
from assisted cellular remodeling. Another inducer of autophagy,
exposure to spermine, also enhances mESC reprogramming effi-
ciency (Chen et al., 2011). Moreover, the reprogramming transcrip-
tion factor Sox2 inhibits mTOR gene expression, which in turn
activates autophagy during iPSC reprogramming. Sox2-induced
mTOR gene repression occurs by recruitment of the nucleosome
remodeling and deacetylase (NuRD) repressor complex to the mTOR
gene promoter (Wang et al., 2013a). Although mTOR activation
impairs iPSC reprogramming, its inhibition with rapamycin does
not disrupt expression of Oct4, Sox2, and Nanog genes in hESCs
and promotes the expression of endoderm and mesoderm
lineage differentiation genes (Zhao et al., 2009b). In contrast, DRP
domain-containing mTOR (DEPTOR)-interacting protein, a negative
regulator of mTORC1/2, maintains pluripotency for mESCs and
hESCs (Agarwal et al., 2014).

The AMPK/mTORC1 pathway regulates mitochondrial biogenesis
in somatic cells, and therefore, its role in iPSC differentiation merits
further consideration as a mechanism to regulate cell type-specific
mitochondrial content and function (Zong et al., 2002; Reinrich &
Shulman, 2006; Morita et al., 2012). In addition, AMPK can directly
regulate gene expression through histone phosphorylation (Baugrand
et al., 2010). Studies to determine the mechanism(s) that regulate
energy-sensing pathway activation and deactivation during iPSC
reprogramming and PSC differentiation may reveal how these
changes occur in vivo to control organoïdal and lineage-specific
development.

Other molecular players

c-Myc is one of the original four reprogramming transcription factors
used in iPSC reprogramming of fibroblasts, but can be removed
and/or replaced by Lin28 or other transfectors (Takahashi et al.,
2007; Yu et al., 2007; Nakagawa et al., 2008; Wernig et al., 2008).
iPSCs reprogrammed with Oct4, Sox2, Klf4, and c-Myc transfectors
show an increase in glycolytic metabolism compared to mIPSC
reprogramming that excludes c-Myc (Folmes et al., 2013b). While
the metabolic influence of c-Myc in PSCs has not been further char-
acterized, c-Myc promotes RNA splicing of PRE21 in cancer cells,
which activates biosynthetic anabolic pathways (David et al., 2010; Chanet & Gottlieb, 2012). c-Myc also has a large role in stimu-
I
ating glutamine metabolism in lymphocytes and cancer cells (Gao
et al., 2009; Le et al., 2012; Liu et al., 2012; Murphy et al., 2013).

Lin28 is an evolutionarily conserved regulator of microRNAs
(miRNAs) that can be used for iPSC reprogramming in combination
with Oct4, Sox2, and Klf4 (Yu et al., 2007; Viswanathan et al.,
2008). Lin28 knockout mice have defects in growth and glucose
metabolism (Shinoda et al., 2013). LIN28 expression is regulated by
let-7, a microRNA that is also post-transcriptionally repressed by
Lin28 in a feedback regulatory loop. Knockdown of let-7 in fibroblasts
enhances iPSC reprogramming (Molto et al., 2010). LIN28 preferentially binds to the miRNAs of metabolic enzymes to control their translation, which influences cell growth and survival. Metabolic enzymes targeted by LIN28s in iPSCs include enzymes involved in glycolysis, cholesterol biosynthesis, and mitochondrial metabolism (Peng et al., 2011). Lin28 also enhances the translation of enzymes involved in OXPHOS during the repair of damaged tissues (Shyr-Chang et al., 2013b). Adenosylmethionine decarboxylase 1 (Armadillo) participates in the biosynthesis of polyamines. Polyamines are positively charged metabolites that can bind to acidic sites of macromolecules including
nucleic acids, proteins, and phospholipids. Elevated levels of Armadillo are required for self-renewal of mESCs. Additionally, transla-
tional inhibition of Armadillo by miR-762 is required for the differen-
tiation of NPCs (Zhang et al., 2012).
changes in the structure, function, and expression of the nucleus. Binding of transcriptional regulators to their target genes before, during, and after these cell fate changing processes depends on dynamic alterations in DNA methylation, histone modifications and variants, chromatin remodelling complex activities, and global and local three-dimensional chromosome topologies (Papp & Math, 2013). Cellular metabolism directly influences the epigenetic landscape of a cell by modulating the level and activity of metabolite cofactors and substrates for enzymes that control at least DNA and histone modifications (Kaelin William & McRight Steven, 2018). Therefore, cellular metabolite levels and flux help determine cellular fate during iPSC reprogramming and PSC differentiation.

DNA and histone methylation is a major regulator of gene expression and chromatin remodelling (Codar & Heggman, 2011). Methyltransferase enzymes that methylate DNA and histones utilize S-adenosyl methionine (SAM) as a methyl donor for transferring methyl groups. Interestingly, SAM levels are elevated in both human and mouse PSCs compared to fibroblasts, but are even higher in hiPSCs when compared to hESCs (Panopoulos et al., 2011; Shy-Chang et al., 2013a). Additionally, SAM levels increase in the late stages of iPSC reprogramming, indicating coordinate accumulation with increasing pluripotent potential (Shy-Chang et al., 2013a). Global DNA methylation is also higher in some hiPSC lines when compared to hESCs, suggesting that SAM levels participate in regulating the extent of global DNA methylation (Deng et al., 2009).

In mESCs, SAM is generated by uptake of extracellular threonine, which is converted to glycine by threonine dehydrogenase (TDH) (Fig. 2). Therefore, threonine uptake and TDH activity are required to maintain high levels of SAM in mESCs. Clearance of glycine, the product of TDH, produces SAM by threonine methylation production, which leads to the conversion of methionine plus ATP into SAM via methionine adenosyltransferases (Fig. 2) (Shy-Chang et al., 2013a). mESCs cannot survive in culture medium lacking threonine (Wang et al., 2009) at least partially because histone 3 lysine 4 di- and trimethylation (H3K4me2 and H3K4me3) is lost in mESCs deprived of threonine, whereas MEFs remain unaffected (Shy-Chang et al., 2013a). The human TDH gene is a non-functional pseudogene due to two splice acceptor mutations and one nonsense mutation. Therefore, threonine cannot be used for SAM production or level regulation in human cells (Wang et al., 2009). hESCs instead depend on the uptake of extracellular methionine for SAM production, with methionine deprivation resulting in a loss of H3K4me3 that predisposes hESCs to differentiation into any of the three embryonic germ layers (Fig. R1 and 2). Leucine: methionine deprivation leads to cell apoptosis through a p53/p21 mitogen-activated protein kinase (MAPK)-mediated stress signaling response (Shiraki et al., 2014). Therefore, adequate methionine in culture media is required to maintain SAM levels and global DNA and histone methylation.

Short-term removal of methionine from hiPSC culture media may be used to inhibit enzymatic methylation reactions, DNA and histone methylations are also susceptible to demethylation reactions. Vitamin C, 2-oxoglutarate (α-ketoglutarate, αKG), and Fe(II) act as cofactors for 2-oxoglutarate (2-OG)-dependent dioxygenases, which include ten-eleven translocation (TET) methylcytosine dioxygenases and Junon) domain-containing (JMJC) histone demethylases (Fig. 2). TET methylcytosine dioxygenases oxidize 5-methylcytosine (5mC) in DNA to form 5-hydroxymethylcytosine (5hmC), which is an initial modification

Vitamin C enhances iPSC reprogramming (Fig. 1), and Tet1 can replace Oct4 in the iPSC reprogramming cocktail because its expression activates the transcription of Oct4 (Esteban et al., 2010; Gao et al., 2013). Interestingly, the absence of vitamin C impairs hiPSC reprogramming by Tet1, whereas the converse is also observed. With vitamin C, Tet1 inhibits the mesenchymal-to-epithelial transition (MET), but in conditions lacking vitamin C, Tet1 promotes reprogramming without activating the MET (Chen et al., 2013). The differences in the role of Tet1 that depend on the level of vitamin C in the culture media highlight the importance of optimizing vitamin C levels during iPSC reprogramming, PSC differentiation, and transdifferentiation protocols. Tet dioxygenases can also promote the MET in combination with thymine DNA glycosylase, which enhances iPSC reprogramming (Hu et al., 2014). Moreover, Tet1 can enhance reprogramming by interacting with Nanog to increase expression of key pluripotency-associated target genes (Costi et al., 2014).

The pattern of DNA 5hmC differs between hESCs and hiPSCs at large hotspots where hiPSCs have been incompletely hydroxymethylated (Wang et al., 2013b). mESCs lose DNA 5hmC when cultured without vitamin C, and addition of vitamin C leads to a rapid Tet1 and Tet2-dependent increase in DNA 5hmC and DNA demethylation (Blaschke et al., 2013; Minor et al., 2013). Resulting vitamin C-induced changes in DNA methylation occur in genomic regions that tend to gain DNA methylation after in vitro culture in contrast to blastocysts in vivo (Blaschke et al., 2013). Consequently, vitamin C may be an important component of culture media to use to more closely replicate the in vivo environment. Vitamin C levels can also modulate the activity of the JmjC class of 2-oxoglutarate (2-OG)-dependent dioxygenases (Fig. 3). JmjC family member proteins ADH1A/B enhance iPSC reprogramming in a vitamin C-dependent manner (Wang et al., 2011). Further investigations on the regulation of other JmjC demethylase family member proteins by vitamin C levels may show similar activities.

Surprisingly, the non-essential amino acid L-proline causes the epigenetic state of PSCs. Culturing of mESCs with L-proline causes a mesenchymal-like invasive phenotype while maintaining expression of pluripotency genes. L-proline-treated cells show augmented levels of H3K36me2 and H3K40me3, which can be reversed by vitamin C exposure (Coevers et al., 2013). Another potential link between cellular metabolism and histone methylation is lysine-specific demethylase 1 (LSD1), which requires L-proline dimedone (PAD1) for enzymatic activity. Therefore, PAD levels, which also serve as a concentration-dependent cofactor for FAO and respiration, can control the activity of LSD1. LSD1 is required for the maintenance of hESC pluripotency and occupies gene promoters that also bind OCT4 and NANOG transcription factors (Adorno et al., 2011).

Histone acetylation is generally associated with gene activation and can often block histone methylation. Sodium butyrate, a histone deacetylase inhibitor, enhances iPSC reprogramming efficiency, emphasizing the importance of dynamic regulation of histone acetylation (Mali et al., 2010; Hou et al., 2013). Simultaneously, a family of NAD-dependent deacetylases, can remove acetyl groups from histones depending on the metabolic state of a cell. The NAD+/NADH ratio is significantly higher in hESCs compared to fibroblasts (Salkyin et al., 2018), and therefore, the systems may
have differential activity in PSCs versus differentiated cells. Sirt1 is required for genomic stability and telomere elongation of iPSCs (De Ronco Maria et al., 2014). SIRT6 can improve the iPSC reprogramming efficiency of fibroblasts obtained from older patients (Sharma et al., 2018).

How intermediate metabolites from programmed patterns of metabolism and environmental influences regulate epigenome-modifying enzyme activities, such as the sirtuins, requires further study in early development and PSCs. Many studies, especially in cancer, have investigated the role of specific epigenetic enzymes, and their mutant forms, through genetic manipulation. The link between acetyl-CoA levels and protein acetylation has been studied in cancer, but its role has not been investigated in pluripotency, iPSC reprogramming, or iPSC differentiation (Fig 2) (Welti et al., 2009; Choudhary et al., 2011). In addition to vitamin C, sKG is an important cofactor for dioxygenases. sKG is a TCA cycle intermediate and can also be produced through conversion from glutamate by aminotransferases involved in other metabolic pathways in the cytoplasm. In some cancers, the majority of sKG is produced by phosphoenolpyruvate carboxykinase 1 (PCK1) (Possemato et al., 2011).

Mitochondrial disease modeling with hiPSCs

mtDNA is maternally inherited and encodes genes for 13 protein subunits in 4 of 5 ETC complexes, 2 rRNAs, and 22 tRNAs. Disease-causing mutations in these mtDNA genes occur in an estimated 1 in 5,000 children and adults (De Marco & Schon, 2009; Schaefer et al., 2003). Mammalian cells may contain up to ~5-10 mtDNAs with sequence variations, a mixture state that is termed heteroplasmy (Legros et al., 2000). The heteroplasmy ratio, or the ratio mtDNA carrying a mutant gene to mtDNA carrying the wild-type (WT) gene, varies in cells of each individual, and affected patients with the same mtDNA mutation can exhibit a very different range and severity of symptoms (Pickrell Allik & Youle Richard, 2013). Cells with the highest energy requirements, such as muscle and brain, are most often affected, but patients may not manifest symptoms until their cells accumulate enough of the disease-causing mutant mtDNA through cell proliferation over time. When the mtDNA mutation burden accumulates to roughly 60-90% of the total mtDNA present in a cell, OXPHOS may become compromised and symptoms ensue (Mishra & Chan, 2014). Accurate mouse models of mtDNA mutations are infrequent, and iPSCs have emerged as a potentially good
model system to study the different cellular manifestations of mutant mtDNA diseases.

The most common mtDNA mutation is a heteroplasmic 3243A>G mutation in the tRNA-Leu(UUR) gene, which can result in two distinct patient phenotypes. Maternally inherited diabetes and deafness (MIDD) is one manifestation of this mutation, whereas the other main manifestation is mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS syndrome) (Goto et al., 1990; Chue et al., 2004). hiPSCs were successfully generated with heteroplasmic mtDNA 3243A>G of variable mutational loads. While mitochondrial transcripts are unaffected in fibroblasts derived from MELAS patients due to their low energy requirements, there was a decrease in mitochondrial transcripts in hiPSCs with high mutational loads (MELAS-high iPSCs) and in neurons derived from the MELAS-high iPSCs. Respiratory ETC complex activity, however, was decreased in MELAS fibroblasts and not in MELAS-high iPSCs or neurons derived from MELAS-iPSCs (Folmes et al., 2013a; Himimlilinen et al., 2013). Interestingly, MELAS-mutant mtDNA and WT mtDNA had a bimodal segregation pattern at the end of hiPSC reprogramming, resulting in hiPSCs containing either more WT or more mutant mtDNA (Fig 3). Therefore, there is a mtDNA “bottleneck” during hiPSC reprogramming (Cherry et al., 2013), perhaps similar to the mtDNA “bottleneck” that occurs during activated oocyte cleavage divisions in early mammalian development (Slonbridge, 2000; Smith et al., 2002; Ove et al., 2008; Carling et al., 2011), and there is no selection for or against the MELAS mutation (Fig 3) (Himimlilinen et al., 2013). The extent of heteroplasmy in hiPSCs decreases with increasing passage number in vitro (Folmes et al., 2013a).

Fibroblasts from mtDNA “mutator” mice, which carry a mutation in the polymerase gamma (Pold) gene, carry a high mtDNA mutation load due to errors in mtDNA replication (Trifunovic et al., 2004). Mutator iPSCs reprogrammed from mutator MEFs with heavy mtDNA mutational loads proliferate at lower rates and reduced ability to form EBs, teratomas, and chimeric mice. EBs generated from mutator iPSCs are more skewed to glycolytic metabolism than are EBs from WT iPSCs, which could account for their decrease in differentiation potential (Wahlestedt et al., 2014).

Defects in the function of mitochondria may also be caused by mutations in nuclear-encoded genes with roles in mitochondrial energy metabolism, mitochondrial dynamics, mitochondrial transport, apoptosis, or mitochondria. One disease associated with mitochondrial dysfunction by perhaps one or more of these mechanisms is the neurodegenerative disease, Parkinson’s disease (PD).

Figure 3. Somatic cell reprogramming to pluripotency causes a mtDNA “bottleneck”. mtDNA undergoes a genetic bottleneck, or reduction in copy number, during de-differentiation, similar to the mtDNA bottleneck that occurs during normal female germ lineogenesis. This reduction has an unresolved mechanism that results in a shift from a heteroplasmic toward a homoplasmic state with no clear preference for wild-type or mutant mtDNA. This shift does not occur during the continuous culturing of fibroblasts in which characteristic levels of heteroplasmy are maintained over time.
iPSCs have been generated from fibroblasts of PD patients caused by multiple different mutations including parkin (PARK2), PTEN-induced putative kinase 1 (PINK1), and leucine-rich kinase 2 (LRK2) (Seillet et al., 2011; Cooper et al., 2012; Inzirilli et al., 2012). PINK1 and Parkin proteins interact to regulate mitophagy, the process of selectively targeting poorly functioning mitochondria with low Δψm for engulfment by an autophagosome and eventual degradation (Clark et al., 2006; Park et al., 2006). PARK2, an E3 ubiquitin ligase, is recruited to damaged mitochondria in a PINK1-dependent manner to polyubiquitinate mitochondrial outer membrane proteins (Narendra et al., 2008, 2010; Chan et al., 2011). Neurons differentiated from PINK1 mutant iPSCs have abnormalities in mtDNA copy number (Seillet et al., 2011). Additionally, neurons differentiated from both mutant PINK1 and LRK2 iPSCs are vulnerable to oxidative stress when exposed to PD-associated toxins. Mitochondria in mutant LRK2 iPSC-differentiated neurons require less and are more mobile than those from healthy subjects. Sensitivity of PD iPSC-differentiated PD neurons to PD-associated toxins is rescued by treatment with either an LRK2 inhibitor, complex I inhibitor, or rapamycin (Cooper et al., 2012). PARK2 mutant iPSC-differentiated neurons show increased oxidative stress, α-synuclein accumulation and Lewy body formation, which are clinical manifestations of PD, providing a model for this aspect of PD pathophysiology (Inzirilli et al., 2012).

Concluding remarks

Shifts in cellular metabolism accompany shifts in cell identity and facilitate changes in cell function. Applications in regenerative medicine will likely require a fuller understanding of metabolic mechanisms that can alter cellular identity, function, and longevity. Glycolytic metabolism generally accommodates a high rate of biosynthesis and cell proliferation, whereas OXPHOS generates ATP more efficiently for functioning differentiated cells. While progress has been made in understanding how cellular energy metabolism is conserved with pluripotent and differentiated states, most cause-and-effect features have not yet been determined. Glycolysis is linked to the primed pluripotent state which is favored in hypoxic environments and by HIF trans factor stabilization. Further work is necessary to identify the transcription factors and signaling pathways that regulate glycolytic flux and overall capacity in iPSCs and during induced differentiation. Additionally, the mitochondria in iPSCs are rudimentary and the mechanism(s) regulating their maturation or return to immaturity are only starting to be discovered. How the mitochondrial fusion/fission machinery is regulated, what causes changes in mitochondrial localization, what senses and instructs lineage-specific mitochondrial mass accumulation and maintenance, and what factors facilitate transitions in metabolism and cell fate remains a significant area of ongoing and future investigations.

PSC metabolism regulates the activities of epigenetic modifying enzymes and therefore influences gene expression patterns, differentiation potential, and functional competence. While the influence of metabolism on stem cell and global metabolism patterns are more broadly studied, the regulation of other key metabolites, such as acetyl-CoA, has not been thoroughly investigated in PSCs and iPSCs. As the connections between energy metabolism and cell fate become understood, methods for manipulating PSC metabolism may be harnessed to improve efficiencies and functional outcomes for nuclear reprogramming, PSC differentiation, and transdifferentiation.

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Conflict of interest

The authors declare that they have no conflicts of interest.

References


Chapter 3:

\(\alpha\)-Ketoglutarate accelerates the initial differentiation of primed human pluripotent
**Cell Metabolism**

*α*-Ketoglutarate Accelerates the Initial Differentiation of Primed Human Pluripotent Stem Cells

**Graphical Abstract**

- αKG
  - naive PSCs
- succinate
  - αKG
  - primed PSCs
  - differentiation stimulus

**Highlights**

- hPSCs produce TCA cycle metabolites despite low OXPHOS
- α-ketoglutarate (αKG) promotes early differentiation of hPSCs
- Accumulation of succinate or depletion of αKG delays differentiation of hPSCs
- αKG/succinate alters histone methylation corresponding to differentiation kinetics

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**In Brief**

α-ketoglutarate (αKG) is an important cofactor for demethylation reactions that helps to maintain naive pluripotent stem cells. ToSlaa et al. show that at later stages of pluripotency, αKG can promote early differentiation, highlighting that the cellular context and potentially the stage of cellular maturity can alter the effect of αKG.
α-Ketoglutamate Accelerates the Initial Differentiation of Primed Human Pluripotent Stem Cells

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SUMMARY

Pluripotent stem cells (PSCs) can self-renew or differentiate from naive or more differentiated, primed, pluripotent states established by specific culture conditions. Increased intracellular α-ketoglutarate (αKG) was shown to favor self-renewal in naive mouse embryonic stem cells (mESCs). The effect of αKG or αKG-succinate levels on differentiation from primed human PSCs (hPSCs) or mouse epiblast stem cells (EpiSCs) remains unknown. We examined primed hPSCs and EpiSCs and show that increased αKG or αKG-succinate ratios accelerate, and elevated succinate levels delay, primed PSC differentiation. αKG has been shown to inhibit the mitochondrial ATP synthase and to regulate epigenome-modifying dioxygenase enzymes. Mitochondrial uncoupling did not impede αKG-accelerated primed PSC differentiation. Instead, αKG induced, and succinate impaired, global histone and DNA demethylation in primed PSCs. The data support αKG promotion of self-renewal or differentiation depending on the pluripotent state.

INTRODUCTION

Human pluripotent stem cells (hPSCs) may self-renew or differentiate into all three germ layers (Thomson et al., 1998), but use in regenerative medicine is limited by generally inefficient differentiation strategies (Blanpain et al., 2012). During in vitro differentiation, hPSCs undergo a metabolic shift that increases respiration and oxidative phosphorylation (OXPHOS) and reduces glycolysis, with inhibition of this transition impeding differentiation (Moussaieff et al., 2015; Zhang et al., 2011; Zhou et al., 2012). Despite the importance of this metabolic shift, differentiation protocols have focused on manipulating key signaling pathways and have overlooked metabolic contributions.

α-Ketoglutarate (αKG), a tricarboxylic acid (TCA) cycle metabolite, is a cofactor for αKG-dependent dioxygenase enzymes, which include JmjC-domain containing histone demethylases (JHDMs) and ten-eleven translocation (TET) enzymes (Kelsoe and McKnight, 2013). αKG can also bind and block the mitochondrial ATP synthase and inhibit mechanistic target of rapamycin (mTOR) signaling (Chen et al., 2014). Addition of cell-penetrable dimethyl-αKG (dm-αKG) to culture media enhances self-renewal and inhibits the differentiation of naive-state mouse embryonic stem cells (mESCs) likely by promoting histone and DNA demethylation (Carey et al., 2013). The hPSCs grown in standard conditions are in a primed, or more developmentally mature, pluripotent state, similar to post-implantation mouse epiblast stem cells (EpiSCs) (Graeber et al., 2010; James et al., 2005; Tesar et al., 2007). A role for αKG in primed mouse or hPSCs has not been explored.

Naive and primed pluripotent stem cells (PSCs) show many molecular differences, including self-renewing conditions, epigenetic states, and metabolism (Graeber et al., 2010; Leitch et al., 2013; Marks et al., 2012; Ware et al., 2014; Zhou et al., 2012). A consensus naive state for hPSCs, however, remains somewhat elusive. Culture conditions that establish naive-like hPSCs yield slightly different transcriptional profiles (Huang et al., 2014). Uncertainty about whether naive hPSCs offer differentiation advantages over traditional primed hPSCs (Tara, 2014).
emphasizes the remaining importance of primed hPSCs as options for potential clinical applications.

Metabolites other than aKG have been shown to play a role in PSC self-renewal and differentiation. Removal of methionine, which provides methyl groups for DNA and histone methylation, potentiates PSC differentiation (Shiraki et al., 2014). Increased acetyl-coenzyme A delays PSC differentiation and histone acetylation and maintains expression of OCT4 (Mouscadet et al., 2013). Oxygen levels can enhance reprogramming to pluripotency or differentiation of hPSCs, depending on environmental context (Mathieu et al., 2014; Xie et al., 2014). The mESCs are dependent on threonine catabolism for histone and DNA methylation (Ghysen-Chang et al., 2013; Wang et al., 2009). Here, we investigate the role for aKG during primed PSC differentiation.

RESULTS

TCA Cycle Metabolite Production in hPSCs

Respiration is reduced in hPSCs compared to their differentiated counterparts, suggesting that TCA cycle metabolite production could be low (Zhang et al., 2011; Zhou et al., 2012). To examine the TCA cycle, stable isotope labeling experiments were performed in Essential 8 (EB) media promoting self-renewal or Essential 6 (E6) media encouraging differentiation (Figures S1A and S1B). Using the E8 or E6 system, culture media differ by only two factors that are excluded from the E6 media; basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β), ensuring differences in metabolism are due to different cell states. A shift in the oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) ratio confirmed a shift toward OXPHOS in E8 media differentiation (Figure 1A). Furthermore, glutamine withdrawal reduced oxygen consumption, implicating glutamine as a TCA cycle fuel in hPSCs (Figure 1B).

Despite a low OCR to ECAR ratio, hPSCs showed a robust contribution of [U-13C]-glucose into TCA cycle metabolites (Figure 1C). Glutamine withdrawal increased the glucose contribution to TCA cycle metabolites aKG, succinate, and malate in E6, but not in E8, culture conditions (Figure 1C). The mass isotopologue distribution (MID) of citrate indicates the contribution of [U-13C]-glucose to the TCA cycle, with m/z 2 and m/z 3 isotopologues indicating initial entry of glucose into the TCA cycle and m/z 4, m/z 5, and m/z 6 isotopologues indicating 13C glucose carbons that have cycled through one or two turns (Figure 1D). In glutamine-sufficient conditions, no differences in the citrate MID in E8 and E6 cultures were detected (Figure 1B), but glutamine withdrawal for 18 hr resulted in an increase in m/z 4 and m/z 6 citrate isotopologues in undifferentiated human embryonic stem cells (hESCs) (E8) (Figure 1F). Thus, glucose-derived carbons are retained through one or two turns of the TCA cycle in the absence of glutamine in self-renewing hPSCs. Consistent with this result, glutamine withdrawal led to a decrease in unlabeled aKG (m/z 0) in E8 conditions (Figure S1C).

The MID of citrate with [U-13C]-glutamine indicates the amount of citrate derived from glutamine after one turn (m/z 0) or two turns (m/z 4) of the TCA cycle (Figure 1G). Increased m/z 4 citrate was detected in E8 compared to E6 conditions, suggesting a lower contribution of glutamine to the TCA cycle in E8 differentiated hPSCs (Figure 1H). However, 40% of glutamate, which is generated directly from glutamine, was unlabeled in E6 conditions (Figure S1D).

Measurement of extracellular glutamate levels revealed net uptake of glutamate by cells cultured in E6 medium (Figure 1I). Detection of [U-13C]-glutamate uptake confirmed these results (Figure 1J), with increased conversion of glutamate into aKG occurring in differentiated cells (E8) only in the absence of glutamine (Figure 1K). The data suggest that glucose and glutamine are the major contributors to the TCA cycle in hPSCs; other metabolites, such as glutamate, fuel the TCA cycle in early differentiated hPSCs.

In proliferative cells, aKG-producing transaminases (TAs), which transfer amine groups from glutamate to α-keto acids to form amino acids (Figure S1B), are high activity (Costa et al., 2016). Glutamine inclusion in hPSC culture medium, which provides glutamate for TAs, increases aKG, alanine, and aspartate levels, products of these TAs (Figures S1F and S1G). To further study TA activity, [13C2]-glutamine was used to quantify the transfer of N from glutamate to amino acids (Figure 1L). Expression of glutamin pyruvic transaminase (GPTs) and glutamic oxaloacetic transaminase (GOTe) was confirmed in hPSCs, verifying their contribution to aKG production (Figure S1H). Robust aKG production in primed hPSCs prompted studies into a role for aKG in PSC differentiation.

aKG Accelerates Multi-lineage Primed PSC Differentiation

Neuroectoderm (NE) differentiation was induced in primed h1, h9, UCLA1, and hPSC2 hPSCs by dual SMAD inhibition (Chambers et al., 2009). The dim-aKG significantly increased the percentage of Pax6, an essential transcription factor for NE specification in humans (Zhang et al., 2016), expressing cells by day 4 of differentiation (Figures 2A–2C). MAP2+ and NESTIN+ positive cells modestly increased with dim-aKG treatment (Figures 2D and 2A). To determine whether this effect was lineage specific, endoderm differentiation was induced by high-concentration activin A exposure (StAmour et al., 2009). On day 2, dim-aKG significantly increased the percentage of Hh cells expressing SOX17, a definitive endoderm transcription factor (Figures S2A and S2A). Combined, the data support that aKG accelerates the early differentiation of multiple hPSC germ lineages.

NE differentiation was examined with added aKG, which unlike dim-aKG, is dependent on membrane transporters for uptake, and resulted in an increase in Pax6-positive cells (Figures 2D and 2F). In contrast to dim-aKG, which shows a dose-dependent increase in cell differentiation, aKG levels beyond 4 mM did not further stimulate NE differentiation (Figures 2D–2F). Added aKG and dim-aKG each increased intracellular aKG and TCA cycle metabolite levels, although only 12 mM dim-aKG reached significance (Figures 2H and 2I).

Consistent with prior results showing that dim-aKG supports naive mESC self-renewal (Carey et al., 2015), an increase in alkaline phosphatase staining was detected in naive mESCs incubated with dim-aKG during 48 hr of leukemia inhibitory factor (LIF) withdrawal (Figure S2D). Because dim-aKG accelerates primed PSC differentiation, we examined the role for aKG in primed mouse PSCs, or EpiScs. Addition of dim-aKG to EpiScs induced to differentiate by withdrawal of bFGF and activin A.

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Figure 1. Production of TCA Cycle Metabolites in hPSCs

(A) Ratio of OCR to BCAAR in hPSCs cultured in medium containing E8 or lacking E6 (EGF and TGF-β).

(B) OCR quantitated in hPSCs grown in media containing glutamine or with glutamine removed for 1 or 18 hr.

(C) Fractional contribution of [U-13C]glucose to [1-13C]glucose after 18 hr, quantitated by UPLC-MS.

(D) Schematic illustrating how the M/D of citrate from [U-13C]glucose reveals the contribution of glucose-labeled metabolites through multiple turns of the TCA cycle.

(E) M/D of citrate in hPSCs from [U-13C]glucose in conditions containing glutamine (E) or lacking glutamine (F).

(G) Schematic of [U-13C]glutamine labeling of the TCA cycle. The m+5 isotope of citrate can be produced by reductive carboxylation of glutamine (blue). The m+4 and m+3 isotope pools contain carbon derived from glutamine after one and two turns of the TCA cycle, respectively.

(H) M/D of citrate from [U-13C]glutamine in hPSCs cultured in E6 or E6 medium.

(I) Measurement of glutamate uptake from culture medium in hPSCs maintained in E6 medium or differentiated in E8 medium.

(J) The m+5 isotope of glutamate in hPSCs from [U-13C]glutamate indicates increased uptake of [U-13C]glutamate from the culture medium in differentiated cells (E8) grown with or without glutamine.

(K) The m+5 isotope pool of AKG in hPSCs grown in [U-13C]glutamate.

(1) The m+1 isotope pool of labeled amino acids in hPSCs grown with [1-13C]glutamine, reflecting the activities of multiple AKG producing TAs.

Data represent mean ± SD of at least three biological replicates. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. The p-values were determined by a paired two-tailed Student’s t test (A and J) or by one-way ANOVA (B) or two-way ANOVA (C, E, F, H, J, and K) with correction for multiple comparisons.
Figure 2. αKG Accelerates Differentiation of Primed PSCs

(A) Flow cytometry analysis of Pax6 transcription factor expression in UCLAl hESCs encouraged to differentiate into NE at 4 or 6 days. The figure shows 12 mM dm-α-KG-induced traces (blue) and control traces (black).

(B) Flow cytometry quantification of the percentage of PA69 expressing cells at 4 days of NE differentiation for H9, UCLAl, and H1 hESCs and HIPS2 human-induced pluripotent stem cells (iPSCs). H9, UCLAl, and H1 cells were incubated with (blue) or without (black) 12 mM dm-α-KG, and HIPS2 iPSCs were incubated with (blue) or without (black) 9 mM dm-α-KG. Lines connect pairs of independent biological replicates.

(C) Immunoblot of ectoderm markers Pax6 and MAP2C after 2, 4, and 6 days of NE differentiation of H1 hESCs.

(D and E) Flow cytometry of Pax6 expression in H9 hESCs on day 4 of NE differentiation, with indicated amounts of α-KG (α) or dm-α-KG (B) added to the culture medium.

(F and G) Percentage of H9 hESCs cells positive for Pax6 expression on day 4 of differentiation plotted against the concentration of added α-KG (F) or dm-α-KG (G). Lines connect independent biological replicates.

(H and I) UPLC-MS quantification of fold change of α-KG levels (H) and other TCA cycle metabolite levels (I) in H9 hESCs incubated with the listed concentrations of α-KG or dm-α-KG. Error bars represent SEM of three biological replicates.

(figure continued on next page)
(Geser et al., 2010) accelerated the rate of O ct inactivation (Figures 3J, 3K, and S2E–S2G). Therefore, sKG can accelerate differentiation of both mouse and human PSCs.

sKG has been shown to bind to and inhibit the mitochondrial ATP synthase subunit 3, leading to mTOR inhibition (Chin et al., 2014). To determine whether ATP synthase inhibition contributes to sKG-accelerated primed PSC differentiation, an OXPHOS uncoupling agent, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazine (FCCP), was used during NE differentiation. FCCP inhibits ATP production as a mitochondrial inner membrane protonophore that dissipates the H⁺ ion electrochemical gradient that runs the ATP synthase. That dim sKG accelerated NE differentiation in iPS cells incubated with FCCP (Figures S2H and S2I) suggested an alternative mechanism. Furthermore, hPSCs treated with an inhibitor of ATP synthase, oligomycin, during NE differentiation showed almost no PAX6 expression after 4 days, despite the addition of pyruvate and uridine to promote cell survival (Figures S2J and S2K (Birday et al., 2015; Sullivan et al., 2015)). Because ATP synthase inhibition delays or inhibits NE differentiation, which opposes the accelerating effect of dim sKG, we conclude that sKG does not accelerate differentiation of primed PSCs through inhibition of ATP synthase.

Succinate Accumulation Delays hPSC Differentiation

A second potential mechanism for sKG-accelerated hPSC differentiation is stimulation of epigenome-modifying dioxygenases. In this event, an sKG-dependent dioxygenase competitive inhibitor, such as succinate (Kato et al., 2012), would impair differentiation. Cell-permeable succinate, dimethyl succinate (dim), resulted in a decreased percentage of PAX6-expressing hPSCs during NE differentiation compared to hPSCs incubated with dim sKG alone (Figures S2L and S2M). Inhibition of succinate dehydrogenase A (SDHA), which converts succinate to fumarate, results in a chemical inhibitor, 3-nitropicolinic acid (NPA), or with small hairpin RNA (shRNA), should also cause succinate accumulation (Figure 3A). NPA treatment decreased the sKG-to-succinate ratio 34-fold, with a 1.4-fold mean increase in succinate and 2.4-fold mean decrease in sKG (Figures 3B and S3A). NPA delayed NE, as indicated by PAX6 expression (Figures 3C and S3B–S3D), and the loss of pluripotency marker SSEA3 in hPSCs differentiated into NE (Figure S3E). Knockdown of SDHA, confirmed by immunoblot and reduced OCR (Figures S3F–S3H), also delayed PAX6 and MAP2B expression during NE differentiation, which was rescued by dim sKG (Figures 3D, 3E, and S3I).

We further assessed the role of succinate in differentiation by embryoid body (EB) formation, which contrasts with lineagedirected differentiation by removal of LIF/FGF rather than by addition of supplements or inhibitors. OCT4 was almost eliminated in EBs expressing a scrambled shRNA, whereas shRNA targeting SDHA maintained elevated OCT4 expression (Figures S3J and S3K). Validating these results, inhibition of SDHA with NPA significantly decreased succinate expression in EB differentiation compared to control (Figure S3L).

TA Inhibition Delays hPSC Differentiation

To decrease sKG levels, chemical inhibitors of sKG-producing TAs were used (Figure 3A). Aminooxyacetic acid (AOA), a pan-TA inhibitor, reduced 15N transfer from [15N]glutamine to alanine, aspartate, isoleucine, serine, and methionine in hPSCs (Figure S3M). L-cycloserine (cyclo), a GPT inhibitor, decreased 15N transfer to alanine in hPSCs treated with cyclo (Figure S3M). Both inhibitors caused a significant decrease in sKG levels and other TCA cycle metabolite levels, but they had no effect on basal respiration (Figures 3F, S3N, and S3O). AOA impaired Pax6 and MAP2B activation during NE differentiation (Figures 3G, 3H, and S3P). A mixture of non-essential amino acids (NEAA) inhibited alanine and cyclo oxidation, indicating a lack of effect on AOA treatment, whereas supplementation with dim sKG rescued the block in differentiation caused by AOA (Figures 3G, 3H, and S3P). Cyclo caused a less dramatic, but significant, decrease in the percentage of MAP2B-positive cells, corresponding to its relatively smaller effect on sKG levels compared to AOA (Figures 3I and 3J). Supplementation with dim sKG restored MAP2B levels in cyclo-treated cells (Figures 3I and 3J). Therefore, decreased sKG levels cause a delay or inhibition of directed differentiation.

