Metabolic Specialization of Mouse Embryonic Stem Cells

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Mouse embryonic stem (ES) cells are endowed with four unusual properties. They are exceedingly small, exhibiting an intracellular volume two to three orders of magnitude smaller than that of normal mammalian cells. Their rate of cell division, wherein cell doubling takes place in only 4–5 h, is more rapid than even the fastest growing cancer cell lines. They do not senesce. Finally, mouse ES cells retain pluripotency adequate to give rise to all cell types present in either gender of adult mice. We have investigated whether some or all of these unusual features might relate to the possibility that mouse ES cells exist in a specialized metabolic state. By evaluating the abundance of common metabolites as a function of the conversion of mouse ES cells into differentiated embryoid bodies, it was observed that the most radical changes in metabolite abundance related to cellular building blocks associated with one carbon metabolism. These observations led to the discovery that mouse ES cells use the threonine dehydrogenase (TDH) enzyme to convert threonine into acetyl-coenzyme A and glycine, thereby facilitating consumption of threonine as a metabolic fuel. Here we describe the results of a combination of nutritional and pharmacological studies, providing evidence that mouse ES cells are critically dependent on both threonine and the TDH enzyme for growth and viability.

All types of living cells are endowed with regulatory capabilities that facilitate adaptation to environmental fluctuation and its associated uncertainty. When a cancer cell is deprived of oxygen, for example, it mobilizes the stabilization and activation of the hypoxia-inducible transcription factor (HIF). HIF, in turn, activates an organized battery of genes that allow the cancer cell to run glycolysis more efficiently (Wang et al. 1995; Ivan et al. 2001). More macroscopically, the HIF pathway also allows its associated tumor tissue to recruit vasculature for the enhanced delivery of blood glucose and oxygen. These sorts of metabolic adaptations abound in nature, ranging from the abilities of microbial organisms to utilize different sources of fuel and nutrients, to the ability of worms to arrest larval growth and enter a state of metabolic quiescence in response to either stress or nutrient limitation, to the ability of certain mammals to enter states of hibernation where body temperature drops to a level as low as 5°C above freezing. Certain cell types, including the budding yeast *Saccharomyces cerevisia*, have evolved regulatory strategies that facilitate robust oscillation between the radically different states of oxidative to glycolytic metabolism (Tu et al. 2005; Li and Klevecz 2006). As described in several chapters included in this volume, metabolic oscillation has also been observed in numerous organisms as a function of circadian rhythm.

In searching for new examples of metabolic specialization, we began experiments 2–3 years ago on mouse embryonic stem (ES) cells. We chose to flip over the stone covering mouse ES cells for three reasons. First, ES cells are unusually small—perhaps taking up only 1:1000 the volume of typical mammalian cells. Second,

their rate of growth, as measured by the time taken for cells to undertake and complete the cell division cycle, is incredibly fast. When grown in culture, mouse ES cells divide once every 4–5 h, constituting a rate of cell division faster than even the most aggressive of cultured cancer cell lines. Finally, mouse ES cells are unique in harboring the pluripotent capacity to differentiate into any somatic cell of an adult mouse, including cells of the germ lineage (Evans and Kaufman 1981; Martin 1981).

Our approach was simple; we grew mouse ES cells under conditions where they either remained pluripotent or were triggered to differentiate and then conducted an unbiased survey of the abundance of roughly 100–200 generic metabolites. Standard methods of liquid chromatography–mass spectrometry facilitated the quantitation of these metabolites in ES cells as compared with embryoid bodies that form in response to differentiation cues (withdrawal of leukemia-inducing factor and simultaneous administration of retinoic acid). Cells associated with embryoid bodies are much larger than mouse ES cells, their rate of cell division is slowed considerably, and they no longer retain pluripotency. This survey revealed three categories of metabolites (Wang et al. 2009). The first class consisted of metabolites, including many essential and nonessential amino acids, whose abundance did not change as a function of the conversion of undifferentiated mouse ES cells into embryoid bodies. The second class consisted of metabolites that decreased in abundance as a function of differentiation. Finally, the third class consisted of metabolites that increased in abundance as the cells were triggered to differentiate into embryoid bodies.

Of the eight metabolites that changed in abundance most substantially as a function of ES cell differentiation, six could be interpreted to be related to one carbon metabolism (Fig. 1). 5-Aminoimidazolecarboxamide-R (AICAR), an intermediate in purine biosynthesis, and folic acid were observed at decreasing levels as ES cells differentiated. In contrast, methyl-tetrahydrofolate (mTHF), inosine, guanosine, and adenosine were observed to increase in abundance as ES cells were converted into embryoid bodies.

