UNIVERSIDAD DE CHILE
FACULTAD DE CIENCIAS FÍSICAS Y MATEMÁTICAS
DEPARTAMENTO DE INGENIERÍA QUÍMICA Y BIOTECNOLOGÍA

METABOLIC ENGINEERING ON IgG PRODUCING CHO CELLS: CONSTRUCTION AND COMPARATIVE ANALYSIS OF CLONES AT A METABOLIC LEVEL

TESIS PARA OPTAR AL GRADO DE DOCTOR EN CIENCIAS DE LA INGENIERIA

MENCION QUIMICA

CAMILA ALEJANDRA WILKENS DÍAZ-MUÑOZ

PROFESORA GUÍA:
Dra. ZIOMARA GERDTZEN HAKIM

MIEMBROS DE LA COMISIÓN:
Dra. CLAUDIA ALTAMIRANO GÓMEZ
Dra. BARBARA ANDREWS FARROW
Dr. JUAN ASENJO DE LEUZE
Dr. ORIANA SALAZAR AGUIRRE

SANTIAGO DE CHILE
2015

RESUMEN DE LA TESIS PARA OPTAR AL GRADO DE: Doctor en Ciencias de la Ingeniería, mención Química
POR: Camila Alejandra Wilkens Díaz-Muñoz
FECHA: 5/01/2015
PORFESORA: Ziomara Gerdtzen Hakim

La venta de biofármacos representa una industria billonaria que ha crecido exponencialmente desde la década de los 70's debido al aumento de la demanda por estas proteínas terapéuticas altamente específicas. Estas proteínas recombinantes son sintetizadas por diferentes líneas celulares, especialmente aquella derivada de ovarios de hámster chino CHO ya que presentan altas tasas específicas de producción de proteína recombinante y son fácilmente adaptables a escalas industriales. Hoy en día, más de la mitad de los anticuerpos monoclonales que se encuentran en el mercado son producidos por células CHO.

Para incrementar el rendimiento de los procesos productivos, diferentes metodologías han sido usadas por investigadores. Resultados positivos han sido obtenidos aplicando principios de ingeniería y utilizando herramientas de biología molecular para modificar reacciones bioquímicas. Proyectos de Ingeniería Metabólica han sido exitosos en reducir la producción de metabolitos indeseados, especialmente lactato, e incrementar la síntesis de proteína recombinante. En este trabajo se estudia el efecto que tienen diferentes estrategias de Ingeniería Metabólica sobre el metabolismo y cultivo de células CHO.

Se ha probado que el metabolismo de carbono de células CHO es altamente ineficiente, consumiendo una cantidad de glucosa mayor de la necesaria para mantener el metabolismo energético y proliferación celular. En este trabajo se construyeron clones de células CHO productoras de IgG recombinante que sobre-expresan PYC2, MDH II y trasportador de fructosa. La expresión de estos genes permitiría aliviar cuellos de botella en el metabolismo central del carbono de las células. En este trabajo se estudiaron y contrastaron los efectos de la sobre-expresión de estos genes sobre la extensión de los cultivos, metabolismo y productividad. Los resultados indican que todos los clones estudiados presentan un metabolismo más eficiente, caracterizado por una menor producción de lactato por glucosa consumida y que no todos mejoraron su proliferación celular y/o productividad específica. Células CHO sobre expresando PYC2 mejoraron su tasa máxima de crecimiento, pero redujeron la tasa de producción de proteína recombinante; la sobre-expresión de MDH II conduce a la reducción del crecimiento celular y síntesis de proteína; finalmente, sobre-expresar el transportador de fructosa aumenta la proliferación celular y síntesis de proteína recombinante en medios con fructosa. Proponemos que las diferencias en producción de proteína se deben a alteraciones del estado RedOx de la célula que afectan en ensamblado de las cadenas peptídicas y la secreción de éstas.

Reducir la expresión del gen Lactato deshidrogenasa A ha sido el objetivo de numerosos trabajos con el fin de reducir la síntesis de lactato e incrementar la producción de proteína recombinante. Utilizando el nuevo sistema para edición genómica CRISPR-Cas logramos interrumpir una de las copias del gen. Resultados del análisis de cultivos fed-batch de estas células indican que el crecimiento celular y el metabolismo fueron afectados y la síntesis específica y volumétrica de la proteína recombinante incrementó considerablemente.

Se realizó un análisis a profundidad del metabolismo de las células deficientes en LDHa. Se midió la razón NAD^+/NADH de éstas y el valor indicó que las células mutadas presentan niveles de NAD^+/NADH menores que las del cultivo control, sugiriendo una mejora en su metabolismo energético debido a la mayor acumulación de NADH. Mediante Análisis de Flujos Metabólicos se estimó los flujos entre las reacciones más importantes del metabolismo central de las células. Los resultados confirmaron que en las células mutantes existen mayores flujos en el ciclo del TCA, debido principalmente a un mayor aporte de carbonos provenientes del catabolismo de amino ácidos. Estas células también presentan un menor flujo en la vía de la glicólisis, lo que se correlaciona con la menor proliferación que estas presentaron, y esto último puede explicar el aumento de síntesis de proteínas.

En este trabajo se aplicaron exitosamente conceptos de Ingeniería Metabólica para la construcción de clones con distintos metabolismos. Este trabajo revela los efectos de varias modificaciones, lo que lo hace una fuente útil información acerca de los efectos que tienen variadas estrategias metabólicas sobre cultivos de células CHO. Finalmente, este trabajo resulta ser un aporte para la comunidad científica contribuyendo con la primera comparación de diferentes clones que sobre-expresan genes claves del metabolismo central del carbono y entregando el primer estudio en profundidad del efecto de reducir la expresión del gen de la LDHa.

THESIS SUMERY TO OBTAIN THE DEGREE: Doctor in Engineering Sciences, mention Chemistry
BY: Camila Alejandra Wilkens Díaz-Muñoz
DATE: 5/01/2015
ADVISOR: Ziomara Gerdtzen Hakim

Biopharmaceuticals represent a multi-billion industry that has been expanding exponentially since the 1970s due to an increased demand for highly specific therapeutic proteins. These recombinant proteins are synthesized by different animal cell lines and among these Chinese hamster ovary (CHO) cells are the most widely used because they produce proteins at high specific rates and are easily adapted to industrial scales, nowadays more than half of the commercially available monoclonal antibodies are produced by CHO cells.

To increase process yields several methodologies have been used by researchers. Positive results have been obtained by using engineering principles to modify specific biochemical reactions using molecular biology tools. Projects using Metabolic Engineering have successfully reduced production of undesirable metabolic products, mainly lactate, and enhanced recombinant protein synthesis. In this work we have taken different Metabolic Engineering approaches in order to assess the impact over culture's performance and metabolism.

CHO cells carbon metabolism has been proven to be inefficient, consuming more glucose that the necessary to sustain their energy metabolism and cell proliferation. Different clones of IgG producing CHO cells overexpressing PYC2, MDH II and fructose transporter were constructed in this work in order to alleviate different limiting reactions of the cells' central carbon metabolism. This work studied and compared the effect of overexpressing these genes on cultures' lifespan, cell metabolism and IgG productivity. Culture performance of engineered clones indicates that even though all studied clones had a more efficient metabolism, characterized by a lower production of lactate per consumed glucose, not all of them show the expected improvement of cell proliferation and/or specific productivity. CHO cells overexpressing PYC2 were able to improve their exponential growth rate but reduced IgG synthesis, MDH II overexpression lead to a reduction in cell growth and protein production, and cells transfected with the fructose transporter gene were able to increase cell density and total volumetric protein production. We propose that a RedOx unbalance caused by the new metabolic flux distribution affected IgG assembly and protein secretion

Lactate Dehydrogenase A gene knock down has been the aim of numerous cell engineering approaches for lactate reduction cultures to improve recombinant protein yield. Using the novel genome editing tool, CRIPR-Cas, one copy of the LDHa was disrupted. Cultures' performances in fed-batch indicate that cell growth and metabolism were affected by LDHa knock down and specific protein rate and volumetric production were greatly enhanced.

Metabolic analyses to comprehend the changes that LDHa knock down were performed. NAD+/NADH ratios were measured and the value indicated that mutant cells had lower NAD^+/NADH levels, suggesting enhanced energy metabolism due to accumulation of NADH. LDHa Knock Down and parental cells' intracellular fluxes were estimated and contrasted using Metabolic Flux Analysis. Results confirmed that LDHa deficient cells have an enhanced energy metabolism, characterized by higher fluxes in the TCA cycle which is achieved due to a larger input of carbons from amino acid catabolism. Mutant cells also have lower fluxes through the glycolytic pathway which correlates to lower cell proliferation, and the latter may explain the increased recombinant protein synthesis.

This work successfully applied Metabolic Engineering concepts to construct CHO cell clones with distinct metabolisms. This work reports the effects of several modifications, making this work a useful source of information regarding different metabolic engineering strategies on CHO cell cultures. Finally, this work is an important contribution to the scientific community delivering the first comparison of different engineered clones overexpressing key genes involved in the central carbon metabolism and the first in depth analysis of the effect of reducing the expression of LDHa.

To Christian

Acknowledgments

This last years would not have been possible without the constant support and encouragement of my advisor Dr. Ziomara Gerdtzen. I am also very thankful of Dr. Wei-Shou Hu for receiving me into his group and guiding me during my internship at the University of Minnesota. Just as important were my fellow lab mates from the Univeristy of Chile and Minnesota with whom I worked these last years and who shared with me their knowledge, skills and friendship. Finally I am greatfull of the support given by my family, specially from Christian, who was always my biggest supporter and fan.

I wish to acknowledge those who kindly shared with me the vectors used in the first part of this work. Dr.Roland Wagner for the PCMVSHE-PYC2 vector. Dr. Miranda Yap for the pcDNA 3.1 Hygro MDHII vector, the “Genetically Engineered Mouse Facility” at the M.D. Anderson Cancer Center por el hygromycin resistance cassette and Dra. Mariella Bollati for the pcDNA3.1(+) Zeo vector. I am also thankful of those who helped me with different experiments of my thesis like Dr. Mauricio Vergara, Dr. Tulio Nuñez and Dr. Victoria Tapia.

I would like to thank Conicyt for it’s support this last years thought the Doctoral Scholarship, International Doctoral Internship Scholarship, Events or Short Course Scholarship and Doctoral Thesis Support Scholarship, and to the Fullbright Comission for their Doctoral Internship Scholarship. The work done for this thesis was financed by the FONDECYT Iniciación 11090268 proyect, V.I.D. UChile Enlace Fondecyt-2013-14116, Proyecto Dirección Académica FCFM - Z.P. Gerdtzen, “Institute for Cell Dynamics and Biotechnology” Millenium Institute, Fondo Basal FB0001 CeBiB and the CHO Consortium.

Table of Contents

Acknowledgments 5

Table of Contents 6

List of abbreviations 9

Introduction 11

Central Carbon Metabolism 13

Glucose and Lactate 14

Cellular and Metabolic Engineering, perspectives and challenges 16

Objectives 18

Bibliography 19

1. CHAPTER 1: Comparative metabolic analysis of CHO cell clones obtained through metabolic engineering, for IgG productivity, growth and cell longevity 21

1.1. Abstract 22

1.2. Introduction 22

1.3. Material and Methods 24

1.3.1. Cell line and culture conditions 24

1.3.2. Metabolite determination 25

1.3.3. Vectors, transfection and selection 25

1.3.4. Specific rate determination 26

1.4. Results and Discussion 26

1.4.1. Engineered clones cultures' performance 27

1.4.2. Analysis of potential metabolic burden of vectors and antibiotic resistance to clones' behavior. 31

1.4.3. Effects on central carbon metabolism 32
1.4.4. Thermodynamic implications 35
1.5. Conclusions 37
1.6. Bibliography 40
1.7. Supplementary Information 43
2. CHAPTER 2: Construction and characterization of a LDHa knock down CHO cell clone generated with the novel genome editing tool CRISPR-Cas 44
2.1. Abstract 45
2.2. Introduction 45
2.3. Material and Methods 48
2.3.1. Cell line and culture conditions 48
2.3.2. Metabolite determinations 49
2.3.3. Specific rate determination 49
2.3.4. Experimental procedure 49
2.3.5. Target selection 50
2.3.6. Vectors, Transfection and cell cloning 51
2.3.7. Mutation Analysis and PCR/Restriction enzyme Assay 52
2.3.8. Genomic DNA extraction from single cell clones in 96 well plates 53
2.3.9. Western Blot and sample preparation 53
2.4. Results and Discussion 54
2.4.1. LDHa Sequence Analysis and Possible Targets Screening 54
2.4.2. LDHa Mutation analysis 56
2.4.3. Gene translation analysis 61

2.4.4. Clone characterization 62

2.5. Conclusions 64

2.6. Bibliography 66

2.7. Supplementary Material 75

2.7.1. CHO cells' complete genomic LDHa sequence 75

2.7.2. CHO cells' lactate dehydrogenase A amino acid sequence aligned against *Mus musculus*'76

3. CHAPTER 3: In depth analysis of the metabolic effects of LDHa knock down over LDHc overexpressing CHO cells in fed-batch culture 78

3.1. Abstract 79

3.2. Introduction 79

3.3. Material and Methods....81

3.3.1. Cell line and culture conditions....81

3.3.2. Metabolite determinations....82

3.3.3. Specific rate determination....82

3.3.4. Metabolic Flux Analysis....82

3.4. Results and Discussion 85

3.4.1. RedOx State Analysis....85

3.4.2. Analysis of cultures' glucose and lactate metabolism 86

3.4.3. Metabolic Flux Analysis....87

3.5. Conclusions 92

3.6. Bibliography 94

3.7. Supplementary Material 104

Conclusions 105

List of abbreviations

αKG:	α-ketoglutarate
ΔG:	Gibbs free-energy
$\Delta G'^{0}$:	Biochemical standard free energy changes
ΔL/ΔG:	ΔLactate/ΔGlucose
ΔL/ΔF:	ΔLactate/ΔFructose
μ_{max}:	Maximum cell growth rate
Aa:	Amino acid
AcCoa:	Acetyl CoA
Asp:	Aspartate
ATP:	Adenosine-5'-triphosphate
BHK cells:	Baby hamster kidney cells
Biom:	Biomass
CER:	Carbon dioxide evolution rate
CHO cells:	Chinese hamster ovary cells
Cit:	Citrate
CMV:	Cytomegalovirus
CO_2:	Carbon dioxide
CoA-SH:	Coenzyme A
DMEM:	Dulbecco's modified Eagle's medium
DSB:	Double strand breaks
FBS:	Fetal bovine serum
Fc-fp:	Fc-fusion protein
Frc:	Fructose
FrcTr:	Fructose transporter
Fum:	Fumarate
gDNA:	Genomic DNA
gRNA:	Guide RNA
Glc:	Glucose
Glu:	Glutamate
GLUT5:	Fructose transporter
GS:	Reduced Glutathione
GSSG:	Oxidized Glutathione
HEK 293 cells:	Human embryonic kidney cells
HR:	Homologous recombination
Hygro:	Hygromycin
IgG:	Inmunoglobulin G
K.D.:	Knock down
K'_{eq}:	Biochemical equilibrium constant
Lac:	Lactate
LDHa:	Lactate dehydrogenase A
Mal:	Malate
m.a. enzyme:	Mutation Analysis enzyme
MDH II:	Malate dehydrogenase II
MTX:	Methotrexate
NAD+:	Oxidized nicotinamide adenine dinucleotide
NADH:	Reduced nicotinamide adenine dinucleotide
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate

NHEJ:	Non-homologous end joining
NLS:	Nuclear localization signal
OAA:	Oxaloacetate
OUR:	Oxygen uptake rate
PAM:	Proto-spacer adjacent motif
PCR:	Polymerase chain reaction
PEI:	Polyethylenimine
PPP:	Pentose phosphate pathway
PYC2:	Pyruvate carboxylase
Pyr:	Pyruvate
q_p:	Specific rate of production
q_c:	Specific rate of consumption
R:	Ideal gas constant (8.315 J mol^{-1} $°K^{-1}$)
R.P.:	Recombinant protein
RQ:	Respiration quotient
Suc:	Succinyl CoA
SucCoA:	Succinyl CoA
T:	Temperature (°K)
TCA:	Tricarboxylic acid cycle
Zeo:	Zeocin

Introduction

During the 1970's the technical advances of mammalian cell culture techniques and molecular biology developed the necessary tools for the production of recombinant proteins using animal cells as hosts. By the mid 1980's the biopharmaceutical industry was born when the FDA approved the first therapeutic protein synthesized in animal cells to be used in humans. These proteins present folding and glycosylation patterns similar to the ones present in proteins expressed by humans giving them higher efficiency at lower doses. Table 1 enumerates some of the most widely used therapeutic proteins (Hu and Zhou, 2012).

Table 1: Commercially available therapeutic proteins

Protein	Treatment	Company	Host
α-galactosidase	Pompe disease	Genzyme	CHO
β-glucocerebrosidase	Gaucher's disease	Genzyme	CHO
Ig-CTLA4 fusion	Rheumatoid arthritis	Bristol-Myers Squibb	CHO
Luteinizing hormone	Infertility	Serono	CHO
Tissue plasminogen activator	Acute myocardial infraction	Genentech	CHO
EPO	Anemia	Amgen/Ortho Biotech	CHO
Deoxyribonuclease I	Cystic fibrosis	Genentech	CHO
Interferon-β	Relapsing multiple sclerosis	Biogen Idec, Serono	CHO
Follicle stimulating hormone	Infertility	Serono	CHO
Factor IX	Hemophillia A	Wyeth	
TNF receptor fusion	Rheumatoid arthritis	Amgen, Wyeth	CHO
Factor VIII	Hemophilia A	Wyeth, Baxter	CHO
Anti-Abciximab	Prevention of blood clots	Centocor	CHO
Anti-CD20 mAb	Non-Hodgkin's lymphoma	Genentech, Biogen, IDEC	SP2/0
Chimeric, anti-α-chain T cell IL-2 receptor	Prophylaxis of acute organ rejection in allogeneic renal transplantation	Novartis	CHO
Anti-CD20 murine mAb	Non-Hodgkins lymphoma	Genentech, Biogen IDEC	CHO
Muromomab CD3	Reversal of acute kidney transplant rejection	Johnson & Johnson	Hybridoma
Anti-TNF- - mAb	Active Crohn's disease	Centocor	SP2/0
Humanized, anti-α-subunit T cell IL-2 receptor	Prevention of acute kidney transplant rejection	Protein Design Labs	NS0

(Hu and Zhou, 2012)

Over the past 3 decades the demand for this type of drugs has increased significantly representing nowadays 70% of the therapeutic protein market (Wurm, 2004b) and a multi-billion dollar market (Walsh, 2010) as seen in Table 2.

Table 2: Top 10 best -selling biopharmaceutical products in 2009

Product	Sales value ($ Billion)	Company
Enbrel (Etanercept)	6.58	Amgen, Wyeth, Takeda Pharmaceuticals
Remicade (Infliximab)	5.93	Centocor (Johnson & Johnson), Schering-Plough, Mitsubishi Tanabe Pharma
Avastin (Bevacizumab)	5.77	Genentech, Roche, Chugai
Rituxan/MabThera (Rituximab)	5.65	Genentech, Biogen-IDEC, Roche
Humira (Adalimumab)	5.48	Abbott, Eisai
Epogen/Procrit/Eprex/ESPO (Epoetin alfa)	5.03	Amgen, Ortho, Janssen-Cilag, Kyowa Hakko Kirin
Herceptin (Trastuzumab)	4.89	Genentech, Chugai, Roche
Lantus (Insulin glargine)	4.18	Sanofi-aventis
Neulasta (Pegfilgrastim)	3.35	Amge
Aranesp/Nespo (Darbepoetin alfa)	2.65	Amgen, Kyowa Hakko Kirin

(Walsh, 2010)

Several animal cell lines have been developed for biopharmaceutical production. Some lines are derived from human cells, such as HEK-293 from human embryonic kidney cells or PER-C6 from retinal cells, and others come from other mammalians like mouse myeloma cells (NS0), baby hamster kidney cells (BHK) and Chinese hamster ovary cells (CHO). The latter are the most widely used, producing more than half of the commercially available monoclonal antibodies. To date, CHO cells are well characterized, they are known to produce recombinant proteins at high specific rates and are easily adapted to industrial scales (Birch and Racher, 2006).

The increasing number of approved biopharmaceuticals and higher demand for them are pushing the companies into improving cultures' yield, which during the last 3 decades has gone from 50 mg/L to 10 g/L. To achieve this several aspects of cell culture have been approached, among them, vector design, culture methods, media design and cell metabolism

(Barnes and Dickson, 2006). Regarding the latter, studies have shown that cells in culture present an inefficient metabolism that negatively affects cell proliferation and protein synthesis (Gambhir et al., 2003a; Glacken et al., 1986a; Korke et al., 2004b; Kurano et al., 1990a). In order to improve metabolism and other cellular traits Cellular Engineering was developed, which makes use of molecular biology tools to alter biochemical reactions within the cell. To date, studies have reported success at improving metabolism, increasing biomass, reducing of unwanted metabolites, and enhancing recombinant protein yield.

Central Carbon Metabolism

Central carbon metabolism consists in three main metabolic pathways, Glycolysis, Pentose Phosphate Pathway (PPP) and Tricarboxylic Acid (TCA) Cycle, which are shown in Figure 1. Through these pathways most carbon molecules consumed by the cells are distributed towards other pathways, biomass precursors are synthesized and important energy molecules, such as ATP, NADH, NADPH and $FADH_2$, are produced.

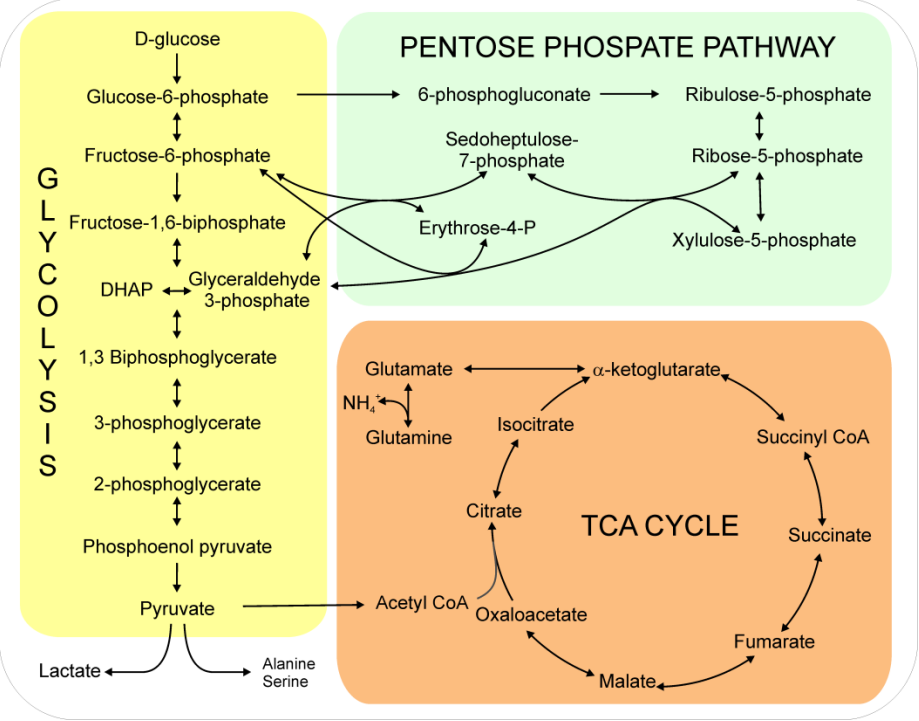


Figure 1: Central carbon metabolism in animal cells

Glycolysis is the main catabolic pathway through which glucose is oxidized and broken down, additionally, phospholipid precursors, ATP and NADPH are produced through its. At the end of this pathway one molecule of glucose can be completely oxidized to produce two pyruvate molecules, which can then enter the TCA cycle to produce more NADH and $FADH_2$ molecules to feed the cell's energy metabolism.

Most glucose-6-phosphate produced during glycolysis continues through the pathway, but some is diverted towards PPP. The ultimate metabolic fate of this pathway is to produce ribose-5-phosphate, which is a key molecule for nucleotide production, making this pathway very important for biomass synthesis.

Part of the pyruvate produced is transported into the mitochondria were it is converted into Acetyl CoA by the enzymatic complex Pyruvate Dehydrogenase (PDH). Acetyl CoA enters the TCA cycle by donating the acetyl group to an oxaloacetate molecule to form citrate, which afterwards loses a CO_2 molecule and is converted into isocitrate. In the next step isocitrate is transformed into α-ketoglutarate which loses a CO_2 molecule to form succinate. This molecule is converted into fumarate, then to malate and finally goes back to oxaloacetate to restart the cycle. This cyclic pathway provides the energy for the metabolism with NADH and $FADH_2$ molecules that participate in ATP production by providing electrons to the electron transport chain. Additionally, many of the aforementioned TCA cycle intermediates are present in the anabolism or catabolism of many amino acids, as well as in the synthesis of sterols and fatty acids.

Glucose and Lactate

Part of the pyruvate that is not transported into the mitochondria is used for the synthesis of other molecules, such as alanine and serine. Nevertheless, most of it is converted into lactate by the Lactate Dehydrogenase enzyme (LDH), which catalyzes the reversible reaction were 1 molecule of pyruvate is reduced into a lactate molecule while oxidizing NADH to NAD^+. Lactate is afterwards transported outside the cell by the symporter monocarboxylate transporter along with H^+ as shown in Figure 2.

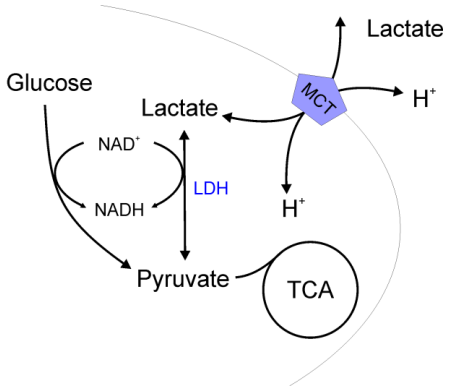


Figure 2: Lactate synthesis and transport

Studies have shown that the presence of lactate in culture and the osmolality increase caused by its transport across the cell membrane restricts cell growth and recombinant protein synthesis (Glacken et al., 1986a; Kurano et al., 1990a). Because of this, many studies and efforts have been made in order to diminish lactate concentration in cultures to improve recombinant protein yield.

To study the metabolic state of a culture and its efficiency the stoichiometric ratio between lactate production and glucose consumption (ΔL/ΔG) is used. The maximum theoretical value for this ratio is 2, but this value is usually not reached during culture because it would mean that there is no carbon input into biomass production or energy metabolism. Several works have reported that cultures with high initial glucose concentration are likely to reach ΔL/ΔG's values close to 1.6, which evidence an inefficient use of consumed glucose, with most of it being eventually transformed into lactate (Europa et al., 2000a; Gambhir et al., 2003a). Europa and collaborators were able to change glucose and glutamine specific consumption rates through controlled feeding in order to reach different metabolic states, each characterized by a different ΔL/ΔG value. By comparing the different states they demonstrated that cultures with low ΔL/ΔGs were able to achieve higher cell densities and protein synthesis while reducing ammonia and lactate production. In a publication by Korke and collaborators, they correlated gene expression of enzymes involved in glycolysis and the TCA cycle with different metabolic states (Korke et al., 2004b). This work concludes that lower fluxes through the studied pathways were related to a reduction of the expressions of the involved enzymes.

Cellular and Metabolic Engineering, perspectives and challenges

Cellular Engineering is a broad discipline that applies engineering and molecular biology principles over cellular and subcellular processes for cell line improvement (Nerem, 1991). On the other hand, Metabolic Engineering a is more a specific discipline, focused on targeting specific biochemical reactions to enhance product formation or cellular properties (Stephanopoulos et al., 1998). Common strategies for these two disciplines include introducing different genetic perturbations, such as increasing a promoter's strength or introduce or remove a gene or a whole pathway. When aiming at generating new clones it is important consider that altering the intended gene is not enough. A previous exhaustive analysis of metabolic pathways associated to the gene of interest is also required. This will help determining the system's properties and limiting steps in reaction pathways and will ultimately indicate which changes have to be done in order to obtain the desired phenotype and help avoid unviable clones or mutations that have no effect on the pathway. After gene and pathway manipulation, differences in phenotype between the mutant and the parental cell line have to be assessed with technics or tools such as Metabolic Flux Analysis (MFA) (Stafford and Stephanopoulos, 2001).

Key aspects of cell culture have been studied and improved by either Cell or Metabolic Engineering in order to increase recombinant protein production. The first is cultures' lifespan, altering cell cycle genes (Fussenegger et al., 1998) or apoptosis genes (Dorai et al., 2010; Mastrangelo et al., 2000; Zustiak et al., 2012) has been shown to prolong high viability. To increase volumetric production protein specific productivity biomass can also be increased. Researchers have been able to improve specific productivity by reducing lactate accumulation (Chen et al., 2001b; Zhou et al., 2011a) and to increase cell density by lowering ammonia production (Park et al., 2000). Other works with positive results in these aspects reduced the uptake rate of the main carbon source by overexpressing fructose transporter (Inoue et al., 2011; Wlaschin and Hu, 2007a), enhanced energy metabolism overexpressing *PYC2* or *MDHII* genes (Chong et al., 2010a; Elias et al., 2003b; Irani et al., 2002a) or increased cellular proliferation by overexpressing the *c-myc* gene (Ifandi and Al-Rubeai, 2003).

