# Multidisciplinary Analysis of the Metabolic Shift to Lactate Consumption in CHO Cell Culture

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# Riassunto

La coltura di cellule di mammifero rappresenta il sistema maggiormente utilizzato per la produzione di proteine terapeutiche a livello industriale. I terreni di coltura impiegati a tale scopo sono, nella maggior parte dei casi, chimicamente definiti e specificamente ottimizzati per evitare la penuria di principi nutritivi essenziali per la produzione. Le cellule mantenute in queste condizioni di coltura, però, possono presentare un metabolismo alterato, in cui i substrati vengono ossidati in modo incompleto causando l'accumulo di prodotti secondari. Tra questi vi è l'acido lattico, che può avere effetti negativi sulla coltura dovuti all'acidificazione del terreno.

Il lattato è normalmente prodotto durante la fase di crescita esponenziale delle cellule, mentre il suo consumo può avvenire al passaggio alla fase stazionaria. Questo cambiamento nel metabolismo del lattato non è però comune a tutte le colture industriali, e nè può essere facilmente controllato, poiché i meccanismi responsabili di tale fenomeno non sono ancora completamenti definiti.

L'obiettivo principale di questo progetto di tesi è lo studio del metabolismo del lattato in coltura di cellule CHO, allo scopo di individuare alcuni dei fattori principali che lo regolano. In particolare, è stato analizzato il ruolo del mitocondrio in questo fenomeno. A tal scopo sono state impiegate come modello delle linee cellulari caratterizzate da opposti profili metabolici del lattato. La fase iniziale di produzione del lattato è stata associata a una rapida consumazione della glutammina in tutte le colture caratterizzate da una rapida crescita cellulare. In seguito all'esaurimento della glutammina, sono stati osservati due scenari differenti. In un primo caso, le cellule consumano il lattato, che quindi diventa una risorsa di energia. Alternativamente, il lattato continua a essere accumulato nel terreno di coltura, causando una riduzione del piruvato che viene completamente ossidato a $CO_2$. Pertanto, si è ipotizzato che la capicità ossidativa del mitocondrio sia una possibile causa dell'eccessivo accumulo di lattato. Per verificare questa teoria, sono stati misurati il potenziale di membrana del mitocondrio e il consumo di ossigeno, evidenziando così una interessante correlazione tra il ridotto metabolismo ossidativo e l'elevata produzione di lattato.

Successivamente, è stata anche valutata l'espressione di geni, specificamente selezionati, in correlazione con il metabolismo del lattato. Una riduzione di espressione significativa ($|FC \geq 2|$) è stata riscontrata in due geni: precisamente, il trasporta-

tore mitocondriale dell'aspartato-glutammato (anche noto come aralar1) e il translocatore della membrana interna del mitocondrio, timm8a. Aralar1 è di particolare interesse poichè è un componente della navetta del malato-aspartato. Questa navetta favorisce l'ossidazione del NADH citosolico, necessaria per evitare un rallentamento del flusso glicolitico. La sua attività riduce pertanto la quantità di NADH che è invece utilizzata nella conversione del piruvato a lattato ad opera della lattato deidrogenasi.

Sulla base di questi dati, sono state generate linee cellulari che overesprimono aralar1 o timm8a. Entrambi i geni hanno favorito il passaggio alla fase di consumazione del lattato nella linea cellulare che normalmente accumulava elevate concentrazioni di questo metabolita. Invece, quando i due geni sono stati overespressi nella linea cellulare che già consumava il lattato, nessun cambiamento metabolico è stato osservato.

I risultati presentati in questa tesi mostrano che il metabolismo mitocondriale svolge un ruolo fondamentale nel controllo della produzione del lattato. Inoltre, sia i terreni di coltura utilizzati che le caratteristiche intrinseche della linea cellulare possono influenzare l'attività mitocondriale. L'espressione di geni specifici può anche essere modulata dal terreno di coltura. In particolare, è stata analizzata l'espressione di aralar1 and timm8a, che vengono proposti come geni promettenti per creare una linea cellulare ad utilizzo industriale, con un migliore metabolismo del lattato.

**Parole chiave:** cellule CHO, lattato, glutamminolisi, potenziale di membrana del mitocondrio, metabolismo ossidativo, aralar, timm8a, navetta malato aspartato, ingegnerizzazione cellulare.

# Abstract

Mammalian cells represent the most widely used host system for the industrial production of recombinant therapeutic proteins. In order to increase productivity, chemically defined media are often used and optimized to provide the cells with the necessary nutrients. However, a deregulated cell metabolism is often observed in these culture conditions. In particular, carbon sources are fast and inefficiently consumed with a consequent accumulation of byproducts, mainly lactate and ammonia.

Lactate, in particular, causes a decrease of the medium pH which can be detrimental for cells growth and productivity. Its metabolic profile is normally characterized by a first production phase, which is associated to the cells exponential growth. Afterwards, when the cells enter into the stationary phase, a shift to net lactate consumption can occur under optimal culture conditions. However, such a metabolic shift is not easily controlled, since the mechanisms modulating lactate production in cell culture are still under investigation.

This work aims to understand which factors could influence the lactate metabolic shift in CHO cell culture, focusing, in particular, on the mitochondrial role. To this purpose, cell lines with opposite lactate profiles were compared. The initial lactate production phase was common to all the fast growing cultures and was concomitant to a rapid glutamine consumption. After glutamine depletion, two different scenario occurred. In one case, the cells started to consume lactate until complete depletion. Alternatively, lactate continued to be accumulated, causing a decrease of media pH. The mitochondrial oxidative capacity was hypothesized as a possible cause of the different lactate profile. Indeed, mitochondrial membrane potential and oxygen consumption measurements highlighted a correlation between a reduced oxidative metabolism and a state of high lactate production. This correlation was confirmed by evaluating other cell lines or media compositions which resulted in the same divergence of lactate metabolism.

Afterwards, the expression of selected genes was analysed in correlation with the observed lactate profile. Among 22 genes, two were identified as significantly downregulated (absolute fold change $\geq 2$) in conditions of high lactate accumulation; namely, the mitochondrial aspartate-glutamate carrier (aralar1) and the translocase

of the inner mitochondrial membrane 8 (timm8a). Aralar1, in particular, is an important component of the malate-aspartate shuttle (MAS). This system promotes the recycling of the cytosolic NADH pool, which is necessary for maintaining the glycolytic flux. Alternatively, NADH can be oxidised through pyruvate conversion into lactate, catalysed by the lactate dehydrogenase enzyme. Therefore, an inefficient MAS activity can engender an increased lactate accumulation.

Finally, stable cell lines, which overexpressed aralar1 or timm8a, were generated. Clones derived from a lactate-producing cell line showed an improvement of lactate metabolism. In particular, they switched more easily to lactate consumption compared to the untransfected control, while maintaining a similar glucose consumption rate. On the other hand, no impact of the transgene expression was observed in the clones derived from the lactate-consuming cell line, since the switch to lactate consumption was maintained and no other effect on cell growth or glucose metabolism was detected. The data obtained indicate that lactate consumption was most likely promoted by a better NADH oxidation through the malate-aspartate shuttle, which resulted in a more efficient link between glycolysis and the mitochondrial oxidative metabolism.

In conclusion, the results presented in this thesis indicate that the mitochondrial oxidative capacity plays a central role in the control of lactate production. Media composition and intrinsic cell characteristics have both an impact on mitochondrial activity. Moreover, the expression of specific genes can also be influenced by the culture media. In particular, aralar1 and timm8a have been identified as promising targets for the generation of a host cell line with an improved lactate metabolism.

**Keywords:** CHO cells, lactate, metabolic shift, glutaminolysis, mitochondrial membrane potential, oxidative metabolism, oxygen consumption, aralar, timm8a, malate aspartate shuttle, cell engineering

# Contents

# List of Figures

# List of Tables

# Abbreviations

Most cited abbreviations are listed below. Other used abbreviations are not included since they appear once in the text and together with the definition.

| | |
|---|---|
| AOAA | Aminooxyacetic acid |
| CCCP | Carbonyl cyanide *m*-chlorophenyl hydrazone |
| CHO | Chinese hamster ovary |
| $\Delta\Psi_m$ | Mitochondrial membrane potential |
| DMSO | Dimethyl sulfoxide |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GS | Glutamine synthetase |
| HPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| IVC | Integral of viable cell density |
| LDH | Lactate dehydrogenase |
| MAS | Malate-aspartate shuttle |
| PGC1$\alpha$ | Peroxisome proliferator-actived receptor $\gamma$ coactivator 1$\alpha$ |
| TMRE | Tetramethylrhodamine ethyl ester perchlorate |
| UCP2 | Uncoupling protein 2 |
| UCP3 | Uncoupling protein 3 |

# Introduction

# 1

# Mammalian cell culture in industrial process

Mammalian cell culture has become the main system for the industrial production of recombinant therapeutic proteins. Indeed, the complex post-translational modifications required to obtain highly effective biopharmaceuticals promoted the switch from bacteria to mammalian cells. Typically used cell lines are chinese hamster ovary cells (CHO), myeloma cell lines (such as NS0 and Sp2/0), baby hamster kidney (BHK) and human embryo kidney (HEK-293) cells, (Wurm, 2004). However, CHO cells represent the host of choice with more than 70 % of commercialized biopharmaceuticals produced within this cell line (Walsh, 2010). Tissue-type plasminogen activator (tPA) was the first FDA approved therapeutic protein produced using a mammalian cell line, precisely CHO cells, in 1987 by Genentech (Ezzell, 1987). The preference for this cell line is mainly due to its safeness, since it does not propagate human viruses, the relatively easy transfer of genes into its genome, its robust and fast growth in industrial culture systems and its ability to produce human-like glycoforms. Furthermore, CHO cells have also acquired a long history of regulatory approval, which is a major advantage too (Hammond et al., 2011).

CHO cells were originally isolated by Puck *et al.* in 1958 as fibroblast-like cells from the ovary of a Chinese hamster (Puck et al., 1958). From this ancestor other cell lines were generated and adapted to grow in suspension culture. The CHO-K1 cells, as an example, were derived from a subcloning process of the parental CHO cell (Kao and Puck, 1968) and afterwards they were mutagenized to generate the CHO-DXB11 cell line, which presents a deletion of one dihydrofolate reductase (*dhfr*) allele and an inactivating mutation of the other (Urlaub and Chasin, 1980). Subsequently another *dhfr* mutant, named DG44, was obtained from a CHO-pro3$^{-}$ strain (proline dependant) and it lacks both dihydrofolate reductase alleles (Urlaub et al., 1983). CHO-DXB11 and DG44 represent probably the most widely used CHO-derived host cell

lines. They are both auxotrophic for glycine, hypoxanthine and thymidine, which are therefore supplemented into the culture media. This characteristic is largely exploited as a selection method to generate stable clones transfected with both the *dhfr* gene and the gene of interest. Moreover, the availability of *dhfr* inhibitors, such as methotrexate (MTX), improves the stringency of selection and promotes the transgene amplification.

Another selection strategy is based on the expression of the glutamine synthetase (GS) gene, even though CHO cells express endogenously this gene. Transgene integration and amplification are promoted using the inhibitor methionine sulphoximine (MSX). This approach has also the advantage of avoiding glutamine addition to the medium, hence reducing ammonia accumulation (Seth et al., 2006).

Recently, the CHO-K1 genome has been sequenced (Xu et al., 2011), thus opening new opportunities to better characterize this cell line and to improve its performance in industrial cell culture by targeting specific genes. However, karyotype studies have highlighted a considerable genomic heterogeneity between the different CHO cell lines and especially after recombinant cells generation (Derouazi et al., 2006). This behaviour complicates industry's efforts of developing a general and standardized platform for the production of recombinant proteins in CHO cells.

Nevertheless, resources are constantly dedicated to the pursuit of a better understanding of CHO cells physiology in industrial bioprocesses. Indeed, since the first FDA approved drug produced in CHO cells using stirred bioreactors, a strong improvement has been done in upstream process, leading to an increase in protein yields to grams/litre range (Walsh, 2010). Main used approaches include cell line engineering to increase productivity, but also to extend culture longevity; media and feed optimization, to avoid the negative impact of limiting components or waste products accumulation on cell growth; and process control (Jain and Kumar, 2008).

In particular the analysis of CHO cells metabolism has gained a strong interest in the past decades as a tool to improve cell growth and reduce byproducts by tailored media and feed design (Ma et al., 2009). The benefit is an increase in culture productivity and process robustness (Jain and Kumar, 2008; Mulukutla et al., 2010).

# 2

# *In vitro* metabolism

Industrial cell cultures are often optimized to maintain high viability for extended period of time in order to increase product yield. Therefore, serum-free media and complex feeds are designed with the aim of avoiding nutrient limitations and sustain a rapid cell proliferation. However, they can often become too rich in glucose and amino acids, which can promote a deregulated metabolisms with a fast and inefficient consumption of carbon sources. The main drawback is a strong accumulation of lactate and ammonia, which has a detrimental impact on cell growth and productivity (Gòdia and Cairó, 2006; Xing et al., 2008). Indeed, a survey of several manufacturing runs at Genentech Inc. clearly highlighted a correlation between low lactate accumulation and optimal productivity (Mulukutla et al., 2012).

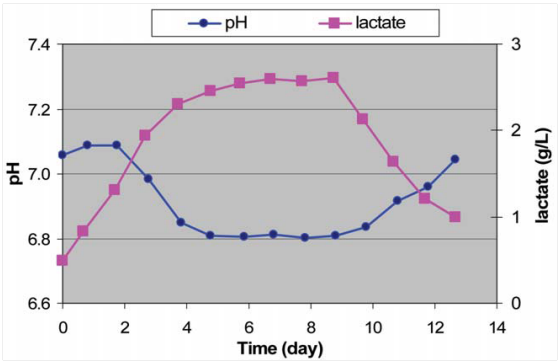


Figure 2.1: An optimal lactate profile is characterized by a shift to consumption late in culture. This improves culture pH as well. From Li et al. (2006)

The major issue of lactate accumulation is the acidification of the medium. In pH controlled bioreactors this problem is normally addressed by base addition, hence inducing an increase in osmolarity that is also negative for cell viability. In optimal conditions, a metabolic switch to lactic acid consumption occurs late in culture (Fig-

ure 2.1).

However, this shift is neither generic nor it can be easily controlled, since the mechanisms underling it are still under investigations. Several approaches have been proposed to tackle this phenomenon and will be presented later (see sections 3.1 and 3.2).

The two main metabolic pathways which can lead to high lactate accumulation are glycolysis and glutaminolysis.

## 2.1 Glucose metabolism

Glucose is a primary source of energy and intermediates for cell culture. It enters into two metabolic pathways: glycolysis (Figure 2.2), which generates intermediates for biosynthesis and energy, and the pentose phosphate cycle, which produces NADPH for cell anabolism and ribose for nucleic acids synthesis.

The end product of glycolysis is pyruvic acid which can be either channelled into the tricarboxylic acid (TCA) cycle, for complete oxidation to $CO_2$, or converted into lactate by the lactate dehydrogenase enzyme (LDH). Three rate limiting enzymes control the glycolytic flux: hexokinase, phosphofructokinase and pyruvate kinase .

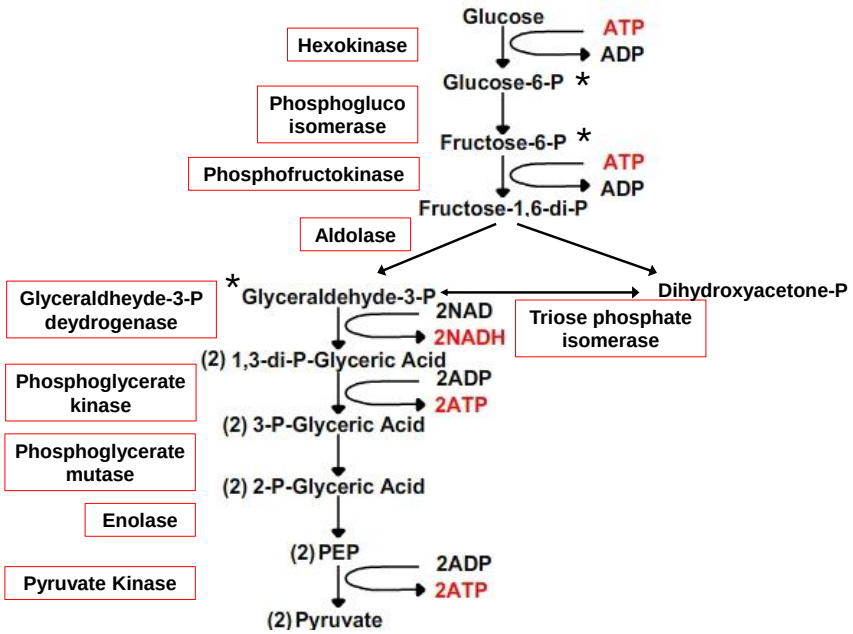


Figure 2.2: Glucose is oxidized in the glycolitc pathway into two molecules of pyruvate. Concomitantly two ATP and two NADH are generated. Involved enzymes are framed. Branch points for the pentose phosphate pathway are indicated by a star.

Fast growing cells rapidly consume high amounts of glucose and convert it mainly to lactate. This phenomenon was first studied in cancer cells and is known as Warburg effect or aerobic glycolysis, since it occurs even in presence of enough oxygen (Warburg, 1956). Different hypothesis have been proposed to explain this behaviour. As illustrated in Figure 2.3, for each molecule of glucose that is converted into pyruvate two molecules of ATP and two of NADH are generated. Previous studies re-

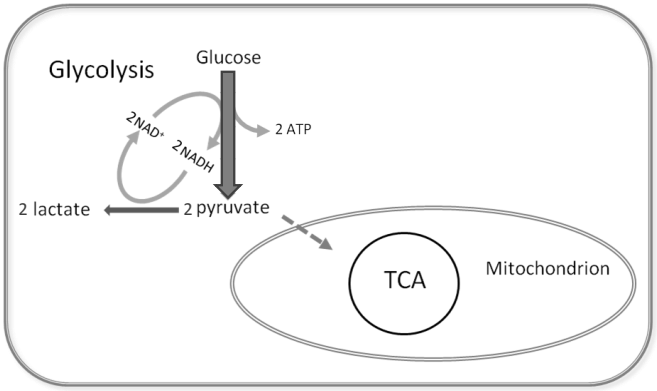


Figure 2.3: Pyruvate conversion to lactate allows NADH recycling but reduces its progression into the TCA cycle.

ported that if the glycolytic flux is sufficiently high, the ATP outcome can rapidly fulfil cell needs without a complete substrate oxidation in the mitochondria (DeBerardinis et al., 2008). Nevertheless, NADH must also be recycled to enable the maintenance of the gycolytic flux. This can often occur through the reduction of pyruvate into lactate. Alternatively, $NAD^+$ can be regenerated in the mitochondria, but this requires the activity of two shuttle systems: the malate/aspartate shuttle or the glycerol-3phosphate shuttle, which could be a bottleneck if the glycolytic rate is too high (Bender et al., 2006; Mulukutla et al., 2010).

Other hypothesis to explain aerobic glycolysis speculate about an extensive glucose uptake that would exceed the oxidative capacity of the TCA cycle (DeBerardinis et al., 2008), thus causing an accumulation of pyruvate and its subsequent conversion into lactate. The deficiency of anaplerotic reactions, such as those catalysed by the pyruvate carboxilase and phosphoenolpyruvate carboxykinase, would result in the same pyruvate accumulation (Godia and Cairo, 2006; Neermann and Wagner, 1996). Overall, the consequence of the restricted pyruvate entry into the TCA cycle would be a reduction of the mitochondrial oxidative activity.

Finally, lactate production has also been proposed as a protection mechanism to

decrease the mitochondrial oxidative metabolism and the consequent reactive oxygen species (ROS) accumulation (Brand, 1997). Indeed, during normal respiration, single electrons can leak to $O_2$ giving rise to the production of reactive oxygen species (Brand, 2010). At high concentration, ROS can induce apoptosis if the detoxification capability of glutathione is exceeded. Therefore, it has been hypothesized that cancer cells engage in high aerobic glycolysis to protect against ROS-mediated apoptosis, while fulfilling their energy need. Indeed, this behaviour is largely studied as a system to therapeutically target cancer cells (Ralph et al., 2010).

In addition, aerobic glycolysis can be induced by the activation of the hypoxia-inducible factor 1$\alpha$ (HIF-1$\alpha$). This transcription factor, normally activated by condition of low oxygen availability, promotes the expression of different genes involved in glycolysis, such as: the glucose transporter GLUT1, the hexokinase, LDH and the pyruvate dehydrogenase kinase (PDK). PDK, in particular, inhibits the pyruvate dehydrogenase enzyme, hence reducing pyruvate progression into the TCA cycle. Moreover, HIF-1$\alpha$ also regulates mitochondrial biogenesis by inhibiting the expression of the peroxisome proliferator-activated receptor gamma coactivator-1$\beta$ (PGC-1$\beta$), (Aragonés et al., 2009; Houtkooper et al., 2012). The activation of this system allows the cells to switch to glycolysis and slow down mitochondrial oxidative metabolism, which is instead oxygen dependent. Interestingly, constitutive HIF-1$\alpha$ expression has been reported in several cancer cell lines and its activity seems to be activated by glucose, pyruvate and lactate (Lu et al., 2002).

In conclusion, lactate production can be the result of a complex regulation of glucose metabolism, which reflects the extraordinary cells adaptability to environmental changes.

## 2.2 TCA cycle and glutamine metabolism

The TCA cycle is a major source of energy for cells since it generates three molecules of NADH, one of $FADH_2$ and one GTP at each cycle (Figure 2.4).

Moreover, it provides necessary intermediates for other metabolic and biosynthetic pathways. For instance, during cell proliferation a part of the carbon that enters the TCA cycle is diverted into lipid synthesis. This pathway requires citrate efflux from the mitochondria and its conversion to oxaloacetic acid (OAA) and the lipogenic precursor acetyl-CoA. OAA is then metabolised to pyruvate in order to produce the NADPH required for anabolic reactions (Figure 2.5). This truncated TCA cycle causes also a reduction of the flux from citrate to $\alpha$-ketoglutarate ($\alpha$-KG).

Glutamine, however, is able to replenish the cycle fulfilling cells energy need. Indeed, glutamine is first deaminated to glutamate, which is then converted to $\alpha$-KG, either by deamination or transaminase reaction. Transaminases catalyse the produc-

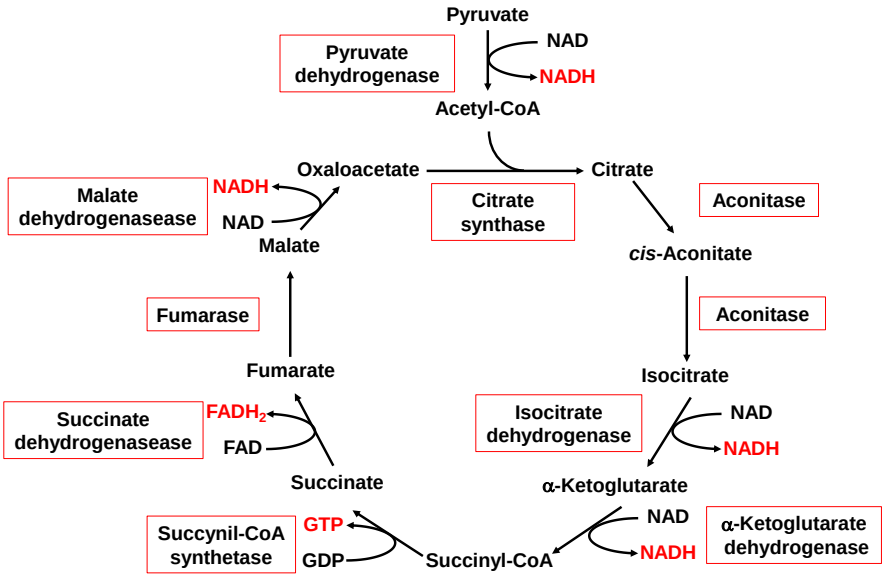


Figure 2.4: Pyruvate enters into the TCA cycle by conversion to Acetyl-CoA. Involved enzymes are framed.

tion of the amino acids alanine or aspartate through glutamate reaction with pyruvate or oxaloacetate, respectively (Godia and Cairo, 2006). Therefore, glutamine is relevant not only for energy production but also for intermediates synthesis.

