Cell culture processes for the production of viral vectors for gene therapy purposes

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Abstract

Gene therapy is a promising technology for the treatment of several acquired and inherited diseases. However, for gene therapy to be a commercial and clinical success, scalable cell culture processes must be in place to produce the required amount of viral vectors to meet market demand. Each type of vector has its own distinct characteristics and consequently its own challenges for production. This article reviews the current technology that has been developed for the efficient, large-scale manufacture of retrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex virus vectors.

Introduction

Gene therapy is a developing technology that has the potential to treat several acquired diseases and of correcting inherited disorders. The premise is that genetic information is delivered to target cells, either through an *in vivo* or *ex vivo* route, by means of a suitable vector. Several strategies have been conceived for the treatment of diseases with an acquired genetic component. Thus, the original concept of gene therapy has evolved to include: (i) Suicide gene therapy – the delivery of genes able to kill the targeted cell in a controlled manner. This approach is often used to target tumor cells; (ii) Suppressive gene therapy – transdominant functions or sequences, like anti-sense molecules and ribosomes, capable of switching off a functional gene, are delivered to cells; (iii) Immune gene

therapy – delivery of genes capable of inducing the immune system to destroy cells expressing specific determinants (Palu et al. 1999).

The key to success for any gene therapy strategy is having a vector able to serve as a safe and efficient gene delivery vehicle (Friedmann 1997). Therapeutic genes can be delivered to cells via either viral or non-viral vectors. Non-viral vectors can be divided into two sub-groups; cationic lipids and molecular conjugates (Gao and Huang 1995; Michael and Curiel 1994). Simple mixing *in vitro* of the synthetic reagent with plasmid DNA leads to the spontaneous formation of DNA/vector aggregates via a self-assembly process. These vectors are relatively easy to make, non-pathogenic and free of adventitious contaminants (Wu and Ataai 2000). However, for efficient gene transfer the vector must survive in the extra cellular medium and reach the

target cells. Once the vectors have reached the target cell several steps, including binding, internalization, endosomal release, uncoating, nuclear escape and expression, are required to achieve efficient gene delivery and expression. While non-viral methods are generally considered to be safer than viral transduction, they are less efficient, and no commercial processes have been developed to meet the potential market demand (Theodossiou et al. 1999). In contrast, viruses have complex and precise structural features, adjusted through natural evolution for efficient transfection of specific host cells or tissues (Navarro et al. 1998).

A number of virus types are currently being investigated for use as gene delivery vectors. These include retroviruses, adenoviruses, adeno-associated viruses, poxviruses, rhabdoviruses, parvoviruses and alpha viruses (Palu et al. 1999). It is unlikely that any one of these vectors will emerge as a suitable vector for all applications. Instead, a range of vectors will be necessary to fulfill the objectives of each treatment (McTaggart and Al-Rubeai 2002). The typical characteristics of the main viral vectors are summarized in Table 1. Although vectors derived from adenovirus, adeno-associated virus and herpes virus are receiving more attention in the field of gene therapy (Vos 1995; Parasrampur 1998; Benihoud et al. 1999) recombinant retroviruses are currently used in the majority of clinical trials (Kang et al. 2000; Wu and Ataai 2000; Wu et al. 2002; Merten 2004). The main limitation of viral vectors is the relatively low harvest from non-optimized processes. In order for viral gene therapy to be a clinical and commercial success, there must be production systems capable of efficient and economical virus manufacture that

can meet market demand. This review focuses on the current cell culture processes used to produce viral vectors and highlights the advantages and the limitations of the current technology.

Retroviral vectors

Retroviruses can be divided into three subfamilies, Oncovirinae, Lentivirinae, and Spumavirinae, according to their pathogenicity and are further classified by their virion structure, cell receptors and oncogenicity (Kim et al. 2000). To date, the majority of clinical trials have used vectors derived from murine leukemia virus (MLV) (Merten 2004), which belong to the oncovirus sub-family, although some believe that adenovirus is currently the most commonly used virus. As a result, the majority of studies on optimization of culture and process conditions have focused on retroviruses. Retroviruses have a number of advantages as gene therapy vectors; gene transfer is highly efficient with permanent integration of the gene into the host genome. They have low immunogenicity, a wide host range and the vector proteins are not expressed in the host. However, vector integration can lead to an elimination of a functional gene when inserted into an ORP. There is the important risk of insertional mutagenesis, which may activate oncogenes, and they can only infect dividing cells, except for lentivirinae, which are discussed separately. In addition, it is difficult to obtain high titers in non-optimized systems (McTaggart and Al Rubeai 2002). Typical virus titers are between 10^6 and 10^7 infectious particles (IP) per ml, whereas theoretical predictions have suggested that 10^8 IP ml⁻¹ of infusion

Table 1. Typical characteristics of viral vectors used in gene therapy applications.

Viral vector	Genome	Insert size	Infectivity	Expression
Retrovirus	ss RNA	7–10 kb	Dividing cells (except lentivirus and spumavirus)	Permanent
Adenovirus	ds DNA	5 kb (single deletion), 10 kb (double deletion), 30 kb (gutless)	Dividing and quiescent cells	Transient
Adeno-associated virus	ss DNA	4.7 kb	Quiescent cells	Long-term
Herpes virus	ds DNA	20–30 kb	Dividing and quiescent cells (Neuron specificity)	Episomal, but latent infection permanent

ss = Single stranded; ds = double stranded.

product would be required to treat a tumor, based on the supposition that a typical tumor contains 10^9 cells g^{-1} and a diseased organ may contain 100 g of tumor. This figure assumes 100% transduction efficiency, no particles will be lost by complement inactivation and that infection is not limited by cell replication. Therefore, the actual viral dose may be 10–1000 times greater (Braas et al. 1996; Lyddiat and O'Sullivan 1998). It was subsequently shown empirically that titers $> 5 \times 10^7$ IP ml^{-1} of infusion product can efficiently obtain *in vivo* gene transfer in animal models of hemophilia, arthritis, cancer, chronic HBV infection, cystic fibrosis and other diseases (Ghivizzani et al. 1997; Karavodin et al. 1998; Sallberg et al. 1998; Wang et al. 1998; Nemunaitis et al. 1999; Van den Driessche et al. 1999). This still leaves a gap between the number of viral particles required and the number that can be produced.

The problem of achieving acceptable virus titers is two-fold; firstly, the cells must be capable of producing a sufficient number of infectious particles. This has led to the development of highly efficient producer cell lines. Production can be further enhanced through optimization of the culture conditions. Secondly, viruses are particles not small molecules, and have a very short half-life, typically 2–8 h at 37 °C (Le Doux et al. 1999; Merten et al. 2001a). Maintaining their capability to infect target cells is essential. McTaggart and Al-Rubeai (2002) compared the potential methods for the production of retroviral vectors and concluded that in order to reduce production cost, critical culture parameters should be optimized and continuously controlled throughout the production process. To optimize a bioreaction process for retrovirus production, the biological potential of the cell must be optimally exploited and the virus stability maximized. The design of an optimal cell culture process must therefore account for three important factors: the type of bioreactor, the mode of operation and the culture conditions.

Producer cell lines

The packaging cell lines used to produce the retrovirus particles contain the virus helper genes (*gag-pol* and *env*) that have been deleted from the vector genome. Producer cell lines are made by

inserting the vector, which has had these genes replaced by a therapeutic gene, into the packaging cell on a separate plasmid (Pear et al. 1993). Hence, the structural and functional proteins required for the synthesis of new virus particles are produced by the cell. Recombinant viruses are released from the cell in a similar fashion to wild type viruses. The use of this split genome approach (separation of the viral gene sequences and the MLV vector = third generation packaging cell line) leads to a considerable improvement of vector safety because recombination events leading to replication competent retroviruses (RCR) are greatly reduced. Packaging cells are unable to produce their own virus particles as the Ψ packaging sequence and the LTRs are only contained on the vector plasmid (Robbins et al. 1998). The mechanism of producing retroviral vectors from packaging cell lines is illustrated in Figure 1.

A very recent development of highly versatile producer cell lines (Flp293A, 293 FLEX), based on HEK293 cells and equipped with FRT sites containing an MLV-GFP vector, allows the efficient Flp recombinase mediated cassette exchange of MLV vectors. After cassette exchange, the tagged RV producer cell clone produced titers of vector containing the transgene of choice at levels similar to those observed for the mother producer cell line (Schucht et al. 2005; Coroadinha et al., 2005).

Historically, packaging cell lines were based on mouse cell lines (NIH 3T3). However, certain limitations of these packaging cell lines have initiated the search to improve them. The primary drawback associated with mouse cell lines is the low titers that they achieve. Furthermore, murine retroviral sequences that are present in murine packaging cells can be selectively packaged into retroviral particles (Torrent et al. 1994) increasing the possibility of generating RCRs. Thus, the use of human cell lines for the establishment of packaging cells is a step towards increased biological safety, because they lack endogenous murine retroviruses (Rigg et al. 1996; Pensiero et al. 1996; Forestell et al. 1997). In fact, viral supernatants or producer cells derived from human cells have never given a positive test result for RCRs in small- or medium-scale assays (Sheridan et al. 2000). A further advantage of using human based packaging cell lines is the fact that the glycosylation of the env protein is of a human type. This is important

Figure 1. The principle mechanism of a third generation MLV packaging cell line used to produce recombinant retroviral vectors for gene therapy.

when the vectors have to be used in an *in vivo* setting. In the case that the vector has a non-human glycosylation, they are inactivated by the human complement system within 20 min after application.

The NIH 3T3 (mouse), and a ferret brain cell line have been used for the establishment of retrovirus vector producer cell lines, as well as the human cell lines HT1080, TE671, 293 (which can grow in suspension), and CEM (which is an obligatory suspension cell line). For more details, the review by Merten (2004) is recommended.

Packaging and producer cell lines are able to continuously release vector particles, making them well suited to large-scale production systems. This gives them a significant advantage over transient transfection or infection systems, such as baculovirus or adenovirus systems, where generation of large volumes of characterized supernatants is difficult (Rigg et al. 1996).

Bioreactor design

The majority of packaging cell lines used to produce retroviral vectors are anchorage-dependent, necessitating a high surface-to-volume ratio to support high density cell growth. Roller bottles have been extensively used for the production of retroviruses. However, scale-up of this system has a number of drawbacks; roller bottles are a multiple process system which means that scale-up requires an increase in unit number as opposed to unit size. This results in a very labor intensive process. Furthermore, roller bottles can only be used in batch, fed-batch or repeated batch mode, which results in extended residence times for virus particles. It is also difficult to control culture parameters, such as dissolved oxygen (DO_2) and pH, which may limit cell production. This has lead researchers to investigate microcarrier cultures as a potential industrial scale process.

