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Metabolomics and Mammalian Cell Culture

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1. Introduction

Since the mid-1950s, when pioneering work of Earle and colleagues (1954) enable routine cell culture, mammalian cell culture has been used in the large-scale production of recombinant protein and monoclonal antibodies. Mammalian cell lines are preferred as production host for many pharmaceuticals, since complex post-translational modifications of the produced proteins (especially glycosylation) are generally not properly performed by microbial systems (Lake-Ee Quek et al., 2010).

Wagburg described that under batch conditions, mammalian cells display an inefficient metabolic phenotype characterized by high rates of glucose to lactate conversion (Warburg, 1956) together with partial oxidation of glutamine to ammonia and non-essential amino acids (Fitzpatrick et al., 1993; Jenkins et al., 1992; Ljunggren and Haggstrom, 1992; Ozturk and Palsson, 1991). The accumulation of fermentation by-products causes a reduction of the culture density and product titer that can be realized (Martinelle et al., 1998).

In order to increase the cell productivity a common optimization approach is to grow cells to moderately high density in fed-batch and the deliberately induce a prolonged, productive stationary phase. While optimization of this perturbed batch strategy is responsible for the increase of monoclonal antibodies titer seen over the past decades it has a number of shortcomings, including:

a. the strategy has to be refined for each new cell line,
b. the ultimate metabolic phenotype during prolonged stationary phase varies between cell lines and it is not always possible to achieve the most productive phenotypes for a given strain
c. volumetric productivity remains relatively low due to moderate cell density (Lake Ee Quek et al., 2010).

Other approaches are related with the changes of cell phenotypes through metabolic engineering or change of culture media conditions in order to manipulate the cellular metabolic behavior.

Although transcriptomics and proteomics have been explored extensively for mammalian cell engineering (Korke et al., 2002; Seow et al., 2001; Seth et al., 2007; Smales et al., 2004; de la Luz et al., 2007, 2008) these tools fall short of generating direct measurements of the physiological state of the cell. It is essential to combine these techniques with metabolic flux

analysis (MFA) a powerful method to quantify the manifestation of a phenotype: the intracellular reaction rates or the fluxome. One of the relatively new "omic" sciences is the field of metabolomics. The metabolome was first described by Oliver and collagues (1998) as being the set of all of low-molecular-mass compounds synthesized by an organism. Metabolomics is therefore the analysis of small molecules that constitute the metabolism, and it offers the closest direct measurements of a cell´s physiological activity (Beecher, 2002; Khoo and Al-Rubeai, 2007). The metabolomic analysis can be considered as "the measurement of the change in the relative concentrations of metabolites as the result of the deletion or overexpression of a gene, should allow the target of a novel gene product to be located on the metabolic map". Another definition of the metabolome states that it consists of "only those native small molecules that are participant's in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell" (Khoo and Al-Rubeai, 2007).

The metabolomic as a new powerful tool to understand the complex processes of large scale mammalian cell cultures for biopharmaceutical production has not been yet embraced during process development and scale-up. This is mostly because metabolites are now not the primary focus and the relationship between metabolites and protein production in different media are not fully understood. It is for this reason that metabolomics can bridge the gap of understanding as to the dynamics of metabolism, cell growth and protein production (Khoo and Al-Rubeai, 2007). The metabolomics can be use to optimize conditions of bioreactors or the development chemically defined media. Characterizing cell lines, culture media and selection of cell lines are a vital step in the process development of biologics. In this chapter, we describe the state-of-the-art of the use of metabolomics tool in mammalian cell lines.

2. Complexity of metabolome analysis

Metabolomics requires the unbiased identification and quantification of all of the metabolites present in a specific biological sample (from an organism or *in vitro*). Metabolites are generally labile species, by their nature are chemically very diverse, and often present in a wide dynamic range. For analysis of mRNA and proteins one "only" needs to know the genome sequence of the organism and exploit this information using nucleic acid hybridization or protein separation followed by MS (although PTM are problematic). However, the analysis of metabolites is not as straightforward. In contrast to transcripts or protein identification, metabolites are not organism specific (that is to say, sequence dependent) (Hollywood et al., 2006).

In addition, their diverse chemical properties make complete metabolite analysis difficult. Genes are composed of a linear four-letter code, whereas proteins have a 20-letters code of primary amino acids. Metabolites do not have any fixed codes, and thus a general method of characterization is difficult. Present methods use the specific chemical properties of these entities to separate, identify and decipher their structures. Combinatorial approaches allow for a greater coverage. An ideal metabolic analysis should provide:

a. Give an instantaneous snapshot of all metabolites in any given system,
b. Use analytical methods that have high recovery, experimental robustness, reproducibility, high resolving power and high sensitivity (Fiehn, 2001) whilst being able to be applied universally,

c. Provide the unambiguous quantification and identification of metabolites and (d) factors to be highlighted while easily being incorporated into biochemical network models (Soo H and Al-Rubeai M, 2007).