In rich media, glutamine can also be partially oxidized to malate which is then converted into pyruvate and lactate in the cytosol (Fig. 2.5). This metabolism, known as glutaminolysis, provides the cells with further NADPH for biosysnthesis. Moreover, it avoids malate build up, if its production is not balanced by an adequate acetyl-CoA availability that is necessary for the TCA cycle progression. Interestingly, in tumour cells glutaminolysis represents the major source of NADPH and more than the half of the glutamine-derived carbon is secreted as lactate and alanine (DeBerardinis et al., 2007).

Glutaminolysis and glycolysis are both considered as hallmarks of cancer cells or fast proliferating cells metabolism. Therefore the regulation of these pathways is of great interest, not only for cancer research to specifically target tumour cells (Gogvadze et al., 2008; Jones and Thompson, 2009), but also for industrial cell culture.

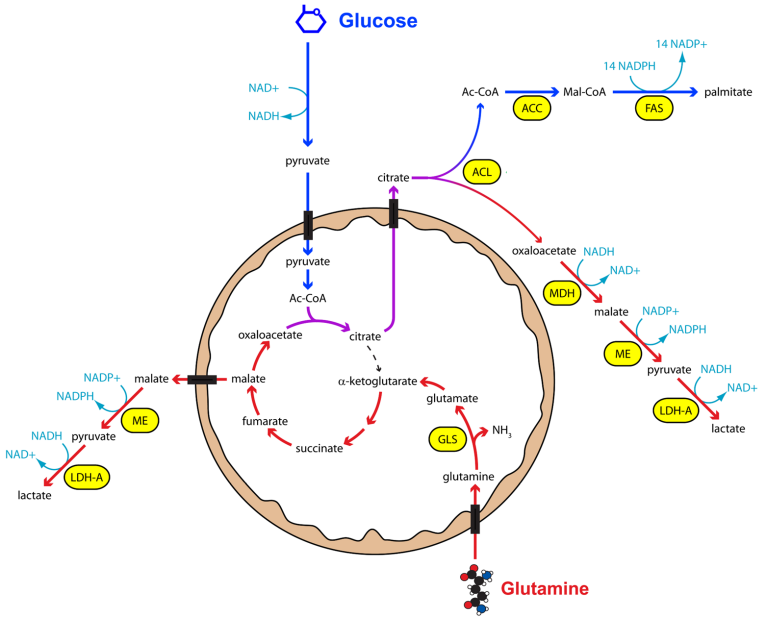


Figure 2.5: The TCA cycle allows lipogenic precursor synthesis through the intermediate citrate. Glutamine can also promote lactate accumulation through the glutaminolytic pathway. ACL, ATP-citrate lyase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ME, malic enzyme; Ac-CoA, acetyl-CoA; Mal-CoA, malonyl-CoA; MDH, malate dehydrogenase; LDH-A, lactate dehydrogenase-A; GLS, glutaminase. Adapted from Deberardinis et al. (2008)

## 2.3 Oxidative phosphorylation

The NADH and $FADH_2$, produced during glycolysis and the TCA cycle, are reoxidised in the mitochondrial respiratory chain (Figure 2.6). Electrons are transferred from these reducing equivalents through four inner-membrane protein complexes to the final acceptor, oxygen, that is then converted into water. Precisely, NADH transfers its electrons to the Complex I/NADH coenzyme Q reductase, while $FADH_2$ to the complex II/Succinate CoQ reductase. Electrons are then transported to the complex III/Cytochrome *c* reductase and Complex IV/cytochrome *c* oxidase. Concurrently, $H^+$ are transferred in the inter-membrane space establishing an electrochemical gradient, known as mitochondrial membrane potential ($\Delta\Psi_m$). Hence, protons are the driving force for ATP production by the $F_0F1$ ATP synthase complex (Fernie et al., 2004). Three molecules of ATP are produced from each oxidized NADH and two from $FADH_2$. Oxidative phosphorylation represents therefore the most efficient metabolism in terms of energy yield.

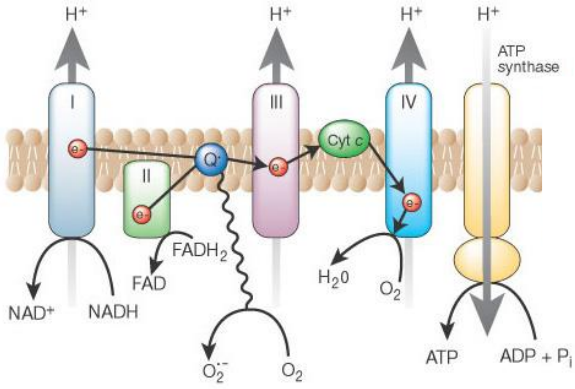


Figure 2.6: The mitochondrial respiratory chain. Adapted from (Brownlee, 2001)

# 3

# Strategies to improve cell metabolism

An optimal culture process should be characterized by a fast growth phase, where most of the energy is used to rapidly reach a high biomass, followed by a sustained stationary phase, where the energy is diverted to the production of the protein of interest. Therefore, the analysis and optimization of cell metabolism becomes fundamental to achieve this goal, while minimizing waste products accumulation and viability drop.

A review of the literature on lactate metabolism in industrial culture has highlighted two main approaches to control its production: media optimization and cell culture engineering.

## 3.1 Media composition can impact lactate profile

Glucose and glutamine, as previously reported, are the major source of energy for a cell but they can also lead to high lactate accumulation. Indeed, Altamirano *et al.* proposed the simultaneous substitution of both components with galactose and glutamate (Altamirano et al., 2006). These alternative carbon sources have the advantage of being more slowly transported into the cell, therefore promoting a more efficient substrate oxidation with less ammonia and lactate accumulation. However, they have the drawback of reducing cell growth. In a previous work, instead, glucose was maintained in the medium, but at low concentration, together with galactose (Altamirano et al., 2000). Cell growth was consequently improved and reached a comparable level to the growth in high glucose alone. More interestingly, lactate metabolism switched from production to consumption, once glucose was depleted. The reported results show the strong dependence of the cells on glycolysis, at least in this culture system. A late switch to alanine consumption was also reported, indicating a state of energy need due to the slow galactose consumption.

Optimization of the feeding strategy can also improve lactate profile (Ma et al.,

2009). Gagnon *et al.* proposed a pH controlled feeding strategy which efficiently reduced lactate accumulation (Gagnon et al., 2011). In this scenario lactate production, as a result of high glycolysis, induces a pH drop that is used to switch off the glucose feeding pump. Consequently, the cells switch to lactate consumption, which promotes pH increase and finally restores the glucose feeding. Cell growth and productivity resulted to be improved. However, the biochemistry changes underlying the switch of lactate metabolism were not clarified.

Metabolic profiling approaches are gaining a strong interest as a strategy to define which metabolites are depleted during a specific cell culture process. The goal is to design tailored feeding regime according to the needs of the cells. With this approach Sellick *et al.* designed a specific pyruvate and amino acids feed for the culture of a CHO-GS cell line, which increased cell growth rate, productivity and promoted the switch to lactate consumption. More interestingly, the switch was maintained even if the feed was complemented with glucose, thus showing that lactate metabolism is not only dependent on the glycolytic rate, but a more balanced media can promote its consumption (Sellick et al., 2011).

In a recent paper published by Genentech, (Luo et al., 2011), a positive impact of higher copper concentration on lactate consumption was reported. Copper is the cofactor of two important enzymes; the cytochrome C oxidase complex, that is part of the electron transport chain, and the superoxide dismutase, which is involved in ROS detoxification. Therefore, a link between mitochondrial oxidative capacity and lactate metabolism was hypothesized. Moreover, in the same study, two different recombinant CHO cell lines were compared which showed an opposite lactate profile, even if maintained in the same culture condition. An impairment of the mitochondrial oxidative metabolism was hypothesized for the lactate producer clone. The work of Luo *et al.* highlights that a mix of medium components and unknown intrinsic cell characteristics can influence lactate profile, thus making more difficult the understanding and optimization of cell metabolism.

Lactate can also be considered as a nutrient in those culture conditions where cells are able to switch to net lactate consumption. Therefore, this substrate may be used as a feed to both supply energy to the cells and control the pH (Li et al., 2012). As reported in the published work, this strategy also avoids alanine consumption, which normally occurs later in culture and has the side effect of increasing NH4+ accumulation. Nevertheless, this approach implies the ability of the cell line to consume lactate. However, this constraint could be bypassed by feeding with pyruvate (Sellick et al., 2011).

## 3.2 Cell engineering to target lactate production

Specific metabolic pathways can be optimized by overexpression or downregulation of key enzymes in order to reduce lactate accumulation. In particular pyruvate entry into mitochondria is considered as a crucial flux constraint.

A direct strategy to reduce pyruvate conversion to lactate is to silence the LDH gene either by homologous recombination (Chen et al., 2001) or by siRNA technology (Kim and Lee, 2007a). While this approach can lead to net reduction of lactate accumulation, it does not entail an increased pyruvate oxidation in the mitochondria. Moreover, reducing LDH activity can have the drawback of decreasing glycolytic flux, as a consequence of the limited NAD regeneration that is normally coupled to the pyruvate-lactate reaction. Indeed, the final lactate per glucose yield can be unchanged, as reported by Kim *et al.*

To overcome this issue Zhou *et al.* reduced the expression of both LDH and pyruvate dehydrogenase kinase (Zhou et al., 2011). The last enzyme suppresses pyruvate dehydrogenase activity and therefore reduces pyruvate progression into the TCA cycle. Transfected cells showed a metabolic switch to lactate consumption and a decreased glucose uptake rate. The measurement of the cellular ATP content showed a slight increase in the transfected cells, indicating a general improvement of the cell energy state.

Another flux constraint is represented by the conversion of pyruvate into oxaloacetate. This anaplerotic reaction is catalysed by pyruvate carboxylase (PC), which was reported to be absent or expressed at very low levels in CHO cells (Neermann and Wagner, 1996). Its overexpression induced a decrease of the lactate production rate and improved cell viability (Irani et al., 1999; Fogolín et al., 2004; Kim and Lee, 2007b). In particular, in the first two cited works, the authors used the yeast PC for the recombinant clone generation. This enzyme is cytosolic and not mitochondrial, as the one of higher eukaryotes. Therefore, the produced oxaloacetate needed to be reduced into malate to enter the mitochondrion. With this elegant strategy, not only pyruvate entry into the TCA cycle was improved, but NADH was also recycled, probably reducing even further the LDH activity. Recombinant clones also showed a lower glucose consumption, probably due to a more efficient use of the carbon sources, and a slight improvement of the lactate/glucose ratio. Nevertheless, the metabolic switch to lactate consumption was not reported.

Malate oxidation to oxalocetic acid, catalysed by the enzyme malate dehydrogenase II (MDH II), also represents a putative bottleneck in the TCA cycle progression. As already reported for glutaminolysis (see section 2.2 and figure 2.5 on page 26), this flux constraint can cause an efflux of malate out of mitochondria and its conversion into lactate. MDHII overexpression in CHO cells, indeed, reduced the specific lactate secretion rate and increased total ATP production (Chong et al., 2010). However, the

impact on the glycolytic flux is unknown, since data about glucose consumption rate were not reported.

Finally, cells engineering to allow growth on slowly metabolised sugars, such as galactose and fructose, has been also proposed (Wlaschin and Hu, 2007; Jiménez et al., 2011).

In conclusion, the presented strategies, while managed to improve lactate profile to a certain extent, are not based on a genomic approach to specifically identify candidates clearly linked to lactate consumption ability. Moreover, cell metabolism is tightly regulated and interconnected, therefore any attempt to modify a specific flux rate can have an impact on another branch. Anaplerotic reactions and redundant enzyme isoforms can jeopardize the cell engineering approaches used, as an example, to downregulate undesired pathways. Therefore, the development of a cell host with an optimal metabolic profile can be a challenging task that requires further investigation with a more system oriented view.

## 3.3 New insights in lactate metabolism

Lactate has for long been considered only as a waste product. However, lactate can also exert a metabolic control over glycolysis, through the inhibition of the phosphofructokinase (PFK) enzyme (Leite et al., 2011). PFK catalyse a rate limiting step of the glycolytic pathway. In a recent paper, published by Mulukutla *et al.*, the metabolic switch of NS0 cells was modelled by integrating metabolic flux data with transcriptome analysis (Mulukutla et al., 2012). In most conditions, lactate net consumption occurs when its level in the medium is high and the glycolytic rate is low. However in some cases, lactate can be consumed even when glucose is still present at relatively high concentrations. Interestingly, this phenomenon was modelled by adding the inhibitory effect of lactate on the PFK enzyme, which would result in a slowing down of the glycolytic flux meanwhile lactate consumption occurs. Nevertheless, the consumption phase can be sustained to a residual lactate concentration that is beyond the inhibitory level. In this phase, the transcriptome analysis identified a reduced activity of the AKT signalling.

AKT is a well known stimulator of glycolysis, therefore the downregulation of its signalling cascade can explain the low glycolytic flux which takes place when lactate inhibition over PFK is no more occurring. In this way, AKT inhibition simulates a condition of low glucose which prompts the cell to consume lactate as alternative source of energy. This study clearly emphasizes the complexity of cell metabolism, the interconnection with unexplored signalling pathways and it opens new opportunities for cell engineering to improve lactate metabolism (Mulukutla et al., 2012).

Finally, recent research has focused on the capability of mitochondria to metabo-

lize lactate. Passarella *et al.* has reported the existence of a mitochondrial LDH in the inner mitochondrial compartment of different mammalian tissues (Passarella et al., 2008). Lactate entry into the mitochondria occurs through three different transporters: a lactate/$H^+$ symporter and a lactate/pyruvate and lactate/oxaloacetate antiporters. Even if the existence of a mitochondrial LDH in CHO cells has not been proven yet, these new findings provide new hints in the understanding, and modelling, of lactate metabolism.

# 4

# Research objectives

The aim of this thesis project is to study lactate metabolism in CHO cells during batch culture in chemically defined media. The main model consists of two non recombinant CHO cell lines, which differ in their lactate profile under the same culture conditions. The objective is to identify the main causes of lactate over-production and provide some tools to control it. The approaches to tackle this phenomenon are divided in four parts:

- The first question to address is the identification of the prominent sources of lactate production. As described before, lactate can mainly arise from glycolysis or from glutamine partial oxidation in the TCA cycle, or from both of them. Therefore the impact of these carbon sources on lactate metabolism in both model cells lines is evaluated.

- Mitochondria play a key role in lactate metabolism and they can be considered as the hub of different pathways which can lead to lactate accumulation. Namely, the pyruvate progression into the TCA cycle, the flux constraints at the level of malate oxidation to oxaloacetate and the NADH shuttling from the cytosol. A general overview of the mitochondrial oxidative capacity can be obtained by measuring the mitochondrial membrane potential and oxygen consumption. This type of analysis, if applied on cell lines with different lactate profiles, can highlight possible mitochondrial impairment and thus focus our attention on this organelle.

- Cell engineering can be an interesting tool to improve cell metabolism. The availability of cell lines or culture conditions with different lactate profiles represents an attractive model to evaluate the expression level of different enzymes and transporters. The aim is to identify specific genes which expression correlates with different lactate profiles. Then the effect of their overexpression on cell metabolism can be evaluated.

- Most of the attempt to improve cell metabolism are specific to the tested cell line or to the culture condition. Therefore, in this project, different media and recombinant cells are tested in order to develop a more general approach and to validate the hypothesis generated with the main culture model.

# Material & Methods

# 5

# Material & Methods

## 5.1 Reagents

Unless differently stated, all chemicals were from Sigma-Aldrich GmbH (Buchs, Switzerland) and suitable for cell culture.

## 5.2 Cell lines

Suspension adapted CHO-S cells, referred in the text as control cell line, were from Life Technologies (Zug Switzerland). The subclone was derived from the parental CHO-S cells by limiting dilution in medium 1. This subcloning test was performed by a different operator and was independent from this thesis work. Recombinant clones G4, C6 and A1 were CHO-GS derived cell lines which expressed the same therapeutic protein. Recombinant clones c#T1, c#T2 and c#T6 were generated from the CHO-S cell line by stable transfection with a timm8a carrying vector. Clones c#A4 and c#A6 were derived from the same parental cell line and overexpressed the aralar1 gene. Stable clones, transfected with either timm8a or aralar1, were also generated from the subclone and named sc#T2, sc#T5, sc#T6 and sc#A1, sc#A4, sc#A5, respectively.

### 5.2.1 Cell counting and IVC

Cell numbers and viability were measured using a Vi-Cell analyzer (Beckman Coulter International S.A., Nyon, Switzerland). This equipment uses the Trypan blue dye exclusion method.

The integral of viable cell density (IVC) was calculated using the following formula:

$$IVC_t = IVC_{t-1} + \left[ VC_{t-1} + \frac{VC_t - VC_{t-1}}{2} \right] \times (t - t_1)$$

Where t is time in days and VC is the viable cell density in $10^6$ cells/mL. Cells were passaged every 2-3 days at a density of $0.2 \times 10^6$ or $0.1 \times 10^6$ cells/mL, respectively.

Unless otherwise mentioned, cells were seeded by centrifugation at a density of $0.05 \times 10^6$ cells/mL for the growth curves. The assays were monitored for 12-14 days, until the viability drop significantly.

## 5.3 Culture conditions and media

Cells were cultivated in 50 mL TubeSpin® (TPP AG, Trasadingen, Switzerland) in orbitally shaken incubator at 37°C, 5% CO2 and 80 % humidity (Adolf Kuhner AG, Birsfelden, Switzerland). All media were chemically defined. Two proprietary media (medium 1 and 2) or alternatively the commercially available CD CHO medium ( Life technologies, Zug, Switzerland) were used. Initial glutamine concentration was set at 4.5 mM in all tested media. After glutamine supplementation, media were sterile filtered and stored at 4°C in the dark for a maximum of three months. Unless differently stated, all the growth assays were performed as batch in 20 mL of medium.

### 5.3.1 Culture conditions for specific tests

For the feeding test, glutamine was diluted in water at a concentration of 200 mM and then supplemented to the cells on day 5 of growth. Two final feed levels were tested: 4 and 6mM.

The factorial design was performed using a from scratch preparation of medium 2 deficient in copper, biotin and alanine. Concentrated stock solution of alanine (600 mM) and copper (1mM) were prepared in water and supplemented, when necessary, to the cells on day 0. Biotin solution (1mM) was instead prepared in 0.01 M NaOH. Data analysis was performed using the Design Expert Software (Version 8.0.1, Stat-Ease Inc, Minneapolis, USA).

The inhibition test with aminooxyacetic acid required first a dose-response curve to identify the best concentration. Control cells were seeded in medium 1 at a density of $0,2 \times 10^6$ cells/mL. The concentration of 0,25 mM was finally selected. This represented the lowest concentration which still impacted lactate metabolism without impairing cell growth. AOAA was added directly to the culture medium on day 0. pH was adjusted by equimolar addition of NaOH.

The fed-batch culture for the gene expression analysis was performed by growing the recombinant cell line A1 in CD CHO medium in spin tubes. The culture volume was set at 30 mL. Two different feeds (feed1 and feed2) were added from day 3 to day 12 or on day 4,7 and 10, respectively. The culture was executed by a different operator.

## 5.4 Metabolites measurement

Glucose, glutamine, lactate, pH and NH4+ in cell culture supernatant were measured using a NOVA Biomedical analyzer (Nova Biomedical GmbH, Rödermark, Germany).

Amino acids concentration was measured in cell culture supernatant on different days of growth. The test was performed by the Analytical group at Merck-Serono. Briefly, samples were harvested on day 5, 7, 10 and 12 of cell culture and centrifuged for 5 minutes at 300 g. The supernatant was then clarified with a 0.45 $\mu$m filter. The amino acids analysis was performed using a commercially available kit, AccQ-Tag Ultra Derivation kit (Waters AG, Montreux-Chailly, Switzerland) with a UPLC column, according to manufacturer's guidelines.

## 5.5 Mitochondrial metabolism

### 5.5.1 Oxygen consumption measurement

Oxygen consumption was measured using the PreSens Oxoplate (Precision Sensing GmbH, Regensburg, Germany). These 96 well plates have a round bottom equipped with an oxygen sensor. Two dyes are included in the sensor: an indicator, which fluorescence is oxygen dependent, and a reference, which is $O_2$ independent and used to normalize the well to well discrepancy. Cells, on different days of culture, were resuspended at a concentration of $10^6$ cells /mL in spent medium. 200 $\mu$l were used for the test. The assay was carried out using a Spectramax Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) and fluorescence emission was monitored for 45', with a reading every minute, at 37°C ( excitation 540 nm; and emission 650 and 590 nm for indicator and reference signal, respectively).

Oxygen $pO_2$ values as % of air saturation were calculated after calibration using the following formula:

$$pO_2 = 100 \times \frac{\frac{K_0}{I_r} - 1}{\frac{K_0}{K_{100}} - 1}$$

Where $I_r$ is the ratio between the indicator and reference fluorescence intensity for each sample. $K_0$ is the $I_r$ value obtained with the 0% oxygen calibrator, which was prepared by diluting 1g of $Na_2SO_3$ in 100 mL of water. $K_{100}$ is the $I_r$ value given by the 100% oxygen calibrator, which was made by agitating 50 mL of water for one minute. $pO_2$ values were plotted over time to calculate the oxygen consumption rate from the slope. Finally, for each day of testing, values were normalized and expressed as percentage of the lactate consuming condition, which was set at 100 % .

Two controls were also used. Oligomycin, an inhibitor of the $F_0$ /F1 ATP syn-

thase, was added at a final concentration of 1 $\mu$M to evaluate the residual oxygen consumption due to proton leaking. Antimycin A, an inhibitor of the Complex III of the respiratory chain, was used at a final concentration of 10 $\mu$g/mL to evaluate residual oxidative reactions not due to mitochondrial respiration. Spent media were also tested to check for possible interference of media components with the fluorescence detection.

### 5.5.2 Mitochondrial membrane potential

Mitochondrial membrane potential was measured in $10^5$ intact cells with 100 nM TMRE (Biotium Inc., Hayward, CA, USA). TMRE stock solution was prepared by dissolving the powder in DMSO at a concentration of 1mM and was stored at -20°C. The protonophore CCCP was dissolved at a concentration of 1 mg/mL in DMSO and stored at 4°C. Working solutions of TMRE and CCCP were prepared freshly in PBS. Cells were harvested on different days of culture, centrifuged for 5 minutes at 300g and resuspended in spent medium for the staining. The incubation was performed in 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany), covered with a gas-permeable seal (Diversified Biotech, Dedham, MA, USA) for 20 minutes at 37°C. The cells were washed in PBS prior to flow cytometry analysis using a Guava PCA 96 equipment (Millipore, Molsheim, France).

TMRE is a lipophilic, cationic fluorescent dye that accumulates within mitochondria proportionally to the membrane potential. At the used concentration TMRE works in a quenched manner, therefore, CCCP addition (final concentration 10 $\mu$M) induces an increase of fluorescence as a result of dye redistribution in the cytosol (Chalmers and McCarron, 2008; Scaduto and Grotyohann, 1999). The ratio of fluorescence increase, under the same incubation conditions, was used as an indication of the mitochondrial membrane potential. For each test, normalized values were calculated as percentage of the lactate consuming condition, which was set at 100 %
.

## 5.6 Gene expression assay

### 5.6.1 RNA extraction and cDNA preparation

RNA was extracted from $5 \times 10^6$ cells harvested on different days of culture. Cells were centrifuged for 5 minutes at 300 g and the total RNA isolation was performed using the RNeasy Mini kit (Qiagen AG, Basel, Switzerland) according to manufacturer's protocol. RNA concentration and quality was estimated spectrophotometrically with a Spectramax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) by

measuring the absorbance at 260 and 280 nm. Samples were stored at -20°C until use. Retrotranscription was performed with the High Capacity RNA-to-cDNA kit (Life Technologies, Zug, Switzerland) using 1 $\mu$g of RNA for each sample. cDNA was stored at -20°C until use.

### 5.6.2 Real-time PCR assay

The gene expression array was performed with a custom TaqMan® Array 96 well Plate, including 3 endogenous control genes (18S, GAPDH and HPRT) and run on a 7500 Fast Real time PCR (Life Technologies, Zug, Switzerland). GAPDH was finally selected as endogenous reference gene. 50 or 100 ng of cDNA were used for each test and were diluted in the TaqMan® gene expression Master Mix, following manufacturer's instructions. The array was charged with murine primers and probes, since CHO specific sequence were not available at the time of the test. However, a high homology between the Chinese hamster and mouse genome has been reported (Yee et al., 2008b). For the individual amplification of aralar1 and timm8a the same primers and probes of the array were used. Primers efficiency was evaluated for these genes and for the reference, GAPDH, by serial 1:10 dilution of the cDNA. The obtained Ct values were plotted versus the logarithm of cDNA concentration and the slope was used to calculate the amplification efficiency, using the following formula:

$$E = 10^{\frac{-1}{slope}} - 1$$

Where E is the efficiency. The required 100±10 % efficiency was obtained for all primers.

