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Genetic Aspects of Cell Line Development from a Synthetic Biology Perspective

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Abstract Animal cells can be regarded as factories for the production of relevant proteins. The advances described in this chapter towards the development of cell lines with higher productivity capacities, certain metabolic and proliferation properties, reduced apoptosis and other features must be regarded in an integrative perspective. The systematic application of systems biology approaches in combination with a synthetic arsenal for targeted modification of endogenous networks are proposed to lead towards the achievement of a predictable and technologically advanced cell system with high biotechnological impact.

Keywords Synthetic biology · Mammalian cells · Controlled genetic engineering · Synthetic expression cassettes · Controlled intervention

Contents

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1 Introduction

Mammalian cell lines have been used for decades for the production of biopharmaceuticals. To date, the development of producer cells mainly focuses on the optimization of the features of the recombinant expression cassettes that encode the biopharmaceutical proteins of interest. Highly potent promoter elements that allow high levels of expression have been identified. In addition, protocols for amplification of these cassettes have been developed. Recent approaches also acknowledge the impact of the chromosomal integration site on the performance of the recombinant expression cassettes.

However, due to the great complexity of mammalian cells and the multifactorial facets of cell productivity, it has so far been difficult to systematically circumvent limitations that still affect the production of recombinant proteins. There is growing evidence that high-producer cell clones must have not only optimal design but also an appropriate chromosomal integration site of the expression cassette. Cells often accumulate random, uncontrolled mutations that render them better producer systems. On the other hand, considerable efforts have been made to increase genomic stability and productivity by manipulating cellular properties such as cell growth or apoptosis.

While the first example of a pure synthetic prokaryotic organism was given recently [58], such an endeavor does not seem to be feasible for mammalian cells in the near future. Due to the greater complexity of the mammalian cell, synthetic approaches focus on modulation of selected pathways or networks. For this purpose, strategies are required that allow us to specifically combine cellular networks or to interfere with them. Examples of such approaches concern cellular metabolic networks and cellular proliferation. While much data and information on relevant pathways have been assembled, a deep systems-biology-based analysis of these networks is not yet available. This is the focus of current work in the field. A systems-biology-driven rational engineering of cells is expected to increase the productivity of cell systems directly or indirectly.

To date, approaches for development of cell systems for production have not been guided by systems biology analysis. They have been based on a patchy knowledge of cellular processes. A consequent systems biology analysis as it is carried out in this rapidly growing field will permit the expansion of these efforts to new targets. In the first part of this review, the state of the art of methods which allow controlled expression of genes is summarized. Genetic engineering of cells is a prerequisite for rationally coupling or interfering with distinct cellular pathways. This section is followed by one that describes selected approaches to inducing specific expression phenotypes. Finally, approaches that have been initiated to alter certain cellular phenotypes are described. The methods presented here are perfect tools with which to intervene in all types of cellular processes and are thus key for challenging or confirming new systems-biology-driven networks.

2 Approaches to Tuning Gene Expression in Mammalian Cells

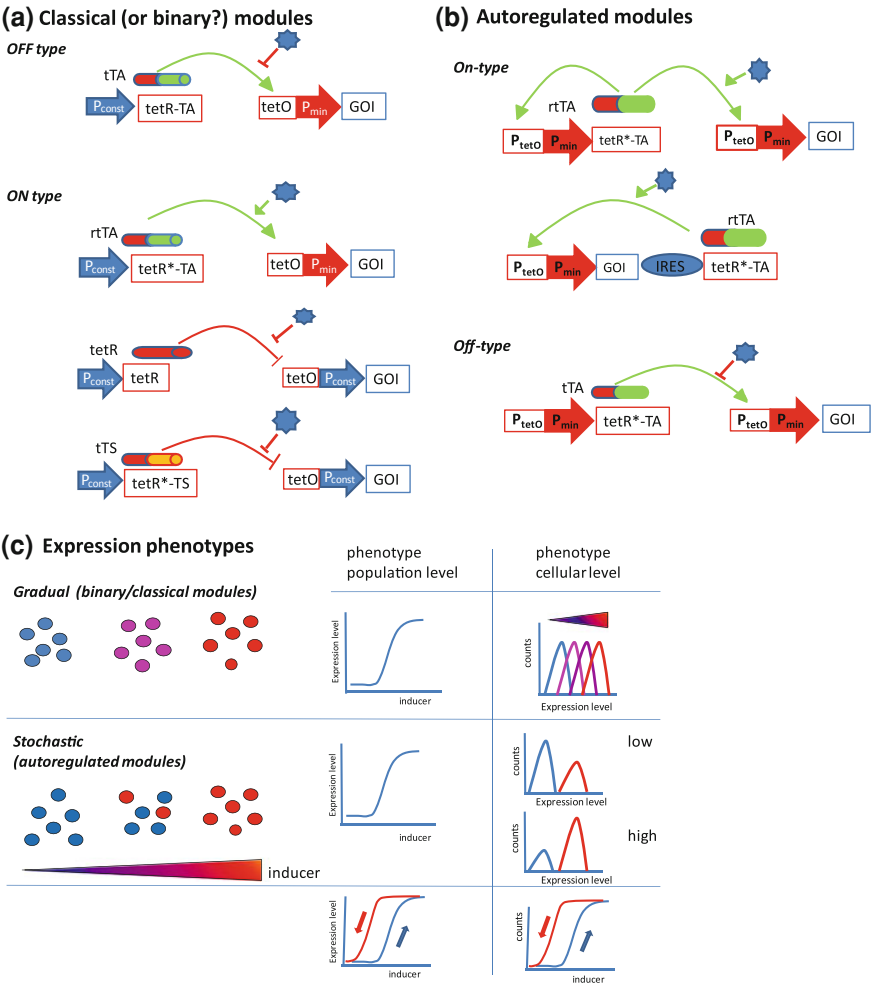
To control expression of genes in mammalian cells, regulatory circuits that are largely independent of the cellular metabolism have been developed. These comprise both transcriptionally regulated modules for tunable expression of transgenes, and post-transcriptionally regulated elements such as the ones that control protein stability. In addition, siRNA-based approaches are used to down-regulate cellular genes. The basic principles of these approaches together with examples will be discussed in the following paragraphs.

2.1 Transcriptional Control

In the last two decades, synthetic systems for controlling transcription in mammalian cells have been developed [50, 119, 191]. These are based on the principles that regulate transcription of synthetic prokaryotic gene-networks. Generally, these systems rely on prokaryotic protein moieties that bind to their cognate DNA operator sequences, an event that is necessarily modulated by external signals (i.e. inducer molecules). In order to adapt these systems for transcriptional control in mammalian cells, prokaryotic DNA binding moieties are fused to eukaryotic protein domains, constituting a chimeric transactivating protein with the capacity to activate or repress the activity of promoters. Further, synthetic regulatable promoters are designed by combining a minimal eukaryotic promoter with prokaryotic DNA elements (operators) that allow binding of the cognate protein moiety. The transcriptional activator or repressor domain can thereby be recruited to the synthetic promoter (Fig. 1a). For these complex systems to be successfully employed in mammalian cells, the following requirements have to be met:

- Binding of the transactivating protein to the promoter tunable in a dose-dependent manner;
- Fast reversibility upon withdrawal of the inducer;
- No or low basal expression in the absence of the inducer;
- No or minimal interference with the host cell system and the endogenous gene network.

