Chapter 18
Cell Engineering for Therapeutic Protein Production

Eric Baek, Che Lin Kim, Jin Hyoung Park, and Gyun Min Lee

Abstract In order to meet the ever-growing demand for therapeutic proteins, high therapeutic protein productivity in mammalian cell culture is necessary. Cell engineering is one of the most effective and powerful ways to improve the production of therapeutic proteins. This chapter describes various strategies of engineering biotechnologically important mammalian cell lines, mainly Chinese hamster ovary (CHO) cells, to achieve high therapeutic protein productivity.

Keywords Chinese hamster ovary cells • Therapeutic proteins • Cell engineering • Specific productivity • Time integral of viable cell concentration

18.1 Introduction

The era of biopharmaceuticals began as the human plasminogen activator (tPA), the first therapeutic protein from recombinant mammalian cells, was approved in 1986 (Wurm 2004). The field of biotechnology and biopharmaceutics has been expanding since. The economic success of therapeutic proteins from mammalian cells has been recognized as the sales of biologics reached $120 billion in the US in 2012 and are expected to increase to $150 billion by 2015 (Butler and Meneses-Acosta 2012). In order to satisfy the ever-growing demand for therapeutic proteins and to maximize biopharmaceutical manufacturing, the establishment of high and stable producers and the optimization of culture conditions is certainly important.

Among the 58 biopharmaceuticals approved from 2006 to 2010, 32 were produced from mammalian cells, such as NS0, baby hamster kidney (BHK), human embryo kidney-293 (HEK-293), and Chinese hamster ovary (CHO) cells (Kim et al. 2012). Among them, CHO cells are the most widely used mammalian host due to several advantages: CHO cells are safe hosts without any adverse effects. They accomplish high specific productivity (q_p) through the gene amplification system, encompass efficient post-translational modification suitable for human therapeutics, and easily adapt to growth in a serum-free suspension culture. All of

E. Baek • C.L. Kim • J.H. Park • G.M. Lee (✉)
Department of Biological Sciences, KAIST, 335 Gwahak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
e-mail: gyunminlee@kaist.ac.kr

M. Al-Rubeai (ed.), *Animal Cell Culture*, Cell Engineering 9,
DOI 10.1007/978-3-319-10320-4_18

these features have contributed to the popularity of CHO cells and their domination in the commercial production of therapeutic proteins. For a few decades, more than 100-fold yield improvement of titers in CHO cell culture was achieved as a result of a tremendous amount of studies and efforts. From cell line development to an omics-based approach, there have been numerous innovative strategies aimed to optimize the culture condition and maximize the production. Among those strategies, this chapter will primarily focus on genetic engineering in CHO cells and discuss current achievements and potential ways to increase q_p through genetic engineering.

18.2 Cell Engineering Strategies

For the commercial production of therapeutic proteins, including monoclonal antibodies and erythropoietin, an achievement of high volumetric productivity and/or high product titer is important to meet profitable standards. A variety of novel and innovative strategies have been designed, manipulated, and combined to optimize the process of mammalian cell culture and to maximize the economical profits of producing therapeutic proteins. The rationale behind the strategies is quite simple: to increase the time integral of viable cell concentration (TIVCC) and/or increase q_p. Correspondingly, cell engineering has been applied to various intracellular mechanisms, such as cell death, metabolism, and cell cycle. Cell engineering has been striving to improve culture characteristics as shown in Fig. 18.1.

Figure 18.1 schematically depicts two variables, q_p and Xv × dt, that contribute to the final product titer. The increase in Xv × dt means that cells that produce therapeutic proteins have remained viable at a higher cell concentration for a longer culture period. Along with, the increase in q_p means the rate at which cells are

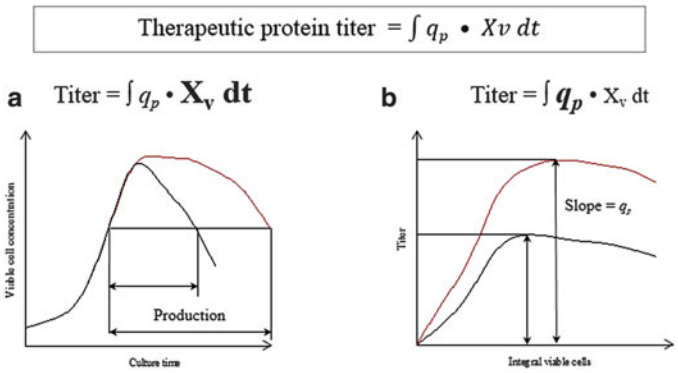

Fig. 18.1 The engineering scheme of CHO cells for improved therapeutic protein production. (**a**) Engineering for improved Xv × dt. (**b**) Engineering for improved q_p. Xv and dt represent viable cell concentration and culture time, respectively

producing therapeutic proteins has increased. These two variables are the keys to achieve a cost-effective level of production. Cell engineering strategies aiming to increase these two variables are discussed in this chapter.

18.3 Improving Viable Cell Concentration

One of the main challenges facing the optimization of therapeutic protein production in mammalian cell cultures is to increase TIVCC. Many studies have revealed various strategies to increase TIVCC by enhancing culture longevity, improving the specific growth rate (μ), and increasing the maximum viable cell concentration. These strategies primarily aim to expand the culture duration, providing more time for mammalian cells to produce therapeutic proteins, thereby maximizing the production in a given time. Many factors are involved in TIVCC in mammalian cell culture. However, programmed cell death (PCD), cell proliferation, and metabolic engineering are three main areas that researchers have been focusing on.

18.3.1 Anti-cell Death Engineering

Cell death is undoubtedly the most important issue in mammalian cell culture. During cell culture, cell death is caused by various stresses such as nutrient depletion, accumulation of toxic by-products, hypoxia, and shear stress (Kim et al. 2012). Cell death occurs in two general forms: necrosis and PCD (Arden and Betenbaugh 2004). Necrosis is a sudden and passive form of cell death that is characterized by the distortion and degradation of organelles and cellular swelling (Danial and Korsmeyer 2004). In this sense, necrosis is a type of cellular injury, while PCD, as the name refers, is tightly programmed under a genetically controlled mechanism. There are two types of PCD: apoptosis and autophagy (Hwang and Lee 2008). Both types of PCD are intimately regulated by active cellular signaling, which is able to be engineered and interfered (Broker et al. 2005). Consequently, many studies have targeted PCD to overcome a variety of stresses and to increase culture longevity.

18.3.1.1 Apoptosis

The term ‘apoptosis’ was first proposed in 1972, in which ‘apoptosis’ was derived from the Greek word apo- (from), ptosis- (falling off) (Kerr et al. 1972). Apoptosis was first observed as a structural breakage and burst of the cell into membrane-bound fragments. It is also further characterized by chromatin condensation and nucleosomal DNA fragmentation (Kerr et al. 1972; Wyllie 1980). Until now, due to its special and significant implications in medical fields such as cancer and

neurodegeneration, apoptosis and its mechanisms have been well studied (Mohan et al. 2009). In the field of therapeutic protein production, however, apoptosis and its implications in cell culture have gained attention in the last few decades. Various commercial cell lines used for large-scale production of therapeutic proteins have shown to undergo apoptosis during their cultivation (Cotter and Al-Rubeai 1995). In the case of CHO cell culture, it is known that the primary cause for the inability to maintain a viable cell culture is apoptosis. In particular, the removal of serum or nutrient deprivation at the end of the cell culture has led cells to undergo apoptosis (Goswami et al. 1999; Singh et al. 1994). Therefore, preventing or alleviating apoptosis is crucial for maintaining a high viable cell concentration throughout the culture.

Mechanism of Apoptosis

Apoptosis is an outcome of the caspase-dependent cascade. Although the cascade and participating pathways are complex, they have been well studied and genetically defined. Consequently, researchers have targeted specific genes involved in apoptotic pathways and limited the activation of the cascade in order to prolong culture longevity and enhance the production of therapeutic proteins. Nevertheless, a lot of undiscovered effects of apoptotic targetable sites have yet remained to be analyzed for the optimization of CHO cell cultures. Therefore, understanding the apoptotic pathway is the key to its further application in CHO cell culture. Two major signaling pathways of apoptosis, intrinsic and extrinsic pathways (Fig. 18.2), are briefly discussed in this section.