Even though a number of different perspectives in Cellular Engineering have proven to be successful for protein production enhancement, there are still pending challenges (Griffin et al., 2007). Among them, is the lack of genomic and bioinformatics resources, were proper analysis of large-scale data could help improve the understanding of the biological system.

There is also a need for the creation of a large number of different clones with different metabolic characteristics, in order to enable a statistical study of significant traits of high producers. Comparison and understanding of the effects of each modification will help scientists make more science-based decisions for new Cell Engineering projects were genomic and proteomic information will propose new single or multigene modifications.

Objectives

The main goal of this thesis is to construct improved CHO cell lines applying a Metabolic Engineering approach and to enhance the understanding of the different metabolic states and traits of high producing cell lines. In order to achieve this three specific aims were outlined:

1. Obtain CHO cell clones overexpressing key genes of the central carbon metabolism, compare cells' culture performance and analyze the metabolic repercussions.

2. Study the effect of LDHa gene knock down and assess its impact over cells' metabolism, cultures' lifespan, cell density and productivity.

3. Expand the understanding of the new metabolic state and improved productivity of LDHa deficient CHO cells using metabolic tools.

Each of these objectives are covered in the following three chapters of this document. Each chapter stands independently with its own Introduction, Material and Methods section, Results and Discussions and Conclusions.

Bibliography

Barnes, L.M., Dickson, A.J., 2006. Mammalian cell factories for efficient and stable protein expression. Current opinion in biotechnology 17, 381-386.

Birch, J.R., Racher, A.J., 2006. Antibody production. Advanced drug delivery reviews 58, 671-685.

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. Biotechnology and bioengineering 72, 55-61.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.-Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. Journal of biotechnology 147, 116-121.

Dorai, H., Ellis, D., Keung, Y.S., Campbell, M., Zhuang, M., Lin, C., Betenbaugh, M.J., 2010. Combining high- throughput screening of caspase activity with anti- apoptosis genes for development of robust CHO production cell lines. Biotechnology progress 26, 1367-1381.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003. Improving glucose and glutamine metabolism of human HEK 293 and trichoplusiani insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnology progress 19, 90-97.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnology and bioengineering 67, 25-34.

Fussenegger, M., Schlatter, S., Dätwyler, D., Mazur, X., Bailey, J.E., 1998. Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. Nature biotechnology 16, 468-472.

Gambhir, A., Korke, R., Lee, J., Fu, P.-C., Europa, A., Hu, W.-S., 2003. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. Journal of Bioscience and Bioengineering 95, 317-327.

Glacken, M., Fleischaker, R., Sinskey, A., 1986. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnology and bioengineering 28, 1376-1389.

Griffin, T.J., Seth, G., Xie, H., Bandhakavi, S., Hu, W.-S., 2007. Advancing mammalian cell culture engineering using genome-scale technologies. Trends in biotechnology 25, 401-408.

Hu, W.-S., Zhou, W., 2012. Cell Culture Bioprocess Engineering. Wei-Shou Hu.

Ifandi, V., Al-Rubeai, M., 2003. Stable transfection of CHO cells with the c-myc gene results in increased proliferation rates, reduces serum dependency, and induces anchorage independence. Cytotechnology 41, 1-10.

Inoue, Y., Inoue, A., Kawahara, H., 2011. Efficient production of recombinant IgG by the GLUT5 co-expression system, In BMC proceedings, BMC proceedings. BioMed Central Ltd, p. P50.

Irani, N., Beccaria, A.J., Wagner, R., 2002. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. Journal of biotechnology 93, 269-282.

Korke, R., Gatti, M.d.L., Lau, A.L.Y., Lim, J.W.E., Seow, T.K., Chung, M.C.M., Hu, W.-S., 2004. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. Journal of biotechnology 107, 1-17.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. Journal of biotechnology 15, 113-128.

Mastrangelo, A.J., Hardwick, J.M., Zou, S., Betenbaugh, M.J., 2000. Part II. Overexpression of bcl- 2 family members enhances survival of mammalian cells in response to various culture insults. Biotechnology and bioengineering 67, 555-564.

Nerem, R.M., 1991. Cellular engineering. Annals of biomedical engineering 19, 529-545.

Park, H.-S., Kim, I.-H., Kim, I.-Y., Kim, K.-H., Kim, H.-J., 2000. Expression of carbamoyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary< i> dhfr</i>-cells decreases accumulation of ammonium ion in culture media. Journal of biotechnology 81, 129-140.

Stafford, D.E., Stephanopoulos, G., 2001. Metabolic engineering as an integrating platform for strain development. Current opinion in microbiology 4, 336-340.

Stephanopoulos, G., Aristidou, A.A., Nielsen, J., 1998. Metabolic engineering: principles and methodologies. Academic press.

Walsh, G., 2010. Biopharmaceutical benchmarks 2010. Nature biotechnology 28, 917.

Wlaschin, K.F., Hu, W.-S., 2007. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. Journal of biotechnology 131, 168-176.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature biotechnology 22, 1393-1398.

Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A.F., Meier, A., Yuk, I.H., Vijayasankaran, N., Leach, K., Joly, J., 2011. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. Journal of biotechnology 153, 27-34.

Zustiak, M.P., Dorai, H., Betenbaugh, M.J., Sauerwald, T.M., 2012. Controlling apoptosis to optimize yields of proteins from mammalian cells, In Protein Expression in Mammalian Cells, Protein Expression in Mammalian Cells. Springer, pp. 111-123.

CHAPTER 1: Comparative metabolic analysis of CHO cell clones obtained through metabolic engineering, for IgG productivity, growth and cell longevity

1.1. Abstract

Cell engineering has been used to improve animal cells' central carbon metabolism. Due to the central carbon metabolism's inefficiency and limiting input of carbons into the TCA cycle, key reactions belonging to these pathways have been targeted to improve cultures' performance. Previous works have shown the positive effects of overexpressing PYC2, MDH II and fructose transporter. Since each of these modifications was performed in different cell lines and culture conditions, no comparisons between these modifications can be made. In this work we aim at contrasting the effect of each of the modifications by comparing pools of transfected IgG producing CHO cells cultivated in batch cultures. Results of the culture performance of engineered clones indicate that even though all studied clones had a more efficient metabolism, not all of them showed the expected improvement on cell proliferation and/or specific productivity. CHO cells overexpressing PYC2 were able to improve their exponential growth rate but IgG synthesis was reduced, MDH II overexpression lead to a reduction in cell growth and protein production, and cells transfected with the fructose transporter gene were able to increase cell density and reach the same volumetric protein production as parental CHO cells in glucose. We propose that a redox unbalance caused by the new metabolic flux distribution could affect IgG assembly and protein secretion. In addition to reaction dynamics, thermodynamic aspects of metabolism are also discussed to further understand the effect of these modifications over central carbon metabolism.

1.2. Introduction

Improving cell metabolism has been a common objective for researchers in the field of cell culture for many years. Previous studies have determined that cells in culture make an inefficient use of glucose, producing high levels of lactate, which has a negative effect on cell proliferation and protein synthesis (Gambhir et al., 2003b; Glacken et al., 1986b; Korke et al., 2004a; Kurano et al., 1990b). A cell engineering approach has been proposed to improve cell metabolism, overexpressing or knocking down key genes involved in the central carbon metabolism (Glacken, 1988; Wurm, 2004a).

A successful study to improve central carbon metabolism was carried out by Irani and collaborators. They overexpressed a copy of the yeast *pyruvate carboxylase* (PYC2) in BHK-21A cells in order to augment the pyruvate input into the TCA cycle (Irani et al., 1999).

Results of this investigation showed that after clonal selection, recombinant cells are able to achieve similar cell densities than the parental cell, while consuming less glucose and glutamine, while producing less lactate, and showing a higher ATP concentration and TCA cycle fluxes. In a posterior work by the same researchers, they studied the impact of PYC2 overexpression on the production of erythropoietin by BHK-21A cells (Irani et al., 2002b). Results showed that in perfusion cultures, engineered cells were able to produce two times more recombinant protein than wild-type cells and achieved higher specific production rate. Due to the impact of these results, other investigators have studied the effect of PYC2 overexpression on other cell lines such as HEK 293, *Trichplusia ni* (Elias et al., 2003a) and CHO cells (Fogolin et al., 2004), reaching similar positive results.

Inefficient glucose metabolism has been linked to high glucose consumption. To control this issue, media design strategies have been proposed. However, the use of most alternative sugars does not lead to high cell density cultures (Altamirano et al., 2000; Eagle et al., 1958). Wlaschin and Hu proposed to overexpress the SLC2A5 gene which translates into the fructose transporter GLUT5 and use fructose as the main carbon source in CHO cells (Wlaschin and Hu, 2007b). Results indicate that selected recombinant clones in fructose were able to reach higher cell densities than the parental cells in glucose. These engineered cells were characterized by a better use of the main carbon source, consuming a lower amount of carbon molecules and producing less lactate. To further investigate the impact of SLC2A5 gene overexpression, in 2010 Inoue and collaborators reported that cells derived from human myeloma overexpressing GLUT5 were able to achieve more than 1.5 times the cell density reached by wild-type cells and produce more than 2 times the amount of recombinant protein (Inoue et al., 2010).

In a work by Chong and collaborators, they observed malate accumulation in the extracellular media and concluded that the conversion of malate into oxaloacetate could act as a bottleneck of the TCA cycle (Chong et al., 2010b). In this same work, the authors proposed to overexpress the *malate dehydrogenase II* (MDH II) gene to improve TCA cycle flux. They observed that engineered selected cells have higher ATP and NADH intracellular concentration, and are able to reach almost twice the cell density that wild-type cells achieve in fed-batch cultures.

In this chapter we aim at gaining a better understanding of the real impact that each of these modifications has over a specific recombinant protein producing cell line. Specifically we

compare cell growth, metabolic efficiency and recombinant protein production on an IgG producing CHO cell line. In order to have a clear assessment of the overall effect of the manipulation over culture's performance and avoid enhancing any previous positive bias that a specific clone could have, in this work we have analyzed cell pools and not cells that have undergone best clone selection.

1.3. Material and Methods

1.3.1. Cell line and culture conditions

The IgG producing cell line CHO DP12 clone#1933 [CHO DP12, clone#1933 IL8.92 NB 28605/12] (ATCC® CRL12444 ™) was grown in adherence in T75 flasks maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Gibco, ME090283L1) medium supplemented with 5% FBS (Hyclone, SH30910.03), 200 nM MTX (Sigma, M8407), 5 mg/L transferrin (Sigma, T8158**)**, 1.7 µL/L 2-mercaptoethanol (Sigma, M3148-250), 0.1 ml/L ethanolamine (Sigma, E0135), 0.11 mM ascorbic acid (Sigma, A4544), 0.18 mg/L putrescine (Sigma, P5780), 29 mM sodium bicarbonate (Sigma, S4019), 28.9 mM sodium selenite (Sigma, S5261), 0.2 g/L pluronic F68 (Sigma, P1300), with a final concentration of glucose (Sigma, G5146) or fructose of 20 mM (Sigma, F3510) and glutamine 4 mM (Sigma, G1517). Selection antibiotics were added for selecting positively transfected cells and for maintaining selective pressure on transfected cultures: 680 µg/mL zeocin (Invitrogen, R250) and 250 µg/mL hygromycin (Invitrogen, 10687-010). Cells were maintained inside a CO_2 incubator (Shel Lab, USA) at 37.0 °C, with 96% relative humidity in a 5% CO_2 enriched air atmosphere.

Growth curve experiments were performed in batch cultures with biological duplicates, monitoring their progress twice a day by determining cell density and sampling the supernatant for further analysis. Cells were inoculated at a concentration of 5.5×10^4 [cells cm^{-2}] (equivalent to 0.14×10^6 [cells mL^{-1}]) with cells from the mid exponential phase of growth. Cell number and viability were determined by the trypan blue exclusion method. After cell counting the supernatant was spun briefly and frozen at -20°C before metabolites analysis.

1.3.2. Metabolite determination

Glucose, fructose and lactate concentrations were determined with the appropriate kit as recommended by the supplier (Randox, GL364; Biovision, K619; Randox, LC2389) and glutamine was determined with a YSI analyzer. IgG concentration was quantified by ELISA. Briefly, the ELISA protocol used goat anti-human IgG Fc-specific (Sigma-Aldrich, Sigma-Aldrich, I3391) and mouse anti-goat IgG Alkaline phosphatase (Sigma-Aldrich, A2064) as primary and secondary detection antibodies. P-nitro-phenyl phosphate was used as the enzyme's substrate (Sigma-Aldrich, N1891). The calibration curve was constructed using human IgG (Pierce, PI31879) as standard. IgG concentration was determined by absorbance reading at 405 nm. NAD^+/NADH ratios were measured using Biovision's NAD^+/NADH Quantification Colorimetric Kit (Biovision, K337-100) following the protocol provided by the supplier.

1.3.3. Vectors, transfection and selection

Vector construction for overexpressing the fructose transporter was carried out as described in the work by Wlaschin and Hu (Wlaschin and Hu, 2007b). Briefly, the full-length Slc2a5 gene in a CMV-Sport6 vector (MM1013, OpenBiosystems) was cloned into the pcDNA3.1(+) vector with zeocin resistance (Invitrogen, V860-20) using NotI and EcoRI restriction sites. For overexpressing the pyruvate carboxylase gene the pCMVSHE-PYC2 vector used by Irani and collaborators (Irani et al., 1999) was cotransfected in a 10:1 ratio with a plasmid containing PGK-hygromycin cassette for selection, provided by GEMF at M.D. Anderson Cancer Center. Overexpression of the malate dehydrogenase II gene was achieved using the vector constructed by Chong and collaborators (Chong et al., 2010b) where the MDH II gene was cloned into the pcDNA3.1(-) vector with hygromycin resistance for selection. The expression of all these genes was controlled by the CMV constitutive promoter. All constructs in this work were verified by PCR and sequencing.

To determine that the changes seen in the studied clones are due to gene overexpression and not to the plasmids or the expression of different resistance genes, control clones were also constructed by transfecting the parental cell line with the empty vectors pcDNA3.1(+) zeocin, pcDNA3.1(-)hygro and the PGK-hygromycin cassette.

Cells were transfected employing PEI (Polysciences, Inc., 24765-2) using a modified version of the protocol described by Jones and collaborators (Jones et al., 2010). The day prior to transfection 0.4×10^6 [cells mL^{-1}] were inoculated in a 60 mm petri dish. The transfection complex was prepared by mixing 5 µg of DNA with 14 µL of 1[µg/µL] PEI solution, adding serum free media to a final volume of 625 µL. The complex was incubated for 20 min at room temperature and then added to the cells. Final volume was completed with 1.875 mL of serum free media. At 4 h post transfection all media was replaced with fresh media supplemented with 5% FBS. Selection of stable clones began after 72 h after transfection by reducing FBS to 2% and adding the appropriate antibiotic concentration, previously determined by a cell death curve. After 1 week cells that had the desired vector were positively selected and FBS was increased to 5%. Cells were maintained under selective pressure for the duration of experiments.

1.3.4. Specific rate determination

The specific rate of production or consumption of cell metabolites was calculated from a cumulative curve constructed by adding the differential amount of a metabolite produced or consumed between two consecutive time points in the culture. The specific rates were calculated as the derivative of the curve fitting this data divided by the cell density evaluated in each time point. This method ensures small error propagation from measurement errors. The value of $\Delta L/\Delta Hexose$ is defined as $-q_{Lac}/q_{Hexose}$ and it was calculated as the quotient between the two specific rates.

1.4. Results and Discussion

Different pools of engineered clones were obtained through transfection. CHO cells overexpressing PYC2, MDH II and fructose transporter are referred as CHO PYC, CHO MDH and CHO FrcTr respectively. The study of these cell pools was done by comparing clones' performance in culture against parental cells grown in glucose and fructose based media. In this work we aim at studying CHO PYC and CHO MDH with glucose as their main carbon source and compare them to CHO cells grown in glucose based media. CHO FrcTr cells are grown in fructose based media and are compared to both control cultures.

To prove that the results from engineered cells can be attributed to gene overexpression and not to the metabolic burden caused by transfected plasmids and/or the resistance gene

expression, control clones expressing only the antibiotic resistance gene were constructed as explained in the Material and Methods section. These cell clones are referred as CHO PGK-Hygro and CHO pcHygro CHO pcZeo and their growth, metabolism and IgG productivity in glucose based media are compared to those of the parental cell line in the same culture condition.

The parental cell line used in this work is a low IgG producer clone. This characteristic was selected in order to assure that protein synthesis would not face limiting factors such as amino acid depletion or endoplasmic reticulum stress due to unfolded protein response. This enables us to isolate the effect of each modification on protein productivity. In order to study the overall effect of each modification over a population of CHO cells, no single cell selection was performed for any of the constructed cells.

1.4.1. Engineered clones cultures' performance

Figure 1 shows cell growth, glucose or fructose consumption, lactate production, glutamine uptake and IgG production for CHO PYC, CHO MDH and CHO FrcTr clones compared to parental cell line cultures. Table 1 presents the parameters that describe the cultures' growth, metabolism and productivity associated to the data presented in Figure 1. Figure 1.A and B and Table 1 provide information regarding growth of the studied clones. Wild-type CHO cells in glucose based media are able to sustain high cell viability for 120 h, proliferating at a maximum rate (μ_{max}) of 1.65×10^{-2} h^{-1} for 62 h, and reaching a cell density of 1.2×10^{6} [cells mL^{-1}]. As expected, when wild-type CHO cells are grown using fructose as their main carbon source their growth is clearly altered, as cultured cells have a lifespan of only 65 h, while exhibiting a μ_{max} similar to the one observed for control cells grown in glucose. The exponential phase lasted for only 23 h leading to a low cell density of 0.65×10^{6} [cells mL^{-1}]. Culture performance of engineered cells differs from the one exhibited by parental cells. CHO PYC cells have an extended lag phase, followed by an 81 h long exponential growth phase characterized by a μ_{max} of 1.76×10^{-2} h^{-1}. By the end of this period CHO PYC cells have reached the same cell density as the control experiment in glucose. After this, the culture is capable of sustaining a long stationary phase before death. CHO cells overexpressing the MDH II enzyme exhibit a higher μ_{max} than control cells but with a shorter exponential phase, which explains the low cell density achieved. Finally, CHO FrcTr cells were grown with fructose as the main carbon source and overexpression of the fructose transporter translated into the highest μ_{max} of the studied cells.

This is the only culture that can be considered as statistically different from the glucose control with a p-value of 0.023. CHO FrcTr cells reached the highest cell density (1.5×10^{6} [cells mL^{-1}]) achieved by any of the studied clones.

Table 1: Parameters for cell growth, $\Delta L/\Delta H$[a] and IgG's specific productivity and their percentage variation vs. their respective control cultures.

	CHO Glc control	CHO Frc control	CHO PYC	CHO FrcTr	CHO MDH
Exponential phase length [h]	62	23	70	62	50
			12.9%	0%	-19.4%
μ_{max} [10^{-2} h^{-1}]	1.65	1.62	1.76	2.35	1.72
			6.7%	42.4%	4.2%
ΔL/ΔH	1.042	0.629	0.79	0.653	0.559
			-24.2%	-37.3%	-46.4%
Specific IgG production [mg IgG/h 10^{6} cells	2.30×10^{-4}	3.79×10^{-4}	1.08×10^{-4}	1.99×10^{-4}	1.71×10^{-5}
			-53.0%	-13.5%	-92.6%

[a]H stands for hexose; depending on the culture's c onditions it can be either glucose (G) or fructose (F).

Results regarding glucose, fructose and glutamine uptake, and lactate production are shown in Figures 1.C, D, E, F, G and H and ΔL/ΔH values are presented in Table 1. By the end of the culture's lifespan, parental cells grown in glucose consumed nearly 65% of the available glucose and all of the available glutamine, and produced a total of 14 mM of lactate with a ΔL/ΔG of 1.04. Parental cells grown in fructose based media consumed only 20% of the available fructose and all the glutamine, while producing only 4 mM on lactate with a ΔL/ΔF of 0.63.

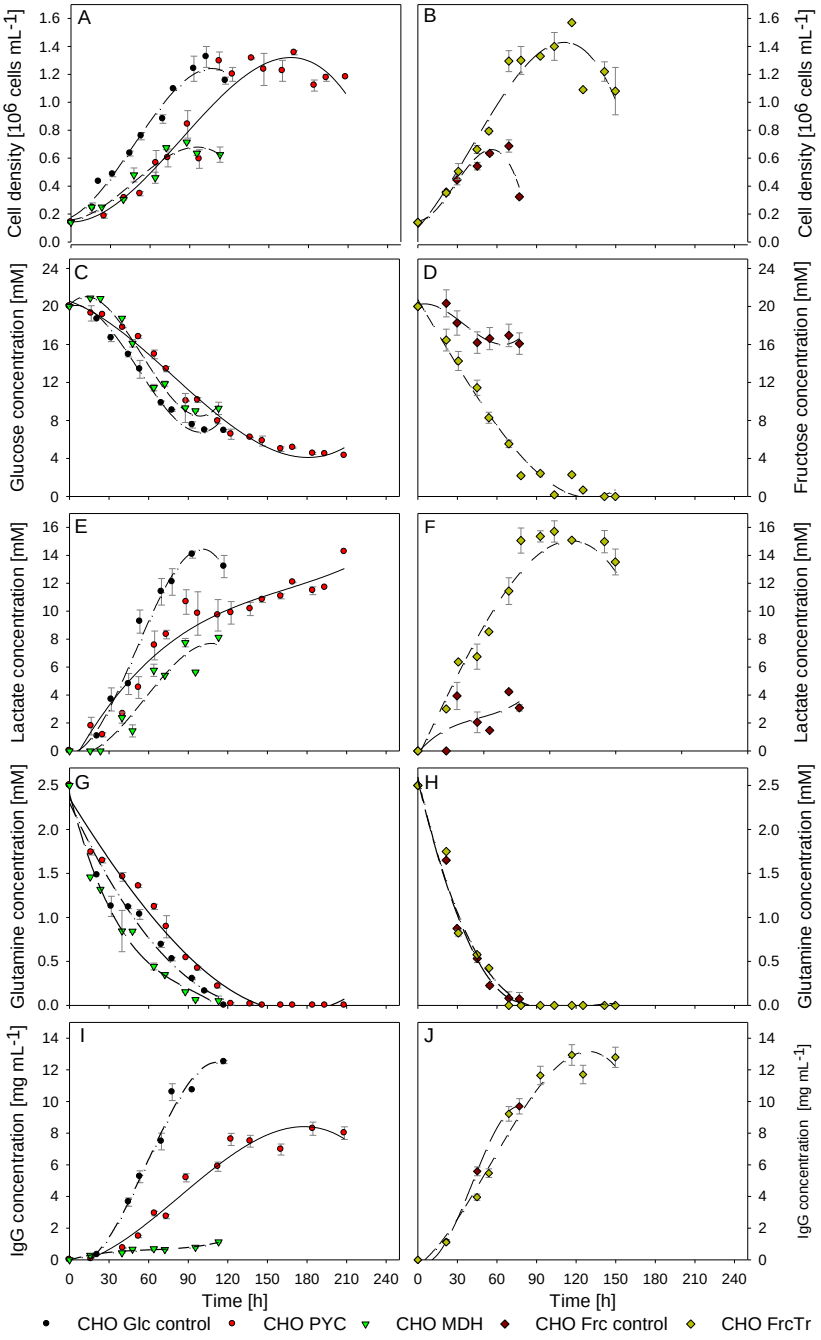


Figure 1: Batch cultures of different CHO cell engineered clones. Graphs to the left describe cultures that use glucose as the main carbon source and graphs to right depicts cultures that use fructose as their main carbon source. CHO Glc control (●); CHO PYC (●);CHO MDH (▼); CHO Frc control (♦); CHO FrcTr (♦). A, B: Viable cells; C, D: main carbon source concentration; E, F: lactate concentration; G, H: glutamine concentration; I, J: IgG concentration.

Results show that engineered cells have a different metabolism than the one exhibited by control cells. CHO PYC cells are able to consume glucose at a high rate during the first 100 h of culture producing lactate with a ΔL/ΔG of 0.79, which indicates a more efficient metabolism with lower lactate production per glucose consumed. At this time glutamine is depleted. In the following stage of the culture, CHO PYC cells consume glucose at a lower rate, which is apparent from the change in the slope of the curve in Figure 1.C. During this low glucose consumption period cells neither consume nor produce any lactate, and are able to sustain high viability without using glutamine as a main carbon source. CHO MDH cells consume 60% of the available glucose, all available glutamine and produce 8 mM of lactate with a ΔL/ΔG of 0.56, showing the highest metabolic efficiency among the studied clones; this ΔL/ΔG value has a significant statistical difference with the glucose control experiment with a p-value of 4.7×10^{-5}. Lastly, overexpression of the fructose transporter in the CHO FrcTr cells enabled them to consume nearly 90% of the fructose present in the culture media at a high rate, as can be observed from to the steep slope of the curve in Figure 1.D. This higher fructose consumption led to a high lactate synthesis producing 16 mM of lactate, but the ΔL/ΔF achieved is only of 0.65, which indicates a more efficient metabolism than control cells in glucose (p-value 0.018). Glutamine depletion in this culture occurred after nearly 70 h of culture, but cells were able to continue proliferating after this for 80 more hours.

Differences regarding IgG production can be observed in Table 1 and Figures 1.I and J. Control cells grown in glucose and fructose were able to produce 12.5 and 9.7 mg/L of IgG respectively at a specific rate of 2.3×10^{-4} and 3.79×10^{-4} [mg h^{-1} 10^{-6} cells]. Unlike what has been reported already regarding cells overexpressing the PYC2 gene, the engineered CHO cells obtained were unable to exceed control cells productivity, producing 7 mg/L at a specific rate of 1.08×10^{-4} [mg h^{-1} 10^{-6} cells] during the exponential growth phase, with no significant recombinant protein synthesis observed during the stationary phase. To this date the effect of MDH II overexpression on productivity has not been reported. Our results evidence a drastic decline in productivity, as CHO MDH cultures reached a final production of 1 mg/L at a specific rate of 1.71×10^{-5} [mg h^{-1} 10^{-6}cells]. CHO FrcTr cells were able to produce a similar amount of IgG at the end of culture's lifespan in comparison to control culture in glucose, reaching an IgG concentration of 12.8 mg/L, but its specific production rate was reduced reaching only 1.99×10^{-4} [mg h^{-1} 10^{-6}cells]. This means that the increased volumetric production of CHO cells grown in

fructose based media observed would be caused mainly by the increase in cell density and culture's lifespan and not due to an improvement in the cells' specific production rate.

Better culture strategies can be proposed based on cultures' performance understanding. The results presented here show that CHO PYC and CHO MDH cultures exhibit no significant improvement in terms of exponential growth rate, culture's lifespan or specific IgG productivity; therefore they are not better candidates for industrial production than the parental cell line. On the other hand, CHO FrcTr batch cultures showed that there was no significant statistical difference with the control CHO cells grown in fructose based media, but there were significant differences in comparison to CHO cells grown with glucose as the main carbon source. CHO FrcTr cells were able to match glucose control culture's performance in terms of volumetric IgG production and surpass parental cells' production in fructose based media. IgG production by CHO FrcTr clones in fructose was improved mainly due to biomass increase, therefore the use of fed-batch or perfusion reactors to further improve volumetric production could be limited by the maximum cell density that these cells can achieve in these reactors.

1.4.2. Analysis of potential metabolic burden of vectors and antibiotic resistance to clones' behavior.

Control cultures were performed in order to determine that all the differences stated above were due to the overexpression of the gene of interest and not from the metabolic burden caused by either the presence and replication of the vector, or the expression of the resistance gene or addition of antibiotic. For this analysis, the percentage variation of important culture parameters for engineered clones and control cell cultures observed are summarized in Table 1 and 2. Exponential phase length is not impacted by transfection of empty vectors whereas engineered cells' growth span changed between -19.4 and 12.9%. The differences in μ_{max} between control cultures and the parental cell culture range between 4.2 and 8.3%, while engineered cells present differences between 4.2 and 42.4%. Glucose consumption and lactate production were also not significantly altered in control cells, exhibiting variations of $\Delta L/\Delta G$ between -11.6 and -2.3%, while engineered clones' $\Delta L/\Delta G$ ranges from -46.6 to -24.2%. Lastly, specific productivity for cells with empty vectors is similar to parental cells' exhibiting changes from -11.4 to 18.7%, which is not as significant as the changes of specific production of engineered clones that goes from -13.5 to 92.6%. All these differences indicate that the changes in parameters observed experimentally are most likely caused by the overexpression of the studied genes.

Table 2: Percentage variation for control cultures' parameters compared to a parental cell culture.