Microcarriers fall into two categories: solid and porous carriers. Both types have been used to culture retrovirus producing cells. The solid carriers Cytodex 1 and 3 have been used to culture PA317 cells (Wu et al. 2002) and the former has been used to culture TE Fly GA18 cells (Merten et al. 2001a). The PA317 cultures were reported as achieving a maximum virus titer of 3.1 and 3.4×10^6 IP ml⁻¹ on Cytodex 1 and 3, respectively, and TE Fly GA18 cells produced 2.0×10^7 IP ml⁻¹. When the agitation rate in the PA317 cultures was optimized, a maximum virus titer of 8.5×10^6 IP ml⁻¹ was achieved on Cytodex 1. These titers for PA317 cells are comparable with stationary, monolayer cultures and do not show a vast improvement, despite the ability to control the culture conditions. Hence, to increase the cell density and subsequently the virus titer, macroporous microcarriers could be used. Porous microcarriers have a significantly greater surface area than solid microcarriers, which allows for a far higher cell density. However, when Cultispher-S carriers were compared with solid carriers the virus titer was 10-fold less, even though similar cell densities were reported. Typical maximum virus titers for both PA317 cells and FlyRD18 cells are in the order of 10^5 IP ml⁻¹ (Gerin et al. 1999b; Cruz et al. 2000; Wu et al. 2002). This loss of virus titer in macroporous carriers in stirred tank reactors is almost certainly due to transport limitations. In solid carrier cultures, cells consume nutrients from the bulk medium and virus particles are released into the vessel. When cells are immobilized within a porous structure, cellular activity is determined by the chemical concentrations in the particle, not in the bulk medium. Cells initially penetrate relatively deeply into the porous matrix; however, over time an outer shell of highly dense cells forms, limiting transfer of oxygen and nutrients to the centre of the particle (Al-Rubeai et al. 1990; Yamaji and Fukuda 1992). Additionally, toxic metabolites are unable to diffuse out of the particle and CO₂ production causes a drop in the local pH. Consequently, this microenvironment induces cell death and a necrotic core develops, reducing the overall cell productivity and viability. Additionally, diffusion of virus particles into the bulk medium is hindered, causing an increase in residence time and a greater degree of inactivation. To overcome this problem with macroporous microcarriers, intraparticle convec-

tion can be increased by culturing cells in either a packed or fluidized bed bioreactor. Figure 2 illustrates the operating principle of the CelliGen packed bed reactor, the CellCube and the Cytopilot Mini fluidized bed bioreactor. These systems have shown the greatest potential for retrovirus production, to date.

Packed bed bioreactors have demonstrated superior performance over microcarrier cultures for both ψ CRIP MFG-lacZ cells and TE Fly GA18 cells (Merten et al. 1996, 2001a). Kang et al. (2000) showed that daily vector production was approximately 30 times greater in a packed bed bioreactor (packed with Fibracel discs) than a microcarrier system utilizing 3 g l⁻¹ Cytodex. Similarly, Merten et al. (2001a) showed a 116% increase in the maximum virus titer and a 400% increase in daily vector production when comparing a microcarrier system (5 g l⁻¹ cytodex 1) and a CelliGen packed bed bioreactor containing 70 g of Fibracel discs. Evaluation of a fluidized bed bioreactor (Cytopilot) was recently carried out by Warnock et al. (2004). The Cytopilot demonstrated good vector production, with a maximum virus titer of 10^7 IP ml⁻¹, and a total viral yield of 5.03×10^{10} IP in 11.4 l. In roller bottle cultures, daily titers of 5×10^6 IP ml⁻¹ were achieved from 50 ml per bottle (total yield: 2.5×10^8 IP per bottle) (unpublished results). Therefore, the total yield obtained from the Cytopilot over 19 days was equivalent to the yield from 10 daily harvests from 20 roller bottles. It was observed that virus titer increased with an increasing cell number. Therefore, as the Cytopilot has the potential for higher cell numbers in longer runs it also has the potential for greater virus titers. In contrast, the cell number in roller bottles reaches its maximum within 3–5 days. Thus, an increase in total viral yield can only be accomplished by an increase in unit number or longer culture periods. Preliminary data recently published by Merten (2004) have also shown the potential of the Cytopilot; the PG13 packaging cell line was cultured on 150 ml of Cytoline 1 for 336 h and produced a total vector harvest of 1.33×10^{10} infectious particles. This was comparable with the standard CellCube process. Culture conditions were not optimized in this study, though, indicating that product yield could be greatly improved.

In addition to packed bed and fluidized bed bioreactors, fixed bed culture systems, such as

Figure 2. Schematic diagrams of different bioreactor systems used in the production of retroviral vectors. (a) CelliGen Plus packed bed bioreactor (Courtesy of New Brunswick Scientific, Edison NJ USA, www.nbsc.com); (b) CellCube, lab version (Courtesy of Corning); (c) Cytopilot Mini Fluidized bed bioreactor (Amersham Biosciences).

the CellCube, have been used for retrovirus production (Kotani et al. 1994; Merten et al. 2001a; Wikström et al. 2004). The performance of the CellCube is reported to be comparable with the Celligen packed bed reactor, with each systems producing an average of 2.1×10^7 and 0.55×10^7 IP ml⁻¹, respectively (Merten 2004). The yield from the CellCube was also reported to be comparable with the automated roller bottle system, RollerCell 40 (Wikström et al. 2004). When the CellCube was run under GMP condi-

tions, the virus titer was between 1.2×10^6 and 3.0×10^6 IP ml⁻¹. This was lower than titers recorded under test conditions due to a higher perfusion rate. While the higher perfusion rate reduced the vector concentration it did increase the titer stability and thus the quality of the vector preparation.

Hollow-Fiber bioreactors have shown some potential for retrovirus production. Pan and Whitley (1999) studied vector production using PA317 cells and observed titers of 2.2×10^7 IP ml⁻¹.

This was a 14.4-fold increase over control dish cultures. Up to 3 l of vector supernatant was generated during a 2-month large-scale production run. There was a potential to double this volume of higher-titer supernatant by increasing the frequency of harvest. Virus titers eight times greater than T-flask cultures were observed using a TE Fly clone (12.8×10^6 IP ml⁻¹); however, this result was inferior to titers obtained in fixed bed bioreactors (Merten 2004).

The primary limitation of packed bed, fixed bed and hollow-fiber bioreactors is their limited scalability. The largest practical packed-bed volume in the configuration of the Celligen Plus reactor (Figure 2a) is approximately 20 l, providing 200–300 l of supernatant (Merten 2004). No industrial processes currently employ packed-bed bioreactors as they have limited linear scale-up due to concentration gradients, the system is non-homogeneous, it is not possible to sample reactor content and the outgrowth of cells leads to channel blockage (Looby and Griffiths 1990). The disadvantage of the CellCube fixed bed bioreactor is that some components, such as the oxygenator and the probes, must be cleaned and re-sterilized between batches. Cleaning validation as well as process validation is relatively difficult (Wikström et al. 2004). Additionally, the largest system available is the module 400, which has a surface area of 340,000 cm².

Hollow-fiber systems are associated with several disadvantages including poor cell viability, large diffusion gradients, which limit scale-up and effect product quality, and difficulties in culturing anchorage dependent cell lines (Warnock and Al-Rubeai 2005). As medium enters the lumen it encounters a positive transmembrane pressure and is able to permeate into the extra-cellular space. As this pressure decreases along the length of the fiber, it becomes negative towards the outlet, causing medium to flow back into the lumen (intra-capillary space). This phenomenon is called Starling flow (Starling 1896). This results in a higher cell density at the inlet end compared to the outlet or, with suspension cells, a packed cell mass accumulating at the outlet end (Griffiths 1988). The resulting presence of large numbers of dead cells limits culture performance in two ways. Firstly, there is a need for high rates of cell proliferation to replace lost cells, which will reduce the specific productivity (Al-Rubeai et al. 1992). Sec-

ondly, the release of proteases, DNA and other cellular components will lead to product degradation and will complicate downstream processing (Fassnacht et al. 1999).

To overcome the problem of scalability, some attention has been given to the development of suspension cell lines. Suspension cells can be easily grown in stirred tank reactors, which can be scaled up to several thousand liters. Murine based packaging cell lines are based on NIH 3T3 cells which are strictly adherent. The human packaging cell lines derived from HT1080 and TE671 cells are also adherent but when they are grown in suspension they form clumps. Clump culture is comparatively inefficient with titers generally being 10–100 times lower than those obtained in adherent cultures (Gerin et al. 1999b; Merten et al. 2001a). A novel cell line has been developed from a T-lymphoblastoid cell line. CEMFLYA19 cells cultured in 250 ml spinner flasks reached a density of $1.5\text{--}3.0 \times 10^7$ cells ml⁻¹, and produced an average of 1.6×10^7 IP ml⁻¹. This was reported to be 2.5–6 fold higher than T-flask cultures (Pizzato et al. 2001). The authors also reported that preliminary results showed that this cell line could produce 5×10^6 IP ml⁻¹ in a 1.4 l perfused reactor. It is expected that after optimization of the culture conditions the titer can be significantly increased. Therefore, the CEMFLYA cell clone has the greatest potential for industrial production of retrovirus particles. A number of packaging cell lines based on human 293 cells have also been produced. These cells can be grown as either adherent or suspension cultures. The virus titers achieved are comparable to other human based producer cells, and range from 10^6 to 10^7 IP ml⁻¹ (Merten 2004). In addition, it has been reported that particles produced from ProPak and 293-SPA clones have a transduction efficiency that is up to 10 times greater than for vectors produced from NIH 3T3 derived produced cells (Forestell et al. 1997; Davis et al. 1997).

Culture conditions

In addition to the type of bioreactor used, culture conditions can have a profound effect on the virus titer. Due to the instability of retrovirus particles at 37 °C, much attention has been given to culture temperature. Virus production in twenty-two pro-

ducer cell lines derived from the murine PA317 cell line was evaluated at 32, 34 and 37 °C. A 5- to 15-fold increase of vectors was produced at 32 °C compared to 37 °C; the vector increase at 34 °C was intermediate. The production of retroviral vectors was scalable achieving similar results in flasks, roller bottles, or a CellCube Bioreactor (Kotani et al. 1994). This group suggested that the increased titer could be due to (i) reduced metabolic activity/growth of the cells, causing an increase in vector production; or (ii) increased stability of virus particles at lower temperatures. However, several studies have shown that vector production is decreased when cellular metabolic activity is reduced (Shen et al. 1996; McTaggart and Al-Rubeai 2001). Kinetic analysis of retrovirus production and decay showed that the rate at which recombinant retroviruses spontaneously lose their activity was a strong function of temperature, decreasing roughly 2-fold for every 5 °C reduction in temperature, whereas the rate at which retroviruses are produced is only weakly affected by temperature, decreasing about 10% for every 5 °C reduction in temperature (Le Doux et al. 1999). Therefore, the increased stability at 32 °C leads to a higher titer. A number of other groups have also demonstrated improved titers and greater virus half life at lower temperatures using both murine and human packaging cells (Lee et al. 1996; Kaptein et al. 1997; McTaggart and Al-Rubeai 2000). However, the advantages of culturing cells at 32 °C could not be corroborated by other investigators (Forestell et al. 1995; Cruz et al. 2000) leading to a more detailed examination of the mechanism of thermal stability in retroviruses. It could be shown that retroviral vectors produced at 37 °C were characterized by a higher thermal stability than those produced at 32 °C (Cruz et al. 2005). It was revealed that virus stability was inversely proportional to the level of cholesterol in the viral membrane; hence, depletion of viral cholesterol resulted in increased thermal stability. Cholesterol levels were associated with the cell line as differences in the cellular plasma membrane determined the lipid composition in the retrovirus shell (Beer et al. 2003b). Furthermore, a shift in cultivation temperature from 37 to 32 °C impacted the expression of a number of genes. Most importantly, the cholesterol biosynthesis/transport pathway was activated, causing an increase in plasma membrane cholesterol levels (Beer et al. 2003a). Thus, virus

particles released at 37 °C are phenotypically different from those synthesized at 32 °C. This accounts for the discrepancy between reports on virus production at various temperatures. In a subsequent study, Electron Paramagnetic Resonance (EPR) was used to show that production temperature exerts an important effect on membrane rigidity. This effect depends on the vector type: retroviral vectors with the amphotropic envelope protein produced at 32 °C by TE FLY cells are more rigid than those produced at 37 °C, the opposite being observed for vectors with the GALV envelope protein. The differences with respect to rigidity, production temperature and inactivation kinetics observed for different producer cell lines and different env proteins can be explained by differences in the activation energy for the inactivation of these vectors (Cruz et al. 2005). Thus, vector thermal stability can be influenced by controlling the individual lipid components in the particle membrane by adjusting cultivation temperature or by the host cell line.