One possible interpretation of these observations is that one carbon metabolism is rate-limiting for the proliferation of mouse ES cells. If so, this might explain why folic acid levels are high in ES cells relative to embryoid bodies and that the opposite is the case for mTHF.

If ES cells cannot adequately “charge” folic acid into the mTHF form required for one carbon metabolism, this might explain why mTHF levels are so low in ES cells relative to differentiated embryoid bodies. Likewise, because AICAR is an intermediate in the purine biosynthetic pathway that requires one carbon metabolism to be converted into the next step of the pathway, if mTHF levels were rate-limiting in ES cells, this might explain the unusually high AICAR levels in ES cells relative to embryoid bodies. Similarly, because the biosynthesis of purines, including inosine, guanosine, and adenosine, is dependent on one carbon metabolism, and because purines must be consumed at a prolific rate for ES cells to complete the cell division cycle in only 4–5 h, it is possible that the substantive increase in these

Figure 1. Coordinated changes in metabolite abundance during embryonic stem (ES) cell differentiation. Bar graphs show the fold change of indicated metabolites. White and gray bars denote quantifications from the measurements of two daughter ions of each metabolite. Experiments were performed in triplicate, with error bars indicating $\pm$ S.D. AICAR, Aminoimidazolecarboxamide-R; acetyl-CoA, acetyl-coenzyme A; THF, tetrahydrofolate.

metabolites as a function of ES cell differentiation could also be interpreted as a consequence of one carbon metabolism being rate-limiting in ES cells.

Pursuing this line of thought, we focused on the mRNA (messenger RNA) abundance of 16 enzymes known to be involved in the control of one carbon metabolism. Quantitative polymerase chain reaction (qPCR) assays were used to monitor the levels of these mRNAs in ES cells as compared with seven tissues prepared from adult mice (liver, kidney, lung, testis, brain, heart, and intestine). As shown in Figure 2, such efforts revealed unusually copious expression of the mRNA encoding threonine

dehydrogenase (TDH) in mouse ES cells. ES cells express the TDH mRNA at levels between 1000- and 2000-fold higher than that observed for the seven adult mouse tissues chosen for comparison. Moreover, as shown in Figure 3, TDH mRNA, protein, and enzymatic activity all disappear precipitously as mouse ES cells are cued to differentiate into embryoid bodies (Wang et al. 2009). Using an antiserum reagent that specifically binds to the TDH enzyme, we further employed immunohistochemistry to study the localization of TDH in undifferentiated mouse ES cells, as well as cultures of ES cells that had been cued to differentiate for different time intervals

Figure 2. Robust expression of threonine dehydrogenase (TDH) mRNA in ES cells. Quantitative polymerase chain reaction (qPCR) analyses of mRNA abundance of 16 enzymes known to be involved in one carbon metabolism in ES cells as compared with seven tissues of the adult mouse. The 16 enzymes comprised serine hydroxymethyltransferase 2 (Shmt2); methylenetetrahydrofolate dehydrogenase 2 (Mthfd2); methylenetetrahydrofolate dehydrogenase 1-like (Mthfd1l); serine hydroxymethyltransferase 1 (Shmt1); methylenetetrahydrofolate dehydrogenase 1 (Mthfd1); 5-methyltetrahydrofolate-homoserine methyltransferase (Mtr); methylenetetrahydrofolate reductase (Mthfr); thymidylate synthetase (Tys); formiminotransferase cyclodeaminase (Ftdc); phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (Gart); 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (Atic); threonine dehydrogenase (Tdh); 2-amino-3-ketobutyrate coenzyme A ligase (Gcat); glycine dehydrogenase (Gldc); aminomethyltransferase (Amt); dihydrolipoamide dehydrogenase (Dld); glycine cleavage system protein H (Gcsh); sarcosine dehydrogenase (Sardh); dimethylglycine dehydrogenase (Dmgdh); and mitochondrial methionyl-tRNA formyltransferase (Mtfmt). Gldc, Amt, Dld, and Gcsh each encode individual subunits for a holoenzyme. For each qPCR comparison, the tissue sample showing the lowest signal was arbitrarily set at a numerical value of 1. Experiments were performed in triplicate, with error bars indicating $\pm$ S.D.