	CHO pcZeo	CHO PGK Hygro	CHO pcHygro
Exponential phase length	0	0	0
μ_{max}	8.3	4.2	8.3
ΔL/ΔH	-2.3	-11.6	-9.3
Specific IgG production	18.7	17.4	-11.4

1.4.3. Effects on central carbon metabolism

The effect of PYC2 overexpression has a clear impact on culture's performance. In previous works done on BHK cells (Irani et al., 2002b; Irani et al., 1999), cultures reached higher cell densities, had an extended lifespan, and cells exhibited a more efficient metabolism producing less lactate per glucose consumed and were able to maintain high viability without glutamine consumption. In another publication by Fogolin and collaborators, PYC2 was overexpressed in CHO cells and they reported a drop in cell density, but cells also exhibited improvements in lifespan, metabolic efficiency and recombinant protein synthesis (Fogolin et al., 2004). Results obtained in this work regarding CHO PYC cells show a reduction of the total recombinant protein production and the specific rate at which it is produced. Figure 2 illustrates some of the most important metabolic pathways that are involved in the central carbon metabolism in animal cells. PYC enzyme converts pyruvate into oxaloacetate, which then turns into malate. This molecule is afterwards transported into the mitochondrial matrix through the malate-aspartate shunt. From the diagram in Figure 2 it is possible to observe that the first consequence that can be inferred from overexpressing PYC is that cells would have a higher input of reducing power from the cytosol into the mitochondria due to the higher flux through the Mal-Asp shuttle, therefore increasing the cell's energy metabolism, which could explain the culture's extended lifespan. Table 3 shows a comparison of NAD^+/NADH ratios of cultured cells. It is possible to observe that CHO PYC's NAD^+/NADH ratio compared to the control culture in glucose based media is significantly lower (p-value 0.003), evidencing an enhanced energy metabolism. The second impact is a reduction in the available pyruvate for other metabolic pathways. As previous work by the author showed, lactate synthesis is related to pyruvate accumulation inside the cell (Wilkens et al., 2011a), PYC2 overexpression should lead to a reduction in pyruvate

concentration in the cytosol therefore reducing lactate synthesis and improving the cells ΔL/ΔG ratio. Finally, CHO PYC cells exhibit a higher growth rate than control cells, which has been shown to negatively influence protein synthesis. Previous works indicate that cells growing at lower rates are able to produce recombinant proteins at higher rates than fast growing cultures due to increased resource availability (Fussenegger et al., 1997b; Terada et al., 1996a). In this work we propose a second mechanism through which faster proliferation can affect protein synthesis. Cells are dependent on NADPH for biosynthetic pathways and for the generation of reduced glutathione. By increasing NADPH use for proliferation, less of this nucleotide would be available for maintaining appropriate GS/GSSG ratios in the cell, specifically in the endoplasmic reticulum. Borth and collaborators showed that intracellular assembly of light and heavy chain is a major limiting factor for overall cell specific IgG productivity (Borth et al., 2005a). We believe that for the cells analyzed in this study, when cellular NADPH requirements increase towards anabolic pathways a GS/GSSG unbalance occurs in the endoplasmic reticulum that affects IgG assembly and therefore reduces protein production. In Irani's and Fogolin's works the recombinant proteins produced were erythropoietin and rhGM-CSF respectively, which consist in only one polypeptide chain (Boissel et al., 1993; Rozwarski et al., 1996) and therefore in those cases protein synthesis may not have encountered assembly problems.

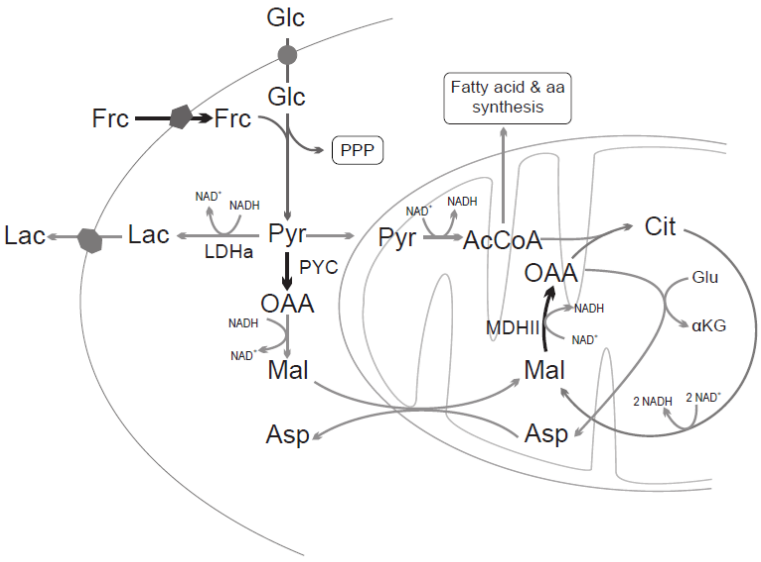


Figure 2: Impact of each of the modifications on CHO cells' metabolism. Reactions catalyzed by everexpressed proteins are represented in bold lines.

Table 3: NAD^+/NADH ratios in studied cultures.

Cell Cultures	NAD^+/NADH
Parental CHO cells Glc control[1]	17.12±2.04
Parental CHO cells Frc control[2]	4.19±1.05
CHO PYC	3.66±0.95
CHO FrcTr	3.64±0.71
CHO MDH	8.57±1.84

[1] Parental cells grown in glucose based media
[2] Parental cells grown in glucose based media

Cells overexpressing the MDH II enzyme are able to alleviate a bottleneck of the TCA cycle, that is the conversion of malate into oxaloacetate. The work by Chong and collaborators showed that by augmenting MDH II activity, the flux through the Mal-Oaa step of the cycle increases (Chong et al., 2010b). As a direct result of this, Chong reported an increase in NADH production that improves the cells energy metabolism; also supported by data in Table 3, were a lower NAD^+/NADH ratio can be seen (p-value 0.03). A second effect is that by rising oxaloacetate concentration the condensation reaction between oxaloacetate and acetyl CoA would exhibit higher rates and therefore all fluxes through the TCA cycle should be improved. By increasing mitochondrial acetyl CoA requirements there is less of this key metabolite for other metabolic pathways such as fatty acid synthesis and other molecules, which could have a direct impact on biomass production, therefore, explaining the lower cell proliferation observed in CHO MDH cultures. Cellular requirements for acetyl CoA could also explain the lower lactate production observed as more pyruvate is converted into acetyl CoA instead of lactate. Finally, the reduced IgG production could be explained by a GS/GSSG unbalance, but in this case it is expected to be due to lower fluxes in the pentose phosphate pathway, which, as Boada and collaborators showed, can be directly correlated to NADPH reduction (Boada et al., 2000).

As shown by the results in Table 1, CHO cells overexpressing the GLUT5 transporter are able to consume fructose at higher rates than the glucose uptake of parental cells, and continue cell proliferation without glutamine in the culture media. This enables a higher pyruvate production rate from the glycolytic pathway and therefore leads to pyruvate accumulation. As indicated above, when pyruvate concentration inside the cell increases, so does lactate's synthesis; nevertheless carbon metabolism of CHO FrcTr in fructose proved to be more efficient than that of CHO cells in glucose, as shown by the lower $\Delta L/\Delta F$ ratio observed for CHO FrcTr. Table 3 shows that CHO FrcTr cells exhibit a lower NAD^+/NADH ratio compared to control CHO cells grown in glucose (p-value 0.014) and similar to control cells in fructose based media. Lower

NAD^+/NADH levels are consistent with lower lactate synthesis since the Pyr-Lac reaction is coupled with NADH-NAD^+ conversion; therefore, lower fluxes in that reaction would enable higher NADH accumulation. CHO FrcTr cultures had an improved total IgG production compared to parental cells, but this fact can only be attributed to the higher cell density achieved by cultures, since specific IgG production rate was reduced compared to the control. This drop can be explained by higher NADPH requirements altering the GS/GSSG balance in the endoplasmic reticulum and negatively affecting IgG assembly in a similar way as proposed above for CHO PYC cells.

1.4.4. Thermodynamic implications

Modifications of any enzyme's expression will likely lead to changes in the intracellular pools of the metabolites involved in the reactions catalyzed by these reactions (Fogolin et al., 2004; Siddiquee et al., 2004; Yamamoto et al., 2012), therefore changing the reaction's available Gibbs free-energy. Table 4 shows the standard free-energies changes ($\Delta G'^0$) for some important reactions discussed in this work and an approximation to their equilibrium constants (K'_{eq}) (Lehninger et al., 2008). When the PYC enzyme is overexpressed it is expected for the cytoplasmic and mitochondrial concentration of pyruvate to decrease as a result of this modification. From the data shown in Table 4 it is possible to observe that the Pyr-AcCoA reaction has a highly negative Gibbs standard free-energy, indicating that the reaction is thermodynamically favorable towards AcCoa synthesis with a high K'_{eq}. The Pyr-AcCoA reaction's Gibbs free-energy is given by the equation:

$$\Delta G = \Delta G'^0 + RT \cdot \ln\left(\frac{[AcCoA][NADH][CO_2]}{[Pyr][NAD^+][CoA-SH]}\right) \quad (1)$$

Based on information available in literature it is possible to estimate the free-energy for this reaction for mammalian cells in culture. The mitochondrial NADH/NAD^+ ratio has been reported as 0.77:1, the CoA-SH/AcCoA is 0.627:1 (Kerbey et al., 1977), pyruvate concentration is 0.085 mM (Kummel et al., 2006; Schuster et al., 1988) and CO_2 concentration is 11.25 mM (Longmore et al., 1969; Sies et al., 1973). The resulting average Gibbs free-energy for this reaction in mammalian cells is -20.3 kJ mol^{-1}. If the available pyruvate for the reaction is reduced by tenfold, the available free-energy would be reduced by 5.9 KJ mol^{-1}, making it less favorable.

Even in this scenario the Pyr-AcCoA reaction is spontaneous and thermodynamically favorable, so no inconsistencies are found in this respect.

Regarding the thermodynamics of lactate synthesis in the context of PYC2 overexpression a similar analysis can be made. According to published data the NAD^+/NADH ratio in the cytoplasm is 700:1 (Williamson et al., 1967) and the Lac/Pyr ratio is 19.5:1 (Zupke et al., 1995), which leads to an estimated Gibbs free-energy for the reaction of -0.56 KJ mol^{-1}. Wild-type CHO cells in culture show a reversed LDH reaction evidenced by lactate's consumption. In a previous study by the authors, lactate synthesis and uptake are proposed to be determined by pyruvate's concentration (Wilkens et al., 2011a). Due to this reported evidence, a low Gibbs free-energy would be expected in order for this reaction to reverse its flux under low pyruvate conditions. As seen from CHO PYC culture's performance, lactate was synthesized throughout the exponential growth phase. This indicates that initially the glucose consumption rate is high enough to produce pyruvate at a high enough rate to feed the different metabolic pathways. A decline in pyruvate concentration leads to a reduction in the available free-energy for the reaction, leading to a lower lactate production rate.

As shown in Table 4 the standard free-energy for the Mal-Oaa reaction is positive, making this a limiting reaction in the TCA cycle, which is consistent with previously reported results (Chong et al., 2010b). In order for the reaction to proceed in the direction of oxaloacetate synthesis, the concentration of malate has to be several orders of magnitude higher than oxaloacetate as shown in both experimental and computer modeling studies (Mooney and Lane, 1982; Wu et al., 2007). Overexpression of MDH II is expected to increase the reactions rate, therefore increasing oxaloacetate's accumulation in the mitochondria, which should augment the condensation rate for the reaction catalyzed by citrate synthase. Considering an average citrate and oxaloacetate mitochondrial concentration of 3.1 and 0.003 mM respectively (Kummel et al., 2006; Robinson et al., 1971), the average Gibbs free-energy for this reaction is -13.1 kJ mol^{-1}. If the concentration of oxaloacetate would increase 10 times, the available free-energy would be -19.0 kJ mol^{-1} boosting the synthesis of citrate and therefore improving the TCA cycle from a thermodynamic point of view.

Results shown in the previous section indicate that in fructose based media, cells that overexpress a fructose transporter are able to consume fructose at a much higher rate than wild-type cells. As a direct result of this, cells produce pyruvate at a higher rate, leading to

pyruvate accumulation. In this scenario, with higher pyruvate concentrations, the Gibbs free-energy available for the reactions that yield lactate and acetyl CoA would be reduced by 5.9 kJ mol^{-1} each. This decrease in the available free-energy enhances the flux of carbon towards lactate and acetyl CoA production. This is consistent with CHO FrcTr cultures' performance, where lactate synthesis is enhanced in comparison to wild-type cells. The enhanced lifespan displayed by the cells could be associated with an improved energy metabolism.

Table 4: Standard Gibbs free-energies for studied reactions and their equilibrium constants.

Reaction	$\Delta G'^{0}$ [kJ mol^{-1}]	K'_{eq}
Pyr + CoA-SH + NAD^+ → AcCoA + NADH + CO_2	-33.4	$7x10^5$ >
OAA + AcCoA + H_2O → Cit + CoA-SH	-32.2	$4x10^5$ >
Mal + NAD^+ ↔ OAA + NADH	29.7	$6x10^{-6}$>
Pyr + NADH ↔ Lac + NAD^+	-25.1	$2x10^4$ >

> Lower limit value

1.5. Conclusions

All modifications studied in this work were aimed at enhancing cultures' performance by improving the cells central carbon metabolism as a result of augmenting the flux through bottleneck reactions or reducing the flux through a reaction that yields an unwanted metabolite. In previous works where these modifications were proposed, experiments were carried out in different cell lines, expressing different proteins and grown in different culture conditions, therefore results could not be compared directly. This work presents the first comparison of the application of different metabolic engineering strategies to mammalian cells.

Each of the modifications studied had a different effect on cultures' performance. Overexpression of PYC2 enabled cells to achieve a higher maximum growth rate and a more efficient metabolism, but product synthesis was reduced. When MDH II was overexpressed the exponential growth rate was also improved in comparison to the control culture, but due to a short exponential phase cells were not able to reach high densities. Even though CHO MDH cells presented the lowest production of lactate per glucose consumed they were neither able to produce IgG at an increased rate, nor to improve the volumetric production of recombinant protein. When cells overexpressed the fructose transporter and were cultured in fructose-based media, they improved their growth in the exponential phase, reaching higher cell densities and increasing their recombinant protein volumetric production. Based on previously published

results it was expected to find high improvement in all cultures studied in this work, mostly due to the improvement in their use of the main carbon source (Europa et al., 2000b). We propose that cells overexpressing PYC2, MDH II or GLUT5 might not present problems in the polymerization of the peptidic chains, but the overexpression of these proteins may cause an unbalance in the NADP/NADPH and GS/GSSG, which would explain the observed experimental results. As shown in the Supplementary Figure 1, an $NADP^+$/NADPH unbalance leads to an unfavorable redox state for light and heavy chain assembly, which would in turn cause a reduced protein production due to lower protein secretion.

The effects of the modifications studied in this work extend to the thermodynamics of the metabolic network. The analysis showed that by changing the expression of each of the studied enzymes, key reactions in the metabolism could be thermodynamically favored or limited. When PYC2 is overexpressed pyruvate accumulation decreases, therefore decreasing the amount of free-energy available for the lactate synthesis reaction. The resulting increase in malate concentration in the mitochondria makes the Mal-Oaa reaction thermodynamically more favorable increasing the reaction's rate, NADH synthesis, and improving cells' energy metabolism. On the other hand, MDH II overexpression enhances oxaloacetate production. This increase in turn increases the available Gibbs free-energy for the condensation reaction through which carbon molecules enter the TCA cycle. CHO FrcTr cells are able to produce more pyruvate. Higher pyruvate levels lead to an increase in available free-energy for lactate and acetyl CoA synthesis.

In conclusion, this work was able to successfully compare the effect of different modifications and evaluate their suitability for industrial use. Each of these modifications has an important impact over carbon metabolism improving the ΔL/ΔHexose that each culture exhibits and reducing NAD^+/NADH ratios. The discussions from a metabolic and thermodynamic point of view presented in this work support previous conclusions regarding improvement of the energy metabolism of these cell modifications. In addition, it explains the drop in productivity that three of the studied clones exhibit by linking the new metabolic flux distribution to changes in the redox state, which have a detrimental effect on IgG's assembly, therefore reducing its secretion. The impact of these modifications on protein synthesis might be different for cells producing single chain recombinant proteins. Due to the complexity of cellular metabolism, the overall effect of a given modification on the central carbon pathway is difficult to predict. However, our results

suggest that by improving energy metabolism and the flux into the TCA cycle, it is possible to improve cultures' performance, provided that appropriate redox conditions can be maintained.

1.6. Bibliography

Altamirano, C., Paredes, C., Cairo, J.J., Godia, F., 2000. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnol Prog 16, 69-75.

Boada, J., Roig, T., Perez, X., Gamez, A., Bartrons, R., Cascante, M., Bermudez, J., 2000. Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress. FEBS Lett 480, 261-264.

Boissel, J.P., Lee, W.R., Presnell, S.R., Cohen, F.E., Bunn, H.F., 1993. Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure. J Biol Chem 268, 15983-15993.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnol Prog 21, 106-111.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. J Biotechnol 147, 116-121.

Eagle, H., Barban, S., Levy, M., Schulze, H.O., 1958. The utilization of carbohydrates by human cell cultures. J Biol Chem 233, 551-558.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003. Improving glucose and glutamine metabolism of human HEK 293 and Trichoplusia ni insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnol Prog 19, 90-97.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnol Bioeng 67, 25-34.

Fogolin, M.B., Wagner, R., Etcheverrigaray, M., Kratje, R., 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J Biotechnol 109, 179-191.

Fussenegger, M., Mazur, X., Bailey, J.E., 1997. A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnol Bioeng 55, 927-939.

Gambhir, A., Korke, R., Lee, J., Fu, P.C., Europa, A., Hu, W.S., 2003. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. J Biosci Bioeng 95, 317-327.

Glacken, M.W., 1988. Catabolic Control of Mammalian Cell Culture. Nature Biotechnology 6, 1041 - 1050.

Glacken, M.W., Fleischaker, R.J., Sinskey, A.J., 1986. Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol Bioeng 28, 1376-1389.

Inoue, Y., Tsukamoto, Y., Yamanaka, M., Nakamura, S., Inoue, A., Nishino, N., Kawahara, H., 2010. Efficient production of recombinant IgG by metabolic control and co-expression with GLUT5 in a fructose-based medium. Cytotechnology 62, 301-306.

Irani, N., Beccaria, A.J., Wagner, R., 2002. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. J Biotechnol 93, 269-282.

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.

Jones, M.L., Seldon, T., Smede, M., Linville, A., Chin, D.Y., Barnard, R., Mahler, S.M., Munster, D., Hart, D., Gray, P.P., Munro, T.P., 2010. A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies. J Immunol Methods 354, 85-90.

Kerbey, A.L., Radcliffe, P.M., Randle, P.J., 1977. Diabetes and the control of pyruvate dehydrogenase in rat heart mitochondria by concentration ratios of adenosine triphosphate/adenosine diphosphate, of reduced/oxidized nicotinamide-adenine dinucleotide and of acetyl-coenzyme A/coenzyme A. Biochem J 164, 509-519.

Korke, R., Gatti Mde, L., Lau, A.L., Lim, J.W., Seow, T.K., Chung, M.C., Hu, W.S., 2004. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. J Biotechnol 107, 1-17.

Kummel, A., Panke, S., Heinemann, M., 2006. Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolome data. Mol Syst Biol 2, 2006 0034.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. J Biotechnol 15, 113-128.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2008. Lehninger principles of biochemistry, 5th ed. W.H. Freeman.

Longmore, W.J., Niethe, C.M., McDaniel, M.L., 1969. Effect of CO2 concentration on intracellular pH and on glycogen synthesis from glycerol and glucose in isolated perfused rat liver. J Biol Chem 244, 6451-6457.

Mooney, R.A., Lane, M.D., 1982. Control of ketogenesis and fatty-acid synthesis at the mitochondrial branch-point for acetyl-CoA in the chick liver cell: effect of adenosine 3',5'-monophosphate. Eur J Biochem 121, 281-287.

Robinson, B.H., Williams, G.R., Halperin, M.L., Leznoff, C.C., 1971. Factors affecting the kinetics and equilibrium of exchange reactions of the citrate-transporting system of rat liver mitochondria. J Biol Chem 246, 5280-5286.

Rozwarski, D.A., Diederichs, K., Hecht, R., Boone, T., Karplus, P.A., 1996. Refined crystal structure and mutagenesis of human granulocyte-macrophage colony-stimulating factor. Proteins 26, 304-313.

Schuster, R., Holzhutter, H.G., Jacobasch, G., 1988. Interrelations between glycolysis and the hexose monophosphate shunt in erythrocytes as studied on the basis of a mathematical model. Biosystems 22, 19-36.

Sies, H., Noack, G., Halder, K.H., 1973. Carbon-dioxide concentration and the distribution of monocarboxylate and H+ ions between intracellular and extracellular spaces of hemoglobin-free perfused rat liver. Eur J Biochem 38, 247-258.

Terada, S., Suzuki, E., Ueda, H., Makishima, F., 1996. Cytokines involving gp130 in signal transduction suppressed growth of a mouse hybridoma cell line and enhanced its antibody production. Cytokine 8, 889-894.

Wilkens, C.A., Altamirano, C., Gerdtzen, Z.P., 2011. Comparative metabolic analysis of lactate for CHO cells in glucose and galactose Biotechnol. Bioprocess Eng 16.

Williamson, D.H., Lund, P., Krebs, H.A., 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 103, 514-527.

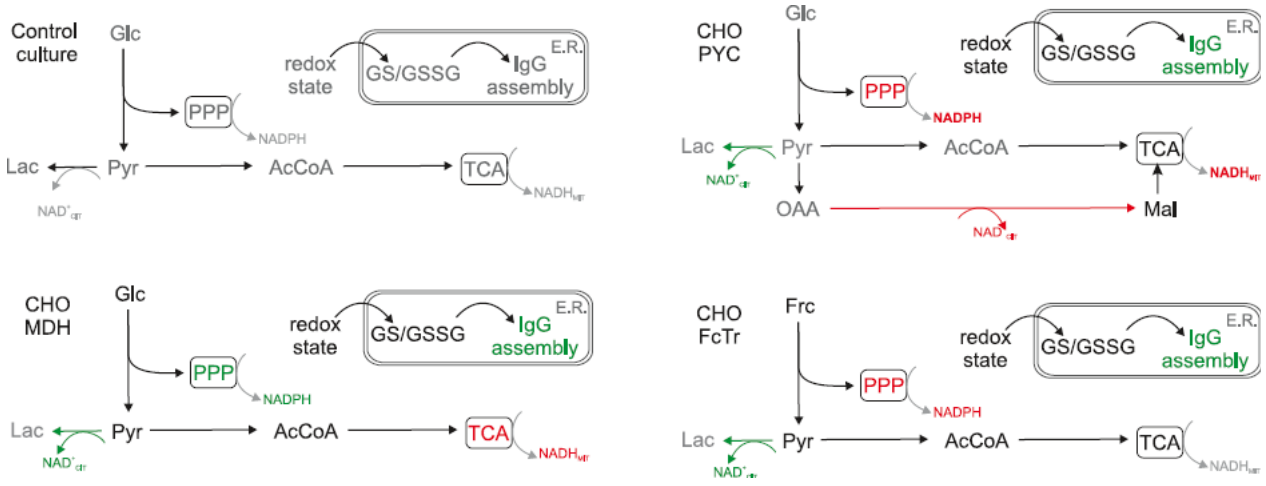
Wlaschin, K.F., Hu, W.S., 2007. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. J Biotechnol 131, 168-176.

Wu, F., Yang, F., Vinnakota, K.C., Beard, D.A., 2007. Computer modeling of mitochondrial tricarboxylic acid cycle, oxidative phosphorylation, metabolite transport, and electrophysiology. J Biol Chem 282, 24525-24537.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22, 1393-1398.

Zupke, C., Sinskey, A.J., Stephanopoulos, G., 1995. Intracellular flux analysis applied to the effect of dissolved oxygen on hybridomas. Appl Microbiol Biotechnol 44, 27-36.

1.7. Supplementary Information



Supplementary Figure 1: Effect of the different modifications over RedOx state and its effect over protein assembly. Green and red text and arrows indicate an increase or decrease in pathway flux or molecule synthesis.

CHAPTER 2: Construction and characterization of a LDHa knock down CHO cell clone generated with the novel genome editing tool CRISPR-Cas

2.1. Abstract

Lactate dehydrogenase A gene knock down (K.D.) has been the aim of numerous cell engineering approaches for lactate reduction in animal cell cultures in order to improve cells' metabolism and recombinant protein yield. Recently a novel genome editing tool, CRIPR-Cas, was developed for inducing double strand breaks in the genome and it has proven to be an efficient new instrument for introducing targeted mutations. In this work we used George Church's CRISPR-Cas system and relied on the non-homologous end join repair system to introduce mutations in one of the LDHa gene copies to obtain an LDHa knocked down CHO cell clone. This chapter describes in detail the CRISPR-Cas system, how target sequences were selected, the high throughput mutant detection assay used and culture performance results of the selected clone, additionally a discussion of the effect that LDHa K.D. exerts on cell metabolism.

Detailed analysis of the LDHa gene's coding region and lactate dehydrogenase's protein's catalytic residues was performed to select target regions. Several possible target sequences were identified within the gene, but only three were considered suitable. Mutation screening results indicate that the employed CRISPR-Cas system did not present a high efficiency. Nevertheless, using a PCR/Restriction enzyme essay it was possible to identify one mutant clone with a loss-of-function mutation on a copy of LDHa, which lead to a reduced gene translation. Fed-batch cultures were conducted in order to compare mutated cells' culture performance against the parental cell line. Results show that cell growth and metabolism were affected by LDHa knock down and specific protein rate and volumetric production were greatly enhanced

2.2. Introduction

After observing that both high lactate concentration and high lactate production per glucose consumed are negative for cultures' performance Chen and collaborators proposed to reduce lactate synthesis by reducing the *lactate dehydrogenase* (LDHa) gene expression (Chen et al., 2001a). Through homologous recombination they were able to knock down this gene in hybridoma cells. After clone selection a 30% reduction of LDHa activity was registered and the best clone proved to have a more efficient metabolism consuming more glucose while producing less lactate. Additionally, cells were able to reach higher cell density and produce 2,5 times the

total amount of recombinant protein than wild-type cells. Using alternative strategies for knocking down gene's expression, such as siRNA (Kim and Lee, 2007a; Zhou et al., 2011b) and antisense DNA (Jeong et al., 2006b), other researches have been able to achieve similar results, demonstrating that cells with reduced LDHa activity are less resistant to oxidative stress and intracellular and extracellular pH changes, but are able to achieve higher cell proliferation and have higher ATP concentrations.

Several publications have recently shown the efficacy of the novel genome engineering tool CRISPR-Cas for introducing mutations on different organisms (Chang et al., 2013; Cho et al., 2013; DiCarlo et al., 2013; Feng et al., 2013; Friedland et al., 2013; Fu et al., 2013; Hockemeyer et al., 2011; Hwang et al., 2013; Jiang et al., 2013; Li et al., 2013a; Shan et al., 2013). This new methodology differs from its most famous predecessors, zinc fingers (ZFN) and TALENs, due to its simple construction method, were only a 23 nucleotide sequence has to be introduced into a vector that contains all other necessary sequences (Bassett et al., 2014; DiCarlo et al., 2013; Jao et al., 2013; Jiang et al., 2013; Mali et al., 2013b; Waaijers et al., 2013), but has the drawback that it is prone to off target mutations (Fu et al., 2013; Hwang et al., 2013; Pattanayak et al., 2013). Additionally, the CRISPR-Cas system shows great potential for regulating transcription by blocking, repressing or activating it (Bikard et al., 2013; Larson et al., 2013; Qi et al., 2013) or delivering effector domains to regulatory regions (Gilbert et al., 2013) or transcription factors (Farzadfard et al., 2013).

The Crispr-Cas system was developed from the bacterial and archaea's adaptive immune system against invaders called type II CRISPR-Cas. The engineered system consists of a Cas9 nuclease with a nuclear localisation signal (NLS) that introduces a double strand breaks (DSB) on a specific target on the genomic DNA (gDNA) as illustrated in Figure 3. The correct positioning of the nuclease is achieved by the presence of a synthetic guide RNA (gRNA) designed to locate it over a desired sequence in the genome. The target sequence consists of a 20 nucleotide DNA sequence beginning with a G called protospacer, flanked by a conserved NGG sequence known as proto-spacer adjacent motif (PAM), resulting in a $G(N)_{19}NGG$ target. Mutations can be introduced during the repair of the DSB by different cellular mechanisms. The first is non-homologous end joining repair (NHEJ), which is an error-prone procedure were two broken ends are rapidly ligated back together but with the insertion or deletion of some nucleotides resulting in frame shift mutations that can knock out genes (Burma et al., 2006; Thompson and Limoli, 2003; Valerie and Povirk, 2003). The second is homologous

recombination (HR) were repair occurs using a homologous sequence as a template for restoration of the DNA (Arnaudeau et al., 2001; Sargent et al., 1997; Takata et al., 1998; Thompson and Schild, 2001). It is known that DSBs significantly increase the chance of repair by HR allowing the introduction of sequences in a specific locus of interest as long as it is placed between two homology arms (Johnson and Jasin, 2001; Johnson et al., 1999; Liang et al., 1998).

