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Metabolic engineering of animal cells

F. Gòdia, J.J. Cairó

Abstract Animal cells are widely used in industrial processes as sophisticated cell factories to obtain a high number of complex proteins with correct post-translation modifications and biological activity, with many applications in diagnostic and therapeutic uses. However, from the bioprocess point of view these are still sub-optimal processes, mainly due to the complex requirements for the in vitro growth of the cells, their metabolic and physiological patterns, and the response of mechanisms developed for in vivo growth to the external conditions found in culture in vitro. Metabolic engineering, combined with the corresponding redesign of the process itself, offers the possibility to the enhance the performance of animal cells grown in in vitro systems, targeting how to redesign the cells themselves to make them more robust, efficient, and productive. This paper reviews efforts made in this direction, and how the metabolic engineering of animal cells has been approached to reshape their profiles in various key aspects, namely: central metabolism, protection of apoptosis, regulation of cell cycle, and finally, the combined engineering of different aspects.

Keywords Animal cells, Metabolic engineering, Metabolism, Apoptosis, Cell cycle

1 Introduction

The potential of animal cell cultures arises from the capability these cells to undertake complex post-translation modifications, enabling them to express, process and secrete proteins with the required biological activity to be used for therapeutic and diagnostic applications. For this reason, animal cells have been the cellular systems selected to produce a wide range of products for human and animal health care applications. The in vitro culture of animal cells is used extensively in the manufacturing of virus vaccines, antibodies, interferons, growth factors, immunoregulators, etc. Recently, new areas of application have emerged, such as gene therapies, tissue engineering, and artificial organ development. This interest is even more evident when the information available on new drugs under development in different stages of clinical trials is examined. Indeed, a very high percentage of the new proteins under development, which will provide the therapeutic arsenal for the coming decades, are obtained by processes based on animal cell technology [1].

However, in spite of its potential from the bioprocess point of view, animal cell technology can still be considered sub-optimal. Frequently, batch or fed-batch culture modes are employed with the use of relatively few advanced control systems. Cell growth patterns show low growth rates, low final cell concentration, and rapid viability decrease after maximum cell density is achieved. Limiting conditions can easily appear due to the complex and delicate nature of this system: nutrient exhaustion or imbalance , accumulation of metabolites that can be inhibitory or even toxic to the cells, lack of oxygen, shear stress and osmolality are some of them. (It is important to mention the very high number of compounds required in the medium formulations, including main carbon and nitrogen sources, vitamins, hormones, amino acids, salts, trace elements, and some specific proteins.)

Two main aspects should be considered when examining this limited bioprocessing performance based on animal cell technology. First, the cells experience a deep change in their environmental conditions when the homeostatic control, as part of a tissue of a pluricellular organism, is altered by the conditions usually found in a bioreactor. The response of the cell mechanisms (developed for in vivo growth) to the external conditions found in culture in vitro can often be opposite to the desired performance of the bioprocess. Second, the complex nature of the intracellular metabolic patterns of animal cells, such as central metabolism, cell-cycle progression, and programmed cell death or apoptosis will dominate the final output of the cell culture, and influence other key events, such as product expression and glycosylation. This latter point is of critical importance in obtaining a product with the desired biological activity. It should be noted that some of these intracellular patterns are still not fully understood and their corresponding molecular mechanisms have not been completely elucidated.

For further progress, improvement of the performance of animal cell technology bioprocesses is required, especially since the generalisation of the developed products will require more optimal and intensive production systems. Such an improvement can be envisaged from

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different angles: bioprocess engineering, cellular or metabolic engineering and molecular engineering. It is important to recognise that the most optimal processes will emerge only when these different levels can be combined. Bioprocess engineering improvements have been achieved in many aspects, as reported by different authors [2, 3, 4, 5], e.g. the development of new bioreactors, especially for high density cell culture, the development of operational strategies, such as fed-batch and perfusion cultures, and the development of on-line instrumentation and control tools to optimise cell culture operation. In most of these developments, however, it is evident that even in improved systems, due to the complexity and sensitivity of the cells an adverse event will trigger the cell death programme. This is the most frequent cause of the decrease in cell viability in the cultures. In this context, the incorporation of the metabolic engineering approach can provide the tools to redesign mammalian cell lines internally , making them more robust, better adapted and self-regulated in culture conditions in vitro and, in consequence, with extended viability and increased productivity. In the following, different efforts in this direction will be reviewed. They have been grouped in four main parts: central metabolism, apoptosis protection, cell-cycle control and multiple gene expression. The last part should be highlighted, since due to the strong links between the different cellular processes, an optimal approach to metabolic engineering should combine the cell modification at different points. Finally, the biological activity and stability of the proteins expressed by animal cells should also be considered as they are linked to their correct post-translation processing, particularly with respect to glycosylation [6]. The last section discusses the use of metabolic engineering approaches to modify glycosylation patterns, which provides the opportunity of obtaining new glycoprotein products.

