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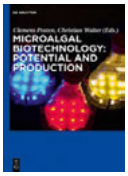

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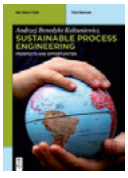

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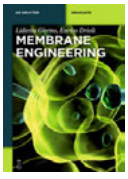

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Animal Cell Biotechnology

In Biologics Production

Edited by
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Preface

It is now more than 15 years ago when the first edition of the book 'Mammalian Cell Biotechnology in Protein Production' was published. At that time, realization of the book was driven by the requirements of academia and industry working on cell-based research and production processes to intensify their biological and technological understanding for bringing their inter-disciplinary knowledge to an applicable know-how and for using their technological capacities to an all-embracing capability.

During the last 20 years the biopharmaceutical market showed a substantial growth and can be described as the age of blockbusters. While at the beginning drugs dominated targeting genetic defects (Antithrombin III or Factor VIII), more and more therapeutics for treatment of cancer, cardiovascular, immunological and neurological diseases have been produced. However, treatment of diseases with a broad diversity and physiological dependency on the genetic background of the host is less predictable. As a consequence, not all patients benefit from blockbusters. Current clinical research increasingly reveals information about the molecular basis of these diseases, especially through reliable omics data. Patient stratification methods lead to a facilitated prediction if a certain drug will be effective or if it is better not used. This generates the "virtual patient" and asks for individualized medicines, leading to reduced use of blockbusters and request for a diversification of medications, even if they are only of interest for smaller patient groups. Thus, the next decade will be characterized by more sophisticated direct-to-consumer distribution channels which will diminish the role of wholesalers. The blockbuster sales model will be more and more replaced by products or combinations of drugs that are primarily focused on specialized medication and treatment procedures.

Meanwhile, industry was able to develop new products of the second and third generation and launched the so-called biosimilars that allow companies to rely, at least in part, on the safety and efficacy data of the reference brand product. Increases in knowledge and the more rational approach to drug discovery have contributed to the discovery of many important new classes of biopharmaceuticals but the costs for licensing a product constantly increased and amounts up to more than 1 billion USD.

The regulatory authorities have continuously strengthened their rules and guidelines. This concern not only the quality of the production process and the product, it also influences the average number of patients enrolled in clinical trials. While in 1970 approximately 2,000 individuals were needed for approval of a certain drug, more than 5,000 have been requested in 1990.

Today, the research and development process typically spans more than a decade and still remains subject to considerable risk and uncertainty. This is reflected by the low probability of success. In 2010, Ernst and Young presented an estimation of 5,000 products in phase I/II development and another 20,000 products in preclinical development. This reflects the failure rate in this step. The high number indicates the potential revenues but as well the requirement for new drugs as stated above. Also,

the high number requests cheaper, faster and more efficient procedures to obtain clinical trial materials. The capacity to develop manufacturing processes in a competitive time period is often not available at the critical phase and the flexibility of the facility is missing. New cheaper and flexible production facilities are necessary to satisfy the increasing demand. At the same time, the considerable increase in the number of new drug candidates, their use in individualized medicine leads to a reduction of the manufacturing volume forcing companies to leave their established routes and set up flexible facilities.

While the above considerations concern recombinant proteins, mainly antibodies, gene and cell therapies represent a new class of drugs that have the potential for cure. As of 2012, over 2,030 clinical gene therapy trials for human and animal health have to be completed or have been ongoing. Around 200 companies are involved in developing gene therapeutics and their number increased 4-fold during the last decade.

The new book encompasses the major aspects for the development and manufacturing of biopharmaceuticals and cell-based processes ranging from the genetic, molecular and cellular issues up to the final product. It is mainly directed to the expression and production of proteins and builds bridges to other biologics like those based on viral genomes. All these therapeutics have particular requirements on purity, potency and safety with tight specification ranges guaranteed by robust processes of highest efficiency run under cGMP conditions. The book also addresses researchers in industry and academia that need higher amounts of recombinant proteins from animal cells for functional tests and structural investigation.

The chapters have been written by outstanding experts in the respective biotechnological areas. The content is aimed at students interested in the blooming field of protein and virus-based biotechnology for the development and manufacturing of biotherapeutics and at researchers working in this scientific field in academics and industry as well as at all scientists interested in specific aspects of the applied animal cell culture-based biotechnology.

Berlin, in March 2014 *Hansjörg Hauser and Roland Wagner*

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1 Introduction

1.1 Industrial Use and Perspectives of Animal Cell Culture

Roland Wagner and Hansjörg Hauser

1.1.1 Introduction

In 2013, it was estimated that there were more than 900 products in clinical phases up to submission and several thousand products in preclinical development [1]. The global pharmaceutical sales increased dramatically during the last 2 decades, e.g., it more than doubled from 2000 to 2009. The USA, with around 37 %, is still the world's biggest single market. Growth in this market, which was above average until the first years of the new millennium, has tended to approximate the moderate dynamics of European markets in the past 3 years. Latin American and Asian markets have grown most strongly. Europe's share of the world market declined in 2009 to 31 % (from 32 % in the previous year), due mainly to the Euro's weaker exchange rate against the U.S. dollar. Germany's global market share also fell very slightly in 2009 from 4.5 to 4.3 %. In real terms, assuming a constant exchange rate, Germany's share of the world market decreased from 5.0 to 3.5 % over the past decade. Additionally, the product diversity is broadened by the placement of gene and viral therapeutics as well as cell therapeutics and antisense products to the portfolio. From the overall 907 products in development, 338 count for the largest group of monoclonal antibodies and 250 for vaccines.

In the mid-1980s the average accumulated protein product concentration ranged at ca. 200 mg/L. For producing today's necessary amount of the anti-cancer drug Avastin® of nearly 3 t per year several million liters bioreactor capacity would have been needed based on such a process. Subsequent developments particularly concerned cell culture nutrient media selection of suitable cell lines, cell line engineering, and adapted modular feeding strategies. A combination of achievements led to substantial extension of the cultivation time and hence the available time period for the enrichment of a product such that the amount of producing cells over the prolonged cultivation interval, the cell integral, based on a distinctly higher amount of viable cells, and cell specific productivity is increased. With these methods antibody product titer of more than 5 g/L and above can be achieved within 2 weeks of culture.

1.1.2 Cells as Bioreactors for the Production of Biologics

With the approval of the tissue plasminogen activator (tPA, Activase®) in 1986 mammalian cell culture and particularly the Chinese hamster ovary (CHO) cell line became the most popular production system for the manufacturing of protein therapeutic products. Even 20 years after tPA approval, CHO cells remained as the preferred mammalian cell line for the production of recombinant protein therapeutic for several reasons. CHO cells are easy to handle and can grow in suspension culture, a prerequisite for a homogenous large-scale culture in the industry. Moreover, and very important, CHO cells pose less risk as few human viruses are able to propagate in them [2]. They can grow in serum-free and chemically defined media, which ensures reproducibility between different batches of cell culture and minimizes risk of contamination and impurities. Last, but not least, CHO cells are capable to perform post-translational modifications to recombinant proteins, which are compatible and bioactive in humans [3]. Specifically, glycosylation of glycoproteins produced by CHO cells are more human-like, with the absence of immunogenic α-galactose epitope [4]. Several gene amplification systems are well established to make use of the genome instability of CHO cells to allow for gene amplification, which ultimately result in higher yield of recombinant protein. Currently, recombinant protein titers from CHO cell culture have reached the gram per liter range, which is a 100-fold improvement over similar processes in the 1980s. The significant improvement of titer can be attributed to progress in establishment of stable and high producing clones as well as optimization of culture process. Due to these reasons, CHO cells are established host cell lines for regulatory approvals of therapeutic glycoprotein products [2, 3, 5]. Beside CHO, there are a handful of other cell lines accepted by the regulatory authorities derived from other the Syrian baby hamster, the Muscovy duck, insects and human tissue.

1.1.3 Protein Expression Technologies

The product is expressed in cells by applying appropriate genetic engineering techniques and transfection of the cells with the expression vector bearing the desired genes of interest. The cellular productivity can be modulated by selecting special DNA regulatory elements carried on the vector and by targeting its integration site on the host cell genome. Productivity can also be increased through improving cell culture characteristics via cell line engineering [6].

Antibodies Make up the Biggest Group

Monoclonal antibodies are the fastest growing category starting from 1 % in 1995 to 14 % in 2001 and more than 70 % in 2013. Based on their molecular structure and the resulting binding properties, they have the skills to specifically recognize antigens

and cellular markers. An incredible amount of variants and their distinct action is the basis for a nearly inexhaustible therapeutic potential. For the year 2015 the market volume of monoclonal antibodies is estimated to 64 billion U.S. dollars covering at least 38 % of the total biotechnology-based pharmaceutical market which will be shared with other proteins and vaccines then encompassing around 170 billion U.S. dollars. From the 38 billion U.S. dollars in 2009 an approximate double in growth for the antibody market is prognosticated [7].

Personalized Medicine Drives Industry to the Development of new Drugs and More Efficient Manufacturing Processes

Personalized medicine will lead to the renunciation from standard therapy approaches looming today, that has been offered for all patients. At the same time this will require a drastic increase in the diversity of therapeutics. Further, this trend will reduce the market share of so-called blockbuster drugs as the therapeutic antibodies Avastin®, Herceptin®, Rituxan®, or Enbrel®. Industry reacts to this situation by efficiency increase in the development of new drugs and by a substantial reduction of the development period and/or by the application of innovative economic manufacturing processes.

The Biological Potential and the Technological Effort Assign the Process Limit

The rapid and straight increase in the productivity of biologics-producing processes during the last 5 years up to now easily tempts to assume that this progress could keep on with practically endless constancy. While the theoretical end of cellular productivity is not yet reached, from the industrial point of view a final titer will be sufficient when a stable platform process at maximum economy is reached. The maximum cellular productivity might be estimated by the production capacity of a natural high producer cell that has developed throughout biologic evolution. An antibody-producing B lymphocyte is able to produce up to 20,000 antibody molecules per second [8]. Related to a contemporary cell culture in a bioreactor this is equivalent to a production of 6 grams per liter and day at a representative cell concentration of 10 million cells per milliliter culture broth. Translating this capacity to a typical fed-batch process over a cultivation period of 14 days, 80 grams antibody product per liter culture would be produced. This considerable amount is still more than 10-fold higher than a state of the art process today (see above).

Increase in Productivity and Process Realization Have to Form a Unit

In general, product maximization leads to a higher yield. However, this advantage is often accompanied by an increase in rather undesired side products and reduction of product quality that take out the shine from the laudable but unreflected titer

numbers. A higher protein concentration imposes additional molecule interactions leading to a higher formation of aggregates and substantially impeding following process steps, which in extreme result in an uneconomic process by a costly and inefficient product purification. Therefore, the economic reason will aim to a balanced situation, which will be adjusted by the competition between the biochemical and cell physiological potency as well as the height of the production capacity and the resulting possibilities. In reality, production bioreactors for cell cultures of today have a working volume of about 10,000 L. Such a volume is integrated in the flow of the total process in a way that the upstream and downstream process form a procedural unit. A significantly higher product concentration will induce new challenges for the purification concept. Chromatographic systems must take enormous dimensions and the purification efficiency has to be substantially increased, such that the higher amount of incompletely or even incorrectly processed, possibly denaturated or aggregated protein will be robustly separated from the desired product in order to guarantee a constant high quality. Primarily, such an additional investment for the manufacturing of new antibody therapeutics would be hardly justifiable. Therefore, the development of cell-based expression systems will preferably focus on the optimization and intensification of the product quality and titers ranging in the one-digit gram range will probably dominate future processes in view of economics. Nevertheless, the request of achieving high product titers will mostly drive the process expectations, especially when competitive biosimilars are developed which shall displace existing products from the market after patent closure.

Biosimilars Require Processes of Highest Robustness

Generics must contain the same active ingredients as the original formulation as they are considered identical in dose, strength, route of administration, safety, efficacy, and intended use (U.S. Food and Drug Administration on generic drugs). This term is only used for small molecule-based drugs produced by simple processes. In contrast, biologics generally exhibit high molecular complexity and are quite sensitive to changes in manufacturing processes since they are made by or derived from a living organism. Differences in impurities and/or breakdown products can have serious health implications. This has created a concern that copies of biologics might perform differently than the original branded version of the product. Consequently, only a few versions of follow-up biologics have been licensed in the regulatory authorities in U.S. (FDA) and Europe (EMA). Examples of those biosimilars are erythropoietin (EPO), human growth hormone (hGH) and granulocyte colony stimulating factor (G-CSF). The development of a biosimilar is only attractive when the following prerequisites are fulfilled:

a) A long ranging market demand.
b) The original product is produced with an inefficient process or organism.
c) No product of a second generation is planned.
d) Patent closure within the next 10 years.

Nevertheless, numerous patents will be closed during the next 5 to 10 years. For proteins of the first generation such as EPO, G-CSF, IFN and FSH the patent protection is already expired or will be expired in the next years. Products of the second generation are blockbuster monoclonal antibodies and/or fusion proteins such as Rituxan®, Herceptin®, Avastin®, and Enbrel® that are most highly attractive for being redesigned as a biosimilar using a more efficient process with a better profitability (see Table 1.1.1). Additionally, products of the third generation are improved mAb/fusion proteins such as Orencia® (Abatacept, extracellular domain of CTLA-4/IgG1-Fc) from Bristol-Myers Squibb or Prolia® (Denosumab) from Amgen and similar new products which are protected by patents beyond 2020. Due to parallel development approaches of different companies the potential of becoming a blockbuster product is comparably lower for this product class. However, the development of a biosimilar is a challenging process. Beside the preclinical data, also the clinical phases, but often in limited extent, have to be passed. Moreover, it takes distinctly more time for market penetration compared to classical generics due to a slower acceptance process of the product by the physicians. Nevertheless, the effort is worthwhile because the development of a new product requires an expenditure amount of 1 billion U.S. dollars and above, which is more than 5 times higher compared to the respective biosimilar.

Table 1.1.1: Blockbuster cell culture-based biologics of the second generation under development as a biosimilar.

