Journal of Biotechnology 153 (2011) 27–34

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases

Meixia Zhou[a], Yongping Crawford[a], Domingos Ng[a], Jack Tung[a], Abigail F.J. Pynn[a], Angela Meier[a], Inn H. Yuk[a], Natarajan Vijayasankaran[b], Kimberly Leach[a], John Joly[a], Bradley Snedecor[a,*], Amy Shen[a,**]

[a] Department of Early Stage Cell Culture, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA
[b] Department of Late Stage Cell Culture, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA

ARTICLE INFO

Article history:
Received 5 August 2010
Received in revised form 24 February 2011
Accepted 1 March 2011
Available online 8 March 2011

Keywords:
CHO
Simultaneous knockdown
siRNA
LDH and PDHK

ABSTRACT

Large-scale fed-batch cell culture processes of CHO cells are the standard platform for the clinical and commercial production of monoclonal antibodies. Lactate is one of the major by-products of CHO fed-batch culture. In pH-controlled bioreactors, accumulation of high levels of lactate is accompanied by high osmolality due to the addition of base to control pH of the cell culture medium, potentially leading to lower cell growth and lower therapeutic protein production during manufacturing. Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of the substrate, pyruvate, into lactate and many factors including pyruvate concentration modulate LDH activity. Alternately, pyruvate can be converted to acetyl-CoA by pyruvate dehydrogenases (PDHs), to be metabolized in the TCA cycle. PDH activity is inhibited when phosphorylated by pyruvate dehydrogenase kinases (PDHKs). In this study, we knocked down the gene expression of lactate dehydrogenase A (LDHa) and PDHKs to investigate the effect on lactate metabolism and protein production. We found that LDHa and PDHKs can be successfully downregulated simultaneously using a single targeting vector carrying small inhibitory RNAs (siRNA) for LDHa and PDHKs. Moreover, our fed-batch shake flask evaluation data using siRNA-mediated LDHa/PDHKs knockdown clones showed that downregulating LDHa and PDHKs in CHO cells expressing a therapeutic monoclonal antibody reduced lactate production, increased specific productivity and volumetric antibody production by approximately 90%, 75% and 68%, respectively, without appreciable impact on cell growth. Similar trends of lower lactate level and higher antibody productivity on average in siRNA clones were also observed from evaluations performed in bioreactors.

1. Introduction

The market for biopharmaceutical protein products is growing rapidly and the industry is projected to reach $70 billion dollars by 2010 (Walsh, 2009). Due to the increase in demand for therapeutic proteins, there is a need to develop technologies to achieve better productivity. Towards this goal, different approaches including host cell engineering have been explored in CHO cells (Kuystermans et al., 2007; O'Callaghan and James, 2008). CHO cells are widely used to produce therapeutic proteins including recombinant monoclonal antibodies using fed-batch processes in bioreactors with controlled pH (Langheinrich and Nienow, 1999). Lactate is one of the main accumulated waste products during fed-batch culture and it has been shown that high lactate levels can inhibit cell growth and protein production (Glacken et al., 1988; Lao and Toth, 1997). In addition, the accumulation of lactate at high levels requires increased alkali addition to the culture medium to control the pH (Dietl et al., 2010; Langheinrich and Nienow, 1999). Lactate secretion and increased addition of alkali to the medium to maintain pH result in increased osmolality which can inhibit cell growth and lead to lower antibody productivity (Cruz et al., 2000; Irani et al., 1999). Hence, reducing the lactate level is desirable for the development of robust and productive antibody production processes.

There are many factors impacting lactate production in mammalian cell culture including intracellular concentration of pyruvate (Liu et al., 2009; Samuvel et al., 2009). Pyruvate is the substrate for both LDH and PDH and is a key branch point that determines whether the consumed carbon source is excreted as lactate after oxidation through glycolysis or metabolized further in the TCA cycle. LDH directly catalyzes the interconversion of pyruvate

* Corresponding author. Tel.: +1 650 225 6446; fax: +1 650 225 2006.
** Corresponding author. Tel.: +1 650 225 1161; fax: +1 650 225 2006.
E-mail addresses: snedecor.brad@gene.com (B. Snedecor), shen.amy@gene.com (A. Shen).

0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.03.003

and lactate with concurrent interconversion of NADH and NAD+. In mammalian cells, LDHs exist as either homo- or heterotetramers consisting of mostly of A and B subunits encoded by the LDHa and LDHb genes (Baumgart et al., 1996; Li et al., 1983). In CHO cells, the LDH isotype has been shown to be an intermediate of the A3B and A2B2 tetramer (Jeong et al., 2001). Previous studies showed that downregulating LDHa in CHO cells by disrupting the gene via homologous recombination (Chen et al., 2001), antisense technology (Jeong et al., 2001) or siRNA (Kim and Lee, 2007) reduced lactate level, but did not achieve appreciable improvement in protein productivity (Kim and Lee, 2007).

The PDH complex is a multienzyme unit consisting of three catalytic enzymes, E1, E2, and E3 (Patel and Korotchkina, 2001). This complex catalyzes the rate-limiting reaction converting pyruvate to acetyl-CoA which is the entry point of the tricarboxylic acid (TCA) cycle. The activity of PDH is regulated by PDHKs and pyruvate dehydrogenase phosphatases (PDHPs). PDHKs phosphorylate PDH to suppress its enzymatic activity whereas PDHPs dephosphorylate and thus activate PDH enzyme activity (Patel and Korotchkina, 2001; Roche and Hiromasa, 2007; Holness and Sugden, 2003). There are four isotypes of PDHK in mammalian cells: PDHK1, PDHK2, PDHK 3 and PDHK4 (Harris et al., 2002). Each of these isotypes has a different tissue-specific distribution (Bowker-Kinley et al., 1998). Since the expression of both LDHa and PDHKs can affect the pyruvate level and it has been shown previously that knocking down LDHa expression can reduce lactate levels (Kim and Lee, 2007), we hypothesize that if we simultaneously reduce the expression of LDHa and PDHKs in CHO cells, more pyruvate may be converted to acetyl-CoA, thereby increasing energy production from the TCA cycle in these cells. Therefore, the downregulation of LDHa and PDHKs may lead not only to reduced lactate but also to increased antibody production in CHO cells.