In order to achieve controlled expression of a protein, initial approaches relied on temperature-sensitive (ts) protein mutants displaying altered activities upon shifting temperature. Today, different kinds of small-molecular-weight inducers are used to achieve controlled expression. These include antibiotics, steroid hormones, *quorum*-sensing molecules, urea, immunosuppressive and anti-diabetic drugs, phloretin, biotin, L-arginine and volatile compounds like acetaldehyde. In addition, light-induced systems have been employed in mammals [100, 203, 209]. For a comprehensive list of inducer molecules used in transgene expression



regulatable systems, see Table 1. In the following, the principle of regulatory synthetic modules will be exemplified by the "Tet-system". This system has been shown to allow tight control of gene expression in many different biological contexts, from diverse primary cells to animal models.

2.1.1 The Tet-System

The tetracycline regulation response in *E.coli* relies on the binding of a protein (tetR) to its cognate operator sequences (tetO) located in the *E.coli* tetracycline operon. Gossen and Bujard developed a fusion protein consisting of the tetR binding domain and the transactivating domain of herpes simplex virus VP16 [63].

◀**Fig. 1** **a** *Binary expression modules*. Binary Tet-dependent expression modules consist of two independent expression units: a constitutive promoter (Pconst) that drives expression of a transactivator (tTA, rtTA) or a transrepressor (tetR, tTS) and a synthetic, inducible promoter driving the gene of interest (GOI). The transacting proteins consist of a DNA binding moiety derived from the bacterial tetR (tR) fused to a transactivating (TA) or silencing (TS) domain. Binding of the transacting molecules to the tetO sequence in synthetic promoters is achieved by the tetR binding domain tR or mutated variants tR*. The transactivating or repressing domains are thereby directed to the synthetic promoters. Doxycycline (Dox) or tetracycline (depicted as a *star*) binds to the transacting molecules and induces a conformational change that modulates binding to the tetO sequence. While Dox inhibits binding of proteins harbouring the tR domain (tTA, tTS and tetR), it is required for binding in the rtTAs that incorporate mutant tR* domains. In Off-type expression cassettes, transcription is switched off on addition of Tet, while On-type expression cassettes require Tet to be induced. **b** *Autoregulated modules*. In these modules, the transacting protein is controlled by a Tet-responsive promoter, thereby generating a positive feedback loop. This can be realized in two independent expression units for the transacting proteins and the GOI, respectively. Expression of these two proteins can be linked in a bicistronic message. Alternatively, bidirectional Tet-responsive promoters can be exploited for this purpose (not depicted). Both On-type and Off-type designs can be realized. **c** Expression phenotypes generated with binary modules result in gradual increase of expression upon administration of increasing concentrations C of the inducer. This is observed on both the population level and the cellular level. In contrast, autoregulated modules result in a stochastic activation of gene expression. In this case, in individual cells expression is either 'On' or 'Off'. Accordingly, two distinct expression states can be maintained which are visible on the single cell level only. Increasing concentrations of the inducer increase the probability that cells express the transgene. This is exemplified for low concentrations of the inducer (c_{low}) and high concentrations (c_{high}). Stochastic gene activation results in hysteresis [90, 120]: while cells need a certain concentration of inducer to switch gene expression on (*blue arrow*), lower concentration is needed to maintain expression (*red arrow*)

This fusion protein, called tTA, can transactivate synthetic promoters in which the Tet operator sequences have been fused to a minimal eukaryotic promoter. Transcription is thereby activated, leading to expression of the gene of interest (GOI) (see Fig. 1a and b). The binding of tetracycline [or its derivatives such as doxycycline (Dox)] to the tetR binding domain induces a conformational change that leads to the release of the protein complex from the promoter, abrogating transcription (Tet-off system). Reverse transactivators (rtTAs) have been developed in which mutations in the prokaryotic domain reverse its binding properties. In these mutants, the binding to the promoter is only achieved in presence of the inducer Dox [64]. In order to increase the stringency of the expression system, these mutants have been significantly improved [10, 183]. Moreover, new transactivator variants have been developed that are optimized for human codon usage [114]. It was shown that HSV VP16 can outcompete binding of transcription factors, thereby inhibiting transcription of cellular genes, an effect called transcriptional squelching [59]. To overcome this side effect, a panel of different transactivating domains from cellular activator proteins such as p65 and E2F4 have been functionally fused to the Tet-binding domain [3, 62, 183, reviewed in 14].

Since the performance of inducible systems is limited by basal expression levels, efforts to modify the Tet-dependent promoter have been undertaken.

Table 1 Transcription regulatable systems

Inducers	References
Antibiotics	
Coumermycin	Zhao et al. [215]
Macrolides (e.g. Erythromycin)	Weber et al. [192]
Streptogramines (e.g. Pristinamycin)	Fussenegger et al. [50]
Tetracycline (and derivatives)	Gossen and Bujard [63]; Gossen et al. [64]; Urlinger et al. [183]
Steroids	
Mifepristone	Wang et al. [187]
Estrogen	Braselmann et al. [24]
Ecdysone/Muristerone A	No et al. [134]; Yao et al. [207]
Physiological molecules	
NADH	Weber et al. [192]
Urea	Kemmer et al. [83]
Acetaldehyde	Weber et al. [192]
Nicotine	Malphettes et al. [111]
Vitamin H (Biotin)	Weber et al. [192]; Weber et al. [195]
Arginine	Hartenbach et al. [68]; Weber et al. [196]; Weber et al. [192]
Environmental signals	
Temperature	Boorsma et al. [20, 21]; Siddiqui et al. [163]; Weber et al. [193]
Light	Wu et al. [203]; Yazawa et al. [209]; Levskaya et al. [100]
Hypoxia	Binley et al. [16]
Radiation	Mezhir et al. [123]
Metal ions (Zinc)	Searle et al. [159]
Quorum sensing signals	
Butyrolactone	Weber et al. [194]
Acylated homoserine lactone	Neddermann et al. [130]
Others	
IPTG	Hu and Davidson [73]
Cumate	Mullick et al. [129]
Rosiglitazone	Tascou et al. [176]
Electricity	Weber et al. [197]
Phloretin	Gitzinger et al. [60]

An increase of stringency in transgene regulatable expression has been achieved by optimizing the Tet-responsive promoter through modifications of heptameric tetO sequences [1] as well as by the use of other minimal promoter elements, such as the ones from MMTV and HIV [31, 70, 166].

With these improvements, tight regulation of a plethora of transgenes has been realized in various cell types. Moreover, simultaneous expression of several transgenes was achieved on coupling these genes in multicistronic expression units or with the help of a bidirectional promoter [9].