Company	**Brand Name**	**Product**	**Cell Line**	**Patent Protection**	**Sales 2011** (in billion USD)
Amgen	Enbrel®	Etanacept, TNFR2-p75/IgG1-Fc	CHO	2012, prolonged to 2022	7.9
AbbVie	Humira®	Adalimumab	CHO	2015	8.2
Genentech/BiogenIdec Roche	Rituxan® MapThera®	Rituximab	CHO	2016	6.8
Centocor	Remicade®	Infliximab	SP2/0	2018	7.2
Genentech	Avastin®	Bevacizumab	CHO	2019	6.0
Genentech	Herceptin®	Trastuzumab	CHO	2019	5.9

Antibody-drug Conjugates Force onward the Market

Antibody-drug conjugates (ADCs) make up one of the growing fields in the biopharmaceutical industry. ADCs are a combination of a monoclonal antibody or its fragment (scFv) and a reactive, mostly cytotoxic chemical that has been fragilely fused via a special chemical linker for assembling the final drug vector. This principle allows for the development of highly targeted therapeutic approaches for a wide range of diseases, particularly oncological and hematological indications. By combining the

specific targeting capability with the cancer-killing ability of a cytotoxic drug, ADCs allow sensitive discrimination between healthy and diseased tissue. The biochemical reaction between the antibody and the cellular target protein triggers a signal in the tumor cell, which then absorbs or internalizes the antibody together with the cytotoxin (Table 1.1.2). After the ADC is internalized, the cytotoxic drug is released and kills the cancer cell [9].

Table 1.1.2: List of highly potential cytotoxic agents used for the formation of antibody-drug conjugates.

Name	Target	Action
α-Amanitin	RNA polymerase II	Inhibition
Monomethyl auristatin E	Tubulin	Inhibits polymerization
Calicheamicin	DNA (minor groove)	Scission
Duocarmycin	DNA (minor groove)	Alkylate adenine at N3
Doxorubicin	DNA	Intercalation
Maytansinoid	Tubulin	Inhibits polymerization
Pyrrolobenzodiazepines	DNA (minor groove)	Cross-linking

The linker is the crucial part or the ADC and relies usually on chemical synthesis [10]. The complexity in structure, development, and manufacturing impose new challenges to the process. Only three ADCs have received market approval so far. However, after a request from the U.S. Food and Drug Administration (FDA), Pfizer/Wyeth, the developer and marketer of the first ADC to receive marketing approval in 2001 for the treatment of patients with acute myelogenous leukemia (Gemtuzumab ozogamicin, trade name Mylotarg®), withdrew the drug from the market in June 2010. As a result, only two ADCs are marketed (2013), including Brentuximab vedotin (trade name Adcetris®, marketed by Seattle Genetics and Millennium/Takeda) and Trastuzumab emtansine (trade name Kadcyla®, marketed by Genentech/Roche).

Targeting Biologicals to Specific Disease Sites

Cytokines are mediators of cell communication. Their therapeutic use often requires high doses to achieve effective local biological levels. However, the clinical use of some cytokines is limited because of their pleiotropism, which can result in unwanted side effects. Thus, protein engineering technologies that overcome these limitations and enable the targeting of cytokines to specific sites have been developed. One such example uses antibody-based recognition to direct the cytokine to a particular tissue [11]. This method requires the target site-specific cleavage of the fusion protein, thereby exploiting the severity of the pathological process to regulate drug delivery.

Because these technologies are based on the expression of fusion proteins, their application can be extended to diverse biologicals.

1.1.4 Vaccines Are Produced in Mammalian Cells

Prevention is better than cure. The mortality of people infected by the aggressive pox virus *Variola major* was 20 to 60 %. Up to 1967, when the World Health Organization (WHO) started a program to exterminate smallpox, yearly 15 million new infections were registered from which 2 million people died. Thirteen years later, smallpox was nearly eliminated by consequent vaccination campaigns. The word "vaccine" originates from the Latin *Variolae vaccinae* (cow pox), which the British physician Edward Jenner finally demonstrated in 1796 after 6 persons successfully used the principle that could prevent smallpox in humans. Today the term "vaccine" applies to all biological preparations, produced from living organisms, that enhance immunity against disease and either prevent (prophylactic vaccines) or, in some cases, treat disease (therapeutic vaccines). Apart form the adjuvants, vaccines consist of proteins, viruses and bacteria, natural or recombinant, which are either nonpathogenic or inactivated, cells or cocktails from those. Early viral vaccines were produced in primary cells.

The worldwide vaccine business is the fastest growing market in biopharmaceuticals counting for nearly 40 billion U.S. dollars in 2013 and expected to increase over 100 billion U.S. dollars in 2023 with a yearly growth rate of 10 % (according to Exane-PNB Paribas [12]). As stated by the WHO [13] merely the worldwide influenza will increase from 2.9 billion U.S. dollars in 2011 to 3.8 billion U.S. dollars in 2018 (see also Table 1.1.3).

Table 1.1.3: Top product vaccine sales in 2010 [17].

Brand Name	Producer	Type/Composition	2010 Sales (in billion USD)
Prevnar-13®	Pfizer	13-valent pneumococcal conjugate vaccine	2.40
Proquad®	Merck/Sanofi-Aventis	Measles-mumps-rubella and varicella combination vaccine (NMR-V)	1.40
Gardasil®	Merck	HPV	1.35
Prevnar®	Pfizer	7-valent pneumococcal conjugate vaccine	1.20
Fluzone®	Sanofi Pasteur	Influenza (seasonal and H1N1 strains	1.20
Infanrix and Pediarix	Glaxo SmithKline	Infanrix = DTaP, Pediarix = DTap-HepB-IPV (combination DPT-based vaccines with acellular pertussis	1.20

1.1.5 Virotherapy and Gene Therapy Open the Door for Cure

Virotherapy uses specific viruses that have been genetically modified such that they exclusively can propagate in highly active dividing cells, as this is the case for cancer cells. Such oncolytic viruses indicate a new class of high-potency therapeutics. Large amounts of potent viruses have to be produced in animal cells cultivated in large scale bioreactors. The first oncolytic virus candidate was H101, a modified adenovirus, which was licensed in 2005 by China's State Food and Drug Administration (SFDA) for nasopharyngeal carcinoma together with chemotherapy. H101 has been engineered to remove a viral defense mechanism that interacts with a normal human gene P53, which is very frequently dysregulated in cancer cells [14]. Systemic therapy is now marketed under the brand name Oncorine® by Shanghai Sunway Biotech.

Gene therapy is generally defined as the transfer of recombinant nucleic acids to humans as well as animals with the aim to regulate, to repair, to replace, to add, or to eliminate a genetic sequence. Principally, gene therapy is only allowed to perform in somatic cells excluding a passage via the germ line. For the *ex vivo* approach cells (e.g., lymphocytes or hematopoietic stem cells) are taken from the patient, modified outside the body by a therapeutic gene transfer, and the corrected cells are then reinjected. Three virus families are currently covering 90 % of the vector systems used for gene therapy: adenoviruses (AdV), adeno-associated viruses (AAV) and retroviruses (RV) including lentiviruses (LV). All viruses are produced with animal cells in culture. AdVs and AAVs yield at an amount of 10,000 to 100,000 particles per cell, whereas only 1 to 10 RV particles are released per cell. Retrovirus vectors are used for *ex vivo* gene therapy and only relatively low amounts are necessary for the treatment. In contrast, virus doses of up to a quadrillion (1×10^{15}) particles per patient are used for one *in vivo* approach. This is 100 times more than the total amount of human cells in a body (10 trillion = 10^{13}). The production of such a high amount of virus needs highly efficient manufacturing processes. The breakthrough in the acceptance of gene therapy was in 2012 when the European Commission approved Glybera®, an AAV1-LDL^{S447X} gene therapy for the treatment of lipoprotein lipase deficiency (LPLD), as first gene therapeutic in the Western world. In order to treat all 5,000 patients worldwide a minimum amount of 1×10^{17} virus genomes has to be produced using at least a 10,000 L bioreactor capacity, which is comparable to those used for the production of recombinant pharmaproteins [15]. According to Global Industry Analysts Inc. [16] the market for gene therapy is expected to reach 794 million U.S. dollars by 2017. The key factors driving growth in the market include a rising demand for new and efficient therapies for cancers treatment, inability to cure some cancer types and other critical diseases, and prospective launch of gene therapies in major global markets.

1.1.6 Cells as Therapeutics

Cell-based therapies are a viable option for an increasing number of diseases. The transplanted cells are either used as mediators of specific actions or as replacements for diseased or lost cells/tissue. Examples for the first application concerns T cells and mesenchymal stem cells to prevent reactivation of latent cytomegalovirus (CMV) in the transient state of immunodeficiency and graft versus host disease after hematopoietic cell transplantation, respectively. The second application mainly concerns the use of stem cells. A number of animal experiments and a few human studies have demonstrated its usefulness. Further, embryonic stem cells and the recent development of induced pluripotent stem (iPS) cells technology demonstrated the potential of cell-based therapy in rodent models. It was shown that such cells are capable to differentiate *in vitro* and *in situ* and respective cell grafts can improve deficiencies. Altogether, the recent findings have shown great promise for developing the foundation of the cell-based therapy. Unfortunately, there are several challenges faced by researchers that must be overcome before stem cell therapies can become a successful reality for those suffering from disease. While T cell-based immunotherapies are now being included in the clinical practice of transplant recipients to prevent and treat infections and complications associated with CMV, AdV and Epstein-Barr virus (EBV), other cell therapies are still in the development or in clinical trials. Although the projected market for cell therapies and regenerative medicine is estimated to amount several hundred billion U.S. dollars in 2020, significant short-term payoffs are not expected. While the biotechnology of cell therapy applications has not been addressed in this book, it is obvious that the expertise researchers and industrialists have gained from protein production will be essential to further fabrication of such cells.

1.1.7 Protein Production in Pharmaceutical Research

In the development of pharmaceuticals expression of proteins in cells is often required. Medium or large scale production concerns mainly the aspects of structural biology. Despite a multitude of recent technical breakthroughs speeding high-resolution structural analysis of biological macromolecules, production of sufficient quantities of well-behaved, active protein continues to represent the rate-limiting step in many structure determination efforts. To determine protein structure milligram amounts of pure protein are needed. If cells are required for the production of such proteins (due to activity, authentic processing, multisubunit complexes, membrane location), the technologies applied for recombinant pharmaceutical protein production are applied. The same technologies are used for functional assessment of certain proteins. In fact, the trials for the successful production of large quantities of pure proteins for structural biology have significantly contributed to the development of recombinant expression systems as they are used today. While the direct application

of these technologies is not directly translated into industrial products, their successful use is often rate-limiting in the development of new drugs.

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2 Cell Lines

2.1 Generation of Cell Lines and Biotechnological Applications

Christoph Lipps, Tobias May, and Dagmar Wirth

2.1.1 Introduction

Mammalian organisms contain more than 200 different cell types that are required to fulfill the various functions. Many of these functions depend on the interaction of different cell types. To investigate the molecular basis of diseases, to develop novel treatment options for intervention, and to screen novel drugs, cell based systems are required that reduce the complexity of the organism. For many of such applications the cells need to maintain physiological relevance to mimic the cells *in vivo* as close as possible. For industrial applications, cell based systems should be robust, reliable, and also cost effective.

For many years, cell based systems have been used in basic research as well as in applied disciplines like protein production, drug discovery, and toxicity testing, and they are also used for therapy in regenerative medicine approaches. These systems are based on either primary cells (primary cell cultures, PCCs) or immortalized cell lines, which are also called continuous cell lines, CCLs.

Primary cells can be isolated from various tissues of the organisms and protocols have been established to cultivate many different cell types *in vitro*. These cells closely reflect the properties of cells *in vivo*. Thus, they are considered to be physiologically relevant. However, primary cells are associated with several drawbacks (Figure 2.1.1). As primary cells have a limited proliferation potential they cannot be expanded *in vitro* to larger cell numbers. Thus, to obtain the desired cell numbers, primary cells are collected from many different donors and/or large tissue samples are required. For many cell types, the isolation procedure is a tedious and time-consuming process. Primary cells from humans are characterized by a high donor-to-donor variability. These differences originate, e.g., from (i) the genetic variability between different individuals, (ii) environmental influences, (iii) diseases and medications to which the donor has been exposed, and (iv) technical reasons like variations during the isolation procedure. Although this high degree of variability is scientifically and commercially interesting (e.g., with respect to personalized medicine) it poses a big hurdle for the development of robust and reliable assays. Moreover, primary cells are attributed with a potential risk for adventitious agents. (For a more detailed discussion of the disadvantages of primary cells for production of vaccines see Chapter 2.3.)

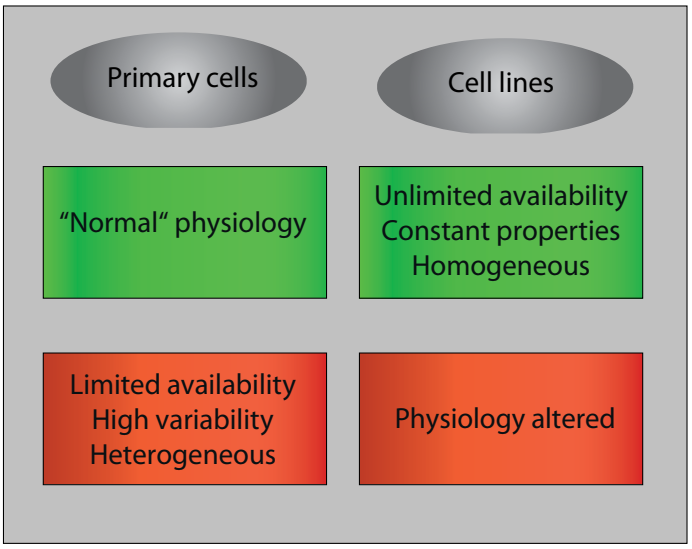

Figure 2.1.1: Comparison of primary cells and cell lines. The major advantages (green) and disadvantages (red) of the respective cell system are given.disadvantages (red) of the respective cell system are given.

As a consequence of the lack of reproducibility of primary cells, many applications rely on unlimitedly proliferating cell lines or CCLs. Cell lines can be either isolated from tumors or generated upon spontaneous or induced immortalization of primary cells. Cell lines by definition show indefinite growth. Thus, they can be easily expanded to the required cell numbers. In addition, if compared to primary cells, they represent a rather homogenous, genetically defined cell population with constant properties. Moreover, they can be genetically modified to display additional features. However, immortalized cells are frequently accompanied with the (partial) loss of relevant physiological properties (Figure 2.1.1). Thus, cell lines lack many features and markers of the tissue they were isolated from.

Since there is no *in vitro* cell system available that meets all the requirements, the selection of a cell system is always a compromise. Accordingly, it strongly depends on the specific application whether physiologically more relevant primary cells or robust cell lines are employed. For example, for the production of recombinant proteins and/or therapeutic viruses and vaccines, cells should show a high productivity, stability, and robustness. Moreover, they should proliferate under defined conditions so that tightly controlled processes can be installed. At the same time, the physiological relevance of the cell system is less important. For such applications, cell lines such as CHO or HEK293 cells proved to be highly instrumental.

In contrast, in drug development the physiological relevance of the employed cell system is of high priority. In this respect, many established cell lines do not meet the

expectations. Thus, in the last years emphasis has been laid on the development of new immortalized cell lines with improved properties from specific tissues that have not yet been available previously and that meet the requirements of physiological relevance.