Intrinsic apoptotic pathway is activated via non-receptor-mediated intracellular signals inside the cell in response to stress, such as DNA damage, lack of nutrients, hypoxia, and detachment from the extracellular matrix (Fulda and Debatin 2006). These kinds of pro-apoptotic stress signals induce mitochondria to release proteins into cytosol to activate a caspase proteolytic cascade in the cytoplasm (Degterev et al. 2003). Cytochrome c, located in the mitochondrial intermembrane space, is one of the important proteins that are released for the initiation of the apoptotic program (Yang et al. 1997). When cytochrome c is released, it triggers a procaspase-activating adaptor protein called Apaf1 to oligomerize into a heptamer called an apoptosome, which then recruits procaspase-9 through a caspase recruitment domain (CARD) (Fulda and Debatin 2006). Eventually, the executioner caspase-3 is activated by apoptosome and cleaves major substrates to run apoptosis, which leads to the destruction of the cell. The fate of the intrinsic pathway lies between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins, which regulate the permeability of the mitochondrial membrane by balancing the release of cytochrome c to the cytosol (Mohan et al. 2009). The Bcl-2 family consists of three families: anti-apoptotic Bcl-2 proteins, pro-apoptotic Bcl-2 homology (BH) 123 proteins, and pro-apoptotic BH 3 only proteins. BH123 proteins include Bax and Bak, and pro-apoptotic BH3 only proteins include Bad, Bim, Bid, Puma, and Noxa (Hengartner 2000). Anti-apoptotic proteins, i.e., Bcl-2

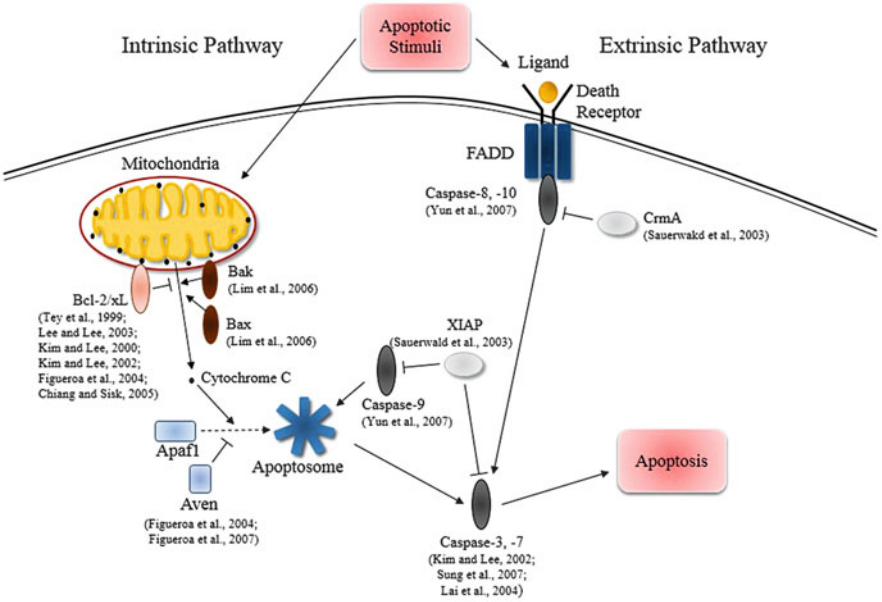

Fig. 18.2 Apoptotic pathway and various strategies for anti-apoptosis cell engineering. *CrmA* cytokine response modifier A, *FADD* Fas-associated death domain, *XIAP* X-linked inhibitor of apoptosis

and Bcl-xL, located on the mitochondrial membrane, not only prevent the release of cytochrome c, but also inhibit the activities of pro-apoptotic proteins.

The extrinsic pathway is regulated within the cell plasma membrane by the binding of ligands to death receptors. These ligands include Apo2L/TRAIL and CD95L/FasL (Ashkenazi 2002). The binding of ligands to death receptors promotes the recruitment of the protein Fas-associated death domain (FADD), which then assembles initiator procaspases, i.e. procaspase-8 and procaspase-10, to form a death-inducing signaling complex (DISC) (Fulda and Debatin 2006). In turn, DISC initiates caspase cascades and executes PCD by eventually activating the downstream effector class of caspases-3, -6, and -7 (Arden and Betenbaugh 2004).

Apoptosis Engineering

Although only the major regulators of apoptosis are described above, there are a lot of key genetic factors that determine either pro- or anti-apoptotic signals. This fact provides us a wide range of cell signals that could be used for genetic modification. However, for therapeutic protein production, the genetic modification to prevent or alleviate apoptosis has mainly focused on the overexpression of anti-apoptotic genes, down regulation of caspases, and the down regulation of pro-apoptotic genes.

The effect of the overexpression of Bcl-2 and Bcl-xL, the anti-apoptotic genes, on CHO cell cultures are the most widely studied topic in the anti-apoptosis approach. In batch cultures, the CHO cell line producing a chimeric antibody with enhanced levels of Bcl-2 showed a 75 % increase in maximum viable cell concentration compared to control cultures. Bcl-2 overexpressing CHO cells also showed better resistance to ammonia toxicity and growth arrest using thymidine (Tey et al. 2000a). The establishment of Bcl-2 overexpressing the dhfr-CHO cell line as the host cell line expedited the developmental process of establishing apoptosis-resistant CHO cell lines, and increased the productivity of therapeutic proteins as well (Lee and Lee 2003). The overexpression of Bcl-2 was also seen in cultures with the addition of sodium butyrate (NaBu). NaBu is known to enhance the expression of foreign genes. Thus, NaBu can improve the level of expression of therapeutic proteins. Despite its positive effect, NaBu has a drawback of causing the cytotoxic effect and inducing apoptosis during cell culture. However, the CHO cells overexpressing Bcl-2 suppressed the NaBu-induced apoptosis and yielded a greater monoclonal antibody concentration than non-overexpressing cells (Kim and Lee 2000). Furthermore, CHO cells overexpressing Bcl-2 showed greater resistance to hyperosmotic stress-induced apoptosis (Kim and Lee 2002a).

The beneficial effect of overexpression Bcl-2 on culture longevity was also seen in other mammalian cell lines, such as NS0 and Burkitt's lymphoma (BL). The overexpression of Bcl-2 in an NS0 cell line suppressed apoptosis and resulted in an approximately 20 % increase in maximum viable cell number. The Bcl-2 transfected NS0 cell line also showed great resistance to nutrient limitation and cytostatic agent, i.e. thymidine (Tey et al. 2000b). In the case of BL cell lines, Bcl-2 transfected cells exhibited a better protection from apoptosis in batch culture as well as in culture under glutamine deprivation (Singh et al. 1996).

Bcl-xL, another widely studied anti-apoptotic gene, also showed positive results in CHO cell culture with its overexpression. The overexpression of Bcl-xL protected cells from apoptosis, and exhibited an increased cell survivability and titer of the products (Figueroa et al. 2004; Chiang and Sisk 2005). Moreover, the overexpression of Bcl-xL suppressed apoptosis upon nutrient depletion in the later stage of batch culture (Kim et al. 2009). The overexpression of Mcl-1, another member of the Bcl-2 family, in CHO cells also showed increased viabilities, suggesting an alternative cell engineering strategy (Reynolds et al. 1994; Majors et al. 2009).

The combinatorial anti-apoptosis engineering of Bcl-2/Bcl-xL with other apoptotic-related genes was investigated. It was found to have displayed synergistic effects against apoptosis. The co-overexpression of Bcl-xL and Aven, a gene known to inhibit the activation of caspases, showed better protection against apoptosis (Figueroa et al. 2004, 2007). The overexpression of Myc-c, a gene related to cell proliferation, with Bcl-2 overexpression resulted in higher proliferation rates and maximum cell concentrations as well as a decreased apoptosis (Ifandi and Al-Rubeai 2005). The co-overexpression of Bcl-2 and Beclin-1, an autophagy inducing gene, yielded a synergistic effect on anti-cell death compared to a single overexpression of Bcl-2 (Lee et al. 2013).

Caspases are members of a family of cysteine protease, which plays a central role in the caspase-cascade system of the induction, transduction, and amplification of intracellular apoptotic signals (Degterev et al. 2003). Thus, the suppression of caspases is a promising strategy of anti-apoptosis engineering. While there are 15 mammalian members of the caspase gene family, genetic engineering in CHO cells has been attempted on two sub-groups of caspases: initiator and effector caspases. Dominant negative mutants of caspase-8 and -9, the initiator caspases, in CHO cells showed an enhanced viability in both batch and fed-batch cultures (Yun et al. 2007). By using antisense RNA and small interfering RNA of caspase-3 and -7, CHO cells were found to be more resistant to NaBu-induced apoptosis (Kim and Lee 2002b; Sung et al. 2007). The cleaved caspase-3 by U6 snRNA promoter-driven ribozyme also resulted in enhanced cell viability and the production of interferon-beta (IFN-β) in low serum cultures (Lai et al. 2004). Another strategy targeting the activity of caspases was using genetic caspase inhibitors, such as X-linked inhibitor of apoptosis (XIAP) and cytokine response modifier A (CrmA). XIAP and CrmA are known to inhibit caspase-9,-3,-7, and caspase-8, respectively. A stable CHO cell line expressing XIAP and CrmA was investigated and showed increased and prolonged viability (Sauerwald et al. 2003).

The down-regulation of caspases may be a beneficial strategy, but a close consideration of the apoptotic pathway and strategy of usage is necessary. Caspases are activated in the downstream of the apoptotic pathway, which is regulated by the upstream release of cytochrome c in mitochondria. Although cells do not cross apoptosis by inhibiting caspase activation in the downstream, cells may compensate for the lack of some effectors by up-regulating the others. Hence, even with the inhibition of caspases, the upstream depolarization of the mitochondrial membrane and the release of cytochrome c may be unavoidable (Sung et al. 2007). Therefore, a meticulous investigation on the interrelationship between apoptotic signals is essential.

Down regulation of pro-apoptotic genes, such as Bax, Bak, Alg-2, and Requiem, was also applied to anti-apoptosis engineering in CHO cells. A stable CHO cell line producing IFN-γ with Bax and Bak genes knocked down displayed an extended culture longevity and higher viable cell densities in fed-batch cultures (Lim et al. 2006). Using small interfering RNA technology, two stable CHO cell lines of Alg-2 and Requiem-silenced successfully yielded higher viable cell densities. In particular, the titer of IFN-γ increased by 2.5-fold in these cell lines (Wong et al. 2006).