Tet-induced repression of promoter activity. A reverse approach to the activation of a minimal promoter consists in the repression of gene expression by binding of a repressor moiety to the regulatable promoter. For this purpose, a chimeric protein consisting of the Krueppel Associated Box (KRAB) domain of the mammalian *Kox1* gene and the TetR binding domain was generated [34, 112]. In the absence of the inducer, this protein binds to the tetO sequence of the regulatable promoter and exerts its epigenetic silencing activity by inducing the formation of heterochromatin. On administration of the inducer, the repressor dissociates from the tetO site leaving it available for its activation. This strategy was successfully employed for reduction of the basal activity of the Tet-inducible promoter. Forster et al. combined the rtTA-dependent activation of the Tet-on system with a specific repression of the uninduced state [45]. For this purpose, a repressor is co-expressed that is inversely activated by the inducer. To avoid heterodimerization of repressor and activator proteins, heterologous elements for dimerization and DNA recognition are employed in the transactivator and repressor proteins, respectively. Basal expression of the system could thereby be reduced and tighter regulation was achieved. Freundlieb et al. employed the epigenetically modulating KRAB domain derived from the human kidney protein Kid-1 for a fusion with the DNA binding moiety of the tetR binding domain to establish a Tet-dependent silencer tTS [49]. This fusion represses basal expression from the Tet-responsive promoter leading to a strong reduction of basal activity, also in vivo [216]. Apart from these artificial fusion proteins that direct repressor domains to synthetic promoters, the prokaryotic tetR protein itself can directly impair transcription of a mammalian promoter [207].

Two-vector versus one-vector systems. Most synthetic transcriptional regulation systems rely on two components: one ensures the expression of the transactivator or repressor; the other contains the inducible cassette with the promoter and the GOI. In most cases, these two elements are delivered by separate vectors (two-vector system). As an alternative, one-vector systems were developed: in this set-up all necessary components are assembled in one vector. The two components might be oriented in the same or in opposite directions with regard to the transcriptional activity. The major advantages of having both elements in one vector are the single gene transfer step and the possibility of packaging in viral vectors. However, the promoters can influence each other's activity due to the close proximity of the regulatory elements, which complicates the vector design.

Autoregulation. One elegant way to overcome promoter interactions is the use of autoregulated systems in which the expression of both the transactivator and the GOI are controlled by a single ligand-responsive promoter. This can be achieved in multicistronic cassette designs or by the use of bidirectional promoters. Such modules can be transduced by retroviral and lentiviral vectors [114, 121, 182]. Autoregulated systems depend on a low level of basal expression that is required for inducibility. It is important to note that this basal expression is low and does not necessarily induce a biological effect [116]. Autoregulated expression cassettes have been shown to result in a stochastic, i.e. bimodal, type of expression. This is in contrast to the classical systems which rely on constitutive transactivator

expression and which give rise to gradual expression response [120]. For a comprehensive distinction between the characteristics associated with autoregulated and constitutive expression systems, the reader is referred to Fig. 1b. In addition, it has been shown that autoregulated expression circuits are characterized by increased expression stability and reduced sensitivity to noise-inducing fluctuations [13].

2.1.2 Steroid-Inducible Systems

Another important group of transcription inducible systems are those relying on steroids. In the human proteome, steroid hormone receptors constitute the largest group of transcription factors. Activation of these cytosolic receptors depends on binding to their cognate ligands, the steroid hormones, which easily cross plasma membranes or epithelial borders. Upon binding, a conformational change is induced that results in release of heat shock proteins and facilitates translocation of the receptor to the nucleus where gene expression is induced by binding of the receptors to their cognate promoters (see [135] for review).

Drawbacks are associated with this kind of system. The respective inducers and/or repressors of GOI expression may modulate not only the transgene, but also endogenous gene expression. Expression of endogenous genes is avoided by using synthetic steroids that do not interact with the cellular machinery [26]. A number of agonists and antagonists of synthetic progesterone receptors were identified and their function as inducers was characterized in more detail [132].

2.2 Post-transcriptional Control Systems

2.2.1 Gene Silencing Via RNA Interference

A new regulatory mechanism of post-transcriptional gene silencing (PTGS) which is based on RNA interference (RNAi) was discovered and explored in the last decade (reviewed in [162]). In mammalian cells, this mechanism is endogenously realized by so-called miRNAs, small RNAs that show homology to endogenous mRNAs. Upon binding to cognate mRNA targets, they can specifically down-regulate expression. miRNAs are encoded in hairpin structures of many cellular mRNAs. This hairpin structure is processed by cellular enzymes Drosha/DGCR8 to pre-miRNAs and further cleaved by Dicer. The resulting short RNA molecule of 21–23 nucleotides (nt) can then bind to homologous sequences in their cognate mRNA in a complex with Argonate proteins; this complex is called RISC, the RNA-induced silencing complex. Depending on the extent of homology, the consequence of this binding is either translational repression or degradation of the mRNA, resulting in a specific gene silencing. The discovery and subsequent

exploitation of this gene-silencing mechanism via RNAi has opened new avenues towards specific manipulation of genes.

To down-regulate target genes, three different options can be followed. Long-term down-regulation is achieved (a) by Pol II-dependent transcription of miRNA encoding mRNAs, thereby exploiting the cellular pathway of processing pre-miRNAs down to the short 21–23 nt entities, (b) by transducing Pol III-dependent expression units to transcribe small hairpin RNAs that rely on cleavage by Dicer to generate the single-stranded RNAs that bind to their cognate target sequences or (c) by transient down-regulation through direct transfection of synthetically produced short (21–23 nt) RNAs, so called siRNAs.

2.2.2 Steroid Receptor Fusion Proteins

Several studies have shown that the regulatory domains of steroid receptors constitute a reversible molecular switch for the post-translational regulation of a wide variety of cytoplasmic and nuclear proteins [140]. Control of gene expression is achieved by exploiting steroid receptors such as the estrogen receptor and the progesterone receptor. Here, the pre-existing protein is inactive and becomes activated by the addition of the steroid. Indeed, it has been shown that upon fusing the ligand-binding domain of steroid receptors to the GOI, nuclear translocation can be controlled. Various examples have been reported. These include cell cycle regulation proteins such as myc, fos and interferon regulatory factor-1 (IRF-1) [38, 85, 172]. This principle has also been successfully employed to regulate recombinases such as Cre, Flp and PhiC31 that allow site-specific DNA modifications including targeted integrations [42, 74, 107, 161].

Mutations in the hormone-binding domain allowed restriction of activation of the fusion proteins to synthetic hormone analogues such as tamoxifen or mifepristone [82, 185]. Cross-activation by endogenous hormones is thereby overcome and also allows application of this principle in vivo. It should be mentioned that these compounds might still influence cell physiology: e.g., tamoxifen acts as an estradiol antagonist.

2.2.3 Control of Protein Stability

Recently, a novel post-translational strategy for controlling the protein level has been introduced [8]. Fusing the protein of interest to a specific destabilization domain leads to rapid degradation of the protein. This degradation can be blocked by administration of drugs that bind to the destabilization domain and thereby shield the protein from degradation. This activity has been shown to be dose-dependent. Meanwhile, a number of applications to various proteins highlight the flexibility of this strategy [8, 61, 142].

A reverse approach was taken by Nishimura et al. [133]. Fusion proteins of the GOI with *Arabidopsis thaliana* auxin/IAA transcription repressor 17 (Aid) are

ubiquitinated and readily degraded by co-expressed TIR-1 in an auxin-dependent manner. In this system, TIR-1 interacts with the mammalian ubiquitination complex SCF. In the presence of auxin, a phytohormone, degradation is induced.

Apart from the obvious advantages for controlling expression of proteins that negatively affect the cells, these strategies will also be of interest for perturbing cells for a limited period of time.