Real-time PCR data were analysed applying the comparative ΔΔCt method according to manufacturer's guidelines. More precisely, the lactate consuming conditions was always used as reference sample and expression differences were reported as fold change. The Data Assist software (Life Technologies, Zug, Switzerland) was also used as support to confirm the data analysis.

## 5.7 Recombinant clone generation

Control and subclone cells were transfected with a pCMV6-AC-GFP vector containing either the mouse aralar1 or timm8a ORF sequence cloned upstream the GFP-tag (ORIGENE Technologies, Rockville, USA). The vector carried also the neomycin resistance gene. The transfection was performed in suspension culture in medium 1. The X-treme GENE HP DNA Transfection Reagent (Roche AG, Rotkreuz, Switzerland) was used as liposome based gene transfer method. A reagent:DNA ratio of 2:1 was selected and 2 $\mu$g of plasmid DNA was used for each transfection.

Cells were counted and resuspended in fresh medium 1 at a concentration of $5 \times 10^6$ cells/mL. The reagent-DNA mix, after 10 minutes of pre-incubation, was added to 600 $\mu$L of cells. After two hours of standard incubation, cells were diluted by adding 6 mL of fresh medium. Two days post transfection, cells were resuspended in medium 1 supplemented with 800 $\mu$g/mL of G418 for stable transfectants selection. The antibiotic concentration was decided by performing a kill curve with untransfected cells. The chosen level was the one that gave complete cell death in around 10 days.

After three weeks of selection, the four stable pools were used for single clones isolation by limiting dilution method. Briefly, cells were diluted in medium 1 containing 0.3% of phenol red, which allowed to monitor the cell growth, and dispensed in 384 wells plates using an automatic sampler. After two weeks, selected colonies were transferred in a stepwise manner to 96, 24 and 6 well plates with almost one week of monitoring for each step. GFP fluorescence was measured during the entire selection process using a Guava PCA 96 flow cytometer (Millipore, Molsheim, France) in order to identify the most stable clones. Finally, colonies were transferred from the 6 wells plate to spin tubes and cultivated according to the standard protocol. The stable clones which adapted to the suspension culture were used for the growth assay.

The flow cytometry graphs were generated using the Flow JO software (Tree Star Inc, Ashland, OR, USA).

## 5.8 Fluorescent microscopy

The colocalization assay was performed by staining the cells with a specific mitochondrial dye, Mitotracker Red CM-$H_2$XRos (Life Technologies, Zug, Switzerland). This dye does not fluoresce until it enters a living cells, where it is then oxidised in the fluorescent probe and sequestered into the mitochondria. A stock solution was prepared by diluting the dye in DMSO at a concentration of 1mM and stored at -20°C. The working solution was then prepared fresh in medium 1. Aralar1 and timm8a proteins were visualized thanks to the GFP tag. Briefly, cells were centrifuged for 5 minutes at 300 g and resuspended in fresh medium at a concentration of $10^6$ cells/mL. The staining was performed with 200 nM of Mitotracker for 15 minutes at 37°C. Afterwards, cells were washed in PBS and drops were spread on a Superfrost plus slide (Fisher Scientific AG, Wohlen, Switzerland). Images were acquired with a motorized Axiovert 200 M fluorescent microscope (Carl Zeiss AG, Feldebach, Switzerland) equipped with a 63x objective. A HBO 100 lamp was used as a fluorescence excitation source. A Zeiss AxioCa HRm camera was mounted on the microscope, and image acquisition was controlled by the Axiovision 4.1 software (Carl Zeiss AG, Feldebach, Switzerland).

# Results & discussion

# 6

# CHO cell metabolism analysis

## 6.1 Introduction to the culture model

The analysis of cell metabolism can be a challenging task since both environmental and cell characteristics can interfere with the investigated pathway. An optimal condition to study lactate metabolism would be to compare two cell lines, which are apparently identical, but show opposite lactate profile when grown in the same culture condition. This approach has the advantage of reducing external contributions, such as changes of media composition, temperature shift or transgene integration, which can also be used to induce a switch to lactate consumption, but at the same time can perturb different metabolic pathways and introduce further variables.

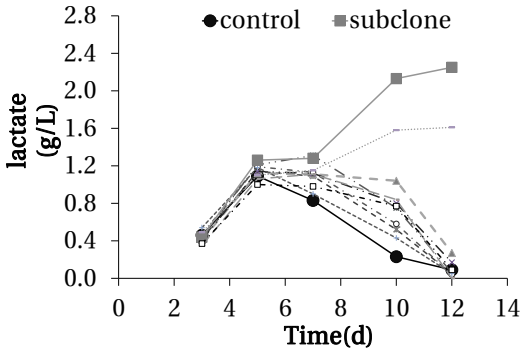


Figure 6.1: Lactate profile of the parental cell line (black circles) and the isolated subclones (dashed lines). The one with the highest lactate production was selected for the project (grey squares).

Hence, in this study two non recombinant CHO cell lines were used, which differ

in their lactate metabolism even if cultivated in the same chemically defined medium (referred next as medium 1). More precisely, one cell line is the commercially available CHO-S cells (referred next as control cell line). The second is a subpopulation of the control (referred next as subclone) obtained during an independent subcloning experiment.

The subcloning was performed by a limited dilution strategy in medium 1 with the aim of isolating a new clone which grew more and with less clumps. Among the ten isolated subclones, eight retained the ability to switch to net lactate consumption, as the control cell line, while two showed a continuous lactate accumulation. Within the last group, the subclone with the highest lactate production was selected for the study (Fig. 6.1, grey squares). This characteristic was retained after freezing and thaw and throughout the entire study.

## 6.2 Growth and metabolic profile

The two cell lines were characterized for their growth and metabolism in medium 1. As reported in figure 6.2 A and B, both the control cells and the subclone showed a similar exponential growth and viability, with a the peak occurring on day 9. The decline phase, however, was less abrupt in the subclone. Glutamine, glucose and glutamate (Fig. 6.2 C, D and G respectively) consumption were identical for both cell lines, whereas lactate profile (Fig. 6.2 E) clearly differed. During the first 5 days of growth, both cell lines produced lactate to the same extent. Afterwards, only the control cells switched to net consumption. On the contrary, the subclone, after a steady phase, continued to accumulate lactate until day 9. A pH drop to 6,8 was also observed during this phase (Fig. 6.2 F). Eventually, even the subclone switched to a partial consumption at the end of the culture.

Interestingly, the metabolic switch in the control cells occured at the time point when glutamine was almost depleted, and glucose and lactate were consumed simultaneously. On the contrary, the subclone preferred to engage in glycolysis and used lactate only when also the residual glucose was at low concentration in the medium. Probably, the higher subclone viability at the end of the culture was supported by this late lactate consumption. A different preference in the use of the available carbon sources was, therefore, assumed for the two cell lines.

The amino acids profile also showed a similar consumption between the two cell lines, with the exception of alanine and serine (Fig. 6.3). Alanine metabolism was characterized by an initial production phase, probably during period of high nutrients availability, and then switched to consumption on day 10, when residual substrates were at lower concentration. Interestingly, while the switch occurred at the same moment for both cell lines, a stronger consumption was observed in the sub-

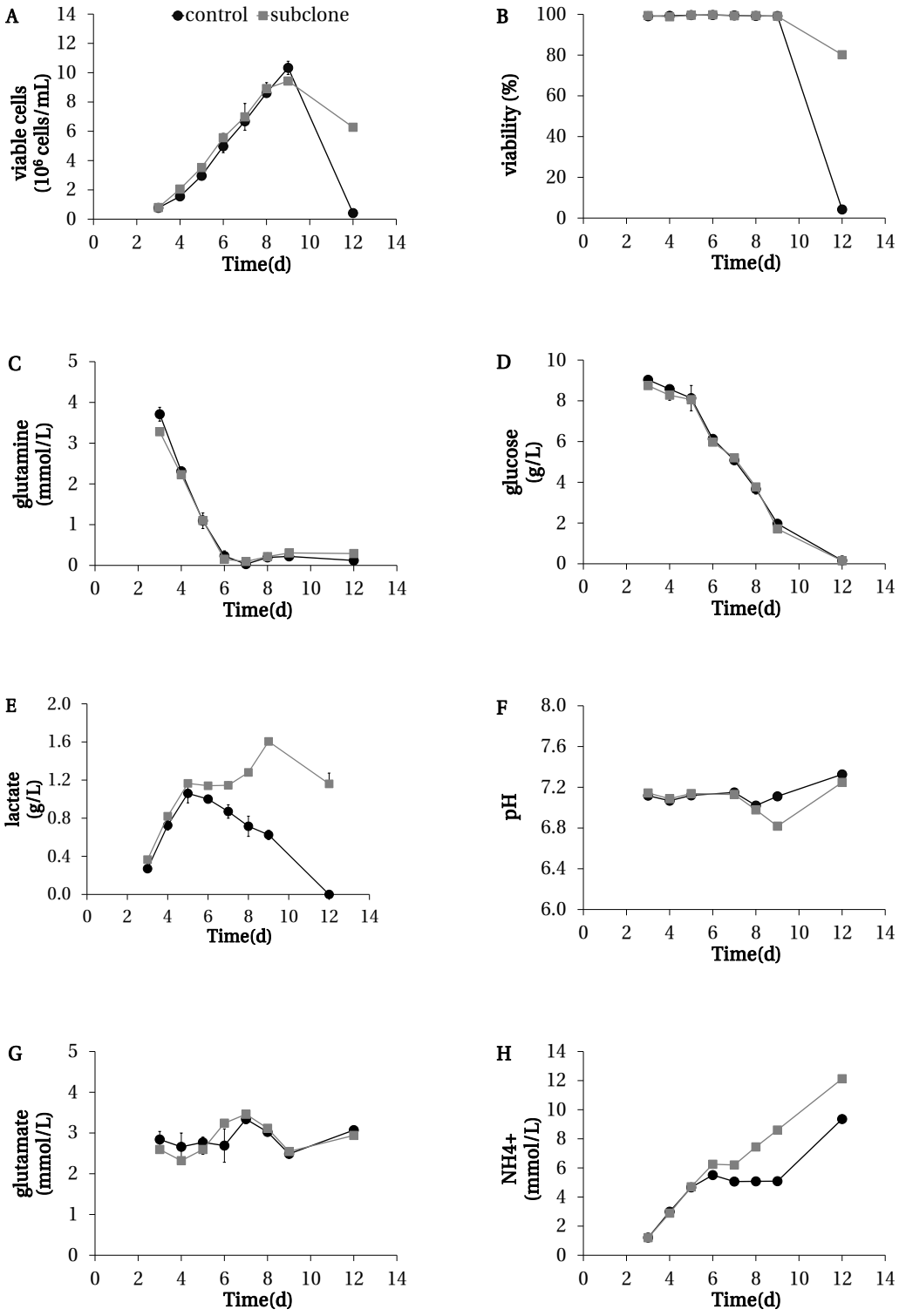


Figure 6.2: Control cell line (circles) and subclone (squares) growth and metabolic profiles in medium 1. Representative graph of several experiments. Mean ± standard deviation from duplicate cultures.

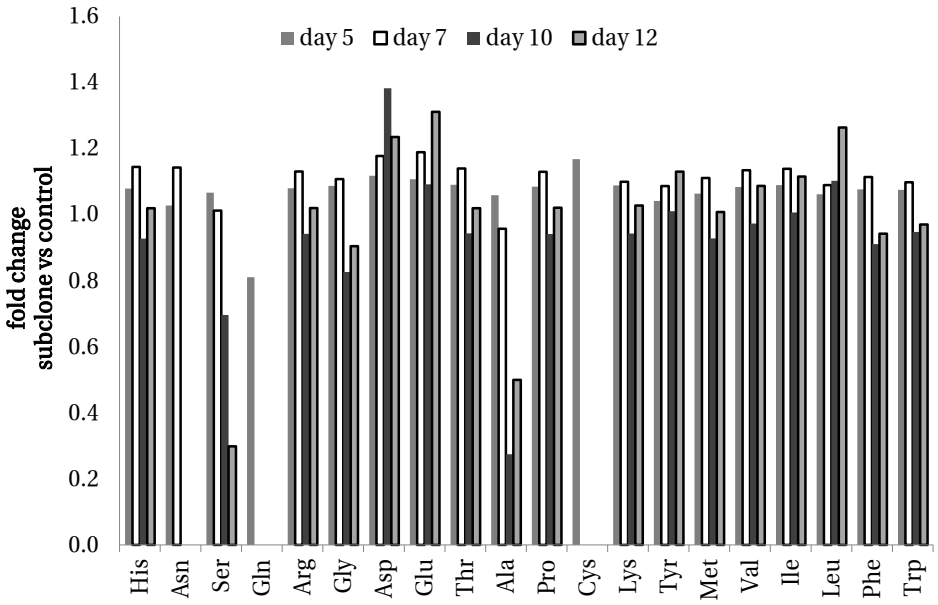


Figure 6.3: Residual amino acids concentrations are reported for the subclone as normalized values against the control cell line on day 5, 7 , 10 and 12 of cell growth in medium 1. Mean from duplicate cultures, standard deviations are not reported for better clarity.

clone. Alanine can be converted to pyruvate by the alanine aminotransferase enzyme and, hence, replenish the TCA cycle instead of lactate. Serine consumption was also more prominent in the subclone after day 7. Serine is also a glucogenic amino acids and is converted to either pyruvate or 3-P-glyceric acid. This higher amino acids consumption could also account for the increased ammonia accumulation observed in the subclone (Fig. 6.2 H). Cysteine, glutamine and asparagine were undetectable after day 5 or 7 in both cell lines supernatant.

## 6.3 Glutamine influences the metabolic shift in control cells

Glutamine depletion was reported to be concurrent with the switch to lactate consumption in the control cell line, as shown in figure 6.2 C and E.

To better investigate its role on lactate metabolism, both cell lines were fed with 4 mM or 6 mM of glutamine on day 5, when its residual concentration was low and the switch normally occured (Fig. 6.4 B, control cell line and F, subclone).

Interestingly, the feeding induced an almost stoichiometric increase of lactate levels and a delay of its consumption in the control cell line (Fig. 6.4 D). Precisely, the measured increase was of 0,32 g/L and 0,5 g/L for the 4mM and 6mM feed, respectively, which fits the 1:1 conversion due to the glutaminolytic pathway. This behaviour clearly indicated glutamine as an important factor in modulating lactate metabolism in this cell line.

A higher lactate production was also observed in the subclone (Fig. 6.4 H), although consumption never occurred, probably due to glycolysis contribution. Glucose consumption rates and cell growth were not significantly impacted by the glutamine feeds in both cell lines (Fig. 6.4 C and G, A and E, respectively).

## 6.4 Glucose impacts lactate metabolism only in the subclone

Glucose can be the main source of lactate production in a cell as a result of glycolysis. In section 6.2, a correlation between glucose consumption and higher lactate accumulation was observed only in the subclone.

In order to better explore this behaviour, both cell lines were cultivated in media with increasing glucose concentrations; precisely with 8, 10 and 12 g/L (Fig. 6.5 B, control cells and F, subclone).

The control cell line was not impacted by this higher glucose availability, since the metabolic switch occurred always on day 5 (Fig. 6.5 C). However, a steady phase was observed towards the end of the culture in the case of the richest media. It is likely that this phenomenon was linked to the extended viability and the depletion of other limiting metabolites.

As expected, the increasing glucose concentrations delayed the lactate metabolic switch in the subclone, precisely from day 9 in the medium containing 8 g/L of glucose, to day 12 in the 10 g/l medium (Fig. 6.5 F and G). In the richest medium a slight consumption was observed on day 14, but due to the strong cell death it was not possible to better characterize it. Interestingly, lactate consumption started always when glucose was almost depleted.

Glucose consumption (Fig. 6.5 B and F) and growth profiles (Fig. 6.5 A and E) were similar in all tested conditions, with the richer media showing a prolonged viability until day 14 in the control cell line. Glutamine profile was not impacted as well (Fig. 6.5 D and H).

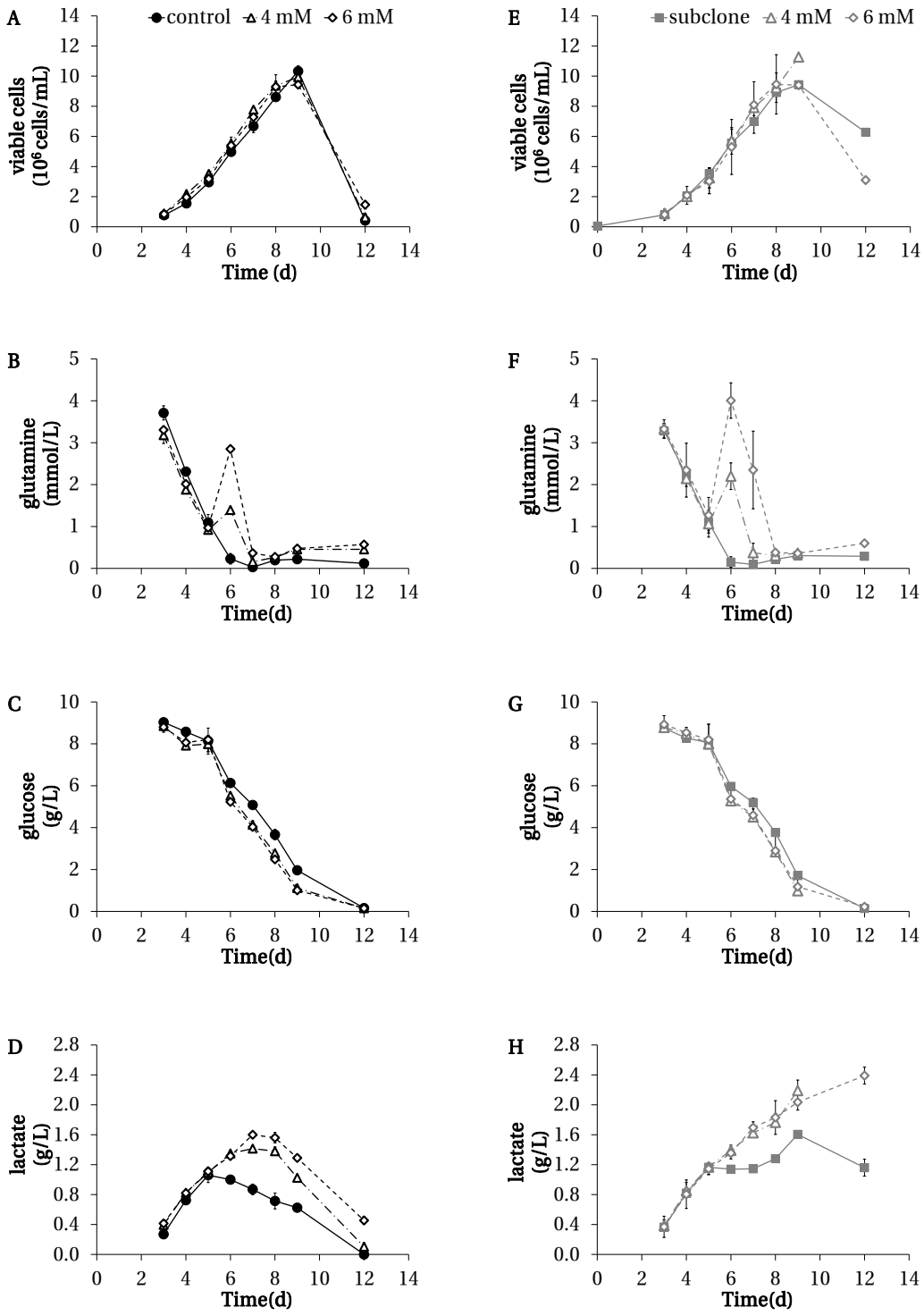


Figure 6.4: Metabolic profile of the control cell line (A to D) and the subclone (E to H) in medium 1 after glutamine feeding. Two feeds were added on day 5: 4 mM (triangles) or 6 mM (diamonds). Control condition is indicated by filled symbols. Mean ± standard deviation from two independent tests.

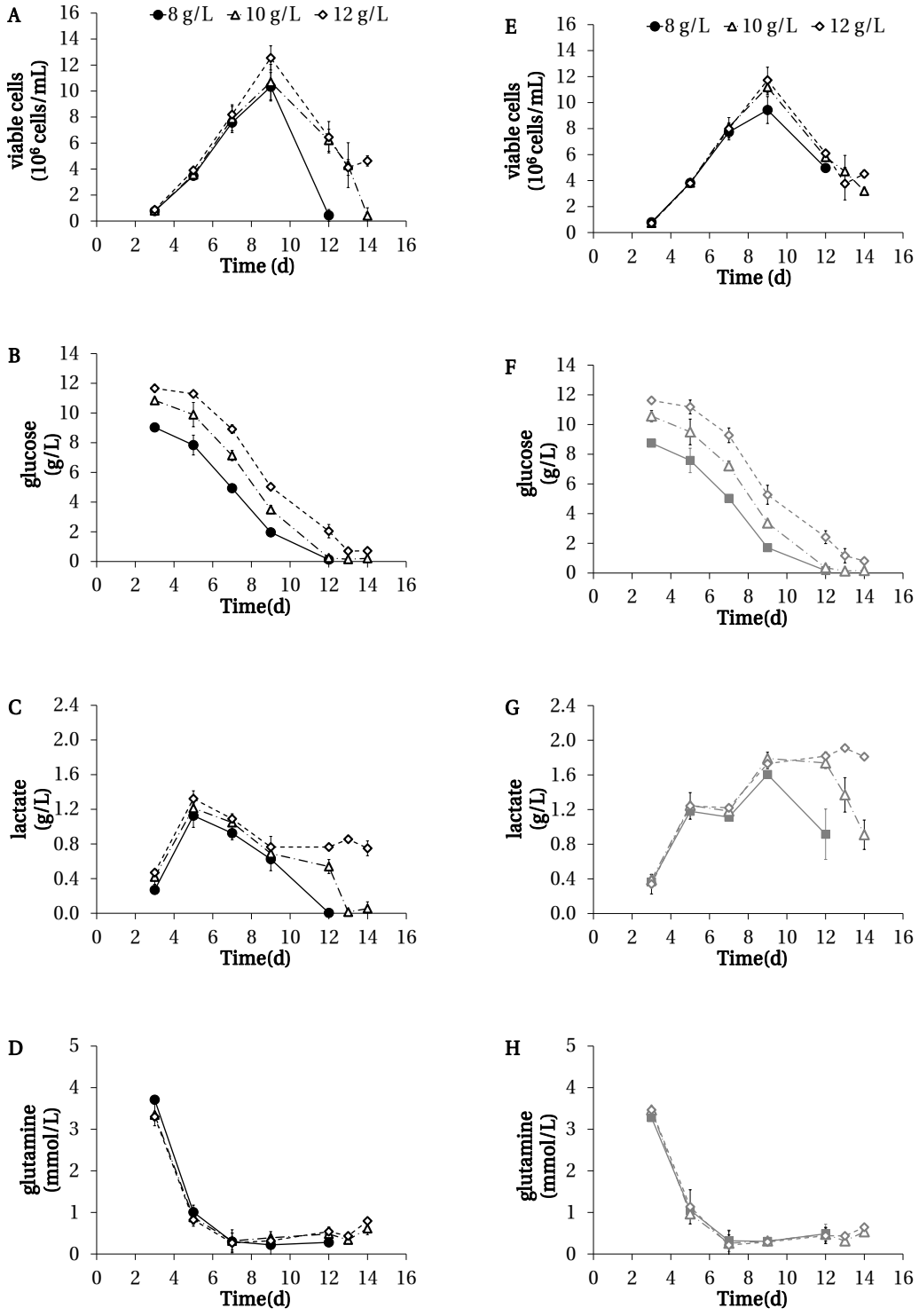


Figure 6.5: Metabolic profile of the control cell line (A to D) and the subclone (E to H) in medium 1 with different initial glucose levels. Three concentrations were tested: 8 (standard concentration, filled symbol), 10 (triangles) and 12 g/L (diamonds). Mean ± standard deviation from two independent experiments performed with duplicates.

These results confirmed the hypothesis that the subclone preferred to use glutamine and glucose as main energy sources and only after their depletion it switched to lactate. Probably, some unknown limiting factors either inhibited lactate consumption or slowed it down to an extent that did not counterbalance its production from glycolysis, thus resulting in a higher accumulation.