Cell engineering on genes that are not directly related to apoptosis, such as telomerase reverse transcriptase (TERT), has been also investigated to enhance cell growth and viable cell density. TERT, which is one of two subunits in telomerase, is responsible for a proper cell division by maintaining the structural integrity of a chromosome and ensuring complete replication of the extreme ends of chromosome termini. The TERT-transfected adherent CHO cell line revealed an increase in telomerase and a better resistance to apoptosis, presumably through a process of healing of DNA breaks (Crea et al. 2006). The role of TERT in producing collagen fibers suggests that the overexpression of TERT strengthened the ability of cells to

adhere to plastic in serum free media and to overcome apoptotic cell death (Crea et al. 2006).

There is no doubt that genetic engineering on apoptosis is beneficial on culture longevity and cell viability. However, a major problem lies with the compensation between the cell survivability and q_p of the therapeutic proteins. In other words, apoptotic engineering does not guarantee its beneficial effects on q_p (Chiang and Sisk 2005; Lee and Lee 2003; Meents et al. 2002a; Tey et al. 2000a). Although there have been many studies that reported positive effects of anti-apoptosis engineering on q_p, there have also been many studies that found no effect on q_p. The clonal and cell line variability is the most reasonable explanation for this variance on q_p. Consequently, the anti-apoptosis engineering is accentuated along with q_p-enhancing factors, such as NaBu and hyperosmolality, even though these factors induce apoptosis. In this regard, the anti-apoptosis engineering, along with q_p-enhancing factors, produces synergistic effects by overwhelming apoptosis induced by q_p-enhancing factors and improving q_p simultaneously.

18.3.1.2 Autophagy

Autophagy, classified as PCD type II, is an evolutionarily conserved catabolic process through the lysosomal-mediated degradation pathway. The term, autophagy, originated from the Greek words meaning 'to eat oneself' (Levine 2005). Upon various stresses, such as nutrient depletion, hypoxia, reactive oxygen species, and DNA damage, the role of autophagy is to commit 'suicide' or to degrade long-lived cytoplasmic organelles to release substrates for biosynthesis and energy generation in order to endure such stress (Kroemer et al. 2010; Rabinowitz and White 2010). Unlike apoptosis, the involvement of autophagy in PCD has been controversial, whether cell death or cell survival, even though opinions are leaning toward a cell survival. While autophagy has been observed in dying cells, the idea of compensation for energy loss suggests that autophagy is a survival mechanism.

Mechanism of Autophagy

Multiple autophagic pathways are involved to form the double-membrane vesicles called autophagosomes, which fuse with lysosome later in the process for the degradation of cytoplasmic organelles and proteins (Fig. 18.3). Many stress sensors are activated through various stresses in order to initiate autophagy.

AMP-activated protein kinase (AMPK) is an energy sensor that is activated following nutrient deprivation, and that triggers autophagy. Activated AMPK phosphorylates the ULK complex, which plays an important role in initiating the autophagic cascade (Egan et al. 2011). The phosphorylated ULK complex dissociates from mTORC1, which is an autophagy inhibiting complex, and forms phagophores, which are the premature forms of autophagosomes (Jimenez-Sanchez

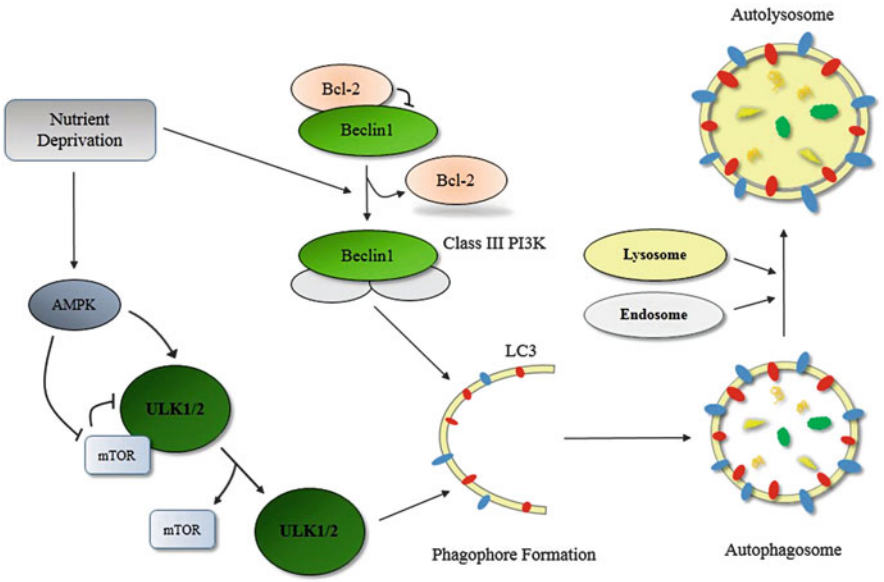

Fig. 18.3 A simplified autophagic pathway from induction by nutrient deprivation to formation of autolysosome. *AMPK* adenosine monophosphate-activated protein kinase, *LC3* microtubule-associated protein light chain 3, *mTORC* mammalian target of rapamycin complex, *PI3K* phosphatidylinositol 3-kinase, *ULK* unc-51-like kinase

et al. 2012). The formation of phagophores requires another critical complex called the Class III phosphatidylinositol 3-kinase (Class III PI3K), while the key protein in the Class III PI3K is Beclin-1, which is responsible for promoting phagopore maturation. The interesting aspect of Beclin-1 is that it is closely related to the anti-apoptotic protein Bcl-2. Under normal conditions or when conditions of nutrient deprivation is not occurring, the binding of Bcl-2 to Beclin-1 inhibits autophagy induction. But under nutrient deprivation, Beclin-1 dissociates from Bcl-2 to induce autophagy (Kroemer et al. 2010).

Autophagy-related proteins (Atgs) undergoes ubiquitin-like reactions to elongate and maturate phagophores to form autophagosomes. Another important protein in the process of autophagosome formation is the microtubule-associated protein 1 light chain 3 (LC3). LC3-I is converted to LC3-II and recruited to phagophore during the maturation of the phagophore. Eventually, LC3-II remains on the autophagosomal membrane until fusion with a lysosome. Thus, the level of LC3-II serves as the bone fide marker for the presence of autophagosome and the level of autophagy (Shvets et al. 2008). The autophagosome undergoes fusion with endosomes and lysosomes. Then, the cytoplasmic contents inside the autophagosome are degraded and the breakdown products are released into the cytosol where the cell utilizes them in various metabolic pathways (Kroemer et al. 2010).

Autophagy Engineering

The role of autophagy has been studied in CHO cell culture for only a few years. The occurrence of autophagy in batch/fed batch cultures and under stressful conditions was observed in 2008 (Hwang and Lee 2008). When glucose and glutamine were limiting towards the end of batch culture, autophagy was observed by the accumulation of LC3-II and TEM images of autophagic vacuoles containing cytoplasmic materials.

In an effort to improve therapeutic protein production, various approaches implying an autophagy pathway have been attempted. These approaches have included using chemical inducers and inhibitors of autophagy, and genetic engineering of core autophagy-related genes. Chemical autophagy inducers and inhibitors appear to exert only partial effects on autophagy and affect a wide range of cellular responses by targeting other cellular pathways. Thus, they are prone to impact the product quality and safety (Kim et al. 2013; Mizhushima and Klionsky 2007; Jardon et al. 2012). Therefore, genetic engineering of autophagy has become a promising strategy to modulate autophagy and enhance the production of therapeutic proteins in CHO cell cultures. However, the genetic approach of autophagy and its effect on therapeutic proteins production has not been widely studied yet.

In 2012, four core autophagy pathway genes (ULK1, Beclin1, Atg7, and Atg9A) were investigated in CHO cell cultures (Lee and Lee 2012). The changes in the mRNA and protein expression of these genes were observed, in which Atg9A was the only gene showing decreased levels of mRNA and protein simultaneously at the late period of the culture. However, the overexpression of Atg9A did not significantly influence the autophagy induction and culture longevity, which suggests that combinatorial regulations of the genes, such as Ulk1 and Beclin-1, would be effective in producing therapeutic proteins (Lee and Lee 2012). Meanwhile, Beclin-1 was co-overexpressed with Bcl-2, which resulted in a more efficient protection of cells from the stressful culture conditions than Bcl-2 alone (Lee et al. 2013). This result provided a potential application of using the synergistic effect of pro-autophagy together with anti-apoptotic engineering in CHO cell cultures.

Although the role of autophagy in CHO cell culture has not been clearly understood yet, the management of autophagy by genetic engineering is promising and opens up novel ways to improve the production of therapeutic proteins (Kim et al. 2013).

18.3.2 Cell Proliferation Engineering

The specific growth rate (μ) of CHO cells is definitely an important feature that needs to be considered when improving TIVCC. Many studies have been targeting genes and chemicals related to cell proliferation in order to increase μ and the maximum viable cell concentration. Although the increase in μ might boost the

maximum viable cell concentration, it is not always the case. The extent of increase in one factor does not correlate with that of the other (Kim et al. 2012).

Cell growth is a thoroughly coordinated process of cell division, which consists of four phases: G1 (growth), S (synthesis), G2 (gap2), and M (mitosis) (Majors et al. 2008). The most important regulators of this pathways are cyclin and cyclin-dependent kinases (CDK), and their role is to drive the transition between each phase of cell cycle (Fussenegger et al. 1998). Many studies have aimed to regulate the process of cell cycle by media modification, feeding materials in a fed-batch culture, and genetic engineering (Altamirano et al. 2000; Bibila and Robinson 1995).

