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Studying Myc's Role in Metabolism Regulation

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Abstract

The *MYC* oncogene encodes a master transcription factor, Myc, which regulates genes involved in ribosome biogenesis, lipid synthesis, nucleic acid synthesis, intermediary metabolism, and cell growth and proliferation. The genomics of Myc target genes has been well-established through global mapping of Myc binding sites in a variety of different cancer cell lines. These studies highlight the importance of Myc in regulating glucose and glutamine metabolism as well as mitochondrial and ribosomal biogenesis. These genomic studies, however, only become relevant with the companion metabolic studies using a variety of methods to measure oxygen consumption, glucose uptake, or metabolic pathways based on ^{13}C -labeled glucose or glutamine uptake. These methods are described herein.

Keywords

MYC; Cancer metabolism; Oxygen consumption; Glucose uptake; Stable isotope resolved metabolomics (SIRM); ^{13}C -labeled glucose or glutamine

1 Introduction

We have contributed to a richer understanding of the function of the *MYC* oncogene over the last several decades [1, 2]. Having established that the oncogenic Myc protein functions mainly as a transcription factor that dimerizes with Max, we have focused on the regulation of Myc target genes and how these genes confer Myc-mediated cellular phenotypes [3]. Studies from the last several decades indicate that Myc is a master regulator of cell growth through its direct regulation of ribosomal biogenesis coupled with regulation of cell metabolism [4, 5]. Once cell size reaches a critical point, Myc collaborates with E2F to trigger entry into S phase through direct regulation of nucleotide metabolism and DNA replication [6, 7]. Upon completion of S phase, cells in G2 phase depend on Myc to traverse through mitosis to complete their duplication [2]. The *MYC* gene is activated by growth factors, is sensitive to nutrient availability, and is diminished by nutrient deprivation and hypoxia [8]. By contrast, dysregulated MYC-induced cancer cells undergo enforced cell mass accumulation and hence are addicted to nutrients, whose withdrawal triggers metabolic catastrophe and death of cells with sustained MYC expression [1]. We believe that this difference provides a therapeutic window for metabolic therapy; however, there are significant similarities between normal activated lymphocyte metabolism and the cancer metabolism of lymphoma cells [9]. As such, an additional therapeutic opportunity could

exist in the window between circadian regulation of normal cell metabolism and the dysregulated, sustained non-circadian cancer metabolism (Fig. 1).

Our laboratory documented through genomic studies that Myc regulates ribosome biogenesis (cell growth) and virtually all genes involved in glycolysis, mediating the conversion of glucose to pyruvate [10]. We first identified that Myc directly regulates lactate dehydrogenase A (LDHA), which converts pyruvate to lactate, conferring aerobic glycolysis or the Warburg effect [11]. The Warburg effect, however, is unable to account for the composition of a growing cancer cell, which depends on more than just glucose for cell mass accumulation and cell division [12 – 14]. In this regard, we recently documented that Myc also regulates glutamine metabolism by inducing key glutamine plasma membrane transporters, such as SLC1A5 (ASCT2), and glutaminase (GLS), which converts glutamine to glutamate, for its further oxidation in the TCA cycle as α -ketoglutarate [15]. We also documented that Myc directly stimulates genes involved in mitochondrial biogenesis and enhances cellular respiration [16]. In this regard, proliferating cells use aerobic glycolysis to shunt carbon skeletons toward ribose and glycerol. They also depend on a hybrid TCA cycle comprising of both glucose and glutamine carbons to provide the necessary building blocks, such as fatty acids, amino acids and nucleotides, for the proliferating cancer cell [17, 18]. Thus, Myc promotes the acquisition of nutrients and energy for cell growth as well as regulating genes directly involved in ribosome biogenesis and cell proliferation [5].