In this study, we demonstrated that the expression of LDHa and PDHK1, 2, and 3 can be decreased simultaneously using a single siRNA targeting vector. We found that concurrent reduction of LDHa and PDHK1, 2, and 3 gene expression can decrease lactate level and increase antibody production without impacting cell growth or product quality attributes.

2. Materials and methods

2.1. Construction of the vector targeting LDHa and PDHK1, 2, 3

The targeting sequence for LDHa was selected according to the paper by Kim and Lee (2007) and the LDHa siRNA sequence is CTCGATTCCGTTATCTGAT. To design the siRNA targeting sequences for PDHKs, partial cDNA sequences for CHO PDHK1, 2, and 3 were cloned by reverse transcription-polymerase chain reaction (RT-PCR) with primers located within the highly conserved regions of PDHKs. Partially cloned sequences were used for siRNA sequence design according to the method described by Elbashir et al. (2002).

PDHK1 siRNA sequence is GCAGTTCCTGGACTTCGGA.
PDHK2 siRNA sequence is CATTCAGTACTTCTTGGAC.
PDHK3 siRNA sequence is TGTAGCTGATGTCGTGAAA.

The single construct containing siRNA sequences targeting LDHa and PDHKs was constructed using the pSilencer 3.1-H1 hygro vector (Cat# AM5766, Applied Biosystems/Ambion, Austin, TX). The LDHa siRNA sequence was inserted into the *KasI* site of pSilencer 3.1, with an addition of an U6 promoter from pSilencer 2.1 at the immediate 5′ end. SiRNA sequences for PDHK1 and 2 were inserted into *BamHI/HindIII* and *HindIII* sites, respectively. A *BglII* site was introduced to the 3′ side of PDHK2 siRNA sequence and used for the insertion of PDHK3 siRNA.

2.2. Cell culture

CHO cells derived from DUXB11 and deficient in dihydrofolate reductase (DHFR) were cultured in a proprietary DMEM/F12-based medium in shake flask vessels at 37 °C and 5% CO_2. Cells were passaged every three to four days.

2.3. Stable siRNA cell line (siRNA clone) development

A CHO cell line resistant to 25 nM methotrexate (MTX) and expressing a recombinant monoclonal antibody was transfected using Lipofectamine 2000 CD (Cat# 12566-014, Invitrogen, Carlsbad, CA) according to manufacturer's recommendation (Invitrogen, Carlsbad, CA). Transfected cells were centrifuged and seeded into DMEM/F-12-based selective (glycine-, hypoxanthine- and thymidine-free) medium containing 25 nM MTX and 400 μg/ml hygromycin (Cat# 10687010, Invitrogen, Carlsbad, CA). Resuspended cells were plated into 96-well plates to generate individual clones. SiRNA clones were derived from transfection with an siRNA plasmid containing targeting sequences for LDHa and PDHKs genes, while mock clones were derived from transfection with a mock plasmid containing a scrambled sequence designed with no appreciable homology to known genes.

2.4. Quantitative real time PCR (qRT-PCR or Taqman) analyses

Total RNA from each individual clone was isolated using the RNeasy 96 kit (Cat# 74181, Qiagen) and was treated with DNase digestion (Cat# 79254, RNase free DNase kit, Qiagen) to remove residual DNA that may be present in the isolated RNA sample. Taqman was performed using a universal qRT-PCR master mix according to the manufacturer's instructions (Cat# 4309169, Applied Biosystems) and expression levels of PDHKs and LDHa were normalized expression to the housekeeping gene β-microglobulin.

Primer and probe sequences used for Taqman analysis were as follows:

PDHK1 forward primer: GCCCATCTCATCGAAAACA
PDHK1 reverse primer: AGCCATCTTTAATGACTTCGACTAC
PDHK1 probe: TCGCAGTTTGGATTTATGCTTCCAATG
PDHK2 forward primer: GATCTGTCCATCAAAATGAGTGA
PDHK2 reverse primer: TGTGGAGTACATGTAGCTGAAGAG
PDHK2 probe: CTCTCAATCTTCCTCAAGGGGACACC
PDHK3 forward primer: CAGCCTGGAGCCTACAAGA
PDHK3 reverse primer: GGCATACAGTCGAGAAATTGG
PDHK3 probe: AAGCCATAACCAAATCCAGCCAAGG
LDHa forward primer: GCCGAGAGCATAATGAAGAA
LDHa reverse primer: CCATAGAGACCCTTAATCATGGTA
LDHa probe: CTTAGGCGGGTGCATCCCATTT
β-Microglobulin forward primer: TCCTCTCAGTGGTCT GCT TGG
β-Microglobulin reverse primer: TGGCGTGTGTAGACTTGCACTT
β-Microglobulin probe: TGCCATCCAGCGTCCCCCA

All primers and probes were synthesized and purified at Genentech.

2.5. Fed-batch shake flask clone evaluation

Twelve siRNA clones and 12 mock clones were seeded into proprietary production medium with a pH of 7.15. These were then cultured employing a 14-day fed-batch culture process with one bolus feed on day 3 and a temperature shift from 37 °C to 33 °C on day 2. Cell viability and viable cell counts were monitored by Trypan blue dye exclusion using a Vi-Cell (Beckman Coulter). Lactate concentrations were measured on day 3, 7, 10 and 14 using a Nova Bioprofile analyzer (Nova biomedical).

2.6. Fed-batch bioreactor operations

Bioreactor experiments were performed in 2 l stirred tank bioreactors (Applikon, Foster City, CA) operated at a 1.5 l working volume. After a concentrated nutrient feed at 72 h post-inoculation, glucose was added as needed during the 14-day fed-batch culture. Dissolved oxygen and agitation were maintained in the bioreactor cultures at setpoints of 30% of air saturation and 275 rpm, respectively. Culture pH was controlled at 7.0 by addition of CO_2 gas or 1 M Na_2CO_3. Culture temperature was maintained at 37 °C for the first 48 h, and shifted to 33 °C thereafter. Process control in each bioreactor was achieved using a Digital Control Unit (DCU) from B. Braun Biotech (Allentown, PA).

