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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 (α KG), a TCA cycle metabolite, acts as a cofactor for α KG-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 α KG-dependent dioxygenases. Therefore, changes in the α KG-to-succinate ratio caused by changes in cellular metabolism impact the activity of α KG-dependent dioxygenases and therefore gene expression. Both JHDMs and TETs have known roles in

pluripotent stem cell self-renewal and differentiation. Recently, α KG 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 α KG in hPSCs has not previously been investigated, we examined the role of α KG in primed hPSC differentiation. We discovered that α KG can accelerate the differentiation of primed hPSCs likely through its action on α KG-dependent dioxygenases. Because α KG promotes self-renewal in mESCs, we investigated whether α KG promotes differentiation of primed mouse pluripotent stem cells derived from the post-implantation embryo called Epiblast stem cells (EpiSCs). α KG also promoted differentiation in mouse EpiSCs which suggests that the role of α KG is dependent on the stage of pluripotency and is not species dependent. To further confirm the role of α KG in hPSC differentiation, we decreased the α KG-to-succinate ratio by inhibition of α KG producing enzymes or succinate consuming enzymes during hPSC differentiation. Both manipulations led to a delay of hPSC differentiation. Finally, manipulation of the α KG-to-succinate ratio led to changes in DNA hydroxymethylation and histone methylation levels suggesting an epigenetic mechanism. Taken together, my data suggests that α KG plays a context specific, differentiation promoting role in primed pluripotent stem cells.

The dissertation of Tara Ann TeSlaa is approved.

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2017

DEDICATION

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

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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 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 (TGF β), 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 differentiate 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 (α KG), to act as a cofactor. This group of enzymes, called α KG 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 formaldehyde 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 α KG to support self-renewal and maintain the demethylation of DNA and histones (Carey et al., 2014).

However, many studies support the role of α KG and α KG-dependent enzymes in promoting differentiation. Knockout of the ten eleven translocation (Tet) enzymes, members of the α KG-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 α KG-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 α KG-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 α KG in primed hPSC differentiation has previously been unexplored despite these numerous connections. Addition of α KG 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, α KG-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).

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Chapter 2:

Pluripotent stem cell energy metabolism: an update

Pluripotent stem cell energy metabolism: an update

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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 extra-cellular factors. Glycolysis is the enzymatic conversion of glucose to pyruvate, which generates 2 net ATP molecules per molecule of glucose. Cells that depend mainly on glycolysis for ATP production further convert pyruvate to lactate, which is excreted. 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 & Troike, 1979; Martin & Leese, 1995; Jansen *et al.*, 2008; Leese, 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 (Leese & Barton, 1984; Houghton *et al.*, 1996; 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; Brison *et al.*, 2004). In concept, *in vivo* 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 allogeneic immune 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 POU5F1, SOX2, KLF4, and MYC (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007) or by other

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Glossary

5hmC	5-hydroxymethylcytosine	MET	mesenchymal-to-epithelial transition
5mC	5-methylcytosine	Mfn1	mitofusin-1
ADP	adenosine diphosphate	Mfn2	mitofusin-2
AMD1	adenosylmethionine decarboxylase 1	miPSCs	mouse-induced pluripotent stem cells
AMP	adenosine monophosphate	MOMP	mitochondrial outer membrane permeabilization
AMPK	AMP-activated protein kinase	mPSCs	mouse pluripotent stem cells
ARNT	aryl hydrocarbon receptor nuclear translocator	mPTP	mitochondrial permeability transition pore
ATP	adenosine triphosphate	mtDNA	mitochondrial DNA
Cited2	CREB-binding protein (CBP)/p300-interacting transactivator with glutamic acid and aspartic acid tail 2	mTOR	mammalian target of rapamycin
DEPTOR	DEP domain-containing mTOR-interacting protein	mTORC1	mammalian target of rapamycin complex 1
Drp1	dynamitin-related protein 1	mTORC2	mammalian target of rapamycin complex 2
EBs	embryoid bodies	NAD	nicotinamide adenine dinucleotide
ETC	electron transport chain	NPCs	neural progenitor cells
FAD	flavin adenosyl dinucleotide	NuRD	nucleosome remodeling and deacetylase
FAO	fatty acid oxidation	OCT4	octamer-binding protein 4
FBS	fetal bovine serum	OPA1	optic atrophy 1
HAT	histone acetyltransferase	OXPHOS	oxidative phosphorylation
hESCs	human embryonic stem cells	PC	pyruvate carboxylase
HIF1α	hypoxia-inducible factor 1 α	PDH	pyruvate dehydrogenase
HIF1β	hypoxia-inducible factor 1 β	PDK1	pyruvate dehydrogenase kinase 1
HIF2α	hypoxia-inducible factor 2 α	PDK3	pyruvate dehydrogenase kinase 3
hiPSCs	human-induced pluripotent stem cells	Phb2	prohibitin 2
hLIF	human leukemia inhibitory factor	PHD	prolyl hydroxylase
HMT	histone methyltransferase	POU5F1	POU class 5 homeobox 1
hPSCs	human pluripotent stem cells	PSCs	pluripotent stem cells
IMS	intermembrane space	pVHL	von Hippel-Lindau tumor suppressor protein
iPSCs	induced pluripotent stem cells	PYGL	glycogen phosphorylase liver
JmjC	Jumonji domain-containing	REX1	reduced expression 1
Jph2	junctophilin 2	ROS	reactive oxygen species
KLF4	Kruppel-like factor 4	SAM	S-adenosyl methionine
LKB1	liver kinase B1	SOX2	SRY (sex-determining region Y)-box 2
LSD1	lysine-specific demethylase 1	TCA cycle	tricarboxylic acid cycle
MEFs	mouse embryonic fibroblasts	TDH	threonine dehydrogenase
mEpiSCs	mouse epiblast stem cells	Tet1	ten eleven translocation
mESCs	mouse embryonic stem cells	Tsc2	tuberous sclerosis 2
		UCP2	uncoupling protein 2

methods (Yu *et al.*, 2007; Huangfu *et al.*, 2008; Lowry *et al.*, 2008; Ichida *et al.*, 2009; Kim *et al.*, 2009; Lin *et al.*, 2009; Lyssiotis *et al.*, 2009; Zhou *et al.*, 2009a; Jia *et al.*, 2010; Warren *et al.*, 2010; Zhu *et al.*, 2010; Anokye-Danso *et al.*, 2011; Hu *et al.*, 2011; Miyoshi *et al.*, 2011; Bayart & Cohen-Haguenauer, 2013; Hou *et al.*, 2013; Sommer & Mostoslavsky, 2013). Both hESCs and human iPSCs (hiPSCs) are markedly glycolytic, secreting abundant lactate, in ambient (~160 mm Hg) O₂ (Zhang *et al.*, 2011; Zhou *et al.*, 2012), which differs substantially from the ~40 mm Hg O₂ partial pressure measured for several mammalian reproductive tracts (Fischer & Bavister, 1993). A similar glycolytic preference in different O₂ environments at first pass suggests a pluripotent stage-specific metabolic program that is relatively insensitive to O₂ levels in chemically defined or undefined culture media. However, hESCs replicate well in 1–5% O₂ and resist spontaneous differentiation compared to culture in 21% O₂, suggesting that O₂ levels influence the factors that maintain pluripotency (Ezashi *et al.*, 2005). Somatic cell reprogramming to hiPSCs or mouse iPSCs (miPSCs) requires a shift from mainly OXPHOS to mainly glycolytic metabolism and high levels of lactate production (Yoshida *et al.*, 2009; Zhou *et al.*, 2012). iPSC production efficiency is enhanced by performing reprogramming in hypoxia or inducing a shift to glycolysis during this process, indicating a role for metabolism in controlling and not just passively responding to de-differentiation (Yoshida *et al.*, 2009; Zhu *et al.*,

2010; Jung *et al.*, 2013). In fact, a shift to glycolysis may occur early in reprogramming before self-renewal and pluripotent gene expression (Folmes Clifford *et al.*, 2011; Mathieu *et al.*, 2014; Prigione *et al.*, 2014). Interestingly, glycolysis-skewed pluripotent stem cells (PSCs), which include ESCs and iPSCs, resemble many cancer cell types that revert to “Warburg metabolism” (aka “aerobic glycolysis”) upon malignant transformation and coupled cellular de-differentiation (Warburg, 1956; Christofk *et al.*, 2008; Figueroa *et al.*, 2010; Lu *et al.*, 2012; Ward Patrick & Thompson Craig, 2012).

OXPHOS is low in hPSCs, which includes both hESCs and hiPSCs, and the mitochondria are perinuclear and less fused into a filamentous network structure with swollen, less mature appearing inner membrane cristae folds than mitochondria in terminally differentiated cell types (Oh *et al.*, 2005; St John *et al.*, 2005, 2006; Houghton, 2006; Suhr *et al.*, 2010; Zeuschner *et al.*, 2010; Folmes Clifford *et al.*, 2011; Zhang *et al.*, 2011). The perinuclear arrangement of mitochondria has also been noted in cleavage stage embryos of several mammalian species including mice and humans and has been suggested as a “stemness” property (Batten *et al.*, 1987; Barnett *et al.*, 1996; Wilding *et al.*, 2001; Squirrell *et al.*, 2003; Lonergan *et al.*, 2006, 2007). Mouse ESCs (mESCs), which like hESCs are obtained from the blastocyst inner cell mass, contain mitochondria that display even less mature morphological and ultrastructural features than hPSCs (Folmes Clifford *et al.*, 2011; Zhou *et al.*, 2012).

However, hPSCs metabolically resemble developmentally more mature, glycolytic mouse epiblast stem cells (mEpiSCs), obtained from the post-implantation epiblast, 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 hPSCs that are “primed”, or more mature, than naïve, or ground state hPSCs. Naïve hPSCs, similar to mESCs that represent the least mature pluripotent stage, have recently been obtained by hPSC 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) (Gafni *et al.*, 2013; Takashima *et al.*, 2014; Theunissen Thorold *et al.*, 2014; Ware *et al.*, 2014). hPSCs reset to a naïve state through transient ectopic expression of *NANOG* and *KLF4* respire at a higher level than “primed” hPSCs, similar to pre-implantation mouse embryos and naïve mESCs (Fig 1) (Takashima *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 (Kaelin William & 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 hPSCs, 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 & Suomalainen, 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 hPSC tissue culture to mimic the hypoxic early embryonic microenvironment *in vivo*. Transcription factors such as hypoxia-inducible factor 1 α (HIF1 α) and 2 α (HIF2 α) control the genomic response to low O₂ tension by promoting the expression of genes such as *pyruvate dehydrogenase kinase 1*

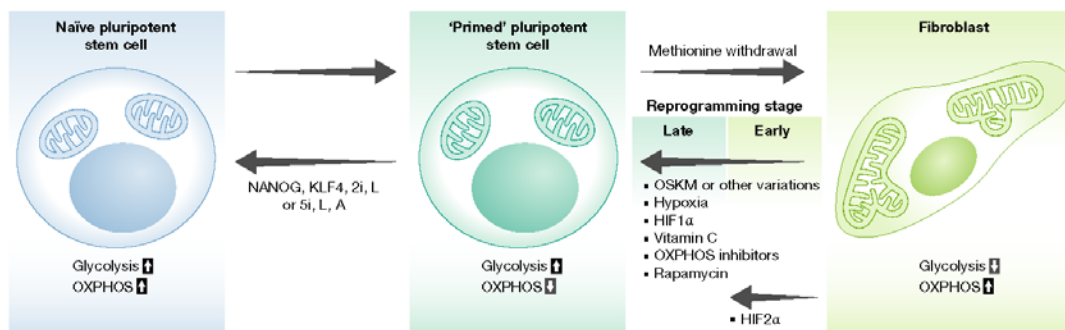


Figure 1. Influence of energy metabolism on pluripotent status.

Naïve human pluripotent stem cells (hPSCs) show an increase in ATP production through oxidative phosphorylation (OXPHOS) compared to more mature, “primed” hPSCs. Primed hPSCs can be converted to the naïve state through ectopic expression of *NANOG* and *KLF4*, inhibition of the ERK pathway by two inhibitors (2i), and stimulation with human leukemia inhibitory factor (L) (Takashima *et al.*, 2014). Alternatively, the naïve state can be induced with a cocktail of five inhibitors and growth factors Activin and hLIF (5i/L/A) (Theunissen Thorold *et al.*, 2014). Somatic cells can be reprogrammed with OCT4, SOX2, KLF4, and c-MYC (OSKM). Fibroblasts are more oxidative than primed hPSCs. 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 hPSC culture, which is required to maintain DNA and histone methylation, promotes differentiation.

(PDK1), lactate dehydrogenase A (LDHA), and glycogen phosphorylase liver (PYGL), which encode for key glycolysis regulating enzymes (Greer *et al*, 2012; Zhou *et al*, 2012). HIF1 α and HIF2 α are degraded in ambient air (~21% O₂ at sea level) by hydroxylation and ubiquitination from O₂-dependent prolyl hydroxylases (PHDs) and the von Hippel-Lindau tumor suppressor protein (pVHL), respectively (Maxwell *et al*, 1999; Ohh *et al*, 2000; Jaakkola *et al*, 2001). In hypoxia, HIF1 α and HIF2 α are stabilized and form a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT; aka HIF1 β). HIF heterodimers accumulate in the nucleus and bind to promoters of genes that regulate cellular adaptation to hypoxia, stimulating their transcription (Wang *et al*, 1995). As noted above, hypoxia inhibits the spontaneous differentiation of hESCs and also increases the efficiency of iPSC reprogramming (Ezashi *et al*, 2005; Yoshida *et al*, 2009). HIF2 α -dependent transactivation of *Oct4* gene expression promotes self-renewal and the maintenance of pluripotency in hypoxia (Niwa *et al*, 2000; Covello *et al*, 2006). HIF1 α stabilization promotes a metabolic shift to increased glycolysis and lactate production during the transition from mESCs to mEpiSCs. Ectopic expression of a non-degradable form of HIF1 α in mESCs is sufficient to induce a mEpiSC-like phenotype with a decrease in OXPHOS and an increase in glycolysis, indicating the importance of HIF transcription factors in early embryonic development (Zhou *et al*, 2012).

Hypoxia causes the re-entry of lineage-committed progenitor cells derived from hESCs back into pluripotency. hESCs transiently induced to lineage non-specific differentiation by fetal bovine serum (FBS) addition instead de-differentiate in 2% O₂, whereas hESCs differentiated with FBS in air (~21% O₂) proceed ahead. As expected, differentiated hESCs shift their metabolic balance from mainly glycolysis to OXPHOS, whereas de-differentiated hESCs remain glycolytic. De-differentiated hESCs are enriched for HIF1 α and HIF2 α target gene expression, suggesting a role for HIFs in promoting re-entry into the pluripotent state (Mathieu *et al*, 2013).

HIF2 α is required early in iPSC reprogramming for shifting from mainly OXPHOS to lactate-producing glycolysis, but stabilization of HIF2 α beyond day 12 of reprogramming is detrimental (Fig 1). Ectopic expression of sequence-stabilized HIF1 α and/or HIF2 α is sufficient to impair OXPHOS in fibroblasts (Mathieu *et al*, 2014). HIF1 α improves iPSC reprogramming efficiency by increasing glycolysis and lactate production through activation of target genes *PDK1*, *pyruvate dehydrogenase kinase 3* (*PDK3*), and *pyruvate kinase isoform M2* (*PKM2*) (Fig 1) (Mathieu *et al*, 2014; Prigione *et al*, 2014). CREB-binding protein (CBP)/p300-interacting transactivator with glutamic acid and aspartic acid tail 2 (*Cited2*) is a HIF1 α antagonist. *Cited2* is expressed and inhibits HIF1 α during lineage non-specific mESC differentiation, with *Cited2* knockout mESCs unable to silence *Oct4* or activate differentiation-related genes. shRNA knockdown of *HIF1 α* in *Cited2*-deficient mESCs partially rescues this defect in lineage non-specific differentiation (Li *et al*, 2014).

HIF transactivation also regulates the lineage-specific differentiation of human neural progenitor cells (NPCs) (Xie *et al*, 2014). Neurons are more oxidative than glial cells (Kasischke *et al*, 2004; Bélanger *et al*, 2011). NPCs generated by changing the hPSC culture media to enable the formation of rosette structures can develop into neurons or glia by directed NPC differentiation. NPCs derived from hESC differentiation are mainly glycolytic (Birket *et al*, 2011), and

proteomic comparisons of hESCs at different stages of neural lineage differentiation show differential expression of enzymes that regulate redox homeostasis (Fathi *et al*, 2014). The differentiation of mixed lineage embryoid bodies (EBs) in 2% O₂ also promotes neurogenesis. Most cells differentiated from hPSC-derived NPCs are neurons with a concomitant small number of glial cells. Remarkably, just shifting the O₂ environment during NPC differentiation from 21 to 2% O₂ strongly shifts the culture toward gliogenesis and away from neurogenesis. This effect can be replicated with HIF stabilizing deferoxamine in ambient O₂ as well. HIF1 α promotes gliogenesis through the inhibition of LIN28a by displacement of MYC on the LIN28 promoter. Remarkably, even a transient low O₂ period during NPC differentiation skews the resulting culture strongly toward gliogenesis, suggesting that the O₂-sensing machinery induces a lasting effect on NPC differentiation potential (Xie *et al*, 2014).

Mitochondria and the electron transport chain

Mammalian cells consume glucose and convert it to pyruvate with ATP production in several enzymatic steps during glycolysis (Teslaa & Teitell, 2014). Pyruvate in turn can be converted to lactate by LDH and will be excreted from the cell. Alternatively, pyruvate can enter mitochondria as acetyl-CoA, through the action of pyruvate dehydrogenase (PDH), or as oxaloacetate, via pyruvate carboxylase (PC), to generate CO₂ and additional ATP through OXPHOS. Pyruvate that enters the TCA cycle regenerates NADH and FADH₂, which subsequently donate electrons to the electron transport chain (ETC) and establish a hydrogen ion gradient, which is used by the F₀F₁ ATP synthase to make ATP from ADP plus inorganic phosphate. ETC activity therefore depends on the ADP/ATP ratio as well as the levels of environmental and internal resources that include TCA cycle carbon substrates and electron acceptors. The levels and functional assemblies of nucleus and mitochondrial DNA (mtDNA) encoded ETC subunits that comprise ETC complexes I through V, excluding nucleus-encoded complex II, and their assemblies into higher order supercomplexes, along with mitochondrial network fusion/fission status, further determines the minimal and maximal respiratory potential of most cells, which remains to be established for PSCs. The complexity of ETC complex regulation has been shown in other systems, including the differential expression of subunits in complex IV and the assembly of complexes I, III, and IV into supercomplexes (Fukuda *et al*, 2007; Chen *et al*, 2012b; Ikeda *et al*, 2013; Lapuente-Brun *et al*, 2013).

Low-level respiration and activity of the ETC in primed PSCs, including hPSCs and mEpiSCs, may at least partially result from the hypoxic microenvironment *in vivo*, immediately post-implantation. In addition, the donation of electrons from NADH and FADH₂ to the ETC may result in oxidative stress through formation of reactive oxygen species (ROS). Primed PSCs may limit ROS to prevent damage to proteins, lipids, and importantly DNA within the cell. However, steady-state ROS levels also increase with PSC differentiation and can help drive differentiation at later stages of precursor cell development (Cho *et al*, 2006; Saretzki *et al*, 2008). The addition of antioxidants to the culture medium of hiPSCs enhances their genomic stability, consistent with a benefit for maintaining low ROS in PSCs (Luo *et al*, 2014). Vitamin C, an antioxidant, also enhances the efficiency of iPSC reprogramming (Esteban *et al*, 2010),

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 hPSCs, 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 substrates for use in OXPHOS (Vozza *et al.*, 2014). Also, nuclear genes encoding multiple subunits of cytochrome C oxidase (complex IV of the ETC), which donates electrons to O₂, are expressed at a lower level in mEpiSCs compared to mESCs (Zhou *et al.*, 2012). DMSO-induced differentiation of mPSCs increases ETC complex I and complex IV activities along with mitochondrial biogenesis to support an increase in mitochondrial ATP production (Han *et al.*, 2014). The ETC maintains the mitochondrial inner membrane electrochemical potential, $\Delta\psi$, 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, $\Delta\psi$ 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 $\Delta\psi$ (Hatefi, 1985). hPSCs have relatively low respiration and ETC activity; therefore, ATP hydrolase activity of the ATP synthase helps to maintain $\Delta\psi$ and sustain cell viability (Zhang *et al.*, 2011). Interestingly, hPSCs maintain a higher $\Delta\psi$ than their differentiated derivatives (Chung *et al.*, 2007; Armstrong *et al.*, 2010; Prigione *et al.*, 2011), which 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 (Mattenberger *et al.*, 2003).

iPSC 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 iPSC 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 PSCs. Cardiomyocyte differentiation induces the expression of nucleus-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; Tohyama *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 (PPAR α), a highly expressed nuclear hormone receptor in the heart associated with FAO, promote cardiomyogenesis of mESCs through increasing ROS production (Sharifpanah *et al.*, 2008).

Differences in carbon substrate types can be used to purify metabolically mature mouse cardiomyocytes following differentiation from mPSCs because of key differences in metabolite handling capacity between mouse cardiomyocytes and mPSCs. Fetal

cardiomyocytes preferentially consume lactate for the production of ATP (Fisher *et al.*, 1981; Werner & Sicard, 1987). Therefore, cardiomyocytes derived *in vitro* from PSCs can utilize lactate in the absence of glucose to produce ATP, whereas mESCs and MEFs 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 (Tohyama *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 (Twig *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 iPSC reprogramming (Vazquez-Martin *et al.*, 2012a), although shRNA knockdown of Drp1, also resulting in mitochondrial fusion, did not impair iPSC 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) is a zinc finger-containing protein that is required to maintain PSC self-renewal and is repressed 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, fission 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 fission associated with pluripotency.

Mitochondrial network fusion requires fusion of the outer mitochondrial membrane, mediated by mitofusin-1 and -2 (MFN1 and MFN2), and fusion 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.*, 2003; Alavi *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 nucleus-encoded mitochondrial protein, is expressed at high levels in mESCs and promotes expression of the long isoforms of Opa1. Ectopic expression of Phb2 in mESCs inhibits lineage-directed differentiation toward neurons and endoderm and causes mitochondrial swelling (Kowno *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 tethers mitochondria to the sarcoplasmic reticulum, which is required for Ca²⁺ signaling and

energy metabolism in cardiomyocytes (Chen *et al.*, 2012a). Junctional protein 2 (Jph2), which is also part of the junctional membrane complexes that physically link mitochondria with the sarcoplasmic reticulum, is required for proper mitochondria function, Ca^{2+} homeostasis, and the differentiation of mESCs into cardiomyocytes (Liang *et al.*, 2012). Opening of the mitochondrial permeability transition pore (PTP) enables macromolecular diffusion across the mitochondrial inner membrane, which inhibits ATP production by OXPHOS (Hunter *et al.*, 1976; Kim *et al.*, 2003). Inhibition of the PTP promotes cardiomyocyte differentiation of mPSCs through increasing mitochondrial function (Hom Jennifer *et al.*, 2011; Cho *et al.*, 2014). Interestingly, antioxidant exposure during PTP inhibition synergistically enhances cardiomyogenesis by an unknown mechanism(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 activate catabolic pathways that generate ATP and inhibit anabolic pathways that consume ATP (Hardie *et al.*, 2012). 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-Martin *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). Concordantly, hyperactivation of mTORC1 by knockout 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 miPSC reprogramming efficiency (Chen *et al.*, 2011). Moreover, the reprogramming transcription 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 also disrupts expression of *OCT4*, *SOX2*, and *NANOG* genes in hESCs and promotes the expression of endoderm and mesoderm lineage differentiation genes (Zhou *et al.*, 2009b). In contrast, DEP domain-containing mTOR (DEPTOR)-interacting protein, a negative

regulator of mTORC1/2, maintains pluripotency for mESCs and hESCs (Agrawal *et al.*, 2014).

The AMPK/mTORC1 pathway regulates mitochondrial biogenesis in somatic cells, and therefore, its role in hPSC differentiation merits further consideration as a mechanism to regulate cell type-specific mitochondrial content and function (Zong *et al.*, 2002; Reznick & Shulman, 2006; Morita *et al.*, 2013). In addition, AMPK can directly regulate gene expression through histone phosphorylation (Bungard *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 organismal 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 it can be removed and/or replaced by Lin28a or other transfectors (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Nakagawa *et al.*, 2008; Wernig *et al.*, 2008). miPSCs 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 characterized, c-Myc promotes RNA splicing of PKM2 in cancer cells, which activates biosynthetic anabolic pathways (David *et al.*, 2010; Chaneton & Gottlieb, 2012). c-Myc also has a large role in stimulating 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 miRNA that is also post-transcriptionally repressed by LIN28a in a feedback regulatory loop. Knockdown of *let-7* in fibroblasts enhances iPSC reprogramming (Melton *et al.*, 2010). LIN28 preferentially binds to the mRNAs of metabolic enzymes to control their translation, which influences cell growth and survival. Metabolic enzymes targeted by LIN28a in hPSCs include enzymes involved in glycolysis, cholesterol biosynthesis, and mitochondrial metabolism (Peng *et al.*, 2011). Lin28a also enhances the translation of enzymes involved in OXPHOS during the repair of damaged tissues (Shyh-Chang *et al.*, 2013b).

Adenosylmethionine decarboxylase 1 (*Amd1*) 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 *Amd1* are required for self-renewal of mESCs. Additionally, translational inhibition of *Amd1* by miR-762 is required for the differentiation of NPCs (Zhang *et al.*, 2012).

Metabolic influence on epigenetics

Differentiation from and reprogramming to pluripotency, along with transdifferentiation between lineage-specific cell types, involves

changes in the structure, function, and expression of the nuclear genome. 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 remodeling complex activities, and global and local three-dimensional chromosome topologies (Papp & Plath, 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 & McKnight Steven, 2013). 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 remodeling (Cedar & Bergman, 2009). 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.*, 2012; Shyh-Chang *et al.*, 2013a). Additionally, SAM levels increase in the late stages of iPSC reprogramming, indicating coordinate accumulation with increasing pluripotent potential (Shyh-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 mPSCs. Cleavage of glycine, the product of Tdh, produces SAM by fueling methionine production, which leads to the conversion of methionine plus ATP into SAM via methionine adenosyltransferases (Fig 2) (Shyh-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 (Shyh-Chang *et al.*, 2013a). The human *TDH* gene is a nonfunctional 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 (Figs 1 and 2). Long-term methionine deprivation leads to cell apoptosis through a p53/p38 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 methyltransferase reactions.

DNA and histone methylations are also susceptible to demethylation reactions. Vitamin C, 2-oxoglutarate (α -ketoglutarate, α KG), and Fe(II) act as cofactors for 2-oxoglutarate(2-OG)-dependent dioxygenases, which include ten-eleven translocation (TET) methylcytosine dioxygenases and Jumonji 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

on the pathway to cytosine demethylation (Delatte *et al.*, 2014). 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 (Costa *et al.*, 2013).

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 2). JmjC family member proteins Jhdmla/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 influences 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 K3K9me3, which can be reversed by vitamin C exposure (Comes *et al.*, 2013). Another potential link between cellular metabolism and histone methylation is lysine-specific demethylase 1 (LSD1), which requires flavin adenosine dinucleotide (FAD) for enzymatic activity. Therefore, FAD 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 trans factors (Adamo *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). Sirtuins, 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 (Salykin *et al.*, 2013), and therefore, the sirtuins may

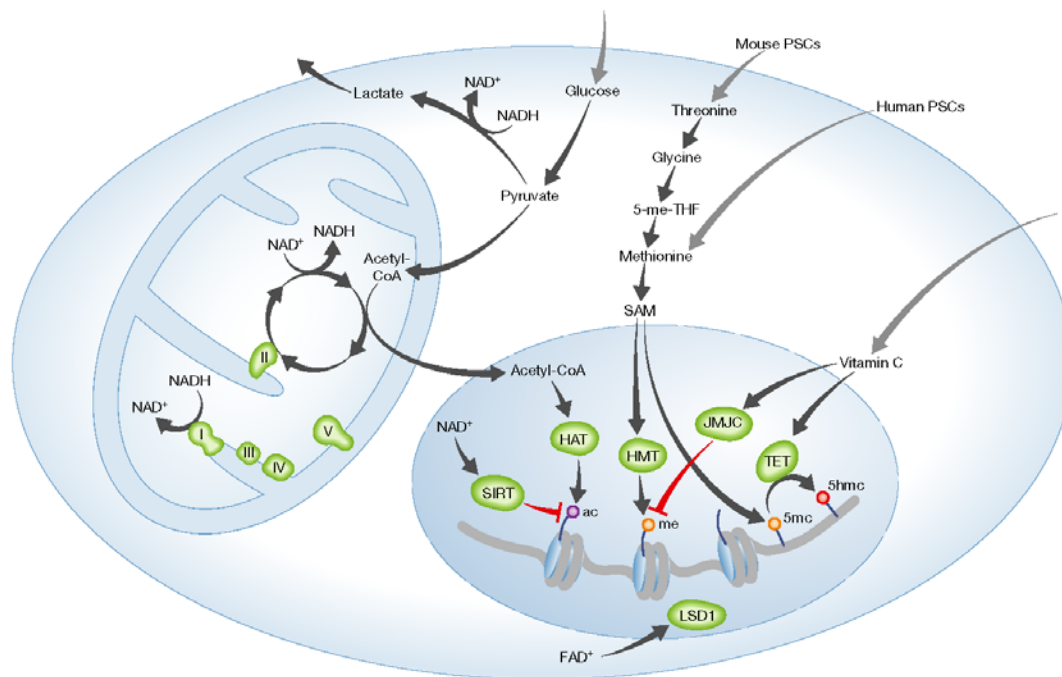


Figure 2. Influence of metabolites on pluripotent stem cell epigenetics. Intermediate metabolism sets and maintains levels of metabolites that serve as substrates or cofactors for epigenetic modifying enzymes. Uptake of threonine and methionine from the culture media is required to maintain S-adenosylmethionine (SAM) levels in mPSCs and hPSCs, respectively. SAM is a methyl donor for histone methyltransferases (HMT) and DNA methyltransferases (DNMTs). Vitamin C is a cofactor for the JMJC family of demethylases and TET methylcytosine dioxygenases (TET). Acetyl-CoA, a TCA cycle intermediate, is an acetyl group donor for histone acetyltransferases (HAT). NAD⁺, generated through glycolysis or by the electron transport chain (ETC), is a cofactor for the sirtuin (SIRT) family of deacetylases.

have differential activity in PSCs versus differentiated cells. Sirt1 is required for genomic stability and telomere elongation of iPSCs (De Bonis Maria *et al.*, 2014). SIRT6 can improve the iPSC reprogramming efficiency of fibroblasts obtained from older patients (Sharma *et al.*, 2013).

