Engineering delivery and release of protein therapeutics for mammalian systems

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In recent years conventional therapeutic strategies using small molecule drugs have been complemented by new approaches in the field of modern biotechnology. Innovative therapeutic strategies include the delivery of genetic information that is capable of replacing inherited genetic defects and the delivery of protein therapeutics such as monoclonal antibodies or recombinant cytokines or cell- and tissue-engineering approaches.

A major hurdle still to overcome in gene-therapy protocols is the efficient delivery of genetic information to a targeted cell and the stringent control of expression of the delivered genes. Currently viral systems are the most promising tools for gene therapy and the most comprehensive pre-clinical and clinical data is available for these systems.

The first chapter of this work summarizes the protocols and compares the production and use of different viral transduction systems for mammalian cells.

The second chapter describes the construction of recombinant adeno-associated viral vectors (rAAV), which are attracting more and more attention for gene therapy applications, for the efficient and safe delivery of transgenes controllable by a small molecule antibiotic. Vectors for the delivery of binary ON- and OFF-type, auto-regulated, self-regulated and bi-directional systems were constructed and functionally tested in various mammalian cell lines. Delivery and regulation of these systems in vivo was confirmed by injecting the engineered rAAV vectors into mice.

Model reporter proteins simplify the analysis of new delivery, release and control scenarios in vitro and in vivo. The third chapter of this work describes the design and evaluation of a novel enzyme-based reporter system for mammalian cells. Engineered bacteria-derived reporter proteins specifically designed for localization either intracellularly or extracellularly were generated. Functional expression of these constructs was confirmed in different cell lines and extensive analysis of their biological and chemical characteristics was performed. These new proteins provide an interesting alternative to currently available reporter systems for mammalian cells due to their simple and sensitive assay formats that require no sample pre-treatment as well as their compactness and high stability.
A major limitation of the use of biologics in therapeutic interventions is that their structural complexity and fragility causes difficulties in safe and efficient administration. Treatments using these factors would need either repeated pulsed administrations or a continued release of the active substance over a prolonged period of time. In the fourth chapter of this work a versatile delivery system based on biocompatible polymer microcapsules is described. This system can be implanted and allows for the trigger-induced release of encapsulated biologics upon an external cue. A release system was designed where sensor cells within the capsules express, upon induction by a chemical cue, an enzyme that degrades the polymer structure and leads to the collapse of the particle. Co-encapsulated biologics can be released “on demand” and by changing the sensor cell line a variety of different cues can be used to trigger the burst.

Overall, this work presents new strategies to improve the delivery, monitoring, expression-control and release of biologics utilizing engineered mammalian cells, viral vectors and polymer capsules.
Zusammenfassung

In den letzten Jahren wurden traditionelle Behandlungsstrategien mittels "small molecule drugs" durch neue Ansätze aus dem Gebiet der modernen Biotechnologie ergänzt. Innovative Strategien beinhalten unter anderem:

(1) Die Administration genetischer Information, welche in der Lage ist vererbte Defekte zu komplementieren.
(2) Die Anwendung von auf Proteinen basierenden Therapeutika wie z.B. monoklonale Antikörper oder rekombinant hergestellte Zytokine.
(3) Neue Ansätze in den Gebieten der Zell- und Gewebetherapie.


Im zweiten Kapitel wird die Konstruktion von rekombinanten Adeno-Assoziierten viralen Vektoren (rAAV), welche für gentherapeutische Anwendungen immer mehr in den Mittelpunkt rücken, beschrieben. Vektoren für die Applikation von binären, auto-regulierten, selbst-regulierten und bidirektionalen Systemen, welche durch das Antibiotikum Erythromycin reguliert werden können, wurden konstruiert und ihre Funktionalität in verschiedenen Säugerzellen getestet. Zusätzlich wurde die kontrollierte Expression eines Glykoproteins nach Administration eines selbst-regulierten rAAV in vivo gezeigt.

Reporterproteine vereinfachen die Analyse von neuen Administrations-, Freisetzungs- und Kontrollsystemen in vitro und in vivo. Das dritte Kapitel befasst sich mit dem Design und der Evaluation eines neuen, enzymbasierenden Reportersystems für Säugerzellen. Es wurden von einem bakteriellen Protein abgeleitete Reporterkonstrukte, speziell konzipiert für die intra- und extrazelluläre Lokalisation, entwickelt. Die funktionelle Expression dieser Enzyme wurde in...
verschiedenen Säugerzellinien gezeigt und die biologischen und chemischen Eigenschaften wurden detailliert analysiert. Diese neuen Reporterproteine stellen, aufgrund ihrer Kompaktheit, der hohen Stabilität, sowie ihrer einfachen und sensitiven Analyseformate, welche keine Vorbehandlung der Proben erfordern, eine Alternative zu gegenwärtig erhältlichen Reportersystemen dar.


Introduction
General Introduction

Over the last 25 years, since human insulin became the first medicine to be produced using recombinant technologies, modern biotechnology has become a promising field for the development of new drugs and therapies. Recombinant protein drugs such as insulin, interferon, growth hormones, recombinant vaccines and therapeutic antibodies have made it to the market over the years as new biotech and pharmaceutical companies expand their research and development investments in the field (Lahteenmaki and Lawrence, 2007). Besides the production of therapeutic proteins, mammalian biotechnology also provides new tools to cure diseases. Gene and cell based therapies provide new strategies to fight diseases that so far have been challenging to treat by existing therapies (Edelstein et al., 2007; Lillicrap et al., 2006; Melo et al., 2004; Mimeault et al., 2007; Sueblinvong et al., 2007).

Gene therapy

In the gene therapy field, more than 1300 clinical trials have been completed since 1989 when Rosenberg et al. conducted the first immunotherapy for patients with advanced melanoma (Rosenberg et al., 1990). Although gene therapy is still regarded as the ultimate cure for genetic diseases by attacking the source of the disease, optimally foregoing the need for a life-long therapy of symptoms, severe set-backs in the field have slowed or even stalled the development of new therapies (Hacein-Bey-Abina et al., 2003; Raper et al., 2003). Extensive risk/benefit examinations have been conducted and research in the field has been directed towards the understanding of potential risks associated with gene therapy protocols (Check, 2003; Gansbacher, 2002). In spite of the setbacks associated with first clinical trials, the effort in bringing gene therapy to a safe and efficient state where it can be used to treat severely ill patients is still prevailing and recent work shows promising results (Morgan et al., 2006; Ott et al., 2006).

The precise delivery of genes and dose-dependant expression of the corresponding protein products are the determining criteria for successful gene therapy applications. Viral vectors are considered the most promising vehicles to efficiently deliver foreign genetic information to a targeted cell-nucleus (Edelstein et al., 2007). Today, the most commonly used viral vector systems are based on oncoretrovirus (Landau and Littman, 1992; Peer et al., 1993), lentivirus (Naldini et al., 1996; Zufferey et al., 1997), adenovirus (Bett et al., 1994; Chartier et al., 1996),
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herpes simplex virus (Krisky et al., 1997) and adeno-associated virus (Xiao et al., 1998). Each of these vectors have unique properties that make them more or less suitable for a particular application, and all of these systems, except the lentiviral vectors, have been evaluated in clinical trials (Edelstein et al., 2007).

Vectors were engineered to exploit the highly efficient viral machinery to deliver genetic information to cells. These viral vectors are able to infect a broad range of different cell types but are incapable of causing a productive infection. After major setbacks using retroviral vectors and adenoviral vectors in clinical trials, much of the hope in the gene therapy community relies on recombinant adeno-associated viral vectors (rAAV). rAAV has shown promising pre-clinical and clinical results (Jiang et al., 2006; Kaplitt et al., 2007; Li et al., 2005; Wang et al., 2000) and has superior safety characteristics among all currently used viral vector systems. rAAV vectors integrate into the host chromosome at a significantly lower frequency than retroviral or lentiviral vectors and more than 95% of transgene expression stems from stable circular episomes in the target cell. This characteristic reduces the risk of insertional mutagenesis and thereby decreases proto-oncogene activation significantly. These characteristics combined with a high transduction efficiency for a broad variety of tissues as well as the stability of rAAV particles makes this system a premium choice for gene therapy applications. A significant shortcoming of rAAV vectors is their low packaging capacity. The delivery of an entire genomic locus including spacious endogenous regulatory sequences is beyond the capacity of currently used rAAV vectors. Even the delivery of smaller promoter-transgene cassettes encounters size constraints in certain applications. As a result, vector design has become crucial for successful rAAV-based gene therapy. Different strategies have been employed to optimize and compress the delivered cassettes or engineer the vector system in such a way that larger amounts of genetic information can be transferred (Duan et al., 2000; Urabe et al., 1997; Yan et al., 2000).

