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# Metabolic flux rewiring in mammalian cell cultures

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Continuous cell lines (CCLs) engage in 'wasteful' glucose and glutamine metabolism that leads to accumulation of inhibitory byproducts, primarily lactate and ammonium. Advances in techniques for mapping intracellular carbon fluxes and profiling global changes in enzyme expression have led to a deeper understanding of the molecular drivers underlying these metabolic alterations. However, recent studies have revealed that CCLs are not necessarily entrenched in a glycolytic or glutaminolytic phenotype, but instead can shift their metabolism toward increased oxidative metabolism as nutrients become depleted and/or growth rate slows. Progress to understand dynamic flux regulation in CCLs has enabled the development of novel strategies to force cultures into desirable metabolic phenotypes, by combining fed-batch feeding strategies with direct metabolic engineering of host cells.

## Addresses

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## Introduction

Continuous cell lines (CCLs) require constant availability of carbon, nitrogen, energy (ATP), and reductant (NADPH) to sustain their anabolic functions. Most CCLs, such as those used in industrial bioprocesses, rely heavily upon aerobic glycolysis to supply the energetic demands of cell growth, which involves rapid conversion of glucose to lactate even in the presence of abundant oxygen [1] (Figure 1a). However, glycolysis provides only 2 moles of ATP per mole of glucose consumed, whereas mitochondrial oxidative phosphorylation (OXPHOS) can provide up to 36 moles of ATP from the same amount of glucose. As a result, aerobic glycolysis is considered 'wasteful' from a bioenergetic and biosynthetic standpoint because it does not make efficient use of glucose to supply either ATP or carbon to the cell [2]. Increased consumption of glutamine is also exhibited by many CCLs, but the nitrogen provided

by this substrate is also 'wasted' due to elevated production of ammonium and alanine [3]. These observations imply that the metabolic phenotypes of CCLs are not programmed to economize their use of carbon, nitrogen, or energetic resources, but instead tend to increase their nutrient uptake beyond what is required for growth [4]. This leads to accumulation of excreted byproducts, primarily lactate and ammonium, which reduce cell viability and recombinant protein yields and introduce unwanted variability into cell culture bioprocesses [2].

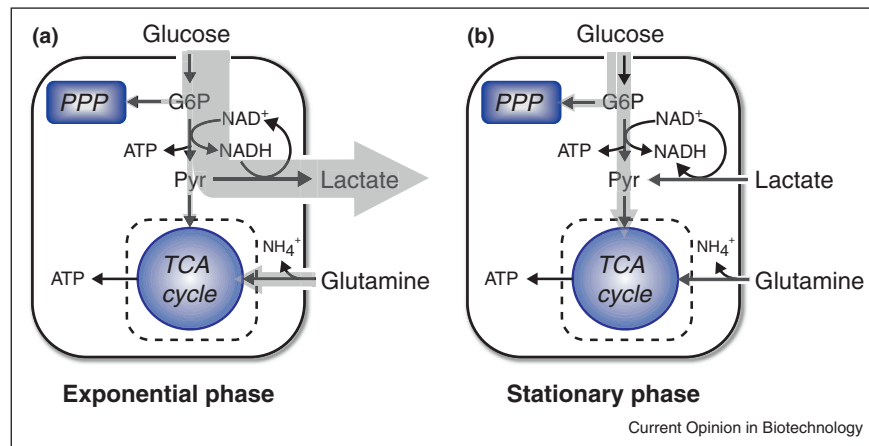
Minimizing wasteful byproduct accumulation has been a goal of the mammalian biotechnology industry for over 25 years, but it still remains a poorly understood and often ill-controlled problem [5]. Furthermore, many production cultures can exhibit dramatic shifts in metabolic phenotype during the course of a typical bioprocess run, yet the molecular mechanisms responsible for dynamic nutrient sensing and metabolic response to changing environmental conditions are only now beginning to emerge [6]. This review aims to present recent progress in understanding the causes and consequences of metabolic reprogramming in mammalian cell cultures, as well as engineering strategies that have been applied to suppress undesirable metabolic phenotypes in industrial bioprocesses. Much of this progress has been enabled by advances in techniques for mapping intracellular carbon fluxes using isotope tracing and metabolic flux analysis (MFA), combined with approaches for profiling global changes in expression and posttranslational modification (PTM) of metabolic enzymes and regulatory proteins.