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 hPSC differentiation (Fig 2) (Wellen *et al.*, 2009; Choudhary *et al.*, 2014). In addition to vitamin C, α KG is an important cofactor for dioxygenases. α KG is a TCA cycle intermediate and can also be produced through conversion from glutamate by aminotransferases involved in other metabolic pathways in the cytosol. In some cancers, the majority of α KG is produced by phosphoserine aminotransferase 1 (PSAT1) (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 (DiMauro & Schon, 2003; Schaefer *et al.*, 2004). Mammalian cells may contain up to ~5–10 mtDNAs with sequence variations, a mixture state that is termed heteroplasmy (Legros *et al.*, 2004). 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 afflicted patients with the same mtDNA mutation can exhibit a very different range and severity of symptoms (Pickrell Alicia & 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; Chae *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; Hämäläinen *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 (Shoubridge, 2000; Smith *et al.*, 2002; Cree *et al.*, 2008; Carling *et al.*, 2011), and there is no selection for or against the MELAS mutation (Fig 3) (Hämäläinen *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 (*Polg*) 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 mitophagy. One disease associated with mitochondrial dysfunction by perhaps one or more of these mechanisms is the neurodegenerative disease, Parkinson’s disease (PD).

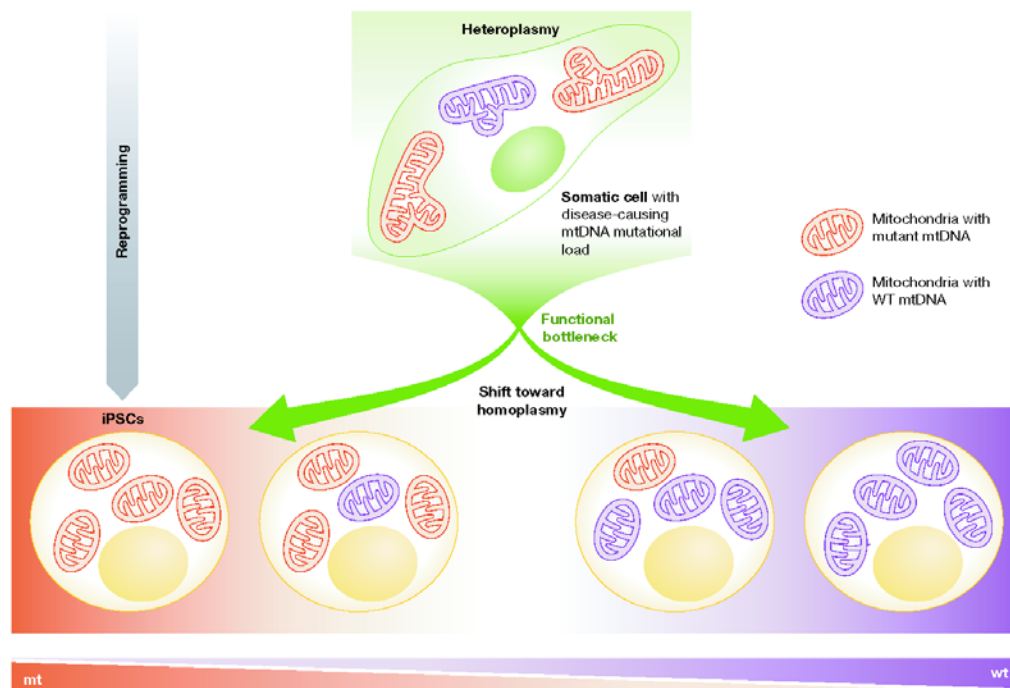


Figure 3. Somatic cell reprogramming to pluripotency causes a mtDNA “bottleneck”.

mtDNA undergoes a genetic bottleneck, or reduction in copy number, during de-differentiation, similar to the mtDNA bottleneck that occurs during normal female germ line oogenesis. 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* (LRRK2) (Seibler *et al.*, 2011; Cooper *et al.*, 2012; Imaizumi *et al.*, 2012). PINK1 and Parkin proteins interact to regulate mitophagy, the process of selectively targeting poorly functioning mitochondria with low $\Delta\psi$ 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 (Seibler *et al.*, 2011). Additionally, neurons differentiated from both mutant PINK1 and LRRK2 hiPSCs are vulnerable to oxidative stress when exposed to PD-associated toxins. Mitochondria in mutant LRRK2 iPSC-differentiated neurons respire less and are more mobile than those from healthy subjects. Sensitivity of PD iPSC-differentiated neurons to PD-associated toxins is rescued by treatment with either an LRRK2 inhibitor, coenzyme Q₁₀, 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 (Imaizumi *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 correlated 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 transfactor stabilization. Further work is necessary to identify the transcription factors and signaling pathways that regulate glycolytic flux and overall capacity in PSCs and during induced differentiation. Additionally, the mitochondria in PSCs 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 differential mitochondrial mass accumulation and maintenance, and what factors facilitate transitions in metabolism and cell fates 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 SAM levels and global methylation patterns is more heavily 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 unearthed, 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 conflict of interest.

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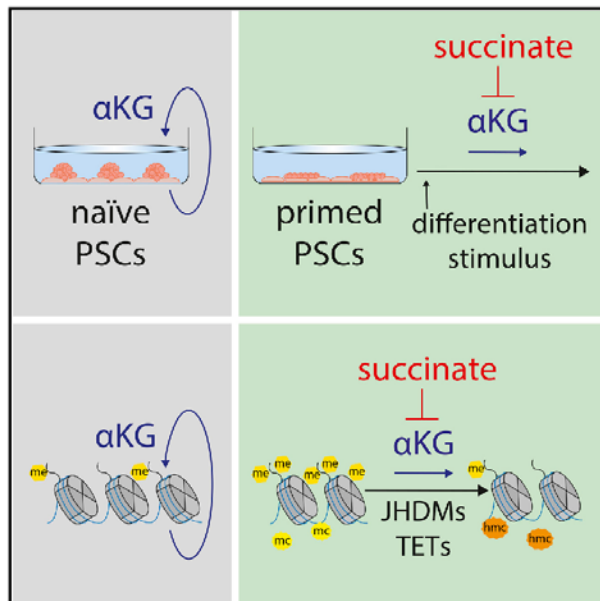
Chapter 3:

α -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



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In Brief

α -ketoglutarate (α KG) is an important cofactor for demethylation reactions that helps to maintain naive pluripotent stem cells. TeSlaa et al. show that at later stages of pluripotency, α KG can promote early differentiation, highlighting that the cellular context and potentially the stage of cellular maturity can alter the effect of α KG.

Highlights

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



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α -Ketoglutarate 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 (α KG) was shown to favor self-renewal in naive mouse embryonic stem cells (mESCs). The effect of α KG or α KG/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 α KG or α KG-to-succinate ratios accelerate, and elevated succinate levels delay, primed PSC differentiation. α KG has been shown to inhibit the mitochondrial ATP synthase and to regulate epigenome-modifying dioxygenase enzymes. Mitochondrial uncoupling did not impede α KG-accelerated primed PSC differentiation. Instead, α KG induced, and succinate impaired, global histone and DNA demethylation in primed PSCs. The data support α KG 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 (Takahashi et al., 2007; 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 (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 (α KG), a tricarboxylic acid (TCA) cycle metabolite, is a cofactor for α KG-dependent dioxygenase enzymes, which include JmjC-domain containing histone demethylases (JHDMs) and ten-eleven translocation (TET) enzymes (Kaelin and McKnight, 2013). α KG can also bind and block the mitochondrial ATP synthase and inhibit mechanistic target of rapamycin (mTOR) signaling (Chin et al., 2014). Addition of cell-permeable dimethyl- α KG (dm- α KG) 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., 2015). 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) (Greber et al., 2010; James et al., 2005; Tesar et al., 2007). A role for α KG 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 (Greber 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 (Pera, 2014)



emphasizes the remaining importance of primed hPSCs as options for potential clinical applications.

Metabolites other than α KG 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 (Moussaieff et al., 2015). 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 (Shyh-Chang et al., 2013; Wang et al., 2009). Here, we investigate the role for α KG 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 (E8) 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 with E6 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 - ^{13}C] glucose into TCA cycle metabolites (Figure 1C). Glutamine withdrawal increased the glucose contribution to TCA cycle metabolites α KG, succinate, and malate in E8, but not in E6, culture conditions (Figure 1C). The mass isotopologue distribution (MID) of citrate indicates the contribution of [U - ^{13}C] glucose to the TCA cycle, with m+2 and m+3 isotopologues indicating initial entry of glucose into the TCA cycle and m+4, m+5, and m+6 isotopologues indicating ^{13}C 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 1E), but glutamine withdrawal for 18 hr resulted in an increase in m+4 and m+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 α KG (m+0) in E8 conditions (Figure S1C).

The MID of citrate with [U - ^{13}C] glutamine indicates the amount of citrate derived from glutamine after one turn (m+4) or two turns (m+2) of the TCA cycle (Figure 1G). Increased m+4 citrate was detected in E8 compared to E6 conditions, suggesting a lower contribution of glutamine to the TCA cycle in E6 differentiated hPSCs (Figure 1H). However, 40% of glutamate, which is gener-

ated 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 - ^{13}C] glutamate uptake confirmed these results (Figure 1J), with increased conversion of glutamate into α KG occurring in differentiated cells (E6) 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, α KG-producing transaminases (TAs), which transfer amine groups from glutamate to α -keto acids to form amino acids (Figure S1E), have high activity (Coloff et al., 2016). Glutamine inclusion in hPSC culture medium, which provides glutamate for TAs, increases α KG, alanine, and aspartate levels, products of these TAs (Figures S1F and S1G). To further study TA activity, [^{15}N] glutamine was used to quantify the transfer of ^{15}N from glutamate to amino acids (Figure S1E), and ^{15}N was detected in alanine, aspartate, isoleucine, serine, and methionine (Figure 1L). Expression of glutamic-pyruvic transaminases (GPTs) and glutamic-oxaloacetic transaminases (GOTs) was confirmed in hPSCs, verifying their contribution to α KG production (Figure S1H). Robust α KG production in primed hPSCs prompted studies into a role for α KG in PSC differentiation.

α KG Accelerates Multi-lineage Primed PSC Differentiation

Neuroectoderm (NE) differentiation was induced in primed H1, H9, UCLA1, and HIPS2 hPSCs by dual SMAD inhibition (Chambers et al., 2009). The dm- α KG significantly increased the percentage of PAX6, an essential transcription factor for NE specification in humans (Zhang et al., 2010), expressing cells by day 4 of differentiation (Figures 2A–2C). MAP2C- and NESTIN-positive cells modestly increased with dm- α KG treatment (Figures 2C and S2A). To determine whether this effect was lineage specific, endoderm differentiation was induced by high-concentration activin A exposure (D'Amour et al., 2005). On day 2, dm- α KG significantly increased the percentage of H9 cells expressing SOX17, a definitive endoderm transcription factor (Figures S2B and S2C). Combined, the data support that α KG accelerates the early differentiation of multiple hPSC germ lineages.

NE differentiation was examined with added α KG, which unlike dm- α KG, is dependent on membrane transporters for uptake, and resulted in an increase in PAX6-positive cells (Figures 2D and 2F). In contrast to dm- α KG, which shows a dose-dependent increase in cell differentiation, α KG levels beyond 4 mM did not further stimulate NE differentiation (Figures 2D–2G). Added α KG and dm- α KG each increased intracellular α KG and TCA cycle metabolite levels, although only 12 mM dm- α KG reached significance (Figures 2H and 2I).

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

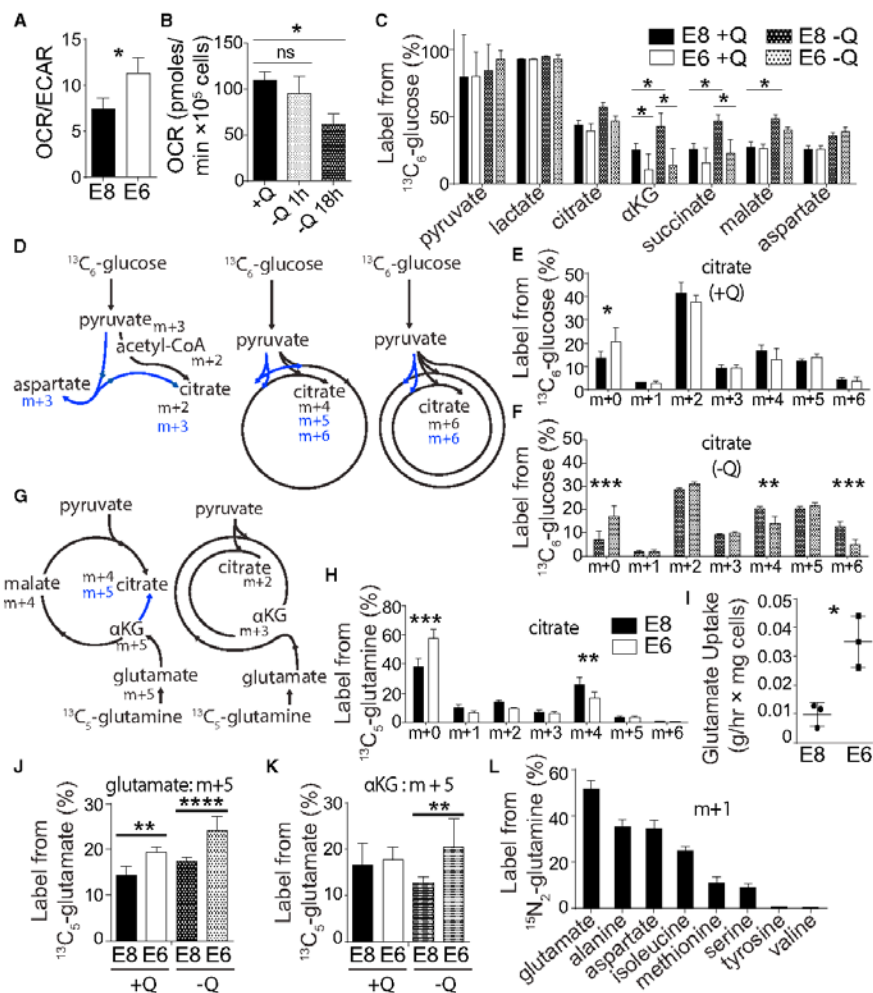


Figure 1. Production of TCA Cycle Metabolites in hPSCs

(A) Ratio of OCR to ECAR in H9 hESCs cultured in medium containing (E8) or lacking (E6) bFGF and TGF- β .
 (B) OCR quantified in H9 hESCs grown in media containing glutamine or with glutamine removed for 1 or 18 hr.
 (C) Fractional contribution of ^{13}C -labeled metabolites from $[\text{U}-^{13}\text{C}]$ glucose after 18 hr, quantified by UHPLC-MS.
 (D) Schematic illustrating how the MID of citrate from $[\text{U}-^{13}\text{C}]$ glucose reveals the contribution of glucose-labeled metabolites through multiple turns of the TCA cycle.
 (E and F) MID of citrate in H9 hESCs from $[\text{U}-^{13}\text{C}]$ glucose in conditions containing glutamine (E) or lacking glutamine (F).
 (G) Schematic of $[\text{U}-^{13}\text{C}]$ glutamine labeling of the TCA cycle. The m+5 isotopologue of citrate can be produced by reductive carboxylation of glutamine (blue). The m+4 and m+2 isotopologues contain carbons derived from glutamine after one and two turns of the TCA cycle, respectively.
 (H) MID of citrate from $[\text{U}-^{13}\text{C}]$ glutamine in H9 hESCs cultured in E8 or E6 medium.
 (I) Measurement of glutamate uptake from culture medium in H9 hESCs maintained in E8 medium or differentiated in E6 medium.
 (J) The m+5 isotopologue of glutamate in H9 hESCs, indicating increased uptake of $[\text{U}-^{13}\text{C}]$ glutamate from the culture medium in differentiated cells (E6) grown with or without glutamine.
 (K) The m+5 isotopologue of αKG in H9 hESCs grown in $[\text{U}-^{13}\text{C}]$ glutamate.
 (L) The m+1 isotopologue of listed amino acids in H9 hESCs grown with $[\text{U}-^{15}\text{N}]$ glutamine, reflecting the activities of multiple αKG -producing TAs.
 Data represent mean $\pm$ 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 an unpaired two-tailed Student's t test (A and I) or by one-way ANOVA (B) or two-way ANOVA (C, E, F, H, J, and K) with correction for multiple comparisons.

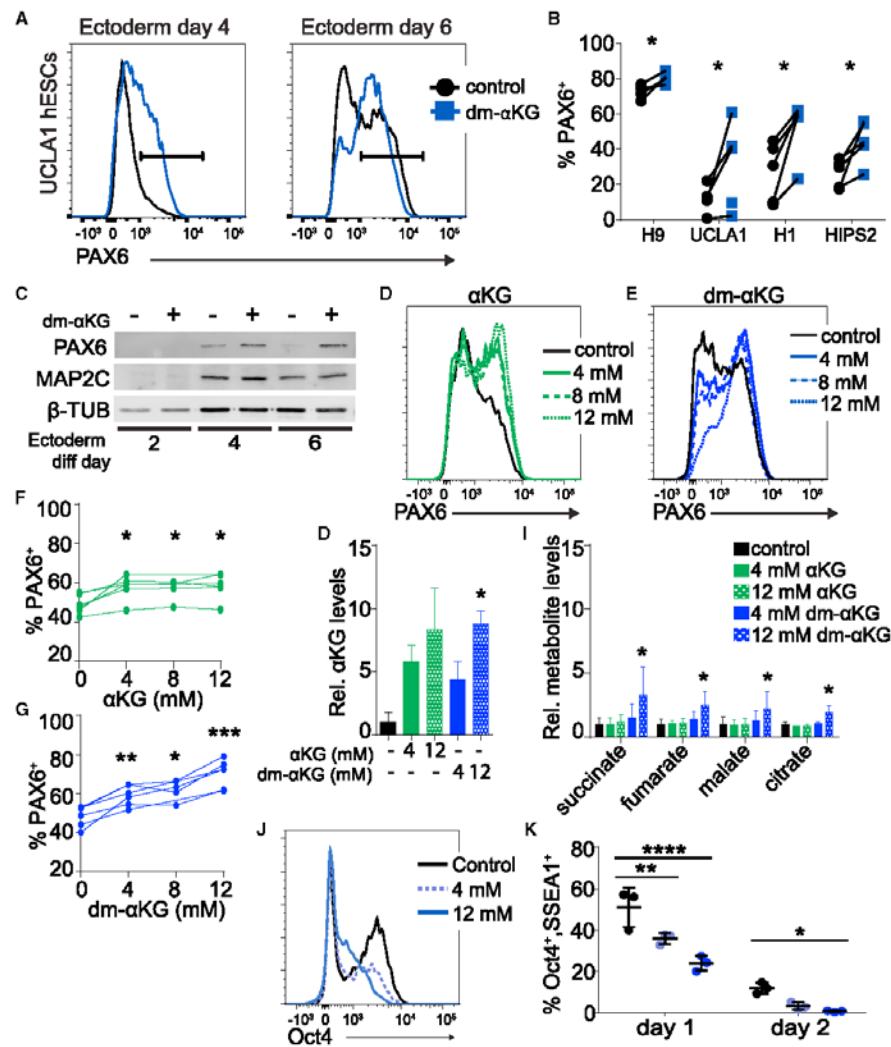


Figure 2. αKG Accelerates Differentiation of Primed PSCs

(A) Flow cytometry analysis of PAX6 transcription factor expression in UCLA1 hESCs encouraged to differentiate into NE at 4 or 6 days. The figure shows 12 mM dm-αKG incubated traces (blue) and control traces (black).
(B) Flow cytometry quantification of the percentage of PAX6-expressing cells at 4 days of NE differentiation for H9, UCLA1, and H1 hESCs and HIPS2 human-induced pluripotent stem cells (hiPSCs). H9, UCLA1, and H1 cells were incubated with (blue) or without (black) 12 mM dm-αKG, and HIPS2 hPSCs were incubated with (blue) or without (black) 6 mM dm-αKG. 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 H9 hESCs.
(D and E) Flow cytometry of PAX6 expression in H9 hESCs on day 4 of NE differentiation, with indicated amounts of αKG (D) or dm-αKG (E) added to the culture medium.
(F and G) Percentage of H9 hESC cells positive for PAX6 expression on day 4 of differentiation plotted against the concentration of added αKG (F) or dm-αKG (G). Lines connect independent biological replicates.
(H and I) UHPLC-MS quantification of fold change of αKG levels (H) and other TCA cycle metabolite levels (I) in H9 hESCs incubated with the listed concentrations of αKG or dm-αKG. Error bars represent SEM of three biological replicates.

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(Greber et al., 2010) accelerated the rate of Oct4 inactivation (Figures 2J, 2K, and S2E–S2G). Therefore, α KG can accelerate differentiation of both mouse and human primed PSCs.

α KG has been shown to bind to and inhibit the mitochondrial ATP synthase subunit β , leading to mTOR inhibition (Chin et al., 2014). To determine whether ATP synthase inhibition contributes to α KG-accelerated primed PSC differentiation, an OXPHOS uncoupling agent, carbonyl cyanide 4-(trifluoromethoxy)-phenyl-hydrazone (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 dm- α KG accelerated NE differentiation in H9 hESCs 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) (Birsoy et al., 2015; Sullivan et al., 2015). Because ATP synthase inhibition delays or inhibits NE differentiation, which opposes the accelerating effect of dm- α KG, we conclude that α KG does not accelerate differentiation of primed PSCs through inhibition of ATP synthase.

Succinate Accumulation Delays hPSC Differentiation

A second potential mechanism for α KG-accelerated hPSC differentiation is stimulation of epigenome-modifying dioxygenases. In this event, an α KG-dependent dioxygenase competitive inhibitor, such as succinate (Xiao et al., 2012), would impair differentiation. Cell-permeable succinate, dimethyl succinate (dms), resulted in a decreased percentage of PAX6-expressing hPSCs during NE differentiation compared to hPSCs incubated with dm- α KG alone (Figures S2L and S2M). Inhibition of succinate dehydrogenase A (SDHA), which converts succinate to fumarate either with a chemical inhibitor, 3-nitropropionic acid (NPA), or with small hairpin RNA (shRNA), should also cause succinate accumulation (Figure 3A). NPA treatment decreased the α KG-to-succinate ratio 34-fold, with a 14-fold mean increase in succinate and 2.4-fold mean decrease in α KG (Figures 3B and S3A). NPA delayed NE-specifying 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 dm- α KG (Figures 3D, 3E, and S3I).

We further assessed the role of succinate in differentiation by embryoid body (EB) formation, which contrasts with lineage-directed differentiation by removal of bFGF 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 impaired OCT4 repression in EB differentiation compared to control (Figure S3L).

TA Inhibition Delays hPSC Differentiation

To decrease α KG levels, chemical inhibitors of α KG-producing TAs were used (Figure 3A). Aminooxyacetic acid (AOA), a pan-TA inhibitor, reduced ^{15}N transfer from [$^{15}N_2$] glutamine to alanine, aspartate, isoleucine, serine, and methionine in hPSCs (Figure S3M). L-cycloserine (cyclo), a GPT inhibitor, decreased ^{15}N transfer to alanine in hPSCs treated with cyclo (Figure S3M). Both inhibitors caused a significant decrease in α KG 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 (NEAAs), including alanine and aspartate, had no effect on AOA treatment, whereas supplementation with dm- α KG 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 α KG levels compared to AOA (Figures 3I and 3J). Supplementation with dm- α KG restored MAP2B levels in cyclo-treated cells (Figures 3I and 3J). Therefore, decreased α KG 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 S4A). Low levels of DCA increased PAX6-positive cells on day 4 of NE differentiation, whereas higher DCA levels had no effect (Figures S4B and S4C). Furthermore, dm- α KG 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 α KG in accelerated hPSC differentiation. Rather, the data indicate that succinate delays and α KG promotes the initial differentiation of primed PSCs, most likely through actions on α KG-dependent dioxygenases.

α KG/Succinate Regulates the Epigenome of Differentiating hPSCs

To evaluate the role of α KG-dependent dioxygenases during hPSC differentiation, an inhibitory α KG mimetic, dimethylxalylglycine (dmog) was used. Exposure to dmog inhibited PAX6 expression at concentrations that did not affect cell number (Figures 4A, 4B, and S4D). To assess the effect of the α KG on TET enzymes, dot blots were performed and levels of 5-hydroxymethylcytosine (5hmc) and 5-methylcytosine (5mc) in DNA were measured from H9 and UCLA1 hESCs after 4 days of NE differentiation. Although dm- α KG exposure caused a 2-fold increase in the 5hmc-to-5mc ratio, NPA caused a significant decrease in this ratio (Figures 4C, 4D, and S4E). The data suggest a significant role for TET enzymes in α KG-accelerated differentiation of primed hPSCs. To evaluate α KG regulation of JHDMs, histone

(J and K) Flow cytometry analysis (J) and quantification (K) of Oct4 expression in EpiSC-1 cells differentiated by growth factor withdrawal for 24 hr under control conditions, with 4 mM dm- α KG or with 12 mM dm- α KG.

Data represent mean $\pm$ SD of three biological replicates unless otherwise noted. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. The p values were determined by a ratio-paired two-tailed Student's t test (B, F, G, and H) or by two-way ANOVA with repeated measures and with correction for multiple comparisons (I and K).

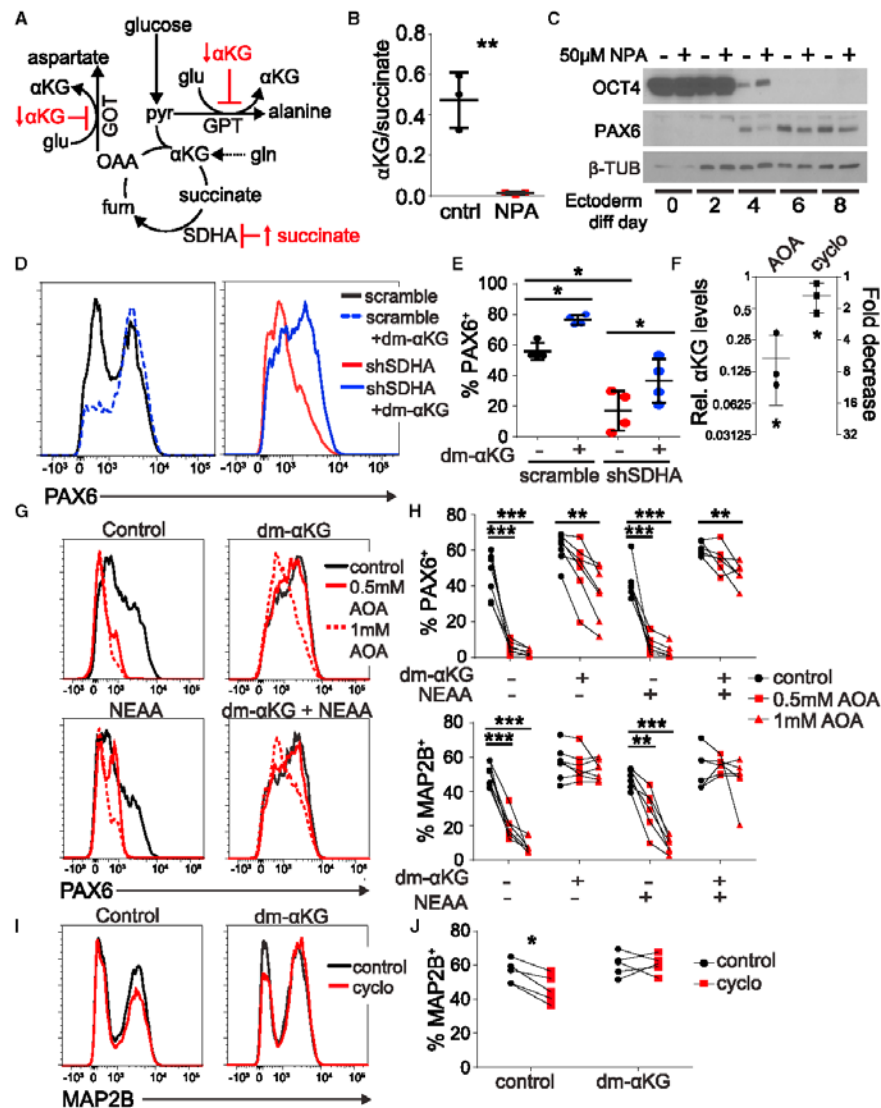


Figure 3. Decrease in the αKG-to-Succinate Ratio Delays hPSC Differentiation

(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 10 μM NPA incubated (red) H9 hESCs for 18 hr, quantified by UHPLC-MS.
 (C) Immunoblot of OCT4 and PAX6 at indicated time points of NE differentiation with or without 50 μM NPA for H9 hESCs. β-TUB, β-tubulin.
 (D and E) Flow cytometry analysis of PAX6 expression in H9 hESCs expressing shRNA targeting SDHA or scramble control shRNA treated with dm-αKG where indicated at day 4 of NE differentiation.
 (F) UHPLC-MS quantification of fold change in αKG levels with TA inhibitors AOA and cyclo compared to drug carrier controls.

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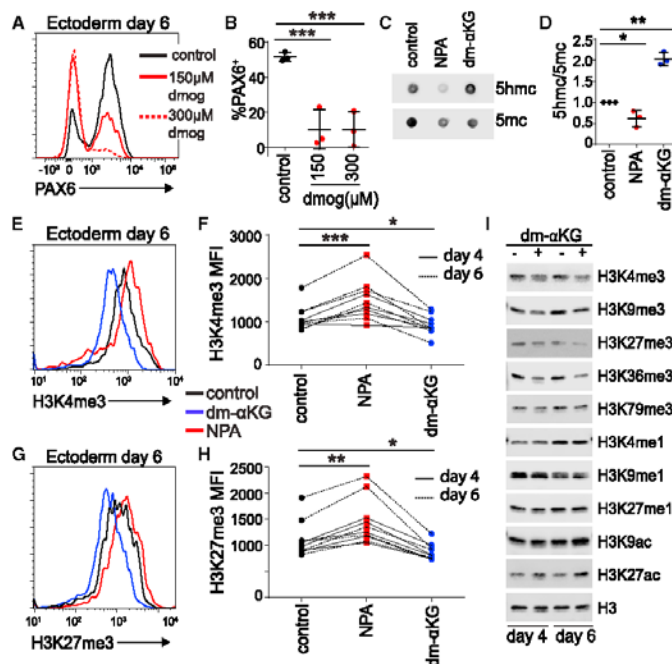


Figure 4. Changes in the α -KG-to-Succinate Ratio Alter DNA Hydroxymethylation and Histone Methylation

(A and B) Flow cytometry analysis of PAX6 expression in H9 hESCs after 6 days of NE differentiation with the indicated concentrations of the α -KG-dependent dioxygenase inhibitor, dmog. (C and D) Dot blot quantification of 5hmC and 5mC in DNA from H9 hESCs after 4 days of NE differentiation under control conditions or with 50 μ M NPA or 12 mM dm- α -KG added.

(E and F) Flow cytometry analysis of the H3K4me3 mark shown as mean fluorescent intensity (MFI) for UCLA1 hESCs at 4 days (solid line) or 6 days (dotted line) of NE differentiation incubated without (black) or with 12 mM dm- α -KG (blue) or 50 μ M NPA (red). Lines connect independent biological replicates.

(G and H) Flow cytometry analysis of the H3K27me3 mark shown as MFI for UCLA1 hESCs at 4 days (solid line) or 6 days (dotted line) of NE differentiation incubated without (black) or with 12 mM dm- α -KG (blue) or 50 μ M NPA (red). Lines connect independent biological replicates.