To determine whether TCA cycle flux affects differentiation, dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), was used. PDK inhibits pyruvate dehydrogenase (PDH) activity. Therefore, DCA increases PDH activity and glucose flux into the TCA cycle, which elevates electron transport chain (ETC) activity (Figure 3A). Low levels of DCA increased PAX6-positive cells on day 4 of NE differentiation, whereas higher DCA levels had no effect (Figures 3B and S4C). Therefore, dim sKG enhanced differentiation at 0 and 3 mM DCA but not at 1 mM (Figures S4B and S4C). These results do not support changes in TCA cycle flux as a mechanism for sKG in accelerated hPSC differentiation. Rather, the data indicate that succinate delays and sKG promotes the initial differentiation of primed PSCs, most likely through actions on sKG-dependent dioxygenases.

sKG/Succinate Regulates the Epigenome of Differentiating hPSCs

To evaluate the role of sKG-dependent dioxygenases during hPSC differentiation, an inhibitory sKG mimetic, dimethylglyoxine (dimox) was used. Exposure to dimox inhibited PAX6 expression at concentrations that did not affect cell number (Figures 4A, 4B, and S4D). To assess the effect of the sKG on TET enzymes, dot blots were performed and levels of 5-hydroxymethylcytosine (hmC) and 5-methylcytosine (mC) in DNA were measured from hPSCs after 4 days of NE differentiation. Although dim sKG exposure caused a 2-fold increase in the bimC-to-mC ratio, NPA caused a significant decrease in this ratio (Figures 4C, 4D, and S4E). These data suggest a significant role for TET enzymes in sKG accelerated differentiation of primed hPSCs. To evaluate sKG regulation of JHDM2, histone
Figure 3. Decrease in the αKG-to-Succinate Ratio Delays hiPSC Differentiation

(A) Inhibition or depletion of SDHA causes succinate accumulation. Inhibition of TAs, such as GPT or GOT, results in a decrease in αKG levels. Both of these manipulations cause a decrease in the αKG-to-succinate ratio, which is predicted to inhibit αKG-dependent dioxygenases.

(B) The αKG-to-succinate ratio in control (black) or 50 μM NPA-treated (red) hiPSCs for 18 hr, quantified by UPLC-MS.

(C) Immunoblot of OCT4 and PAX6 at indicated time points of hiPSC differentiation with or without 50 μM NTA for 5–9 hiPSCs. β-TUB, β-tubulin. (D) and (E) Flow cytometry analysis of PAX6 expression in hiPSCs expressing shRNA targeting SDHA or scramble control shRNA treated with dm-αKG where indicated at day 4 of NE differentiation.

(F) UPLC-MS quantification of histone change in αKG levels with TA inhibitors AOA and cyclo compared to drug control controls.

(legend continued on next page)
lysine 4 trimethylation (H3K4me3) and histone lysine 27 trimethylation (H3K27me3) were assessed by intracellular flow cytometry. In general, NPA treatment during NE differentiation in UCLA1 hESCs led to an increase in histone marks, whereas dim-αKG led to a decrease (Figures 4E–H and S4F–S4J). Analysis of an array of histone post-translational modifications by immunoblot revealed an overall repressive effect of dim-αKG on global lysine trimethylation but little effect on global monomethylation or acetylation marks (Figure 4l).

Overall, the data suggest that an increased αKG-to-succinate ratio accelerates, and a decreased αKG-to-succinate ratio retards, initial primed PSC differentiation by threed cofactor/inhibitor activities on epigenome remodeling enzymes, including TET enzymes and JHDMs. This interpretation is consistent with the proposed αKG/succinate mechanism for maintained naïve pluripotency in mESCs through epigenetic regulation (Casey et al., 2015), only with an inverse cellular outcome in the primed pluripotent state with induced differentiation.

**DISCUSSION**

This study reveals an unanticipated differentiation-promoting role for αKG in primed PSCs. Results have shown that αKG supports self-renewal of naïve mESCs, potentially by promoting the demethylation of histones and DNA (Casey et al., 2015). Our data support a similar mechanism with an opposite outcome in the context of primed PSCs induced to differentiate. Consistent with a context-specific role for αKG, TET enzymes and JHDMs have dual roles in the self-renewal and differentiation of mESCs. TET1 promotes reprogramming to naïve pluripotency, whereas triple knockout of the TET enzymes impedes differentiation (Costa et al., 2013; Davlatov et al., 2014). JHDMs are a large class of enzymes that have functions in both naïve pluripotency and differentiation. For example, JMJD3 and UTX promote naïve pluripotency (Casey et al., 2015), whereas Lsd1 is involved in neural differentiation (Schmitz et al., 2011). The data suggest that αKG enhances the activity of a large family of enzymes that may both maintain low DNA and histone methylation levels, favoring the naïve PSC state, and promote epigenome remodeling during induced primed PSC differentiation. These opposing outcomes are consistent with the state-dependent effects of oxygen levels in maintaining pluripotency or promoting differentiation (Mathieu et al., 2014; Xie et al., 2014). Both DNA and histone modification levels are lower in naïve compared to primed PSCs (Hackett and Surani, 2014; Leitch et al., 2018). These epigenome differences, along with metabolome differences

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(i–j) Flow cytometry analysis of PAX6 and MAP2 expression in hESCs on day 2 of NE differentiation co-incubated with AOA (0 and 50 μM) or 50 μM cyclo (i and j) supplemented with 1 μM dim-αKG, 1 μM N6EAAs, or both where indicated. Data are represented as box plots at least three biological replicates. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. The p values were determined by unpaired Student's t test (i and j) or by one-way ANOVA (i) or two-way ANOVA (j) with correction for multiple comparisons.
between naive and primed pluripotent states, could support a differential role for sGK in the study. Our results suggest a model for sGK promotion of induced differentiation by primed PSCs from demethylation reactions that help silence pluripotency genes and activate lineage-specific genes to accelerate induced multi-lineage differentiation. A key goal of hPSC research is to develop more mature and functional cells for regenerative medicine. Increased extracellular sGK could be useful for improving primed state PSC differentiation.

EXPERIMENTAL PROCEDURES

Standard procedures were followed for immunoblotting, confocal microscopy, and qRT-PCR analysis, as described in the Supplemental Experimental Procedures. Maintenance of cell viability, immunodepletion of ESCs/EBs, and Biological Safety was ensured by K.H. and M.A.T.

Cell Culture

P10 hPSCs were passaged onto feeder-free culture (Felber Scientific) in mTeSR1 medium with Gentle Cell Dissociation Reagent (STEMCELL) for most experiments. For experiments performed in EBs or EB-media (STEMCELL), hPSCs were switched from mTeSR1 to appropriate media at passage. EBs were grown in feeder-free conditions in medium containing bFGF and 10 ng/mL LIF. Further details are provided in the Supplemental Experimental Procedures.

Seahorse Measurements

OCR and ECAR assays were performed as previously described (Zhang et al., 2015). The hPSCs were plated onto an XF96 microplate (Seahorse Biosciences) at 10^5 or 10^6 cells/well in 10 µL of XF24 Medium (Seahorse Biosciences) supplemented with 17.5 mM glucose. Cell metabolic rates were measured using an XF96 Extracellular Flux Analyzer (Seahorse Biosciences). Basal respiration was determined by equalizing OCR before and after the addition of 1 µM rotenone and 1 µM antimycin A (Sigma).

Metabolite Extraction and Analysis

Cellular metabolites were extracted with 90% ice-cold methanol, and ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS) measurements of metabolite levels were performed and analyzed as previously described (Thu et al., 2014). Details are provided in the Supplemental Experimental Procedures.

Glutamate Uptake

Levels of glutamate in culture media were measured using a BioPlex Pro Basic Analyzer (Bio-Rad Medical). Details are provided in the Supplemental Experimental Procedures.

NE Differentiation

Differentiation was performed as previously reported (Chambers et al., 2009). Details are provided in the Supplemental Experimental Procedures.

Flow Cytometry Analysis

Cells were collected with Gentle Cell Dissociation Reagent and processed using the CytoFLEX Cytometer Kit (BD Biosciences). Cells were analyzed with or without LSRRI and LSRFB (BD Biosciences).

Dot Blot Analysis and Quantification

DNA was collected with the QIAGEN Blood and Tissue Kit (QIAGEN) and quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). DNA was denatured at 95°C for 5 min, put on ice, and neutralized by adding ammonium acetate to a final concentration of 0.12 M. Then, 400 ng of each sample were spotted on Amersham Hybond-N+ (Fisher) nylon membranes and baked at 80°C for 2 hr. Membranes were blocked with 5% skim milk for 3 hr and incubated with primary antibody overnight. The immunoblot procedure was followed. Blots were imaged with an Odyssey Fc and quantified with Image Studio v.5.2.5 (LI-COD Biosciences).

Statistical Analysis

Values are presented as mean ± SEM. Data were analyzed with Prism (GraphPad). Parameters were analyzed using two-tailed Student’s t test. Other data were analyzed using one-way or two-way ANOVA with correction for multiple comparisons. In all cases, p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.07.062.

AUTHOR CONTRIBUTIONS

T.T., A.C.C., S.L.E., and D.B. performed experiments. L.L. and K.H. provided key reagents and expertise. T.T., J.H., T.G.O., D.B., and M.A.T. participated in setting goals for designing the study and analyzing data. T.T. and M.A.T. wrote the paper with help from T.G.O. and D.B. Validation of cell line and cell line and cell line compliance, including ESCs, EBs, and Biological Safety, was overseen by K.H. and M.A.T.

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REFERENCES


Supplemental Information

α-Ketoglutarate Accelerates the Initial Differentiation of Primed Human Pluripotent Stem Cells

Tara TeSlaa, Andrea C. Chaikovsky, Inna Lipchina, Sandra L. Escobar, Konrad Hochedlinger, Jing Huang, Thomas G. Graef, Daniel Braas, and Michael A. Teitell
**Figure S1. Production of TCA cycle metabolites in hPSCs (related to Figure 1).**

(A) Expression of POU5F1 (encoding OCT4 protein) and NANOG core pluripotency transcription factors in H9 hESCs grown in E8 medium and after 4 days of differentiation in E6 medium, which lacks bFGF and TGFβ, quantified by qRT-PCR. Data represents technical replicates of a representative experiment.

(B) Immunoblot of OCT4 levels in H9 hESCs cultured in E8 or E6 medium at 4 days.
(C) Mass isotopologue distribution (MID) of αKG from [U-13C] glucose incorporation. Black and white bars represent H9 hESCs cultured with glutamine (+Q) in E8 medium or differentiated in E6 medium, which lacks bFGF and TGFβ, respectively, for 16h. Dark and light gray bars represent H9 hESCs cultured without glutamine (+Q) in E8 medium or differentiated in E6 medium, respectively.

(D) MID of glutamine and glutamate from [U-13C] glutamine incorporation. Black and white bars represent H9 hESCs cultured in E8 medium or differentiated in E6 medium, which lacks bFGF and TGFβ, respectively, for 16h.

(E) Schematic depicting αKG production from glutamate by transaminase enzymes.

(F, G) UHPLC-MS quantification of fold changes in metabolites in H9 hESCs cultured with (+Q) or without (-Q) glutamine for 18h.

(H) qRT-PCR quantification of transaminase gene expression on days 0, 2, and 4 of neuroectoderm (NE) differentiation from H9 hESCs.

Error bars represent mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001.
P values were determined by paired two-tailed Student’s t-test (F, G), or by two-way ANOVA with correction for multiple comparisons (C, D).
Figure S2. αKG accelerates differentiation of primed pluripotent stem cells
(related to Figure 2).

(A) Quantification of flow cytometry analysis of ectoderm marker NESTIN in H9 hPSCs after 4 days of NE differentiation.

(B) Flow cytometry analysis of SOX17 definitive endoderm transcription factor and SSEA3 surface pluripotency biomarker expression at 2 days of endoderm differentiation for H9 hESCs.

(C) Quantification of flow cytometry analysis of the percent of cells positive for SOX17 transcription factor expression at 2 days of endoderm differentiation in H9 hESCs.

(D) Flow cytometry analysis of Alkaline Phosphatase (AP) expression in v6.5 mESCs differentiated by LIF withdrawal for 48h with or without 4mM dm-αKG.

(E, G) Flow cytometry analysis of Oct4 expression in EpiSC-9 cells after 1 day of differentiation by growth factor withdrawal. The dotted black line represents Oct4 expression in undifferentiated EpiSC-9 cells, whereas the solid black and blue lines represent differentiated control cells and cells differentiated with 12mM dm-αKG, respectively.

(F) Immunofluorescence of Oct4 and DNA stain Hoechst 33528 in undifferentiated EpiSC-9 cells and EpiSC-9 cells differentiated by growth factor withdrawal for 24h. Scale bars are 50μm.

(H, I) Flow cytometry analysis of PAX6 expression in H9 hESCs differentiated into NE. Dm-αKG plus 300nM FCCP (blue) exposure increased PAX6 expressing cells compared to hESCs differentiated with 300nM FCCP (gray) or DMSO (black) only.
(J) Coomassie brilliant blue stain to quantify H9 hESC survival after differentiation into NE with or without the indicated amount of added oligomycin.

(K) Flow cytometry analysis of PAX6 expression in H9 hESCs differentiated into NE either with exposure to 1nM oligomycin (red) or 0.01% ethanol carrier control (black).

(L-M) Flow cytometry analysis of PAX6 expression in H9 hESCs differentiated into NE at 4 days under control conditions, with 12mM dm-\(\alpha\)KG, or with both 12mM dm-\(\alpha\)KG and 16mM dimethyl succinate (dms). Percent PAX6 expressing H9 hESCs at 4 and 6 days of NE differentiation is shown in (M).

Error bars represent mean ± SD. *p<0.05, **p<0.001.

P values were determined by paired two-tailed Student's t-test (C, G, I), or by two-way ANOVA with correction for multiple comparisons (M).
Figure S3. Decrease in the αKG/succinate ratio delays hPSC differentiation (related to Figure 3).

(A) UHPLC-MS quantification of succinate (succ) and αKG in H9 hESCs treated with 10μM NPA, an inhibitor of SDHA.

(B-D) Flow cytometry plots (B) and quantification (C, D) of cells expressing PAX6 transcription factor in H9 (B, C) and UCLA1 (D) hESCs differentiated into NE for 4 or 6 days, as indicated. Red lines and data points indicate co-incubation with 50μM NPA. (C, D) Lines connect independent biological replicates.

(E) Flow cytometry analysis of SSEA3 pluripotency biomarker at 4 days of NE differentiation for UCLA1 hESCs. Flow cytometry quantification of the percent SSEA3 expressing UCLA1 hESCs incubated with (red) or without (black) 50μM NPA addition. Lines connect pairs of independent biological replicates.

(F, G) Immunoblot for SDHA in H9 (F) and H1 (G) hESCs expressing shRNA targeting SDHA or a scramble control. β-TUB, β-TUBULIN; H3, histone 3.

(H) Basal respiration of H9 scramble control and SDHA knockdown hESCs quantified by Seahorse extracellular flux (XF) Analyzer. OCR, oxygen consumption rate.

(I) Flow cytometry analysis of MAP2B expression in H9 hESCs expressing scramble (control) shRNA, shRNA targeting SDHA, or treated with 12mM dm-αKG after 4 days of NE differentiation.

(J-K) Immunoblot of OCT4 expression in H1 embryoid bodies (EBs) (J) and H9 EBs (K) at 21 days of differentiation expressing either a shRNA targeting SDHA or a control (scr) shRNA.
(L) Immunoblot of OCT4 expression in H9 EBs at 21 days of differentiation with or without SDHA inhibitor NPA.

(M) M + 1 isotopologue of amino acids in H9 hESCs cultured with [15N2] glutamine indicating the activities of various αKG-producing transaminases. hESCs were treated with 0.5mM aminoxyacetic acid (AOA), 0.05% DMSO, or 50μM L-cycloserine (cyclo), as indicated.

(N) UHPLC-MS quantification of fold change in TCA cycle metabolites relative to drug carrier control for H9 hESCs treated with either 0.5mM AOA or 50μM cyclo. Error bars represent SEM of 3 biological replicates.

(O) OCR quantified in H9 hESCs treated with 0.5mM AOA, 0.05% drug carrier DMSO, or 50μM cyclo, as indicated.

(P) Flow cytometry analysis of MAP2B expression in H9 hESCs after 4 days of NE differentiation treated with AOA, 12mM dm-αKG, or 1mM non-essential amino acids (NEAA), as indicated.

Error bars represent mean ± SD unless otherwise indicated. *p≤0.05; **p≤0.01; ***p≤0.001.

P values were determined by paired two-tailed Student’s t-test (A, C, D, E, H), by one-way analysis of variance (ANOVA) (O) with correction for multiple comparisons or by two-way analysis of variance (ANOVA) with correction for multiple comparisons (N, M).
Figure 4. Changes in the α-KG/succinate ratio alter DNA hydroxymethylation and histone methylation (related to Figure 4).

(A) Ratio of oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) in H9 hESCs treated with pyruvate dehydrogenase kinase (PDK) inhibitor, dichloroacetate (DCA). Error bars represent mean ± SD.
(B, C) Flow cytometry analysis of PAX6 expression (B) and quantification of percent PAX6 positive cells (C) on day 4 of NE differentiation in H9 hESCs treated with DCA or 12mM dm-αKG, as indicated. Error bars represent SEM, n=3.

(D) Coomassie brilliant blue stain to quantify H9 hESCs after differentiation into NE with the indicated concentrations of dimethylxalylgycine (dmxog).

(E) Dot blot for 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mc) in DNA of UCLA1 cells differentiated into NE for 4 days under control conditions or with the addition of 12mM dm-αKG.

(F, G) Flow cytometry analysis of H3K4me3 shown as mean fluorescent intensity (MFI) for H9 hESCs at 6 days of NE differentiation incubated without (black) or with 12mM dm-αKG (blue) or 50μM NPA (red). Lines connect pairs of independent biological replicates.

(H, I) Flow cytometry analysis of H3K27me3 shown as MFI for H9 hESCs at 6 days of NE differentiation incubated without (black) or with 12mM dm-αKG (blue) or 50μM NPA (red). Lines connect pairs of independent biological replicates.

(J) Immunoblot of H3K4me3 and H3K27me3 in H9 hESCs differentiated into NE at 4 days with GSK-J4, a histone demethylase inhibitor (control) or the indicated amount of NPA incubation.

*p≤0.05; **p≤0.01.

P values were determined by one-way (G, I) or two-way (C) ANOVA with correction for multiple comparisons.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

hPSC Culture
Primed hPSCs (H9, H1, UCLA1, HIPS2) (Lowry et al., 2008; Perez et al., 2012; Thomson et al., 1998) were maintained on CF-1 irradiated MEF feeder cells (Global Stem) in DMEM/F12 supplemented with 20% KnockOut serum replacement (Life Technologies), 1% Glutamax, 1% Pen/Strep, 1% non-essential amino acids, 0.1mM 2-mercaptoethanol, and 10ng/ml bFGF (R&D Systems) and passaged with 1mg/ml collagenase IV (Life Technologies). For experiments, hPSCs were shifted onto feeder-free Matrigel (Fisher) in mTeSR1 medium (Stem Cell Technologies) and passaged with Gentle Cell Dissociation Reagent (Stem Cell Technologies).

mEpiSC Culture and Differentiation
Two mouse EpiSC lines were used. One mouse EpiSC line was a gift from Paul Tesar (Najm et al., 2011; Tesar et al., 2007) and was labeled EpiSC-9, whereas the other line was isolated as previously reported (Chenoweth and Tesar, 2010) and labeled mEpiSC-1. Briefly, mouse embryos were collected at E6.5. Embryonic fragments were isolated in FHM Hepes-buffered medium (Millipore), and the visceral endoderm was dissociated by 5 min incubation in 0.5% trypsin and 2.5% pancreatin. Epiblasts were plated on fibronectin in EpiSC media (DMEM/F12 with 0.5% N-2 Supplement (Fisher), 1% B27 supplement (Fisher), 50μg/ml bovine serum albumin fraction V (Fisher), 12ng/ml FGF2 (Peprotech), 20ng/ml Activin A (Peprotech), 0.11 nM 2-mercaptoethanol (Fisher), 1%
non-essential amino acids, 1% GlutaMax (Fisher), and 1% Pen/Strep) and passaged two days later.

mEpiSCs were maintained on culture dishes coated with human plasma purified fibronectin (EMD Millipore) in EpiSC media, which was replaced daily during maintenance of pluripotency. Differentiation was performed by removing FGF2 and Activin A from the culture medium.

**mESC Culture and Differentiation**

Naïve v6.5 mESCs were maintained on MEFs in standard Serum/LIF culture conditions. Briefly, serum/LIF medium contained Knockout DMEM (ThermoFisher), 15% ES-qualified FBS (ThermoFisher), 1% Pen/Strep, 1% NEAA, 1% GlutaMax (ThermoFisher), 50μM β-mercaptoethanol (ThermoFisher), and 10ng/ml hLIF (Peprotech). To passage and plate for differentiation, mESCs were dissociated with 0.25% trypsin. For differentiation, mESCs were plated onto gelatin coated plates and cultured in serum/LIF medium lacking LIF.

**Sample Preparation for UHPLC-MS Analysis**

Media of primed H9 hESCs and differentiated H9 hESCs, grown in Essential 6 and Essential 6 medium (Stem Cell Technologies), respectively, was changed 18h prior to metabolite extraction into DMEM/F12 medium without pyruvate, glucose, and glutamine (US Biologicals) supplemented with 1% StemPro hESC supplement (Life Technologies), 1.8% BSA, 0.1mM 2-mercaptoethanol, 10ng/ml bFGF, 17.5 mM [U-13C] glucose (Cambridge Isotope Laboratories) or unlabeled glucose (Sigma). For
experiments including glutamine, 2mM glutamine (Life Technologies), [U-\textsuperscript{13}C] glutamine (Cambridge Isotope Laboratories), or \textsuperscript{15}N\textsubscript{2} glutamine was added to the medium. For experiments with aminoxyacetic acid (AOA) and L-cycloserine (cyclo), cells were incubated with medium containing or lacking \textsuperscript{15}N\textsubscript{2}-glutamine for 6h. For glutamate tracing, 0.5 mM [U-\textsuperscript{13}C] glutamate (Cambridge Isotope Laboratories) was added. Metabolite extraction and mass spectrometry analysis was performed as previously described (Thai et al., 2014). To extract intracellular metabolites, cells were briefly rinsed with cold 150 mM ammonium acetate (pH 7.3), followed by addition of 1 ml cold 80% MeOH in water, whereupon cell suspensions were transferred into Eppendorf tubes. To the cell suspensions, 10 nmol D/L-norvaline were added and rigorously mixed three times on ice followed by centrifugation (1.3 x 10\textsuperscript{4} rpm, 4\textdegree C). The supernatant was transferred into a glass vial, metabolites dried down under vacuum, and finally resuspended in 70% acetonitrile. For mass spectrometry analysis of a sample, 5 \textmu l was injected onto a Luna NH\textsubscript{2} (150 mm x 2 mm, Phenomenex) column. Samples were analyzed with an UltiMate 3000RS LC (Thermo Scientific) coupled to a Q Exactive mass spectrometer (Thermo Scientific). The Q Exactive was run with polarity switching (+3.00 kV / -2.25 kV) in full scan mode with an m/z range of 70-1050. Separation was achieved using A) 5 mM NH\textsubscript{4}AcO (pH 9.9) and B) ACN. The gradient started with 15% A) going to 90% A) over 18 min, followed by an isocratic step for 9 min and reversal to the initial 15% A) for 7 min. Metabolites and isotopomers were quantified with TraceFinder 3.1 using accurate mass measurements (± 3 ppm) and retention times. For isotopologue distribution measurements, data was corrected for naturally occurring \textsuperscript{13}C as described previously (Moseley, 2010). Data analysis was performed using the formula statistical
language R. Fractional contributions were calculated using the formula described previously, where \( m_i \) denotes the intensity of the isotopologue, and \( n \) marks the number of carbons in the metabolite (Fendt et al., 2013).

**Glutamate uptake measurements**

Glutamate levels in cell culture media were measured using a BioProfile Basic Analyzer (Nova Biomedical). hPSCs were cultured in 6-well plates and either maintained in E8 media or differentiated in E6 media for 4 days. On day 4 the media was changed to DMEM/F12 without pyruvate, glucose, and glutamine (US Biologicals) with 1% StemPro hESC supplement (Life Technologies), 1.8% BSA, 0.1 mM 2-mercaptoethanol, 10ng/ml bFGF, 17.5 mM unlabeled glucose (Sigma) and 2 mM glutamine (Life Technologies).

After 18h, 1 ml of media was removed from each sample and analyzed on the Nova BioProfile analyzer. Measurements were normalized to protein concentration measured by BCA assay (Pierce).

**Chemicals and Antibodies**

3-nitroproionic acid (NPA), dimethyl succinate (dms), dimethyl 2-oxoglutarate (dm-\( \alpha \)KG), oligomycin, L-cycloserine (cyclo), dimethylxaloylglycine (dmog), and dichloroacetate (DCA) were purchased from Sigma. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was from Abcam. Aminoxyacetic acid hydrochloride (AOA) was purchased from MP Biomedicals. Stock dm-\( \alpha \)KG was added to DMEM/F12 at a concentration of 800 mM and the pH was adjusted to 7.2 before
adding to culture medium. GSK-J4 was from Tocris. Antibodies used in this study are listed in the table below.

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**Lentivirus-mediated shRNA Knockdown**

pLKO.1 vectors containing shRNA targeting SDHA RNA transcripts were purchased from Sigma-Aldrich (TRCN0000028085 (shSDHA#1), TRCN0000028043 (shSDHA#2), and TRCN0000028093 (shSDHA#5). Vectors were transfected along with packaging vectors pCMV-dR8.9 and pCMV-VSVG, into 293FT cells using Fugene HD (Promega). The next day sodium butyrate was added for 8h followed by addition of fresh media. To harvest lentivirus, media was collected 48h and 72h after transfection. Virus was concentrated using a Lenti-X Concentrator (Clontech) and suspended in OptiMEM (Life Technologies). For hESC transduction, colonies on matrigel were transduced with 25 µl of 1×10⁷ TU/ml of viral particles and 7 µg/ml polybrene for 8h on day 1. Transduction was repeated on days 2, 4, and 5. Selection with 1µg/ml puromycin started on days 7-10 post first transduction.

**Ectoderm and Endoderm Differentiation**

hPSCs were passaged as single cells using Gentle Cell Dissociation Reagent (Stem Cell Technologies) and 2 x 10⁶ cells were plated per well in a six-well plate in mTeSR1 media with 10 µM Y-27632 (BioPioneer). NE differentiation was performed as previously reported (Chambers et al., 2009; Shiraki et al., 2014). Briefly, media was changed 1 day after plating (on day 0) to RPMI 1640 (Life Technologies) supplemented with 2% B27 supplement (Life Technologies), 1% N-2 supplement (Life Technologies), 10 µM SB431542 (Stemgent), 0.2 µM Dorsomorphin (Stemgent), 1% glutamax, and 1% non-
essential amino acids (ThermoFisher). In AOA and cyclo experiments, non-essential amino acids (ThermoFisher) were excluded except when indicated. Media was changed daily throughout differentiation. Endoderm differentiation was performed as previously described (D'Amour et al., 2005). Briefly, media was changed 1 day after plating (on day 0) to RPMI supplemented with 0.1% FBS (Omega), 1% glutamax (Life Technologies), and 100ng/ml Activin A (Life Technologies). Two days after plating (day 1), the concentration of FBS in the medium was increased to 2%. Treatment with NPA, dm-αKG, dms, FCCP, oligomycin, AOA, cyclo, or DCA was started on day 0 of differentiation and maintained in culture medium until cells were collected for analysis.

**Embryoid Body Formation**

hESCs were dissociated into single cells by incubating in Gentle Cell Dissociation Reagent (Stem Cell Technologies) for 15 min. Cells were plated in Aggrewell Media (Stem Cell Technologies) with 10 μM Y-27632 (BioPioneer) in Aggrewell plates (Stem Cell Technologies). The next day, embryoid bodies were transferred to a 10 cm polystyrene petri dish (not tissue culture treated) and grown in DMEM/F12 (Life Technologies) supplemented with 20% KnockOut serum replacement (Life Technologies), 1% Glutamax, 1% Pen/Strep, 1% non-essential amino acids, and 0.1 mM 2-mercaptoethanol. Media was changed every 2-3 days throughout differentiation.

**Flow cytometry**

Primed and differentiated hPSCs were incubated at room temperature for 15 min in Gentle Cell Dissociation Reagent (Stem Cell Technologies). Cells were then centrifuged
at 300 × g and suspended in 100 μl BD Perm/Fix Buffer (BD Bioscience). Cells were incubated for 15 min on ice. Next, cells were washed twice with 1 ml 1 × BD Perm/Wash Buffer (BD Bioscience). After the second wash, cells were incubated in 50 μl 1 × BD Perm/Wash Buffer containing primary antibodies or conjugated antibodies for 45 min on ice. Cells were then washed with 1 ml 1 × BD Perm/Wash Buffer. When secondary antibodies were required, cells were suspended in 50 μl 1 × BD Perm/Wash Buffer with secondary antibodies (1:1000). Cells were washed once with 1 × BD Perm/Wash Buffer and suspended in 300 μl 2% FBS/PBS for analysis. Data collection was performed on either an LSR II (BD Bioscience) or an LSR Fortessa (BD Bioscience). Data analysis was performed with FlowJo software (Treestar).

**Immunoblot Analysis**

Cells were harvested in SDS buffer (40 mM Tris-HCl pH 6.8, 3% glycerol, 1% SDS).

Protein concentrations were quantified using a BCA assay (Pierce). When HRP-conjugated secondary antibodies were used, immunodetection was performed with ECL reagent (GE Healthcare) or SuperSignal West Pico Chemiluminescent Substrate (Life Technologies). Alternatively, IRDye-conjugated secondary antibodies were used and images captured with an Odyssey Fc (Licor).

**Immunofluorescence Confocal Microscopy**

Cells were grown on 4-well μ-slides Ph+ (ibidi) coated with fibronectin (EMD Millipore).

Cells were then fixed for 10 min with 4% formaldehyde in 1× PBS, pH 7.4, and permeabilized for 15 min with 0.5% Triton X-100 in PBS. Incubation with primary
antibodies was performed overnight in 1% BSA in 1x PBS, pH 7.4. After incubation with Alexa-Fluor conjugated secondary antibodies, images were acquired with a LSM 780 confocal microscope (Zeiss).

**Coomassie Brilliant Blue Staining**

Cells were fixed for 10 min with 4% formaldehyde in 1x PBS, pH 7.4. After PBS washes, coomassie brilliant blue stain solution (50% methanol, 10% acetic acid, 0.05% Brilliant blue R 250 (Sigma) in water) was added to the cells and shaken for 10 min.

Cells were washed with 1x PBS, pH 7.4, and imaged.

**qRT-PCR**

Cultured cells were harvested with TRIzol reagent (Life Technologies). RNA was extracted according to the manufacturer's instructions. RNA was converted to cDNA with iScript (Bio-Rad). qRT-PCR was performed on a LightCycler480 (Roche) using SYBR green (Roche). The primers used are shown in the table below.

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SUPPLEMENTAL REFERENCES


Chapter 4:

Low oxygen tension inhibits human pluripotent stem cell differentiation
INTRODUCTION

Hypoxic conditions can promote self-renewal of human pluripotent stem cells (hPSCs) and reprogramming of somatic cells to pluripotency (Ezashi et al., 2005; Forristal et al., 2013; Forristal et al., 2010; Forsyth et al., 2006; Mathieu et al., 2013; Mathieu et al., 2014; Prasad et al., 2009; Yoshida et al., 2009; Zhou et al., 2012). One mechanism by which low oxygen tension is thought to promote pluripotency is through hypoxia inducible factor 1α (HIF1α) and hypoxia inducible factor 2α (HIF2α). HIF2α has been shown to promote expression of stem cell transcription factor Oct4 (Covello et al., 2006), while both HIF1α and HIF2α can promote a shift to glycolysis during reprogramming (Mathieu et al., 2014; Prigione et al., 2014). The stability of HIF enzymes in response to oxygen tension is regulated by prolyl hydroxylase enzymes that depend on O2, Fe2+, ascorbate and α-ketoglutarate (αKG) for their activity (Bruick and McKnight, 2001; Jaakkola et al., 2001; Yu et al., 2001).

Oxygen tension can also play a role in promoting the differentiation process. Low oxygen environments favor ectoderm differentiation in hPSCs and promote gliogenesis in human neural progenitor cells (Xie et al., 2014). Furthermore, hypoxia can improve human embryonic stem cell (hESC) differentiation into retinal progenitors and endothelium (Bae et al., 2011; Prado-Lopez et al., 2010). In the mouse system, physiologic hypoxia has been shown to be required for proliferation and survival of hematopoietic precursors during development (Adelman et al., 1999). In summary, low oxygen environments can both enhance self-renewal of hPSCs and promote their differentiation in some contexts.
TCA cycle metabolite αKG can promote differentiation of hPCs probably through the activation of αKG-dependent dioxygenases including Jumonji domain containing histone demethylases (JHDMs) and ten-eleven translocation (TET) methylcytosine oxidases (TeSlaa et al., 2016). Like HIF prolyl hydroxylases, these enzymes are also dependent on O₂, Fe²⁺, ascorbate and α-ketoglutarate (αKG), and can be inhibited by metabolites with chemical structures similar to αKG, such as succinate and L-2-hydroxyglutarate (L-2HG). L-2HG has recently been shown to be produced in low oxygen environments through promiscuous activity of lactate dehydrogenase A (LDHA) and malate dehydrogenase (MDH) (Intlekofer et al., 2015; Intlekofer et al., 2017; Oldham et al., 2015; Xu et al., 2011). Therefore, hypoxia may affect self-renewal and differentiation through three mechanisms: 1) direction inhibition of αKG-dependent dioxygenases, including JHDMs, TET, and PHDs due to low oxygen availability, 2) inhibition of αKG-dependent dioxygenases by L-2HG or 3) stabilization of HIF leading to gene expression changes.

In this chapter, the role of hypoxia in hPSC differentiation is investigated. We show that very low oxygen environments cause HIF-independent inhibition of hPSC differentiation and investigate whether this is due to 2HG accumulation or direct inhibition of αKG-dependent dioxygenases.

MATERIALS AND METHODS

hPSC Culture

hPSCs (H9, UCLA1, HIPS2) (Diaz Perez et al., 2012; Lowry et al., 2008; Thomson et al., 1998) were cultured on CF-1 irradiated MEF (Global Stem) feeder layer in DMEM/F12 supplemented
with 20% KnockOut serum replacement (ThermoFisher), 1% Glutamax, 1% Pen/Strep, 1% non-
essential amino acids, 0.1mM 2-mercaptoethanol, and 10ng/ml bFGF (R&D Systems). Cells
were passaged with collagenase IV (Life Technologies) and converted to feeder-free conditions
prior to differentiation or exposure to hypoxia. Feeder-free hPSCs were grown on growth factor
reduced Matrigel (Corning) in mTeSR1 (Stem Cell Technologies) and passaged using gentle cell
dissociation reagent (Stem Cell Technologies). Oxygen level was controlled using a HeraCell
150i Tri-Gas Incubator (ThermoFisher Scientific).

Lentivirus-mediated shRNA Knockdown

pLKO.1 vectors were obtained from Sigma-Aldrich containing either shRNA targeting ARNT
RNA transcripts (TRCN0000003819 (shARNT #19) and TRCN0000003816 (shARNT#16)) or a
scrambled control sequence. Lentivirus production was achieved by co-transfection of each
vector with pCMV-dR8.9 and pCMV-VSVG into HEK 293FT cells using TransIT-293
Transfection Reagent (Mirus Bio). Transfected HEK 293FT cells were treated with 5mM sodium
butyrate (Sigma) for 8 hours on the day following transfection. Culture medium was collected 48
and 72 hours post-transfection and lentivirus was concentrated from culture media using Lenti-X
Concentrator (Clonetech). Lentivirus was aliquoted and stored at -80°C until use on hPSCs.
Feeder-free hPSCs were transduced by addition of 25μl of 1 x 10^7 TU/ml of viral particles
overnight. Transduction was repeated 4 times before initiation of selection by treatment with
1μg/ml puromycin. Initially puromycin was maintained in the medium for 3 passages and
thereafter added to the medium for two days prior to passaging of the cells.

Chemicals and Antibodies
Dimethyl 2-oxoglutarate (dm-αKG) was purchased from Sigma. Dm-αKG was diluted in DMEM/F12 to 800mM and the pH was adjusted to 7.2 before addition to cell culture and final concentration. Antibodies used in this study are listed in the table below.

<table>
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**Neuroectoderm Differentiation**

For differentiation, hPSCs were dissociated into single cells by exposure to gentle cell dissociation reagent (Stem Cell Technologies) for 15 minutes. Cells plated in mTeSR (Stem Cell Technologies) with 10μM Y-27632 (BioPioneer) at a concentration of 4 x 10^5 or 8 x 10^5 cells per well of a 12 or 24 well plate, respectively. One day after plating (day 0), medium was changed to RPMI (ThermoFisher) supplemented with 2% B27 (ThermoFisher), 1% N-2 supplement (ThermoFisher), 10μM SB431542 (Stemgent), 0.2μM Dorsomorphin (Stemgent), 1% glutamax, and 1% non-essential amino acids (ThermoFisher). In experiments with low oxygen tensions, cells were transferred to hypoxic incubator after medium change on day 0 and maintained in the hypoxic incubator throughout differentiation.