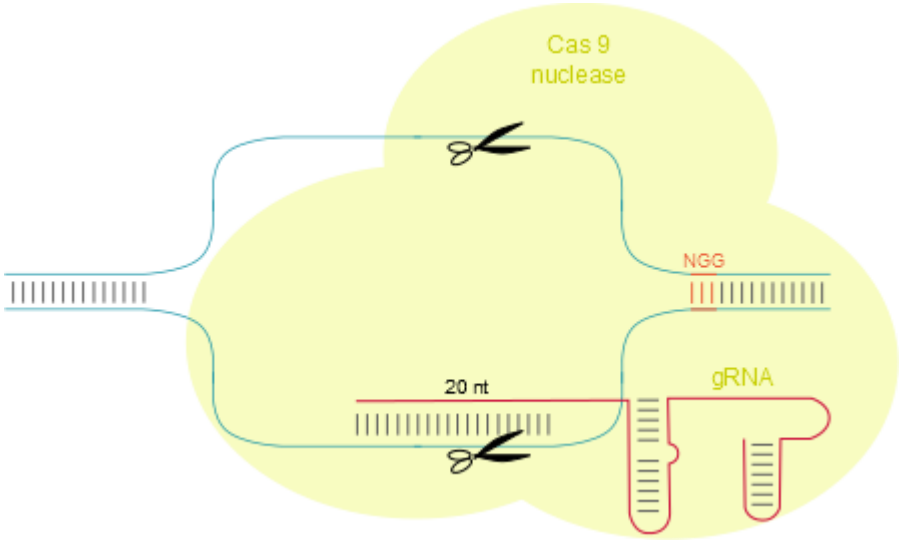


Figure 3: CRISPR-Cas system. Blue lines represent genomic DNA, synthetic gRNA is shown in red and the light green figure is the Cas9 nuclease

In this work we aim at using the CRISPR-Cas system to knock down or knock out the lactate dehydrogenase A (LDHa) gene in order to obtain more efficient recombinant protein producing CHO cell cultures. This subject is of interest, for industrial purposes, to reduce lactate synthesis, since its presence and production rate have a negative effect on cell growth and protein synthesis (Europa et al., 2000a; Glacken et al., 1986a; Kurano et al., 1990a). Previous works have been able to successfully knock down LDHa with short interference RNA (Kim and Lee, 2007b; Zhou et al., 2011a) and antisense mRNA (Jeong et al., 2006a; Jeong et al., 2001), showing that cultures exhibited a more efficient metabolism consuming more glucose while producing less lactate. Additionally, cells were able to reach higher cell densities and produce more recombinant protein than parental cells.

To provide results and definitive proof of successful gene knock down researchers have used Western Blot to compare expression levels of the targeted protein. This technique has been used to confirm knock down of the lamin A/C gene in S2 cells using siRNA (Elbashir et al., 2001), Bax and Bak knock down using RNAi in CHO cells (Lim et al., 2006) and DHFR knock out in CHO cells employing zinc finger nucleases (Santiago et al., 2008).

To the authors knowledge LDHa knock out is likely to be lethal for cells because of the importance of the enzyme in cell metabolism and RedOx maintenance. To this date there have not been any publications reporting viable cells without measurable LDHa activity. In order to attempt loss-of-function mutation in all alleles of LDHa the experiments will be performed in a CHO cell line that overexpresses LDHc. This isoform of LDH is exclusively expressed in testis (Goldberg et al., 2010) and differs from the other isoforms in its kinetic parameters with a higher affinity for lactate (Gupta, 1999). We speculate that in case of achieving a successful K.O., these cells would still be able to produce lactate and oxidize NADH at a suitable rate that will enable cells to survive.

2.3. Material and Methods

2.3.1. Cell line and culture conditions

Recombinant Fc-fusion protein producing DXB-11 CHO cells overexpressing *Mus musculus'* LDHc were maintained at 37°C, 96% humidity and in a 5% CO_2 enriched air atmosphere. Culture media consisted in DMEM/F12 (1:1) (Gibco, ME090283L1) supplemented with 5 mg/L transferrin (Sigma, T8158), 1.7 µL/L 2-mercaptoethanol (Sigma, M3148-250), 0.1 ml/L ethanolamine (Sigma, E0135), 0.11 mM ascorbic acid (Sigma, A4544), 0.18mg/L putrescine (Sigma, P5780), 29 mM sodium bicarbonate (Sigma, S4019), 28.9 mM sodium selenite (Sigma, S5261), 0.2 g/L pluronic F68 (Sigma, P1300), with a 22.22 mM final concentration of glucose (Sigma, G5146) and 4 mM glutamine (Sigma, G1517). During transfection, cell cloning and high-throughput clone analysis cells were grown in adherence adding 5% FBS to the culture media. For clone characterization, cells were maintained in suspension in spinner flasks with 5% serum and cultured using a fed-batch strategy.

Suspension fed-batch experiments were performed in biological duplicates. Starting on day 0 cells were inoculated at 0.5×10^6 cells mL^{-1}. Sampling was done twice a day from day 0

until culture's viability descended from 75%. From day 2 a chemically defined feeding media was fed to the spinner once a day adjusting the final glucose concentration to 22.22 mM. Cell number and viability were determined by the trypan blue exclusion method. After cell counting cells were spinned down and the supernatant was stored at -20°C until further analysis.

2.3.2. Metabolite determinations

Glucose and lactate concentrations were determined using a Glucose and Lactate Assay kit respectively (GL3981, LC2389, Randox) and recombinant protein concentration in the supernatant was quantified with an ELISA test.

2.3.3. Specific rate determination

For calculating the specific rate of production (q_p) or consumption (q_c) of all metabolites present in the culture media, a cumulative curve is constructed by adding the amount of that metabolite produced or consumed between two consecutive time points of culture. The specific rates are calculated as the derivative of the polynomial curve that fits the curve divided by the cell density evaluated in each time point. This method ensures small fluctuations in the results that may be caused by any measurements errors. The value of $\Delta Lactate/\Delta Glucose$ is defined as $-q_{Lac}/q_{Glc}$ therefore, it is calculated as the quotient between the two specific rates.

2.3.4. Experimental procedure

For the methodology used in this work, mutation occurrence relies on errors taken place during the NHEJ repair of the DSB caused by the Cas9 nuclease. This repair system adds or deletes some base pairs around the cutting site, which in the CRISPR-Cas case sits 3 bp upstream from the PAM sequence (Jinek et al., 2012), as seen in Figure 4.a. In this work the mutation analysis consists in a PCR/Restriction enzyme assay that screens for the loss of a restriction site similar to the one employed by Shan and collaborators (Shan et al., 2013). Therefore one of the considerations for choosing a target sequence is the presence of a restriction enzyme recognition site as close as possible to the expected cutting site, which should be destroyed after the DSB repair. In this work the enzyme that recognizes this site will be referred as *m.a. enzyme*.

The experimental procedure consists in:

1. Target selection
2. Mutant generation by delivering the CRISPR-Cas system
3. Genomic DNA mutation analysis from pool of treated cells
4. Single cell cloning
5. Genomic DNA mutation analysis from individual clones

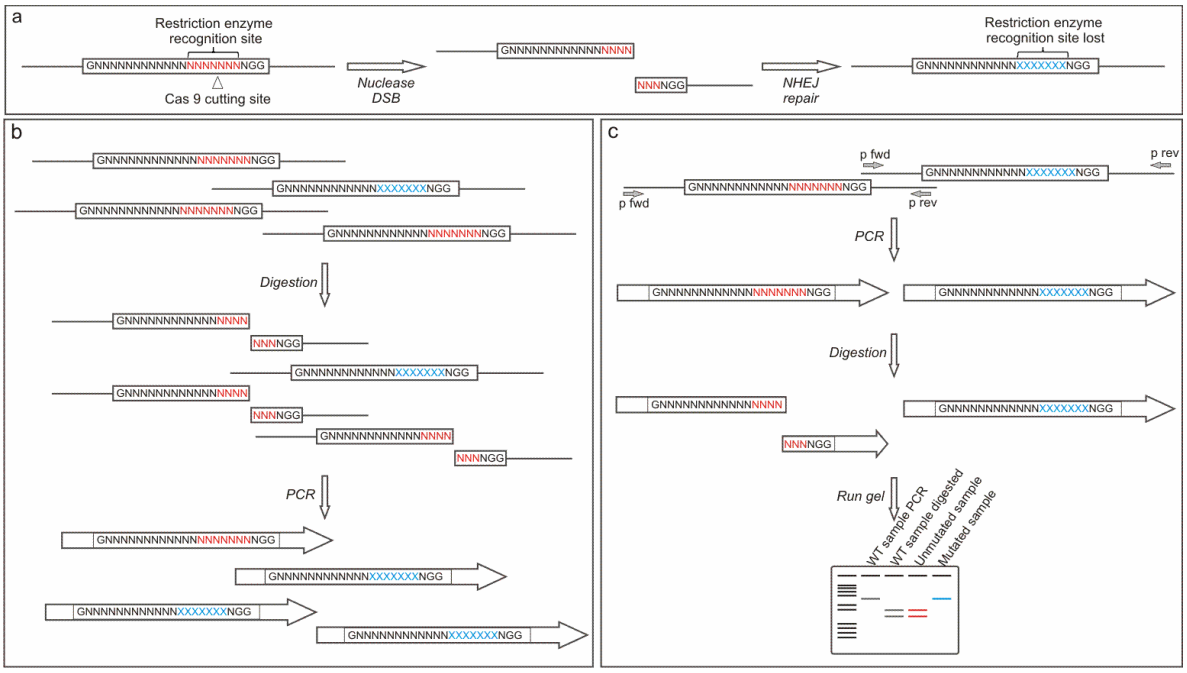


Figure 4: Schematic representation of mutation screening. a.- mutation occurrence after nuclease DSB and NHEJ repair; b.- digestion and PCR procedure to enrich the sample in mutated sequences; c.- PCR/restriction enzyme assay to determine if a DNA sample contains the desired mutation

2.3.5. Target selection

Previous to target selection different elements within the LDHa gene had to be identified, such as introns, exons and nucleotides that transcribe catalytic amino acid residues of the lactate dehydrogenase enzyme. Target sequences for the CRISPR-Cas system should have a

$G(N)_{19}NGG$ pattern, which can be either on the 5'-3' or 3'-5' strand. Sequences had to be found within each exon of the LDHa gene so the possible mutation would affect coding sequences. For selecting the best possible targets different characteristics were assessed for potential target sequences; such as position in the coding sequence, GC content, secondary structures, possible off targets and unique restriction sites over Cas9 cutting site. Position is crucial for the mutation to occur upstream from catalytic amino acids in order for the resulting enzyme to lose catalytic activity. Knowing GC content is important because it has been shown to influence the stability of RNA and DNA alignments (Sugimoto et al., 1995) and secondary structures enables us to avoid possible issues with the gRNAs' structure. GC content and secondary structures were determined using an online oligonucleotide property calculator (Kibbe, 2007). Possible off targets were identified by comparing the target sequence against *Cricetulus griseus'* genome using the CHOgenome.org's BLAST tool (Hammond et al., 2012) and looking at the amount of high homology alignments. Correct activity of the CRISPR-Cas is more sensitive to mismatches in the 3' end of the gRNA (Fu et al., 2013), therefore possible off targets were considered to be all those sequences with a $M_7N_{14}GG$ pattern, were M_7 corresponds to any 7 nucleotide sequences at the 5' end and the $N_{14}GG$ stands for a sequence that perfectly aligns with the 3'end of the intended target. Finally, unique restriction sites were identified using NEBcutter, a web based digestion pattern finder (Vincze et al., 2003).

2.3.6. Vectors, Transfection and cell cloning

The CRISPR-Cas system employed in this work was developed by Church and collaborators (Mali et al., 2013a). The system consists of 2 vectors that are cotransfected into the cells. The first plasmid expresses a human codon-optimized Cas9 nuclease and the other transcribes the synthetic guide RNA. The desired gRNA vector was constructed as instructed by annealing two 60 bp oligomers that contain the target sequence using Phusion polymerase (M0530, New England Biolabs) to obtain a 100 bp DNA fragment. Afterwards this sequence was cloned into the empty linearized gRNA cloning vector using the Gibson Assembly method (E5510, New England Biolabs). After transformation, colony isolation and miniprep all resulting vectors were verified by sequencing.

Cells were transfected using Lipofectamine 2000 (11668, Invitrogen) following the manufacturer's indications. The day prior to transfection 0.4×10^6 cells mL^{-1} were inoculated in a 6 well plate with 2.1 mL of media. The transfection cocktail was prepared mixing a tube that

contained 5 µg of a 1:1 molar mixture of the Cas9 and gRNA vectors in serum free DMEM/F12 up to 250 µL with another tube that contained 10 µL of Lipofectamine and serum free DMEM/F12 to a final volume of 250 µL and incubated for 20 min. Afterwards cells are washed with PBS and 1.6 mL of media and the DNA-lipofectamine mixture is added to each corresponding well. After 4-6 hours of incubation at 37°C the media is removed and replaced with fresh media containing FBS.

Cell cloning was carried out 72 h post transfection by plating close to 100 cells at a 0.5 cells mL^{-1} concentration in 96 well plates. The remaining cells were used for genomic DNA extraction using the DNeasy Blood & Tissue Kit (69504, Qiagen).

2.3.7. Mutation Analysis and PCR/Restriction enzyme Assay

The first mutation analysis is performed over genomic DNA extracted from the pool of treated cells using a PCR/restriction enzyme assay. Because the mutant proportion in this pool is unknown and could be very low, enrichment of the mutated sequences in the sample can be performed. Figure 4.b shows this procedure which consists in the digestion of the genomic DNA with the *m.a.enzyme* (4 h at 37°C) followed by a PCR amplification were primers were designed to amplify a 200-250 bp region containing the target sequence.

The procedure for the PCR/restriction enzyme assay, used to determine if a DNA sample contains the desired mutation, is explained in Figure 4.c. From treated and untreated samples a 200-250 bp region containing the target sequence is amplified by PCR, using GoTaq polymerase (M300, Promega), and digested (16 h at 37°C) with the *m.a. enzyme*. In a 2% agarose gel samples of untreated cells' PCR and digested samples are run as controls besides treated cells' digested samples. It is expected that samples from cells that were not mutated would show the same digestion pattern as the one presented by the untreated digestion sample. If cells were mutated the restriction enzyme recognition site would have been destroyed, therefore the PCR product would not be digested and migrate as the control's PCR product. In the case of samples from the pool it is expected to find a mixture of these two results for the treated samples. For single cell clone analysis in case of a homozygous mutation a single undigested band is expected, if only one allele was mutated the three band pattern is expected and if no mutation occurred the digestion pattern of untreated cells is anticipated.

Potential mutants were confirmed with Sanger sequencing. After PCR using Phusion polymerase the fragments were TA cloned into the PCR2.1TOPO® TA vector using the TOPO cloning system and transformed into One Shot® TOP10 chemically competent cells (K4500, Invitrogen).

2.3.8. Genomic DNA extraction from single cell clones in 96 well plates

The 10x stock of the lysis buffer consists of 100 mM TrisCl pH8, 10 mM EDTA pH8, 4.5% Tween-20 and 4.5% Triton-x 100. Before each procedure fresh 1x buffer was prepared and supplement with 200 µg/mL Proteinase K (19131, Qiagen). Cells were washed with PBS and then 20-30 µL of lysis buffer were added to each well and left at room temperature for 10 minutes to insure lysis. Afterwards, the buffer was transferred to a 96-well PCR plate and incubated in a thermocycler for 1 hour at 55 °C, then 15 min at 95 °C. The resulting solution was used directly in PCR assays.

2.3.9. Western Blot and sample preparation

Protein extraction was performed by incubating 6×10^6 cells in RIPA lysis buffer (Tris-HCl 20 mM, NaCl 150 mM, EDTA 5 mM, Nonidet P-40 1 %, SDS 0.1 %, sodium deoxycholate 0.5 % and PMSF 1 mM, pH 7.5) with the commercial protease inhibiting cocktail Complete®, Mini EDTA-free (11836170001, Roche). 2 mL of the lysis buffer was added to pelleted cells, and then shaken at 4°C for 15 min. Finally, the sample was centrifuged at 14.160 RCF for 15 min and the supernatant containing proteins was collected for further analysis. Protein concentration was determined using the commercial kit Bicinchoninic Acid™ (23227, Pierce Thermo Scientific), based on the Lowry method, using a 1 mg/mL BSA solution as a standard.

Equal mass quantities of each protein sample were resolved in a 10% PAGE-SDS gel. A discontinuous polyacrylamide gel was prepared and later run in the commercially available Mini-PROTEAN® II Electrophoresis Cell (165-2940, Bio-Rad) system using fresh Tris/Glycine/SDS running buffer (Tris-HCl 25 mM, glycine 192 mM y SDS 0.1 %). Samples were prepared by mixing them with a 5X loading buffer (Tris-HCl 60 mM, glycerol 25 %, SDS 2 %, 2-mercaptoethanol 14.4 mM and bromophenol blue 0.1 %, pH 6.8) and boiled at 95°C for 5 min. The Spectra™ Multicolor Broad Range Protein Ladder (11874544, Fermentas) was used as a molecular weight marker. After loading the samples into the gel, proteins were separated by

running the gel at 80 mV for 30 min (or until the sample reached the separating gel) and afterwards increasing the voltage to 100 mV for 90 min (or until bromophenol blue marker falls from the gel).

The Mini Trans-Blot® Electrophoretic Transfer Cell system (170-3930, Bio-Rad) was used for transferring the protein to a Protran Nitrocellulose Hybridization Transfer Membrane (NBA083S001EA, Whatman®, PerkinElmer™), using freshly prepared and cold transference buffer (Tris-HCl 25 mM, glycine 192 mM, methanol 20 % y SDS 0.01 %). The assembled system was run at 4°C and 300 mA for 90 min. To verify protein transfer quality the membrane was stained using Ponceau S stain (Ponceau S 0.2% (w/v), glacial acetic acid 5%) and afterwards rinsed off with three washes of TBST buffer (Tris-HCl 10 mM, NaCl 150 mM and Tween20 0.1 %, pH 7.5).

Prior to inmunodetection, the membrane was blocked by incubation in TBST buffer with 5% non-fat dry milk overnight at 4°C. The next day the membrane was washed with TBST buffer and incubated for 1 hr at room temperature with the primary antibody in TBST buffer with 1% non-fat dry milk. Subsequently membrane was washed 3 times with 30 mL of TBST buffer for 15 minutes each time. Afterwards, the membrane was exposed to the secondary antibody in the same manner as with the primary antibody and washed the same way. Finally, detection was performed using the SuperSignal® West Pico Chemiluminiscent Substrate (34077, Pierce, Thermo Scientific). Image analysis was performed using the open source image processing program ImageJ.

For LDHa detection, the Anti-LDHA antibody produced in rabbit (AV54777, Sigma) was used as primary antibody and Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate as the secondary antibody (31466, ThermoScientific). Actin was used as a housekeeping control and was detected with Monoclonal Anti-β-Actin antibody produced in mouse (A5441, Sigma) and Rabbit anti-Mouse IgG (H+L) Secondary Antibody (31450, ThermoScientific).

2.4. Results and Discussion

2.4.1. LDHa Sequence Analysis and Possible Targets Screening

LDHa's coding sequence with its most important features is presented below. By aligning CHO cells' LDHa gDNA and mRNA sequences, obtained from CHOgenome.org, with *Mus musculus'*

LDHa sequence (Fukasawa and Li, 1987) 7 exons were be identified, each highlighted with a different color in Figure 5. Analysis of the highly conserved mammalian lactate dehydrogenase A amino acid sequence allowed to identify codons that codify for the catalytic residues (Holmes and Goldberg, 2009); these are presented in bold font and underlined in Figure 5. The catalytic residues are His 193, which is the active site's proton acceptor; Arg99 and Asn138, which bind the coenzyme; and Arg106, Arg169 and Thr248 that attach the substrate to the enzyme (Read et al., 2001). In the supplementary section the complete gDNA and protein sequences are provided.

>NM_001244050.1_CHOg
ATGGCAACACTCAAGGACCAGCTGATTGTGAATCTACTTAAGGAAGAACAGACCCCCCAGAACAAGATTACGATTGTT
GGGGTTGGTGCTGTTGGCATGGCTTGTGCCATCAGTATCCTCATGAAGGACTTAGCAGATGAGCTTGCCCTTGTTGAT
GTCATGGAAGACAAGCTGAAGGGGGAAATGATGGATCTCCAGCATGGCAGCCTTTTCCTCAGAACACCAAAAATTGTC
TCTGGCAAAGACTATAGTGTGACAGCAAACTCCAAGCTGGTCATTGTCACAGCGGGGGCC**CGT**CAGCAAGAGGGAGAG
AGC**CGA**CTCAATCTGGTCCAACGAAACGTGAACATCTTCAAGTTCATCATTCCCAACGTTGTGAAATACAGTCCAGAC
TGCAAGCTGCTTATTGTTTCA**AAC**CCAGTGGATATCTTGACCTACGTGGCTTGGAAAATAAGTGGCTTTCCCAAAAAC
CGAGTTATTGGGAGTGGCTGCAATTTGGATTCTGCT**CGA**TTCCGTTATCTGATGGGAGAAAGGTTGGGTGTTCACCCA
CTAAGCTGTCATGGATGGGTCCTAGGGGAG**CAT**GGAGACTCCAGTGTGCCTGTGTGGAGTGGAGTGAATGTAGCTGGT
GTCTCCCTGAAGAATCTGAATCCAGAGCTGGGCACTGATACCGACAAGGAGCAGTGGAATGAGGTTCACAAGCAGGTG
GTTGACAGTGCCTATGAGGTGATCAAACTGAAGGGTTAT**ACA**TCCTGGGCCATTGGTCTGTCTGTGGCTGACTTGGCC
GAGAGCATAATGAAGAATCTTAGGCGGGTGCATCCCATTTCTACCATGATTAAGGGTCTCTATGGAATCAAGGATGAT
GTCTTCCTCAGTGTCCCTTGTGTCCTGGGACAAAATGGAATCTCCGATGTTGTGAAGGTGACTCTAACTTCTGAAGAG
GAGGCCCGCTTGAAGAAGAGTGCAGATACACTATGGGGGATCCAGAAAGAGCTGCAGTTCTGA

Figure 5: LDHa coding sequence in CHO cells

Table 3 summarizes the information gathered for all potential target sites. Based on this targets 7 and 8 were chosen since they exhibited desired qualities such as position regarding catalytic residues, a restriction enzyme cut site close to Cas9's cutting site and fewest possible off-targets.

Table 3: Possible CRISPR-Cas targets.

ID #	Protospacer	PAM	position DNA	Exon	Strand	GC content (%)	Possible off targets	Restriction enzyme	Secondary structures
1	GAACAAGATTACGATTGTTG	GGG	60	1	+	43	0		p.h.[1]
2	GCTTGCCCTTGTTGATGTCA	TGG	141	2	+	52	3		
3	GTCATGGAAGACAAGCTGAA	GGG	157	2	+	52	3		
4	GAAATGATGGATCTCCAGCA	TGG	181	2	+	48	6		
5	GCTGGTCATTGTCACAGCGG	GGG	270	3	+	65	1		
6	GCGGGGGCCCGTCAGCAAGA	GGG	286	3	+	78	1		p.h. + s.a.[2]
7	**GGGAGAGAGCCGACTCAATC**	**TGG**	**306**	**3**	**+**	**61**	**0**	**MlyI**	
8	**GTTCACCCACTAAGCTGTCA**	**TGG**	**538**	**4**	**+**	**52**	**3**	**AluI**	
9	GCTGTCATGGATGGGTCCTA	GGG	551	4	+	61	0	AvaII	
10	GGATGGGTCCTAGGGGAGCA	TGG	559	4	+	65	0		
11	GAAGAATCTGAA**TCCAGA**GC	TGG	633	5	+	48	7	Hpy188III	
12	GCTGGGCACTGATACCGACA	AGG	651	5	+	61	0		
13	GTGGAATGAGGTTCACAAGC	AGG	678	5	+	52	8		
14	GAATGAGGTTCACAA**GCAGG**	**T**GG	681	5	+	52	4	BspMI	
15	GCATAATGAAGAATCTTAGG	CGG	785	6	+	43	2		
16	GGTGACTCTAACTTCTGAAG	AGG	915	7	+	48	3		
17	GACTCTAACTTCTGAAGAGG	AGG	918	7	+	48	6		p.h.
18	GAAGAGTGCAGATACACTAT	GGG	951	7	+	48	0		p.h. + s.a.
19	GTCAGCCACAGACAGACCAA	TGG	752	6	-	57	10		
20	GCTCTGGATTCAGATTCTTC	AGG	630	5	-	48	9		p.h. + s.a.
21	GGCTCTCTCCCTCTTGCTGA	CGG	294	3	-	65	2		

[1] p.h.:potential hairpin
[2] s.a.:self annealing

To amplify the 200-250 bp region flanking target sequences by PCR, primers were designed in order to amplify the exons in which the targets are located and are presented in Table 4.

Table 4: Primers for exon amplification.

Primer name	Sequence
exon 3 fwd	CTAGACTATAGTGTGACAGCAAAC
exon 3 rev	CTGGGTTTGAAACAATAAGCAGC
exon 4 fwd	TGGATATCTTGACCTACGTGGCTTG
exon 4 rev	ACTGGAGTCTCCATGCTCCCC

2.4.2. LDHa Mutation analysis

Mutation analysis was performed over genomic DNA samples extracted from the pool of transfected cells with the CRISPR–Cas system as described in the Material and Methods section. Agarose gel pictures are shown below in Figure 6 and no clear undigested bands can be seen in either gel, which indicated that little to no mutation occurred among the treated cells.

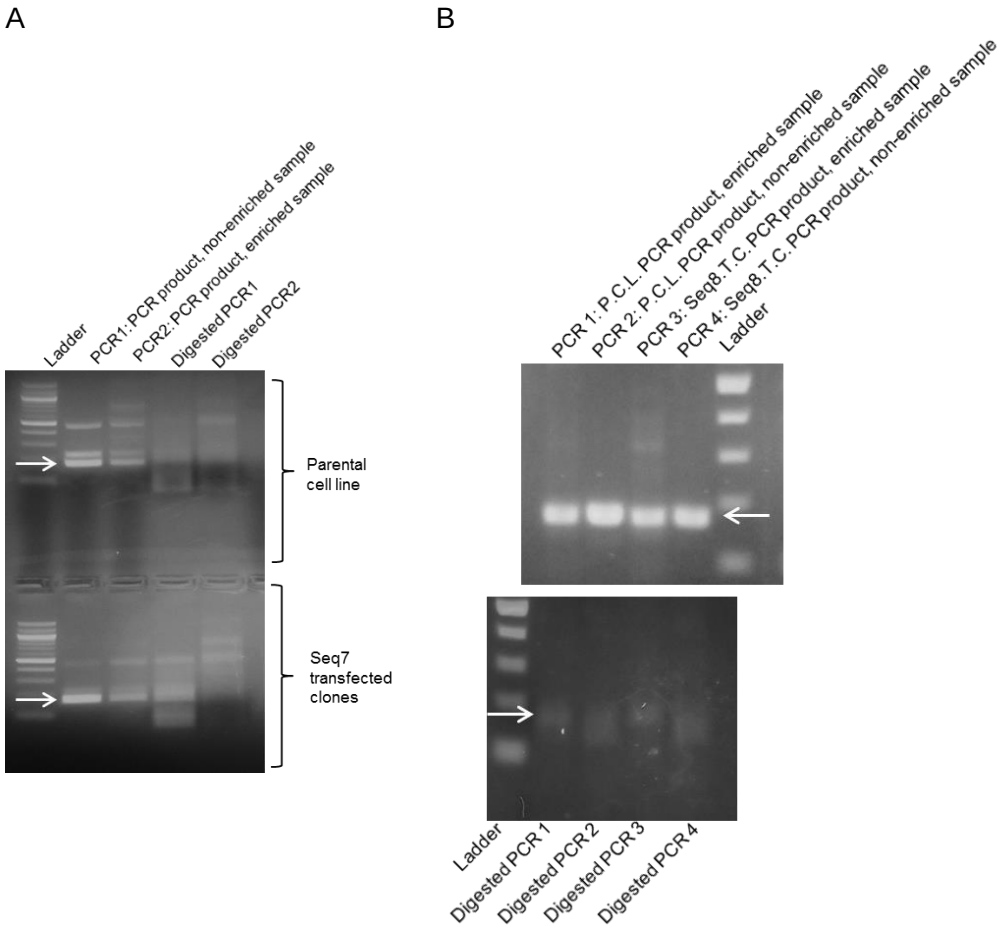


Figure 6: Agarose Gel pictures of mutation analysis from pool samples. A: PCR and digestion products in control cells (parental cell line) and target 7 mutation; B: PCR and digestion products of parental cell line (P.C.L) as control and clones transfected with Seq 8 (T.C.).

After genomic DNA extraction from single cell clones, a PCR/restriction enzyme assay was performed over every sample recovered as described above. Pictures from the agarose gels are shown in Figure 7 and Figure 8.