2.7. Calculation of specific lactate production or glucose uptake rates

The average cell specific metabolite (lactate, glucose) production or uptake rate, q_S, is calculated as the slope of the graph of integrated total cell number, and the cumulative metabolite produced, $[S_t - S_0]$, based on the mass balance equation formulated over the whole culture volume:

$$S_t - S_0 = q_S \int_0^t X\,dt$$

where S_t is the total amount of the metabolite in the culture volume (mg) at time t, S_0 is the total amount of the metabolite in the culture volume (mg) at time $t = 0$, X is the total number of cells in the culture volume at any given time t, and q_S is the specific production or uptake rate in mg/cell/day. These rates are calculated at sampling time points by writing the above mass balance equation over the preceding ($t = 0$) and succeeding sampling time points ($t = t$). Average rates are calculated over an extended time interval, t, as follows:

$$[q_S]_{\text{Average}} = \frac{1}{t}\int_0^t q_S\,dt$$

Per the convention used in this work, if more metabolite is produced than consumed by the cell, then the value of q_S is positive.

2.8. Intracellular ATP measurement

Intracellular ATP level was determined using a CellTiter-Glo Assay kit from Promega (Cat# G7571), according to manufacturer's description. Luminescence was recorded with Perkin Elmer EnVision 2104 Multilabel Reader. The luminescence from each sample was measured in triplicate and the relative ATP level or luminescence was plotted.

2.9. Sample analyses

Antibody titer was determined using conventional protein A affinity chromatography with UV detection (Fahrner et al., 1999). Culture samples were analyzed for viable cell concentration and viability by Vi-Cell AS cell counter (Beckman Coulter, Fullerton, CA), pH and lactate by Bioprofile 400 bioanalyzer (Nova Biomedical, Waltham, MA), and osmolality by a multi-sample osmometer (Advanced Instruments, Norwood, MA).

2.10. Statistical analysis

Two tailed student t-tests were performed using JMP 8.0 software (SAS Institute, Cary, NC).

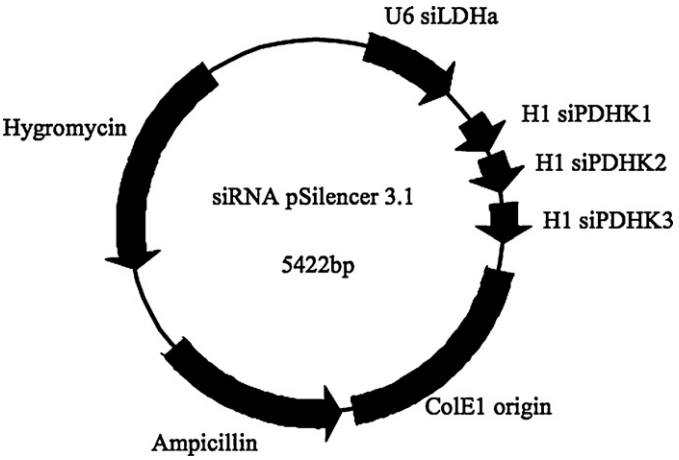

Fig. 1. The siRNA construct targeting LDHa/PDHK1, 2, 3. Small interfering RNAs targeting LDHa, PDHK1, PDHK2 and PDHK3 were cloned into single pSilencer 3.1 hygromycin vector. The siRNA targeting sequence for LDHa was under U6 promoter regulation whereas the siRNA targeting sequences for PDHK1, 2, and 3 were under H1 promoter control.

3. Results

3.1. Construction of an siRNA vector targeting PDHKs and LDHa

There are four PDHK genes reported (Harris et al., 2002) in mammalian cells. To determine if all four PDHK genes are expressed in CHO cells, four sets of RT-PCR primers were designed based on the conserved regions between human and mouse PDHK sequences. Our PCR results revealed that even though all four PDHK mRNAs can be detected in CHO cells, the PDHK4 mRNA level is minimal and much lower than other 3 PDHKs (data not shown) in DHFR-deficient CHO cells. Therefore, we decided to knock down the expression of PDHK1, 2, and 3 genes along with LDHa gene. For each PDHK gene, three siRNA sequences were designed and tested to choose the siRNA sequence exhibiting best reduction of the target gene (data not shown). The best siRNA sequence for LDHa was selected based on the findings by Kim and Lee (2007). The siRNA sequences for LDHa and PDHKs were constructed in a single vector where the siRNA for LDHa is under the control of U6 promoter, whereas siRNAs for each PDHK are driven by H1 promoters (Fig. 1).

3.2. Generation of siRNA clones with reduced expression of PDHK1, 2, 3 and LDHa

The siRNA construct targeting PDHKs and LDHa was transfected into CHO cells expressing a monoclonal antibody. Individual clones were assayed for the mRNA expression of four genes, PDHK1, 2, 3 and LDHa, using Taqman analysis. Twelve clones that exhibited decreased expression of those four genes were identified (Fig. 2) for further analysis. The mock vector containing a scrambled sequence was also transfected into the same antibody expressing cells. Twelve mock clones were chosen randomly to be used as controls and their mRNA expression levels of LDHa and PDHK1, 2, and 3 genes were also analyzed by Taqman. On average, the mRNA expression levels for LDHa, PDHK1, 2, and 3 in the 12 selected siRNA clones were reduced by 90%, 32%, 83%, and 70%, respectively, compared to the mock clones (Fig. 2).