The genetic engineering of cell proliferation in CHO cells has been conducted with genes such as cyclin-dependent kinase like 3 (cdkl1), E2F-1, and cyclin E (Jaluria et al. 2007; Majors et al. 2008; Renner et al. 1995). The enhanced expression of cdkl3, which is involved in G1 to S phase transition, elevated cell proliferation in CHO cells as well as other mammalian cell lines such as HeLa and HEK-293 (Jaluria et al. 2007). The overexpression of E2F-1, which is an important link between phases in the cell cycle, also increased the viable cell concentration in a batch culture of the CHO cells. However, there were no significant changes in monoclonal antibody production (Majors et al. 2008). The high expression level of Cyclin E also stimulated cell proliferation in CHO cells (Renner et al. 1995). A typical oncogenic protein, c-myc, was also found to have positive effects on cell proliferation and, consequently, enhanced μ and the maximum viable cell concentration (Kuystermans and Al-Rubeai 2009). Many other potential engineering targets of genes contributing to the enhancement of cell proliferation, such as valosin-containing protein, requiem, Alg-2, and malate dehydrogenase II, have been discovered via the omics-based approach (Chong et al. 2010; Doolan et al. 2010; Wong et al. 2006).

In an attempt to increase μ in CHO cell culture, the ironic relationship between μ and q_p was found. Although it is difficult to evaluate the definite relationship between μ and q_p, q_p seems to unexpectedly decrease in high μ in CHO cells. Therefore, it is important to interpret the results of cell proliferation engineering with consideration of other unanticipated factors.

18.3.3 Metabolic Engineering

Metabolic engineering is a challenge to indirectly improve the cell growth and TIVCC by mitigating the adverse effects of toxic metabolic by-products, i.e. ammonia and lactate. Ammonia, a product of cellular metabolism and the chemical decomposition of glutamine in the medium, accumulates during the culture. The high level of ammonia accumulation causes the inhibition of cell growth and a decline in TIVCC (Yang and Butler 2000). Lactate, another major waste in CHO cells, is also a troublesome metabolic product during culture. CHO cells have the disadvantage of being unable to completely oxidize glucose to CO_2

and H_20. Therefore, most of the glucose is oxidized to pyruvate, which is eventually converted to lactate by lactate dehydrogenase (LDH) (Kim and Lee 2007a). The accumulated lactate causes acidification, which inhibits cell growth and decreases TIVCC.

In order to reduce the accumulation of waste products, genetic engineering has been used to modify energy metabolism and to redirect cells into pathways utilizing energy more efficiently. In 2006, Zhang et al. investigated metabolism in CHO cells expressing glutamine synthetase (GS), which catalyzes glutamate with ammonia to yield glutamine. When glutamate, a less ammoniagenic substrate, was substituted for glutamine, the ammonia accumulation was reduced (Zhang et al. 2006). With a similar approach to reduce ammonium accumulation, carbamoyl phosphate synthetase I (CPS I) and ornithine transcarbamoylase (OTC) were overexpressed. These genes are related to the urea cycle that eliminates and converts ammonia into urea. The overexpression of CPS I and OTC successfully enhanced cell growth and alleviated the adverse effects of ammonia (Park et al. 2000).

In an attempt to reduce lactate accumulation, pyruvate carboxylase was expressed in CHO cells. Pyruvate carboxylase catalyzes the adenosine triphosphate (ATP)-dependent irreversible carboxylation of pyruvate to form oxaloacetate by bypassing lactate production. Compared to control cells, CHO cells expressing pyruvate carboxylase had 21–39 % decrease in lactate production and a higher cell viability (Kim and Lee 2007b).

Lactate dehydrogenase, which is a major pro-regulator of the conversion between pyruvate and lactate, is definitely a promising target in metabolic engineering. Accordingly, the down-regulation of lactate dehydrogenase-A (LDHa) by siRNAs reduced lactate production with no impairment in cell proliferation and therapeutic protein production (Kim and Lee 2007a). Similar results were also seen in co-knocked down of LDHa and pyruvate dehydrogenase kinases (PDHKs) by siRNAs. The down-regulation of LDHa and PDHKs reduced lactate production, while it increased q_p and volumetric monoclonal antibody production (Zhou et al. 2011).

The effect of fructose-specific transporter (GLUT5) was also investigated in order to reduce lactate accumulation. GLUT5 expression allowed the utilization of fructose as an alternative to glucose and avoided the overflow of excess carbon to lactate by supplying sugar to cells at a more moderate rate. Although an appropriate and precise expression level of GLUT5 is needed to reduce lactate production, the metabolic engineering of transporters for the uptake of nutrients may be a strategy to reduce lactate (Wlaschin and Hu 2007).

Throughout the cultures, ammonia and lactate have always been inconvenient by-products that inhibit cellular growth and apoptosis. The strategies that manipulate the metabolism of CHO cells and reduce toxic waste products demonstrate an alternative way to enhance TIVCC. Rather than targeting cell death or proliferation that might directly influence the TIVCC of CHO cells, metabolic engineering aims to indirectly improve TIVCC by targeting the metabolic characteristics of CHO cells.

18.4 Improving Specific Productivity

To achieve a high product titer in CHO cells, there have also been many attempts to increase q_p as well as to increase TIVCC. The q_p is a key indicator of the high producer cell lines. In accordance with an improved ability to isolate high producers, the typical q_p has increased fivefold over the last decade (Butler and Meneses-Acosta 2012). Along with the enhanced culture longevity, an increased q_p has resulted in at least 20-fold higher product titer over the last two decades (Lim et al. 2010).

Generally, it has been observed that μ and q_p are inversely related. CHO cell cultures under a low culture temperature (Yoon et al. 2003) or hyperosmotic pressure (Kim and Lee 2002a; Lee and Lee 2000) have resulted in an enhanced q_p accompanied by a retarded growth rate. The basis of the relationship between μ and q_p remains elusive because of their effects on multiple cellular processes. For example, the adaptation of CHO cells to a low culture temperature was expected to have increased q_p and μ, but resulted in a decrease in the q_p (Yoon et al. 2006). Generally, high producer cell lines have slow growth rates due to the additional metabolic burden thrust on them (Mohan et al. 2008).

The relationship between cell size, cell cycle and q_p has also been studied. The cell clones expressing a high level of thrombopoietin (TPO) were larger in size and had morphologies resembling non-transfected dhfr-CHO parental cells (Chung et al. 2000). The studies of centrifugal elutriated fractions of a particular cell cycle phase revealed that the cell size is the major cellular determinant of recombinant protein productivity (Lloyd et al. 2000). Recently, a strong positive correlation between cell size and productivity has been reported by transcriptomic and proteomic analysis of CHO cell lines producing monoclonal antibodies (Kang et al. 2013).

With an aim to improve q_p, the bottlenecks in the process of protein secretion need to be identified and resolved. The components in the regulation of cell cycle, folding, secretion, and transport are considered as key regulators. This section will describe these four engineering approaches to resolve those bottlenecks.

18.4.1 Cell Cycle Engineering

A common feature of q_p-enhancing conditions, such as low culture temperature, hyperosmolality, and chemical (NaBu) treatment, is cell cycle arrest (Sunley and Butler 2010). Based on this consensus, one of the strategies to increase q_p in CHO cells is to control proliferation. The proliferation of mammalian cells is controlled by a series of checkpoints that are regulated by a complex network of signaling molecules to ensure that events of cell division do not occur prior to the completion of necessary preceding steps.

The cell cycle itself can be divided into two different phases: an interphase, in which cells are growing and accumulating the nutrients for mitosis and DNA

duplication, and an M-phase, in which cells are split into two daughter cells. The interphase has three sub-divisions, which are G1, S, and G2, and the transitions between the sub-divisions are tightly regulated (Fig. 18.4). During the S phase of interphase, the amounts of DNA in the cells are duplicated for cell division. G1, the first phase within the interphase, and G2, directly after S phase, are called gap preparing for either the replication of DNA (S phase) or cell division (M phase), respectively. The M phase, which consists of mitosis and cytokinesis, is the process of cell separation into two daughter cells that start the cell cycle again. Non-proliferating cells, which are arrested and remained for a long period of time in the G1 phase, may enter the quiescent G0 phase. These cells are maintained in G0 because of the phosphorylation of key cell cycle regulators (Afshari and Barrett 1994). However, they can re-enter the cell cycle process if provided with an appropriate condition.

To enhance the therapeutic protein production in CHO cells, the genetic manipulation of the cell cycle related genes, especially G1/S cell cycle arrest, was actively studied in biotechnology industries. While cell cycle arrest can also occur in the G2/M phase, the induction of arrest in the G1/S phase is more common (Kumar et al. 2007), as summarized in Table 18.1. It has been observed that cell cycle arrest at G1 phase was usually more metabolically activated, increased the cell size, and actively expressed many genes related to ribosome biosynthesis (Bi et al. 2004; Carvalhal et al. 2003; Dez and Tollervey 2004). Genetic targets that induce cell growth arrest have included members of the cyclin-dependent kinase inhibitor (CKI) family such as $p21^{Cip1}$ and $p27^{Kip1}$.