Before any metabolome measurements are taken it is essential that metabolism is stopped as quickly as possible, especially because the enzymes are active. For animal cells liquid N_2 is used to snap freeze the sample, followed by mechanical disruption in order to release the metabolites (Viant et al., 2005). The next stage of the analysis is to extract the metabolites. There are many different methods (Tweeddale et al., 1998; Buchholz et al., 2001; Villas-Boas et al., 2005) and the most common ones are:

- Acid extraction using perchloric acid, followed by freeze thawing, then neutralization with potassium hydroxide
- Alkali extraction typically using sodium hydroxide, followed by heating (80^{o}C)
- Ethanolic extraction by boiling the sampling in ethanol at 80^{o}C

When the extract is finally ready, the choice of the analytical tool is based on the level of chemical information required about the metabolites, remembering that there will be a chemical bias with respect to that method, and the speed of analysis is also another consideration. The figure 1 shows different methods and approaches used for the metabolomic analysis.

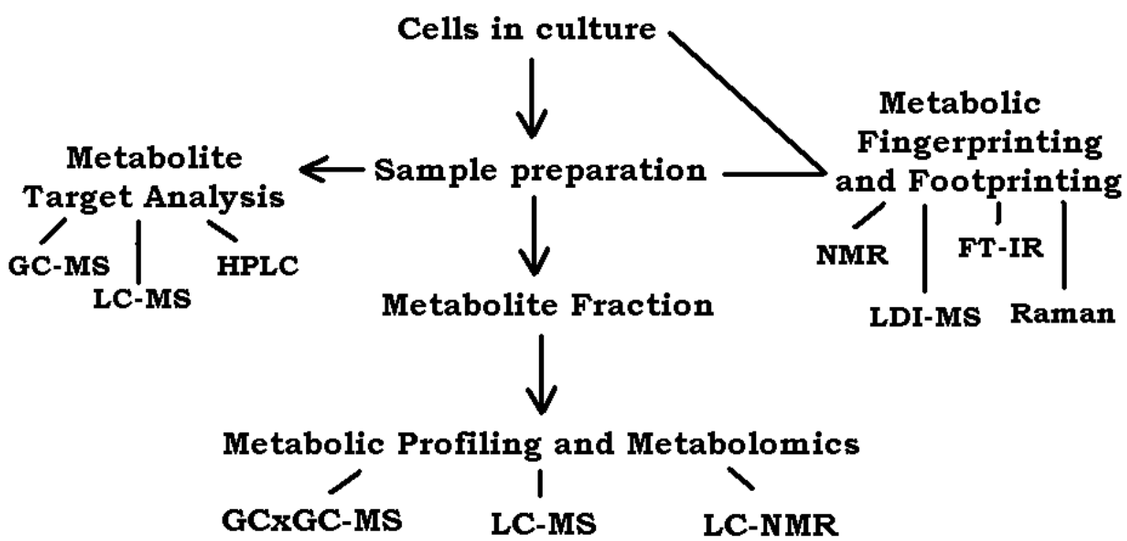

Fig. 1. Technologies for metabolome analysis. GC-MS: gas chromatography mass spectrometry, GCxGC-MS: 2 dimensional GC coupled to mass spectrometry, LC-MS: liquid chromatography mass spectrometry, HPLC: high performance liquid chromatography, LC-NMR: liquid chromatography coupled to nuclear magnetic resonance, NMR: nuclear magnetic resonance, LDI-MS: laser desorption ionization mass spectrometry, FT-IR: Fourier transform infrared spectroscopy

The most popular approaches are:

1. Metabolite target analysis: which is an approach that is restricted to metabolites for example a particular enzyme system that would be directly affected by any perturbation
2. Metabolite profiling: which is focused on a specific group of metabolites (for example lipids) or those associated with a specific pathway; within clinical and pharmaceutical analysis, this is often called metabolic profiling, which is used to trace the fate of a drug or metabolite
3. Metabolomics: is the comprehensive analysis of the entire metabolome, under a given set of conditions
4. Metabonomics: which seeks to measure the fingerprint of biochemical perturbations caused by disease, drug and toxins.
5. Metabolic fingerprinting: is used to classify samples based on provenance of either their biological relevance or origin by using a fingerprinting technology that is rapid but does not necessarily give specific metabolite information.

3. Experimental design

3.1 Cell culture growth, stimulation

The differences in optimized cell culture growth conditions present another major concern for cell line metabolomics. This is particularly an issue is studies involving comparative analysis of several different cell types, all of which might contain different levels of glucose, glutamine and lactate, as well as other nutrients and additives, which will probably lead to differences in the metabolome of the cells. If possible, it is recommended to use the same growth medium for all cell lines in the study to reduce variance in metabolic profile that can be caused by the medium (Cuperlovic-Culf et al., 2010). The standard enhancement of cell culture medium with serum of animal origin can add another level of complexity in cell growth condition optimization. Variations in serum can lead to contamination with exogenous metabolites and alterations of endogenous cell metabolite.

In order to minimize the influence of different cell culture conditions in the metabolomic final results, proper experimental designs are crucial. Nonetheless, more effort is required in the future for the determination of metabolic differences caused by various growth conditions, cell culture age and/or passage number for different cell lines.