**Flow Cytometry Analysis**

To collect cells for flow cytometry analysis, gentle cell dissociation reagent (Stem Cell Technologies) was used to single cell dissociation. Cells were then fixed and permeablized using
BD Cytofix/Cytoperm Kit (BD Biosciences) according to manufacturer instructions. Briefly, cells were fixed for 15 minutes using BD Cytofix/Cytoperm solution and washed twice in 1x BD Perm/Wash Buffer. Cells were incubated on ice with primary antibodies for 1 hour before another wash with BD Cytofix/Cytoperm. Finally, cells were suspended in PBS with 2% fetal bovine serum (Omega) and data was collected with either an LSRII (BD Biosciences) or an LSRFortessa (BD Bioscience). Data analysis was performed with FlowJo software (Treestar).

**Immunoblot**

For ARNT/HIF1beta immunoblots, nuclear fractions of cells were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher) according to manufacturer instructions. For whole cell lysates cells were harvested in SDS buffer (40mM Tris-HCl pH 6.8, 3% glycerol, 1% SDS). For all immunoblots protein concentrations were quantified using a BCA assay kit (Pierce). IRDye-conjugated secondary antibodies were used and images captured with an Odyssey Fc (Licor).

**Metabolite Extraction and Analysis**

To extract intracellular metabolites, cells were washed with cold D-PBS followed by a wash with cold 150mM ammonium acetate (pH 7.3). Next, 1ml of -80°C 80% methanol in water (v/v) was added to each well of a 12-well plate. Cells were scraped off of the plate and transferred to cold 1.5 ml Eppendorf tubes. 10 nmol of D/L norvaline was added to cell suspension before centrifugation at (1.3 x 1.4 rpm, 4°C). Supernatant was transferred to a glass vial. To harvest any remaining metabolites, 200μl was added to the cell mixture which was vortexed three times for 5 seconds. Centrifugation was repeated and supernatant was transferred into glass vial.
Samples were dried with the EZ-Elite Personal Evaporator aqueous program for 2 hours and 40 minutes. Metabolites were resuspended in 70% acetonitrile. For mass spectrometry analysis of a sample, 5μl was injected onto a Luna NH2 (150 mm x 2 mm, Phenomenex) column. Samples were analyzed with an UltiMate 3000RSLC (Thermo Scientific) coupled to a Q Exactive mass spectrometer (Thermo Scientific). The Q Exactive was run with polarity switching (+3.00 kV / -2.25 kV) in full scan mode with an m/z range of 70-1050. Separation was achieved using A) 5 mM NH4AcO (pH 9.9) and B) ACN. The gradient started with 15% A) going to 90% A) over 18 min, followed by an isocratic step for 9 min and reversal to the initial 15% A) for 7 min. Metabolites and isotopomers were quantified with TraceFinder 3.1 using accurate mass measurements (≤ 3 ppm) and retention times. For isotopologue distribution measurements, data was corrected for naturally occurring 13C as described previously (Moseley, 2010). Data analysis was performed using the formula statistical language R. Fractional contributions were calculated using the formula described previously, where mi denotes the intensity of the isotopologue, and n marks the number of carbons in the metabolite (Fendt et al., 2013).

**Statistical Analysis**

Data were analyzed with Prism (GraphPad). Data were analyzed using two-way ANOVA with correction for multiple comparisons. In all cases, p < 0.05 was considered significant.

**Results**

Low oxygen tension (1% O2) inhibits hPSC neuroectoderm differentiation independent of HIF transcriptional activity.
Low oxygen environments have been shown to both improve reprogramming to pluripotency and to promote neuroectoderm differentiation (Mathieu et al., 2014; Xie et al., 2014; Yoshida et al., 2009). To investigate the role of low oxygen levels in hPSC differentiation, we cultured hPSCs in 1% O2 or 21% O2 throughout neuroectoderm differentiation triggered by dual SMAD inhibition (Chambers et al., 2009). Differentiation was severely impaired in 1% oxygen environment when compared to normoxic conditions (Figure 1A and 1B). We hypothesized that this inhibition could be due to stabilization of HIF1α and HIF2α, which has been shown to activate OCT4 expression and improve early reprogramming (Covello et al., 2006; Mathieu et al., 2014). Therefore, we knocked down expression of HIF1β/ARNT, the heterodimeric partner for transcriptional activity of both HIF1α and HIF2 α, by stable lentiviral shRNA expression (Figure 1C). Hypoxia inhibited differentiation in both the scramble control and ARNT knockdown hPSCs suggesting that activation of HIF by hypoxia is not required to inhibit differentiation (Figure 1D and 1E). Alternatively, 2-hydroxyglutarate (2HG) produced by lactate dehydrogenase and malate dehydrogenase enzymes in hypoxic conditions could play a role in inhibiting differentiation (Intlekofer et al., 2015; Oldham et al., 2015). αKG-dependent dioxygenases play an important role in hPSC differentiation, and L-2HG is an inhibitor of many of the αKG-dependent dioxygenase enzymes (Figueroa et al., 2010; Lu et al., 2012; TeSlaa et al., 2016).

**αKG does not promote differentiation in hypoxic conditions**

Because 2HG competitively inhibits αKG-dependent dioxygenases, we hypothesized that addition of αKG may rescue differentiation. To determine whether αKG could rescue the effect of low oxygen tension during differentiation, hPSCs were differentiated into neuroectoderm in
the presence and absence of dm-αKG in 1% or 21% O₂ conditions. Dm-αKG was unable to increase the number of Pax6 positive cells after differentiation under low oxygen tension (Figure 2A and 2B). Treatment with dm-αKG under hypoxic conditions has been shown to increase 2HG levels in cancer cell lines (Intlekofer et al., 2015). This data suggests either that addition of αKG causes increased L-2HG accumulation, or that αKG-dependent dioxygenases are directly inhibited by low oxygen tension in the context of hPSC differentiation at 1% O₂.

**HIF is not required for dm-αKG accelerated differentiation**

Dm-αKG can cause the stabilization of HIFs through inhibition of HIF prolyl hydroxylase 2 (PHD2) despite its metabolism into αKG (Hou et al., 2014). Because dm-αKG can promote neuroectoderm differentiation of hPSCs and hypoxia skews differentiation toward ectoderm, we hypothesized that HIF could play a role in the effect of dm-αKG on differentiation (TeSlaa et al., 2016; Xie et al., 2014). Knockdown of HIF1β/ARNT, however, had no effect on dm-αKG accelerated differentiation (Figure 2C). Furthermore, low oxygen inhibited differentiation in HIF1β/ARNT knockdown hPSCs regardless of dm-αKG supplementation. This data suggests that dm-αKG accelerates differentiation independent from its influence on HIF stabilization.

**Discussion**

Hypoxia can influence cell fate through multiple mechanisms including stabilization of HIF and inhibition of αKG-dependent dioxygenases through direct inhibition or L-2HG accumulation. Our data suggests that 1% oxygen inhibits differentiation through inhibition of these dioxygenases. Measurement of L-2HG levels should be done to determine if L-2HG is high in differentiating hPSCs in 1% O₂. Furthermore, differentiation at 2% O₂ and 5% O₂ should be
done with shHIF1β/ARNT hPSCs to determine whether HIF can promote neuroectoderm differentiation in more moderate hypoxic conditions and whether L-2HG accumulates in these conditions. Interestingly, L-2HG dehydrogenase (L2HGDH), which converts L-2HG back to αKG, is expressed at high levels in human iPSCs when compared with fibroblasts, while D-2-hydroxyglutarate dehydrogenase (D2HGDH), which converts D-2HG to αKG is higher in fibroblasts (data not shown). D-2HG, unlike L-2HG, does not increase in hypoxic conditions (Intlekofer et al., 2015; Oldham et al., 2015). This suggests that L2HGDH may be important for differentiation of tissue exposed to hypoxic conditions during development. Rare autosomal recessive mutations in L2HGDH cause only neurological defects including psychomotor retardation, cerebellar ataxia, macrocephaly, or epilepsy (Steenweg et al., 2010). This leads to the hypothesis that moderate hypoxia promotes neuroectoderm development while L2HGDH limits L-2HG accumulation that could inhibit αKG dependent dioxygenases. Furthermore, this suggests that early metabolic changes during differentiation may be lineage specific and play a role in tissue specification.

Another issue that arises from this work is common practice of maintaining hPSCs in hypoxic environments to promote self-renewal (Ludwig et al., 2006a; Ludwig et al., 2006b). An outstanding question is whether long term culture in moderate hypoxia can alter L-2HG levels or inhibit αKG dependent dioxygenases enough to alter hPSC differentiation or lineage preference. Furthermore, culture of hPSCs in feeder free conditions, specifically Essential 8 media (E8), has been shown to cause a modest increase 2HG levels (Zhang et al., 2016). It would be interesting to see if feeder free conditions cause decreased ability to differentiate through 2HG.
accumulation. It is unclear, however, which enantiomer of 2HG increases in feeder free conditions.
TeSlaa et al Figure 2

A

21% O₂

1% O₂

--- control
--- 4mM dm-αKG
--- 12mM dm-αKG

PAX6

B

% PAX6⁺

21% O₂

1% O₂

--- control
--- 4mM dm-αKG
--- 12mM dm-αKG

C

% PAX6⁺

21% O₂

1% O₂

--- control
--- 12mM dm-αKG

scramble
shARNT 16
shARNT 19

scramble
shARNT 16
shARNT 19
Figure Legends

Figure 1. Severe hypoxia inhibits hPSC differentiation

(A,B) Flow plot and quantification of the percent PAX6 positive cells in differentiated hPSC measured by flow cytometry after 4 days of neuroectoderm differentiation in either normoxic (21% O₂) or hypoxic conditions (1% O₂).

(C) Immunoblot of ARNT/HIF1β and nuclear loading control HDAC1 in nuclear extracts of H9 hESCs with lentiviral expression of shRNA targeting ARNT/HIF1β or scramble control.

(D) Quantification of flow cytometry data of the percent PAX6 positive cells in H9 hESCs expressing either shRNA targeting ARNT/HIF1β or scramble control after 4 days of differentiation in either normoxic (21% O₂) or hypoxic conditions (1% O₂).

*p≤0.05; ***p≤0.001. P values were determined by two-way analysis of variance (ANOVA) with correction for multiple comparisons.

Figure 2. αKG cannot rescue severe hypoxia inhibited differentiation and promotes differentiation independent of HIF.

(A,B) Flow plot and quantification of the percent PAX6 positive cells in differentiated HIPS2 hPSCs measured by flow cytometry after 4 days of neuroectoderm differentiation in either normoxic (21% O₂) or hypoxic conditions (1% O₂) with addition of dm-αKG where indicated.

(C) Flow plot and quantification of the percent PAX6 positive cells in differentiated H9 hPSCs expressing either shRNA targeting ARNT/HIF1β or scramble control measured by flow cytometry after 4 days of neuroectoderm differentiation in either normoxic (21% O₂) or hypoxic conditions (1% O₂) with addition of dm-αKG where indicated.
*p≤0.05. P values were determined by two-way analysis of variance (ANOVA) with correction for multiple comparisons.
References


Chapter 5:

Conclusion
Pluripotent stem cells can be cultured in two distinct developmental states: the naïve pluripotent state which corresponds to the inner cell mass of the preimplantation blastocyst and the primed pluripotent state which corresponds to the epiblast post uterine implantation (Nichols and Smith, 2009). These different developmental stages are characterized by differences in their response to signaling pathways, their epigenetic landscape and their metabolism (Greber et al., 2010; Hayashi et al., 2008; Leitch et al., 2013; Marks et al., 2012; Zhou et al., 2012). While mouse pluripotent cells can be derived from either the pre- or post-implantation embryo to generate naïve or primed pluripotent stem cells, respectively, human pluripotent stem cells (hPSCs) have traditionally been cultured in primed pluripotent conditions despite being derived from the preimplantation epiblast (Nichols and Smith, 2009).

α-Ketoglutarate (αKG) can promote self-renewal of the naïve pluripotent state by promoting the activity of αKG-dependent enzymes that maintain low DNA and histone methylation levels characteristic of the naïve state (Carey et al., 2014). hPSCs, which are maintained in the primed pluripotent state, however, have relatively higher levels of both DNA and histone methylation (Liao et al., 2015). Therefore, we hypothesized that αKG may play a different role in hPSCs. Furthermore, inhibition of αKG-dependent dioxygenases is often seen to block differentiation in cancer, and activity of several JHDMs and TET enzymes are required for differentiation of PSCs (Dawlaty et al., 2014; Lu et al., 2012). Despite these connections, the role of αKG in primed hPSC differentiation has not been previously investigated.

To determine the role of αKG in hPSCs, we first investigated how TCA cycle metabolites including αKG are produced in hPSCs in comparison to hPSC-derived differentiated cells. To do this we fed hPSCs and differentiated cells 13C-labeled glucose, glutamine and glutamate and measured intracellular metabolites using ultra high pressure liquid chromatography combined with mass spectrometry (UHPLC-MS). Our results showed that hPSCs have active TCA cycles with carbons from glucose and glutamine robustly contributing to TCA cycle metabolite production. Furthermore, despite low respiration in hPSCs compared to differentiated cells, more carbon from glucose and glutamine was seen in TCA cycle
metabolites in hPSCs. This suggested that other carbon sources may contribute to respiration upon differentiation. We then measured glutamate uptake and incorporation and saw that upon differentiation, cells became metabolically flexible gaining the ability to use glutamate in their TCA cycle. Metabolic flexibility is important for normal cellular function and the response to metabolic stress. Limitations in cellular and organismal metabolic flexibility is often seen in disease states such as cancer or diabetes (Goodpaster and Sparks; Olson et al., 2016). It seems that hPSCs are metabolically less flexible when compared to differentiated cells. This idea is supported by other work showing that hPSCs are dependent on glutamine for their survival (Tohyama et al., 2016). This could be one additional way in which disease states such as cancer resemble a stem cell like or less differentiated state.

Next, we more specifically investigated αKG production which can be made either by glutamate dehydrogenase or transaminase enzymes. In proliferative cells transaminase enzymes are often active to conserve nitrogen by producing amino acids rather than releasing the nitrogen as ammonia (Coloff et al., 2016). To measure transaminase activity, we used $^{15}$N$_2$-glutamine to measure amine group transfer from glutamate to amino acids. We found robust activity of many transaminases including the glutamate pyruvate transaminases and glutamate oxaloacetate transaminases suggesting that αKG is robustly produced by these enzymes in hPSCs. High activity of transaminase enzymes serves to help support the biosynthetic demands of proliferative cells by producing amino acids important for both nucleotide biosynthesis, such as aspartate, and protein translation. In addition, these enzymes also produce αKG which can alter gene expression through αKG-dependent dioxygenases. Therefore, these enzymes may serve as a link between proliferation and epigenetic regulation coordinating biosynthesis and gene expression.

Because hPSCs robustly produce αKG, we hypothesized that it may be important for αKG-dependent enzyme function during differentiation. To determine whether αKG can affect differentiation, ectoderm and endoderm differentiations were performed with supplementation of αKG or cell permeable dimethyl-
αKG (dm-αKG) in the media. Both dm-αKG and αKG supplementation increased the percent of cells positive for lineage specific markers early in differentiation. From this data, we concluded that αKG accelerates the differentiation of primed hPSCs. This differentiation-promoting role of αKG is distinct from its role in supporting self-renewal of naïve mESCs (Carey et al., 2014). Based on our results we hypothesized that this difference is due differences in the pluripotent states that primed hPSCs and naïve mESCs exist in rather than species-dependent differences. To test this hypothesis, we obtained mouse primed pluripotent cells, called epiblast stem cells (EpiSCs), and differentiated them in the presence of dm-αKG. Dm-αKG accelerated the differentiation of mouse EpiSCs suggesting that αKG plays a different role in different pluripotent states. To further confirm the role of αKG in primed hPSCs, we next tried to decrease the activity of the αKG-dependent dioxygenases either by decreasing αKG levels or by increasing levels of succinate, a competitive inhibitor of the dioxygenase enzymes. Two different transaminase inhibitors were used to decrease αKG levels, aminooxyacetate and L-cycloserine, and a succinate dehydrogenase inhibitor, 3-nitropropionic acid. All three inhibitors caused a significant delay or inhibition of neuroectoderm differentiation. This data was consistent with our conclusion that αKG promotes primed hPSC differentiation.

Finally, we looked at the epigenetic targets of αKG-dependent dioxygenases, the methylation of histone tails and cytosine methylation of the DNA. During primed hPSC differentiation, dm-αKG causes a decrease in histone trimethyl marks and an increase in the ratio of 5-hydroxymethylcytosin to 5-methylcytosine. Combined these data suggest a differentiation promoting role for αKG in primed hPSCs probably through its action on epigenetic members of the αKG-dependent dioxygenase family. These results highlight the metabolic, epigenetic, and functional differences that exist between the naïve and primed pluripotent states. Primed pluripotent cells are prepared for differentiation while naïve pluripotent cells seem to be in a more stable self-renewing state. This is consistent with epigenetic studies that have shown that ablation of epigenetic repressors reinforces the self-renewal of naïve PSCs, while ablation of the same epigenetic repressors in the primed PSCs promotes differentiation (Weinberger et al., 2016).
To follow up the study of αKG in hPSC differentiation, I briefly investigated the role of hypoxia inducible factors (HIFs) and oxygen tension in early differentiation. One motivation for this work was to determine whether dm-αKG acts through stabilization of HIFs by inhibiting prolyl-4-hydroxylase 2 (PHD2) (Hou et al., 2014). To investigate this question, HIF1β/ARNT was knocked down in hPSCs. Depletion of HIF1β/ARNT does not negate the effect of dm-αKG suggesting that HIFs do not have a driving role in dm-αKG driven differentiation. This is consistent with our results in which αKG, which activates PHD2, promoted primed hPSC differentiation.

Interestingly, while studying the role of HIF in αKG-promoted differentiation, I found that low oxygen (1% O₂) environments inhibit differentiation of hPSCs into neuroectoderm. Furthermore, I found that this inhibition occurs through a HIF independent pathway. αKG-dependent dioxygenases, which are important in early differentiation, require oxygen for their activity and can be inhibited by 2-hydroxyglutarate (2-HG) (Loenarz and Schofield, 2011; Xu et al., 2011). L-2-hydroxyglutarate levels increase in hypoxic environments and therefore this could be a mechanism by which differentiation is inhibited by very low oxygen tensions in hPSCs (Intlekofer et al., 2015; Oldham et al., 2015). More work needs to be done to understand how varying low oxygen tensions can promote self-renewal and inhibit differentiation in some contexts and promote ectoderm differentiation in others (Ezashi et al., 2005; Xie et al., 2014). These differences may be due to differences in the levels of oxygen. In this model, severe hypoxia may inhibit differentiation while moderate hypoxia may promote differentiation into neuroectoderm.

Combined with other current results in the field, my work demonstrates the context-specific role that metabolites can have in regulating cellular fate. Furthermore, it suggests that each component of differentiation medium as well as other environmental factors can influence the efficiency, completeness, and speed of cellular maturation. Therefore, one goal of studying hPSC metabolism could be to
manipulate metabolism to improve differentiation of hPSCs. To achieve this purpose, however, systematic studies will need to be performed in which metabolic perturbations are made during periods of differentiation of hPSCs into multiple lineages. A deeper understanding of the metabolic requirements of each germ layer particularly during early differentiation has the potential to inform differentiation protocols.

While studies focused on single metabolites or pathways are informative, a broader approach would accelerate progress in the field. Therefore, another strategy to improve the production of mature and functional differentiated cells would be to carefully design differentiation media and the extracellular matrix throughout the differentiation to simulate the environment that a cell is exposed to during normal development. However, increased understanding of human development in vivo is required for this strategy. Because studies of human development of this nature are not feasible, studies in hPSCs will probably need to be guided by developmental biology in model organisms.

A more practical application of this idea could be to design a better in vitro environment for the culture of naïve hPSCs. While naïve culture conditions for hPSCs have been established, many questions still exist regarding the physiologic relevance and usefulness of naïve hPSCs maintained in these conditions. Designing novel culture conditions that better model the environment that the ICM is exposed to in vivo could improve the culture of naïve hPSCs and reduce the need for the numerous small molecule inhibitors currently used for this purpose. The ICM of the blastocyst is exposed to both trophectoderm cells and the blastocoel fluid that exists in the cavity of the embryo. The volume of the blastocoel fluid is minimal which may present limitations for metabolomic studies. Some metabolic characterization of the blastocoel fluid has been done in bovine blastocysts, however. Interestingly lactate, aspartate, and glycine are all very high in bovine blastocoel fluid and are taken up by the inner cell mass (Gopichandran and Leese, 2003). It would be interesting to see how these metabolites change hPSC metabolism, self-renewal and differentiation. A pitfall to this approach is that the ICM does not self-renew in vivo, but continues to
differentiate. Therefore, modeling of the \textit{in vivo} environment of the ICM may not improve self-renewal of the human naïve PSCs. Feeder layers can impact cellular metabolism and the differentiation potential of hPSCs (Gu et al., 2016; Lee et al., 2015; Ojala et al., 2012; Zhang et al., 2016). \textit{In vivo} the trophectoderm secretes metabolites and other factors into the blastocoel fluid providing the environment for the ICM. Therefore, study of the metabolism of the human trophectoderm could lead to a deeper understanding of how naïve and primed pluripotency can be supported. In the bovine system, the trophectoderm secretes more lactate than the ICM (Gopichandran and Leese, 2003).

Another approach to investigating the metabolism of the naïve pluripotent state is to reprogram primed pluripotent stem cells to naïve pluripotent cells while altering the metabolism through addition of metabolites, small molecule inhibitors, or other metabolic supplements. Because of its role in supporting the self-renewal of naïve PSCs (Carey et al., 2014), αKG is a candidate metabolite that could improve reprogramming to the naïve pluripotent state. Oxidative phosphorylation (OXPHOS) promotes conversion of mouse EpiSCs into naïve PSCs (Sone et al., 2017), however it remains to be seen if OXPHOS will also promote the naïve state in human PSCs. It may be interesting to add metabolites that are enriched in the blastocoel fluid during the reprogramming process to determine if they improve conversion to the naïve state.

Beyond using metabolism to improve methods for culturing and differentiating hPSCs, much work can be done to better understand the mechanisms that regulate mitochondrial metabolism, morphology, homeostasis, and apoptosis in primed hPSCs. Primed hPSCs exhibit low levels of OXPHOS and respire at their maximum capacity despite having numerous mitochondria and fairly active TCA cycles (Zhang et al., 2011). While high levels of uncoupling protein 2 (UCP2) and pyruvate dehydrogenase kinase (PDK) promote the shunting of TCA cycle metabolites away from OXPHOS, there are probably other mechanisms limiting OXPHOS activity in hPSCs. Furthermore, mitochondria in hPSCs have an immature morphology exhibited by perinuclear and punctate structure with swollen cristae (Suhr et al., 2010; Zhang
et al., 2011). However, despite many descriptive studies, very little work has been done to identify mechanistic regulators of mitochondrial morphology and function in hPSCs. Interestingly, hPSCs are more sensitive to mitochondria mediated apoptosis in response to many cellular stresses when compared to differentiated cell types (TeSlaa et al., 2016). Rapid degradation of p53 and activated Bax sequestered to the golgi apparatus in hPSCs are partially responsible for their sensitivity to apoptotic stimuli (Dumitru et al.; Liu et al., 2013; Setoguchi et al., 2016). Mechanisms related to the differences in mitochondrial morphology and turnover, however, remain to be identified.
References


Appendix I:

Pulse laser triggered high speed microfluidic fluorescence activated cell sorter
Pulsed laser triggered high speed microfluidic fluorescence activated cell sorter†‡

Ting-Hsiang Wu, a,b Yue Chen,a Sung-Yong Park, c Jason Hong, b Tara Teslan, b Jiang F. Zhong, e Dino Di Carlo, d Michael A. Teitell c and Pei-Yu Chiu a

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We report a high speed and high purity pulsed laser triggered fluorescence activated cell sorter (PLACS) with a sorting throughput up to 20,000 mammalian cells s⁻¹ with 37% sorting purity, 50% cell viability in enrichment mode, and >90% purity in high purity mode at 1500 cells s⁻¹ or 3000 beads s⁻¹. Fast switching (30 μs) and a small perturbation volume (~90 pl) is achieved by a unique sorting mechanism in which explosive vapor bubbles are generated using focused laser pulses in a single layer microfluidic PDMS channel.

Introduction

Microfluidic-based fluorescence activated cell sorters (μFACS) have several advantages over conventional electrostatic droplet-based cell sorters. A closed detection and sorting environment prevents aerosols that can potentially contaminate equipment, personnel, and subsequent sorting experiments. This advantage is critical when sorted cells are re-cultured or used for PCR analysis as the contaminant can be exponentially expanded, resulting in artifacts. Micrometre-sized channel dimensions are also suitable for handling small sample volumes and low cell numbers with high yield. Microfluidic sorting chips can be made disposable, which offers improved biosafety for sorting pathogenic samples. Furthermore, additional functionalities such as population expansion, drug screening, single-cell DNA and RNA analysis can be integrated directly downstream of the sorting region on the same chip, minimizing sample loss and time delay between sorting and subsequent processes. Although various on-chip μFACS mechanisms have been demonstrated, sorting live mammalian cells at high speeds with high sort purity and cell viability remains a challenge. By using electro-osmotic flow switching, E. coli cells were sorted at 20 cells s⁻¹. An integrated microfabricated PDMS valve was demonstrated a sorting speed of 44 cells s⁻¹. By dynamically plugging the channel with a thermo-reversible gelation polymer, sorting of E. coli was shown at 5 cells s⁻¹. Using optical force routing, high sort purities >90% was achieved with HeLa cells at a sorting speed of ~100 cells s⁻¹. Under the enrichment mode of sorter operation, a throughput of 12,000 cells s⁻¹ was demonstrated using an off-chip flow switching valve but with a low sorted purity of 0.2%. Alternatively, an enrichment of ~230-fold (sort purity ~66%) at 1000 mammalian cells s⁻¹ was reported using an integrated piezoelectric actuator (PZT)-driven polysiloxanohelastone (PDMS) membrane valve. Slow cell switching mechanisms and large fluid perturbation volumes are the two major factors limiting the throughput and purity of current microfluidic FACS devices. Reported switching times range from 500 msec using dielectrophoretic force switching. 3 msec using flow switching with a thermo-reversible gelation polymer or with optical force switching, and 0.1 msec for the PZT-driven membrane valve. These mechanisms are orders of magnitude slower than the speed of standard electrostatic-droplet-based cell sorters. Microfluidic based droplet sorters on the other hand demonstrated 0.5 msec switching time with speeds up to 2000 droplets s⁻¹. A faster cell sorting rate is limited by droplet breakup and cell encapsulation efficiency. Two-phase (aqueous droplets in an oil medium) systems also increase the complexity in subsequent cell recovery and processing. A micromachined, silicon-based bubble jet sorter showed switching of microparticles at 49 μs switching time. However, sorting of mixed populations of live cells has not been demonstrated.

Pulsed laser microbeams have been shown to induce bubble cavitation in solution on the microscale for high-speed droplet
generation,5 enhancing fluid mixing,6 microfluidic switching,7 and gene transfection.8 When a laser pulse with energy surpassing a threshold is focused in a liquid medium (e.g., water), the intense optical field generates heated plasma within the focal volume. The sharp temperature increase and thermoelastic stress built-up induces a cavitation bubble at the laser focus within nanoseconds. The bubble lifetime and bubble volume are determined by the laser wavelength, laser fluence at the focal spot, and pulse duration.

Here we demonstrate a microfluidic-based, high-speed and high-purity pulsed laser activated cell sorter termed PLACS which utilizes small volume liquid jets induced by pulse laser triggered cavitation bubbles for sample switching. A complete cycle in PLACS, as characterized by the bubble lifetime, is 30 μs. Bubble expansion and collapse transiently switches fluid flow inside the microchannels. The actuated fluid volume and the actuation location can be precisely controlled by the deposited laser pulse energy and laser focus position. By using all optical switching and eliminating any on-chip active components, the microfluidic chip is made of a single-layer PDMS channel bonded to a glass cover slide, which can be low cost and disposable. Furthermore, PLACS is compatible with the vast repertoire of PDMS-based microfluidic structures for performing additional upstream or downstream functions and analyses.

**Principle of PLACS**

The design of PLACS is depicted in Fig. 1a. The microfluidic chip consists of a main channel with two outlets, collection and waste. The pulsed channel runs in parallel with the main channel and is connected to the main channel through a straight narrow nozzle at the tip of the Y junction. Using hydrodynamic focusing, the input samples are focused to a narrow stream and flow into the left waste outlet. As a desired object flows through the fluorescence detection region upstream of the Y junction, a laser pulse is triggered and focused through a high NA objective lens into the pulsed channel after an optimal delay time. This induces an explosive cavitation bubble which expands and displaces the surrounding fluid creating a high speed liquid jet through the nozzle into the main channel. This liquid jet deflects the desired object into the collection channel. A rapid switching cycle of 30 μs is demonstrated based on the expansion and collapse rate of a cavitation bubble (Fig. 2). Using a nozzle to focus the deflecting liquid jet narrows the switching region in the sample stream and minimizes the disturbance range on neighboring objects to 60 μm (Fig. 3). This is essential for achieving high throughput and high sorting purity. Previously, we demonstrated that a laser-induced cavitation bubble can deform an elastic membrane for switching an adjacent fluid or particle flow.10 In that prior case, the membrane deformation extended over hundreds of microns in length, which perturbed a large fluid volume and affected sample flows upstream of the switching region, yielding lower sort purity. In this work, the volume of liquid jet delivered into the sample channel as captured by the time resolved image is ~90 pl (Fig. 2 at 15 μs). Combined with the capability of operating at high sample flow speeds of ~2 ml s⁻¹, PLACS can achieve mammalian cell sorting at high speeds with high purities.

![Fig 1](image_url)  **Fig. 1** PLACS operation. (a) Schematic of the cell sorter. The sample flow is hydrodynamically focused into the waste channel. As the desired object flows through the fluorescence detection region upstream from the Y junction, the laser pulse is triggered after an optimum delay and induces a cavitation bubble in the adjacent pulsed channel. The bubble expansion produces a high-speed liquid jet that deflects the desired sample towards the collection channel for sorting. (b) Time-resolved images of the cavitation bubble generated by the focused pulsed laser beam in the microfluidic cell sorter. (c) Fluorescent particle switching in PLACS. Without switching, the particle flows towards the left waste outlet. With fluorescence activated switching, a laser pulse was triggered and the particle was sorted into the collection outlet on the right.

**Materials and methods**

**Experiment setup and device fabrication**

As shown in Fig. 4, the pulsed laser system was a Q-switched Nd:YVO4 laser (EKSPLA, Jazz 20) operating at 532 nm wavelength, 8 ns pulse width, and a repetition rate up to 100 kHz. The pulsed laser beam was expanded and focused by an objective lens (100×, NA 0.9) through the glass substrate into the pulsed channel. The laser focus was positioned 10 μm away from the connection nozzle to the main channel. The laser pulse energy deposited into the channel was adjusted using a half-wave plate followed by a polarizing beam splitter and was set at 3.1 μJ per pulse for switching. For sample fluorescence excitation, a 10 mW, 488 nm solid state laser (CrytalLaser, DL-488-010) was reflected by a dichroic mirror (Chroma, zd188rcd) and lightly focused into the main channel through a 25×, NA 0.4 objective lens from the PDMS side of the microfluidic chip. The emitted sample fluorescence was collected by the same objective lens.
and detected using a photomultiplier tube (SensTech, F01CWADS-01) after passing through a bandpass filter (Chroma, HQ510/20m) matching the fluorescence emission spectrum. An aperture preceded the PMT and was placed at the sample conjugate image plane. The aperture opening defined the fluorescence detection area to be \( \sim 30 \times 30 \) \( \mu \)m covering the entire width of the sample channel and blocked scattered light from the laser pulse and plasma emission. The PMT signal was integrated using a DAQ card (National Instruments, PCI-7831R) at 100 kHz. FPGA logic was programmed using LabView (National Instruments) to perform real-time detection, threshold comparisons, and timed triggering of the pulsed laser. To image and characterize the fast dynamics of the cavitation bubble, a flashlight (High-Speed Photo-Systeme, Nanolite KL-M) with 11 ms flash duration was used as the illumination for time-resolved photography. Images were taken by a CCD camera (Zeiss, AxioCam MRm) and the flash delay from the laser pulse was controlled by LabView.

The device was fabricated using a conventional replica molding technique. Microchannel features were photolithographically defined in SU-8 (MicroChem 2025) photosensitive resin on a silicon wafer and then replicated onto a PDMS (Sylgard 184) layer. The PDMS replica was bonded to a glass cover slide substrate after oxygen plasma treatment. The measured microchannel height was 30 \( \mu \)m. Sheath flow channels were 80 \( \mu \)m wide. The sample channel width was 80 \( \mu \)m, which separated into 40 \( \mu \)m wide collection and waste outlet channels at the Y junction. The pulsed channel width was 300 \( \mu \)m and the straight nozzle connecting the sample and pulsed channel had a channel width of 20 \( \mu \)m and length of 100 \( \mu \)m.

Sorting of microbeads and mammalian cells

For particle sorting experiments, 10 \( \mu \)m green fluorescent beads (Thermo Fisher Scientific, G1000) and 10 \( \mu \)m non-fluorescent beads (Polysciences, 17136-5) were suspended in deionized water with 3% w/v Tween 80 (Sigma-Aldrich) to the desired concentrations. Sheath flows contained deionized water with 3% w/v Tween 80. All channel flows were driven by syringe pumps (Harvard Apparatus, PHD2000). Allura Red dye (concentration 67 mg ml\(^{-1}\), Sigma-Aldrich) was added in the pulsed channel flow.

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Fig. 2 A cavitation bubble was generated within nanoseconds after laser pulse arrival. The bubble grew to a maximum diameter of 230 \( \mu \)m in the major axis and 205 \( \mu \)m in the minor axis within 3 \( \mu \)s and began to collapse. During bubble expansion, the surrounding fluid was displaced at the speed of the bubble front and a high-speed liquid jet was injected in the connecting nozzle into the main sample channel, deflecting the target object towards the collection channel. The liquid injected into the sample channel is 100 \( \mu \)m (liquid jet length) \( \times \) 20 \( \mu \)m (nozzle width) \( \times \) 30 \( \mu \)m (liquid height) \( \sim \) 90 \( \mu \)l. (see 15 \( \mu \)s). The bubble collapsed completely by 30 \( \mu \)s after the laser pulse. Allura Red dye (concentration 67 mg ml\(^{-1}\), Sigma-Aldrich) was added in the pulsed channel flow. Laser pulse energy was 31 \( \mu \)J.

Fig. 3 Switching window and optimum object switching delay. Particle switching efficiency was measured at different laser pulse delays ranging from 5 to 75 \( \mu \)s after detection. The highest switching efficiency of 99% was obtained at a delay of 35 \( \mu \)s and decreased to 0% at 75 \( \mu \)s. The perturbation range of neighboring objects is \( \sim \) 60 \( \mu \)m, estimated by the particle speed (10 \( \mu \)m s\(^{-1}\)) and the 75 \( \mu \)s window. The distance between the detection region and Y junction was 50 \( \mu \)m. The sorting efficiency was obtained by analyzing particle traces from 50 switching events for each delay condition.

Fig. 4 PLACS experimental setup. For cavitation bubble induction, we used a Q-switched, 8 ns pulsewidth Nd:YVO4 laser. The pulsed laser beam was focused by an objective lens (100\%, NA 0.9) into the pulsed channel. Sample fluorescence was detected by a photomultiplier tube and sampled using a DAQ card and integrated every 10 \( \mu \)s. FPGA logic was programmed using LabView to perform real-time detection, threshold comparison and timed triggering of the pulsed laser.
to reduce the laser energy threshold for bubble generation. The sample flow rate was 1.2 ml h⁻¹ and the average sample speed was 0.8 m s⁻¹ in the main channel. For cell sorting, human Nalm-6 pre-B cells or B lymphoma Ramos cells cultured in RPMI 1640 culture media with usual supplements were washed and resuspended in phosphate buffered saline (1x PBS, pH 7.4) with 2% w/v bovine serum albumin (BSA) to the desired concentration. Fluorescent cell samples were obtained by staining Nalm-6 or Ramos cells with Calcein AM (Invitrogen). After sorting, the collected cells were incubated at 37 °C, 5% CO₂ for 30 min in PBS before addition of propidium iodide (Invitrogen) for viability evaluation. The sample flow rate and flow speed settings were the same as the particle sorting experiments.

Sort purity analyses by flow cytometry

Sorted samples purities were analyzed by a flow cytometer (BD, FACSCanto II). Positive and negative control samples were used to confirm the fluorescence gating conditions. For each sort condition, the average sort purity was obtained from measurements of 3 experiments. In each measurement, the total number particles analyzed were ~5000. Cell viability based on propidium iodide exclusion was measured on the same flow cytometer with a sample size of ~5000 cells for each sorting experiment.

Gene expression quantification

Human Nalm-6 pre-B cells were suspended in sorting buffer (1x PBS, pH 7.4, with 2% w/v BSA) to a concentration of ~1 x 10⁶ ml⁻¹. The mixture ratio of Calcein-AM stained cells versus unstained cells was 1:1. Cells were sorted through PLACs at room temperature and sorted cells were retrieved from both the collection and waste outlets (~1 x 10⁶ cells in each sample). Unsorted cells were incubated in sorting buffer at room temperature for the same duration as the sorted cells. RNA was extracted from retrieved cells immediately after sorting using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. Real-time qPCR was performed according to manufacturer’s protocol on the Roche LightCycler 480 Real-Time PCR System with SYBER Green I Master mix (Roche). For baseline gene expression evaluation, cells were incubated in culture media at 37 °C, 5% CO₂ before mRNA extraction. For positive controls, Nalm-6 cells were incubated in media at 42 °C for one hour for HSPA46 evaluation, whereas for POS evaluation, cells were treated with 10 µg ml⁻¹ cycloheximide (Sigma-Aldrich) for two hours. Primers for gene expression were generated using Roche’s Universal Probe Library Assay Design Center online. 