3.3. Fed-batch shake flask evaluation of siRNA and mock clones

3.3.1. Reduced lactate levels and higher pH in culture media in siRNA clones

To evaluate the effect of siRNA-mediated downregulation of LDHa and PDHKs on lactate production, 12 siRNA and 12 mock

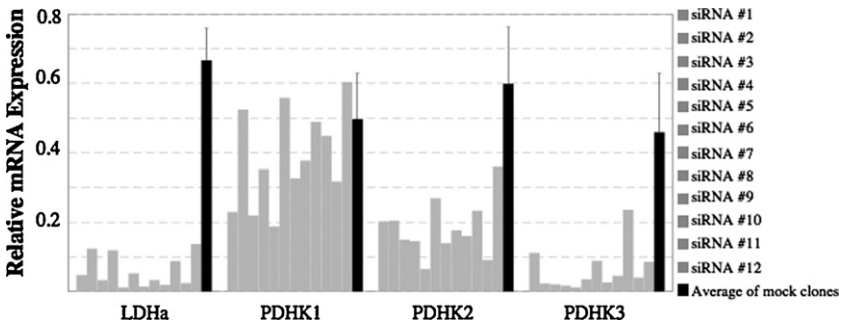

Fig. 2. Relative LDHa, PDHK1, 2, and 3 mRNA expression levels in 12 selected siRNA clones. Expression levels of mRNAs for LDHa and PDHKs were normalized to that of the housekeeping gene β-microglobulin. The average and relative mRNA expression level from 12 mock clones is shown in black. The relative mRNA expression levels from 12 individual siRNA clones are shown in grey.

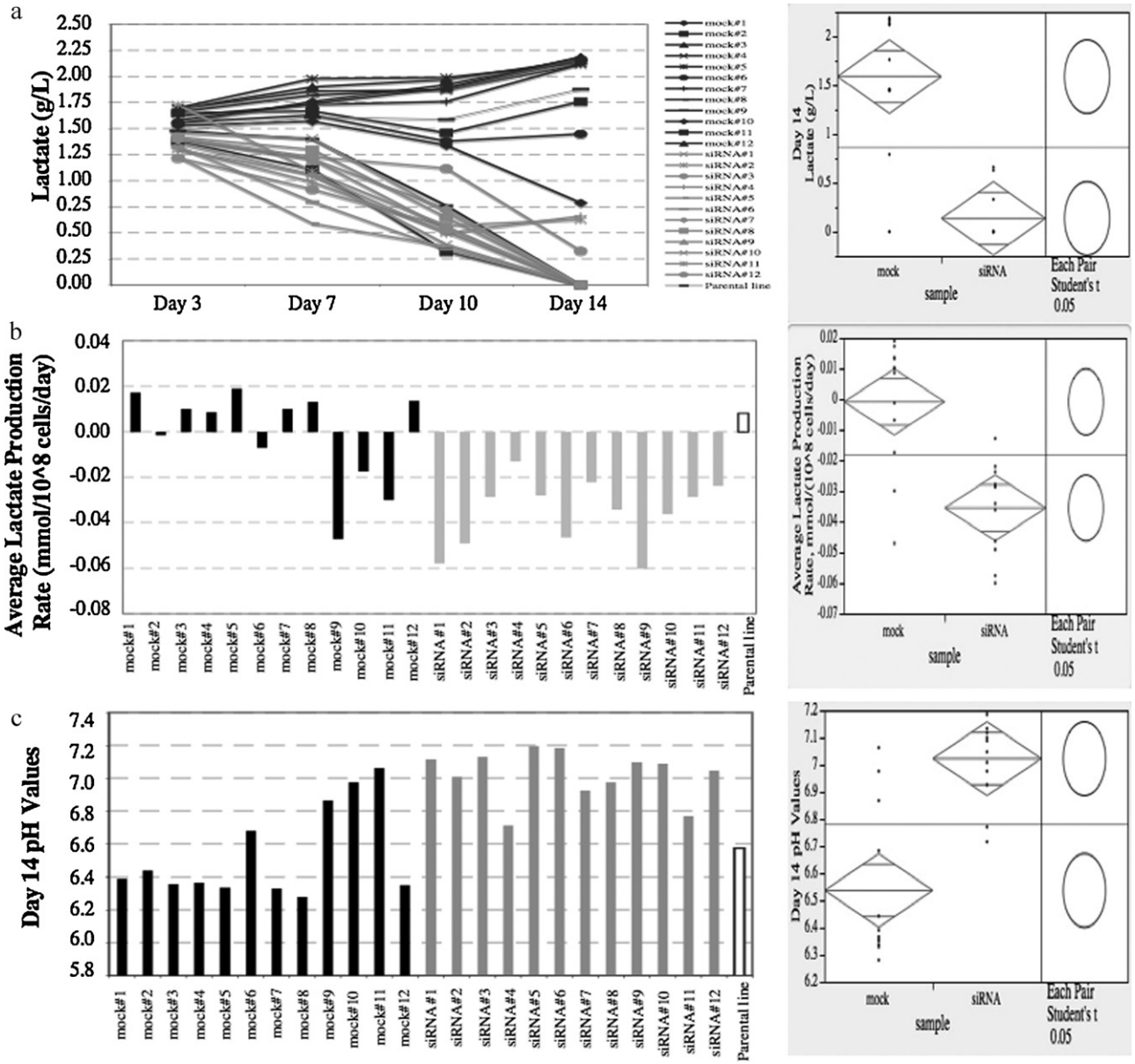

Fig. 3. Lactate profiles, average lactate production rates, and day 14 pH values in fed-batch shake flask evaluation. Lactate concentrations were measured on day 3, 7, 10 and 14 during a 14-day shake flask evaluation. The fed-batch shake flask experiments were performed in triplicate and the data shown is from one experiment. Data from mock clones is shown in black; data from siRNA clones is shown in grey; the parental clone is shown as indicated. (a) Lactate profile; (b) average lactate production rate between day 3 and 14 (mmol/10^8 cells/day); (c) Day 14 pH values.

clones were evaluated in shake flask vessels in our proprietary medium employing a 14-day, fed-batch process. The experiment has been repeated three times and similar results were observed. Here we show the results from one set of experiments. Compared to mock clones, the siRNA clones generally had lower lactate levels (Fig. 3). By day 14, the siRNA clones produced approximately 90% less lactate on average than mock clones ($p < 0.0001$) (Fig. 3a). Consistent with the lower lactate levels in siRNA clones over the 14-day production period, for siRNA clones the average lactate production rate between days 3 and 14 was negative 0.02 mg/10^6 cells/day, indicating net lactate consumption. In contrast, for mock clones the average lactate production rate was 0.01 mg/10^6 cells/day, indicating the overall lactate synthesis rate was higher than the consumption rate for these clones. This difference in lactate production rate between siRNA and mock clones was statistically significant ($p < 0.002$) (Fig. 3b). Since each clone had different expression levels of mRNA for LDHa and PDHKs, we observed variations in lactate production. Nevertheless, the average lactate level in the siRNA group was lower than that in mock group, leading to a lower average pH for mock clones than that of siRNA clones in fed-batch shake flask cultures. By day 14, the average pH for mock clones dropped to 6.54, whereas the average pH for siRNA clones was 7.04 (Fig. 3c). The observed lower average pH is in agreement with the higher average lactate level for the mock clones.