(I) Immunoblot of histone methylation and acetylation marks in UCLA1 hESCs differentiated into NE at 4 or 6 days with or without dm- α -KG incubation.

Data represent mean $\pm$ SD of at least three biological replicates. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. The p values were determined by one-way ANOVA (B and C) or two-way ANOVA (F and H), with repeated measures and correction for multiple comparisons.

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 dm- α -KG led to a decrease (Figures 4E–4H and S4F–S4J). Analysis of an array of histone post-translational modifications by immunoblot revealed an overall repressive effect of dm- α -KG on global lysine trimethylation but little effect on global monomethylation or acetylation marks (Figure 4I).

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

DISCUSSION

This study reveals an unanticipated differentiation-promoting role for α -KG in primed PSCs. Results have shown that α -KG sup-

ports self-renewal of naive mESCs, potentially by promoting the demethylation of histones and DNA (Carey 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 α -KG, TET enzymes and JHDMs have dual roles in the self-renewal and differentiation of mESCs. TET1 promotes reprogramming to naive pluripotency, whereas triple knockout of the TET enzymes impedes differentiation (Costa et al., 2013; Dawlaty et al., 2014). JHDMs are a large class of enzymes that have functions in both naive pluripotency and differentiation. For example, JMJD3 and UTX promote naive pluripotency (Carey et al., 2015), whereas Jarid1b is involved in neural differentiation (Schmitz et al., 2011). The data suggest that α -KG enhances the activity of a large family of enzymes that may both maintain low DNA and histone methylation levels, favoring the naive 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 methylation levels are lower in naive compared to primed PSCs (Hackett and Surani, 2014; Leitch et al., 2013). These epigenome differences, along with metabolome differences

(G–J) Flow cytometry analysis of PAX6 and MAP2B expression in H9 hESCs on day 4 of NE differentiation co-incubated with AOA (G and H) or 50 μ M cyclo (I and J) supplemented with 12 mM dm- α -KG, 1 mM NEAAs, or both where indicated. Data represent mean $\pm$ SD of at least three biological replicates. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. The p values were determined by unpaired Student's t test (B and F) or by one-way ANOVA (E) or two-way ANOVA (H and J) with correction for multiple comparisons.

between naive and primed pluripotent states, could support a differential role for α KG identified in this study. Our results suggest a model for α KG 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 mature and functional cells for regenerative medicine. Increased α KG could be useful for improving primed-state PSC differentiation.

EXPERIMENTAL PROCEDURES

Standard procedures were followed for immunoblotting, confocal microscopy, and shRNA knockdown, as described in the [Supplemental Experimental Procedures](#). Maintenance of university compliance, including ESCRO, IRB, and Biological Safety, was overseen by K.H. and M.A.T.

Cell Culture

Primed hPSCs were passaged onto feeder-free matrigel (Fisher Scientific) in mTeSR1 medium with Gentle Cell Dissociation Reagent (STEMCELL) for most experiments. For experiments performed in E8 or E6 media (STEMCELL), hPSCs were switched from mTeSR1 to appropriate media at passage. EpiSCs were grown in feeder-free conditions in medium containing bFGF and activin A on fibronectin. Further details are provided in the [Supplemental Experimental Procedures](#).

Seahorse Measurements

OCR and ECAR assays were performed as previously described ([Zhang et al., 2012](#)). The hPSCs were plated onto an XF24 microplate (Seahorse Bioscience) at 10^4 or 10^5 cells/well with 10 μ M Y-27632 (BioPioneer). The next day, 1 hr before the assay, the medium was changed to XF Media (Seahorse Bioscience) supplemented with 17.5 mM glucose. Cell metabolic rates were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Basal respiration was determined by quantifying OCR before and after the addition of 1 μ M rotenone and 1 μ M antimycin A (Sigma).

Metabolite Extraction and Analysis

Cellular metabolites were extracted with 80% ice-cold methanol, and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) measurements of metabolite levels were performed and analyzed as previously described ([Thai et al., 2014](#)). Details are provided in the [Supplemental Experimental Procedures](#).

Glutamate Uptake

Levels of glutamate in culture media were measured using a BioProfile Basic Analyzer (Nova Biomedical). Details are provided in the [Supplemental Experimental Procedures](#).

NE Differentiation

NE differentiation was performed as previously reported ([Chambers et al., 2009](#); [Shiraki et al., 2014](#)). Details are provided in the [Supplemental Experimental Procedures](#).

Flow Cytometry Analysis

Cells were collected with Gentle Cell Dissociation Reagent and processed using the Cytofix/Cytoperm Kit (BD Biosciences). Cells were analyzed with either LSRII or LSRFortessa (BD Biosciences).

Dot Blot Analysis and Quantification

DNA was collected with the DNeasy Blood and Tissue Kit (QIAGEN) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA was denatured at 99°C for 5 min, put on ice, and neutralized by adding ammonium acetate to a final concentration of 0.66 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 proce-

dures were followed. Blots were imaged with an Odyssey Fc and quantified with Image Studio v.5.2.5 (LI-COR Biosciences).

Statistical Analysis

Values are presented as mean $\pm$ SD or mean $\pm$ SEM. Data were analyzed with Prism (GraphPad). Pairwise comparisons 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.cmet.2016.07.002>.

AUTHOR CONTRIBUTIONS

T.T., A.C.C., S.L.E., and D.B. performed experiments. I.L. and K.H. provided key reagents and expert guidance. T.T., J.H., T.G.G., D.B., and M.A.T. participated at differing levels in designing the study and analyzing data. T.T. and M.A.T. wrote the paper with help from T.G.G. and D.B. Maintenance of university compliance, including ESCRO, IRB, and Biological Safety, was overseen by K.H. and M.A.T.

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Cell Metabolism, Volume 24

Supplemental Information

α -Ketoglutarate Accelerates the Initial

Differentiation of Primed Human

Pluripotent Stem Cells

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SUPPLEMENTAL FIGURES

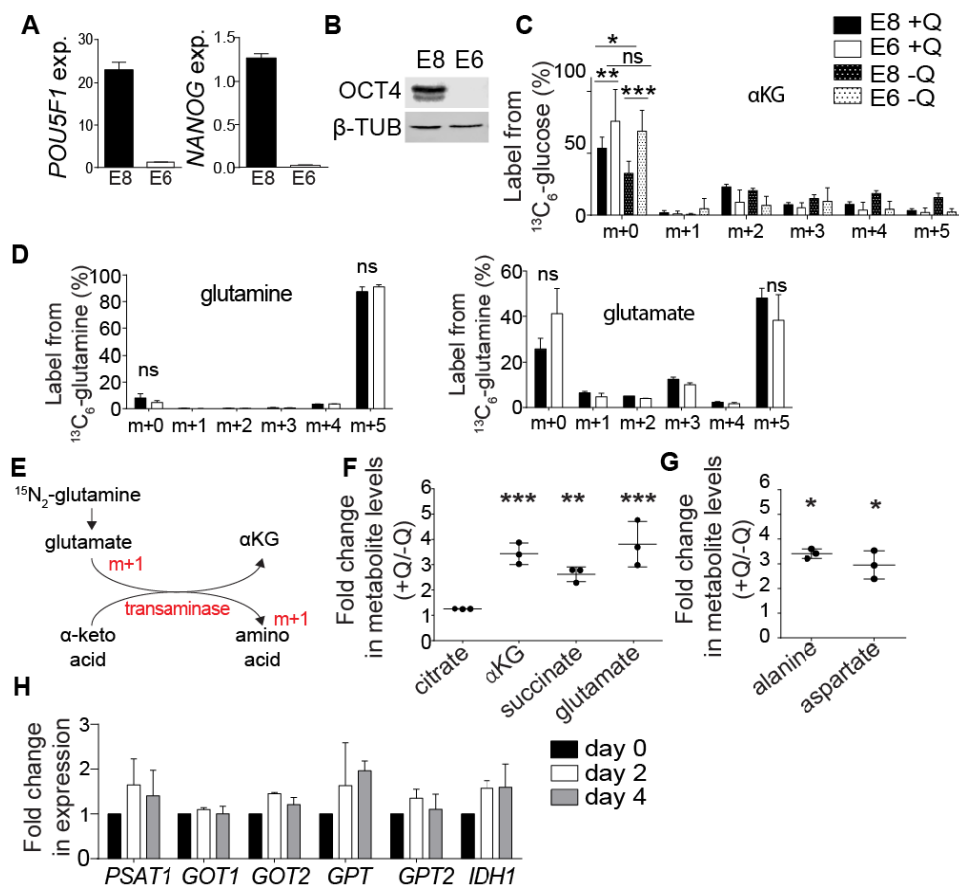


Figure S1. Production of TCA cycle metabolites in hPSCs (related to Figure 1).

(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 TGF β , 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 α KG from [U- 13 C] 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 TGF β , 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- 13 C] glutamine incorporation. Black and white bars represent H9 hESCs cultured in E8 medium or differentiated in E6 medium, which lacks bFGF and TGF β , respectively, for 16h.

(E) Schematic depicting α KG 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 $\pm$ SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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).

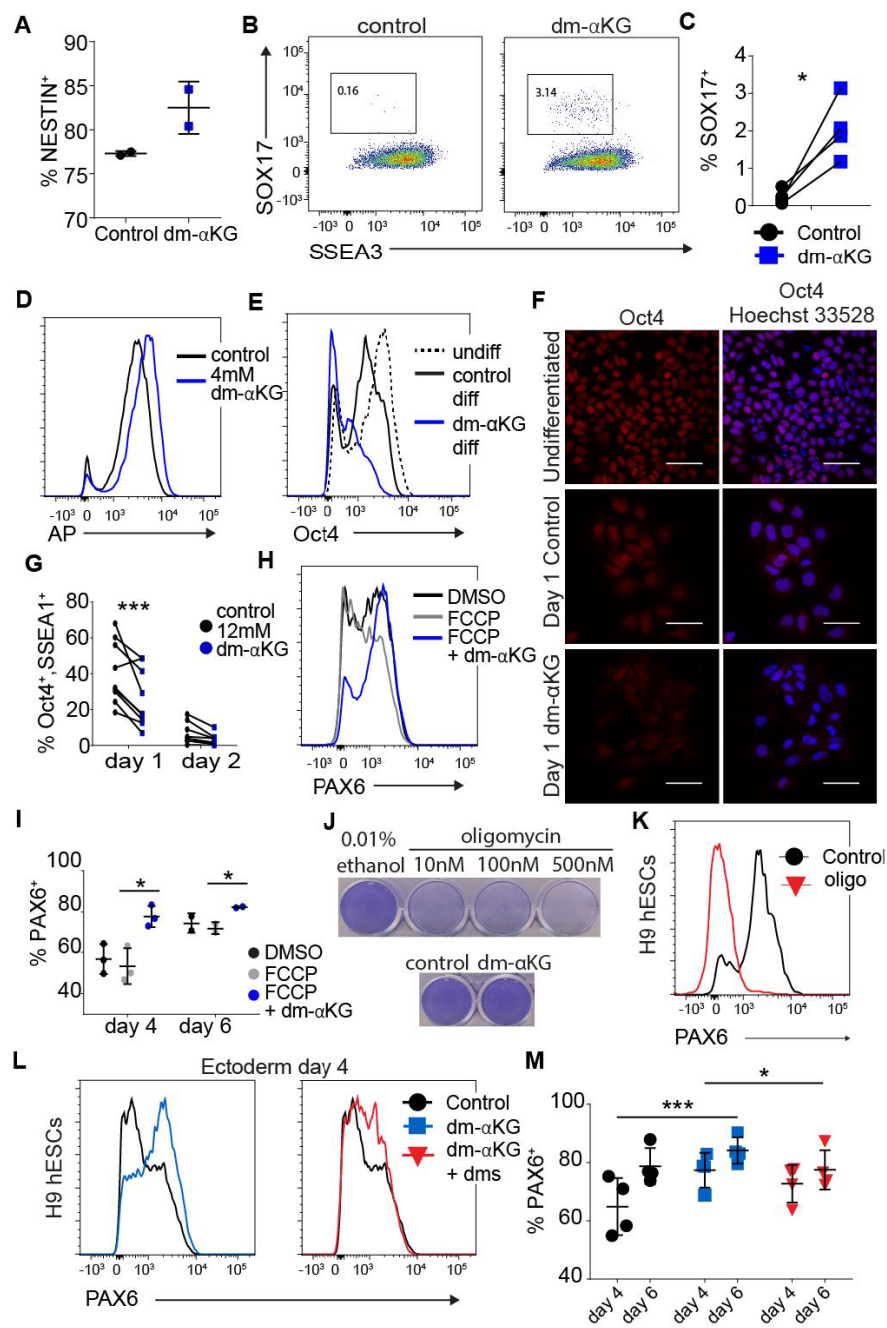


Figure S2. α KG accelerates differentiation of primed pluripotent stem cells (related to Figure 2).

(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- α KG.

(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- α KG, 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 50 μ m.

(H, I) Flow cytometry analysis of PAX6 expression in H9 hESCs differentiated into NE. Dm- α KG 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- α KG, or with both 12mM dm- α 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 $\pm$ SD. * $p \leq 0.05$, *** $p \leq 0.001$.

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).

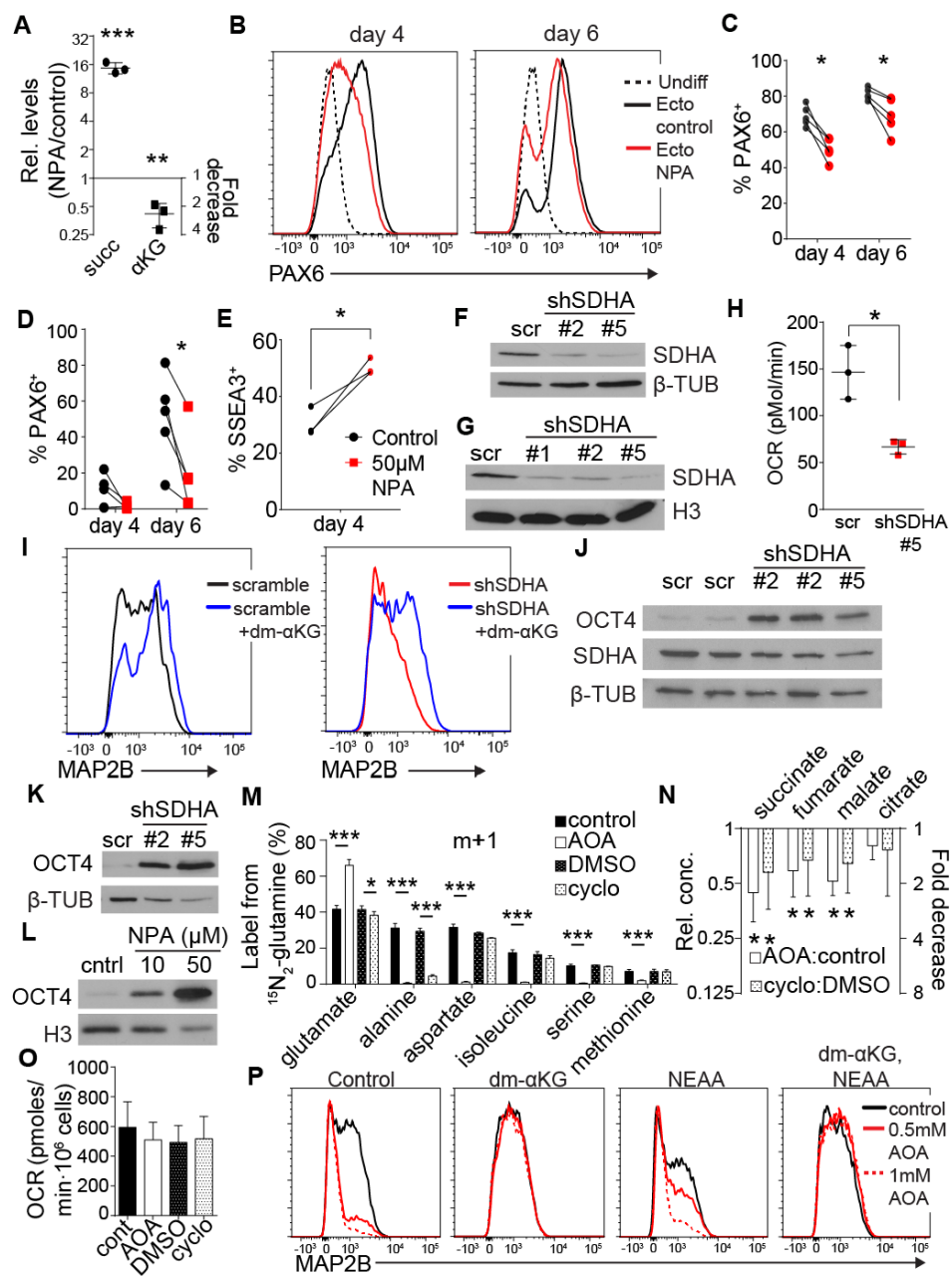


Figure S3. Decrease in the α KG/succinate ratio delays hPSC differentiation (related to Figure 3).

(A) UHPLC-MS quantification of succinate (succ) and α KG in H9 hESCs treated with 10 μ M 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 50 μ M 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) 50 μ M 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- α KG 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 [$^{15}\text{N}_2$] glutamine indicating the activities of various α KG-producing transaminases. hESCs were treated with 0.5mM aminooxyacetic acid (AOA), 0.05% DMSO, or 50 μ M 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 50 μ M 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 50 μ M cyclo, as indicated.

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

Error bars represent mean $\pm$ SD unless otherwise indicated. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

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).

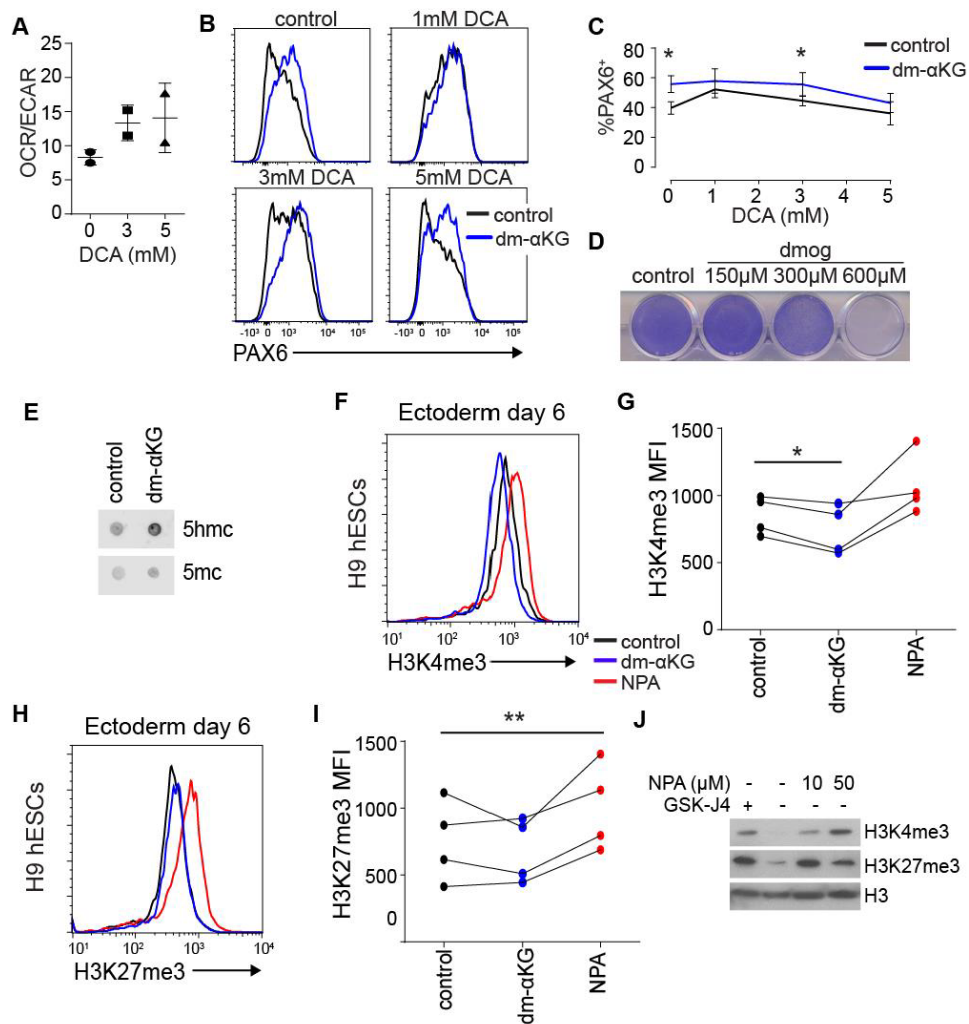


Figure 4. Changes in the α -KG/succinate ratio alter DNA hydroxymethylation and histone methylation (related to Figure 4).

(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 $\pm$ 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- α KG, 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 dimethyloxallylglycine (dmog).

(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- α KG.

(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- α KG (blue) or 50 μ M 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- α KG (blue) or 50 μ M 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 $\leq$ 0.05; **p $\leq$ 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/ul 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), 50 μ M 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 8 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-¹³C] glutamine (Cambridge Isotope Laboratories), or [¹⁵N₂] glutamine was added to the medium. For experiments with aminooxyacetic acid (AOA) and L-cycloserine (cyclo), cells were incubated with medium containing or lacking [¹⁵N₂]-glutamine for 6h. For glutamate tracing, 0.5 mM [U-¹³C]-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 10⁴ rpm, 4°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 µl 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 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-nitropropionic acid (NPA), dimethyl succinate (dms), dimethyl 2-oxoglutarate (dm- α KG), oligomycin, L-cycloserine (cyclo), dimethyloxaloylglycine (dmog), and dichloroacetate (DCA) were purchased from Sigma. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was from Abcam. Aminooxyacetic acid hydrochloride (AOA) was purchased from MP Biomedicals. Stock dm- α 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.

Primary Antibodies (Unconjugated)			
Antigen	Application	Source (catalog number)	Dilution
Pax6	Western blot	Santa Cruz (sc-7750)	1:50
Oct3/4	Western blot, IF	Santa Cruz (sc-9081)	WB: 1:1000; IF: 1:500
SDHA	Western blot	Cell Signaling (5839)	1:500
Alkaline Phosphatase	Flow Cytometry	Abcam (ab108337)	1:1000
H3	Western blot	Cell Signaling (9715)	1:1000
β -TUBULIN	Western blot	Sigma (T4026)	1:1000
H3K4me3	Western blot, Flow cytometry	Cell Signaling (9751)	1:500
H3K27me3	Western blot, Flow cytometry	Abcam (6002)	1:1000
H3K9me3	Western blot	Cell Signaling (5327)	1:1000
H3K36me3	Western blot	Cell Signaling (9763)	1:1000
H3K79me3	Western blot	Abcam (2621)	1:1000
MAP2	Western blot	Millipore (MAB3418)	1:500
H3K4me1	Western blot	Millipore (07-436)	1:500
H3K9me1	Western blot	Millipore (07-448)	1:1000
H3K27me1	Western blot	Millipore (07-450)	1:1000
5-methylcytosine	Dot blot	Active Motif (39649)	1:1000
5-hydroxymethylcytosine	Dot blot	Active Motif (39769)	1:1000
H3K9ac	Western blot	Cell Signaling (4353)	1:1000
H3K27ac	Western blot	Cell Signaling (9649)	1:1000
Primary Antibodies (Conjugated for flow cytometry)			
Antigen	Conjugation	Source (catalog number)	Dilution
SSEA3	BV421	BD Bioscience (562706)	5 μ l/sample
SOX17	AlexaFluor488	BD Bioscience (562205)	5 μ l/sample
PAX6	PerCPCy5.5	BD Bioscience (562388)	5 μ l/sample
MAP2B	Alexa488	BD Bioscience (560399)	20 μ l/sample
NESTIN	Alexa 647	BD Bioscience (560393)	20 μ l/sample
OCT3/4	AlexaFluor488	BD Bioscience (561628)	5 μ l/sample
SSEA1	AlexaFluor647	BD Bioscience (560120)	5 μ l/sample
Secondary Antibodies			
Antigen	Conjugation	Source (catalog number)	Dilution
Mouse IgG	AlexaFluor488	ThermoFisher (A-21202)	1:1000
Rabbit IgG	AlexaFluor647	ThermoFisher (1-31573)	1:1000
Rabbit IgG	HRP	Jackson (711-035-152)	1:3000
Goat IgG	HRP	Santa Cruz (sc-2020)	1:3000
Mouse IgG	HRP	Cell Signaling (7076)	1:3000
Goat IgG	IRDye800CW	Licor (926-32214)	1:15000

Mouse IgG	IRDye680RD	Licor (926-68072)	1:15000
Mouse IgG	IRDye680RD	Licor (925-68070)	1:15000
Rabbit IgG	IRDye800CW	Licor (925-32211)	1:15000

Lentivirus-mediated shRNA Knockdown

pLKO.1 vectors containing shRNA targeting *SDHA* RNA transcripts were purchased from Sigma-Aldrich (TRCN000028085 (shSDHA#1), TRCN000028043 (shSDHA#2), and TRCN000028093 (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 μ l of 1×10^7 TU/ml of viral particles and 7 μ g/ml polybrene for 8h on day 1. Transduction was repeated on days 2, 4, and 5. Selection with 1 μ g/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 2×10^6 cells were plated per well in a six-well plate in mTeSR1 media with 10 μ M 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 μ M SB431542 (Stemgent), 0.2 μ M 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- α KG, 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 μ M 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 1ml 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 1ml 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 300µl 2% FBS/PBS for analysis. Data collection was performed on either an LSRII (BD Bioscience) or an LSRFortessa (BD Bioscience). Data analysis was performed with FlowJo software (Treestar).

Immunoblot Analysis

Cell 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 Chemillumincent 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 1x 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.

GENE	PRIMERS
<i>POU5F1</i>	5' TGG TCC GAG TGT GGT TCT GTA A 3'
	5' TGT GCA TAG TCG CTG CTT GAT 3'
<i>NANOG</i>	5' TCC AGC AGA TGC AAG AAC TC 3'
	5' TTG CTA TTC TTC GGC CAG TT 3'
<i>EIF2B2</i>	5' TTG AGG CGA TTA ATG AGC TG 3'
	5' TGG AGT GAA TGT GCT CCA GA 3'
<i>PSAT1</i>	5' CGG TCC TGG AAT ACA AGG TG 3'
	5' AAC CAA GCC CAT GAC GTA 3'
<i>GOT1</i>	5' ATC TGT GCT TCT CGT CTT GC 3'
	5' AGA TTG CAC ACC TCC TAC CC 3'
<i>GOT2</i>	5' GAC CAA ATT GGC ATG TTC TGT 3'
	5' CGG CCA TCT TTT GTC ATG TA 3'
<i>GPT1</i>	5' CAA CAA CGT CTT CCT GTC 3'

	5' GGA TGG GGA TGA GCA CAC 3'
<i>GPT2</i>	5' GGA TCT TCA TTC CTG CCA AA 3'
	5' ACA TGT CTG GAG CCA TTT GA 3'
<i>IDH1</i>	5' ACG TGG AAT TGG ATC TAC ATA GC 3'
	5' TGA CGC CAA CAT TAT GCT TC 3'
<i>RPLP0</i>	5' TCT ACA ACC CTG AAG TGC TTG AT 3'
	5' CAA TCT GCA GAC AGA CAC TGG 3'

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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 1 α (HIF1 α) and hypoxia inducible factor 2 α (HIF2 α). HIF2 α has been shown to promote expression of stem cell transcription factor Oct4 (Covello et al., 2006), while both HIF1 α and HIF2 α 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 (α KG) 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 α KG can promote differentiation of hPSCs probably through the activation of α KG-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 O_2 , Fe^{2+} , ascorbate and α -ketoglutarate (α KG), and can be inhibited by metabolites with chemical structures similar to α KG, 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) direct inhibition of α KG-dependent dioxygenases, including JHDMs, TET, and PHDs due to low oxygen availability, 2) inhibition of α KG-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 α KG-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 (Clontech). Lentivirus was aliquoted and stored at -80°C until use on hPSCs. Feeder-free hPSCs were transduced by addition of 25µl of 1×10^7 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- α KG) was purchased from Sigma. Dm- α KG 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.

Primary Antibodies				
Antigen	Application	Conjugation	Source (catalog number)	Dilution
PAX6	Flow cytometry	PerCP-Cy5.5	BD Bioscience (562388)	5 μ l/sample
ARNT/HIF1beta	Immunoblot	n/a	Novus Biologicals (NB100-124)	1:500
HDAC1	Immunoblot	n/a	Santa Cruz Biotech	1:1000

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 10 μ M Y-27632 (BioPioneer) at a concentration of 4 x 10⁵ or 8 x 10⁵ 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), 10 μ M SB431542 (Stemgent), 0.2 μ M 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/HIF1 β 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, 200 μ l 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 was 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, 5 μ l 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 14 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).

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 HIF1 α and HIF2 α , 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 HIF1 β /ARNT, the heterodimeric partner for transcriptional activity of both HIF1 α 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). α KG-dependent dioxygenases play an important role in hPSC differentiation, and L-2HG is an inhibitor of many of the α KG-dependent dioxygenase enzymes (Figuerola et al., 2010; Lu et al., 2012; TeSlaa et al., 2016).

α KG does not promote differentiation in hypoxic conditions

Because 2HG competitively inhibits α KG-dependent dioxygenases, we hypothesized that addition of α KG may rescue differentiation. To determine whether α KG could rescue the effect of low oxygen tension during differentiation, hPSCs were differentiated into neuroectoderm in

the presence and absence of dm- α KG in 1% or 21% O₂ conditions. Dm- α KG was unable to increase the number of Pax6 positive cells after differentiation under low oxygen tension (Figure 2A and 2B). Treatment with dm- α KG 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 α KG causes increased L-2HG accumulation, or that α KG-dependent dioxygenases are directly inhibited by low oxygen tension in the context of hPSC differentiation at 1% O₂.

HIF is not required for dm- α KG accelerated differentiation

Dm- α KG can cause the stabilization of HIFs through inhibition of HIF prolyl hydroxylase 2 (PHD2) despite its metabolism into α KG (Hou et al., 2014). Because dm- α KG 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- α KG on differentiation (TeSlaa et al., 2016; Xie et al., 2014). Knockdown of HIF1 β /ARNT, however, had no effect on dm- α KG accelerated differentiation (Figure 2C). Furthermore, low oxygen inhibited differentiation in HIF1 β /ARNT knockdown hPSCs regardless of dm- α KG supplementation. This data suggests that dm- α KG accelerates differentiation independent from its influence on HIF stabilization.