3.2 Sample preparation and metabolite extraction

The goal of metabolomics is to analyze all or, at least, as many as possible different metabolites without selectivity for any particular molecular type and/or characteristics. The correct sample preparation is the first step in order to ensure the detection of a large number of metabolites. As metabolic processes may be rapid, varying from milliseconds to minutes (Gerdtzen et al., 2004; Taoka and Banerjee, 2002), the first necessary step is to rapidly stop any inherent enzymatic activity or any changes in the metabolite levels. The time and method of sampling are important issues to be considered to ensure reproducibility in the analytical sample, especially since a large number of biological replicates is commonly used (Khoo and Al-Rubeai, 2007).

These methods include freeze-clamping (with lower-temperature receptacles), immediate freezing in liquid nitrogen or by acidic treatments (ap Rees and Hill, 1994). Freezing in liquid nitrogen is generally considered to be the easiest way of stopping enzyme activity provided that cells or tissues are not allowed to partially thaw before extracting metabolites. In order to prevent this from happening, enzyme activity is inhibited by freezing-drying or by immediate addition of organic solvents while applying heat. Cells are subsequently disrupted, releasing the metabolites. Frozen samples may be ground down by sonication, homogenization by mechanical means in pre-chilled holders (Fiehn et al., 2000) or directly in an extraction solvent (Orth et al., 1999). The mixture of cell debris, protein, nucleotides and the desired metabolites need to be separated; this can be done by centrifugation or filtration.

For the complete analysis of a cell culture, it is important to measure both extracellular (footprint) and intracellular (fingerprint) metabolic profiles. Metabolic footprinting is technically simple because it requires only centrifugation to separate culture media and cells before the analysis. Metabolic fingerprinting, although much more technically challenging because it requires metabolite extraction from cells, provides more complete information about cellular metabolic processes (Cuperlovic-Culf et al., 2010). Recently a study related with different metabolite extraction protocols for mammalian cell culture was published (Dietmair et al., 2010). In this study, the authors compared 12 different extraction methods, according to their results; extraction in cold 50% aqueous acetonitrile was superior to other methods.

3.3 Analytical Instruments platforms

Currently, the main analytical techniques used for the analysis of the metabolome are nuclear magnetic resonance spectroscopy (NMR) and hyphenated techniques such as gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS). In addition other combinations are possible, e.g. capillary electrophoresis (CE) coupled to MS or LC coupled to electrochemical detection. Alternatively, Fourier transform infrared spectroscopy (FTIR) and direct infusion mass spectrometry (DIMS) have been applied (Dunn and Ellis, 2005; van Greef et al., 2004; Lindon et al., 2007; Koek et al., 2010) without any prior separation, except for eventual sample preparation. NMR, FTIR and DIMS are high throughput methods and require minimal sample preparation and may be preferred techniques for metabolic fingerprint. However, the obtained spectra are composed of the signals of very many metabolites and elucidation of these complex spectra can be very complicated. In addition, detection limits for NMR and FTIR are much higher that for MS-based techniques, limiting the application range to metabolites present in higher concentrations. Therefore, GC, LC and CE coupled to MS are generally preferred in metabolomics to allow quantification and identification of as many as possible metabolites.

The general requirements for metabolomic instruments are:

- Excellent sensitivity and resolution for a wide range of molecules types
- The ability to handle a large range of concentrations (from pM to mM) for different molecular types
- The ability to identify and quantify different molecules
- Short analysis time
- To enable the measurement of many samples without sample degradation during the measurement
- Reproducible measurement across different centers and time

Several reviews have dealt with the application of NMR and MS in metabolomics (Ala-Korpela, 2008; Detmer et al., 2007; Griffin, 2003). NMR is a non-invasive, non-destructive, highly discriminatory and fast method that can analyze rather crude samples. NMR spectroscopy can be performed without extensive sample preprocessing and separation and provides several different experimental protocols optimized for mixture analysis and molecular formula or structure determination. The results of NMR measurements have proven highly replicable across centers and instruments (Viant et al., 2005). NMR can provide measurements for different types and sizes of both polar and non-polar molecules through analysis of different spectral windows. In addition, NMR instruments are highly versatile and with only minor changes in probes, users can obtain spectral information for different nuclei (^{1}H, ^{13}C, ^{15}N, and ^{32}P among others) in solvent or solid samples and even *in vivo* (Griffin, 2003). NMR is also the only method used in metabolomics that currently enables direct measurements of molecular diffusion, interactions and chemical exchange. Several databases and methods are being developed that enable metabolite identification and quantification from NMR spectra (Table 1). The major problem with NMR technology as applied to metabolomics is its low sensitivity, which limits the majority of currently available instruments to measurement of fewer than 100 metabolites.