**RESULTS AND DISCUSSION**

**Bubble dynamics and fluid perturbation volume estimation**

In PLACs, a desired object was detected, a nanosecond laser (332 nm wavelength, 8 ns pulsewidth) induced a bubble that expanded to a maximum diameter (major axis) of 2.2 µm in 3 µs after laser pulse arrival (Fig. 1b). Fig. 1c shows a fluorescent particle trace that was successfully switched into the collection outlet on the right by a pulsed laser triggered high speed bubble flow. Perturbation volume was estimated by measuring the maximum liquid jet volume injected into the main sample channel. As captured by time-resolved imaging, the liquid jet flow reached the center of the main sample channel while the bubble front reached the boundary of the pulsed channel (Fig. 2, 15 µm). Estimated liquid jet volume is 140 nm (liquid jet length) x 20 µm (channel height) x 30 µm (channel height) ~90 pl.

**Switching window characterization and optimizing object switching delay after detection**

The switching window or perturbation range was determined by measuring particle switching efficiency at different time delays after detection. Particles were detected at a distance of 50 µm upstream of the "Y" junction. Switching efficiency was measured for particles at different locations with respect to the nozzle (translating to different time delays after detection) as the laser pulse was triggered. The highest efficiency was 96% obtained at a delay of 35 µs and the efficiency decreased to 0% at a delay of 75 µs and ~40% at 5 µs (Fig. 3). Particles outside of this region (75 µs × particle speed 0.8 m s⁻¹ = 60 µm) were not switched and followed the original flow focusing streamline into the waste channel. The switching window and optimal delay time between triggering laser pulse for switching and object detection depend on the sample flow speed and the detection region distance to the Y junction. Variable delay time between object detection by the PMT and laser triggering was controlled by FPGA programmed in LabView (see Experimental setup section). For different delay times ranging from 5 to 75 µs, fluorescent particle traces were recorded and analyzed. Switching efficiency was obtained by measuring the percentage of successful switching events (particle trace going into the collection channel) in a total of 50 sampled fluorescent particle images.

**Sorting of mixed polystyrene microspheres**

We characterized the sorter performance at different object throughputs and initial mix ratios. Samples were prepared by mixing 10 µm green fluorescent beads with non-fluorescent polystyrene beads. Throughputs ranging from 3000 to 10000 particle/s were obtained by keeping the green bead concentration at 5.7 x 10⁶ ml⁻¹ while increasing the non-fluorescent bead concentration accordingly. High purity (>90%) of the sorted beads were obtained at a sorting throughput of 3000 particles s⁻¹ by analyzing the collection sample using a commercial flow cytometer (Fig. 5a,b and Supplementary Movie 1). The fluorescent beads were enriched from an initial mix ratio of 0.0887 to a final ratio of 9.61, corresponding to a 1105-fold enrichment. At higher throughputs the sort purity decreases as a result of the narrowing of the average distance between adjacent particles (Fig. 5c). At a throughput of 10000 particles s⁻¹ the collection purity was 45%, equivalent to a 426-fold enrichment. To evaluate sorter performance in switching individual particles at high frequencies compared to rare event sorting, bead samples with initial mix ratios ranging from 0.01 to 0.27 (unsorted purity...
purity with ~90% cell viability were obtained for sorting fluorescent Nalm-6 cells (Table 1), a performance comparable to similar experiments performed on a high-end commercial FACS. For high-speed cell enrichment, Ramos cells were sorted at 10,000 cells s⁻¹ and 20,000 cells s⁻¹. The obtained sort purity were 48.6% and 37.4%, corresponding to enrichment factors of 473 and 298.5 respectively (Table 1). Under these sorting conditions, cells sorted into the collection or waste channels exhibited the same stress levels seen in unsorted cells, as measured by HSP70 and FOS stress-response gene expression (Fig. 6). These results showed that hydrodynamic cell focusing and cavitation bubble triggered liquid jet switching did not exert additional stress on the cells being sorted.

Device reliability

The reproducibility of PLACS sorting process under repeated laser pulsing and bubble cycles was tested. We verified that the microchannels remained intact and exhibited no burning or leakage after 100 million (10⁶) actuations (Fig. 7). Cavitation bubbles after 100 million cycles showed no discernible changes in

![Diagram](image)

Fig. 5 PLACS sorting results. (a) Mixed 10 µm green fluorescent and non-fluorescent polystyrene microparticles before sorting. Initial mix ratio = 6.9897. (b) Collected sample after sorting with a final mix ratio = 9.61. Fluorescent microparticle concentration was enriched by a factor of 1105. (c) Sort purity at different sorting speeds ranging from 3000 to 10,000 microparticles s⁻¹. High purity (90 ± 3% (mean ± s.d.)) of the sorted particles were obtained at a sorting throughput of 3000 particles s⁻¹. The collection purity measured at the highest speed tested (10,000 particles s⁻¹) was 45 ± 15%. The sort purity decreased at higher throughputs as the average distance between adjacent particles shortened. In these experiments, green fluorescent particle concentrations were kept constant (5.7 × 10⁴ ml⁻¹) while non-fluorescent particle concentrations were increased accordingly to obtain the target throughput. (d) Sort purity at different initial mix ratios ranging from 0.001 to 0.27 (unsorted purity 1% to 21%). At the initial mix ratio of 0.27, measured collection target particle purity was 84 ± 3% and waste target particle purity was 1 ± 0%. Sorting speed was kept at 3000 particles s⁻¹. 1% to 21% were sorted at a throughput of 3000 particles s⁻¹. High collection object purity (94%) with low target object waste (1%) were maintained even at the highest initial mix ratio tested.

Sorting of mammalian cells

To evaluate mammalian cell sorting, human pre-B Nalm-6 or B lymphoma Ramos cells were stained with calcine AM (green fluorescence) and mixed with untreated cells at the desired ratio. Results showed at sorting speeds of 500 and 1500 cells s⁻¹, >90%

![Diagram](image)

Fig. 6 Evaluation of stress levels of Nalm-6 cells after PLACS sorting. (a, b) The HSP70 gene expression level indicates the cellular response to heat shock, and FOS gene expression reflects heat shock and fluid shear stress. Gene expression levels were normalized to a GAPDH housekeeping gene for all cell samples under different conditions. Sorted cells retrieved from the collection and waste outlets showed expression levels that were statistically similar to the unsorted cells (incubated in sorting buffer at room temperature for the sort duration). A positive control for HSP70 expression was provided by incubating Nalm-6 cells in culture media at 42 °C for one hour. For FOS expression, a positive control was provided by treating Nalm-6 cells with cycloheximide (CHX) for two hours.

### Table 1 Results of sorting Nalm-6 human pre-B cells and B lymphoma Ramos cells at different sorting throughputs (Col: Collection sample; W: Waste sample)

<table>
<thead>
<tr>
<th>Throughput (cells s⁻¹)</th>
<th>Cell Type</th>
<th>Before sort</th>
<th>After sort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial green cell percentage (%)</td>
<td>Cell density (×10⁶ cells ml⁻¹)</td>
</tr>
<tr>
<td>500</td>
<td>Nalm-6</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>1560</td>
<td>Nalm-6</td>
<td>0.8</td>
<td>4.6</td>
</tr>
<tr>
<td>10 000</td>
<td>Ramos</td>
<td>0.2</td>
<td>30.6</td>
</tr>
<tr>
<td>20 000</td>
<td>Ramos</td>
<td>0.2</td>
<td>61.3</td>
</tr>
</tbody>
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*Lab Chip, 2012, 12, 1378–1383* This is a journal of the Royal Society of Chemistry 2012
the bubble pattern, further ensuring reliable switching over long periods of device operation.

Conclusion

PLACS overcomes the limit of microfluidic-based fluorescence-activated cell sorting mechanisms in achieving simultaneously high speed, high purity, and high viability sorting. The bubble jet switching mechanism actuates a small and well-controlled fluidic volume for sample sorting which allows sorting of multiple cell types in any biological medium. Our current device operates with a bubble cycle time of 30 ps. Shorter switching times are possible by utilizing a smaller bubble actuation volume and modified channel design. PLACS has the potential to bridge the sorting throughput gap between current microfluidic PACS and conventional electrostatic-droplet-based cell sorters.

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References

Appendix II:

Nanoblade Delivery and Incorporation of Quantum Dot Conjugates into Tubulin Networks in Live Cells
Nanoblade Delivery and Incorporation of Quantum Dot Conjugates into Tubulin Networks in Live Cells

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Supporting Information

ABSTRACT: Quantum dots (QDs) have not been used to label cytoskeleton structure of live cells owing to limitations in delivery strategies, and QDs conjugation methods and issues with nonspecific binding. We conjugated tubulin to QDs and applied the emerging method of photothermal nanoblade to deliver QD-tubulin conjugates into live HeLa cells. This method will open new opportunities for cytosolic targeting of QDs in live cells.

KEYWORDS: Nanoblade delivery, QD-tubulin, cytosolic targeting

Since their introduction as biological imaging probes,1,2 quantum dots (QDs) have gained prominence in various imaging applications due to their unique attributes such as high brightness, broad excitation spectrum, narrow emission spectrum, and excellent photostability.3,4 However, QDs also suffer from several shortcomings such as relatively large size5,6 non-specific binding (due to the charge of surface coatings),7 large anisotropy8,9 and cytotoxicity10. These shortcomings have restricted the general utility for quantum dots to only a subset of imaging applications.

An important, but elusive, goal has been the delivery and labeling of QDs to cytosolic targets. Such a capability would enable multiplexed detection and single particle tracking of transiently interacting proteins and dynamic cellular machines over long periods of times. Successful cytosolic targeting requires, however, an efficient delivery that escapes the endocytic pathway, supplying QDs to the cytosol that is not engulfed in membranous organelles (endosome-free QDs). In addition, once delivered, QDs should have minimal steric hindrance (i.e., small size) and minimal nonspecific binding so that they can freely diffuse and sample the full cytosolic volume in order to find and specifically bind to their target(s).

Although extensive efforts have been vested in developing cytosolic targeting methods and many reports claim to have achieved successful targeting,11,12 a general and widely applicable protocol that offers infallible demonstrations of specific cytosolic targeting of fine structures such as the cytoskeleton are still lacking. Conventional methods can be classified into two main categories: (i) Facilitated delivery strategies such as, using cell penetrating peptides (CPP),13-15 protein sponge, polymer carriers,16-19 phototoxic19,10 and transfection reagents.20 Methods belonging to this category provide high throughput delivery, but suffer from low efficiency release of endosome-free QDs (i.e., most QDs end up trapped in endosomes). (ii) Active delivery methods include electroporation11 and microinjection.22 Electroporation offers an efficient way of delivery by temporarily destabilizing the plasma membrane to create transient pores using high voltage electrical pulses. However, this method suffers from low cell viability, aggregation of the payload, and low uptake of large objects.23 The other active method, microinjection, is the most efficient and direct way to deliver QDs into the cytoplasm. The delivery is done via a sharp glass microcapillary tip (with a diameter <0.5 μm) that mechanically penetrates the cell membrane while maintaining reasonable cell viability.24,25 However, the injection of large cargo (>0.5 μm) or aggregate-prone objects such as QDs and QDs-protein conjugates is difficult due to repeated tip dogging.

Here we applied the photothermal nanoblade technique26-27 to deliver tubulin–QDs conjugates into the cytoplasm of HeLa cells. As shown in Figure 1a, a laser pulse is used to excite surface plasmons in the thin titanium coating on the tip of a glass capillary pipette; the plasmon absorption conducts heat into the liquid medium in close proximity to the metal which in turn produces nanosecond-short explosive vapor bubbles right next to the cell membrane. These bubbles produce large transient cuts or pores in the cell membrane. Concurrently, by pressurizing the capillary, a transient liquid flow is generated, enabling the delivery of the payload into the cytosol. In contrast to traditional microinjection, the photothermal nanoblade is brought in gentle contact with the cell membrane, eliminating the need for damaging, mechanical puncturing. It also allows use of a...
relatively large tip orifice (up to ~2 μm) and injection of relatively large objects such as bacteria.\textsuperscript{15}

To demonstrate successful QD delivery and cytosolic targeting, we incorporated QDs-tubulin conjugates into growing microtubules in live cells. The large size of QDs could possibly hinder the polymerization of the conjugates in growing filaments. To circumvent this, a multistep scheme was therefore devised (Figure 1b): (i) amine-derivatized, PEG-coated QDs were reacted with [bis(2-aminoethyl)aminod] dibenzyl borate (B3) cross-linker followed by a gel filtration step (to remove excess B3); (ii) tubulin monomers were polymerized at 37 °C in the presence of GTP and DMNO; (iii) when the tubulin solution became turbid (due to microtubule polymerization), precartivated QDs were added to react with amine groups on polymerized tubulin molecules; and (iv) the reaction solution was quenched with hydroxyamine and centrifuged at 15,000g at 4 °C for 15 min. The pellet was then depolymerized at 4 °C in a DMNO-free buffer and purified by a 100K molecular weight cutoff (MWCO) centrifuge filter. As a control, a random conjugation method was also implemented (Figure 1c). The precartivated QDs were mixed with tubulin dimers, resulting in nonspecific conjugation via amine groups.

Following photothermal nanoblade delivery of depolymerized tubulin-QD conjugates (Figure 1b) to HeLa cells, filamentous structures were observed by wide-field fluorescence microscopy for long periods of times (Figure 2a,b), indicating successful delivery and targeting. Nonspecific background and incomplete filamentous structures were observed after nanoblade delivery of conjugates prepared according to the scheme described in Figure 1c, possibly due to conjugation-induced blocking of the tubulin binding site (Figure 2c). Also, nanoblade-based delivery of bare amine-derivatized PEG-coated QDs into HeLa cells resulted in interspersed punctuated spots over a uniform staining of the cytosol (Figure 2e), possibly indicating aggregation of probes in the cytosol. The above representative images (more data in the Supporting Information) showed the successful cytosolic staining on microtubules. However, the effect of QDs size on cyto-staining still remains for further study. Although it has been reported that large size of QDs probes may result in multivalent conjugation, cross-linking, static hindrance, reduced diffusion, and potential alternation of function of

Figure 2. Images of live HeLa cells after photothermal nanoblade delivery of (a) tubulin-QD conjugates prepared with the three-step conjugation strategy (schema in Figure 1b); (b) zoom-in of boxed area in panel a; (c) tubulin-QD conjugates prepared with the single-step conjugation strategy (Figure 1c); (d) zoom-in of boxed area in panel c; (e) bare amine-derivatized PEG-coated QDs. Scale bar: 8 μm.
bimolecules, a thorough and systemic study is still needed in the future.

In order to confirm specific targeting and QD incorporation into growing filaments, cells with QD-stained filamentous structures were fixed and sequentially labeled with annexin V–labeled QDs followed by Alexa 647–labeled IgG (mouse) followed by Alexa 488–labeled IgG (goat). Cells injected with tubulin–QD conjugates (Figure 1b) displayed good colocalization between the QDs and the Alexa 647 channels (Figures 3d–f), whereas cells injected with bare amine-derivatized PEG-coated QDs (Figures 3g–i) did not show good colocalization (Figures 3d–f).

PEG-coated QDs displayed filamentous structures only on the Alexa 647 channel and poor colocalization (Figures 3d–f).

Figure 3. Images of fixed HeLa cells after photothermal nanoblade delivery of tubulin–QD conjugates (green) and annexin V–labeled labeling of tubulin (red). (a) tubulin–QD conjugates (scheme in Figure 1b); (b) immunocytochemistry image of same cell as in panel a; (c) overlay of panels a and b; (d) bare amine-derivatized PEG-coated QDs; (e) immunocytochemistry image of same cell as in panel a; (f) overlay of panels d and e. Scale bar: 8 μm.

Figure 4. Examples of cell morphology after (a) microinjection and (b) photothermal nanoblade (tip ~1 μm) delivery of tubulin–QD conjugates. (c) Cell viability postphotothermal nanoblade and conventional microinjections of bare amine-derivatized PEG-coated QDs and tubulin–QD conjugates (scheme in Figure 1b).

QD conjugates with high cell viabilities albeit at a low throughput. Nanotechnology-based solutions that are inspired by the photothermal nanoblade could potentially provide higher throughput delivery to cells in the future.

ASSOCIATED CONTENT

Supporting Information
Additional information about materials and methods, sample preparation, and more images. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Notes
The authors declare no competing financial interest.

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Appendix III:

Wnt signaling directs a metabolic program of glycolysis and angiogenesis in colon cancer
Wnt signaling directs a metabolic program of glycolysis and angiogenesis in colon cancer

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Abstract

Much of the mechanism by which Wnt signaling drives proliferation during oncogenesis is attributed to its regulation of the cell cycle. Here, we show how Wnt signaling in colon cancer cells reduces glycolytic metabolism and results in small, poorly perfused tumors. We identify pyruvate dehydrogenase kinase 3 (PDK3) as an important direct target within a larger gene program for metabolism. PDK3 inhibits pyruvate flux to mitochondrial respiration and a rescue of its expression in Wnt-inhibited cancer cells rescues glycolysis as well as vessel growth in the tumor microenvironment. Thus, we identify an important mechanism by which Wnt-driven Warburg metabolism directs the use of glucose for cancer cell proliferation and links it to vessel delivery of oxygen and nutrients.

Keywords: angiogenesis; colonic cancer; fluorescence lifetime imaging; metabolism; Wnt.

Introduction

Normal patterns of Wnt signaling are necessary for tissue development and maintenance, but aberrant Wnt signaling is implicated in many cancers, especially colon cancer (Blenz & Clevers, 2009). Overactive Wnt signaling leads to constitutively active β-catenin, which via LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors leads to inappropriate activation of Wnt target genes (Clevers, 2006; Klaus & Birchmeier, 2008). Previous studies have identified multiple functional outputs of oncogenic Wnt signaling, including proliferation, EMT induction, angiogenesis, migration, and cell survival (Braak et al., 2005). Here, we propose a novel function in the regulation of cancer metabolism.

Cancer metabolism is quickly regaining a foremost position in research as its role in epigenetics, proliferation, and survival is understood to be fundamentally connected. Otto Warburg first recognized that cancer cells ferment much of their glucose supply into lactate regardless of the presence of oxygen, a phenomenon termed the Warburg effect, or aerobic glycolysis (Warburg, 1956). It is now appreciated that cancer cells have different metabolic demands than normal cells and they therefore modify their use of metabolites to meet those demands. Instead of a dominant program for efficient production of ATP, proliferating tumor cells rely on a metabolic program of glycolysis to support anabolic production of biomass (Deterzkirn et al., 2008; Vander Heiden et al., 2009). An emphasis on glycolysis is thought to be driven not only by exposure to hypoxic conditions (mostly through stabilized HIF1α) but also by oncogenic signaling pathways, such as PI3K/AKT (Bisgrove et al., 2004; Wizel & Thompson, 2012). Although Wnt plays a well-known role in homeostatic liver metabolism (Liu et al., 2011) and can cross-talk with metabolic pathways in normal cells such as differentiating osteoblasts (Seidler & Vidal-Puig, 2010; Ben et al., 2013), there are little data implicating Wnt directly in the metabolic reprogramming of cancer.

Here, we probe the contribution of LEF/TCF/β-catenin activity to the metabolic programming of cancer cells. We block the downstream activity of Wnt through the disruption of β-catenin/LEF/TCF complexes by overexpressing dominant negative (dn) LEF/TCF isoforms that lack the β-catenin binding domain. We utilize both standard metabolomics analyses and a state-of-the-art microscopy technique for monitoring changes in the metabolism of living cells and tissues. The microscopy technique, called the phase approach...
to fluorescence lifetime imaging microscopy (FLIM), utilizes two-photon microscopy for label-free detection of intrinsically autofluorescent molecules (Digranes et al., 2008; Stringari et al., 2011). In our study, we used FLIM to monitor the metabolic corezyme nicotinamide adenine dinucleotide (NADH), the principle electron acceptor in glycolysis and electron donor in oxidative phosphorylation. NADH FLIM is especially powerful as it provides a non-invasive, rapid, and sensitive readout of the metabolic status of single cells within their native microenvironment. We recently used FLIM analysis to discover that stem cells at the base of intestinal crypts are highly glycolytic and that a metabolic trajectory of glycolysis-to-oxidative phosphorylation tracks with a gradient of strong-to-weak Wnt signaling in intestinal crypts (Stringari et al., 2012). Here, we show through FLIM detection of free and bound NADH in vitro in living cancer cells and in vivo in living perfused tumors that blocking Wnt alters the metabolic program of cancer cells and leads to reduced use of aerobic glycolysis. We identify pyruvate dehydrogenase kinase (PDHK) as a novel Wnt target gene that promotes this effect, and we note that a cell-extrinsic effect of PDHK-driven glycolysis is enhanced tumor angiogenesis.

Results

Blocking Wnt alters the expression of metabolism-linked genes

Although multiple roles of Wnt signaling in colon cancer have been well studied, such as proliferation via regulation of the G1 phase of the cell cycle (van de Wetering et al., 2002), there are other functions that have yet to be identified. To reveal new roles of Wnt in colon cancer, we performed Gene Ontology analysis of our recently published microarray data set (Hoevetier et al., 2012). This data set reflected the change in global gene expression when three different dominant negative (dn) LEF/TCF isoforms were individually and rapidly induced with doxycycline in DLD-1 colon cancer cells (Fig 1A). Induction of dnLEF/TCFs reduces Wnt signaling through interference with endogenous β-catenin/TCF and β-catenin/LEF complexes, and we therefore focused our analysis on genes that were downregulated within 8 and 22 h after induction. The ontology analysis was carried out using PANTHER software which classified the downregulated genes into different categories of biological processes (Thomas, 2003; Thomas et al., 2006). A consistently large subset of regulated genes fell under the category of metabolism (Fig 1B and C; Supplementary Table S1). In fact, Panther binomial statistical analysis revealed that Wnt target genes connected to metabolism were the most highly overrepresented category compared to baseline representation of these genes in the human genome. These data, along with the notable downregulation of two key genes critical to cancer metabolism, pyruvate dehydrogenase kinase (PDK1) and the lactate transporter, MCT-1 (SLC16A1), led us to hypothesize that Wnt specifically and directly regulates a program of cellular metabolism in colon cancer cells.

dnLEF-1 and dnTCF-1Emut do not alter the cell cycle or proliferation in vitro

To test this hypothesis, we used dnLEF/TCF overexpression to block Wnt target gene regulation in colon cancer cell lines and examined resultant changes in metabolism. Knowing that overexpression of the potent dnTCF-1Emut isdnLEF-1 Emut halts cell cycle progression and cell proliferation (van de Wetering et al., 2002), while isoforms lacking the alternatively spliced Cterminal Emut avoid this stall (Naishiro et al., 2001; Atch a et al., 2007), we induced the expression of dnLEF-1 in which naturally lacks Emut sequences. In parallel, we also induced the expression of dnTCF-1Emut, a mutated form of dnTCF-1Emut with a five-amino acid substitution in the auxiliary C- clamp DNA binding domain in the Emut that eliminates the control of proliferation (Atch a et al., 2007). Use of these less potent dominant negative isoforms enabled an uncoupling of our test for effects on cell metabolism from effects on cycle progression. We utilized two different expression systems in two different colon cancer cell lines. In two independent clonal lines of DLD-1 colon cancer cells, we used a stably integrated doxycycline inducible system to express Flag-tagged dnLEF-1 (dnLEF-1[1]) and dnLEF-1[2]). In a second cell line (SW480), we used a lentiviral system to express physiological levels of Flag-dnLEF-1 or Flag-dnTCF-1Emut. In all cases, transgene expression was assessed by Western blot (Supplementary Fig S1A and E), and functional disruption of Wnt activity was confirmed by repression of the luciferase reporter Targe TOFFFlank (STOP) (Supplementary Fig S1B and F). We also confirmed that dnLEF-1 and dnTCF-1Emut had no impact on cell proliferation (Supplementary Fig S1C and G) or any parameter of the cell cycle profile (Supplementary Fig S1D and H). Cell cycle analysis performed at even longer time points of dnLEF/TCF expression also showed no change in the profile (data not shown). Overall, these data show that dnLEF-1 and dnTCF-1Emut expression have no effect on the cell cycle or the intrinsic abilities of the cells to proliferate and can therefore reveal phenotypes of blocking Wnt that are uncoupled from these functions.

Blocking Wnt alters the metabolic program of colon cancer cells

One of the most recognized hallmarks of cancer is Warburg metabolism, or a tendency for cancer cells to utilize glycolysis rather than oxidative phosphorylation regardless of the availability of oxygen (Warburg, 1956). We tested whether blocking Wnt alters carbohydrate metabolism by testing for changes in lactate (a byproduct of glycolysis) and ATP production (most efficiently produced via oxidative phosphorylation). A measure of lactate levels in the media of mock-infected SW480 cells compared to cells infected with lentiviruses expressing dnLEF-1 or dnTCF-1Emut revealed a significant decrease in lactate production (Fig 2A and B). This was true for both 2D conventional cultures and 3D suspension cultures in soft agar which were accompanied by a visual color change of the media (due to the secretion of excess lactic acid; Fig 2B). The drop in lactate production occurred independently of cell number, which was not significantly altered because of the lack of any effect of dnLEF/TCF expression on proliferation (Fig 2A). Similar analyses were performed in four different colon cancer cell lines (Supplementary Fig S2). We also interfered with Wnt by application of the drug XAV939. This small molecule inhibitor targets poly-ADP-ribose translesionases tankyrase 1, 2 to destabilize β-catenin, an action that works even in colon cancer cells where its destruction complex components are defective (Huang et al., 2009). We observed similar decreases in lactate production upon XAV939 application, a change consistent with a decrease in Warburg-type metabolism (Fig 2C).
Another hallmark of Warburg is less efficient production of ATP (since oxidative phosphorylation produces more ATP per molecule of glucose than glycolysis). We therefore measured changes in ATP levels in SW480 cells infected with dM6C/TCF-expressing lentivirus. We observed that any interference with Wnt signaling triggered increases in ATP production, suggesting an increased utilization of oxidative phosphorylation versus glycolysis (Fig 2B). We also tested for rates of glucose consumption, and as expected, rates were lower when either dM6C/TCFs or XAV939 was used to disrupt Wnt signaling (Fig 2E-G). For more precise measurements, we used Seahorse technologies to measure the glycolytic flux (extracellular acidification rate or ECAR), and mitochondrial respiration (oxygen consumption rate or OCR) of colon cancer cells subjected to our conditions. We observed that expression of dM6C/TCF in SW480 cells lowered the rate of glycolysis, but did not significantly affect the rates of respiration (Fig 2H and I). XAV939 treatment induced a similar effect, which we report as a 30–40% increase in the rates of oxidative phosphorylation relative to glycolysis (OCR/ECAR ratio, Fig 2J).

While the above technologies are quantitative, they are single-end-point measurements of an entire population of cells. Recognizing that cancer metabolism can be heterogeneous and is best evaluated in living cells, we utilized the phaser approach to fluorescence lifetime imaging microscopy (FLIM) to evaluate metabolism. Specifically, we used FLIM to monitor dynamic shifts in patterns of aerobe glycolysis versus oxidative phosphorylation by following the signature of the metabolic and autofluorescent marker NADH. NADH autofluorescence can be excited at a specific wavelength (740 nm), and the pattern of the decay of this fluorescence differs depending on its bound or unbound state (Lakowicz et al., 1992). Glycolytic cells have a predominance of free, unbound NADH. Respiring cells have high rates of oxidative phosphorylation and a predominance of bound NADH (e.g., NADH bound to mitochondrial enzymes; Monticci, et al., 2003; Greve, et al., 2004). The signature of fluorescence decay of NADH can be graphically represented on a 2D phaser plot where the decay rates for the pure free or bound species of NADH occupy very different positions (Becker et al., 2004; Colyer et al., 2008; Desman et al., 2008; Stringari et al., 2011). In a complex cellular environment where combinations of free and bound NADH co-exist, fluorescence signatures of decay map to experimental points between the extreme phaser plot posi-
Figure 2. Blocking Wnt alters the metabolic program of colon cancer cells.

A. Lactate levels are reduced with transient transduction of dnLEF-1 or dnTCF-1Emu in SW480 cells growing under standard culture conditions for 20 days. Representative graph of three replicates is shown with error bars representing the SEM between three internal replicates.

B. Fold change of lactate levels produced in 3D cultures. Images are of representative wells for each condition. Measurements performed on media collected from SW480 cells grown in soft agar after 22 days. Representative graph of three replicates is shown with error bars representing the SEM between three internal replicates.

C. Fold change in lactate levels of SW480 cells treated with Wnt inhibitor XAV939 (10 μM) for a minimum of 4 days. Data represent the average of six independent trials ± SEM.

D. ATP levels in SW480 cells collected 7 days post-transduction. Data represent the average of three independent trials ± SEM.

E-G. Fold changes in glucose consumption in SW480 cells expressing dnLeF1/TCF1 (I), DLD-1 cells (dnLeF1-18)) treated with doxycycline to induce dnLEF-1 expression (F), and SW480 cells treated with XAV939 (10 μM) (G). Data represent the average of four trials ± SEM.

H. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in SW480 cells stimulated with Mock, dnLEF1, or dnTCF-1Emu virus. Data represent the average of three independent trials ± SEM.

I. Data from (E) represented as an OCR/ECAR ratio.

J. OCR/ECAR ratio of SW480 cells treated with XAV939 (0.0 μM). Data represent the average of three independent trials ± SEM.

Data information: *p value < 0.05, **p value < 0.01, ***p value < 0.001.
Figure 3. Fluorescence lifetime analysis of NADH reveals shifts in glycolysis upon expression of dnLEF/TCF.

A) Phase plot of a FLIM analysis showing DLD-1 cells before and after a 1-hr treatment with 4 mM potassium cyanide (KCN). The phase position of pure free NADH is also shown.
B, C) Top panel in (B) shows the autofluorescence intensity at 780 nm. Bottom panels in (B) correspond to the free/bound NADH coloring of the same field of cells according to the color map shown on the phase plot in (A). Changes indicate an increase in free-bound NADH after KCN treatment. This is also shown in (C), a scatterplot analysis of average phase positions of individual cells before and after KCN treatment.
D) Phase position of DLD-1 (dnLEF/TCF) cells, and a color map to represent the gradient of relative levels of free and bound NADH.
E-H) The top row of images show time-phase fluorescence intensity images excited at 740 nm for two clones of DLD-1, dnLEF/TCF stably cells [dnLEF/TCF] and dnLEF/TCF mutant SW480 cells. The bottom row shows free/bound NADH color mapping as indicated by the color map in (D). The bottom panels show scatterplots where each point represents the average phase position from one cell. Induction of dnLEF/TCF or dnLEF/TCF mutant expression or treatment with the XAV939 inhibitor XAV939 results in a shift toward bound NADH. All images and measurements were taken 5 days after seeding under confluent conditions. Representative data from single dishes are shown from among at least three replicate experiments for each cell line. Each treatment resulted in a population on the scatterplot distinct from mock cells with P < 0.0002.

an overall cancer type of metabolism (Bird et al., 2009; Skala et al., 2007; Yuan et al., 2007; Yu & Heikal, 2009).

To demonstrate the relationship between a phase shift and a change in metabolism, cells were treated with potassium cyanide (KCN) for 1 hr to block mitochondrial respiration and trigger an increase in reduced, free NADH. FLIM analysis (using two-photon microscopy at 740 nm to excite bound and free NADH) before and after KCN treatment moved the phase distribution closer to the pure free NADH position, indicating a release of NADH from mitochondria and an increase in the ratio of free to bound NADH (Fig. 3A). This result is also visually confirmed through a false-color mapping of free/bound NADH overlaid pixel-by-pixel on the original images taken of the cells, where pink/red and blue/white represent, respectively, higher and lower concentrations of free NADH relative to bound NADH (Fig. 3B). A scatterplot display of the data enables a comparison of the average position of individual cells for each set of conditions and therefore reveals the spread of the data between replicates of the same conditions as well as between different experimental conditions. In this case, the phase positions of each pixel within one cell are averaged and plotted as a single point (Fig. 3C).

To assess the impact of blocking Wnt target gene activation on the ratio of free to bound NADH, we performed FLIM analysis on normoxic cells expressing dnLEF/TCF or cells treated with the XAV939 inhibitor. Doxycycline induction of dnLEF/TCF in two different clonal DLD-1 cell lines caused the average phase position to shift away from free NADH to the upper left of the scatterplot, toward bound NADH (Fig. 3D, E and F). This shift represents a decrease in free/bound NADH, a change that was also evident in the color mapping of NADH states in the DLD-1 cells. Parental
DLD-1 cells treated with the same concentration of doxycycline showed no significant change in the pharyn positions, indicating that doxycycline alone does not alter the metabolic signature (Supplementary Fig S3D). To confirm this result in other colon cancer cells, we performed FLIM analysis on SW480 cells infected with empty virus or lentivirus carrying dmeLEF-1N or dmeTCF-1Emut expression cassettes (Fig S5G, additional dmeTCF isoforms in Supplementary Fig S4A and C), as well as analysis of cells treated overnight with XAV939, the Wnt signaling inhibitor (Fig 3E). We also tested four other colon cancer cell lines that varied with respect to their endogenous level of Wnt signaling (Supplementary Fig S3C). Shifts in metabolism correlated directly with the ability to reduce Wnt signaling. These data suggest that blocking Wnt signaling in colon cancer cells results in a decrease in the ratio of free to bound NADH, which indicates a decrease in glycolysis. Taken together, our assessment of lactate production, ATP production, ECAR, OCR, glucose consumption, and FLIM signatures of NADH all indicate that oncogenic Wnt signaling promotes a glycolytic form of metabolism.

Blocking Wnt reduces PDK1 levels via regulation of transcription

We next asked whether the changes in metabolism induced by blocking Wnt could be attributed to any of the metabolism-linked target genes identified in the DLD-1 microarray experiments. One common target gene downregulated by all of the dmeLEF-1C proteins was pyruvate dehydrogenase kinase (PDHK1). This kinase phosphorylates and inhibits the pyruvate dehydrogenase (PDH) complex in mitochondria (Roche et al, 2001). Inhibition of PDH1 in turn, reduces the conversion of pyruvate to acetyl-CoA for entry into the TCA cycle and oxidative phosphorylation. Therefore, PDHK1 plays an important modulatory role in the promotion of a glycolytic phenotype and is therefore not surprisingly found to be upregulated in a number of cancers (Kousouridou et al, 2006; Wegiel et al, 2008, Banumath et al, 2012).

To validate the microarray data and test whether PDK1 levels are modulated by β-catenin and LEF/TCF proteins, Western blot analysis was performed for both DLD-1 and SW480 cells expressing dmeLEF-1 or dmeTCF-1Emut. PDK1 protein levels were reduced 53–75% for all cell lines (Fig 4A). To confirm that this reduction is evident at the mRNA level, RT-qPCR analysis of PDK1 mRNA was performed for doxycycline-treated dmeLEF-1-DLD-1 cells after 24 or 120 h of expression. At both time points, PDK1 mRNA levels were reduced to 70%, similar to the reduction observed at the protein level (Fig 4B and C). Since there are four members of the PDK family (PDK1-4), we asked whether any of the additional PDK family members were also downregulated after blocking Wnt. RT-qPCR analysis of PDK2-4 levels in DLD-1 cells expressing dmeLEF-1 indicates that neither PDK2 nor PDK3 levels were altered, while PDK4 showed a modest, but significant decrease from its already low basal level (Fig 4B). Overall, these data suggest that PDK1 and possibly PDK4 are regulated by Wnt/β-catenin signaling in colon cancer cells.

A possible connection between Wnt and metabolism occurs through the Wnt target gene, c-Myc, and in fact, previous reports suggest that under certain conditions, c-Myc can potentiate the upregulation of PDK1 levels (Kim et al, 2007). We examined c-Myc levels after expression of dmeLEF-1/TCF. With the exception of a slight decrease in c-Myc protein after 96 h of dmeLEF-1 expression in DLD-1 cells (Fig 5A), there is little to no difference in c-Myc mRNA or protein levels even after 120 h (Fig 4A and C). This result also matches the previously published microarray, which showed no change in c-Myc mRNA after expression of dmeLEF-1. Therefore, the change in PDK1 levels observed is independent of Wnt regulation of c-Myc.

To determine the kinetics of PDK1 transcription upon disruption of Wnt signaling, we used a 4-thiouridine labeling procedure to identify actively transcribed mRNAs (Dillman et al, 2008). After induction of dmeTCF-1Emut for 2 h, DLD-1 cells were incubated with a pulse of 4-thiouridine for 30 min to incorporate the nucleotide label into nascently transcribed RNA. After the pulse, cells were harvested and labeled RNA was purifi ed via a histone/protein/steric-assisted pull-down procedure (Dillman et al, 2008). Semi-quantitative RT-PCR for PDK1 mRNA demonstrated that transcription of the PDK1 locus was immediately increased upon the induction of dmeTCF1E-mut expression (Fig 4D). The known Wnt target gene AXIN2 served as a positive control, and constitutive, ubiquitous UBA12 served as a negative control. Induction of dmeTCF-1Emut reduced transcription by at least 50% for both PDK1 and AXIN2, demonstrating that PDK1 is likely to be a direct Wnt target gene. We have also performed a genome-wide ChiP-seq study of dmeTCF-1E binding (N.P. Hovey, M.D. Zeller, M.M. McQuade, A. Garibaldi, A. Busch, K. Hertel, P. Baldi and M.L. Waterman, in preparation) and discovered in the genome data set that TCF-1 binds to distal upstream (‘Peak 1’) and downstream (‘Peak 2’) sites surrounding the PDK1 locus (Fig 4E). Each genomic region of TCF-1 occupancy contains three putative Wnt response elements (Supplementary Fig S4). We designed PCR primers specific to these two regions as well as the PDK1 promoter and an internal site in the locus as a negative control. Using chromatin immunoprecipitation procedures, we determined that TCF-1 directly binds to the distal sites but not the promoter (Fig 4E). To test whether these sites confer transcription regulation in colon cancer cells, we subcloned each distal region next to the heterologous thymidine kinase promoter and luciferase open reading frame. Transient transfection assays showed that both fragments increased promoter activity in SW480 colon cancer cells and induction of either dmeLEF-1 or treatment with XAV939 eliminated this regulation (Fig 4F). We also tested these fragments for their ability to regulate the PDK1 promoter (Supplementary Fig S4). While both regions conferred a distinct, modest level of activation, the PDK1 promoter itself was markedly sensitive to downregulation by dmeLEF-1 (Supplementary Fig S4). These data demonstrate that PDK1 is a direct Wnt target gene and that regulation occurs through distal regulatory sites upstream downstream of the locus.