## 6.5 Discussion

Reported results indicate that the two cell lines have a different preference for the available energy sources. In particular:

- During the first 5 days of culture, both the control and the subclone behave similarly and accumulate lactate. The production phase persists as long as glutamine is present in the medium. Glutaminolysis was already described as a relevant pathway for lactate production in cancer cells. This metabolism requires the efflux of malate out of the mitochondria and its conversion to pyruvate, and finally into lactate, with production of NADPH (DeBerardinis et al., 2007). Most likely, our fast growing cells undergo the same metabolic pathway in order to fulfill their NADPH need for anabolic processes, which are particularly active during the exponential growth phase. Other amino acids can also contribute to the malate build up and indeed the amount of lactate accumulated on day 5 is not stoichiometric with the glutamine consumption alone. Glucose consumption is quite limited till day 5, however its contribution to lactate accumulation can not be excluded (Fig. 6.2).

- After glutamine depletion, the two cell lines show an opposite behaviour. The control cells switch to net lactate consumption, which is likely used to replenish the TCA cycle. Glucose is also simultaneously consumed. Interestingly, the metabolic switch occurs when the cells are still in the exponential growth phase and is concomitant with glutamine depletion. Therefore, glutamine seems to strongly modulate lactate metabolism, as it was also demonstrated with the feeding experiment (Fig. 6.4). This finding differs from literature examples, where lactate consumption is often associated to cell entry into the stationary phase and to a reduction of glucose consumption (Li et al., 2012; Ma et al., 2009; Mulukutla et al., 2012).

- Glucose promotes higher lactate accumulation only in the subclone. In this cell line the metabolic shift to consumption occurs only when both glucose and glutamine are depleted and the cells stop to grow. This behaviour is more in agreement with the previously reported literature examples. This greater

lactate production most probably reduces the amount of pyruvate which proceeds into the TCA cycle, with a consequent impact on the mitochondrial oxidative metabolism.

Different causes can be postulated: a saturation of the TCA cycle or carriers impairment which reduces the intermediates entry into the mitochondria. A higher expression of the LDH-A subunit, which has been associated to lactate production instead of oxidation (Kim and Lee, 2007a), could be hypothesized as well. Overall, all these conditions would most likely induce a reduced mitochondrial activity in the subclone.

Other substrates could be used by the subclone to overcome the lower pyruvate entry in the TCA cycle. Alanine could be an example. Indeed, this amino acids is normally produced and excreted in situation of high nutrients availability. Afterwards, when the preferred carbon sources are depleted, cells start to consume alanine through conversion into pyruvate (Li et al., 2012; Gambhir et al., 2003). Indeed, a stronger alanine consumption was observed for the subclone, after day 7, compared to the control cells. Nevertheless, the difference was not enough to compensate for the amount of pyruvate diverted toward lactate production. Fatty acids oxidation could also be used to compensate for the reduced pyruvate oxidation in the subclone mitochondria. However, considering that the cells are in exponential growth and medium 1 contains only an unsaturated fatty acid in $\mu$g amount, it is more likely that lipids are produced in this phase, for the build up of new cells membrane, than oxidised (Schaub et al., 2010).

In conclusion, a condition of reduced mitochondrial activity in the subclone seemed to be an attractive hypothsis which needed further investigations.

# 7

# Mitochondrial metabolism analysis

## 7.1 Introduction

Mitochondria are double membranes organelles which have a central role in different cell processes, such as: energy and intermediates production, apoptosis, reactive oxygen species (ROS) generation and $Ca^{2+}$ homeostasis. In the last decades, mitochondria have been extensively studied, in particular in association to several diseases, as neurodegenerative disorders, diabetes and normal tissues ageing, which have been linked to specific mitochondria dysfunctions (Greaves et al., 2012; Maechler et al., 2010).

According to the endosymbiotic theory, this organelle is the result of the incorporation of an aerobic bacterium by the primordial eukaryotic cell. Indeed, mitochondria contain their own genome, a double stranded circular molecule of 16 Kb, which is considered as the residual of the bacterial DNA. The mitochondrial DNA (mtDNA) contains 37 genes, which encode for subunits of the respiratory chain complexes I, III and IV, the ATP synthase, 22 tRNA and 2 rRNA, needed for the translation of the mtDNA transcripts (Chan, 2006). The other proteins required for mitochondria functioning are encoded by the nuclear genome and transported to their final location by a complex translocation system (Neupert and Herrmann, 2007).

Mitochondria are also highly dynamic organelles. Indeed, they undergo a complex turnover process, which includes fission-fusion, biogenesis of new mitochondria and autophagy (Grandemange et al., 2009; Diaz and Moraes, 2008). All these mechanisms are strongly regulated by the cells energy requirements, but the signaling pathways behind these phenomena, and especially the interconnection between mitochondria dynamics and cell physiology, are still under investigation.

In this work, mitochondria have been studied for their key role in energy production. Several approaches can be used to evaluate mitochondrial oxidative activity. The measurement of TCA cycle enzymes rate is an example (Zhang et al., 2006; Neer-

mann and Wagner, 1996), although it can be a time consuming strategy since several reactions should be evaluated to get a complete view of the fluxes. Metabolite profiling can also be used to monitor both extracellular and intracellular intermediates throughout the cell culture, thus providing information about limiting nutrients or reactions (Ma et al., 2009). Alternatively, radiolabeled carbon sources allow to monitor flux distribution and, specifically, the amount of glucose and glutamine that are completely oxidized to $CO_2$ in mitochondria (Goudar et al., 2010; Butler et al., 1997). However, to obtain a more general view of the oxidative capacity of the mitochondria, the oxygen consumption rate is often measured, since it is a simple and informative approach (Gohil et al., 2010). Indeed, $O_2$ is the final acceptor of the mitochondrial electron transport chain, where it is converted to water. Moreover, concomitant with the electron flow, a proton gradient ($\Delta\Psi_m$) is also generated between the mitochondrial matrix and the intermembrane space. $\Delta\Psi_m$ is finally the driving force for ATP production (see paragraph 2.3 on page 27).

In this chapter, the oxygen consumption rate and the mitochondrial membrane potential of the two model cell lines have been compared. The aim has been to evaluate if the higher lactate production in the subclone could be linked to a reduced mitochondrial metabolic activity.

## 7.2 Mitochondrial oxidative metabolism is reduced in the subclone

### 7.2.1 Oxygen consumption rate

$O_2$ consumption was monitored in both cell lines at different moments of culture in medium 1. More precisely samples were harvested on day 2, when the TCA cycle was fuelled by the high glutamine availability; on day 5, when glutamine was depleted and the control cells switched to lactate consumption; on day 7 and 9, when the difference in lactate metabolism was more evident between the two cell lines.

The assays to monitor the mitochondrial metabolism were not performed after day 9 to avoid misinterpretation due to the decrease in cell viability and possible occurrence of apoptotic events (Grandemange et al., 2009; Tiefenthaler et al., 2001). Indeed, the mitochondrial apoptotic pathway begins with the permeabilisation of the outer membrane, followed by a collapse of the proton gradient and release of cytochrome-*c* (Frezza et al., 2007). Consequently, the $\Delta\Psi_m$ and the $O_2$ measurements would be negatively impacted.

As shown in figure 7.1, the oxygen consumption in the subclone significantly declined after the first 5 days of culture, reaching about 64% and 55 % of the control cell line on day 7 and 9, respectively. Two controls were also added to the test.

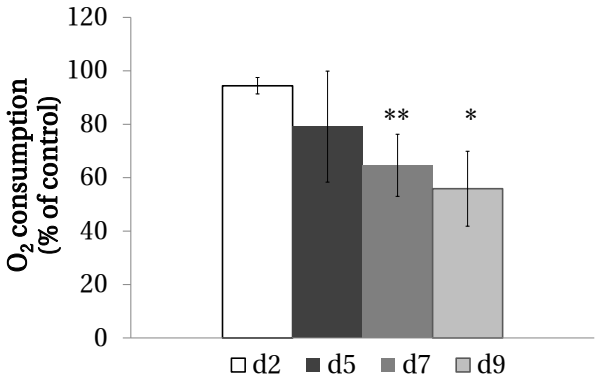


Figure 7.1: Oxygen consumption was measured in both cell lines on different days of culture in medium 1. The PreSens Oxoplate were used to monitor $O_2$ levels for 45 minutes, with a reading every minute, at 37°C in a flurometer. Only subclone data are reported as a percentage of the control cell line (set at 100 %) on each day of analysis. Mean ± standard deviation from four independent tests performed with duplicates. *, $p$ <0,05 and **, $p$ <0.01, Student's $t$-test.

Oligomycin, an inhibitor of the ATP synthase, allowed to evaluate the extent of oxygen consumption due to proton leaking. The inhibitor of the electron chain complex III, antimycin A, was instead used to block mitochondrial respiration. In both cases, the resulting oxygen consumption curves of the two cell lines were super-imposable (data not shown). This confirmed that the results reported in figure 7.1 were due to a reduced mitochondrial oxidative capacity in the subclone.

### 7.2.2 Mitochondrial membrane potential

The mitochondrial membrane potential was also measured in both cell lines to confirm the observations of the previous test.

As shown in figure 7.2, the mitochondrial membrane potential in the subclone was comparable to the control cell line during the first 5 days of culture, while it decreased significantly and reached around 80% of the control on day 7, and 60% on day 9.

Interestingly, the reduction of the mitochondrial oxidative metabolism in the subclone was evident only after day 5 in both tests. At this time point, glutamine was depleted in medium 1 (Fig. 6.2 on page 49). Therefore, other metabolites should be used to replenish the TCA cycle. While the control cells probably oxidised lactate to this purpose, the subclone, instead, diverted a part of the pyruvate pool to lactate production. Therefore, the carbons which entered into the TCA cycle were most

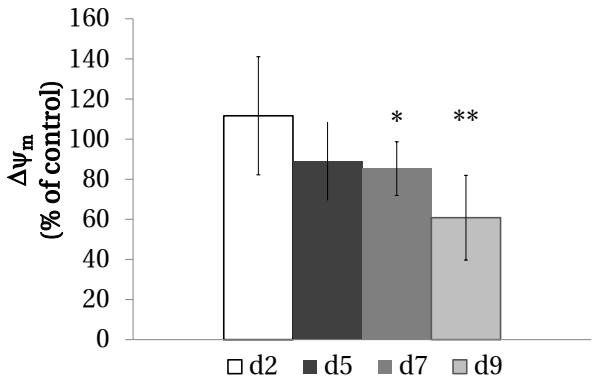


Figure 7.2: $\Delta\Psi_m$ was measured in both cell lines by flow cytometry, after staining with TMRE, on different days of culture in medium 1. Only subclone measures are reported as a percentage of the control cell line (set at 100 %) on each day of analysis. Mean ± standard deviation from five independent tests performed with duplicates. *, $p$ <0,05 and **, $p$ <0.01, Student's $t$-test.

likely reduced compared to the control cell line. Hence, the metabolic behaviour observed in the subclone resulted into a lower mitochondrial activity, as confimed by the $\Delta\Psi_m$ and the oxygen consumption evaluation.

## 7.3 Media impact on mitochondrial metabolism

Two other chemically defined media were tested on both model cell lines. The goal was to evaluate whether intrinsic cell properties were the only cause for the higher lactate accumulation in the subclone, or media composition could also influence this metabolism. In particular, a different proprietary medium (referred next as medium 2) and the commercially available CD CHO medium were used.

Surprisingly, in medium 2, also the subclone became able to switch to lactate consumption after day 5 (Fig. 7.3 D). The shift occurred again at the time point when glutamine was depleted, and both cell lines consumed glucose and lactate simultaneously (Fig. 7.3 B and C). The growth profile was similar to the one reported in medium 1 (Fig. 6.2 on page 49), except for a earlier cell death in medium 2 probably due to nutrient depletion (Fig. 7.3 A and C). Lactate concentration on day 5 was higher than in medium 2, however the consumption phase was faster than in medium 1 (Fig. 7.3 D and 6.2 E). Accordingly, the mitochondrial membrane potential and the oxygen consumption in the subclone were comparable to the control cell line, as shown in figure 7.3 E and F.

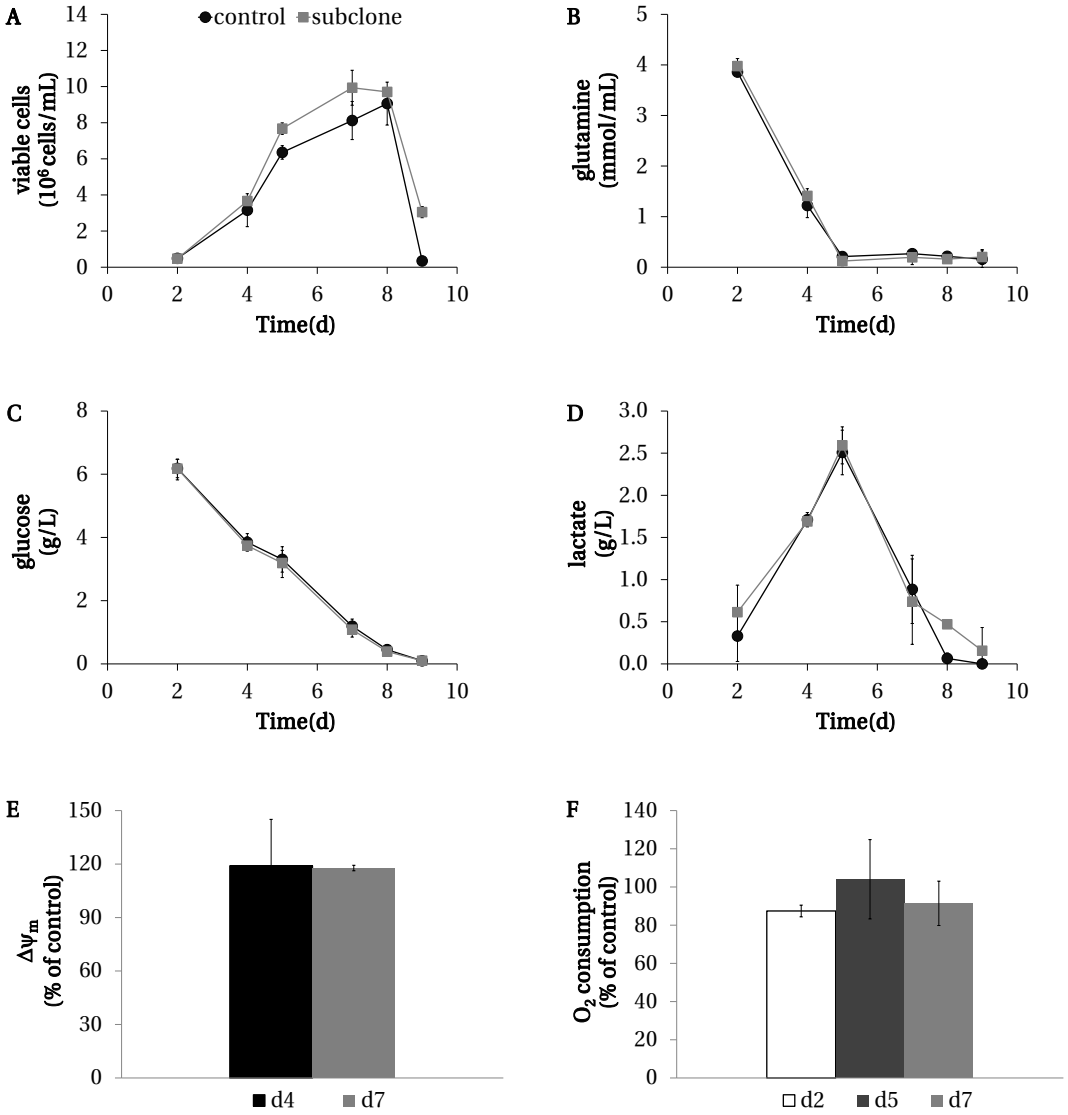


Figure 7.3: Control cell line (circles) and subclone (squares) growth (A) and metabolic profiles (B, C and D) in medium 2. The mitochondrial membrane potential (E) and oxygen consumption (F) in the subclone are expressed as a percentage of the control cell line (set at 100 %) on each day of analysis. Mean ± standard deviation from three independent tests performed with duplicates.

Based on these data, it can be excluded that the lower mitochondrial activity reported for the subclone in medium 1 is caused by an irreversible cell characteristic. The results indicate that a combination of both cell properties and media composition affects lactate metabolism and the mitochondrial oxidative capacity.

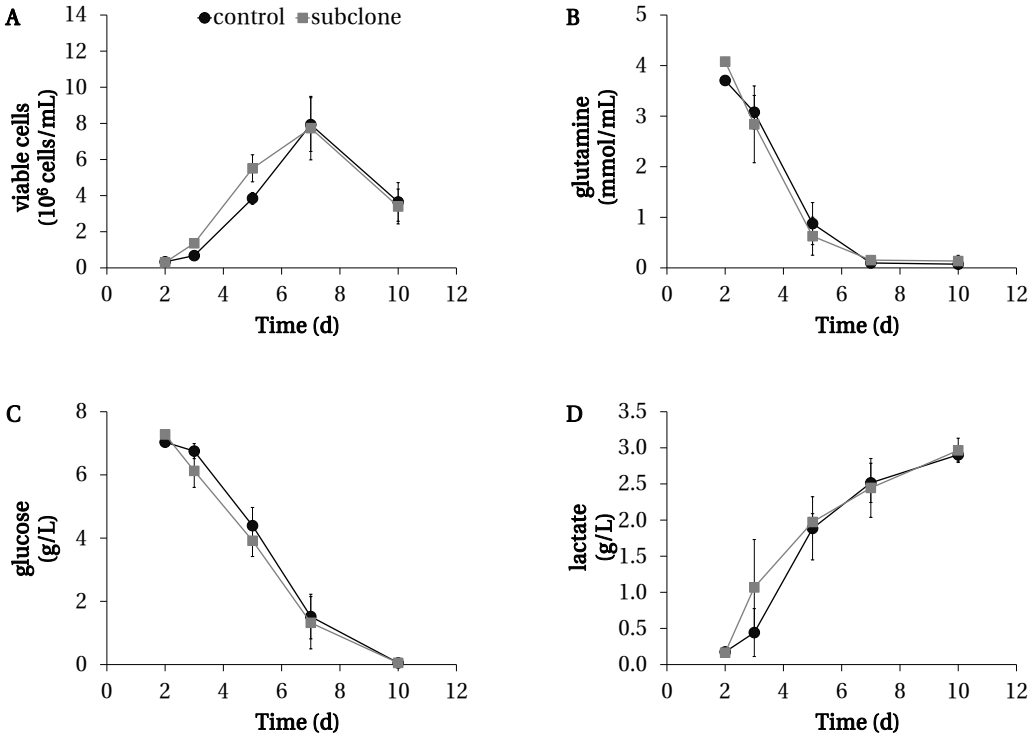


Figure 7.4: Control cell line (circles) and subclone (squares) growth (A), metabolic profiles (B, C and D) in CD CHO medium. Mean ± standard deviation from three independent tests performed with duplicates.*, $p$ <0.005 , Student's $t$-test.

This conclusion was further validated by growing the cells in CD CHO medium. Indeed, CD CHO triggered a higher lactate production in the control cell line, preventing the metabolic switch (Fig. 7.4 D). Cell growth , glutamine and glucose profiles (7.4 A, B and C respectively) were similar for both cell lines and also comparable to those observed in medium 1. However, the peak of growth was reached earlier in CD CHO than in medium 1 and cells death was also anticipated due to glucose depletion.

The mitochondrial membrane potential and the oxygen consumption of the control cell line in CD CHO were compared to the values obtained in medium 1. A significant decrease on day 7 was observed for the culture in CD CHO (Fig. 7.5 A and B, respectively), which correlated with the higher lactate accumulation observed in this medium.

In conclusion, data hitherto reported clearly suggested that the culture medium

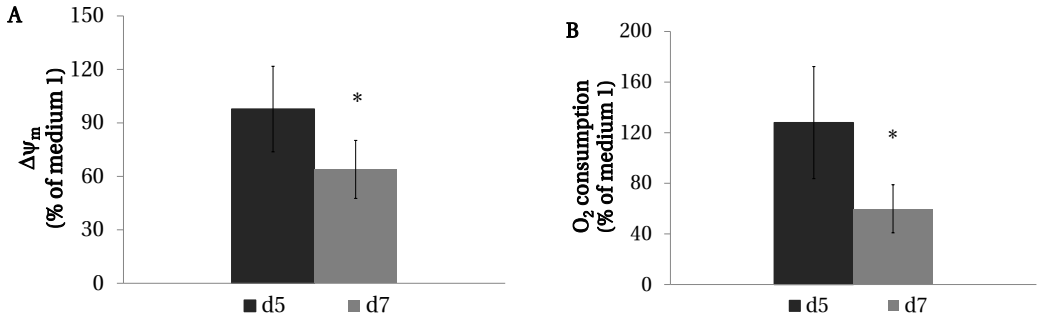


Figure 7.5: The mitochondrial membrane potential (A) and oxygen consumption (B) of the control cells in CD CHO are expressed as a percentage of the values obtained for the same cell line in medium 1 (set at 100 %). Mean ± standard deviation from three independent tests performed with duplicates.*, $p$ <0.005 , Student's $t$-test.

impacts lactate production. Moreover, the mitochondrial oxidative metabolism is involved in the observed phenomenon.

## 7.4 Key media components impact lactate metabolism

Medium 1 and 2 composition were compared in order to identify which components were differently present and could have an impact on lactate profile. The same analysis could not be done on CD CHO medium since its composition is not known.

Three elements had significantly different concentrations in the two media: alanine, which was absent in medium 2, copper and biotin, which were more concentrated in medium 2.

In order to better investigate their effect on lactate metabolism, a full factorial design was performed. Medium 2, deficient in alanine, copper and biotin, was used as a basal medium. The three identified elements were then added according to the levels reported in table 7.1 to obtain the final experimental matrix shown in table 7.2. Standard medium 2 was also included and used as a control (run 7 in table 7.2).

All tested conditions showed similar growth (Fig. 7.6 A and D), glucose and glutamine consumption profiles (data not shown). Lactate, instead, was significantly influenced by alanine and copper levels. In particular, while the metabolic switch on day 5 was maintained, the extent of consumption varied. Ammonia final concentration was also impacted.

As shown in figure 7.6 C and F, alanine supplementation increased $NH_4^+$ level at the end of the culture in both cell lines from around 4 to 6 mmol/L (circles versus triangles, $p$ value <0.0001, ANOVA analysis). It also rose residual lactate concentration,

Table 7.1: Each component was added to the basal medium at the concentration normally present in medium 1 or 2. Normalized concentrations are reported as percentage.

| Component | Medium 1 | Medium 2 |
|---|---|---|
| Alanine | 100 % | 0% |
| Copper | 20% | 100% |
| Biotin | 6% | 100% |

Table 7.2: Experimental matrix for the factorial design. Level 1 represents the high and -1 the low concentration. Each run corresponds to a test

| | Levels | | |
|---|---|---|---|
| **Run** | **Alanine** | **Copper** | **Biotin** |
| 1 | 1 | -1 | 1 |
| 2 | 1 | -1 | -1 |
| 3 | 1 | 1 | 1 |
| 4 | 1 | 1 | -1 |
| 5 | -1 | -1 | 1 |
| 6 | -1 | -1 | -1 |
| 7 | -1 | 1 | 1 |
| 8 | -1 | 1 | -1 |

but this was significant only in the subclone (Fig.7.6 E, $p < 0.005$, ANOVA analysis). A reduced copper concentration also caused an increase of lactate levels on day 9 compared to medium 2. However, its role was more significant in the subclone (Fig. 7.6, squares versus triangles. $p$ value < 0.0005, for the subclone (E) and < 0.05, for the control (B), ANOVA analysis). The combination of the two components increased even further the residual lactate concentration in the subclone, but the effect was less significant (Fig.7.6 E, diamonds. $p$<0.05, ANOVA analysis). Biotin had no major effect.

In conclusion, alanine and copper were identified as key elements to take into account when designing a medium. However, even if relevant, they were not sufficient to control lactate metabolism, since the switch to consumption still occurred and was concomitant to glutamine depletion. Furthermore, their effect was more significant in the subclone than in control cells.