The effect of pH on vector titer was assessed by McTaggart and Al-Rubeai (2000) using the human packaging cell line, FLYRD18. The optimal pH was determined to be 7.2; cell viability was not affected at lower pH levels with the exception of pH 6, when cell viability fell to 85%. The virus titer at 6.8 and below was significantly decreased compared to cultures at pH 7.2. At a pH of 7.8 the virus titer was 50% of the titer at 7.2 (McTaggart and Al-Rubeai 2000). Dissolved oxygen levels were also studied but were not found to be limiting to cell growth or virus production between 20 and 80% saturation.

While several studies have focused on the effect of physical conditions, few studies have addressed the effect of chemical factors. Olsen and Sechelski (1995) reported an increase in virus production between 20- and 1000-fold in the presence of sodium butyrate using PA317 murine cells. Interestingly, this group reported a 50-fold increase in LTR driven transcripts but only a 4-fold increase in SV40 promoter driven transcripts in the same cell line. Hence, while sodium butyrate may increase RNA transcription, the actual responsiveness is dependent on the promoter type. Pages et al. (1995) examined a number of activators known to enhance the expression of MoMLV genes. They observed a 5-fold increase in virus production from ψ CRIP cells in the presence of 5 mM sodium

butyrate. However, when combined with dexamethasone (1 μ M), a 10-fold increase was detected.

Previous studies on the effects of serum have shown conflicting results. Shen et al. (1996) showed increasing cell growth and vector production in PMFG/ ψ CRIP murine cell cultures when the serum concentration was increased from 1 to 10%. It was concluded that a strong promotion of vector production by cell growth was the reason for increased titers with increasing serum concentration. However, Lee et al. (1996) demonstrated no effect of decreasing serum from 20 to 1% in culture medium used to produce retrovirus from ψ CRIP cells. In contrast, serum was shown to have a negative dose dependent effect on retrovirus production in the human cell lines FLYRD18 (Gerin et al. 1999a, b; McTaggart and Al-Rubeai 2000) and TE Fly RD (Warnock and Al-Rubeai 2004). This phenomenon was sustainable in excess of 6 days. In order to confirm that the negative effect of serum was not due to virus inactivation, vectors were incubated with and without 5% serum at both 32 and 37 °C. Vector decay rates were comparable at both temperatures indicating that serum components were not responsible for virus inactivation (McTaggart 2000). The negative effect of serum was attributed to protease inhibitors present in serum, which have an adverse effect on virus assembly and budding (Gerin et al. 1999a). While the different findings may be explained by the origin of the cell line (murine or human), it may also signify that serum has opposing roles; certain components are able to promote vector synthesis while other elements inhibit virus assembly.

It is known that elevated concentrations of ammonia and lactate can be toxic to mammalian cells and that continuous cell lines use glucose inefficiently which results in the production of large amounts of lactate. It has been proven that lactate concentrations above 5 mM have a profound negative effect on TE FLY GALV cell growth; however, specific vector production is significantly increased after the addition of 5–20 mM lactate. Ammonia only has minor effects on TE FLY cell growth and vector production, with an IC₅₀ (50% inhibition of cellular growth) being in excess of 8 mM (Merten et al. 2001b). In order to reduce the production of lactate, glucose can be replaced by fructose as the main carbon source. This substitution results in an increase in

viral titer by approximately 5-fold in a range of MLV producer cells, as shown in Figure 3. This phenomenon can be observed in T-flask and bioreactor cultures; TE FLY cells cultured in an NBS basket reactor produced a cumulative titer of 6.96×10^{10} IP using fructose compared to 1.49×10^{10} IP with glucose (Gény-Fiamma et al. 2004). This increase in product is due to an increased cell number (7.1×10^9 vs. 4.34×10^9 cells/reactor) and an increase in the specific production by a factor of 2.81 in fructose.

Mode of operation

As previously mentioned, the half-life of retroviruses is between 2 and 8 h at 37 °C, which is often the culture temperature. When particles are produced in multiple unit processes, such as T-flasks or roller bottles, harvesting is done discontinuously. The actual harvest time will vary depending on the virus characteristics, but in general will be performed twice daily. At larger scales this becomes a laborious task and increases the risk of contamination. Consequently, perfusion culture offers the best option for the production of retroviral vectors (Merten 2004). Accordingly, it was determined that the optimal bioreaction mode for retrovirus production was batch mode at 37 °C for 53 h (to allow for cell growth) followed by perfusion at 32 °C with the addition of 0.1 μ M dexamethasone and 5 mM sodium butyrate. This produced 2682 IP cm⁻³ h⁻¹ which was a 3.5-fold increase over batch cultures under the same conditions (Cruz et al. 2000). The optimal perfusion rate required to support high density cell cultures and maximize vector harvest is regarded as three to four reactor volumes per day. This supposition is supported by the data published by Merten et al. (2001a).

Lentiviral vectors

Lentiviruses are members of the retrovirus family, yet they possess a number of distinct characteristics that are absent in other retroviruses. In addition to the conventional three open reading frames of *gag*, *pol* and *env*, which encode the capsid proteins, viral enzymes and the envelope glycoproteins, respectively, they have a varying complement of regulatory and accessory genes that control their

Figure 3. Comparison of the average specific growth rate (a) and the vector production (b) in different producer cells cultured in 4.5 g l^{-1} glucose or 15 g l^{-1} fructose in T-flasks (* = 20 g l^{-1} fructose). Data adapted from Gény-Fiamma et al. (2004).

own gene expression. This allows temporal separation into early and late phases, and enables them to manipulate the host cell for both virus production and entry and integration of their genetic material into the cell genome as the provirus (Lever et al. 2004). Therefore, lentiviruses are able to infect non-dividing cells unlike other retroviruses which require host cells to be actively dividing. This makes them suitable for the treatment of such diseases as those of the nervous system, cardiac and skeletal muscle, and the liver. The most prominent member

of the lentivirus sub-family is the human pathogen HIV. The extensive body of knowledge that has been generated on this virus has allowed researchers to reengineer it as a gene vector. HIV-1 remains the best studied vector (Lever et al. 2004), although recent work has revealed the potential of HIV-2 (Sadaie et al. 1998) SIV (Schnell et al. 2000; Stitz et al. 2001), EIAV (equine infectious anemia virus) (Olsen 1998; Mitrophanous et al. 1999; Ikeda et al. 2003), FIV (feline immunodeficiency virus) (Johnston et al. 1999; Curran et al. 2000; Brooks et al.

2002; Loewen et al. 2003) and BIV (bovine immunodeficiency virus) (Takahashi et al. 2002) as vectors.

One of the major problems in the production of lentiviruses has been the development of a packaging cell line. Stable expression of lentivirus particles has proven to be more difficult than for oncoviruses (Carroll et al. 1994; Kafri et al. 1999), partly due to the expression of such proteins as REV and viral protease, which appear to be toxic to cells (Haselhorst et al. 1998). Consequently, lentiviral vectors have been produced by transient transfection of high-expressing cell lines such as COS (Imren et al. 2002) and 293T (Coleman et al. 2003; Sena-Esteves et al. 2004) using generally four different plasmids (gag-pol, env, rev, and the LV-vector). This method was also used to produce vectors for the first clinical trial, demonstrating that scale-up for instance using CellFactory 10 stacks is practicable (Schonely et al. 2003, Merten personal communication).

It has been reported that optimized transient production of lentiviral vectors from these cells can yield titers in excess of 10^8 IP ml^{-1} for VSV-G pseudotyped vectors (Sena-Esteves et al. 2004).

Research into the large-scale production of lentiviral vectors is still in its infancy. Two recent reports have used the Cell Factory system (NunclonTM) to scale-up virus production. Geraerts et al. (2005) produced a serum-free HIV-1 vector using the Cell Factory and tangential flow filtration prior to centrifugation. Using this method, they were able to routinely generate titers of 10^9 – 10^{10} transducing units (TU) ml^{-1} from 2 l of culture medium. Furthermore, they observed large transduction volumes in mouse brain and no immune response (Geraerts et al. 2005). In a separate study, Ni et al. (2005) developed a three-level cascade gene regulation system designed to remove tetracycline transactivator (tTA) from cytomegalovirus immediate early promoter (CMV)-controlled expression to reduce cytotoxicity from constitutive expression of tTA and leaky expression of packaging genes. A one-step integration of the three packaging plasmids was also performed to shorten the culture time for clonal selection. Producer cells yielded remarkably stable vector production over a period greater than 11 days with the highest titer 3.5×10^7 TU ml^{-1} and 300 ng p24 ml^{-1} , yielding 2.2×10^{11} TU and 1.8 mg p24 from one cell factory (Ni et al. 2005). Cell Factories

are designed for large scale cell culture and production of biologics such as vaccines, monoclonal antibodies, or recombinant proteins. They provide a large growth surface in limited space areas and are available with 1, 2, 10 and 40 trays. However, as with the CellCube, they have limited scale-up potential as the 40 tray system has a maximum surface area of 25,284 cm^2 , and it is not possible to sterilize the trays by autoclave.