3.3.2. Increased antibody titer and specific productivity (Qp) in siRNA clones

To investigate whether knocking down the gene expression of PDHKs and LDHa affects antibody production, we collected samples from fed-batch shake flask experiments on days 3, 7, 10 and 14 to measure antibody titers by protein A chromatography. On average, the siRNA clones produced around 68% more antibody than the mock clones (Fig. 4a, $p < 0.022$), and the average Qp for the siRNA clones was approximately 75% higher than the mock clones (Fig. 4b, $p < 0.006$). To evaluate cell growth, shake flask samples were collected on days 3, 7, 10, and 14 to measure viable cell counts and viabilities to calculate integrated viable cell count (IVCC). In

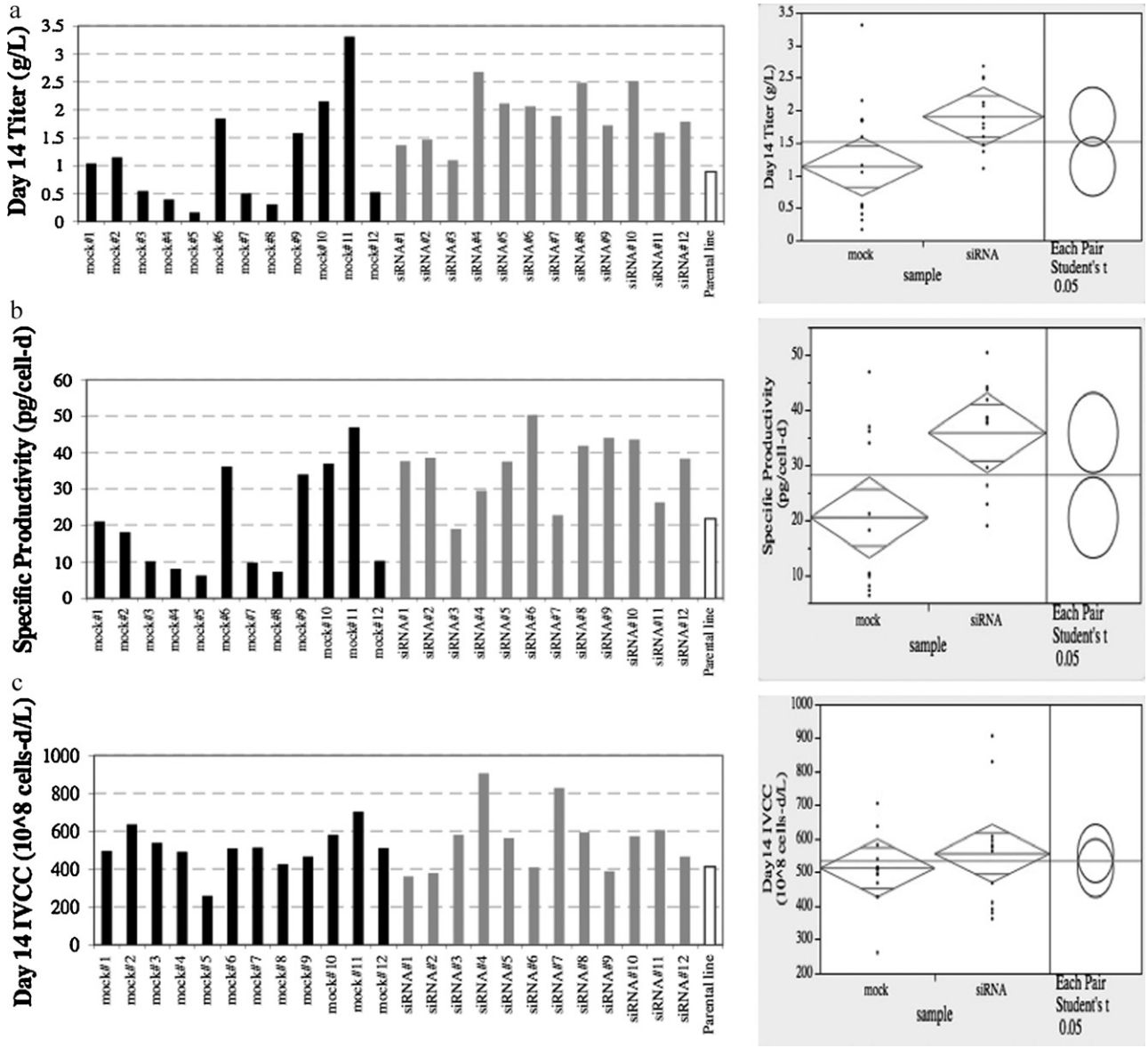

Fig. 4. Titer, Qp, and cell growth profiles in fed-batch shake flask evaluations. The fed-batch shake flask experiments were performed three times and the data shown is from one experiment. Data from the mock clones is shown in black, data from the siRNA clones is shown in grey, and the parental clone is shown in white. (a) D14 titer in g/L; (b) specific productivity or Qp in pg/cell/day; (c) cell growth measured by integrated viable cell count (IVCC) in 100 millions of cells-day per liter.

contrast to antibody titers and Qps, no appreciable cell growth differences were observed between the two groups (Fig. 4c). Antibody product quality attributes including glycan profiles, charged variants and percentage of aggregates were comparable between the siRNA and mock clones (data not shown).