Particularly in settings where the expression of delivered transgenes has to be regulated, a compact architecture is a prerequisite to encoding of all the elements of the regulation system. Many gene regulation systems for mammalian cells are available. These regulation systems respond to an array of varying stimuli and allow the precise control of expression levels of delivered transgenes (Weber and Fussenegger, 2006). Vector designs encoding compact control systems, mainly the tetracycline-responsive TET system (Gossen and Bujard, 1992), have been
extensively studied in oncoretroviral, lentiviral systems (Kafri et al., 2000; Koponen et al., 2003; Regulier et al., 2002; Reiser et al., 2000; Unsinger et al., 2001), and to a lesser extent in rAAV (Chtarto et al., 2003; Gafni et al., 2004; Jiang et al., 2004). Although the building blocks for safe delivery and the subsequent regulation of transgene expression are established, a long road of improved safety and efficiency lays ahead before these gene therapy techniques can be utilized as efficient weapons for the treatment of diseases.

**Reporter Proteins**

Reporter proteins are valuable tools for quick, precise and sensitive analysis of gene expression in biological systems. Fluorescent proteins such as GFP and its derivatives (Hadjantonakis et al., 2003), luciferases (de Wet et al., 1985; Lorenz et al., 1991), various enzymatic reporters (An et al., 1982; Gorman et al., 1982; Tjuvajev et al., 1995) or secreted reporter genes (Berger et al., 1988; Schlatter et al., 2002) changed the landscape of many domains of modern biology; allowing the detection and quantification of effects that have not been analyzed before. Many aspects of the development of biologics have to be closely monitored in order to optimize the design, production, delivery and expression of the factors. Reporter proteins provide an approach to monitor these crucial steps and aid in the simplification and streamlining of the development process. Reporter proteins are commonly used and provide important information on the optimization of production processes (Reischer et al., 2004), the engineering of producer cell lines (Choe et al., 2005; Sleiman et al., 2008), the tracing of implanted cells (Rome et al., 2007), the quantification of expression levels upon delivery (Bartoli et al., 2006; Wang et al., 2001) and the fate of certain cells following implantation (Schiedlmeier et al., 2003).

One group of reporter proteins is secreted from cells through conventional pathways and then analyzed extracellularly. These reporter proteins represent the final product of a complex cascade of events including transcription, transcript processing, mRNA export from the nucleus, translation, posttranslational modification and protein export. Utilizing secreted reporter proteins allows one to measure these parameters and therefore they are a powerful tool for applications such as metabolic engineering (Choi et al., 2006; Park et al., 2005), gene regulation (Weber et al., 2002), synthetic biology (Kramer and Fussenegger, 2005; Weber et al., 2007), gene therapy (Gronevik et al., 2005; Lamartina et al., 2003) or glycosylation monitoring (Lipscomb et al., 2005).
A limitation of certain secreted reporter proteins is the difficulty to distinguish the activity of the engineered factor from signals arising from cognate endogenous proteins. This often leads to lower signal to background ratios and thus reduces the sensitivity of the assay. For some reporter systems, such as human placental secreted alkaline phosphatase (SEAP), these ratios can be improved by inactivating endogenous signals by heat treatment or specific inhibitors (Berger et al., 1988; Schlatter et al., 2002). Other reporter protein, such as human growth hormone or erythropoietin, have the intrinsic limitation that direct activity assays are nonexistent and expression levels have to be quantified via labor intensive methods, such as an ELISA (Muramatsu et al., 2001; Selden et al., 1986). For some applications gene size restraints exist, such as for viral delivery or multi-cistronic expression systems, so the length of the coding region of the reporter protein becomes crucial. To overcome these limitations compact systems have been developed to free space for other elements. The development of new secreted reporter proteins that are heterologous, compact, sensitive, stable, high-throughput analysis capable and compatible to existing reporter platforms represent a useful complement to existing systems.

**Drug delivery**

Apart from gene therapy, other fields of modern biotechnology have shown promising results for the treatment of various severe diseases such as cancer, multiple sclerosis, Alzheimer, Parkinson disease and autoimmune diseases. Protein therapeutics employing monoclonal antibodies, hormones or cell-based platforms have shown promising pre-clinical and clinical results (Dove, 2002; Leader et al., 2008).

Due to their chemical and biological properties, protein and cell therapeutics have to be administered by injection or released from special implanted devices. Although repeated injection is currently, and will likely be in the future, the major route of administration of biologics due to its convenience for many treatments other delivery modes would be more advantageous in certain applications. The annual growth in the field of drug delivery systems has been significant and an increasing number of companies enter the market with innovative strategies for drug delivery (Orive et al., 2003c). Systems for oral (Veronese and Harris, 2002), transdermal (Langer, 2003) and transmucosal drug delivery (Illum et al., 2001) have been developed over the last few years. In addition to these advancements in administration and dosing of therapeutics, another promising approach is the immunoisolation and in
situ production of biologics. Cells expressing biologics are isolated from the immune system by polymeric membranes that enable the free transfer of nutrients and waste products. These membranes also prevent the access of the different players of the immune system to the contents of the capsule, be they allogeneic or xenogeneic cells (Orive et al., 2004). Various promising research initiatives have been initiated recently using encapsulation protocols (Orive et al., 2003a). Polymer capsules have been used to encapsulate different types of biologics including protein therapeutics (Blanco-Prieto et al., 2000; Han et al., 2001), vaccines (Rosas et al., 2002; Sturesson et al., 1999), liposomes (Dhoot and Wheatley, 2003; Kibat et al., 1990; Lee et al., 2003), DNA (Alexakis et al., 1995; Lengsfeld et al., 2002; Zhang et al., 2004), viral vectors (Sailaja et al., 2002) and whole cells/tissues (Luca et al., 2007; Read et al., 2001; Springer et al., 2000; Wang et al., 1997).

There are various modes of release for therapeutics depending on needs of the system, these include: (i) continuous release over time (Hasse et al., 1997; Rinsch et al., 2002), (ii) initial burst followed by decreasing release over time (Chretien and Chaumeil, 2005; Pelegrin et al., 1998; Wang et al., 1997), (iii) pulsed release patterns (Bussemer et al., 2003; De Geest et al., 2005) and (iv) inducible release triggered by internal and external cues (Angelatos et al., 2005; Fischel-Ghodsian et al., 1988; Skirtach et al., 2004). In particular the rupture of polymer capsules, and release of their contents, “on-demand” represents an appealing approach to deliver therapeutic molecules when they are needed in the body. Different approaches have been investigated for the release of therapeutics on demand comprising inducible capsule rupture and release of encapsulated cargo by triggers such as an electric field (Kiser et al., 1998), IR light (Angelatos et al., 2005), a magnetic field (Lu et al., 2005), a pH change (Dejugnat and Sukhorukov, 2004), swelling or osmotic bursting of the capsule (De Geest et al., 2005; Iskakov et al., 2002), enzymatic degradation of the polymer capsule (De Geest et al., 2006; Itoh et al., 2006) or destabilization of the capsule induced by changes in salt and redox states (Haynie et al., 2005; Schuler and Caruso, 2001). Although significant progress in the field of delivery of biologic drugs has been made there still remain numerous obstacles to overcome. These obstacles include the mechanical and chemical stability of capsules, biosafety, long-term survival of capsules, biocompatibility and long-term functionality (Orive et al., 2003b).
Due to the complexity of gene- and cell-therapies and protein therapeutics, efficient administration, dosage and formulation is much more complex than for traditional small molecule drugs. To harness the potential of these new classes of therapeutics novel methods of biologic delivery have to be developed and existing technologies have to be improved and adapted.

**Contributions of this work**

**Chapter 1: Transduction Technologies**

This chapter presents basic methodologies for the production and use of the most commonly utilized virus-based vectors for the delivery of genetic information to mammalian cells. Optimized production protocols for viral delivery vectors namely, oncoretrovirus, lentivirus, adenovirus and adeno-associated virus are provided. The intention was to provide a thorough groundwork from where one can choose an optimal transduction system for a given application. Each viral system has its inherent advantages and disadvantages. The choice of a virus will depend on the required characteristics such as the ability to integrate genetic material into the host genome, the tropism, the packaging capacity, safety profiles, immunogenicity and available production and concentration protocols.

The optimized protocols allow for the production of both crude and highly pure vector preparations suitable for *in vitro* and *in vivo* use. The last section of the chapter consists of a case study partly derived from chapter 2 of this work describing the application of rAAV vectors in the transduction and regulated expression of secreted and intracellular transgenes in mammalian cells.

The chapter is written in a protocol-based style as it has been published in a methodology book for the establishment of cell lines for biotechnology applications.