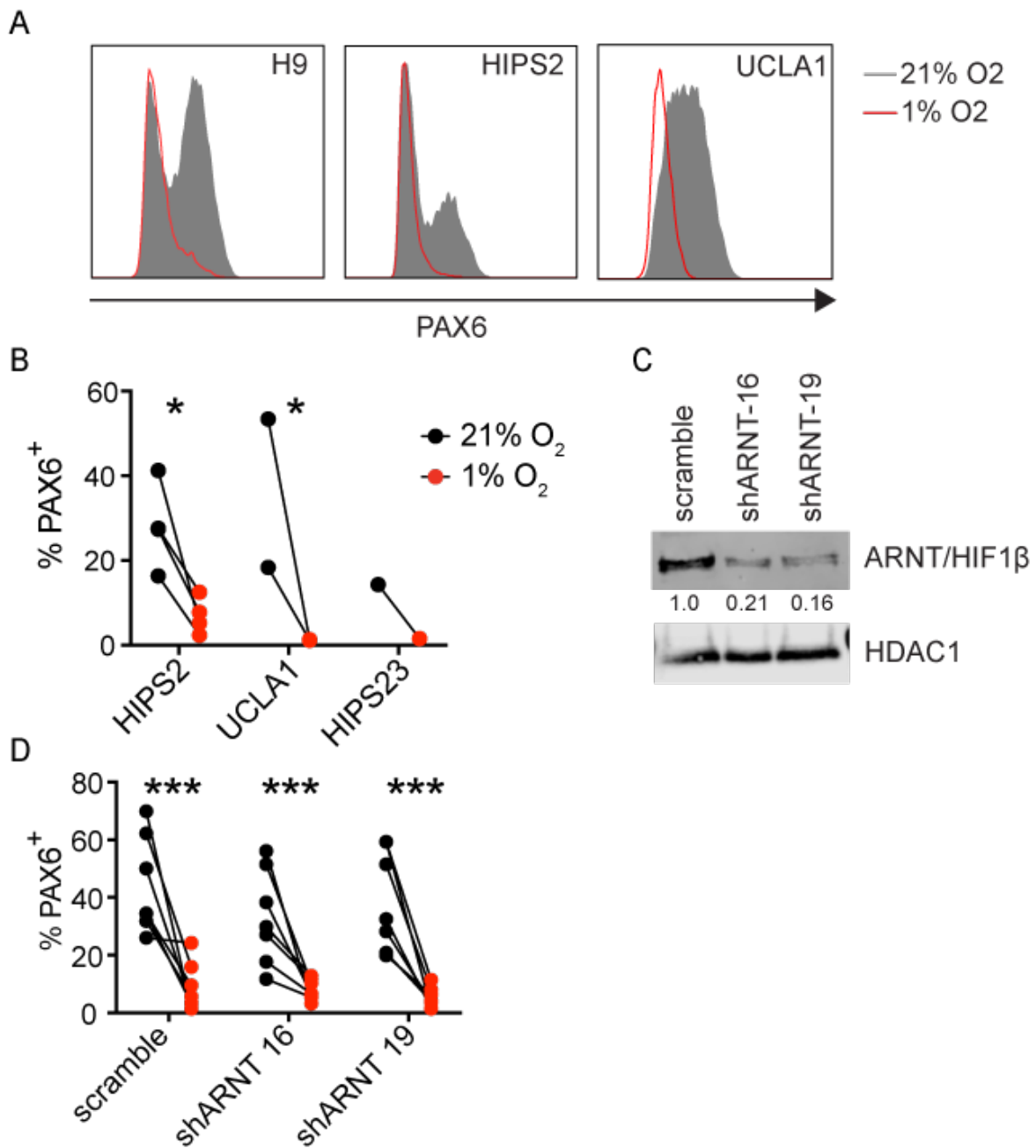
Discussion

Hypoxia can influence cell fate through multiple mechanisms including stabilization of HIF and inhibition of α KG-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 shHIF1 β /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 α KG, is expressed at high levels in human iPSCs when compared with fibroblasts, while D-2-hydroxyglutarate dehydrogenase (D2HGDH), which converts D-2HG to α KG 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 α KG 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 α KG 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.



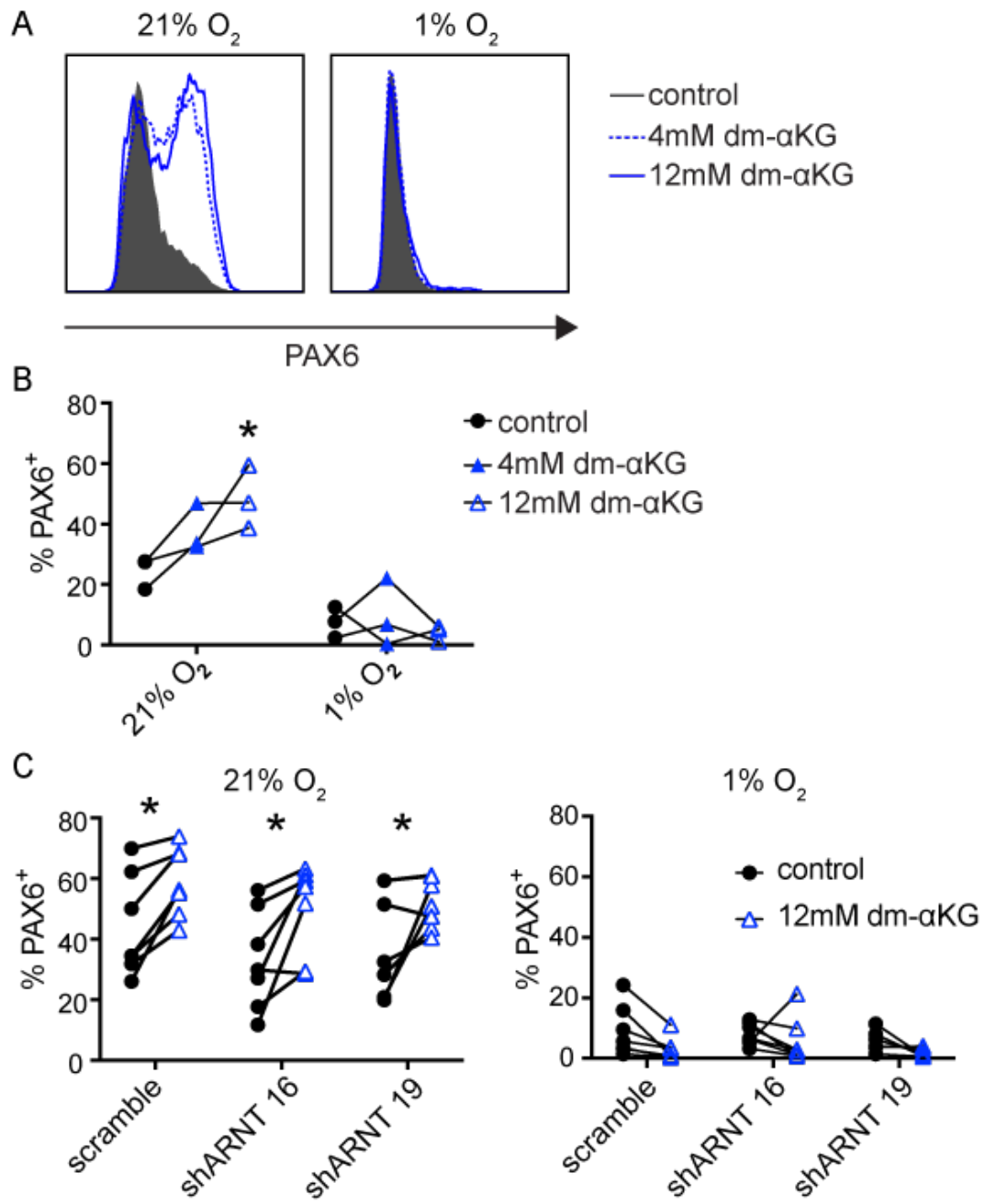


Figure Legends

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/HIF1 β and nuclear loading control HDAC1 in nuclear extracts of H9 hESCs with lentiviral expression of shRNA targeting ARNT/HIF1 β or scramble control.

(D) Quantification of flow cytometry data of the percent PAX6 positive cells in H9 hESCs expressing either shRNA targeting ARNT/HIF1 β or scramble control after 4 days of differentiation in either normoxic (21% O₂) or hypoxic conditions (1% O₂).

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

Figure 2. α KG 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- α KG where indicated.

(C) Flow plot and quantification of the percent PAX6 positive cells in differentiated H9 hPSCs expressing either shRNA targeting ARNT/HIF1 β 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- α KG where indicated.

* $p \leq 0.05$. P values were determined by two-way analysis of variance (ANOVA) with correction for multiple comparisons.

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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 (α KG) can promote self-renewal of the naïve pluripotent state by promoting the activity of α KG-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 α KG may play a different role in hPSCs. Furthermore, inhibition of α KG-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 α KG in primed hPSC differentiation has not been previously investigated.

To determine the role of α KG in hPSCs, we first investigated how TCA cycle metabolites including α KG are produced in hPSCs in comparison to hPSC-derived differentiated cells. To do this we fed hPSCs and differentiated cells ^{13}C -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 α KG 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 $^{15}\text{N}_2$ -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 α KG 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 α KG which can alter gene expression through α KG-dependent dioxygenases. Therefore, these enzymes may serve as a link between proliferation and epigenetic regulation coordinating biosynthesis and gene expression.

Because hPSCs robustly produce α KG, we hypothesized that it may be important for α KG-dependent enzyme function during differentiation. To determine whether α KG can affect differentiation, ectoderm and endoderm differentiations were performed with supplementation of α KG or cell permeable dimethyl-

α KG (dm- α KG) in the media. Both dm- α KG and α KG supplementation increased the percent of cells positive for lineage specific markers early in differentiation. From this data, we concluded that α KG accelerates the differentiation of primed hPSCs. This differentiation-promoting role of α KG 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- α KG. Dm- α KG accelerated the differentiation of mouse EpiSCs suggesting that α KG plays a different role in different pluripotent states. To further confirm the role of α KG in primed hPSCs, we next tried to decrease the activity of the α KG-dependent dioxygenases either by decreasing α KG levels or by increasing levels of succinate, a competitive inhibitor of the dioxygenase enzymes. Two different transaminase inhibitors were used to decrease α KG 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 α KG promotes primed hPSC differentiation.

Finally, we looked at the epigenetic targets of α KG-dependent dioxygenases, the methylation of histone tails and cytosine methylation of the DNA. During primed hPSC differentiation, dm- α KG causes a decrease in histone trimethyl marks and an increase in the ratio of 5-hydroxymethylcytosine to 5-methylcytosine. Combined these data suggest a differentiation promoting role for α KG in primed hPSCs probably through its action on epigenetic members of the α KG-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 α KG 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- α KG acts through stabilization of HIFs by inhibiting prolyl-4-hydroxylase 2 (PHD2) (Hou et al., 2014). To investigate this question, HIF1 β /ARNT was knocked down in hPSCs. Depletion of HIF1 β /ARNT does not negate the effect of dm- α KG suggesting that HIFs do not have a driving role in dm- α KG driven differentiation. This is consistent with our results in which α KG, which activates PHD2, promoted primed hPSC differentiation.

Interestingly, while studying the role of HIF in α KG-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. α KG-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 *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). *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), α KG 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.

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Appendix I:

Pulse laser triggered high speed
microfluidic fluorescence activated cell sorter

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PAPER

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

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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, 90% 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.¹ Micrometre-sized channel dimensions are also suitable for handling small sample volumes and low cell numbers with high yield.² Microfluidic sorting chips can be made disposable, which offers improved biosafety for sorting pathogenic samples. Furthermore, additional functionalities such as population expansion,³ drug screening,⁴ single-cell DNA⁵ and RNA⁶ analyses 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⁻¹.⁷ An integrated microfabricated PDMS valve⁸ demonstrated a sorting speed of 44 cells s⁻¹. By dynamically plugging the channel with a thermo-reversible gelation polymer,⁹ sorting of *E. coli* was shown at 5 cells s⁻¹. Using optical force routing,² 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%.¹⁰ Alternatively, an enrichment of ~230-fold (sort purity ~65%) at 1000 mammalian cells s⁻¹ was reported using an integrated piezoelectric actuator (PZT)-driven polydimethylsiloxane (PDMS) membrane valve.¹¹ 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,¹² 3 msec using flow switching with a thermo-reversible gelation polymer⁹ or with optical force switching,² and 0.1 msec for the PZT-driven membrane valve.¹¹ These mechanisms are orders of magnitude slower than the speed of standard electrostatic-droplet-based cell sorters. Microfluidic based droplet sorters¹³ 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 breakage 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 microspheres at 4.9 μs switching time.¹⁴ 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

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generation,¹⁵ enhancing fluid mixing,¹⁶ microfluidic switching,¹⁷ and gene transfection.¹⁸ 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 switch 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 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.¹⁷ 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 ~ 1 m s⁻¹, PLACS can achieve mammalian cell sorting at high speeds with high purities.

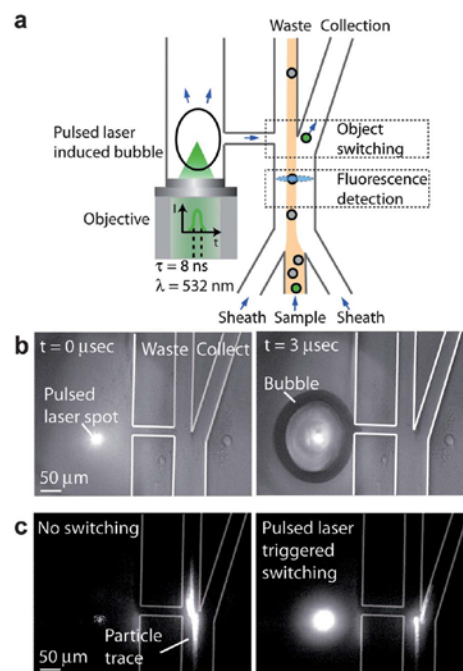


Fig. 1 PLACS operation. (a) Schematic of the cell sorter. The sample flow is hydrodynamically focused into the waste channel. As the desired object flows through the fluorescence detection region upstream from the Y junction, the laser pulse is triggered after an optimum delay and induces a cavitation 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 pulsewidth, and a repetition rate up to 100 kHz. The pulsed laser beam was expanded and focused by an objective lens (100 $\times$, NA 0.9) through the glass substrate into the pulsed channel. The laser focus was positioned 100 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 31 μJ per pulse for switching. For sample fluorescence excitation, a 10 mW, 488 nm solid state laser (CyrtaLaser, DL-488-010) was reflected by a dichroic mirror (Chroma, z488rdc) and lightly focused into the main channel through a 25 $\times$, NA 0.4 objective lens from the PDMS side of the microfluidic chip. The emitted sample fluorescence was collected by the same objective lens

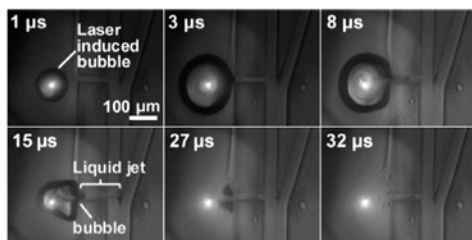


Fig. 2 A cavitation bubble was generated within nanoseconds after laser pulse arrival. The bubble grew to a maximum diameter of 230 μm in the major axis and 205 μm in the minor axis within 3 μs and began to collapse. During bubble expansion, the surrounding fluid was displaced at the speed of the bubble front and a high-speed liquid jet was induced in the connecting nozzle into the main sample channel, deflecting the targeted object towards the collection channel. The liquid injected into the sample channel is 140 μm (liquid jet length) $\times$ 20 μm (nozzle width) $\times$ 30 μm (channel height) $\sim$ 90 pL (see 15 μs). The bubble collapsed completely by 30 μs 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 μJ .

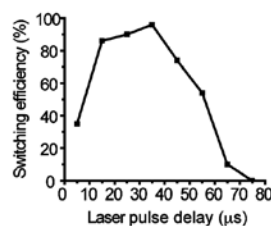


Fig. 3 Switching window and optimum object switching delay. Particle switching efficiency was measured at different laser pulse delays ranging from 5 to 75 μs after detection. The highest switching efficiency of 96% was obtained at a delay of 35 μs and decreased to 0% at 75 μs . The perturbation range of neighboring objects is $\sim$ 60 μm , estimated by the particle speed (0.8 m s^{-1}) and the 75 μs window. The distance between the detection region and Y junction was 50 μm . The sorting efficiency was obtained by analyzing particle traces from 50 switching events for each delay condition.

and detected using a photomultiplier tube (Sens-Tech, P30CWAD5-01) 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 $\times$ 80 μm 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 flashlamp (High-Speed Photo-Systeme, Nanolite KL-M) with 11 ns 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.¹⁹ Microchannel features were photolithographically defined in SU-8 (MicroChem 2025) photoresist molds 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 μm . Sheath flow channels were 80 μm wide. The sample channel width was 80 μm , which separated into 40 μm wide collection and waste outlet channels at the Y junction. The pulsed channel width was 300 μm and the straight nozzle connecting the sample and pulsed channel had a channel width of 20 μm and length of 100 μm .

Sorting of microbeads and mammalian cells

For particle sorting experiments, 10 μm green fluorescent beads (Thermo Fisher Scientific, G1000) and 10 μm 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

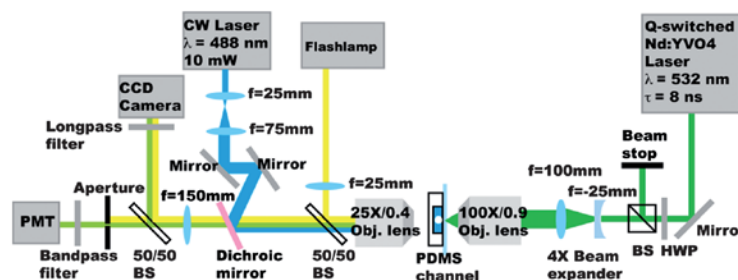


Fig. 4 PLACS experimental setup. For cavitation bubble induction, we used a Q-switched, 8 ns pulsewidth Nd:YVO4 laser. The pulsed laser beam was focused by an objective lens (100 $\times$, NA 0.9) into the pulsed channel. Sample fluorescence was detected by a photomultiplier tube and sampled using a DAQ card and integrated every 10 μs . 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 160 culture media with usual supplements were washed and re-suspended 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% CO_2 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, FACSCantoII). Positive and negative control samples were used to configure 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 $\sim 1 \times 10^6 \text{ ml}^{-1}$. 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 ($\sim 1 \times 10^6$ 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 protocols. 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 base level gene expression evaluation, cells were incubated in culture media at 37°C , 5% CO_2 before mRNA extraction. For positive controls, Nalm-6 cells were incubated in media at 42°C for one hour for *HSPA6* evaluation, whereas for *FOS* evaluation, cells were treated with $10 \mu\text{g ml}^{-1}$ cycloheximide (Sigma-Aldrich) for two hours. Primers for gene expression were generated using Roche's Universal Probe Library Assay Design Center online. *FOS* (*c-fos*) Fwd: (CTACCACTCACCCGACAGACT) Rev: (AGGTCCGTGCAGAAGTCCT). *HSPA6* Fwd: (TCATGAAGCCGAGCAGTACA) Rev: (GTTTTTGGCAGCCACTCTGT). *GAPDH* Fwd: (GCTCTCTGCTCCTCCTGTTTC) Rev: (ACGACCAAATCCGTTGACTC).

Results and discussion

Bubble dynamics and fluid perturbation volume estimation

In PLACS, once a desired object was detected, a nanosecond laser (532 nm wavelength, 8 ns pulsewidth) induced a bubble that

expanded to a maximum diameter (major axis) of $230 \mu\text{m}$ in $3 \mu\text{s}$ 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, $15 \mu\text{s}$). Estimated liquid jet volume is $140 \mu\text{m}$ (liquid jet length) $\times$ $20 \mu\text{m}$ (nozzle width) $\times$ $30 \mu\text{m}$ (channel height) $\sim 90 \text{ pL}$.

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 $50 \mu\text{m}$ 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 $35 \mu\text{s}$ and the efficiency decreased to 0% at a delay of $75 \mu\text{s}$ and $<40\%$ at $5 \mu\text{s}$ (Fig. 3). Particles outside of this region ($75 \mu\text{s} \times$ particle speed $0.8 \text{ m s}^{-1} = 60 \mu\text{m}$) were not switched and followed the original flow focusing streamline into the waste channel. The switching window and optimal delay time between triggering laser pulsing 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 $75 \mu\text{s}$, 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 $10 \mu\text{m}$ green fluorescent beads with non-fluorescent polystyrene beads. Throughputs ranging from 3000 to 10 000 particles/s were obtained by keeping the green bead concentration at $5.7 \times 10^4 \text{ ml}^{-1}$ while increasing the non-fluorescent bead concentration accordingly. High purities ($>90\%$) 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 0.0087 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 10 000 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

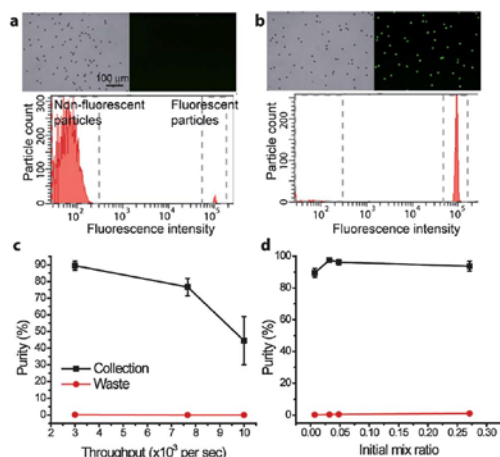


Fig. 5 PLACS sorting results. (a) Mixed 10 μm green fluorescent and non-fluorescent polystyrene microspheres before sorting. Initial mix ratio = 0.0087. (b) Collected sample after sorting with a final mix ratio = 9.61. Fluorescent microsphere concentration was enriched by a factor of 1105. (c) Sort purity at different sorting speeds ranging from 3000 to 10 000 microspheres s^{-1} . High purities ($90 \pm 3\%$ (mean $\pm$ 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 $45 \pm 15\%$. 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 ($5.7 \times 10^4 \text{ ml}^{-1}$) 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 $94 \pm 3\%$ and waste target particle purity was $1 \pm 0\%$. 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 calcein AM (green fluorescence) and mixed with untreated cells at the desired ratios. Results showed at sorting speeds of 560 and 1500 cells s^{-1} , >90%

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.¹¹ For high-speed cell enrichment, Ramos cells were sorted at 10 000 cells s^{-1} and 20 000 cells s^{-1} . The obtained sort purities 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 *HSPA6* and *FOS* stress-response gene expression² (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 (10^8) actuations (Fig. 7). Cavitation bubbles after 100 million cycles showed no discernible changes in

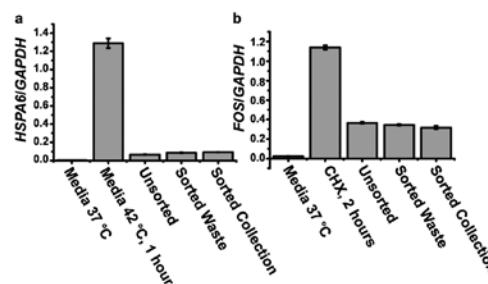


Fig. 6 Evaluation of stress levels of Nalm-6 cells after PLACS sorting. (a,b) The *HSPA6* gene expression level indicates the cellular response to heat shock, and *FOS* gene expression reflects heat shock and fluidicshear stress. Gene expression levels were normalized to a GAPDH house-keeping gene for 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 *HSPA6* 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)

Throughput (cells s^{-1})	Cell Type	Before sort			After sort			
		Initial green cell percentage (%)	Cell density ($\times 10^6 \text{ cells ml}^{-1}$)	Viability (%)	Col. purity (%)	W. purity (%)	Col. viability (%)	W. viability (%)
560	Nalm-6	1.2	1	94.1	91.5	0.3	90	84.4
1500	Nalm-6	0.8	4.6	93.4	97.9	0.3	89.4	90.2
10 000	Ramos	0.2	30.6	98	48.6	0.1	87.6	97
20 000	Ramos	0.2	61.3	98.5	37.4	0.0	98.8	98.1

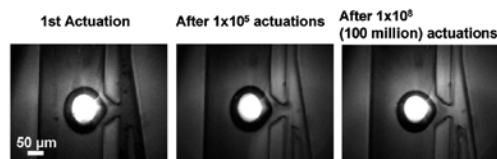


Fig. 7 PLACS reliability evaluation. A laser-induced cavitation bubble (imaged at 3 μ s after laser pulse arrival) could be repeatedly produced >100 million times without showing significant bubble pattern alteration or microchannel degradation. The pulsed laser repetition rate was 10 kHz.

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 μ s. 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 FACS and conventional electrostatic-droplet-based cell sorters.

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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

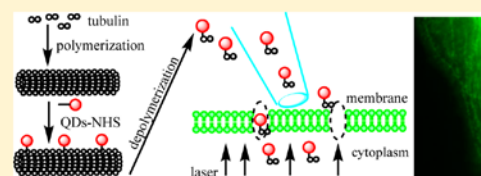
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Supporting Information

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.^{3–5} However, QDs also suffer from several shortcomings such as relatively large size,⁶ nonspecific binding (due to the charge of surface coatings),^{7,8} large avidity,⁹ blinking,^{10,11} and cytotoxicity.¹² 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 endocytotic 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,¹³ 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),^{14,15} proton sponge polymer carriers,^{16,17} pinocytosis,^{18,19} and transfection reagents.¹⁸ 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 electroporation^{20,21} 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.¹⁸ 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.^{23,24} However, the injection of large cargo (>0.5 μm) or aggregation-prone objects such as QDs and QDs–protein conjugates is difficult due to repeated tip clogging.

Here we applied the photothermal nanoblade technique^{25–27} 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 pipet; 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

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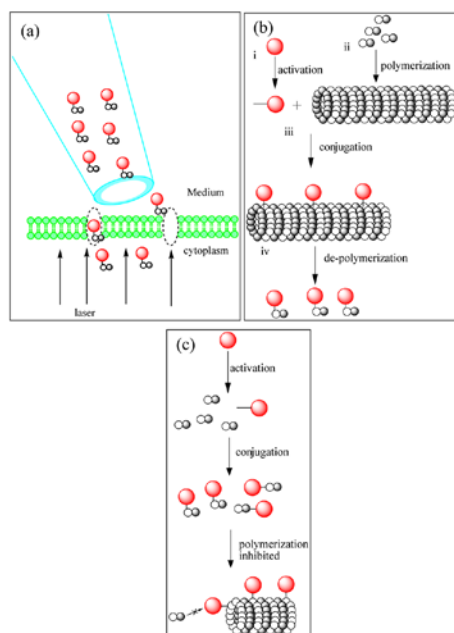


Figure 1. (a) Photothermal nanoblade delivery of tubulin-QD conjugates into the cytosol; (b) three-step tubulin-QDs conjugation strategy; (c) single-step tubulin-QD conjugation strategy.

relatively large tip orifice (up to $\sim 2 \mu\text{m}$) and injection of relatively large objects such as bacteria.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[sulfosuccinimidyl] suberate (BS3) cross-linker followed by a gel filtration step (to remove excess BS3); (ii) tubulin monomers were polymerized at 37 °C in the presence of GTP and DMSO; (iii) when the tubulin solution became turbid (due to microtubule polymerization), preactivated QDs were added to react with amine groups on polymerized tubulin molecules; and (iv) the reaction solution was quenched with hydroxylamine and centrifugation at 15 000g at 4 °C for 15 min. The pellet was then depolymerized at 4 °C in a DMSO-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 preactivated 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-

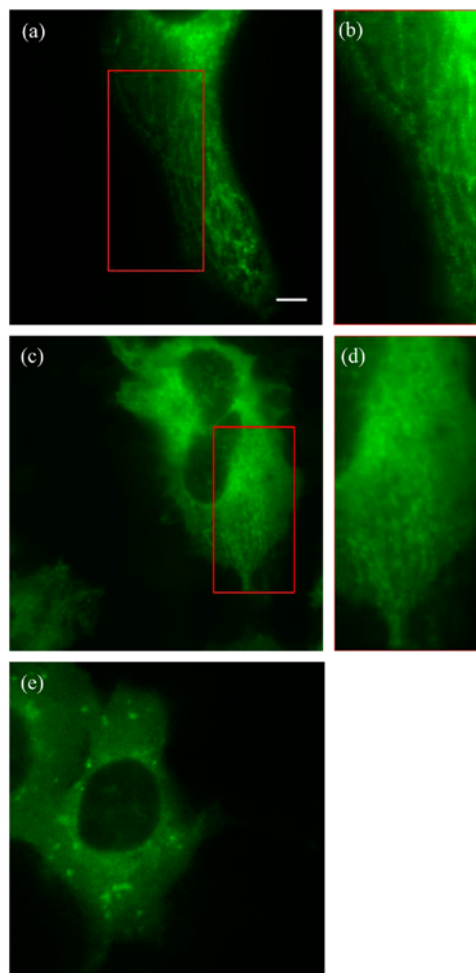


Figure 2. Images of live HeLa cells after photothermal nanoblade delivery of (a) tubulin-QD conjugates prepared with the three-step conjugation strategy (scheme in Figure 1b); (b) zoom-in of boxed area in panel a; (c) tubulin-QD conjugates prepared with the single-step conjugation strategy (Figure 1c);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

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 cytosol 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, steric hindrance, reduced diffusion, and potential alternation of function of

biomolecules,^{28–30} 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 antitubulin IgG (mouse) followed by alexa-647 antimouse IgG (goat). Cells injected with tubulin-QDs conjugates (Figure 1b) displayed good colocalization between the QDs and the alexa-647 channels (Figures 3a–c), whereas cells injected with bare amine-derivatized

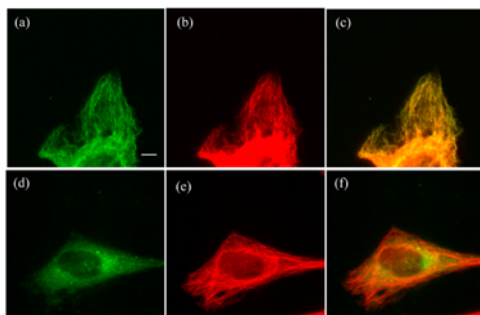


Figure 3. Images of fixed HeLa cells after photothermal nanoblade delivery of tubulin–QD conjugates (green) and immunocytochemistry labeling of tubulin (red): (a) tubulin–QD conjugates (scheme in Figure 1b); (b) immunocytochemistry image of same cell as in panel a; (c) overlay of panels a and b; (d)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

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

Figure 3 provides evidence for successful delivery and targeting of QDs to a cytosolic target, i.e., the polymerizing microtubule network. We also tested the delivery of the same conjugates using the conventional microinjection method. The two delivery methods were compared with regard to successful targeting and cell viability post injection (Figure 4). Cell viability was estimated by surface morphology 1 hr after injection. Conventional microinjection of amine-derivatized PEG-coated QDs was difficult (due to aggregation and clogging) but still possible. Cell viabilities post deliveries of these QDs were comparable for both methods (Figure 4). Conventional microinjection of tubulin–QDs conjugates was not at all possible for ~ 0.5 μ m microcapillary tips due to severe aggregation and clogging. When tip diameters were increased to ~ 1 μ m, some successful injections could be carried out but with greater difficulty in penetrating the cell membrane (higher mechanic force was needed to puncture the cell membrane) and with significant reduction in cell viability post injection. In contrast, photothermal nanoblade delivery was as efficient for tubulin–QDs conjugates as for amine-derivatized PEG-coated QDs, while maintaining good cell viability post injection (Figure 4). Furthermore, photothermal nanoblade delivery is significantly easier as it requires only a gentle contact on the cell membrane and therefore is devoid of failed attempts that result in broken pipet tips.