Figure 3. Measurements of TDH enzyme activity, mRNA abundance, and protein abundance, as a function of ES cell differentiation. (A) Western blotting assays of 293T cells stably transformed with an expression vector encoding a flag-tagged version of TDH. Western blotting revealed no TDH signal in parental 293T cells, an intermediate signal in transformed clone 1, and a higher signal in transformed clone 2. (B) TDH enzyme activity assays for mouse ES cells (ES), untransformed 293T cells (293T), transformed 293T clone 1 cells (C1), and transformed 293T clone 2 cells (C2). Mitochondria were isolated from mouse ES cells and the three 293T clones by differential centrifugation (Wang et al. 2009). Equal amounts (10 mg) of mitochondrial protein were subjected to an assay reaction containing 100 mM Tris-HCl (pH 8.0), 50 mM NaCl, 25 mM threonine, and 5 mM NAD⁺ (nicotinamide adenine dinucleotide) at 25°C. Absorbance at 340 nm was recorded over time on a microplate reader to monitor conversion of NAD⁺ to NADH. (C) TDH enzyme activity present in mitochondrial extracts from undifferentiated ES cells and days 3, 5, and 7 embryoid body cells as determined under the same reaction conditions as in B. (D) Western blotting signals for Oct4, Nanog, TDH, and actin in protein samples prepared from undifferentiated ES cells and days 3, 5, and 7 embryoid body cells. (E) TDH mRNA levels as monitored by qPCR in ES cells and days 3, 5, and 7 embryoid body cells. (Reprinted, with permission, from Wang et al. 2009, ©AAAS.)

(via withdrawal of leukemia-inhibiting factor and exposure to retinoic acid). Immunohistochemical staining revealed exclusively mitochondrial localization of the TDH enzyme and further confirmed that the TDH enzyme disappears almost immediately as ES cells differentiate (Fig. 4).

TDH is the rate-limiting enzyme that catalyzes the conversion of threonine into glycine and acetyl-coenzyme A (CoA; Dale 1978). It is localized within mitochondria, allowing its products to feed directly into their respective metabolic pathways. The glycine generated by TDH-mediated degradation of threonine feeds the glycine cleavage system, thereby facilitating production of 5,10-methylene-THF. The acetyl-CoA produced by TDH is readily available for entry into the tricarboxylic acid (TCA) cycle. The observations outlined thus far give an indication that mouse ES cells might consume threonine as a metabolic fuel in a manner not unlike rapidly growing bacterial cells (Almaas et al. 2004). If TDH were indeed responsible for helping to establish a specialized metabolic state for mouse ES cells, this might explain why acetyl-CoA levels drop as a function of ES

cell differentiation and why threonine levels increase as a function of differentiation (Fig. 1).

As an initial means of testing whether mouse ES cells utilize threonine as a metabolic fuel, we carried out the simple experiment of testing their growth in culture media tailored to be devoid of a single amino acid. "Drop-out" tissue culture media individually missing a single one of the 20 amino acids were prepared and supplemented with 10% fetal bovine serum (FBS). The FBS provided some residual levels of amino acids as well as protein constituents that can be hydrolyzed by cells to produce amino acids. As such, the dropout media were not formally devoid of individual amino acids but probably contained at least 100-fold lower levels of the specified amino acid. With the exception of glutamine, normal tissue culture medium is supplemented with 400 μ M of each of the essential and nonessential amino acids. Glutamine is typically supplemented at the 4 mM level (Dulbecco and Freeman 1959). As shown in Figure 5, ES cell colonies were observed to grow on 19 of the individual dropout culture media in a manner indistinguishable from normal tissue culture medium. The one exception

Figure 4. Immunohistochemical staining of TDH in mouse ES cells as a function of differentiation. Mouse ES cells were grown in chamber slides and subjected to leukemia-inducing factor withdrawal-mediated differentiation. (*Top panels*) Images of an undifferentiated ES colony; (*middle panels*) images of a partially differentiated colony; (*bottom panels*) images of an extensively differentiated colony. Cells were fixed and stained with antibodies specific to the mouse TDH enzyme (Wang et al. 2009). TDH immunoreactivity was visualized using Alexa488-labeled goat–antirabbit secondary antibodies (green). Before fixation, cells were incubated with Mito-tracker dye, allowing visual localization of mitochondria (red). DIC, Differential interference contrast. (Reprinted, with permission, from Wang et al. 2009, ©AAAS.)

was the dropout medium missing threonine. No ES colonies were observed to grow on threonine-deprived culture medium (Fig. 5).