2
Metabolic engineering of central metabolism

When animal cells are cultured in vitro some general patterns can be observed when their central metabolism is analysed. The main pathways of the central metabolism have been described in detail [7] and they are depicted schematically in Fig. 1, including the catabolism of the two main carbon and energy sources for animal cells, glucose

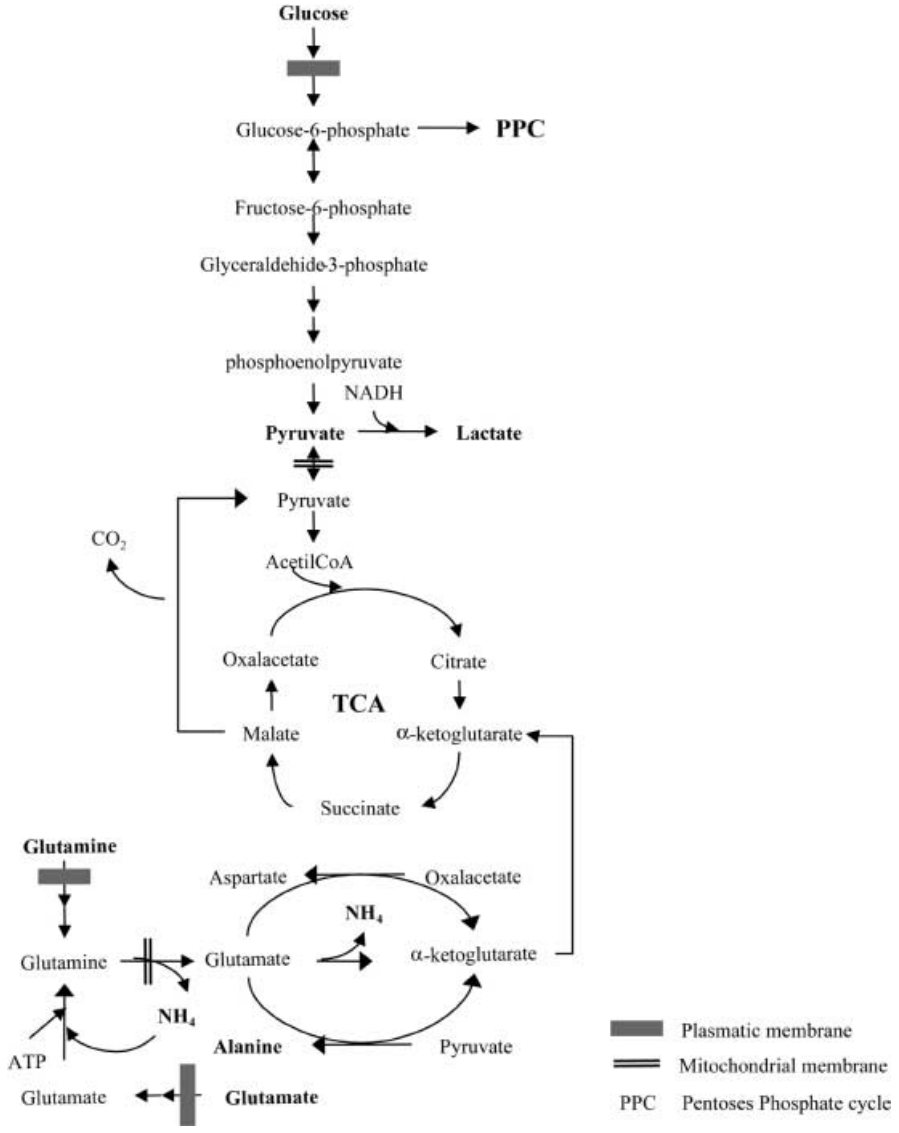

Fig. 1. General scheme of the main metabolic pathways of the central metabolism of mammalian cells

and glutamine (denominated as glucolysis and glutaminolysis respectively), the TCA cycle and the pentose phosphate cycle. Other authors have observed that different types of cell lines exhibit very high rates of both glucolysis and glutaminolysis when grown in vitro, such as CHO [8], hybridomas [9, 10, 11, 12] and BHK [13, 14], as revealed in the corresponding analyses of intracellular metabolic distribution. However, these high rates of consumption are not strictly required for the cell metabolism [15, 16] and the dysfunction between cellular requirements and consumption rates generates the accumulation of various end-metabolites that the cells use as compensation mechanisms. These metabolites are primarily lactate, ammonium and some amino acids, such as alanine and proline [17]. It is noted that the percentage of glucose deviated into lactate can be as high as 95%, even in fully aerobic growth conditions. In addition to a very low metabolic efficiency in the use of the main substrates, this situation often leads to limiting situations, either because the inhibiting or toxic nature of these compounds for some lines when they reach high concentrations [18], or in a more indirect manner, such as the high osmolarity reached in the medium [19]. For example, the generation of lactate may not be directly toxic, but the amount of basis required to neutralise it would cause an important increase in osmolarity.

This deregulated central metabolism reflects how the cellular behaviour changes from the very fine tune control in the homeostatic conditions of any animal tissue, to the conditions experienced in culture systems in vitro. The specific mechanisms involved in this change are still not fully understood. One possible explanation is that the NADH transport system into the mitochondrion, based on the malate–aspartate shuttle, is not active enough to incorporate the NADH generated in the first steps of glycolysis and regenerates NAD^+ in an energetically efficient way through oxidative phosphorylation [16]. In this situation, the only possibility for the cells to recover their redox balance is to regenerate NAD^+ in the cytosol in a less efficient way through the lactate dehydrogenase-catalysed reaction, thus producing high amounts of lactate. Another possibility that would explain this behaviour is the lack of specific enzyme activity connecting glycolysis with the TCA cycle, like pyruvate dehydrogenase complex, phosphoenolpyruvate carboxykinase or pyruvate carboxylase [20], directly causing an imbalance between glycolysis and TCA rates. In consequence, glucose is degraded mainly via oxidative glycolysis, generating the output of excess material in the form of lactate.