The role of MS in metabolomic research is constantly expanding, whether the focus is on profiling (targeted analysis) or pattern-based analysis (Hollywood et al., 2006). Recent technological advances in separation science, ion sources and mass analyzers have considerably increased the sensitivity, selectivity, specificity and speed of metabolite detection and identification by MS. There are five important considerations that need to be dealt with in any global metabolite analysis by MS:

1. The efficient and unbiased extraction of metabolites from the sample matrix
2. Separation or fractionation of the analytes by chromatography
3. Ionization of the analyte metabolite
4. Detection of mass signals
5. Analyte identification

Name and availability	Instrument	Additional information
Human Metabolome Project (http:/www.hmdb.ca)	NMR, MS	Biological data; chemical and clinical data specific to humans
BMRD (http:/www.bmrb.wisc.edu)	NMR	Database search for NMR peaks assignment
Prime (http:/prime.psc.riken.jp)	MS, NMR	
Glom metabolome database (http:/csbdb.mpimp-glom.mpg.de)	MS	Specific to plants
METLIN metabolite database (http:/metlin.scrpps.edu)	MS	Drug and drug metabolites; specific to humans
NIST chemistry WebBook (http:/WebBook.nist.gov/chemistry)	NMR, MS, IR	
Madison metabolomics database (http:/mmcd.nmrfarm.wisc.edu)	MS, NMR	
NMR Lab of biomolecules (http:/spinportal.magnet.fsu.edu)	NMR	Databse search for NMR peaks assigment

Table 1. Databases of metabolomic standard data for quantification and assignment

Separation of analytes before MS detection is an important step leading to detection of more features, effectively increasing the overall "peak capacity" of the analytical platform. Separation methods include condensed-phase separation methods and gas-phase analyte separation. DIMS relies solely on the mass spectrometer to perform separation and offers an advantage in terms of speed and sample throughput. The number of identified features in a MS measurements can also be increased by changing the polarity of the ion source. Positive ion mode electrospray is optimal for basic metabolites (e.g. amines). Negative ion mode provides optimal measurement for acidic metabolites. Knowledge of the empirical formula based on exact mass can often be used to assign one or a few putative identifications that can then be used for searching metabolic or chemical databases (Table 1).

A comparative outline of the characteristics of NMR and MS methodologies as applied to metabolomics is provided in Table 2. The two methods are highly compatible and, thus ideal approach is to combine the results from NMR and MS measurements.

The most common forms of chromatography are GC and LC. GC runs are relative long, at about 60 min or more (Gummer et al., 2009); however, deconvolution software allows for the decrease in run times. In LC there is a shift from standard HPLC to UPLC (ultra-performance liquid chromatography), which can significantly increase resolution sensitivity and peak capacity (Gummer et al., 2009) due to the reduced particle size, while decreasing sample volumes and mobile phases. UPLC systems operate at high operating pressures and use sub-2-µm porous packing. Unlike pressured systems such as LC, CE (capillary electrophoresis) makes use of an electric field to move molecules towards the detector, much like gel electrophoresis. CE coupled with UV or LIF (laser-induced fluorescence) detectors are highly sensitive, but lack selectivity.

GC coupled to MS is one of the most common instrument platforms to be used in metabolomics experiments. GC-MS instruments using linear quadrupole analysers have been available for decades providing a robust technology that is amenable to automation. The identification of a wide range of primary metabolites (often after derivitisation) is greatly facilitated by the high resolution of capillary GC, the reproducible fragmentation of metabolites in the mass spectrometer and the ready availability of large mass spectral libraries (Roessner et al., 2001). Recent developments in GC-MS have resulted in improvements in both the GC and MS capabilities of this platform and a move towards the use of high mass accuracy/ high mass resolution instruments. The requirement for high throughput has led to the use of nominal GC-time-of-flight TOF-MS with much faster scan rates. High scan rates allow rapid temperature gradient programs, resulting in shorter run times and increased sensitivity (Gummer et al., 2009). Alternatively, the combination of two-dimensional GC with TOF-MS has resulted in the development of very high resolution fast MS that can be used to detect more metabolites than is possible using single queadrupole (Q), TOF and ion trap mass analyzer.

One if the drawbacks of many GC-MS metabolomics analyses is the need to derivitise metabolites before analysis. In contrast, many classes of polar metabolites can be analyzed directly by LC-MS without derivitisation. LC systems interfaced with TOF mass analyzer are now commonly used in metabolomics analyzes, delivering high throughput and high

mass resolution analysis capability with mass accuracy approaching single digit ppm. Recently developed instruments also allow rapid polarity switching between positive and negative mode within a single run, reducing the need for multiple runs and cost per sample (Gummer et al., 2009).

Analysis	**NMR**	**MS**
High throughput-metabolites	No	Medium
High throughput-samples; automation	Yes	No
Quantitative	Yes	Yes
Availability in clinic	No	No
Equipment cost	High	High
Maintenance cost	Medium	High
Per sample cost	Low	High
Required technical skills	Yes	Yes
Sensitivity	Medium	High
Reproducibility	High	Low
Data analysis automation	Yes	Yes
Identification of new metabolites	Difficult	Possible
Chemical exchange analysis	Yes	No
In vivo measueremnt	Possible	Impossible

Table 2. Comparison of characteristics of major experimental methods for metabolomic analysis

LC-MS linear quadrupole, triple quadrupole (QQQ), QTrap and io trap mass analyzers have also been utilized for global and targete metabolomics, but may be limited by mass accuracy and mass resolution in identifying metabolites. However, the use of triple quadrupole and QTrap mass analyzers in various selective ion scaning modes can be used to detect specific metabolites or metabolite classes with high sensitivity and are particulary useful for targeted metabolomic analysis.

CE-MS offers a complementary approach to LC-MS for analyzing anions, cations, and neutral particles in a single run. Metabolites can be analyzed directly without derivitisation and the chromatographic resolution and sensitivity of CE is very high. However, CE is less frequently used for metabolomic analyzes tha LC-MS.