Adenoviral vectors

Packaging cell lines and vector construction

Adenoviral vectors are believed to have currently surpassed retrovirus as the most commonly used vectors for gene transfer. Adenoviruses have a number of advantages as a gene delivery vehicle. They are able to infect both dividing and quiescent cells, insertional mutagenesis is unlikely to occur, they have a high transduction efficiency and high titers can be easily achieved (McTaggart and Al-Rubeai 2002). In contrast to retroviruses, adenoviruses are non-enveloped DNA particles. The most commonly used human adenoviruses (type 2 and type 5) consist of a linear 36 kb double stranded DNA molecule. Both strands are transcribed and nearly all transcripts are heavily spliced. Viral transcription is conveniently defined as early (encoding E1, E2, E3, and E4), delayed early (encoding transcripts IX and IVa), and late genes, depending on their temporal expression relative to the onset of viral DNA replication (Shenk 1996). First generation recombinant vectors are usually deleted in their E1A or E1B regions, therefore providing space for the insertion of a therapeutic gene up to 5 kb (Breyer et al. 2001). Expression of E1A initiates adenoviral replication and activates adenoviral transcription (Chuah et al. 2003); therefore, an adenovirus-transformed cell line that provides the missing E1A and E1B genes *in trans* is required to grow the replication defective virus (Graham et al. 1977). However, expression of the therapeutic gene is transient and, due to the expression of viral genes/proteins in the transduced cells, adenovirus vectors can be potentially immunogenic, causing a direct cytopathic effect (Nadeau and Kamen 2003). The most common and best documented packaging cell line for adenovirus production is the Human

Embryonic Kidney 293 cell line, which contains the E1 region of the adenovirus (Graham et al. 1977). Packaging cells based on the 293 cell line are versatile, and can be grown as adherent cultures or as suspension cultures in serum-containing low Ca^{2+} media (Garnier et al. 1994) and serum-free low Ca^{2+} media (Côté et al. 1998; Peshwa et al. 1993). Unfortunately, homologous recombination between the left terminus of first-generation Ad vector or helper-virus DNA and partially overlapping E1 sequences in the genome of 293 cells may lead to the emergence of replicative competent adenoviruses (RCA), which contain E1 genes (Lochmuller et al. 1994). To overcome this problem, alternative host cell lines have been developed by reducing these overlapping sequences, as for 911 cells, or by eliminating any overlap, as for N52.E6 (Schiedner et al. 2000) or PER.C6 (Fallaux et al. 1998) cells. The 911 cell line exhibits similar frequencies of homologous recombination to 293 cells, although vector yields are three times higher in 911 cells (Fallaux et al. 1996). The PER.C6[®] cell line, derived from the Human Embryonic Retinal cell, has been established for industrial applications (e.g. full tracability available) and is a good producer. The cell population is clonal and the cell line was designed to minimize RCA production. Unfortunately, this cell line is only available through licensing. Yields from the PER.C6 cell line are similar to those achieved in 293 and 911 cells; however, as overlap between the genome and the E1 sequence has been eliminated, this clone, as well as the N52.E6 cell line, does not generate RCAs (Fallaux et al. 1998; Schiedner et al. 2000). Another cell line is A549, a lung carcinoma cell. This cell line has been engineered with low recombination homology and is used to support the replication of E1-deleted adenovirus vectors (Imler et al. 1996).

More recently, non-RCA vectors have been developed by double deletions (E1 and E3) or triple deletions (E1, E3 and E4). These vectors have the added advantage that they can accommodate up to 10 kb of foreign DNA (Gao et al. 1996; He et al. 1998). The generation of gutless adenoviral vectors has also been accomplished; these vectors are devoid of viral proteins and consequently display reduced immunogenicity and long-term transgene expression (Parks et al. 1996). Additionally, this system has the capability of accommodating up to 35 kb of exogenous DNA

and is considered the most advanced vector system to date (Breyer et al. 2001).

Production methods

Conventional methods for producing small volumes of viral vectors involve culturing cells in stationary, adherent cultures, such as T-flasks or roller bottles (Wu et al. 2002). Adherent cell cultures have a higher cell specific productivity compared to suspension cells (Iyer et al. 1999); however, scale-up is limited by available surface area. To date, very few studies have investigated cell growth and virus production using microcarriers as a potential means of scaling-up production. As with retrovirus production, solid microcarriers have been shown to be superior to macroporous carriers, with Cytodex 3 offering the best results. However, the maximum specific virus titer remains inferior to T-flask cultures (Wu et al. 2002). Hydrodynamic shear forces are believed to affect cellular receptor levels and thus hinder virus entry into 293 cells at high MOI. Additionally, 293 cells have been cited as having a low ability to colonize microcarriers due to poor attachment (Nadeau and Kamen 2003).

Suspension cultures are more appealing for large-scale production due to the ease of operation and scale-up. Per.C6[®] and 293 cells can be grown in stirred tank bioreactors and, depending on the mode of operation, cell densities are in excess of 5×10^6 cells ml^{-1} at scales from 3 to 20 l (Nadeau et al. 1996; Côté et al. 1998; Kamen and Henry 2004). Typical titers are in the range of 1×10^{10} to 6×10^{10} virus particles (VP) ml^{-1} . A recent study using the HeLaS3 human tumor cell line for propagating recombinant adenoviruses, demonstrated yields of 6×10^{11} VP ml^{-1} and showed that this productivity could be maintained at pilot (70 l) scales (Yuk et al. 2004). Successful large-scale production for all cell lines is dependent on optimization of the culture conditions, and determining the best mode of operation.

Optimization of physicochemical conditions

Optimization of the physicochemical conditions in the bioreactor can greatly improve the viral titer. The pH, temperature and pCO_2 have all been shown to be important parameters in cell

growth, metabolism and adenovirus production. When uninfected PER.C6[®] cells were grown at pH ranging from 7.1 to 7.6 no significant difference was seen in the growth rate, with doubling time calculated as being approximately 31 h. At a pH of 6.8 a longer lag phase was observed and the doubling time was calculated to be 50 h. The maximum viable cell concentration was seen to be inversely proportional to pH, with the highest concentration obtained at pH 6.8 and the lowest obtained at pH 7.6. Glucose consumption and lactate production were significantly lower under more acidic conditions and this may explain the differences in the maximum cell concentration (Xie et al. 2002). There was a 40% difference in cell concentration between these two extremes in pH. The same authors reported an optimal pH of 7.3 for virus productivity which decreased by 50% at pH of 6.7 and 7.7 (Xie et al. 2002). Jardon and Garnier (2003) reported a 4-fold increase in virus titer at pH 7.2 compared to pH 6.7 and 7.7 using 293 cells. Infectivity of the virus was not deemed to be responsible for this difference as the increase of infected cells was similar at all pH. However, the pH was not found to have a significant effect on viable cell concentrations of 293 cells in the range of 6.7–7.7; although 293 metabolism was slower at a higher pH.

Temperature has an effect on both cell metabolism and virus titer. Low temperature prolongs viability of 293S cells and slows the progression of the infection. At 39 °C negligible amounts of virus was produced; at 37 °C virus titer reached a maximum at 30 h post-infection (hpi). Higher titers were obtained at 35 and 32 °C, with maximum titers being reached at 40 and 74 hpi, respectively. The highest titers were produced at 35 °C, which were 3.4-fold higher than at 37 °C (Jardon and Garnier 2003). This finding was confirmed by Cortin et al. (2004) who showed a 2.4-fold increase in viral titer when 293S cells were cultured at 35 °C compared to 37 °C.

The pCO₂ does not have an effect on viable cell concentration; however, as with pH, virus productivity is significantly affected by changes in pCO₂. The virus infection phase is prolonged with increasing pCO₂, which also decreases the virus yield. The maximum titer at 0.2 atm was 2-fold lower than the maximum titer at 0.05 atm (Jardon and Garnier 2003).

One of the challenges for efficient propagation of PER.C6[®] cells at large scale is to overcome the sensitivity of virus infected cells to gas sparging, which is required for oxygenation and CO₂ removal. Productivity of an adenovirus vector is significantly reduced under sparging conditions as compared to nonsparged. This is a common problem which has been thoroughly investigated during the 90s in insect cell-baculovirus culture (Kioukia et al. 1996). Investigations have suggested a buffer containing surfactant (Polysorbate-80, PS-80) that was included in the virus seed stock formulation and introduced through virus infection into the culture at a very low concentration as the cause of the reduced virus productivity. To alleviate the deleterious effects of sparging, the virus seed stock was prepared in the absence of the buffer containing PS-80. At the same time, the concentration of Pluronic-F68 (PF-68) in the serum-free medium was increased to 1 g/l, at which cell growth and metabolism were unaffected; this measure alone did not result in virus productivity improvement. Only by implementing the two measures together was virus productivity loss completely eliminated under sparging conditions. This adenovirus propagation process was successfully scaled up to 250 l (Xie et al. 2003).

In contrast to retroviral producer cell lines, 293 and PER.C6 cells are routinely grown in serum-free medium. A clone of the 293S cell line was developed by Côté et al. (1998) that was able to grow in low calcium serum free medium with a similar growth profile to cells grown in low calcium medium containing 5% bovine serum. In batch culture the 293S cells grew to 5×10^6 cells ml⁻¹ whereas the 293SF-3F6 clone grew to 8×10^6 cells ml⁻¹. When the culture was scaled-up to a 2.8 l bioreactor, densities approached 10^7 cells ml⁻¹ without cell aggregation, and a doubling time of 24 h. There was no significant difference in specific infectious virus production between 293S cells and 293SF-3F6 cells in 125 ml shake flask culture, but a three-fold increase was observed when 293SF-3F6 cells were cultured in a 2.8 l bioreactor, relative to 293S shake flask controls (Côté et al. 1998). A number of proprietary formulations are available for the production of adenoviral vectors including those from JRH Biosciences (Lenexa, KS), Sigma Aldrich (St. Louis, MO), Cambrex (East Rutherford, NJ), and Gibco Life Sciences (Carlsbad, CA). It has been

reported that Sigma's G9916 medium, which is used for the culture of PER.C6 cells, is capable of producing densities of 5.6×10^6 cells ml^{-1} , with an adenovirus titer of 1.5×10^9 particles ml^{-1} when using 125 ml spinner flasks (www.sigmaaldrich.com/img/assets/11200/sg_ls_cc_g9916_chart.jpg). JRH Biosciences have reported that their EX-CELLTM VPRO medium, which is also used to culture PER.C6 cells, can attain densities of 6×10^6 cells ml^{-1} from roller bottle production. The adenovirus titers obtained were 5×10^9 ID₅₀ ml^{-1} (www.jrhbio.com). The EX-CELLTM 293 serum free medium was capable of average densities of 2.5×10^6 cells ml^{-1} with an average doubling time of 33 h. Virus titers were up to 3×10^{10} ID₅₀ ml^{-1} . 293 cells grown in 293 SFM II medium (Gibco Life Sciences) demonstrate a doubling time in the range of 26–40 h and typically achieve cell densities of 3×10^6 cells ml^{-1} in shaker or spinner culture and 4×10^6 cells ml^{-1} in bioreactor culture (www.invitrogen.com/content/sfs/manuals/3919.pdf).