Although different strategies have been used in order to prevent this imbalance by regulating the feeding of glucose and glutamine in the media in order to reduce their consumption rates [5, 21, 22], these types of strategy are based on on-line instrumentation and control systems in order to correctly manipulate the cell environment and specifically the concentrations of the main compounds. Metabolic engineering strategies are targeted directly to the redirection of the central metabolism at the cellular level and different results have shown that this is a valid approach. A BHK cell line was genetically modified introducing the cytosolic pyruvate carboxylase (PYC) from the yeast *Saccharomyces cerevisiae* [23]. The metabolic changes introduced by this modification are shown in Fig. 2. In the non-transformed cells pyruvate incorporation into TCA is limited and NAD^+ regeneration is obtained through lactate dehydrogenase, and then lactate is produced. In the transformed cells pyruvate carboxylase competes with lactate dehydrogenase for the cytoplasmatic pyruvate pool and the oxaloacetate so obtained can be further converted to malate by means of cytoplasm malate dehydrogenase. This reaction is important, since it allows malate to be incorporated into the TCA and simultaneously renders reduced NAD^+. In addition, the backward reaction from malate to pyruvate, catalysed by the malic enzyme, allows the cell to produce NADPH (as shown in Fig. 2). The results obtained with PYC-modified BHK cells show that the expression of the gene has no deleterious effect on cell growth, as cells grow better either in batch or perfused cultures. Also, a more efficient and balanced metabolism is achieved, as reflected in the decreased glucose consumption (1.4–4 fold, depending of the specific clone), decreased lactate production by a factor of 2.5, reduction of glutamine utilisation, increased oxygen consumption rate and lower lactate to glucose yield [23].

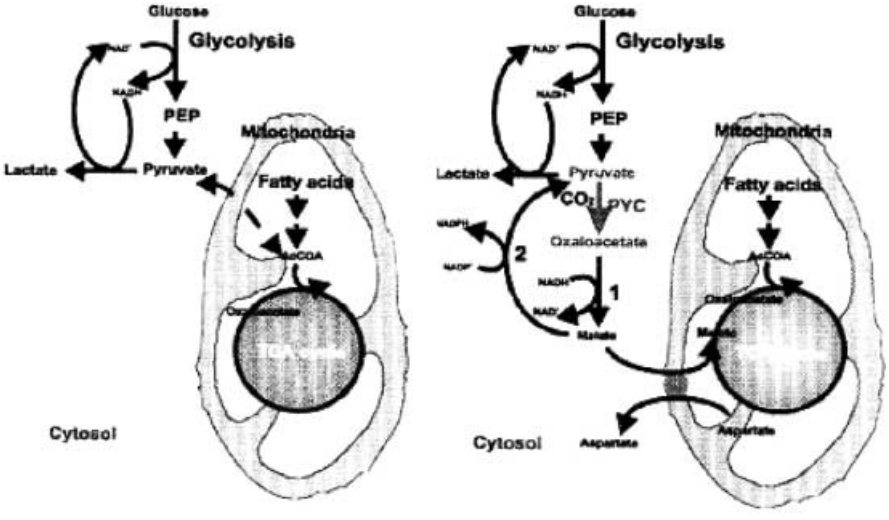

Fig. 2a, b. Schematic outline of the usual **a** and modified **b** pathway of glucose oxidation in mammalian cell lines (according to [23]). In non-modified cells, the glucose metabolism is governed by the low rate of pyruvate conversion to AcCoA and entry to TCA cycle, with the concomitant excretion of high amounts of lactate. In cells modified with the cytosolic pyruvate carboxylase gene (PYC), pyruvate is transformed to oxaloacetate and then to malate by cytosolic malate dehydrogenase (1). Malate can be then incorporated to the mitochondrion and to the TCA. In addition, the malic enzyme (2) provides also a backward reaction from malate to pyruvate that allows NADPH production

The central metabolism has also been modified using antisense RNA approaches, by which the transcription of a gene is partially blocked by the hybridisation of the antisense RNA transcripts to the target mRNA [24]. In this case the glycolysis rate in a hybridoma cell line has been reduced by lowering the level of expression of a key enzyme in the pathway [25], the two enzymes targeted particularly being the glucose transporter into the cell and the enolase enzyme. This results in a reduction of almost 50% in the specific glucose consumption rate in cells transfected with antisense RNA constructions against the glucose transporter GLUT1, and in a reduction of 22% for enolase. Although the specific constructions developed were not stable over a number of sub-cultures, these results demonstrate the possibilities of this approach.