**Chapter 2: Adeno-associated viral vectors engineered for macrolide-adjustable transgene expression in mammalian cells and mice**

We have combined the efficiency and safety of recombinant adeno-associated viral vectors of serotype 2 (rAAV2) with the effectiveness of a gene regulation system (Weber et al., 2002) that responds to a clinically licensed antibiotic in order to deliver and control transgenes *in vitro* as well as *in vivo*. 
rAAV vectors were constructed for binary ON-type and OFF-type systems expressing either intracellular or secreted reporter protein under the control of erythromycin responsive promoters. These engineered rAAV vectors were able to regulate reporter expression in an array of different mammalian cell lines. Binary systems have the advantage that relatively large transgenes can be delivered, which is crucial for viral vector system having a small packaging capacity such as rAAV. A considerable disadvantage of binary systems is the requirement that both parts of the system i.e. both vectors have to infect the same cell in order to obtain functional regulatable expression. To circumvent this we designed compact single vectors that were auto-regulated, self-regulated and bi-directional which harbored all of the necessary elements for functional regulation together with the gene of interest (GOI). Although good induction factors were obtained for all three types of one-vector systems, only the self-regulated rAAV2 vector was able to obtain high transduction rates. Using the self-regulated one-vector rAAV2 system mice were transduced and regulatable expression of a secreted reporter gene was observed in vivo.

Chapter 3: InXy and SeXy, compact heterologous reporter proteins for mammalian cells

Intracellular (InXy) and secreted (SeXy) bacterial xylanase-derived reporter proteins were created allowing for a quick and sensitive quantification of activity in the supernatant and cell pellet of mammalian cells. InXy and SeXy are entirely heterologous proteins in mammalian cells and direct measurement of their activity from cell culture supernatants or lysed cell pellets without pretreatment is possible. Both reporters are robust and maintain activity after incubation at a broad range of temperatures, pH conditions and after long term incubation in complex biological mixtures such as human serum. InXy and SeXy reporters are compatible with other commonly used reporter proteins thus permitting parallel analysis. SeXy is extensively glycosylated when secreted from various mammalian cell lines and could be utilized to monitor biopharmaceutical processes since most of the currently produced biopharmaceuticals are glycoproteins. In vivo SeXy was shown to circulate in the blood stream of mice after being infected with rAAV particles hosting a SeXy expression cassette.
Chapter 4: A novel versatile system for stimulus-controlled drug release from polymer capsules

The tailored degradation of polymer capsules, upon the action of certain cues, is a promising approach to design delivery scenarios for stimulus-dependent release of therapeutic molecules.

A new, flexible system was created that was able to respond to selectable triggers either applied from the outside, such as antibiotics, hormones, amino acids, biotin, or directly linked to endogenous stimuli, such as oxygen levels or quorum-sensing molecules at the site of infection.

Clinically tested cellulose sulfate (CS)/poly-dimethyl-diallylammonium chloride (pDADMAC) encapsulation technology (Merten et al., 1991) was combined with the controlled inducible expression of a bacteria-derived cellulase that destabilizes the capsule resulting in the release of its therapeutic cargo. The functionality of the modified secreted cellulase (SecCell) was tested in various mammalian cell types and a stable cell line was created, using HEK293-T, which expressed SecCell under the control of either a doxycycline- or an erythromycin-responsive promoter. The molecular weight cut-off of the capsule system was determined to be around 40 kDa which is in an attractive range for the delivery of biologics. The trigger-induced release of the model glycoprotein SEAP was illustrated by co-encapsulating a sensor cell population, which expresses SecCell that is repressed by the presence of doxycycline, together with a cell line that constitutively produces SEAP. In the presence of doxycycline SEAP was observed to be exclusively accumulating inside the capsule. In the absence of this molecule SecCell was expressed by the sensor cells, disrupted the capsule and SEAP was released into the supernatant. Micrographs and time-lapse images reveal a collapse of the capsule structure upon SecCell expression. Release characteristics could be modulated by varying the time of induction, the number of sensor cells encapsulated or by titrating SecCell induction levels with different concentrations of inducer. It was also shown that the capsules were exclusively ruptured when SecCell was expressed internally. This characteristic of the capsules allows for the co-administration of varying capsule-populations containing different sensor cells responsive to changing stimuli. One can imagine applications where multiple capsule-populations containing specific biologics co-encapsulated with different stimuli-responsive sensor cells could be administered...
at once. Then these different biologics could be released at desired points in time according to the presence of different stimuli.

Moreover, it was shown by injecting capsules containing both a model glycoprotein and doxycycline-responsive sensor cells into mice that an \textit{in vivo} inducible release of the glycoprotein into the bloodstream is possible.
References


Introduction


Introduction


CHAPTER 1

Transduction Technologies

Abstract

Safe and reliable transfer of genetic information at high frequencies into desired target cells remains one of the major challenges in therapeutic life sciences. Multiply attenuated viral vector systems, which are able to transfer heterologous DNA or RNA into almost any cell phenotype as well as in vivo, emerged as the most efficient transgene delivery systems in modern biology. We provide a comprehensive technical overview on the most commonly used viral transduction systems and compare their specific characteristics in order for researchers to make the best choice in a particular scientific setting.

1. Introduction

The successful transfer of genetic material to target cells still represents a major experimental challenge in all aspects of molecular life sciences including biotechnology and therapeutic initiatives. A promising delivery method involves the use of engineered animal and human viruses. Such vectors are designed to transfer a desired transgene by exploiting the efficient delivery machinery of the wild-type virus while at the same time minimizing the risk of pathogenicity.

Different systems, based on animal as well as human viruses, have recently been developed and, in some cases, advanced to clinical application. Worldwide more than 400 clinical trials using viral vectors have been, or are currently being, conducted (Edelstein et al., 2004). The majority of these trials have been performed using retroviral vectors, followed by adenoviral and other viral vector systems, among them adeno-associated viral (AAV) vectors. Due to the high transfer efficiency, and reasonably straightforward production protocols, viral vectors have also become increasingly popular for routine applications in various fields of life sciences.

The majority of viral vector systems have been designed to retain, and even extend, the viruses’ cell tropism while ensuring high biosafety characteristics. For some viruses the ability to transduce many cell types is intrinsically present (e.g. adenovirus or AAV). Other viruses have a far narrower host range (e.g. lentiviruses which are mainly restricted to CD4-expressing cells). Such viruses must be altered through pseudotyping with envelope or capsid proteins from other compatible virus
species thereby enabling the recombinant vector to transduce a broader variety of cell types.

To ensure biosafety most vectors are rendered replication incompetent through selective deletion of wild type viral genetic information, thereby preventing productive viral life cycles within the target cell (Somia and Verma, 2000). In addition to the removal of non-essential sequences from the wild-type genome, a fundamental principle underlying the production of recombinant viral particles is the redistribution of indispensable elements to either helper plasmids or producer cell lines. The resulting free space flanked by the viral inverted/long terminal repeats (ITRs/LTRs) is then employed to accommodate the heterologous DNA to be delivered. For a number of vector types only one viral open reading frame (ORF) is replaced and delivered in trans (e.g. first generation adenovirus) (Danthinne and Werth, 2000), whereas for other types the entire viral coding sequence is replaced by foreign DNA with only minimal viral elements retained (e.g. adeno-associated virus, “gutless” adenovirus) (Morsy et al., 1998; Xiao et al., 1998).

The architecture of a viral vector has significant impact on the safety of the transduction system (see Figure 1). Typically, the more the viral genome is segregated and lacks overlapping sequences, the smaller is the probability for recombination-based generation of replication-competent revertants during the production process. Since wild-type virus is on the whole highly optimized and adapted to its environment, altering its organization and expression patterns, as done for the construction of viral vectors, is likely to decrease production efficiency. This often manifests in lower titers, poor encapsidation of viral genomes or changes in transduction efficiency of recombinant viral particles. It is therefore crucial to find an optimal balance between high-safety profiles while retaining high efficiency and quality of viral particle production.

Every viral transduction system has its unique characteristics which render it more suitable for one or another application. Crucial parameters influencing selection of appropriate transduction systems are outlined in Table 1. For every application one should carefully consider the advantages and drawbacks of each system, and decide according to the experimental requirements.

This chapter provides detailed protocols and guidelines for the production and purification of the four most common viral transduction systems; namely, oncoretroviral, lentiviral, adenoviral and adeno-associated viral vectors. At the end of
the chapter we provide an example in which adeno-associated viral vectors have been engineered to deliver adjustable reporter genes \textit{in vitro} to exemplify the convenience of viral delivery systems for various applications.