To summarize, we conjugated tubulin to QDs and successfully delivered and targeted them into growing microtubule structures inside live cells using the photothermal nanoblade delivery method. This method achieved efficient delivery of endosome-free

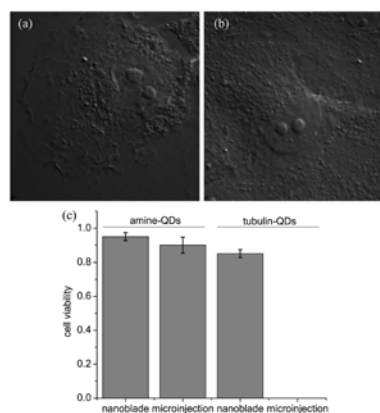


Figure 4. Examples of cell morphology after (a) microinjection and (b) photothermal nanoblade (tip ~ 1 μ m) delivery of tubulin–QD conjugates. (c) Cell viability postphotothermal nanoblade and conventional microinjections of bare amine-derivatized PEG-coated QDs and tubulin–QD conjugates (scheme in Figure 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/ β -catenin signaling directs another hallmark of tumorigenesis, namely Warburg metabolism. Using biochemical assays and fluorescence lifetime imaging microscopy (FLIM) to probe metabolism *in vitro* and in living tumors, we observe that interference with Wnt signaling in colon cancer cells reduces glycolytic metabolism and results in small, poorly perfused tumors. We identify pyruvate dehydrogenase kinase 1 (PDK1) as an important direct target within a larger gene program for metabolism. PDK1 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; colon cancer; fluorescence lifetime imaging; metabolism; Wnt

Subject Categories Cancer; Metabolism; Molecular Biology of Disease

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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 (Bienz & Clevers, 2000). 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 (Brabletz *et al.*, 2005). Here, we propose a novel function in the regulation of cancer metabolism.

Cancer metabolism is quickly regaining a forefront 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 (DeBerardinis *et al.*, 2008; Vander Heiden *et al.*, 2009). An emphasis on glycolysis is thought to be driven not only from exposure to hypoxic conditions [mostly through stabilized HIF1 α (Semenza, 2010)] but also by oncogenic signaling pathways, such as PI3K/AKT (Elstrom *et al.*, 2004; Ward & Thompson, 2012). Although Wnt plays a well-known role in homeostatic liver metabolism (Liu *et al.*, 2011) and can crosstalk with metabolic pathways in normal cells such as differentiating osteoblasts (Sethi & Vidal-Puig, 2010; Esen *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 phasor approach

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to fluorescence lifetime imaging microscopy (FLIM), utilizes two-photon microscopy for label-free detection of intrinsically autofluorescent molecules (Digman *et al*, 2008; Stringari *et al*, 2011). In our study, we used FLIM to monitor the metabolic coenzyme 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 (PDK1) as a novel Wnt target gene that promotes this effect, and we note that a cell-extrinsic effect of PDK1-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 (Hoverter *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.

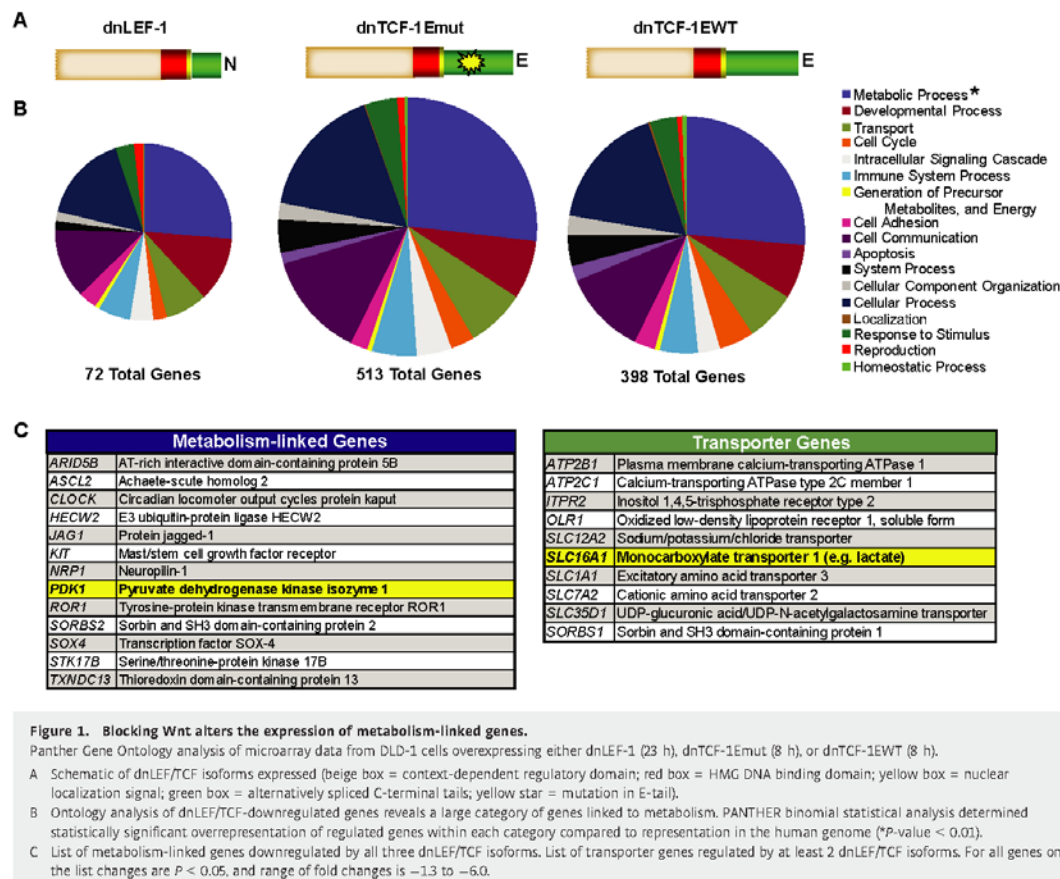
dnLEF-1 and dnTCF-1Emut do not alter the cell cycle or proliferation *in vitro*

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-1E or dnTCF-4E isoforms halts cell cycle progression and cell proliferation (van de Wetering *et al*, 2002), while isoforms lacking the alternatively spliced C-terminal E-tail avoid this stall (Naishiro *et al*, 2001; Atcha *et al*, 2007), we induced the expression of dnLEF-1N which naturally lacks E-tail sequences. In parallel, we also induced the expression of dnTCF-1Emut, a mutated form of dnTCF-1E with a five-amino acid substitution in the auxiliary C-clamp DNA binding domain in the E-tail that eliminates the control of proliferation (Atcha *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 Super TOPFlash (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-1N and dnTCF-1Emut expression have no effect on the cell cycle or the intrinsic ability 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 lentivirus 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-ribosyltransferases 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 dnLEF/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 2D). We also tested for rates of glucose consumption, and as expected, rates were lower when either dnLEF/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 dnLEF/dnTCF 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 phasor approach to fluorescence lifetime imaging microscopy (FLIM) to evaluate metabolism. Specifically, we used FLIM to monitor dynamic shifts in patterns of aerobic glycolysis versus oxidative phosphorylation by following the signature of the metabolic and autofluorescent cofactor 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; Monici *et al*, 2003; Croce *et al*, 2004). The signature of fluorescence decay of NADH can be graphically represented on a 2D phasor 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; Digman *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 phasor plot posi-

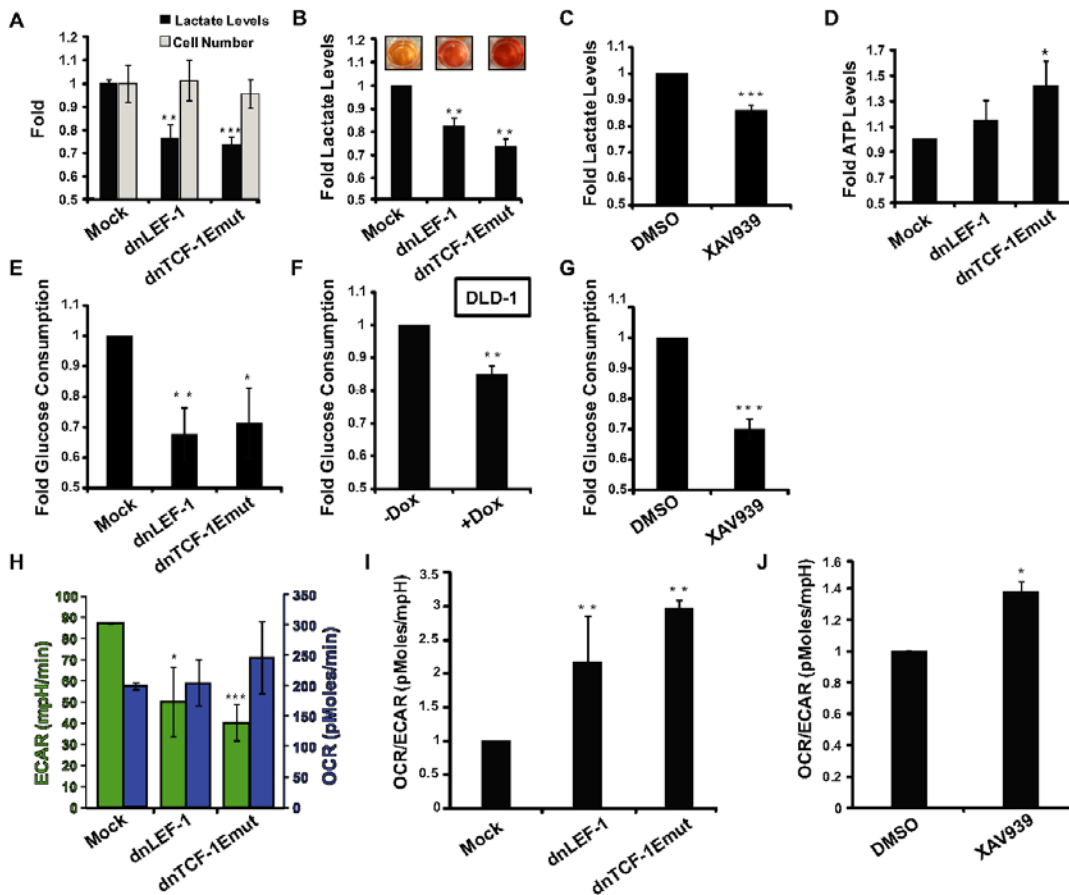


Figure 2. Blocking Wnt alters the metabolic program of colon cancer cells.

- A** Lactate levels are reduced with lentiviral transduction of dnLEF-1 or dnTCF-1Emut in SW480 cells growing under standard culture conditions for 10 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 μ M) for a minimum of 4 days. Data represent the average of six independent trials ($\pm$ SEM).
- D** ATP levels in SW480 cells collected 7 days post-transduction. Data represent the average of three independent trials ($\pm$ SEM).
- E–G** Fold changes in glucose consumption in SW480 cells expressing dnLEF/TCFs (**E**), DLD-1 cells [dnLEF-1(2)] treated with doxycycline to induce dnLEF-1 expression (**F**), and SW480 cells treated with XAV939 (10 μ M) (**G**). Data represent the average of four trials ($\pm$ SEM).
- H** Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in SW480 cells transduced with MOCK, dnLEF-1, or dnTCF-1Emut virus. Data represent the average of three independent trials ($\pm$ SD).
- I** Data from (**H**) represented as an OCR/ECAR ratio.
- J** OCR/ECAR ratio of SW480 cells treated with XAV939 (10 μ M). Data represent the average of three independent trials ($\pm$ SD).

Data information: *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

tions of the pure free and bound species (Fig 3A). In other words, experimental positions on the phasor plot indicate the relative ratio of free to bound NADH and therefore the relative levels of glycolysis

and oxidative phosphorylation. In our analysis, increases in the free/bound NADH ratio (rightward and downward shift of experimental points in the phasor plot) indicate more glycolysis and

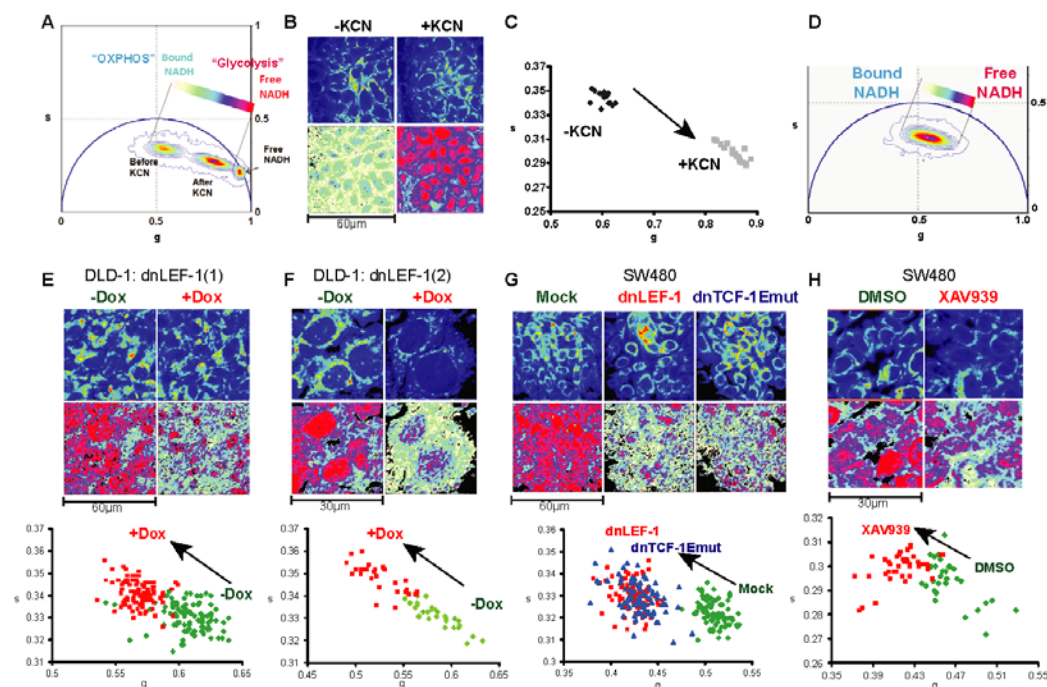


Figure 3. Fluorescence lifetime analysis of NADH reveals shifts in glycolysis upon expression of dnLEF/TCF.

A Phasor plot of a FLIM analysis showing DLD-1 cells before and after a 1-min treatment with 4 mM potassium cyanide (KCN). The phasor position of pure free NADH is also shown.
B, C Top panel of images in (B) shows the autofluorescent intensity at 740 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 phasor 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 phasor positions of individual cells before and after KCN treatment.
D Phasor position of DLD-1/dnLEF-1(1) 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 two-photon fluorescence intensity images excited at 740 nm for two clones of DLD-1 dnLEF-1 stable cells [dnLEF-1(1) and dnLEF-1(2)] and 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 phasor position from one cell. Induction of dnLEF-1 or dnTCF-1Emut expression, or treatment with the Wnt inhibitor XAV939, results in a phasor shift toward bound NADH. All images and measurements were taken 5 days after seeding under confluent conditions. Representative data from single trials 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 $P < 0.0001$.

an overall cancer type of metabolism (Bird *et al*, 2005; Skala *et al*, 2007; Yuan *et al*, 2007; Yu & Heikal, 2009).

To demonstrate the relationship between a phasor shift and a change in metabolism, cells were treated with potassium cyanide (KCN) for 1 min 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 phasor 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 phasor 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/TCFs or cells treated with the XAV939 inhibitor. Doxycycline induction of dnLEF-1N in two different clonal DLD-1 cell lines caused the average phasor 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 phasor 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 dnLEF-1N or dnTCF-1Emut expression cassettes (Fig 3G; additional dnTCF isoforms in Supplementary Fig S3A and C), as well as analysis of cells treated overnight with XAV939, the Wnt signaling inhibitor (Fig 3H). 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 indicates 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 dnLEF/TCF proteins was pyruvate dehydrogenase kinase (PDK1). This kinase phosphorylates and inhibits the pyruvate dehydrogenase (PDH) complex in mitochondria (Roche et al, 2001). Inhibition of PDH, in turn, reduces the conversion of pyruvate to acetyl-CoA for entry into the TCA cycle and oxidative phosphorylation. Therefore, PDK1 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 (Koukourakis et al, 2006; Wigfield et al, 2008; Baumunk et al, 2012).

To validate the microarray data and test whether PDK1 levels are modulated by β -catenin and LEF/TCFs, Western blot analysis was performed for both DLD-1 and SW480 cells expressing dnLEF-1 or dnTCF-1Emut. PDK1 protein levels were reduced 50–75% 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 dnLEF-1 DLD-1 cells after 24 or 120 h of expression. At both time points, *PDK1* mRNA levels were reduced to 60%, 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 dnLEF-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.

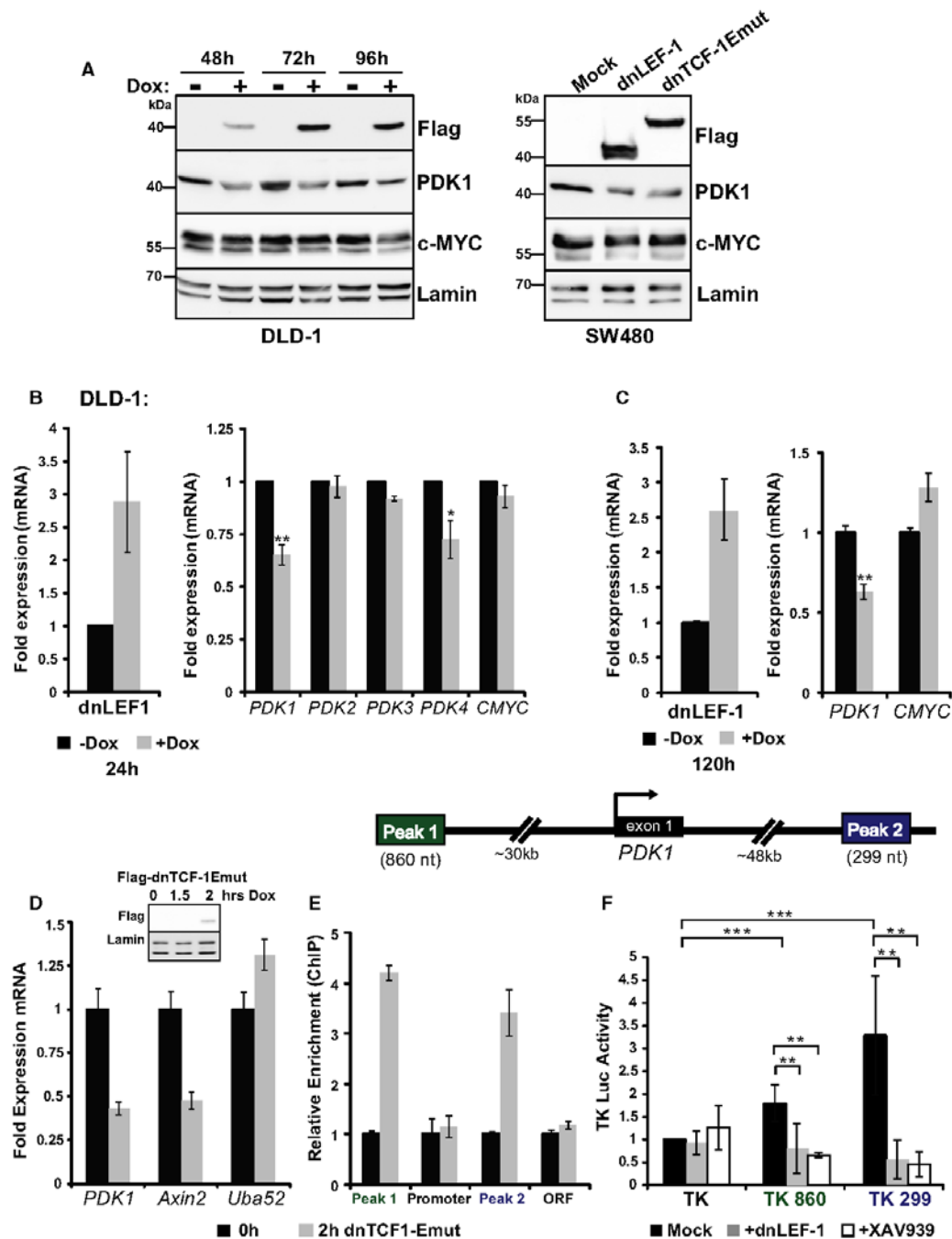
A possible connection between Wnt and metabolism occurs through the Wnt target gene, c-Myc, and in fact, previous reports suggest that under certain conditions, c-Myc can potentiate the upregulation of PDK1 levels (Kim et al, 2007). We examined c-Myc levels after expression of dnLEF/TCF. With the exception of a slight decrease in c-Myc protein after 96 h of dnLEF-1 expression in DLD-1

cells (Fig 4A), 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 dnLEF-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 (Dölken et al, 2008). After induction of dnTCF-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 purified via a biotinylation/streptavidin pull-down procedure (Dölken et al, 2008). Semi-quantitative RT-PCR for *PDK1* mRNA demonstrated that transcription of the *PDK1* locus was immediately reduced upon the induction of dnTCF1E-mut expression (Fig 4D). The known Wnt target gene *AXIN2* served as a positive control, and constitutive, ubiquitous *UBA52* served as a negative control. Induction of dnTCF-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 dnTCF-1E binding (N.P. Hoverter, M.D. Zeller, M.M. McQuade, A. Garibaldi, A. Busch, K.J. 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 dnLEF-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 dnLEF-1 (Supplementary Fig S4). These data demonstrate that *PDK1* is a direct Wnt target gene and that regulation occurs through distal regulatory sites upstream and 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 dnLEF-1. We used lentiviral infection of DLD-1 cells to restore physiological levels of PDK1. As shown in Fig 5A, dnLEF-1 expression alone led to a decrease in endogenous PDK1 levels and a decrease in glycolysis (a shift in the FLIM phasor position away from free NADH). In contrast, lentiviral restoration of normal PDK1 levels rescued the FLIM signature of glycolysis (a return of the phasor 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 PDK1. When repeated in the second DLD-1 clonal 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 PDK1 could be a major reason for the metabolic shift triggered by dnLEF/TCF interference with Wnt/ β -catenin signaling.

Given that we propose PDK1 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 PDK1 (Whitehouse et al, 1974). A 48-h treatment of DCA led to the same characteristic shift in the phasor plot toward bound NADH that was seen with expression of dnLEF/TCF, again indicating a decrease in a glycolytic phenotype (Fig 5B). The FLIM shift corresponded to an increase in the ratio of oxygen consumption (oxidative phosphorylation) relative to the extracellular acidification rate (OCR/ECAR, Fig 5C). These changes in metabolic metrics match previous reports that DCA treatment decreases lactate excretion (Bonnet 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 (Cairns et al, 2007; Stockwin et al, 2010; Tong 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 μ M irinotecan nor dnLEF-1 alone had much effect on cell proliferation and 20 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 5D). These data suggest that dnLEF/TCF inhibition of PDK1 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 PDK family, not just PDK1. We therefore used lentiviral shRNA knockdown to test for the specific significance of PDK1 (Supplementary Fig S5B and C). Western blot analysis of multiple knockdown lines revealed reduced PDK1 protein levels and significant reduction of phosphorylation of its main substrate, mitochondrial pyruvate dehydrogenase (PDH β Ser293, Supplementary Fig S5B). FLIM analysis of each knockdown cell line revealed signif-

icant shifts in the free/bound NADH signature that mimic the decrease in glycolysis observed with dnLEF1 or dnTCF1 expression or DCA treatment (Supplementary Fig S5C). These results indicate that PDK1 is an important Wnt target gene and that it contributes major activities to glycolysis. However, we also observed the knock-down cultures to revert to a glycolysis mode of metabolism several days later. Western blot analysis of the reverted cultures showed that while PDK1 protein levels remained low, phosphorylation of the target substrate pyruvate dehydrogenase had recovered, a sign of compensatory rescue via other PDK family members or other kinases (data not shown). Thus, the more stable shift in metabolism observed with DCA treatment or dnLEF1/dnTCF expression suggests that total cellular PDK activity is targeted by Wnt signaling beyond single, selective regulation of PDK1. We conclude that PDK1 is a major Wnt target gene, but that it is coordinately regulated within an entire gene program for glycolysis.

Blocking Wnt 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 dnLEF-1 or dnTCF-1Emut in SW480 cells injected into immune-deficient NOG mice drastically decreased tumor growth by up to 90%. 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 vivo* with the previous results *in vitro*, we used Western blot analysis to examine levels of PDK1 and c-Myc. PDK1 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-1Emut tumors (Fig 6C). These results are consistent with the previously described microarray which showed a decrease in c-MYC RNA levels with dnTCF-1Emut, but not dnLEF-1 (Hovert et al, 2012). Western blot analysis also revealed stable reduction of pyruvate dehydrogenase phosphorylation in both dnLEF/TCF tumor types (PDH β Ser293; Fig 6C). Overall, the tumor analysis suggests that while tumors expressing dnLEF/TCF show similar reductions in PDK1 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 PDK1 levels via regulation of transcription.

- A Whole-cell lysates from DLD-1 dnLEF-1(1) cells were collected 48, 72, and 96 h after 0.01 μ g/ml doxycycline treatment and were probed with the antibodies shown. SW480 cells were harvested 48 h post-transduction.
- B, C RT-qPCR analysis was performed on RNA collected from DLD-1 dnLEF-1(2) cells harvested 24 h (B) or 120 h (C) after the addition of doxycycline. Graphs shown represent the average of three trials ($\pm$ SEM).
- D RT-qPCR analysis was performed on 4-thiouridine-labeled RNA isolated from a 30-min pulse in the presence/absence of dnTCF-1Emut, induced by 2-h doxycycline treatment in DLD-1 cells. Known Wnt target genes *Axin2* and *Uba52* were used as positive and negative controls, respectively. A representative graph is shown of two replicates, with error bars representing the SD among three internal replicates.
- E RT-qPCR analysis of chromatin immunoprecipitated from DLD-1 cells with or without induction of FLAG-dnTCF-1Emut, using anti-FLAG antibody. PCR primers designed to detect the indicated PDK1 genomic regions show that dnTCF-1Emut associates with distal regions that flank the PDK1 locus. A representative graph is shown of two replicates, with error bars representing the SD among three internal replicates.
- F Luciferase reporter activity in SW480 cells shows that Peak 1 and Peak 2 regions confer elevated transcription activity to the heterologous thymidine kinase (TK) promoter. Expression of transduced dnLEF-1, or treatment with the Wnt inhibitor XAV939 (10 μ M), eliminates the regulatory activity of these fragments. Graph shown represents the average of three independent replicates ($\pm$ SD).

Data information: *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.
Source data are available online for this figure.

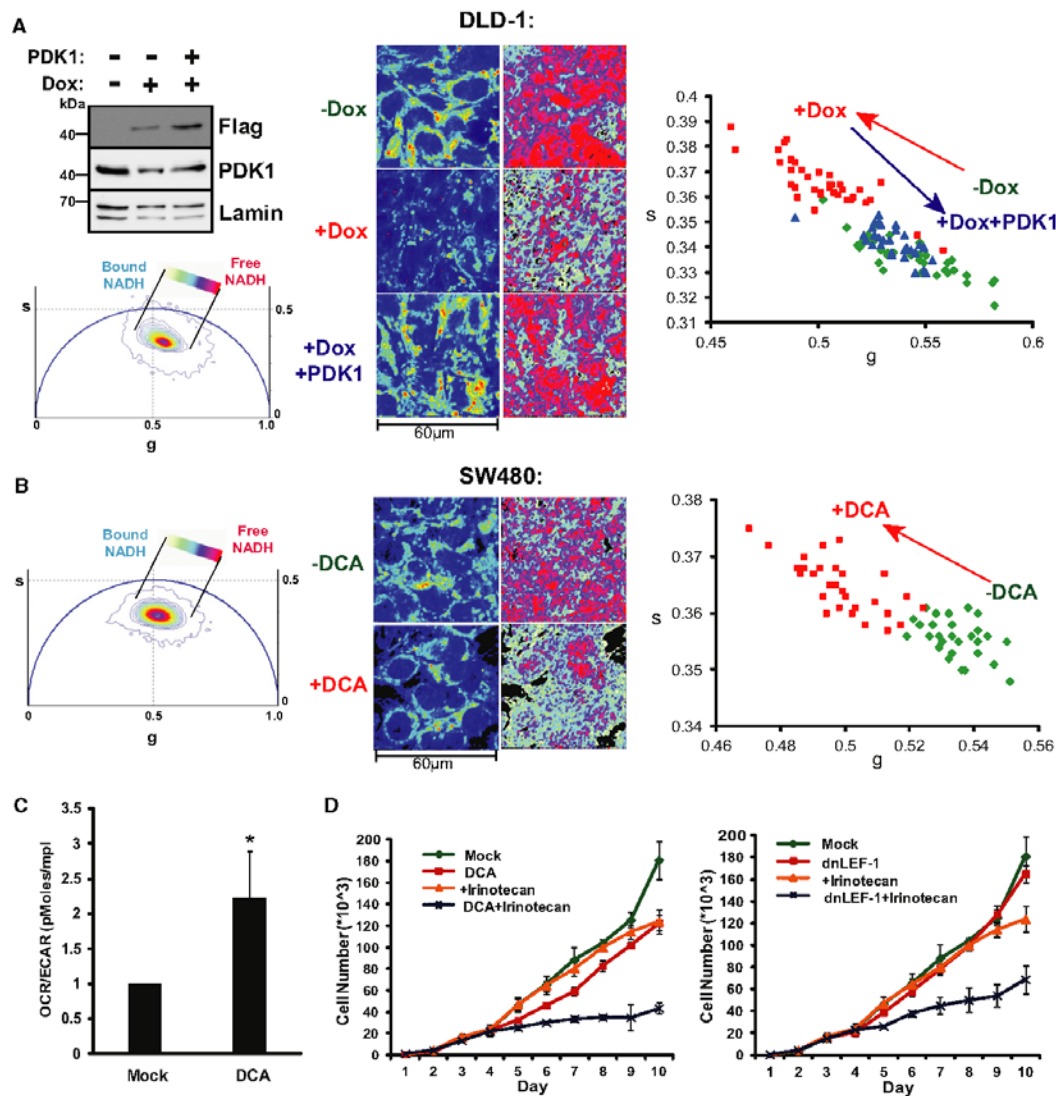


Figure 5. PDK1 overexpression rescues the altered metabolic phenotype induced by blocking Wnt.

A Western blot analysis of lysates collected from DLD-1 dnLEF-1(2) cells treated with or without 0.01 μ g/ml doxycycline for 24 h and with or without PDK1 lentivirus for 72 h. FUM imaging was performed at confluency (96 h doxycycline and 7 days post-transduction). Intensity images are shown on the left. FUM results, shown through the free/bound NADH color mapping (right), as well as the scatterplot, show that doxycycline induction of dnLEF-1 shifted the phasor 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 phasor shift toward bound NADH ($P < 0.0001$ comparing +DCA to mock).