A third approach in the modification of the central metabolism is focused on the glutaminolysis pathway. As previously mentioned glutamine is the second major compound in medium formulations. Its metabolisation, partly because of its spontaneous decomposition, leads to the generation of high amounts of ammonium ions. The substitution of glutamine by other compounds resulting in the generation of fewer ammonium ions, such as glutamate, is possible in some cell lines such as CHO. However, hybridoma cell lines require metabolic engineering to provide them with the glutamine synthetase gene, because this gene is not expressed constitutively [26]. This modification has been used extensively in a number of cell lines [25, 27, 28]. Its use allows the culture of cells without ammonium generation and, interestingly, a marked reduction in the glucose consumption rate is also observed. This describes the interaction among the main pathways shown in Fig. 1, i.e. when the glutamine metabolism decreases, the glutaminolysis rate also decreases in order to balance the TCA cycle circulation. A further consequence of this metabolic re-distribution is that the cells do not export any malate from the mitochondrion for conversion to pyruvate and further to alanine, as the cells do not require transamination reactions to pump out part of the accumulation of ammonium ions. The comparison of the metabolic fluxes for a GS transformed and a non-transformed hybridoma cell line are presented in Fig. 3, together with the values for the main specific rates [25]. The transformation of animal cell lines with the glutamine synthetase gene was combined with various control strategies in order to design optimised processes, mainly based in fed-batch strategies for the culture of hybridoma cells, with very positive results [3, 27, 29], including final cell concentrations up to 6.6×10^6 cells/mL and final monoclonal antibody concentrations up to 2.7 g/L.

3
Metabolic engineering for the protection from apoptosis

The process of programmed cell death or apoptosis is a critical issue in the in vitro culture of animal cells, since this is the death mechanism most often responsible of the loss of cell viability and therefore causing the termination of the culture. Apoptosis provides very clear evidence of how the internal mechanisms that animal cells possess as part of multicellular organisms can become a serious drawback when the cells are cultured out of their natural environment. Indeed, physiological control of cell death is an intrinsic part of life maintenance in multicellular organisms [30] as has been seen in various events such as: development, maintenance of homeostasis, protection from oncogene deregulation, DNA damage, stress response and pathogen invasion [31, 32]. Different physiological events characterise programmed cell death: cell shrinkage, chromatin condensation and membrane blebbing. By these steps the cell is dismantled and can be recognised in the organism by the phagocytic cells that will finally eliminate it. Apoptosis is a very complex process and not fully understood, with many different pathways

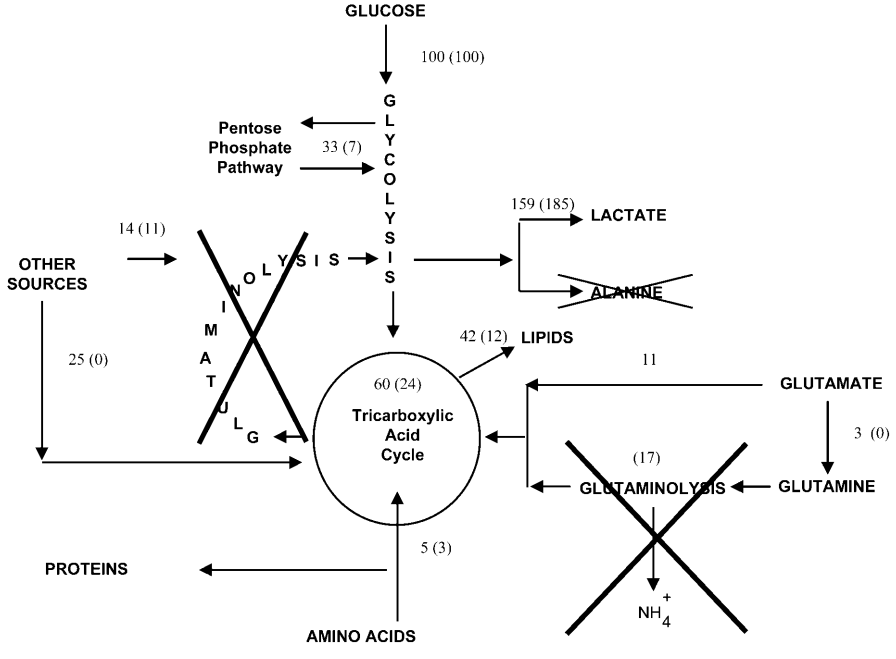

Fig. 3. Scheme with the values of the main metabolic fluxes provided for a hybridoma cell line and for the same cells transfected with glutamine synthetase genes (according to [25]). Values inside parentheses correspond to untransformed cells. Values shown are relative units as a percentage of the specific uptake rate

and signals being involved. It is now accepted that every single cell has its own death programme, but this is not executed if the cell receives signals to remain alive. Therefore, the cell is continuously in a balance between live and death stimuli, submitted to a high number of regulation mechanisms. The effects of this imbalance are the basis of the development of important diseases, and for this reason apoptosis research is receiving tremendous interest. Indeed, the consequence of an insufficient cell death is neoplasia, and autoimmune and degenerative diseases are developed when excessive cell death is present.

From the point of view of the in vitro culture of animal cells for manufacturing purposes, the advent of cell death is a clear limitation, since the irreversible death programme can be initiated by multiple causes [33, 34, 35, 36]. It is important to stress the genetically encoded nature of the process. Once triggered because a certain death stimuli has occurred (for example, exhaustion of a key nutrient, such as glutamine), it can not be reversed by a late action correcting the initial signal (i.e. feeding the culture with more glutamine will not recover the cells that are already progressing through the apoptosis programme). There are many causes for the onset of apoptosis in cell cultures and it is extremely difficult to externally control each specific variable involved due their high number, and the fact that the possibility would always exist for cells to escape from the control and start the apoptosis process foetal calf serum in the medium formulation. Although completely necessary from the point of view of safe manufacturing and regulation aspects, the growth on serum- or protein-free media makes the cells more fragile, with a greater possibility that their death programme will be initiated.