Mode of operation

Adenovirus productivity can be further enhanced by optimizing the process operation. The most simplistic method is batch culture, as no feeding is required and there is a low risk of contamination. As mentioned before in "Production Methods" cell densities of 293 and PER.C6[®] cells can reach densities in the range of $3\text{--}9 \times 10^6$ cells ml^{-1} in suspension bioreactor cultures at scales ranging from 3 to 20 l (Nadeau et al. 1996; Côté et al. 1998; Kamen and Henry 2004). However, despite the greater cell densities, the virus yield is unchanged which is the result of a lower cell specific productivity. The optimal density for virus production has been reported to be 5×10^5 cells ml^{-1} , which is an order of magnitude lower than typical cell densities (Kamen and Henry 2004). The "cell density effect" is probably a result of nutrient limitation and/or byproduct accumulation. Therefore, fed-batch and perfusion cultures have been implemented in order to support high cell densities and maintain specific productivity.

Cell viability in fed-batch cultures is maintained by the addition of nutrients such as glucose, L-glutamine and essential amino acids (EAA). This addition also helps to dilute the concentration of

toxic byproducts such as lactate and ammonia. Fed-batch addition of glucose and EAA after 24 h after the first medium replacement allowed virus production to proceed at cell densities of 2×10^6 cells ml^{-1} ; however, the specific productivity was lower than at an infection density of 10^6 cells ml^{-1} (Nadeau et al. 2000; Nadeau et al. 2002a). Low-glutamine fed batch cultures were able to sustain cell densities of approximately 4.5×10^6 cells ml^{-1} , which was a two-fold increase over batch cultures. By controlling the glutamine concentration at 0.1 mM, a 10-fold increase in virus production was observed in fed-batch over batch cultures. However, cell specific productivity was not reported (Lee et al. 2003).

Perfusion cultures are possibly the most efficient means of virus production, whereby nutrients are continuously replenished and toxic metabolites are removed. Cells are retained in the bioreactor by the use of such instruments as spin filters, acoustic separators, or hollow fiber devices. The density of 293S cells at infection can reach an average of 8×10^6 cells ml^{-1} , with a perfusion rate of 1 reactor volume of medium per day, although the density does decrease after infection to 5.75×10^6 cell ml^{-1} . This, however, is eight times greater than for batch cultures. By reducing the culture temperature to 35 °C the viral yield is reported to be 7.8×10^9 IP ml^{-1} . Furthermore, the specific virus productivity was comparable to batch cultures that were infected at a cell density of 6×10^5 cells ml^{-1} (Cortin et al. 2004). Perfusion experiments with HeLaS3 cells showed a similar trend, with the cell density at infection being $5\text{--}14 \times 10^6$ cells ml^{-1} , and the maximum virus titer being an order of magnitude higher than in fed-batch cultures. It was also found that cell-specific productivity did not change as the cell density increased (Yuk et al. 2004). A comparison between different modes of production for adenovirus using 293 cells is presented in Figure 4.

Adeno-associated viral vectors

Recombinant adeno-associated viruses (rAAV) are non-pathogenic viruses that carry a 4.8 kb single stranded DNA molecule (Lai et al. 2002). Replication requires the functions provided by a helper virus, such as adenovirus or herpes simplex virus, making them naturally replication deficient. rAAV

Figure 4. Production data from published literature and conferences for adenovirus production in batch (Nadeau et al. 2001), fed-batch (Nadeau et al. 2002a) and perfusion culture in a 0.5 l reactor (Garnier et al. 2002) and a 10 l reactor (Nadeau et al. 2002b) using 293 cells.

are attractive vehicles for gene therapy as they efficiently transduce a wide variety of cells including dividing and non-dividing cells, such as muscle and neurons, and they provide long-term gene expression. Unlike adenoviruses, rAAV vectors do not induce a host immune response and they are characterized by excellent safety profiles. However, the size of the genome limits the size of the therapeutic sequence it can carry (Wu and Ataai 2000).

rAAV production requires three essential elements; the vector containing the DNA transgene flanked by AAV inverted terminal repeats, and the AAV *rep* and *cap* genes, which code for replication and structural proteins, respectively. The latter two genes can be supplied *in trans* (Merten et al. 2005). In addition, helper functions derived from adenovirus or herpes simplex virus have to be provided in order to induce rAAV replication. The two most commonly used methods to produce rAAV vectors are transient transfection and the use of stable cell lines containing the required genes. Transient transfection employs the use of 293 or A549 cells that have been cotransfected with two plasmids that contain the rAAV vector and the *rep* and *cap* genes, followed by an infection with helper virus to induce the replication of rAAV. The other possibility is that the cells are cotransfected with three plasmids, that contain the rAAV vector, the *rep* and *cap* genes, and the adenovirus helper genes (Grimm et al. 1998; Xiao

et al. 1998). The use of producer cell lines has mainly focused on HeLa cells, although some investigators have evaluated the use of 293 and A549 cells. These cells contain the rAAV vector and the *rep* and *cap* genes of AAV. For inducing the production of rAAV, the cells have to be infected with the helper virus. Thus, generation of stable cell lines is better suited to large-scale production than transient transfection; nevertheless, generation of such cells can be tedious and time consuming. Furthermore, the highest virus titers for these production methods are typically about 10^7 IP ml^{-1} . It has been estimated that 10^{12} to 10^{14} rAAV particles are required for clinical human use (Clark 2002). In order to overcome these limitations, recent studies have focused on producing rAAV vectors in insect cell cultures, using the recombinant baculovirus system (Urabe et al. 2002). Production of rAAV particles is achieved by co-infecting Sf9 cells with three baculovirus vectors, BacRep, BacCap, and Bac-rAAV. These encode the respective components of the rAAV production machinery. This system lends itself to large-scale production under serum-free conditions, as Sf9 cells are grown in suspension. Infection of Sf9 cells at an MOI of 5 and a ratio of 1:1:1 for all three baculoviruses was able to yield a total of 2.2×10^{12} IP in a 3 l bioreactor. The process was scaled-up to 20 l without loss of productivity and demonstrated that quantities sufficient to meet clinical demand could be met (Meghrouis et al.

Table 2. Production yields of rAAV using different production systems.

Production method	Yield (vg/cell)	Scale-up	Reference
293, triple transfection	$10\text{--}10^3$	Small scale	Salveti et al. (1998); Xiao et al. (1998), Matsushita et al. (1998), Grimm et al. (1999), Collaco et al. (1999).
HeLa based producer cell, rAAV production induced by infection with wt Ad5	$10^4\text{--}10^6$	Reactor scale possible	Blouin et al. (2004), Jenny et al. (2005)
A549 based producer cell, rAAV production induced by infection with a Ad ts	10^5	Reactor scale: 15 l, Larger scale possible	Gao et al. (2002); Farson et al. (2004)
Baculovirus system: Sf9 infected with three different rec. baculoviruses	$10^4\text{--}10^5$	Reactor scale: 20 l, larger scale possible	Meghrou et al. (2005)

2005). Further optimization of this system is required to improve productivity at high cell densities.

Although the baculovirus process has great potential there are still a number of challenges to overcome. The baculovirus has to be carefully eliminated during downstream processing and the toxicity associated with the virus or viral proteins has to be evaluated. Additionally, the baculovirus is genetically unstable and it may be necessary to reduce the amount of baculovirus used in production by designing a single baculovirus which bears all the genetic elements required (Merten et al. 2005). For further information on baculovirus biology and its application in biopharmaceutical research and production the reader is referred to the excellent reviews by Kost et al. (2000) and Mannix and Jarman (2000).

A comparison of the different production systems is presented in Table 2. Further details on production issues of rAAV can be found in a review by Merten et al. (2005).

Herpes simplex virus

Herpes simplex virus (HSV) is a double stranded DNA virus that has ~150 kb genome encoding 80 viral genes. HSV has the largest loading capacity of all viral vectors and is capable of carrying approximately 30 foreign genes (Wu and Ataii 2000). HSV vectors are able to proceed to a latent state of infection, when most of the viral genes are transcriptionally inactivated. This makes HSV an ideal candidate for gene transfer to neurons.

Moreover, the HSV genome remains episomal after infection, which eliminates the problem of insertional mutagenesis and opportunistic malignancy (Mellerick and Fraser 1987).

Production of replication defective HSV vectors in culture is associated with a wide range of cytopathogenic effects, which include cytoplasmic blebbing, chromosomal aberration and host cell DNA fragmentation (Johnson et al. 1992). It has been well established that the immediate-early (IE) genes are responsible for cytotoxicity and IE gene deletion mutants have decreased toxicity, although they still demonstrate some cytopathogenic effects (Wu et al. 1996; Krisky et al. 1998). Unfortunately, HSV vectors with deactivated genes have yields that can be up to 1000 times lower than wild type viruses when produced in T-flask culture. They can also display syncytia, a fusion event causing the formation of polykaryotes. Certain HSV mutants favor cell to cell fusion and spread of their particles as oppose to release into the supernatant. However, by optimizing the length of the growth period, the amount of medium perfused before and after infection and the post-infection period, it was possible to increase the viral yield in a CellCube bioreactor compared to production in either roller bottles or T-flasks. The total virus collected was 3.89×10^9 IP from 7.2 l of medium, which is still significantly lower than wild-type virus (Ozuer et al. 2002). Further optimization of the culture conditions revealed that virus yield showed a 2-fold increase with infection at 33 °C, however, complementing cells had a longer doubling time at 33 °C than at 37 °C and showed a 16–24 h lag in virus production. Con-

Table 3. Optimal culture conditions for the production of retrovirus, adenovirus and herpes virus vectors used in gene therapy applications.

Viral vector	Temperature (°C)	pH	pO ₂ (%)	pCO ₂ (atm)	References
Retrovirus	32–34	7.2	20–80	–	Kotani et al. (1994), Le Doux et al. (1999), McTaggart and Al-Rubeai (2000), Merten (2004)
Adenovirus	35	7.2 (293) 7.3 (PER.C6®)	–	0.05	Jardon and Garnier (2003), Xie et al. (2002)
Herpes virus	37, 33; 24 h post-infection	–	–	–	Wechuck et al. (2002)

sequently, a two stage process was developed where cells were cultured and infected at 37 °C, before lowering the temperature to 33 °C, 24 h post-infection. Extracellular virus production under these conditions increased 3-fold in early passage cells, and up to 10-fold in late passage cells (Wechuck et al. 2002).