Cell growth requires the generation of new proteins, lipid membranes, complex carbohydrates, and nucleic acids. As such, Myc appears to be involved in all these processes, by stimulating the expression of a plethora of metabolic genes prior to DNA replication [3]. Acute in vivo adenoviral-mediated overexpression of MYC in hepatocytes results in massive cellular hypertrophy with little evidence of cell proliferation, indicating that Myc is sufficient to induce ribosome biogenesis, increase cell size, and trigger G1 checkpoint, such as a p53-dependent one [19]. Biomass accumulation, however, should be accompanied by a commensurate amount of nutrients and bioenergetics, such that constitutive biomass accumulation enforced by ectopic Myc renders cells addicted to a constant supply of bioenergetic substrate. Indeed, we documented that ectopic Myc expression induces glucose-dependent apoptosis, and Yuneva et al. demonstrated that human cells overexpressing MYC are addicted to glutamine [20, 21]. These observations suggest that inhibition of enzymes involved in glucose or glutamine metabolism should be toxic to Myc-dependent cancer cells. In this regard, we provided proof-of-concept experiments that inhibition of LDHA with FX11 or glutaminase with BPTES or 968 could curb tumor progression in vivo [18, 22, 23].

Because normal lymphocytes (and presumably adult tissue stem cells) depend on Myc-mediated glycolysis and glutaminolysis for mitogenesis [9], pharmacological inhibition of metabolic enzymes could have undesirable immunosuppressive or myelotoxic effects [24]. Normal leukocytes, however, do display circadian gene expression, which is disrupted in chronic myelogenous leukemia cells [25]. In this regard, we began to determine whether ectopic Myc might disrupt cellular circadian rhythm in cancer cells, rendering them continuously susceptible to metabolic therapies while sparing normal cells at time points when normal metabolic activity is lowest. Thus, we hypothesize that the metabolic

therapeutic window would be widened at certain times of the day when normal circadian-responsive tissues are at their lowest metabolic rates and hence would be spared from the toxic effects of metabolic inhibitors (Fig. 1).

To fully understand the role of Myc in metabolism, we have determined the role of Myc in mitochondrial biogenesis and established that Myc could increase oxygen consumption and glucose uptake [16, 22, 26]. Further, we have used ^{13}C -glucose or ^{13}C , ^{15}N -glutamine coupled with stable isotope resolved metabolomics (SIRM) methods for tracking the fate of individual atoms from ^{13}C labeled precursors through various metabolic pathways [18, 27]. SIRM employs complementary analytical platforms, such as high resolution NMR, FT-ICR-MS, and GC-MS, to detect the magnetic or mass difference between ^{12}C and ^{13}C and other stable isotopes such as ^{15}N . NMR quantifies positional isotopomer distributions (the number and location of ^{13}C or ^{15}N atoms in a molecule), whereas MS methods determine the mass isotopologue distributions (numbers of ^{13}C or ^{15}N atoms in a molecule). In this chapter, we will provide protocols for measuring oxygen consumption, glucose uptake, and preparing isotopically labeled cellular extracts for metabolomics.

2 Materials

2.1 Measuring Oxygen Consumption

1. P493 human lymphoma B cells.
2. RPMI 1640 (Life Technologies).
3. Fetal bovine serum.
4. Penicillin-Streptomycin (P/S).
5. Oxytherm System (Hansatech Instruments Ltd).

2.2 Measuring NBD-Glucose Uptake by Flow Cytometry

1. 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG): 5 mg (Life Technologies).
2. Phosphate buffered saline (PBS).
3. Optional: Propidium Iodide (PI) (Life Technologies).

2.3 Labeling P493 Cells with ^{13}C Glucose or Glutamine

1. D-Glucose- $^{13}\text{C}_6$ (Sigma).
2. L-Glutamine- $^{13}\text{C}_5$, $^{15}\text{N}_2$ (Sigma).
3. RPMI without glucose, with glutamine (Life Technologies).
4. RPMI without glutamine, with glucose (Life Technologies).
5. RPMI without glutamine, without glucose (Teknova, or from Rainbow Scientific, Inc).
6. Dialyzed fetal bovine serum 10,000 MW ready to use (Life Technologies).