In such a scenario, the development of cell lines engineered in a way that they are protected against apoptosis would enable to have more robust cells for the culture processes. It is important to realise that a complete protection against apoptosis or its complete inhibition is very improbable to attain, but the protection from apoptosis during a significant time window would allow to correct the culture event causing the problem and to recover the cells, bringing them back to normal proliferation. In brief, metabolic engineering for apoptosis protection would provide more robust, self-protected cells to be used in culture systems in vitro. This should have a direct impact in prolonging cell viability of the cultures, high number of cells, and therefore improved production processes. Therefore understanding the different pathways and steps involved in the apoptosis process, and how they are regulated by a number of events and gene expression is a necessary step in order to identify possible targets for metabolic engineering of the cells.

The overall process of apoptosis involves a number of events [37, 38], as depicted in Fig. 4. In the initiation step, the cell receives a stimuli that activates apoptosis, either induced by specific receptors (in an interaction mediated by death receptors belonging to the tumour necrosis factor (TNF) receptor gene superfamily, in which the death domain of a cell surface receptor interacts with the death domain of other cellular proteins) or by many stress situations to which the cell can be exposed (nutrient deprivation, oxygen depletion, etc.). After initiation, a second step (signalling), brings by means of a number of parallel individual pathways the apoptotic signal to a reduced number of central pathways in what is called the effector phase. This central part of the whole process has received a lot of attention and many genes with different actions have been identified. If the cell progresses through this phase, it will reach the final step of apoptosis (degradation), characterised by the destruction of the DNA and the restructuring of the cytoskeleton to proceed to dismantle the cell. The knowledge on the mechanisms and genes involved in the different phases of apoptosis has increased greatly although it is still not fully understood. The main aspects in this process are outlined in Fig. 5 [39], but recent publications give a more detailed review of the different mechanisms[31, 40, 41, 42, 43].

In the central effector phase of apoptosis, the genes of the *bcl-2* family play an important role. Some of these genes prevent cell death: *bcl-2*, *bcl-x*$_L$, *mcl-1*, as some others promote cell death: *bax*, *bcl-x*$_S$, *bad* and *bak*. Another central role in this effector phase is that of the caspase (cysteine-containing aspartase-specific proteases) protein family. Caspases are present in the cell in an inactive form, known as pro-caspases, that become active after the cell detects some apoptotic stimuli. Then caspases act by cutting specific target proteins in the apoptosis progress. Different types of caspases act during the apoptotic cascade. In the first instance, after a given death stimuli, the cell activates the so-called initiator caspases, mainly caspase 8 in the case of receptor-induced activation and caspase 9 in the case of a stress-induced activator. In the second phase, these initiator caspases activate the so-called executioner caspases, like caspase 3, 6 and 7, that follow on cutting their target proteins in the apoptosis cascade. Even at this late stage the cell still has some final mechanisms to stop the apoptosis process, such as p35, CrmA and the so-called IAPs (inhibition of apoptosis proteins). Finally, the involvement of mitochondrion in the effector phase of apoptosis should be highlighted, particularly by its release of some specific proteins

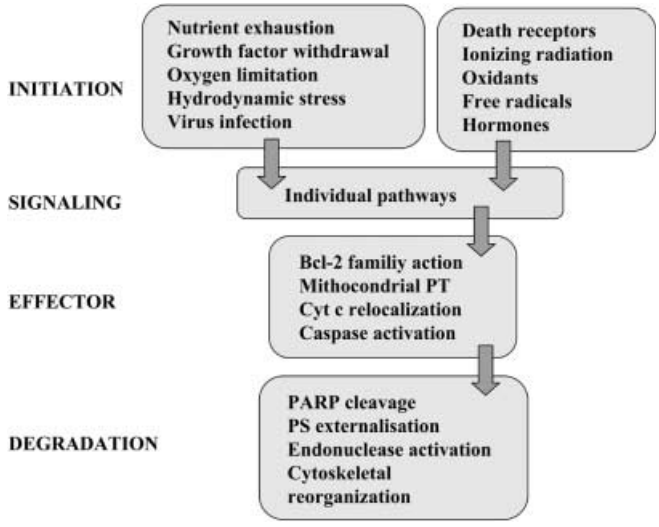

Fig. 4. Main events in the apoptotic cascade (adapted from [37])