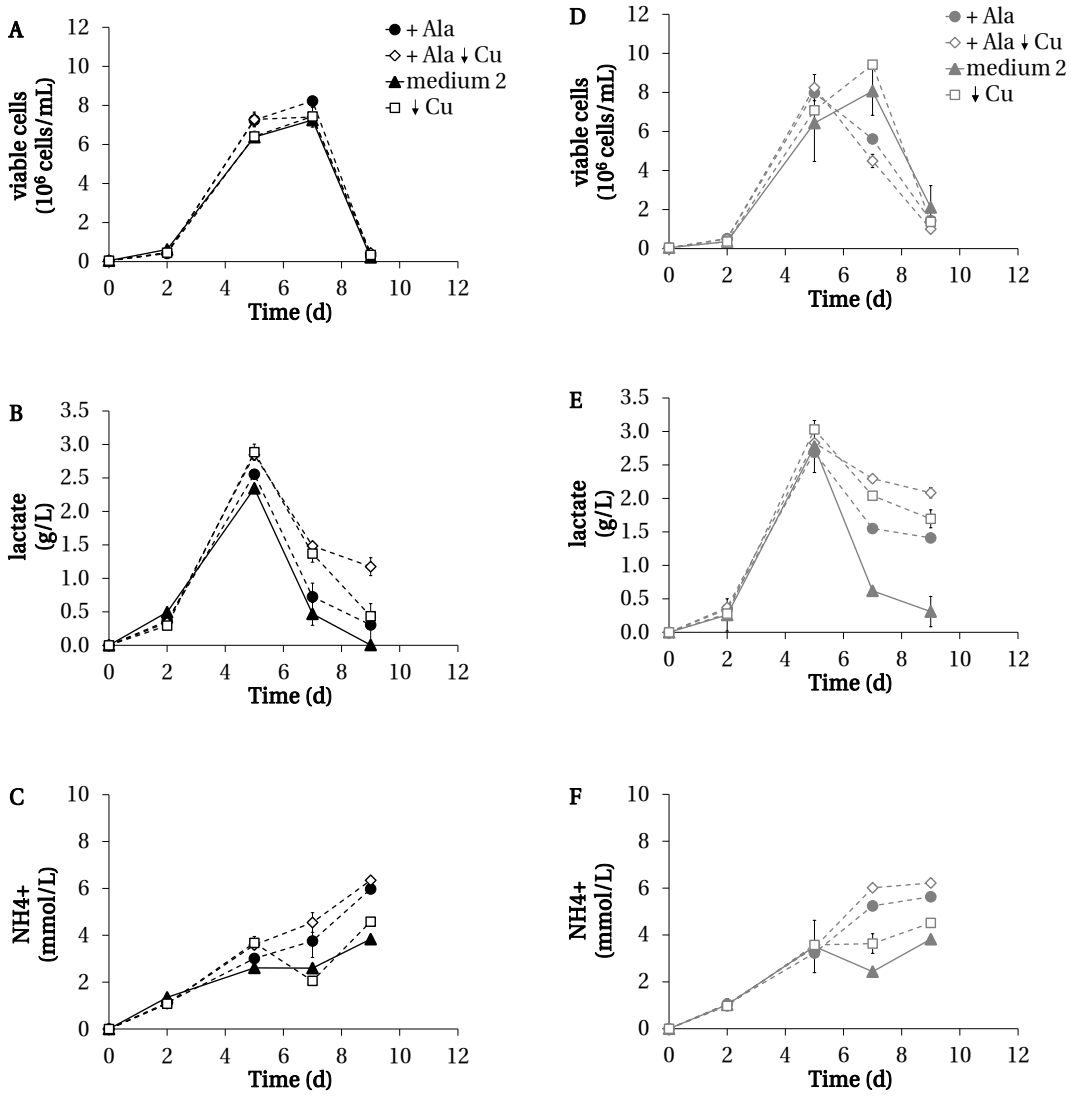


Figure 7.6: Growth, lactate and ammonia profiles for the control cell line (A to C) and the subclone (D to F). Standard medium 2 (triangles, run 7 and 8), alanine supplementation (circles, run 3 and 4), low copper concentration (squares, run 5 and 6) and the mix of these two conditions (diamonds, run 1 and 2) are reported. Biotin had no effect, therefore data were lumped according to the level of the other two components. Mean ± standard deviation from duplicate cultures.

## 7.5 Discussion

The mitochondrial membrane potential and the cell oxygen consumption depend on the activity of the electron transport chain. The respiratory chain, in turn, is conditioned by the NADH availability in the mitochondria, either produced in situ or transported from the cytosol through the shuttle systems.

Data shown in figure 7.1 and 7.2 clearly highlight the presence of an impaired mitochondrial metabolism in the subclone, when compared to the control cell line. Interestingly, the reduction of $\Delta\psi_m$ and $O_2$ consumption in the subclone is concomitant with the divergence in lactate metabolism between the two cell lines. Different hypothesis can be postulated to explain the observed behaviour.

An alteration of the electron transport chain, as an example, can cause a reduction in $\Delta\psi_m$ and $O_2$ consumption. However, this should be detectable already at the beginning of the culture, when the TCA cycle is mostly fuelled by glutamine.

The expression of an uncoupling protein can be hypothesized as well. These proteins allow protons return through the mitochondrial membrane, bypassing the coupling with ATP production. The result is a decrease of the mitochondrial membrane potential, while the respiratory chain activity and oxygen consumption would be stimulated as a mechanism to compensate for the lower ATP availability (Criscuolo et al., 2006). Reported data, instead, showed a reduction of $O_2$ consumption in conditions of high lactate production, which, therefore, excluded the hypothesis of uncoupling activity.

A reduced NADH/$FADH_2$ availability in mitochondria is likely to be the main cause, either due to a flux constraint in the TCA cycle or to a lower intermediates availability. Indeed, the stronger lactate production in the subclone likely diverts a part of the glycolysis-derived pyruvate from the progression into the TCA cycle. In contrast, the control cells not only consume glucose at a rate similar to the subclone, but they also consume lactate, thus increasing the fuelling of the mitochondrial oxidative metabolism. However, the reason why the subclone prefers to convert part of the pyruvate pool into lactate is still unclear.

An impaired pyridine nucleotides shuttling into the mitochondria may be hypothesized. Indeed, in this condition, pyruvate reduction into lactate would be used as an alternative NADH recycling system, needed to enable the proceeding of the glycolytic flux (as reported in figure 2.3 on page 23).

A combination of intrinsic cell characteristics and media composition has been reported to influence lactate metabolism. Indeed, presented data show that medium 2 fosters lactate consumption in the subclone. Accordingly, subclone $\Delta\psi_m$ and $O_2$ consumption are comparable to the control cells (Fig. 7.3). In the CD CHO medium, instead, even the control cells accumulate higher amounts of lactate. Consequently,

the mitochondrial membrane potential and oxygen consumption are reduced on day 7, when compared to the same cell line in medium 1 (Fig. 7.4).

Considering these data, the measurement of the mitochondrial membrane potential, or the oxygen consumption rate, can be proposed as a marker of an impaired lactate metabolism in CHO cells. This could be of interest either in the clones selection process, as already proposed by Hinterkörner *et al.* (Hinterkörner et al., 2007), or in media optimization, to identify key metabolites which can boost oxidative metabolism.

Three candidate components have been identified which are present at significantly different concentrations in medium 1 and 2. Among these, alanine and copper are able to impact both lactate and $NH_4^+$ metabolism (Fig. 7.6).

Alanine is normally used by the cells when other carbon sources are depleted, as already discussed in section 6.2 on page 48. In medium 1, a higher alanine consumption has been reported for the subclone after day 7, compared to the control cells (Fig. 6.3 on page 50). Interestingly, medium 2 supplementation with alanine increased the residual lactate concentration in the subclone culture (Fig.7.6E , circles versus triangles). The obtained result indicates that the subclones, even if it is able to consume lactate, prefers to use alternative sources of energy, such as alanine, when they are available. The observed increase in $NH_4^+$ concentration can also be ascribed to alanine metabolism, either due to the alanine dehydrogenase activity, which directly converts alanine to pyruvate with production of $NH_4^+$ and NADH, or to the alanine transaminase enzyme. This enzyme catalyses the conversion of alanine plus $\alpha$-ketoglutarate to pyruvate and glutamate. The latter is then recycled back to $\alpha$-ketoglutarate via glutamate dehydrogenase with the release of $NH_4^+$. Li *et al.* also reported a link between lactate, alanine and ammonia metabolism (Li et al., 2012). Indeed, they observed a phase of alanine consumption which occurred late in culture after lactate depletion and caused a rise of ammonia level in the medium. Therefore, lactate feeding was proposed as a strategy to prevent the use of alanine. In this work, on the contrary, the opposite behaviour has been observed in medium 1. Indeed, the subclone consumes first alanine, while lactate is slightly oxidised only at the end of the culture. Therefore, the lactate feeding strategy, although interesting, can be applied only to cell cultures that show a strong lactate consumption capacity, as our control cell line, but not for those cells that behave like the subclone.

The copper effect, instead, is visible only on lactate metabolism. Indeed, a reduced copper concentration results in higher lactate amount at the end of the culture, compared to the standard medium 2 (Fig. 7.6E, squares versus triangles). The role of copper on lactate metabolism has already been described in literature (Qian et al., 2011; Luo et al., 2011) . Indeed, copper is a cofactor of the cytochrome c oxydase complex, which is a component of the electron transport chain. Therefore, sup-

plementing the cells with higher copper most probably boosts the oxidative capacity of the mitochondria; thus increasing pyruvate, and consequently lactate, oxidation. Nevertheless, a too high copper concentration can be toxic as well (Scheiber and Dringen, 2011). Moreover, copper plays also an important role in the function of the superoxide dismutase. This enzyme promotes the detoxification of superoxides, which are normal by-products of the mitochondrial respiration. Superoxides, as the other reactive oxygen species, can cause cell damages and death if they accumulate beyond a certain extent (Brand, 2010).

However, when medium 1 was supplemented with higher copper concentration, no impact on lactate metabolism in the subclone was observed (data not shown). Therefore, copper seems to be important, but not sufficient, to control lactate accumulation. This result is also in agreement with data reported by Luo *et al.* (Luo et al., 2011)

Biotin, instead, does not impact lactate metabolism in our model, despite its role as a cofactor of pyruvate carboxilase. PC catalyses pyruvate conversion into oxaloacetate and represents an important anaplerotic reaction for the TCA cycle. However, its expression has been reported to be at very low levels in CHO cells (Fogolín et al., 2004; Kim and Lee, 2007b), which may explain the absence of a significant effect of biotin supplementation on lactate metabolism.

It is noteworthy that alanine and copper effects are stronger in the subclone than in the control cells, thus showing that the cell line variable still plays a key role. Therefore, both media composition and cell line characteristics should be taken into account when designing a strategy to optimize cell metabolism.

In conclusion, the reported data confirm our hypothesis that the mitochondrial oxidative capacity plays a key role in the control of lactate metabolism. Moreover, copper and alanine have been clearly identified as key components to take into consideration when designing a medium for CHO cell culture.

# 8

# Gene expression analysis

## 8.1 Introduction

Omic approaches are gaining a strong interest as strategies to improve bioprocess performances (Schaub et al., 2012). Metabolomic, in particular, is widely used to identify limiting nutrients, which can be then added into the basal medium or supplemented as feeds (Sellick et al., 2011; Ma et al., 2009). Furthermore, this approach can also be used to identify possible flux constraints which can be overcome by overexpressing a specific enzyme. For instance, Chong *et. al* reported a strong extracellular malate accumulation in bioreactor cultures of recombinant CHO cells, which they hypothesized was linked to an enzymatic bottleneck at the mitochondrial malate dehydrogenase. Its overexpression, indeed, not only reduced malate secretion, but it also boosted the oxidative metabolism and ATP production (Chong et al., 2010).

Other omic strategies include transcriptomic and proteomic assays. The common goal is to find markers, normally enzymes or transporters, which can be linked to an optimal growth, productivity or metabolism (Carlage et al., 2009; Yee et al., 2008a; Schaub et al., 2010). These approaches have also been proposed as tools to acquire a better understanding of the lactate metabolic shift in CHO cell culture (Pascoe et al., 2007). For instance, the analysis of hybridoma cells which underwent the metabolic shift to lactate consumption showed a down-regulation of the glycolytic enzymes. However, in this case the slowing down of the glycolytic flux was most probably the result of the culture system change from fed-batch to continuous, where the glucose concentration was maintained at a very low level (Korke et al., 2004a).

In a recent paper, Mulukutla et al. highlighted the importance of the signaling pathways in the control of lactate metabolism. In particular, they reported a down-regulation of several AKT1-regulated genes during the lactate consumption phase. AKT1 is a known stimulator of glucose uptake and metabolism, therefore the reduc-

tion of its activity would results in a slowdown in glycolysis. Consequently, the cells would start to consume lactate as an alternative source of energy, even in presence of high glucose concentration. Although these interesting results shed light on new candidates for the control of lactate metabolism, the factors that control AKT activity and promote the metabolic change still need to be identified (Mulukutla et al., 2012).

In the previuos chapter, the mitochondrial oxidative capacity has been identified as a key factor in the modulation of lactate metabolism in our model. To better investigate this phenomenon, the expression levels of specific genes was evaluated in conditions of opposite lactate metabolism.

## 8.2 Gene targets selection

The gene expression assay was performed using a custom made Real-Time PCR array with mouse primers and probes, since CHO-specific primers were not available at the moment of the test. Indeed, the CHO-K1 genome has been published only recently (Xu et al., 2011). Nevertheless, a high homology between the mouse and chinese hamster genome has been reported (of around 92 %), which supports the cross-hybridization between the two species (Kantardjieff et al., 2009; Yee et al., 2008b).

The targets selected for the array included 44 different genes, reported in table 8.1, and 3 controls.

Table 8.1: Selected targets for the gene expression array

| ***Gene symbol*** | ***Gene name*** |
|---|---|
| ***Glycolysis*** | |
| *Hk1* | *hexokinase 1* |
| *Hk2* | *hexokinase 2* |
| *Pfkl* | *phosphofructokinase, liver* |
| *Pfkm* | *phosphofructokinase, muscle* |
| *Pkm2* | *pyruvate kinase, muscle 2* |
| *Pklr* | *pyruvate kinase, L R isoform* |
| ***Anaplerotic reaction and miscellaneous*** | |
| *Pck1* | *phosphoenolpyruvate carboxykinase 1, cytosolic* |
| *Pck2* | *phosphoenolpyruvate carboxykinase 2, mitochondrial* |
| *Pc* | *pyruvate carboxylase* |
| *Pdha1* | *pyruvate dehydrogenase, E1-alpha polypeptide* |
| *Pdk2* | *pyruvate dehydrogenase kinase, isoenzyme 2* |
| *Ldha* | *lactate dehydrogenase a* |
| *Acly* | *ATP citrate lyase* |

*continuing form the previous page*

| ***Gene symbol*** | ***Gene name*** |
|---|---|
| *Me1* | *malic enzyme 1, NADP(+) dependent cytosolic* |
| *Me2* | *malic enzyme 2, NAD(+) dependent mitochondrial* |
| *Me3* | *malic enzyme 3, NADP(+) dependent mitochondrial* |
| *Mdh1* | *malate dehydrogenase 1, cytosolic* |
| *Got1* | *glutamate oxaloacetate transaminase, cytolsolic* |
| *Got2* | *glutamate oxaloacetate transaminase, mitochondrial* |
| ***TCA cycle*** | |
| *Cs* | *citrate synthase* |
| *Mdh2* | *malate dehydrogenase 2, mitochondrial* |
| *Sdha* | *succinate dehydrogenase complex, subunit a* |
| **Respiratory chain components** | |
| *Cox4ai1* | *cytochrome c oxidase, subunit IV, isoform 1* |
| *Uqcrc2a* | *ubiquinol-cytochrome c reductase core protein II* |
| *Atp5b* | *ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit* |
| ***Uncoupling proteins and controller of mitochondrial metabolism*** | |
| *Ucp2* | *uncoupling protein 2* |
| *Ucp3* | *uncoupling protein 3* |
| *Ppargc1a* | *peroxisome proliferator-actived receptor $\gamma$, coactivator 1$\alpha$* |
| *Slc16a1* | *monocarboxylic acid transporter-1* |
| ***Mitochondrial transporters*** | |
| *Slc25a10* | *dicarboxylate ion carrier* |
| *Slc25a12* | *aralar, aspartate glutamate carrier 1* |
| *Slc25a19* | *mitochondrial thiamine pyrophosphate carrier* |
| *Slc25a20* | *carnitine-acylcarnitine translocase* |
| *Slc25a21* | *mitochondrial oxodicarboxylate carrier* |
| *Slc25a22* | *glutamate carrier 1* |
| *Slc25a31* | *adenine nucleotide translocator* |
| *Tomm20* | *translocase of the outer mitochondrial membrane 20* |
| *Timm8a* | *translocase of the inner mitochondrial membrane 8a* |
| *Timm9* | *translocase of the inner mitochondrial membrane 9* |
| *Timm13* | *translocase of the inner mitochondrial membrane 13* |
| *Timm22* | *translocase of the inner mitochondrial membrane 22* |
| ***Control genes*** | |
| *18s* | *18s ribosomal RNA* |
| *Gapdh* | *Glyceraldehyde 3-phosphate dehydrogenase* |
| *Hprt1* | *Hypoxanthine-guanine phosphoribosyltransferase 1* |

The criteria for the selection of the glycolysis related targets was based on their known role as rate limiting enzymes. In particular, the hexokinase isoform 2 and the muscle pyruvate kinase were of great interest since they have been associated to a high glycolytic rate in tumour cells (Mathupala et al., 1997; Christofk et al., 2008). The anaplerotic reactions group includes enzymes which promote pyruvate, or its precursor phosphoenolpyruvate, oxidation into the TCA cycle (PCK, PC and PDH) thus reducing its conversion to lactate (Kim and Lee, 2007b; Neermann and Wagner, 1996). Pyruvate dehydrogenase kinase, on the contrary, inhibits the activity of PDH by phosphorilation, therefore blocking the oxidation of pyruvate.

The malic enzyme converts malic acid into pyruvate, diverting it from the TCA cycle. Malate accumulation is often observed in cell culture, probably due to a bottleneck in the Krebs cycle (Ma et al., 2009; Sidorenko et al., 2008). Therefore, ME1 was of particular interest for the model presented in this work to better investigate the initial phase of lactate production. For the same reason, MDH II was also introduced in the test. The ATP citrate lyase catalyse the formation of AcetilCoA from citrate in the cytosol, thus providing the building block for lipid synthesis. MDH and GOT exist as mitochondrial and cytosolic isoforms. They are relevant not only for intermediates production but also as part of the malate-aspartate shuttle (Barron et al., 1998).

The uncoupling proteins are mitochondrial proton transportes which dissipate the electrochemical gradients necessary for ATP production. While UCP1 is the only one clearly identified as able to mediate the non shivering thermogenesis, the function of UCP2 and 3 is still under investigation (Azzu and Brand, 2010). Interestingly, Pecqueur *et al.* reported that UCP2 knock out cells depend more on glycolysis for their energy production (Pecqueur et al., 2008). Moreover, its expression is promoted by glutamine at the translational level (Nübel et al., 2008). Therefore, it appeared to be a good candidate for the control of lactate metabolic switch. UCP3, instead, has been linked to the control of ROS production, but there also hypothesis that it could work as a pyruvate uniporter (Criscuolo et al., 2006; Mozo et al., 2006).

The peroxisome proliferator-actived receptor $\gamma$ coactivator $1\alpha$ (PGC$1\alpha$) is a major coordinator of different mitochondrial processes such as biogenesis, fatty acid oxidation and respiration. Interestingly, it is enriched in tissues with an high oxidative activity and its expression is regulated by the cell energy demand in a complex manner (Fernandez-Marcos and Auwerx, 2011; Ventura-Clapier et al., 2008). Interestingly, lactate has been reported to increase the expression of PGC$1\alpha$ and the monocarboxylate transporter-1 (MCT1) in L6 cells (Hashimoto et al., 2007). MCT1, in particular, mediates the transfer of pyruvate and lactate across the cell membrane.

Finally, the last group reported in table 8.1 includes the solute carriers family (*slc*), which transports different metabolic intermediates across the mitochondrial membrane. In more detail, *Slc25a10* transports dicarboxylates such as malate and suc-

cinate. Aralar1 promotes the exchange of glutamate with aspartate, and it is also a component of the malate-aspartate shuttle (Rubi et al., 2004; Neupert and Herrmann, 2007). *Slc25a19* translocates the thiamine pyrophosphate, which is the cofactor of decarboxylase enzymes, such as PDH and $\alpha$-ketoglutarate dehydrogenase. The carnitine-acylcarnitine translocase promotes the transfers of fatty acids into the mitochondria for oxidation. Lastly the group of translocases of the inner and outer mitochondrial membrane allows the correct sorting and localization of the proteins targeted to the mitochondria (Arco and Satrústegui, 2005; Rehling et al., 2003).

## 8.3 Gene expression array results

The described array was used to monitor gene expression in the subclone versus the control cell line during the culture in medium 1. Samples were harvested on day 6, when the control cells probably start a metabolic reorganization for the shift to lactate consumption; and on day 9, when the lactate profile is strongly different between the two cell lines. The real-time PCR data were analysed using the comparative $\Delta\Delta$Ct method, with the lactate consuming condition considered as reference sample. GAPDH was selected as endogenous control, since it showed the lowest variability during the culture and between the two cell lines compared to the others (18S and Hprt1). In table 8.2, only the sequences that gave rise to a reliable amplification signal are reported.

In order to confirm the observed results and to have a more general view, two additional cell lines were included in the test. More precisely, the recombinant clone G4, which was able to switch to lactate consumption as the control cell line, and the recombinant clone C6, which instead had a lactate profile similar to the subclone (Fig. 8.1 D)[1]. It should be noted, however, that clone G4 had a limited growth in this culture condition, but still consumed glucose faster than clone C6 (Fig. 8.1 A and C). Both recombinant clones were derived from a CHO-GS cell line, produced the same protein and were kept under the standard culture conditions (batch culture in tube spins with medium 1).

When a stringent fold change cut off of |FC≥ 2| was applied, only two genes, aralar1 and timm8a, resulted to be significantly downregulated in the subclone on day 6 ($p$ value <0.001, Student's $t$-test from four independent tests). On day 9, only aralar1 fitted the cut off requirement. Nevertheless, if a more relaxed cut off of |FC≥ 1,5| was considered, timm8a down-regulation would be significant also on day 9 ($p$ value <0.005, Student's $t$-test). According to this cut off, other genes would show a significant change of expression on day 6. However, they were not considered further, since on this time point the lactate profile was not strongly different between

[1] For control and subclone lactate profile refer to figure 6.2 on page 49

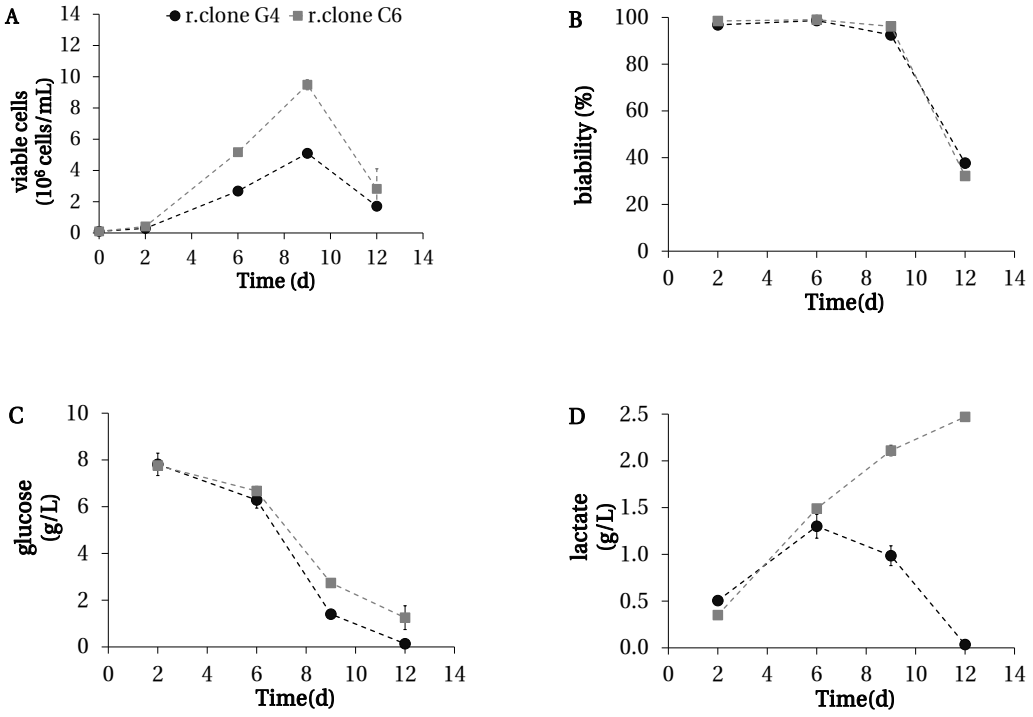


Figure 8.1: Recombinant clone G4 (circles) and C6 (squares) growth and metabolic profiles in medium 1. Mean ± standard deviations from two independent cultures.