PDK1 overexpression rescues the altered metabolic phenotype induced by blocking Wnt

To determine how much of the Wnt-driven metabolic signature depends on PDK1, we asked whether restored expression could rescue the metabolic shift induced by dmeLEF-1. We used lentiviral infection of DLD-1 cells to restore physiological levels of PDK1. As shown in Fig 5A, dmeLEF-1 expression alone led to a decrease in endogenous PDK1 levels and a decrease in glycolysis (a shift in the FLIM phase position away from free NADH). In contrast, lentiviral restoration of normal PDK1 levels rescued the FLIM signature of glycolysis (a return of the phase position back to that of cells not
expressing dnLEF-1 (Fig 5A). This rescue is also evident in the free/ bound NADH color mapping of the cells, showing similar levels of free/bound NADH in cells without doxycycline compared to cells with doxycycline and PKD1. When repeated in the second DLD-1 clone line expressing dnLEF-1, a rescue of the FLIM signature was similar, indicating at least a partial rescue in the metabolic shift (Supplementary Fig S5). Taken together, these results suggest that reduction of PKD1 could be a major reason for the metabolic shift triggered by dnLEF/TCF interference with Wnt/β-catenin signaling.

Given that we propose PKD1 expression as a major Wnt target for glycolysis, we compared the effects of dnLEF/TCF to dichloroacetate (DCA), a well-known small molecule inhibitor of PKD1 (Whitehouse et al, 1974). A 48-h treatment of DCA led to the same characteristic shift in the phase plot toward bound NADH that was seen with expression of dnLEF/TCF, again indicating a decrease in a glycolytic phenotype (Fig S8). The FLIM shift corresponded to an increase in the ratio of oxygen consumption (oxidative phosphorylation) to the extracellular acidification rate (OCR/ECAR, Fig S2). These changes in metabolic matrix match previous reports that DCA treatment decreases lactate excretion (Bonnert et al, 2007) and increases ATP levels (Sun et al, 2011) similar to our measurements with dnLEF/TCF expression. Another reported phenotype of DCA treatment in cancer cells is an increase in sensitivity to chemotherapy treatment (Caïns et al, 2007; Stockwell et al, 2015; Xiong et al, 2011). Therefore, we examined whether dnLEF-1 forced similar increases in sensitivity to the chemotherapeutic agent irinotecan. We treated SW480 cells with DCA alone, dnLEF-1 alone, DCA with irinotecan, or dnLEF-1 with irinotecan. Neither 2.5 μM irinotecan nor dnLEF-1 alone had much effect on cell proliferation and 10 mM DCA had only a very slight impact. In contrast, both DCA and dnLEF-1 greatly enhanced the sensitivity of the cells to irinotecan, showing a synergistic decrease in the proliferation rate (Fig SD). These data suggest that dnLEF/TCF inhibition of PKD1 in many ways mimics the effects of DCA. However, while there was congruence between dnLEF/TCF and the DCA phenotypes, DCA targets all four members of the PKD family, not just PKD1. We therefore utilized lentiviral shRNA knockdown to test for the specific significance of PKD1 (Supplementary Fig S8 and C). Western blot analysis of multiple knockdown lines revealed reduced PKD1 protein levels and significant reduction of phosphorylation of its downstream substrate, mitochondrial pyruvate dehydrogenase (PDH) (Fig S9). FLIM analysis of each knockdown cell line revealed significant shifts in the free/bound NADH signature that mimics the decrease in glycolysis observed with dnLEF1 or dnTCF1 expression or DCA treatment (Supplementary Fig S5C). These results indicate that PKD1 is an important Wnt target gene and that it contributes major activities to glycolysis. However, we also observed the knockdown cultures revert to a glycolysis mode of metabolism several days later. Western blot analysis of the reverted cultures showed that while PKD1 protein levels remained low, phosphorylation of the target substrate pyruvate dehydrogenase had recovered, a sign of compensatory rescue via other PKD family members or other kinases (data not shown). Thus, the more stable shift in metabolism observed with DCA treatment or dnLEF/dnTCF expression suggests that total cellular PKD activity is targeted by Wnt signaling beyond single, selective regulation of PKD1. We conclude that PKD1 is a major Wnt target gene, but that it is coordinately regulated within an entire gene program for glycolysis.

Blocking Wnt7 reduces in vivo tumor growth

Since growing cells in vitro (on plastic) does not accurately mimic the metabolic demands of an in vivo tumor, we tested the impact of dnLEF/TCF expression on cancer cells in a xenograft tumor model. As shown in Fig 6A, expression of dnLEF1 or dnTCF1/Emut in SW480 cells injected into immune-deficient NOD mice drastically decreased tumor growth by up to 97%. The tumors created from cells expressing dnLEF/TCF had 20–30% fewer proliferating cells as measured by Ki67 staining (Fig 6B). To compare the changes in protein expression in vitro with the previous results in vitro, we used Western blot analysis to examine levels of PKD1 and c-Myc. PKD1 protein was reduced in both dnLEF/TCF tumor types. Levels of c-Myc protein were variable but overall similar between the dnLEF-1-expressing tumors compared to mock, while c-Myc protein was significantly reduced in the dnTCF-1/Emut tumors (Fig 6C). These results are consistent with the previously described micromass showing a decrease in c-Myc RNA levels with dnTCF1/Emut, but not dnLEF1 (Hervert et al, 2012). Western blot analysis also revealed subtle reduction of pyruvate dehydrogenase phosphorylation in both dnLEF/TCF tumor types (Supplementary Fig S9). Overall, the tumor analysis suggests that while tumors expressing dnLEF/TCF show similar reductions in PKD1 as cells in vitro, the expression of dnLEF/TCF has a much different effect on cell growth: no change in vitro but a strong, negative effect on growth in vivo.

Figure 4. Blocking Wnt directly reduces PKD1 levels via regulation of transcription.

A Whole-cell lysates from DLD-1 or dnLEF-1 cells were collected 48, 72, and 96 h after 0.05 μg/ml doxycycline treatment and were probed with the antibodies shown. Western blot analysis was performed as described in Methods.

B C RT-qPCR analysis was performed on RNA isolated from DLD-1 or dnLEF-1 cells harvested 24 h or 48 h after the addition of doxycycline. Graphs shown represent the average of three trials (± SEM). RT-qPCR analysis was performed on 4-chloro-1-naphthol RNA isolated from a 30-min pulse in the presence/absence of dnTCF1/Emut, induced by 2 μg/ml doxycycline treatment in DLD-1 cells. A Western blot was probed with the antibodies shown. The antibody that detects the indicated PKD family member shows that dnTCF1/Emut associates with detectable levels in DLD-1 cells. A representative graph is shown of two replicates, with error bars representing the SD among three internal replicates.

D Western blot analysis of PYKD1 expression normalized to β-actin from DLD-1 cells with or without induction of FLAG-dnTCF1/Emut using anti-FLAG antibody. A representative graph is shown of two replicates, with error bars representing the SD among three internal replicates.

E F Luciferase reporter activity in SW480 cells shows that Peak 2 and Peak 2 regions confer decreased transcription activity to the heterologous TATA boxes (TE) promoter. Expression of transcriptional DNA (dnLEF) or treatment with the Wnt inhibitor XAV939 (6 μM) in DLD-1 cells increases the regulatory activity of these fragments. Graph shows the average of three independent replicates (± SEM). Data information: *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

Source data are available online for this figure.
Figure 5. PDK1 overexpression rescues the altered metabolic phenotype induced by blocking Wnt.

A. Western blot analysis of lysates extracted from DLD-1 (dualFLAG) cells treated with or without 200 ng/ml dextran sulfate for 48 h and with or without PDK1 lentivirus for 72 h. FUM imaging was performed at confounding 96 h dextran sulfate and 7 days post-transduction. Intensity images are shown on the left. FUM results shown through the dextran sulfate staining (right), and the scatterplot show that dextran sulfate induction of dLEF-1 shifted the position toward bound NADH, while PDK1 rescue shifted it back to its original position (P < 0.0001 comparing -Dox to +Dox and comparing +Dox to +Dox+PDK1).

B. FUM analysis of SW480 cells treated with 50 mM DCA for 48 h shows a phase shift toward bound NADH (P < 0.0001 comparing +DCA to mock).

C. OCR/ECAR ratio of DCA (20 mM)-treated SW480 cells shows increased oxygen consumption rate (mitochondrial activity) relative to the extracellular acidification rate (glycolysis produced lactate). Data shown represent the average of three independent trials (± SD, *P*-value < 0.05).

D. Cell proliferation assay of SW480 cells treated with or without 20 mM dextran, 20 mM DCA, and dLEF-1 lentivirus shows an increased sensitivity to induction when treated with DCA or dLEF-1. A representative graph of two trials is shown. Error bars represent the standard deviation between eight technical replicates.

Source data are available online for this figure.
To test whether the same change in metabolism that we detected in vivo by FLIM analysis is also detected in vitro, we adapted FLIM analysis for living, actively perfused xenograft tumors. For all tumors, tail vein injection of fluorescent dextran (FITC dextran) was used to continuously image live blood cell flow through the vasculature of the tumor. Mice were anesthetized and intact xenograft tumors were exposed (with feeder vessels preserved) followed by immediate stabilization of the mice over the microscope objective in a temperature-controlled environment. Non-invasive, confocal FLIM imaging was performed at depths between 50 and 200 μm around the subcapsular region of the tumors (see Supplementary Fig S6 for a schematic of this in vivo FLIM). A similar metabolic response to Wnt signaling inhibition was observed with these analyses. That is, dnLEF1/ΔTCF1-expressing tumors were less glycolytic (phaser position shifted toward bound NADH; Fig 6D). A notable difference compared to cells grown in monolayer in vitro was that xenograft tumors had a greater degree of variability in FLIM profile, reflecting the existence of a more complex tumor microenvironment (additional replicates shown in Supplementary Fig S7). To test whether PDK1 expression could rescue this effect, we imaged xenograft tumors of dnLEF1 cells that had been transiently transduced with a PDK1 expression vector prior to injection into the mice. PDK1 expression rescued the metabolic signature, shifting the phaser plot back toward the free NADH position (Fig 6D; Supplementary Fig S7).

Blocking Wnt reduces tumor vessel density, while reintroduction of PDK1 restores it

Much of the cost and benefit analysis of glycolytic metabolism has focused on intracellular changes in metabolites and biosynthetic
intermediaries. However, imaging live tumors highlighted a striking extrinsic benefit of glycolytic metabolism on the tumor microenvironment. FITC-dextran labeling of the vasculature revealed that the density of blood vessels was greatly diminished in dnLEF/TCF-expressing tumors (Fig 7A). Thus, one important reason these dnLEF/dnTCF-expressing tumors were reduced in size was because of poor nutrient delivery. Interestingly, re-expression of PDK1 strictly in the injected tumor cells appeared to restore vessel density to levels equivalent to that of the mock tumors (Fig 7A). To quantify these changes, we stained tumors sections for the endothelial marker CD31 and counted vessel density in the same subcapsular region where the PLIM analysis was performed. Vessel density was greatly diminished in the tumors expressing dnLEF-1 or dnTCF-1Enuc, but rescue expression of PDK1 returned vessel density back to the original level observed in the mock tumors (Fig 7B). A few studies have shown that enhanced glycolysis can promote angiogenesis through the stabilization of HIF1α (Hunt et al., 2007; Miloradova et al., 2008; Sunetricka et al., 2012). We therefore evaluated HIF1α protein levels in the xenograft tumors. Interestingly, HIF1α protein levels were reduced upon dnLEF/TCF expression, and levels were at least partially restored with reintroduction of PDK1 (Fig 7C). HIF1α mRNA levels were unaffected and stable in the tumors expressing dnLEF-1 or dnTCF-1Enuc.
all conditions (Fig 7D), suggesting that regulation occurs at a post-transcriptional step. We also note that while the well-known angiogenic factor VEGF was downregulated in the dLLEP/TCF-expressing tumors, addition of PDK1 did not restore these levels (Fig 7D), suggesting that the PDK1 rescue of angiogenesis is VEGF independent. Overall, dLLEP/TCF expression in vivo reduced tumor growth, reduced PDK1 levels, reduced the glycolytic metabolic signature (including tumor lactate levels, Fig 7F), and reduced the density of blood vessels feeding the tumor. Restoration of PDK1 expression rescued both the change in metabolism and the reduction in vessel density, a striking demonstration of cell-autonomous and non-autonomous effects of a metabolic enzyme.

Discussion

Here, we report that Wnt/β-catenin signaling directs a metabolic program of glycolysis in colon cancer cells, a common cancer phenotype known as the Warburg effect. This metabolic change is accompanied by non-autonomous effects on the microenvironment in the form of increased vessel development. A direct Wnt target gene, PDK1, is identified to participate in both of these Wnt-driven, cancer-supporting phenotypes (model in Fig 8A). We also describe for the first time the use of FLIM imaging to detect metabolic changes in living, actively perfused xenograft tumors. We suggest that effects on metabolism are a common, core function of all LLEP/TCF family members and isoforms, even those isoforms previously understudied due to their lack of regulation of proliferation and the cell cycle.

A prevailing theme in the field of cancer metabolism is that the same oncogenic pathways that drive transformation must also favor metabolic pathways that support this process (Ward & Thompson, 2012). In fact, given that Wnt signaling plays a critical role in embryonic development, tissue remodeling, and cancer biology, and given that the success of these processes depends on tight coordination with the metabolic pathways that support them, it has been suggested that Wnt is a prime candidate for directing this
coordination (Setali & Vidal-Puig, 2010). Our study supports this paradigm as we demonstrate that blocking Wnt target gene activation alters cancer cell metabolism by reducing aerobic glycolysis. Two recent studies also support this conclusion. One study in breast cancer cells found that activation of Wnt signaling enhanced glycolysis through indirect actions of the transcriptional repressor Snail on cytochrome c oxidase, a component of the electron transport chain (Lee et al., 2012). A second, more recent study found that Wnt stimulates a fast-acting, short-term: Wnt/β-catenin/Rac1-mTORC2-Akt signal to upregulate protein and activity levels of glycolytic enzymes, including PKD1 (Eben et al., 2013). Regulation occurred within 10-20 min of Wnt stimulation and was independent of β-catenin, LDL/TCFs, and transcription. Our findings contrast with this system, because we find that β-catenin and LDL/TCF are required to target PKD1 transcription. Indeed, our test for a connection to mTORC2 was negative as inhibition of β-catenin with either dmLef/TCF expression or XAV939 treatment for as long as 8 h had no effect on mTORC2 activity in DL1-1 and SW80 cells and inhibition of mTORC2 for 24 h with the specific mTOR inhibitor PP242 had no effect on PKD1 mRNA levels (Supplementary Fig S9). We propose that Wnt signals have short- and long-term modes of metabolic regulation. Short-term modes trigger an mTOR kinase cascade that affects protein stability of glycolytic enzymes. Long-term modes use β-catenin to change gene expression of at least some of these enzymes as well as other metabolically relevant components (Fig IC, Supplementary Materials and Methods). This latter mode is of primary importance in cancers that harbor aberrant, chronic, elevated levels of Wnt signaling with or without Wnt ligands. Chronic Wnt signaling could transform the metabolic potential of cancer cells by elevating the overall levels of key components for glycolysis and thereby set the stage for robust Warburg metabolism in the face of whatever environment the transformed cells encounter.

PKD1 is a well-known, key metabolic regulator of glycolysis. This kinase phosphorylates the pyruvate dehydrogenase (PDH) complex to inhibit the first step of converting pyruvate to acetyl-CoA (Roche et al., 2001), an important regulatory nexus between glycolysis and respiration. PKD1 phosphorylation of PDH suppresses entry of fuel into mitochondrial and instead promotes glucose fermentation to lactate in the cytoplasm, an important activity for promoting the Warburg effect in cancer. Indeed, PKD1 is upregulated in numerous cancers, including colon cancer (Koukouraki et al., 2006; Wigfield et al., 2008; Baumunk et al., 2012). Here, we propose that cancer relevant levels of Wnt signaling use LDL/TCFs for direct regulation of PKD1. PKD1 mRNA was identified through microarray analysis as downregulated after blocking Wnt in colon cancer cells (Fig 1). Our chromatin immunoprecipitation, 4-thiouridine labeling, and transient transfection analyses show that this downregulation is direct (Fig 4D-F, Supplementary Fig S4). Genome-wide ChIP-seq studies by others (Endo et al., 2012) also show that the PKD1 gene locus is occupied by TCF-4 (TCF7L2) in multiple cancer cell lines (Supplementary Fig S8). Interestingly, peaks of occupancy are also present in the promoter regions of the other PKD family members, especially for PKD2 and PKD4. It is possible that multiple PKD isoforms have the capacity to respond to Wnt signaling, a possibility underscored by our observation of compensatory recovery of PDH phosphorylation when PKD1 was selectively targeted for knockdown by shRNAs.

Our experiments demonstrate that PKD1 is a pivotal mediator of Wnt effects on metabolism as rescue of its expression restored glycolysis and vessel density in tumors. However, we expect that PKD1 is only one target of an entire gene program driven by Wnt to promote cancer-supporting metabolism. For example, while PKD1 knockdown caused a significant shift of metabolism in cultures, the shift was not as stable as the dmLef/TCF treatments. Also, PKD1 rescue expression did not entirely restore tumor mass. It is possible that knockdown of a single Wnt target gene out of the context of an entire gene program is not sufficient to shift metabolism and tumor proliferation phenotypes. Ontology analysis identified additional metabolism-linked genes that were downregulated by dmLef/TCF treatments (Fig 1 and Supplementary Table S1). This includes genes that encode nutrient and small molecule transporter genes that affect metabolic pathways. For example, the isocitrate transporter MCT-1 (monocarboxylate transporter 1 or SLC16A1) is a critical player in tumor metabolism and is upregulated in colon cancer (Pinheiro et al., 2008). MCT-1 was downregulated with dmLef/TCF expression, both by microarray analysis and by RT-qPCR analysis of xenograft tumors (Supplementary Fig S8). The MCT-1 promoter is occupied by TCF-4, especially in HCT116 colon cancer cells (Supplementary Fig S8), implicating MCT-1 as a Wnt target gene. Previous studies have identified other metabolism-connected Wnt target genes. For example, the well-known Wnt target gene c-Myc plays pivotal roles in cancer metabolism, driving both aerobic glycolysis and glutaminolysis (Ono et al., 2000; Wise et al., 2008; Deng, 2010). In fact, c-Myc has been shown to enhance HIF-1α-induced regulation of PKD1 (Kim et al., 2007). We note here, however, that c-Myc is not required in our system to mediate changes in metabolism as its levels are not altered with the expression of dmLef/1 (supported by our microarray, Western blot, and RT-qPCR analyses). However, as Wnt/β-catenin signaling is the main driver of colon cancer, we expect that the Wnt target c-Myc should cooperate with the genes identified in our study to collectively drive strong, glycolytic signatures of Warburg metabolism. Additional Wnt target genes linked to glycolysis have also been identified in the liver. For example, Charley et al. show that Wnt augments LDH (lactate dehydrogenase) activity and downregulates two mitochondrial ATPase subunits in hepatocytes, thus driving glycolysis at the expense of oxidative phosphorylation (Charley et al., 2009). While it is not clear whether these effects are direct or indirect, these data along with the other evidence above suggest that Wnt signaling promotes a gene program that supports a cancer-promoting metabolic profile of enhanced aerobic glycolysis.

Given the strong evidence for direct regulation of PKD1 transcription, we asked whether Wnt and glycolysis gene programs were correlated in primary human tumors. We used publicly available gene expression data from The Cancer Genome Atlas Data Portal to test for correlations between PKD1 expression and signatures of overactive Wnt signaling (TCGA, https://tcga-data.nci.nih.gov/tcga/). No correlation was identified, a finding consistent with recent studies of large tumor data sets (>2,500) that found confounding levels of heterogeneity for gene signatures of metabolism, and consistent with our observation of compensatory PDK activities in PKD1 shRNA knockdown lines (Hu et al., 2013). Nevertheless, a different method of analysis detected links between Wnt signaling and glycolysis in tumors (Supplementary Fig S10). In this analysis, a comparison of the expression level of Wnt and glycolysis genes defined by Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for unsupervised clustering of 248 human tumors into nine tumor groups with one group of 21 tumors strongly
clustered (group 4, Supplementary Fig S1A). This same cluster was detected when gene signatures were compared via distance measures within the cluster versus the other eight tumor groups (Supplementary Fig S1B). Strong similarities (correlations and anti-correlations) between Wnt signaling components and glycolysis genes were detected within this group, relationships that distinguished the cluster from the remaining clusters. Importantly, PDK1 ranked as a highly correlated gene in the 21 tumors, its expression most often having negative, anti-correlations with Wnt components (see Supplementary Materials and Methods). The majority of anti-correlating genes encode antagonists of Wnt signaling or components of non-canonical Wnt signaling, and interestingly, the STRING algorithm for protein interaction networks revealed that most of these anti-correlating components engage in direct or indirect interactions with the Wnt component Dishevelled (Supplementary Fig S1C; Szklarczyk et al., 2011). These data are compelling because any kind of clustering of colon tumors on the basis of gene expression suggests a promising approach to probe differences between tumors for diagnosis and/or prognosis and they most certainly warrant further investigation and validation.

Most tumorigenic actions of signaling pathways like Wnt represent distorted use of normal functions crucial to cellular homeostasis. How might Wnt promotion of aerobic glycolysis be beneficial to normal tissues? One organ that illustrates this benefit is the liver where Wnt signaling regulates liver function, including functional and anatomical liver zonation, hepatocyte maturation, and glycolytic metabolism (Tan et al., 2008; Thompson & Monga, 2007; Colletti et al., 2009; Gehardt & Herbermann, 2010). The pancreas is also central to whole-body glucose homeostasis, a tissue that exhibits important links between Wnt signaling and disease. In fact, polymorphisms in the TCF7L2 (TCF-4) locus encode the strongest links to risk of diabetes of any SNP (Grant et al., 2006; Lyons et al., 2007; Cadigan & Waterman, 2012). Most relevant to our study is that Wnt regulation of glycolysis is important to normal intestinal tissue. We recently used FLIM analysis to identify a metabolic gradient along the crypt-villus axis of the mouse small intestine (Sintirgui et al., 2012). Consistent with our finding that Wnt signals drive a glycolytic phenotype, we observed the strongest glycolytic signatures at the base of the crypt and lowest at the top of the villi, correlating with high and low Wnt activity, respectively. According to our model (Fig 8A), Wnt promotes a glycolytic signature through the regulation of PDK1, which then promotes HIF1α stabilization. Consistent with this model, we observe high levels of PDK1 protein and its target, phospho-PDK1 at the bottom of human intestinal crypts (Fig 8B). Stabilized HIF1α is also detected in crypt bases (in addition to the hypothesis region at the tops of crypts as has been reported previously) (Giles et al., 2006; Fig 8B). Wnt-stimulated HIF1α stabilization could contribute to a positive feedback loop to support glycolysis in the intestinal stem cell niche as HIF1α targets several components of glycolysis. Wnt also plays an important direct role in the maintenance of stem cells (Kemnitz et al., 1998; Nussle, 2008), which utilize aerobic glycolysis as a metabolic resource (Suda et al., 2011; Zhou et al., 2012). The benefits of this type of metabolism in stem cells include support for proliferation as well as reduction in ROS (reactive oxygen species) production to protect the integrity of the genome (Kondoh et al., 2007; Prigione et al., 2010).

In the present study, we identify a new link between Wnt signaling and tumor angiogenesis. Although Wnt has been connected to angiogenesis through direct regulation of VEGF expression (Zhang et al., 2001), we report for the first time the role of Wnt-directed PDK1 expression in this process. Indeed, PDK1 and PDK2 have recently been shown to enhance angiogenesis (McFate et al., 2008; Suinendra et al., 2012). We suggest that one mechanism for this phenomenon is increased lactate production because levels of this metabolite were increased in PDK1 rescue tumors (Fig 7F). Lactate has been associated with increased angiogenesis via reduced ADP ribosylation and stabilization of HIF-1α protein (Lu et al., 2002; Hunt et al., 2007; Miletanová et al., 2008). Stabilized HIF-1α could enhance angiogenesis at least partly through upregulation of VEGF (Freer et al., 1996; Pugh & Ratcliffe, 2003) and SDF-1 (stromal cell-derived factor-1), Krasheninnikov et al., 2007). While we observe changes in HIF1α levels that correlate with tumor vessel density, we did not observe concomitant changes in VEGF levels, suggesting that other angiogenic factors such as SDF-1 might be relevant (Fig 7C–E). Even though the exact mechanism linking Wnt and PDK1 to angiogenesis is not known, our discovery that PDK1 rescues vascularization highlights how the metabolic status of cancer cells communicates to the tumor microenvironment.

Consistent with our finding that oncogenic Wnt regulates PDK1, blocking Wnt mimics the effects of small molecule inhibition of the PDK family of kinases through DCA. DCA treatment reduces glycolysis, increases oxidative phosphorylation, decreases lactate production, increases cancer cell sensitivity to chemotherapy drugs, and reduces xenograft tumor mass and vascularity (Bonnet et al., 2007; Papandreou et al., 2011). A DCA trial in human glioblastoma patients resulted in promising tumor regression, as well as enhanced tumor apoptosis, and to note decreased tumor vascularity (Michalakis et al., 2010). Collectively, results from multiple studies suggest that the effects of DCA are much more robust in vivo than in vitro (Papandreou et al., 2011), mirroring our lack of growth phenotype when blocking Wnt in culture, as opposed to the drastic reduction in xenograft tumor size in vivo. This could be attributed to the excess nutrients available in in vitro culture which can mask dependencies on specific metabolic pathways. More intriguing is the possibility that DCA and ddLEF-1 expression are more effective in vitro because blocking aerobic glycolysis has profound effects on the tumor microenvironment, such as reduced vascularity. Our study therefore has important implications for the development of cancer therapies targeting the Wnt pathway and for testing Wnt inhibitors in vivo, as inhibitors that do not block proliferation or cell cycle progression in artificial culture conditions in vitro may still be effective at limiting tumor growth in vivo via their effects on the more sensitive Wnt metabolism program. In fact, partial interference with oncogenic Wnt signaling may be an effective treatment strategy as it might normalize metabolic activity in tumor cells while preserving essential Wnt signaling functions in normal cells such as cell cycle and cell differentiation programs.

Materials and Methods

Cell lines/constructs

Colon cancer cell lines, SW480 and DLD-1, were cultured in either Dulbecco’s modified Eagle’s medium (DMEM; Gibco 11960) or RPMI-1640 medium (Cellgro 15-040), supplemented with 10% fetal...
bovine serum (FBS) and 2 mM glutamine. Unless otherwise stated, experiments were performed in the same media, DLD-1 (dulBEF) and dntTFC-1 (dntTFC-1) stable cell were created by transfecting Tet- inducible dntLEF-1 or dntTICF-1 into DLD-1 or T87 cells (a generous gift from M. van de Wetering and H. Clevers) as previously described (Yokoyama et al., 2010; Howes et al., 2012). Cells were maintained in 500 μg/ml zeocin and 10 μg/ml blasticidin. However, it was found that dntLEF/TICF expression in the presence of zeocin reduces cell proliferation (Yokoyama et al., 2010, and data not shown). Therefore, in order to avoid any contribution from zeocin, all experiments were performed in the absence of antibiotics. Induction of dntLEF-1 or dntTICF-1 was achieved through the addition of 0.01 or 0.1 mM doxycycline to the media, respectively. Lentiviral constructs were cloned via Cold Fusion (System Biosciences) by inserting coding sequences for Flag-tagged dntLEF-1, Flag-tagged dntTICF-1 (Atcha et al., 2007), or Flag-tagged PKD1 (a gift of Jean Zhao (Booth et al., 2007); Adigenes plasmid e20544) into pCDH lentivector (System Biosciences; SBI-CDS6AA-2). See Supplementary Materials and Methods for cloning information regarding TRK and PKD1 receptors.

**Lentiviral preparation and infection**

Lentivirus was prepared and target cells were transduced using System Biosciences lentivirus technology according to their specifications (see Supplementary Materials and Methods for details). Cells were harvested for subsequent assays 72 h post-transduction unless otherwise stated.

**Lactate assay**

Lactate measurements were performed on media collected from both cells grown in suspension (soft agar) and cells grown on solid support (plastic). SW480 cells were embedded in soft agar 72 h post-transduction at a concentration of 1,000 cells in 2.5 ml of 0.3% agar in DMEM with 10% FBS. Fresh media were added once a week. After 22 days of growth (5 days since last media change), images of wells were taken and media were collected. For cells grown on plastic, SW480 cells (72 h post-transduction) were seeded in a 96-well plate at 5,000 cells per well. Media were changed after 72 h and then left unchanged until media were collected after 7 days. Media were collected from three triplicate wells, while the number of cells in three wells were determined using the SRI assay (described below). SW480 cells treated with XAV939 (Sigma X5001) were treated with either 10 μM XAV939 or DMSO for at least 4 days prior to media collection. For lactate measurements of xenograft tumors, pre-weighted flash-frozen xenograft tumors were homogenized in a Precellys 24 homogenizer in the presence of 10 μl HBS (hank’s balanced salt solution) per mg tissue at 60,000 rpm, twice, for 15 s each. Tissue extractions and lactate assay were performed according to L-Lactate Assay kit (Enzo Biocience #1200011002). All measurements were performed in triplicate.

**Sulforhodamine B cell growth (SBRB) assay**

Cells were seeded in 96-well plates at 5,000 cells per well with eight replicates for each condition and time point. Media were refreshed every day (with or without 0.01 μg/ml doxycycline, 2.5 μM (rotenone), or 20 mM DCA as needed). Cells were fixed and stained according to published protocols (Shekhar et al., 1990) for a 10-day period. Optical density readings were performed at 450 nm.

**ATP assay**

SW480 cells were seeded at 125,000 cells per well (12-well plate) 72 h post-transduction and harvested 96 h after seeding. ATP measurements were performed according to the ENLIFEN ATP Assay System (Promega #F2000). All measurements were performed in triplicate.

**Glucose consumption assay**

Cells were seeded at 5,000 cells per well in a 96-well plate and media were harvested 6 d later. Low glucose media (DMEM or RPMI with 5.5 mM glucose) were added 24 h (SW480) or 12 h (DLD-1) prior to harvest. XAV939 (10 μM) treatment was performed for 4 days, while doxycycline was added throughout the experiment to induce dntLEF-1 in DLD-1 cells. Glucose concentration in the media was determined according to glucose HK (kit Sigma GAFK20).

**XF24 Extracellular Flux Assay**

Metabolic rates of oxygen consumption (OCR) and extracellular acidification (ECAR) were measured using an XF24 Extracellular Flux Assay ( Seahorse BioScience) as described previously (Zhang et al., 2012). Cells were plated at a density of 100,000 cells per well in a XF24 Cell Culture Microplate (Seahorse #100777-OEM). One hour before the assay, growth media were replaced by XF Assay Medium (Seahorse #012065-108) supplemented with 5.5 mM glucose and adjusted to pH 7.4. Oligomycin, rotenone, and antimycin A were prepared for final concentrations of 1 μM, and FCCP was prepared for a final concentration of 200 nM. Inhibitors were injected during the measurements: 2 min of mixing, 2 min of incubation, and 4 min of measurement. After the assay, protein was collected and measured with BCA Protein Assay Reagent (Thermo #23225) for data normalization. XAV939 and DCA were added 16 h before assay at concentrations of 10 μM and 50 mM, respectively, and were maintained in the XF Assay Medium during the measurements. To exclude non-mitochondrial oxygen consumption, OCR values were calculated by taking the difference between the OCR before the addition of inhibitors and the OCR after the addition of rotenone and antimycin A.

**Fluorescence lifetime imaging microscopy (FLIM) of cells in vitro**

Cells were seeded at 150,000 cells per plate (85-mm glass bottom dishes). Infection with lentivirus was performed 72 h prior to seeding. Treatment with 0.01 μg/ml doxycycline was performed starting at the time of seeding. FLI imaging was performed 5 days after seeding (under confluent conditions). Both XAV939 (10 μM) and DCA (50 mM) were added 48 h prior to imaging. For KCN treatment, cells were treated with 4 mM KCN 24 h after seeding. Cells were imaged by FLIM before the addition of KCN, as well as 1 min after treatment. Fluorescence lifetime images were acquired with a two-photon microscope coupled with a Becker and H Hick 880 card (Becker and Hickl, Berlin). See Supplementary Materials and
Methods for additional description of FLIM hardware, software, and analysis.

**Western blot analysis**

Thirty micrograms of lysate was analyzed by Western blot using the following antibodies: Flag (1:1,000, Cell Signaling #2368), lamin (1:1,000 Cell Signaling #2204), phospho-Akt (Ser473; 1:1,000 Cell Signaling #9271), and β-actin (1:10,000, Scront). Blots were imaged on the Fujifilm LAS-5000 Imaging System. For the preparation of xenograft lysates, tumors were homogenized in a Precellys 24 homogenizer in the presence of lysis buffer at 6,000 rpm, twice, for 15 s each.

**Real-time PCR**

Total RNA was isolated with Trizol from DLD-1 cells after treatment with doxycycline for either 24 h or 120 h. Xenograft tumors were homogenized in a Precellys 24 homogenizer in the presence of Trizol at 6,000 rpm, twice, for 15 s each. A total of 2 μg of RNA was reverse-transcribed using random primers according to the High Capacity cDNA Reverse Transcription Kit (Invitrogen #4368814). Real-time quantitative PCR (RT-qPCR) was performed with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas #K0222). Relative change in gene expression was calculated using the ΔΔCt method using GAPDH expression for normalization. See Supplementary Materials and Methods for a list of primer sequences.

*4′ Thiouridine labeling and isolation of nascent RNA*

4′ Thiouridine labeling was performed in duplicate following 2 h of mock (no doxycycline) or 1 μg/ml doxycycline treatment of DLD-1 colon cancer cells. 500 μM 4′ thiouridine (Sigma) was added to cells for 30 min at 37°C. Collected cells were resuspended in 4 ml Trizol reagent and total RNA purified. Labeled RNA was chemically biotinylated and purified using streptavidin-coated magnetic beads as described (Dillken et al., 2008). Briefly, 4′ thiouridine-labeled RNA was biotinylated with 2 μl biotin-HIDP (Pierce; 1 mg/ml dissolved in dimethylformamide)/3 μg RNA added to 1 μl 1X biotinylation buffer (100 mM Tris pH 7.4, 10 mM EDTA, and 7 μl water) at 1.5 h at room temperature with rotation following RNA precipitation. Biotinylated RNA was separated from bulk, unlabeled RNA using streptavidin-coated magnetic beads (μMacs Streptavidin kit).

**Chromatin Immunoprecipitation (ChIP)**

DLD-1 cells with or without 2 h doxycycline treatment (to induce expression of FLAG-dTet-TCF-1-Estet) were cross-linked with 1% formaldehyde in 1 × PBS for 15 min at room temperature. Cross-linking was quenched with 125 mM glycine for 5 min, and recovered cells were washed with 1 × PBS. Cellular lysates were balanced for immuno-precipitation by Bradford assay (500 arbitrary units (AU) per sample) and pre-cleared with 30 μl His-magnetic beads (Invitrogen 100323) for 30 min prior to the addition of 50 μl of FLAG antibody-conjugated magnetic beads (Sigma A2220). FLAG antibody beads were pre-blocked by 5 washes with 1 ml 1 × PBS/BSA solution (5 mg/ml BSA fraction V in 1 × PBS). Immunoprecipitations were carried out overnight at 4°C followed by magnetic separation and two 5-min washes with 1 ml LiCl buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate). Beads were washed twice with cold 1 × PBS before suspension in proteinase K buffer (80 mM Tris–HCl pH 8.0, 5 μl of proteinase K (20 mg/ml)) and 300 mM NaCl for an overnight incubation at 65°C. ChIP DNA was recovered with the Fermentas GeneJET PCR Purification Kit in preparation for PCR. Real-time PCR (RT-qPCR) was used to quantify enrichment of HDK1 gene locus regions. Primers specific to the genomic regions are provided in Supplementary Materials and Methods.

**Luciferase assay**

Cells were seeded at 2 × 10³ per 6-well 24 h prior to transfection. Each well was transfected with 0.1 μg SuperFect® transfection reagent (Qiagen) and 0.1 μg firefly luciferase reporter and 0.1 μg thymidine kinase β-galactosidase plasmid using BioT transfection reagent (Biotrend). Cells were treated with 0.01 μg/ml doxycycline or 10 μM XAV939 at the time of transfection where indicated. Cells were harvested 24 h post-transfection and assayed for luciferase activity and β-galactosidase activity (for normalization).