7. ^{13}C Glucose labeling medium: 0.2 % ^{13}C (make the stock 25% in PBS and sterile filter), 10 % dialyzed FBS, 1 % penicillin-streptomycin. Bring up the remaining volume with RPMI with glutamine without glucose.
8. ^{13}C Glutamine labeling medium: 2 mM ^{13}C -Glutamine (make the stock 200 mM in PBS and sterile filter), 10 % dialyzed FBS (make the stock 2 M in PBS and sterile filter), 1 % Penicillin-Streptomycin (P/S). Bring up the remaining volume with RPMI with glucose without glutamine.

3 Methods

See Note 1.

3.1 Measuring Oxygen Consumption

Oxygen consumption is measured based on oxygen diffusing through the membrane, which traps a thin layer of electrolyte (KCl solution) on an electrode disc. The generated current bears a direct stoichiometric relation to the oxygen reduced and is converted to a digital signal.

1. Maintain P493 human lymphoma B cells at density of 10^5 cells/ml in RPMI 1640 with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin.
2. Collect 5×10^6 cells in 1 ml medium after a designed treatment time for measuring oxygen consumption using a Clark-type oxygen electrode (Oxytherm System).
3. Set up the Oxytherm System according to the manufacturer's instructions.
4. Pipette cells and medium into the chamber above a membrane that is permeable to oxygen.
5. Record the oxygen consumption using the provided software.

3.2 Measuring NBD-Glucose Uptake by Flow Cytometry

2-NBDG is a fluorescent glucose analog and is used to monitor glucose uptake in live cells. Its molecular formula is $\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_8$ and its molecular weight is 342.26. NBD fluorescence typically displays excitation/emission maxima of ~465/540 nm which can be detected by flow cytometry.

1. Treat the cells for a designed time (*see* Note 2).
2. Add fluorescent 100 μM 2-NBDG, with and without the designed treatment.
3. Return plates in the incubator for 30 min and keep them in the dark before flow cytometry analysis.
4. Remove the incubation medium and wash the cells twice with pre-cold PBS.
5. Resuspend cells in 200 μl pre-cold PBS.

¹Store all stock solutions at -20°C .

²Dissolve 2-NBDG in distilled and filtered water at a concentration of 5 mM.

6. Optional: Add PI at a final concentration of 1 µg/ml.
7. Place samples on ice and perform flow cytometry analysis within 30 min.
8. Measure FL1 and FL3 fluorescence intensity (2-NBDG and PI, respectively) of cells. Glucose uptake is assessed on the sub-population that has 2-NBDG positive and PI negative.

3.3 Labeling P493 Cells with ¹³C Glucose or Glutamine

1. Prepare ¹³C labeling glucose or glutamine medium.
2. Remove the incubation medium and wash the cells twice with PBS.
3. Add ¹³C labeled glucose or glutamine to cells and let them grow for 24 h (*see Note 3*).
4. Use stable isotope resolved metabolomics (SIRM) methods, such as high resolution NMR, FT-ICR-MS, and GC-MS to track the fate of ¹³C glucose or glutamine.