Table 8.2: Expression fold changes for the lactate producer cell line versus the lactate consumer in medium 1. Values in bold fit the |FC≥ 2| cut off, while italics values fit the |FC≥ 1,5| cut off.

| Gene name | subclone vs control | | clone C6 vs clone G4 | |
|---|---|---|---|---|
| | day 6 | day 9 | day 6 | day 9 |
| *Hexokinase 2* | (–) 1.40 | (+) 1.46 | (+) 1.12 | (–) 1.14 |
| *Phosphofructokinase liver* | *(–) 1.90* | (+) 1.06 | (+) 1.18 | *(–) 1.54* |
| *Phosphofructokinase muscle* | (+) 1.08 | (+) 1.43 | | |
| *Pyruvate dehydrogenase a1 subunit* | (–) 1.35 | (–)1.30 | | |
| *Pyruvate dehydrogenase kinase 2* | (+) 1.34 | (–) 1.01 | (+) 1.21 | (–)1.15 |
| *Citrate syntase* | (–) 1.01 | (+) 1.17 | | |
| *Citrate lyase* | (–) 1.11 | (+) 1.08 | (+) 1.06 | *(–)1.75* |
| *Succinate dehydrogenase* | *(–) 1.60* | (+) 1.46 | **(–) 2.22** | (–)1.15 |
| *Malate dehydrogenase 2* | (–) 1.17 | (+) 1.04 | (–) 1.11 | (–) 1.22 |
| *Malate dehydrogenase 1* | (–) 1.23 | (+) 1.27 | (–) 1.16 | (–) 1.12 |
| *Malic enzyme 1* | *(–) 1.69* | (+) 1.10 | (-) 1.11 | (–) 1.23 |
| *Glutamate-oxaloacetate transaminase* 2 | (–) 1.45 | (+) 1.09 | (+) 1.14 | (–) 1.37 |
| *Aralar1* | **(–) 2.77** | **(–) 2.08** | **(–) 3.23** | **(–) 2.44** |
| *Mitochondrial dicarboxylate carrier* | *(–)1.80* | (+) 1.05 | **(–) 4.35** | *(–) 1.53* |
| *Mitochondrial glutamate carrier* | (–) 1.07 | (+) 1.16 | (+) 1.23 | *(–) 1.72* |
| *Monocarboxylic acid transporter* | *(–) 1.65* | (+) 1.42 | (–) 1.23 | (–) 1.32 |
| *Timm 22* | *(–) 1.54* | (–) 1.11 | | *(–) 1.56* |
| *Timm 8a* | **(–) 2.43** | *(–) 1.75* | (–)1.40 | *(–) 1.72* |
| *Timm 13* | (+) 1.02 | (–) 1.04 | (+) 1.02 | (–) 1.19 |

upregulated sequences, (+); downregulated sequences (–)

the two cell lines.

Interestingly, the recombinant clone C6 showed also a significant reduction of aralar1 expression on both days of test. Timm8a, instead, would be included only on day 9, according to the |FC≥ 1,5| cut off. Succinate dehydrogenase and the mitochondrial dicarboxylate carrier were also down-regulated in the recombinant clone C6, but only on day 6. Again, considering that the difference in the lactate profile was evident on day 9, these two genes were not investigated further. Moreover, the expression of these two genes seemed to be more specific to the recombinant cell line, since their levels were not significantly changed in the subclone. The same conclusion applied to other genes that fitted the |FC≥ 1,5| cut off on day 9, but only in the recombinant cell lines.

## 8.4 Aralar1 and timm8a expression in high lactate conditions

### 8.4.1 Medium 2 versus medium 1

Aralar1 and timm8a expression was evaluated in other culture conditions or clones, which showed differences of lactate metabolism, in order to verify if a general correlation could be postulated.

As reported in figure 7.3 on page 61, the subclone became able to consume lactate when cultivated in medium 2. This condition represented an interesting system to investigate if aralar1 and timm8a expression correlated with the changed lactate profile, suggesting a possible impact of media composition on these two genes.

For each cell line, the results obtained in medium 2 were compared with those reported for medium 1. The test was performed only on day 8, which corresponded to the growth peak in medium 2.

Table 8.3: Gene expression fold changes on day 8.

| Gene name | Medium 2 vs medium 1 | |
|---|---|---|
| | control | subclone |
| *aralar1* | (+)1.20 | *(+)1.56* |
| *timm8a* | (+)1.00 | **(+)2.04** |

Aralar1 showed a significant increase of expression when the subclone was cultivated in medium 2, if the |FC≥ 1,5| cut off was applied ( Table 8.3, $p$ value < 0.001, Student's $t$-test on three independent assays). Timm8a was also upregulated but the analysis was affected by a high variability, which weakened its significance ($p$ value = 0.06).

The two genes expression, instead, was not impacted by the change of media in the control cell line. Accordingly, the lactate profile was the same in both medium 1 and 2.

### 8.4.2 CD CHO medium versus medium 1

In section 7.3 it has been reported that CD CHO medium is able to prevent the switch to lactate consumption in the control cell line (Fig.7.4 on page 62).

Table 8.4: Gene expression fold changes on day 7.

| Gene name | CD CHO vs medium 1 | |
|---|---|---|
| | control | subclone |
| *aralar1* | **(–)6.90** | **(-)2.52** |
| *timm8a* | **(–)2.70** | (–)1.18 |

Therefore, aralar1 and timm8a expression was measured in CD CHO cultures of both cell lines and was compared to the values obtained in medium 1. Samples were harvested on day 7 of cultures, which corresponded to the growth peak in CD CHO.

Indeed, as shown in table 8.4, a strong decrease of both gene expression was detected in the control cell line when cultivated in CD CHO medium ($p$ value < 0,001, Student's $t$-test form two independent assays). Even the subclone showed a decrease of gene expression in CD CHO medium compared to medium 1. However, this was valid only for aralar1.

### 8.4.3 Fed-batch culture

Finally, aralar1 and timm8a expression was also evaluated in fed-batch cultures. The recombinant cell line used for the test (referred next as recombinant clone A1) was generated similarly to the G4 and C6 clones, but represented a different colony.

Table 8.5: Gene expression fold changes on day 10.

| Gene name | Feed 2 vs feed 1 |
|---|---|
| *aralar1* | *(–)1.92* |
| *timm8a* | **(–)2.42** |

Cells were grown in CD CHO medium and supplemented with two different feeding regimes (referred next as feed 1 and feed 2), which differently influenced the ability to shift to lactate consumption (Fig. 8.2 B). Samples were recovered on day 10 of culture and the lactate consuming condition was taken as reference sample for the

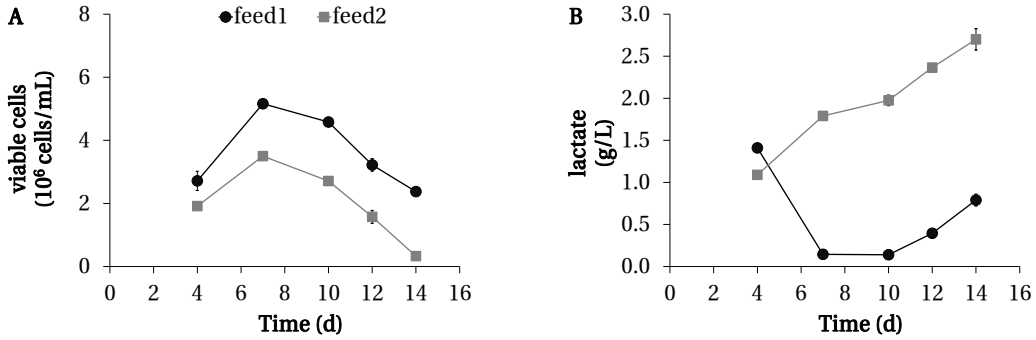


Figure 8.2: Recombinant clone A1 growth (A) and lactate profile (B) in CD CHO medium. The culture medium was supplemented with feed 1 (circles) or feed 2 (squares) on different days during the growth. Mean ± standard deviations from duplicate cultures.

data analysis. It should be noted that although cells were no more in the exponential growth phase on the day of the test, both feeding conditions showed a similar decrease in cell viability.

As reported in table 8.5, aralar1 and timm8a expression was downregulated when the cells were supplemented with feed 2. Accordingly, this feeding regime induced a higher lactate accumulation compared to feed 1.

In conclusion, the reported results indicated a correlation between a strong lactate production and *aralar1* and *timm8a* downregulation in the tested conditions.

## 8.5 Discussion

Gene array can be an informative tool to correlate specific genes expression with the observed metabolic state and provide promising targets for host cell engineering (Wlaschin et al., 2006). For this reason, the expression of selected genes has been evaluated in our culture model, and also in different cell lines or culture conditions which showed opposite lactate profiles. The targets have been specifically chosen for their possible relation with the mitochondrial oxidative activity and lactate production.

From the gene expression analysis, two gene resulted to be down-regulated in conditions of high lactate production: aralar1[2] and timm8a (Table 8.2). In particular aralar1 shows a stronger down regulation which is maintained until the end of the

[2]The predicted CHO-K1 sequence for aralar1 became available during the writing of this manuscript. The homology with the mouse cDNA was of 92 % (XM 003495954.1 and NM 172436.3)

growing phase. More interestingly, the same result has been obtained not only for the model cell lines, but also in two recombinant clones which also differ for their lactate profile (clone G4 and C6). Moreover, it should be noted that the cut off selected for the study (|FC≥ 2|) is considered as a rather stringent for gene expression comparison in mammalian cell cultures. Indeed, it has been reported that only a small percentage of genes show a more than two fold expression change in microarray analysis, even when butyrate treated cells are compared to untreated (Wlaschin et al., 2006; Schaub et al., 2010). A more relaxed cut off of (|FC≥ 1.5|) is indeed often used (Qian et al., 2011; Yee et al., 2008a; Korke et al., 2004a). Therefore, it was also included in the study.

Several culture conditions have been tested to further verify if a possible correlation between the expression of these two genes and lactate metabolism could be confirmed. For instance, medium 2 and CD CHO represented an interesting condition to validate our hypothesis, since they are able to differently influence the metabolic shift. Interestingly, aralar1 and timm8a expression correlates with the observed lactate profile in these media (Table 8.3 and 8.4). Finally, the same correlation has also been observed in fed-batch cultures with two feeding strategies that differently impact lactate metabolism (Table 8.5).

The reported results also indicate that unknown media components, or more probably a more balanced metabolism, can influence the expression of these two genes. However, even if tantalising, the mechanisms that control aralar1 and timm8a expression in our culture conditions were not investigated further.

### 8.5.1 Aralar and timm8a role in cell metabolism

The connection between the identified genes and the cell lactate metabolism is well described in the literature.

*Aralar1* encodes for the mitochondrial aspartate-glutamate carrier (AGC), which is a component of the malate-aspartate shuttle (MAS, Figure 8.3) and is localised in the inner mitochondrial membrane. This system transfers reducing equivalents from the cytosol to the mitochondria and its activity is necessary to regenerate the cytosolic $NAD^+$ pool required for the progression of glycolysis. Indeed, the cytosolic reduction of oxaloacetate into malate releases $NAD^+$. Malate is then transported, in exchange for $\alpha$-ketoglutarate, into the mitochondrial matrix where it is oxidised back to oxaolacetate, with NADH production. NADH is then oxidised in the electron transport chain to generate ATP. To complete the shuttle, oxaloacetate reacts with glutamate to give aspartate and $\alpha$-ketoglutarate. Aspartate is hence exported to the cytosol in exchange for glutamate. Finally, the inverse transaminase reaction occurs in the cytosol to give back oxaloacetate.

Another shuttle system exists: the glycerol-3-phosphate shuttle (GPS). Two iso-

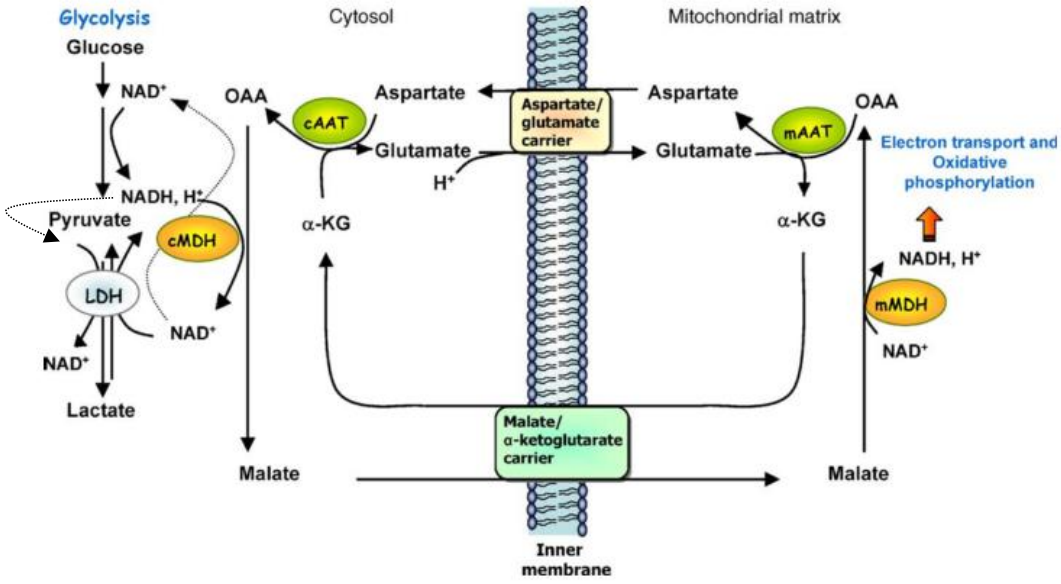


Figure 8.3: The malate-aspartate shuttle. cAAT and mAAT, cytosolic and mitochondrial aspartate aminotransferase; cMDH and mMDH, cytosolic and mitochondrial malate dehydrogenase; LDH, lactate dehydrogenase; OAA, oxaloacetate; $\alpha$-KG, $\alpha$-ketoglutarate. Modified from McKenna et al. (2006)

forms of the enzyme glycerol-3-phosphate dehydrogenase (G3PDH) are involved in the process. The cytosolic G3PDH converts dihydroxyacetone phosphate to glycerol-3-phosphate realising $NAD^+$. Afterwards, glycerol-3-phosphate is reconverted into dihydroxyacetone phosphate by the mitochondrial G3PDH. This enzyme is localized on the outer surface of the inner mitochondrial membrane and is FAD dependent (Bender et al., 2006). Therefore, GPS has a lower energy yield compared to MAS. Moreover, it has been reported that GPS activity is insufficient to compensate for an impaired MAS functioning or in conditions of high cytosolic NADH levels, such as during lactate consumption (Barron et al., 1998). In general, according to the literature analysis, MAS activity seems to be of greater importance than GPS, especially when lactate is used as carbon source (Satrústegui et al., 2007). Therefore, our study was focused more on the malate-aspartate shuttle.

The aspartate-glutamate carrier, specifically, promotes the aspartate efflux to the cytosol, in exchange with glutamate plus a proton. As a consequence of its electrogenic activity, AGC functioning is favoured in active mitochondria (Palmieri et al., 2001). Two isoforms of this carrier have been identified: AGC1 (aralar1) and AGC2 (citrin), which belong to the calcium-binding mitochondrial carriers family. Aralar1 is expressed in most tissues, mainly pancreatic islets, muscle and brain; while citrin is more enriched in the liver, where it is also involved in the urea cycle (Satrústegui et al., 2007). Indeed, mutations of citrin have been associated to the type II citrullinaemia disease, which is characterized by ammonia build up in the blood (Kobayashi et al., 1999).

AGC plays a crucial role in the functioning of the shuttle, since it promotes the only irreversible step of the process (Satrústegui et al., 2007). Its activity is calcium stimulated. Indeed, the amino-terminal extension harbours EF-hand $Ca^{2+}$-binding motifs, which face the inter-membrane space. In this way, the extramitochondrial calcium level can regulate AGC activity (Palmieri et al., 2001; Lasorsa et al., 2003; Gellerich et al., 2010).

MAS activity has been extensively studied in $\beta$-cells, where insulin secretion is linked to the glycolytic flux, respiratory chain activity and the consequent rise of ATP levels (Bender et al., 2006). Indeed, in these cells glycolysis is closely connected to the mitochondrial metabolism, in order to fulfil the ATP requirement for insulin secretion. Therefore, the malate aspartate shuttle plays a key role to support an efficient transfer of reducing equivalents into the mitochondria. Moreover, LDH activity in $\beta$-cells is quite low (Sekine et al., 1994), therefore the amount of NADH that is used to reduce pyruvate into lactate is limited. It has also been shown that aralar1 overexpression in $\beta$-cells can elicit an increase of glucose oxidation to $CO_2$, boost ATP production and insulin secretion. More interestingly, lactate release was also reduced, even if the total amount of consumed glucose was unchanged. This suggests that

MAS activity modulates the fraction of pyruvate which is oxidised into the mitochondria or reduced to lactate (Rubi et al., 2004). Conversely, AGC1 silencing resulted in the opposite effects, at least on tumour-derived insulin secreting cells (Casimir et al., 2009).

Another site of particular interest for aralar1 activity is represented by the brain which shows peculiar characteristics in terms of lactate homoeostasis and shuttle expression. It has been reported that, after the glutamate stimulus, astrocytes engage mostly in aerobic glycolysis and release lactate. The latter is then used by neurons as energy source. This type of cell-cell lactate shuttle, known as the neuron-glia coupling (Magistretti, 2006), has been reported also for other tissues (Brooks, 2009). Interestingly, aralar1 is highly enriched in neurons, while lower levels can be detected in astrocytes (McKenna et al., 2006), thus confirming its crucial role in lactate oxidation.

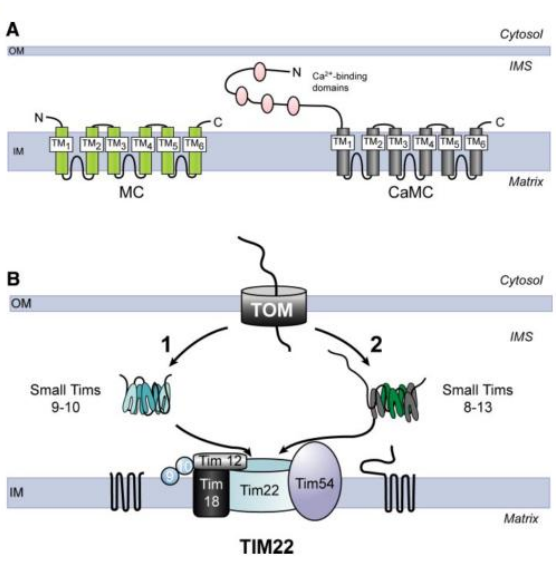


Figure 8.4: The mitochondrial membrane translocation system. (A) substrates of the small Timm complex: MC, mitochondrial carrier and CaMC, calcium-binding mitochondrial carriers. (B)Translocation pathway for the MC proteins (1) and the CaMC (2). IMS= intermembrane space, OM= outer membrane, IM= inner membrane. From Arco and Satrústegui (2005)

The other identified gene, timm8a, is a component of the mitochondrial translocase system. This complex of proteins favours the import from the cytosol and correct localization of the mitochondrial proteins (Neupert and Herrmann, 2007). In particular, timm8a, together with its partner timm13, act as chaperone-like proteins. Their role is to facilitate the transport of proteins belonging to the family of calcium-binding carriers, such as aralar1, across the inter-membrane space to the TIMM22

complex. The latter mediates the carrier insertion into the inner mitochondrial membrane (Fig. 8.4). Timm8 mutations have been associated to the deafness/dystonia syndrome. Decreased mitochondrial NADH levels were observed in lymphoblasts derived from patients cultivated in presence of lactate. This was caused by the impaired AGC translocation, which did not allow a correct shuttling of the NADH produced during lactate oxidation (Roesch et al., 2004).

Interestingly, Korke *et al.* reported a 1.4 fold upregulation of timm13 in hybridoma that underwent the metabolic shift to lactate consumption (Korke et al., 2004b). However, no great attention was paid to this gene and further investigation has not been reported.

It is noteworthy that Timm8a expression levels vary among different tissues, with a prominent enrichment in neurons and liver. Interestingly, its tissue distribution correlates also with aralar1 and citrin expression, respectively (Roesch et al., 2004).

In conclusion, considering the gene expression data and the literature review it was speculated that the higher lactate production, observed in some culture conditions, could be linked to an impaired NADH shuttling into the mitochondria. In this situation, LDH activity would be favoured in the direction of lactate production, as alternative system to regenerate the cytosolic NAD pool.

Lastly, even though the mechanisms underlying the observed downregulations are not clear yet, aralar1 and timm8a seem to be promising candidates for CHO cell engineering to improve lactate metabolism.

# 9

# Aralar1 and timm8a effect on lactate metabolism

## 9.1 Introduction

In this chapter, the potential impact of the two genes, aralar1 and timm8a, on lactate metabolism was investigated. At first, an inhibitor of the malate-aspartate shuttle was tested in the control cells to evaluate if the switch to lactate consumption was affected. Afterwards, aralar1 and timm8a were overexpressed in both control cells and subclone to assess their effect on lactate profile and cell growth.

## 9.2 Malate-aspartate shuttle inhibition

Aminooxyacetic acid (AOAA) is an inhibitor of the pyridoxal-dependent enzymes, such as the aspartate aminotransferase that is a key enzyme of the malate-aspartate shuttle (DaVanzoa et al., 1966). Therefore, even if not strictly specific, AOAA is often used to study the effect of MAS inhibition on cell physiology (Kauppinen et al., 1987; Barron et al., 1998; McKenna et al., 2006; Contreras and Satrústegui, 2009).

Interestingly, when control cells were treated with 0.25 mM of AOAA, the switch to lactate consumption after day 5 was prevented and a further accumulation occurred (Fig. 9.1 D). A slight lactate consumption was observed at the end of the culture, at the time point when glucose was depleted. The obtained lactate profile was therefore comparable to the one normally observed for the subclone. Treated cells showed also a slower cell death and a slight increase of $NH_4^+$ production (Fig. 9.1 A and E). On the other hand, glucose and glutamine profile were not impacted (Fig. 9.1 B and C). The mitochondrial membrane potential was also measured and , as expected, treated cells showed a significant decrease of $\Delta\Psi_m$ on day 7 (Fig. 9.1 F).