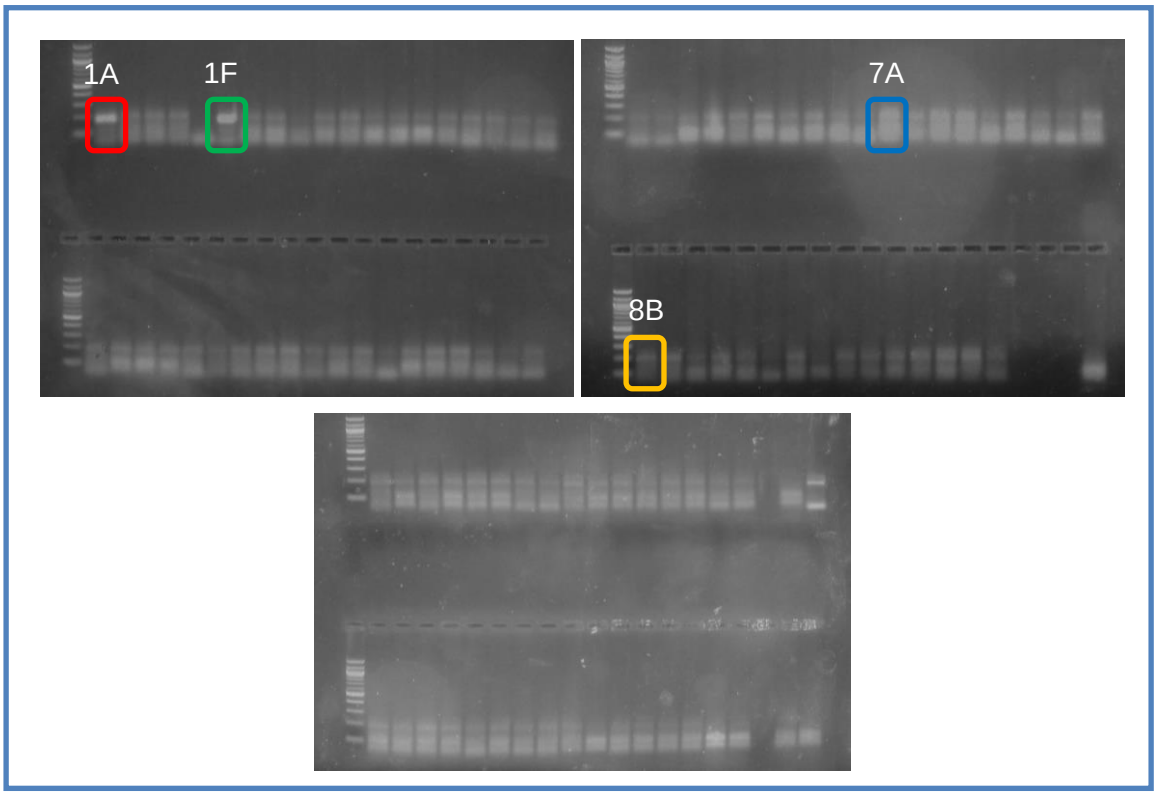


Figure 7: PCR/Restriction enzyme Assay from target 7 mutation samples

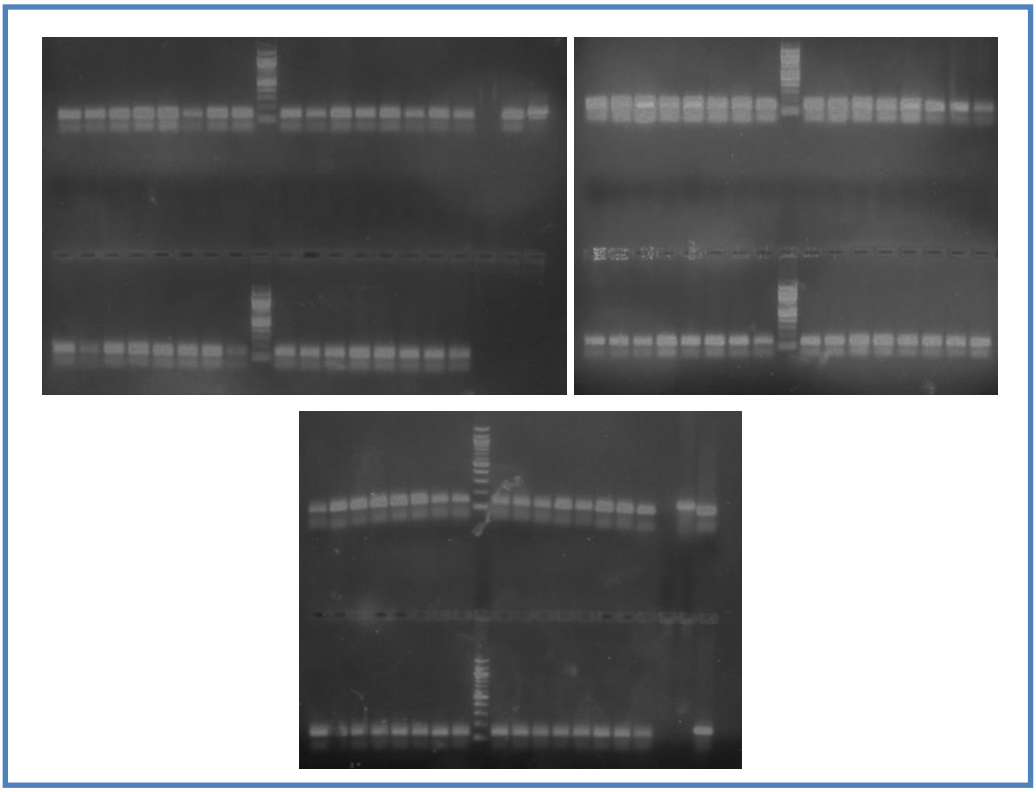


Figure 8: PCR/Restriction enzyme Assay from target 8 mutation samples

As seen in the pictures, only samples 1A, 1F, 7A and 8B from Figure 7, corresponding to single cell clones from target 7 mutation experiments were confirmed as possible mutants. The low number of possible mutants is consistent with the results obtained from pool analysis. It is clear that the CRISPR-Cas system used in this work presented a very low efficiency in CHO cells, probably because the Cas9 nuclease was codon optimized for human cells. A more recent work by Ronda and collaborators proved that when the Cas9 codon sequence is specifically optimized for CHO cells, higher efficiency can be achieved using the CRISPR-Cas system (Ronda et al., 2014).

Sequence analysis results of clones 1A, 7A, 8B and control cells and confirmed that no mutation took place in those cells; therefore, electrophoretic bands corresponding to undigested DNA must have been present due to incomplete digestion. Additionally, all these cells presented two slightly different LDHa alleles in a 1:1 ratio across all sequencing results (Figure 9). It is observed that Allele 1 had the expected sequence, whereas Allele 2 presented a silent point mutation, located 4 bp upstream from the target 7 sequence (underlined), that changed an adenine for a guanine (highlighted in pink).

Sequencing results for 1F clones, shown in Figure 9, evidenced the existence of one mutated LDHa allele, whose sequence is shown below with point mutations highlighted in light blue. Results indicate that this particular clone could have 3 LDHa alleles because the ratios between Allele 1, Allele 2 and the Mutated Allele copies were approximately 1:1:1; the existence of an extra copy of LDHa has been previously reported by Yip and collaborators (Yip et al., 2014) .

```
LDHa Allele 1    CAAAGACTATAGTGTGACAGCAAACTCCAAGCTGGTCATTGTCACAGCGGGGGCCCGTCA 299
LDHa Allele 2    TCTAGACTATAGTGTGACAGCAAACTCCAAGCTGGTCATTGTCACAGCGGGGGCCCGTCA 165
LDHa 1F mutant   TCTAGACTATAGTGTGACAGCAAACTCCAAGCCGGTCATTATCACAACGGGG-CCCGACA 142
                                              ▼
LDHa Allele 1    GCAAGAGGGAGAGAGCCGACTCAATCTGGTCCAACGAAACGTGAACATCTTCAAGTTCAT 359
LDHa Allele 2    GCAAGAGGGAGAGAGCCGACTCAATCTGGTCCAGCGAAACGTGAACATCTTCAAGTTCAT 225
LDHa 1F mutant   GCAAGAGGGAGAGAGCTGACTCATTCTGCTCCAGAGAAATGTGAACATCTTCAAGTTCAT 202
                                              ▲
LDHa Allele 1    CATTCCCAACGTTGTGAAATACAGTCCAGACTGCAAGCTGCTTATTGTTTCAAACCCAGT 419
LDHa Allele 2    CATTCCCAACGTTGTGAAATACAGTCCAGACTGCAAGCTGCTTATTGTTTCAAACCCAGA 285
LDHa 1F mutant   CATTCCCAAAGTTGTGAAGTATAGTCCAAACTGCAAGCTGCTTATTGTTTCAAACCCAGA 262
```

▼ ▲: Indicates Cas9 cutting site

Figure 9: Sequencing results of clone 1F

As seen above, only the adenine to thymine mutation in position 166 of LDHa 1F mutant sequence took place around Cas 9 cutting site and no mutation effectively disrupted MlyI recognition site (in bold) and all other mutations were distributed evenly between 52 bp upstream and 65 bp downstream from the nuclease cutting site. Since MlyI recognition sequence was not destroyed after DSB repair the undigested band seen in the agarose gel must correspond to undigested amplicons, like the results from the other sequenced clones. The presence of point mutation found away from the expected site might be due to a defective HR repair, but no evidence exists to support this thesis. Nonetheless mutations like these have been previously reported in different organisms such as human cells (Fu et al., 2013), zebrafish (Hwang et al., 2013), plants (Fauser et al., 2014; Li et al., 2013b) and bacteria (Jiang et al., 2013), but no explanations have been proposed to explain them.

LDHa's amino acid sequence of parental cells compared to 1F mutant's is shown below in Figure 10, catalytic residues are highlighted in yellow. It is possible to observe that the point mutations of clone 1F's nucleotide sequence caused changes in the amino acid sequence of the protein and a reading frame shift that caused the apparition of an early stop codon. All these changes should have caused functional loss of LDHa for two reasons. First, no catalytic amino acids would be present in the resulting protein if protein synthesis would have occurred. Secondly, because more than half of the protein was not translated the protein would not have been able to go through the appropriate post translational modifications, such as folding, phosphorylation, acylation or glycosylation, therefore the peptide chain would be degraded by the Unfolded Protein Response (Walter and Ron, 2011).

```
CHO LDHa        MATLKDQLIVNLLKEEQTPQNKITIVGVGAVGMACAISILMKDLADELALVDVMEDKLKG 60
Mutant 1F       MATLKDQLIVNLLKEEQTPQNKITIVGVGAVGMACAISILMKDLADELALVDVMEDKLKG 60
                ************************************************************

CHO LDHa        EMMDLQHGSLFLRTPKIVSGKDYSVTANSKLVIVTAGARQQEGESRLNLVQRNVNIFKFI 120
Mutant 1F       EMMDLQHGSLFLRTPKIVSGKDYSVTANSKPVIITTGP---------------------- 98
                **************************** **:*:*.

CHO LDHa        IPNVVKYSPDCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGV 180
Mutant 1F       ---------------------------------------------DSKR----------- 102
                                                             ** *

CHO LDHa        HPLSCHGWVLGEHGDSSVPVWSGVNVAGVSLKNLNPELGTDTDKEQWNEVHKQVVDSAYE 240
Mutant 1F       -----------ERADS---IWS-----------------SET------------------ 113
                          *:.**   :**                ::*

CHO LDHa        VIKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGIKDDVFLSVPCVLGQNGI 300
Mutant 1F       ------------------------------------------------------------

CHO LDHa        SDVVKVTLTSEEEARLKKSADTLWGIQKELQF 332
Mutant 1F       --------------------------------
```

Figure 10: Comparison of CHO cells' and 1F mutant LDHa sequence

2.4.3. Gene translation analysis

Western blot was performed in order to assess the LDHa protein levels in the mutants and wild type cells, in order to LDHa mutant cells, results are shown in Figure 11.

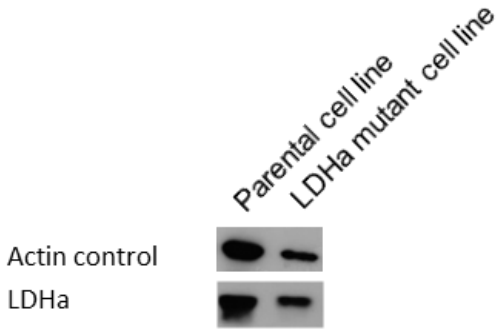


Figure 11: Western blot analysis of LDHa gene expression

For correct comparison of the results, image analysis was performed to quantify the intensity of the bands and obtain a normalized value of the present LDHa that allows the values to be compared between studied cultures. Normalization was performed by dividing the LDHa band intensity by actin's band intensity. Results in

Table **5** show that LDHa expression in control cells is higher than in LDHa knock down cells by 28.5%. This result is consistent with what was expected, as one of the three copies of the LDHa gene was knocked down so a reduction close to 33.3% was anticipated.

Table 5: Normalized intensity of gene bands in Western blot.

	Band Intensity		
Gene	LDHa	Actin	LDHa normalized
Parental cell line	4.20×10^{7}	3.93×10^{7}	1.12
LDHa mutant cell line	1.87×10^{7}	1.59×10^{7}	0.80

2.4.4. Clone characterization

To study the effect of the LDHa knock down over culture performance and cell metabolism fed-batch cultures of the parental and the mutant cell lines growing in suspension were performed as described in the Material and Methods section. Figure 12 shows cell growth, recombinant protein (R.P.) production, glucose consumption and lactate production for both cell lines, Table 6 summarizes important culture parameters calculated from the data obtained.

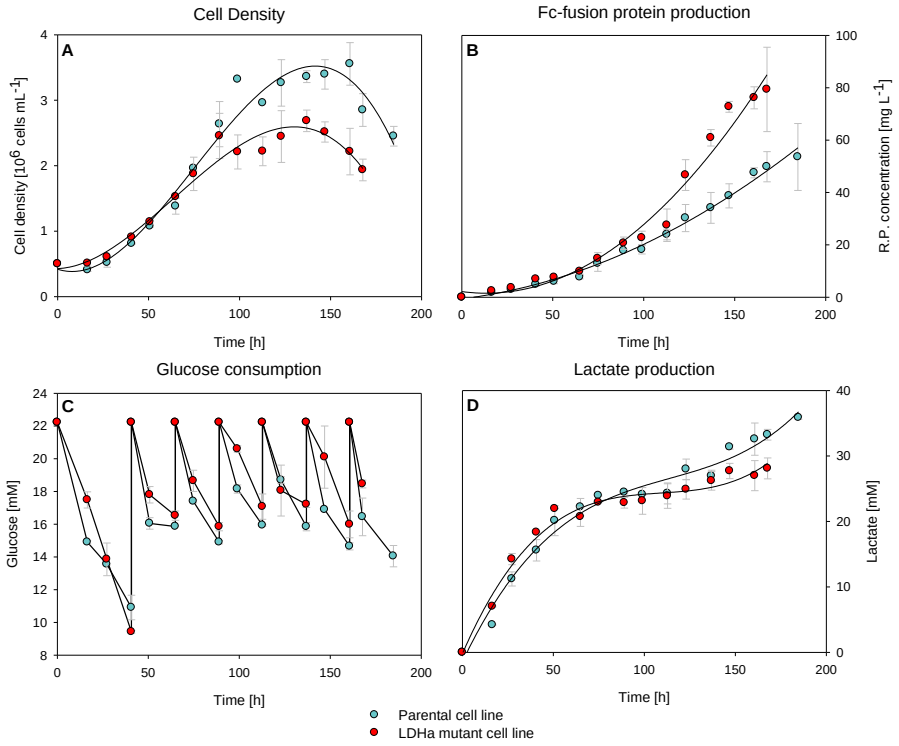


Figure 12: Effect of LDHa K.D. on culture performance.

Figure 12.A and Table 6 show how culture's growth is clearly affected by the mutation. It is possible to observe that LDHa mutant cells' culture reaches a lower cell density than control cells', and also has a lower maximum cell growth rate (μ_{max}). For both cultures the exponential growth phase lasts for about 73 h, after which cell growth stops and cultures begin a stationary phase that continues for close to 100 h. Regarding cell metabolism. Figure 9.C, 9.D and the data in Table 6 show that during the batch phase of the culture control and mutant cells were able to reach similar ΔL/ΔG, with mutants' ΔL/ΔG only a 13% higher than the controls'. Once feeding begins LDHA K.D. cells had a 30% lower production of lactate per consumed glucose. Neither of the cultures showed lactate consumption. As seen in Figure 12.B volumetric recombinant protein production is higher for LDHa mutant cells and data from Table 6 evidences improvement regarding specific production. The specific rate during exponential growth is 70% higher for mutant cells than for control cells. Protein production increases significantly after cell growth stops; control cells are able to improve their specific production by 70% while mutant cells' specific rate increases 3.88 times.

Table 6: Cultures' performance parameters

		Control	LDHa KD
[10^{-2} h^{-1}]		2.56	2.22
ΔL/ΔG	Batch	1.31	1.48
	Feeding	0.66	0.46
Specific R.P. production [mg R.P h^{-1} 10^{-6} cells]	Exponential growth phase	1.0 x 10^{-4}	1.7 x 10^{-4}
	Stationary phase	1.7 x 10^{-4}	6.6 x 10^{-4}

LDHa K.D. in LDHc overexpressing CHO cells leads to an improved metabolic efficiency. However, cell proliferation was reduced as can be seen by the lower μ_{max} and cell density achieved by CHO LDHa K.D. cultures. This is probably due to the altered metabolism that these cells exhibit. A direct result of knocking down LDHa enzyme would be the decrease of lactate synthesis, which augments the available pyruvate for other pathways such as amino acid metabolism, and increase pyruvate transport into the mitochondria as well as acetyl CoA synthesis. The increase of available acetyl CoA in the mitochondria would allow higher fluxes in the TCA cycle, therefore improving the energy metabolism.

A previous study by Borth and collaborators established that disulfide formation in the endoplasmic reticulum for the assembly of light and heavy chain is a major limiting step over cell's IgG specific productivity (Borth et al., 2005b). The reduction in lactate synthesis rate observed for CHO LDHa K.D. cultures impacts the RedOx state of the cell because this reaction is one of the most important for the oxidation of NADH outside the mitochondria. This change in the balance might have a positive effect over the RedOx state in other cell compartments such as the endoplasmic reticulum improving the assembly of the two chains that form the fusion protein. The RedOx alteration due to the diminished lactate production would explain the high protein specific production rate observed in these cultures.

2.5. Conclusions

George Church's CRISPR-Cas system, although proven to be useful in human cells, does not present high efficiency of mutation in CHO cells. Issues might have arisen due to the codon optimization of the Cas 9 nuclease. Nevertheless, CRISPR-Cas continues to be an important

tool to introduce targeted mutations into any type of cells. Potential uses of this tool include targeted knock outs/downs and introducing genes at specific loci of the genome.

We were able to show that a loss-of-function mutation in one of the copies of LDHa in an LDHc overexpressing CHO cell line resulted in enhanced specific and volumetric IgG productivity and reduced production of lactate during the feeding period of a fed-batch culture. LDHa knock down mutants exhibited a slower exponential growth, and reached a lower cell density than control cells. The mutated cell line in fed-batch culture compared to the control culture produced a similar amount of lactate per consumed glucose during the batch period of culture, but showed a reduction of $\Delta L/\Delta G$ during the feeding phase of fed-batch at high glucose concentration. This may explain the improved productivity observed since, as reported previously by other investigators (Fussenegger et al., 1997a; Terada et al., 1996b) when proliferation is inhibited cells increase their recombinant protein productivity because cellular resources that would have been directed towards biomass production can be diverted towards protein synthesis.

In conclusion, in this work we were able to successfully use the CRISPR-Cas system to generate a CHO cell clone that overexpresses LDHc with a disruption in one of its LDHa gene copies. To better understand the effect of this mutation on cell culture, a metabolic characterization was done by studying culture's performance in fed-batch cultures in terms of cell growth, recombinant protein production, and glucose and lactate metabolism. Experiments showed a significant reduction in cell growth and increased recombinant protein production. The results and discussions presented in this work support previous conclusions regarding lactate reduction and/or LDHa gene expression. Our results suggest that modifications in the lactate metabolism lead towards an improved energy metabolism and enhanced energy and RedOx conditions for protein synthesis.

2.6. Bibliography

Altamirano, C., Paredes, C., Cairo, J.J., Godia, F., 2000. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnol Prog 16, 69-75.

Arnaudeau, C., Lundin, C., Helleday, T., 2001. DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. Journal of molecular biology 307, 1235-1245.

Barnes, L.M., Dickson, A.J., 2006. Mammalian cell factories for efficient and stable protein expression. Current opinion in biotechnology 17, 381-386.

Bassett, A.R., Tibbit, C., Ponting, C.P., Liu, J.-L., 2014. Mutagenesis and homologous recombination in Drosophila cell lines using CRISPR/Cas9. Biology open 3, 42-49.

Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., Marraffini, L.A., 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic acids research 41, 7429-7437.

Birch, J.R., Racher, A.J., 2006. Antibody production. Advanced drug delivery reviews 58, 671-685.

Boada, J., Roig, T., Perez, X., Gamez, A., Bartrons, R., Cascante, M., Bermudez, J., 2000. Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress. FEBS Lett 480, 261-264.

Boissel, J.P., Lee, W.R., Presnell, S.R., Cohen, F.E., Bunn, H.F., 1993. Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure. J Biol Chem 268, 15983-15993.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005a. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnology progress 21, 106-111.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005b. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnol Prog 21, 106-111.

Burma, S., Chen, B.P., Chen, D.J., 2006. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA repair 5, 1042-1048.

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J.-W., Xi, J.J., 2013. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell research 23, 465-472.

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. Biotechnology and bioengineering 72, 55-61.

Cho, S.W., Kim, S., Kim, J.M., Kim, J.-S., 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nature biotechnology 31, 230-232.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.-Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010a. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. Journal of biotechnology 147, 116-121.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010b. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. J Biotechnol 147, 116-121.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., Church, G.M., 2013. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic acids research 41, 4336-4343.

Dorai, H., Ellis, D., Keung, Y.S., Campbell, M., Zhuang, M., Lin, C., Betenbaugh, M.J., 2010. Combining high-throughput screening of caspase activity with anti-apoptosis genes for development of robust CHO production cell lines. Biotechnology progress 26, 1367-1381.

Eagle, H., Barban, S., Levy, M., Schulze, H.O., 1958. The utilization of carbohydrates by human cell cultures. J Biol Chem 233, 551-558.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003a. Improving glucose and glutamine metabolism of human HEK 293 and Trichoplusia ni insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnol Prog 19, 90-97.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003b. Improving glucose and glutamine metabolism of human HEK 293 and trichoplusiani insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnology progress 19, 90-97.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000a. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnology and bioengineering 67, 25-34.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000b. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnol Bioeng 67, 25-34.

Farzadfard, F., Perli, S.D., Lu, T.K., 2013. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. ACS synthetic biology 2, 604-613.

Fauser, F., Schiml, S., Puchta, H., 2014. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. The Plant Journal.

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D.-L., Wei, P., Cao, F., Zhu, S., Zhang, F., Mao, Y., 2013. Efficient genome editing in plants using a CRISPR/Cas system. Cell research 23, 1229.

Fogolin, M.B., Wagner, R., Etcheverrigaray, M., Kratje, R., 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J Biotechnol 109, 179-191.

Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J.A., 2013. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nature methods 10, 741-743.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature biotechnology 31, 822-826.

Fukasawa, K.M., Li, S.S.-L., 1987. Complete nucleotide sequence of the mouse lactate dehydrogenase-A functional gene: comparison of the exon-intron organization of dehydrogenase genes. Genetics 116, 99-105.

Fussenegger, M., Mazur, X., Bailey, J.E., 1997a. A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnol Bioeng 55, 927-939.

Fussenegger, M., Mazur, X., Bailey, J.E., 1997b. A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnology and bioengineering 55, 927-939.

Fussenegger, M., Schlatter, S., Dätwyler, D., Mazur, X., Bailey, J.E., 1998. Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. Nature biotechnology 16, 468-472.

Gambhir, A., Korke, R., Lee, J., Fu, P.-C., Europa, A., Hu, W.-S., 2003a. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. Journal of Bioscience and Bioengineering 95, 317-327.

Gambhir, A., Korke, R., Lee, J., Fu, P.C., Europa, A., Hu, W.S., 2003b. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. J Biosci Bioeng 95, 317-327.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442-451.

Glacken, M., Fleischaker, R., Sinskey, A., 1986a. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnology and bioengineering 28, 1376-1389.

Glacken, M.W., 1988. Catabolic Control of Mammalian Cell Culture. Nature Biotechnology 6, 1041 - 1050.

Glacken, M.W., Fleischaker, R.J., Sinskey, A.J., 1986b. Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol Bioeng 28, 1376-1389.

Goldberg, E., Eddy, E.M., Duan, C., Odet, F., 2010. LDHC: The Ultimate Testis-Specific Gene. Journal of andrology 31, 86-94.

Griffin, T.J., Seth, G., Xie, H., Bandhakavi, S., Hu, W.-S., 2007. Advancing mammalian cell culture engineering using genome-scale technologies. Trends in biotechnology 25, 401-408.

Gupta, G., 1999. LDH-C4: a unique target of mammalian spermatozoa. Critical reviews in biochemistry and molecular biology 34, 361-385.

Hammond, S., Kaplarevic, M., Borth, N., Betenbaugh, M.J., Lee, K.H., 2012. Chinese hamster genome database: an online resource for the CHO community at www. CHOgenome. org. Biotechnology and bioengineering 109, 1353-1356.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., 2011. Genetic engineering of human pluripotent cells using TALE nucleases. Nature biotechnology 29, 731-734.

Holmes, R.S., Goldberg, E., 2009. Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Computational biology and chemistry 33, 379-385.

Hu, W.-S., Zhou, W., 2012. Cell Culture Bioprocess Engineering. Wei-Shou Hu.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology 31, 227-229.

Ifandi, V., Al-Rubeai, M., 2003. Stable transfection of CHO cells with the c-myc gene results in increased proliferation rates, reduces serum dependency, and induces anchorage independence. Cytotechnology 41, 1-10.

Inoue, Y., Inoue, A., Kawahara, H., 2011. Efficient production of recombinant IgG by the GLUT5 co-expression system, In BMC proceedings, BMC proceedings. BioMed Central Ltd, p. P50.

Inoue, Y., Tsukamoto, Y., Yamanaka, M., Nakamura, S., Inoue, A., Nishino, N., Kawahara, H., 2010. Efficient production of recombinant IgG by metabolic control and co-expression with GLUT5 in a fructose-based medium. Cytotechnology 62, 301-306.

Irani, N., Beccaria, A.J., Wagner, R., 2002a. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. Journal of biotechnology 93, 269-282.

Irani, N., Beccaria, A.J., Wagner, R., 2002b. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. J Biotechnol 93, 269-282.

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.

Jao, L.-E., Wente, S.R., Chen, W., 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proceedings of the National Academy of Sciences 110, 13904-13909.

Jeong, D.-w., Cho, I.T., Kim, T.S., Bae, G.W., Kim, I.-H., Kim, I.Y., 2006. Effects of lactate dehydrogenase suppression and glycerol-3-phosphate dehydrogenase overexpression on cellular metabolism. Molecular and cellular biochemistry 284, 1-8.

Jeong, D.-w., 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. Biochemical and biophysical research communications 289, 1141-1149.

Jiang, W., Bikard, D., Cox, D., Zhang, F., Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature biotechnology 31, 233-239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816-821.

Johnson, R., Jasin, M., 2001. Double-strand-break-induced homologous recombination in mammalian cells. Biochemical Society Transactions 29, 196-200.

Johnson, R.D., Liu, N., Jasin, M., 1999. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature 401, 397-399.

Jones, M.L., Seldon, T., Smede, M., Linville, A., Chin, D.Y., Barnard, R., Mahler, S.M., Munster, D., Hart, D., Gray, P.P., Munro, T.P., 2010. A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies. J Immunol Methods 354, 85-90.

Kerbey, A.L., Radcliffe, P.M., Randle, P.J., 1977. Diabetes and the control of pyruvate dehydrogenase in rat heart mitochondria by concentration ratios of adenosine triphosphate/adenosine diphosphate, of reduced/oxidized nicotinamide-adenine dinucleotide and of acetyl-coenzyme A/coenzyme A. Biochem J 164, 509-519.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic acids research 35, W43-W46.

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. Applied microbiology and biotechnology 74, 152-159.

Korke, R., Gatti Mde, L., Lau, A.L., Lim, J.W., Seow, T.K., Chung, M.C., Hu, W.S., 2004a. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. J Biotechnol 107, 1-17.

Korke, R., Gatti, M.d.L., Lau, A.L.Y., Lim, J.W.E., Seow, T.K., Chung, M.C.M., Hu, W.-S., 2004b. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. Journal of biotechnology 107, 1-17.

Kummel, A., Panke, S., Heinemann, M., 2006. Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolome data. Mol Syst Biol 2, 2006 0034.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990a. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. Journal of biotechnology 15, 113-128.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990b. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. J Biotechnol 15, 113-128.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nature protocols 8, 2180-2196.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2008. Lehninger principles of biochemistry, 5th ed. W.H. Freeman.

Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., Li, Y., Gao, N., Wang, L., Lu, X., 2013a. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nature biotechnology 31, 681-683.

Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., Sheen, J., 2013b. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nature biotechnology 31, 688-691.

Liang, F., Han, M., Romanienko, P.J., Jasin, M., 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proceedings of the National Academy of Sciences 95, 5172-5177.

Longmore, W.J., Niethe, C.M., McDaniel, M.L., 1969. Effect of CO2 concentration on intracellular pH and on glycogen synthesis from glycerol and glucose in isolated perfused rat liver. J Biol Chem 244, 6451-6457.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013a. RNA-guided human genome engineering via Cas9. Science 339, 823-826.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013b. RNA-guided human genome engineering via Cas9. Science 339, 823-826.

Mastrangelo, A.J., Hardwick, J.M., Zou, S., Betenbaugh, M.J., 2000. Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. Biotechnology and bioengineering 67, 555-564.

Mooney, R.A., Lane, M.D., 1982. Control of ketogenesis and fatty-acid synthesis at the mitochondrial branch-point for acetyl-CoA in the chick liver cell: effect of adenosine 3',5'-monophosphate. Eur J Biochem 121, 281-287.

Nerem, R.M., 1991. Cellular engineering. Annals of biomedical engineering 19, 529-545.

Park, H.-S., Kim, I.-H., Kim, I.-Y., Kim, K.-H., Kim, H.-J., 2000. Expression of carbamoyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary< i>

dhfr</i>-cells decreases accumulation of ammonium ion in culture media. Journal of biotechnology 81, 129-140.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., Liu, D.R., 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature biotechnology 31, 839-843.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173-1183.

Read, J., Winter, V., Eszes, C., Sessions, R., Brady, R., 2001. Structural basis for altered activity of M-and H-isozyme forms of human lactate dehydrogenase. Proteins: Structure, Function, and Bioinformatics 43, 175-185.