References

1. Dang CV. MYC on the path to cancer. *Cell*. 2012; 149:22–35. [PubMed: 22464321]
2. Eilers M, Eisenman RN. Myc's broad reach. *Genes Dev*. 2008; 22:2755–2766. [PubMed: 18923074]
3. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. *Semin Cancer Biol*. 2006; 16:253–264. [PubMed: 16904903]
4. Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. Drosophila myc regulates cellular growth during development. *Cell*. 1999; 98:779–790. [PubMed: 10499795]
5. van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer*. 2010; 10:301–309. [PubMed: 20332779]
6. Zeller KI, Zhao X, Lee CW, Chiu KP, Yao F, Yustein JT, Ooi HS, Orlov YL, Shahab A, Yong HC, Fu Y, Weng Z, Kuznetsov VA, Sung WK, Ruan Y, Dang CV, Wei CL. Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proc Natl Acad Sci USA*. 2006; 103:17834–17839. [PubMed: 17093053]
7. Liu YC, Li F, Handler J, Huang CR, Xiang Y, Neretti N, Sedivy JM, Zeller KI, Dang CV. Global regulation of nucleotide bio-synthetic genes by c-Myc. *PLoS One*. 2008; 3:e2722. [PubMed: 18628958]
8. Okuyama H, Endo H, Akashika T, Kato K, Inoue M. Downregulation of c-MYC protein levels contributes to cancer cell survival under dual deficiency of oxygen and glucose. *Cancer Res*. 2010; 70:10213–10223. [PubMed: 20980433]
9. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, McCormick LL, Fitzgerald P, Chi H, Munger J, Green DR. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity*. 2011; 35(6):871–882. [PubMed: 22195744]
10. Dang CV, Kim JW, Gao P, Yustein J. The interplay between MYC and HIF in cancer. *Nat Rev Cancer*. 2008; 8:51–56. [PubMed: 18046334]
11. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci USA*. 1997; 94:6658–6663. [PubMed: 9192621]
12. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009; 324:1029–1033. [PubMed: 19460998]

³Adherent cell density in the plate should be about 80–95 % confluence in a 10 cm cell culture. Suspension cells should grow at 10⁵ cells/ml.

13. Dang CV. Links between metabolism and cancer. *Genes Dev.* 2012; 26:877–890. [PubMed: 22549953]
14. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer.* 2011; 11:325–337. [PubMed: 21508971]
15. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature.* 2009; 458:762–765. [PubMed: 19219026]
16. Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, Kim JW, Yustein JT, Lee LA, Dang CV. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. *Mol Cell Biol.* 2005; 25:6225–6234. [PubMed: 15988031]
17. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA.* 2007; 104:19345–19350. [PubMed: 18032601]
18. Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, Tsukamoto T, Rojas CJ, Slusher BS, Zhang H, Zimmerman LJ, Liebler DC, Slebos RJ, Lorkiewicz PK, Higashi RM, Fan TW, Dang CV. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* 2012; 15:110–121. [PubMed: 22225880]
19. Kim S, Li Q, Dang CV, Lee LA. Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. *Proc Natl Acad Sci USA.* 2000; 97:11198–11202. [PubMed: 11005843]
20. Shim H, Chun YS, Lewis BC, Dang CV. A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc Natl Acad Sci USA.* 1998; 95:1511–1516. [PubMed: 9465046]
21. Yuneva M, Zamboni N, Oefner P, Sachidanandam R, Lazebnik Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol.* 2007; 178:93–105. [PubMed: 17606868]
22. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci USA.* 2010; 107:2037–2042. [PubMed: 20133848]
23. Wang JB, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, Wilson KF, Ambrosio AL, Dias SM, Dang CV, Cerione RA. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell.* 2010; 18:207–219. [PubMed: 20832749]
24. Gerriets VA, Rathmell JC. Metabolic pathways in T cell fate and function. *Trends Immunol.* 2012; 33:168–173. [PubMed: 22342741]
25. Yang MY, Yang WC, Lin PM, Hsu JF, Hsiao HH, Liu YC, Tsai HJ, Chang CS, Lin SF. Altered expression of circadian clock genes in human chronic myeloid leukemia. *J Biol Rhythms.* 2011; 26:136–148. [PubMed: 21454294]
26. Kim JW, Gao P, Liu YC, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol.* 2007; 27:7381–7393. [PubMed: 17785433]
27. Liu W, Le A, Hancock C, Lane AN, Dang CV, Fan TW, Phang JM. Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. *Proc Natl Acad Sci USA.* 2012; 109:8983–8988. [PubMed: 22615405]

Fig. 1.
Hypothetical therapeutic window of MYC-induced cancer