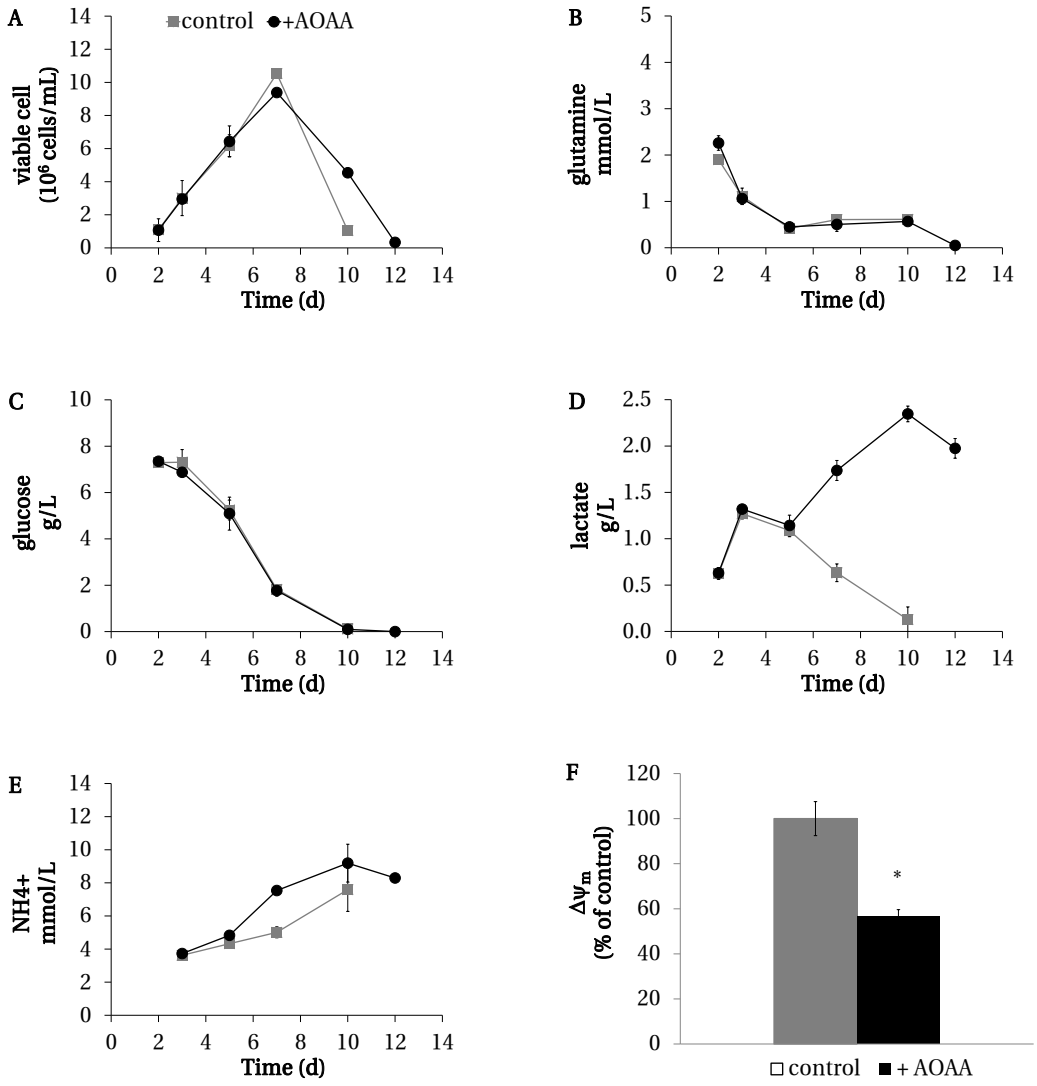


Figure 9.1: Aminooxyacetic acid effect on lactate metabolism in control cells. Cells were seeded at 0.2*$10^6$ cells/mL in medium 1 and treated with 0.25 mM of AOAA from day 0 (circles). Not treated cells (squares). (A) growth, (B) glutamine, (C) glucose, (D) lactate, and (E) $NH_4^+$ profiles. Mitochondrial membrane potential (F) was measured on day 7 and values are expressed as a percentage of the non treated cells (*, $p$ value < 0.05, Student's $t$-test ). Mean ± standard deviation from two independent cultures with duplicates.

Overall, AOAA modified control cells metabolism making it more similar to the subclone one (see Fig. 6.2 on page 49). Therefore, these results supported the hypothesis that an inefficient NADH shuttling might represent a constraint to lactate oxidation.

## 9.3 Aralar1 and timm8a overexpression

### 9.3.1 Clones selection and characterization

Stable clones overexpressing aralar1 or timm8a were obtained using a vector carrying the mouse ORF with a C-terminal GFP tag. The GFP signal was used to screen stable clones by flow cytometry. Positive clones were also further selected for their capacity to grow in suspension cultures without formation of cell aggregates.

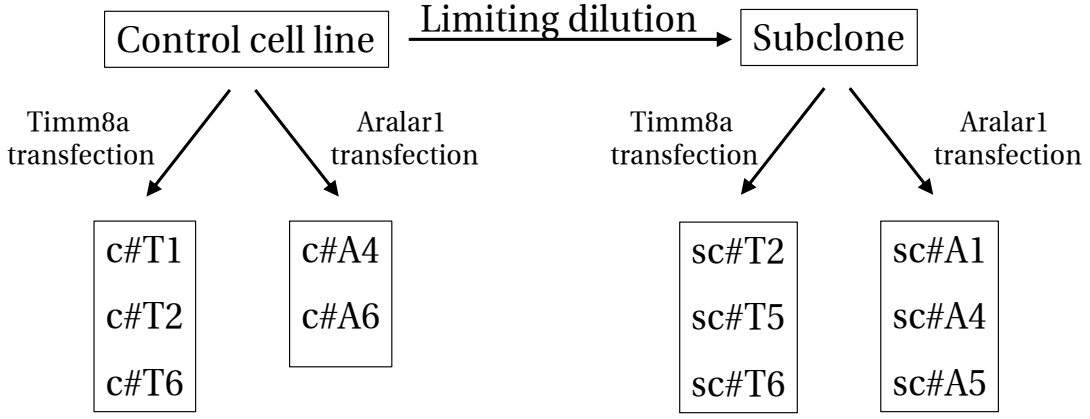


Figure 9.2: Stable transfected clones nomenclature and original parental cell lines are described for better clarity.

Finally, eleven clones were kept for further characterization: five were generated from the control cell line transfection and overexpressed either timm8a-GFP (referred next as c#T1, c#T2 and c#T6) or aralar1-GFP (referred next as c#A4 and c#A6). Six clones were derived from the subclone transfection: three timm8a-expressing clones (referred next as sc#T2, sc#T5 and sc#T6) and three aralar-expressing clones were selected (referred next as sc#a1, sc#A4 and sc#A5). For better clarity, cell lines interrelations are summarized in figure 9.2.

GFP signal was monitored for 30 days to ensure the stability of expression (Fig. 9.3) and the correct subcellular localization was validated by co-staining with the

mitochondrial-specific dye, Mitotracker red (Fig.9.4).

### 9.3.2 Clones growth and metabolic profile

Selected clones were cultivated in medium 1 to evaluate the transgenes impact on growth and metabolism. Since high lactate production is often associated to fast proliferation, as in cancer cells (Vazquez et al., 2010; Jones and Thompson, 2009), we postulated that improving the cell metabolism could have a potential negative impact on cell growth.

Conversely, as shown in figure 9.5 and 9.6 A, the growth of the control cell line-derived clones was generally comparable to the untransfected control. Only c#T1 behaved differently with a maximum cell density around $5 \times 10^6$ cells/mL. Interestingly, all clones maintained the switch to lactate consumption after day 5, which still corresponded to the time point when residual glutamine was low in the medium (Fig. 9.5 and 9.6 C and D).

Clone c#A6, exceptionally, did not engage in net lactate consumption after day 5 and also showed a big variability of the measured lactate values. Moreover, this clone was also characterized by a faster glucose consumption and cell growth, with the decline phase starting earlier than the other clones. Therefore, it can be speculated that this different behaviour could reflect the depletion of important substrates towards the end of the culture, which resulted in a higher residual lactate concentration and variability.

In conclusion, timm8a or aralar1 overexpression did not strongly modify the characteristic phenotype of the control cell line.

The recombinant clones generated from subclone transfection showed a more variable growth (Fig. 9.7 and 9.8 A). However, most of them reached a growth peak of at least $8 \times 10^6$ cells/mL, except for sc#T6. Glucose and glutamine profiles were similar to the untrasfected subclone (Fig. 9.7 and 9.8 B and C, respectively). Remarkably, the recombinant clones switched to net lactate consumption after day 5 and simultaneously used lactate and glucose as sources of energy (Fig. 9.7 and 9.8 D). The metabolic shift again correlated with the cell culture phase when glutamine was almost depleted. Therefore, the resulting metabolic behaviour was more similar to the control cell line.

For better comparison, recombinant clones and parental cell lines IVC (integral of viable cell density) was also calculated and reported in graph with the lactate concentration on day 11 (Fig. 9.9). The control cell-derived clones showed no difference in the residual lactate level compared to the untrasfected cell line, except for c#A6 as already discussed. Moreover, no significant correlation between cell growth and lactate profiles could be highlighted.

Even though lactate was slightly consumed at the end of the subclone culture, its

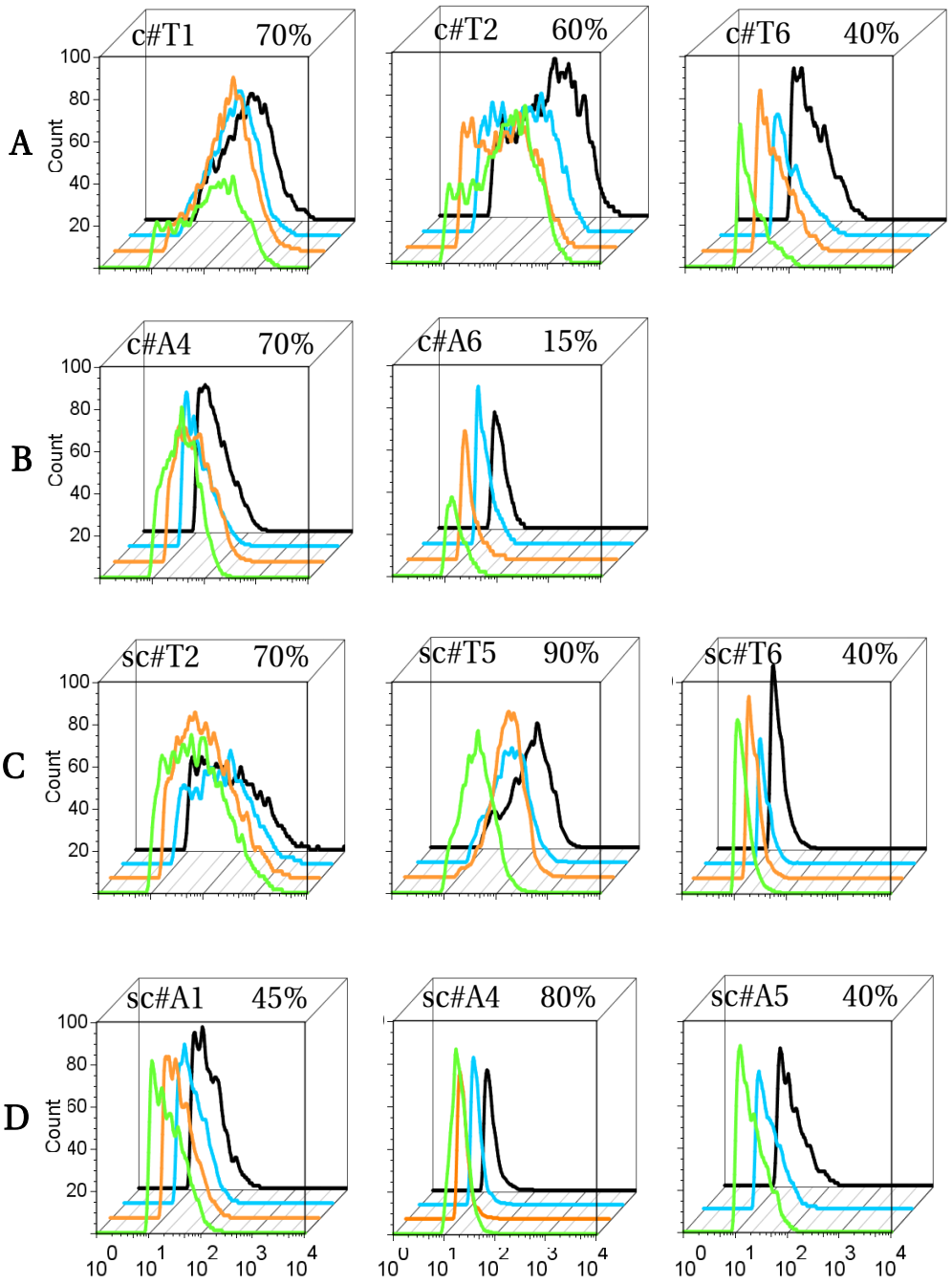


Figure 9.3: Clones stability was validated by monitoring the GFP expression for one month. (A and B) control cell line-derived clones, (C and D) subclone-derived clones. Green fluorescence intensity is reported for positive gated populations (indicated as %). Black line, day 8; blue line, day 17; orange line, day 23; green line, day 30.

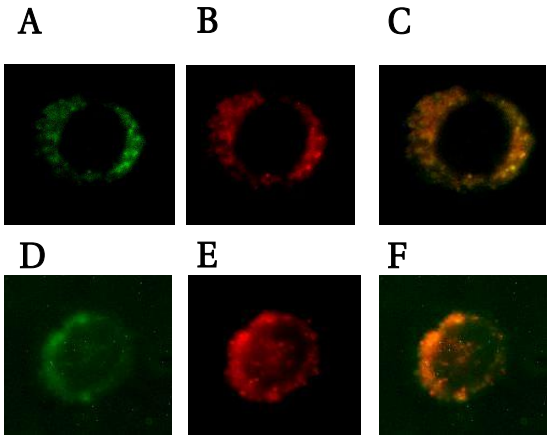


Figure 9.4: The correct mitochondrial localization of timm8a (A to C) an aralar1 (D to F) was evaluated by using the specific mitochondrial-dye, Mitotracker red. (A and D) GFP, (B and E) Mitotracker red and (C and F) merge of both signals.

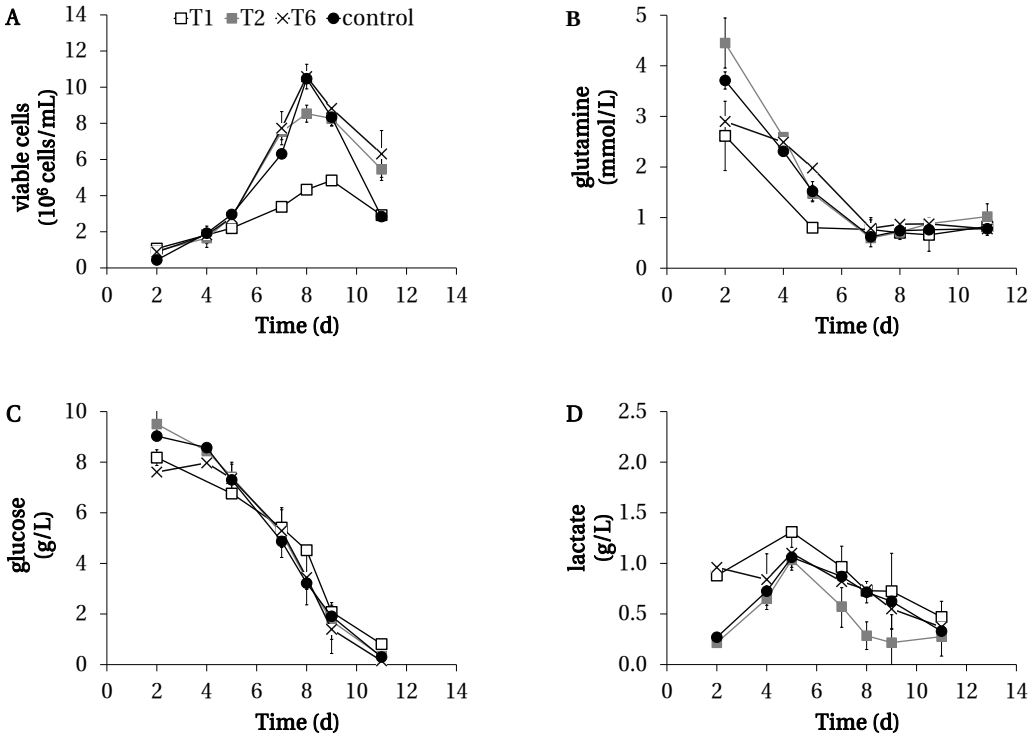


Figure 9.5: Control-derived timm8a overexpressing clones cultivated in medium 1. Growth (A), glutamine (B), glucose (C) and lactate metabolism (D) of the clones (c#T1, empty squares; c#T2, grey squares and c#T6, cross) and control cells (circles). Mean ± standard deviation from three independent cultures.

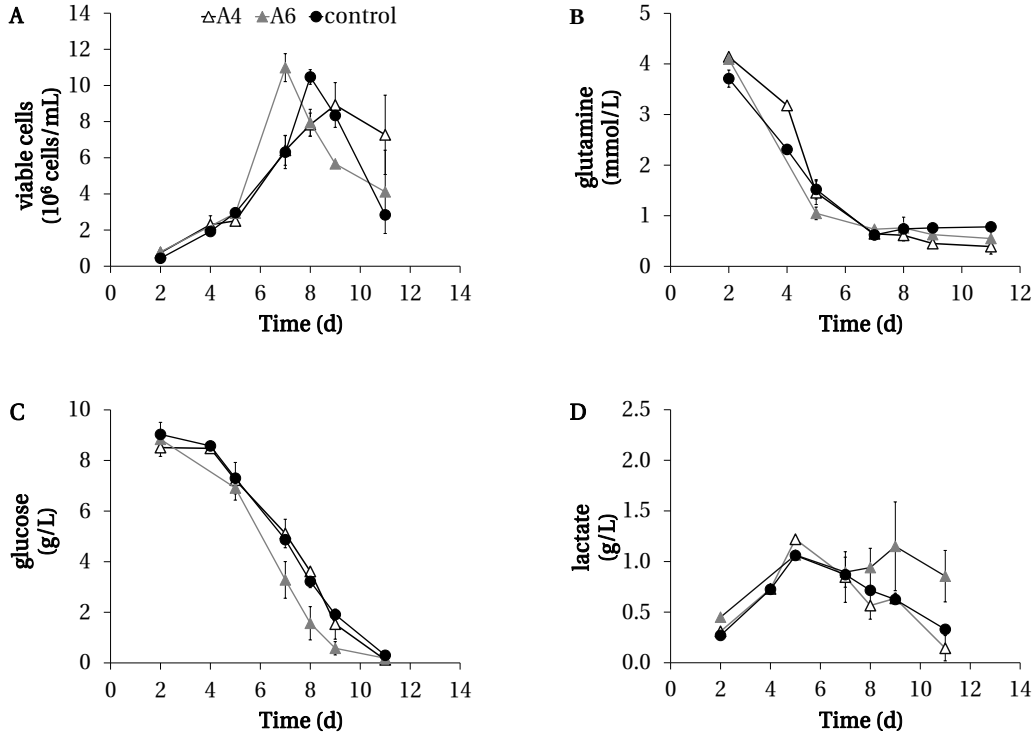


Figure 9.6: Control-derived aralar1 overexpressing clones cultivated in medium 1. Growth (A), glutamine (B), glucose (C) and lactate metabolism (D) of the clones (c#A4, empty triangles; c#A6, filled triangles) and control cells (circles). Mean ± standard deviation from three independent cultures.

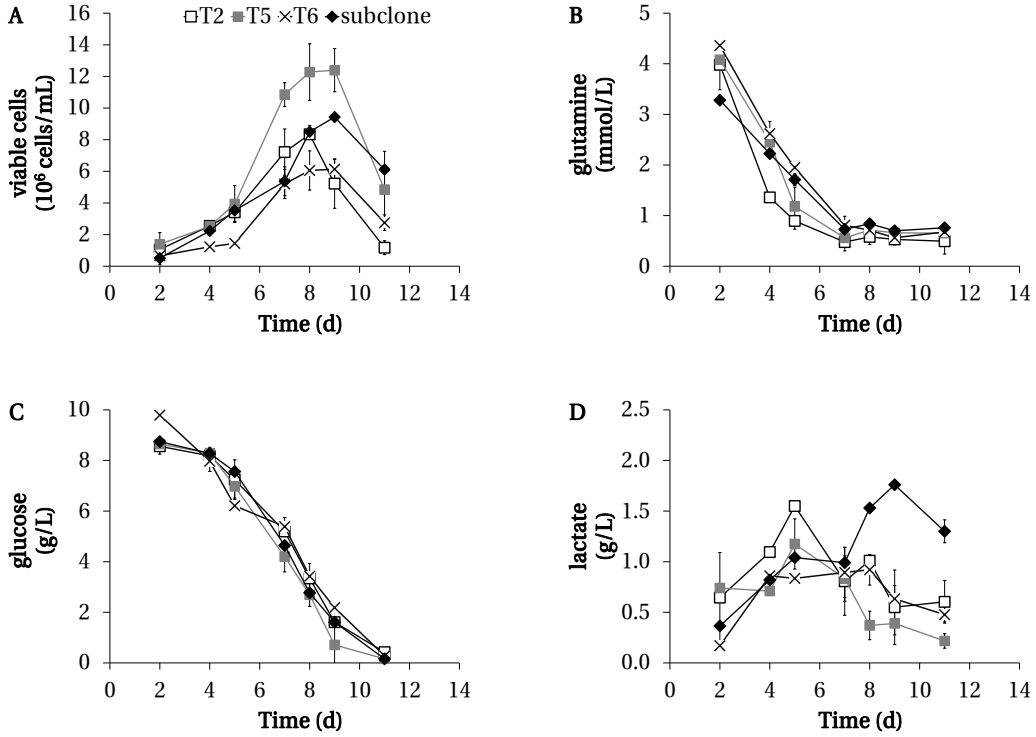


Figure 9.7: Subclone-derived timm8a overexpressing clones. Growth (A), glutamine (B), glucose (C) and lactate metabolism (D) of the clones (sc#T2, empty squares; sc#T5, grey squares and sc#T6, cross) and the untransfected subclone (diamonds) in medium 1. Mean ± standard deviation from three independent cultures.

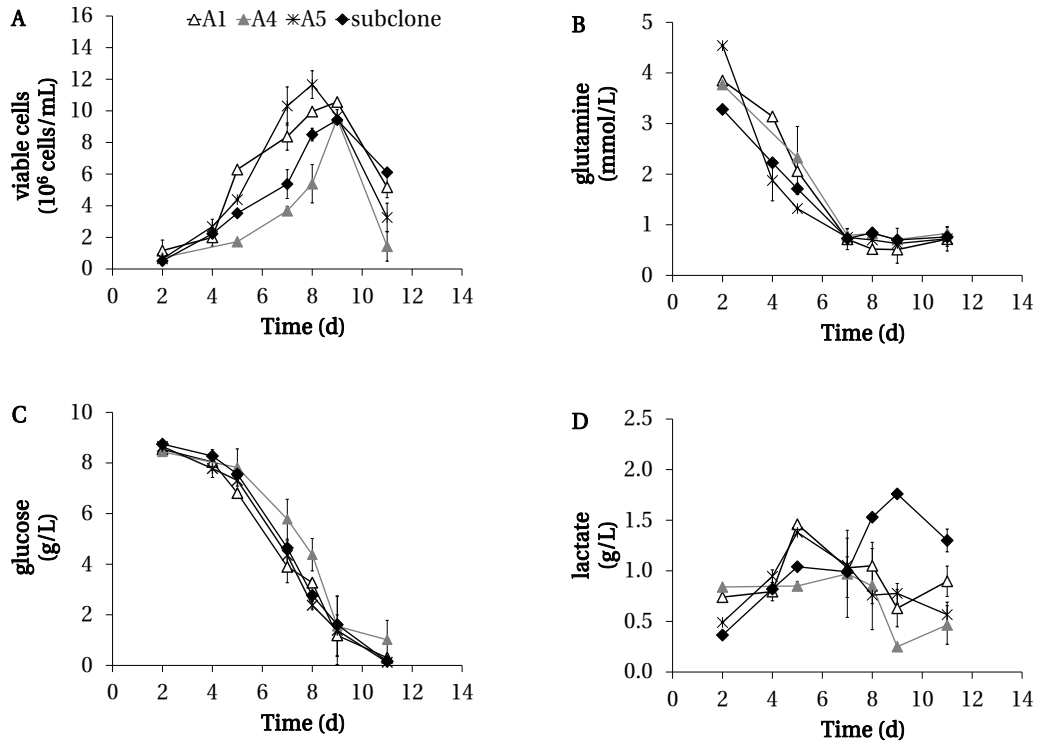


Figure 9.8: Subclone-derived aralar1 overexpressing clones. Growth (A), glutamine (B), glucose (C) and lactate metabolism (D) of clones (sc#A1, empty triangles; sc#A4, filled triangles and sc#A5, stars) and the untransfected subclone (diamonds) in medium 1. Mean ± standard deviation from three independent cultures.