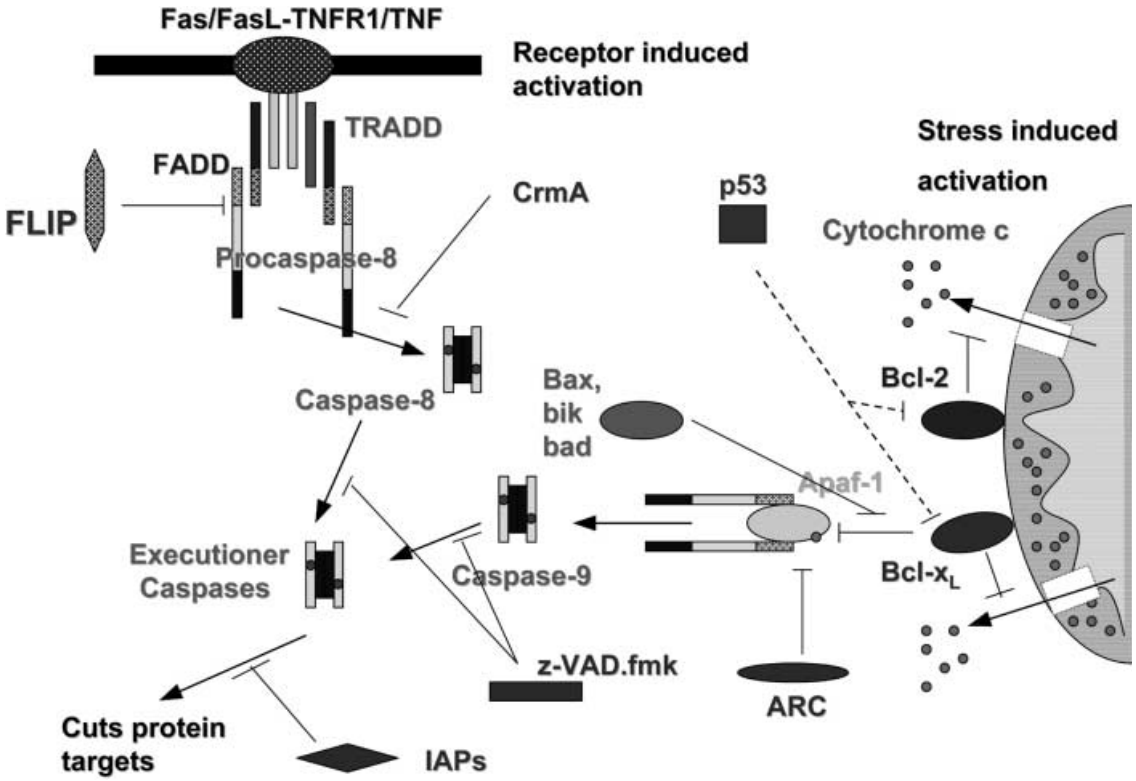

Fig. 5. A more detailed scheme of some of the key events and molecules involved in the apoptosis process, via receptor induced activation and stress-induced activation, illustrating the comments given in the text (adapted from [39])

involved in the activation of caspases [41], particularly cytochrome-c. An illustrative example of how the *bcl-2* family proteins, caspases, and the released cytochrome-c act together in order to initiate apoptosis is the mechanism proposed for the activation of caspase 9 [45]. In a non-apoptotic situation, cytochrome-c is present in the mitochondrion, pro-caspase 9 is not activated and Apaf-1, one of the proteins also responsible for caspase activation is bound to an anti-apoptotic protein, *bcl-x_L*. After the advent of the apoptotic stimulus, cytochrome-c is released from the mitochondrion due to the alteration of the membrane permeability, and a pro-apoptotic member of the *bcl-2* family, like *Bik* can interact with *bcl-x_L*, releasing *Apaf-1*. In the presence of cytochrome-c, *Apaf-1* can bind to pro-caspase 9 and promote its dimerisation and activation. This model still has problems to be solved, but it is a representative example of how, in order to prevent apoptosis, deeper knowledge gained in this process enables the selection of specific targets, such as over-expression on anti-apoptotic *bcl-2* family genes, inhibition of pro-apoptotic genes, prevention of the permeability transition in the mitochondrion membranes, inhibition of caspases (for example with CrmA, p35, IAPs, or even specific peptides) (reviewed in reference [32]). Another consequence of this knowledge is that the number of genes implicated and the regulation mechanisms are so wide that it is necessary to introduce various modifications simultaneously in order to obtain marked positive effects, as will be discussed later in this work.

The first target and most studied one for metabolic engineering derived from knowledge on apoptosis is the over-expression of *bcl-2* gene. Many positive results of prevention of apoptosis induced by different insults have been reported in hybridoma cells [36, 44, 45, 46] and CHO cells [47]. These results have confirmed the important role of *bcl-2* in apoptosis development and have enabled culture profiles with enhanced viability to be obtained, prolonged duration, and increased final cell number and product concentration. Also, it was shown that *bcl-2* transfected hybridoma cells can be cultured in intensive high cell density perfusion systems for prolonged times, showing higher robustness and productivities than non-transfected cells, enabling an almost 100% increase in final antibody titters in the perfusion medium to be obtained [48]. CHO cells transfected with *bcl-2* have been cultured in fixed-bed reactors for extended periods with increased robustness [49]. It was also shown that the protective effect of different anti-apoptotic genes may differ for different cell lines [50]: in a study carried out to investigate the protection of CHO and BHK cells by over-expression of *bcl-2* and *bcl-x_L* upon infection with alphavirus vectors, it was observed that CHO cells were more protected by *bcl-x_L*, while BHK cells received more protection by *bcl-2* over-expression. It has also been reported that *bcl-2* may not have any protection effect for some cell lines [51]. *bcl-2* has also been co-expressed together with the anti-apoptotic gene *bag-1* and an increased protective effect has been observed on hybridoma cell growth and productivity when compared to the transfection with only *bcl-2* [52]. Also, antisense technology has been employed to prevent apoptosis, by transfecting cells with an antisense directed towards a pro-apoptotic gene, particularly *c-jun*. In this

case, the new cell lines expressing the *c-jun* antisense were also cell-arrested and the cells could be maintained alive for a longer time at the non-proliferative stage, but could still actively produce the product protein [53]. This last result shows the strong connection between apoptosis and cell-cycle progression, as will be emphasised below.