## Metabolic physiology of mammalian cell cultures

### Why do CCLs rely on aerobic glycolysis for proliferation?

Although hypotheses abound as to the adaptive advantage provided by aerobic glycolysis, little consensus has been achieved in the 85 years since this paradoxical metabolic shift was first reported by the German biochemist Otto Warburg through his studies of rat tumor tissues [7]. It is important to note that this effect is not restricted to mammalian cells, but is in fact analogous to the well-known Crabtree effect whereby yeast shift to aerobic ethanol production during rapid growth on glucose [8] or in response to cell-cycle dysregulation [9]. One explanation for this metabolic flux rewiring is that, while quiescent cells utilize mitochondria chiefly as a catabolic engine to produce ATP, proliferating cells must repurpose their mitochondria to supply biosynthetic intermediates: citrate is exported to supply carbon for lipid biosynthesis, oxaloacetate and alpha-ketoglutarate may be withdrawn for amino acid or nucleotide biosynthesis,

Figure 1



Typical metabolic phenotypes of proliferating and non-proliferating CCLs. **(a)** Exponentially growing cultures exhibit aerobic glycolysis and rely on elevated glutamine consumption to fuel mitochondrial metabolism. This results in increased lactate and ammonium production as cells rewire their metabolism to maintain carbon, nitrogen, and redox balance. **(b)** Stationary phase cultures metabolize glucose mainly by oxidation in the TCA cycle, which provides much higher ATP yields and reduced byproduct accumulation. An increased proportion of incoming glucose is diverted into the oxidative pentose phosphate pathway to maintain NADPH levels.

and flux rerouting via malic enzyme (ME) and isocitrate dehydrogenase (IDH) reactions can be used to supply anabolic reducing power in the form of NADPH [10] (Figure 2). Indeed, <sup>13</sup>C MFA studies have recently shown that flux leaving the TCA cycle as citrate can at times exceed the flux entering it from pyruvate, with the additional citrate supplied by reductive carboxylation of glutamine via IDH operating in the retrograde direction [11,12\*] (Figure 3b). With their mitochondria redirected toward anabolic processes that utilize glutamine as a major carbon substrate, proliferating cultures shift toward aerobic glycolysis to satisfy their cellular demands for ATP production.