In this chapter, different strategies to establish immortalized cell lines are summarized. This comprises the description of the commonly used immortalizing genes including their proposed molecular action, protocols for cell type specific immortalization, as well as recently emerged conditional immortalization strategies to control cell proliferation. In addition, a noncomprehensive overview is given on biotechnologically relevant cell lines as well as their applications. Emphasis is also given on cell lines used in the drug development process.

2.1.2 Principles of Immortalization

In primary cells, proliferation is tightly controlled. In this surveillance, the tumor suppressor genes p53 and pRb play a pivotal role. These genes control cell cycle progression and contribute to DNA integrity. Moreover, they are involved in the control of cellular senescence and apoptosis, which represent the ultimate mechanisms to eliminate cells that display aberrant proliferation control (for review see, e.g., [1-3]).

In immortal, i.e., infinitively growing cells, this well-balanced regulatory network is perturbed and immortalized cells have lost the original capacity to control cell proliferation. A natural source of immortal cells are tumors in which specific cellular control genes are usually found to be mutated. Tumor derived cell lines are often characterized by genetic instability, which contributes to the generation of additional mutations that lead to immortalization. Tumor derived cell lines display an aberrant growth control and often are tumorigenic. This can be demonstrated by their capacity to form tumors upon injection into immune-compromised mice (see also Chapter 2.3).

As an alternative to tumor derived cell lines, immortal cell lines are generated upon manipulation of primary cells isolated from healthy tissues. For certain cell types like mouse embryo fibroblasts (MEFs), immortalization of cells can simply be achieved upon continuous passaging of primary cells following a regular splitting protocol. In a report by Todaro and Green, MEFs were split every three days and replated with a low density of 3×10^5 per plate (3T3 protocol) [4]. By this protocol, multiple random genetic and epigenetic changes that confer a proliferative advantage are accumulated and finally result in immortalization of cells [5]. Due to the randomness of this process, it is not specified which mutations are induced through this protocol. In later studies the role of the tumor suppressor p53 was investigated, however with nonconsistent results. In one study clear indication was given that p53 is the major driver of the immortalization process [6]. In contrast, another study demonstrated that p53 is only involved in spontaneous immortalization of MEFs if a modified protocol (the 3T12 protocol) but not if the 3T3 protocol is used [7]. This highlights the major

disadvantage of spontaneous immortalization protocols: since the genetic alteration that is provoked is not controllable the cells differ in their properties and an extensive screening has to be performed to identify the desired cell clone with the desired properties. Another important drawback of such a random immortalization by cultivation is the fact that it is restricted to certain cell types such as mouse fibroblasts while most cell types cannot become immortalized by this method.

Over the last decades, knowledge has been accumulating on specific genetic manipulations that result in the immortalization of cells. While in the beginning mainly random procedures were employed, in the recent years novel strategies have been emerging that are directed to achieve the rational and producible development of such designer cell lines (Figure 2.1.2). It has to be noted that the various protocols for immortalization are not generally applicable but are usually restricted to certain cell types and/or species. This is exemplified by the recent observation, namely that human cells usually require immortalization strategies that are different to cells from other mammals like, e.g., murine cells [8].

The most important and promising approaches towards a rational development of cell lines are summarized in the following sections.

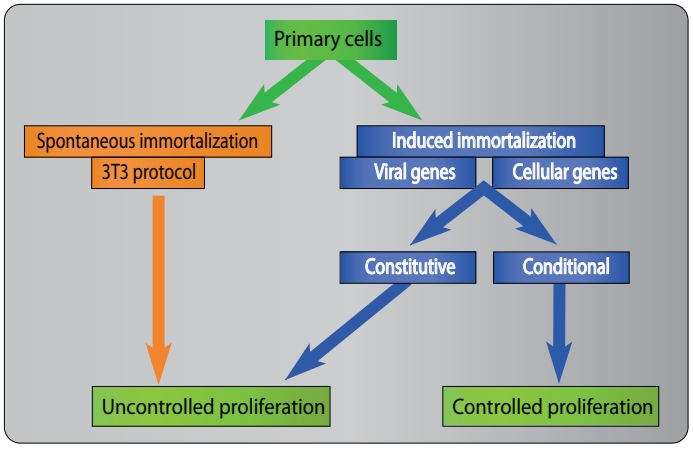

Figure 2.1.2: Schematic representation of the different immortalization approaches. Immortalization can be achieved through spontaneous events like, e.g., outgrowth from tumor material or through defined cultivation regimens (3T3 protocol). A more controlled approach is the introduction of immortalization genes into primary cells of choice. If the immortalization gene is controllable by the exogenous means a growth controlled immortalized cell line is established. In this case proliferation can be controlled by exogenous stimuli.

2.1.2.1 Immortalization of cells based on viral oncogenes

The first cell lines created *in vitro* were derived upon infection of primary cells with viruses that have an intrinsic oncogenic potential (tumor viruses). Various viruses are known to confer infinitive growth of cells by transducing oncogenes that deregulate the cellular growth control. A highly successful strategy for development of cell lines by viral infection represents the use of Epstein-Barr virus (EBV) for the immortalization of B lymphocytes.

Epstein-Barr Virus (EBV) genes

EBV is now in routine use for the establishment of B cell lines that are of particular interest for the development and generation of human monoclonal antibodies (for review see [9]). The gene or the genes from EBV that are responsible for the immortalization of B cells are not yet defined. However, it has been shown that the EBV nuclear antigen 1 (EBNA1) strongly enhances the immortalization process by several orders of magnitude [10]. This indicates that the concerted action of several EBV genes induces immortalization of primary B cells. The relevant pathways of EBNA1 based immortalization include the downregulation of p53 and the induction of reactive oxygen species (ROS). EBNA1 lowers the cellular p53 level indirectly by binding to the herpes virus-associated ubiquitin-specific protease (HAUSP) [11]. This complex binds to the p53-Mdm2 complex and is considered to induce deubiquitination of Mdm2 which in turn leads to the inactivation of p53 [12]. The accumulation of ROS after transduction of EBNA1 is probably induced through the induction of a NADPH oxidase (Nox2) [13]. High ROS levels induce DNA damage and the associated elevated mutation rates are considered to contribute to the immortalization of primary B cells.

Rather than employing replication competent wild type viruses, more controlled immortalization can be achieved by the transfer of the relevant subgenomic viral tumor genes. For this purpose, expression cassettes are generated in which viral or nonviral promoters are used to express the viral genes. These cassettes can be introduced into primary cells by nonviral methods. However, an increase of the efficiency of gene transfer and thus immortalization is often required. Thus, recombinant viruses such as retroviruses or lentiviruses that integrate the immortalizing genes into the cellular genome are of interest. Since lentiviruses can also infect arrested cells, this strategy is currently the method of choice for immortalization of primary cells with limited proliferation capacity.

Human Papillomavirus (HPV) E6 and E7

Out of the 100 human papillomaviruses 15 types are so called high-risk papillomaviruses that are considered to be the causative agents of most cervical cancers (for review see [14]). The major tumor promoting potential of these viruses is based on the viral E6 and E7 proteins. These proteins have been shown to interfere with cell cycle

control and the regulation of apoptosis. E7 inhibits the function of various pRb family members by binding and thereby facilitating cell cycle progression (reviewed by [15]). E6 on the other hand is known to promote the degradation of p53 and thereby disrupts the growth control by p53. Another function of E6 is the induction of telomerase activity which supports the immortalization of cells by maintaining telomere length (see below) [16]. For the efficient immortalization of human cells both proteins – E6 and E7 – are required. In certain settings the immortalization of human primary cells can also be achieved through expression of only a single HPV gene. It was demonstrated that both E7 and E6 are able to immortalize primary human foreskin keratinocytes when transferred alone. However, the efficiency was dramatically increased when E6 and E7 were used in combination [17] or when a variant of the E6 gene was employed [18]. It has to be noted that foreskin derived keratinocytes have a high intrinsic proliferation potential which probably contributes to the fact that this cell type is comparably easy to immortalize. Generally, the combination of E6 and E7 has been shown to be particularly efficient for the immortalization of epithelial cells [19].

Adenoviral E1A and E1B

The family of the adenoviruses comprises more than 50 serotypes (which fall into six different groups). Although adenoviruses per se do not induce cancer formation, it has been demonstrated early that the proteins E1A and E1B are able to immortalize primary cells *in vitro* [20]. The E1A gene is the dominant oncogene [21]. E1A is able to immortalize primary cells per se, albeit with a much lower frequency when compared to immortalization regimens which utilize E1A and E1B together [22].

The E1A protein has been shown to interact with retinoblastoma family members (pRb, p107 and p130) (for review see [23]). In the normal setting the tumor suppressor pRb regulates the transition from the G1 to the S phase of the cell cycle. The binding and inactivation of pRb through E1A therefore leads to activation of the S phase genes, which is mainly accomplished through the transcription factor E2F [15, 23]. Another important feature of E1A which contributes to its potential concerning immortalization is the interaction between E1A and p300/CBP. The precise mechanism is not yet completely understood but seems to involve transcriptional deregulation of various genes through epigenetic modulation such as histone deacetylation. One prominent target gene which is activated through this mechanism is the oncogene c-myc [24]. The action of E1B protein is mediated by its interaction and neutralization of p53 [25]. Interestingly, the inactivation of p53 is achieved without degradation of this tumor suppressor protein. As a consequence of this inactivation the initiation of the apoptosis program usually triggered by p53 seems to be prevented [26].

Simian Virus 40 Large T Antigen (TAg)

The simian virus 40 (SV40), a member of the polyomavirus group, is a monkey virus that has been shown to induce various types of tumors in rodents. Whether SV40 large T antigen (TAg) also causes tumors in humans is still under debate. Because of an accidental contamination of poliovirus vaccines in the 1960s, millions of people were exposed to SV40 virus [27]. Fortunately, this did not lead to a significant increase in tumor incidence, which suggests that the tumorigenic potential of the SV40 virus is low. The major protein that is responsible for the immortalization of primary cells is the large T antigen (TAg). For immortalization of cells, a temperature-sensitive mutant of SV40 TAg has gained particular relevance (see below). TAg is known to modulate the activity of a number of cellular proteins but amongst them, p53 and pRb are regarded as the most important ones for immortalization (for review see [28]). The binding of TAg to p53 leads to the inactivation of the tumor suppressor protein, which is essential for the block of its function as a transcription factor and the immortalization capacity of TAg [29]. In addition, TAg mediated immortalization is contributed by the inactivation of pRb. This interaction is mediated through a conserved motif (LXCXE motif) which is also found in "large T proteins" from other polyomaviruses [30].

While TAg alone can be successfully employed for the establishment of rodent cell lines, the immortalization of primary human cells by TAg is a very inefficient process. Transduction of human cells with TAg usually leads to an extension of the cellular life span. However, the TAg-transduced cells still enter into crisis in which most of the cells die and only a very small fraction of cells evade [31]. Importantly, the cells that went through crisis display a grossly altered phenotype when the established cell line is compared to their primary counterparts which is also reflected by an altered karyotype [32]. Therefore, an efficient immortalization of primary human cells is only achieved with a concerted action of TAg with a second immortalizing gene such as c-myc or human telomerase (hTert, see below).

2.1.2.2 Immortalization by hTert and the Impact of Telomeres

A critical part during genome duplication during S phase is the replication of the very ends of chromosomes; the repetitive DNA stretches which are called telomeres. These telomeres consist of repeats of the sequence TTAGGG/CCCTAA. In germ cells, a total of about 15 kb of telomeric repeats are found. In contrast, in differentiated cells the telomere length is significantly shorter. This reduction of telomeric ends is a consequence of the fact that during DNA replication the telomere ends cannot be fully replicated ("end replication problem") [33]. As a consequence the telomeres progressively shorten with every replication cycle.

In human stem cells, telomerase can antagonize this effect via its reverse transcriptase subunit hTert. However, telomerase is not expressed in human somatic cells. Only in tumor cells the activity of hTert is restored leading to the stabilization of

the telomeres. It could be shown that ectopic expression of hTert can lead to immortalization of human cells [34]. Since this initial study many human cell types have been successfully immortalized with hTert. In many cases, these cell lines display features of primary cells. This is highlighted by studies that successfully employed the hTert-immortalized cell lines in regenerative medicine approaches, e.g., with bovine adrenocortical cells [35], with human dermal endothelial cells [36], and human mesenchymal stem cells [37]. The use of hTert alone is restricted to certain human cell types, as others need the concerted action of several genes for efficient immortalization [38]. Importantly, prolonged constitutive expression of hTert induces changes in gene expression that lead to a premalignant phenotype [39].

Of note, telomerase is not applicable for the establishment of murine cell lines. This is probably due to the fact that laboratory mouse strains have long telomeres and murine primary cells often exhibit an endogenous telomerase activity [40].

2.1.3 Targeted Immortalization of Specific Cell Types

While the above mentioned genes act in a broader spectrum of cell types, the immortalization capacity of certain genes is restricted to specific cell types (reviewed in [41]). This can be exploited to preferentially immortalize cells in crude preparations of tissue cells that display a mixture of various cell types (e.g., see [42]). Indeed, a number of studies demonstrate that the transfer of certain genes can selectively immortalize specific cell types (Figure 2.1.3).

One example represents the immortalization of murine monocytes from the bone marrow-derived cell populations by v-myc [43, 44]. Another example concerns the specific immortalization of murine embryonic endothelial cells from a crude mixture of embryonic primary mouse cells by polyomavirus middle T antigen (PymT) [45].

Also specific immortalization of various human cell types has been reported. Human keratinocytes were evaluated for specific immortalization by the inhibitor of differentiation 1 (Id1). Cell lines could be generated that show increased telomerase activity and modulations in the two major tumor suppressor pathways pRb and p53 as highlighted by an impaired p53-mediated DNA-damage response. In addition, these cell lines have lost the differentiation potential normally seen in primary keratinocytes [46]. How robust this approach is remains to be shown since another study using this gene could only observe an extended lifespan of primary keratinocytes but not immortalization [47]. Differences in the experimental set-up might contribute to the contradicting observations.

Specific immortalization has also been shown for human hepatocytes. In the study of Ray et al. (2000), the hepatitis C virus (HCV) core protein was applied [48]. However, compared to primary hepatocytes the cell lines displayed an altered cell morphology suggesting that immortalization was accompanied with loss of the differentiated phenotype.