Cyclin dependent kinases (CDKs) are central components of the pathway that regulate cell cycle transition (Sugimoto et al. 2002) and therefore are an important target to attain cell cycle control. CDK activity was directly influenced by cyclins, which are phosphorylated and formed ternary complexes with CKIs such as $p21^{Cip1}$

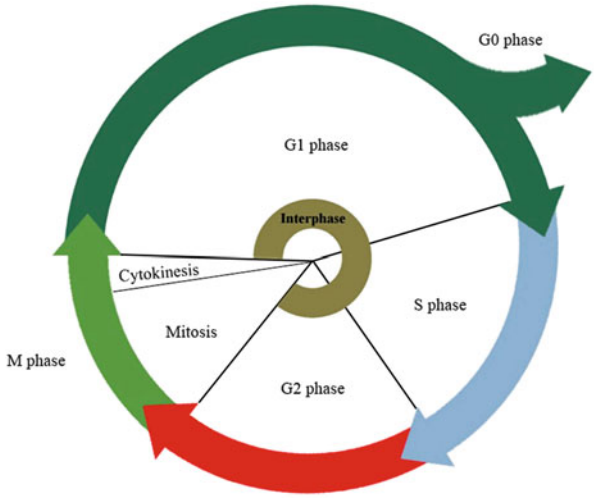

Fig. 18.4 The phases of the mammalian cell cycle, including three sub-divisions of interphase (G1-, S-, G2-phases) and M-phase

and $p27^{Kip1}$ (Grana and Reddy 1995). Based on this consensus, $p21^{Cip1}$ and $p27^{Kip1}$, the cell cycle regulating factors, have been used as engineering targets of mammalian cell culture to obtain a similar effect. The overexpression of the CKIs ($p21^{Cip1}$ and $p27^{Kip1}$) induced growth arrest through the inhibition of CDK activity, blocking the downstream phosphorylation of the Rb protein and consequently arresting the cells in the G1 phase of the cell cycle. Engineering for growth arrest, however, allows only for the overexpression of a few targeted genes such as p53, $p21^{Cip1}$, and $p27^{Kip1}$.

Transiently introducing $p21^{Cip1}$, $p27^{Kip1}$, or p53175P (a p53 mutant showing specific loss of apoptotic function) into secreted alkaline phosphatase (SEAP) producing CHO cell lines achieved approximately fourfold greater productivity than that of the unmodified cell line by G1 phase cell cycle arrest (Fussenegger et al. 1997). The use of G1 cell cycle arrest in CHO cells by stably overexpressing $p27^{Kip1}$ showed a dramatic increase of q_p, for not only SEAP (Carvalhal et al. 2003; Mazur et al. 1998), but also for soluble intracellular adhesion molecule-1 (sICAM) (Meents et al. 2002b). The overexpression of $p21^{Cip1}$ induced the cell cycle arrest and led to about a fourfold increase in q_p of IgG4 producing CHO cell lines (Bi et al. 2004). Similarly, IPTG-inducible expression of $p21^{Cip1}$ resulted in fourfold higher q_p in IgG4 producing NS0 myeloma cells (Watanabe et al. 2002). The combinatorial strategy with $p21^{Cip1}$, anti-apoptotic protein, Bcl-xL, or differentiation factor, CCAAT/enhancer binding protein α (C/EBPα), resulted in a further increase of cell growth and about 10–15 times increase in SEAP production in CHO cells (Fussenegger et al. 1998). Also, an attempt to engineer the combination of $p21^{Cip1}$ and another representative anti-apoptotic protein, Bcl-2, resulted in a greater enhancement in q_p of CHO cells and NS0 cells (Astley and Al-Rubeai 2008; Ibarra et al. 2003). Moreover, it has been suggested that $p21^{Cip1}$-induced cell cycle arrest can shorten the time required for adaptation of cell lines to suspension and protein-free environments (Astley et al. 2007).

The overexpression of transcription factor c-Myc has been recently investigated to enhance the proliferation of CHO cells (Kuystermans and Al-Rubeai 2009). This finding that the therapeutic protein production was increased through enhanced cell growth rather than cell growth arrest showed another target for proliferation engineering, as predicted through a study of histone deacetylase (HDAC)-inhibited growth arrest (Jiang and Sharfstein 2008). However, the effect of genetic engineering for inducing cell growth arrest is hindered by the intrinsic complexity to the biological system. A high-throughput combinatorial screening of cell cycle regulating genes would provide significant benefits to target gene selection for cell engineering by determining gene sets that are able to repress apoptotic triggers in dysregulated cell cycle control.

Table 18.1 Effect of genetic engineering for G1-phase arrest on q_p in mammalian cells

Engineered gene	Cell	Therapeutic protein	Expression system	Effect on q_p	References
$p21^{Cip1}$	CHO	SEAP	Transient	4.6-fold increase	Fussenegger et al. (1997)
$p27^{Kip1}$	CHO	SEAP	Transient	3.9-fold increase	Fussenegger et al. (1997)
p56175P (p53 mutant)	CHO	SEAP	Transient	3.9-fold increase	Fussenegger et al. (1997)
$p27^{Kip1}$	CHO	SEAP	Stable	4-fold increase	Carvalhal et al. (2003)
$p27^{Kip1}$	CHO	sICAM	Stable	5-fold increase	Meents et al. (2002b)
$p21^{Cip1}$	CHO	IgG4	Stable	4-fold increase	Bi et al. (2004)
$p21^{Cip1}$	NS0	IgG4	Controlled[a]	4-fold increase	Watanabe et al. (2002)
$p27^{Kip1}$ and p53175P (p53 mutant)	CHO	SEAP	Stable	10–15-fold increase	Mazur et al. (1998)
$p21^{Cip1}$ and C/EBPα	CHO	SEAP	Controlled[b]	10–15-fold increase	Fussenegger et al. (1998)
$p27^{Kip1}$ and Bcl-xL	CHO	SEAP	Controlled[b]	30-fold increase	Fussenegger et al. (1998)
$p21^{Cip1}$ and Bcl-2	CHO	Monoclonal Antibody (mAb)	Stable	2-fold increase	Astley and Al-Rubeai (2008)
$p21^{Cip1}$ and Bcl-2	NS0	IgG4	Controlled[a]	4-fold increase	Ibarra et al. (2003)

C/EBP α CCAAT/enhancer binding protein α, *SEAP* secreted alkaline phosphatase, *sICAM* soluble intracellular adhesion molecule

[a]IPTG-inducible Lacswitch II system was used

[b]Tetracycline-regulated coexpression system was used; the multicistronic expression unit which is driven by the tetracycline-repressible promoter was used so that gene expression could be simultaneously induced upon withdrawal of tetracycline from the cell culture medium

18.4.2 Chaperone Engineering

Since the therapeutic proteins are secreted proteins, the proteins resident in the endoplasmic reticulum (ER) that has an essential role in the secretory pathway are thought to be a good resource for cell engineering. It has been reported that the amount of heterologous protein secretion does not increase proportionally with the gene copy number or even the intracellular amount of heterologous protein (Schröder 2007). Therefore, the rate-limiting steps in enhancing q_p are believed to be translational or post-translational processes. Some studies have demonstrated that the up-regulation of the ER-resident proteins can increase the production of therapeutic proteins.

Among the ER-resident proteins that have a major role in protein folding, the chaperones machineries are mainly targeted to enhance q_p. The molecular chaperones are ubiquitous proteins that assist polypeptides to reach a proper conformation or cellular location without affecting folding rates or becoming part of the final structure (Baneyx 2004). The heat shock protein (Hsp) family, protein disulfide isomerase (PDI) family, and lectin binding enzymes are three of the common molecular chaperones. Members of these families take part in various functions of the cells.

Numerous studies dealing with chaperone engineering have revealed that the effects of molecular chaperones on protein production are dependent on several factors, including the chaperones concerned, the target therapeutic proteins, and expression systems (Mohan et al. 2008). For example, the effects of PDI overexpression have been mixed, resulting in either enhanced, decreased, or even unaffected q_p. It was discovered that controlled overexpression of ERp57, an isoform of PDI, showed a twofold increase in q_p (Hwang et al. 2003). In another study, PDI overexpression had no effect on the q_p for an interleukin-15 producing cell line, whereas PDI overexpression appeared to negatively affect the q_p for a tumor necrosis factor receptor:Fc fusion protein (TNFR:Fc) producing cell line due to its co-localization and intracellular retention (Davis et al. 2000). It was also observed that the controlled overexpression of PDI showed no effect on the q_p for the TPO producing cell line, while it only moderately enhanced the q_p in a monoclonal antibody producing cell line (Mohan et al. 2007). PDI is the most widely studied chaperone for CHO cell engineering and could enhance q_p, depending on the target therapeutic protein. Recently, it has been reported that the transient expression of PDI family proteins, PDI, ERp72, or pancreatic PDI (PDIp), did not show any improvement in the q_p (Hayes et al. 2010).

Other chaperones have also been studied and showed their relation to increase the q_p. The overexpression of immunoglobulin heavy chain-binding proteins (BiP) appeared to be positively related to an increased productivity in mammalian cells (Jones et al. 2005). However, BiP overexpression in combinations with PDI resulted in a decreased q_p (Borth et al. 2005) and its down-regulation rather than its overexpression, resulted in an improved q_p (Dorner et al. 1988). Simultaneous overexpression of both calnexin and calreticulin, which are lectin binding chaperones, resulted in an enhanced q_p with no inhibiting effect on the cell growth (Chung et al. 2004).

Employing an inducible chaperone expression system seems superior to the constitutive overexpression of chaperones for determining the effect of chaperone expression on q_p, because the occurrence of clonal variability is excluded (Mohan et al. 2008). Fundamentally, the effects of chaperones on protein production depend on several factors such as the employment of an expression system, the target therapeutic proteins, and the overexpressed chaperones. This can be described by previous studies shown in Table 18.2.