#### Metabolic flux rewiring is associated with altered expression and activity of metabolic enzymes

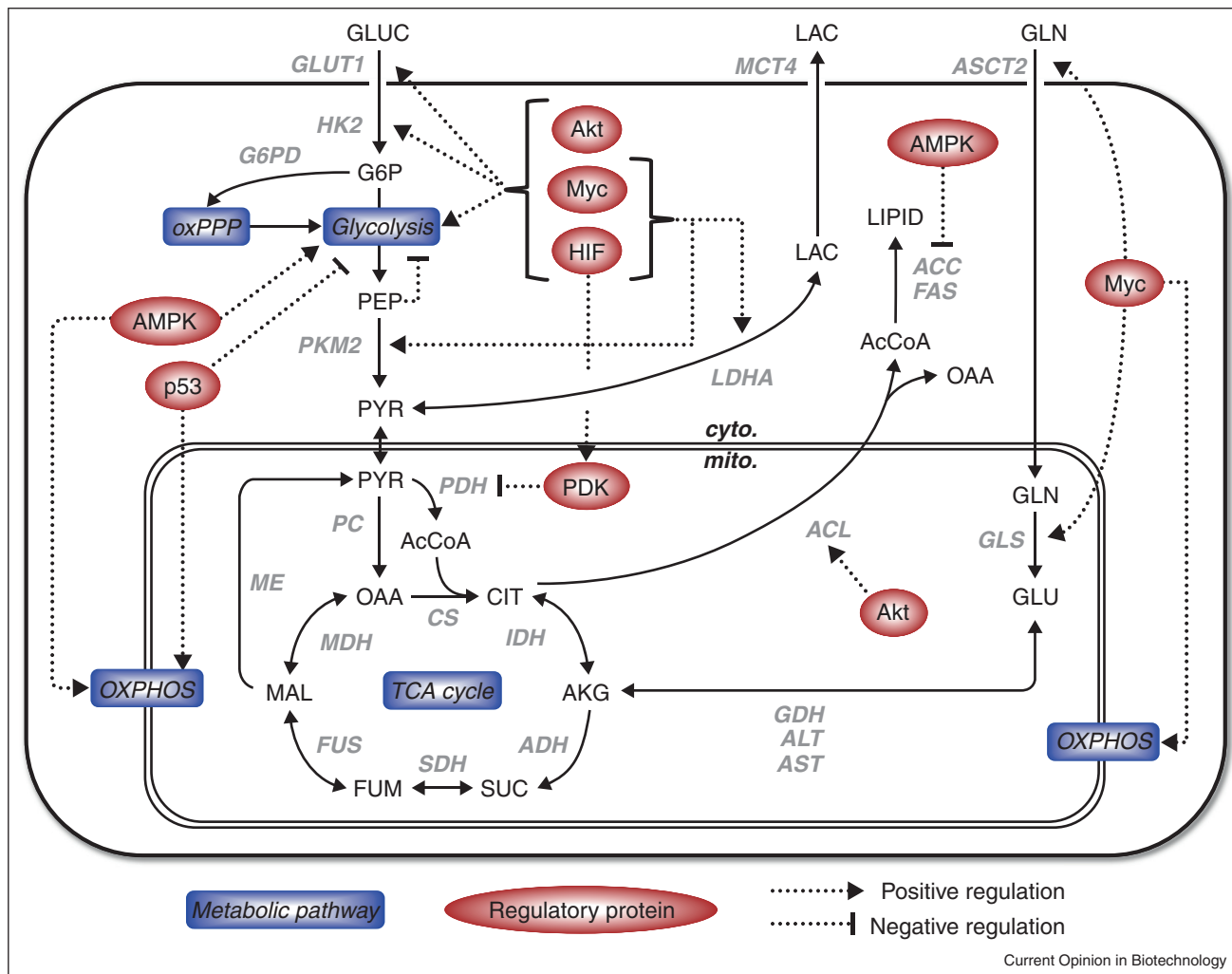
Although the shift to aerobic glycolysis has been a scientific paradox—and a stumbling block of the mammalian biotech industry—for some time, the underlying molecular alterations associated with metabolic flux rewiring in CCLs have been largely undefined. However, recent studies have identified transcriptional and proteomic signatures associated with increased aerobic glycolysis that are conserved across several different cell and tissue types [13]. Overexpression of the high-affinity glucose transporter GLUT1 and the initial glycolytic enzyme hexokinase 2 (HK2) is frequently observed in cancer cells and transformed cell lines [14,15] (Figure 2). Studies in hybridoma, Chinese hamster ovary (CHO), and baby hamster kidney (BHK) cells found that hexokinase activity was consistently lowest among all glycolytic enzymes examined, suggesting that it may be the

rate-limiting enzyme of glycolysis in many industrial cell lines [16,17].