Robinson, B.H., Williams, G.R., Halperin, M.L., Leznoff, C.C., 1971. Factors affecting the kinetics and equilibrium of exchange reactions of the citrate-transporting system of rat liver mitochondria. J Biol Chem 246, 5280-5286.

Ronda, C., Pedersen, L.E., Hansen, H.G., Kallehauge, T.B., Betenbaugh, M.J., Nielsen, A.T., Kildegaard, H.F., 2014. Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool. Biotechnology and Bioengineering, n/a-n/a.

Rozwarski, D.A., Diederichs, K., Hecht, R., Boone, T., Karplus, P.A., 1996. Refined crystal structure and mutagenesis of human granulocyte-macrophage colony-stimulating factor. Proteins 26, 304-313.

Sargent, R.G., Brenneman, M.A., Wilson, J.H., 1997. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Molecular and cellular biology 17, 267-277.

Schuster, R., Holzhutter, H.G., Jacobasch, G., 1988. Interrelations between glycolysis and the hexose monophosphate shunt in erythrocytes as studied on the basis of a mathematical model. Biosystems 22, 19-36.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.-L., 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. Nature biotechnology 31, 686-688.

Sies, H., Noack, G., Halder, K.H., 1973. Carbon-dioxide concentration and the distribution of monocarboxylate and H+ ions between intracellular and extracellular spaces of hemoglobin-free perfused rat liver. Eur J Biochem 38, 247-258.

Stafford, D.E., Stephanopoulos, G., 2001. Metabolic engineering as an integrating platform for strain development. Current opinion in microbiology 4, 336-340.

Stephanopoulos, G., Aristidou, A.A., Nielsen, J., 1998. Metabolic engineering: principles and methodologies. Academic press.

Sugimoto, N., Nakano, S.-i., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M., Sasaki, M., 1995. Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. Biochemistry 34, 11211-11216.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., Takeda, S., 1998. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. The EMBO journal 17, 5497-5508.

Terada, S., Suzuki, E., Ueda, H., Makishima, F., 1996a. Cytokines involving gp130 in signal transduction suppressed growth of a mouse hybridoma cell line and enhanced its antibody production. Cytokine 8, 889-894.

Terada, S., Suzuki, E., Ueda, H., Makishima, F., 1996b. Cytokines involving gp130 in signal transduction suppressed growth of a mouse hybridoma cell line and enhanced its antibody production. Cytokine 8, 889-894.

Thompson, L.H., Limoli, C.L., 2003. Origin, recognition, signaling, and repair of DNA double-strand breaks in mammalian cells. Eukaryotic DNA damage surveillance and repair, 107-145.

Thompson, L.H., Schild, D., 2001. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 477, 131-153.

Valerie, K., Povirk, L.F., 2003. Regulation and mechanisms of mammalian double-strand break repair. Oncogene 22, 5792-5812.

Vincze, T., Posfai, J., Roberts, R.J., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic acids research 31, 3688-3691.

Waaijers, S., Portegijs, V., Kerver, J., Lemmens, B.B., Tijsterman, M., van den Heuvel, S., Boxem, M., 2013. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. Genetics 195, 1187-1191.

Walsh, G., 2010. Biopharmaceutical benchmarks 2010. Nature biotechnology 28, 917.

Walter, P., Ron, D., 2011. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. Science 334, 1081-1086.

Wilkens, C.A., Altamirano, C., Gerdtzen, Z.P., 2011. Comparative metabolic analysis of lactate for CHO cells in glucose and galactose Biotechnol. Bioprocess Eng 16.

Williamson, D.H., Lund, P., Krebs, H.A., 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 103, 514-527.

Wlaschin, K.F., Hu, W.-S., 2007a. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. Journal of biotechnology 131, 168-176.

Wlaschin, K.F., Hu, W.S., 2007b. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. J Biotechnol 131, 168-176.

Wu, F., Yang, F., Vinnakota, K.C., Beard, D.A., 2007. Computer modeling of mitochondrial tricarboxylic acid cycle, oxidative phosphorylation, metabolite transport, and electrophysiology. J Biol Chem 282, 24525-24537.

Wurm, F.M., 2004a. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature biotechnology 22, 1393-1398.

Wurm, F.M., 2004b. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22, 1393-1398.

Yip, S.S., Zhou, M., Joly, J., Snedecor, B., Shen, A., Crawford, Y., 2014. Complete Knockout of the Lactate Dehydrogenase A Gene is Lethal in Pyruvate Dehydrogenase Kinase 1, 2, 3 Down-Regulated CHO Cells. Molecular biotechnology, 1-6.

Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A.F., Meier, A., Yuk, I.H., Vijayasankaran, N., Leach, K., Joly, J., 2011. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. Journal of biotechnology 153, 27-34.

Zupke, C., Sinskey, A.J., Stephanopoulos, G., 1995. Intracellular flux analysis applied to the effect of dissolved oxygen on hybridomas. Appl Microbiol Biotechnol 44, 27-36.

Zustiak, M.P., Dorai, H., Betenbaugh, M.J., Sauerwald, T.M., 2012. Controlling apoptosis to optimize yields of proteins from mammalian cells, In Protein Expression in Mammalian Cells, Protein Expression in Mammalian Cells. Springer, pp. 111-123.

2.7. Supplementary Material

2.7.1. CHO cells' complete genomic LDHa sequence

>gi|351516859|ref|NW_003614690.1|:176871-185211 Cricetulus griseus unplaced genomic scaffold, CriGri_1.0 scaffold1048, whole genome shotgun sequence

ATGGCAACACTCAAGGACCAGCTGATTGTGAATCTACTTAAGGAAGAACAGACCCCCCAGAACAAGATTACGATTGTTGGGGTTGGT
GCTGTTGGCATGGCTTGTGCCATCAGTATCCTCATGAAGGTAAGTGGGGATCCTTCAGGTCACAAGCCCAAGCATTGGGAGGCCCTA
CATTGTCACATTGTATATAAAACTATCAAGTTTCAGGCACTCATTCAAGAGAGCCTTCTATGAAACATTTTGCAACATGGTGATGCA
CAAAGGATTATCCAAAGTAACATTATAAAAGGTTAGCAGACTGAGGCCTTTTTAAAATGCTCTACAGTATGTTAGCATGCCCTACCA
GCAAGAAAGAATGCAGGGAGTTGAAGGAACCTAGGGTCTCCCATATGGTAGGTAACCATTGGAGCTGTATGCCCAGCTCTTAAGTAA
TTTTTAATTGAAATGTATATGTACCTGTGGTAAAAATTAATATCCTACCGAATGGGATATCTTGAATTTCTATCCAATCCCTAATGT
TCCCTGACTTATAATAGTTTTCCTTTGAGAAAAAAGTGTGTGTGTGTGTATGAGCACACTGGGTAACTGAGGGTAATTGGCTTCTCT
CCTACCTTGTGGGTTCTGGGGGTCAAACTCAGCTCACCAGGCTTGTGCAGGAATTGCTTTCCCCTCGAGCCATCTCAATCAGAGCTA
AAATTTAATTGGTCAGATAACCACATAATTGCATTAGAAAACACTTGCTGAGACAGGGTAAATTTTTTTTTTAAGATTTTATTTATTT
ATTATGTATACAACATTCTGCTTCCATATATATCTACACACCAGAAGAGGGCACCAGATCTCATAACGGGTGGTTGTGAGGCACTAT
GTGGTTTCTGGGAATTGAACTCATGACCTCTGGAAGAGCAGTCAGTGCTCTTAACCTCAGAGCCATTTCTCCAGCCCTCAGGGTAAA
TGTTAATATATATATTTTTATATATGGGGCTAAGACAGGGTTTCTCTTTGTAACCCAGGCTGTTCTGGAACTCATTCTGTAGACCAG
ACTGGCCTCAAACTCATCTGCCTGCCTTTGCCTCCCAAATATTAAAAATAAAGTTTTAAGGACAGGGTTTCCTCCTGTGCAGCACTC
ATAAATTGATCCACACCTGTATTTTTTTCTCTTGGAGATGGGATTTGACTGTATGTGCCTCCCAACTGCTGTCATTAAAGGCATGCA
TTACTGTGCCCAGCTTTGCAGTTCATTAATTCAGAGTCCTCTCCCCCATCTTAAGGTCTCACTCATGCTAGGCAACAGTCTACCACT
GTTTCAAGCAGTGGTACTAACCAATAGTGACTAACAGCCTCCCCCCACAGAAGCTCTTCCAGTGGGGAACAGATTCGACTCTGCATG
GGGCCATCTGGGAGGCAGTGGGTTTGAATGCCCTGCCTTTTTGATGCCTGAAATACAGAAAATTGCATGTCCTGAAAACCCTCAAAA
TAAGTTTCCCTTTTAACTCATACTTGTCTTTTAGGACTTAGCAGATGAGCTTGCCCTTGTTGATGTCATGGAAGACAAGCTGAAGGG
GGAAATGATGGATCTCCAGCATGGCAGCCTTTTCCTCAGAACACCAAAAATTGTCTCTGGCAAAGGTTGGTTGGGGCCAACTTCCCT
TTGTCTGTATTAGGCTTTCAGTTTTAAGATCTTCCTCAGGCCTGAAATCCTATCTGTAATTCTGTCTTTGATTCAAGACAGAAGGCC
TTGGGATACTTTTGGTCTCAAAACCTATCAAAGCTTCAGTTGCTAGTAAGATTTTAAGTGGAAAACTGATTGTACAAATATGAAAAT
CCTCTGAATTAGAAGATAGTGAGATCTGAATGTACATTGTTTCAAGTGTATATTGCTACGATGGAGCCCAGGGCCTTGTGCACACCA
GGCAAGTGCTCTGGCTGCTCACCTTTCCAGCTTTGCTGCATGTTTAAATGTTCTTCCACACTGCCCACATCCTATACTGACCCAGAC
TTTAGAATTTCATGTTAGAGACCTGTGCAGCAGATTCTTGACCTTTTTACACTTAACTGGCACCAGCCAGGAATCCAGGGTTGAAAG
TCTTTGACCTCTTACCTGATGGGCATCTCTTTGGGTCAGCTGCCCTGTACCACGGGGTCTACAGAAGAATGAGAGCAAGGAGAAGAT
TGCTGGGCTCTCAGCAATGATTAAGTGGCTAGCTAAAAAGTAAGAAAATGCCTGGAATGGGGAGTATGTCTCAAGTGGAGGAGGGGA
GAAAGAGAATATCCTATGTTAAGGTAGAAATATACACATAAGTAATTGATAGAGGATCAGATATATTGTATTCCGTCCTGTTATAAT
AAAATGTGACTAAATGTGCAGTATGAGAAAGGCATCAATATTATTCCCATCCTTAAGAGACCACAGTTAGAAGAGTGCATCCAGAGG
GAAGGTATCAGTGTCAATAGATCTCCATCTGCTATGGACAGCGGTTTATAGAACAAGTTTTTGCTGCGTAGGTAAAAGTTCCCCAAA
GGGACCTTCATTTCTGGTCTTTTCTCTAGACTATAGTGTGACAGCAAACTCCAAGCTGGTCATTGTCACAGCGGGGGCCCGTCAGCA
AGAGGGAGAGAGCCGACTCAATCTGGTCCAGCGAAACGTGAACATCTTCAAGTTCATCATTCCCAACGTTGTGAAATACAGTCCAGA
CTGCAAGCTGCTTATTGTTTCAAACCCAGTGGATATCTTGACCTACGTGGCTTGGAAAATAAGTGGCTTTCCCAAAAACCGAGTTAT
TGGGAGTGGCTGCAATCTGGATTCTGCTTCTGGCCTCGAACTCATAGAGATCCACCTACCTCTGCCTTCTGAGTGCTGGATAAAAAG
CGTGCGCCATCAATGCACGGCCCCAGGTGAGGCTTTTAAAGTGCAGAAGCAGACACCCTGCTTATCCTGATGATGTGTTATTAGTTT
CCTGTAGCTTTGTGTTTTTATAAAATAGTTCATTTAAGAATGTTAAAGGGGCTGGAGAGATGATGGCACAGCAGTTAAGAGCACTGG
CTGCTCTTCCACAGGACACAGGTTCAATTCCCAGCACCCTTACTCAGACACACATGCAGGCAGGATACCAGTGTACATAAAATAAAA
GTAAATCTTAAAAAAAAAAGAATGTTGAAGGCTTCTAAATGGAGTTGCAAAGTTTAGAGTTTCTTGTGATCTTGGAGAAGCAAGTAA
CTATGAGTAATTCTATCTTAATTGGTGCACTGTTAGTTTTAAATAGTACTTTGAATATTTGGACATGAGTTACAGTAAAGATCTTAA
CAATACTGATCCATCTTCTGTTATCTAAATTTTTGTGGGTTTTTTTTTTTTTTTTTTTGTAGTGGATATCTTGACCTACGTGGCTT
GGAAAATAAGTGGCTTTCCCAAAAACCGAGTTATTGGGAGTGGCTGCAATTTGGATTCTGCTCGATTCCGTTATCTGATGGGAGAAA
GGTTGGGTGTTCACCCACTAAGCTGTCATGGATGGGTCCTAGGGGAGCATGGAGACTCCAGTGGTAAGTCTTTAGTATCAGTTTTTC
TCTTTTTGGAAACAGAAAAATAGTGTAAAATGTCTCTAGGCATTTCTTTACTAAATAAAAGAAAGATGGCTCTGTTAAGAACACTGG
TTGGCTGCTGTTCAGAGGTCTTATTATCAGAGGTCCTGATACTGAGTTCAATTCTTAGCAACCACAAGGCAGCTCCCAACCATCTAT
ATTGAGATCTGATGCCCTCTTCTGGTGTGTAAGCAAACATGCAAGCAGAACACATACATAAGACACTGTATATCTACATAGTAAATC
AATCTCTTTTTTAGAAAAGTCTTAACTCAAGTATCCATGTTATATGGCATTTATCACAGGGAAATAATAAGCTTGGATATGTTAATT
TTTTTAATATTGAGACAGGGTCTACCATTGTAGCCCAGGCTGGCTTGGAACTCAACAGTCCAGGCTTTGTCTTCTGGCTAGGGAATT
TCTGGCATGAGCTACTGTACACAGCCTATTCATTCATTTATTTGTTCATTAATTTTTTTAAGATTTTATTTATTTATTATGTATACA
ACATTCTGCTTCCATGTATATCTGCATACCAGAAGAGGGAATCAGATCTCATAACGGATGGTTGTGAGCCACCATGTGGTTGCTTGG
AATTGAACTCAGGACCTCTGGAAGAGCAGTCAGTGCTCTTAACCTCTGAGCCATCTCTCCAGCCCCTGTTCATTAATCTTGATGCTA
GTGATTGAACCCAGGATCCTCACATGTGCTAAGCACAGTCTACTAAGCTATGCTCTCAGTCCCTAGATGGACGTGTTAGCCATGGTT

CTTAGGATAACTTCAGTCATGTAATTCATTCACCAGTTTAAACTTGGCTTGAATCGCCTGCCCATGACAAGATCAGCCATGTCAGGT
GGGGGGAAACATTGTTAGGTTGTAGTTGAGAAGGTTATATTCATGCAATTCTCAGATGCCAGTGAAACCCCCTTAAAGTGTGAATTT
GGTGTTATTGATTGTAAATACATAGCCTACACTTTAGTGGGTTAGGAAAATGTGTTAGAATATACAGATCTTGGGGCTGGAGAGATG
GCTCAGAGGTTAAGAGCACCAACTGCTCTTCCAGAGGTCCTGAGTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCTGTTA
TGAGATCTGGTACCTTCTTCTGGTGTGCAGATATCCATGGAAGCAGAATGTTGTATATATAGCAAATAAATAAAATCTTTTAAAAAA
TGAATATGCAGATCTTGGTTTTATGCACTGTAAAATTCAAGGCTTTGGTAATCTTGGCCTTCCTCCACAAATGAGGGTAGAGGGCAG
GTGAATGGTTACTGTTCTGTGCTCTCCTGTGGAAACATTGTTGTAAGCTGCTTGTTTTTTCCAGTGCCTGTGTGGAGTGGAGTGAAT
GTAGCTGGTGTCTCCCTGAAGAATCTGAATCCAGAGCTGGGCACTGATACCGACAAGGAGCAGTGGAATGAGGTTCACAAGCAGGTG
GTTGACAGGTGAGGTGAGCCTTCGTGATTGGCAGCATGGAAACTGTGTTTAGATTTTCATAGCACTTCATTTGTGTGCTTTTGTTTA
AAATTTTTCTTCAGCTTTGTTAATCATAATGCTATATATATATAAAGACTAAAATGCAACACCAAATACAGGTAAGAGGGGCTGATG
CTGGTTTCAGTCTCCCATTTGTAAAATGTAACACCTTGGGAATATAAGGTGGTACCATCTCCTAGGAAAATAGTTTGGCAGTTCTTT
TACAACTGAAAGCAGATCTGCCATACAAAAAGATCTTTTTAAAATCAAGATCAAATAAATTTAGTTCTACCCTTGACAACATATTAA
TTCACTCAGAAGGATCTTTTTTATAGGATGTATTTTGGGGTGTATGGATGTTCTGCCTTCCTTTGTGTCTGTACTACATGGATGCAG
TATCCGTGTAGTCCAGAAGAGGGCACAGGATTCCTTGGAACTAGAGTTAGACTTGTGAACCATCTTGTTGGTGTTGTGAATCAAACA
TAGGTCCTCTTAACCATTGAGCCACCTCTGCAGCTCCCAGAAATAAGATAGCAAAACTTTTTATTTTATTTATACTAATAGAAGTAC
GGCCAACTTCTTTATTTGGATAATATAGGTATAAGTATGGTCTGGAGTCTTTCATTTGTTTTTGTGGGGGGGGTTGTTTATTTTAGA
CAGGGTTTTCTCTGTGGCCCTGGCTTGTCCTGGAACTTGCTTTGCAGACCAGGCTGGCTTTGAACTCAGAAATCTCCCTGCCTCTGC
CTCCCGAGTACAGGGTTTAAAGGCCTGTGCCACCATGGCCTGGATCATTTTGTTTTTGTCTTTAGAGACAGGGTTTCTCTGTGTAGT
CCTGGCTGTCCTGAAACCCTGTAGACCAGGATGGCCTCAGACTCAGAGATCTATTTGCCCGCCTCTGCCGCCCAAATGCTGGGATTA
AAGGCCACCGCCGCCGCCACCTGGCTTTGGTTCTTACACAAATTCTAGATTTGTTTGTTTGTTTGTTTTGTTTTTTCATGACAGGGT
TTCTCTGTGGCTTTTGGAGGCTGCCCTGGAACTAGCTCTTGTAGACCAGGCTGGTCTCGAACTCTCAGAGATCCACCTGCCTCTGCC
TCCCGAGAGCTGAGACTAAAGGCGTGGGCCACCAACGCCTGGCCAAATTCTAGATTCTTAAGTAAATATTATAACTGCTATAAGTGA
ACTTTGCTTCTCAGCCTGTTTCACTTGAATGACCTTGATCCAACTGTGCTGTGTGTAAGCCTCATGGATTTTGTATTAGTGACTTTC
TGTTGGCTGTGATAAAAGGTACCACCACCAAACCAATTTCCTGAAGAAAGTTTGTTTTGGGCTTATGGTTCCCAAGGTTTAGAGTTC
ATCCATGGTGGTGGAGCCAGAGGTATGGGTAGCAGGAGGCAGGCCGCTTCAGAAGTAGCTGAGAGCTTATCCTAACCACATACAGGA
AGAAGAGAGCTAAATGTTGCTTGTCTTTTGAAACCTCAAAGCCTGTTCCCAGTGACATGCTTCCTCACCAAGCAGCTATCAACTGGG
GACCAGATCTTCAAATATCCTATACTTATGGAGGACATCTCGTTCAAAACACCTCACATGGGCCTGGAGAGATGGCTCAGTGAGGAG
AGATGGCTCAGAGGTCCTGAGTTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCCATTATGAGATCTGGTGCCCTCTTCTG
GTGTGCAGATAGATACACATGGAAGCAGAATATTGTATACATAATAAATAAATAAAACCCATACAGTTTCTGAGGTGCTGGGAATGG
AACTCAGACTCTCTTGAGTCCTAGGAACACAGGATACATTTCGGTCCGGTCATTATTTCACTTTTGTTTTTCTGCCACTAACCATAG
TCTTAGCTATCTCACCAGCCCCTCCATATTTCATTTCTGAGAATTTTTGTTGTTTTGACTAAGTAATATTCCCTCTGTCAATGTACA
AGACTCCATGGAGGGGAGGGCCAAAGTTTGCCCTTCCCGGCAAAGGTCATGCTTAACTATATAACATGTCTTGGTGGTTTTTATTGT
AAAATATGTTCAGTAAAACATTTATTTTACTTGTTTTTTCCCTGCCCTTCACCCTTTTCCCATTCTTCCTATTCCACAGTGCCTATG
AGGTGATCAAACTGAAGGGTTATACATCCTGGGCCATTGGTCTGTCTGTGGCTGACTTGGCCGAGAGCATAATGAAGAATCTTAGGC
GGGTGCATCCCATTTCTACCATGATTAAGGTAATGGATTTGCAGAGAAGCATGTTGTATGGTTTATTTCTTGGCTTTACAAAGTCTT
AAGAAGACTAATGGAAATTCTCAGTCACTGACTGAAGTAAATTCATCCGTTCATGCTTTTCTTTTGGAAAGAAATAGCTGACCTATG
GAATTAATTAATGTCCTATCAGCACTGAAGGTATCTAAGAGTTATGCTGATCAAATGGAAGACACTGCTCAGTTACCAGTCTTAGAG
AAATGGGGAAGTGTAATTTATTGTGTACATTACTGAGGCTTGTGGGTTTGTGGGCTTTTTTTTTTGGTTTTGTTTTTTGGGGGGTTT
TTTTGGTGTTTTTTTGAGACAGGGTTTCTCTGTGTAGCTTTGGAGCCTATCCTGGCAGTCGCTCTGGAGACCAGGCTGGCCTCCAAC
TCATAGAGATCCACCTGCCTCTGCCTCCCCAGTGCTGGGATTAAAGGCGTGTGCCACCAAAGCCCAGCTTTTTTTCTGTTTTGTTTT
TGTTGTTGGGTTGTTTTTTGTTTTTTTGTTTTCTGTTTTTTTGAGGCAGGGGTCTTAGGTAATGCTAGCTGGCCTGGCCTTGAACTT
GAGATATATATTTTTGATGATAACCTTGAAGTACCTCTAGCTTCCATGTGCTCGGGTAACAGACATGTGCCAAGCCTAGCTGTTGCT
GTCTCAATGACTTTCTATTACACTGTCCATTGTTACATCATACCTGGGCATCTCTGGAACAGACTAGATCAGCACGCTTGTTTTAAA
AAGGCTTTATAACTGTGTAAGAGCTGAATCCCAGGAAACCTGAGGATGTGTAGGCAAAATGTAATCTCTTTCATTTCGTGTTCAGGG
TCTCTATGGAATCAAGGATGATGTCTTCCTCAGTGTCCCTTGTGTCCTGGGACAAAATGGAATCTCCGATGTTGTGAAGGTGACTCT
AACTTCTGAAGAGGAGGCCCGCTTGAAGAAGAGTGCAGATACACTATGGGGGATCCAGAAAGAGCTGCAGTTCTGA

2.7.2. CHO cells' lactate dehydrogenase A amino acid sequence aligned against *Mus musculus*'

95.2% identity in 336 residues overlap; Score: 1647.0; Gap frequency: 0.0%

```
CHO             1 MATLKDQLIVNLLKEEQTPQNKITIVGVGAVGMACAISILMKDLADELALVDVMEDKLKG
mouse,          1 MATLKDQLIVNLLKEEQAPQNKITVVGVGAVGMACAISILMKDLADELALVDVMEDKLKG
                  ***************** ****** **********************************
```

```
CHO            61 EMMDLQHGSLFLRTPKIVSGKDYSVTANSKLVIVTAGARQQEGESRLNLVQRNVNIFKFI
mouse,         61 EMMDLQHGSLFLKTPKIVSSKDYCVTANSKLVIITAGARQQEGESRLNLVQRNVNIFKFI
                  ************ ****** *** ********* ************************

CHO           121 IPNVVKYSPDCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGV
mouse,        121 IPNIVKYSPHCKLLIVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGV
                  *** ***** **************************************************

CHO           181 HPLSCHGWVLGEHGDSSVPVWSGVNVAGVSLKNLNPELGTDTDKEQWNEVHKQVVDSAYE
mouse,        181 HALSCHGWVLGEHGDSSVPVWSGVNVAGVSLKSLNPELGTDADKEQWKEVHKQVVDSAYE
                  * ****************************** ******** ***** ************

CHO           241 VIKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGIKDDVFLSVPCVLGQNGI
mouse,        241 VIKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTMIKGLYGINEDVFLSVPCILGQNGI
                  ****************************************  ******** ******

CHO           301 SDVVKVTLTSEEEARLKKSADTLWGIQKELQFSTOP
mouse,        301 SDVVKVTLTPEEEARLKKSADTLWGIQKELQFSTOP
                  ********* *************************
```

CHAPTER 3: In depth analysis of the metabolic effects of LDHa knock down over LDHc overexpressing CHO cells in fed-batch culture

3.1. Abstract

Metabolic Flux Analysis (MFA) is a useful mathematical tool to gain insights into the metabolism of cells or organisms, it allows scientist to estimate and compare metabolic fluxes of different experiments. In this work MFA, and other tools were used to comprehend metabolic differences between Fc-fusion protein producing CHO cells overexpressing LDHc with an LDHa knock down clone generated from that cell line. Previous results showed that the mutant cell line in fed-batch culture reached lower cell densities, produced similar concentrations of lactate but exhibited a more efficient metabolism characterized by a lower production of lactate per consumed glucose and were able to surpass parental cell recombinant protein production.

Results showed differences between parental and mutant cells' RedOx state, with the latter having lower NAD^+/NADH levels, suggesting an enhanced energy metabolism. ΔL/ΔG values in time show that under constant feeding, LDHa deficient cells have a more efficient metabolism with lower production of lactate per consumed glucose. Finally, metabolic flux analysis reveals higher fluxes in the TCA cycle, proving that LDHa K.D. cells have an enhanced energy metabolism with higher fluxes in the TCA, which are mainly attributed to higher catabolism of amino acids.

3.2. Introduction

Lactate reduction in mammalian cell cultures has been the aim of researchers both in industry and academia, because its production and presence in culture have a negative effect protein production and cell proliferation (Glacken et al., 1986b; Kurano et al., 1990b). To overcome this issue different approaches have been proposed such as alternative culture strategies, media design and metabolic engineering. Regarding this last approach, protein production and metabolic enhancement have been reported on works where lactate dehydrogenase (LDHa) gene expression was reduced.

Chen and collaborators disrupted one of LDHa gene copies through homologous recombination (Chen et al., 2001a); after cell cloning a 30% reduction of LDHa activity was registered and the best selected clone proved to have an improved metabolism that consumed more glucose while producing less lactate. Additionally, cultures were able to reach higher cell

densities and produce 2.5 times the total amount of recombinant protein than wild-type cells. Using alternative strategies for knocking down gene's expression, such as siRNA (Kim and Lee, 2007a; Zhou et al., 2011b) and antisense DNA (Jeong et al., 2006b), researches have been able to achieve similar results, demonstrating that cells with reduced LDHa activity are less resistant to oxidative stress and alterations of intracellular and extracellular pH, but are able to achieve higher cell proliferation and ATP concentrations. In the previous chapter of this thesis an LDHa deficient CHO cell line was constructed from LDHc overexpressing CHO cells using the CRISPR-Cas system. Results showed that in fed-batch culture cells were able to improve their metabolism during the feeding stage of culture and achieved a 45% improvement in volumetric production; even a more impressive fold change in specific productivity (70-388%) was produced.

Even though effects of LDHa knock down over cultures' performance and other characteristics have been described in each of the previous published works, a more profound understanding of metabolic consequences of this change are yet to be achieved. In the past decades, metabolic models have been widely used for understanding biological systems. Metabolic Flux Analysis (MFA) is a mathematical tool that allows the estimation of intracellular fluxes under metabolic steady state or pseudo steady state by measuring extracellular accumulation and consumption rates of nutrients and cellular products like glucose, amino acids, lactate, biomass and recombinant protein (Stephanopoulos et al., 1998; Varma and Palsson, 1994). MFA uses the mass conservation principle to set a collection of lineal equations that connect intracellular fluxes with specific production and consumption rates of a metabolic network representative of the studied organism. Using linear algebra principles the values of the unknown vector of intracellular fluxes is solved.

The insights given by MFA have proven useful for a number of purposes that have expanded the understanding of cells' metabolism. For example, it has been used to observe factors that lead to metabolic shift towards lactate consumption like reduced glycolytic flux (Mulukutla et al., 2012; Wilkens et al., 2011b). In the work by Europa and collaborators MFA showed that a higher input of pyruvate into the TCA reflects in a more efficient metabolism characterized by a lower production of lactate per consumed glucose in continuous culture (Europa et al., 2000a). MFA was performed to compare energy metabolism of hybridoma cells in culture with 3 distinct metabolisms; results suggested that glycolytic flux is reduced in cultures characterized by low $\Delta L/\Delta Gs$ and that amino acid metabolism has a direct impact over lactate production. (Gambhir et al., 2003a). This tool has also been used to improve media composition

for increased production of both proteins in CHO cells in continuous culture and virus in HEK 293 cells in batch culture (Martinez et al., 2010; Xing et al., 2011).