**Xenograft tumors**

SW480 stable transfectants for xenograft injection were prepared through lentiviral infection with pCDM vector alone, dntTCF-1/ or dntTCF-1-Estet, with or without PD1K, followed by selection with 500 μg/ml G418. Cells (2.5 × 10³) were injected subcutaneously into immune-deficient NOD mice. Tumors were removed and measured 4 weeks after injection. Xenograft tumors for FLIM analysis were injected at 5 × 10⁶ cells per tumor and allowed to grow for 5 weeks.

**Fluorescence lifetime imaging microscopy (FLIM) of in vivo xenograft tumors**

All animal procedures were approved by the UC Irvine IACUC. Nine-week-old NOD mice (Jackson Labs) with 3-week-old xenograft tumors were anesthetized with 100 mg/kg ketamine/10 mg/kg xylazine. FITC or TRITC dextran (155 kDa; 12.5 mg/ml) was injected into the tail vein to label the tumor vasculature. Xenografts were exposed via a skin flap cut so as to avoid severing the xenograft’s feeder vessels. The mice were positioned on the stage in an environment chamber maintained at a constant 27°C. Images were acquired at an average depth of 50–200 μm, using FITC/ TRITC labeling of vessels to distinguish tumor cells from metabolically distinct endothelial and blood cells. At least three 78-μm fields of view were imaged for each tumor. Tumors were examined from eight mice. Fluorescence lifetime images were acquired with a Zeiss 710 microscope (see Supplementary Materials and Methods for additional description of FLIM hardware, software, and analysis).
Immunohistochemistry

For Ki67 staining, deparaffinized 3.5-μm sections of formalin-fixed paraffin-embedded (FFPE) tumor xenografts were stained for Ki67 (Dako #M7230, 1:300 dilution) using a Ventana Benchmark Ultra autostainer with peroxidase-based detection. For each section, the number of positive nuclei per 4-10 fields (at a 20× magnification) was manually enumerated and the mean ± SEM was calculated. For CD31 staining, sections of FFPE tumor xenografts were blocked in avidin-biotin and a Mouse-On-Mouse kit (Vector Labs, Burlingame, CA) and subjected to antigen retrieval (citrate buffer pH 6.0 with steaming) and then incubated in 1:50 dilution of anti-mouse CD31 (Dako rat anti-mouse CD31, #31604, followed by peroxidase development. Enumeration of vessel density was done by counting vessels in every cross-section within the subcapsular region of each tumor sample and calculating mean ± SEM. For FDG staining in small intestine and phospho-PDK1 and HIF-1α staining in colon, following pressure cooker antigen retrieval in citrate buffer, sections were blocked in 3 % H2O2, goat serum, and avidin-biotin-blocking reagent (Vector Labs). Sections were incubated in primary antibodies such as anti-PDK1 (Santa Cruz sc-28783, 1:1000), anti-PDK1 (Cell Signaling #2564, 1:200), and anti-HIF-1α (Thermo PA1-16601, 1:500), followed by biotinylated secondary antibodies and visualization using a peroxidase-conjugated avidin-biotin Vectastain protocol. Slides were then counterstained with hematoxylin and mounted.

Bioinformatic analysis of human adenocarcinoma gene expression

Analysis of Wnt signaling and glycolysis pathway gene expression in human colon cancer used the publicly available colon adenocarcinoma (COAD) mRNA expression data from The Cancer Genome Atlas (TCGA) Data Portal (https://tcga-data.nci.nih.gov/tcga/). Gene memberships of the Wnt signaling and glycolysis pathways were used as defined by Kyoto Encyclopedia of Genes and Genomes (KEGG). Hierarchical clustering of log2-transformed normalized expression of 238 tumors was used to identify groups of tumor samples showing similar expression profiles for the 185 analyzed genes as well as direct tumor-to-tumor correlations for the expression of pathway components. STRING analysis (http://string-db.org; Szklarczyk et al., 2011) was used to display interactions among the PDK1 correlating genes. For additional details, see Supplementary Materials and Methods.

Statistical analysis

Statistical evaluation was performed by Student’s unpaired t-test. P < 0.05 was considered statistically significant.

Supplementary information for this article is available online:
http://embj.embjournals.com

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Author contributions

KTP and MLW conceived the project. MLW, KTP, C.S., E.C., T.T., M.T., and B.B. designed experiments, with C.S. and E.C. providing direction on human and mouse experiments. KTP, C.S., and E.C. performed all experiments and analyzed data. K.W. and P.M. performed mouse husbandry and mouse manipulation for FLiM. T.T. carried out the Seahorse experiments. KTP and MLW wrote the manuscript. KTP, C.S., E.C., T.T., M.T., N.I., M.T., and MLW provided critical editing of the manuscript. E.C., B.B., M.T., and MLW supervised research.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix IV:

Techniques to Monitor Glycolysis
CHAPTER FIVE

Techniques to Monitor Glycolysis

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Abstract

An increased flux through glycolysis supports the proliferation of cancer cells by providing additional energy in the form of ATP as well as glucose-derived metabolic intermediates for nucleotide, lipid, and protein biosynthesis. Thus, glycolysis and other metabolic pathways that control cell proliferation may represent valuable targets for therapeutic interventions and diagnostic procedures. In this context, the measurement of glucose uptake and lactate excretion by malignant cells may be useful to detect shifts in glucose catabolism, while determining the activity of rate-limiting glycolytic enzymes can provide insights into points of metabolic regulation. Moreover, metabolomic studies can be used to generate large, integrated datasets to track changes in carbon flux through glycolysis and its collateral anabolic pathways. As discussed here, these approaches can reveal and quantify the metabolic alterations that underlie malignant cell proliferation.
1. INTRODUCTION

Glycolysis is the intracellular biochemical conversion of one molecule of glucose into two molecules of pyruvate with the concurrent generation of two molecules of ATP. Pyruvate is a metabolic intermediate with several potential fates including entrance into the tricarboxylic acid (TCA) cycle within mitochondria to produce NADH and FADH₂. These reducing agents subsequently donate electrons to the mitochondrial electron transport chain (ETC), which when fully coupled to the complex V ATP synthase of the mitochondrial inner membrane generates an additional 34 molecules of ATP per glucose. Alternatively, pyruvate can be converted into lactate in the cytosol by lactate dehydrogenase with concurrent regeneration of NAD⁺ from NADH. Conversion of pyruvate to lactate blocks further ATP production, but the resultant increase in NAD⁺ drives the first biochemical step in glycolysis (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008). An increase in the flow of carbon metabolites through the glycolytic pathway, or glycolytic flux, can increase the rate of ATP production within cells despite being markedly less efficient at generating ATP compared to oxidative phosphorylation (Pfeiffer, Schuster, & Bonhoeffer, 2001).

In addition to generating ATP, glycolysis also supplies biosynthetic intermediates for cell growth and proliferation. For example, glucose-6-phosphate, the first cytosolic product of glucose metabolism, can shunt into the pentose phosphate pathway to drive NADPH generation from NAPD⁺. NADPH reduces reactive oxygen species produced mainly by respiration to maintain cellular redox balance and to protect the genome from mutations. Carbon flux through the pentose phosphate pathway supplies metabolites for nucleotide biosynthesis that is required for DNA replication and RNA transcription. Another example is 3-phosphoglycerate, a glycolytic metabolite used to synthesize serine, glycine, and cysteine, which in turn supplies one carbon metabolism. Folate and methionine cycles, the components of one carbon metabolism, provide metabolites that support diverse cellular processes including methylation reactions, antioxidant defenses, lipid head group modifications, and nucleotide metabolism (Locasale, 2013).

Warburg (1956) first observed that proliferating tumor cells augment aerobic glycolysis, the conversion of glucose to lactate in the presence of oxygen, in contrast to nonmalignant cells that mainly respire when oxygen is available. This mitochondrial bypass, called the Warburg effect, occurs in rapidly proliferating cells including cancer cells, activated lymphocytes, and
pluripotent stem cells. While the Warburg effect is energy inefficient, it is offset by an increased glycolytic flux to provide additional biosynthetic precursors to support rapid cancer cell proliferation (DeBerardinis et al., 2008). This energy compromise supports higher rates of nucleotide synthesis for DNA replication and RNA transcription, phospholipids for membrane production, and amino acids for protein translation to support increased cell division. The Warburg effect has been exploited for clinical diagnostic tests that use positron emission tomography (PET) scanning to identify increased cellular uptake of fluorinated glucose analogs such as $^{18}$F-deoxyglucose.

Not all tumors, however, shift to glycolysis for energy production. Some diffuse large B cell lymphomas and glioblastomas remain dependent on oxidative phosphorylation for energy production (Caro et al., 2012; Marin-Valencia et al., 2012). Metabolic enzyme activity is heterogeneous between different tumors even within tumor classes, and glycolytic enzymes can be either increased or decreased in their expression (Hu et al., 2013). Glutamine and fatty acids can also be used by cancers as alternative sources of fuel to make ATP through oxidative phosphorylation (Le et al., 2012; Zaugg et al., 2011).

Although Warburg made his observations over 75 years ago, the detailed mechanisms and consequences of shifting metabolism toward glycolysis are only starting to be revealed. Pyruvate kinase isoenzyme M2 (PKM2), an embryonic splice variant of the glycolytic enzyme pyruvate kinase (PK), is highly expressed in several types of cancer (Christofk, Vander Heiden, Harris, et al., 2008; Lim et al., 2012). PKM2 shows a decreased kinase activity that helps shunt glycolytic intermediates through biosynthetic pathways at the expense of respiration to CO$_2$ (Christofk, Vander Heiden, Harris, et al., 2008; Hitosugi et al., 2012). Phosphorylation of Tyr-105 of PKM2 causes the release of the allosteric activator of PKM2, 1,6-bisphosphate, which decreases its activity (Hitosugi et al., 2012). Another glycolytic enzyme, phosphoglycerate dehydrogenase, is amplified in human tumors and directs glycolytic carbon flux into serine biosynthesis instead of continued catabolism to pyruvate (Locasale et al., 2011; Possenrotto et al., 2011). An increased carbon flux through the serine biosynthesis pathway also supports glycine production, which is used for nucleotide biosynthesis and regulates cell proliferation (Jain et al., 2012).

### 2. MEASURING GLUCOSE UPTAKE AND LACTATE PRODUCTION

For cells in culture, glycolytic flux can be quantified by measuring glucose uptake and lactate excretion. Glucose uptake into the cell is through
glucose transporters (Glut1–Glut4), whereas lactate excretion is through monocarboxylate transporters (MCT1–MCT4) at the cell membrane. A caveat to relatively straightforward measurements of extracellular fluid concentrations of glucose and lactate is that they do not provide information on other possible fates for glucose-derived carbons (Fig. 5.1). Extracellular measurements of glucose and lactate in cell culture media are relatively simple to perform, cell nondestructive, and provide a reasonable estimate of glycolytic flux (Table 5.1).

Glucose uptake into cells provides an intracellular estimate of glycolytic flux and can be quantified through detection of trapped glucose analogs. This approach can quantify glucose uptake at the level of a single cell, but is cell destructive.

2.1. Extracellular glucose and lactate

Commercially available kits and instruments are available to quantify glucose and lactate levels within cell culture media. When using these methods, cell number must be considered as this can greatly affect results. Kit detection methods are usually colorimetric or fluorometric and are compatible with standard lab equipment such as spectrophotometers.

BioProfile Analyzers (NovaBiomedical) or Biochemistry Analyzers (YSI Life Sciences) can measure levels of both glucose and lactate in cell culture media. GlucCell (Cesco BioProducts) can measure only glucose levels in cell culture media. While each commercial method has a different detection protocol, the collection of culture media for analysis is the same. Generally for both extracellular glucose and lactate measurements, cells should be plated at equal densities with the culture media changed 24 h prior to collection. To account for differences in the rates of cell proliferation or death,
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**Table 5.1: Methods to detect glucose uptake and lactate excretion.**
cells should be counted at the time of media collection for normalization of results to cell number.

2.1.1 Extracellular acidification rate by Seahorse XF analyzer

The Seahorse extracellular flux (XF) analyzer (Seahorse Bioscience) is a powerful tool for measuring glycolysis and oxidative phosphorylation (through oxygen consumption) simultaneously in the same cells. Glycolysis is determined through measurements of the extracellular acidification rate (ECAR) of the surrounding media, which is predominately from the excretion of lactic acid per unit time after its conversion from pyruvate (Wu et al., 2007). However, additional metabolic processes in cells, such as CO$_2$ production by the TCA cycle, can change the pH of the media complicating this analysis. Because ECAR is essentially a measurement of pH, a buffering agent, such as sodium bicarbonate, is not included in the assay medium. In addition, bicarbonate and media pH can play a role in regulating glycolysis, which can confound measurements of ECAR. To increase measurement accuracy, chemical inhibitors can be used to determine pH changes that are from lactate excretion versus other sources of media acidification (Fig. 5.2). The Seahorse XF analyzer requires only small cell numbers per assay condition and has injection ports for the addition of up to four inhibitors in each experiment. Available detectors use either a 24-well (XF24) or a 96-well (XF96) assay plate format.

To obtain reproducible data within the linear detection range of the XF analyzer, optimization of the cell number and inhibitor concentration is required. The XF24 analyzer has a two-step cell seeding protocol and generally requires $2 \times 10^4$–$1 \times 10^5$ cells per well. Experimental cell density should be chosen based on three main factors: (1) an ECAR value of at least 20 mP H/ min, which is the low end of a potential linear detection range, (2) retained morphology of the plated cells by visual inspection, and (3) a cell density that lies within the linear range of a graph of cell seeding density versus ECAR values (Fig. 5.3A). Metabolic inhibitors used in assays should be titrated to obtain the optimal concentration for maximal response for each cell type without toxicity, as assessed by ECAR and oxygen consumption rate (OCR) measurements (Fig. 5.3B). Loosely adherent and suspension cells can be attached to XF V7 cell culture assay plates by coating these plates with Cell-Tak (BD Bioscience). The example protocol below is for adherent cells used with the XF24 format.

To probe glycolysis, an assay called the glycolysis stress test is often used. In this assay, glucose, oligomycin, and 2-deoxyglucose (2-DG) are inserted through injection ports sequentially while measurements are being made.
Figure 5.2 Chemical inhibitors of glycolysis. Inhibitors of glycolytic enzymes are used to quantify metabolite fluxes within the glycolytic pathway. Inhibition of glycolysis with 2-deoxy-o-glucose (2-DG) is a common tool for measuring glycolysis (e.g., glycolysis stress test).

Glucose is supplied to feed glycolysis, and the difference between ECAR before and after addition of glucose is a measure of the glycolytic rate. Oligomycin inhibits ATP synthase in the ETC, which decreases the ATP/ADP ratio and drives glycolysis. The difference between ECAR before and after oligomycin addition is equal to the glycolytic reserve capacity of cells. 2-DG inhibits glycolysis and therefore provides a baseline ECAR measurement. ECAR after 2-DG addition accounts for the nonglycolytic ECAR of cells (Fig. 5.3C).

2.1.1.1 Protocol for ECAR measurement by Seahorse XF analyzer
Day 1: Seed cells and prepare a sensor cartridge
For adherent cells, wash cells with 1 x PBS, pH 7.4, and add 1 x trypsin until cells begin to visually detach. Add culture media with serum to
deactivate trypsin and pipette up and down to create a uniform cell suspension. Count cells (e.g., hemocytometer) and resuspend the total so that the number of desired cells per well is within 100 µl (e.g., for 5 x 10^4 cells per well, suspend cells at a concentration of 5 x 10^5 cells per ml). Pipette 100 µl of cell suspension into each well of 24-well V7 plate (Seahorse Bioscience #100777-004). For accurate reproducible measurements, pipette up and down to make sure that cells are uniformly spread throughout the well. One well in each V7 plate row or column should lack cells to be used as a blank control. Put the seeded plate into a 37 °C, 5% CO_2 incubator to allow cells to adhere for 1–5 h. Add an additional 150 µl of media and allow cells to grow overnight in a 37 °C, 5% CO_2 incubator.

The XF24 and XF96 sensor cartridges (Seahorse Bioscience #100850-001) must be hydrated overnight for consistent results. Add XF calibrant, pH 7.4 (Seahorse Bioscience #100840-000) to each well of a Seahorse 24-well or 96-well plate. Put a sensor cartridge on top of the plate with sensors submerged in calibrant solution. Hydrate at 37 °C without CO_2 for up to 72 h. Turn on the Seahorse XF Analyzer to allow the instrument to warm to 37 °C before an assay.

Day 2: Prepare a cell plate for analysis
Warm XF DMEM media (Seahorse Bioscience # 102353-100) to 37 °C in a water bath, add L-glutamine to a final concentration of 2 mM and
adjust the pH of the media to 7.4. Remove all but 50–100 µl of media from each well and add 1 ml of 37 °C XF DMEM media, pH 7.4. It is important during this process to always leave a small amount of media in each well so that cells do not dry out and the cell monolayer is not disrupted. Then remove all but 50 µl of media from each well again and add the desired volume of 37 °C XF DMEM media. Place a 24- or 96-well plate in a 37 °C incubator without CO₂ for 1 h before running an assay. CO₂ will alter the pH of the media and disrupt ECAR measurements.

Inhibitors should be adjusted to the proper concentration in the XF DMEM media. Since inhibitors will be added through injection ports in the cartridge, they should be at a concentration of 10 × higher than the final dilution. Prepare 2 ml stocks of 100 mM glucose, 1 M 2-DG, and the optimized oligomycin concentration chosen by titration. Pipette 61.5 µl of glucose stock solution, 67.5 µl of oligomycin stock solution, and 75 µl of 2-DG stock solution into injection ports A, B, and C, respectively. Make sure to fill all A, B, and C injection ports including blank wells.

The XF24 and XF96 analyzers require the input of a protocol command sequence. Important parameters of this protocol are mix time, wait, and measure time. Cell lines with higher metabolic activity require shorter measurement times than cells with less metabolic activity. A good protocol to start with is a 3-min mix time, 3-min wait, and 3-min measure time. If OCR or ECAR values are too low, a protocol of 2-min mix time, 2-min wait, and 4- or 5-min measure time can improve the data quality.

After running the glycolysis stress test, normalize ECAR data to cell number or protein concentration and plot ECAR against time. Glycolysis, glycolytic capacity, and glycolytic reserve can be calculated with normalized ECAR values using the equations given below (Fig. 5.3C):

\[
\text{Glycolysis} = \text{ECAR after addition of glucose} - \text{ECAR after 2} - \text{DG treatment}
\]

\[
\text{Glycolytic capacity} = \text{ECAR after oligomycin treatment} - \text{ECAR after 2} - \text{DG treatment}
\]

\[
\text{Glycolytic reserve} = \text{ECAR after oligomycin treatment} - \text{ECAR after addition of glucose}
\]

\[
\text{Nonglycolytic acidification} = \text{ECAR after 2} - \text{DG treatment}
\]
2.2. Glucose analog uptake

To determine the glucose uptake rate by cells, a labeled isoform of glucose can be added to the cell culture media and then measured within cells after a given period of time. Two types of glucose analogs are typically used for these studies: radioactive glucose analogs, such as 2-deoxy-o-[1,2-\(^3\)H]-glucose, 2-deoxy-o-[1-\(^{14}\)C]-glucose, or 2-deoxy-2-(\(^{18}\)F)-fluoro-o-glucose (\(^{18}\)FDG), or fluorescent glucose analogs, such as 2-[\(N-(7\)-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). Measurements of radioactive glucose analog uptake require a scintillation counter, whereas 2-NBDG uptake is usually measured by flow cytometry or fluorescent microscopy. In contrast to extracellular approaches, these techniques require cell harvest and termination of the cell culture.

2.2.1 2-Deoxy-o-[1,2-\(^3\)H]-glucose or 2-deoxy-o-[1-\(^{14}\)C]-glucose

Glucose analog, 2-DG, is brought into cells by a glucose transporter and is phosphorylated within the cytosol by hexokinase (Jenkins, Furler, & Kraegen, 1986). Further metabolism of 2-DG is slow and phosphorylation by hexokinase traps 2-DG within a cell (Hansen, Gulve, & Holloszy, 1994). By using radioactive 2-deoxy-o-[1,2-\(^3\)H]-glucose or 2-deoxy-o-[1-\(^{14}\)C]-glucose, the accumulation of 2-DG can be measured to quantify glucose uptake.

2.2.1.1 Protocol for 2-deoxy-o-[1,2-\(^3\)H]-glucose or 2-deoxy-o-[1-\(^{14}\)C]-glucose uptake

Plate approximately 5 \( \times \) 10\(^5\) cells per well in a 24-well plate. Allow time for the cells to attach and spread on the plate and switch to glucose-free or glucose low media. Add 1 \( \mu \)Ci 2-deoxy-o-[1,2-\(^3\)H]-glucose (MP Biomedicals #0127088) or 2-deoxy-o-[1-\(^{14}\)C]-glucose (MP Biomedicals #0111012) and incubate for at least 20 min. Wash cells with 1 \( \times \) PBS, pH 7.4 (Life Technologies #10010-023), and lyse cells with 1% SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris, pH 7.5). Use a scintillation counter to quantify \([^3H]\) or \([^{14}C]\) and normalize readings to cell number.

2.2.2 2-Deoxy-2-(\(^{18}\)F)-fluoro-o-glucose

\(^{18}\)FDG PET scanning has been used in clinical cancer diagnostics since the 1990s, but use of \(^{18}\)FDG for in vitro research protocols has been more limited, likely because of the short half-life of the radioactive fluoride (109.7 min) and limited access to a cyclotron for its manufacture for most research labs. Like other 2-DG analogs, \(^{18}\)FDG is phosphorylated after transport into the
cell and cannot be exported (Smith, 2000). $^{18}$FDG accumulation occurs in many tumors, or other rapidly dividing cells, due to the Warburg effect, and increased analog trapping contrasts with surrounding normal tissues to provide signal discrimination for in vivo PET imaging. Use of $^{18}$FDG for in vitro experiments requires consideration of factors that can influence metabolism including cell density and growth media (Mertens, Mees, Lambert, Van de Wiele, & Goethals, 2012). $^{18}$FDG requires instrumentation to measured gamma radiation and increased safety procedures for working with radiation. Because the measurement $^{18}$FDG uptake in vitro is uncommon and an alternate method involving fluorescent detection of glucose uptake is available, a detailed work protocol for $^{18}$FDG uptake measurements is not provided here.

2.2.3 2-[N-(7-Nitrobenz-2-oxa-1,3-dioxol-4-yl)amino]-2-deoxyglucose

2-NBDG is taken into cells through glucose transporters and phosphorylated by hexokinase. However, 2-NBDG can be dephosphorylated, which can result in efflux from cells, but this analog still provides a good approximation of glucose uptake (O’Neil, Wu, & Mullani, 2005). Because 2-NBDG and glucose are both imported by glucose transporters, unlabeled glucose in the media will impact 2-NBDG uptake (O’Neil et al., 2005; Zou, Wang, & Shen, 2005). Therefore, the use of low-glucose media is optimal when tolerated by cells and available. 2-NBDG can be excited to fluoresce by 465 nm wavelength light and yields a 540-nm wavelength emission, which can be detected using a fluorescence detector channel or filter typically used for green fluorophores. A negative control sample should be included that is not incubated with 2-NBDG to set flow cytometer gating of negative and positive events and compensation when necessary (Fig. 5.4).

2.2.3.1 Protocol for 2-NBDG uptake

Approximately $5 \times 10^3$ cells should be plated per well. Cell confluence can affect metabolism and should be considered and adjusted appropriately. For each condition to be examined, count and plate cells at equal densities and allow time for cells, if they are adherent, to fully attach to the plate surface or substrate. Wash cells with $1 \times$ PBS, pH 7.4, followed by the addition of culture media (low glucose when possible) supplemented with 10–100 µM 2-NBDG (Life Technologies #N13195), and incubate for 1–12 h at 37 °C with 5% CO₂. A negative control should be incubated with the same culture media without adding 2-NBDG. The cell density, concentration of 2-NBDG, and time of incubation can be adjusted for optimal results.
Figure 5.4 Measurement of 2-NBDG uptake by flow cytometry. (A) A negative control that has not been cultured with 2-NBDG. (B) A sample cultured with 2-NBDG.

For analysis by flow cytometry, add 1× trypsin (Life Technologies #25300054), harvest cells, wash twice with ice cold 1× PBS, pH 7.4, at 4°C, and keep on ice. Keep the samples on ice to inhibit dephosphorylation and export of 2-NBDG from cells. Analyze the collected cells by flow cytometry within 30 min of harvest. A control sample lacking 2-NBDG should
be used to set the flow cytometer compensation and gate parameters for 2-NBDG positive and negative events (Fig. 5.4).

3. MEASURING THE ACTIVITY OF RATE-LIMITING GLYCOLYTIC ENZYMES

While the activity of any single glycolytic enzyme is not a proxy for carbon flux through the entire pathway, specific enzymes limit the rate of glycolysis, and therefore, their activities control the maximum possible flux. Hexokinase, phosphofructokinase, and PK are the main rate-limiting enzymes in glycolysis.

Conversion from NAD$^+$ to NADH or NADP$^+$ to NADPH is often used in enzymatic assays because NADH and NADPH absorb light at 340 nm, while NAD$^+$ and NADP$^+$ lack absorbance at this wavelength (McComb, Bond, Burnett, Keech, & Bowers, 1976). By coupling the enzyme of interest with an NAD$^+/$/NADH- or NADP$^+/$/NADPH-dependent enzyme, the activity of the enzyme can be measured by the change in absorbance at 340 nm over time. Hexokinase, phosphofructokinase, and PK can be coupled with NAD$^+/$/NADH- or NADP$^+/$/NADPH-dependent enzymes by using enzymes that react with their products.

3.1. Hexokinase

Hexokinases phosphorylate the 6-hydroxyl of hexose using the $\gamma$-PO$_4$ of ATP as a donor group (Wilson, 1995). In humans, hexokinase 4 (HK4) is more specific to glucose than other hexoses and is referred to as glucokinase. An ADP-dependent glucokinase (ADPGK) has been recently discovered in mouse and human genomes, which transfers a phosphoryl group from ADP to glucose (Richter et al., 2012). Hexokinase 1 (HK1), hexokinase 2 (HK2), and hexokinase 3 (HK3) are allosterically inhibited by their product, glucose-6-phosphate, but HK4 is not sensitive to glucose-6-phosphate concentrations (Wilson, 1995). Hexokinase is the first key enzyme in glycolysis because it traps glucose within cells.

High expression of HK2 is associated with poor prognosis in hepatocellular carcinoma and brain metastasis (Palmieri et al., 2009; Peng, Lai, Pan, Hsiao, & Hsu, 2008). HK2 is required for tumor growth in multiple mouse models of cancer and is a potential target for therapeutics (Patra et al., 2013). Breast cancer cells regulate HK2 through miR–155, which increases expression of HK2 through multiple mechanisms (Jiang et al., 2012). To measure hexokinase activity, glucose is provided as a substrate, and glucose-6-phosphate
dehydrogenase (G6PD) further metabolizes glucose-6-phosphate in a NADP\(^+\) dependent manner. The absorbance of NADPH at 340 nm will increase as G6PD converts glucose-6-phosphate to fructose-6-phosphate.

### 3.1.1 Protocol for hexokinase activity assay

Wash cells with 1× PBS, pH 7.4, and incubate in lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM DTT, protease inhibitor cocktail) for 30 min on ice to lyse cells. Prepare reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl\(_2\), 0.6 mM ATP (Sigma #A2383), 100 mM glucose (Sigma #G8270), 0.2 mM NADP\(^+\) (Sigma #0505), and 0.1 units of glucose-6-phosphate dehydrogenase (Sigma #G4134)) per ml. Add 20 μg of fresh cell lysate to 1 ml of reaction buffer. A negative and positive control should be included without cell lysate and with 0.05 units of hexokinase (Sigma #H5000) added. Mix, incubate at 37 °C, and measure optical absorbance of the reaction at 340 nm with a spectrophotometer every 15 s for 10 min (Yi et al., 2012). Enzyme activity can be represented as the change in absorbance per minute, which should be calculated on a linear portion of the obtained curve.

### 3.2. Phosphofructokinase

Glucose-6-phosphate can enter the pentose phosphate pathway or continue through the glycolytic pathway. Phosphofructokinase 1 (PFK1) provides the first enzymatic step at which a glucose molecule becomes committed to glycolysis and therefore is subject to regulation (Nelson & Cox, 2008). PFK1 activity depends on the concentrations of AMP, ADP, and ATP with allosteric activation by AMP and ADP and allosteric inhibition by ATP. Additional allosteric inhibition is provided by citrate and activation by fructose 2,6-bisphosphate (Nelson & Cox, 2008).

PFK1 activity has been linked to the Warburg effect through the p53 target protein, TP-53-induced glycolysis and apoptosis regulator (TIGAR). TIGAR inhibits glycolysis and promotes the pentose phosphate pathway by reducing levels of fructose-2,6-bisphosphate, thereby reducing PFK1 activity (Bensaad et al., 2006). Glycosylation of the fructose-2,6-bisphosphate binding site on PFK1 also inhibits glycolytic flux which decreases cell proliferation, thereby reducing cancer aggressiveness (Yi et al., 2012). Measurement of phosphofructokinase activity can be achieved by providing other glycolytic enzymes, aldolase, triosephosphate isomerase, and glyceraldehyde 3-phosphate dehydrogenase, which are
NAD$^+$ dependent. An increase of NADH absorbance at 340 nm can be observed as fructose 1,6-bisphosphate is converted to 1,3-bisphosphoglycerate.

3.2.1 Protocol for phosphofructokinase activity assay
Wash cells with 1 x PBS, pH 7.4, and incubate in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM DTT, protease inhibitor cocktail) for 30 min on ice to lyse cells. Prepare reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 5 mM ATP (Sigma #A2383), 0.2 mM NADH (Sigma #N4505), 100 mM KCl, 5 mM Na$_2$HPO$_4$, 5 mM MgCl$_2$, 0.01 AMP (Sigma #01930), 5 mM fructose-6-phosphate (Sigma #F3627), 5 units of triosephosphate isomerase (Sigma #T2507) per ml, 1 unit of aldolase (Sigma #A2714) per ml, and 10 units of glyceraldehyde-3-phosphate dehydrogenase (Sigma #G2267)) per ml. Add 20 µg of fresh cell lysate to 1 ml of reaction buffer. A negative and positive control should be included without cell lysate and with 0.05 units phosphofructokinase (Sigma #F0137) added. Measure optical absorbance of the reaction at 340 nm at room temperature every 15 s for 10 min with a spectrophotometer (Yi et al., 2012). Activity can be represented as the change in absorbance per minute, which should be calculated in a linear portion of the obtained curve.

3.3. Pyruvate kinase
PK catalyzes the final step in glycolysis by transferring a phosphoryl group from phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP. PK is active as a tetramer protein and is allosterically inhibited by ATP, acetyl-CoA, and long-chain fatty acids (Nelson & Cox, 2008). There are four PK isoforms including PKL, PKR, PKM1, and PKM2. PKL and PKR, the dominant PK isoforms in the liver and red blood cells, undergo allosteric activation by fructose-1,6-bisphosphate (Carbonell, Marco, Felin, & Sols, 1973). Fructose-1,6-bisphosphate is also an allosteric activator of PKM2, which is a splice variant of the constitutively active PKM1 enzyme isoform (Dombraucks, Santarsiero, & Mesecar, 2005). The product of PK, pyruvate, can be converted to lactate by lactate dehydrogenase, which is NADH dependent. The activity of PK can then be detected by a loss of absorbance at 340 nm.

3.3.1 Protocol for PK activity assay
Wash cells with 1 x PBS, pH 7.4, and incubate in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM DTT,
protease inhibitor cocktail) for 30 min on ice to lyse cells. Prepare reaction buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.6 mM ADP (Sigma #A2754), 0.5 mM PEP (Sigma #860077), 0.18 mM NAD⁺ (Sigma #N1636), 10 μM fructose-1,6-bisphosphate (Sigma #F6803), and 10 units of lactate dehydrogenase (Sigma #59747)) per ml. Add 10 μg of fresh cell lysate to 1 ml of reaction buffer. Measure optical absorbance of the reaction at 340 nm every 15 s for 10 min with a spectrophotometer (Christofk, Vander Heiden, Wu, Asara, & Cantley, 2008; Yi et al., 2012). A positive and a negative control should be run with no cell lysate and 0.05 units of PK (Sigma #P7768) added. Activity can be represented as the change in absorbance per minute, which should be calculated in a linear portion of the obtained curve.

4. METABOLITE MEASUREMENTS AND GLUCOSE TRACING

Unlabeled metabolites or 13C-labeled metabolites can be quantified by mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectrometry. Measurements of unlabeled metabolites provide steady-state data on the levels of all detectable metabolites within the cell. Using metabolite concentrations for extrapolation of flux information is difficult, however, because changes in the steady-state level of a metabolite can be due to increased or decreased flux through any of its associated metabolic pathways. Absolute quantification of an unlabeled metabolite is possible by adding a labeled standard for the metabolite of interest before MS analysis. Metabolite profiling was used to identify decreased serine biosynthesis as a result of PKM2 silencing (Chaneton et al., 2012).

The use of 13C-labeled glucose provides glycolytic flux data by quantifying 13C glucose-derived carbons in downstream metabolites. However, labeled substrates are costly and the label can be diluted by other carbon sources, making measurements challenging for some metabolites. However, detection of 13C-glucose-derived carbons in glycolytic intermediates is robust; making it a good method for analyzing glycolysis.

While chromatography combined with MS is becoming the most widely used approach for metabolomics, NMR spectroscopy also has advantages for detecting and quantifying metabolites. NMR spectroscopy is quantitative, fast, and reproducible but has a detection limit in the micromolar range (Pan & Raftery, 2007; Want, Cravatt, & Siuzdak, 2005). By contrast, MS
is exceedingly sensitive in the nanomolar range, making it useful for complex biosamples. MS analysis, however, requires metabolite separation with gas chromatography (GC) or liquid chromatography (LC), which reduces the speed of data acquisition. GC/MS combines the separation of complex biosamples with the retained sensitivity of MS (Want et al., 2005). However, GC/MS has an additional derivatization step of sample preparation that is required for detecting nonvolatile or large, polar macromolecules (Dietmair, Timmins, Gray, Niehen, & Krömer, 2010; Want et al., 2005). By contrast, LC/MS detects a broad range of metabolites with simple sample preparation. Following the separation of metabolites by LC, they must be ionized for analysis by MS. Liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) combines an automated ionization method, electrospray ionization, with the simplicity of LC and the sensitivity of MS. Therefore, we provide metabolomics protocols that are suitable for use with LC/ESI-MS. Fourier transform ion cyclotron resonance MS (FT-ICR-MS), orbitrap MS, and multipass time of flight MS (multipass TOF-MS) have all been used for metabolomic analysis (Aharoni et al., 2002; Lei, Huhman, & Sumner, 2011). FT-ICR-MS has ultrahigh resolution and mass accuracy below 1 ppm, while orbitrap MS also has high resolving power and mass accuracy between 1 and 5 ppm (Lei et al., 2011).

4.1. Metabolite measurements

Metabolite extraction must be performed to obtain metabolites from cultured cells for LC/ESI-MS analysis. Metabolite extraction generally has two parts: quenching and extraction. Quenching first limits further metabolic activity and removes contaminants, which is then followed by a metabolite extraction step (Sellick, Hansen, Stephens, Goodacre, & Dickson, 2011). Metabolites with a high turnover rate, such as ATP, depend on complete and quick quenching methods for accurate detection. Chemical and physical properties of each metabolite require different methods of quenching and different extraction solutions making it challenging to find one method that works for all metabolites (Dietmair et al., 2010). Glucose-6-phosphate is particularly unstable and requires effective quenching for accurate measurements. Enzymatic assays to measure glucose-6-phosphate can be used to check on the success of quenching and extraction steps. For optimization of extraction, temperature and solvent can be adjusted in both the quenching and extraction step.
4.1.1 Protocol for metabolite extraction of adherent cells for LC/ESI-MS analysis

Approximately $1 \times 10^6$ cells are minimally required for detection of metabolites by LC/ESI-MS. Cells can be grown in six-well plates for direct extraction from each well. For normalization to cell number or protein concentration, an extra well of each condition should be plated in parallel for cell counting or protein quantification. Add fresh media to cells 24 h prior to metabolite extraction. Place the six-well plate on ice and wash cells with ice cold $1 \times$ PBS, pH 7.4. Add 400 μl of ice cold HPLC grade methanol (Fisher #A454-4). To maximize quenching speed, methanol can be cooled on dry ice to achieve a temperature as low as $-40^\circ$C. Add an equal volume of Milli Q water and gently shake the plate to ensure mixing. Scrape cells off the plate and transfer to a 1.5-ml eppendorf tube. For separation of polar from nonpolar metabolites, add 400 μl of HPLC grade chloroform (Fisher C607-4). Briefly vortex each sample every 5 min for 15–30 min. Transfer the top layer, which is the aqueous phase containing polar molecules, to a new tube. The remaining bottom layer contains nonpolar metabolites, which can also be transferred into a fresh tube. Glycolytic intermediates are polar molecules and should be in the top layer. Dry the contents of both tubes with a vacuum concentrator. Samples can then be stored at $-80^\circ$C until analysis. Alternatively, samples can be suspended in 50 μl of initial LC mobile phase solution for immediate LC/ESI-MS analysis. MS measurements should be performed according to the methods described in the instrument documentation.