Vibrational spectroscopies are relative insensitive, but FTIR allows for high throughput screening of biological samples in an unbiased fashion. Similar to NMR, water signals pose a problem and must be subtracted electronically or attenuated total reflectance may be used. Compared with the other methods it is one of the least sensitive, but its unbiasness to compounds and ability to analyse large numbers of sample in a day makes it a plausible method for screening purposes (Khoo and Al-Rubeai, 2007).

LC-MS-based instruments can be operated in direct infusion mode with no chromatographic separation for measurement of the total mass spectrum for the mixture. The infusion can be performed with either the LC autosampler or with and offline syringe pump. Ion trap, TOF, Q-TOF, Orbitrap and FT-ICR-MS mass analyzers have been used with this mode of sample delivery. This approach relies totally on the mass analyser to resolve isobaric metabolites such as leucine or isoleucine. The key advantange of direct infusion analysis is the potential

for automated high throughput sample analysis with both low and high mass resolution mass analyzers.

Another beneficial experimental method for cell culture metabolomics analysis involves stable isotope labeling followed by either MS or NMR measurement. This approach enables pathway tracing, easier metabolite assignment and metabolic flux measurements. Isotopic labeling has previously enabled detailed determination of pathways leading to the production of specific metabolites and the development of the highly accurate mathematical models of these pathways (Hollywood et al., 2006).

4. Aplications in mammalian cell culture – Study cases

4.1 Analysis of molecular mechanisms associated to the adaptation of NS0 myeloma cell line to protein-free medium

The NS0 mouse myeloma cell line has become one of the most popular systems for large-scale heterologous protein expression. For reasons of regulatory compliance, cost, batch consistency, downstream processing, and material availability, industrial applications of NS0 has moved towards serum or protein-free medium platforms (Barnes et al., 2000). For serum- or protein-free cultivation, the cell culture medium is often supplemented with lipids (derived from plant or synthetic sources) in addition to other protein supplements. The effect of lipid supplementation on the physiology of hybridomas and myelomas has been reported (Jenkins et al., 1992). NS0 cells are naturally cholesterol-dependent; not only is their growth greatly facilitated by lipid supplementation, but is also dependent on provision of cholesterol. NS0 cells capable of cholesterol-free growth can be isolated by selecting mutant clones or by adaptation. Adaptation generally involves passaging cells over a time period during which the serum concentration is decreased gradually (Sinacore et al., 2000). Eventually, the resulting population develops the capability to grow in the absence of serum. Different mechanisms underlying a cholesterol-dependent phenotype could include the absence (or mutation) of a gene or a segment of gene along the cholesterol biosynthesis pathway. There could be changes in the expression level of some proteins of the pathway due to gene regulation or other control mechanisms. In addition to the specific gene expression alterations along the cholesterol and lipid metabolism pathways, cholesterol dependence could also be the result of insufficient precursor supply (Spens and Haggstrom, 2005).

The molecular mechanisms of host and recombinant NS0 cell lines that could be related to the adaptation to protein-free medium are studied in this work. A quantitative study of proteins with differential expression levels in four conditions (host NS0 cell line adapted and non-adapted to protein-free medium, and a monoclonal antibody (Mab) transfectoma producer NS0 adapted and non-adapted to the same protein-free medium) is reported. The study is based on the use of the combination of two-dimensional electrophoresis and mass spectrometry, and a novel quantitative proteomic approach, isobaric tagging for relative and absolute quantification (iTRAQ). The metabolic study of these cell lines cultured in different nutrient conditions is also reported. Taking into account the proteomic results and metabolic analysis, a possible mechanism related to the adaptation of NS0 cell line to protein-free medium is proposed.

4.1.1 Results: Proteomic analysis

To characterize the changes associated with the adaptation to the protein-free medium 2DE gels of protein extracts from the cell line cultured in PFHM II with or without 1% (v/v) FBS were compared over a pI range of 3 to 10. Following adaptation to the protein-free medium, 78 spots changed their intensity by a factor of ≥2 from 1200 detected spots/ treatment. Interestingly, the majority of differentially expressed proteins decreased their expression in cells adapted to the protein-free medium. Fifty eight proteins were characterized by MALDI-MS and/or LC-ESI-MS-MS. The identified proteins were grouped according to their molecular function. Four major cellular pathways seem to be involved in the adaptation to the protein-free medium: i) carbohydrate metabolism and energy production, especially glycolysis and the Krebs cycle, ii) protein synthesis and folding; iii) membrane transport, and iv) cell proliferation (de la Luz et al., 2007).

In order to increase the number of proteins related with cell cycle regulation, DNA replication and lipids synthesis we used another strategy based in the isobaric labeling and the subcellular fractionation. iTRAQ reagent technology is a newly developed method for relative quantification of proteins from up to four samples. It has immense potential to improve the sensitivity and quality of mass spectrometric analysis of the proteome. We were able to identify and quantify 575 proteins simultaneously from the four states of culture. Among these 575 proteins, 43% were identified by a single significant peptide per protein; the rest were identified by at least two significant peptide of the same protein. The method used in our case to analyze the differential expression levels between two or more conditions was the locfdr. This method is a new approach to the problem of multiple comparisons and controls the number of false positive differentially expressed proteins below the user-specified threshold.