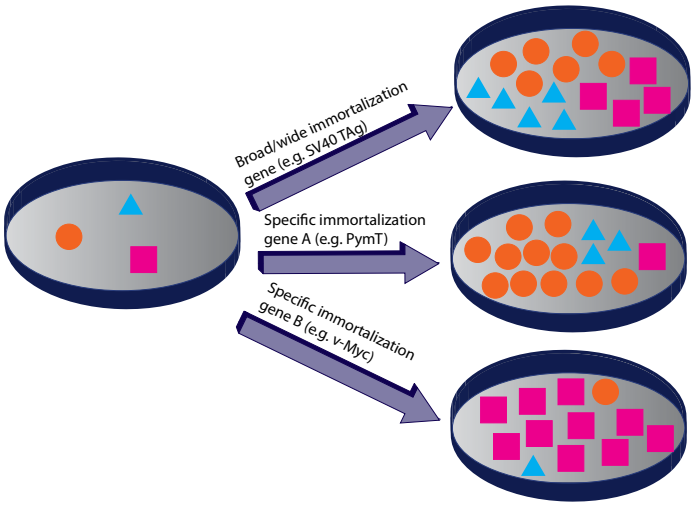

Figure 2.1.3: Schematic representation of cell type specific immortalization. Broadly acting immortalization genes like SV40 large T antigen immortalize primary cells widely independent of the cell type. Therefore if the starting material is a mixture of different primary cell types an immortalization approach with TAg leads to cell lines from the different cell types. In contrast, cell type specific immortalization genes like PymT or v-myc only lead to the immortalization of certain cell types. Therefore, from a mixture of different cell types only those will be immortalized that are amenable to the immortalization of the respective cell type specific immortalization gene.

A promising cell type for specific immortalization are memory B cells which can be immortalized by the ectopic expression of the apoptosis modulating gene Bcl-xl together with Bcl6. Bcl6 is a transcriptional repressor that prevents differentiation of B cells into plasma cells by downregulating p53 and facilitates the expansion of the B cells. Importantly, the resulting cell lines are fully functional with respect to antibody secretion [49].

A specific cell type of interest is the human CD34+ hematopoietic stem cell. It was found that the MLL-AF9 fusion gene, which is predominantly associated with myeloid leukaemias, was able to immortalize this cell type. These cells are still able to differentiate either to the myeloid or to the lymphoid lineage [50].

While these reports represent promising examples for exploitation of the cell type specific activities of immortalizing genes, it has to be emphasized that the potential of the individual genes is hardly predictable and thus has to be evaluated experimentally. A rational exploitation of immortalizing genes is also hampered by the limited knowledge of the signaling pathways that govern cell type specific proliferation.

2.1.4 Growth Controlled Cell Lines

For various applications it is of interest to control the proliferation of immortal cells over time. This concerns, e.g., biotechnological production processes where it seems advisable to block the increase of cell mass to dedicate the energy resources of a cell to the synthesis of a secreted protein of choice over long time.

Moreover, for some cell types it has been suggested that the differentiated state is restricted to a nonproliferative state while in the proliferative state cell type specific pathways are downregulated. Such a finding has been reported, e.g., for hepatocytes *in vivo* [51]. To control proliferation, different regulation strategies have been exploited. These comprise the use of post-translational, transcriptional and genetic control of immortalizing genes. These strategies are briefly described in the following paragraphs.

2.1.4.1 Controlled Proliferation by Post-translational Control of Immortalizing Genes

Growth control can be achieved by the controlled expression of immortalizing genes. Such a conditional immortalization of cells was first achieved with the help of a temperature-sensitive mutant of TAg (tsTAg). This mutant protein is fully active and the cells are cultivated at 33 °C. At this permissive temperature the tsTAg is comparable in its function to the wild type TAg and leads to proliferation and immortalization. Proliferation arrest is achieved by shifting the cells from 33 to 37-39 °C. Exposed to elevated temperatures the mutant tsTAg is misfolded and thereby inactivated and subjected to degradation [52]. tsTAg has proven to be instrumental for establishing various temperature-dependent cell lines. Growth-controlled cell lines have been established by retroviral transfer of tsTAg into primary cells (e.g., [53]) and also by isolation of cells from a tsTAg transgenic mouse, the so-called Immortomouse® [54, 55]. Examples for tsTAg-based conditionally immortalized cell types encompass myogenic cell lines [56], hepatocyte cell lines [57, 58], tissue specific microvascular endothelial cells [59], an astrocyte cell line [60], and more recently an adrenal medullary cell line [61] as well as stroma cell lines [62]. For a more comprehensive overview please see review by Obinata (1997) [63]. tsTAg-immortalized cell lines were used for a multitude of applications (see overview in [64, 65]). However, it has to be noted that the temperature shift itself imposes a significant change in cellular properties [66], which complicates the interpretation of cellular behavior regarding reversion of immortalization. In addition, one report indicates that the regulation control through the tsTAg is not stringent [67]. If tsTAg-immortalized rat hepatocytes were maintained for extended time periods at the nonpermissive temperature outgrowth of cells was observed. Still, in these cells tsTAg protein was temperature-controlled. This indicates that the immortalized cells acquired spontaneous mutations if maintained at the nonpermissive temperature. These mutations in turn induced proliferation independent of

tsTAg and consequently growth control was lost [67]. This kind of adaptive mutation might apply for other cell types and systems. However, immortalization systems have been described that retain the conditional phenotype long-term [68, 69].

Since temperature-sensitive mutants are not available for other immortalizing genes, more generic protocols to control activity of these genes have been explored. One example concerns the post-translational control of immortalizing genes upon fusion to the estrogen receptor moiety (ER). In ER fusion proteins the protein activity is structurally impaired. Binding of 4-hydroxy-tamoxifen to the ER domain changes the conformation of the fusion protein and restores the activity. Based on such an approach employing HoxB8-ER, murine macrophage, neutrophil and hematopoietic progenitor cell lines were generated [70].

2.1.4.2 Controlled Proliferation by Transcriptional Control of Immortalizing Genes

Besides post-translational control also transcriptional control of immortalizing genes has been exploited. For this purpose, synthetic, so-called orthogonal regulation systems have been implemented, that allow specific control of a synthetic promoter while not affecting the cellular regulatory network (reviewed by [71]). An extensively explored regulatory system concerns the "tetracycline system" (Tet-system) in which a recombinant transactivator protein specifically binds to a synthetic promoter and thereby induces transcription. The binding of the transactivator is modulated by tetracycline (Tet) or its derivatives such as doxycycline (Dox). Thus, addition or withdrawal of Tet or Dox results in control of transcription.

In the original study from Gossen and Bujard (1992) [72] the Tet-off system was used in which in absence of Tet the transactivator tTA binds to and activates the cognate promoter while in presence of Tet binding is impaired. Further developments in this field comprise the development of a Tet-on system [73], a bidirectional promoter which facilitates the expression of two cistrons [74] and a Tet-dependent promoter with a reduced basal activity [75] (for review see [76]).

While the classical set-up with a constitutive expression of the transactivator results in a gradual, dose-response, an auto-regulated set-up leads to stochastic activation. In such a setting the transactivator is controlled by the synthetic promoter, which allows implementing all components required for regulation and transgene expression in a single expression cassette [77, 78].

Based on an auto-regulated Tet system a highly efficient method for conditional immortalization was established [69]. In these cells, cell proliferation is strictly dependent on Dox. Due to the compactness of the expression cassettes they can be packaged into lentiviral vectors which facilitates an efficient gene transfer of the immortalizing genes in a broad range of cell types [68]. By Tet-controlled expression of SV40 TAg, PymT, hTert, and c-Myc, various types of proliferation-controlled cell lines have been established. Examples of growth-controlled cell lines include mouse fibroblasts [69], lung microvascular derived endothelial cells [68] and liver sinusoi-

dal endothelial cells [79], but also human cell lines such as umbilical vein-derived endothelial cells [68], as well as human mesenchymal stem cells [80, 81].

2.1.4.3 Genetic Control of Immortalization

Finally, also genetic switches have been utilized to control immortalizing genes and thereby revert the immortalization state. For this purpose, site-specific recombinases such as Cre or Flp recombinase were successfully employed to facilitate excision of the immortalizing gene(s) flanked with the cognate recombination target sites (e.g., loxP or FRT sites) [82-85]. However, in contrast to the previously described transcriptional and post-translational control systems, the reversion of the genetic switches is irreversible since the immortalizing gene is eliminated. Efficient transfer of the recombinase by adenoviral transduction combined with an extensive selection process can eliminate nonrecombined cells which would overgrow the population of reverted cells [86]. To further improve this critical step, more recent approaches involve inducible mutants of the recombinase, which are stably integrated into the cells and activated on demand [87]. The use of recombinases implements the risk for unintended genetic rearrangements if multiple copies of the immortalizing genes are integrated at various chromosomal sites. Such rearrangements could also involve cellular chromosomal regions, which can potentially affect cellular properties. Thus, recombinase-based control of proliferation requires additional controls to ensure the cellular phenotype.

2.1.4.4 Controlled Cell Proliferation in Protein Production Processes

Control of cell proliferation is an interesting option to dedicate the cell's capacity to the synthesis of recombinant proteins rather than to the increase of cell mass. Accordingly, controlled proliferation was evaluated with respect to the cellular productivity. To this end, the proliferation of immortalized cell lines was blocked by controlled expression of genes that stop cell cycle progression. One example concerns expression of the cyclin-dependent kinase inhibitors p21 or p27 which stop the cell cycle predominantly in the G1 phase. Interestingly, in the growth arrested state cell lines showed an enhanced production of the protein of interest. For Chinese hamster ovary cell (CHO) a 4- to 30-fold increase in antibody production could be observed in the growth arrested state if compared to proliferating cells [88-90], reviewed by [91]. Also mouse fibroblasts conditionally immortalized with SV40 TAg were analyzed for their productivity in the proliferating and in the nonproliferating state. In this study, the overall increase in production was 2-fold higher in the growth-arrested cells. Importantly, this ratio remained constant throughout the cultivation period [92].

These studies indicate that controlled proliferation enables a higher productivity of producer cells. Moreover, growth-controlled producer cells allow to continuously harvest secreted proteins over extended time periods, which makes this strategy

attractive for production. However, to date this technology has not exploited in a certified process of therapeutic protein production.

2.1.5 Cell Lines for Biotechnological Applications

In industrial research cell lines are mainly used either for drug development or for producing biologicals. In the following sections a noncomprehensive list of the most important cell lines for these two industrial applications is provided (Figure 2.1.4).

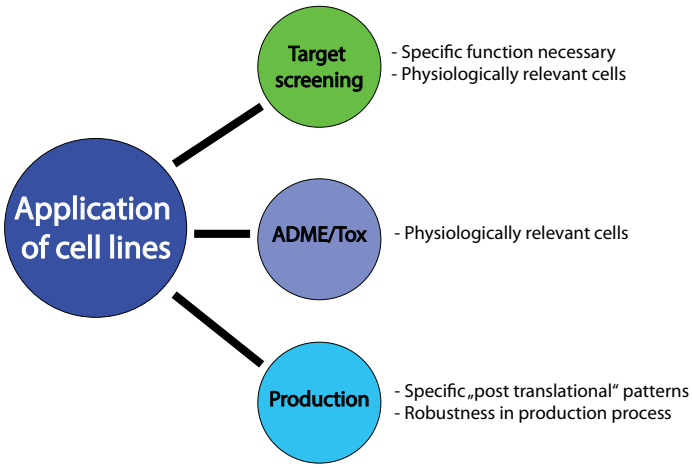

Figure 2.1.4: Applications and requirements of cell lines in biotechnology. The major industrial applications of cell lines are depicted. In addition, the desired properties of the cell lines in these applications are given.

2.1.5.1 Production Cell Lines

One major application of cell lines is the production of biotechnologically relevant proteins, such as antibodies, and other therapeutic proteins, such as human α-antithrombin (hAAT), blood coagulation factors, erythropoietin or follitropin, as well as their use for vaccine production. Also, recombinant viruses for gene therapeutic approaches are produced with the help of continuously growing cell lines that act as helper cells [93-96].

Such cell lines require a number of specific features. Besides a high overall productivity and genetic stability of the cell, a critical property concerns the type of glycosylation that is provided by the producer cell. Generally, a glycosylation pattern is required that confers protein activity and resembles best the glycosylation type in humans. In particular, it should not induce an antigenic response in humans. Further,

for protein producer cells also practical issues such as a high efficiency of genetic manipulation of these cell lines is of importance.

Currently, the production of proteins is mainly based on few cell lines that have been shown to provide high productivity, safety, acceptable stability, of recombinant protein production and can be subjected to fermentation processes under serum free conditions. Many production protocols are based on cell lines such as Chinese hamster ovary cells (CHO) [97, 98] and HEK293 cells [99] that have been isolated decades ago. (The application of these cell lines in vaccine production is highlighted in Chapter 2.3). In the recent years, the demand for well-characterized, traceable and rapidly expandable cell lines with defined and improved properties increases. Accordingly, an increasing number of cell lines has been generated by rational transfer of defined immortalizing genes to characterized primary cells of different species and tissues, resulting in the establishment of so-called designer cells. In the following, we give an overview about the history of some of these various cell lines that are or may become relevant for research and biotechnological applications. Since Chapter 2.2 is dedicated to the CHO cell line, it is not included in this compilation.

2.1.5.2 The HEK293 Cell Line and Its Derivatives

One frequently used recombinant protein production cell line is HEK293. This cell line has been generated upon transfection of a mixture of sheared DNA obtained from adenovirus Ad5 into human embryonic cells derived from kidney [99]. In depth characterization revealed that HEK293 cells contain a stably integrated single copy of the left part of the adenoviral genome (nucleotides 1 to 4,344) of Ad5 [100]. This region includes the adenoviral E1A and E1B genes controlled by the viral promoters. Later, it was found that HEK293 cells unexpectedly display a number of neuronal markers suggesting that the immortalized parental cell was of embryonic neuronal origin rather than representing a classical kidney epithelial cell [101]. Indeed, experimental evidence was given that adenoviral Ad5- and also Ad12-based immortalization protocols are more efficient on human embryonic neurons rather than kidney cells [101, 102]. This is probably due to a certain resistance of kidney epithelial cells to immortalization and/or a preference of adenoviral E1A/E1B immortalization for neuronal cells. This example shows that even low numbers of 'contaminating' cells in a cell preparation can give rise to a preferential immortalization while the cell type of interest may not be targeted.

Due to the fact that HEK293 cells express E1A and E1B proteins this cell line has been broadly used as a helper cell line for E1A/E1B-deleted recombinant adenoviral vectors (for review see [103, 104]). Upon transfer of recombinant adenoviral vectors to HEK293 cells, the E1A/E1B function is provided *in trans* and complements the formation of replication-defective viral particles. Further, upon transfection of EBNA1 or SV40 T antigen encoding expression cassettes, derivatives of 293 cells were generated so-called 293E and 293T cells, respectively [105]. These cells support the amplification

of episomal plasmids that harbor the respective origin of replication [106]. Moreover, 293T and also 293E cells have been shown to provide high transfection rates. This property makes the cell lines as an efficient tool for various applications and in particular for small and large scale transient expression of proteins. This is also reflected by the fact that a number of pharmaceuticals are produced with this cell type [107]. Moreover, 293 cells and also the SV40 T antigen expressing 293T cells derived thereof are routinely used for the production of recombinant γ-retroviral and lentiviral vectors (reviewed in [108, 109]). Finally, these cells serve as production systems for various viruses such as influenza virus [110] and vaccinia virus [111].