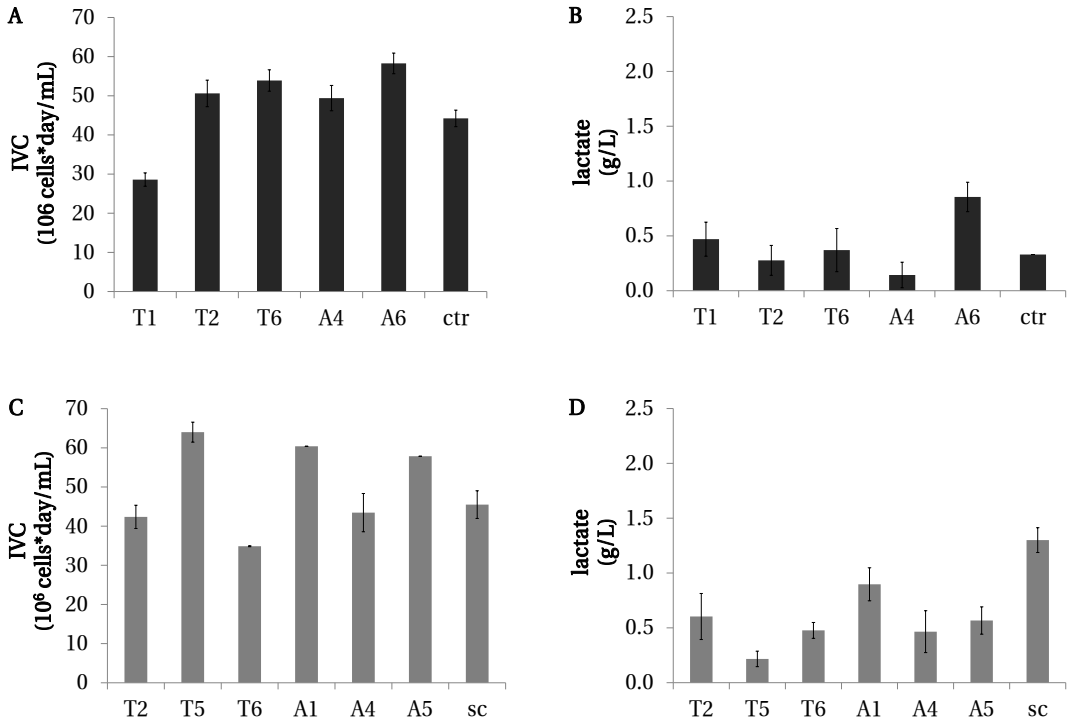


Figure 9.9: Integral of viable cell density and residual lactate concentration of the recombinant clones derived from the control cell line (A and B) and the subclone (C and D) on day 11. The untrasfected cell lines are referred as ctr, for the control cells, and sc for the subclone. Mean ± standard deviation from three independent cultures.

residual concentration was still higher than in the recombinant clones. In particular, timm8a clones showed a reduction of lactate levels of around 60% , for sc#T2 and #T6, and of 80%, for sc#T5, compared to the untransfected subclone. For the aralar clones, the residual lactate concentration decreased of 30%, for sc#A1, and of 50% , for sc#A4 and A5 (Fig. 9.9 D). It should also be noted that a weak increase of lactate levels occurred at the end of culture for clones sc#A1 and #A4, which also impacted the reported differences.

Clones sc#T5 and sc#A4, in particular, showed a more evident lactate consumption after day 5, compared to the other clones (Fig. 9.7 D and 9.8 D). Interestingly, this two clones were the most homogeneous population with the highest % of positive cells (Fig.9.10). Therefore, a correlation between the level of aralar or timm8a expression with the extent of lactate consumption could be hypothesized, even if the number of tested clones was not high enough to have statical significance.

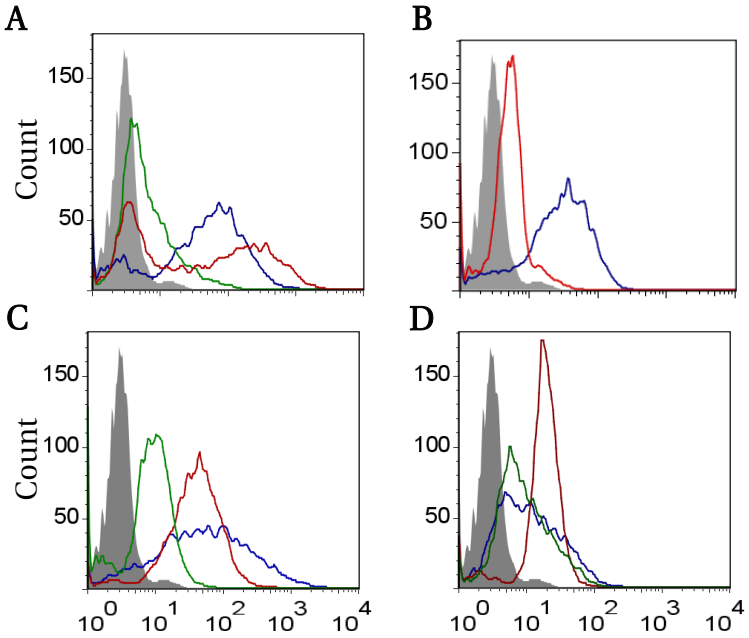


Figure 9.10: GFP fluorescence signal in recombinant clones. The untrasfected cell line is in grey in all graphs. (A) blue, c#T1; red, c#T2; green, c#T6. (B) blue, c#A4; red, c#A6. (C) blue, sc#T2; red, sc#T5; green, sc#T6. (D) blue, sc#A1; red, sc#A4; green, sc#A6. Green fluorescence intensity is reported on X-axis in logarithmic scale.

### 9.3.3 Mitochondrial metabolism in recombinant clones

The induction of the metabolic shift to lactate consumption in the subclone-derived clones most probably resulted in an increased pyruvate oxidation in the TCA cycle,

together with a more efficient NADH shuttling. In order to verify if the mitochondrial oxidative metabolism was indeed improved, the oxygen consumption rate was measured in the recombinant clones.

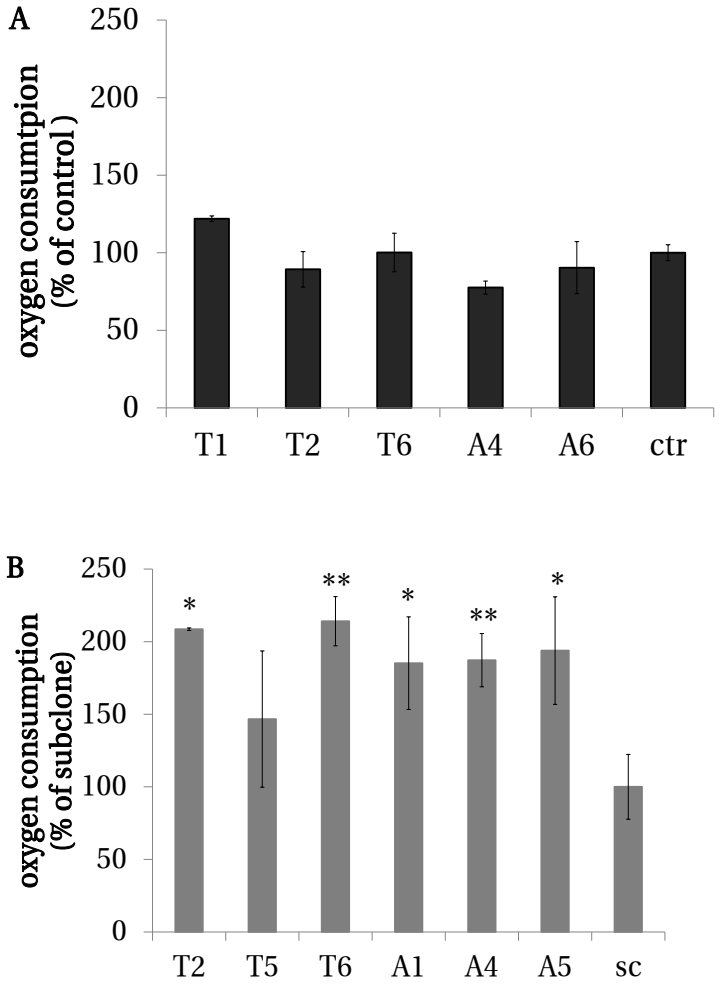


Figure 9.11: Oxygen consumption in control-derived clones (A) and in subclone-based clones (B). Normalized values are reported as percentage of the untransfected cell lines.The test was performed on day 8 of culture in medium 1. Mean± standard deviations from two independent tests. *, $p$ value < 0.05; **, $p$ value < 0.005 Student's $t$-test.

As expected, all sc# clones showed a significant increase in oxygen consumption compared to the untransfected subclone (Fig. 9.11 B). The only exception was sc#T5 which showed a more variable oxygen consumption. On the other hand, transgene expression did not impact the $O_2$ consumption in clones derived from the control cell

line (Fig.9.11 A) and this correlated with the unchanged metabolic profile observed in this group of recombinant clones.

The mitochondrial membrane potential of the recombinant clones was also evaluated. All clones showed a $\Delta\Psi_m$ value comparable or higher than the control cell line, thus confirming a state of increased mitochondria activity. However, a high variability was observed, which was most probably due to growth phase and viability differences among clones, and to GFP interference. Therefore, these data have not been included.

## 9.4 Discussion

Data reported in this chapter confirmed aralar1 and timm8a as potential targets to improve CHO lactate metabolism in industrial cell culture.

The malate-aspartate shuttle role was investigated by both inhibiting and promoting its activity. Indeed, MAS activity inhibition with aminooxyacetate prevented the switch to lactate consumption in the control cell line. Interestingly, the lactate over production persisted until glucose depletion. Afterwards a slight consumption occurred, as already observed in the subclone. Glucose profile, instead, was not impacted by the treatment. The resulting behaviour clearly indicates that the higher lactate accumulation can be due to a less efficient MAS functioning, which is no more able to handle the NADH produced during glycolysis. Therefore, a part of it needs to be recycled by pyruvate reduction. After glucose depletion, the NADH generated during lactate oxidation can instead be re-oxidised by the residual MAS activity.

Aralar and timm8a overexpression resulted in an improved lactate metabolism. Indeed, the clones derived from subclone transfection became able to switch to lactate consumption after day 5, and oxidised glucose and lactate simultaneously (Fig. 9.7 and 9.8). Glucose metabolism remained unchanged, thus indicating that lactate consumption was not due to a slowing down of the glycolytic flux, but it was promoted by a better NADH oxidation through the malate-aspartate shuttle. Interestingly, aralar1 and timm8a overexpression did not modify the metabolism of the control cell line, since the shift to lactate consumption was maintained ( Fig.9.5 and 9.6). Accordingly, the oxygen consumption increased in the subclone-derived clones, while it remained mostly unchanged in the others (Fig.9.11).

No strong difference has been observed between timm8a and aralar1 clones, indicating that both genes positively impacted cell metabolism. However, while the improvement observed for the aralar1 clones can be ascribed only to MAS activity, timm8a expression can have a wider downstream impact. Indeed, substrates of timm8/timm13 complex include the aspartate/glutamate carrier, but also timm23 and most probably the ATP-Mg/Pi carrier.

The ATP-Mg/Pi carrier is also a calcium activated transporter, as aralar1, and its activity is modulated by the cytosolic $Ca^{2+}$ levels (Arco and Satrústegui, 2005). It catalyses the reversible transport of ATP-Mg in exchange for phosphate, through the mitochondrial membrane, thus controlling the adenine nucleotide content in the mitochondria (Gellerich et al., 2010). However, its exact role and how it interacts with the other adenine translocators are still elusive.

Timm23, instead, is a component of the mitochondrial translocation machinery and mediates the correct sorting of protein both directed to the matrix and the inner membrane (Rehling et al., 2003). Therefore, an impairment of timm8a functioning can impact the correct sorting of multiple proteins, which are timm23 substrates. As already discussed, the deafness/dystonia disease has been linked to a timm8a mutation. Its symptoms, indeed, are hypothesized to be the result of the reduced timm23 translocation, which ultimately impacts the cell oxidative capacity (Roesch et al., 2002).

In conclusion, aralar1 and timm8a seem to be good candidates to develop a host cell line with an improved lactate metabolism. Indeed, their overexpression can ensure a more balanced link between glycolysis and mitochondrial metabolism and favour lactate consumption, by promoting an efficient NADH shuttling into the mitochondria.

# Conclusions & perspectives

# 10

# Conclusions & perspectives

## 10.1 Conclusions

An optimal cell metabolism is of paramount importance in industrial process in order to obtain a sustained growth and high productivity. However, *in vitro* metabolism is often deregulated with a fast and inefficient nutrient oxidation and byproducts accumulation. Lactate, as a waste product, can be detrimental for its impact on pH and osmolarity, consequently to base addition (Lao and Toth, 1997). Therefore, an excessive accumulation of lactate must be avoided and efforts are still made to better understand and control lactate metabolism in industrial cell culture (Neermann and Wagner, 1996; Altamirano et al., 2000; Chong et al., 2010).

In this study, lactate metabolism in CHO cell culture has been analysed with two different but complementary approaches. At first, different media and cell lines were compared to evaluate their impact on the mitochondrial oxidative metabolism. Then, the expression of selected genes was analysed to identify possible correlations with the observed lactate profile.

Lactate is most often considered as the end product of glucose consumption and its production is generally associated to the exponential growth phase (Mulukutla et al., 2010). In this work, instead, it was showed that glutaminolysis also plays an important role. This metabolic pathway is characteristic of fast proliferating cells, since it provides the NADPH used for lipid synthesis (DeBerardinis et al., 2007). In our model glutamine was rapidly consumed during the first 5-6 days to fuel the TCA cycle. This phase was accompanied by a high lactate production. Probably, malate efflux out of mitochondria and its conversion to pyruvate contributed to the observed lactate build up. Later in culture, glutamine depletion induced the control cells to use lactate as an alternative source of energy to replenish the TCA cycle. This hypothesis is further supported by the fact that glutamine feeding on day 5 resulted in an

almost stoichiometric lactate increase in the control cells. The switch to lactate consumption still occurred, when also the extra glutamine was depleted. Glucose was still at high concentration in the medium on the day of the shift and was consumed simultaneously to lactate.

The isolation of a subclone of the control cells, which instead accumulated higher amount of lactate in a glucose-dependent way, allowed a more in depth investigation of the metabolic shift. The impact of the cell line and media composition was also evaluated. Low alanine and high copper concentrations were identified as important factors to maintain the metabolic switch. The role of copper was also highlighted by Genentech (Luo et al., 2011). Nevertheless, the cell line still represented an important parameter which impacted the magnitude of alanine and copper effect.

The mitochondrial oxidative capacity was also evaluated by measuring the mitochondrial membrane potential and the oxygen consumption. Both parameters showed a significant reduction in the conditions of high lactate accumulation. Most probably, pyruvate diversion to lactate production caused a decrease of the mitochondrial oxidative metabolism, which was not counteracted by the oxidation of alternative nutrients. Hence, these results confirmed that mitochondria play a key role in the control of lactate oxidation.

The gene expression assay was, therefore, mostly focused on the analysis of mitochondrial metabolism and intermediates uptake. Among the 22 amplified genes, two , *aralar1* and *timm8a*, were down-regulated in conditions of strong lactate accumulation. Aralar1 is a key component of the malate-aspartate shuttle (MAS), which allows for the oxidation of the cytosolic NADH (Bender et al., 2006). During glycolysis, two molecule of NADH are produced at each cycle and they need to be recycled to maintain the glycolytic flux. This can occur either through the malate-aspartate shuttle or by pyruvate reduction into lactate. Therefore, if MAS activity is impaired, as in the case of low aralar1 expression, the LDH pathway would be fostered.

In this work, it has been shown that MAS inhibition with aminooxyacetate blocked the metabolic switch in the control cells. Moreover, the resulting metabolic phenotype was highly similar to the subclone one.

Aralar1 overexpression, instead, promoted the switch to lactate consumption in the subclone. On the other hand, the already efficient lactate metabolism of the control cells was not perturbed by the transgene expression.

The other gene, timm8a, is a component of the mitochondrial translocation machinery and promotes the correct sorting of the proteins targeted to the inner membrane, such as aralar1 (Roesch et al., 2004). Its overexpression induced the same improvement of lactate metabolism as aralar1. However, since timm8a can impact the localization of other mitochondrial proteins , its effect can not be univocally ascribed to an improved MAS functioning. To better elucidate this point the mitochondrial

fraction should be analysed to evaluate the enrichment of timm8a and timm23 substrates. However, this was not performed since it was beyond the scope of this thesis.

To summarize the studied model, it was postulated that during the first phase of culture, the malate-aspartate shuttle is able to handle the NADH produced during glycolysis, and lactate is mainly the result of a truncated TCA cycle (Fig. 10.1 A). Pyruvate reduction into lactate also contributes to NAD regeneration. After glutamine depletion, the metabolic switch can occur only if MAS activity is sufficient to counteract the extra NADH production, resulting from lactate oxidation (Fig. 10.1 C). Otherwise, a stronger lactate accumulation would occur (Fig. 10.1 B).

MAS functioning, ultimately, depends directly on aralar1 expression level and ,indirectly, on timm8a translocation activity.

## 10.2 Perspectives

This study highlighted the importance of mitochondrial oxidative capacity in lactate metabolism. Optimized media can boost the mitochondrial metabolism, resulting in an improved lactate profile. However, intrinsic cell characteristics, such as aralar1 expression, can also have an impact. Therefore, the monitoring of oxygen consumption could be proposed as a tool to select clones with an optimal metabolism, or for nutrients screening to improve media composition. However, further optimization is needed to fulfil the high throughput level required for an industrial application.

It should be noted that oxygen probes are normally present in all culture bioreactors. Nevertheless, the generated data are often not regarded as indicators of the mitochondrial oxidative state, while they could be highly informative, for instance, to evaluate the impact of a feed on the mitochondrial metabolism.

Aralar1 and timm8a were identified as promising targets for the generation of a host cell line with improved lactate metabolism. However, their impact on productivity still need to be evaluated. Nevertheless, it can be speculated that a more efficient substrates consumption in the recombinant clones would provide more energy for the production of the therapeutic protein.

Moreover, it would be of interest to evaluate an inducible system for the overexpression of aralar1. Indeed, the possibility to switch from fast lactate accumulation to consumption could be advantageous to exploit the rapid cell proliferation often associated to the lactate production phase. Afterwards, the transgene activation would promote the lactate switch from waste product to energy source. This flexible system could also be useful in case of optimized media where lactate consumption is already favoured.

In this work, an impact of medium composition on aralar1 and timm8a expres-

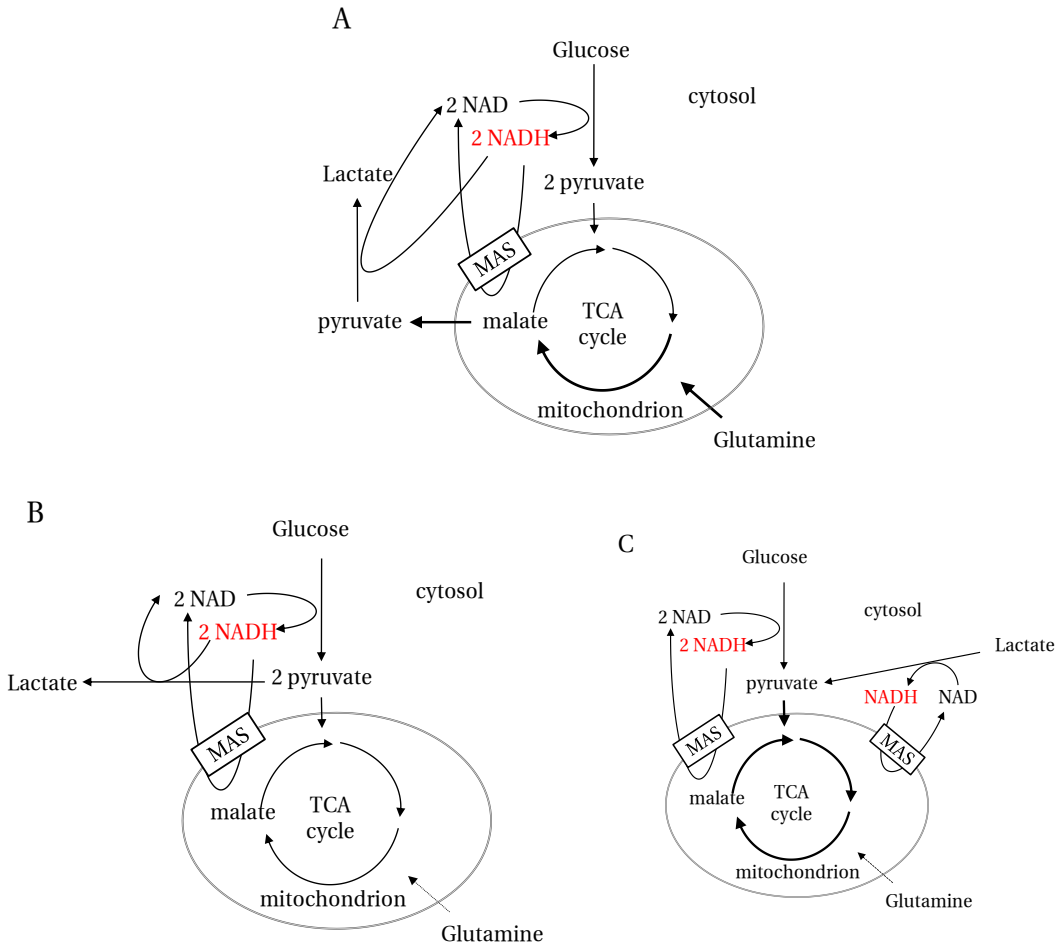


Figure 10.1: Hypothesized glucose and glutamine metabolism in the studied model cell line. (A) Initial lactate production phase is common to the control and subclone cells. After glutamine depletion only control cells consume lactate due to the higher MAS expression (C), while the subclone accumulates lactate as alternative reaction to reoxidise NADH (B).

sion has been highlighted. Further analysis are needed to identify the key components and the signaling pathways that are involved, which could open new possibilities for both media optimization and cell engineering. The CHO-K1 genome availability provides possibilities to more precisely study other candidate genes, such as PGC1$\alpha$, which was, indeed, included in the study but no reliable signal was obtained (Xu et al., 2011). PGC1$\alpha$, in particular, regulate mitochondrial biogenesis and metabolism and its expression and activity are finely regulated (Fernandez-Marcos and Auwerx, 2011; Cantó and Auwerx, 2012). Another interesting target could be the uncoupling proteins, UCP2 and 3. Interestingly, UCP2 expression is influenced by glutamine at the translational level and it is hypothesized that it could have a role in the metabolism of this amino acid (Criscuolo et al., 2006). UCP3 also seems to be involved in the control of mitochondrial metabolism in presence of glucose and, in particular, an activity in promoting pyruvate efflux out of the mitochondria has been postulated (García-Martinez et al., 2001; Mozo et al., 2006).

In conclusion, this thesis work has shown that the integration of metabolic analysis and transcriptome profiling can reveal new interesting insights to elucidate mammalian cell behaviour in industrial cell cultures and can provide tools to control it.

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# Curriculum vitae

**Curriculum vitae**
Francesca Zagari

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**Work experience**
March 2009 – present **PhD in Biotechnology and Bioengineering**, *École polytechnique Fédérale Lausanne (EPFL)* and *Merck Serono*, Fenil-sur-Corsier (Switzerland). Research project on analysis of mammalian cells metabolism in industrial bioprocesses. Focus on media impact on lactate production, gene expression analysis and cell engineering.

July 2007– February 2009 **Technical Service Engineer**, *Millipore Corporation*, Molsheim (France). Main roles: technical problem solving and customers and sales force support.

March 2006 — July 2007 **Product Specialist**, *Globe Diagnostic*, Milan (Italy). Main roles: development and validation of new applications for automated ELISA analyzers and customer support.

September 2005 – March 2006 **Internship**, *GREPI laboratory, CHU-Joseph Fourier University*, Grenoble (France). Research project on the development of new immunogenic vectors for the immunotherapy of melanoma.

July 2004 — June 2005 **Internship**, *Molecular Pharmacology Laboratory, University of Milan.* Research project: Vectors development for the generation of animal and cell models for *in vivo* imaging

**Education**
March 2009- **PhD in Biotechnology and Bioengineering**, *EPFL*, Lausanne, Switzerland

September 1998 - March 2004 - **Master Science Degree in Pharmaceutical Biotechnology**, *University of Milan*, Italy. Master thesis internship in the Laboratory of Molecular pharmacology, Department of Pharmacological Science. Research project on the transcriptional regulation of the oestrogen receptor expression and SNP correlation with oestrogen-dependent disease.

**Foreign languages**
English: fluent oral and written. Cambridge First Certificate in English (FCE), June 2005.
French: fluent oral and good level written

**Posters**

F Zagari, M Jordan, B Kleuser, L Baldi, D Hacker, M Stettler, FM Wurm , H Broly. *Mitochondrial membrane potential and ROS levels correlate with different lactate profiles in CHO cells.*
FEBS 2011, Turin, Italy

F Zagari, M Jordan, B Kleuser, D Beattie, H Broly1 , FM Wurm. *Analysis of CHO cells metabolism in a chemically defined medium* .
Life Science Symposium 2010, Lausanne, Switzerland

P. Ciana, F. Zagari, and A. Maggi. *Regulation of ERα expression and activity during cell cycle.*
CASCADE Summer School on Nuclear Receptors 2004, Ecole Normale Supérieure de Lyon, France.

**Publications**

Francesca Zagari, Martin Jordan, Matthieu Stettler, Hervé Broly, Florian M. Wurm. *Lactate metabolism shift in CHO cells culture: the role of mitochondrial oxidative activity.* Submitted to New Biotechnology.