Expression of both GLUT1 and HK2 is controlled by the transcription factors HIF1 and c-Myc [18] and the signaling protein Akt [19], which are often dysregulated in immortalized cells. These same proteins also control expression of several other key glycolytic enzymes that are commonly upregulated in proliferating CCLs (Figure 2): phosphofructokinase 1 (PFK1), the bifunctional enzyme phosphofructokinase 2/fructose-2,6-bisphosphatase (PFK2/FBPase), lactate dehydrogenase A (LDHA), and the M2 isoform of pyruvate kinase (PK) [13]. The connection between increased expression of glycolytic enzymes and cell immortalization is further strengthened by the finding that either spontaneously immortalized mouse embryonic fibroblasts (MEFs) or oncogene-induced human fibroblasts increase their glycolytic rate, and inhibition of any one of many different glycolytic enzymes can induce MEF senescence [20,21].

Upregulation of glycolysis in CCLs is typically accompanied by downregulation of enzymes that facilitate mitochondrial translocation and oxidation of glucose-derived pyruvate. For example, Neermann and Wagner [16] measured no detectable level of pyruvate dehydrogenase (PDH) or pyruvate carboxylase (PC) activity in a wide range of CCL cultures, and similarly Fitzpatrick *et al.* [17] reported no detectable PDH activity in an antibody-secreting murine hybridoma cell line (Figure 2). In contrast, activities of both PDH and PC were detectable in insect cell lines and primary liver cells [16]. Low activity of PDH in CCLs may be attributable to

Figure 2



Major pathways of central carbon metabolism and key regulatory proteins that control enzyme expression and activation. The enzymes PFK1 and PFK2/FBPase (discussed in the text) have been lumped under *Glycolysis* and are not shown explicitly. See list of abbreviations for explanation of nomenclature.

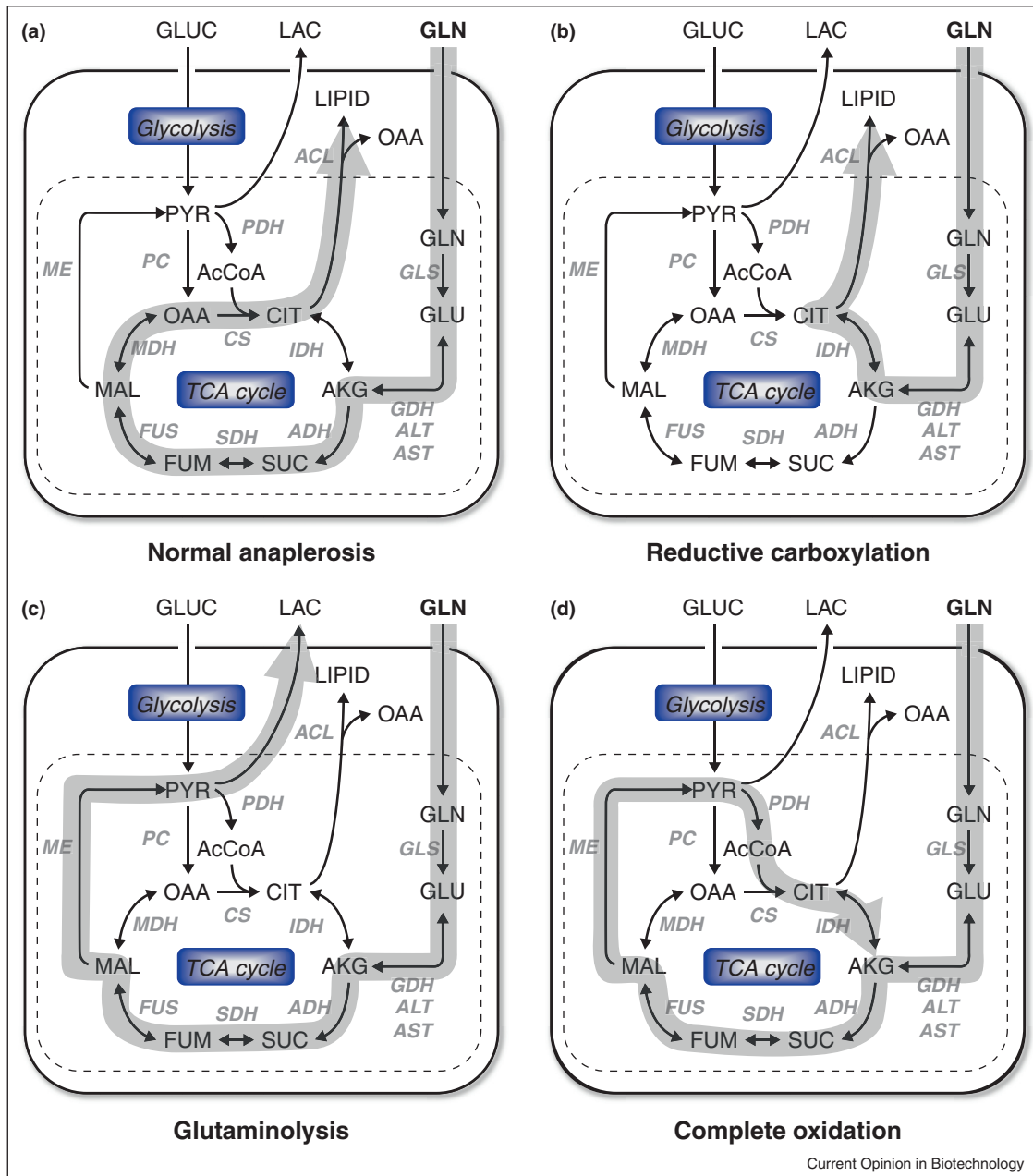
phosphorylation of its E1 $\alpha$  subunit by one of four different pyruvate dehydrogenase kinase (PDK) isoforms, of which PDK1 is known to be a direct transcriptional target of HIF1 [22]. With entry of pyruvate into mitochondria inhibited, CCLs rely on alternative carbon sources — primarily glutamine and, to a lesser extent, asparagine and branched-chain amino acids (BCAAs) — to maintain mitochondrial biosynthetic and bioenergetic functions. Interestingly, recent work has shown that glutamine metabolism is under direct control of c-Myc, providing a molecular explanation for increased glutamine consumption in Myc-overexpressing cells [23,24].