3.4. Bioreactor fed-batch culture evaluation of siRNA and mock clones

Since small-scale fed-batch bioreactor cultures in which pH and dissolved oxygen are controlled are commonly used as scale-down models for larger scale manufacturing bioreactors, we further investigated the performance of some siRNA and mock clones in bioreactors. We selected two siRNA clones and two mock clones which best represented the average productivity for each group in the shake flask evaluation to minimize selection bias, along with the parental line used for siRNA and mock plasmid transfections, for bioreactor evaluation. Cell culture samples were collected daily (except on days 6 and 13) for lactate, glucose, osmolality, cell size, and titer analysis. The lactate levels for the siRNA clones generally remained flat after day 4, whereas the lactate levels for mock and parental clones continued to increase during the 14-day production period. On day 14, the two siRNA clones had approximately 86% lower lactate levels on average than the mock clones or the parental clone (Fig. 5a). On average, siRNA clones had a lower lactate production rate (Fig. 5b), a lower glucose uptake rate (Fig. 5c), and a lower ratio of lactate produced to glucose consumed compared to the mock and parental clones (Fig. 5d) indicating that less lactate was produced for each consumed glucose in siRNA clones than those in mock and parental clones. These data suggest that more pyruvate enters the TCA cycle instead of converting to lactate in siRNA clones for each consumed glucose.

The osmolalities for the siRNA clones remained around 300 mOsm whereas the osmolalities for mock clones or the parental clone continued to increase due to more addition of alkali during the 14-day production period. On day 14, the average osmolality for the two siRNA clones was approximately 60% lower than those of the mock and parent clones (Fig. 5e). Importantly, on day 14, the average antibody titer was increased by approximately 125% and the average Qp was increased by approximately 59% in the siRNA clones (Fig. 6). As was observed in the fed-batch shake flask evaluation, the siRNA and mock clones have comparable viabilities, cell sizes, and cell growth in the bioreactors (data not shown).

3.5. Intracellular ATP content analysis

To further understand the possible underlying mechanism for the observed antibody productivity improvement in siRNA clones, the intracellular ATP content was analyzed for five clones evaluated in 2 l bioreactors. As shown in Fig. 7, the parental line and two mock clones have comparable cellular ATP contents, whereas the siRNA clones have significantly higher ATP levels ($p < 0.001$) suggesting that more pyruvate was channeled into TCA cycle generating more ATP.

4. Discussion

It has been demonstrated previously that reducing LDHa gene expression alone was able to reduce lactate production (Kim and Lee, 2007). However, despite the 45–79% reduction in lactate level, there was no appreciable improvement in Qp and product titer, suggesting that knocking down LDHa alone in CHO cell is not sufficient to improve Qp and product yield efficiently. We observed that simultaneously decreasing PDHK1, 2, and 3 gene expression was neither sufficient to reduce lactate level nor to increase antibody productivity (our unpublished observation). The only way for cells

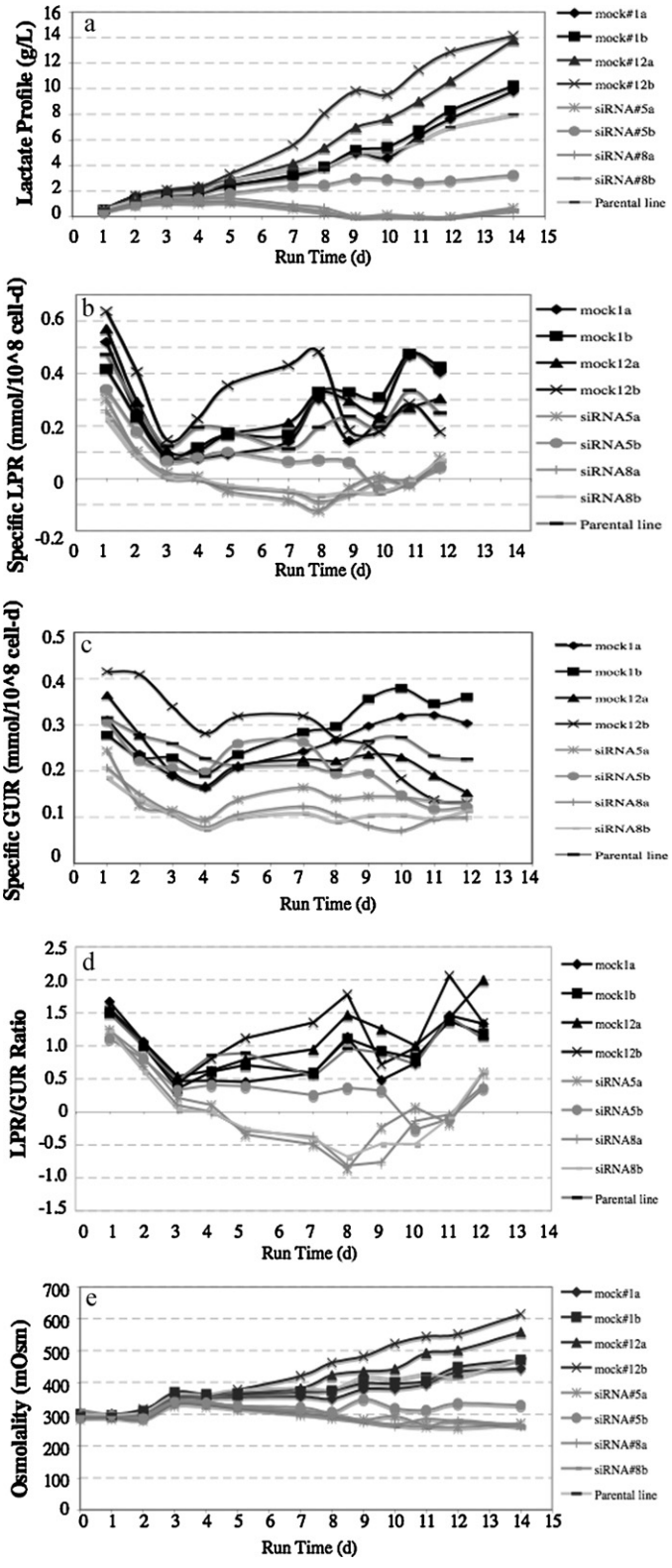

Fig. 5. Lactate profiles, specific lactate production rates (LPR), specific glucose uptake rates (GUR), ratios of lactate produced to glucose consumed, and osmolality profiles in a bioreactor evaluation. Each clone was run as a duplicate except the parental clone was run as a singlet. Data from the mock clones is shown in black, data from the siRNA clones is shown in grey, and the parental clone is shown as indicated. (a) Lactate profiles; (b) specific lactate production rates; (c) specific glucose update rate; (d) ratios of lactate produced to glucose consumed; and (e) osmolality profiles.