Summary

The commercial success of viral gene therapy is dependent on the efficient and reliable production of vectors. To date, the majority of gene therapy protocols have employed the use of retroviral vectors. These are normally produced from adherent cell lines, such as FLYRD, TE FLY and PA317. The most favorable option for their large-scale production is by perfusion fixed-bed reactors, although these systems have inherent limitations in their scalability. Fluidized bed bioreactors have also shown great potential for large-scale operations but the development of these processes is still in its infancy. A suspension packaging cell line has been developed for retrovirus particles, and this may offer the best long-term option for large-scale production. Lentiviral vectors, which are a sub-family of retroviruses, are presently produced by transient transfection in 293 cells although packaging cells are developed in order to improve production conditions and safety issues. Therefore, production processes may resemble those for adenoviral vectors more than for retroviral vectors. Non-replication competent adenoviruses can be transiently produced by infection of 293, PER.C6®, A549, and most recently HeLa cells with a viral vector stock. Although some investigators have used adherent cell lines in microcarrier culture, the majority of production processes now rely on suspension cultures in stirred tank bioreactors. Viral productivity

is limited by cell number in batch reactions; for that reason, perfusion cultures have been implemented for optimized production. Adeno-associated viruses are a more attractive gene vehicle than adenoviruses although the gene insert size is very limited. The most efficient and scalable system for their production is insect cell culture using baculovirus infection. Herpes simplex virus is also an attractive vector as it can hold multiple genes. However, the low viral yields obtained from non-cytotoxic particles are problematic. Production has been achieved in CellCube bioreactors. The optimal culture conditions reported in the literature for retrovirus, adenovirus and herpes simplex virus vectors have been summarized in Table 3.

Although significant improvements have been made in the production of viral vectors, a considerable amount of work still needs to be done if cell culture processes are indeed going to meet the market demand. Further process development is especially needed in the improvement of suspension cell lines for retrovirus production, and in the large-scale production of lentivirus, recombinant adeno-associated virus and herpes simplex virus.

References

- Al-Rubeai M., Emery A.N., Chalder S. and Jan D.C. 1992. Specific monoclonal antibody productivity and the cell cycle-comparisons of batch, continuous and perfusion cultures. *Cytotechnology* 9: 85–97.
- Al-Rubeai M., Rookes S. and Emery A. N. 1990. Studies of cell proliferation and monoclonal antibody synthesis and secretion in alginate-entrapped hybridoma cells. In: de Bont J.A.M., Visser J., Mattiasson B. and Tramper J. (eds), *Physiology of Immobilized cells*. 10–12–1989. Elsevier Science Publishers Cells, Amsterdam, The Netherlands, pp. 181–188.
- Beer C., Buhr P., Hahn H., Laubner D. and Wirth M. 2003a. Gene expression analysis of murine cells producing amphotropic mouse leukaemia virus at a cultivation temperature of 32 and 37 °C. *J. Gen. Virol.* 84: 1677–1686.

- Beer C., Meyer A., Muller K. and Wirth M. 2003b. The temperature stability of mouse retroviruses depends on the cholesterol levels of viral lipid shell and cellular plasma membrane. *Virology* 308: 137–146.
- Benihoud K., Yeh P. and Perricaudet M. 1999. Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* 10: 440–447.
- Blouin V., Brument N., Toublanc E., Raimbaud I., Moullier P. and Salvetti A. (2004). Improving rAAV production and purification: towards the definition of a scaleable process. *J Gene Med.* 6(1): s223–s228.
- Braas G., Searle P.F., Slater N.K.H. and Lydiatt A. 1996. Strategies for the isolation and purification of retroviral vectors for human gene therapy. *Bioseparation* 6: 211–228.
- Breyer B., Jiang W., Cheng H., Zhou L., Paul R., Feng T. and He T.C. 2001. Adenoviral vector-mediated gene transfer for human gene therapy. *Curr. Gene Ther.* 1: 149–162.
- Brooks A.I., Stein C.S., Hughes S.M., Heth J., Mccray P.M., Sauter S.L., Johnston J.C., Cory-Slechta D.A., Federoff H.J. and Davidson B.L. 2002. Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors. *Proc. Natl. Acad. Sci. USA* 99: 6216–6221.
- Carroll R., Lin J.T., Dacquel E.J., Mosca J.D., Burke D.S. and St Louis D.C. 1994. A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system utilizing stable HIV-1 packaging cell lines. *J. Virol.* 68: 6047–6051.
- Chuah M.K., Collen D. and Vanden Driessche T. 2003. Biosafety of adenoviral vectors. *Curr. Gene Ther.* 3: 527–543.
- Clark K.R. 2002. Recent Advances in Recombinant Adeno-Associated Virus Vector Production. *Kidney Int.* 61: 9–15.
- Coleman J.E., Huentelman M.J., Kasparov S., Metcalfe B.L., Paton J.F., Katovich M.J., Semple-Rowland S.L. and Raizada M.K. 2003. Efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use in vivo. *Physiol. Genom.* 12: 221–228.
- Collaco R.F., Cao X. and Trempe J.P. 1999. A helper virus-free packaging system for recombinant adeno-associated virus vectors. *Gene* 238: 397–405.
- Coroadinha A.S., Schuch R., Gama-Norton L., Wirth D., Hauser H. and Carrondo M.J.T. 2005. The use of recombinase Cassette Exchange in retroviral Vector Producer Cell Lines: predictability and efficiency in transgene replacement. *J. Biotechnol.* Submitted.
- Cortin V., Thibault J., Jacob D. and Garnier A. 2004. High-titer adenovirus vector production in 293S cell perfusion culture. *Biotechnol. Prog.* 20: 858–863.
- Côté J., Garnier A., Massie B. and Kamen A. 1998. Serum-free Production of Recombinant Proteins and Adenoviral Vectors by 293SF-3F6 Cells. *Biotechnol. Bioeng.* 59: 567–575.
- Cruz P.E., Almeida J.S., Murphy P.N., Moreira J.L. and Carrondo M.J. 2000. Modeling retrovirus production for gene therapy. I. Determination of optimal bioreaction mode and harvest strategy. *Biotechnol. Prog.* 16: 213–221.
- Cruz P.E., Carmo M., Coroadinha A.S., Bengala A., Goncalves D., Teixeira M., Merten O.-W., Gény-Fiamma C. and Carrondo M.J.T. 2005. Retroviral vector stability: inactivation kinetics and membrane properties. In: Godia F. and Fussenegger M. (eds), *Animal Cell Technology meets Genomics*. Springer, Dordrecht/NL, pp. 303–308.
- Curran M.A., Kaiser S.M., Achacoso P.L. and Nolan G.P. 2000. Efficient transduction of nondividing cells by optimized feline immunodeficiency virus vectors. *Mol. Ther.* 1: 31–38.
- Davis J.L., Witt R.M., Gross P.R., Hokanson C.A., Jungles S., Cohen L.K., Danos O. and Spratt S.K. 1997. Retroviral particles produced from a stable human-derived packaging cell line transduce target cells with very high efficiencies. *Hum. Gene Ther.* 8: 1459–1467.
- Fallaux F.J., Kranenburg O., Cramer S.J., Houweling A., Van Ormondt H., Hoebe R.C. and Van Der Eb A.J. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* 7(2): 215–222.
- Fallaux F.J., Bout A., VanDer Velde I., VanDen Wollenberg D.J., Hehir K.M., Keegan J., Auger C., Cramer S.J., Van Ormondt H., Van Der Eb A.J., Valerio D. and Hoebe R.C. 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum. Gene Ther.* 9(13): 1909–1917.
- Farson D., Harding T.C., Tao L., Liu J., Powell S., Vimal V., Yendluri S., Koprivnikar K., Ho K., Twitty C., Husak P., Lin A., Snyder R.O. and Donahue B.A. 2004. Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. *J. Gene Med.* 6(12): 1369–1381.
- Fassnacht D., Rössing S., Singh R.P., Al Rubeai M. and Pörtner R. 1999. Influence of bcl-2 on antibody productivity in high cell density perfusion cultures of hybridoma. *Cytotechnology* 30: 95–105.
- Forestell S.P., Bohnlein E. and Rigg R.J. 1995. Retroviral end-point titer is not predictive of gene transfer efficiency: implications for vector production. *Gene Ther.* 2: 723–730.
- Forestell S.P., Dando J.S., Chen J., de Vries P., Bohnlein E. and Rigg R.J. 1997. Novel retroviral packaging cell lines: complementary tropisms and improved vector production for efficient gene transfer. *Gene Ther.* 4: 600–610.
- Friedmann T. 1997. Overcoming the obstacles to gene therapy. *Scientific American*, 80–85.
- Gao G.P., Yang Y. and Wilson J.M. 1996. Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J. Virol.* 70: 8934–8943.
- Gao G.P., Lu F., Sanmiguel J.C., Tran P.T., Abbas Z., Lynd K.S., Marsh J., Spinner N.B. and Wilson J.M. 2002. Rep/Cap gene amplification and high-yield production of AAV in an A549 cell line expressing Rep/Cap. *Mol. Ther.* 5: 644–649.
- Gao X. and Huang L. 1995. Cationic liposome-mediated gene transfer. *Gene Ther.* 2: 710–722.
- Garnier A., Cortin V., Thibault J. and Jacob D. 2002. Production of Recombinant Adenovirus by 293 Cells Cultures in Perfusion. *Cell Culture Engineering VIII*, Snowmass, Colorado, April 1–6.
- Garnier A., Cote J., Nadeau I., Kamen A. and Massie B. 1994. Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytotechnology* 15: 145–155.
- Gény-Fiamma C., Millot L., Rocca C., Danos O. and Merten O.W. 2004. Optimization of the production of retroviral vectors: influences of the sugar source. In: Yagasaki K., Miura Y., Hatori M. and Nomura Y. (eds), *Animal Cell*