2.1.5.3 The PerC.6 Cell Line

Learned from the lesson of HEK293 cells, human embryonic retinoblasts were used to establish the PerC.6 cell line [112]. For this purpose, the adenoviral genes E1A and E1B (nucleotides 459 to 3,510) controlled by the PGK promoter or the endogenous promoter, respectively, were transferred. In contrast to the HEK293 cell line the employed adenoviral region of the PerC.6 cell line excludes viral sequences that are homologous to E1A/E1B-deleted, recombinant adenoviral vectors. Accordingly, the risk for homologous recombination towards replication competent adenoviruses is significantly reduced in PerC.6 cells. The construction of this cell line was done under well-documented good manufactory practice (GMP) conditions which facilitated its use for pharmaceutical production. Therefore, besides its use for recombinant adenovirus and adeno-associated virus (AAV) production, this cell line proved to be efficient for production of vaccines including influenza (reviewed in [113, 114]), poliovirus [115] and also for protein production [107].

2.1.5.4 Recent Developments

Recent developments of cell lines for biotechnological purposes include the brain-derived cell line AGE1.HN which was generated upon transfer of an expression cassette encoding the E1A and E1B genes driven by the PGK promoter and the E1B-dependent viral promoter, respectively. In addition to these immortalizing genes the adenoviral gene pIX was introduced into this cell line, which alters the cell metabolism and enhances productivity for secreted proteins [116]. The AGE1.HN cell line has been shown to display neuronal but not glial cell markers and was already applied for protein production [117].

Similarly, duck cells were immortalized using the E1A and E1B adenoviral genes with or without pIX (AGE1.CR.pIX and AGE1.CR, respectively). These cell lines have been shown to be suitable for propagation of influenza and vaccinia virus [118, 119].

Another approach was based on primary cells isolated from amnion fluid. Upon immortalization with the abovementioned adenoviral genes E1A/E1B and pIX a cell line was established designated as CAP [120, 121]. Although the specific cell type that

is immortalized by this procedure has not yet been defined, these cells have been shown to give rise to high levels of protein [121, 122] as well as influenza virus [123].

2.1.5.5 Cell Lines Used in the Drug Development Process

In the drug development process cell lines are used in high throughput screenings as tools to identify drug candidates. In this early developmental stage, considerable cell numbers ($> 1 \times 10^9$ cells) are required to perform automated screenings. Due to this fact robust cell systems are mandatory and therefore CHO and HEK293 cells are often used that overexpress the desired drug target [124, 125]. The identified hits are further characterized in more relevant cell systems, e.g., in cell lines displaying a special phenotype or even in primary cells. As this characterization is dependent on the drug target and/or the desired indication there is no golden rule which cell systems are used in this stage of the drug development process.

After narrowing down the number of potential drug candidates they are optimized with respect to efficacy and to pharmacokinetic properties like absorption, distribution, metabolism, excretion, and their toxicity profile (ADMET profiling). For some of these parameters robust cell based *in vitro* test systems have been established.

2.1.5.6 Caco-2 Cells

An important pharmacological property is absorption. Drugs are preferably applied through the oral route. Therefore they have to stand the acidic surrounding of the stomach but also have to be efficiently absorbed in the small intestine where they enter the blood stream and are distributed to the target location. Thus, if the absorption process is inefficient, the dose has to be increased. High drug concentrations are more likely to generate toxic side effects. Accordingly, the optimization of the absorption characteristics is a critical parameter of drug development. For this purpose an excellent *in vitro* model – the Caco-2 cell line – was established. This cell line was derived in the 1970s from a colorectal adenocarcinoma [126]. About ten years later it was recognized that this cell line was able to spontaneously differentiate into a cell type that is similar to small intestinal enterocytes [127]. Today, Caco-2 cells are widespreadly used in various labs working in disciplines like nutrition, pharmacology, and toxicology.

Among the relevant features of this cell line are the expression of specific small intestinal marker proteins and tight junction complexes. Importantly, also the Caco-2 cells form microvilli on the apical side (facing the gut lumen) when cultured on cell culture inserts (e.g., in transwell plates). For drug development purposes the most important property is that Caco-2 cells form a tight barrier. The formation of the barrier is induced when the cells are plated onto cell culture inserts and are maintained for 3 weeks [128]. During this time a tight barrier develops which limits passive transport through the cell layer. It was shown that for such passive transport processes the

Caco-2 cells show a permeability coefficient that is comparable to the *in vivo* situation [129].

Also active transporters play important roles in the absorption of drugs. The Caco-2 cells do express several intestinal transport systems albeit to a lesser extent than in the small intestine *in vivo*. This is probably the reason for the slower transport through the Caco-2 cells compared to the *in vivo* situation. Another difficulty is that the cellular properties change with continuous passaging. Currently, different subclones have arisen from the parental Caco-2 cells. Among the properties that change with continuous passaging are an increase in TEER (trans epithelial electrical resistance) and accordingly a decrease in permeability [130]. Further, the cells change in the cell proliferation rate, in the activity of metabolic enzymes (Cyp3A4), and the cells seem to lose the contact inhibition [131].

2.1.5.7 Keratinocyte Cell Lines (HaCaT, NIKS)

Although not frequently used in the drug development process keratinocyte cell lines play an important role in industrial research. The evaluation of keratinocyte cell lines as a surrogate for skin was promoted by an EU regulation (REACH) which requests that every chemical substance has to be tested for its toxicological profile. So far, this substance testing is mainly performed *in vivo*, which is costly and harbors ethical concerns. As an alternative cell based surrogates for skin already have been developed. *In vitro* three-dimensional skin equivalents can be established and are used for substance testing, e.g., in the cosmetic industry. So far, these skin equivalents are composed of primary keratinocytes and in some cases also other primary cell types like fibroblasts or macrophages [132].

Probably the most famous and still the most frequently used keratinocyte cell line is HaCaT. This cell line was established from adult skin of a donor who came down with melanoma. Cells were isolated from the distant periphery of the tumor and the cell line was established by spontaneous immortalization, which was probably facilitated through cultivation of the cells at elevated temperatures (38.5 °C), a temperature known to increase the proliferation capacity of primary keratinocytes. HaCaT cells are not tumorigenic upon transplantation into nude mice. Importantly, these cells are able to differentiate into stratified epithelia *in vitro*. This, however, happens at a slower rate than with normal primary human keratinocytes [133].

Recently, a novel spontaneously immortalized human keratinocyte cell line, NIKS, was described. NIKS cells are immortal and also nontumorigenic. Importantly, they form a fully stratified squamous epithelium *in vitro* when cultivated in organotypic cultures. NIKS cells were isolated from juvenile foreskin keratinocytes and cultivated for the cell line generation on MEF feeders. The resulting cell line is still fully responsive to growth factor treatment as, e.g., epidermal growth factor (EGF) leads to increased proliferation whereas TGFb1 treatment leads to inhibition of proliferation [134]. NIKS cells even differentiate to the same extent as their primary counter-

parts when cultivated in organotypic cultures together with dermal fibroblasts. In the meantime companies use this progenitor cell line to create skin equivalents, which can be used for substance testing but are also tested for regenerative purposes (e.g., treating severe burns).

2.1.5.8 Hepatocyte Cell Lines

For drug development hepatocytes are one of the key cell types. This is due the fact that the liver is the major site of drug metabolism. Hepatocytes are highly specialized cells with unique properties including the detoxification of metabolites. Thus, they are of pivotal interest as screening systems for drug development but also for routine toxicology studies.

Human hepatocellular carcinoma tissues represent a source for the establishment of immortal hepatic cell lines. Several cell lines have been established (Mz-Hep.1, KYN-2, BC2) which display some of the hepatic markers (for overview see [135, 136]). The most widely used hepatoma cell line is the human HepG2 cell line which was derived from a well differentiated hepatocellular carcinoma of a 15-year-old Caucasian male [137]. Interestingly, this cell line can synthesize many human plasma proteins including albumin and α-fetoprotein [137]. While albumin is expressed in adult hepatocytes, α-fetoprotein is a marker of the progenitor phenotype and is not expressed in mature hepatocytes. Although HepG2 cells show many liver-specific functions, they lack the functional expression of almost all relevant human cytochrome P450 family members [138]. This limits the use of HepG2 cells as an *in vitro* model for metabolism or toxicology studies. Certain protocols such as 1a,25-dihydroxyvitamin D3-based induction of Cyp3A4 [139] or the cultivation on a nanotechnology-based three-dimensional scaffold [140] provide evidence that this cell line can be used for drug metabolism studies although only a limited number of settings. In addition, the drug-metabolizing potential has to be characterized for each assay before the produced data can be interpreted [141].

Due to the obvious limitations of the HepG2 cell line there is still a big demand for novel hepatic cell lines which was at least partially solved with the establishment of the Huh-7 [142] and HepaRG cell line [143]. The Huh-7 and its subclone Huh7.5 are particularly suited as *in vitro* model systems for studying HCV biology whereas it is not suited for toxicology tests due to its limited hepatocyte functionality (for overview see [144]). The HepaRG cell line on the other hand retains an undifferentiated morphology under normal culture conditions but can be differentiated through modifying the culture conditions (cell density, DMSO addition). This differentiation process induces the typical hepatocyte morphology as well as the expression of various cytochrome P450s, phase II enzymes and drug transporters. Therefore the HepaRG cell line is currently considered as an alternative to primary hepatocytes for toxicology tests.

The obvious drawback associated with the generation of hepatic cell lines from hepatocarcinomas is the unpredictability. Therefore, regimens were developed to

standardize the immortalization process of hepatocytes. To this end it was shown that primary human hepatocytes can be immortalized by the transfer of immortalizing genes such as TAg, hTert, E6/E7, or as well the gene encoding the core protein of HCV. Several cell lines like the PH5CH [145], the Fa2N-4 [146], the HepLi5 [147], the HuS-E [148], or the NKNT-3 cell line [149] have been established. For these approaches either TAg alone (PH5CH, Fa2N-4, HepLi5), or hTert in combination with TAg (NKNT-3) or E6/E7 (HuS-E) were employed. However, all these cell lines still show significant differences when compared to primary hepatocytes, which limit their use as predictive models [150, 151].

Another approach was used to establish the cBAL111 cell line. In this case, human fetal liver cells were immortalized by recombinant expression of hTert [152]. Importantly, this cell line has the potential to differentiate to a more mature phenotype so that these cells show a hepatic phenotype which includes albumin expression and urea synthesis. However, also in the case of this cell line, the levels are not comparable to primary mature hepatocytes (overview see [152]).

2.1.6 Outlook

In the last decades, mammalian cell lines gained increasing relevance for biotechnology. This concerns their role as potent production systems for a plethora of biologicals. In addition, recent advances in the development of immortalization protocols allowed the generation of highly specialized cell lines. These cell lines can already partly substitute primary cells for applications that rely on complex properties of specialized cell types. It can be envisioned that the refinement of the protocols, together with the increasing knowledge in the signaling pathways relevant for specialized cells, will allow to further improve the methods for expansion of cells with complex phenotypes.

Functional complementation of immortalized cell lines could be a future option. Based on synthetic biology approaches, relevant properties could be restored. Missing or silenced regulatory cascades could be reactivated or reassembled by implementing genetic circuits, respectively. This might concern the implementation of specific modification patterns (such as genes for distinct glycosylation profiles) but also more complex features such as the detoxification properties of hepatocytes.

Finally, reliable and reproducible methods for immortalizing patient-derived cells might pave the way for their application in regenerative medicine. Certainly, cell lines that reflect the various genetic settings found in the human population will also have a high potential for the establishment of novel *in vitro* screening/test systems.

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2.2 CHO History, CHO Evolution and CHO Genomics – an Unsolvable Enigma?

Florian M. Wurm

2.2.1 Introduction

Immortalized Chinese hamster ovary (CHO) cells are the source of multiton quantities of protein pharmaceuticals. Such cells are characterized by diversity in genetics and phenotypes. Preexisting diversities in cell populations are further enhanced by selective forces when such populations become separated from each other so that the sharing and mixing of a given gene pool (of a species) is prevented (Darwin). In nature, such spreading of groups of individuals without gene pool sharing results in speciation. The distribution of CHO cell lines to hundreds of laboratories that grow these cells under (very diverse, but practical) conditions represents a strong case of such evolutionary selections.

CHO cells have been used in cell culture for research and for industrial applications for more than 50 years and a bewildering number of differently named cell lines can be found in the literature. This chapter tries to represent, in a cursory and not necessarily comprehensive way, the history of CHO cells, particularly the origin and subsequent fate of key industrially used cell lines. It is, in the opinion of the author, not a radical proposal to suggest that the name CHO represents many truly different cell "species", based on their inherent genetic diversity and their highly dynamic rate of genetic change. The remodeling of the many genomic structures in clonal or nonclonal cell populations, further enhanced by the nonstandardized culture conditions in hundreds of different labs, renders CHO cells a typical case for "groups of quasispecies". This term was coined in the 1970s for families of related (genomic) sequences exposed to high mutation rate environments where a large fraction of offspring is expected to carry one or more mutations. The implications of the quasispecies concept for CHO cells used in protein manufacturing processes are significant. CHO genomics/transcriptomics will provide only limited insights when analysis is performed on a small number of "old" and poorly (historically and otherwise) characterized CHO cell lines. Strictly speaking, any individual cell in a culture appears to carry a peculiar genomic structure. Only screening of many clonal cell lines derived directly and under controlled culture conditions from one relatively well-defined starting material may reveal a narrow diversity of phenotypes with respect to physiological/metabolic activities, and thus, that allows more precise and reliable predictions of the potential of a clone for high-yielding manufacturing processes.

The dominance of Chinese hamster ovary (CHO) cells for manufacture of complex therapeutic proteins is based on a number of characteristics of these cells that had been recognized in the early 1980s by groups of researchers, mostly in early biotech-

nology start-ups. At Genentech Inc. in South San Francisco, these cells were grown at a very large scale in suspension cultures, resulting in the first recombinant protein from animal cells in bioreactors approved for human therapy, in 1987. Subsequent research and development work in process, science with these cells continued since then to further improve both, protein quality and quantity from CHO cultures in bioreactors. Proteins like etanercept (Enbrel®, a TNF inhibitor for rheumatoid arthritis treatment) and trastuzumab (Herceptin®, an anti-HER-2 breast cancer antibody) are each produced at more than 1 t per year and tens of thousands of patients benefit from these protein drugs. In spite of these very impressive achievements in both, clinical success and manufacturing from cells in large bioreactors, recent publications have expressed hope that a detailed knowledge of DNA sequences and transcription patterns of CHO cell lines could provide urgently needed tools to improve the manufacture of protein pharmaceuticals [1]. However, in the opinion of the author, this research does not take sufficiently into account a profoundly adverse problem with CHO cells that could very much limit "omics" approaches and/or deliver entirely irrelevant data.