**MFA studies provide a global picture of metabolic flux rewiring**

Intracellular pathway fluxes are the functional end points of metabolism and can be precisely assessed using isotope

tracing and comprehensive MFA experiments [25,26]. Studies of hybridoma [17,27,28], CHO [29,30], BHK [16], and human [3,31,32,33] cell lines confirm that >75% of glucose carbon is typically converted to lactate during exponential phase growth, with <10% diverted into the pentose phosphate pathway to supply nucleotide precursors and <10% oxidized to CO<sub>2</sub> in the TCA cycle (Figure 1a). Glutamine uptake ranged from 10 to 50% of glucose uptake during these studies and was largely metabolized through entry into the TCA cycle via conversion to alpha-ketoglutarate. Glutamine carbon entering the TCA cycle has three possible fates (Figure 3): firstly, conversion to lipids or other macromolecule precursors; secondly, glutaminolysis to form lactate via a truncated TCA cycle; or thirdly, complete oxidation to CO<sub>2</sub>. Due in large part to these glutamine-fueled modes of TCA cycle operation, it has been estimated that

Figure 3



Alternative fates of glutamine carbon entering the TCA cycle. Glutamine carbon can be converted to lipids or other macromolecular building blocks through either **(a)** normal anaplerosis (in the oxidative direction) or **(b)** reductive carboxylation. On the other hand, glutamine can be used to supply ATP and/or NADPH without retention of carbon by either **(c)** glutaminolysis to form lactate + CO<sub>2</sub> or **(d)** complete oxidation to CO<sub>2</sub>.

mitochondrial OXPHOS still contributes  $\geq 50\%$  of cellular ATP production in proliferating CCLs, despite marked upregulation of glycolysis [17,34,35]. In fact, *Le et al.* [36] have recently shown that a human B-cell line can grow in total absence of glucose by relying on complete oxidation of glutamine to generate ATP.