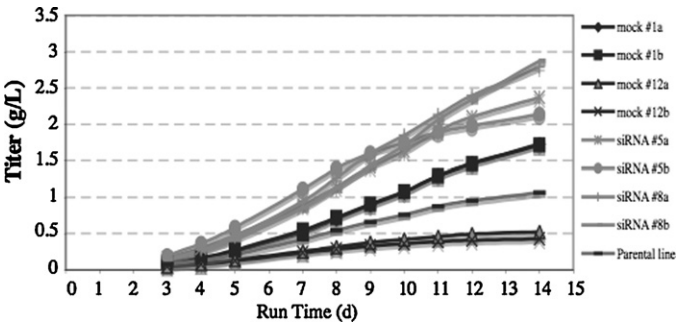

Fig. 6. Productivities in a bioreactor evaluation. Each clone was run as a duplicate except the parental clone was run as a singlet. Data from the mock clones is shown in black, data from the siRNA clones is shown in grey, and the parental clone is indicated.

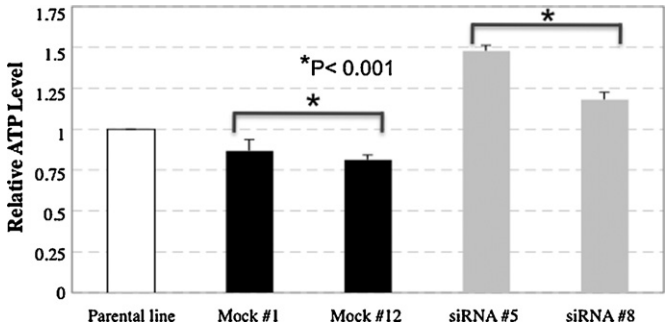

Fig. 7. Intracellular ATP measurement. Each clone was run as a triplicate and the arrow bar is shown as one standard deviation. ATP content is normalized to the parental line. Data from the mock clones is shown in black, and data from the siRNA clones is shown in grey, and the parental clone is shown as indicated.

to generate lactate is through pyruvate reduction and pyruvate can not only be converted to lactate by LDH but can also be converted to acetyl-CoA by PDH to enter the TCA cycle to be oxidized. Therefore, it is likely that reducing lactate production by knocking down LDHa expression as well as promoting pyruvate entry into the TCA cycle by knocking down PDHKs may synergize to reduce lactate level and to provide cells with more energy from the TCA cycle as fuel for cells to use, leading to increased antibody production. More work is underway to measure internal levels of acetyl-CoA and pyruvate in order to further understand the underlying mechanism(s) of the observed improvement in antibody productivity.

We substantially reduced the expression of LDHa and PDHK2 and 3 mRNA and moderately reduced the expression of PDHK1 mRNA in all siRNA clones tested. The moderate reduction in PDHK1 mRNA expression is likely due to a non-optimum siRNA targeting sequence since moderate mRNA reduction was observed with three PDHK1 siRNA sequences tested transiently (data not shown). The observed differences in titers and lactate levels among mock clones may indicate that the parental clone is heterogeneous in antibody productivity and cellular metabolism even though the parental cell line was derived from a single clone. We evaluated 12 mock clones to take the clonal variation into consideration. Some mock clones (6, 9, 10, and 11) have higher titers than the rest of the mock clones, comparable to siRNA clones. One common feature of these mock clones including clone 11 is that their average lactate production rates are either 0 or negative indicating lactate consumption is necessary for good productivity for these clones. The expression levels of PDHKs/LDHa mRNAs in these mock clones are higher than in most siRNA clones and also higher than the average level of the 12 mock clones (data not shown), suggesting that in addition to PDHKs/LDHa levels, other factors may have contributed to their productivity leading to clonal variation. SiRNA clones were identified based on PDHKs/LDHa mRNA levels and as a group, siRNA clones have lower levels of lactate and higher titers than the group of mock clones. The average lactate production rates are negative for all siRNA clones, whereas only 3 mock clones have negative average lactate production rates and those 3 mock clones have comparable titers as siRNA clones.

Not surprisingly, we noted that there was a good inverse relationship between day 14 titers and lactate levels among mock clones, but not among siRNA clones (data not shown). Our data indicate that knocking down LDHa and PDHKs simultaneously decreases lactate level and increases antibody production in CHO cells. Hence, simultaneous reduction of both LDHa and PDHKs may provide an efficient approach for the development of robust and productive antibody production processes.

We saw similar results when we further investigated the performance of two mock and two siRNA clones in bioreactors. Given the limitation in bioreactor availability as well as experimental complexity, it was not practical to run 12 siRNA and 12 mock clones in duplicate. Hence, four clones were selected to best represent the average productivity in each group based on fed-batch shake flask evaluations. Similar to the observations from shake flask experiments, the siRNA clones had lower lactate levels and higher titers than mock clones in bioreactor evaluation. SiRNA clones had lower glucose uptake rate and lower ratio of lactate produced to glucose consumed, supporting the hypothesis that more pyruvate is channeled into the TCA cycle for each consumed glucose. Perhaps siRNA clones have used TCA cycles more extensively than parental and mock clones to provide energy cells need, hence less glucose is needed for siRNA clones. Given that pH is controlled in fed-batch bioreactors, it is not surprising to observe that mock cultures exhibited increased osmolality compared to siRNA cultures since higher lactate levels in the mock clones required more alkali to be added to maintain the setpoint pH.