CHO cells – whether being referred to as K1, DG44, DX B11, CHO-Toronto, CHOpro3-, or CHO-S – are members of a widely distributed family of related, but profoundly different cell lines, since their individual behaviors/phenotypes (responsiveness to different environmental conditions) in cell culture differ quite significantly. CHO cells are immortalized cells, and the extent of their relatedness in genome structure, genomic sequence composition, and in transcription patterns has not been studied so far. Thus, reasonable conclusions on their similarities are therefore not available. However, in view of the published information on CHO cells and taking into consideration generally available information on fluidity of genomes of immortalized cell populations, it is proposed that each one of the above-mentioned CHO cell lines could be considered a "quasispecies". This term was first coined by Eigen and Schuster in the late 1970s in a series of landmark papers describing a high mutation rate environment where a group or "cloud" of related offspring exists and where one would expect that a large fraction of the offspring carries at least one mutation [2-4]. Since then, the term has been extensively discussed and applied in virology (reviewed in [5]) and, most recently, in the population dynamics of cancer cells [6].

Understanding and appreciating the above reasoning requires a "complete" history of CHO cells prior to their use in modern biotechnology. Fortunately, most of the history of the cells has been recorded in an enormous wealth of publications resulting from research executed with CHO cells from the 1960s to the 1980s. In addition, approximately at the time of the emergence of interest for CHO cells in the biotechnology industry, in 1985 Gottesman [7] edited a 900-page compendium entitled *Molecular Cell Genetics* that contains CHO main work exclusively dedicated to the Chinese hamster and the cells derived from this species. (Unfortunately, this compendium is out of print today but was available to the author for this chapter.)

2.2.2 CHO Cells in Metabolic and Genetic Studies

Today, few users of CHO cells know that they were extremely popular for fundamental research in molecular and classical cell genetics for a 30-year period prior to their use in pharmaceutical biotechnology. Their popularity was based on their practicality in cell culture, their few (11 pairs) and large chromosomes (in comparison to human ones), and the ease with which metabolic and other mutations could be generated or found and subsequently studied in elegant experimental approaches. During this period, the identification of the genes for the encoded enzymes and their expression profiles was a "hot topic". CHO cells became the preferred study object for gene and genome-based research in a mammalian host system. Induced and spontaneous mutations lead with a high success rate to physiological deficiencies (metabolic mutants) that could easily be identified through culture in selective media. Mammalian cell culture technology had matured at the time to an extent that media with partially defined or even serum-free compositions could be generated with ease [8-12]. These procedures permitted the application of concepts of microbial genetics to mammalian cells.

2.2.3 The Early History of CHO Cells

In 1957 CHO cells were established in the laboratory of Puck [13], then at the Eleanor Roosevelt Institute for Cancer Research and later at the Department of Biochemistry of the University of Colorado in Denver, from 0.1 g of ovary tissue from a Chinese hamster. The outbred hamster was provided by Yerganian from the Boston Children's Cancer Research Foundation [14]. It is important to note that out-breeding tries to avoid homozygosity, i.e., reducing the number of identical alleles in the genome, and thus maintains a typically vigorous and diverse genetic background of diploid animals or plants. The issue of allelic diversity in diploid systems will be discussed later again, since it appears that loss of heterozygosity (LOH) is a genetic trend for genomes of immortalized cells: they frequently loose entire chromosomes or fragments of chromosomes and thus increase the degree in homozygosity over time.

From the trypsinized ovary tissue a culture emerged that appeared to be predominantly of a fibroblast type and had a near diploid karyotype with only 1 % of the cell population differing in chromosome number by one less or more from the expected chromosome number of 22 (11 pairs) [13]. However, this small diversion from the strictly diploid character of primary cells is not observed in primary cells of human origin where a fully diploid karyotype is prevalent until senescence occurs ("Hayflick limit") after about 50 population doublings and the cells die [15].

The fibroblast morphology of the primary ovary cells was maintained in culture for more than 10 months, longer than the time limit being established for human fetal cells by Hayflick. At an unknown time thereafter, the morphology of some cells

changed, and these cells overgrew the fibroblast cell type. It appears that cells in culture derived from the hamster ovary were liberated from the typical constraint in cell population doublings (i.e., they may have already experienced some type of spontaneous immortalization) while remaining close to a diploid character. However, the mentioning of additional morphological changes after 10 months in culture points towards additional modifications, most likely with a genetic cause whose origin is (still) not understood today. Subsequent recloning of these cells with a modified morphology resulted in the cell line that is now called CHO. The change of morphology from a fibroblast type of culture to a more epitheloid morphology of cells is mentioned in Puck [16, 17]. Unfortunately, no information on the specifics of the cloning step or the potential diversity of CHO cell lines is available. For clarity in this text, the author refers to these original CHO cells as CHO-ori (see Figure 2.2.1).

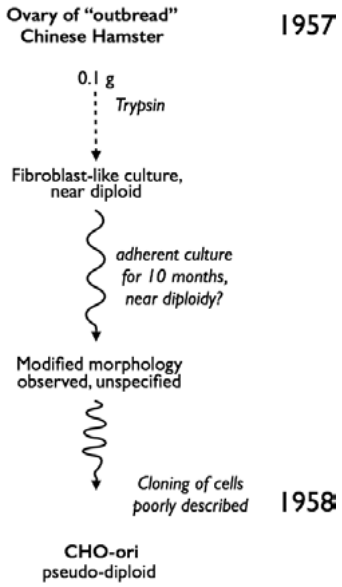

Figure 2.2.1: A scheme representing the generation of the early "original" CHO cell line.

The scheme in Figure 2.2.1 depicts the little knowledge (as available at a time distance of more than 45 years) about the origin, history, and early handling of CHO cells. Obviously, under today's standards of scrutiny in our industry, one can only say that this description is unsatisfying. It is highly unlikely that "cloning" was done at the time in Puck's laboratory with the attention to details we apply today in industry to this step. In retrospect, several pertinent questions arise, unfortunately without a chance to obtain answers. Particularly striking are issues around the immortalization of these cells, their assumed genomic constitution, and their apparent phenotypic change in culture.

Subcultures of these CHO-ori cells, now a CHO cell line (to be distinguished from cell strains with a limited life span, according to Hayflick's terminology), were passed on to many laboratories. They were described as "hardy", growing very well, and fast in adherent culture. They had a high cloning efficiency (always done in an adherent mode in dishes or flasks) even at very low fetal bovine serum concentrations in the culture medium. 10 to 20 % fetal bovine serum (FBS) in commercial media was standard in cell culture at the time. However, Hamilton and Ham [11] already reported in 1977 the growth of these cells in serum-free media. The cells required the addition of proline to the culture medium, and, to the author's knowledge, today all CHO cell lines require it. The first reference to this is from 1963 [16]. Thus, it appears that loss of competence for proline synthesis is an early event in the history of these cells.

The following text does not follow the actual history of passing cells from one laboratory to another, but gives names and pedigree according to the importance for the emergence of the biopharmaceutical industries that uses CHO cell lines today.

2.2.3.1 CHO-DXB11

The very first product made by CHO cells, and thus the starting point of the biotechnology era involving recombinant mammalian cells, employed a cell line called CHO-DXB11. This line was generated at Columbia University by Urlaub and Chasin, who were interested in dihydrofolate reductase (DHFR) [17]. The cells in Chasin's lab were derived from CHO-K1 after a coworker in Puck's lab (Kao) had "cloned" the CHO-ori cells. According to Chasin (personal communication), the CHO-K1 subclone was established in the late 1960s, perhaps almost 10 years after CHO-ori cells had emerged and passed on to others as an immortalized cell line. Similar to CHO-ori cells, CHO-K1 cells were also supplied to many laboratories around the world and experiments with these cells were described in numerous publications. Vials of frozen CHO-K1 cells were also deposited at the American Type Culture Collection (ATCC). The history of these deposited cells was not available to the author at the time of writing this chapter.

Chasin established the cell line CHO-DXB11 (also called DUK-XB11, see Figure 2.2.2). The purpose of the work was to delete DHFR activity. These cells carry a deletion of one locus for DHFR and a missense mutation (T137R) of the second DFHR locus, rendering the cells incapable of reducing folate, a precursor for thymidine and hypoxanthine synthesis [17]. The cell line is not named in the mentioned paper, but it is one of the gamma-ray-induced mutants described. It is interesting to note that this cell line, the first to become a host system for the production of hundreds of kilograms of human tissue plasminogen activator (tPA), was the product of (severe) mutagenesis. The reasons why this and not any other CHO cell line became the pioneering cell line in biotechnology is rapidly explained: the dual inactivation of the DHFR locus rendered this cell line very useful for transgenesis with a functional DHFR gene [18]. Transfer of a functional DHFR gene via plasmid transfection could repair the DHFR deficiency and allow easy selection of recombinant cells in well-defined media. In

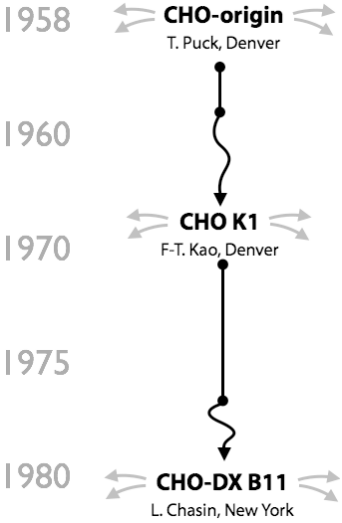

Figure 2.2.2: The origin of CHO-DX B11 cells. A straight line indicates transfers of cells without intentional modification of the cell line. A line with multiple curves indicates intended experimental steps that are expected to change the genotype/phenotype of the cell population. The grey arrows next to the name of the cells indicate the fact that many cells (as frozen vials or as active cultures) were distributed to many labs over extended periods of time. No information on such transfers is available today.

addition, a second, unrelated gene of interest (GOI), encoded by the same plasmid vector, could easily be transferred simultaneously and recombinant clones expressing both the functional transgenic DHFR gene and the desired cotransferred GOI could be recovered [19]. This 1983 publication is the first to describe cotransfer of two genes into CHO cells whereby the two corresponding DNA sequences were provided on two separate plasmids. They were simply cotransfected at different ratios. In the case of the paper quoted, a 1:10 ratio of the DHFR plasmid to the gamma-interferon plasmid gave the highest yielding clones.

The DHFR-negative cells were grown in media containing 5 to 10 % fetal bovine serum (FBS). These cells were then transfected, using calcium phosphate as a reagent, and recombinant clones were identified after exposure of the transfected population in media lacking glycine, hypoxanthine, and thymidine (GHT-minus medium and dialyzed fetal bovine serum). A risk factor mediated by the use of sera from cows, bovine spongiformous encephalopathy (BSE), became an important consideration for pharmaceutical manufacturing in the 1990s. In industry, sera were generally obtained from BSE-free sources (Australia, New Zealand). Whether this practice was followed in academic labs is difficult to assess. Transfection and cloning occurred in an adherent mode, whereby cloning was done by using “cloning rings” or cotton-swabs. It must be assumed that these techniques were also used during the early phases of the CHO history. In all cases, an identified colony, visible to the naked eye, was targeted and many cells from such a colony were transferred into a well of a multiwell plate. Regulatory concerns requires today “single cell cloning”, frequently done even twice in order to “prove” clonality. This is the most stringent “population bottleneck” one can imagine – an issue which has important consequences on the genomic constitution(s) of the emerging clonal cell population.

2.2.3.2 CHO-DG44

For the researchers of metabolic studies with these cells, a low rate of reversion to DHFR activity in CHO-DXB11 cells presented a problem. In order to fully eliminate this possibility and to provide also a better DHFR-negative host system for eventual gene transfer, Chasin engaged in another round of DHFR elimination, but not with cells derived from the K1 populations. Figure 2.2.3, established to the best knowledge available to the author, tries to establish the history of CHO-DG44 and other cell lines of relevance to the industry.

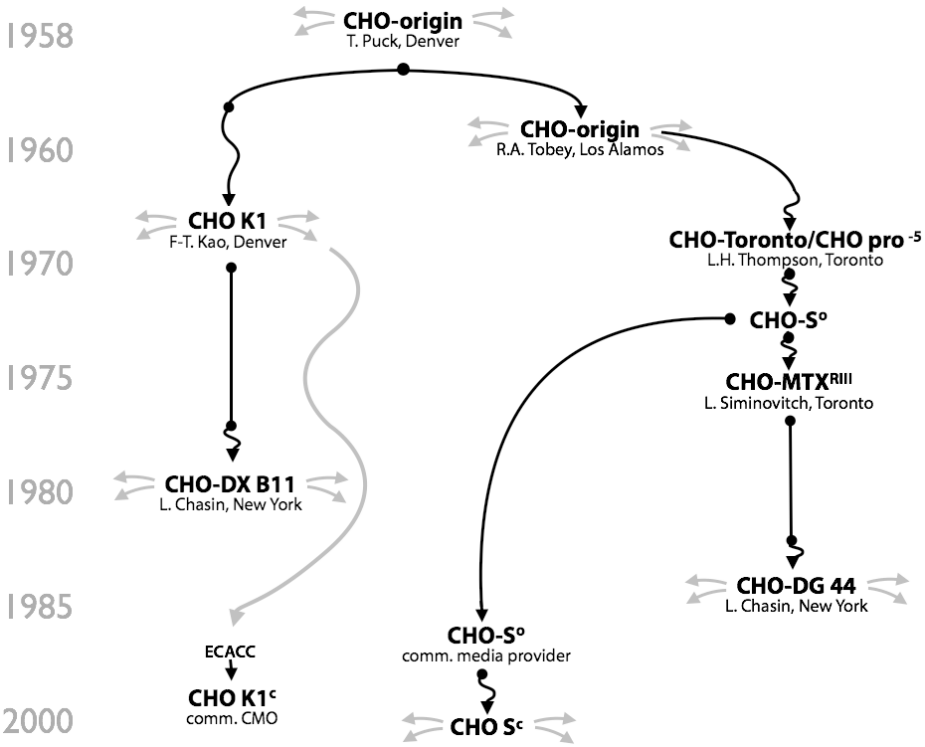

Figure 2.2.3: Origin of CHO-DX B11, CHO-DG 44 cells, commercial CHO S^c cells and commercial CHO $K1^c$ cells. A short multicurved line (black) indicates literature describing experimental steps that are expected to change the genotype/phenotype of the cell population. Year indications are best estimates. Small grey arrows next to a given cell name indicate multiple distributions of cells to different labs and companies. The grey, multicurved line indicates uncertainties or undescribed fates of the CHO $K1^c$ cells.