- Technology: Basic & Applied Aspects. Kluwer Academic Publishers, Netherlands, pp. 89–97.
- Geraerts M., Michiels M., Baekelandt V., Debyser Z. and Gijssbers R. 2005. Upscaling of lentiviral vector production by tangential flow filtration. *J. Gene Med.* Published online May 20, 2005. <http://dx.doi.org/10.1002/jgm.778>.
- Gerin P.A., Gilligan M.G., Searle P.F. and Al-Rubeai M. 1999a. Improved titers of retroviral vectors from the human FLYRD18 packaging cell line in serum- and protein-free medium. *Hum. Gene Ther.* 10: 1965–1974.
- Gerin P.A., Searle P.F. and Al-Rubeai M. 1999b. Production of retroviral vectors for gene therapy with the human packaging cell line FLYRD18. *Biotechnol. Prog.* 15: 941–948.
- Ghivizzani S.C., Lechman E.R., Tio C., Mule K.M., Chada S., McCormack J.E., Evans C.H. and Robbins P.D. 1997. Direct retrovirus-mediated gene transfer to the synovium of the rabbit knee: implications for arthritis gene therapy. *Gene Ther.* 4: 977–982.
- Graham F.L., Smiley J., Russell W.C. and Nairn R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36: 59–74.
- Griffiths J.B. 1988. Overview of cell culture systems and their Scale-up. In: Spier R.E. and Griffiths J.B. (eds), *Animal Cell Biotechnology*, vol. 3. Academic Press Limited, London, pp. 179–220.
- Grimm D., Kern A., Rittner K. and Kleinschmidt J.A. 1998. Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum. Gene Ther.* 9: 2745–2760.
- Grimm D. et al. 1999. Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Ther.* 6: 1322–1330.
- Haselhorst D., Kaye J.F. and Lever A.M. 1998. Development of cell lines stably expressing human immunodeficiency virus type 1 proteins for studies in encapsidation and gene transfer. *J. Gen. Virol.* 79(Pt 2): 231–237.
- He T.C., Zhou S., da Costa L.T., Yu J., Kinzler K.W. and Vogelstein B. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95: 2509–2514.
- Kost T.A., Klein J.L. and Condreay J.P. 2000. Application of recombinant baculoviruses in biopharmaceutical research. In: Al-Rubeai M. (ed.), *Cell Engineering*, Vol. 2: Transient Expression. Kluwer Academic Publications, pp.1–28.
- Ikeda Y., Takeuchi Y., Martin F., Cosset F.L., Mitrophanous K. and Collins M. 2003. Continuous high-titer HIV-1 vector production. *Nat. Biotechnol.* 21: 569–572.
- Imler J.L., Chartier C., Dreyer D., Dieterle A., Sainte-Marie M., Faure T., Pavirani A. and Mehtali M. 1996. Novel complementation cell lines derived from human lung carcinoma A549 cells support the growth of E1-deleted adenovirus vectors. *Gene Ther.* 3: 75–84.
- Imren S., Payen E., Westerman K.A., Pawliuk R., Fabry M.E., Eaves C.J., Cavilla B., Wadsworth L.D., Beuzard Y., Bouhassira E.E., Russell R., London I.M., Nagel R.L., Leboulch P. and Humphries R.K. 2002. Permanent and panerythroid correction of murine beta thalassemia by multiple lentiviral integration in hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* 99: 14380–14385.
- Iyer P., Ostrove J.M. and Vacante D. 1999. Comparison of manufacturing techniques for adenovirus production. *Cytotechnology* 30: 169–172.
- Jardon M. and Garnier A. 2003. pH, pCO₂, and temperature effect on r-adenovirus production. *Biotechnol. Prog.* 19: 202–208.
- Jenny C., Toubanc E., Danos O. and Merten O.-W. 2005. Evaluation of a serum-free medium for the production of rAAV-2 using HeLa derived producer cells. *Cytotechnology*. In press.
- Johnson P.A., Yoshida K., Gage F.H. and Friedmann T. 1992. Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. *Brain Res Mol. Brain Res.* 12: 95–102.
- Johnston J.C., Gasmi M., Lim L.E., Elder J.H., Yee J.K., Jolly D.J., Campbell K.P., Davidson B.L. and Sauter S.L. 1999. Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J. Virol.* 73: 4991–5000.
- Kafri T., van Praag H., Ouyang L., Gage F.H. and Verma I.M. 1999. A packaging cell line for lentivirus vectors. *J. Virol.* 73: 576–584.
- Kamen A. and Henry O. 2004. Development and optimization of an adenovirus production process. *J. Gene Med.* 6: S184–S192.
- Kang S.-H., Lee G.M. and Kim B.-G. 2000. Justification of continuous packed-bed reactor for retroviral vector production from amphotopic ψ CRIP murine producer cell. *Cytotechnology* 34: 151–158.
- Kaptein L.C., Greijer A.E., Valerio D. and van Beusechem V.W. 1997. Optimized conditions for the production of recombinant amphotropic retroviral vector preparations. *Gene Ther.* 4: 172–176.
- Karavodin L.M., Robbins J., Chong K., Hsu D., Ibanez C., Mento S., Jolly D. and Fong T.C. 1998. Generation of a systemic antitumor response with regional intratumoral injections of interferon gamma retroviral vector. *Hum. Gene Ther.* 9: 2231–2241.
- Kioukia N., Nienow A.W., Al-Rubeai M. and Emery A.N. 1996. Influence of agitation and sparging on the growth rate and infection of insect cells in bioreactors and comparison with hybridoma culture. *Biotechnol. Prog.* 12: 779–785.
- Kim S.H., Kim S. and Robbins P.D. 2000. Retroviral vectors. *Adv. Virus Res.* 55: 545–563.
- Kotani H., Newton P.B.III, Zhang S., Chiang Y.L., Otto E., Weaver L., Blaese R.M., Anderson W.F. and McGarrity G.J. 1994. Improved methods of retroviral vector transduction and production for gene therapy. *Hum. Gene Ther.* 5: 19–28.
- Krisky D.M., Wolfe D., Goins W.F., Marconi P.C., Ramakrishnan R., Mata M., Rouse R.J., Fink D.J. and Glorioso J.C. 1998. Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther.* 5: 1593–1603.
- Lai C.M., Lai Y.K.Y. and Rakoczy P.E. 2002. Adenovirus and adeno-associated virus vectors. *DNA Cell Biol.* 21: 895–913.
- Le Doux J.M., Davis H.E., Morgan J.R. and Yarmush M.L. 1999. Kinetics of retrovirus production and decay. *Biotechnol. Bioeng.* 63: 654–662.
- Lee S.G., Kim S., Robbins P.D. and Kim B.G. 1996. Optimization of environmental factors for the production and handling of recombinant retrovirus. *Appl. Microbiol. Biotechnol.* 45: 477–483.

- Lee Y.-Y., Yap M.G., Hu W.S. and Wong K.T. 2003. Low-glutamine fed-batch cultures of 293-HEK serum-free suspension cells for adenovirus production. *Biotechnol. Prog.* 19: 501–509.
- Lever A.M.L., Strappe P.M. and Zhao J. 2004. Lentiviral Vectors. *J. Biomed. Sci.* 11: 439–449.
- Lochmuller H., Jani A., Huard J., Prescott S., Simoneau M., Massie B., Karpati G. and Acsadi G. 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum. Gene Ther.* 5: 1485–1491.
- Loewen N., Leske D.A., Chen Y., Teo W.L., Saenz D.T., Peretz M., Holmes J.M. and Poeschla E.M. 2003. Comparison of wild-type and class I integrase mutant-FIV vectors in retina demonstrates sustained expression of integrated transgenes in retinal pigment epithelium. *J. Gene Med.* 5: 1009–1017.
- Looby D. and Griffiths B. 1990. Immobilisation of animal cells in porous carrier culture. *Trends Biotechnol.* 8: 204–209.
- Lyddiat A. and O'Sullivan D.A. 1998. Biochemical recovery and purification of gene therapy vectors. *Curr. Opin. Biotechnol.* 9: 177–185.
- Mannix C. and Jarman R.F. 2000. A guide to successful scale-up of the baculovirus expression system. In: Al-Rubeai M. (ed.), *Cell Engineering, Vol. 2: Transient Expression*. Kluwer Academic Publications, pp. 43–55.
- Matsushita T., Elliger S., Elliger C., Podsakoff G., Villarreal L., Kurtzman G.J., Iwaki Y. and Colosi P. 1998. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 5: 938–945.
- McTaggart S. 2000. Retroviral Vector Production for Gene Therapy Applications. Ph. D. Thesis, University of Birmingham.
- McTaggart S. and Al-Rubeai M. 2000. Effects of culture parameters on the production of retroviral vectors by a human packaging cell line. *Biotechnol. Prog.* 16: 859–865.
- McTaggart S. and Al-Rubeai M. 2001. Relationship between cell proliferation, cell-cycle phase, and retroviral vector production in FLYRD18 human packaging cells. *Biotechnol. Bioeng.* 76: 52–60.
- McTaggart S. and Al-Rubeai M. 2002. Retroviral vectors for human gene delivery. *Biotechnol. Adv.* 20: 1–31.
- Meghrouh J., Aucoin M.G., Jacob D., Chahal P.S., Arcand N. and Kamen A.A. 2005. Production of recombinant adeno-associated viral vectors using a baculovirus/insect cell suspension culture system: from shake flasks to a 20-L bioreactor. *Biotechnol. Prog.* 21: 154–160.
- Mellerick D.M. and Fraser N. 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. *Virology* 158: 265–275.
- Merten O.-W. 2004. State-of-the-art of the production of retroviral vectors. *J. Gene Med.* 6(Suppl 1): S105–S124.
- Merten O.-W., Cornet V., Petres S., Couvé E. and Heard J.M. 1996. Large scale production of retrovirus vectors (abstract). *Cytotechnology* 21: 8.
- Merten O.-W., Cruz P.E., Rochette C., Gény-Fiamma C., Bouquet C., Goncalves D., Danos O. and Carrondo M.J.T. 2001a. Comparison of different bioreactor systems for the production of high titer retroviral vectors. *Biotechnol. Prog.* 17: 326–335.
- Merten O.-W., Gény-Fiamma C. and Douar A.M. 2005. Current issues in adeno-associated viral vectors production. *Gene Ther.* 12: S51–S61.
- Merten O.-W., Landric L. and Danos O. 2001b. Influence of the metabolic status of packaging cells on retroviral vector production. In: Merten O.-W., Mattanovich D., Lang C., Larsson G., Neubauer P., Porro D., Postma P., Teixeira de Mattos J. and Cole J.A. (eds), *Recombinant Protein Production with Prokaryotic and Eukaryotic Cells A Comparative View on Host Physiology*. Kluwer Academic Publishers, Netherlands, pp. 303–318.
- Michael S.I. and Curiel D.T. 1994. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Ther.* 1: 223–232.
- Mitrophanous K., Yoon S., Rohll J., Patil D., Wilkes F., Kim V., Kingsman S., Kingsman A. and Mazarakis N. 1999. Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther.* 6: 1808–1818.
- Nadeau I., Garnier A., Cote J., Massie B., Chavarie C. and Kamen A. 1996. Improvement of recombinant protein production with the human adenovirus/293S expression system using fed-batch strategies. *Biotechnol. Bioeng.* 51: 613–623.
- Nadeau I., Gilbert P.A., Jacob D., Perrier M. and Kamen A. 2002a. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. *Biotechnol. Bioeng.* 77: 91–104.
- Nadeau I., Jacob D., Perrier M. and Kamen A. 2000. 293SF metabolic flux analysis during cell growth and infection with an adenoviral vector. *Biotechnol. Prog.* 16: 872–884.
- Nadeau I. and Kamen A. 2003. Production of adenovirus vector for gene therapy. *Biotechnol. Adv.* 20: 475–489.
- Nadeau I., Seanez G. and Wu F. 2001. Adenovirus production in 293 cells: a comparative study between a suspension cell and an adherent cell process. The 17th ESACT Meeting, Tylosand, Sweden, June 10–14.
- Nadeau I., Seanez G. and Wu F. 2002b. Optimization of a 293 suspension process for adenovirus production. *Cell Culture Engineering VIII, Snowmass, Colorado*, April 1–6.
- Navarro J., Oudrhiri N., Fabrega S. and Lehn P. 1998. Gene delivery systems: bridging the gap between recombinant viruses and artificial vectors. *Adv. Drug Deliv. Rev.* 30: 5–11.
- Nemunaitis J., Fong T., Burrows F., Bruce J., Peters G., Ognoskie N., Meyer W., Wynne D., Kerr R., Pippen J., Oldham F. and Ando D. 1999. Phase I trial of interferon gamma retroviral vector administered intratumorally with multiple courses in patients with metastatic melanoma. *Hum. Gene Ther.* 10: 1289–1298.
- Ni Y., Sun S., Oparaocha I., Humeau L., Davis B., Cohen R., Binder G., Chang Y.N., Slepishkin V. and Dropulic B. 2005. Generation of a packaging cell line for prolonged large-scale production of high-titer HIV-1-based lentiviral vector. *J. Gene Med.* 7(6): 818–834.
- Olsen J.C. 1998. Gene transfer vectors derived from equine infectious anemia virus. *Gene Ther.* 5: 1481–1487.
- Olsen J.C. and Sechelski J. 1995. Use of sodium butyrate to enhance production of retroviral vectors expressing CFTR cDNA. *Hum. Gene Ther.* 6: 1195–1202.
- Ozuer A., Wechuck J.B., Goins W.F., Wolfe D., Glorioso J.C. and Ataai M.M. 2002. Effect of genetic background and