In this chapter we aim at providing insights into the effect of LDHa knock down over cultures' performance that may lead to explain improved protein production by analyzing overall metabolism. Studies were performed on different time points during a fed-batch culture of the engineered CHO cells, described in the previous chapter, and control cells. In order to achieve this, ΔL/ΔG values were studied, NAD^+/NADH ratios were quantified and Metabolic Flux Analysis was used to estimate their intracellular fluxes in the central carbon metabolism and biomass and protein synthesis pathways.

3.3. Material and Methods

3.3.1. Cell line and culture conditions

In this study, recombinant Fc-fusion protein producing DXB-11 CHO cells overexpressing *Mus musculus' LDHc* with one copy of the *LDHa* gene knocked down, constructed in the previous chapter, were used. Cells were maintained in an incubator at 37°C, 96% humidity and with a 5% CO_2 enriched air atmosphere. Culture media was DMEM/F12 (Gibco, ME090283L1) supplemented with (Sigma, T8158), 1.7 µL/L 2-mercaptoethanol (Sigma, M3148-250), 0.1 ml/L ethanolamine (Sigma, E0135), 0.11 mM ascorbic acid (Sigma, A4544), 0.18 mg/L putrescine (Sigma, P5780), 29 mM sodium bicarbonate (Sigma, S4019), 28.9 mM sodium selenite (Sigma, S5261), 0.2 g/L pluronic F68 (Sigma, P1300), 22.22 mM glucose (Sigma, G5146), 4 mM glutamine (Sigma, G1517) and 5% FBS (Hyclone, SH30910.03).

Growth curves were performed in suspension fed-batches with biological duplicates. On day 0 cells were inoculated at 0.5×10^6 cells mL^{-1}, and samples were taken twice a day until while culture's viability was higher than 75%. Starting on day 2 a proprietary chemically defined feeding media was fed to the spinner once a day adjusting the final glucose concentration to 22.22 mM. Cell number and viability were determined by the trypan blue exclusion method. After cell counting the supernatant was frozen for further analysis.

3.3.2. Metabolite determinations

Glucose and lactate concentrations were determined using Randox's Glucose and Lactate Assay kit respectively (GL3981, LC2389, Randox) and Fc-fusion protein concentration in the supernatant was quantified by ELISA. Amino acid concentration was measured by HPLC with a method based on a previously reported amino acid detection method that uses diethyl ethoxymethylenemalonate as a derivatization agent and an analytical column Synergi Hydro-RP 80A (4.60 mm × 250 mm, 4 µm) (00G-4375-E0, Phenomenex) (Rebane and Herodes, 2010). NAD^+/NADH ratios were measured using Biovision's NAD^+/NADH Quantification Colorimetric Kit (Biovision, K337-100) following the protocol provided by the supplier.

3.3.3. Specific rate determination

Specific rate of production (q_p) or consumption (q_c) of any metabolite at any time point in was calculated as the derivative of the polynomial curve that fits the metabolite's cumulative curve divided by the cell density at the desired time point. A metabolite's cumulative curve is constructed by adding the amount of metabolite produced or consumed between two consecutive time points of culture. This method ensures small fluctuations in the results that may be caused by any measurements errors. The value of $\Delta Lactate/\Delta Glucose$ ($\Delta L/\Delta G$) is defined as $-q_{Lac}/q_{Glc}$ and it is calculated as the negative quotient between the two specific rates.

Carbon dioxide evolution rate (CER) was calculated from the measured oxygen uptake rate (OUR) in order to obtain CO_2 production rate. A previous work by Bonarius and collaborators estimated that the respiration quotient (RQ = CER/OUR) for mammalian cells is close to 1, indicating that for every mole of O_2 consumed 1 mole of CO_2 is released (Bonarius et al., 1995). Oxygen consumption rate was measured by withdrawing a 20 mL sample from the spinner and transferring it into a 20 mL flask with a magnetic stirrer. This flask was kept at 37°C with a dissolved oxygen probe (Mettler Toledo) and tightly sealed to avoid air exchange. Oxygen concentration was monitored for 30 min and the OUR was estimated in [mmol O_2 hr^{-1} 10^{-9} cells] as the quotient between oxygen's reduction rate by the number of cells in the flask.

3.3.4. Metabolic Flux Analysis

Metabolic flux analysis (MFA) was performed to estimate and contrast the different metabolic flux distributions during the exponential growth and stationary phases of control cells and LDHa

deficient CHO cells. The metabolic network considered consists of 30 reactions and 32 metabolites that belong to the central carbon and amino acid metabolism, biomass production and recombinant protein synthesis as shown, in Figure 13 and Table 7. The biomass synthesis reaction was obtained from the average elementary composition of an animal cell proposed by Gambhir and collaborators, where glucose and amino acids are considered as the main molecules for biomass production (Gambhir et al., 2003b), which was used in a previous work (Wilkens et al., 2011b), and the Fc-Fusion protein synthesis reaction was developed from the amino acid sequence of an Fc TNF-alpha fusion protein (personal communication with Dr. Wei-Shou Hu).

A total of 23 specific rates were measured and are listed below in Table 9, these correspond to species that represent the largest carbon and nitrogen uptake and production fluxes; among them are biomass, recombinant protein, CO_2 and amino acids accumulation rates. The amino acids measured represent 93.8% of the total amino acids supplemented in the culture media. Asparagine and proline, cysteine and tryptophan cannot be measured using the amino acid quantification HPLC technique described before. The specific uptake rates of species such as acetyl CoA, fumarate, malate, oxaloacetate, pyruvate, succinyl CoA and α-ketoglutarate are low and were assumed to be null.

Intracellular flux values were calculated by solving the linear equation system $x(t)=Ar(t)$ where A is the 32x30 stoichiometric matrix that describes the simplified metabolic system, $x(t)$ is vector the represents the 30 unknown intracellular fluxes and $r(t)$ is a vector containing the known accumulation rates of the 32 species considered in the system. To solve the over determined system the least-square method was used (Bonarius et al., 1996; Vallino and Stephanopoulos, 1993).

The matrix A that describes the metabolic network is nonsingular and well posed, with a condition number of 31.24 which indicates a low error propagation (Sánchez et al., 2014). Furthermore, sensitivity analysis over the stoichiometric matrix indicates that only the fluxes of reactions 3, 4, 5, 6, 9 and 14 in Table 1 are more sensitive to measurement errors in the specific rates of leucine, lysine and methionine. The consumption rate of these amino acids is very low and given accuracy of the measuring technique the amplification error is expected to remain low.

Table 7: Biochemical reactions considered for Metabolic Flux Analysis.

N°	Reaction	Pathway
1	Glc → 2Pyr	Glycolysis
2	Pyr ↔ Lac	Lactate synthesis
3	Pyr → AcCoA + CO_2	TCA cycle
4	AcCoA + OAA → αKGT + CO_2	TCA cycle
5	αKGT → SucCoA + CO_2	TCA cycle
6	SucCoA ↔ Fum	TCA cycle
7	Fum ↔ Mal	TCA cycle
8	Mal ↔ OAA	TCA cycle
9	Gln → Glu + NH_3	Glutaminolysis
10	Glu ↔ αKGT + NH_3	Glutaminolysis
11	Mal ↔Pyr + CO_2	Glutaminolysis
12	Pyr + Glu ↔ αKGT + Ala	Amino acid metabolism
13	Ser → Pyr + NH_3	Amino acid metabolism
14	2Gly → Ser + CO_2 + NH_3	Amino acid metabolism
15	Cys → Pyr + NH_3	Amino acid metabolism
16	Asp + αKGT → Glu + OAA	Amino acid metabolism
17	Asn → Asp + NH_3	Amino acid metabolism
18	His → Glu + CO_2 + NH_3	Amino acid metabolism
19	Pro → Glu	Amino acid metabolism
20	Arg + αKGT → 2Glu + CO_2 + NH_3	Amino acid metabolism
21	Ile + αKGT → Glu + SucCoA + AcCoA	Amino acid metabolism
22	Met + Ser + αKGT → Glu + Cys + SucCoA	Amino acid metabolism
23	Thr → SucCoA + NH_3	Amino acid metabolism
24	Val + αKGT → Glu + 2AcCoA + CO_2	Amino acid metabolism
25	Phe → Tyr	Amino acid metabolism
26	Tyr + αKGT → Glu + Fum + 2AcCoA + CO_2	Amino acid metabolism
27	Lys + 2αKGT → 2Glu + 2AcCoA + CO_2	Amino acid metabolism
28	Leu + αKGT → Glu + 3AcCoA	Amino acid metabolism
29	0.0208Glc + 0.0377Gln + 0.0133Ala+ =.0070Arg + 0.0261Asp + 0.004Cys + 0.0006Glu + 0.0165Gly + 0.0033His + 0.0084Ile + 0.0133Leu + 0.0101Lys + 0.0033Met + 0.0055Phe + 0.0081Pro + 0.0099Ser + 0.0080Thr + 0.0040Tyr + 0.0096Val → Biomass ($CH_{1.975}N_{0.2605}O_{0.489}$)	Biomass synthesis
30	0.0049Gln + 0.0054Ala + 0.0043Arg + 0.0036Asn + 0.0043Asp + 0.0065Cys +0.0061 + 0.0054 Gly + 0.0025His + 0.0081Ile + 0.0061Leu + 0.061Lys + 0.0016Met + 0.00027Phe + 0.0110Pro + 0.0106Ser + 0.0094Thr + 0.0031Tyr + 0.0081Val → Fc-fusion Protein ($CH_{1.561}N_{0.278}O_{0.315}$)	Fc-fusion protein synthesis

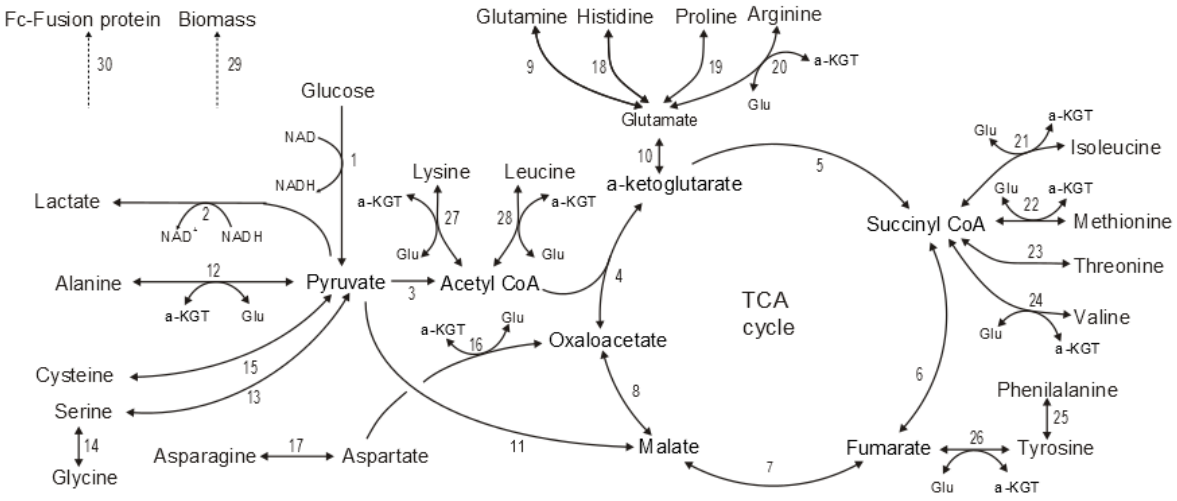


Figure 13: Metabolic Network considered for MFA analysis.

3.4. Results and Discussion

The fed-batch culture in this section is the one previously described in Chapter 2. Results showed that LDHa mutant cells reached a lower cell density and proliferated at a slower maximum cell growth rate (μ_{max}) than control cells. Cell metabolism for both cultures had a similar $\Delta L/\Delta G$ throughout the batch phase of culture, but during feeding LDHa K.D. cells presented more efficient metabolism reflected in a 30% lower $\Delta L/\Delta G$. Fc-fusion protein production was greatly enhanced by LDHa knock down increasing the volumetric production by 45%. Its specific accumulation rate was 70% higher than control cells' during exponential growth phase and over 380% higher through stationary phase. The following results provide a deeper analysis and understanding of the effect of LDHa knock down in LDHc overexpressing CHO cells.

3.4.1. RedOx State Analysis

It is expected that LDHa K.D. would have an impact over cells' RedOx state, as conversion of pyruvate to lactate is coupled with the oxidation of NADH. Flux reduction through that reaction would favor accumulation of NADH. This leads towards an enhanced energy metabolism because of a bigger supply of reducing equivalents for transportation to the mitochondria through the malate-aspartate shunt.

Table 8: NADH+/NADH ratios of studied clones.

	NAD^+/NADH
Parental cell line	6.33
LDHa mutant cell line	5.66

Measurements of NAD^+/NADH ratios of parental cells and LDHa deficient cells during the batch stage of culture are presented in Table 8. Results indicate that mutant cells have a lower NAD^+/NADH level, confirming the expected results of lower NADH oxidation and loss of reducing power within the cell accompanied by an enhanced energy metabolism.

3.4.2. Analysis of cultures' glucose and lactate metabolism

Figure 14 describes the evolution of the ΔL/ΔG parameter of the studied cultures in time. It is possible to observe that control cells increase their ΔL/ΔG during the batch phase of culture, but the quotient begins to drop once feeding begins, whereas LDHa K.D. culture exhibits a constant reduction of the ΔL/ΔG parameter in time. Initially the ΔL/ΔG for LDHa K.D. is 50% higher than that of control cells but by the end of the first phase of the culture it is 14% lower. During the feeding stage LDHa K.D's ΔL/ΔG remains constantly lower than the one of control cells. Further data analysis during feeding indicates that the reduction percentage of ΔL/ΔG values between two consecutive time points is higher for mutated cells than for control cells.

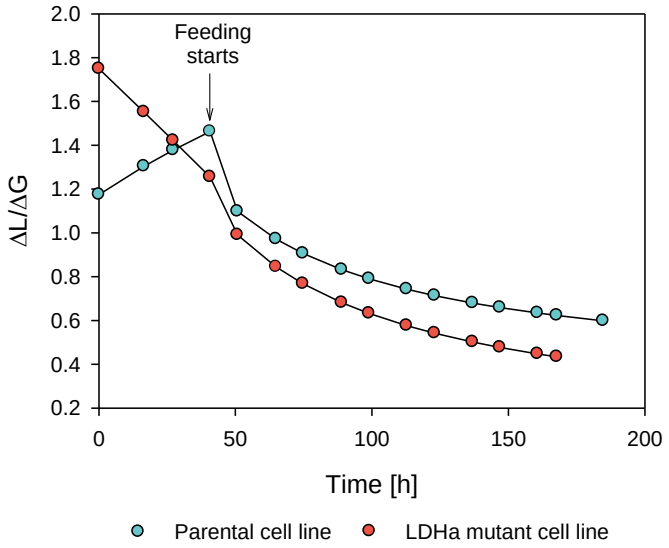


Figure 14: ΔL/ΔG evolution through culture's lifespan.

3.4.3. Metabolic Flux Analysis

Metabolic flux analysis was performed to estimate and contrast intracellular flux distribution from the mid exponential growth phase of the LDHa K.D. cell line against its parental cell line in fed batch culture. Cells were considered to be at mid exponential after 64.9 h of culture; accumulation rates of all measured species were calculated and used to obtain specific production/consumption rates at that time point, which are presented in Table 9

Table 9: Specific rates used for metabolic flux analysis.

	Specific Rates (mmol 10^{-9} cell h^{-1})	
Species	**Parental cell line**	**LDHa mutant cell line**
fc-Fusion protein	$5{,}19\times10^{-3}$	$5{,}84\times10^{-3}$
Alanine	$2{,}90\times10^{-2}$	$2{,}75\times10^{-2}$
Arginine	$-3{,}94\times10^{-3}$	$-3{,}63\times10^{-3}$
Asparagine	$-8{,}39\times10^{-3}$	$-8{,}39\times10^{-3}$
Aspartate	$4{,}10\times10^{-5}$	$2{,}88\times10^{-4}$
Biomass	$3{,}11\times10^{-1}$	$1{,}68\times10^{-1}$
CO_2	$1{,}70\times10^{-1}$	$2{,}74\times10^{-1}$
Glucose	$-8{,}72\times10^{-2}$	$-7{,}64\times10^{-2}$
Glutamine	$-4{,}90\times10^{-2}$	$-4{,}53\times10^{-2}$
Glutamate	$-6{,}05\times10^{-3}$	$-4{,}02\times10^{-3}$
Glycine	$-1{,}88\times10^{-3}$	$-2{,}52\times10^{-3}$
Histidine	$-1{,}30\times10^{-3}$	$-1{,}27\times10^{-3}$
Isoleucine	$-4{,}62\times10^{-3}$	$-4{,}26\times10^{-3}$
Lactate	$1{,}20\times10^{-1}$	$1{,}00\times10^{-1}$
Leucine	$-8{,}06\times10^{-3}$	$-7{,}22\times10^{-3}$
Lysine	$-3{,}68\times10^{-3}$	$-4{,}73\times10^{-3}$
Methionine	$-2{,}68\times10^{-3}$	$-2{,}25\times10^{-3}$
NH_3	$1{,}14\times10^{-1}$	$1{,}45\times10^{-1}$
Phenilalanine	$-3{,}48\times10^{-3}$	$-4{,}56\times10^{-3}$
Serine	$-6{,}62\times10^{-3}$	$-7{,}18\times10^{-3}$
Threonine	$-4{,}08\times10^{-3}$	$-5{,}36\times10^{-3}$
Tyrosine	$-6{,}13\times10^{-3}$	$-7{,}18\times10^{-3}$
Valine	$-5{,}88\times10^{-3}$	$-5{,}38\times10^{-3}$

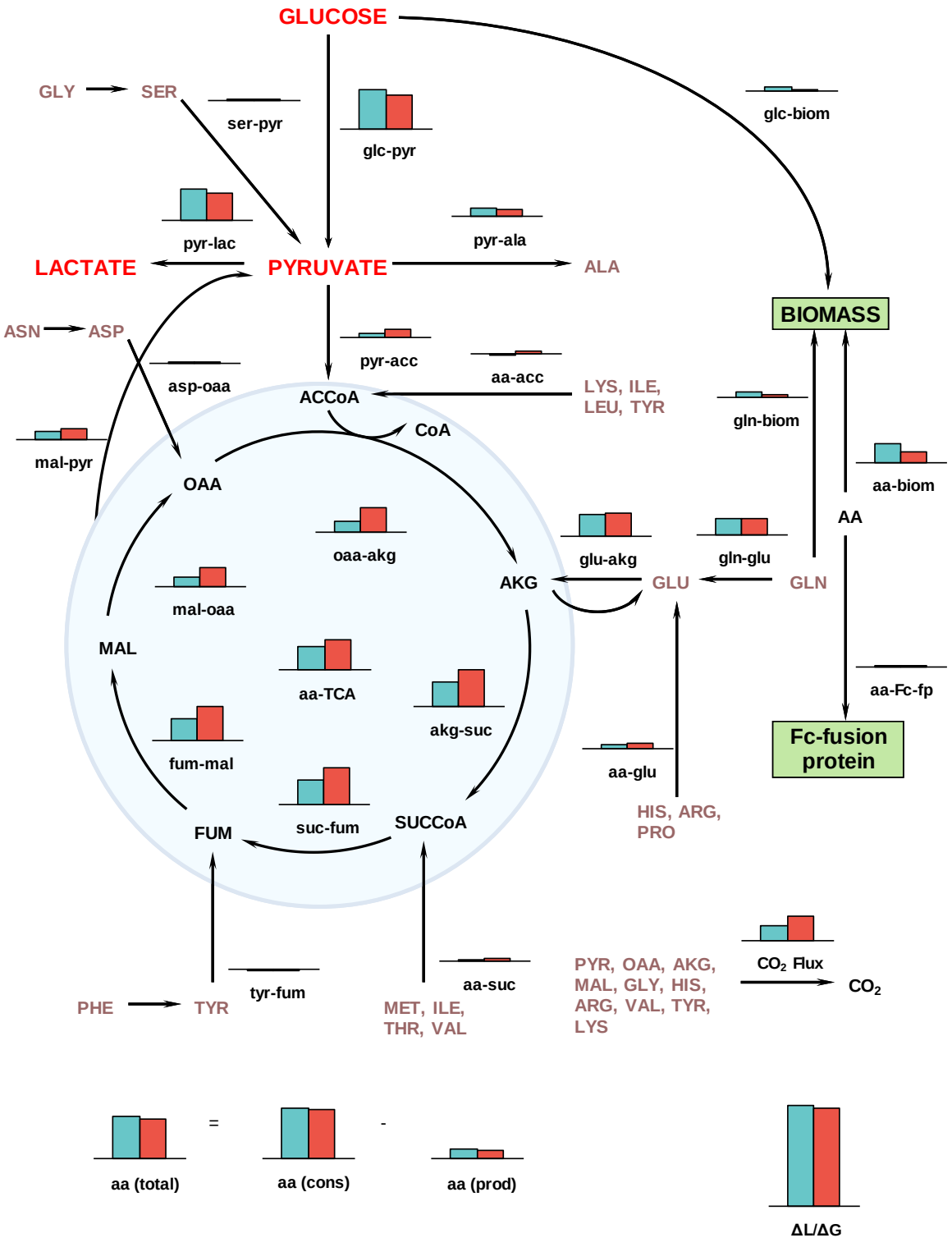


Figure 15: Comparison of metabolic flux distribution between the parental cell line ■ and LDHA mutant cells ■.

The calculated intracellular fluxes associated to the simplified metabolic network considered in this work are presented in Figure 15 in mmol C 10^{-9} cells h^{-1}. Fluxes corresponding to parental cells and mutant LDHa clones are shown in light blue and red respectively. Detailed values are provided in Table 10 in the supplementary section. From Figure 15 it is possible to observe strong metabolic differences between the two cultures studied, especially in reactions linked to the pyruvate node, TCA cycle and biomass synthesis. Graphs associated to the pyruvate node in Figure 15 show that in both experiments pyruvate is produced mainly by glucose oxidation, followed by pyruvate synthesis from the *mal-pyr* shunt and from serine conversion. Results show that in LDHA K.D cells there is a lower flux from glucose to pyruvate and a higher contribution from *mal-pyr* and *ser-pyr* reactions for pyruvate synthesis than in parental cells, given that fluxes in these reactions are increased 25% and 300% respectively. Pyruvate is then used for lactate, alanine and acetyl CoA production. LDHa mutant cells exhibit lower fluxes towards lactate and alanine synthesis, which is consistent with their lower specific production rates and increased acetyl CoA production, indicating a higher input of carbons from glycolysis into the TCA cycle.

Even though ΔL/ΔG has been used to describe carbon metabolism's efficiency by quantifying the amount of lactate that was produced per consumed glucose, MFA analysis enables us to perform a deeper analysis of the metabolic state of the cells. Despite parental cells having a higher glycolytic flux than LDHa K.D cells, MFA results indicate that both cultures have a similar net carbon influx towards pyruvate of 0.56 and 0.55 [mmol C 10^{-9} cells hr^{-1}] respectively. But regardless of that, both cultures differ in their lactate yield per produced pyruvate. Parental cells' yield is 0.66 [mol of lactate/mol of pyruvate] and LDHa K.D cells' is 0.56, from a theoretical maximum of 1. Additionally, their acetyl CoA yield from pyruvate was 0.10 and 0.17 [mol of acetyl CoA/mol of pyruvate] respectively. These numbers give further insights on the higher metabolic efficiency of cells with reduced LDHa expression were less consumed carbon molecules are used for the production of lactate and more are directed towards sustaining energy metabolism.

Higher fluxes in reactions of the TCA cycle in LDHa K.D cells evidence an enhanced energy metabolism in comparison to parental cells, and are consistent with the RedOX results discussed above. The increased fluxes can be correlated to higher CO_2 production rates obtained, since CO_2 is produced by the oxidative decarboxylation reactions on the Krebs cycle.

Higher fluxes are due not only to an increased input of carbon molecules from glycolysis, as discussed above, but also to amino acid metabolism. The *aa-TCA* graph shows that LDHa deficient cells have a higher input of carbons into the TCA cycle from amino acids than control cells. LDHa K.D. cells exhibit higher fluxes in the anaplerotic reactions that feed the Krebs cycle through different intermediaries such as α-ketoglutarate, oxaloacetate and succinyl CoA, and also increased acetyl CoA synthesis from amino acids. Higher TCA fluxes also explain the increased conversion of malate to pyruvate by the malic enzyme, reaction that deflects molecules from the cycle regulating carbon flux and avoiding excessive flux.

Due to its high concentration, glutamine is considered to be the most important nitrogen source and the second main carbon source of the culture, among the supplemented nutrients. Carbons from this molecule enter the TCA cycle after glutamine transformation to glutamate through transamination or deamination reactions. Glutamate can be later converted to α-ketoglutarate and enter the Krebs cycle. As can be seen in the *gln-glu* graph, the glutamine input is very similar in parental and LDHa K.D. cells. In both cases these fluxes surpass the carbon input from glycolysis making glutamine the biggest contributor to energy metabolism in both of the cases studied.

The graphs for the reactions *glc-biom*, *gln-biom* and *aa-biom* reveal that LDHa K.D. cells have lower fluxes towards biomass synthesis, which is consistent with the reduced cell proliferation observed experimentally. In a previous study decreased glycolytic fluxes were correlated to reduced biomass production since any decrease in the main carbon source uptake led to a higher reduction in the flux through the Pentose Phosphate Pathway, which produces important precursors for biomass such as nucleotides. Therefore the reduced glucose uptake of LDHa mutant cells might explain their lower cell proliferation.

Aa-biom graphs show higher fluxes towards recombinant protein synthesis, as expected by the higher protein production rate previously reported. Enhanced protein synthesis is correlated to reduced cell growth as cells' resources that are not used for biomass synthesis are available for protein productions (Takagi et al., 2001).

Figure 16 shows fluxes of reactions involved in the main carbon metabolism normalized by the glycolytic flux (*glc-pyr*). This data provides further evidence that LDHa K.D. cells have a lower dependence on glucose to sustain cellular metabolism and cell growth.

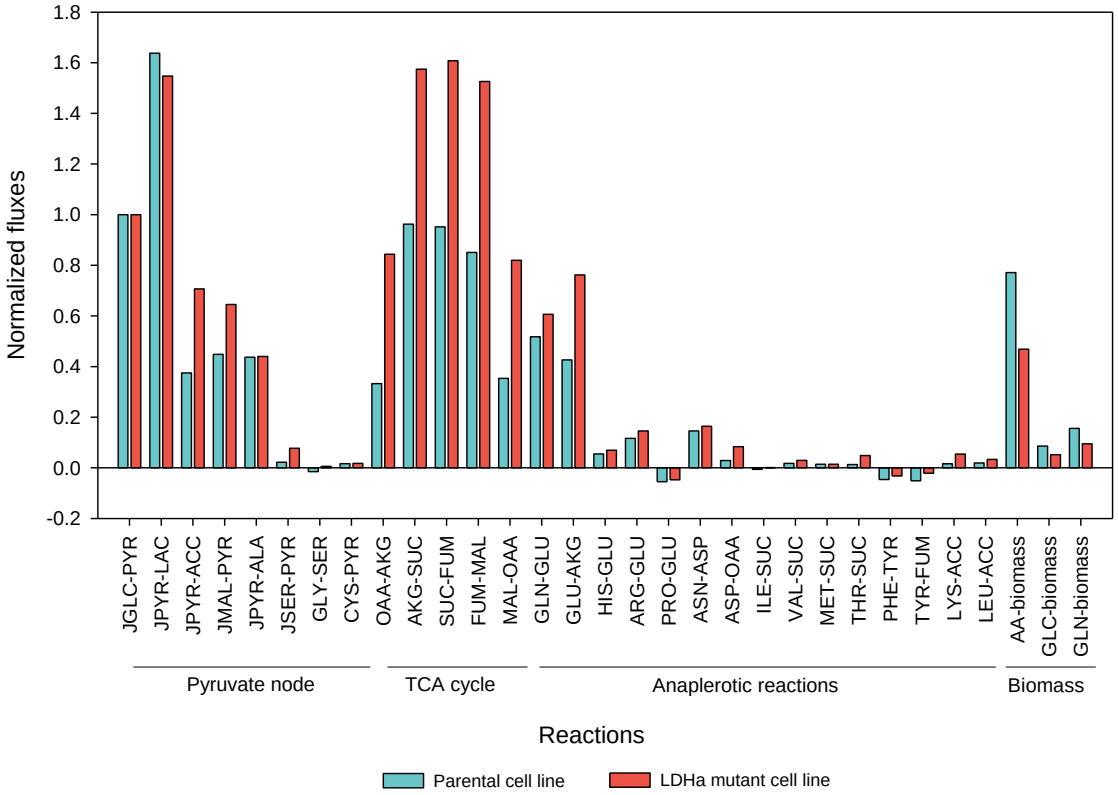


Figure 16: Comparison of Central Fluxes normalized by glycolytic flux

An overall analysis of the graph confirms that LDHa K.D cells have higher fluxes in most metabolic reactions per consumed glucose. Pyruvate synthesis in LDHa K.D cells has a bigger dependency on fluxes from serine, glycine and cysteine than the control experiment. Additionally, fluxes from anaplerotic reactions are higher in mutant cells suggesting that these cells rely more on amino acid catabolism to sustain high fluxes in the TCA cycle in order to obtain energy than parental cells. Finally, glucose and amino acids fluxes towards biomass are lower in the LDHa deficient cells than in the parental cells, which is consistent with their lower cell proliferation rate.