Flintoff, a coworker of Siminovitch, had generated a useful mutant, named CHO-Mtx^{RIII} derived initially from CHO-ori cells, but other cell lines mentioned in the literature (such as CHO pro5- and CHO-S^o) may have been part of its history as well. This

gene-amplified mutant proved to be suitable for deletion of both DHFR alleles [20]. In the same year (1976), Siminovitch published a highly instructive minireview on genetic diversity of cultured somatic (immortalized) cells, based on the many years of insights gained mostly with CHO cells [21].

Chasin and Urlaub used the CHO Mtx^{RIII} cell line to delete the amplified DHFR containing regions of the CHO chromosomes. Their elegant work showing the full deletion of the two DHFR loci on chromosome 2 (actually on chromosome 2 and on a shortened marker chromosome variant Z2) resulted in the now widely-used CHO-DG44 cells, obtained after two "traditional" cloning steps [22, 23, personal information from Chasin].

2.2.3.3 Gene Amplification

The availability of these two DHFR-negative cell lines (CHO-DXB11 and CHO-DG44) allowed an approach to amplify genes with the help of a chemical antagonist of DHFR, methotrexate (MTX). The isolation of "amplified" recombinant cell lines occurred using stepwise increases in the MTX concentration in the culture medium over several subcultivations. Such induced gene amplification usually increases the productivity of cells containing the GOI [24-26]. It is based on selections of mutated cells that have amplified the DHFR containing sections of the genome of the cells, which occurs at the level of large chromosomal sections and thus coamplifies any other DNA as well, in the case of cotransfected DNA the plasmid sequences that contain the GOI. This approach was a key approach for enhancing protein production in clonal subpopulations of transfected CHO cell lines over a 20-year period in the biotechnology industry. During this period, most of the recombinant protein products were derived from CHO cells that had undergone MTX-induced gene amplification. Gene amplification results in large genomic reorganizations, visible to the eye when karyotyping cells [27, 28].

Briefly, new chromosomal structures, known as homogeneously staining regions (HSRs), can be found in metaphases of MTX selected human (cancer) derived cells, as well as in CHO cells. These regions show multiple (up to thousands) repetitions of smaller chromosomal regions (amplicons), all containing DNA containing, at least in part, DHFR genes. In fluorescence *in situ* hybridizations on recombinant CHO cells, large chromosomes were found that contained entire arms and long segments within chromosomal arms, hybridizing with DHFR sequences. Copy-number analysis of such cell lines revealed hundreds and thousands of copies of DHFR in these cells [26-28]. Mechanisms of gene amplification have been studied both in human (cancer) cells and human cell lines [27, 30], as well as in hamster [31, 32] and in mouse cell lines [33, 34], and several mechanisms have been proposed including principles that go back to "fragile chromosomes" and gene transposition as proposed by the work of McClintock in *Zea mays* [35]. The genetic stability of these unusual chromosome structures within a given cell population is poorly understood [29].

2.2.3.4 CHO-K1

In Gottesman [7] we find the following statement: "One subline of the original isolate, called CHO-K1 (ATCC CCL 61) was maintained in Denver by Puck and Kao, whereas another subline was sent to Tobey at Los Alamos. This latter line was adapted to suspension growth by Thompson at the University of Toronto (CHO-S) in 1971 and has given rise to a number of Toronto subclones with similar properties including the line CHO Pro^{-5} used extensively by Siminovitch and numerous colleagues in Toronto, CHO GAT of McBurney and Whitmore, subline 10001 of Gottesman at the NIH, and subline AA8 of Thompson. There are some differences in the karyotypes of the CHO-K1 and CHO-S cell lines, and CHO-S grows well in spinner and suspension culture, whereas CHO-K 1 does not. Both sublines seem to give rise readily to mutant phenotypes." This shows the handling of CHO cells by many laboratories, their diversity in phenotypes (one grows the other not in suspension), and the reason for their popularity at the time. The line "give rise readily to mutant phenotypes" would have to be avoided when presenting cell hosts to regulatory agencies. However, today's popularity of CHO-K1 cells is based on the successful and reliable use of these cells by a well-known contract manufacturing company for the production of high-value pharmaceuticals. Licenses for these cells, now suspension-adapted, in connection with a unique gene transfer system based on the enzyme glutamine synthetase (GS-system), have been taken by a number of their client companies. The GS system was originally designed for NS0 cells (a murine myeloma-derived cell line also used for the fusion with B cells in the generation of hybridomas) [36] and was quickly applied to CHO cells as well. The origin of the CHO-K1 cells in the hands of the above mentioned contract manufacturer goes back to a vial of frozen cells derived in November 1989 from the European Collection of Animal Cell Cultures (ECACC). How and when these cells arrived at ECACC is not known to the author. A serum-free, suspension culture was frozen in the year 2000 (11 years later) as a "development bank". Eventually, a subline was generated that gave rise in October 2002 to a "CHO K1 SV" master cell bank under "protein-free" conditions (Metcalfe, personal communication). Worldwide, at the time of writing of this chapter, there are five licensed pharmaceutical products that were made with the help of the GS system in combination with CHO-K1 cells.

Briefly, recombinant CHO-K1 cells can be obtained after cotransfection with a functional glutamine synthetase gene together with the GOI on the same plasmid followed by selection in the absence of glutamine in the medium. In addition, the application of a GS inhibitor (methionine sulphoximine, MSX) allows either an increase of the stringency of selection or the selection for subpopulations of cells with an amplified copy number of the GS gene and the GOI. One has to assume that the principles of gene amplification with the GS system are similar to the ones discussed above for the DHFR system. Unfortunately, no publications with respect to karyotypic characterization of GS/MSX amplified sequences in CHO cells have been published so far.

2.2.3.5 CHO-S

About 50 years ago it was recognized that some (variants of) CHO cell lines have the capacity to grow in singlecell suspension culture [37]. In 1973, Thompson and coworkers described suspension cultures of CHO cells [38] and CHO-S cells were mentioned (see quote from Gottesman above). Thompson's CHO-S cells (in Figure 2.2.3: CHO-S^o) were derived from the CHO-Toronto cell line, also referred to as CHO pro^{-5}. Unfortunately, there is confusion about the origin and lack of due credit for CHO-S cells. It appears that the CHO-S cells from Thompson's lab, possibly through Tobey, were provided in the late 1980s to a cell culture media and cell host providing company. The company obtained a vial of CHO cells from Tobey at the Los Alamos National Laboratory. These cells were further adapted to a specific medium provided by that company marketed since 2002 as CHO-S. Here, the author will call the CHO-S from Thompson's lab CHO-S^o (o = original) and CHO-S from that company CHO-S^c (c = commercial). In view of their culture history it can be assured that these two cell lines will differ with respect to optimal culture conditions and other phenotypic/genetic features.

Neither of these CHO-S cell lines was used at Genentech in the mid 1980s for culture in singlecell suspension. Instead, recombinant clonal subpopulations derived from CHO-DUXB11, first established in adherent cultures with FBS in the medium, were individually adapted to suspension. The suspension adapted, serum-free subpopulations were not recloned prior to generation of master cell banks (personal information provided by the author). This appears surprising but is scientifically defendable, since "stability" and "identity" of a recombinant cell population has a higher chance of being maintained when cloning is avoided (see also the discussion on stability and microevolution below). The approach to suspension-adapted clonally derived cell lines, grown prior and during cloning in serum-containing media, without another recloning step was the basis for the first large-scale (10,000 L) stirred-tank bioreactor-based culture of CHO cells for the production of human recombinant tPA, and it was also used for other products developed by Genentech in the 1990s.

2.2.4 Diversity of Culture Conditions and the Cytogenetics of CHO Cells

CHO cells have been maintained by hundreds of different laboratories under highly diverse conditions. Therefore, the fluidity of genomic structures in immortalized cells will have to be considered. Decades of research into culture of immortalized cells have taught one important lesson: any culture of clonal or nonclonal cell lines will have a dramatic and lasting effect on the diversity of genotypes exhibited by the cell population. Insights into the persistent and continuing fluidity of genomes of immortalized cells go back to the 1960s. In 1961, Hsu, a highly recognized leader in the cytogenetics of mammalian cells, published a landmark paper entitled "Chromosomal Evolution in Cell Populations" [39] that summarized more than a decade of work after the visual analysis of chromosome structures, and their identification had become a standard

technique. Chromosomes could be counted and identified and thus provided a suitable means to begin to understand the genomic organization of plants and animals. However, in contrast to the clearly recognizable and stable (in structure) chromosomes of diploid animals and plants, chromosomes of animal-derived immortalized cells showed a strong tendency to be nonidentical in number (from cell to cell) and apparently were able to change their organizational structures. Chromosomes of cell lines were not stable, in contrast to the overall unchanged structures and numbers of chromosomes found in wild-type biological species. The extremely rapid genome modifying impact of immortalization is strikingly visible in the unique and highly unusual chromosomal structures of such cells. For CHO cell lines, nothing different was seen. An example of this is given in Figure 2.2.4, which shows the diploid chromosomes of the Chinese hamster in comparison to those of an ancestral CHO-K1 cell line, based on the karyotyping work of Deaven and Peterson [40].

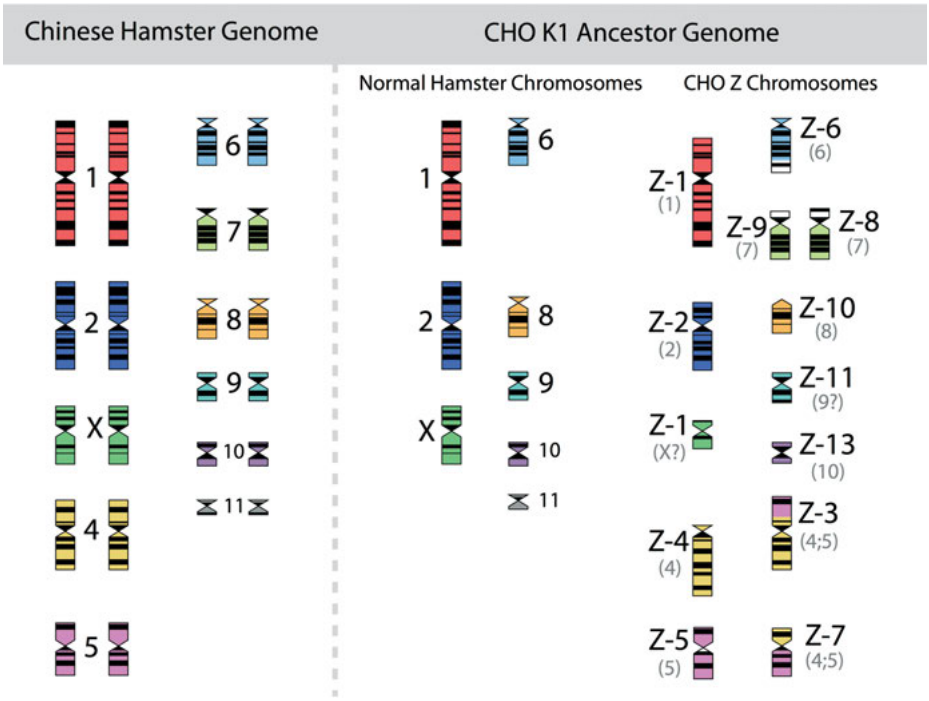

Figure 2.2.4: The 22 chromosomes of the Chinese hamster and the 21 chromosomes of CHO-K1 as identified by G-banding techniques (modified from [40]). A part of this figure was first published in [58] (with permission by Nature Publishing Group).

More recently, Omasa and his group constructed genomic BAC (bacterial artificial chromosome) libraries of available CHO-K1 and CHO-DG44 cell lines in order to

establish a map of the hamster chromosomes as fragments of them are distributed in the chromosomes of those cell lines. The BAC-based maps solidify the earlier made karyotyping based findings by Deaven and Peterson: dramatic rearrangement of chromosomal fragments as compared to the diploid (hamster) genome in both cases. Also, only few structures appear "stable" when comparing DG44 and K1 cells [41]. All immortalized cells present similar restructuring of their genomic DNA. What is not shown is the fact that the genomic structure of CHO-K1 shown in Figure 2.2.4 is only one of many different genomic organizations present in a population of CHO-K1 cells. Other cells may show certain similarities to this pattern, but they will rarely (or maybe never) exhibit one that is identical when studying 50 karyotypes of individual cells. Deaven and Peterson observed a distribution of chromosome numbers per cell, ranging from 19 to 23. 60 to 70 % of the cells had 21 chromosomes. Although CHO-K1 cells do not have the 11 pairs of chromosomes of the hamster genome, the majority of the chromosome structures of the hamster genome are present albeit rearranged with only a few elements (G-banding pattern fragments) not clearly accounted for. Much more recently (2006), in the PhD work by Hazelwood [42] done under the guidance of Dickson (University of Manchester), a similarly complex genomic situation of K1 cells as they are/were used by the above mentioned contract manufacturer was revealed: a CHO-K1 cell line showed metaphase spreads over a broad chromosome number range with 16 to 30 chromosomes (100 cells studied), with 18, 23 and 18 % of cells showing 19, 20 or 21 chromosomes, respectively. A CHO-K1 SV cell line, grown under protein-free conditions (mentioned earlier), showed also a very broad chromosome number distribution of 10 to 30 chromosomes. In this instance, 10, 13, 17, 7 and 12 % (total 59 %) of cells showed 16, 17, 18, 19 or 20 chromosomes, respectively. Studies with clonal subpopulations of these cells revealed similar complexities of the karyotypes – none of them matching even approximately the statistics shown for the "parental" cells [42]. The American Type Culture Collection describes the CHO-K1 cell line's genotype as "chromosome frequency distribution 50 cells: 2n = 22. Stemline number is hypodiploid." Thus, the name "CHO-K1" refers to very different cell populations, most clearly represented by their karyotype.

This is not surprising: in cultivated, immortalized animal cells, singlecell cloning, with and without prior gene transfer, but also just modification of cell culture conditions for a given cell population, leads to new and genetically diverse cells as pointed out by Hsu [39]. Each of these populations of cells represent a new quasispecies family in the terminology of Eigen and Schuster [43-45]. If cloning is performed, as is now essential for a manufacturing cell lines, we will never know the genomic composition of **the one** cell that gives rise to the resulting population of cells. For example, we do not even know whether cloning efficiencies of cells with 19 or 20 or 21 chromosomes are different (however, it is not unreasonable to assume that they are different). Whenever we have a chance to do karyotype analysis on a clonal population, we find diversity in chromosome structures. Clonal cell populations analyzed posttransfection and subjected to stringent selective forces show a bewildering genomic restruc-

turing, as judged by simple karyotyping or chromosome counting. Each clonal population analyzed is different. The modal chromosome numbers vary and individual, recognizable chromosomes show rearrangements [46].