The standard deviation for each peptide value is obtained from the iTracker estimates, while in the case of proteins the variant of t-statistic suggested by Efron was used (Jung et al., 2006) to skip the inconvenience of those proteins that were identified by a single peptide and have standard deviation zero. In all analysis the condition locfdr ≥0.2 was used to select differentially expressed proteins. This threshold is a general accepted standard in fdr applications; it means that to consider a protein as differentially expressed the corresponding probability of being a false positive must be below 20%.

Following this approach we have found a set of 102 differentially expressed proteins. These proteins were classified in different functions and locations according to the KEGG database (de la Luz et al., 2008). According with the previous results four major cellular pathways seem to be involved in the adaptation to the protein-free medium (Figure 2).

4.1.2 Results: Kinetic and metabolic analysis

The host and recombinant NS0 cell line were culture in serum-supplemented and protein-free medium during 140 hours. Total cell number, viable cell number and viability were determined. The specific growth rate is different between both cells, but there was a clear decrease when both cell lines were cultured in absence of serum (Table 3). Intracellular metabolite concentrations were calculated during exponential growth phase. In contrast with previous reports, we found a lost of cholesterol auxotrophy in the host and recombinant NS0 cell line adapted to PFHM. Other metabolites such as phospholipids and

fructosamine involved in specific cellular processes like membrane biogenesis and glycolipid metabolism changed their expression levels in adapted versus non-adapted cell line. In order to check if the intracellular cholesterol concentrations increase in the adapted cell line is a reversible process, cells were cultured in a medium supplemented with serum, and the initial cholesterol levels were determined (de la Luz et al., 2008).

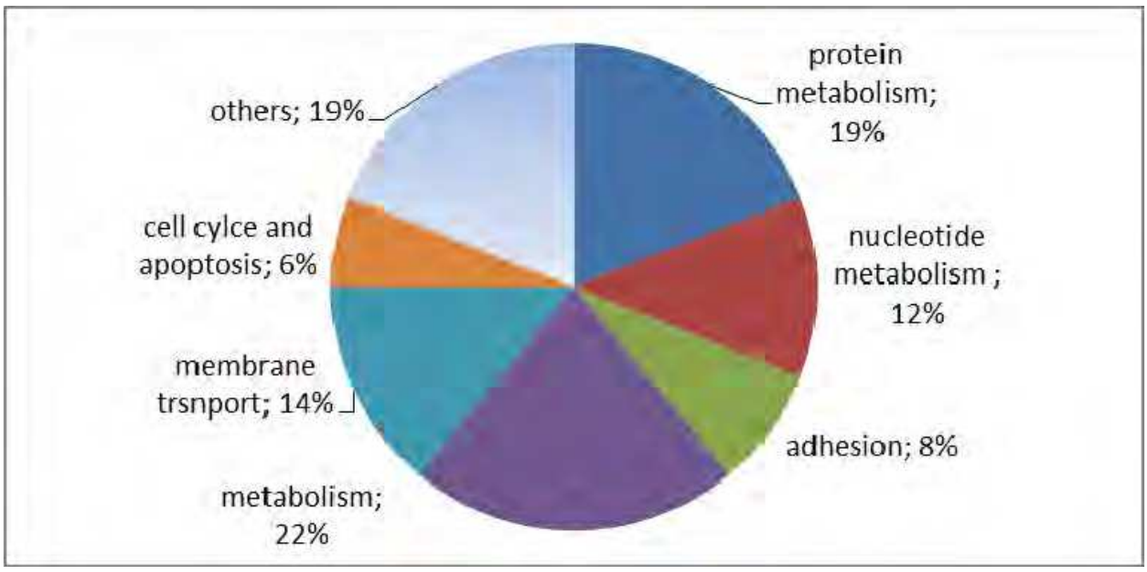

Fig. 2. Distribution of identified proteins taking in account their biological function

Cellular line	Xv_{max} (cell×mL^{-1})	Xt_{max} (cell×mL^{-1})	μ_{max} (cell×mL^{-1}×h^{-1})	IgG_{max} (µg×mL)	qIgG (µg×mL^{-1}×h^{-1})
Host NS0 (FBS)	2.14e^{+06}	2.79e^{+06}	0.034	–	–
Host NS0 (PFHM)	1.55e^{+06}	2.23e^{+06}	0.036	–	–
NS0/hR3 (FBS)	1.26e^{+06}	1.38e^{+06}	0.025	10.70	2.31e^{-07}
NS0/hR3 (PFHM)	1.51e^{+06}	1.58e^{+06}	0.037	41.50	4.73e^{-07}

Xv_{max}: maximum viable cell concentration, Xt_{max}: maximum total cell concentration, μ_{max}: maximum growth rate, IgG_{max}: maximum IgG production, q_{IgG}: specific IgG production rate.