3 Stable and Long-Term Expression of Synthetic Cassettes in Mammalian Cells

Gene transfer is a prerequisite for modification of cells. For the implementation of synthetic cassettes into mammalian cells, standard physicochemical transfection methods can be used. Biotechnologically relevant cell lines such as BHK, Chinese hamster ovary (CHO) and HEK 293 cells can also be robustly and reliably manipulated using standard protocols for liposome-based transfer and electroporation-based protocols [138, 141, 198]. Other cell types and also many primary cells require more efficient transduction systems. In particular, viral transduction methods have been proven to efficiently transduce cassettes. This includes adenoviral vectors [186], adenovirus-associated viral vectors [25], and retroviral and lentiviral vectors [11, 17, 115].

While most gene transfer procedures give rise to a transient expression period, stable gene expression is less frequent. In most cases, it is achieved by integration of the transgene into the chromosomal DNA of the host. Stable expression normally allows the cloning of transgenic cells and thus enables studies with homogenous cell populations that can be further characterized. The following section is focused on stable gene transfer and highlights gamma-retroviral and lentiviral transduction as well as transposon-based methods for efficient and stable genome engineering.

3.1 Gamma-Retroviral and Lentiviral Vectors

Gene transduction mediated by gamma-retroviral and lentiviral vectors is a highly efficient method for stably modifying cells of different species [17, 165]. For production of these vectors, safe packaging systems that rely on helper cell lines or transient packaging systems have been established [57, 124, 157, 189]. For both systems, so-called self-inactivating (SIN) vectors are available, in which the viral promoter/enhancer activity is eliminated upon infection [94, 153, 214]. SIN vectors allow the transduction of cassettes without potentially interfering viral regulatory sequences; accordingly, these are the vector types of choice for transduction of synthetic expression modules.

Gamma-retroviral and lentiviral vectors can accommodate all elements necessary for Tet-dependent expression. In particular, those encoding modules for graded expression [70, 72, 105] have been described. Apart from this, autoregulated modules have also been successfully transduced by gamma-retroviral [96, 181] and lentiviral vectors [113, 114, 120]. Appropriate vector systems have also been developed for the coumermycin system, a system that is similar to the Tet system (see Table 1) [215]. Moreover by lentiviral transduction autoregulated cassettes bimodal expression characteristics have been achieved [120].

3.2 Transposon-Mediated Integration of DNA

In recent years, transposon-mediated integration of transgene cassettes has emerged as a highly efficient method of achieving long-term expression [78]. Various transposon-based systems have been described [48, 77, 88, 125]. Transposable elements derived from natural transposons are non-viral gene delivery vehicles capable of efficient genomic insertion. Briefly, they rely on a transposase that efficiently recombines specific inverted repeat sequences that flank a plasmid-based cassette, resulting in its integration into chromosomes. The transposase integrates plasmid-based DNA into the chromosomal DNA through a precise, recombinase-mediated mechanism. Long-term expression of the GOI is thereby achieved. The transposase can be provided on the same plasmid (*cis*) or in another plasmid (*trans*), but also can be transferred as mRNA or as protein. Different methods can be employed to transfer the transposon plasmids to the cells. The enzymatic efficiency of transposases has been recently improved, now reaching the efficiency of viral transduction [78]. Recent reports include the successful application of this method for the chromosomal integration of Tet-dependent expression modules [70].

3.3 Episomal Vectors

As an alternative to genomic integration, extra-chromosomal maintenance of replicating vectors can provide long-term gene expression, even in proliferating cells. Such vectors are derived from viral entities such as Epstein–Barr virus but can also be based on non-viral episomally replicating vectors (reviewed in [108]). These episomal vectors can persist in the nucleus without integrating into the cellular genome and are not thereby affected by position effects. Episomal vectors comprising synthetic modules have been reported and shown to be efficiently replicated with tightly controlled expression proprieties [7, 22]. In this regard, they are an attractive tool for genetic engineering of cells, including therapeutic applications [37, 202].

3.4 Strategies for Predictable and Stable Expression of Synthetic Modules in Mammalian Cells: Targeted Integration

Most gene transfer protocols—including the above-mentioned protocols employing viruses and transposons—rely on the stable integration of the modules into the genome of the host cell via undirected, i.e. sequence-independent, illegitimate recombination, which is a largely random process. Accordingly, the sites of integration are mostly spread all over the genome [204]. As a consequence, individual cell clones are characterized by specific integration site(s) of the module(s).

Once integrated into the cellular DNA, transgenic modules are affected by neighboring chromosomal elements that modulate promoters to a large extent (see [199] for a recent review). Enhancers and silencers directly affect the synthetic promoters of individual modules in *cis*. Besides this, chromatin-modeling elements such as locus control regions and S/MARs significantly influence the transgene expression level [18, 101, 199]. Finally, evidence has been provided that proximal promoter elements can also interact with incoming promoters (promoter crosstalk) resulting in their down-regulation (e.g. by promoter occlusion) or potentiation [66]. These interactions of the synthetic modules with the chromosomal flanking regions can affect both the basal activity of a synthetic module and its regulation capacity, a phenomenon described as "position effect". Thus, on random integration, individual cell clones display a highly heterogeneous expression pattern of the synthetic module and have to be screened for appropriate, i.e. tightly controlled, expression. Accordingly, the reproducibility is often difficult on random integration of expression cassettes. On the other hand, the heterogeneity might be of advantage if infrequent phenotypes in individual cells have to be studied.

Homologous recombination is used in stem cells for targeting transgenes to specific loci. This method allows the optimization of the performance of a Tet-dependent cassette in a characterized integration site [167]. However, in differentiated cells, homologous recombination is a very infrequent event. For these cells, recombinase-based methods such as targeted integration or recombinase-mediated cassette exchange (RMCE) (reviewed in [54, 200]) represent potent tools for targeting individual chromosomal sites for various applications in order to overcome the limitations of random integrations. Indeed, such approaches have been successfully performed for reliable modification of the mouse genome [136, 149]. In addition, it has been exploited for the generation of production cells [55, 106, 131].

As an alternative to site-specific recombinases, strategies have been followed to increase the frequency of homologous recombination by specific induction of double-strand breaks. For this purpose, zinc (Zn) finger-based nucleases are designed to induce DNA double-strand breaks at specific sites of the genome [87, 184]. Triggered by this DNA break, co-transfected expression cassettes are integrated through cellular repair enzymes. Targeted integration of cassettes into virtually any chromosomal site can thereby be achieved.

4 Expression Characteristics Created by Synthetic Modules

While pioneering work in prokaryotes and lower eukaryotes has provided proof of concept for implementation of even complex synthetic systems [40], synthetic networks have also been designed to create new functionalities in mammalian cells. Strategies for establishing complex artificial circuits in mammalian cells have been summarized in a number of excellent reviews [6, 65, 191, 192]. It is anticipated that new functionalities that mimic endogenous functions can be generated with the help of complex synthetic circuits. One example concerns the engineering of signal transduction pathways. The challenges in specific engineering of signal transduction pathways by synthetic tools have been highlighted [84]. These concern (a) the fact that signalling transduction pathways are much faster than transcriptional responses generated with synthetic circuits, as recently reviewed [143], (b) the impact of subcellular localization of signalling proteins, and (c) the inherent noise of genetic circuits in contrast to signalling molecules whose actions are less stochastic due to the large number of molecules involved.