The 20 different amino acid dropout media were further evaluated in cell growth experiments using HeLa cells and NIH-3T3 cells. As shown in Figure 6A, HeLa cell colonies were observed to grow in all 20 culture conditions. HeLa cell colony sizes were observed to be reduced in culture media deprived of either leucine or arginine. In contrast to the effects of threonine deprivation to mouse ES cells, where absolutely no colonies were observed to grow in threonine-depleted culture medium, HeLa cell colony size was indistinguishable upon comparison of threonine-deficient medium to culture medium supplemented with all essential and nonessential amino acids. It was similarly observed that threonine deprivation failed to impede the growth of NIH-3T3 cells (Fig. 6B). These control experiments provided evidence that mouse ES cells are considerably

more dependent on threonine as a supplement to tissue culture medium than HeLa cells or NIH-3T3 cells.

By evaluating the incorporation of tritiated thymidine into DNA synthesis, as a function of both time of threonine deprivation and amount of threonine supplemented into the culture medium, it was possible to demonstrate that threonine deprivation arrests DNA synthesis in mouse ES cells almost immediately after cells are shifted away from normal tissue culture medium and that DNA synthesis requires that culture medium be supplemented with a minimum level of 30–100 μM threonine (Wang et al. 2009). We tentatively conclude that mouse ES cells cease cell division upon threonine deprivation, at least in part, owing to impediments in one carbon metabolism that are codependent on both threonine and the TDH enzyme that catabolizes this amino acid into glycine and acetyl-CoA.

As a second means of testing whether the growth of mouse ES cells might be dependent on TDH-mediated

Figure 5. Growth dependence of mouse ES cells on tissue culture media selectively deprived of individual amino acids. Following plating at single cell density and growth on gelatinized dishes for 6 h, cells of the E14Tg2A line of mouse ES cells were exposed for 36 h to complete culture medium or medium prepared to be missing a single amino acid. Colonies were stained with an alkaline phosphatase detection kit (Chemicon) and photographed under a Zeiss AxioObserver microscope using bright-field optics. The histogram at bottom right reveals colony numbers per plate in complete culture medium or in medium prepared to be missing a single amino acid. (Histogram reprinted, with permission, from Wang et al. 2009, ©AAAS.)

Figure 6. Effects of amino acid dropout on the growth of HeLa and 3T3 cells. HeLa (*A*) and NIH-3T3 (*B*) cells were grown in the indicated media for either 1 wk (HeLa cells) or 3 d (3T3 cells). Cells were visualized by phase contrast microscopy and photographed with a Zeiss AxioObserver microscope. (Figure 6 continued on following page.)

catabolism of threonine, we sought to identify potent and specific chemical inhibitors of the TDH enzyme via high-throughput drug screening. Because ES cells express the TDH mRNA and enzyme at a level approximately three orders of magnitude higher than other cells or tissues of adult mice, we reasoned that selective chemical inhibitors of the enzyme might impede the growth of mouse ES cells without affecting the growth of other cell types. Recombinant TDH enzyme missing its mitochondrial signal sequence was expressed, purified, and characterized for substrate requirements. The K_m for NAD^+ and threonine were 180 and 14 μM , respectively, and the turnover number was roughly 60,000 molecules of threonine consumed per second. Using the high-throughput screening center at University of Texas

Southwestern Medical Center (UTSWMC), we performed a screen for TDH inhibitors by evaluating roughly 200,000 synthetic chemicals present in the library (Alexander et al. 2011). Inhibitors showing a dose-dependent inhibition of TDH enzyme activity in the range of 1–10 μM were counterscreened against hydroxysteroid dehydrogenase (HSDH), the enzyme that is most closely related to TDH in primary amino acid sequence among all dehydrogenase enzymes available in public databases. Compounds that inhibited both TDH and HSDH were eliminated from further study.

These efforts led to the discovery of six closely related quinazolinecarboxamide (Qc) compounds (Fig. 7A). None of the Qc compounds affected the enzymatic activity of hydroxysteroid dehydrogenase, alcohol

Fig. 6. Continued.

dehydrogenase, lactate dehydrogenase, or glucose-6-phosphate dehydrogenase at a concentration of 10 μM . To determine the IC_{50} values of the Qc compounds against TDH, titrations were carried out between 10 nM and 10 μM . As shown in Figure 7B, the Qc compounds revealed IC_{50} values of 500 nM. By constructing Lineweaver–Burk plots for both substrates in the presence and absence of the Qc1 inhibitor, it was possible to show that both K_m and V_{max} were altered, leading to the conclusion that this class of chemicals act via a mixed, noncompetitive mode of enzyme inhibition. Finally, it was possible to show that the Qc compounds represent reversible enzyme inhibitors. After exposure

of recombinant TDH to the Qc1 inhibitor, dialysis of the sample led to full restoration of enzyme activity (Alexander et al. 2011).