Targeting a single component of the secretory pathway may not be the best strategy for increasing the q_p since the gene regulation system in mammalian cells is complicated. A detailed understanding of protein folding in the ER should be

Table 18.2 Effect of chaperone engineering on q_p of various target proteins produced in CHO cells

Chaperone	Expression system	Therapeutic protein	Effect on q_p	Reference
PDI	Overexpression	mAb	Positive	Borth et al. (2005)
PDI	Overexpression	Interleukin-15	No effect	Davis et al. (2000)
PDI	Overexpression	TNFR:Fc	Negative	Davis et al. (2000)
PDI	Controlled expression[a]	Thrombopoietin	No effect	Mohan et al. (2007)
PDI	Controlled expression[a]	mAb	Moderate effect	Mohan et al. (2007)
BiP	Overexpression	mAb	Negative	Borth et al. (2005)
BiP and PDI	Overexpression	mAb	Negative	Borth et al. (2005)
ERp57	Controlled expression[a]	Thrombopoietin	Positive	Hwang et al. (2003)
Calnexin and calreticulin	Controlled expression[a]	Thrombopoietin	Positive	Chung et al. (2004)

BiP immunoglobulin heavy chain-binding protein, *mAb* monoclonal antibodies, *PDI* protein disulfide isomerase, *TNFR: Fc* a tumor necrosis factor receptor: Fc fusion protein
[a]Doxycycline-regulated expression system (Tet-Off system) was used

preceded so that a more delicate chaperone regulation could enhance the productivity. An overexpression of several chaperones, co-chaperones, holdases and/or foldases, along with a functionally meaningful ratio to modulate the folding environment might be a better approach (Mohan et al. 2008).

18.4.3 Secretion Engineering

Secretion of therapeutic proteins in mammalian cells is mediated by membrane-bound transport vesicles. An effective regulation of this complex trafficking machinery might be one of the strategies to achieve an increased secretion and ultimately improved productivity. To do that, detailed understanding of the membrane fusion between transport vesicles and the target membrane is necessary. In vesicle trafficking, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that are anchored to both transport vesicles and their target membranes trigger membrane fusion (Jahn and Scheller 2006). In the process of SNARE-mediated membrane fusion, the interaction of Sec1/Munc18 (SM) family proteins with SNAREs is essential.

In order to increase q_p by relieving the bottleneck in secretion, key components of membrane fusion events have been introduced for cell engineering. Stable expression of SNAREs and SM protein in mammalian cells increased the production of therapeutic proteins. While other SNAREs related to exocytosis have had no effect or a negative effect, SNAP-23 and VMAP8 specifically showed a positive effect on the q_p when ectopically and stably expressed in mammalian cells (Peng et al. 2011). Also, Sly1- and Munc18-based vesicle traffic engineering resulted in an increased secretory capacity of different therapeutic proteins in CHO cells. Sly1 and Munc18 emerged as SM proteins modulate ER-to-Golgi- and Golgi-to-plasma membrane-addressed exocytosis (Peng and Fussenegger 2009).

While a lot of efforts have been made to develop transcription- and translation-based engineering strategies for improved production of therapeutic proteins, little work has been done on the posttranslational capacity of mammalian cells (Barnes and Dickson 2006; Fussenegger and Hauser 2007; Weber and Fussenegger 2007; Wurm 2004). As some of the strategies have targeted the posttranslational process, boosting the secretion by up-regulating proteins involved in trafficking machinery is a novel engineering strategy to increase q_p.

18.4.4 Unfolded Protein Response-Based Engineering

In mammalian cells, secreted therapeutic proteins are post- or co-translationally translocate into the lumen of the ER for protein folding and maturation. The ER provides an oxidizing environment and enzymes required for protein modification (Ellgaard and Helenius 2003). In the ER, a protein must have reached a correctly folded conformation. If the folding and maturation process fails, the protein is not transported to its final destination, and is eventually degraded.

When the ectopic expression of therapeutic proteins reaches the ER protein-folding capacity, unfolded proteins are accumulated in the lumen and cause ER stress. This leads to the activation of intracellular signal transduction pathways for an unfolded protein response (UPR) to maintain cellular homeostasis (Ron and Walter 2007). In mammalian cells, the UPR signaling pathway activates four processes: (i) attenuation in protein translation (Harding et al. 1999; Ron 2002), (ii) induction of chaperone expression (Haze et al. 1999; Mori 2000), (iii) ER-associated degradation (ERAD) of misfolded proteins (Hosokawa et al. 2001; Yoshida et al. 2003), and (iv) apoptosis (McCullough et al. 2001; Fig. 18.5). The regulation of components in the UPR signaling pathway has been suggested to increase the secretory capacity of cells.

X-box binding protein 1 (XBP-1) has been widely studied in UPR-based engineering. The sliced form of XBP-1 (XBP-1s) functions as a transcription activator and up-regulates many ER chaperones. It has been revealed that the overexpression of XBP-1 can increase the q_p of various therapeutic proteins, whereas XBP-1u has no effect on the q_p (Tigges and Fussenegger 2006). Also, in fed-batch cultivation, the heterologous expression of XBP-1 led to an increase in ER content and

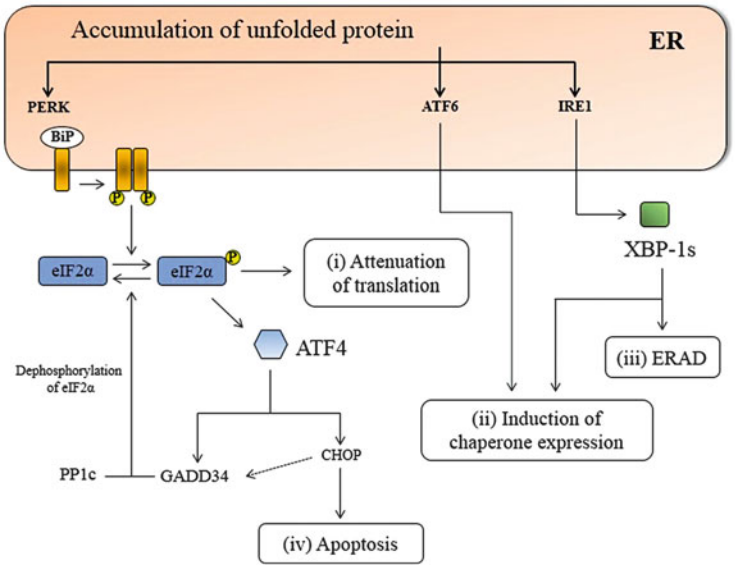

Fig. 18.5 Three different unfolded protein response (*UPR*) signaling pathways that are mediated by inositol-requiring protein-1 (*IRE1*), activating transcription factor-6 (*ATF6*) or protein kinase RNA (*PKR*)-like ER kinase (*PERK*). *ERAD* ER-associated degradation, *XBP* X-box binding protein

enhanced q_p in monoclonal antibody producing cells (Becker et al. 2008). In another study, it has been suggested that the effects of the overexpression of XBP-1 on q_p depends on the expression levels of recombinant proteins (Ku et al. 2008). The overexpression of XBP-1 was particularly effective in CHO cells experiencing secretion bottleneck.

Another strategy in UPR-based engineering was to restore the attenuation in protein translation. Activating transcription factor (ATF4), one of the key regulators in UPR system, releases the translational attenuation by the dephosphorylation of eukaryotic initiation factor-2α via growth arrest and DNA damage inducible protein 34 (GADD34) (Ron 2002). The overexpression of ATF4 and GADD34 showed an enhanced q_p of recombinant antithrombin III (AT-III) (Ohya et al. 2008; Omasa et al. 2008).

Conclusions

We have discussed the two keys to achieve a cost-effective level of production: the specific productivity and time integral of viable cell concentration. Many different cellular mechanisms and characteristics of CHO cells including cell death, metabolism, cell cycling, and protein folding are involved to increase these two variables. Therefore, understanding these mechanisms is

(continued)

certainly important to magnify the benefits of genetic engineering and to propose additional applications. Primarily by expressing specific targeted genes, many studies have increased q_p and TIVCC. Recently, not only the productivity of therapeutic proteins is a significant issue in CHO cell cultures, but also the quality of them is of great concern. With a clear evaluation of the elusive characteristics of CHO cells, CHO cell engineering will efficiently maximize the production of therapeutic proteins.