In Table 2, synthetic approaches are exemplified that allow the establishment of defined expression characteristics. Here, we focus on several elegant studies.

One of the first synthetic circuits created was a synthetic three-step transcription cascade. This was realized by interconnecting the regulatory modules responsive to tetracyclines, erythromycin and streptogramin in a sequential manner [91]. This cascade was shown to be controlled individually at the various levels and provided four defined levels of expression in response to the three antibiotics. Furthermore, functional coupling of endogenous signals and synthetic cassettes was recently realized. In this cascade, hypoxic conditions induced translocation of endogenous HIF-1alpha to the nucleus, which resulted in activation of an artificial multi-step regulatory module initiated by a HIF-1-responsive artificial module [93].

By combining two antibiotic-dependent Off-type switches in a mutually repressing manner, a biological toggle switch could be created [92]. In this study, the authors used two independently controlled synthetic modules to express mutual repressor proteins that switch off the other expression unit. For this purpose they relied on the KRAB transrepressor that epigenetically silences the neighbouring DNA region [127] (for details please consult Sect. 2). Binding of the KRAB domain to the respective promoter was realized upon fusion of the KRAB domain to the erythromycin- or pristinamycin-regulated protein domains, thereby directing the KRAB domain to the respective synthetic promoter. This binding can be impaired by addition of the respective small molecule drugs pristinamycin and erythromycin. Upon releasing one of the repressors by addition of the antibiotic, expression of the second regulatory module is initiated. This expression is maintained even in presence of the repressor, thus providing a toggle-switch-like expression phenotype. Furthermore, with this setting a bimodal (On/Off) expression phenotype was created.

In the bimodal, “bistable” or “switch-like” expression pattern, the concentration of the inducer increases the probability that a gene will be expressed. Once it

Table 2 Switches in mammalian cells realized by synthetic cassettes

Synthetic switches	References
Boolean gates: AND, OR, NOR,…	Kramer et al. [92]; Rinaudo et al. [144]
Synthetic transcription cascade	Kramer et al. [91]
Semi-synthetic transcription cascade	Kramer et al. [93]
Coupled transcription/ translation cascades	Malphettes and Fussenegger [110]; Deans et al. [33]; Greber and Fussenegger [65]
Toggle switch	Kramer et al. [92]; Greber and Fussenegger [65]
Hysteresis	May et al. [120]; Kramer et al. [93]
Semi-synthetic oscillator	Toettcher [179]
Synthetic oscillator	Swinburne et al. [173]; Tigges et al. [177]; Tigges et al. [178]
Epigenetic memory device	Kramer et al. [92]; Greber and Fussenegger [65]
Time delay expression	Weber et al. [192]

is turned on, expression is always maximal. Natural examples for this switch include E2F activation of Rb [208] and the response of mouse embryonic stem cells to leukemia inhibitory factor (LIF) [32]. Bimodally expressed genes in the mouse genome were identified based on microarray data [41]. In an engineered system, bimodal expression has been realized by implementing autoregulated switches [90, 120].

Bimodal expression can result in stochastic activation of gene expression. Moreover, such expression patterns have been shown to result in hysteresis. In natural systems, hysteretic switches are considered to make gene expression more robust and largely independent of noise. This is due to the fact that subtle changes in the concentration of the inducers do not affect the expression phenotype. Indeed, such an expression pattern is also predicted by modelling approaches based on stochastic mathematical tools [120].

More complex expression phenotypes are represented by oscillators. Oscillating gene expression can be achieved by interconnecting positive and negative feedback loops. Periodic gene expression results in the proper timing of events in periodic processes. The first oscillating gene network in mammalian cells has recently been introduced [173]. This approach employed a tetR-based negative feedback loop which was shown to be modulated by the length of the intronic region preceding the tetR coding sequence. By increasing the intron size up to 16 kb, higher oscillation periods were created. A more recent study [177] combined a positive feedback loop with a time-delayed negative feedback loop and could thereby generate tunable and sustained oscillation as exemplified by expression of fluorescent proteins. By adjusting the concentration of the inducer, periods could be manipulated from 140 to 330 min. Significantly slower oscillation periods were achieved by combining a tTA-dependent positive feedback loop with a delayed negative feedback loop. This was realized by implementation of an intronically encoded siRNA that negatively regulates expression of an autoregulated tTA loop.

Interestingly, in contrast to the above-mentioned rapid oscillation in this system, prolonged periods of about 25 h were realized.

While the oscillatory examples are highly promising, to date they could only be realized in transient expression systems. It seems that only a fraction of the transduced cells react in the desired way because the composition of the individual elements must match a certain stoichiometry. This has so far limited the application.

5 Applications of Synthetic Modules for Alteration of Cellular Function

To increase cellular productivity, a number of studies have been performed that aim at the modification of certain cellular features that directly or indirectly regulate production of relevant biomolecules. This includes specific modulation of cellular pathways such as metabolic pathways, proliferation or survival. Here, we focus on strategies for controlling cell proliferation of immortalized cell lines as well as novel approaches for controlled expansion by conditional immortalization. In addition, we discuss approaches towards controlled product secretion.

5.1 Control of Cell Proliferation

Control of cell proliferation is of central interest in mammalian cell biotechnology. The motivation for controlling cell cycle progression and cell proliferation emerged for two different reasons. Firstly, fed-batch production schemes are limited by the fact that ongoing cell proliferation and accompanying increase in cell mass eventually leads to a cellular/system collapse and thus defines the end of the production phase. Therefore, based on the fact that cell proliferation consumes a significant portion of cellular energy, it was hypothesized that control of cell proliferation would allow redirection of this energy to production of the desired product, thereby increasing productivity.

Initial cell engineering strategies developed to increase cell-specific recombinant protein production rate by manipulation of cell cycle progression remained below expectations. The first evidence that recombinant protein production might be uncoupled from cell proliferation was obtained in CHO cells and was described 20 years ago [69]. Since then, the development of strategies that lead to an increase of cellular productivity by manipulating cell cycle progression has become a major area in the metabolic engineering field. These first results opened avenues for improvement of strategies that aim at a more balanced and controlled cell growth in a biotechnological perspective, being an active area of cell engineering research nowadays.

5.1.1 Controlled Growth of Immortalized Cells

Proliferation control of immortalized cell lines has been accomplished by at least three different main strategies. The first involves cell growth arrest based on the engineering of molecular networks that control progression of the mammalian cell cycle [51, 52, 56, 122]. The second is based on cultivation at sub-physiological temperatures (i.e. below 37° C) [19, 46, 47, 81, 154, 210–213]. The third approach consists in the retardation of proliferation by chemical induction of cell cycle arrest [43, 71, 103].

The following paragraph describes approaches towards increased productivity of recombinant proteins by engineering cellular pathways that control the cell cycle progression of immortalized mammalian cell lines such as CHO or HEK293.

Early studies on the mammalian cell cycle and cancer research uncovered a multitude of proteins whose function relies on negative effects on cell cycle progression. The control of the cell cycle of immortalized cell lines predominantly dealt with arrest at the G1 phase, considered to be the ideal time period in which the productivity of the protein of interest can reach its maximal levels ([15, 28, 75, 170, 180, 210, 211] and [170] for a recent review). Nevertheless, other reports described the production of recombinant proteins when the cell cycle is arrested in S phase [47] or even irrespective of cell cycle phase [104].