4 Metabolic engineering for the control of cell-cycle progress

Cell-cycle control is a central event in a cell, where decisions on growth (therefore, division), differentiation and apoptosis triggering are taken, as a consequence of the detection of certain stimuli, insults or events. Cell-cycle regulation and its interaction with apoptosis in mammalian cells have been extensively reviewed recently [54]. Clearly, uncontrolled cell proliferation is one essential mechanism to understand the basis of very important diseases such as cancer and will play a key role in the further development of gene and cell therapy and tissue engineering [55]. A simplified scheme of cell-cycle control is presented in Fig. 6. The cell-cycle progression is divided in the following phases: G1 (where G means gap phase), S (DNA synthesis), G2, and M (cell division or mitosis). Cells in the so-called G0 state do not divide, but will start to divide when some extra cellular signals are detected. When the cells enter the cell cycle, each phase is executed in a coordinated order, to ensure that all the events associated to a given phase are completed before entering the new phase. This progress is controlled at specific checkpoints. Three main checkpoints are known: the transition between G1 and S phases, which is also known as the restriction point or start; the entry into mitosis; and, finally, the exit from mitosis. The regulation at these checkpoints is carried out by specific complexes involving a number of protein kinases, known as cyclin-dependent kinases (CDKs), each one of which needs to be associated to a small protein called cyclin in order to develop its specific action [56]. The CDK2-cyclin E complex plays a major goal in the regulation of the transition between G1- and S-phase, as highlighted in Fig. 6, and this point has received special attention from the metabolic engineering approach of cell proliferation, as G1-S transition is a key target to attempt cell arrest.

A number of cyclin-dependent kinase inhibitors (CDIs) were identified to interact in the regulation at these checkpoints [54]. One of them, p21, inhibits the action of CDK2, as well as other kinases involved in the G1-S transition. p21 expression can be induced by two main mechanisms. One is through the transcriptional factor p53, that plays a major role as ultimate decision maker in cellular events related to DNA damage – DNA repair, cell-cycle arrest or apoptosis [57, 58] – and the other is through the CCAAT/enhancer-binding protein α (C/EBPα) [59], as represented in Fig. 6. A number of different CDIs were identified, such as the members of the p21 family, p27 and p57, affecting various CDK-cyclin complexes [54].

It is evident that as more progress will be made in the identification of cell-cycle control mechanisms, the possibilities for metabolic engineering at this level will increase. The different efforts in this direction have been extensively reviewed [57], and here some relevant examples will be discussed to demonstrate its potential. From the bioprocess point of view, the main interest in manipulating the cell machinery at the level of cell cycle is to arrest cell proliferation, maintaining simultaneously the cell viability and activity, particularly the expression of the product protein. This would allow the design of biphasic processes in which cells would grow to a desired level in a first phase, as observed in any conventional cell culture process. After this growth phase and before any event related to the onset of apoptosis can take place, the expression of genes related to cell-cycle arrest are induced to provide a cytostatic second phase where cells can be growth arrested and therefore can direct most of their metabolism towards cell maintenance and protein expression, rather than to cell growth, for extended periods of time in the culture. In brief, the metabolic engineering approach is directed to decouple cell growth and protein production phases. It should be stressed that such an approach requires the expression of the gene of interest at the point of the culture where the control of cell proliferation is needed and this can be achieved by means of a number of regulated gene expression systems [60].

Cell proliferation engineering has been applied to BHK cells through the expression of the interferon-responsive factor 1 (IRF-1). This transcription factor is critical for the regulation of the interferon system inhibiting cell growth [61] and, in order to regulate its expression, it was fused to the estrogen receptor that is activated in the presence of the hormone estradiol. In this way, the BHK cell transfected with IRF-1-ER fusion can be growth arrested by the addition of estradiol to the culture medium [62]. One interesting strategy is to place the expression of the heterologous protein also under the control of promoters activated by estradiol. In this way, the presence of estradiol acts simultaneously to control cell proliferation and stimulate protein production [63],

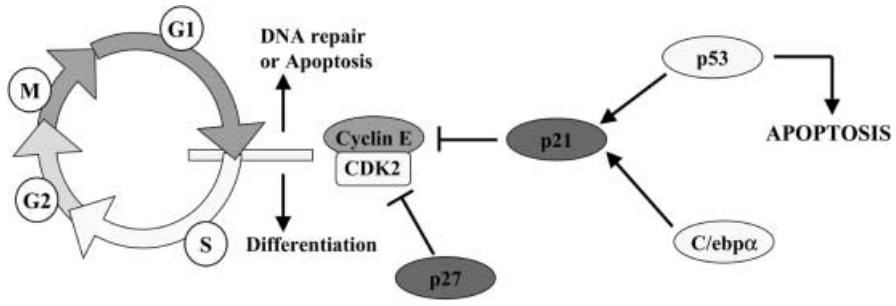

Fig. 6. Schematic representation of the main molecular pathways regulating the cell-cycle progression, particularly the G1-S transition (adapted from [57])