Although controlling cell metabolism during exponential phase is important for maximizing viable cell density

(VCD), specific productivity of recombinant proteins typically does not peak until after the culture has transitioned into stationary phase. For this reason, many industrial bioprocesses involve first growing cells to high density followed by a second phase where growth is slowed but protein production is maintained [37]. As cultures shift to stationary phase, they undergo a dramatic departure from the canonical aerobic glycolysis phenotype observed during exponential phase. This involves

firstly, reduction of specific glucose and glutamine uptake rates while maintaining a similar or elevated TCA cycle flux; secondly, upregulation of oxidative pentose phosphate pathway (oxPPP) flux; thirdly, near complete channeling of glucose-derived pyruvate into mitochondria; and fourthly, in some instances, a full reversal of lactate flux from production to consumption [29<sup>\*\*</sup>,30<sup>\*\*</sup>,38,39] (Figure 1b). As a whole, these observations imply that CCLs transition toward a more oxidative metabolic state as their growth rate slows, which may in turn trigger increased oxPPP flux as an adaptive response to control oxidative stress [39]. This dynamic flux rewiring is likely due, at least in part, to activation of stress signaling pathways involving AMP-activated protein kinase (AMPK) and p53 [13,40,41,42<sup>\*</sup>].

### Impact on current bioprocessing strategies

#### Current mammalian bioprocessing strategies minimize lactate formation by limiting nutrient availability.

Unlike many other cellular processes that are regulated primarily by changes in protein expression, metabolic pathways are able to respond rapidly to changing environmental conditions through dynamic PTM and allosteric control of enzymes. For example, Mulukutla *et al.* [6<sup>\*\*</sup>] recently applied a kinetic model of central carbon metabolism to show that feedback inhibition of PFK1 by lactate can explain the shift from lactate production to lactate consumption that is observed in some fed-batch cultures. Similarly, allosteric regulation of PFK1 could also

underlie bistable switching between high-lactate and low-lactate steady states that has been previously reported in continuous hybridoma cultures [43,44]. The standard industry approach to control this ‘metabolic shift’ and thereby limit lactate accumulation involves expansion of cells in a glucose-limited culture followed by fed-batch feeding in which glucose is restricted to very low levels for the duration of an extended production phase [45]. Closed-loop bioreactor control strategies have been implemented that effectively reduce lactate formation by adjusting the glucose feed rate in response to online pH [46<sup>\*</sup>] or oxygen uptake rate (OUR) measurements [47]. The latter strategy was extended to include simultaneous control of glucose and glutamine feeding, which reduced both lactate and ammonium accumulation and improved peak VCD in fed-batch hybridoma cultures [48].

#### Metabolic engineering can be applied to reduce byproduct accumulation and enhance product titer by genetic manipulation of host cells.

While optimization of nutrient feeding strategies and bioreactor operation has been responsible for much of the progress in mammalian cell culture over the past two decades, metabolic engineering provides an alternative approach to redirect cell physiology toward desired phenotypes. This has potential to minimize the time and cost required to develop customized culture conditions for each new cell line, to eliminate sources of cell-to-cell and