One of the first studies aimed at controlling cell growth by genetic engineering implicated the over-expression of IRF-1. Besides the pleiotropic effects of IRF-1 in activating several cellular genes (reviewed in [95]) ranging from IFN-β to IFN-stimulated genes, it was shown that IRF-1 also acts as a tumor suppressor [174, 175] and, importantly, acts as a negative regulator of cell proliferation [85, 145]. It was shown that the downstream effects of IRF-1 converge, at least partially, in the induction of anti-proliferative genes, such as *p21* [137, 175] leading to a G1 cell cycle-specific arrest. Thus, the control of cell cycle progression by expressing IRF-1 can be regarded as a multigenic approach towards an increase of recombinant protein production by modulating the cellular transcriptome.

The activation of IRF-1 function was achieved by transcriptional activation of the IRF-1 transgene and by post-translational activation. Pioneering studies on growth manipulation of recombinant mammalian cells were performed by establishing a murine C243-based cellular system in which proliferation was controlled by inducible expression and activation of IRF-1 protein [89]. In this proof-of-principle study, the transcription of IRF-1 is driven by a Tet-repressible promoter and thus IRF-1 is expressed only in the absence of the inducer. Growth inhibition could be demonstrated by omitting the inducer from the growth medium. The second approach employed a fusion of IRF-1 to the estrogen receptor. In this case, the function of the constitutively expressed IRF-1 fusion protein is only activated by the addition of β-estradiol. The biotechnological relevance of this achievement was later demonstrated by modulating the IRF-1-responsive cellular system towards production of a relevant model protein [56]. The authors showed that proliferation control of a BHK-derived cell line in a perfused cell-culture process was effectively regulated for over 7 weeks. Moreover,

heterodimeric IgG antibody productivity increased up to sixfold during growth arrest triggered by inducible expression of IRF-1. The drawback of efficient and rapid cell growth inhibition by IRF-1 over-expression in BHK-derived cells is related to a decrease in the cell viability over time. In an attempt to keep cell viability at satisfactory levels, it was shown that manipulation of cycles by addition and removal of estradiol modulates IRF-1 activity, thus overcoming the loss in cell viability of IRF-1 growth-arrested BHK cells [27].

Other studies were performed by over-expressing proteins that cause interventions in the cell cycle control. Such a "one gene metabolic engineering" approach was first performed and described by Bailey et al. [51], over-expressing inhibitors of cell cycle progression transiently transfected into CHO-derived cells. The authors achieved an enhanced productivity in CHO-derived cells upon transient expression of *p21*, *p27* or the *p53*-derived mutant *p53-175P*, (mutant showing specific loss of programmed cell death function, [146]). Importantly, the expression of these genes was coordinated with expression of a model protein, secreted alkaline phosphatase (SEAP), and expression of both genes was driven by a Tet-repressible promoter. Upon triggering expression of each of these inhibitors of cell cycle progression, growth arrest was achieved with concomitant increase in recombinant protein production. Moreover, the growth capacity of the cell lines was rescued when the expression of cell cycle inhibitor proteins was repressed. The concept was further extended by modulating the "one gene metabolic engineering" by constitutive expression of cytostatic genes [122]. The authors found that conditional expression of these genes in stable clones does not entirely recapitulate what was observed in transient expression settings [51]. Stable integration of *p21* did not lead to generation of clones with growth arrest capacity and *p53-175P* expression was related to induction of apoptosis in growth-arrested cells. Nevertheless, the authors succeeded in generating CHO-derived clones expressing *p27* with cell growth arrest properties in a regulatable manner and with a concomitant 10–15-fold increase in relevant protein production per cell.

Cell cycle control is an intricate network of cellular proteins that act in an orchestrated manner in regulatory circuits. This includes some intervening molecules that can be overcome by the function of other proteins. The redundancy of such pathways in controlling cell cycle progression was believed to account for the impairment in establishing stable integrated mutants with cell arrest properties on inducible over-expression of certain cytostatic proteins such as *p21* [122]. In an attempt to circumvent this limitation, Fussenegger et al. described a "one-step multigene metabolic engineering technology" in CHO cells [53] as a means to regulate the multifactorial process of cell cycle progression. In order to increase the levels of active *p21* in the cells, the authors developed a strategy in which *p21* was coordinatedly expressed with the transcription factor C/EBPα (a protein that increases *p21* production and protein half-life). Taking advantage of Tet-controlled tricistronic expression vectors [52], the regulated and coordinated expression of *p21*, *C/EPBα* and the model protein with concomitant cell cycle arrest in stable cell clones were achieved [53]. The authors showed that regulation

of cell cycle progression could be achieved in a multifactorial manner, confirming that an individual cell is more productive in a cell-cycle-arrested state.

The concept of cell growth arrest by regulatable over-expression of cytostatic proteins was further extended towards increase of recombinant protein production. It proved to be applicable not only to other cell lines, and different regulation systems but to expression of proteins with high potential application value [75, 190]. The authors established a low-density batch culture of NS0-derived cell line expressing *p21* in an inducible manner. One of the major achievements in this work was the use of a regulatable promoter whose expression is triggered by the addition of an inducer (On-system). The authors describe the G1-phase arrest in response to high levels of *p21* expression and concomitant increase of IgG4 antibody by more than fourfold in the arrested state.

In another study, the approach described above was extended to a high-cell-density continuous perfusion culture [75]. A multigenic manipulation of the cell cycle was evaluated by expression of the *p21* gene and the anti-apoptotic protein *Bcl-2*. Although the authors could confirm the role of *p21* in the arrest of cell cycle progression and increased productivity, *Bcl-2* expression had no effect on cell viability of the arrested cells. The coordinated expression of anti-apoptotic proteins (such as *Bcl-2* and $BclX_1$), cytostatic genes (*p27*, *p21*) and a GOI was earlier described in CHO-derived cell lines [53]. The authors found that only with coordinated expression of $BclX_1$ and *p27* cell cycle arrest was possible in G1 phase. Stable cell clones with G1 growth arrest proprieties were generated and no signs of apoptosis during growth-arrested periods were observed (for a further detailed description of cellular engineering strategies that aim at the prolongation of cell viability by over-expression of anti-apoptotic proteins, the reader is referred to the Sect. 5.2).

The positive relationship between cell cycle arrest in the G1 phase and the increase in recombinant protein productivity was found in different cell lines [51, 53, 56, 75, 122, 190]. Importantly, the physiological basis of this effect was revealed by different groups [15, 28, 104]. Bi et al. were able to show that in CHO-derived cells expressing inducible *p21*, the increase in productivity of the model protein (GOI) is related to an increased cell size, mitochondrial mass and activity, and ribosome biogenesis [15]. Furthermore, they showed that these alterations were uncoupled from the cell cycle, i.e. these physiological changes occur in the cell despite the abolition of cell cycle progression and division. Characterization of cellular metabolism of a CHO cell line over-expressing the cytostatic *p27* protein showed the same line of evidence [27]. The cell cycle arrest in G1 phase was found to be related to an increase in cell protein content and concomitantly with a higher expenditure of cell energy.