\textbf{Figure 1.} Schematic representation of the most popular viral transduction systems. Elements originating from the wild-type virus, which are retained in transfer vectors, are underlined. Only essential viral elements are shown and not drawn to scale. Abbreviations: cap, gene coding for structural AAV proteins; cPPT, central polypurine tract; E1-E4, adenovirus early transcription units 1-4; env, gene encoding envelope protein; gag, gene encoding core proteins (capsid, matrix, nucleocapsid); ITR, inverted terminal repeat; pol, gene encoding viral reverse transcriptase and integrase proteins; L1-L5, adenovirus late transcription units 1-5; LTR, long terminal repeat; rep, gene coding for replication and multiple other AAV functions; RRE, rev responsive element; nef, rev, tat, vif, vpr, vpu, genes encoding for accessory lentiviral proteins; VA, gene encoding viral associated RNA; \(\psi\), packaging signal.
Table 1. Selection criteria for viral transduction systems

<table>
<thead>
<tr>
<th>Feature</th>
<th>Oncoretroviral vectors</th>
<th>Lentiviral vectors</th>
<th>Adenoviral vectors</th>
<th>Adeno-associated viral vectors</th>
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<tr>
<td>Transduction of non-dividing cells</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Stability of viral particle</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Episomal maintenance</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Integration into the chromosome</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Extensive packaging capacity</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fast production protocols</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>High safety profile</td>
<td>+/-</td>
<td>+/-</td>
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<td>+</td>
</tr>
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<td>Low immunogenicity</td>
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<tr>
<td>Clinical data available</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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2. Materials

2.1.1 General Materials

1. HEK293-T cells (Mitta et al., 2002)

2. DMEM complete medium: DMEM medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany, cat. no. 3302-P231902) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO, cat. no. P4458-100)
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3. Trypsin solution: Trypsin-EDTA solution in PBS (PAN Biotech GmbH, Aidenbach, Germany, cat. no. P10-023100)

4. Sterile CaCl₂ (1M) solution

5. Sterile 2X HeBS solution (HeBS; 0.28M NaCl, 0.05M HEPES, 1.5mM Na₂HPO₄, pH 7.08)

6. Phosphate-buffered saline solution without magnesium and calcium, pH 7.2 (PBS, Sigma, cat. no. D-5652)

7. Cell culture-certified disposable plastic ware: Petri dishes, 6-well plates, 24-well plates (TPP, Trasadingen, Switzerland), pipets, Falcon tubes 15ml and Eppendorf tubes 1.5ml (Greiner bio-one, Kremsmuenster, Austria),

8. Ultracentrifuge tubes (e.g. Beckman Quick-Seal tubes [Beckman cat. no. 342 413] or Sorvall, centrifuge tubes [Sorvall, cat. no. 03141 ])

9. 0.45µm filters (Schleicher & Schuell GmbH, Dassel, Germany)

10. Plasmid DNA purification kits (Jet-Star 2.0 Midiprep, Genomed AG, Bad Oeynhausen, Germany; Wizard mini-prep kit, Promega, Madison, USA)

11. Cell counting device (Casy1® counter, Schaerfe System, Reutlingen, Germany; alternatively, a hemacytometer can be used)

12. Incubator (e.g. Hereaus, HERAcell, Langenselbold, Germany) for cultivation of mammalian cells at 37°C in a humidified atmosphere containing 5% CO₂

13. Ultracentrifuge equipped with swing-out rotor (e.g. Sorvall, Hanau, Germany)

2.1.2 Materials for Oncoretroviral/Lentiviral Vectors

1. GP-293 cells (Clontech, Palo Alto, CA, USA, cat. no. K1063-1)

2. pVSV-G (Clontech)

3. pLEGFP-N1 (Clontech)

4. pMF365 (Mitta et al., 2002)

5. pCD/NL-BH* (Mochizuki et al., 1998)

6. pLTR-G (Reiser et al., 1996)

7. Advanced DMEM (Gibco, cat. No. 12491-015)

8. Chemically defined lipid concentrate (Gibco, cat. no. 11905-031)

9. Egg lecithin (Serva Electrophoresis GmbH, cat. no. 27608)

10. Cholesterol (Sigma, cat. no. C-4951, diluted in PBS)
2.1.3 Materials for Adenoviral Vectors

1. Buffer A (10mM Tris-HCl, 1mM MgCl₂, 135mM NaCl, pH7.5)
2. Buffer B (buffer A +10% Glycerol)
3. Cesium chloride (Gibco, cat. no. 15507-023)
4. Agarose (Brunschwig, Basel, Switzerland, cat. no. 8008)
5. Phenol/chloroform/isoamylalcohol 25:24:1 saturated with 10mM Tris/1mM EDTA (Sigma, cat. no. P-3803)
6. Ethanol 96%
7. TE Buffer (10mM Tris, 1mM EDTA)
8. Glycerol (Fluka, cat. no. 49767)
9. 16G needles (Becton Dickinson, Franklin Lakes, NJ, USA)
10. Dialysis tubing (Sigma, cat. no. D9777-100FT)
11. Dialysis closures (Sigma, cat. no. Z370959-10EA)
12. HEK293 (Microbix, Toronto, Canada)
13. Adenoviral type 5 genomic vector pJM17 (Microbix)
14. Adenovector encoding desired transgene (e.g. pVN31 (Gonzalez-Nicolini and Fussenegger, 2005))
15. Proteinase K (Qiagen GmbH, Hilden, Germany, cat. no. 19131)

2.1.4 Materials for Adeno-Associated Viral Vectors

1. pDG (Grimm et al., 2003)
2. Spin columns MWCO 300’000 (Vivaspin, Vivascience, Germany)
3. Heparin affinity columns (HiTrap heparin HP, Amersham Biosciences, Sweden)
4. Pump, allowing flow rates of 2ml/min
5. Liquid nitrogen or dry ice/ethanol bath
6. Binding buffer (10mM Na₂PO₄, pH 7)
7. Elution buffer (10mM Na₂PO₄, 1M NaCl, pH 7)
8. Storage buffer (binding buffer containing 20% ethanol)
9. PBS-MK buffer (PBS 1x containing 1mM MgCl₂ and 2.5mM KCl)
10. Benzonase (Sigma, cat. no. E1014)
11. Optiprep™ iodixanol solution (Axis-Shield, Rodelokka, Norway)
12. Phenol Red solution 0.5% (Sigma, cat. no. P0290)

2.1.5 Materials for Trigger Inducible Transgene Expression

1. HT-1080 cells (ATCC, CCL-121)
2. AAV vector encoding desired transgene (e.g. pDF141, Figure 2)
3. Erythromycin (Fluka, Buchs, Switzerland, cat. no. 4573) stock solution of 1mg/ml in 96% ethanol
4. 2x SEAP buffer: (20mM homoarginine, 1mM MgCl₂ and 21% (v/v) diethanolamine/HCl, pH 9.8.)
5. pNPP solution (120mM para-nitrophenylphosphate [pNPP hexahydrate, Chemie Brunschwig, Basel, Switzerland, cat. no. 12886-0100]) in 2x SEAP buffer
6. Microplate reader (GeniusPro, Tecan Group Ltd., Maennedorf, Switzerland)
7. Fluorescence microscope (DMI6000B, Leica Inc., Heerbrugg, Switzerland) equipped with CFP and YFP-specific filters

3. Methods

All of the viral vectors introduced in this manual are classified as biosafety level 2 according to the National Institutes of Health (NIH). Working with these systems therefore requires appropriate safety equipment and governmental regulations may apply. Additional precautions must be followed if working with vectors hosting toxic or oncogenic inserts.

The production protocols for all of the following viral vectors are based on comparable techniques. Nevertheless, the protocols for each viral vector system have been separately provided (apart from general procedures which are discussed in the first chapter) to ensure simplicity for the user and to provide comprehensive instructions for each virus type.
3.1. General Procedures

All plasmids were propagated in *Escherichia coli* DH5α. Standard cloning techniques were performed to engineer plasmids (Sambrook and Russell, 2001). The DNA used for virus production was purified using a silica-based anion-exchange DNA purification kit (Genomed) or with “Wizard” mini-prep kits (Promega) (see Note 1), according to the manufacturers’ protocols. DNA concentrations were measured with an UV spectrometer (BioPhotometer, Eppendorf, Hamburg, Germany) and only DNA with a 260/280 coefficient of greater than 1.75 was used for transfections. Unless otherwise specified, HEK293 cells were cultivated in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin solution in a humidified atmosphere at 37°C containing 5% CO₂ (see Note 2).

3.2. Oncoretroviral/Lentiviral vectors

In this chapter oncoretroviral and lentiviral vectors are introduced together as the two systems are closely related and share numerous features. However, separate production protocols are provided for each vector system to simplify their utilization.