4.2. Metabolic flux analysis with stable glucose isotopes

$^{13}$C can replace any of the six carbons within a glucose molecule. [U-$^{13}$C$_6$]-glucose is $^{13}$C labeled at all six carbons and is often used to trace the glucose contribution to the TCA cycle. [1,2-$^{13}$C$_2$]-glucose is the best tracer for glycolytic pathway metabolite measurements (Metallo, Walther, & Stephanopoulos, 2009). In addition, carbon flux through the pentose phosphate pathway is not well defined by [U-$^{13}$C$_6$]-glucose. Instead, [1,2-$^{13}$C$_2$]-glucose will lose one $^{13}$C when it goes through the pentose phosphate pathway, which distinguishes metabolites that have gone through this pathway from those that have gone directly through glycolysis (Fig. 5.5).

Labeled glucose will compete for uptake into the cell with unlabeled glucose within the media. Therefore, glucose-free or low-glucose media is used for labeled glucose tracing flux experiments. Regular culture media should be replaced with [U-$^{13}$C$_6$]-glucose (Cambridge Isotope Labs #CLM-1396) or [1,2-$^{13}$C$_2$]-glucose (Cambridge Isotope Labs #CLM-504) supplemented
media 24 h prior to metabolite extraction. The same protocol can be used for extraction of metabolites for metabolic flux analysis as for measurement of unlabeled metabolites. Data analysis, however, is more complex and requires specialized software. FiatFlux, an open source software for use with isotopic tracer experiments, is an enabling tool for data analysis (Zamboni, Fischer, & Sauer, 2005). OpenFLUX and 13CFlux are also software packages available for use for with 13C metabolic flux analysis (Quek, Wittmann, Nielsen, & Krömer, 2009; Weitzen et al., 2013).

4.3. Release of $^3$H$_2$O from [5-$^3$H]-glucose

The release of $^3$H$_2$O from [5-$^3$H]-glucose is a method to quantify glycolytic flux (Neely, Denton, England, & Randle, 1972). A single tritium at the C5
Figure 5.6 Glycolytic flux measurements with [5-3H]-glucose. A single tritium present on 5C of glucose is released as water in the ninth step of glycolysis catalyzed by enolase.

of glucose is removed by a condensation reaction in the ninth step of glycolysis, which is catalyzed by enolase (Fig. 5.6). The resulting $^3$H$_2$O diffuses freely into the cell culture media and is quantified by scintillation counting. The complete conversion of glucose to pyruvate is not measured by this reaction.

For detection of $^3$H$_2$O using a liquid scintillation counter, the [5-3H]-glucose must be removed from the cell supernatant. One method for separation is to allow $^3$H$_2$O to diffuse into a cold H$_2$O solution while inhibiting the diffusion of [5-3H]-glucose (Vander Heiden et al., 2011). An alternative method is to use ion chromatography to remove the glucose from the cell supernatant (Støttrup et al., 2010).

4.3.1 Protocol for measuring the release of $^3$H$_2$O from [5-3H]-glucose

Wash 1 x $10^6$ cells with 1 x PBS, pH 7.4, and resuspend in 1 ml Krebs buffer (126 mM NaCl, 2.5 mM KCl, 25 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgCl$_2$, and 2.5 mM CaCl$_2$). Incubate at 37 °C, 5% CO$_2$ for 30 min and then replace with Krebs buffer containing 10 μCi [5-3H]-glucose (Perkin Elmer #NET531005MC) and 10 mM unlabeled glucose.
Table 5.2 Glucose tracers used to measure glycolysis and related metabolic pathways

<table>
<thead>
<tr>
<th>Glucose tracer</th>
<th>Detection method</th>
<th>Glycolysis</th>
<th>PPP</th>
<th>TCA cycle</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-(^{13})C(_6)]-glucose</td>
<td>Mass spectrometry</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>$</td>
</tr>
<tr>
<td>[1,2-(^{13})C(_2)]-glucose</td>
<td>Mass spectrometry</td>
<td>High</td>
<td>High</td>
<td>Ok</td>
<td>$$</td>
</tr>
<tr>
<td>[2-(^{13})C(_4)]-glucose</td>
<td>Mass spectrometry</td>
<td>High</td>
<td>High</td>
<td>Ok</td>
<td>$$</td>
</tr>
<tr>
<td>[5-(^3)H]-glucose</td>
<td>Scintillation counter</td>
<td>High</td>
<td>No</td>
<td>No</td>
<td>$$$</td>
</tr>
</tbody>
</table>

PPP, pentose phosphate pathway.

Incubate at 37 °C, 5% CO\(_2\) for 1 h. Spin down the sample and remove 500 µl of cell supernatant. Add 500 µl of 0.2 N HCl. Pack a 2-ml Poly-Prep chromatography column (Biorad #731-1550) with 2 ml of AG 1-X8 resin, 200–400 mesh, formate form (Biorad #140-1454). Wash the resin with 20 ml of 1 N NaOH and then with 10 ml of distilled water. Add 1 ml of supernatant to the column and collect the sample after glucose removal. Dissolve \(^{3}\)H\(_2\)O into scintillation solution and quantify by beta-scintillation counting (Støttrup et al., 2010; Vander Heiden et al., 2010).

5. SUMMARY

Each of the discussed methods provides data on the glycolytic flux in mammalian cells, including cancer. Selecting which technique or combinations of techniques are appropriate for a given study depends on equipment availability, budget, and the type of information required. Extracellular glucose and lactate measurements and enzymatic activity assays can be performed with standard laboratory equipment but provide a limited amount of insight. Metabolomics approaches provide data on glycolysis as well as its offshoot pathways but require expensive equipment and laborious data analyses (Table 5.2). All of these methods can be used in combination to obtain a comprehensive assessment of glycolytic flux and downstream metabolic pathways in cancer cells.

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Appendix V:

P53 regulates rapid apoptosis in human pluripotent stem cells
P53 Regulates Rapid Apoptosis in Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSCs) are sensitive to DNA damage and undergo rapid apoptosis compared to their differentiated progeny cells. Here, we explore the underlying mechanisms for the increased apoptotic sensitivity of hPSCs that helps to determine pluripotent stem cell fate. Apoptosis was induced by exposure to actinomycin D, etoposide, or tunicamycin, with each agent triggering a distinct apoptotic pathway. We show that hPSCs are more sensitive to all three types of apoptosis induction than are lineage-non-specific, retinoic-acid-differentiated hPSCs. Also, Bax activation and pro-apoptotic mitochondrial intermembrane space protein release, which are required to initiate the mitochondria-mediated apoptosis pathway, are more rapid in hPSCs than in retinoic-acid-differentiated hPSCs. Surprisingly, Bak and not Bax is essential for actinomycin-D-induced apoptosis in human embryonic stem cells. Finally, P53 is degraded rapidly in an ubiquitin-proteasome-dependent pathway in hPSCs at steady state but quickly accumulates and induces apoptosis when Mdm2 function is impaired. Rapid degradation of P53 ensures the survival of healthy hPSCs but avails these cells for immediate apoptosis upon cellular damage by P53 stabilization. Altogether, we provide an underlying, interconnected molecular mechanism that primes hPSCs for quick clearance by apoptosis to eliminate hPSCs with unrepaired genome alterations and preserves organismal genomic integrity during the early critical stages of human embryonic development.

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Introduction

Human pluripotent stem cells (hPSCs) have both the ability to self-renew and the ability to differentiate into all cell types in the human body [1,2]. Genomic integrity of hPSCs in utero and in tissue culture is essential for cell lineage fidelity during further development and differentiation, and therefore, a rapid response to cellular damage is required. Human embryonic stem cells (hESCs) have been shown to undergo rapid apoptosis in response to repair in response to DNA damage when compared to differentiated cells [3–8]. The mechanisms, however, that potentiate the rapid response of DNA-damaged hESCs toward apoptosis instead of repair are still not fully understood. The tumor suppressor protein P53 is a known regulator of apoptosis in response to DNA damage in hESCs [3–5,7,8]. In somatic cells, following
DNA damage, P53 contributes to apoptosis induction and execution through transcriptional activation of pro-apoptotic genes, through sequestration of anti-apoptotic proteins, or by interacting with and positively influencing pro-apoptotic proteins. Bax and Bak, in the cytosol [9-15]. Knockdown of P53 in hESCs eliminates the apoptotic response to DNA damage. hESCs expressing P53 lacking a nuclear localization signal can activate apoptosis in response to DNA damage, indicating that cytosol-localized P53, in addition to nuclear P53, contributes to apoptosis in hESCs [5]. Inhibition of cyclin-dependent kinase CDK1 can selectively induce the DNA damage response and P53-dependent apoptosis in hESCs, in contrast to only causing transient cell cycle arrest during DNA repair in differentiated cells [16]. Expression of P53 target genes is rapidly induced in response to DNA damage in embryonic stem cells, but this rapid response is also seen in differentiated cells [5]. Whereas P53 plays a large and potentially distinct role in the DNA damage responses of hESCs and somatic cells, no actual differences in the apoptosis-inducing behavior of P53 or its regulation have yet been identified between hPSCs and differentiated cells. Instead, what has been reported is that the mitochondria in hPSCs are primed for apoptosis due to a difference in the balance between pro-apoptotic and anti-apoptotic proteins, leading to a higher sensitivity and lower apoptotic threshold for hESCs compared to differentiated cells [5,17].

Activation of apoptosis by cell intrinsic stimuli, such as DNA damage, occurs through mitochondrial outer membrane permeabilization (MOMP), which requires the activation of pro-apoptotic BCL-2 family member proteins Bax or Bak [11,16,19]. Some hESC lines show constitutively activated pro-apoptotic Bax localized to the Golgi apparatus during S phase, where it is unable to activate apoptosis until DNA damage induces its translocation to the mitochondria to induce MOMP [20]. Knockdown of Bax in hESCs decreases apoptosis in response to DNA damage. Additionally, P53 is required for the translocation of Bax from the Golgi apparatus to the mitochondria with DNA damage in hESCs [20]. In other cell types and hESC lines, Bax is localized to the cytosol in an inactive state. Once activated by Bax-only proteins, Bax undergoes a conformational change and translocation into the mitochondrial outer membrane [18,21]. Nonetheless, since activated Bax is not detectable in the Golgi apparatus of most hESC lines, this potential sensitizing mechanism cannot be exclusively responsible for the rapid activation of apoptosis in response to DNA damage in these hESC lines [20].

Here, we further investigate apoptotic mechanisms in hPSCs and discover that differential regulation of P53 stability sensitizes hPSCs to apoptosis. Initially, we evaluated the similarities and differences in the apoptotic machinery between hPSCs and differentiated cells to elucidate the pathways underlying the rapid activation of apoptosis in hPSCs. We discovered that hPSCs activate apoptosis rapidly not only in response to DNA damage but also in response to transcriptional inhibition and the induction of endoplasmic reticulum (ER) stress. In addition, we identified important roles for the mitochondrial fusion protein Drp1 and pro-apoptotic BCL-2 family member protein Bak in hESC apoptotic hypersensitivity. Finally, we report that P53 is rapidly degraded at steady state in hPSCs, but inhibition of ubiquitin-proteasome-dependent degradation by Mdm2 causes prompt stabilization of P53 and the induction of apoptosis in hESCs.

Results

hESCs are hypersensitive to diverse mitochondria-mediated apoptotic stimuli

In addition to rapid apoptosis in response to DNA damage, hPSCs also undergo mitochondria-dependent apoptosis upon dissociation into single cells, which can be suppressed by Rho-dependent protein kinase inhibitors [22-23]. Therefore, we considered whether hPSCs are more generally hypersensitive to mitochondria-mediated apoptosis by evaluating whether hPSCs are more sensitive to multiple intrinsic apoptotic stimuli than their differentiated counterparts. We used retinoic acid (RA)–induced differentiation of hPSCs as a differentiated comparative cell derivative of hPSCs [24-26]. RA is an important morphogen during development and is commonly used for neuronal differentiation [27-28]. RA treatment of monolayer cultures of hESCs causes preferential differentiation into ectodermal and mesodermal lineages [30-32]. Undifferentiated hPSCs and RA-differentiated cells were evaluated for apoptosis by incubation with various apoptosis-stimulating agents. Exposure of H9 hESCs to RA reduced the expression of Nanog and Oct4, key transcription factors for maintaining hESC self-renewal, to undetectable levels by days 3 and 5, respectively (Fig. 1a). Actinomycin D, a potent inducer of apoptosis through transcriptional repression, was used to induce apoptosis in H9 and H1 hESCs and in a human induced pluripotent stem cell (hiPSC) line, HIPS2, on days 0, 1, 3, and 5 of RA-induced differentiation (Fig. 1b and Fig. S1b and c). The fragmentation of cells into apoptotic bodies is carried out by a family of intracellular cysteine-dependent, aspartate-directed proteases (caspases). Upon activation of mitochondria-mediated apoptosis and MOMP, procaspase-3 is cleaved and in turn cleaved caspase-3 executes apoptosis through digestion of other intracellular proteins including the DNA repair protein poly(ADP-ribose) polymerase (PARP) [33-35]. Therefore, apoptosis was evaluated
by Western blot analysis of cleaved caspase-3 and cleaved PARP. Undifferentiated hPSCs showed a robust time-dependent cleavage of caspase-3 and PARP after actinomycin D exposure (Fig. 1b and Fig. S1b and c). Although uncleaved caspase-3 showed only slightly decrease during differentiation, hPSCs that were differentiated for 5 days, however, had only low levels of caspase-3 and PARP cleavage after actinomycin D treatment (Fig. 1b and Fig. S1a–c).

Next, DNA damage was induced in undifferentiated and differentiated hPSCs by treatment with etoposide, which causes DNA double-strand breaks through the inhibition of DNA topoisomerase II [38]. Undifferentiated H9 and HPS2 cells again show robust,
time-dependent cleavage of caspase-3 and PARP, but hPSCs differentiated for 5 days have no detectable cleavage of caspase-3 or PARP (Fig. 1c and Fig. S1d).

Apoptosis induced by ER stress is also mediated through the mitochondria [37–40]. To determine whether hPSCs are sensitive to ER stress-induced apoptosis, we treated undifferentiated and differentiated hESCs with tunicamycin, which induces ER stress through the inhibition of N-linked glycosylation [41]. Whereas high levels of cleaved caspase-3 and cleaved PARP were detected in undifferentiated hESCs, there was an absence of these cleaved apoptotic signature proteins in cells differentiated for 5 days (Fig. 1d).

Because cleavage of caspase-3 and PARP is a late event in apoptosis, we next evaluated the release of pro-apoptotic intermembrane space (IMS) proteins cytochrome c and Smac from the mitochondrial IMS upon MOMP [42–44]. Cytochrome c and Smac release was detected by imaging co-localization with the mitochondrial marker TOMM20 using confocal immunofluorescence microscopy following timed actinomycin D exposure. Cytochrome c and Smac were released into the cytosol from a greater percentage of undifferentiated hESCs than from differentiated cells (Fig. 1e and f and Fig. S2a and b). Bax recruitment to the mitochondria can lead to MOMP [11,18], and it precedes the release of IMS proteins from the mitochondria into the cytosol and caspase activation. Therefore, Bax localization to the mitochondria was also measured by immunofluorescence microscopy. Timed actinomycin D exposure induced the mitochondrial localization of Bax in a greater percentage of undifferentiated hESCs than in differentiated cells (Fig. 1g and Fig. S3a and b).

Mitochondrial elongation reduces the sensitivity of hPSCs to apoptosis

Mitochondria are fused, interlinking network structures that extend peripherally in the cytosol of most differentiated cell types, in contrast to the fragmented, punctate, perinuclear appearance of mitochondria in

![Image](image_url)

**Fig. 2.** Knockdown of Drp1 induces mitochondria elongation and suppressed apoptosis sensitivity in hESCs. (a) Knockdown efficiency of Drp1 in h9 hESCs was confirmed by immunoblot. (b) Mitochondrial morphology in h9 hESCs transduced with Drp1 or control shRNAs was analyzed by immunofluorescence confocal microscopy using anti-TOMM20 antibody. (c) h9 hESCs transduced with Drp1 or scramble shRNAs were exposed to actinomycin D for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies for cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). TOMM40 was included as a loading control.
hPSCs [24,45]. Mitochondrial fragmentation occurs during apoptosis [46-48], and therefore, cells with a more fragmented network structure may be more sensitive to apoptotic stimuli. The dynamin-related GTPase Drp1 mediates fission of the mitochondrial network and localizes to the mitochondrial outer membrane at sites of fission [46,49,50]. In somatic cells, Drp1 depletion retards the usual rate of cytochrome c release and caspase activation during apoptosis [46]. To determine whether the fragmented mitochondrial morphology seen in hESCs influences their hypersensitivity to intrinsic apoptotic stimuli, we inhibited mitochondrial fission by shRNA knockdown of Drp1 (Fig. 2a). Immunofluorescent confocal imaging of TOMM20, a mitochondrial outer membrane translocon protein, in Drp1 knockdown hESCs revealed mitochondria that were more elongated when compared to scramble control hESCs (Fig. 2b). Therefore, Drp1 depletion induces mitochondrial network fusion in hESCs. To determine whether an elongated mitochondrial morphology decreases the apoptotic hypersensitivity of hESCs, we induced apoptosis by actinomycin D treatment in Drp1 knockdown and scramble control hESCs. Timed actinomycin D exposure resulted in less cleavage of caspase-3 and PARP in Drp1 knockdown hESCs when compared to scramble control (Fig. 2c). Thus, the fragmented mitochondrial structure in hESCs helps to promote their sensitivity to apoptosis induction.

Bak has a crucial role in hPSC hypersensitivity to apoptosis

Activation of BCL-2 family member proteins Bak and Bax causes their oligomerization and initiates MOMP, which releases IMS proteins into the cytosol and activates the caspase cascade [19,51,52]. Bak and Bax have overlapping roles during development. Bak-deficient mice develop normally, and Bax-deficient mice have limited abnormalities. Double knockouts for both Bak and Bax, however, die perinatally with multiple developmental defects related to defective apoptosis induction, such as persistent interdigital webs and excessive cells in the central nervous and hematopoietic systems [53]. In addition, Bak has been shown to be important for the apoptotic response to etoposide in hESCs, but the roles of Bak and Bax have not been analyzed in response to other apoptotic stimuli [20]. Therefore, because Bak or Bax is essential for mitochondrial-mediated apoptosis and their contribution to apoptosis in hESCs is controversial, we next investigated whether either protein had a more dominant role in mediating hESC hypersensitivity to apoptosis. Bak, Bax, and BCL-XL protein levels are not markedly changed during RA-induced differentiation (Fig. 3a). To further assess the role of Bak and Bax in hESC sensitivity to apoptosis, we separately knocked down the expression of Bak and Bax with shRNA in H1 and H9 hESCs (Fig. 3b and Fig. 4a). The expression of pluripotency biomarker proteins SSEA4, Lin28, Nanog, and Oct4 was not affected by knockdown of either Bak or Bax (Fig. 3c). Activation of apoptosis by exposure to actinomycin D was almost completely inhibited in Bak knockdown cells (Fig. 3d and Fig. S4a and c). Therefore, Bak is an important component contributing to the hypersensitivity of hESCs to mitochondria-mediated apoptosis. In contrast, knockdown of Bax only slightly decreased the induction of apoptosis in hESCs (Fig. 3d and e and Fig. S4b and c). With etoposide treatment, however, Bax played a more crucial role as previously reported (Fig. S4d) [20]. While Bak has an important role in hESC response to etoposide, Bax is important for the response to actinomycin D. This suggests that both Bak and Bax play important roles in hESC apoptosis.

P53 turnover is rapid in hPSCs

Tumor suppressor P53 is a central regulator of growth arrest, senescence, and apoptosis in response to a broad array of cellular damage [10,13,54-56]. Regulation of P53 is mainly accomplished through ubiquitin-proteasome-dependent degradation [57]. Surprisingly, while P53 has been carefully studied in hPSCs, differences in P53 protein stability have not been examined as the potential cause of hPSC hypersensitivity. Mdm2 is the primary E3 ubiquitin ligase that negatively regulates P53 by degradation or by masking the transactivation domain of P53 [58-60]. Mdm2, in turn, is degraded following DNA damage that stabilizes P53 [51,62].

In H1 hESCs, Mdm2 protein levels decrease after RA-induced differentiation, whereas P53 protein levels do not change (Fig. 4a). To determine whether the decrease in Mdm2 was regulated transcriptionally, we performed quantitative real-time PCR to measure Mdm2 mRNA levels. No change in Mdm2 expression was detected between hESCs and RA-differentiated cells (Fig. S5a). P53 expression was 2-fold higher in H9 hESCs than in RA-differentiated hESCs, but no change was seen in H1 cells (Fig. S6a). To determine whether the p53 protein that we detected was wild-type p53, we compared the molecular weight of p53 in H1 hESC cell lysate with recombinant p53 and observed equal molecular weights (Fig. S5c).

To determine whether p53 protein stability is differentially regulated in hESCs and their differentiated counterparts, we treated cells with an Mdm2 inhibitor, nutlin-3a [63]. Timed exposure of hESCs to nutlin-3a rapidly stabilizes high levels of P53 (Fig. 4b). Treatment of differentiated cells with nutlin-3a, however, stabilizes only a small amount of p53 (Fig. 4b). Furthermore, treatment of hPSCs with nutlin-3a activates apoptosis, but apoptosis is
Fig. 3. Bak has a dominant role in actinomycin-D-induced apoptosis of hESCs. (a) Immunoblot of BCL-2 family proteins in H1 hESCs differentiated with 1 μM RA for 0, 3, and 5 days. (b) Immunoblot showing Bak and Bax protein levels in scramble, Bak, and Bax knockdown H1 hESCs. Tubulin was included as a loading control. (c) Immunofluorescent detection of pluripotency biomarker proteins from scramble, Bak, and Bax knockdown H1 hESCs. Scale bar is 200 μm. (d) Scramble, Bak, and Bax knockdown H1 hESCs were exposed to actinomycin D for the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies for cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP). TOMM40 was included as a loading control. (e) H1 hESCs were exposed to actinomycin D in the presence of the pan-caspase inhibitor Q-VD-OPH for the indicated times. Cells were fixed and analyzed by immunofluorescence confocal microscopy using antibodies against cytochrome c. Data were obtained from three independent experiments. Error bars represent SD.

Not detectable after nulin-3α treatment of RA differentiated cells (Fig. 4b). Therefore, p53 is rapidly degraded in hPSCs through ubiquitination by Mdm2. To further probe the difference in p53 stability, we treated H1, H9, and HIPS2 hPSCs with the protein translation inhibitor cycloheximide (CHX). After 2 h of CHX exposure, p53 levels were diminished (Fig. 4c and d, and Fig. S5d and i). In contrast, p53 slowly decreases after CHX exposure in RA-differentiated hPSCs (Fig. 4e and f, and Fig. S5e and g). Additional treatment with the
Fig. 4. P53 turnover is rapid in hESCs. (a) Immunoblot of Mdm2 and P53 in H1 hESCs and day 5 RA-induced H1 differentiated cells. (b) Immunoblot of P53, cleaved PARP (c-PARP), and cleaved caspase-3 (c-caspase-3) in H1 hESCs and RA-differentiated H1 cells after 1 µM etoposide or 4 µM nutlin-3a for the indicated times. (c and e) P53 protein stability analyzed by immunoblot in undifferentiated H1 hESCs (c) and H1 cells differentiated with RA for 5 days (e) were examined by CHX exposure for the indicated times in the presence of DMSO or MG132. (d and f) Band intensities corresponding to P53 protein levels in undifferentiated H1 hESCs (d) and H1 cells differentiated with RA for 5 days (f) were quantified by ImageJ and shown as relative amounts. Protein levels at 0 h were set as 1.0.

proteasome inhibitor MG132 slowed the decrease in levels of P53 in both hPSCs and RA-differentiated hPSCs, indicating that P53 degradation is dependent on ubiquitin proteasome (Fig. 4c and f and Fig. S5d–g). This indicates that P53 undergoes Mdm2-initiated, ubiquitin-proteasome-dependent degradation at a rate higher in hPSCs than in hPSCs induced to undergo differentiation.

Discussion

hESCs and hPSCs have immense potential in regenerative medicine because of their ability to self-renew and to differentiate into every cell type in the human body. Therefore, maintenance of genomic integrity is essential to avoid defects that may cause aberrant development or cancer. Apoptosis is one available mechanism for eliminating damaged cells following DNA or other forms of cellular damage [64]. Recent studies have shown that hESCs undergo rapid apoptosis; however, the mechanisms underlying this enhanced apoptosis sensitivity still remain unclear [3–5,20].

Here, we show that hPSCs undergo rapid mitochondria-dependent apoptosis in response to three independent apoptotic stimuli. Furthermore, we investigated each step of the cell death cascade and found hPSCs to be hypersensitive at all stages. We further showed the importance of mitochondria in rapid apoptosis by inducing elongated mitochondria by Drp1 depletion. The inhibition of rapid apoptosis in hESCs by Drp1 depletion emphasizes the involvement of mitochondria in this unique event. We further examined the contribution of Bax and Bak, two main pro-apoptotic members of the BCL-2 protein family that directly mediate the mitochondrial
apoptosis pathway [39]. Although a recent study showed that etoposide-induced hESC apoptosis is Bax dependent and is Bak independent, our data showed a crucial role for Bak in actinomycin-D-induced apoptosis, suggesting that both Bax and Bak have an important role in hESC apoptosis that is regulated by many apoptotic factors in a complex manner.

Mdm2, a negative regulator of P53, is inactivated following DNA damage, leading to P53 stabilization, which can initiate apoptosis [61,62]. We discovered that a rapid accumulation of P53 caused by Mdm2 inhibitor nutlin-3a is sufficient to induce apoptosis in hESCs, but only has a modest effect in differentiated cells. However, the P53 protein level in steady-state cells is similar in both hESCs and RA-differentiated cells. Therefore, hESCs rapidly turn over P53 in an ubiquitin-proteasome-dependent manner that correlates with high expression of Mdm2. A previous study reported that P53 exhibits no difference in apoptosis-inducing behavior in both differentiated and undifferentiated hESCs and, therefore, the rapid apoptosis in hESCs is due to their highly mitochondrial primed state [5]. However, because P53 interacts with mitochondrial proteins [7], P53 could be a key factor that primes the mitochondria for rapid induction of apoptosis in hESCs. Given that suppression of P53 increases the efficiency of hiPSC generation up to 10- to 20-fold [65], inactivation of P53 by Mdm2 in healthy hPSCs may be crucial for their survival and maintenance. Our study identifies a unique regulation of P53 in hESCs that prepares them to induce rapid apoptosis to maintain genome integrity while assuring survival at steady state.

Materials and Methods

Cell culture

H1, H9 [1], and H9FS2 [66] hiPSCs were grown on matrigel and fed daily with mTeSR-1 medium (Stemcell Technologies), maintained at 37 °C and 5% CO2. The medium was changed daily and passed with Dispase (Stemcell Technologies) before confluence. Differentiation of hiPSCs was induced by 1 μM RA treatment, with a fresh medium change every other day.

Antibodies and reagents

Antibodies were used against Oct4 (Santa Cruz Biotechnology), Nanog (Millipore), SSEA4 (Millipore), Lin28 (Cell Signaling), Tubulin (Sigma), cytochrome c (BD Biosciences), SmadDiablo (BD Biosciences), Bax-NP (Millipore), Bak-NP (Millipore), Dp1 (BD Biosciences), Mdm2 (Santa Cruz Biotechnology), TOMM20 (Santa Cruz Biotechnology), BCL-XL (Cell Signaling), c-caspase-3 (Cell Signaling), full-length caspase-3 (Cell Signaling), c-PARP (Cell Signaling), and P38 (Cell Signaling) proteins. A polyclonal antibody against recombinant TGN90 (Peprotech) was described previously [67]. CHX, MG132, actinomycin D, and etoposide were obtained from Sigma; O-VD-OPH was obtained from SM Biochemicals; recombinant human P53 protein was obtained from BD Pharmingen; and nutlin-3a was obtained from Cayman Chemical.

Western blotting

Cultured cells were harvested into SDS buffer (40 mM Tris–HCl [pH 6.8], 3% glycerol, and 1% SDS). Protein concentrations were quantified using the BCA assay (Pierce). Immunodetection was performed with ECL reagent (GE Healthcare). For protein turnover analyses, cells were incubated with 50 μg/ml CHX with or without 10 μM MG132 for 6 h, as indicated in each figure legend.

Lentivirus-mediated shRNA knockdown in hiPSCs

To generate hESCs that are knocked down for Dp1, Bak, and Bax, we obtained shRNAs that specifically target Dp1 (TRCN0000001097), Bak (TRCN00000033468), and Bax (TRCN00000033472) from Sigma. On day 1, hESC colonies on matrigel were transduced with 25 μl of 1 × 10^4 TU/ml of viral particles and 2 μg/ml polybrene for 4 h. On days 2 and 3, 75 μl of 1 × 10^4 TU/ml of viral particles was also added. Selection with 1 μg/ml puromycin was started on days 5–7 post-infection. Cell culture medium supplemented with puromycin was replaced daily.

Immunofluorescence confocal microscopy

Cells were grown on matrigel-coated chambered cover glass (Thermo Scientific). For cytochrome cSmac release assays, as well as for Bax targeting analysis, cells were exposed to apoptosis stimuli in the presence of 25 μM O-VD-OPH, a broad caspase inhibitor. Cells were fixed for 15 min at room temperature with pre-warmed 4% PFA and then permeabilized for 5 min with 1% Triton X-100. hiPSCs were stained by incubation with primary antibodies. After incubation with AlexaFluor-conjugated secondary antibodies (Invitrogen), images were acquired with either LSM 5 PASCAL or LSM 780 confocal microscopes (Carl Zeiss).

Quantitative real-time PCR

Cultured cells were harvested with CellReagent reagent (Life Technologies). RNA was extracted according to manufacturer’s instructions. RNA was converted into cDNA with iScript (Bio-Rad). Quantitative real-time PCR was performed on a LightCycler480 (Roche) using SYBR green (Roche). Primers used to detect P53 were ggt ggc aaag cag gag att cgg caa cag cac cag aca cgt gga gaa aag cga aat aag ccc ctc tgt gac tgc aag tgt. Primers used to detect Mdm2 were gac gcc agg cag gag aac and ggt ggt tac agc acc aat agt. Primers used to detect TGFβ2 were tgt agg cga tta aag ctc ttc gcg tgc aag tgt. Primers used to detect TGFβ2 were tgt agg cga tta aag ctc ttc gcg tgc aag tgt.

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Conflict of Interest: The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.07.019.

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Keywords: mitochondrial; Bax/Bak; pluripotency; differentiation; cell fate

Abbreviations used:
CHX, cycloheximide; ER, endoplasmic reticulum; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; iPS, human induced pluripotent stem cell; IMS, intermembrane space; MOMP, mitochondrial outer membrane permeabilization; PARP, poly(ADP-ribose) polymerase; RA, retinoic acid.

References

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Appendix VI:

Mitochondria in human pluripotent stem cell apoptosis
Mitochondria in human pluripotent stem cell apoptosis

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ABSTRACT

Human pluripotent stem cells (hPSCs) have great potential in regenerative medicine because they can differentiate into any cell type in the body. Genome integrity is vital for human development and for high fidelity passage of genetic information across generations through the germ line. To ensure genome stability, hPSCs maintain a lower rate of mutation than somatic cells and undergo rapid apoptosis in response to DNA damage and additional cell stresses. Furthermore, cellular metabolism and the cell cycle are also differentially regulated between cells in pluripotent and differentiated states and can aid in protecting hPSCs against DNA damage and damaged cell propagation. Despite these safeguards, clinical use of hPSC derivatives could be compromised by tumorigenic potential and possible malignant transformation from failed to differentiate cells. Since hPSCs and mature cells differentially respond to cell stress, it may be possible to specifically target undifferentiated cells for rapid apoptosis in mixed cell populations to enable safer use of hPSC-differentiated cells in patients.

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1. Introduction

New possibilities in regenerative medicine have been enabled by the production of human embryonic stem cells (hESCs) and the reprogramming of somatic cells into human induced pluripotent stem cells (iPSCs) [1-4]. Both cell types classify as human pluripotent stem cells (hPSCs) and each can self-renew indefinitely in culture. hPSCs also have the potential to develop into all of the mature cells in our bodies, including the germ cells that pass genetic information on to our progeny. Therefore, protection against harmful DNA mutations and other heritable lesions is vital for propagation of our species. Consistent with this fidelity, hPSCs exhibit a slow rate of DNA mutation due to enhanced mechanisms of DNA damage protection and repair [5-9]. In the event that irreparable DNA or other forms of heritable damage do occur, however, suboptimal hPSCs are prevented from replicating by sensitive and rapid apoptotic mechanisms [10-13]. hPSCs also uniquely regulate the cell cycle and cell cycle checkpoints to prevent damaged cells from further differentiation or cell division [14-16]. In this review we will examine and discuss the mechanisms employed by hPSCs to prevent the propagation of damaged cells, which centers on increased sensitivity to apoptosis triggers. Furthermore, teratoma formation in a rodent is the current gold standard test for hPSCs, but this occurrence also presents a potential tumorigenic threat for using hPSC derivative cells clinically [17]. An increased sensitivity to apoptotic stimuli for hPSCs could enable the selective removal of cells which fail to differentiate from a therapeutic cell population. Metabolic differences between hPSCs and their differentiated progeny are also substantial [18-23], potentially enabling metabolic selection against hPSCs in cell mixtures. Therefore, we also examine emerging methods for selective elimination of hPSCs that do not harm healthy populations of differentiated cells intended for cell therapies.

2. hPSCs show increased sensitivity to apoptosis

2.1. Mitochondria and BCL-2 family proteins in apoptosis

Apoptosis can be regulated by BCL-2 (B cell lymphoma-2) family proteins and their interactions with the mitochondria. BCL-2 family proteins can be divided into three functional groups: pro-apoptotic effector proteins BAX (BCL-2 antagonist/killer) and BAX (BCL-2 associated X protein), a set of pro-apoptotic BIM (BCL-2 homology domain 3) only proteins, and a set of pro-survival proteins that include BCL-2 itself [24]. BAX and BAX can initiate the apoptosis cascade by induction of mitochondrial outer membrane permeabilization (MOMP) [25,26]. BAK and BAX are activated through protein interactions that induce structural changes and their homo-oligomerization. Activation of BAX causes exposure of its N-terminal domain and insertion of its C-terminal domain into the mitochondrial outer membrane, which contrasts with BAX, which is localized to the mitochondrial outer membrane in both inactive and active conformations [27-29]. Homo-oligomerized BAX or BAX forms a pore that causes MOMP [25,26,30]. MOMP subsequently leads to the release of pro-apoptotic proteins in the mitochondrial intermembrane space (IMS), including cytochrome c, which activates caspase-9 protease in the cytosol [28,31,32]. In turn, caspase-9 activates additional effector caspases, unleashing a cascade of proteases that culminates in apoptotic cell death [24,33,34]. BAK and BAX have overlapping roles in apoptosis, but differ in their mechanisms of activation [26-28]. BAX or BAX knockout mice have limited abnormalities, whereas a double-knockout of both BAX and BAX is perinatal lethal due to defects in apoptosis induction [35]. In somatic cells, inactive BAX is located in the cytosol until an apoptotic stimulus causes BAX to interact with p53 or BH3-only proteins, activating BAX [27,30-34]. BH3-only proteins can directly bind and activate BAX or BAX, or they can bind and neutralize pro-survival BCL-2 family member proteins. When pro-survival BCL-2 proteins are not bound by BH3-only proteins, they can bind directly to activated BAX or BAX, inhibiting their pro-apoptotic activities [24]. Additional proteins can also interact with BCL-2 family member proteins to sensitize or deaden cellular responses to apoptosis induction [40,42-44]. Therefore, the cellular apoptotic threshold is a complex balance between pro-survival and pro-apoptotic proteins and their interactions.

2.2. Differential roles of BAX and BAX

Some hESC lines show a unique pattern of BAX regulation during S-phase of the cell cycle, pointing these lines for rapid apoptosis induction upon DNA damage. In these lines, during S-phase BAX is sequestered in the Golgi apparatus in its activated conformation, held away from the mitochondrion. Cell stress from DNA damage causes a rapid p53-dependent translocation of active BAX from the Golgi to the mitochondrion by an unknown mechanism, causing apoptosis (Fig. 1). By maintaining BAX in its active conformation in the Golgi, certain hESC lines bypass the BAX activation step and are ‘pruned’ for a cell death response [11]. It is not yet known, however, how active BAX is localized to the Golgi and what holds it there prior to p53-dependent translocation to the mitochondrion. Active BAX forms homo-oligomers and pores activating MOMP [23] making it unclear whether active BAX also causes pore formation in the Golgi membrane stack and, if not, what prevents pore formation or oligomerization. Active BAX is detected by an antibody that recognizes its exposed N-terminal domain [11], but it is not known whether this antibody-detected conformational change is sufficient for BAX activity. The H1 hESC line lacks Golgi-localized active BAX but still exhibits hypomycetism to apoptosis induction that is typical of other hESC lines [10,11,14,15]. One interpretation of this data is that hIPSC hypersensitivity to apoptosis may be independent of active BAX in the Golgi, or there may be additional mechanisms of hypersensitivity. In addition, whereas BAX has a key role in the apoptotic response of hPSCs to DNA damage, BAX is actually more important in response to other apoptotic insults and cell stressors. For example, the induction of rapid apoptosis in hIPSCs by transcriptional inhibition using aminopyrine D relies more on BAX than BAX [10]. Overall, some hESC lines localize active BAX to the Golgi [11] but this unique localization is not required for apoptosis hypersensitivity in all hESC lines, raising questions about its role in mitochondrial pruning of hPSCs for apoptosis.