Table 3. Cellular growth parameters from host NS0 and recombinant NS0 cell lines adapted and non-adapted to protein-free medium

Glycolysis is one of the most important metabolic pathways providing a source of precursors and energy for the cell. Previous analysis by DNA microarray studies have revealed a large number of genes involved in glycolysis, the pentose phosphate pathway and the Krebs cycle to be down-regulated in host NS0 cell line cultured in the absence of cholesterol (Seth et al., 2005). Ten proteins from glycolysis were found up-regulated in non adapted NS0 cell line with respect to adapted. This result could indicate that the glycolysis is a source of molecular precursors (cholesterol and phospholipids), especially in the adapted cell line (Figure 3). The lactate production increase after the adaptation process could be related with the higher lactate dehydrogenase enzyme activity (especific enzyme activity NS0 adapted: 20.85 U*mL^{-1}*cell^{-1}, non-adapted: 12.31 U*mL^{-1}*cell^{-1}).

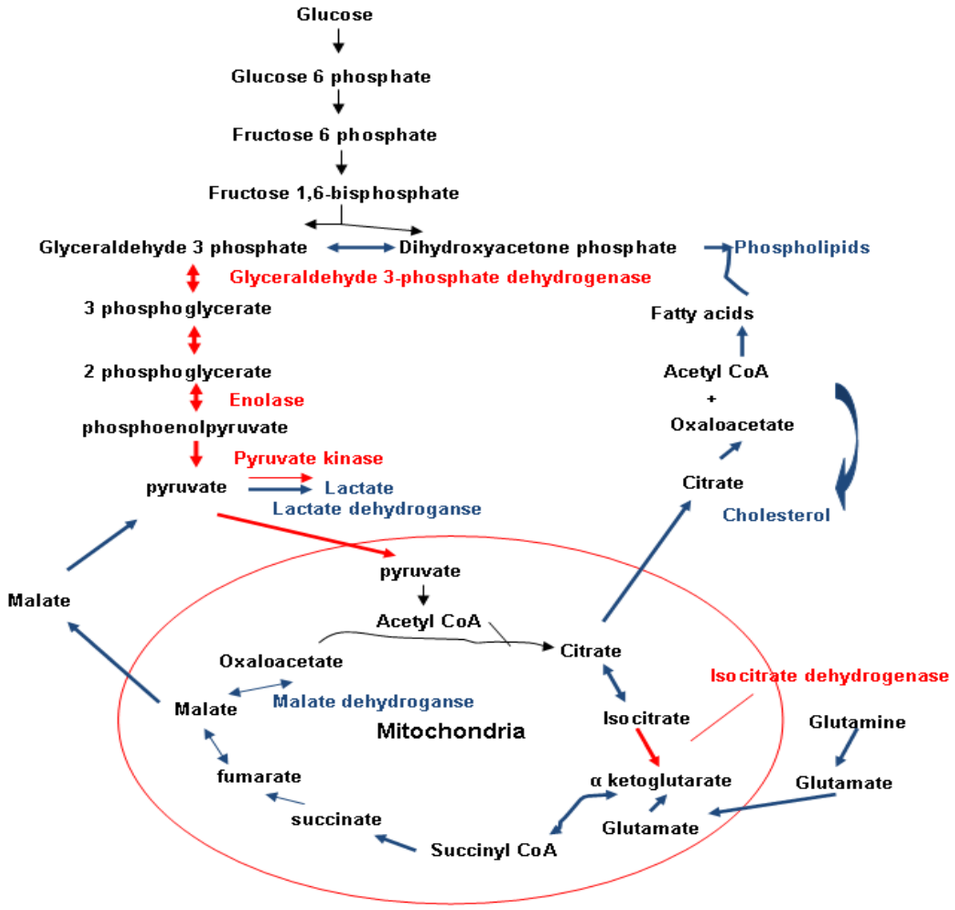

Fig. 3. Metabolic model of the adaptation of NS0 cel line to protein free-medium obtained from proteomic and metabolites analysis. Red: sub-expressed pathways. Blue: over-expressed pathways.

With the aim to calculate the relative rate of glycolysis and glutaminolysis, intracellular concentration of lactate and glucose were determined in the batch culture and the relationships between lactate production and glucose consumption (qL/qG) were calculated (figure 4). These results indicated that the lactate produced depend of the glycolysis and the glutaminolysis. Taken into account protemic and metabolic results we have proposed a metabolic mechanism where the glucose is used for the precursors synthesis. On the other hand, the cell obtain the energy from glutamine degradation.

We used the flux balance analysis (FBA) in order to compare the results obtained with an empiric metabolic network with the experimental results. In this study we used a reported metabolic network (Ma and Zeng, 2003) with changes in the cholesterol reactions, where the cholesterol synthesis pathways was eliminated in NS0 non-adapted. The comparison between adapted and non adapted metabolic network showed changes in carbohydrate and lipid metabolism, very similar with our previous experimental results. Also we analyzed the metabolites that have influence in cellular growth when they are not present in the medium. Glycine, tryptophan, phenylalanine, adenine, palmitic acid, glutamic acid, methinonine and asparagine are relatefd with the increase of cellular biomass (data not shown).