Both, oncoretroviral and lentiviral vectors are enveloped RNA viruses packaging two copies of the viral RNA genome. Oncoretroviral vectors have been derived from different animal viruses such as murine leukemia virus (MLV), rous sarcoma virus (RSV) or avian leucosis virus (ALV) and were among the first viral transduction systems used in gene therapy. The most commonly used lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1) (Mitta et al., 2002; Mochizuki et al., 1998). However, members of the lentivirus family, such as simian immunodeficiency viruses (SIV’s), feline immunodeficiency virus (FIV) or equine infectious anaemia virus (EIAV), also serve as parental viruses for the generation of vectors for gene therapy purposes (Curran and Nolan, 2002). Oncoretroviruses and lentiviruses randomly integrate as proviruses into target chromosomes. In contrast to oncoretroviruses, lentiviruses encode accessory genes, which enable transduction of mitotically inert target cells.

Oncoretroviral vector design is based upon the replacement of the viral gag, pol and env open reading frames (ORFs) by the desired transgene; either under the control of a heterologous promoter, or the viral long terminal repeat (LTR). The only
elements required in cis are the extended packaging signal (Ψ+) and the viral LTRs together with adjacent regions essential for reverse transcription and integration (Mann et al., 1983; Miller et al., 1993).

The design of lentivectors is very similar to oncoretroviral vectors but requires additional viral elements provided in trans such as the Rev and Tat proteins. Besides the LTRs and the packaging signal, lentiviral vectors also contain the Rev-responsive element (RRE) and the central polypurine tract (cPPT) to enable efficient nuclear transport and second DNA strand priming, respectively.

Both oncoretroviral and lentiviral vectors can be efficiently pseudotyped with envelope proteins from other virus species enabling the relatively narrow host-range of the wild-type virus to be broadened to a wider variety of cell types. The envelope protein most frequently used for pseudotyping is derived from the vesicular stomatitis virus glycoprotein (VSV-G), which shows fusogenic activity and uses ubiquitous cell-surface receptors (Naldini et al., 1996; Reiser et al., 1996).

Retroviral vectors have become very attractive for gene therapy initiatives due to their stable integration of genetic information combined with low immunogenicity, improved safety profiles through use of self-inactivating third-generation vectors (Bukovsky et al., 1999) and simple production protocols. Nonetheless, safety concerns remain due to random transgene integration resulting in the risk of proto-oncogene activation or insertional mutagenesis.

In the following chapter we cover the production of small-scale crude oncoretroviral and lentiviral particle preparations by transient transfection methods and provide simple purification and concentration protocols using ultracentrifugation.

### 3.2.1 Retroviral Vector Production

Day 1: Seeding of cells for transfection:

1. Seed GP-293 cells at a concentration of 300’000 cells per 6 well and cultivate at 37°C in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin solution
Day 2: Calcium phosphate transfection of GP-293:

1. Prepare Eppendorf tube containing 80µl of HeBS solution

2. Prepare Eppendorf tube containing 2µg pVSV-G, and 2µg of plasmid encoding the retroviral expression construct (e.g. pLEGFP-N1) in 60µl ddH₂O. Add 20µl of 1M CaCl₂ solution

3. Add DNA mix drop-wise to HeBS solution while bubbling with a pipette, vortex 5s and let sit for 2min. Meanwhile, add chloroquine at a final concentration of 25µM to the cells. Add precipitates to the cells and let sit for 6h

4. Replace medium with fresh complete DMEM and incubate for another 48h

Day 4: Virus harvest:

1. Collect virus-containing supernatant from GP-293 culture and filter through a 0.45µm filter (Schleicher & Schuell)

2. Aliquot the viral particle preparation and freeze at -80°C

3. Concentrate viral stocks by ultracentrifugation for 3.5h at 4°C and 70’000g in an ultracentrifuge equipped with a swing-out rotor and optiseal tubes (Beckman Instruments inc., CA; cat. no. 361625)

4. Discard supernatant and resuspend the pellet in an appropriate volume of PBS and store aliquots at -80°C or in liquid nitrogen (see Note 5)

### 3.2.2 Lentiviral Vector Production

Day 1: Seeding of cells for transfection:

1. Seed 500’000 HEK293-T cells in one well of a 6-well plate and cultivate at 37°C in Advanced DMEM supplemented with 2% FCS, 0.01mM cholesterol, 0.01mM egg lecithin and 1x chemically defined lipid concentrate (see Note 3)
Day 2: Calcium phosphate transfection of HEK293-T:

1. Prepare Eppendorf tube containing 80µl of HeBS solution

2. Prepare Eppendorf tube containing 1µg pCD-NL (helper construct), 1µg pLTR-G (construct encoding the VSV-G gene) and 1µg of the vector encoding the lentiviral expression construct (e.g. pMF365) in 60µl ddH2O. Add 20µl of 1M CaCl2 solution

3. Add DNA mix drop-wise to HeBS solution while bubbling with a pipette, vortex 5s and let sit for 2min. Meanwhile, add chloroquine at a final concentration of 25µM to the cells. Add precipitates and let sit for 5h

4. Replace medium with fresh Advanced DMEM containing 2% FCS, cholesterol, egg lecithin and chemically defined lipid concentrate. Let sit for a further 48h

Day 4: Virus harvest:

1. Collect virus-containing medium from HEK293-T cells and filter through a 0.45µm filter (Schleicher & Schuell) (see Note 4)

2. Aliquot the viral particle preparation and freeze at -80°C or in liquid nitrogen (see Note 5)

3. Concentration of viral stocks: lentiviral particles can be purified using the protocol for oncoretroviral vector ultracentrifugation

3.3. Adenovirus

Adenoviral vectors are among the oldest and most widely used vectors for gene therapy applications. The advantages of this vector type are the extensive packaging capacity, the ability to transduce a variety of different cell types (including non-dividing cells), and its potential for high-titer preparations. Adenovirus is a non-enveloped icosahedral structure, and for its genome contains a double-stranded DNA molecule of 30-40kb. The genome can be classified according to the expression time
point of two major overlapping regions; the early E and the late L open reading frames. More than 50 distinct human adenovirus serotypes are known of which some can cause respiratory, intestinal or eye infections.

Adenoviral vectors have been generated by deleting E1 and E3 (Danthinne and Imperiale, 2000) (first generation), E1, E3 and E2/4 (Wang et al., 1995) (second generation), and adenoviruses devoid of any viral genes containing only the packaging signal and the viral ITRs (high capacity, “gutless” vectors) (Kochanek et al., 2001). First-generation vectors are able to host transgenes of up to 8kb. Following deletion of additional information in 2nd- and 3rd-generation vectors the packaging capacity has been increased up to 35kb for gutless vectors which was associated with a significant reduction in immunogenicity.

Several different production systems for first-generation adenoviral vectors are available. Older systems use homologous recombination in HEK293 cells to generate recombinant adenoviral vectors. Homologous recombination between sequences on the left-end of the adenovirus genome, and sequences on the shuttle plasmid containing the transgene as well as the right end of the adenoviral genome, lead to recombinant virus (Bett et al., 1994). Newer systems take advantage of recombination-triggering enzymes, such as the phage P1-derived enzyme Cre, to enable efficient recombination of the viral components (i) in vitro (Wang et al., 1995) (ii) in bacteria (Aoki et al., 1999; Chartier et al., 1996) or (iii) in a Cre-expressing helper cell line (Ng et al., 1999).

The following protocol is a standard method for producing first-generation adenoviral vectors by homologous recombination in HEK293 cells. This protocol can be adapted to other production systems by changing helper plasmids and production cell lines.
3.3.1 Production of Adenoviral Particles

3.3.1.1 Production of First-Round Virus

Day 1: Seed $8 \times 10^5$ HEK293 (Microbix) cells in a 6cm culture dish

Day 2: CaCl$_2$ transfection for virus production:

1. Prepare Eppendorf tube containing 240µl of HeBS solution

2. Prepare Eppendorf tube containing 3µg of pJM17 (genomic plasmid) and 3µg of shuttle plasmid (e.g. pVN31 (Gonzalez-Nicolini and Fussenegger, 2005)) encoding the transgene in 180µl ddH$_2$O. Add 60µl of 1M CaCl$_2$ solution

3. Add the DNA mix drop-wise to the HeBS solution while bubbling with a pipette, vortex 5s and let sit for 2min. Add the precipitates to the cells and let sit for 6h.