Two recent papers on the genomic landscapes of HeLa cells [47, 48], the immortalized cell line [49] that was the foundation of animal cell culture technologies used today, shed a revealing light on the dynamics of genome remodeling under continuous cultivation. A remarkably high level of aneuploidy and numerous large structural variants were found at unprecedented resolution. A fifth of the HeLa cell line genome showed "loss of heterzygosity" (47). The original genome of Henrietta Lacks, the unfortunate woman who developed cervical cancer and whose cells were the source of the many HeLa cell lines being studied over the last 50 years, would be considered near 100 % heterozygote and thus would show significant sequence variations between the allelic DNAs representing the two sets of the 23 chromosomes. In one of the two genome papers on HeLa cells the average chromosome number of these cells is given as 64 and many segments of chromosome have a ploidy status ranging from triploid to octoploid. For example, one large homozygote fragment of chromosome 5 with a size of about 40 million bp is apparently present with 8 copies in HeLa cells. Another fragment, about 90 million bp, essentially the entire q-arm of chromosome 3 is present as 3 copies. These karyotype features result obviously from losses of fragments or arms of chromosomes while the corresponding allelic fragments are duplicated or multiplied. In [47] it is stated: "The extensive genomic rearrangements are indicative of catastrophic chromosome shattering". Up to 2,000 genes in HeLa cells are expressed at higher ranges than those seen in human tissues. More than 700 large deletions and almost 15,000 small deletions (as compared to the human genome) were detected. Most interesting, in view of the major chromosomal rearrangements in CHO, are the results of multiplex fluorescent *in situ* hybridizations (MFISH), a chromosome-painting method. Unfortunately, only 12 metaphase spreads were analyzed in this way. As with CHO, these 12 metaphase spreads show common structural rearrangements of the karyotype but also a number of "single cell events". The latter show unique translocations of chromosomal fragments, not seen in any of the other cells, indicative of a continuing dynamic of restructuring of the HeLa genome. Since HeLa cells usually do not undergo single cell cloning as CHO cells do, the fate of cells with unique rearrangements is difficult to predict. They may be passed on as a constant and small part of the entire population if they do not negatively affect the duplication of a cell. Unfortunately, the term "unique" and "single cell observations" are not to be taken at face value. An analysis of the karyotype of 12 cells does not provide a sufficient basis to make conclusions about populations of hundreds of millions of dividing cells. In the publication by Adey et al. [48] sequence analysis of two widely used HeLa cell lines (CCL-2 and S3, the latter being cloned from the former four years after the establishment of CCL-2) and 8 additional lines were performed. The cell lines were referred to as hypertriploid (without an assessment of chromosome number). Gains and losses of entire chromosome arms were observed, but also more frequent ampli-

fications and deletions. Evidence of "chromothripsis" (chromosome shattering) is provided in both papers [47, 48], indicating an early event, potentially inherited from the original cancer. This phenomenon, poorly understood, results from a fragmentation of one or more chromosomes that subsequently "heal" or become repaired as a new and differently organized chromosome structure. The analysis of these two landmark papers [47, 48] on the genomics of the widely used HeLa cells requires an extensive knowledge of the complexities of sequence analysis techniques and of the likely biology (population dynamics of replicating units, bottlenecks, gene-copy number effects, etc.). In all humbleness, the author of this chapter does not claim to having fully grasped the genomic structures of the populations of HeLa cells studied by the two groups. In discussing these papers with the lead author of one of the two papers [47], many unanswered questions remain on timing of the origin and the dynamics of restructuring of the mammalian genome in such cells and, most importantly, the individuality of cells in terms of chromosome/genome structures can not be resolved with the presently used sequencing techniques when applied to the entire populations.

However, because of the importance of these phenomena apparently linked to cancerogenesis, mechanisms that result in these unusual chromosome structure and ploidy changes within an emergent population are being studied since a short time. Telling, the title of one of these papers is "Cancer genomes evolve by pulverizing single chromosomes" [50-52]. The findings appear to mirror those karyotype-based observations of chromosomal instability seen in immortalized cells over many decades. Comparing the recent findings in HeLa and human cancers (cells) *in vivo* and *in vitro* with so far available CHO data (karyotypes, BAC hybridizations, genome studies) we find a striking similarity.

2.2.5 Stability, Gene Pools and Microevolution

In the context of pharmaceutical production, stability is defined as the reproducible protein yield and quality from a given cell line over extended periods of time, from thawing of the cell line from a master cell bank until a given time point that is considered the longest period allowed for production. The minimal accepted period for requested proof of stability is about 3 months, based on the fact that manufacturing processes at large scale take significant time and will involve many cell population doublings. The expansion of cells towards the large-scale production vessel from a frozen vial can take up to 4 weeks. Subsequently, the production phase in the large-scale reactor can take up to 3 weeks for a fed-batch process and even longer for a perfusion-based manufacturing approach. Since several batches of product are typically being produced sequentially from one thawed vial of cells, a 3-month time window for this work is calculated very tightly. For approved protein products, stability studies cover at least 6 months of culture are standard.

It is difficult to imagine the huge number of cells that can be generated within a 3-month time window. The author needs to elaborate, in order to highlight the occurring genetic "bottlenecking", CHO cells double their number about once a day (and shorter times have been reported), thus within 3 months about 90 doublings of the initial cell population will occur. Cell banks, the starting population of cells in vials for pharmaceutical manufacturing, are typically made with 4 to 6×10^6 cells per vial, corresponding to about 30 µL of cell biomass (compacted cells). If unrestricted for subsequent growth after thaw, this biomass could theoretically multiply within the 3-month time window to a biomass volume of approximately 3×10^{22} L. However, a single 10,000 L bioreactor will contain "only" about 10^{13} cells (corresponding to a biomass of about 300 L). Thus, any large-scale production run will only use **a minute fraction of the progeny of cells derived from the starting culture after thawing the cells.** Thus scale-up is, in biological terms, equivalent to the expansion of a single invading species into an unexploited environment (where most progeny die/are selected against). In scale-up and maintenance of cells, many restrictions on the growth of these cells occur and thus a new population of quasispecies will evolve. Due to the genetic diversity of the invading population of quasispecies, the final bioreactor will certainly contain a quasispecies different from the starting one deposited in the master cell bank.

Independent from the diversity in CHO populations, the stability of the transgene(s) within these populations represents another problem that is not sufficiently studied and understood. Due to the lack of control over the site(s) of integration of the GOI within a single CHO cell, the issue of its stability within the genome is an unresolved problem. (In this context, it is noteworthy that regulators and some companies are insisting more and more on "true" clonality and that a single cell cloning exercise is not satisfactory. In view of the discussion above, this level of scrutiny is difficult to justify.) In spite of decades of research in this field, no controllable and reproducible gene transfer system has been developed for CHO cells so far. For this reason, manufacturers screen thousands of clonal cell populations (all of which are to be considered quasispecies populations) and study them in extended subcultivations in order to predict with a reasonable probability that the productivity is maintained (a) over time at small scale and (b) after transfer to large scale for manufacturing. Essentially, each time we clone cells, we generate a founder population that undergoes microevolution while we optimize and scale up our cells into large bioreactors. The diversity of these founder populations must be significant, since cloning efficiencies in CHO cells are high (> 80 %), yet cells differ dramatically in their individual genomic composition. The enormous hardiness of these cells, however, allows rapid expansion in number of cells derived from the **one** genome structure of the founder cell while rapidly restructuring it, as has already been shown by Hsu in 1961 [39]. A recent and important paper in this context on CHO cell populations verifies the expected genetic (heritability) diversity with 199 clonal cell populations derived from a CHO K1 parental host system [53].

Since true clonality cannot be preserved and thus does **not solve the stability problem,** the best approach for maintaining a balanced gene pool in a given quasispecies population is to minimize growth-restricting (selective) conditions. Clearly, for commercial pharmaceutical manufacturing, the maintenance of the gene pool composition of the cells in a master cell bank must be assured by all means. By keeping cell populations under conditions with little environmental changes, one can hope that a trend towards a modified gene pool would be minimized. Unfortunately, many standard cell culture techniques are possibly favoring or selecting for modifications of a given gene pool in a quasispecies population of CHO cells. For example, the shift of cells from adherent culture to suspension cultures represents a major environmental modification and thus will lead to the selection of subpopulations. Also, the composition of media that either prevent or allow cells to grow to high density can be considered a selective condition. Finally, even work with controlled bioreactors may be a cause for a population bottleneck. For example, certain reactors have poor gas exchange capacities and thus need to be stirred or otherwise mixed vigorously and, frequently in addition, need to be sparged with pure oxygen gas to maintain basic metabolic activities for the cells. Such conditions can kill sensitive cells and will select for populations of cells that are adapted to these harsher conditions. Other bioreactor systems, for example OrbShake bioreactors [54, 55] are known to have higher gas transfer rates than stirred-tank bioreactors and thus require less energy (correlated to shear stress and liquid turbulence) in order to distribute oxygen to cells. The milder conditions of such bioreactors would be expected to maintain sensitive cells and thus would not apply a restrictive/selective pressure on a population of cells that is scaled from milliliter cultures to hundreds and thousands of liters.

Awareness of the importance of environmental conditions in cell culture for maintaining stability has only recently been discussed in groups of scientists who deal with manufacturing issues. In this context, however, the complexity of genomic compositions and the diversity of genomes in cell populations have not been discussed.

2.2.6 Concluding Remarks

The dilemma faced by the industry using CHO cells is enormous. CHO cells have been known for decades to be highly robust **and flexible**, one of the main reasons for their popularity in manufacturing. Their gold standard status is rooted in the many successful products that have been made in them without risk to transmit unknown infectious agents. The quality of these products and, more importantly, the volumetric yields obtained for such products have surpassed the productivity of microbial systems. This further encourages the use of these cells.

However, the long and convoluted historic pathway of these cells, resulting undoubtedly in an enormous genetic and physiological diversity, combined with a large variety of culture conditions used in hundreds of labs represent a problem in

assessing the genomic composition of any cell line analyzed and its relevance for others. Each population of cells can be considered a **quasispecies**. Each cell line will represent its own unique genome/transcriptome/proteome and, therefore, an evaluation of data in comparison to other cell lines will be difficult if not done within a consortium of most interested parties that are ready to share their data. However, the wish to share data is even more complicated by the fact that companies will have their own, undisclosed ways to engineer their cell lines for more efficient protein manufacturing and are eager to protect them. Each of these efforts will generate population bottlenecks of quasispecies. Also, the work in companies involves different culture media, transfection approaches, reagents, selection steps, bioreactors, freezing and thawing protocols, cloning approaches, and bioprocesses, many of them proprietary.

What we can hope when working with cell lines of CHO origin in a given laboratory over many years is learning to appreciate this diversity and to address it with a large panel of standardized methods. It is a pragmatic approach and continues to be dependent on **screening for favorable phenotypes**. In essence, the genetic diversity problem can be managed by finding the best conditions for a cell population and then keeping the environmental conditions constant in order to minimize subsequent gene pool drifts in the populations.

What role for CHO genomics? A few important papers have been published very recently on sequence compositions and "genomic landscapes" of individual CHO cell lines and of the hamster genome [1, 56, 57]. Data in these papers were generated with cell populations derived from the CHO-K1, DG-44 and CHO-S^c lineages. They shed a first light on the complexities of CHO genomes, and the papers show a considerable level of sequence heterogeneity in comparison to the hamster genome and with respect to each other. In the papers by the Palsson group (1, 57) it was found that mutations appear to accumulate rapidly and are unique to a given cell line. For one antibody producing cell line, more than 300,000 single nucleotide polymorphisms (SNPs) arose, representing 9 % of all SNPs in this line. Notably, the authors claim that such nonuniform distribution of mutations have phenotypic relevance. More work has to be done on CHO, since the papers published so far lack quite important information of relevance to the industry:

- What is the degree of individual chromosome ploidy?
- Have entire chromosomes disappeared that generate long segments of homozygosity (as seen in HeLa)?
- Are such losses common among different CHO cell lines?

It appears that such major sequence variations may be shared within a branch of the CHO families, but not across the different branches. These publications have to be considered a beginning of work necessary to unravel the true genetic diversity of the branches and twigs of the CHO tree [58]. To take CHO K1 cells and its genomes: comparison of data sets derived from CHO K1- cells, grown in adherent culture using DMEM and fetal bovine serum, in comparison to K1 suspension cells in chemically defined

media, and their respective single cell cloned, but subsequently emerged cell populations, etc., will have to be established. The author could imagine tens if not hundreds of such sequence assessments that could lead to very useful insights eventually. The reader is reminded of the fact that the one female hamster, chosen for the origin of CHO cells, was an "outbred" hamster, whose degree of homozygosity was expected to be low. Did the same happen to CHO cells? Is this the reason why CHO were so useful for mammalian genetics studies in the 1960s to the 1980s? In the process of using CHO cells and mutagenizing them for cytogenetic studies and for the application of microbial genetics to mammalian cells, chromosome losses and rearrangements and copy-number compensations occurred that would favor a higher degree of homozygosity in **each of the descendant cell populations used in our industry**. The representation of sequence data from a CHO cell line in connection with a corresponding karyotype map would be useful, preferably linking Giemsa banding data with gene loci. Identifying those chromosomes that match the typical diploid hamster chromosomes and linking them with sequence data would possibly provide a concept for the more stable parts of the CHO genome whose losses would make the cells nonfunctional. The paper of Borth and colleagues that provides sequences of sorted hamster chromosomes is aligned with this wish [55]. For example, chromosome 1, chromosome Z1, chromosome 2, and chromosome Z2 (see Figure 2.2.2) appear to be relatively stable structures, at least from the perspective of all the karyotype maps available so far. Active gene loci and their structural and functional organization within the heterochromatin on these chromosomes are an important resource. Other smaller chromosomes or fragments of chromosomes, if recognizable, may fall into the same category. This, the author is hopeful, would represent a **CHO core genome** providing a highly useful and readily applicable starting point for more fine-tuned studies.

Eventually, we should hope for insights how a single cell founder genome shapes the resulting quasispecies genomes in clonal subpopulations, all expressing the same gene of interest. Practitioners in industry will assure that profound differences in the metabolic behavior of clones from a single transfection event for the production of one particular protein can be seen frequently. The founder genome, derived from the clonal event, is expected to shape the expression profiles, the physiology, the physical and chemical sensitivity of the resulting cells under production conditions. Possibly, a more profound set of data, for example from a 1,000 CHO genomes project, would further enhance our understanding in this context.

Conflict of Interest

The author is founder and member of the executive team of the company ExcellGene SA, a company that offers services to the industry in the context of recombinant protein production from animal cells in bioreactors.

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