Intracellular ATP content analysis showed that siRNA clones have higher intracellular ATP than the parental line or mock clones, indicating that more pyruvate is channeled into the TCA cycle for each consumed glucose to produce more ATP for cells to use in siRNA clones. Increase in pyruvate dehydrogenase activity is likely the cause for higher pyruvate channeling into mitochondria and increased TCA activity in siRNA clones.

In summary, our data from fed-batch shake flask and bioreactor evaluations demonstrated that simultaneous knockdown of LDHa and PDHK1, 2, and 3 in CHO cells is effective in reducing lactate levels and in increasing antibody titers without noticeable impact on cell growth and product quality.

Acknowledgements

We thank the Analytical Operations Department especially Yun Tang, Reneen Yang, Mansour Jazayri, Kevin Lin, Belen Tadesse, and Martin Vanderlaan for performing titer and product quality assays, and the Media Preparation Group for all the media and solutions used during cell culture. We also thank the Cell Banking Group led by Marcia Coyne for their excellent service and support.

References

Baumgart, E., Fahimi, H.D., Stich, A., Volkl, A., 1996. L-Lactate dehydrogenase A4- and A3B isoforms are bona fide peroxisomal enzymes in rat liver. Evidence for involvement in intraperoxisomal NADH reoxidation. J. Biol. Chem. 271, 3846–3855.

Bowker-Kinley, M.M., Davis, W.I., Wu, P., Harris, R.A., Popov, K.M., 1998. Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. Biochem. J. 329, 191–196.

Chen, K., Liu, Q., Xie, L., Sharp, P.A., Wang, D.I., 2001. Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production. Biotechnol. Bioeng. 72, 55–61.

Cruz, H.J., Freitas, C.M., Alves, P.M., Moreira, J.L., Carrondo, M.J., 2000. Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells. Enzyme Microb. Technol. 27, 43–52.

Dietl, K., Renner, K., Dettmer, K., Timischl, B., Eberhart, K., Dorn, C., Hellerbrand, C., Kastenberger, M., Kunz-Schughart, L.A., Oefner, P.J., Andreesen, R., Gottfried, E., Kreutz, M.P., 2010. Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. J. Immunol. 184, 1200–1209.

Elbashir, S.M., Harborth, J., Weber, K., Tuschl, T., 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26, 199–213.

Fahrner, R.L., Whitney, D.H., Vanderlaan, M., Blank, G.S., 1999. Performance comparison of protein A affinity-chromatography sorbents for purifying recombinant monoclonal antibodies. Biotechnol. Appl. Biochem. 30, 121–128.

Glacken, M.W., Adema, E., Sinskey, A.J., 1988. Mathematic descriptions of hybridoma culture kinetics: I. Initial metabolic rates. Biotechnol. Bioeng. 32, 491–506.

Harris, R.A., Bowker-Kinley, M.M., Huang, B., Wu, P., 2002. Regulation of the activity of the pyruvate dehydrogenase complex. Adv. Enzyme Regul. 42, 249–259.

Holness, M.J., Sugden, M.C., 2003. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem. Soc. Trans. 31, 1143–1151.

Irani, N., Wirth, M., van Den Heuvel, J., Wagner, R., 1999. Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction. Biotechnol. Bioeng. 66, 238–246.

Jeong, D., Kim, T.S., Lee, J.W., Kim, K.T., Kim, H.J., Kim, I.H., Kim, I.Y., 2001. Blocking of acidosis-mediated apoptosis by a reduction of lactate dehydrogenase activity through antisense mRNA expression. Biochem. Biophys. Res. Commun. 289, 1141–1149.

Kim, S.H., Lee, G.M., 2007. Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. Appl. Microbiol. Biotechnol. 74, 152–159.

Kuystermans, D., Krampe, B., Swiderek, H., Al-Rubeai, M., 2007. Using cell engineering and omic tools for the improvement of cell culture processes. Cytotechnology 53, 3–22.

Langheinrich, C., Nienow, A.W., 1999. Control of pH in large-scale, free suspension animal cell bioreactors: alkali addition and pH excursions. Biotechnol. Bioeng. 66, 171–179.

Lao, M., Toth, D., 1997. Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. Biotechnol. Prog. 13, 688–691.

Li, S.S., Fitch, W.M., Pan, Y.C., Sharief, F.S., 1983. Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A4 (muscle), B4 (heart), and C4 (testis). J. Biol. Chem. 258, 7029–7032.

Liu, C., Wu, J., Zhu, J., Kuei, C., Yu, J., Shelton, J., Sutton, S.W., Li, X., Yun, S.J., Mirzadegan, T., Mazur, C., Kamme, F., Lovenberg, T.W., 2009. Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. J. Biol. Chem. 284, 2811–2822.

O'Callaghan, P.M., James, D.C., 2008. Systems biotechnology of mammalian cell factories. Brief. Funct. Genomic Proteomic 7, 95–110.

Patel, M.S., Korotchkina, L.G., 2001. Regulation of mammalian pyruvate dehydrogenase complex by phosphorylation: complexity of multiple phosphorylation sites and kinases. Exp. Mol. Med. 33, 191–197.

Roche, T.E., Hiromasa, Y., 2007. Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer. Cell. Mol. Life Sci. 64, 830–849.

Samuvel, D.J., Sundararaj, K.P., Nareika, A., Lopes-Virella, M.F., Huang, Y., 2009. Lactate boosts TLR4 signaling and NF-kappaB pathway-mediated gene transcription in macrophages via monocarboxylate transporters and MD-2 up-regulation. J. Immunol. 182, 2476–2484.

Walsh, G., 2009. Market development of biopharmaceuticals. In: Engelhard, M., Hagen, K., Boysen, M. (Eds.), Genetic Engineering in Livestock. New Applications and Interdisciplinary Perspectives. Springer-Verlag, Berlin, ISBN 978-3-540-85842-3, pp. 69–89.