4. Remove medium and overlay cells with a mix of complete DMEM containing 0.5% agarose (see Note 6)

Day 5-10: Plaques should appear within 5-10 days

1. Pick plaque with sterile Pasteur pipette and transfer to 1ml of PBS containing 10% glycerol (optionally freeze at -80°C until use)

3.3.1.2 Analysis of Viral DNA by Test Digestion

It is recommended that restriction analysis of the backbone is performed prior to amplification of the virus:

Day 1: Seed $8 \times 10^5$ HEK293 (Microbix) cells in a 6cm dish

Day 2: Transduction:

1. Add 200µl of virus suspension from 3.3.1.1. to the cells (cells should be at 80% confluence) and adsorb virus for 30min by shaking plate from time to time to disperse solution over cells

2. Add 5ml of DMEM +10% FCS and incubate at 37°C

3. Cytopathic effect (CPE) should be visible after 3-4 days.
Day 3-4: DNA isolation and restriction digests:

1. Remove medium gently by avoiding the transfer of cells, transfer to Falcon tube and freeze at -80°C
2. Remove remaining medium on the cells and add 360µl of TE buffer containing 0.5% SDS and 0.2mg/ml of Proteinase K and digest overnight
3. Transfer lysate to Eppendorf tube and extract 1x with saturated phenol. Transfer aqueous phase and transfer to new tube
4. Add 1ml of ethanol and vortex briefly
5. Wash twice with ethanol to remove phenol
6. Dry pellet and resuspend in 50µl TE buffer
7. Digest with appropriate restriction enzyme and run on agarose gel (see Note 7)

3.3.1.3 Amplification of Adenoviral Particle Stock

After vector verification, the initial stock can be used for further amplification steps:

Day 1: Seed desired numbers of HEK293 cells in 10cm dishes.

Day 2: When cells reach 80-90% confluence:

1. Remove medium and infect at a MOI of 1-10 (see Note 8) per cell.
2. After 30-60min remove virus solution and re-feed with DMEM +10% FCS. Incubate in humidified atmosphere and check daily for CPE

Day 4-6: When most of the cells are rounded up but not detached:

1. Scrape the cells from the plate with a cell-scraper and transfer cells with the medium to a falcon tube.
2. Centrifuge at 2000g for 15min, remove supernatant and resuspend pellet in desired volume of PBS and freeze at -80°
3. Freeze-thaw 3x by shuttling the sample between liquid nitrogen and 37°C water bath
4. Centrifuge the cell lysate for 15 minutes at 3000rpm to pellet cell debris
5. Transfer supernatant to new tube \(\rightarrow\) crude viral stock (see Note 5)
3.3.1.4 Purification of Particles by Caesium Chloride (CsCl) Gradient

For certain applications (mostly *in vivo* experiments) subsequent purification of crude adenoviral preparations is required. Presented below is a standard purification method using CsCl centrifugation:

1. Add 1.5ml of a 1.45g/ml caesium chloride solution and 2.5ml of a 1.33g/ml of a cesium chloride solution to a 7ml ultraclear tube (place the least dense layer at the bottom of the tube, float this layer on top of the more dense layer again by placing the tip of the syringe at the bottom of the tube)

2. Thaw crude viral stock and layer the virus solution on top of the gradient

3. Add 2mm of mineral oil on top of the virus solution and equilibrate tubes

4. Centrifuge for 2h at 90’000g (at 4°C)

5. Remove the lowest of the three visible white bands by side-wall puncture with a 16G needle (Becton Dickinson)

6. Dilute the virus fraction with 0.5 volumes of TE buffer pH 7.8

7. Layer the solution on top of a second gradient prepared as above but this time with 1ml of 1.45g/ml and 1.5ml of 1.33g/ml cesium chloride and proceed as in step 1c, 2 and 3

8. Recover the virus particle-containing band, transfer to dialysis tubing, seal with clamps and dialyse for 1h against 1l of buffer A

9. Transfer tubing to another beaker containing 1l of buffer B (buffer A + 10% glycerol) and dialyse for another 2h

10. Aliquot virus and freeze at -80°C (see Note 5)

3.4. Adeno-Associated Virus (AAV)

In recent years adeno-associated viral vectors have become widely used in clinical initiatives due to their unique safety properties. So far, no disease has been attributed to the wild-type virus even though a majority of the human population is sero-positive for one or more of the different subtypes. For productive infection AAV is dependent on a helper virus, which is either adenovirus or herpes simplex virus. In the absence of helper functions the AAV enters a non-productive life cycle and integrates site specifically into a locus on human chromosome 19 (Kotin et al., 1990; Samulski et al., 1991). Recombinant adeno-associated viruses lack this characteristic
and remain predominantly episomal (ca. 90%), although a small proportion does still randomly integrate into the host chromosome (ca. 10%) (Nakai et al., 2001).

The unique design of recombinant adeno-associated viral vectors (rAAVs), where all viral open reading frames have been replaced and only the ITRs are delivered \textit{in cis}, renders this transduction system exceptionally safe. Furthermore, the ability of adeno-associated viral vectors to transduce non-dividing cells and provide sustained expression of the transgene in a wide variety of tissues makes AAVs one of the most promising candidates for clinical applications.

Standard production protocols use transient transfection of plasmids encoding the desired transgene(s) flanked by viral ITRs together with a plasmid providing the AAV Rep and Cap proteins \textit{in trans}. HEK293 production cells already provide the crucial adenoviral functions performed by viral E1A and E1B. In addition, other adenoviral proteins such as E2A, E4orf6 and VA are required for efficient production of AAVs. In older protocols these functions are provided by helper adenovirus infection of producer cells (Hermonat and Muzyczka, 1984; Samulski et al., 1989). A major drawback of such protocols is the contamination of rAAV stocks with wild-type helper adenovirus. State-of-the-art protocols replace helper virus infection completely by providing the essential proteins on engineered helper plasmids.

In this section we provide a production protocol where all adenoviral helper functions (except E1) are combined with Rep/Cap on a single plasmid (Grimm et al., 2003). Even though titers obtained with such helper virus-free production protocols are occasionally lower, the advantages in respect of purity and safety outbalance the drawbacks.

Also provided in this section are detailed protocols for the production and purification of crude adeno-associated viral stocks, as well as purification and concentration strategies for use in animal studies.
3.4.1 Production of Crude Adeno-Associated Viral Vector Preparations

Day 1: Seeding of cells for transfection:

1. Seed 300'000 HEK293-T cells per well of a 6-well plate (or for medium-scale production seed 2.5 Mio cells into a 10cm culture dish)

Day 2: Calcium phosphate transfection of HEK293-T:

1. Prepare tube containing 80µl (400µl) of HeBS solution
2. Prepare tube containing 1.2µg (8µg) plasmid containing transgene of interest flanked by the viral ITRs and 2.4µg (16µg) pDG<sup>28</sup> (helper plasmid containing all required adenovirus functions plus AAV rep and cap ORFs) in 60µl (300µl) ddH<sub>2</sub>O. Add 20µl (100µl) of 1M CaCl<sub>2</sub> solution
3. Add DNA drop-wise to HeBS solution while bubbling the solution using a pipette, vortex the mix for 5s and let sit for 2min. Add the precipitates drop-wise to the cells and let sit for another 6h
4. Replace medium with fresh complete medium and incubate for 60h

Day 5: Virus harvest:

1. In the morning, remove medium from cells, wash carefully with 400µl (2ml) PBS and add another 500µl (2ml) of PBS to the cells
2. Detach cells by pipetting with a 1000µl micropipet, transfer cell-suspension to an Eppendorf (15ml Falcon) tube and vortex vigorously
3. Freeze-thaw 3x by shuttling the sample between 37°C water bath and liquid nitrogen (vortex vigorously after each thawing step)
4. Centrifuge for 5min at 8000g
5. Transfer supernatant to new tube (➔ crude viral stock) and store at -80°C (see Note 5)

3.4.2 Virus Purification (for Medium-Scale High-Purity Virus Produced in 10cm Culture Dishes)

1. Add 50U/ml Benzonase (Sigma) to crude viral stock and incubate for 30min at 37°C
2. Dilute crude viral stock in PBS to a final volume of 12ml
3. In an ultracentrifuge tube prepare an iodixanol step gradient by pipetting the crude viral preparation onto a solution containing 15% iodixanol and 1M NaCl in PBS-MK buffer. Continue to sequentially pipet 5ml of a 25%, 4ml of a 40% and 4ml of a 60% iodixanol solution (all in PBS-MK) under the 15%
iodixanol solution. For better distinction of the iodixanol layers, add 2.5µl/ml of a 0.5% Phenol Red stock solution to the 60% and 25% iodixanol layers.

4. Centrifuge for 3.5h at 150’000g (18°C)

5. Harvest the clear 40% iodixanol fraction after puncturing the tube on the side with a 16G needle equipped with a syringe.