C OCR/ECAR ratio of DCA (10 mM)-treated SW480 cells shows that blocking PDK1 activity doubles the oxygen consumption rate (mitochondrial activity) relative to the extracellular acidification rate (glycolysis-produced lactate). Data shown represent the average of three independent trials ($\pm$ SD; * P -value < 0.05).

D Sulforhodamine B cell proliferation assay of SW480 cells treated with or without 2.5 μ M Irinotecan, 20 mM DCA, and dnLEF-1 lentivirus shows an increased sensitivity to Irinotecan when treated with DCA or dnLEF-1. A representative graph of two trials is shown. Error bars represent the standard deviation between eight internal replicates.

Source data are available online for this figure.

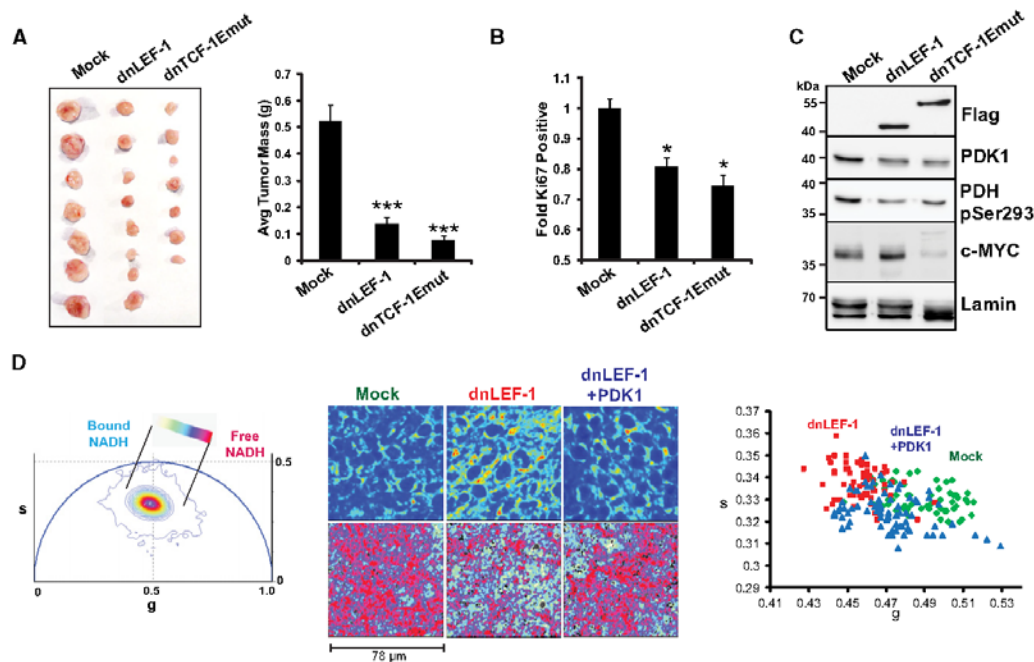


Figure 6. Blocking Wnt reduces *in vivo* tumor growth.

A Expression of dnLEF-1 or dnTCF-1Emut in SW480 cells results in smaller xenograft tumors. Images of tumors are shown with quantification of the tumor mass and volume at the time of harvest. Data include measurements of eight tumors for each condition. Error bars represent the SEM among eight replicates (****P*-value < 0.001). **B** Ki67 staining of paraffin-embedded sections from xenograft tumors shows fewer Ki67-positive cells with dnLEF-1/dnTCF-1Emut expression. Data shown represent the average of counts from at least eight fields. Error bars represent the SEM among at least eight replicates (**P*-value < 0.05). **C** Western blot was performed on protein lysates prepared from freshly extracted xenograft tumors. Western blot analysis of endogenous PDK1 and its target, pyruvate dehydrogenase (pSer293-PDH), shows decreases when dnLEF-1 or dnTCF-1Emut tumors are expressed. cMYC expression shows variable levels of expression. **D** Phasor plot representation for the color mapping of *in vivo* tumor FLIM analysis, fluorescence intensity (top image panels), FUM color mapping (bottom panels), and scatterplot analysis are as described in Fig 5 except that these analyses were performed on living, surgically exposed, yet still actively perfused, xenograft tumors. Both the free/bound NADH color mapping (bottom row) and scatterplot (average phasor position of individual cells within each tumor) show a shift in the phasor position toward bound NADH with dnLEF-1 expression and a return to free NADH with PDK1 overexpression (*P* < 0.0001 comparing dnLEF-1 to mock and comparing dnLEF-1+PDK1 to dnLEF-1). A minimum of three fields of view per tumor were analyzed. Data shown are from one mouse representative of eight replicates (additional tumor analysis in Supplementary Fig S7).

Source data are available online for this figure.

To test whether the same change in metabolism that we detected *in vitro* by FLIM analysis is also detected *in vivo*, 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 μ m around the subcapsular region of the tumors (see Supplementary Fig S6 for a schematic of *in vivo* FLIM). A similar metabolic response to Wnt signaling inhibition was observed with these analyses. That is, dnLEF/dnTCF1-expressing tumors were less glycolytic (phasor position shifted toward bound NADH; Fig 6D). A notable difference

compared to cells grown in monoculture *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 dnLEF-1 cells that had been lentivirally transduced with a PDK1 expression vector prior to injection into the mice. PDK1 expression rescued the metabolic signature, shifting the phasor 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

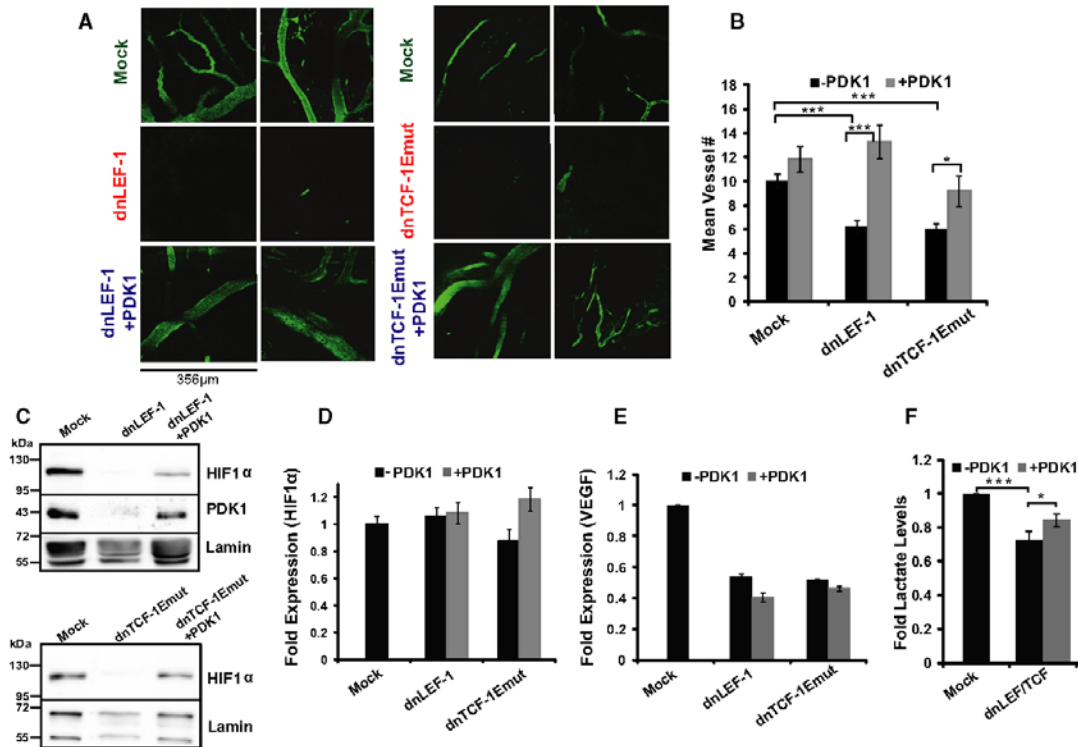


Figure 7. Blocking Wnt reduces tumor vessel density, while reintroduction of PDK1 restores it.

A Tumor vessel density within the subcapsular region is greatly diminished with dnLEF/TCF, but is restored with PDK1 expression. Images show FITC dextran-labeled vasculature in the xenograft tumors (~50–200 μm deep).
B Quantification of vessel density derived from the CD31 staining of paraffin-embedded sections of each tumor. For quantification, the number of vessels per field of view (40× objective) was averaged from every cross-section (at least 20 for each condition) within the subcapsular region for each tumor. Error bars represent the SEM among at least 20 replicate fields.
C Western blot analysis was performed on protein lysates prepared from freshly extracted xenograft tumors.
D, E RT-qPCR analysis of (D) *HIF-1α* and (E) *VEGF* expression in xenograft tumor RNA. *HIF-1α* mRNA levels do not change among the various tumors (normalized to *GAPDH*), but *VEGF* expression levels are downregulated in the dnLEF-1 and dnTCF-1Emut tumors. However, PDK1 rescue expression does not change the levels of either *HIF-1α* or *VEGF* mRNA. RT-qPCR data shown are representative of four replicate tumor sets. Error bars represent the standard error between three internal replicates.
F Lactate levels in dnLEF/TCF-expressing tumors are reduced compared to MOCK levels and are partially restored with PDK1 rescue expression. Data show the average fold lactate levels (compared to mock) in five tumors for each condition, with error bars representing the SEM (**P*-value < 0.05; ****P*-value < 0.001).

Source data are available online for this figure.

intermediates. 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 tumor sections for the endothelial marker CD31 and counted vessel density in the same subcapsular

region where the FLIM analysis was performed. Vessel density was greatly diminished in the tumors expressing dnLEF-1 or dnTCF-1Emut, 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; Milovanova *et al*, 2008; Sutendra *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

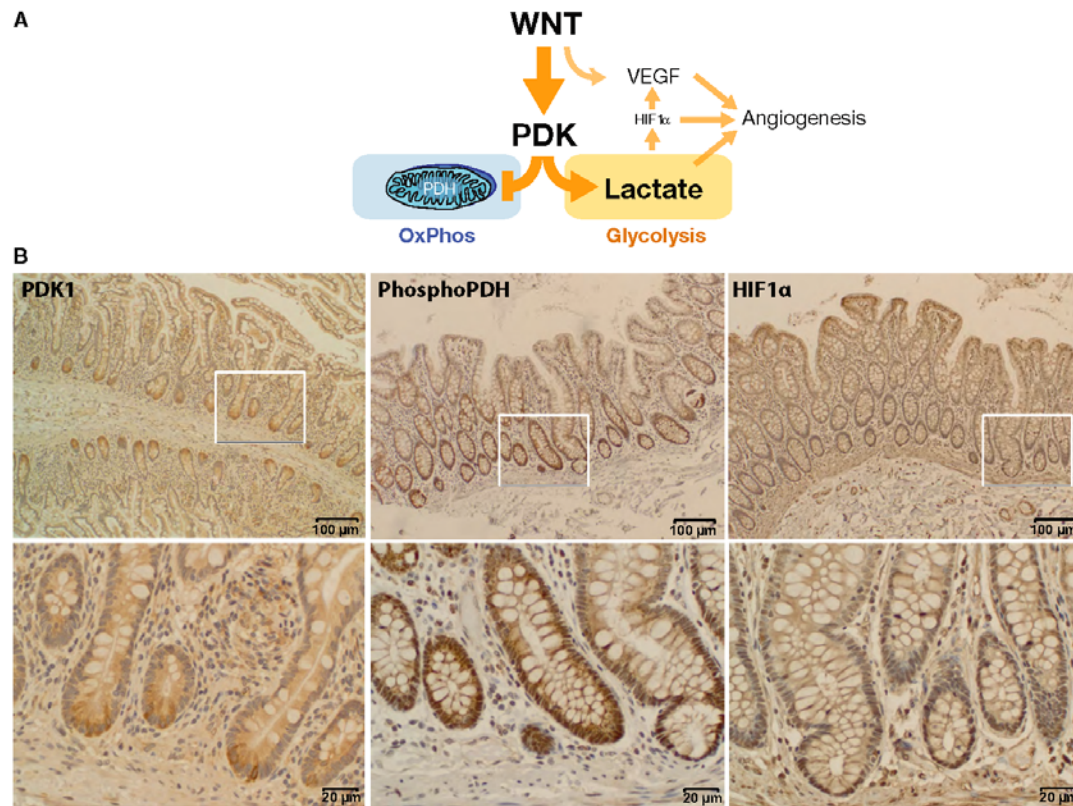


Figure 8. Wnt promotes aerobic glycolysis through PDK1.

A Model of Wnt regulation of cancer metabolism and angiogenesis through PDK1.

B Immunohistochemical staining of normal human small intestine (PDK1) and normal human colon (phospho-PDK and HIF1 α) shows correlations between high levels of Wnt signaling, PDK1 activity, and HIF1 α levels. Bottom row shows higher-power images of boxed portions in the top row.

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 dnLEF/TCF-expressing tumors, addition of PDK1 did not restore these levels (Fig 7E), suggesting that the PDK1 rescue of angiogenesis is VEGF independent. Overall, dnLEF/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 LEF/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 involvement 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 (Sethi & 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-Lrp5-Rac1-mTORC2-Akt signal to upregulate protein and activity levels of glycolytic enzymes, including PDK1 (Esen *et al.*, 2013). Regulation occurred within 10–20 min of Wnt stimulation and was independent of β -catenin, LEF/TCFs, and transcription. Our findings contrast with this system because we find that β -catenin and LEF/TCF are required to directly target *PDK1* transcription. Indeed, our test for a connection to mTORC2 was negative as inhibition of β -catenin with either dnLEF/TCF expression or XAV939 treatment for as long as 3 h had no effect on mTORC2 activity in DLD-1 and SW480 cells and inhibition of mTORC2 for 24 h with the specific mTOR inhibitor PP242 had no effect on *PDK1* mRNA levels (Supplementary Fig S9).

We propose that Wnt signals have short-term 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 1C, 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.

PDK1 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. PDK1 phosphorylation of PDH suppresses entry of fuel into mitochondria and instead promotes glucose fermentation to lactate in the cytoplasm, an important activity for promoting the Warburg effect in cancer. Indeed, PDK1 is upregulated in numerous cancers, including colon cancer (Koukourakis *et al.*, 2006; Wigfield *et al.*, 2008; Baumunk *et al.*, 2012). Here, we propose that cancer relevant levels of Wnt signaling use LEF/TCFs for direct regulation of PDK1. *PDK1* 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 (ENCODE; Kent *et al.*, 2002) also show that the *PDK1* 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 PDK family members, especially for *PDK2* and *PDK4*. It is possible that multiple PDK isoforms have the capacity to respond to Wnt signaling, a possibility underscored by our observation of compensatory recovery of PDH phosphorylation when PDK1 was selectively targeted for knockdown by shRNAs.

Our experiments demonstrate that PDK1 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 PDK1 is only one target of an entire gene program driven by Wnt to promote cancer-supporting metabolism. For example, while PDK1 knockdown caused a significant shift of metabolism in cultures, the shift was not as stable as the dnLEF/TCF treatments. Also, PDK1 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 dnLEF/TCFs (Fig 1 and Supplementary Table S1). This includes genes that encode nutrient and small molecule transporter genes that affect metabolic pathways. For example, the lactate 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 dnLEF/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 (Oshtus *et al.*, 2000; Wise *et al.*, 2008; Dang, 2010). In fact, c-Myc has been shown to enhance HIF-1 α -mediated regulation of PDK1 (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 dnLEF-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, Chafey *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 (Chafey *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 PDK1 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 PDK1 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 PDK1 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 238 human tumors into nine tumor groups with one group of 21 tumors strongly

clustered (group 4, Supplementary Fig S10A). This same cluster was detected when gene signatures were compared via distance measures within the cluster versus the other eight tumor groups (Supplementary Fig S10B). 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 S10C; 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*, 2006; Thompson & Monga, 2007; Colletti *et al*, 2009; Gebhardt & Hovhannisyan, 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; Lyssenko *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 (Stringari *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 HIF1 α stabilization. Consistent with this model, we observe high levels of PDK1 protein and its target, phospho-PDH, at the bottom of human intestinal crypts (Fig 8B). Stabilized HIF1 α is also detected in crypt bases (in addition to the hypoxic region at the tops of crypts as has been reported previously) (Giles *et al*, 2006; Fig 8B). Wnt-stimulated HIF1 α stabilization could contribute to a positive feedback loop to support glycolysis in the intestinal stem cell niche as HIF1 α targets several components of glycolysis. Wnt also plays an important direct role in the maintenance of stem cells (Korinek *et al*, 1998; Nusse, 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; Sutendra *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-1 α protein (Lu *et al*, 2002; Hunt *et al*, 2007; Milovanova *et al*, 2008). Stabilized HIF-1 α could enhance angiogenesis at least partly through upregulation of VEGF (Forsythe *et al*, 1996; Pugh & Ratcliffe, 2003) and SDF-1 (stromal cell-derived factor-1; Karshovska *et al*, 2007). While we observe changes in HIF1 α 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 (Michelakis *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 dnLEF-1 expression are more effective *in vivo* 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 dnLEF-1 and dnTCF-1Emut stable cells were created by transfecting Tet-inducible dnLEF-1N or dnTCF-1Emut into DLD-1 TR7 cells (a generous gift from M. van de Wetering and H. Clevers) as previously described (Yokoyama *et al.*, 2010; Hoverter *et al.*, 2012). Cells were maintained in 500 µg/ml zeocin and 10 µg/ml blasticidin. However, it was found that dnLEF/TCF 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 dnLEF-1 or dnTCF-1Emut was achieved through the addition of 0.01 or 1.0 µg/ml doxycycline to the media, respectively. Lentiviral constructs were cloned via Cold Fusion (System Biosciences) by inserting coding sequences for Flag-tagged dnLEF-1N, Flag-tagged dnTCF-1Emut (Atcha *et al.*, 2007), or Flag-tagged PDK1 [a gift of Jean Zhao (Boehm *et al.*, 2007); Addgene plasmid #20564] into pCDH lentivector (System Biosciences; SBI #CD533A-2). See Supplementary Materials and Methods for cloning information regarding TK and PDK1 reporters.

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 20% 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 was determined using the SRB assay (described below). SW480 cells treated with XAV939 (Sigma X3004) 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-weighed flash-frozen xenograft tumors were homogenized in a Precellys 24 homogenizer in the presence of 10 µl HBSS (Hank's balanced salt solution) per mg tissue at 6,000 rpm, twice, for 15 s each. Tissue extraction and lactate assay were performed according to L-Lactate Assay kit (Eton Bioscience #1200011002). All measurements were performed in triplicate.

Sulforhodamine B cell growth (SRB) 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

irinotecan, or 20 mM DCA as needed). Cells were fixed and stained according to published protocols (Skehan *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 ENLITEN ATP Assay System (Promega #FF2000). 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 dnLEF-1 in DLD-1 cells. Glucose concentration in the media was determined according to Glucose (HK) kit (Sigma GAHK20).

XF24 Extracellular Flux Analyser

Metabolic rates of oxygen consumption (OCR) and extracellular acidification (ECAR) were measured using an XF24 Extracellular Flux Analyser (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 #100777-004). One hour before the assay, growth media were replaced by XF Assay Medium (Seahorse #102365-100) 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 10 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 (35-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. FLIM 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 Hickl 830 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 #2032), PDHK1 (1:1,000 Cell Signaling #3820), c-Myc (1:1,000 Cell Signaling #5605), PDH β Ser293 (1:1,000 Calbiochem #AP1062), HIF1 α (1:1,000 Genetex #GTX127309), AKT (1:1,000 Cell Signaling #9272), phospho-Akt (Ser473; 1:1,000 Cell Signaling #9271), and secondary antibody (1:15,000 anti-rabbit IgG-horseradish peroxidase; Amersham). Blots were imaged on the FujiFilm LAS4000 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 μ g of RNA was reverse-transcribed using random primers according to the High Capacity cDNA Reverse Transcription kit (Invitrogen #4374966). 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 $\Delta\Delta C_t$ 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 1.0 μ g/ml doxycycline treatment of DLD-1 colon cancer cells. 500 μ M 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 (Dölken *et al.*, 2008). Briefly, 4' thiouridine-labeled RNA was biotinylated with 2 μ l biotin-HPDP [Pierce: 1 mg/ml dissolved in dimethylformamide]/1 μ g RNA added to 1 μ l 10 $\times$ biotinylation buffer (100 mM Tris pH 7.4, 10 mM EDTA) and 7 μ l water for 1.5 h at room temperature with rotation followed by RNA precipitation. Biotinylated RNA was separated from bulk, unlabeled RNA using streptavidin-coated magnetic beads (μ MacS Streptavidin kit).

Chromatin Immunoprecipitation (ChIP)

DLD-1 cells with or without 2 h doxycycline treatment (to induce expression of FLAG-dnTCF-1Emut) were cross-linked with 1% formaldehyde in 1 $\times$ PBS for 12 min at room temperature. Cross-linking was quenched with 125 mM glycine for 5 min, and recovered cells were washed with 1 $\times$ PBS. Cellular lysates were balanced for immunoprecipitation by Bradford assay (500 arbitrary units (AU) per sample) and pre-cleared with 30 μ l His-magnetic beads (Invitrogen

10103D) for 30 min prior to the addition of 50 μ l of FLAG antibody-conjugated magnetic beads (Sigma A2220). FLAG antibody beads were pre-blocked by 3 washes with 1 ml 1 $\times$ PBS/BSA solution (5 mg/ml BSA fraction V in 1 $\times$ 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 $\times$ TBS before suspension in proteinase K buffer [30 mM Tris-HCl pH 8.0; 5 μ l 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-PCR) was used to quantify enrichment of *PDK1* gene locus regions. Primers specific to the genomic regions are provided in Supplementary Materials and Methods.

Luciferase assay

Cells were seeded at 2.5×10^5 per 6-well 24 h prior to transfection. Each well was transfected with 0.1 μ g Super8xTopflash (kind gift from Dr RT Moon) or TK or PDK1 luciferase reporters and 0.1 μ g thymidine kinase β -galactosidase plasmid using BioT transfection reagent (Bioland Scientific). Cells were treated with 0.01 μ g/ml doxycycline or 10 μ M 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 transductants for xenograft injection were prepared through lentiviral infection with pCDH vector alone, dnLEF-1 or dnTCF-1Emut, with or without PDK1, followed by selection with 500 μ g/ml G418. Cells (2.5×10^6) were injected subcutaneously into immune-deficient NOG mice. Tumors were removed and measured 4 weeks after injection. Xenograft tumors for FLIM analysis were injected at 5×10^6 cells per tumor and allowed to grow for 3 weeks.

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

All animal procedures were approved by the UC Irvine IACUC. Nine-week-old NOG 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 xenografts' 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 μ m, using FITC/TRITC labeling of vessels to distinguish tumor cells from metabolically distinct endothelial and blood cells. At least three 78- μ m 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- μ m sections of formalin-fixed paraffin-embedded (FFPE) tumor xenografts were stained for Ki67 (Dako #M7240, 1:400 dilution) using a Ventana Benchmark Ultra autostainer with peroxidase-based detection. For each section, the number of positive nuclei per 8–10 fields (at a 20 $\times$ magnification) was manually enumerated and the mean $\pm$ 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 a 1:50 dilution of anti-mouse CD31 (Dianova rat anti-mouse CD-31, #DIA-310), 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 $\pm$ SEM. For PDK staining in small intestine and phospho-PDH and HIF-1 α 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-PDK (Santa Cruz sc-28783, 1:1,000), anti-PDHpSer293 (Calbiochem #AP1062, 1:200), and anti-HIF-1 α (Thermo PA1-16601, 1:500), followed by biotinylated secondary antibodies and visualization using a peroxidase-conjugated avidin-based 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://emboj.embopress.org>

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Author contributions

KTP and MLW conceived of the project. MLW, KTP, CS, EG, TT, MT, and RAE designed experiments, with CS and EG providing direction on FLIM and TT and MT providing Seahorse expertise. CG performed the bioinformatics analysis. KTP carried out experiments on all aspects of the project with assistance from SS-T, NH, and MM. CS performed FLIM experiments and analysis. KW and RAE performed mouse husbandry and mouse manipulation for FLIM. TT carried out the Seahorse experiments. KTP and MLW wrote the manuscript. KTP, CS, RAE, EG, TT, SS-T, MM, NH, MT, and MLW provided critical editing of manuscript. EG, RAE, MT, 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



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 NADP⁺. 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 isoform 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; Possemato 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 entrapped 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 (Nova Biomedical) 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,

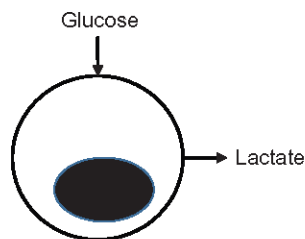


Figure 5.1 *Glucose uptake and lactate excretion approximate glycolytic flux. Additional metabolic processes that siphon or add metabolic intermediates to the glycolytic pathway remain undefined, which may impact glycolytic flux measurements and complicate data interpretations.*

Table 5.1 Methods to detect glucose uptake and lactate excretion						
Method	Detectable metabolite		Equipment	Cell destructive?	Radioactive?	Advantages and disadvantages
	Glucose	Lactate				
Extracellular glucose or lactate kit	Y	Y	Spectrophotometer	N	N	Simple, standard equipment
Extracellular bioanalyzer	Y	Y	Bioanalyzer	N	N	Simple
ECAR measurement	N	Y	Seahorse XF analyzer	N	N	Coupled with oxygen consumption
[³ H]-2-DG or [¹⁴ C]-2-DG uptake	Y	N	Scintillation counter	Y	Y	Expensive tracers
¹⁸ FDG uptake	Y	N	Scintillation counter/ cyclotron	Y	Y	Short half-life
2-NBDG uptake	Y	N	Flow cytometer or fluorescent microscopy	Y	N	Single cell analysis

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 CO₂ 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 2×10^4 – 1×10^5 cells per well. Experimental cell density should be chosen based on three main factors: (1) an ECAR value of at least 20 mpH/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.

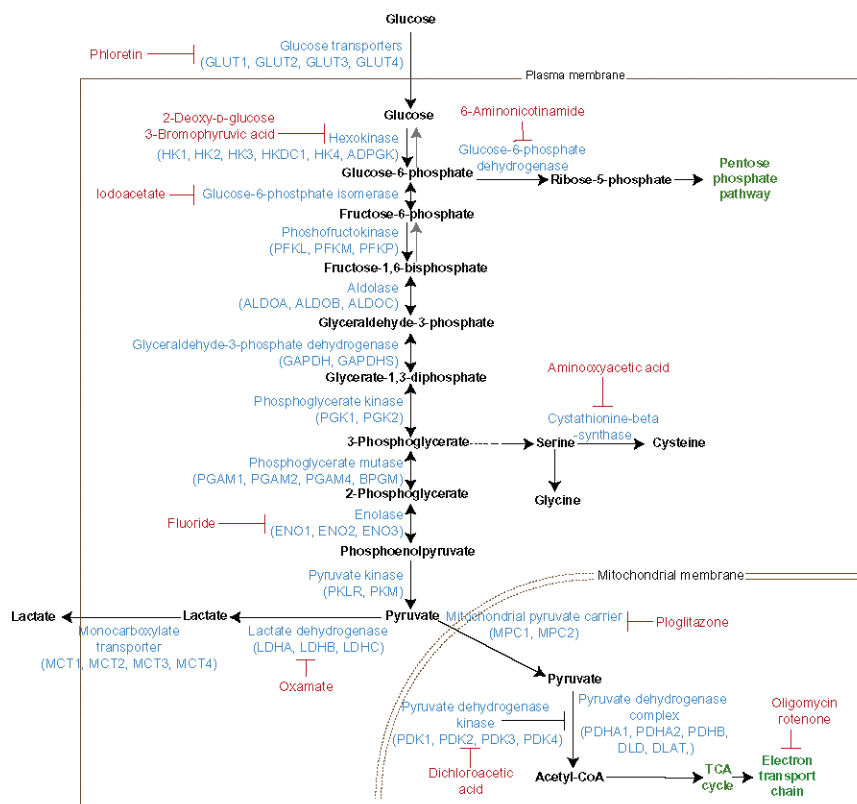


Figure 5.2 Chemical inhibitors of glycolysis. Inhibitors of glycolytic enzymes are used to quantify metabolite fluxes within the glycolytic pathway. Inhibition of glycolysis with 2-deoxy-D-glucose (2-DG) is a common tool for measuring glycolysis (e.g., glycolysis stress test).

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 $1 \times$ PBS, pH 7.4, and add $1 \times$ trypsin until cells begin to visually detach. Add culture media with serum to

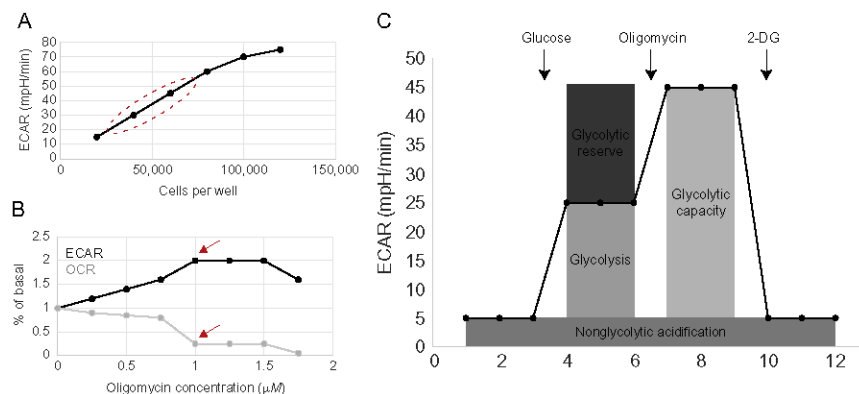


Figure 5.3 ECAR measurements with a Seahorse XF analyzer. (A) Cell number optimization for XF assays. Circle identifies region with acceptable cell densities. (B, C) Oligomycin optimization for glycolysis stress test. Shown are ECAR (B) and OCR (C) with an oligomycin drug titration. 1 μ M is the optimal oligomycin concentration in this case because it stimulates the maximum ECAR (arrow) and inhibits OCR (arrow). (C) Glycolysis, glycolytic capacity, glycolytic reserve, and nonglycolytic acidification can be measured with the glycolysis stress test.

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 μ l (e.g., for 5×10^4 cells per well, suspend cells at a concentration of 5×10^5 cells per ml). Pipette 100 μ l 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% CO₂ incubator to allow cells to adhere for 1–5 h. Add an additional 150 μ l of media and allow cells to grow overnight in a 37 °C, 5% CO₂ 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 CO₂ 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 CO_2 for 1 h before running an assay. CO_2 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 $10\times$ 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):

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

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

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

$$\text{Nonglycolytic acidification} = \text{ECAR after 2 - DG treatment}$$

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-D-[1,2-³H]-glucose, 2-deoxy-D-[1-¹⁴C]-glucose, or 2-deoxy-2-(¹⁸F)-fluoro-D-glucose (¹⁸FDG), or fluorescent glucose analogs, such as 2-[N-(7-nitrobenz-2-oxa-1,3-dioxol-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-D-[1,2-³H]-glucose or 2-deoxy-D-[1-¹⁴C]-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-D-[1,2-³H]-glucose or 2-deoxy-D-[1-¹⁴C]-glucose, the accumulation of 2-DG can be measured to quantify glucose uptake.