- culture conditions on the production of herpesvirus-based gene therapy vectors. *Biotechnol. Bioeng.* 77: 685–692.
- Pages J.C., Loux N., Farge D., Briand P. and Weber A. 1995. Activation of Moloney murine leukemia virus LTR enhances the titer of recombinant retrovirus in psi CRIP packaging cells. *Gene Ther.* 2: 547–551.
- Palu G., Bonaguro R. and Marcello A. 1999. In pursuit of new developments for gene therapy of human diseases. *J. Biotechnol.* 68: 1–13.
- Pan D. and Whitley C.B. 1999. Closed hollow-fiber bioreactor: a new approach to retroviral vector production. *J. Gene Med.* 1: 433–440.
- Parasrampur D.A. and Hunt C.A. 1998. Therapeutic delivery issues in gene therapy, Part 1: Vectors. *BioPharm.* 38–45.
- Parks R.J., Chen L., Anton M., Sankar U., Rudnicki M.A. and Graham F.L. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. USA* 93: 13565–13570.
- Pear W.S., Nolan G.P., Scott M.L. and Baltimore D. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 90: 8392–8396.
- Pensiero M.N., Wysocki C.A., Nader K. and Kikuchi G.H. 1996. Development of amphotropic murine retrovirus vectors resistant to inactivation by human serum. *Hum. Gene Ther.* 7: 1095–1101.
- Peshwa M.V., Kyung Y.-S., McClure D.B. and Hu W.-S. 1993. Cultivation of mammalian cells as aggregates in bioreactors: effect of calcium concentration on spatial distribution of viability. *Biotechnol. Bioeng.* 41: 179–187.
- Pizzato M., Merten O.W., Blair E.D. and Takeuchi Y. 2001. Development of a suspension packaging cell line for production of high titre, serum-resistant murine leukemia virus vectors. *Gene Ther.* 8: 737–745.
- Rigg R.J., Chen J., Dando J.S., Forestell S.P., Plavec I. and Bohnlein E. 1996. A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. *Virology* 218: 290–295.
- Robbins P.D., Hideaki T. and Ghivizzani S.C. 1998. Viral vectors for gene therapy. *Trends Biotechnol.* 16: 35–40.
- Sadaie M.R., Zamani M., Whang S., Sistrion N. and Arya S.K. 1998. Towards developing HIV-2 lentivirus-based retroviral vectors for gene therapy: dual gene expression in the context of HIV-2 LTR and Tat. *J. Med. Virol* 54: 118–128.
- Sallberg M., Hughes J., Javadian A., Ronlov G., Hultgren C., Townsend K., Anderson C.G., O'Dea J., Alfonso J., Eason R., Murthy K.K., Jolly D.J., Chang S.M., Mento S.J., Milich D. and Lee W.T. 1998. Genetic immunization of chimpanzees chronically infected with the hepatitis B virus using a recombinant retroviral vector encoding the hepatitis B virus core antigen. *Hum. Gene Ther.* 9: 1719–1729.
- Salveti A., Orevé S., Chadeuf G., Favre D., Cherel Y., Champion-Arnaud P., David-Ameline J. and Moullier P. 1998. Factors influencing recombinant adeno-associated virus production. *Hum. Gene Ther.* 9: 695–706.
- Schiedner G., Hertel S. and Kochanek S. 2000. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum. Gene Ther.* 11: 2105–2116.
- Schnell T., Foley P., Wirth M., Munch J. and Uberla K. 2000. Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. *Hum. Gene Ther.* 11: 439–447.
- Schonely K., Afable C., Slepishkin V., Lu X., Andre K., Boehmer J., Bengtson K., Doud M., Cohen R., Berlinger D., Slepishkina T., Chen Z., Li Y., Binder D., Davis B., Humeau L. and Dropulic B. 2003. QC release testing of an HIV-1 based lentiviral vector lot and transduced cellular product. *Bioproc. J.* 2: 29–47.
- Schucht R., Coroadinha A.S., Zanta-Boussif M.A., Carrondo M., Hauser H. and Wirth D. 2005. A new generation of retroviral producer cells: predictable and stable virus production by Flp mediated site-specific integration of retroviral vectors. *Mol. Thera.* Submitted.
- Sena-Esteves M., Tebbets J.C., Steffens S., Crombleholme T. and Flake A.W. 2004. Optimized large-scale production of high titer lentivirus vector pseudotypes. *J. Virol. Methods* 122: 131–139.
- Shen B.Q., Clarke M.F. and Palsson D.O. 1996. Kinetics of retroviral production from the amphotropic ψ CRIP murine producer cell line. *Cytotechnology* 22: 185–195.
- Shenk T. 1996. Adenoviridae: the viruses and their replication. In: Fields B.N., Knipe D.M., Howley P.M., Chanock R.M., Melnick J.L., Monath T.P., Roizman B. and Straus S.E. (eds), *Fields Virology*. Philadelphia, Lippincott pp.2111–2148.
- Sheridan P. L., Bodner M., Lynn A., Phuong T.K., DePolo N.J., la Vega D.J.Jr., O'Dea, J., Nguyen K., McCormack J.E., Driver D.A., Townsend K., Ibanez C.E., Sajjadi N.C., Greengard J.S., Moore M.D., Respass J., Chang S.M., Dubensky T.W.Jr., Jolly D.J. and Sauter S.L. 2000. Generation of retroviral packaging and producer cell lines for large-scale vector production and clinical application: improved safety and high titer. *Mol. Ther.* 2: 262–275.
- Starling E.H. 1896. On the absorption of fluids from the connective tissue space. *J. Physiol.* 19: 312–326.
- Stütz J., Muhlebach M.D., Blomer U., Scherr M., Selbert M., Wehner P., Steidl S., Schmitt I., König R., Schweizer M. and Cichutek K. 2001. A novel lentivirus vector derived from apathogenic simian immunodeficiency virus. *Virology* 291: 191–197.
- Takahashi K., Luo T.C., Saishin Y., Saishin Y., Sung J., Hackett S., Brazzell R.K., Kaleko M. and Campochiaro P.A. 2002. Sustained transduction of ocular cells with a bovine immunodeficiency viral vector. *Hum. Gene Ther.* 13: 1305–1316.
- Theodossiou I., Thomas O.R.T. and Dunnill R. 1999. Methods for enhancing the recovery of plasmid genes from neutralised cell lysate. *Bioproc. Eng.* 20: 147–156.
- Torrent C., Bordet T. and Darlix J.L. 1994. Analytical study of rat retrotransposon VL30 RNA dimerization in vitro and packaging in murine leukemia virus. *J. Biol. Med.* 240: 434–444.
- Urabe M., Ding C. and Kotin R.M. 2002. Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene Ther.* 13: 1925–1943.
- Van Den Driessche T., Vanslembrouck V., Goovaerts I., Zwinnen H., Vanderhaeghen M.L., Collen D. and Chuah M.K. 1999. Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. *Proc. Natl. Acad. Sci. USA* 96: 10379–10384.
- Vos J.-M. H. 1995. *Viruses in Human Gene Therapy*. Chapman and Hall.

- Wang G., Davidson B.L., Melchert P., Slepishkin V.A., van Es H.H., Bodner M., Jolly D.J. and McCray P.B.Jr. 1998. Influence of cell polarity on retrovirus-mediated gene transfer to differentiated human airway epithelia. *J. Virol.* 72: 9818–9826.
- Warnock J.N. and Al-Rubeai M. 2004. Influence of serum concentration on cell growth and retrovirus production and decay kinetics. In: Yagasaki K., Miura Y., Hatori M. and Nomura Y. (eds), *Animal Cell Technology: Basic & Applied Aspects*. Kluwer Academic Publishers.
- Warnock J. N. and Al-Rubeai M. 2005. Production of Biologics from Animal Cell Cultures. In: Nedovic V. and Willaert R. (eds), *Applications of Cell Immobilisation Biotechnology*. Springer, Berlin, Heidelberg, New York.
- Warnock J.N., Price T., Slade A. and Al-Rubeai M. 2004. Use of a Fluidised Bed Bioreactor for the Production of Retroviral Vectors for Gene Therapy Applications. *Bioproc. J.* 3: 41–45.
- Wechuck J.B., Ozuer A., Goins W.F., Wolfe D., Oligino T., Glorioso J.C. and Ataai M.M. 2002. Effect of temperature medium composition and cell passage on production of herpes-based viral vectors. *Biotechnol. Bioeng.* 79: 112–119.
- Wikström K., Blomberg P. and Islam K.B. 2004. Clinical grade vector production: analysis of yield, stability, and storage of gmp-produced retroviral vectors for gene therapy. *Biotechnol. Prog.* 20: 1198–1203.
- Wu N. and Ataai M.M. 2000. Production of viral vectors for gene therapy applications. *Curr. Opin. Biotechnol.* 11: 205–208.
- Wu N., Watkins S.C., Schaffer P.A. and DeLuca N.A. 1996. Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J. Virol.* 70: 6358–6369.
- Wu S.C., Huang G.Y. and Liu J.H. 2002. Production of retrovirus and adenovirus vectors for gene therapy: a comparative study using microcarrier and stationary cell culture. *Biotechnol. Prog.* 18: 617–622.
- Xiao X., Li J. and Samulski R.J. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* 72: 2224–2232.
- Xie L., Metallo C., Warren J., Pilbrough W., Peltier J., Zhong T., Pikus L., Yancy A., Leung J., Aunins J.G. and Zhou W. 2003. Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6 cell culture under sparging conditions. *Biotechnol. Bioeng.* 83: 45–52.
- Xie L., Pilbrough W., Metallo C., Zhong T., Pikus L., Leung J., Aunins J.G. and Zhou W. 2002. Serum-free suspension cultivation of PER.C6(R) cells and recombinant adenovirus production under different pH conditions. *Biotechnol. Bioeng.* 80: 569–579.
- Yamaji H. and Fukuda H. 1992. Growth and death behavior of anchorage-independent animal-cells immobilized within porous support matrices. *App. Micro. Biotechnol.* 37: 244–251.
- Yuk I.H.Y., Olsen M.M., Geyer S. and Forestell S.P. 2004. Perfusion cultures of human tumor cells: A scalable production platform for oncolytic adenoviral vectors. *Biotechnol. Bioeng.* 86: 637–642.