6. Equilibrate heparin affinity column with 5-10 bed volumes of binding buffer

7. Filter gradient-purified virus preparation through a 0.45µm filter (Schleicher & Schuell), and run it over the column at a flow rate of approximately 1.5ml/min

8. Wash with 10 bed volumes of binding buffer

9. Elute bound virus with 5 bed volumes of elution buffer

10. Transfer eluate to a vivaspin column and spin for 20min at 3800g, resuspend in 5ml PBS and spin another 20min at 3800g

11. Resuspend in the desired volume of PBS, aliquot and freeze at -80°C

### 3.5. Trigger-Inducible Transgene Transduction Using Engineered AAV

Systems enabling regulated transgene expression are invaluable tools in different aspects of molecular life sciences. Delivery of a desired transgene coupled with the capability to tightly regulate its expression is essential for various applications (Clacksion, 1997; Fussenegger, 2001; Fussenegger et al., 1998; Weber and Fussenegger, 2002). Presented above are different methods for efficient delivery of transgenes to target cells capitalizing on viral transduction systems. Here we provide an application example using adeno-associated viral vectors to transfer and regulate intracellular and secreted reporter transgenes. This protocol may be amenable to other combinations of viral vectors, transgenes and transgene control systems.
Figure 2. Plasmid maps of adeno-associated viral vectors engineered for macrolide-responsive expression of transduced transgenes. Vectors encoding (i) the macrolide-dependent transactivator ET1 driven by a constitutive promoter $P_{hCMV}$ (pDF51), (ii) SEAP driven by the erythromycin-responsive promoter $P_{ETR}$ (pDF77) and (iii) a macrolide-responsive self-regulated one-vector configuration combining $P_{SV40}$-driven ET1 and $P_{ETR}$-driven EYFP expression (pDF141). Abbreviations: bla, beta lactamase; ET1, erythromycin transactivator; EYFP, enhanced yellow fluorescent protein; ITR, inverted terminal repeat; ORI, origin of replication; $pA_{hgh}$, human growth hormone polyadenylation signal; $pA_{SV40}$, simian virus 40 polyadenylation signal; $P_{hCMV}$, human immediate early cytomegalovirus promoter; $P_{ETR}$, erythromycin-responsive promoter; $P_{SV40}$, simian virus 40 promoter; SEAP, secreted alkaline phosphatase.
3.5.1 Viral Transduction using adeno-associated virus

Day 1: Seeding of cells:
1. Seed 40'000 HT-1080 cells per well of a 24-well plate (9 wells) and 200’000 HT-1080 cells per well of a 6-well plate (2 wells)

Day 2: Transduction:
1. Thaw vector stocks in a 37°C water bath and vortex tubes briefly
2. Add pDF141-derived AAVs at a multiplicity of infection (MOI) of 5 (see Note 8) to 2 wells of 6-well plate and pDF77- and pDF51-derived AAVs at identical MOIs to 6 wells of a 24-well plate (see Figure 2 for plasmid maps)
3. Add Erythromycin (EM) at 50ng/ml to one of the wells transduced with pDF141 and to 3 of the wells containing pDF143
4. Incubate overnight at 37°C in a humidified atmosphere containing 5% CO₂

Day 3: Medium change:
1. Change medium the following day and add new DMEM complete, supplemented with 50ng/ml EM where transgene expression should be repressed
2. Incubate for another 48h in a humidified atmosphere containing 5% CO₂

Day 5: Analysis:

Fluorescence microscopy:
Analyze samples using a fluorescence microscope (Leica) equipped with appropriate filters (CFP/YFP filter cube, Leica) (see Figure 3)

SEAP quantification:
1. Harvest 120µl supernatants per well from pDF77/51-transduced cells and 120µl from non-transduced cells as a negative control
2. Heat inactivate supernatants for 20min at 65°C
3. Incubate for 5min on ice and then spin down at top speed for 5min
4. Prepare 9 wells of a 96-well assay plate with 100µl of 2x SEAP assay buffer, pre-warm at 37°C
5. Transfer 80µl culture supernatant to wells containing 2x SEAP buffer
6. Add 20µl substrate solution to each well
7. Quantify absorbance at 405nm for up to 1h in a microplate reader (Tecan) (see Figure 3).

1. Subtract values of the blank from sample values
2. Calculate enzymatic activity

\[
EA \ [U/L] = \frac{\Delta \text{Abs/min} \cdot \nu \cdot \varepsilon \cdot d}{10^6}
\]

Where:
- Dilution factor \( n \): volume measurement/volume sample = 200/80
- Absorption factor of \( \varepsilon \) = 18600 M\(^{-1}\) cm\(^{-1}\)
- Lightpath \( d \) = 0.5cm

**Figure 3.** Macrolide-responsive transgene transduction using engineered adeno-associated viral particles. (A) SEAP expression levels of HT-1080 cells co-transduced with pDF51- and pDF77-derived AAV particles (see Figure 2). (B) Fluorescence micrographs of HT-1080 transduced with pDF141-derived AAV particles delivering a self-regulated macrolide-responsive EYFP expression unit and grown in the presence (+EM) and absence of erythromycin (-EM). Abbreviations: EM, erythromycin; SEAP, secreted alkaline phosphatase
4. Notes

1. To perform transfections we recommend using ethanol or iso-propanol-precipitated DNA for sterility reasons. For constructs used in larger amounts (e.g. helper constructs) we recommend performing midi- or maxi- DNA preparations in order to minimize batch-to-batch variations of virus preparations. For small-scale virus preparations, DNA purification methods based on rapid precipitation-free protocols (e.g. Wizard mini DNA purification system) are sufficient.

2. We observed decreasing efficiency of viral particle production upon transient transfection when using HEK293 cells at higher passage number and recommend therefore to work with low-passage cell populations.

3. Production of lentiviral particle stock is feasible using standard DMEM containing 10% FCS without providing cholesterol, egg lecithin and chemically defined lipid concentrate but infectious titers are significantly lower (Mitta et al., 2005).

4. It is crucial to use low protein binding filters.

5. Storage at -80° (up to 6 months) is sufficient. For long-term storage we recommend liquid nitrogen. Oncoretroviral and lentiviral vectors should be aliquoted in small volumes since preparations loose approximately half of their infectivity with every freeze-thaw step.

6. Some protocols forgo this step by directly cultivating the cells in liquid medium and then harvesting the entire supernatant for further procedures. We recommend initial plaque purification and analysis of the first produced viral stocks to ensure consistent starting material for further steps.

7. Upon complete CPE the DNA preparation should be sufficiently pure for restriction analysis. Nevertheless, genomic DNA will be visible as background smear and some enzymes (e.g. HindIII) repeatedly cut genomic DNA yielding bands which are not to be confused with viral DNA.

8. Vectors encoding for fluorescent proteins are titrated by applying serial dilutions of vector preparations to target cells in 96-well plates (at low density) and subsequent counting of transgene-expressing cells by fluorescence microscopy.
5. References


CHAPTER 2

Adeno-Associated Viral Vectors Engineered For
Macrolide-Adjustable Transgene Expression In
Mammalian Cells and Mice

David A. Fluri, Marie Daoud-El Baba and Martin Fussenegger (2007).
BMC Biotechnology, Nov 6;7:75
Abstract

Background

Adjustable gene expression is crucial in a number of applications such as de- or transdifferentiation of cell phenotypes, tissue engineering, various production processes as well as gene-therapy initiatives. Viral vectors, based on the Adeno-Associated Virus (AAV) type 2, have emerged as one of the most promising types of vectors for therapeutic applications due to excellent transduction efficiencies of a broad variety of dividing and mitotically inert cell types and due to their unique safety features.

Results

We designed recombinant adeno-associated virus (rAAV) vectors for the regulated expression of transgenes in different configurations. We integrated the macrolide-responsive E.REX systems (E_{ON} and E_{OFF}) into rAAV backbones and investigated the delivery and expression of intracellular as well as secreted transgenes for binary set-ups and for self- and auto-regulated one-vector configurations. Extensive quantitative analysis of an array of vectors revealed a high level of adjustability as well as tight transgene regulation with low levels of leaky expression, both crucial for therapeutical applications. We tested the performance of the different vectors in selected biotechnologically and therapeutically relevant cell types (CHO-K1, HT-1080, NHDF, MCF-7). Moreover, we investigated key characteristics of the systems, such as reversibility and adjustability to the regulating agent, to determine promising candidates for in vivo studies. To validate the functionality of delivery and regulation we performed in vivo studies by injecting particles, coding for compact self-regulated expression units, into mice and adjusting transgene expression.

Conclusion

Capitalizing on established safety features and a track record of high transduction efficiencies of mammalian cells, adeno- associated virus type 2 were successfully engineered to provide new powerful tools for macrolide-adjustable transgene expression in mammalian cells as well as in mice.

Background

An array of different viral transduction systems are being used currently in pre-clinical and clinical trials (Edelstein et al., 2004; Tomanin and Scarpa, 2004; Vandendriessche, 2002). Among these, vectors based on the replication-defective adeno-associated virus type 2 have attracted special attention as tools for clinical gene transfer. Different characteristics, such as
(i) the ability to transduce dividing as well as non-dividing cells, (ii) high transduction rates in a wide range of tissues, and notably, (iii) the unique safety properties, make AAVs a promising vector in gene therapy initiatives (Flotte et al., 1992; Grimm and Kay, 2003; Kaplitt et al., 1994; Li et al., 2005; Nakai et al., 2001; Wang et al., 2000; Xu et al., 2000).