2.2.1.1 Protocol for 2-deoxy-D-[1,2-³H]-glucose or 2-deoxy-D-[1-¹⁴C]-glucose uptake

Plate approximately 5×10^4 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 μ Ci 2-deoxy-D-[1,2-³H]-glucose (MP Biomedicals #0127088) or 2-deoxy-D-[1-¹⁴C]-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 [¹⁴C] and normalize readings to cell number.

2.2.2 2-Deoxy-2-(¹⁸F)-fluoro-D-glucose

¹⁸FDG PET scanning has been used in clinical cancer diagnostics since the 1990s, but use of ¹⁸FDG for *in vitro* research protocols has been more limited, likely because of the short half-life of the radioactive fluorine (109.7 min) and limited access to a cyclotron for its manufacture for most research labs. Like other 2-DG analogs, ¹⁸FDG is phosphorylated after transport into the

cell and cannot be exported (Smith, 2000). ^{18}F 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}F 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}F FDG requires instrumentation to measure gamma radiation and increased safety procedures for working with radiation. Because the measurement of ^{18}F FDG uptake *in vitro* is uncommon and an alternate method involving fluorescent detection of glucose uptake is available, a detailed work protocol for ^{18}F 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 5×10^4 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 $1 \times \text{PBS}$, 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% CO_2 . 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.

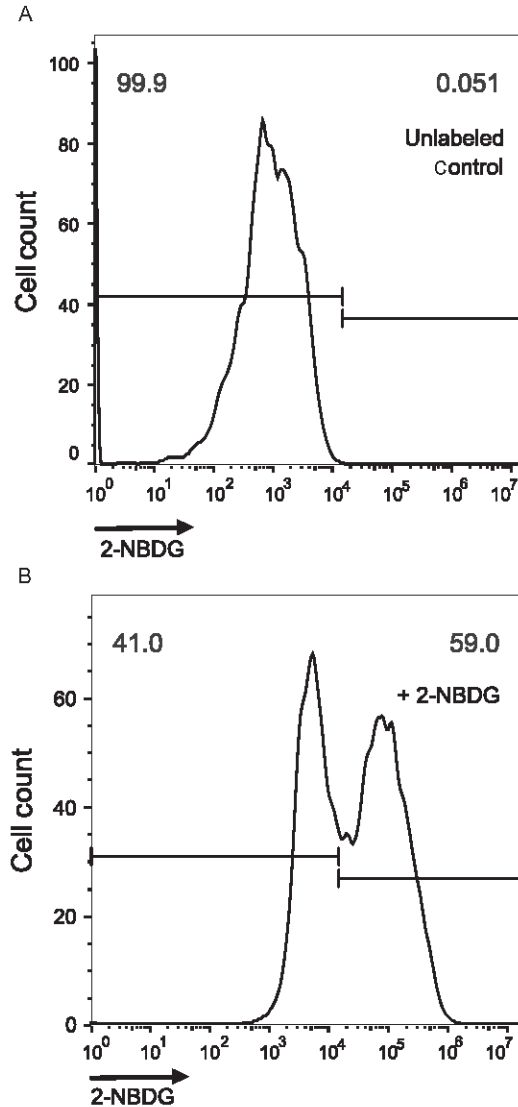


Figure 5.4 Measurement of 2-NBDG uptake by flow cytometry. (A) A negative control that has not been cultured with 2-NBDG. (B) A sample cultured with 2-NBDG.

For analysis by flow cytometry, add $1 \times$ trypsin (Life Technologies #25300054), harvest cells, wash twice with ice cold $1 \times$ 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 $\gamma\text{-PO}_4$ 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 $1 \times$ 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 × 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 MgCl₂, 5 mM ATP (Sigma #A2383), 0.2 mM NADH (Sigma #N4505), 100 mM KCl, 5 mM Na₂HPO₄, 5 mM MgCl₂, 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, Feliú, & 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 (Dombrauckas, 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 × 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 is 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, Nielsen, & 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 1×10^6 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 μ l 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°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 μ l 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°C until analysis. Alternatively, samples can be suspended in 50 μ l 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. $[\text{U}-^{13}\text{C}_6]$ -glucose is ^{13}C labeled at all six carbons and is often used to trace the glucose contribution to the TCA cycle. $[1,2-^{13}\text{C}_2]$ -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 $[\text{U}-^{13}\text{C}_6]$ -glucose. Instead, $[1,2-^{13}\text{C}_2]$ -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 $[\text{U}-^{13}\text{C}_6]$ -glucose (Cambridge Isotope Labs #CLM-1396) or $[1,2-^{13}\text{C}_2]$ -glucose (Cambridge Isotope Labs #CLM-504) supplemented

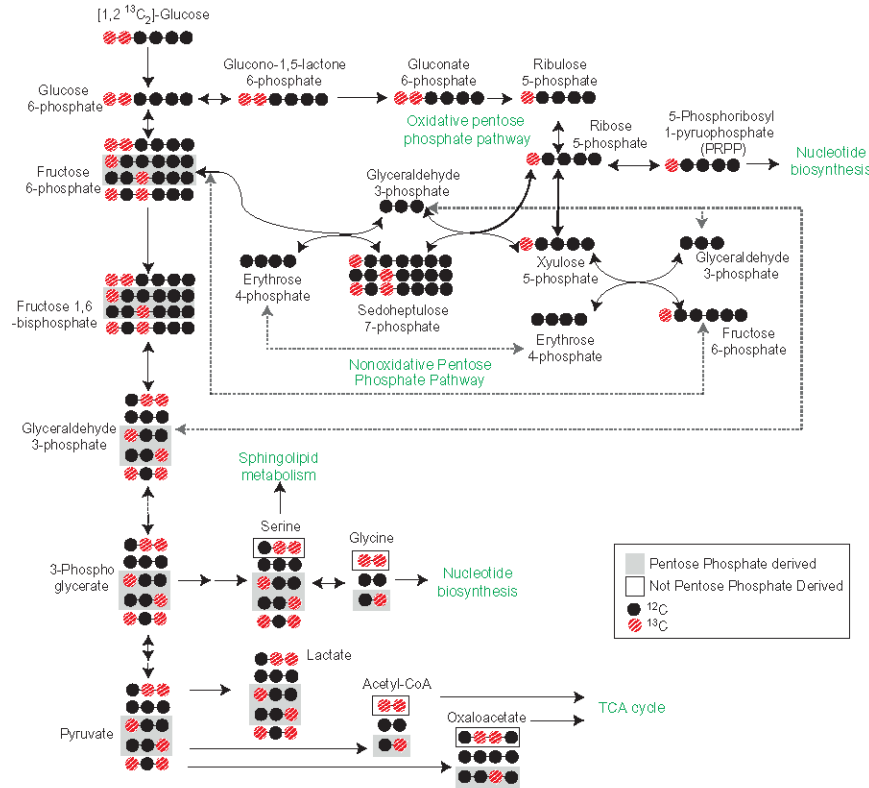


Figure 5.5 Glycolytic pathway metabolite flux analysis with [1,2-¹³C]-glucose. Metabolites derived from the pentose phosphate pathway (gray boxes) can be distinguished from metabolites derived directly from glycolysis (black boxes). For simplicity, not all possible permutations of ¹²C and ¹³C are provided.

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; Weitzel et al., 2013).

4.3. Release of ³H₂O from [5-³H]-glucose

The release of ³H₂O from [5-³H]-glucose is a method to quantify glycolytic flux (Neely, Denton, England, & Randle, 1972). A single tritium at the C5

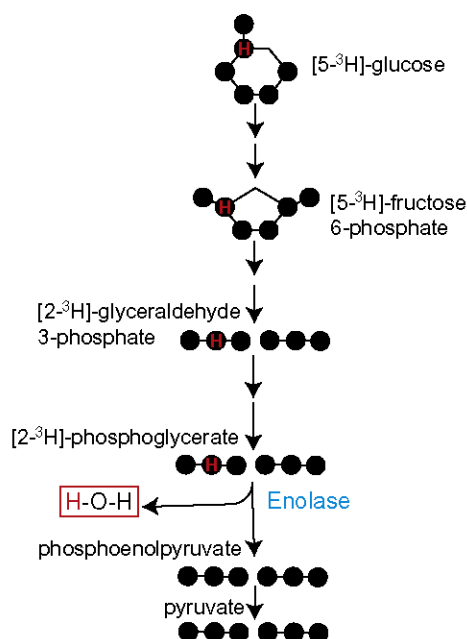


Figure 5.6 Glycolytic flux measurements with [5-³H]-glucose. A single tritium present on 5C of glucose is released as water in the ninth step of glycolysis catalyzed by enolase.

of glucose is removed by a condensation reaction in the ninth step of glycolysis, which is catalyzed by enolase (Fig. 5.6). The resulting ³H₂O 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 ³H₂O using a liquid scintillation counter, the [5-³H]-glucose must be removed from the cell supernatant. One method for separation is to allow ³H₂O to diffuse into a cold H₂O 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 ³H₂O from [5-³H]-glucose

Wash 1×10^6 cells with $1 \times$ PBS, pH 7.4, and resuspend in 1 ml Krebs buffer (126 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, and 2.5 mM CaCl₂). Incubate at 37 °C, 5% CO₂ for 30 min and then replace with Krebs buffer containing 10 μ Ci [5-³H]-glucose (Perkin Elmer #NET531005MC) and 10 mM unlabeled glucose.

Table 5.2 Glucose tracers used to measure glycolysis and related metabolic pathways

Glucose tracer	Detection method	Detection precision			
		Glycolysis	PPP	TCA cycle	Price
[U- ¹³ C ₆]-glucose	Mass spectrometry	Low	Low	High	\$
[1,2- ¹³ C ₂]-glucose	Mass spectrometry	High	High	Ok	\$\$
[2- ¹³ C ₁]-glucose	Mass spectrometry	High	High	Ok	\$\$
[5- ³ H]-glucose	Scintillation counter	High	No	No	\$\$\$

PPP, pentose phosphate pathway.

Incubate at 37 °C, 5% CO₂ 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 solution to the column and collect the sample after glucose removal. Dissolve ³H₂O 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 preference 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 protein Bax or Bak [11,18,19]. 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 BH3-only proteins, Bax undergoes a conformational change and insertion 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 fission 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 [27–29]. RA treatment of monolayer culture 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 hPSC 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

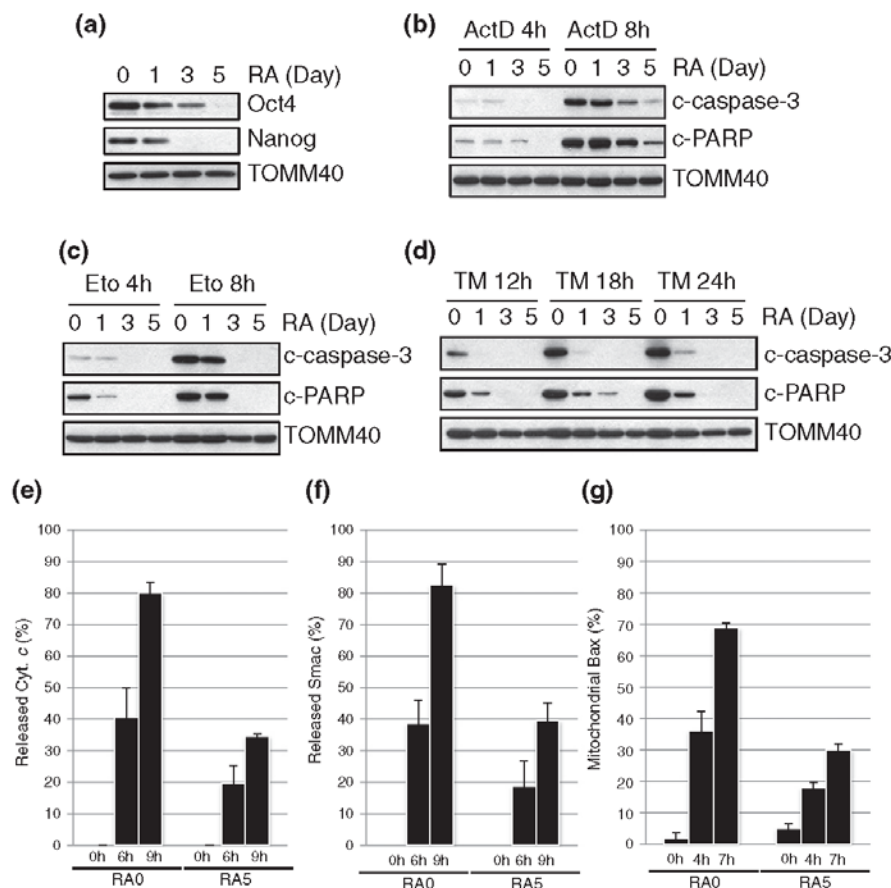


Fig. 1. Rapid apoptosis induction in hESCs. (a) Immunoblot of Oct4 and Nanog in H9 hESCs differentiated with 1 μ M RA for 0, 1, 3, and 5 days. TOMM40 was included as a loading control. (b–d) H9 hESCs differentiated with 1 μ M RA for 0, 1, 3, and 5 days were treated with multiple intrinsic apoptosis inducers: 1 μ M actinomycin D (b), 1 μ M etoposide (c), and 1 μ g/ml tunicamycin (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 and f) H1 hESCs were treated with actinomycin D in the presence of pan-caspase inhibitor Q-VD-OPH. Cells were fixed and IMS protein release of cytochrome c (e) and Smac (f) were detected by immunofluorescence confocal microscopy. Data were obtained from 100 cells in each of three independent experiments. Error bars represent standard deviation (SD). (g) Mitochondria-targeted Bax was detected by immunofluorescence confocal microscopy using anti-Bax and anti-Mortalin (mitochondrial chaperone) antibodies. Cells were treated as in (e) and (f) and Bax and TOMM20 co-localization were counted in each of three independent experiments. Error bars represent SD.

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 [36]. Undifferentiated H9 and HIPS2 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, interlacing network structures that extend peripherally in the cytosol of most differentiated cell types, in contrast to the fragmented, punctate, perinuclear appearance of mitochondria in

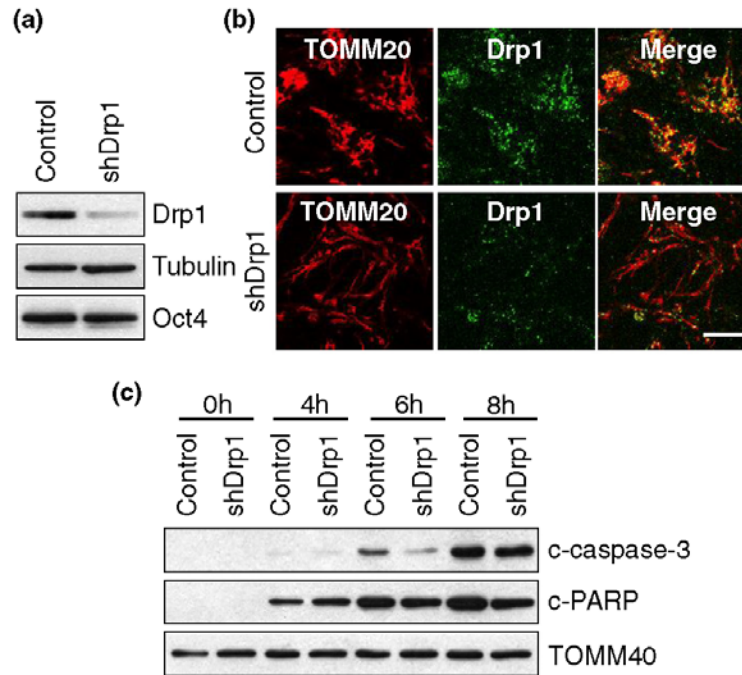


Fig. 2. Knockdown of Drp1 induces mitochondria elongation and suppressed apoptosis sensitivity in hESCs. (a) Knockdown efficiency of Drp1 in H9 hESCs was confirmed by immunoblot. (b) Mitochondrial morphology in H9 hESCs transduced with *Drp1* or control shRNAs was analyzed by immunofluorescence confocal 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 Bax and Bak, 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, Bax has been shown to be important for the apoptotic response to etoposide in hESCs, but the roles of Bax and Bak have not been analyzed in response to other apoptotic stimuli [20]. Therefore, because Bax or Bak is essential for mitochondria-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 Bax and Bak in hESC sensitivity to apoptosis, we separately knocked down the expression of *Bax* and *Bak* with shRNA

in H1 and H9 hESCs (Fig. 3b and Fig. S4a). The expression of pluripotency biomarker proteins SSEA4, Lin28, Nanog, and Oct4 was not affected by knockdown of either *Bax* or *Bak* (Fig. 3c). Activation of apoptosis by exposure to actinomycin D was almost completely inhibited in *Bak* knockdown cells (Fig. 3d and Fig. S4b 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 Bax has an important role in hESC response to etoposide, Bak is important for the response to actinomycin D. This suggests that both Bax and Bak play important roles in hPSC 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 [61,62].

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 H9s, but no change was seen in H1 cells (Fig. S5b). 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

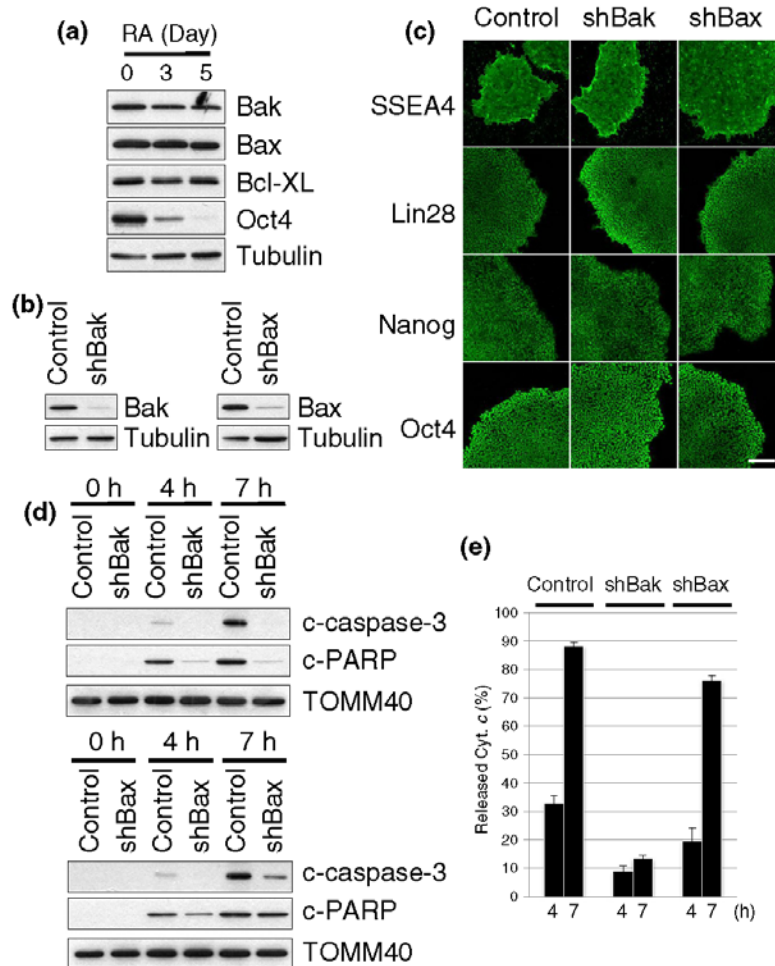


Fig. 3. Bak has a dominant role in actinomycin-D-induced apoptosis of hESCs. (a) Immunoblots of BCL-2 family proteins in H1 hESCs differentiated with 1 μ M RA for 0, 3, and 5 days. (b) Immunoblots showing Bak and Bax protein levels in scramble, *Bak*, and *Bax* knockdown H1 hESCs. Tubulin was included as a loading control. (c) Immunofluorescent detection of pluripotency biomarker proteins from scramble, *Bak*, and *Bax* knockdown H1 hESCs. Scale bar is 200 μ m. (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 nutlin-3a 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 HPS2 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 f). 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

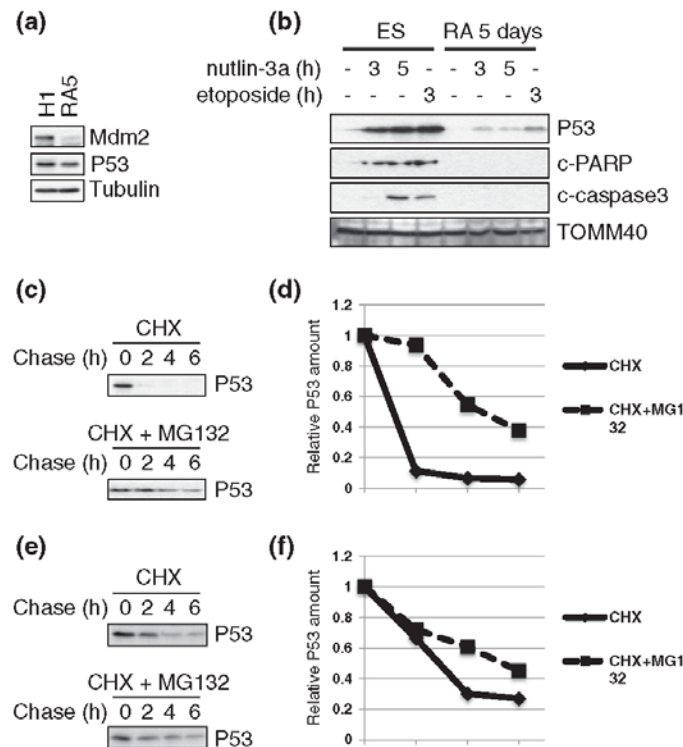


Fig. 4. P53 turnover is rapid in hESCs. (a) Immunoblot of Mdm2 and P53 in H1 hESCs and day 5 RA-induced H1 differentiated cells. (b) Immunoblot of P53, cleaved PARP (c-PARP), and cleaved caspase-3 (c-caspase-3) in H1 hESCs and RA-differentiated H1 cells after 1 μ M etoposide or 4 μ M nutlin-3a for the 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 hiPSCs 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 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 HIPS2 [66] hPSCs 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 passaged with Dispase (Stemcell Technologies) before confluent. Differentiation of hPSCs 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), Smac/Diablo (BD Biosciences), Bax-NT (Millipore), Bak-NT (Millipore), Drp1 (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 P53 (Cell Signaling) proteins. A polyclonal antibody against recombinant TOMM40 (Pacific Immunology) was described previously [67]. CHX, MG132, actinomycin D, and

etoposide were obtained from Sigma; Q-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 the times indicated in each figure legend.

Lentivirus-mediated shRNA knockdown in hESCs

To generate hESCs that are knocked down for *Drp1*, *Bak*, and *Bax*, we obtained shRNAs that specifically target *Drp1* (TRCN0000001097), *Bak* (TRCN0000033466), and *Bax* (TRCN0000033472) from Sigma. On day 1, hESC colonies on matrigel were transduced with 25 μl of 1×10^7 TU/ml of viral particles and 7 μg/ml polybrene for 8 h. On days 2 and 3, 75 μl of 1×10^7 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 *c*/Smac release assays, as well as for Bax targeting analysis, cells were exposed to apoptosis stimuli in the presence of 25 μM Q-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 in PBS followed 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 TRIzol 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 agg cct tgg aac tca agg at and ccc ttg tgc gac ttc agg tg. Primers used to detect *Mdm2* were gac tcc aag cgc gaa aac and ggt ggt tac agc acc atc agt. Primers used to detect *Eifb2* were ttg agg cga tta atg agc tg and tgg agt gaa tgt gct cca ga.

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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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cell fate

Abbreviations used:

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

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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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Abbreviations: BAK, BCL-2 antagonist killer; BAX, BCL-2 associated X protein; CDK1, cyclin dependent kinase 1; CLDN-6, claudin-6; CM, cardiomyocyte; CPE, *Clostridium perfringens* endotoxin; GPX2, glutathione peroxidase-2; GSH, glutathione; GSR, glutathione reductase; GSSG, glutathione disulfide; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; IMS, intermembrane space; MDM2, mouse double minute 2 homolog; MMR, mismatch repair; MOMP, mitochondrial outer membrane permeabilization; NAMPT, nicotinamide phosphoribosyltransferase; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; ROCK, Rho-associated kinase; ROS, reactive oxygen species; SCD1, stearoyl-coA desaturase; UCP2, uncoupling protein 2.

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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 (hiPSCs) [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 low 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 portends 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 mitochondrion. BCL-2 family proteins can be divided into three functional groups: pro-apoptotic effector proteins BAK (BCL-2 antagonist/killer) and BAX (BCL-2 associated X protein), a set of pro-apoptotic BH3 (BCL-2 homology domain 3)-only proteins, and a set of pro-survival proteins that include BCL-2 itself [24]. BAK 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 BAK, which is localized to the mitochondrial outer membrane in both inactive and active conformations [27–29]. Homo-oligomerized BAK 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 [36–38]. *Bak* or *Bax* knockout mice have limited abnormalities, whereas a double knockout of both *Bak* 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 [27,39–41]. BH3-only proteins can directly bind and activate BAK 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 BAK 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 BAK and BAX

Some hESC lines show a unique pattern of BAX regulation during S-phase of the cell cycle, poising 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 mitochondria 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 'primed' 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 [25] 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 hypersensitivity to apoptosis induction that is typical of other hPSC lines [10,11,45]. One interpretation of this data is that hPSC 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, BAK is actually more important in response to other apoptotic insults and cell stresses. For example, the induction of rapid apoptosis in hPSCs by transcriptional inhibition using actinomycin D depends more on BAK than BAX [10]. Overall, some hPSC lines localize active BAX to the Golgi [11], but this unique localization is not required for apoptosis hypersensitivity in all hPSC lines, raising questions about its role in mitochondrial priming 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-

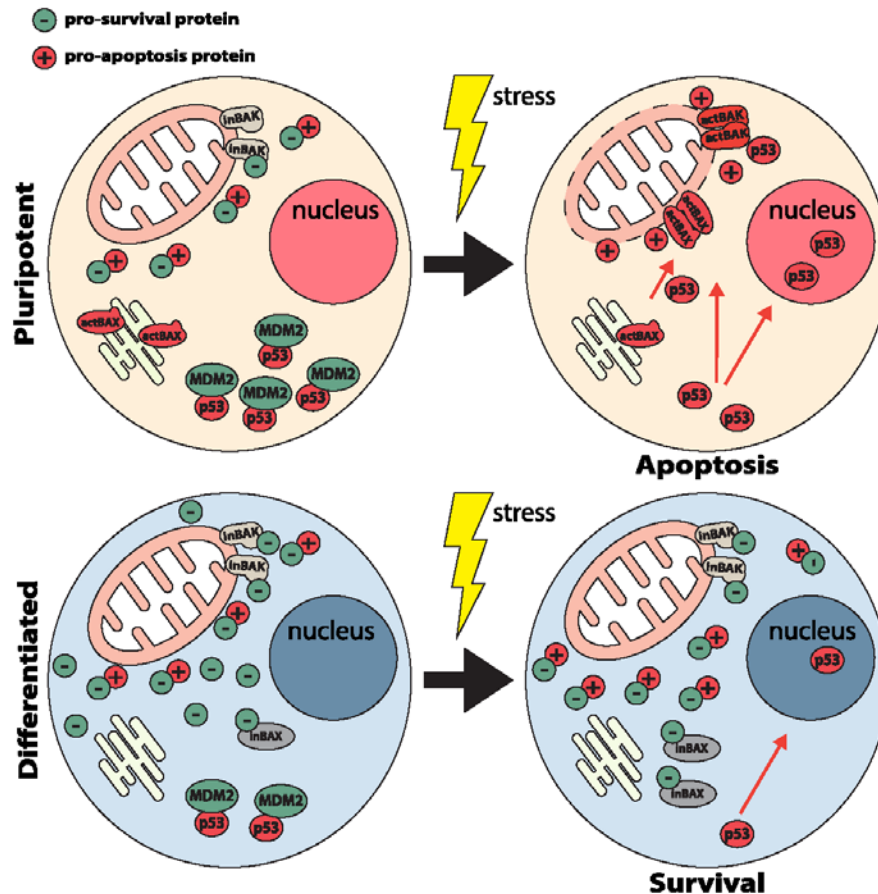


Fig. 1. Mechanisms of hPSC hypersensitivity to apoptosis. hPSCs (top) and differentiated human cells (bottom) are shown before (left) and after (right) exposure to a cellular stress (yellow "lightning bolt"). hPSCs initiate apoptosis (top right) in response to a stress, such as DNA damage, whereas differentiated cells may survive a similar stress (bottom right). Certain hPSC lines contain activated BAX (actBAX) located at the Golgi apparatus. DNA damage causes p53-dependent translocation of actBAX to the mitochondria to initiate MOMP and begin the apoptosis cascade. However, in differentiated cells BAX remains in the cytosol and is maintained in an inactive conformation (inBAX) through interactions with pro-survival proteins (green circles) and requires activation by interactions with BH3-only pro-apoptotic proteins (red circles). hPSCs also show mitochondrial "priming", indicating that the balance between pro-apoptotic and pro-survival mechanisms have been tipped to favor apoptosis compared to differentiated cells. Pro-apoptotic (red circles) and pro-survival (green circles) proteins depict mitochondrial "priming" ratio in hPSCs (top left), in contrast to a more pro-survival protein ratio (bottom left) in differentiated cells. p53 is more rapidly degraded in hPSCs than in differentiated cells. Upon induction of apoptosis, hPSCs rapidly stabilize high concentrations of p53 which induces apoptosis through transcriptional and cytoplasmic/mitochondrial mechanisms of action.

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]. hPSCs 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 hPSCs 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 hESCs 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 hPSCs

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 BAK or BAX oligomerization at the mitochondria [40,43,44,50–52]. 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, hPSCs can still undergo apoptosis induction without p53 transcriptional activity [12]. Therefore, both cytoplasmic and nuclear roles for p53 in apoptosis induction are functioning in hPSCs.

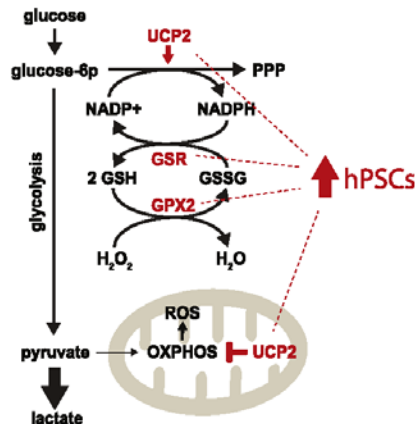


Fig. 2. Elevated expression of antioxidant enzymes drive robust GSH production in hPSCs. hPSCs express high levels of enzymes involved in GSH metabolism, including GPX2 and GSR. GPX2 catalyzes the reduction of hydrogen peroxide to water via the oxidation of GSH to GSSG. To refresh the GSH pool, GSR reduces GSSG to GSH through the oxidation of NADPH to NADP⁺. hPSCs have a high carbon flux through the PPP and low OXPHOS, which is at least partially due to high expression of UCP2 in hPSCs. The PPP produces NADPH which fuels glutathione metabolism. Low OXPHOS limits the ROS produced by the electron transport chain (ETC) of the mitochondrial inner membrane.

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, deacetylation 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.