5.1.2 Conditional Cell Expansion

Many immortal cell lines have been either isolated from tumor tissue or have resulted from spontaneous immortalization. In addition, cell lines have been

Table 3 Conditional and reversible immortalization approaches

Immortalized cell lines	Types of immortalizations	Types of switches	References
Murine			
Embryonic fibroblasts	Conditional	Tet-system	May et al. [116]
Ear fibroblasts (adult)	Conditional	Tet-system	May et al. [118]
Lung microvascular endothelial cells	Conditional	Tet-system	May et al. [121]
Myogenic clonal cell lines	Conditional	Temperature	Morgan et al. [128]
Hepatocytes	Conditional	Temperature	Yanai et al. [205]
Tissue specific microvascular endothelial cells	Conditional	Temperature	Langley et al. [98]
Astrocytes	Conditional	Temperature	Langley et al. [99]
Cardiomyocytes	Reversible	Cre-recombinase	Rybkin et al. [147]
Hematopoietic cells	Reversible	Estrogen	Ito et al. [76]; Wang et al. [188]
Human			
Umbilical vein endothelial cells (HUVEC)	Conditional	Tet-system	May et al. [121]
Liver endothelial cells	Reversible	Cre-recombinase	Salmon et al. [148]

successfully established by introduction of immortalizing genes into primary cells. However, in many cases, such constitutively immortalized cells have limited benefit. They often do not reflect the properties of the primary cells they have been derived from. One issue concerns the expression of the immortalization gene per se, which has a significant impact on the cellular phenotype. In recent years, strategies have been followed aiming at a systematic construction of new cell lines from primary cells. The emphasis of these approaches is to restrict expression of the immortalizing gene(s) to the period of cell expansion, in other words controlled expansion of primary cells. This is achieved by reverting or switching off the immortalizing genes (see Table 3 for an overview of recent approaches). With such approaches, current limitations of immortalized cells which are related to prolonged constitutive expression of the immortalizing gene(s) causing changes in cellular properties might be overcome. The resulting cells may be used for analytic issues, the production of recombinant proteins, and gene and cell therapies.

Conditional cell expansion is achieved by conditional expression of specific genes. One example concerns the gene encoding the simian virus 40 large T antigen (TAg). TAg is a powerful oncogene able to inactivate cellular key tumor suppressors like p53 and retinoblastoma proteins, and is therefore frequently used in generation of immortalized cell lines. There is evidence that the transforming potential of TAg is also attributed to an anti-apoptotic activity which is not related to the activation of p53 [4]. In a pioneering study aimed at generating conditionally immortalized cell lines, Jat et al. created a transgenic mouse called ImmortoMouse for which they developed a thermo-labile mutant of the SV40 TAg [79].

The thermo-labile mutant of SV40 TAg is inactive at the physiological body temperature and permissive at 33 °C. In the ImmortoMouse the thermo-labile mutant of SV40 TAg is under the control of the mouse major histocompatibility complex H-2K^b promoter which is expressed in a broad range of tissues and is induced by interferon. On isolation of cells from the ImmortoMouse, several conditionally immortalized cell lines have been established, including myogenic cell lines [128], hepatocyte cell lines [205], tissue-specific micro-vascular endothelial cells [98] and, more recently, an astrocyte cell line [99]. In these cell lines, permissive conditions (33 °C in the presence of medium containing interferon-γ) turn on the expression and activity of SV40 TAg leading to exponential proliferation of the cells. Importantly, in this proliferative state the established cell lines retained most of their tissue-specific morphological and biochemical properties. When the cells were transferred to non-permissive temperatures (37 °C or higher) they either ceased proliferation or started to differentiate.

While the cell lines developed from the ImmortoMouse gave first proof-of-concept for de-novo establishment of growth-controlled cell lines, the approach is limited to the temperature-labile mutant of SV40 TAg. Obviously, such an approach is not generic or easily translatable to other immortalizing genes. Thus, another approach was employed that makes use of specific excision of the immortalizing gene(s) mediated by DNA recombinases such as Cre or Flp, resulting in reversible expression of the immortalizing gene. Salmon et al. showed that human liver endothelial cells were immortalized through lentiviral-mediated gene transfer of SV40 large T and telomerase. Cre-mediated excision of the immortalizing genes resulted in complete growth arrest within 2 days [148].

In a similar study, Rybkin et al. used TAg to create cardiomyocyte cell lines capable of proliferating and reversibly withdrawing from the cell cycle [147]. Cre recombinase was used to switch the cells from a proliferative to a quiescent state. Although the reversible Cre/loxP approach enables conditional immortalization, this method is challenging since high transduction efficiency of the recombinase is hard to achieve. In addition to this, the selection schemes must be very strict to ensure a homogenously reverted cell line in which the non-transduced cells are not overgrown [121].

However, recent developments in control of gene expression (see Sect. 2) have allowed the principle of conditional expression to be employed in more generic approaches. This was shown both for transcriptionally and post-transcriptionally controlled approaches.

The estrogen receptor was used to construct a selective amplifier gene for controllable expansion of genetically modified hematopoietic cells. Specifically, the fusion of a steroid receptor hormone-binding domain to the growth factor G-CSF exerts a reversible activation in a steroid-dependent manner [76]. Therefore, the growth signal is active only upon estrogen treatment of the transduced murine hematopoietic stem cells, and most of the cells expand even if some of them enter the differentiation pathway. This strategy is applicable to the in vivo expansion of genetically modified hematopoietic stem cells [97]. In another study, the principle of estrogen receptor-mediated control was successfully applied to hematopoietic

progenitor cells using tamoxifen-controlled expression of HoxB8 [188], giving rise to expandable stem cells that are still susceptible to differentiation.

More recent studies have shown that cell proliferation control can be achieved by an autoregulated, Dox-dependent transcriptional immortalization strategy [116]. Autoregulation imposes a bimodal decision on the cells. Thus, only fully induced cells receive the proliferation signal while non-induced cells remain un-induced and are overgrown by the proliferating cells. The installation of the positive feedback loop requires a low basal expression of the transactivator and is initiated through administration of Dox. In the presence of Dox, the system is turned on and the immortalizing gene is expressed, leading to the exponential proliferation of cells [158]. In the absence of Dox, the induced effects are reverted and the cells stop proliferating.

Through lentiviral transduction of the autoregulated expression cassette, a broad range of cell types can be transduced. Lentiviral transduction is much more efficient than plasmid transfer, thereby dramatically increasing the immortalization efficiency up to 1,000-fold [121].

A series of such lentiviral vectors were constructed that can be distinguished by the expression of the proliferator gene such as *SV40 TAg*, *PymT*, *hTert* and *c-Myc*. This allows the simultaneous transduction of cells with different proliferator genes. Indeed, the combination of such genes [86, 206] led to the expansion of cell types that were not previously accessible (Table 1). Examples include lung micro-vascular-derived and HUVEC-derived endothelial cells from mouse and human origin [121]. Importantly, these cells expressed cell-type-specific markers and, in the case of endothelial cells, showed de-novo vessel formation in vitro and in vivo. Controlled proliferation was achieved due to strict co-regulation of these cells which harbor several proliferator genes. Importantly, the conditionally immortalized cells did not induce oncogenic transformation [118].