2.3. Enhanced mitochondrial priming in hPSCs

hPSCs also undergo rapid apoptosis with activation of the unfolded protein response and transcriptional inhibition [10,12,13]. These data strongly suggest that hPSCs are hypersensitive to multiple and perhaps all activators of mitochondrial-
mediated apoptosis. The balance between pro- and anti-apoptotic BCL-2 family proteins controls the sensitivity of cells to mitochondrial-mediated apoptotic stimuli [46,47]. hiPSCs are reportedly in a high mitochondrial priming state in which an elevated ratio of pro-apoptotic to pro-survival BCL-2 proteins are present in comparison to their differentiated progeny (Fig. 1) [12]. However, the mechanistic underpinning for how mitochondrial priming is differentially regulated between hiPSCs and differentiated cells remains unclear. Increased expression of pro-apoptotic BCL-2 family proteins occurs with the reprogramming of fibroblasts to hiPSCs, which could increase mitochondrial priming [13,48]. In addition, decreased expression of pro-survival BCL-2 itself has been reported in hiPSCs compared to differentiated cell types [12,48]. Finally, p53 can impact the activities of BCL-2 family proteins and MOMP, which is discussed next.

3. p53, DNA damage, and cell cycle regulation

3.1. p53 in hiPSCs

The p53 tumor suppressor is activated by cell stress in both pluripotent and differentiated cells [12,49]. p53 is a key component of the DNA damage response through its roles as a transcription factor in the nucleus and through activation of BAX or BAK oligomerization at the mitochondrial [45,49,50,51,52]. The timing of induction of p53 target genes in response to DNA damage is similar in pluripotent and differentiated cells. When p53 is localized only to the cytoplasm, hiPSCs can still undergo apoptosis induction without p53 transcriptional activity [12]. Therefore, both cytoplasmic and nuclear roles for p53 in apoptosis induction are functioning in hiPSCs.
p53 protein stability is tightly regulated by the E3 ubiquitin ligase mouse double minute 2 homolog (MDM2), which triggers p53 degradation by mono-ubiquitination and degradation in the proteasome [53-55]. Disruption of a p53-MDM2 interaction in response to DNA damage is crucial for stabilizing p53 and its activities [53-54]. In hPSCs, inhibition of MDM2 causes rapid p53 stabilization. Interestingly, inhibition of protein translation with cyclohexamide causes a fast proteasome-dependent decrease in p53 levels in hPSCs that does not occur in differentiated cells [10]. Therefore, p53 is unstable in hPSCs due to proteasome degradation. Additionally, decapping of p53, which promotes p53 degradation, is promoted by OCT4-dependent expression of SIRT1 [56]. Together the data suggest that hPSCs rapidly produce and degrade p53 in the absence of cellular stress, which enables quick p53 stabilization upon DNA damage or with other pro-apoptotic stimuli (Fig. 1). Since p53 can directly regulate MOMP through interactions with BAK and BAX [40,43,44,51], differences in p53 stability could account for differences in mitochondrial priming between hPSCs and differentiated cells. Contradicting this idea, mitochondrial priming in hPSCs was unaffected by p53 knockdown, suggesting that other mechanisms contribute to mitochondrial priming [12]. Overall, p53 has an early role in apoptosis for both hPSCs and differentiated cells, but differences in its turnover and stabilization rates increase apoptosis sensitivity for hPSCs with DNA damage.

3.3. Unique cell cycle regulation in hPSCs

hPSCs show an abbreviated cell cycle due to a shortened G1-phase and they undergo p21-dependent cell cycle arrest in G2-phase with DNA damage [58,62,63]. As hPSCs differentiate, there is a concurrent p53-dependent increase in the duration of G1-phase [84]. Propagation of damaged hPSCs is also prevented by blocking differentiation during DNA replication and cell division periods of the cell cycle with an increased probability for DNA damage. During G1-phase, hPSCs are more likely to differentiate, whereas maintained pluripotency is favored during replicative stress in S- and G2/M-phases of the cell cycle [14-16]. Interestingly, hPSCs selectively differentiate into endoderm during early G1-phase, whereas hPSCs preferentially differentiate into ectoderm during late G1-phase [16]. Promotion of pluripotency during DNA replication prevents cell fate changes and the propagation of cells with an increased probability of damaged DNA. Additionally, pluripotency maintenance during S-phase decreases the probability of DNA damage repair and/or apoptosis, which are heightened responses in hPSCs.

4. Dissociation induced apoptosis in hPSCs

Single cell dissociation of hPSCs that normally grow in tightly packed colonies causes apoptosis, which can limit the use of techniques that require clonal isolation of hPSCs [16,5]. To overcome this limitation, inhibition of rho-associated kinase (ROCK) increases both hPSC survival after dissociation and clonogenic efficiency [16]. Single cell dissociation of hPSCs disrupts E-cadherin mediated intercellular contact, resulting in immediate membrane blebbing by ROCK hyperactivation of myosin [67,68]. Myosin activation contracts the actomyosin network and is required for dissociation-induced hPSC apoptosis [67-69]. Although dissociation-mediated apoptosis leads to MOMP and cytochrome c release from mitochondria, the mechanism connecting actomyosin network contraction and MOMP initiation remains unknown [70].
5. Selective apoptosis of hPSCs in therapeutic cell mixtures

5.1. Transformation of hPSCs

A safety concern in developing hPSC derivatives for use in humans is their tumorigenic potential [17,71]. Teratoma formation as the main test for pluripotency illustrates the inherent tumorigenic potential of hPSCs [12]. Furthermore, despite increased protection from DNA damage, extended in vitro culture of hPSCs somewhat paradoxically causes the accumulation of genetic lesions [72-74]. The most prevalent karyotypic change for hPSC lines is trisomy 12, which increases hPSC proliferation rate and tumorigenic potential [72,75-77]. In addition, hPSCs can acquire genetic and epigenetic abnormalities during reprogramming to pluripotency [17,78]. A comparison of teratoma formation between hESCs and hiPSCs revealed that hiPSCs form teratomas more efficiently and more quickly than hESCs [79]. Therefore, therapeutic applications of hiPSC differentiated derivatives will require increased control over innate tumorigenicity and possible malignant transformation of failed to differentiate cells.

In vitro differentiation to desired cell types remains inefficient, with some percentage of undifferentiated cells persisting within the population of differentiated cells to be transferred into patients [80]. Failure to differentiate is a common feature for cancers that maintain a high proliferative capacity and a potential sign of concern for malignant transformation [81-83]. Therefore, strategies to make differentiation closer to 100% efficient, which currently seems unlikely, or to remove pluripotent residual cells while leaving healthier differentiated cells unharmed are of therapeutic interest. Current strategies include introduction of pluripotency-specific suicide genes [84-88], antibody-based enrichment or depletion of cell types [89,90], lentiviral-targeted toxins [91], and selective induction of apoptosis by small molecule inhibitors that target hPSCs.

Here, we examine small molecule inhibitors that may selectively remove hPSCs from heterogeneous cell populations. Many of these strategies take advantage of differences between hPSCs and differentiated cells, including apoptosis hypersensitivity and unique forms of cell metabolism.

5.2. Exploiting hiPSC hypersensitivity to apoptosis

The balance of pro-apoptotic and pro-survival BCL-2 family proteins is different in pluripotent and differentiated cells, which makes strategies targeting this difference potentially useful for selective induction of apoptosis [12,13]. Since many cancers up-regulate pro-survival BCL-2 family proteins, inhibitors targeting these proteins are in development and could find use in regenerative medicine [24]. Also, non-BCL-2 family pro-survival proteins, such as BCL-10 and SURVIVIN, are highly expressed in hESCs than in adult stem/progenitor cells. Since adult stem/progenitor cells express other pro-survival proteins, such as BCL-2, chemical inhibition of BCL-10 or SURVIVIN causes apoptosis of hESCs, but not these more differentiated adult stem/progenitor cells. Hence, pre-treatment of mixed populations of hESCs and differentiated cells with SURVIVIN inhibitors has been shown to repress teratoma formation in mice [57].

As discussed in Section 3.3, there is differential regulation of the cell cycle between hPSCs and differentiated cells. Systematic depletion of key cell cycle-regulatory proteins revealed that inhibition of cyclin dependent kinase 1 (CDK1) activates apoptosis in hPSCs but not in differentiated cells. Selective apoptosis of hPSCs by CDK1 inhibition is mediated by a p53-dependent increase in NOXA, a BIM-only pro-apoptotic protein [92].

As the hypersensitivity of hPSCs to apoptosis becomes better understood, additional strategies for removing hPSCs from differentiated cell mixtures will likely be identified. Since p53 may have a key role in hPSC apoptosis, MDM2 inhibitors may provide an interesting approach. Strategies that increase oxidative stress or DNA damage could provide selective cell death for hPSCs but at a price that seems unacceptable as the potential for increased DNA damage in differentiated cell types also increases.

5.3. Metabolic selection against hPSCs

hPSCs produce more ATP by glycolysis and transition to OXPHOS for most ATP production during differentiation [93,94]. Cardiomyocytes (CMs), for example, produce energy using glucose, fatty acids, and lactate by OXPHOS [85]. By culturing hPSCs and CMs in a mixture in conditions lacking glucose but supplemented with lactate, a population of CMs can be purified that lacks teratoma forming cells [95]. However, many other differentiated cell types cannot uptake and metabolize lactate making this strategy specific for CMs.

A small molecule inhibitor that can selectively kill hPSCs is STF-31. There are conflicting reports that STF-31 kills hPSCs by inhibiting either glucose transporters [96] or nicotinamide phosphoribosyltransferase (NAMPT) [97]. Facilitated glucose transporters are abundant at the hPSC cell membrane, which led to the identification of STF-31 [96]. The timing of STF-31-induced hPSC death, however, is distinct from the kinetics of hPSC death caused by WZB117, a glucose transporter-1 inhibitor. These discrepant temporal results leave open the possibility that STF-31 is acting through a different mechanism. An alternative potential mechanism is through inhibition of NAMPT, which is part of the NAD+ salvage pathway. STF-31 treatment causes a decrease in NAD+ levels before it causes a decrease in glucose uptake. Apoptosis of hPSCs by STF-31 treatment can be rescued by supplementation of nicotinic acid, supporting inhibition of NAMPT as a mechanism for STF-31 killing of hPSCs [97].

A small molecule inhibitor screen with readout for selective killing of hPSCs yielded an inhibitor of stearoyl-CoA desaturase (SCD1), which catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids [98]. Treatment of hPSCs with the SCD1 inhibitor, PuriSin #1, led to apoptosis after the induction of endoplasmic reticulum (ER) stress, which can be caused by saturated fatty acids [99-102]. PuriSin #1 treatment of a mixed population of pluripotent and differentiated cells for 48 h prevented teratoma formation in mice [88]. SCD1 inhibitors may prevent cellular damage and oxidative stress because adding the saturated fatty acid palmitate can cause apoptosis, ER stress, mitochondrial ROS, and mitochondrial DNA damage [101,103].

5.4. Additional strategies for selective hPSC apoptosis

Mitochondria in hPSCs appear granular and spherical with morphologically immature inner membrane cristae [100,104,105]. Whereas there are many characterized differences in metabolism between pluripotent and differentiated cells, very little is known about the differential regulation of mitochondrial biogenesis, dynamics, morphology, and function between these cell states. Mitolock-6, an inhibitor of the mitochondrial redox protein Erv1/ALR, is toxic to hPSCs but has no effect on differentiated cells [106]. Erv1/ALR cooperates with Mia40 to import proteins into the mitochondrial IMS via a disulfide relay system with cytochrome c acting as the electron acceptor [107-109]. It remains unclear how hPSCs are specifically sensitive to Mitolock-6 treatment.

 Tight junction protein claudin-6 (CLDN-6) is expressed specifically in hPSCs and not in differentiated cells. Although CLDN-6 is not required for hPSC survival and self-renewal, it can be targeted with Chromobacterium violaceum enterotoxin (CPE), which binds to six claudin family member proteins, including CLDN-6, to induce cell death. CPE treatment inhibits teratoma formation of hPSCs in mice.
However, CPE also targets CLDN-3, CLDN-4, CLDN-7, CLDN-8 and CLDN-14, which are expressed in various epithelial and endothelial cells. CPE, useful therefore, is limited to cell mixtures with differentiated tissue types that lack expression of these claudin family proteins [90].

6. Concluding remarks

hPSCs exhibit multiple molecular mechanisms for maintaining genome integrity and preventing cellular and organismal damage. However, many of the details for these mechanisms remain to be discovered. Mitochondria, which are central to the apoptotic process, are morphologically and functionally distinct between pluripotent and differentiated cells. Very little, however, is known about how these morphological and functional differences impact apoptosis sensitivity. Additionally, cellular metabolism, which is known to influence apoptosis sensitivity in somatic cells, also differs between the pluripotent and differentiated states [18–23,110].

Pluripotent stem cells and cancer cells share many features including indefinite self-renewal. Cancer cells, however, typically evolve mechanisms to avoid apoptosis despite significant DNA damage and other cell stresses [132]. By contrast, hPSCs are predisposed to apoptosis to maintain genome integrity. In many ways, including patterns of cell metabolism and proliferation, pluripotent stem cells and cancer cells resemble each other [111]. However, for pluripotent cells to survive, these cells must adapt to their new environment. Understanding how cell death mechanisms differ between cancer and pluripotent stem cells can potentially be leveraged to inform therapeutics developments for the benefit of both areas in human health and disease.

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References

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Appendix VII:

Mitochondria transfer by photothermal nanoblade restores metabolite profile in mammalian cells
Cell Metabolism

Mitochondrial Transfer by Photothermal Nanoblade Restores Metabolite Profile in Mammalian Cells

Graphical Abstract

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In Brief
Optimizing mtDNA transfer into mammalian cells is an important step for basic studies and mitochondrial disease therapies. Using a photothermal nanoblade, Wu et al. are able to deliver isolated mitochondria into respiration-deficient cells. Rescued cell lines recover mitochondrial respiration and reset cellular metabolism to the parental cell level.

Highlights
- Proof-of-principle photothermal nanoblade transfer of mitochondria is reported
- Transfer into 143BTK- p0 cells generated rescue clones with recovered respiration
- Mitochondrial transfer reset metabolic enzyme gene expression patterns
- Two of three rescue clones showed metabolite profiles similar to 143BTK- parent cells

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CellPress
Mitochondrial Transfer by Photothermal Nanobrade Restores Metabolite Profile in Mammalian Cells

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SUMMARY

mtDNA sequence alterations are challenging to generate but desirable for basic studies and potential correction of mtDNA diseases. Here, we report a new method for transferring isolated mitochondria into somatic mammalian cells using a photothermal nanoblade, which bypasses endocytosis and cell fusion. The nanoblade rescued the pyrimidine auxotrophic phenotype and respiration of p0 cells that lack mtDNA. Three stable isogenic nanoblade-rescued clones grown in uridine-free medium showed distinct bioenergetic profiles. Rescue lines 1 and 3 reestablished nucleus-encoded anaplerotic and catecholautoic enzyme gene expression patterns and had metabolite profiles similar to the parent cells from which the p0 recipient cells were derived. By contrast, rescue line 2 retained a p0 cell metabolic phenotype despite growth in uridine-free selection. The known influence of metabolite levels on cellular processes, including epigenome modifications and gene expression, suggests metabolite profiling can help assess the quality and function of mtDNA-modified cells.

INTRODUCTION

Mitochondria are double-membrane eukaryotic organelles of α-proteobacterial origin (Sagan, 1967) that are maternally inherited and help produce energy (ATP) and intermediate metabolites, reducing agents (NADH and FADH2), Fe-S clusters, heme, and steroids. They also generate reactive oxygen species during respiration and regulate apoptosis, Ca2+ homeostasis, and intracellular signaling (McBride et al., 2009). Mitochondria exist in an equilibrium between fused and fragmented morphologies that maintain their shape, size, number, and quality, and they contain their own non-nuclear genome (mtDNA) (Chan, 2012). In human, the 16.6 kb circular mtDNA encodes for 13 respiratory chain proteins, 22 tRNAs, and 2 rRNAs. Each nucleated cell contains a few to 100,000 copies of mtDNA that reside in nucleoids (Garcia-Pedriguez, 2007). mtDNA mutations (http://www.mtdna.org/MTDNAmap) can be silent or cause inborn familial diseases that affect high-energy tissues, including brain, heart, and muscle (Taylor and Turnbull, 2009). Cell dysfunction and disease may arise by a critical reduction in mtDNA number or by exceeding a threshold ratio between mutant and wild-type mtDNA, creating a heteroplastic state.

Unlike the nuclear genome, strategies for altering mtDNA are limited. Work to overcome the transmission of inherited mtDNA diseases has turned to preimplantation genetic diagnosis to evaluate risk. For women at risk, the transfer of the mitotic spindles chromosome complex or a polar body or a donor oocyte, or the transfer of pronuclei to a donor egg offers the potential for offspring with healthy mtDNA (Richardson et al., 2013). The generation of “three-parent” embryos as an assisted reproduction strategy has generated interest and debate, although these techniques cannot be used for somatic cells or after birth (Richardson et al., 2015; Vogel, 2014).
Alternative strategies for changing the mtDNA content in germ or somatic cells include somatic cell nuclear transfer (McClelland et al., 2011; Tachioka et al., 2013) and manipulating the cellular heteroplasmy ratio. Heteroplasmy reduction through a “bottleneck” occurs naturally in early mammalian development and may occur with reprogramming somatic cells to pluripotency (Testa and Teitel, 2015). The bottleneck mechanism(s) for reducing heteroplasmy remain unresolved, and not all mtDNA haplotypes appear to survive. Mitochondrial targeted nucleases, including restriction endonucleases, zinc finger nucleases, and TALE nucleases, can enrich for specific mtDNA by incomplete cleavage of target mtDNA in a mixture, shifting the heteroplasmy ratio (Bacmac et al., 2013).

The insertion or replacement of mtDNA sequences by genome editing tools or mitochondrial-targeted adenovirus vectors (Yu et al., 2012) may also reduce specific mtDNA haplotypes. However, success for these procedures requires DNA repair by non-homologous end joining or homologous recombination, which occur infrequently in mammalian mitochondria (Aynayev et al., 2013). Therefore, the acquisition of desired mtDNA haplotypes can be only accomplished by transferring mitochondria containing pre-existing mtDNAs into target cells. Successful approaches include cytoplasmic fusion between unmito-microbe mitochondria donor cells and mtDNA-eliminated p0 cells to generate trans-mitochondrial hybrid cell lines (Moraus et al., 2001). Also, direct microinjection of isolated mitochondria into somatic cells or oocytes (Kong and Altar, 1986; Yang and Koob, 2012) and the transfer of isolated mitochondria, or mitochondrial transfer between cells, in vivo or in co-culture have been reported (Caciolo et al., 2015; Islam et al., 2012; Liu et al., 2014; Spasojevic et al., 2008). However, microinjection is inconsistent, and remains unclear whether transferring nanoblade transfer or the “spontaneous” uptake of isolated mitochondria are general phenomena or condition/cell-type specific mitochondrial transfer mechanisms.

Recently, we developed a photothermal nanoblade for efficient transfer of small and large objects into mammalian cells by direct cytoplasmic delivery (French et al., 2011; Wu et al., 2011, 2010). Here, we present a proof-of-principle study for nanoblade transfer of isolated mitochondria into p0 cells. Metabolomics analyses show the nanoblade is a controlled, reproducible, and general approach for changing the mtDNA haplotype in somatic mammalian cells and may be a potential first step toward reverse mitochondrial genetics.

RESULTS

Photothermal Nanoblade Configuration and Optimization

The apparatus consists of an inverted microscope with a 542 nm nanosecond pulsed laser that illuminates the field of view. A nanoblade delivery microinjection is mounted on a microinjection manipulator and connected to an external pressure source (Figures S1A and S1B). The micropipette tip is coated with a light-absorbing titanium thin film (~100 nm thick (Wu et al., 2011, 2010)). This coated tip is positioned to lightly touch the plasma membrane of a cell using the joystick controller. A transient membrane opening is induced by an ultrafast (~0.30 ns) and localized (~0.4 μm from micropipette tip) cavitation bubble, which forms from a laser pulse that rapidly heats the titanium thin film, causing vaporization of adjacent water layers in the culture media (Wu et al., 2011). Bubble expansion and collapse locally punctures the membrane and creates a several micron-long passageway for large cargo delivery that is rapidly repaired (Yamane et al., 2014). Pressure-driven fluid flow synchronized to the laser pulse transports cargo into the recipient cell cytosol. The micropipette does not penetrate the cell as in microinjection, is not sealed on the membrane, and membrane disruption is localized to the bubble nucleation site (Wu et al., 2010).

To deliver ~2 μM × 1 μM sized mitochondria, the nanoblade was fabricated with a 3 μm bore tip insert (Figures S1C). This inserted orifices prevents clogging and avoids excessive mitochondrial shearing (Figures S2A and S2B). The pulsed laser energy must surpass the superheating threshold for the nanoblade to generate a delivery portal without causing excessive damage and cell death. Optimization of plasma membrane opening efficiency and post-delivery cell viability is cell-type specific and established by titration of the laser pulse fluence (μJ/μm2) prior to mitochondria delivery (Wu et al., 2011, 2010). For 143BTK−/− p0 human osteosarcoma cells, a laser fluence of 156 μJ/μm2 yields 64% membrane opening efficiency and 50% viability at 24 hr (Figure S2C). Human cells require 67% more laser energy (180 μJ/μm2) for efficient membrane opening and 80% cell viability. Although photo-irradiation by laser illumination can deplete mitochondria and cause their clearance by mitophagy (Kim and Lamastre, 2013), minimal to no loss of mitochondrial membrane potential (V(M)) occurred in isolated or whole-cell mitochondria, respectively, with repeated 108 μJ/μm2 laser pulses (Figure S2D).

Mitochondrial Transfer

Recipient 143BTK−/− p0 cells were labeled with membrane potential-insensitive Mitotracker Green and seeded onto a 400 μm × 400 μm square on a patterned glass coverslip to simplify tracking (Figure 1A). HEK293T cells expressing mitochondrial-targeted DsRed fluorescent proteins (pMtDsRed) were generated. ~0.5 mg/ml of isolated DsRed mitochondria were loaded into the nanoblade micropipette and delivered into p0 cells at ~100 cells/μl (Figures 1B). Confocal microscopy with z-stack reconstruction showed donor DsRed and recipient Mitotracker Green-labeled mitochondria, intermixed in the cytosol of recipient p0 cells 4 hr after delivery (Figure 1C).

MDA-MB-435 human breast carcinoma cells have genomic and mitochondrial DNA sequence polymorphisms and a unique MHC haplotype compared to 143BTK−/− p0 cells, providing distinguishing tag. Oxygen consumption rate (OCR) studies showed isolated mitochondria from 143BTK−/− p0 cells, whereas MDA-MB-435 and 143BTK−/− p0 mitochondria had OCRs of 5.1 and 14.7, respectively, whereas 143BTK−/− p0 mitochondria had a negligible OCR.

p0 cells are priminivide autophagosomes that require uridine supplementation to grow because of an inactive dehydrogenase dehydrogenase (DHODH) enzyme resulting from a non-functional
ETC (Gregoire et al., 1986). MDA-MB-453 mitochondria were loaded and nanobrade delivered into ~30 143BTK−/−p0 cells grown in uridine-added medium. 4 days post-delivery, cells were shifted to uridine-free medium, with the emergence of respiratory “rescue” clones starting at ~2 weeks (Figure 1E). The frequency of stable, rescue clone generation was 2.1% ± 3.1%, ~10-fold higher than microinjection (Table S1) (King and Attardi, 1986).

Clone Validation and Bioenergetic Analyses

Three nanobrade rescue clones were evaluated. Although ~10% of cells in rescue clones 2 and 3 had PicoGreen mtDNA staining after 2 weeks in uridine-free medium, >80% of cells in all three clones had mtDNA staining after 4 weeks (Figure 2A). Total DNA from donor, parent, p0 recipient, and clone 1-3 cells were PCR amplified with primers for mtDNA D-loop hypervariable control region (Figure 2B) and a single nucleotide polymorphism, m2981T/G2, in the nucleus-encoded PGFR2 gene. Sequencing confirmed all three rescue clones contained exclusively donor mtDNA and p0 recipient genomic DNA (Figures 2C and 2D).

OXPHOS, was variable (Robinson, 1996), suggesting the clones were not metabolically equivalent. Steady-state ATP levels in clones 1 and 3 were at or above the donor and parent cell levels, respectively, but clone 2 had reduced ATP (Figure 2D). OCR showed basal, maximal respiration, and specific ETC complex I, II, and IV activities for clones 1 and 3 were similar to parent and donor lines, but clone 2 respiration was lower and p0 cell respiration absent (Figure 3C). Combined proliferation rate, ATP level, respiratory, and ETC complex activity data suggest rescue clones 1 and 3 are energetically distinct from clone 2 and more similar to the parent cells than to donor or p0 recipient cells. The data reveal a range of functional reconstitution of the nanobrade transfer of mitochondria and the restoration of transferred mtDNA.

A respiration-independent measure of mitochondrial biomass was provided by citrate synthase enzymatic activity (Zhang et al., 2011), which was similar between rescue clones 1–3, donor, recipient, and parent cells (Figure 3E). p0 recipient cells have a granular mitochondrial network (Mangin et al., 2002) that is retained in rescue clone 1 cells (Figure S3A). Ultrastructure

Figure 1. Generating Mitochondrial Rescue Clones by Photothermal Nanobrade

(A) Recipient 143BTK−/−p0 cells were seeded on a 406 μm x 460 μm square to facilitate nanobrade delivery, tracking, and dead-cell selection. (B) Schematic of mitochondrial delivery by photothermal nanobrade. A 3 μm inner diameter glass micropipette tip coated externally with titanium is positioned to lightly contact the cell surface. A 532 nm pulsed laser illumination triggers a cavitation bubble to open the membrane with coordinated delivery of donor mitochondria into a cell using a fluid pump. (C) Representative confocal image of two focal of Datta label donor mitochondria from HEK293T cells in the cytosol of a single 143BTK−/−p0 cell whose emitters in mitochondria are stained with Milotex Red Green (upper left quadrant). (D) Isolated VDAC in 143BTK−/−p0 donor and 143BTK−/−parent cell mitochondria remain functional and coupled. Mean ± SD ($\mu$m = 3). (E) 2 weeks post-nanobrade delivery, donor VDAC in 143BTK−/−parent, not shown mitochondria transfected into 143BTK−/−p0 recipient cells generated “rescue” clones that emerged in uridine-free dialyzed media (left). 143BTK−/−p0 control cells (left 143BTK−/−p0 cells that received 143BTK−/−donor mitochondria, not shown) died and necrotized from the plate when grown in uridine-free dialyzed media (right).

MHC haplotype analysis showed rescue clone 1 contained the recipient cell nucleus (Figure 2E).

Rescue clones 1–3 proliferated at a similar rate to 143BTK−/−parent cells in uridine-free medium, suggesting the recovery of ETC and DHOD functions (Figure 3A). None of the lines proliferated in 2-deoxyglucose, which blocks glycolysis, but growth in galactose, which favors

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Figure 2. Recipient gDNA–Donor mtDNA Pairing Validates Rescue Clones
A: immunofluorescence staining of mDNA in the cytoplasm of 143BTK−p0 cells containing nanoblade-transferred MDA-MB-453 mitochondria at 2 (top row) and 4 (bottom row) weeks post delivery. 143BTK−p0 cells lack mDNA, do not survive for 4 weeks in uridine−medium, and show only nuclear staining. All 2 weeks in uridine−medium, ~16% of rescue clones 2 and 3 cells have mDNA, which appear as green puncta in the cytoplasm. By 4 weeks of uridine−selection, ~46% of cells in rescue clones 1−3 have mDNA. Mean ± SD.
B: PCR of mDNA D-loop hyper-variable region from rescue clones 1−3 with controls.
C: Sequencing of mDNA D-loop hyper-variable region revealed multiple single nucleotide polymorphisms (SNPs, red color, arrows) present in rescue clones 1−3 and donor MDA-MB-453.

Legend continued on next page
analysis by transmission electron microscopy showed that rescue clone 1 mitochondria had tightly stacked cristae with elevated electron density (Figures S1B–S3D). mtDNA levels by qPCR varied >10-fold between rescue clones 1–3 and did not correlate with proliferation rate, ATP level, OCR, or ETC complex activities (Figure 3E). Also, mitochondrial NDA1 and ND2 transcript expression did not correlate with mtDNA content for clones 1–3, suggesting that differences in mtDNA expression are not the source of variable rescue quality or function (Figure 3F). Finally, ΔΨ′ was quantified with tetramethylrhodamine methyl ester (TMRM), ϕ0 cells hydrolyze ATP in mitochondria to maintain viability and showed a low ΔΨ′ (Hatate, 1996). Rescue clones 1 and 3 had a ΔΨ′ equivalent to parent cells, but rescue clone 2 showed ΔΨ′ restoration in between the parent and ϕ0 recipient cells (Figure 3G). Thus, ΔΨ′ provided a potentially superior biomarker for mitochondrial function in rescue clones from nanobrade transfer.

**Restoring Metabolism-Related Gene Expression**

The introduction of mtDNA into ϕ0 cells could affect nuclear gene expression directly, by impacting transcription factors, or indirectly, by changing metabolic levels that regulate signal transduction and epigenetics (Kessel and McKnight, 2013; Teasla and Teitel, 2016). Changes to nuclear-encoded gene expression patterns by rescuing ϕ0 cells through the transfer of isolated mitochondria have not yet been assessed. Therefore, the steady-state expression of 33 genes encoding amipuric or catalytic enzymes was quantified by qRT-PCR (Figures S4A and S4B). An unbiased, systems-level assessment of steady-state gene expression was obtained by principle component analysis (PCA), which investigated relationships among all six cell lines (Figure 4A). Rescue clones 1 and 3 had gene expression profiles similar to the parent, whereas rescue clone 2 was similar to ϕ0 recipient. Donor cells showed a distinct gene expression profile compared to the other cell lines. The data indicate that nanobrade transfer of mtDNA resets the nuclear-encoded metabolic enzyme gene expression pattern partially (clone 2) or almost completely (clones 1 and 3 to the parent), and not the donor cell nucleus in an isogenic nuclear background.

**Metabolite Profiling**

Intracellular metabolite levels have not been quantified for mtDNA-modified systems. A liquid chromatography-mass spectrometry (LC-MS) based metabolomics assay assessed TCA cycle-related and select cytoplasmic metabolites that could impact gene expression and cellular biophysical functions. Of 12 TCA cycle-related metabolites (aconitate, α-ketoglutarate, succinate, and citrate were highly variable), α-ketoglutarate (α-KG) and citrate (Ct) stood out as strongly depleted in ϕ0 recipient cells compared to parent cells (Figure 4B). All three rescue clones showed increased α-KG and citrate above those in the ϕ0 recipient. In contrast, ϕ0 recipient cells accumulated the oncometabolite 2-hydroxyglutarate (2-HG) and succinate (Suc) relative to parent cells. All three rescue clones rescued the block at succinate dehydrogenase (ETC complex II), although clone 2 retained elevated 2-HG levels in contrast to clones 1 and 3. An unbiased assessment of 96 metabolites, including amino acids and nucleotides, was obtained by hierarchical clustering (Figure 4C) and PCA (Figure S4C) to evaluate the systems level rescue of ϕ0 recipient cells by nanobrade. Rescue clones 1 and 3 clustered with parent cells, whereas rescue clone 2 grouped together with ϕ0 recipient cells, and donor cells stood apart from both of these clusters. Similar to nucleus-encoded gene expression, nanobrade transfer of mtDNA restores the steady-state metabolite pattern of ϕ0 recipient cells partially (clone 2) or almost completely (clones 1 and 3) to the parent and not the donor cells in an isogenic nuclear system.

The activities of metabolic pathways and the contributions of nutrients to specific intracellular metabolites were examined with stable isotope labeling. Fully labeled L-[13C] glucose (GLC) and glutamine (Gln) provided the fractional contribution to network metabolites for recipient, parent, donor, and rescue clone 1-3 lines (Figure S4D). Rescue clones 1 and 3 grouped with the parent line by PCA, while rescue clone 2 was metabolically more similar to ϕ0 recipient cells and the donor line was metabolically distinct from all other analyzed cell lines. Overall, the data suggested that gene expression, metabolic network activity, and metabolite levels in ϕ0 recipient cells were almost completely restored to the parent cell levels in rescue clones 1 and 3, but not in rescue clone 2.

**DISCUSSION**

In a proof-of-principle study, we demonstrated that the photothermal nanobrade can transfer isolated mitochondria from an allogeneic cell type to restore metabolic gene expression, global energetic, and metabolite profiles. The nanobrade restores comparisons of different mitochondria and their metabolic performance in an isogenic nuclear background. The mtDNA level, mtRNA expression, mitochondrial biomass, and ultrastructure did not correlate with rescue clone performance. A broad range of rescue clone respiration and respiratory capacity unrelated to mtDNA or mtRNA levels was also reported for clones previously generated via microinjection (King and Attardi, 1989) and hybrid fusion (Chorny et al., 1994). Instead, system-wide activities as reflected by ΔΨ′ and global metabolites recovery were a better predictor of rescue clone quality and function. Hierarchical clustering and PCA of steady-state metabolite levels and the fractional contribution of glucose and glutamine to isotopologues indicate that rescue clones 1 and 3 are restored to the parental metabolic profile. Interestingly, the partially rescued clone 2 survived uridine-free selection and still remained most similar to ϕ0 cells, possibly by sufficient recovery of ETC function to increase DHOD activity and uridine production. This idea is supported from the reversal of the ETC complex II (succinate dehydrogenase) block and full recovery of parent succinate levels for all three rescue clones. All three rescue clones adopted features of the recipient and not the donor.

(Q) Rescue clones 1–3 contained the same SNP in the POLR2 gene as 1438T>C– parent and 1438T>C– ϕ0 cells (brown), but a distinct SNP from mitochondrial donor VDA-435 cells.

(R) Human leukocyte antigen (HLA) analysis of rescue 1 shows the same major histocompatibility complex (MHC) I loci as 1438T>C– parent and 1438T>C– ϕ0 cells.

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Figure 3. Bioenergetic Profile of Rescue Clones
(A) Proliferation of 143BTK- parent, MDA-MB-453 donor, 143BTK- pO, and rescue clone 1-3 cells in the indicated media formulations. Mean ± SD (n = 3).
(B) Stable state intracellular ATP levels in arbitrary units. Mean ± SD (n = 3).
(C) Mitochondria oxygen assay (left) and electron flow assay (right) as measured by Seahorse XF24 Analyzer. Mean ± SD (n = 3).
(D) Ratio of citrate synthase enzyme activity to total cellular protein, normalized to 1.0 for the 143BTK- parent line. Mean ± SD (n = 3).
(E) mtDNA quantification by qPCR, normalized to 1.0 for the 143BTK- parent line. Mean ± SD (n = 3).
(F) mtDNA-associated ND1 and ND2 transcript quantification by qRT-PCR, normalized to 1.0 for the 143BTK- parent line. Mean ± SD (n = 3).
(G) Representative mitochondrial membrane potential quantified by TMRE staining and flow cytometry.

Compared to cell fusion, the photothermal nanoblade can deliver washed, isolated mitochondria, minimizing the transfer of other cytosolic biomolecules that could impact biological functions in cells, such as microRNAs, metabolites, and signaling molecules. Initial applications for the nanoblade approach include more detailed studies of mtDNA expansion kinetics with or without manipulations of regulators of mtDNA replication or mitochondrial fusion/fission. A defined ratio of mixed donor mitochondria can also be simultaneously transferred into a cell, which aids in studies of heteroplasmic mtDNA composition, nuclear/mitochondrial genome compatibility, and kinetics of metabolic rewiring. Finally, the nanoblade could dissect mechanisms of inefficient, uncontrolled, and spontaneous cellular uptake of mitochondria without cell fusion (Katarzyn et al., 2007; Katarzyn et al., 2014; Matsuoka et al., 2013).

Mitochondria transfer by photothermal nanoblade is ~2% efficient, which is higher than cell fusion (0.0001%-0.5%) (Tables S2 and S3). However, the nanoblade is low throughput because mitochondria are transferred into successive individual cells. Due to variable recovery of recipient cell function seen with all reported mitochondrial transfer approaches, a larger number of clones in each experiment are needed to obtain a spectrum of clone performance and enable the selection of optimal clones for a particular purpose. A potential solution is the recent development of a biophotonic laser-assisted surgery tool (BLAST) for the massively parallel transfer of large cargo into mammalian cells using the same biophysical principle as the one-cell-at-a-time nanoblade, with appropriate modifications and optimization for mitochondrial transfer reactions (Wu et al., 2015).
Figure 4. Metabolic Gene Expression and Metabolite Profile of Rescue Clones

(A) Expression of 38 genes involved in TCA cycle metabolites quantified by qRT-PCR and normalized to the ribosomal 36B4 gene (see Figure S4E). PCA shows the grouped relationships between the six cell lines stacked.

(B) Relative levels of 12 TCA cycle proximate metabolites quantified by LC-MS/MS and normalized to the 143BTK parental line. Mean ± SD (n = 3). Color code consistent with rest of subfigures.

(C) Heatmap of 56 metabolites measured with an ANOVA p-value equal to or less than 0.05. Samples were clustered using a Pearson correlation metric.

(D) PCA using the fractional contribution from U-13C glucose to all measured metabolites.

(E) PCA using the fractional contribution from U-13C glutamine to all measured metabolites.
EXPERIMENTAL PROCEDURES

Standard procedures were followed for cell culture, sequencing, oxygen consumption studies, ATP quantification, microscopy, cytochemistry, and Western assays, eGFP and mCherry, and subcellular fractionation using a magnetic neuronal sorting system (Sartorius, Discovery 500).

Photothermolysis Nanoparticle

Spectroscopic measurements were carried out in triplicate, and data represent the mean ± SD. Student's t-test was used to determine p values.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1616/j.head.2016.5.3.047.

AUTHOR CONTRIBUTIONS


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Statistical Analysis

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