References

Afshari CA, Barrett JC (1994) Disruption of G0-G1 arrest in quiescent and senescent cells treated with phosphatase inhibitors. Cancer Res 54:2317–2321

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

Arden N, Betenbaugh MJ (2004) Life and death in mammalian cell culture: strategies for apoptosis inhibition. Trends Biotechnol 22:174–180

Ashkenazi A (2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2:420–430

Astley K, Al-Rubeai M (2008) The role of Bcl-2 and its combined effect with p21CIP1 in adaptation of CHO cells to suspension and protein-free culture. Appl Microbiol Biotechnol 78:391–399

Astley K, Naciri M, Racher A, Al-Rubeai M (2007) The role of p21cip1 in adaptation of CHO cells to suspension and protein-free culture. J Biotechnol 130:282–290

Baneyx F (2004) Keeping up with protein folding. Microb Cell Fact 3:6

Barnes LM, Dickson AJ (2006) Mammalian cell factories for efficient and stable protein expression. Curr Opin Biotechnol 17:381–386

Becker E, Florin L, Pfizenmaier K, Kaufmann H (2008) An XBP-1 dependent bottle-neck in production of IgG subtype antibodies in chemically defined serum-free Chinese hamster ovary (CHO) fed-batch processes. J Biotechnol 135:217–223

Bi JX, Shuttleworth J, Al-Rubeai M (2004) Uncoupling of cell growth and proliferation results in enhancement of productivity in p21CIP1-arrested CHO cells. Biotechnol Bioeng 85:741–749

Bibila TA, Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotechnol Prog 11:1–13

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

Broker LE, Kruyt FAE, Giaccone G (2005) Cell death independent of caspases: a review. Clin Cancer Res 11:3155–3162

Butler M, Meneses-Acosta A (2012) Recent advances in technology supporting biopharmaceutical production from mammalian cells. Appl Microbiol Biotechnol 96:885–894

Carvalhal AV, Marcelino I, Carrondo MJ (2003) Metabolic changes during cell growth inhibition by p27 overexpression. Appl Microbiol Biotechnol 63:164–173

Chiang GG, Sisk WP (2005) Bcl-xL mediates increased production of humanized monoclonal antibodies in Chinese hamster ovary cells. Biotechnol Bioeng 91:779–792

Chong WP, Reddy SG, Yusufi FN, Lee DY, Wong NS, Heng CK, Yap MG, Ho YS (2010) Metabolomics-driven approach for the improvement of Chinese hamster ovary cell growth: overexpression of malate dehydrogenase II. J Biotechnol 147:116–121

Chung JY, Kim TK, Lee GM (2000) Morphological selection of parental Chinese hamster ovary cell clones exhibiting high-level expression of recombinant protein. Biotechniques 29:768–774

Chung JY, Lim SW, Hong YJ, Hwang SO, Lee GM (2004) Effect of doxycycline-regulated calnexin and calreticulin expression on specific thrombopoietin productivity of recombinant Chinese hamster ovary cells. Biotechnol Bioeng 85:539–546

Cotter T, Al-Rubeai M (1995) Cell death (apoptosis) in cell culture systems. Trends Biotechnol 13: 150–155

Crea F, Sarti D, Falciani F, Al-Rubeai M (2006) Over-expression of hTERT in CHO K1 results in decreased apoptosis and reduced serum dependency. J Biotechnol 121:109–123

Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116:205–219

Davis R, Schooley K, Rasmussen B, Thomas J, Reddy P (2000) Effect of PDI overexpression on recombinant protein secretion in CHO cells. Biotechnol Prog 16:736–743

Degterev A, Boyce M, Yuan J (2003) A decade of caspases. Oncogene 22:8543–8567

Dez C, Tollervey D (2004) Ribosome synthesis meets the cell cycle. Curr Opin Microbiol 7: 631–637

Doolan P, Meleady P, Barron N, Henry M, Gallagher R, Gammell P, Melville M, Sinacore M, McCarthy K, Leonard M, Charlebois T, Clynes M (2010) Microarray and proteomics expression profiling identifies several candidates, including the valosin-containing protein (VCP), involved in regulating high cellular growth rate in production CHO cell lines. Biotechnol Bioeng 106:42–56

Dorner AJ, Krane MG, Kaufman RJ (1988) Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. Mol Cell Biol 8:4063–4070

Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrik J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ (2011) Phosphorylation of ULK1 (hATG1) by AMP-Activated protein kinase connects energy sensing to mitophagy. Science 331:456–461

Ellgaard L, Helenius A (2003) Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol 4:181–191

Figueroa B, Chen S, Oyler GA, Hardwick JM, Betenbaugh MJ (2004) Aven and Bcl-xL enhance protection against apoptosis for mammalian cells exposed to various culture conditions. Biotechnol Bioeng 85:589–600

Figueroa B, Ailor E, Osborne D, Hardwick JM, Reff M, Betenbaugh MJ (2007) Enhanced cell culture performance using inducible anti-apoptotic genes E1B-19 K and Aven in the production of a monoclonal antibody with Chinese hamster ovary cells. Biotechnol Bioeng 97: 877–892

Fulda S, Debatin KM (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25:4798–4811

Fussenegger M, Hauser H (2007) Protein expression by engineering of yeast, plant and animal cells. Curr Opin Biotechnol 18:385–386

Fussenegger M, Mazur X, Bailey JE (1997) A novel cytostatic process enhances the productivity of Chinese hamster ovary cells. Biotechnol Bioeng 55:927–939

Fussenegger M, Schlatter S, Datwyler D, Mazur Z, Bailey JE (1998) Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. Nat Biotechnol 16:468–472

Goswami J, Sinskey AJ, Steller H, Stephanopoulos GN, Wang DIC (1999) Apoptosis in batch cultures of Chinese hamster ovary cells. Biotechnol Bioeng 62:632–640

Grana X, Reddy EP (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11:211–219

Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271–274

Hayes NV, Smales CM, Klappa P (2010) Protein disulfide isomerase does not control recombinant IgG4 productivity in mammalian cell lines. Biotechnol Bioeng 105:770–779

Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10:3787–3799

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776

Hosokawa N, Wada I, Hasegawa K, Yorihuzi T, Tremblay LO, Herscovics A, Nagata K (2001) A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep 2:415–422

Hwang SO, Lee GM (2008) Nutrient deprivation induces autophagy as well as apoptosis in Chinese hamster ovary cell culture. Biotechnol Bioeng 99:678–685

Hwang SO, Chung JY, Lee GM (2003) Effect of doxycycline-regulated ERp57 expression on specific thrombopoietin productivity of recombinant CHO cells. Biotechnol Prog 19:179–184

Ibarra N, Watanabe S, Bi JX, Shuttleworth J, Al-Rubeai M (2003) Modulation of cell cycle for enhancement of antibody productivity in perfusion culture of NS0 cells. Biotechnol Prog 19: 224–228

Ifandi V, Al-Rubeai M (2005) Regulation of cell proliferation and apoptosis in CHO-K1 cells by the coexpression of c-Myc and Bcl-2. Biotechnol Prog 32:671–677

Jahn R, Scheller RH (2006) SNAREs–engines for membrane fusion. Nat Rev Mol Cell Biol 7:631–643

Jaluria P, Betenbaugh M, Konstantopoulos K, Shiloach J (2007) Enhancement of cell proliferation in various mammalian cell lines by gene insertion of a cyclin-dependent kinase homolog. BMC Biotechnol 7:71–82

Jardon MA, Sattha B, Braasch K, Leung AO, Côté HC, Butler M, Gorski SM, Piret JM (2012) Inhibition of glutamine-dependent autophagy increases t-PA production in CHO cell fed-batch processes. Biotechnol Bioeng 109:1228–1238

Jiang Z, Sharfstein ST (2008) Sodium butyrate stimulates monoclonal antibody over-expression in CHO cells by improving gene accessibility. Biotechnol Bioeng 100:189–194

Jimenez-Sanchez M, Tomson F, Zavodszky E, Rubinsztein DC (2012) Autophagy and polyglutamine diseases. Prog Neurobiol 97:67–82

Jones J, Nivitchanyong T, Giblin C, Ciccarone V, Judd D, Gorfien S, Krag SS, Betenbaugh MJ (2005) Optimization of tetracycline-responsive recombinant protein production and effect on cell growth and ER stress in mammalian cells. Biotechnol Bioeng 91:722–732

Kang S, Ren D, Xiao G, Daris K, Buck L, Enyenihi AA, Zubarev R, Bondarenko PV, Deshpande R (2013) Cell line profiling to improve monoclonal antibody production. Biotechnol Bioeng 9999:1–13

Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257

Kim NS, Lee GM (2000) Overexpression of bcl-2 inhibits sodium butyrate-induced apoptosis in Chinese hamster ovary cells resulting in enhanced humanized antibody production. Biotechnol Bioeng 71:184–193

Kim NS, Lee GM (2002a) Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression. J Biotechnol 95:237–248

Kim NS, Lee GM (2002b) Inhibition of sodium butyrate-induced apoptosis in recombinant Chinese hamster ovary cells by constitutively expressing antisense RNA of caspase-3. Biotechnol Bioeng 78:217–228

Kim SH, Lee GM (2007a) Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. Appl Microbiol Biotechnol 74:152–159

Kim SH, Lee GM (2007b) Functional expression of human pyruvate carboxylase for reduced lactic acid formation of Chinese hamster ovary cells (DG44). Appl Microbiol Biotechnol 76: 659–665

Kim YG, Kim JY, Mohan C, Lee GM (2009) Effect of Bcl-xL overexpression on apoptosis and autophagy in recombinant Chinese hamster ovary cells under nutrient-deprived condition. Biotechnol Bioeng 103:757–766