Armed with specific and relatively potent chemical inhibitors of the TDH enzyme, we next asked what effect they might have on the growth of mouse ES cells, which express TDH at exceptionally high levels, as compared with NIH-3T3 cells and HeLa cells. NIH-3T3 cells express the TDH gene, as measured by qPCR analysis of its encoded mRNA, at a level more than 1000-fold lower than mouse ES cells (Wang et al. 2009). HeLa cells do not express detectable levels of TDH mRNA or protein, and it is known that during the course of

Figure 7. Chemical structures and potency of TDH inhibitors. (A) Structures of the six most selective and potent chemicals derived from the TDH inhibitor screen. The compounds contain a quinazolinecarboxamide (Qc) scaffold with various peripheral modifications. (B) IC₅₀ values were determined by titrating the compounds from 10 nM to 10 μ M and measuring TDH activity (Alexander et al. 2011). The approximate IC₅₀ for all six compounds was 0.5 μ M. (C,D) Lineweaver–Burk analysis of enzyme inhibition. TDH enzyme activity was assayed in the absence and presence of the Qc inhibitor at the NAD⁺ and threonine concentrations shown. Blue curves depict data obtained in the absence of inhibitor, and red curves depict data obtained in the presence of inhibitor. Both $V_{\max}$ (y intercept) and K_m (x intercept) were altered in the presence of inhibitor, indicative of mixed noncompetitive inhibition.

evolution, the human TDH gene has been inactivated by at least three different mutations (Edgar 2002). As such, one would not anticipate a selective inhibitor of the TDH enzyme to impede the growth of either NIH-3T3 cells or HeLa cells. When tested at 10 μ M, all six of the Qc chemicals completely blocked the growth of ES cell colonies cultured under feederless conditions (Fig. 8). In contrast, even when NIH-3T3 cells and HeLa cells were exposed to a 30-fold higher level (300 μ M) of the Qc class of TDH inhibitors, no impediment of mitotic cell growth was observed (Alexander et al. 2011).

Knowing that TDH gene expression and enzyme activity decline to nearly undetectable levels when mouse ES cells are triggered to differentiate into embryoid bodies,

Figure 8. Effect of TDH inhibition on ES cell growth. Feederless mouse ES cells were cultured on glass chamber slides and imaged using phase contrast microscopy. When treated with vehicle (DMSO) alone, ES cell colonies rapidly grew in size. Upon exposure to the TDH inhibitor, ES cells failed to proliferate, with colony size remaining unchanged for the first 12 h. After 24 h, clusters of densely packed cells became apparent at the surface of the colonies, indicative of cell death.

we tested whether the Qc class of TDH enzyme inhibitors might affect the growth or viability of embryoid bodies. As shown in Figure 9, the Qc1 chemical failed to affect the growth or viability of embryoid bodies after prolonged exposure at 10, 30 and 90 μ M concentrations of the compound. These observations provide evidence that the only cell type that is growth-inhibited by the Qc class of TDH inhibitors is mouse ES cells. Coupled with the earlier data derived from the study of various

Figure 9. Effect of TDH inhibition on embryoid body morphology. Mouse ES cells were grown in suspension without leukemia-inducing factor for 10 d to allow differentiation into embryoid bodies (EB). EBs were then treated for 24 h with vehicle or Qc1 at indicated concentrations. Even when tested at 90 μ M, the Qc1 inhibitor of the TDH enzyme exerted no detrimental effect on EB growth or viability.

dropout culture media, these data provide evidence that mouse ES cells are critically dependent on TDH-mediated catabolism of threonine.