3.2. Heightened DNA damage protection and repair in hPSCs

Compared to differentiated cells, hPSCs exhibit robust protection from DNA damage and heightened repair mechanisms [5,8,13,58,59]. For example, hPSCs have reduced DNA-damaging mitochondrial reactive oxygen species (ROS), with hiPSCs accumulating less nuclear and mitochondrial DNA damage when exposed to UV light or hydrogen peroxide compared to fibroblasts and most cancer cell lines [13,60]. Glutathione (GSH), a key intracellular antioxidant, is present at higher steady-state levels in hPSCs than fibroblasts, likely from increased expression of many antioxidant and detoxification enzymes (Fig. 2) [13,59,60]. Glutathione

peroxidase-2 (GPX2), which prevents oxidative damage by reducing free peroxides, is highly expressed in hPSCs. GPX2 generates glutathione disulfide (GSSG), which can be reduced back to antioxidant GSH by glutathione reductase (GSR), which is also highly expressed in hPSCs [13]. GSR, however, requires NADPH as a reducing agent cofactor for this reaction. Uncoupling protein 2 (UCP2), which is highly expressed in hPSCs, increases carbon flux through the pentose phosphate pathway (PPP), which produces NADPH (Fig. 2) [18]. Nutrient metabolism also protects hPSCs from oxidative stress by favoring glycolysis over oxidative phosphorylation (OXPHOS) for ATP production, which results in lower ROS production from respiration [18,60]. UCP2 appears well positioned to regulate these activities by reducing electron transport chain activity via transport of C-4 tricarboxylic acid cycle intermediate metabolites out of the mitochondria [18,61].

Generally, hPSCs express DNA damage signaling and repair genes at higher levels than differentiated cells [5,8,59]. hiPSCs also have a more efficient DNA mismatch repair (MMR) pathway than fibroblasts due to higher expression of MMR proteins. Induction of a MMR-dependent response by alkylating DNA damage in hPSCs causes rapid p53 activation and apoptosis in the first cell cycle S-phase as contrasted with an arrest seen in the second cell cycle G2/M-phase after alkylating damage in somatic cell types [9]. Altogether, hPSCs limit DNA damage with reduced oxidative metabolism, high level antioxidant defense mechanisms, and robust DNA damage repair, but when these mechanisms fail, p53 is rapidly stabilized and hypersensitive apoptosis pathway activation is initiated.

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 [64]. 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 most 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 increases 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 [1,65]. To overcome this limitation, inhibition of Rho-associated kinase (ROCK) increases both hPSC survival after dissociation and cloning efficiency [66]. 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. Tumorigenicity 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 [1,2]. 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, hiPSCs 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 hPSC 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 close to 100% efficient, which currently seem unlikely, or to remove pluripotent residual cells while leaving healthy 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], lectin-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 hPSC 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 more 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, pretreatment 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 BH3-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 most ATP by glycolysis and transition to OXPHOS for most ATP production during differentiation [93,94]. Cardiomyocytes (CMs), for example, produce most energy using glucose, fatty acids, and lactate by OXPHOS [95]. 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, PluriSln #1, led to apoptosis after the induction of endoplasmic reticulum (ER) stress, which can be caused by saturated fatty acids [98–102]. PluriSln #1 treatment of a mixed population of pluripotent and differentiated cells for 48 h prevented teratoma formation in mice [98]. SCD1 in hPSCs 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 [60,104,105]. 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. MitoBloCK-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 MitoBloCK-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 *Clostridium perfringens* 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 endoderm cells. CPE utility, 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 [82]. 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 programmed cell death, these cells seem to have diverged. Understanding how cell death mechanisms differ between cancer and pluripotent stem cells can potentially be leveraged to inform therapeutic developments for the benefit of both areas in human health and disease.

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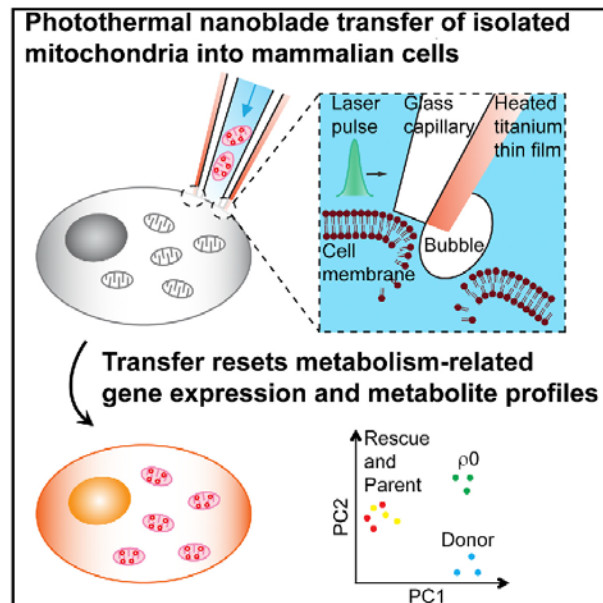
Appendix VII:

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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Mitochondrial Transfer by Photothermal Nanoblade 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 auxotroph phenotype and respiration of p0 cells that lack mtDNA. Three stable isogenic nanoblade-rescued clones grown in uridine-free medium showed distinct bioenergetics profiles. Rescue lines 1 and 3 reestablished nucleus-encoded anapleurotic and cataleurotic 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 in-

herited 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., 2006). 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 humans, 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 (García-Rodríguez, 2007). mtDNA mutations (<http://www.mitomap.org/MITOMAP>) can be silent or cause incurable familial diseases that affect high-energy tissues, including brain, heart, and muscle (Taylor and Turnbull, 2005). Cell dysfunction and disease may arise by a critical reduction in mtDNA number or by exceeding a threshold ratio between mutant and wild-type mtDNAs, creating a heteroplasmic 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 meiotic spindle-chromosomal complex or a polar body to a donor oocyte, or the transfer of pronuclei to a donor egg offers the potential for offspring with healthy mtDNA (Richardson et al., 2015). 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 (Ma et al., 2015; Tachibana 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 (Teslaa and Teitell, 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 mtDNAs by incomplete cleavage of target mtDNAs in a mixture, shifting the heteroplasmy ratio (Bacman et al., 2013).

The insertion or replacement of mtDNA sequences by genome editing tools or mitochondrial-targeted adeno-associated viruses (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 (Alexeyev et al., 2013). Therefore, the acquisition of desired mtDNA haplotypes can only be accomplished by transferring mitochondria containing pre-existing mtDNAs into target cells. Successful approaches include cytoplasmic fusion between enucleated mitochondria donor cells and mtDNA eliminated ρ 0 cells to generate trans-mitochondrial hybrid cell lines (Moraes et al., 2001). Also, direct microinjection of isolated mitochondria into somatic cells or oocytes (King and Attardi, 1988; Yang and Koob, 2012) and the transfer of isolated mitochondria, or mitochondrial transfer between cells, in vivo or in co-culture have been reported (Caicedo et al., 2015; Islam et al., 2012; Liu et al., 2014; Spees et al., 2006). However, microinjection is inefficient, and it remains unclear whether tunneling nanotube 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 ρ 0 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 532 nm nanosecond pulsed laser that illuminates the field of view through the objective lens. A nanoblade delivery micropipette is mounted on a micromanipulator and connected to an external pressure source (Figures S1A and S1B). The micropipette tip is coated with a light-absorbing titanium thin-film $\sim$ 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 ultra-fast (<300 ns) and localized (<0.4 μ m from micropipette rim) cavi-

tation 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 μ m $\times$ 1 μ m-sized mitochondria, the nanoblade was fabricated with a 3 μ m bore tip inner diameter. This wide orifice 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 (mJ/cm²) prior to mitochondria delivery (Wu et al., 2011, 2010). For 143BTK- ρ 0 human osteosarcoma cells, a laser fluence of 108 mJ/cm² yields 64% membrane opening efficiency and 50% viability at 24 hr (Figure S2C). HeLa cells require 67% more laser energy (180 mJ/cm²) for efficient membrane opening and 80% cell viability. Although photo-irradiation by laser illumination can depolarize mitochondria and cause their clearance by mitophagy (Kim and Lemasters, 2011), minimal to no loss of mitochondrial membrane potential ($\Delta\Psi$) occurred in isolated or whole-cell mitochondria, respectively, with repeated 108 mJ/cm² laser pulses (Figure S2D).

Mitochondrial Transfer

Recipient 143BTK- ρ 0 cells were labeled with membrane potential-insensitive MitoTracker Green and seeded onto a 400 μ m $\times$ 400 μ m square on a patterned glass coverslip to simplify tracking (Figure 1A). HEK293T cells expressing mitochondria-targeted DsRed fluorescent protein (pMitoDsRed) were generated. ~ 0.5 mg/ml of isolated DsRed mitochondria were loaded into the nanoblade micropipette and delivered into ρ 0 cells at ~ 100 cells/hr (Figure 1B). Confocal microscopy with z stack reconstruction showed donor DsRed and recipient MitoTracker Green-labeled mitochondria intermixed in the cytosol of recipient ρ 0 cells 4 hr after delivery (Figure 1C).

MDA-MB-453 human breast carcinoma cells have genomic and mitochondrial DNA sequence polymorphisms and a unique MHC haplotype compared to 143BTK- ρ 0 cells, providing distinguishing tags. Oxygen consumption rate (OCR) studies showed isolated mitochondria from 143BTK- parent and MDA-MB-453 cells respire, unlike 143BTK- ρ 0 mitochondria (Figure 1D). Electron transport chain (ETC) coupling to oxidative phosphorylation (OXPHOS) expressed as the respiratory control ratio (RCR, state 3/state 4o respiration) provides an estimate of mitochondrial function. MDA-MB-453 and 143BTK- mitochondria had RCRs of 5.1 and 14.7, respectively, whereas 143BTK- ρ 0 mitochondria had a negligible RCR.

ρ 0 cells are pyrimidine auxotrophs that require uridine supplementation to grow because of an inactive dihydroorotate dehydrogenase (DHOD) enzyme resulting from a non-functional

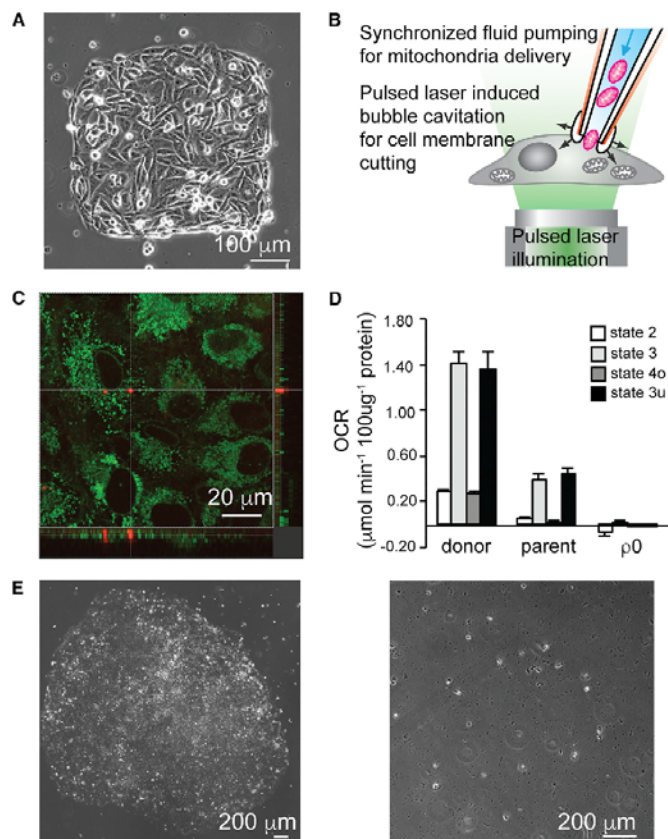


Figure 1. Generating Mitochondrial Rescue Clones by Photothermal Nanoblade

(A) Recipient 143BTK- ρ 0 cells were seeded on a 400 μ m $\times$ 400 μ m square to facilitate nanoblade delivery, tracking, and clonal selection.

(B) Schematic of mitochondrial delivery by photothermal nanoblade. A 3 μ m inner diameter glass microcapillary pipette 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 foci of DsRed-labeled donor mitochondria from HEK293T cells in the cytosol of a single 143BTK- ρ 0 cell whose endogenous mitochondria are stained with MitoTracker Green (upper left quadrant).

(D) Isolated MDA-MB-453 donor and 143BTK- ρ 0 parent cell mitochondria remain functional and coupled. Mean $\pm$ SD (n = 3).

(E) 2 weeks post-nanoblade delivery, donor MDA-MB-453 (and 143BTK- ρ 0 parent, not shown) mitochondria transferred into 143BTK- ρ 0 recipient cells generated "rescue" clones that emerged in uridine-free dialyzed media (left). 143BTK- ρ 0 control cells (or 143BTK- ρ 0 cells that received 143BTK- ρ 0 donor mitochondria, not shown) died and detached 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- ρ 0 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

ETC (Grégoire et al., 1984). MDA-MB-453 mitochondria were loaded and nanoblade delivered into $\sim$ 30 143BTK- ρ 0 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 $\sim$ 2 weeks (Figure 1E). The frequency of stable, rescue clone generation was $2.1\% \pm 3.1\%$, $\sim$ 10-fold higher than microinjection (Table S1) (King and Attardi, 1988).

Clone Validation and Bioenergetic Analyses

Three nanoblade rescue clones were evaluated. Although $\sim$ 15% of cells in rescue clones 2 and 3 had PicoGreen mtDNA staining after 2 weeks in uridine-free medium, $>85\%$ of cells in all three clones had mtDNA staining after 4 weeks (Figure 2A). Total DNA from donor, parent, ρ 0 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, rs2981582, in the nucleus-encoded *FGFR2* gene. Sequencing confirmed all three rescue clones contained exclusively donor mtDNA and ρ 0 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 3B). 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 ρ 0 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 ρ 0 recipient cells. The data reveal a range of functional reconstitution by the nanoblade 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 3D). ρ 0 recipient cells have a granular mitochondrial network (Margineantu et al., 2002) that is retained in rescue clone 1 cells (Figure S3A). Ultrastructure

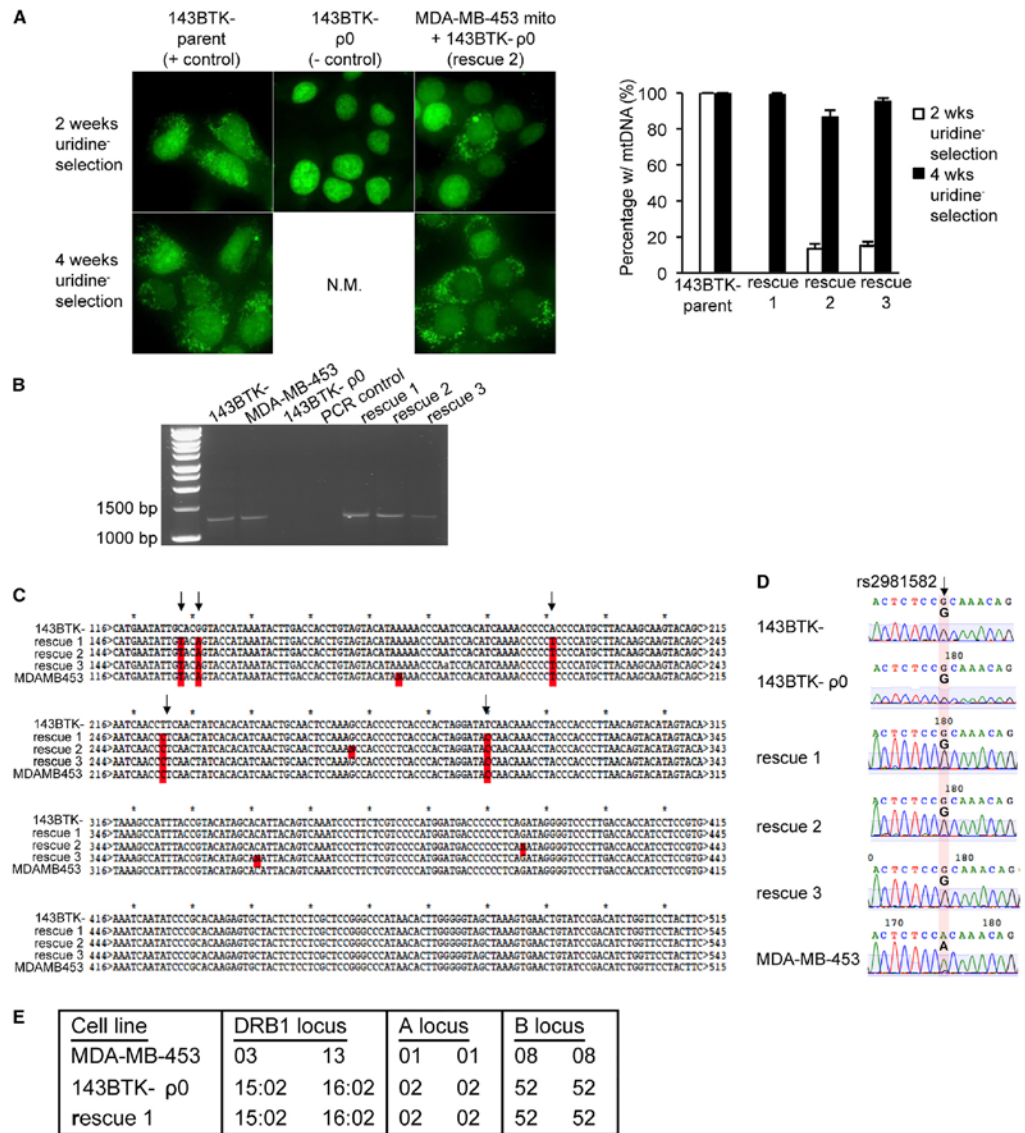


Figure 2. Recipient gDNA-Donor mtDNA Pairing Validates Rescue Clones

(A) PicoGreen staining of mtDNA 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 mtDNA, do not survive for 4 weeks in uridine- medium, and show only nuclear staining. At 2 weeks in uridine- medium, ~15% of rescue clones 2 and 3 cells have mtDNA, which appear as green puncta in the cytoplasm. By 4 weeks of uridine- selection, >85% of cells in rescue clones 1-3 have mtDNA. Mean $\pm$ SD.

(B) PCR of mtDNA D-loop hypervariable region from rescue clones 1-3 with controls.

(C) Sequencing of mtDNA D-loop hypervariable 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 S3B–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 *ND1* 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, $\Delta\Psi$ was quantified with tetramethylrhodamine methyl ester (TMRM). $\rho 0$ cells hydrolyze ATP in mitochondria to maintain viability and showed a low $\Delta\Psi$ (Hatefi, 1985). Rescue clones 1 and 3 had fully restored $\Delta\Psi$ equivalent to parent cells, but rescue clone 2 showed $\Delta\Psi$ restoration in between the parent and $\rho 0$ recipient cells (Figure 3G). Thus, $\Delta\Psi$ provided a potentially superior biomarker for mitochondrial function in rescue clones from nanoblade transfer.

Restoring Metabolism-Related Gene Expression

The introduction of mtDNA into $\rho 0$ cells could affect nuclear gene expression directly, by impacting transcription factors, or indirectly, by changing metabolite levels that regulate signal transduction and epigenetics (Kaelin and McKnight, 2013; Teslaa and Teitell, 2015). Changes to nucleus-encoded gene expression patterns by rescuing $\rho 0$ cells through the transfer of isolated mitochondria have not yet been assessed. Therefore, the steady-state expression of 33 genes encoding anapleurotic or catapleurotic 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 $\rho 0$ recipient. Donor cells showed a distinct gene expression profile compared to the other cell lines. The data indicate that nanoblade transfer of mtDNA resets the nucleus-encoded metabolic enzyme gene expression pattern partially (clone 2) or almost completely (clones 1 and 3) to the parental, 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 cytosolic metabolites that could impact gene expression and cellular biosynthetic functions. Of 12 TCA cycle-related metabolites (sans pyruvate, whose quantification was highly variable), α -ketoglutarate (α -KG) and citrate (Cit) stood out as strongly depleted in $\rho 0$ recipient cells compared to parent cells (Figure 4B). All three rescue clones showed increased α -KG and citrate above those in the $\rho 0$ recipient. In contrast, $\rho 0$ recipient cells accumulated the oncometabolite 2-hydroxyglutarate (2-HG) and succinate (Succ) relative to parent cells. All three rescue clones resolved 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 $\rho 0$ recipient cells by nanoblade. Rescue clones 1 and 3 clustered with parent cells, whereas rescue clone 2 grouped together with $\rho 0$ recipient cells, and donor cells stood apart from both of these clusters. Similar to nucleus-encoded gene expression, nanoblade transfer of mtDNA restores the steady-state metabolite pattern of $\rho 0$ 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 [U - ^{13}C] 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 $\rho 0$ 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 $\rho 0$ 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 photo-thermal nanoblade can transfer isolated mitochondria from an allogeneic cell type to restore metabolic gene expression, global energetic, and metabolite profiles. The nanoblade enables 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 cybrid fusion (Chomyn et al., 1994). Instead, system-wide activities as reflected by $\Delta\Psi$ and global metabolite 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 $\rho 0$ 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.

(D) Rescue clones 1–3 contained the same SNP in the *FGFR2* nuclear gene as 143BTK– parent and 143BTK– $\rho 0$ cells (arrow), but a distinct SNP from mitochondrial donor MDA-MB-453 cells.

(E) Human leukocyte antigen (HLA) analysis of rescue 1 shows the same major histocompatibility complex (MHC) loci as 143BTK– parent and 143BTK– $\rho 0$ cells.

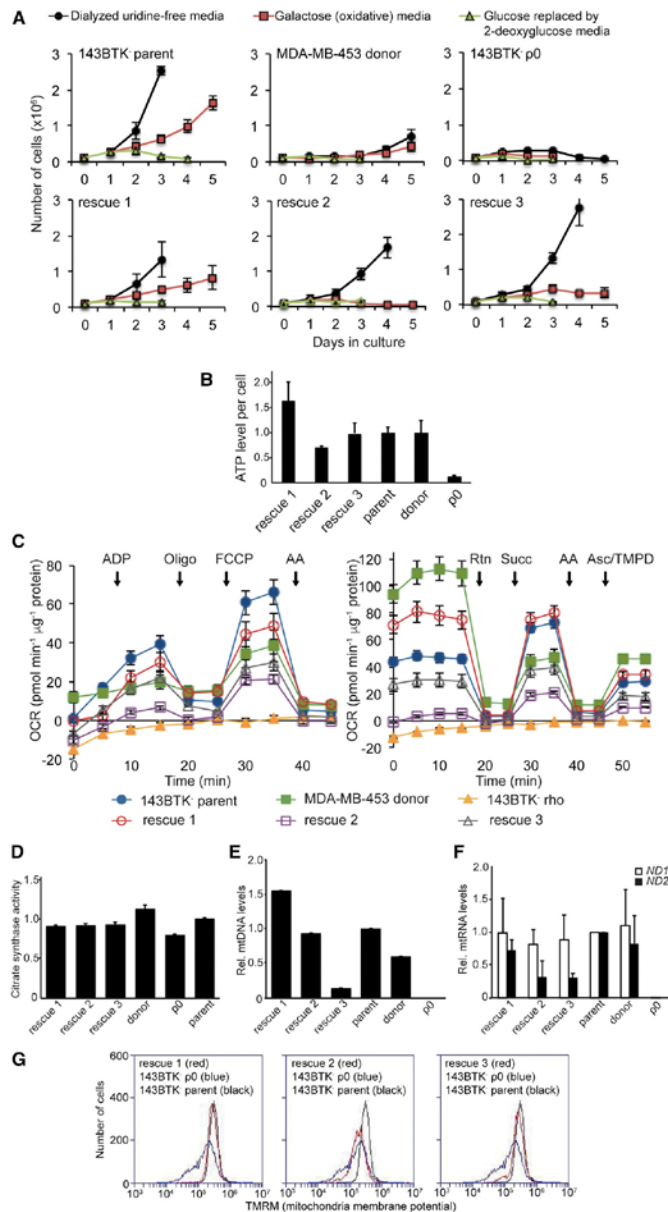


Figure 3. Bioenergetic Profile of Rescue Clones

(A) Proliferation of 143BTK–parent, MDA-MB-453 donor, 143BTK–p0, and rescue clone 1–3 cells in the indicated media formulations. Mean $\pm$ SD ($n = 3$).

(B) Steady-state intracellular ATP levels in arbitrary units. Mean $\pm$ SD ($n = 3$).

(C) Mitochondria coupling assay (left) and electron flow assay (right) as measured by Seahorse XF24 Analyzer. Mean $\pm$ SD ($n = 3$).

(D) Ratio of citrate synthase enzyme activity to total cellular protein, normalized to 1.0 for the 143BTK–parent line. Mean $\pm$ SD ($n = 3$).

(E) mtDNA quantification by qPCR, normalized to 1.0 for the 143BTK–parent line. Mean $\pm$ SD ($n = 3$).

(F) mtDNA-encoded *ND1* and *ND2* transcript quantification by qRT-PCR, normalized to 1.0 for the 143BTK–parent line. Mean $\pm$ SD ($n = 3$).

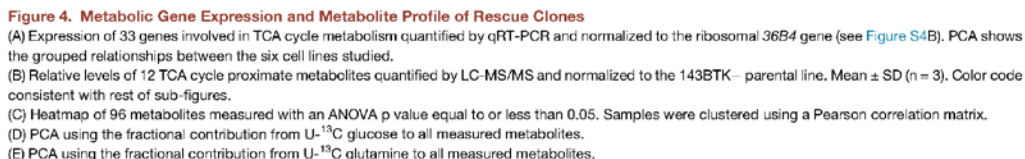
(G) Representative mitochondrial membrane potential quantified by TMRM staining and flow cytometry.

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 heteroplasmy mtDNA competition, 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 (Katrangi et al., 2007; Kitani et al., 2014; Masuzawa et al., 2013).

Mitochondria transfer by photothermal nanoblade is $\sim 2\%$ 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

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

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).



EXPERIMENTAL PROCEDURES

Standard procedures were followed for cell culture, sequencing, oxygen consumption studies, ATP quantification, microscopy, citrate synthase and $\Delta\Psi$ assays, qPCR and qRT-PCR, and metabolomics, as described in the [Supplemental Experimental Procedures](#).

Photothermal Nanoblade

Borosilicate glass micropipettes with a tip $\sim 3\ \mu\text{m}$ in diameter and $\sim 5\ \text{mm}$ long were generated using a micropipette puller (Sutter Instrument, P-97). An $\sim 100\ \text{nm}$ titanium thin film was deposited on the tip and outer walls of pulled micropipettes using a magnetron sputterer deposition system (Denton Vacuum, Discovery 550).

Photothermal Nanoblade Operation and Optimization

The photothermal nanoblade delivery system ([Figures S1A and S1B](#)) uses an inverted microscope (Zeiss, AxioObserver) anchored to an optical table equipped with vibration damping control (Newport, RS Series). A $40\times$ 0.8 NA objective lens was used to view target cell-micropipette tip placement and to channel a pulsed laser beam onto the sample plane. The laser is a Q-switched, frequency-doubled Nd:YAG laser (Continuum, Minilite) with a linearly polarized laser pulse output at 532 nm in wavelength and 6 ns in pulse width. A half wave plate (Newport, 10RP12-16) and a polarizing beam splitter (Newport, 05BC15PH.3) were installed in the laser beam path to adjust the laser energy. The beam was aligned into the epi-fluorescence port of the microscope and reflected into the back aperture of the objective lens onto the sample plane by a dichroic mirror (Chroma, Z532RDC). A long-pass filter (Chroma, HQ570LP) was used to block any back-scattered light from reaching an imaging camera (Hamamatsu, ORCA). An optical diffuser (Edmund Optics, NT55-848) and two convex lenses, $f_1 = 25\ \text{mm}$ and $f_2 = 60\ \text{mm}$, were placed in the beam path to smoothen the laser intensity profile and to control the dimension of the laser spot size at the sample/imaging plane. The laser spot diameter at the imaging plane was $260\ \mu\text{m}$ and covered the entire field of view. A motorized micromanipulator (Eppendorf, InjectMan NI 2) was mounted adjacent to the microscope stage to enable positioning of the titanium-coated micropipette tip using a joystick. A programmable pressure source (Eppendorf, Femtojet) drove liquid and suspended mitochondria into cells. A double pole, single throw switch (Digi-Key, P8101S-ND) was used to coordinately trigger laser pulsing and Femtojet delivery activities.

To optimize the laser intensity for mitochondria delivery, fluorescent dextran ($200\ \mu\text{g}/\text{ml}$ in $1\times$ PBS [pH 7.4]) (Invitrogen, D-3305) was delivered into 20–30 cells at each laser power spanning a range from 72 to $216\ \text{mJ}/\text{cm}^2$. Cell membrane opening efficiency was scored as the number of fluorescent cells divided by the number of delivered cells ([Figure S2C](#)). Cell viability was determined as the number of dextran-containing cells that exclude propidium iodide out of the total number of delivered cells at 2 hr post-nanoblade delivery. The compensation pressure (pc) was set to 5 hPa on the Femtojet so that a trace amount of fluorescent dye discharged from the tip. The injection pressure (pi) was set to 15 hPa, and injection time (ti) was $\sim 0.1\ \text{s}$ to minimize cell lysis from the delivered fluid volume.

Mitochondrial Isolation and Delivery

Mitochondria isolation was performed using standard methods ([Frezza et al., 2007](#)). Briefly, MDA-MB-453 cells were detached from an 80%–90% confluent 15 cm petri dish using a cell scraper and suspended in 1 ml ice-cold isolation buffer, IB_c (10 mM Tris/MOPS, 5 mM EGTA/Tris and 0.2 M sucrose, 1 mM protease inhibitor [Sigma, P8340] and 0.2% BSA, adjusted to pH 7.4). Cell suspension was homogenized using a Teflon pestle in a glass potter with 30 strokes on ice and the mitochondrial fraction obtained after centrifugation washes. Isolated mitochondria pellet was resuspended in ice-cold experimental buffer, EB_c (125 mM KCl, 10 mM Tris/MOPS, 1 mM EGTA/Tris, 0.1 M sucrose, 1 mM KH_2PO_4 , 1 mM protease inhibitor, and 0.2% BSA, adjusted to pH 7.4). Resuspended mitochondria (0.5 mg/ml protein concentration) were kept on ice until delivery. $\sim 8\ \mu\text{l}$ of isolated mitochondria suspension was loaded into a nanoblade micropipette. Nanoblade deliveries were done into ~ 30 cells per experiment ([Table S1](#)), and successful delivery was visualized by transient cytosol volume expansion.

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Statistical Analysis

Experiments were carried out in triplicate, and data represent the mean $\pm$ 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/10.1016/j.cmet.2016.04.007>.

AUTHOR CONTRIBUTIONS

T.-H.W. and E.S. engineered the photothermal nanoblade platform. T.-H.W., E.S., D.C., X.Z., J.S.H., A.N.P., J.M.M., and K.N. generated mitochondrial rescue cell lines and molecular and cellular data. Y.L., T.T., D.B., and T.G.G. generated and analyzed metabolite profiling data. T.-H.W., C.M.K., P.-Y.C., and M.A.T. designed the research and/or analyzed data with help from all other authors. T.-H.W. and M.A.T. wrote the paper with help from E.S., D.C., D.B., C.M.K., and P.-Y.C.

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