Kim JY, Kim YG, Lee GM (2012) CHO cells in biotechnology for production of recombinant proteins: current state and further potential. Appl Microbiol Biotechnol 93:917–930
Kim YJ, Baek E, Lee JS, Lee GM (2013) Autophagy and its implication in Chinese hamster ovary cell culture. Biotechnol Lett 35:1753–1763
Kroemer G, Marino G, Levine B (2010) Autophagy and the integrated stress response. Mol Cell 40:280–293
Ku SC, Ng DT, Yap MG, Chao SH (2008) Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells. Biotechnol Bioeng 99:155–164
Kumar N, Gammell P, Clynes M (2007) Proliferation control strategies to improve productivity and survival during CHO based production culture: a summary of recent methods employed and the effects of proliferation control in product secreting CHO cell lines. Cytotechnology 53: 33–46
Kuystermans D, Al-Rubeai M (2009) cMyc increases cell number through uncoupling of cell division from cell size in CHO cells. BMC Biotechnol 9:76–88
Lai D, Fu L, Weng S, Qi L, Yu C, Yu T, Wang H, Chen W (2004) Blocking caspase-3 activity with a U6 SnRNA promoter-driven ribozyme enhances survivability of CHO cells cultured in low serum medium and production of interferon-β. Biotechnol Bioeng 85:20–28
Lee MS, Lee GM (2000) Hyperosmotic pressure enhances immunoglobulin transcription rates and secretion rates of KR12H-2 transfectoma. Biotechnol Bioeng 68:260–268
Lee SK, Lee GM (2003) Development of apoptosis-resistant dihydrofolate reductase-deficient Chinese hamster ovary cell line. Biotechnol Bioeng 82:872–876
Lee JS, Lee GM (2012) Estimation of autophagy pathway genes for autophagy induction: overexpression of Atg9A does not induce autophagy in recombinant Chinese hamster ovary cells. Biochem Eng J 68:221–226
Lee JS, Ha TK, Park JH, Lee GM (2013) Anti-cell death engineering of CHO cells: co-overexpression of Bcl-2 for apoptosis inhibition, Beclin-1 for autophagy induction. Biotechnol Bioeng 110:2195–2207
Levine B (2005) Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell 120:159–162
Lim SF, Chuan KH, Liu S, Loh SOH, Chung BYF, Ong CC, Song Z (2006) RNAi suppression of Bax and Bak enhances viability in fed-batch cultures of CHO cells. Metab Eng 8:509–522
Lim Y, Wong NS, Lee YY, Ku SC, Wong DC, Yap MG (2010) Engineering mammalian cells in bioprocessing – current achievements and future perspectives. Biotechnol Appl Biochem 55: 175–189
Lloyd DR, Holmes P, Jackson LP, Emery AN, Al-Rubeai M (2000) Relationship between cell size, cell cycle and specific recombinant protein productivity. Cytotechnology 34:59–70
Majors BS, Arden N, Oyler GA, Chiang GG, Pederson NE, Betenbaugh MJ (2008) E2F-1 overexpression increases viable cell density in batch cultures of Chinese hamster ovary cells. J Biotechnol 138:103–106
Majors BS, Betenbaugh MJ, Pederson NE, Chiang GG (2009) Mcl-1 overexpression leads to higher viabilities and increased production of humanized monoclonal antibody in Chinese hamster ovary cells. Biotechnol Prog 25:1161–1168
Mazur X, Fussenegger M, Renner WA, Bailey JE (1998) Higher productivity of growth-arrested Chinese hamster ovary cells expressing the cyclin-dependent kinase inhibitor p27. Biotechnol Prog 14:705–713
McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21:1249–1259
Meents H, Enenkel B, Eppenberger HM, Werner RG, Fussenegger M (2002a) Impact of coexpression and coamplification of sICAM and antiapoptosis determinants bcl-2/bcl-xL on productivity, cell survival, and mitochondria number in CHO-DG44 grown in suspension and serum-free media. Biotechnol Bioeng 80:706–716

Meents H, Enenkel B, Werner RG, Fussenegger M (2002b) p27Kip1-mediated controlled proliferation technology increases constitutive sICAM production in CHO-DUKX adapted for growth in suspension and serum-free media. Biotechnol Bioeng 79:619–627
Mizhushima N, Klionsky DJ (2007) Protein turnover via autophagy: implication for metabolism. Annu Rev Nutr 27:19–40
Mohan C, Park SH, Chung JY, Lee GM (2007) Effect of doxycycline-regulated protein disulfide isomerase expression on the specific productivity of recombinant CHO cells: thrombopoietin and antibody. Biotechnol Bioeng 98:611–615
Mohan C, Kim YG, Koo J, Lee GM (2008) Assessment of cell engineering strategies for improved therapeutic protein production in CHO cells. Biotechnol J 3:624–630
Mohan C, Kim YG, Lee GM (2009) Apoptosis and autophagy cell engineering. Cell Line Dev 6: 195–216
Mori K (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101:451–454
Ohya T, Hayashi T, Kiyama E, Nishii H, Miki H, Kobayashi K, Honda K, Omasa T, Ohtake H (2008) Improved production of recombinant human antithrombin III in Chinese hamster ovary cells by ATF4 overexpression. Biotechnol Bioeng 100:317–324
Omasa T, Takami T, Ohya T, Kiyama E, Hayashi T, Nishii H, Miki H, Kobayashi K, Honda K, Ohtake H (2008) Overexpression of GADD34 enhances production of recombinant human antithrombin III in Chinese hamster ovary cells. J Biosci Bioeng 106:568–573
Park HS, Kim IK, Kim IY, Kim KH, Kim HJ (2000) Expression of carbamyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary dhfr-cells decreases accumulation of ammonium ion in culture media. J Biotechnol 81:129–140
Peng RW, Fussenegger M (2009) Molecular engineering of exocytic vesicle traffic enhances the productivity of Chinese hamster ovary cells. Biotechnol Bioeng 102:1170–1181
Peng RW, Abellan E, Fussenegger M (2011) Differential effect of exocytic SNAREs on the production of recombinant proteins in mammalian cells. Biotechnol Bioeng 108:611–620
Rabinowitz JD, White E (2010) Autophagy and metabolism. Science 330:1344–1348
Renner WA, Lee KH, Hatzimanikatis V, Bailey JE, Eppenberger HM (1995) Recombinant cyclin E expression activates proliferation and obviates surface attachment of Chinese hamster ovary (CHO) cells in protein-free medium. Biotechnol Bioeng 47:476–482
Reynolds JE, Yang T, Qian L (1994) Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer Res 54:6348–6352
Ron D (2002) Translational control in the endoplasmic reticulum stress response. J Clin Invest 110:1383–1388
Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8:519–529
Sauerwald TM, Oyler GA, Betenbaugh MJ (2003) Study of caspase inhibitors for limiting death in mammalian cell culture. Biotechnol Bioeng 81:329–340
Schröder M (2007) The cellular response to protein unfolding stress. In: Robson GD, van West P, Gadd GM (eds) Exploitation of fungi, vol 26, British mycological society symposium series. Cambridge University Press, Leiden, pp 117–139
Shvets E, Fass E, Elazar Z (2008) Utilizing flow cytometry to monitor autophagy in living mammalian cells. Autophagy 4:621–628
Singh RP, Al-Rubeai M, Gregory CD, Emery AN (1994) Cell death in bioreactors: a role for apoptosis. Biotechnol Bioeng 44:720–726
Singh RP, Emery AN, Al-Rubeai M (1996) Enhancement of survivability of mammalian cells by overexpression of the apoptosis-suppressor gene bcl-2. Biotechnol Bioeng 52:166–175
Sugimoto M, Martin N, Wilks DP, Tamai K, Huot TJ, Pantoja C, Okumura K, Serrano M, Hara E (2002) Activation of cyclin D1-kinase in murine fibroblasts lacking both p21(Cip1) and p27(Kip1). Oncogene 21:8067–8074

Sung YH, Lee JS, Park SH, Koo J, Lee GM (2007) Influence of co-down-regulation of caspase-3 and caspase-7 by siRNAs on sodium butyrate-induced apoptotic cell death of Chinese hamster ovary cells producing thrombopoietin. Metab Eng 9:452–464

Sunley K, Butler M (2010) Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. Biotechnol Adv 28:385–394

Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M (2000a) Influence of Bcl-2 on cell death during the cultivation of a Chinese hamster ovary cell line expressing a chimeric antibody. Biotechnol Bioeng 68:31–43

Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M (2000b) Bcl-2 mediated suppression of apoptosis in myeloma NS0 cultures. J Biotechnol 79:147–159

Tigges M, Fussenegger M (2006) Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells. Metab Eng 8:264–272

Watanabe S, Shuttleworth J, Al-Rubeai M (2002) Regulation of cell cycle and productivity in NS0 cells by the over-expression of p21CIP1. Biotechnol Bioeng 77:1–7

Weber W, Fussenegger M (2007) Inducible product gene expression technology tailored to bioprocess engineering. Curr Opin Biotechnol 18:399–410

Wlaschin KF, Hu WS (2007) Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. J Biotechnol 131:168–176

Wong DC, Wong KT, Nissom PM, Heng CK, Yap MG (2006) Targeting early apoptotic genes in batch and fed-batch CHO cell cultures. Biotechnol Bioeng 95:350–361

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22:1393–1398

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555–556

Yang M, Butler M (2000) Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol Bioeng 68:370–380

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275:1129–1132

Yoon SK, Song JY, Lee GM (2003) Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng 82:289–298

Yoon SK, Hong JK, Choo SH, Song JY, Park HW, Lee GM (2006) Adaptation of Chinese hamster ovary cells to low culture temperature: cell growth and recombinant protein production. J Biotechnol 122:463–472

Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K, Mori K (2003) A time-dependent phase shift in the mammalian unfolded protein response. Dev Cell 4:265–271

Yun CY, Liu S, Lim SF, Wang TW, Chung BYF, Teo JJ, Chuan KH, Soon ASC, Goh KS, Song Z (2007) Specific inhibition of caspase-8 and -9 in CHO cells enhances cell viability in batch and fed-batch cultures. Metab Eng 9:406–418

Zhang F, Sun X, Yi X, Zhang Y (2006) Metabolic characteristics of recombinant Chinese hamster ovary cells expressing glutamine synthetase in presence and absence of glutamine. Cytotechnology 51:21–28

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