The analysis presented above evidences a more efficient metabolism of LDH K.D cells in contrast to the control experiment. Throughout most of the exponential growth and all the stationary phase of mutant cells' culture they had a lower production of lactate per consumed glucose. In depth analysis using metabolic flux analysis showed that even though mutant's

pyruvate production comes mainly from glucose oxidation, there is a higher contribution from other sources like serine and malate. Furthermore, LDHa K.D. cells have a lower yield of lactate from pyruvate and a higher yield of acetyl CoA showing that in their metabolism pyruvate has a larger conversion towards acetyl CoA for TCA cycle feeding in comparison to parental cells. Even though *pyr-acc* flux is higher in LDHa K.D. cells, the biggest input of carbons to the Krebs cycle comes from glutamate, produced mainly from glutamine, making this amino acid the biggest contributing molecule to energy metabolism. Additionally, amino acid catabolism has a more relevant participation in LDHa K.D. metabolism by feeding the TCA cycle through its intermediaries. Lower biomass levels obtained for LDHa K.D. cells can be correlated with the decrease in glucose uptake as fewer molecules are diverted towards biomass precursors. Finally, comparison of parental cells and LDHa K.D cells' normalized fluxes indicate that mutant cells rely more on amino acid catabolism for sustaining energy metabolism as seen by the higher fluxes in anaplerotic reactions.

3.5. Conclusions

In depth analyses of the effects of LDHa knock down over the metabolism of LDHc overexpressing CHO cells grown in fed-batch reveal the impact of this mutation. Study of ΔL/ΔG values in time show that mutated cells' metabolism has a lower production of lactate per consumed glucose during the feeding phase of culture. This gives the first indication that these cells have a more efficient metabolism, were less of the consumed glucose is used for the production of lactate, which is an undesirable metabolite of mammalian cultures, and more carbon molecules are used for other cell functions such as sustaining energy metabolism. Measurements of NAD^+/NADH ratios reveal the RedOx state of the cells is also affected by LDHa knock down. LDHa deficient cells have a lower NAD^+/NADH level which prove higher accumulation of reducing power, indicating an improvement in energy metabolism.

Metabolic flux analysis was performed in order to calculate and compare metabolic fluxes of the mutant cell line against parental cells'. Results confirm that LDHa K.D. cells have a different metabolic state than the control experiment. Their metabolism is characterized by lower fluxes in the glycolysis, which may explain reduced cell growth and higher protein synthesis. Nevertheless net flux towards pyruvate synthesis is higher and also present enhanced conversion of pyruvate to acetyl CoA. TCA cycle fluxes are higher ratifying the improved metabolism that previous results suggested. Increased fluxes are due to more available acetyl

CoA as well as higher input of carbons from amino acid catabolism, especially glutamine. Finally, LDHa K.D. cells' metabolism has higher fluxes in most pathways per consumed glucose, evidencing that these cells rely more on amino acid metabolism to sustain high viability.

In conclusion, results presented in this work indicate that LDHa knock down has positive effects over cells metabolism and recombinant protein productivity, by improving use of the main carbon source, reducing production of unwanted metabolites and increasing energy metabolism.

3.6. Bibliography

Altamirano, C., Paredes, C., Cairo, J.J., Godia, F., 2000. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnol Prog 16, 69-75.

Arnaudeau, C., Lundin, C., Helleday, T., 2001. DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. Journal of molecular biology 307, 1235-1245.

Barnes, L.M., Dickson, A.J., 2006. Mammalian cell factories for efficient and stable protein expression. Current opinion in biotechnology 17, 381-386.

Bassett, A.R., Tibbit, C., Ponting, C.P., Liu, J.-L., 2014. Mutagenesis and homologous recombination in Drosophila cell lines using CRISPR/Cas9. Biology open 3, 42-49.

Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., Marraffini, L.A., 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic acids research 41, 7429-7437.

Birch, J.R., Racher, A.J., 2006. Antibody production. Advanced drug delivery reviews 58, 671-685.

Boada, J., Roig, T., Perez, X., Gamez, A., Bartrons, R., Cascante, M., Bermudez, J., 2000. Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress. FEBS Lett 480, 261-264.

Boissel, J.P., Lee, W.R., Presnell, S.R., Cohen, F.E., Bunn, H.F., 1993. Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure. J Biol Chem 268, 15983-15993.

Bonarius, H.P., de Gooijer, C.D., Tramper, J., Schmid, G., 1995. Determination of the respiration quotient in mammalian cell culture in bicarbonate buffered media. Biotechnol Bioeng 45, 524-535.

Bonarius, H.P., Hatzimanikatis, V., Meesters, K.P., de Gooijer, C.D., Schmid, G., Tramper, J., 1996. Metabolic flux analysis of hybridoma cells in different culture media using mass balances. Biotechnol Bioeng 50, 299-318.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005a. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnol Prog 21, 106-111.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005b. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnology progress 21, 106-111.

Burma, S., Chen, B.P., Chen, D.J., 2006. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA repair 5, 1042-1048.

Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J.-W., Xi, J.J., 2013. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell research 23, 465-472.

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

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

Cho, S.W., Kim, S., Kim, J.M., Kim, J.-S., 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nature biotechnology 31, 230-232.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.-Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010a. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. Journal of biotechnology 147, 116-121.

Chong, W.P., Reddy, S.G., Yusufi, F.N., Lee, D.Y., Wong, N.S., Heng, C.K., Yap, M.G., Ho, Y.S., 2010b. Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. J Biotechnol 147, 116-121.

DiCarlo, J.E., Norville, J.E., Mali, P., Rios, X., Aach, J., Church, G.M., 2013. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic acids research 41, 4336-4343.

Dorai, H., Ellis, D., Keung, Y.S., Campbell, M., Zhuang, M., Lin, C., Betenbaugh, M.J., 2010. Combining high-throughput screening of caspase activity with anti-apoptosis genes for development of robust CHO production cell lines. Biotechnology progress 26, 1367-1381.

Eagle, H., Barban, S., Levy, M., Schulze, H.O., 1958. The utilization of carbohydrates by human cell cultures. J Biol Chem 233, 551-558.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. nature 411, 494-498.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003a. Improving glucose and glutamine metabolism of human HEK 293 and Trichoplusia ni insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnol Prog 19, 90-97.

Elias, C.B., Carpentier, E., Durocher, Y., Bisson, L., Wagner, R., Kamen, A., 2003b. Improving glucose and glutamine metabolism of human HEK 293 and trichoplusiani insect cells engineered to express a cytosolic pyruvate carboxylase enzyme. Biotechnology progress 19, 90-97.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000a. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnology and bioengineering 67, 25-34.

Europa, A.F., Gambhir, A., Fu, P.C., Hu, W.S., 2000b. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. Biotechnol Bioeng 67, 25-34.

Farzadfard, F., Perli, S.D., Lu, T.K., 2013. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. ACS synthetic biology 2, 604-613.

Fauser, F., Schiml, S., Puchta, H., 2014. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. The Plant Journal.

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D.-L., Wei, P., Cao, F., Zhu, S., Zhang, F., Mao, Y., 2013. Efficient genome editing in plants using a CRISPR/Cas system. Cell research 23, 1229.

Fogolin, M.B., Wagner, R., Etcheverrigaray, M., Kratje, R., 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J Biotechnol 109, 179-191.

Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J.A., 2013. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nature methods 10, 741-743.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature biotechnology 31, 822-826.

Fukasawa, K.M., Li, S.S.-L., 1987. Complete nucleotide sequence of the mouse lactate dehydrogenase-A functional gene: comparison of the exon-intron organization of dehydrogenase genes. Genetics 116, 99-105.

Fussenegger, M., Mazur, X., Bailey, J.E., 1997a. A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnology and bioengineering 55, 927-939.

Fussenegger, M., Mazur, X., Bailey, J.E., 1997b. A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnol Bioeng 55, 927-939.

Fussenegger, M., Schlatter, S., Dätwyler, D., Mazur, X., Bailey, J.E., 1998. Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. Nature biotechnology 16, 468-472.

Gambhir, A., Korke, R., Lee, J., Fu, P.-C., Europa, A., Hu, W.-S., 2003a. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. Journal of Bioscience and Bioengineering 95, 317-327.

Gambhir, A., Korke, R., Lee, J., Fu, P.C., Europa, A., Hu, W.S., 2003b. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. J Biosci Bioeng 95, 317-327.

Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442-451.

Glacken, M., Fleischaker, R., Sinskey, A., 1986a. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnology and bioengineering 28, 1376-1389.

Glacken, M.W., 1988. Catabolic Control of Mammalian Cell Culture. Nat Biotechnol 6, 1041 - 1050.

Glacken, M.W., Fleischaker, R.J., Sinskey, A.J., 1986b. Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol Bioeng 28, 1376-1389.

Goldberg, E., Eddy, E.M., Duan, C., Odet, F., 2010. LDHC: The Ultimate Testis-Specific Gene. Journal of andrology 31, 86-94.

Griffin, T.J., Seth, G., Xie, H., Bandhakavi, S., Hu, W.-S., 2007. Advancing mammalian cell culture engineering using genome-scale technologies. Trends in biotechnology 25, 401-408.

Gupta, G., 1999. LDH-C4: a unique target of mammalian spermatozoa. Critical reviews in biochemistry and molecular biology 34, 361-385.

Hammond, S., Kaplarevic, M., Borth, N., Betenbaugh, M.J., Lee, K.H., 2012. Chinese hamster genome database: an online resource for the CHO community at www. CHOgenome. org. Biotechnology and bioengineering 109, 1353-1356.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., 2011. Genetic engineering of human pluripotent cells using TALE nucleases. Nature biotechnology 29, 731-734.

Holmes, R.S., Goldberg, E., 2009. Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Computational biology and chemistry 33, 379-385.

Hu, W.-S., Zhou, W., 2012. Cell Culture Bioprocess Engineering. Wei-Shou Hu.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology 31, 227-229.

Ifandi, V., Al-Rubeai, M., 2003. Stable transfection of CHO cells with the c-myc gene results in increased proliferation rates, reduces serum dependency, and induces anchorage independence. Cytotechnology 41, 1-10.

Inoue, Y., Inoue, A., Kawahara, H., 2011. Efficient production of recombinant IgG by the GLUT5 co-expression system, In BMC proceedings, BMC proceedings. BioMed Central Ltd, p. P50.

Inoue, Y., Tsukamoto, Y., Yamanaka, M., Nakamura, S., Inoue, A., Nishino, N., Kawahara, H., 2010. Efficient production of recombinant IgG by metabolic control and co-expression with GLUT5 in a fructose-based medium. Cytotechnology 62, 301-306.

Irani, N., Beccaria, A.J., Wagner, R., 2002a. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. Journal of biotechnology 93, 269-282.

Irani, N., Beccaria, A.J., Wagner, R., 2002b. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. J Biotechnol 93, 269-282.

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.

Jao, L.-E., Wente, S.R., Chen, W., 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proceedings of the National Academy of Sciences 110, 13904-13909.

Jeong, D.-w., Cho, I.T., Kim, T.S., Bae, G.W., Kim, I.-H., Kim, I.Y., 2006a. Effects of lactate dehydrogenase suppression and glycerol-3-phosphate dehydrogenase overexpression on cellular metabolism. Molecular and cellular biochemistry 284, 1-8.

Jeong, D.-w., 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. Biochemical and biophysical research communications 289, 1141-1149.

Jeong, D.W., Cho, I.T., Kim, T.S., Bae, G.W., Kim, I.H., Kim, I.Y., 2006b. Effects of lactate dehydrogenase suppression and glycerol-3-phosphate dehydrogenase overexpression on cellular metabolism. Mol Cell Biochem 284, 1-8.

Jiang, W., Bikard, D., Cox, D., Zhang, F., Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature biotechnology 31, 233-239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816-821.

Johnson, R., Jasin, M., 2001. Double-strand-break-induced homologous recombination in mammalian cells. Biochemical Society Transactions 29, 196-200.

Johnson, R.D., Liu, N., Jasin, M., 1999. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature 401, 397-399.

Jones, M.L., Seldon, T., Smede, M., Linville, A., Chin, D.Y., Barnard, R., Mahler, S.M., Munster, D., Hart, D., Gray, P.P., Munro, T.P., 2010. A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies. J Immunol Methods 354, 85-90.

Kerbey, A.L., Radcliffe, P.M., Randle, P.J., 1977. Diabetes and the control of pyruvate dehydrogenase in rat heart mitochondria by concentration ratios of adenosine triphosphate/adenosine diphosphate, of reduced/oxidized nicotinamide-adenine dinucleotide and of acetyl-coenzyme A/coenzyme A. Biochem J 164, 509-519.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic acids research 35, W43-W46.

Kim, S.H., Lee, G.M., 2007a. 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.

Kim, S.H., Lee, G.M., 2007b. Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. Applied microbiology and biotechnology 74, 152-159.

Korke, R., Gatti Mde, L., Lau, A.L., Lim, J.W., Seow, T.K., Chung, M.C., Hu, W.S., 2004a. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. J Biotechnol 107, 1-17.

Korke, R., Gatti, M.d.L., Lau, A.L.Y., Lim, J.W.E., Seow, T.K., Chung, M.C.M., Hu, W.-S., 2004b. Large scale gene expression profiling of metabolic shift of mammalian cells in culture. Journal of biotechnology 107, 1-17.

Kummel, A., Panke, S., Heinemann, M., 2006. Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolome data. Mol Syst Biol 2, 2006 0034.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990a. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. Journal of biotechnology 15, 113-128.

Kurano, N., Leist, C., Messi, F., Kurano, S., Fiechter, A., 1990b. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. J Biotechnol 15, 113-128.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nature protocols 8, 2180-2196.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2008. Lehninger principles of biochemistry, 5th ed. W.H. Freeman.

Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., Li, Y., Gao, N., Wang, L., Lu, X., 2013a. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nature biotechnology 31, 681-683.

Li, J.-F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M., Sheen, J., 2013b. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nature biotechnology 31, 688-691.

Liang, F., Han, M., Romanienko, P.J., Jasin, M., 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proceedings of the National Academy of Sciences 95, 5172-5177.

Lim, S.F., Chuan, K.H., Liu, S., Loh, S.O., Chung, B.Y., Ong, C.C., Song, Z., 2006. RNAi suppression of Bax and Bak enhances viability in fed-batch cultures of CHO cells. Metabolic engineering 8, 509-522.

Longmore, W.J., Niethe, C.M., McDaniel, M.L., 1969. Effect of CO2 concentration on intracellular pH and on glycogen synthesis from glycerol and glucose in isolated perfused rat liver. J Biol Chem 244, 6451-6457.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013a. RNA-guided human genome engineering via Cas9. Science 339, 823-826.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013b. RNA-guided human genome engineering via Cas9. Science 339, 823-826.

Martinez, V., Gerdtzen, Z., Andrews, B.A., Asenjo, J.A., 2010. Viral vectors for the treatment of alcoholism: use of metabolic flux analysis for cell cultivation and vector production. Metabolic engineering 12, 129-137.

Mastrangelo, A.J., Hardwick, J.M., Zou, S., Betenbaugh, M.J., 2000. Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. Biotechnology and bioengineering 67, 555-564.

Mooney, R.A., Lane, M.D., 1982. Control of ketogenesis and fatty-acid synthesis at the mitochondrial branch-point for acetyl-CoA in the chick liver cell: effect of adenosine 3',5'-monophosphate. Eur J Biochem 121, 281-287.

Mulukutla, B.C., Gramer, M., Hu, W.-S., 2012. On metabolic shift to lactate consumption in fed-batch culture of mammalian cells. Metabolic engineering 14, 138-149.

Nerem, R.M., 1991. Cellular engineering. Annals of biomedical engineering 19, 529-545.

Park, H.-S., Kim, I.-H., Kim, I.-Y., Kim, K.-H., Kim, H.-J., 2000. Expression of carbamoyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary< i> dhfr</i>-cells decreases accumulation of ammonium ion in culture media. Journal of biotechnology 81, 129-140.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., Liu, D.R., 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature biotechnology 31, 839-843.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173-1183.

Read, J., Winter, V., Eszes, C., Sessions, R., Brady, R., 2001. Structural basis for altered activity of M-and H-isozyme forms of human lactate dehydrogenase. Proteins: Structure, Function, and Bioinformatics 43, 175-185.

Rebane, R., Herodes, K., 2010. A sensitive method for free amino acids analysis by liquid chromatography with ultraviolet and mass spectrometric detection using precolumn

derivatization with diethyl ethoxymethylenemalonate: Application to the honey analysis. Analytica chimica acta 672, 79-84.

Robinson, B.H., Williams, G.R., Halperin, M.L., Leznoff, C.C., 1971. Factors affecting the kinetics and equilibrium of exchange reactions of the citrate-transporting system of rat liver mitochondria. J Biol Chem 246, 5280-5286.

Ronda, C., Pedersen, L.E., Hansen, H.G., Kallehauge, T.B., Betenbaugh, M.J., Nielsen, A.T., Kildegaard, H.F., 2014. Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool. Biotechnology and Bioengineering, n/a-n/a.

Rozwarski, D.A., Diederichs, K., Hecht, R., Boone, T., Karplus, P.A., 1996. Refined crystal structure and mutagenesis of human granulocyte-macrophage colony-stimulating factor. Proteins 26, 304-313.

Sánchez, C., Gómez, N., Quintero, J.C., Ochoa, S., Rios, R., 2014. A Combined Sensitivity and Metabolic Flux Analysis Unravel the Importance of Amino Acid Feeding Strategies in Clavulanic Acid Biosynthesis, In Advances in Computational Biology, Advances in Computational Biology. Springer, pp. 169-175.

Santiago, Y., Chan, E., Liu, P.-Q., Orlando, S., Zhang, L., Urnov, F.D., Holmes, M.C., Guschin, D., Waite, A., Miller, J.C., 2008. Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. Proceedings of the National Academy of Sciences 105, 5809-5814.

Sargent, R.G., Brenneman, M.A., Wilson, J.H., 1997. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Molecular and cellular biology 17, 267-277.

Schuster, R., Holzhutter, H.G., Jacobasch, G., 1988. Interrelations between glycolysis and the hexose monophosphate shunt in erythrocytes as studied on the basis of a mathematical model. Biosystems 22, 19-36.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.-L., 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. Nature biotechnology 31, 686-688.

Siddiquee, K.A.Z., Arauzo-Bravo, M., Shimizu, K., 2004. Metabolic flux analysis of pykF gene knockout Escherichia coli based on 13C-labeling experiments together with measurements of enzyme activities and intracellular metabolite concentrations. Appl. Microbiol. Biotechnol. 63, 407-417.

Sies, H., Noack, G., Halder, K.H., 1973. Carbon-dioxide concentration and the distribution of monocarboxylate and H+ ions between intracellular and extracellular spaces of hemoglobin-free perfused rat liver. Eur J Biochem 38, 247-258.

Stafford, D.E., Stephanopoulos, G., 2001. Metabolic engineering as an integrating platform for strain development. Current opinion in microbiology 4, 336-340.

Stephanopoulos, G., Aristidou, A.A., Nielsen, J., 1998. Metabolic engineering: principles and methodologies. Academic press.

Sugimoto, N., Nakano, S.-i., Katoh, M., Matsumura, A., Nakamuta, H., Ohmichi, T., Yoneyama, M., Sasaki, M., 1995. Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. Biochemistry 34, 11211-11216.

Takagi, M., Hia, H.C., Jang, J.H., Yoshida, T., 2001. Effects of high concentrations of energy sources and metabolites on suspension culture of Chinese hamster ovary cells producing tissue plasminogen activator. Journal of bioscience and bioengineering 91, 515-521.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., Takeda, S., 1998. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. The EMBO journal 17, 5497-5508.

Terada, S., Suzuki, E., Ueda, H., Makishima, F., 1996a. Cytokines involving gp130 in signal transduction suppressed growth of a mouse hybridoma cell line and enhanced its antibody production. Cytokine 8, 889-894.

Terada, S., Suzuki, E., Ueda, H., Makishima, F., 1996b. Cytokines involving gp130 in signal transduction suppressed growth of a mouse hybridoma cell line and enhanced its antibody production. Cytokine 8, 889-894.

Thompson, L.H., Limoli, C.L., 2003. Origin, recognition, signaling, and repair of DNA double-strand breaks in mammalian cells. Eukaryotic DNA damage surveillance and repair, 107-145.

Thompson, L.H., Schild, D., 2001. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 477, 131-153.

Valerie, K., Povirk, L.F., 2003. Regulation and mechanisms of mammalian double-strand break repair. Oncogene 22, 5792-5812.

Vallino, J.J., Stephanopoulos, G., 1993. Metabolic flux distributions in Corynebacterium glutamicum during growth and lysine overproduction. Biotechnol Bioeng 41, 633-646.

Varma, A., Palsson, B.O., 1994. Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use. Bio/technology 12.

Vincze, T., Posfai, J., Roberts, R.J., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic acids research 31, 3688-3691.

Waaijers, S., Portegijs, V., Kerver, J., Lemmens, B.B., Tijsterman, M., van den Heuvel, S., Boxem, M., 2013. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. Genetics 195, 1187-1191.

Walsh, G., 2010. Biopharmaceutical benchmarks 2010. Nature biotechnology 28, 917.

Walter, P., Ron, D., 2011. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. Science 334, 1081-1086.

Wilkens, C.A., Altamirano, C., Gerdtzen, Z.P., 2011a. Comparative metabolic analysis of lactate for CHO cells in glucose and galactose Biotechnol. Bioprocess Eng 16.

Wilkens, C.A., Altamirano, C., Gerdtzen, Z.P., 2011b. Comparative metabolic analysis of lactate for CHO cells in glucose and galactose. Biotechnology and Bioprocess Engineering 16, 714-724.

Williamson, D.H., Lund, P., Krebs, H.A., 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. Biochem J 103, 514-527.

Wlaschin, K.F., Hu, W.-S., 2007a. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. Journal of biotechnology 131, 168-176.

Wlaschin, K.F., Hu, W.S., 2007b. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. J Biotechnol 131, 168-176.

Wu, F., Yang, F., Vinnakota, K.C., Beard, D.A., 2007. Computer modeling of mitochondrial tricarboxylic acid cycle, oxidative phosphorylation, metabolite transport, and electrophysiology. J Biol Chem 282, 24525-24537.

Wurm, F.M., 2004a. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22, 1393-1398.

Wurm, F.M., 2004b. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature biotechnology 22, 1393-1398.

Xing, Z., Kenty, B., Koyrakh, I., Borys, M., Pan, S.-H., Li, Z.J., 2011. Optimizing amino acid composition of CHO cell culture media for a fusion protein production. Process Biochemistry 46, 1423-1429.

Yamamoto, S., Gunji, W., Suzuki, H., Toda, H., Suda, M., Jojima, T., Inui, M., Yukawa, H., 2012. Overexpression of genes encoding glycolytic enzymes in Corynebacterium glutamicum enhances glucose metabolism and alanine production under oxygen deprivation conditions. Appl. Environ. Microbiol. 78, 4447-4457.

Yip, S.S., Zhou, M., Joly, J., Snedecor, B., Shen, A., Crawford, Y., 2014. Complete Knockout of the Lactate Dehydrogenase A Gene is Lethal in Pyruvate Dehydrogenase Kinase 1, 2, 3 Down-Regulated CHO Cells. Molecular biotechnology, 1-6.

Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A.F., Meier, A., Yuk, I.H., Vijayasankaran, N., Leach, K., Joly, J., 2011a. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. Journal of biotechnology 153, 27-34.

Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A.F., Meier, A., Yuk, I.H., Vijayasankaran, N., Leach, K., Joly, J., Snedecor, B., Shen, A., 2011b. Decreasing lactate level and increasing antibody production in Chinese Hamster Ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases. J Biotechnol 153, 27-34.

Zupke, C., Sinskey, A.J., Stephanopoulos, G., 1995. Intracellular flux analysis applied to the effect of dissolved oxygen on hybridomas. Appl Microbiol Biotechnol 44, 27-36.

Zustiak, M.P., Dorai, H., Betenbaugh, M.J., Sauerwald, T.M., 2012. Controlling apoptosis to optimize yields of proteins from mammalian cells, In Protein Expression in Mammalian Cells, Protein Expression in Mammalian Cells. Springer, pp. 111-123.

3.7. Supplementary Material

Table 10: Calculated MFA carbon fluxes

Reactions	Carbon Flux [mmol C 10^{-9} cells hr^{-1}] Parental cell line	LDHa mutant cell line
JGLC-PYR	0,4511	0,4012
JPYR-LAC	0,3694	0,3104
JPYR-ACC	0,0564	0,0945
JOAA-AKG	0,1251	0,2819
JAKG-SUC	0,2895	0,4210
JSUC-FUM	0,2862	0,4301
JFUM-MAL	0,2559	0,4081
JMAL-OAA	0,1062	0,2193
JGLN-GLU	0,1946	0,2027
JGLU-AKG	0,2601	0,2697
JMAL-PYR	0,1011	0,1294
JPYR-ALA	0,0985	0,0883
JSER-PYR	0,0050	0,0155
JGLY-SER	-0,0034	0,0011
JCYS-PYR	0,0038	0,0035
JASP-OAA	0,0087	0,0224
JASN-ASP	0,0438	0,0440
JHIS-GLU	0,0206	0,0233
JARG-GLU	0,0437	0,0487
JPRO-GLU	-0,0205	-0,0157
JILE-SUC	-0,0018	0,0004
JVAL-SUC	0,0053	0,0080
JMET-SUC	0,0042	0,0039
JTHR-SUC	0,0039	0,0130
JPHE-TYR	-0,0313	-0,0193
JTYR-FUM	-0,0154	-0,0057
JLYS-ACC	0,0049	0,0145
JLEU-ACC	0,0073	0,0217
JBIOMASS	0,3107	0,1679
JFC-FUSION PROTEIN	0,0051	0,0058
JAA-biomass	0,2192	0,1185
JGLC-biomass	0,0387	0,0209
JGLN-biomass	0,0587	0,0318

Conclusions

In this Ph.D. thesis it was possible to successfully construct, study and compare four clones with different modifications that altered CHO cells' central carbon metabolism. This allowed us to increase the understanding of the different metabolic states and traits that influences cells' productivity.

The first chapter of this thesis describes the analysis of IgG producing CHO cells overexpressing PYC2, MDHII or GLUT5. Culture's performance of each clone was analyzed and compared to each other and a control experiment. Results showed that these modifications had an important impact over carbon metabolism, lowering cultures' ΔL/ΔHexose and NAD^+/NADH ratios. The discussions from a metabolic and thermodynamic point of view presented in this work support previous conclusions regarding improvement of the energy metabolism of these cell modifications. In addition, we explain the drop in recombinant protein productivity in the studied clones exhibit by linking the new metabolic flux distribution to changes in the redox state, which have a detrimental effect on IgG's assembly, therefore reducing its secretion. Our results suggest that by improving energy metabolism and the flux into the TCA cycle, it is possible to improve cultures' performance, provided that appropriate redox conditions can be maintained.

Chapter 2 describes the construction of an LDHa deficient, LDHc overexpressing, Fc-fusion protein producing CHO cell line using the novel CRISPR-Cas genome editing tool. Lower LDHa enzyme content was confirmed using Western Blott Analysis and metabolic characterization was performed by studying culture's performance in fed-batch cultures in terms of cell growth, recombinant protein production, and glucose and lactate metabolism. Experiments showed a significant reduction in cell growth and increased recombinant protein production for LDHa K.D. clones. Our results suggest that modifications in the lactate metabolism lead towards an improved energy metabolism and enhanced energy and RedOx conditions for protein synthesis.

The final chapter consists of an in depth analysis of the LDHa deficient clone constructed in chapter 2. Mutated cells had a lower production of lactate per consumed glucose during the feeding phase of culture, evidencing a more efficient metabolism. Measurements of NAD^+/NADH ratios reveal the RedOx state of the cells is also affected, characterized by a lower NAD^+/NADH

ratio, evidencing a higher accumulation of reducing power. Metabolic flux analysis was performed in order to calculate and compare metabolic fluxes of the mutant cell line against parental cells'. Results confirm that LDHa K.D. cells have a different metabolic state than the control experiment. Their metabolism is characterized by lower fluxes in glycolysis, which may explain the reduced cell growth and higher protein synthesis observed. Nevertheless, net flux towards pyruvate synthesis is higher and also present enhanced conversion of pyruvate to acetyl CoA. TCA cycle fluxes are higher ratifying the improved metabolism that previous results suggested. Increased fluxes are due to more available acetyl CoA as well as higher input of carbons from amino acid catabolism, especially glutamine. Finally, LDHa K.D. cells' metabolism has higher fluxes in most pathways per consumed glucose, evidencing that these cells rely more on amino acid metabolism to sustain high viability. Results presented in this work indicate that LDHa knock down has positive effects over cells metabolism and recombinant protein productivity, by improving use of the main carbon source, reducing production of unwanted metabolites and increasing energy metabolism.

In conclusion, this work provides the scientific community with the first comparison of different Metabolic Engineering approaches that aim at improving fluxes through bottleneck reactions to improve CHO cells' central carbon metabolism by overexpressing key genes of this pathways. Obtaining this information is especially relevant as it is important for the Metabolic Engineering discipline to collect as much information as possible in order to propose and pursue new targets. Additionally, in this work, the novel genome editing tool CRISPR-Cas was successfully used to introduce a loss-of-function mutation in a copy of the LDHa gene of an Fc-fusion protein producing CHO cell line. Finally, this clone was used to study the effect of LDHa knock down on cells' metabolism and productivity. Previous works that were able to reduce LDHa expression only reported the effect over culture's performance; the analysis shown in this work is the first to study in depth the effects of this mutation, using Metabolic Flux Analysis to estimate and compare intracellular fluxes.