Proliferation-controlled fibroblast cells were used to express recombinant erythropoietin (EPO) without any signs of degradation, while expression was higher in the growth-arrested state [117].

5.2 Apoptosis

Apoptosis is a form of regulated or programmed cell death controlled by signaling pathways in mitochondria, endoplasmic reticulum (ER), or cellular surface death receptor(s). A series of cysteine proteases (caspases) execute the cell death program [5, 109]. Apoptosis is an inherited property of cells derived from their original function in the multi-cellular organism which is mostly non-beneficial in technological cell culture. Many mammalian cell lines are susceptible to apoptosis under conditions of typical bioreactor growth cultivation [139, 164], thereby decreasing the overall productivity achievable. Inhibiting or controlling apoptosis is thus a major target of cell engineering, e.g. by blocking pro-apoptotic mechanisms or by over-expressing apoptosis-counteracting proteins.

Apoptosis can be initiated by two general mechanisms, which are characterized by extrinsic or intrinsic pathways. Extrinsically induced apoptosis is initiated through the binding of ligands (e.g. FasL) to receptors of the tumor necrosis factor (TNF) family. FasL binding induces the formation of the death-inducing signaling complex (DISC) which contains the Fas-associated death domain (Fadd), caspase-8 and caspase-10. The intrinsic mechanism of apoptosis initiation is the answer to cellular stress. DNA damage, for instance, causes the activation of *p53* which induces the expression of pro-apoptotic members of the *Bcl-2* family (*Bax*, *Bak*, *Bok*, *Bik*, *Bad*, and *Bid*). These factors are responsible for the release of cytochrome c from the mitochondria which binds to the protease activation factor 1 (Apaf-1). Apaf-1 in turn is now able to activate procaspase-9.

Among the intrinsic factors leading to apoptosis, the ER stress-induced death pathway is certainly of great significance in high-expression bioprocesses. It is caused by an unfolded protein response (UPR), hypoxia, changes in intracellular calcium levels or lack of glucose. Enhanced cell survival in bioreactors has been achieved by the supplementation of chemicals (e.g. the polysulfated naphthylurea Suramin) and certain recombinant proteins. Examples are the expression of the insulin-like growth factor receptor along with its ligand IGF-I and transferrin added to the medium [171], and elevated concentrations of amino acids which have shown to protect cells from environmental stress [35].

Genetic strategies for preventing apoptosis in cell culture can be divided in the over-expression of anti-apoptotic factors and the knockdown of pro-apoptotic factors. The over-expression of anti-apoptotic genes like *bcl-2* and *bcl-*x_L in NS0, CHO, BHK or hybridoma cells was shown to improve cell viability when exposed to cellular stress such as starvation or toxins (see [5] for review). Caspases-9, -3 and -7 are targets of the X-linked inhibitor of apoptosis (XIAP) which can be used for over-expression [151]. Cho et al. demonstrated that the over-expression of the Ca^{2+}-dependent enzyme transglutaminase 2 (TG2) inhibits apoptosis through suppression of caspase-3 and -9 activities [29].

Since apoptosis is a complex biological program with redundancy, a combination of several factors involved in different stages of apoptosis is more effective than the over-expression of a single one. This was demonstrated by the co-expression of *Bcl-XL* and *XIAPΔ* in CHO and myeloma cell lines [150–152]. Dorai et al. have systematically tested the combination of a set of anti-apoptotic genes in a CHO cell line. The best results in terms of viability and productivity could be achieved by over-expression of either single anti-apoptotic genes (*Bcl-2Δ* or *Bcl-XL*), or a combination of two or three anti-apoptotic genes (*E1B-19K*, *Aven*, and *XIAPΔ*) [36].

The knockdown of pro-apoptotic factors represents another genetic engineering strategy to circumvent apoptosis. Silencing *Bax* and *Bak* in CHO cells using shRNA vectors was reported to increase cell viability and improve the production of recombinant interferon-γ in producer CHO cell clones [102]. Silencing caspases-3 and -7 expression, but not caspase-3 alone, can improve cell viability and recombinant thrombopoietin production in CHO cells following treatment with sodium butyrate [168, 169]. Silencing the apoptosis-linked gene 2 (*Alg-2*) and

the Zn-finger protein transcriptional factor *Requiem* in CHO cells was also reported to improve cell viability and recombinant protein production [201]. The disadvantage of these knockdown approaches is that usually less than 100% of the target
mRNA is silenced, leading to a remaining pro-apoptotic function. Using Zn-finger nucleases, Cost et al. established a double knockout of both genes *Bak* and *Bax* in a CHO cell line and could improve the production of IgG up to fivefold under starvation conditions and higher cell densities under normal conditions compared to wild-type cells.

5.3 Secretion Engineering

The majority of therapeutic proteins which are produced by mammalian cells in bioreactors are secreted into the medium. Consequently, efforts have been made to improve cellular productivity by the introduction of biological modules which enhance protein transport and post-translational modifications. All secreted proteins are co-translationally targeted to the endoplasmic reticulum (ER) and then translocated across the membrane of the ER to the Golgi apparatus. The proteins are further processed within the trans-Golgi network before packed into secretory vesicles.

The transport and modification of heterologous proteins through the secretory machinery can be seen as a bottle-neck of protein production. In the ER, cellular quality control of proteins is performed and only "perfect" products pass this barrier [39]. Misfolded, unfolded or unassembled proteins will be degraded and mechanisms are activated to lower the biosynthetic burden of the ER and to protect it. As mentioned above, this unfolded protein response can induce apoptosis; consequently, strategies have been developed to engineer cell lines by altering processes in the ER [156].

Florin et al. generated stable CHO cell lines expressing heterologous ceramide transfer protein (CERT) [44]. CERT mediates ATP-dependent ceramide transport from the ER to the Golgi complex [67]. These cells showed significantly higher specific productivities of the protein HSA and enhanced monoclonal antibody secretion. The expression of the transcription factor X-box binding protein-1 (XBP-1) was shown to increase the ER content of a therapeutic antibody. This, in turn, led to a 40% higher productivity of a CHO cell line [12]. XBP-1 regulates this process by binding to the ER stress-responsive elements within the promoters of a wide spectrum of secretory pathway genes, resulting in enhanced total protein synthesis [160].

Chaperones have been linked to many ER functions such as protein translocation, folding, and oligomerization [2, 155]. The lack of co-chaperones seems to be rate-limiting. However, engineering chaperone systems by over-expression of a single component of the ER secretion machinery has yielded mixed results regarding productivity [80, 126]. It was possible to increase antibody productivity

in a CHO line by over-expression of PDI, but this failed to increase thrombopoietin secretion. PDI is an ER enzyme that catalyses the formation and breakage of disulfide bonds between thiol groups of cysteine residues using the substrate glutathione. It operates as a chaperone to inhibit the aggregation of misfolded proteins. Another group was able to demonstrate that the over-expression of BiP, a member of the hsp70 family, decreased the secretion of a recombinant antibody in CHO cells [23].

In contrast, over-expression of the calreticulin and calnexin chaperones was found to nearly double the specific productivity of thrombopoietin in recombinant CHO cultures [30]. The example of PDI over-expression demonstrates that the engineering of complex systems such as the chaperone system may need more sophisticated engineering to improve secretion rates.

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