a heterodimeric IgG antibody. However, the proliferation control based on this system causes an important decrease in cell viability due to the presence of estradiol in the culture medium. Although the reasons for this estradiol-unrelated cell death process are not fully understood, they could be directly related to the continuous IRF-1 mediated induction of the interferon system. To mitigate this drawback, bioprocess operation design can be combined with metabolic engineering. Indeed, an operation of repeated cycles of estradiol induction, followed by estradiol withdrawal allows sufficient IRF-1 induction to control cell proliferation, but minimising cell death. The growth of BHK-21 cells on microcarrier particles facilitates the realisation of these cycles, as the liquid medium and the solid particles are easily separated. In a different approach, the over-expression of different CDKs has been investigated, particularly p21, p27 and also the related tumour suppressor gene p53 [64]. It has been demonstrated that they all allowed cell arrest at the G1 phase in CHO cells. Additionally, increased productivities of the heterologous protein being expressed (human secreted alkaline phosphatase, SEAP), were obtained when a transient tetracycline-repressible over-expression was used. However, the intracellular effective level for p21 must be higher to have the same effect as p27 and p53. When these genes were introduced in a stable genetic configuration very different effects were observed [65]. While p27 confirmed its potential to accomplish cell growth arrest and increased SEAP productivities up to 10–15 fold, the attained levels for p21 were too low to observe the targeted effect. More surprising was the effect of p53 over-expression, since in addition to not obtaining the desired effect, a clear apoptotic cell death pattern was observed even when expression of an apoptosis-deficient p53 mutant was induced. The high complexity of mammalian cell machinery and its regulation is clearly evident and the induction of the expression of a specific gene may be an over-simplified approach in the metabolic engineering of this type of cell. The following section addresses specifically the expression of multiple genes as a way to make further advances in this direction.

5
Metabolic engineering through the coordinated expression of multiple genes

The extremely complicated nature of mammalian cells has been reflected throughout this paper and it has also been shown how the different parts of the cell machinery that can be considered for cell engineering (central metabolism, apoptosis, cell cycle, product expression) are related one to each other. In consequence, metabolic engineering strategies focussing on one single modification can provide only partial approaches and therefore limited responses. Advanced metabolic engineering of animal cells should envisage multiple gene expression in a coordinated manner, directed to different targets in the cell machinery. Also important in the design of further metabolic engineering approaches for animal cells is the development of expression vectors with regulable induction of gene expression, allowing to turn on the desired gene activity at a specific point of the evolution of the culture process. In this direction the development of multicistronic vectors, containing internal ribosomal entry sites (IRES), is particularly relevant [66] and opens a number of new possibilities. The results obtained using tricistronic systems to engineer CHO cells [67] are very demonstrative of this kind of approach. Indeed, in order to overcome the previously described low activity of the expression of p21 for growth arrest, p21 was expressed together with the differentiation factor CCAAT/enhancer-binding protein α, that has an induction and stabilisation effect on p21, and SEAP as a model for the heterologous protein. The results showed a marked cell growth arrest and higher productivities of the protein were obtained with the growth-arrested non-proliferating cells, up to 10–15 times that of the control cells, a proliferation-competent cell line. In a second multicistronic construction, the fact that cell proliferation arrest by cell engineering could possibly also generate apoptosis was considered. The expression of p27, a gene that had showed previously good results regarding cell-cycle arrest, was combined with the apoptosis protective gene, *bcl-x*$_L$, and the SEAP protein. Again, very good results were attained with this construction and SEAP production was increased up to 30 times. Advances related to multigene metabolic engineering are foreseen in the future in various areas of application of animal cell technology [68].

6
Metabolic engineering of glycosylation

In addition to the previous strategies directed towards the engineering of animal cells to improve their profile in culture and, therefore, leading to more efficient and productive processes, the correct structure of the product, normally a glycoprotein, is critical to ensure its biological activity and stability [69]. It should also be considered that culture conditions can directly affect the protein glycosylation pattern, especially through ammonium accumulation [70]. The glycosylation patterns of the most relevant cell lines are well known [71], and therefore metabolic engineering of the cells, particularly introducing genes encoding glycosyltransferases and glycosidases, can modify the glycosylation profile of a protein. A second metabolic strategy to modify glycosylation is the use of antisense technology, previously described, in order to block a specific biosynthesis activity [72]. The potential of this approach is seen in two examples. In one of them [73], the incomplete or inconsistent sialylation and galactosylation of two recombinant proteins, TNRF-IgG and TNK-tPA, obtained in culture was corrected by the maximisation of the sialic acid content. For this, the CHO cell line employed was engineered to express human β1,4-galactosyltransferase and α2,3-sialyltransferase. The N-linked oligosacharide structures obtained by the engineered cells had a greater homogeneity compared to control cells and, importantly, they had better stability when injected in a rabbit model for pharmacokinetic studies. In the second example [74], a multiple gene expression is introduced to modify the O-glycosylation pathway of CHO cells, following the approach of

multicistronic constructions discussed in the previous section. The significance of this multiple gene expression is that it is designed to over-express one given glycosyltransferase (human UDP-GlcNAc:Galβ1,3GalNAc-R β1,6-N-acetylglucosaminyl-transferase) and to inhibit a second one (CMP-sialic acid:Galβ1,3GalNAcα2,3-sialyltransferase), by means of antisense technology.

7 Conclusion

The metabolic engineering approaches to mammalian cells offer a high potential for the redistribution of different metabolic patterns, as has been demonstrated in many applications. Metabolic engineering approaches must be often combined with bioprocess engineering approaches in order to improve animal cell culture. Further development of metabolic engineering applications to animal cell technology should arise from the deeper knowledge to be gained on basic cellular mechanisms and new technological insights such as the coordinated and conditional expression of multiple genes.

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