To investigate how the Qc1 inhibitor of TDH might affect the growth of mouse ES cells, feederless cultures of undifferentiated cells were exposed to a level of the chemical that had been observed to fully arrest mitotic growth (10 μ M). Lysates were then prepared in 50% methanol:water at 1, 2, 3, and 4 h postexposure to the Qc1 compound. Samples were subjected to liquid chromatography–mass spectrometry (LC-MS/MS), enabling multiple-reaction monitoring of scores of metabolites (Tu et al. 2007). Little change was observed in the majority of metabolites sampled. Among all metabolites decreasing in abundance as a function of time of exposure to the chemical inhibitor of the TDH enzyme, acetyl-CoA and mTHF were at the top of the list (Fig. 10). These

changes are consistent with the fact that TDH-mediated catabolism of threonine directly yields acetyl-CoA and glycine, with glycine feeding the mitochondrial glycine cleavage enzymes to yield mTHF. Among all metabolites increasing in abundance as a function of Qc1-mediated inhibition of the TDH enzyme, AICAR and threonine topped the list. Because AICAR is an intermediate in purine biosynthesis that cannot proceed to the next step in the purine biosynthetic pathway without one carbon metabolism, we hypothesize that AICAR levels increase because the Qc1 compound blocks the charging of tetrahydrofolate (because TDH is impeded in its production of mitochondrial glycine). The increase in intracellular levels of threonine in response to chemical inhibition of the TDH enzyme is interpreted to reflect the fact that threonine, as the direct enzyme substrate, is poorly catabolized in the presence of the inhibitor.

Figure 10. Accumulation of threonine and AICAR, and depletion of acetyl-CoA and mTHF in ES cells treated with TDH inhibitors. Feederless ES cells were treated with 10 μ M of the Qc1 TDH inhibitor for 0, 1, 2, 3, and 4 h before extraction of metabolites in 50% aqueous methanol and subsequent LC-MS/MS analysis (Tu et al. 2007). Metabolites increasing in abundance as a function of exposure to the Qc1 inhibitor of TDH are shown in red. Metabolites decreasing in abundance are shown in green.

After prolonged exposure of mouse ES cells to the Qc1 chemical inhibitor of the TDH enzyme, ES cells were observed to detach from their associated colony and die. Western blot assays were undertaken using antibodies to cleaved caspase 3 as a means of determining whether the cells might undergo programmed cell death after prolonged inhibition of threonine catabolism. Although cleaved caspase 3 could be detected upon exposure of mouse ES cells to a classical chemical inducer of apoptosis (staurosporin), the Qc1 compound failed to enhance the levels of cleaved caspase 3 (Alexander et al. 2011). In contrast, western blotting assays led to a shift in the lipidation of the LC3 protein, enhancing the ratio of the LC3-II form of the protein relative to LC3-I. A similar shift in lipidation of LC3 was observed upon serum starvation of mouse ES cells. This shift in LC3 lipidation has been firmly implicated in autophagy (Kabeya et al. 2000). As such, it would appear that deprivation of TDH-mediated threonine catabolism induces autophagy in mouse ES cells. Consistent with this interpretation, electron microscopic evaluation of normal ES cells compared with cells exposed for 24 h to the Qc1 inhibitor of TDH led to the formation of membrane-bound organelles having the hallmark features of autophagic vesicles (Alexander et al. 2011).

We conclude by offering the hypothesis that mouse ES cells consume threonine as a metabolic fuel by use of the TDH enzyme. If mouse ES cells are deprived of threonine, they arrest DNA synthesis, discontinue mitotic growth, and die. Likewise, if mouse ES cells are exposed to a chemical inhibitor of the TDH enzyme, they become growth-arrested, enter an autophagic state, and eventually die. We have prepared laboratory mice bearing a targeted disruption in the gene encoding TDH and are now poised to study the role of this specialized metabolic pathway via a genetic approach. The combination of nutritional, chemical, and genetic approaches promises to fully resolve the role of threonine catabolism in mouse ES cells. More detailed studies may help to understand how and why the use of threonine as a metabolic fuel might act to keep mouse ES cells from senescence and how this metabolic state might allow mouse ES cells to retain pluripotency. Finally, we offer the hope that it might some day be possible to engineer the expression of TDH into ES cells derived from other species. Were it possible to program other ES cells to be capable of utilizing threonine as a metabolic fuel, it is possible that this might endow such cells with the unusual properties of pluripotency and absence of senescence that are currently limited to mouse ES cells.

ACKNOWLEDGMENTS

We thank Bruce Posner and his staff within the high-throughput screening core at UTSWMC for help in the discovery of the Qc class of TDH inhibitors, LeeJu Wu for extensive technical assistance, Kosaku Uyeda for advice on one carbon metabolism, Benjamin Tu for help with LC-MS/MS assays, and Andrea Roth for help with preparation of the manuscript. We also acknowledge the financial support provided to S.L.M. by an anonymous donor.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2011 76: 183-193 originally published online November 9, 2011

Access the most recent version at doi:[10.1101/sqb.2011.76.010835](https://doi.org/10.1101/sqb.2011.76.010835)

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