**Table 1**

**Summary of genetic manipulations that have been reported to improve metabolic phenotypes of industrial cell lines.**

Host cell	Genetic manipulation	Phenotypic outcome	Reference
Hybridoma	GLUT1 KD (ASO)	Reduced glucose uptake but clones were unstable	[54]
CHO	OX of GLUT5 fructose transporter	Reduced sugar uptake and lactate production when grown on fructose	[55]
Hybridoma	LDHA KD (HR)	Reduced glycolytic flux; improved VCD and IgG titer	[50]
CHO	LDHA KD (ASO)	Reduced lactate production; suppressed apoptosis	[56]
CHO	LDHA KD (siRNA)	Reduced glycolytic flux; no reduction in growth rate	[57]
CHO	LDHA KD + PDK KD (siRNA)	Reduced lactate production; improved antibody productivity	[58]
BHK	Cytosolic PC OX	Reduced glucose and glutamine uptake; reduced lactate production; increased glucose oxidation and ATP content; improved EPO production	[59,60]
HEK	Cytosolic PC OX	Reduced glutamine consumption; reduced lactate and ammonium production	[61,62]
CHO	Cytosolic PC OX	Reduced lactate production and improved recombinant protein titer; impaired growth	[63]
CHO	Mitochondrial PC OX	Reduced glycolysis; no growth impairment	[64]
CHO and NS0 myeloma	GS OX	Conferred ability to grow in glutamine-free medium, thus reducing ammonium production	[65,66]
CHO	OX of urea cycle enzymes	Reduced ammonium formation; improved growth rate	[67]
CHO	OX of Vitreoscilla hemoglobin	tPA production doubled despite a reduction in growth rate; no metabolic assays reported	[68]
CHO	OX of anti-apoptotic proteins Aven, E1B-19K, and XIAP	Cells switched to lactate consumption during exponential phase, resulting in reduced ammonium formation and improved VCD and mAb titer	[69]

run-to-run variability, and to enable higher VCDs and productivities by relaxing the requirement for strict nutrient limitation [49]. Unfortunately, only a limited number of genetic targets have been explored to date with mixed results (Table 1). For example, partial knockdown of LDHA activity in hybridoma cells was successful at reducing lactate formation, but glutamine consumption remained high while glucose consumption fell, indicating that increased conversion of glucose-derived pyruvate into mitochondria was not achieved [50].

One industrially relevant breakthrough has been the use of the glutamine synthetase (GS) enzyme as a selection marker for amplification of heterologous genes in host cells [51]. Because most CCLs express low levels of GS, which is needed to convert glutamate to glutamine, glutamine is an 'essential' nutrient in mammalian cell cultures. However, GS transfection followed by selection on glutamine-free medium will confer a glutamine-independent phenotype to high-expressing clones. This not only eliminates the cellular requirement for glutamine, but effectively abolishes ammonium production within the culture. Overall, the examples summarized in Table 1 illustrate the promise of metabolic engineering for enhancing cell culture bioprocesses through overexpression of heterologous proteins or knockdown of native enzymes. However, apart from the GS selection system, none of these approaches have found widespread adoption in industry to date [49].

## Conclusions

A recent resurgence of interest in the metabolic adaptations of transformed cell lines and other rapidly proliferating mammalian cells has led to a deeper understanding of the molecular drivers behind their paradoxical flux rewiring. However, CCLs are not generally 'locked' in a glycolytic or glutaminolytic phenotype, but instead can shift their metabolism toward increased OXPHOS or to the use of alternative substrates in response to nutrient depletion, environmental perturbations, or genetic manipulations. This apparent plasticity of cell metabolism has been the source of consternation in the mammalian biotech industry, as it represents a source of cell-to-cell and run-to-run process variability. On the other hand, it also holds promise for strategies that might target flexible metabolic nodes to 'corral' cells into desirable phenotypes, either by clever manipulation of culture conditions and feeding strategies or by direct metabolic engineering of bioenergetic pathways. Indeed, steady progress has been made over the past 30 years to mitigate undesirable metabolic phenotypes, and typical antibody titers have increased by up to three orders of magnitude [2]. Continued progress will depend upon improved understanding of metabolic flux regulation in CCLs and also a deeper appreciation of the tradeoffs inherent to engineering cells to simultaneously achieve

high product titer, high specific production rate, and high product quality [52]. Given the dynamic nature of mammalian metabolic networks, it seems unlikely that static genetic manipulations will provide optimal performance over an entire production run. Instead, it may be necessary to combine programmable gene switches [53\*\*] with closed-loop nutrient feeding strategies to achieve maximum cell culture performance.

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