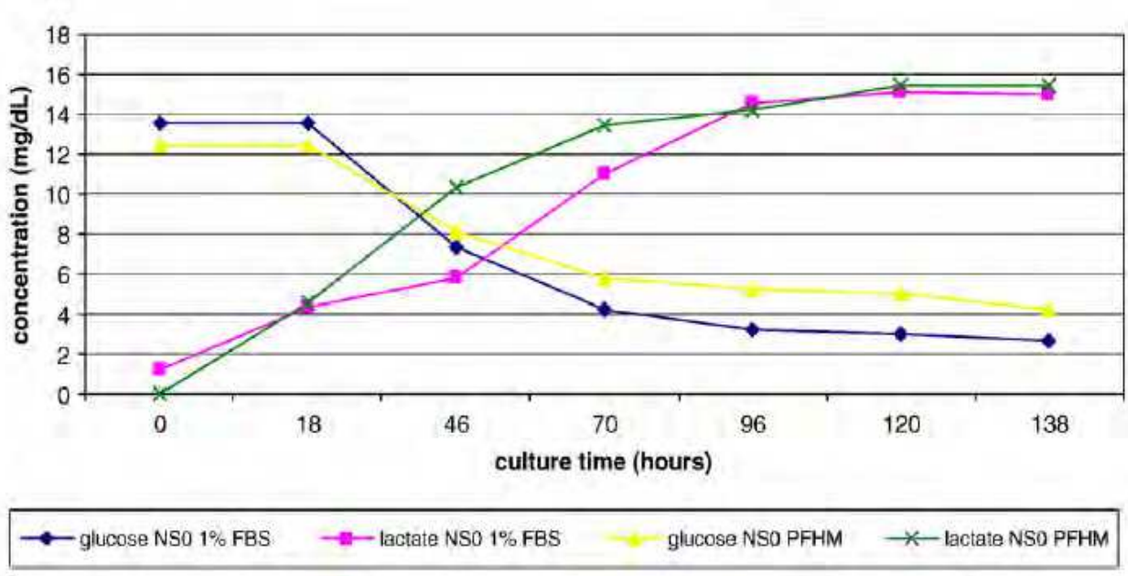

Fig. 4. Glucose consumption and lactate production during a batch culture of myeloma cell line in presence and absence of serum. During the culture period, samples were taken periodically for off-line analysis and media metabolites concentration were determined. The relation between lactate production and glucose consumption is representative of the cellular metabolic state, especially of the glycolysis efficiency

5. Conclusion

Data integration is not limited to flux data. Systems biology encompasses a holistic approach to the study of biology and the objective is to simultaneously monitor all biological processes operating as an integrated system. The use of the data obtained from studies with different "omics" techniques is not simple. In addition, a single gene may code for isoenzymes reacting with multiple metabolite substrates. The difficulty in determining the timing of different events, that it, transcription and protein activity, also contribute to the difficulty in integrating data. Hence in order for metabolomics to be used in systems biology, novel strategies will need to be created. One step forward in such an integration process is the functional assignments between protein/gene and metabolite within a system of interest. This can be done by creating models where basic biochemical pathways are modelled using static data (Khoo and Al-Rubeai, 2007). Second, time-dependent concentrations of other types of components (transcriptomics and/or proteomics) will then be incorporated followed by the reconstruction of the model with statistic data.

In contrast with previous results, changes in metabolic rates and biosynthetic machinery with respect to the presence or not of serum in the culture medium were observed in this study. The analysis was performed by two different ways. First, using iTRAQ reagents, proteins with differential expression levels in two myeloma cell lines cultured in serum-supplemented and serum-free medium were detected. These proteins belong to major pathways related with glycolysis, protein synthesis and membrane transport. These results are in accordance with previous results obtained using 2DE and the study of a revertant

NS0 cholesterol-independent (Seth et al., 2005). Second, the determination of consumption and production of different metabolites like glucose, lactate, cholesterol, phospholipids and phosphorous was performed. Differences in qL/qG were found between adapted and non-adapted cell lines, similar to the results obtained by proteomics. A significant increase was

observed in the intracellular cholesterol concentration in the adapted cell lines. However, when these cell lines adapted to PFHM were cultured in presence of serum, the intracellular cholesterol levels decreased down to the initial conditions, indicating a possible epigenetic mechanism.

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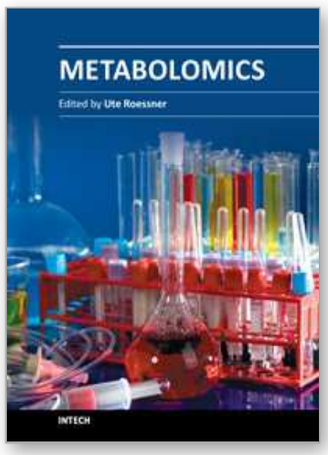

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Metabolomics is a rapidly emerging field in life sciences, which aims to identify and quantify metabolites in a biological system. Analytical chemistry is combined with sophisticated informatics and statistics tools to determine and understand metabolic changes upon genetic or environmental perturbations. Together with other 'omics analyses, such as genomics and proteomics, metabolomics plays an important role in functional genomics and systems biology studies in any biological science. This book will provide the reader with summaries of the state-of-the-art of technologies and methodologies, especially in the data analysis and interpretation approaches, as well as give insights into exciting applications of metabolomics in human health studies, safety assessments, and plant and microbial research.

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