Over the past few years, extensive studies have been carried out on different systems to regulate transgenes with small-molecule stimuli, preferentially clinically licensed agents. Started by the tetracycline-responsive TET system (Gossen and Bujard, 1992; Gossen et al., 1995), numerous other control modalities have followed including those responsive to streptogramin (Fussenegger et al., 2000), macrolide (Weber et al., 2002a) and aminocoumarines (Zhao et al., 2003), immunosuppressive agents (rapamycin) (Rivera et al., 1996), hormones (Beerli et al., 2000; Braselmann et al., 1993; No et al., 1996), or susceptible to temperature (Boorsma et al., 2000; Weber et al., 2003), quorum sensing molecules (Neddermann et al., 2003; Weber et al., 2003) and gaseous acetaldehyde (Weber et al., 2004).

To date, most of the experimental work with AAV vectors in the gene-therapy field has focused on (i) the expression of therapeutic transgenes driven by strong constitutive promoters (Acland et al., 2005; Flotte et al., 1996; Haberman et al., 2003), (ii) the regulated expression of transgenes based on the tetracycline-responsive TET$^{\text{ON}}$ and TET$^{\text{OFF}}$ systems (Bohl et al., 1998; Chtarto et al., 2003; Gafni et al., 2004; Jiang et al., 2004) and, to lesser extent, (iii) the regulated expression of transgenes by rapamycin-controlled transgene expression (Rivera et al., 2005; Wang et al., 2005).

Until recently, in-vivo studies using recombinant AAV particles have been limited by the production of high-titer and helper virus-free preparations. However, with the development of helper-free production methods (Grimm et al., 1998; Xiao et al., 1998) and improved purification and concentration protocols (Clark et al., 1999; Zolotukhin et al., 1999), high-titer production of AAV particles in the absence of helper-virus contamination was achieved, thereby opening the way to in vivo and clinical studies with AAV-derived particles.

We report the design and validation of different AAV type 2-based expression vectors, which enable macrolide-controlled transgene expression capitalizing on the recently developed erythromycin-responsive expression technology (E.REX) (Weber et al., 2002a). E.REX systems exist in two different configurations: (i) the E$_{\text{OFF}}$ arrangement consisting of a macrolide-dependent transactivator (ET, a fusion of the Escherichia coli MphR(A) repressor protein [E] and the Herpes simplex virus VP16 transactivation domain) which binds and activates chimeric promoters (P$^{\text{ETR}}$) assembled by placing ET-specific operator modules 5' of
a minimal eukaryotic promoter in a macrolide-responsive manner. Since the presence of erythromycin turns transgene expression off by abolishing the ET-\(P_{ETR}\) interaction these control modalities are known as OFF-type or \(E_{OFF}\) systems (Weber et al., 2002a). (ii) The \(E_{ON}\) technology consists of a macrolide-dependent transrepressor (E-KRAB (ET4, (Weber et al., 2002a)), a fusion of the \(E. coli\) MphR(A) repressor protein [E] and the KRAB (Krueppel-associated box) transsilencing domain of the human kox-1 gene), which binds and represses chimeric promoters (\(P_{ETR\text{ON}}\)) assembled by placing E-KRAB-specific operator modules 3’ of a constitutive eukaryotic promoter in a macrolide-inducible manner. Since the presence of erythromycin turns transgene expression ON by releasing E-KRAB from \(P_{ETR\text{ON}}\), these control arrangements are known as ON-type or \(E_{ON}\) systems (Weber et al., 2002a).

We have designed a set of recombinant AAV vectors harboring \(E_{OFF}\) or \(E_{ON}\)-controlled expression units (i) on two independent vectors (binary system), (ii) on a single vector expressing the transgene and the transactivator in a dicistronic or bidirectional configuration and (iii) on a single vector containing a constitutive promoter, driving transactivator expression, and the regulatable \(P_{ETR}\) promoter, driving the gene of interest. We have optimized performance, delivery and regulation, analyzed key characteristics such as reversibility and adjustability of the systems and validated the results with quantitative \textit{in-vitro} as well as mouse studies.

**Results**

**Design of recombinant adeno-associated viral particles for transduction of \(E_{OFF}\)-controlled transgene expression**

We have engineered serotype 2-based adeno-associated viral particles for transduction of macrolide-responsive expression of the enhanced yellow fluorescent protein (EYFP). The generic design consisted of a set of two vectors: pDF51 (ITR-\(P_{h\text{CMV}}\)-intron\(\beta\)-globin-ET1-p\(A_{HGH}\)-ITR) harboring an ITR (inverted terminal repeats)-flanked \(P_{h\text{CMV}}\) (human cytomegalovirus immediate early promoter) driven and p\(A_{\text{hgh}}\) (human growth hormone-derived polyadenylation site) terminated ET1 (macrolide-dependent transactivator) expression unit containing a \(\beta\)-globin intron (intron\(\beta\)-globin) reported to increase transcript processing and overall ET1 production levels and pDF54 (ITR-\(P_{ETR}\)-EYFP-p\(A_{SV40}\)-ITR) containing an ITR-flanked expression unit encoding \(P_{ETR}\) (macrolide-responsive promoter) driven and p\(A_{SV40}\) (simian virus 40-derived polyadenylation site) terminated EYFP (enhanced yellow fluorescent protein-encoding gene) expression cassette (Figure 1A). Transgenic AAV-derived
particles produced by transient co-transfection of either pDF60, pDF51 or pDF54 with the helper construct pDG providing constitutive processing and assembly functions (adenovirus E2A, E4 and VA as well as AAV rep and cap genes; (Grimm et al., 2003)) in trans into HEK293-T were validated for regulated EYFP expression by co-transduction of pDF51- and pDF54-derived AAV particles into representative cell types such as Chinese hamster ovary cells (CHO-K1), human fibrosarcoma cells (HT-1080), primary normal human dermal fibroblasts (NHDF) and human breast cancer cells (MCF-7) cultivated in the presence (+EM) or absence (-EM) of the macrolide antibiotic erythromycin (EM). Fluorescence micrographs of transduced populations maintained for 48h in erythromycin-free medium revealed bright green fluorescence in all cell types. Transduced populations cultivated for two days in the presence of 1µg/ml erythromycin showed no important EYFP expression. As a positive control all cell types were transduced with AAV particles harboring EYFP driven by a constitutive human cytomegolovirus immediate early promoter (P_{hCMV}) (Figure 1B).

**One vector-based macrolide-responsive AAV expression vectors**

In order to enable delivery of macrolide-responsive transgene expression in a most compact format and a single transduction event we engineered expression of the macrolide-dependent transactivator ET1 and the desired target gene into a single AAV vector configuration. The pioneering one-vector design concept consisted of tandem constitutive ET1 (P_{SV40}-ET1-pA_{SV40}) and macrolide-responsive EYFP expression units (P_{ETR}-EYFP-pA_{SV40}) placed between two ITRs (Figure 2A). pDF141- (ITR-P_{SV40}-ET1-pA_{SV40}-P_{ETR}-EYFP-pA_{SV40}-ITR) derived AAV particles efficiently transduced HT-1080 and MCF-7 and mediated high-level EYFP expression when transgenic populations were grown for 48h in the absence of erythromycin (Figure 2B). To prevent any deregulation of P_{ETR} by promoters or enhancers encoded in cis and to minimize the overall size of the transgene unit, we assembled (i) pDF124 (ITR-P_{ETR}-EYFP-IRES_{EMCV}-ET1-pA_{SV40}-ITR) a dicistronic autoregulated P_{ETR}-driven AAV expression configuration harboring an ITR-flanked P_{ETR}-driven dicistronic expression cassette with EYFP in the first and ET1 in the second cistron separated by a internal ribosome entry site of encephalomyocarditisviral origin (IRES_{EMCV}) and (ii) pDF89 (ITR-pA1-EYFP\ensuremath{\leftarrow}P_{hCMVmin}\ensuremath{\rightarrow}EYFP-P_{HSP70min}ET1-pA_{SV40}-ITR) containing a bidirectional expression unit consisting of a central ET1-specific operator ETR flanked by P_{ETR} driving EYFP expression in one direction and a P_{HSP70min} (minimal version of the Drosophila melanogaster heat-shock protein 70 promoter) driving ET1 in the opposite direction.
Figure 1. AAV type 2-based macrolide-responsive EYFP expression. (A) Schematic representation of AAV type 2-based vectors encoding ET1 under control of the constitutive P<sub>hCMV</sub> promoter (pDF51) and EYFP driven by P<sub>ETR</sub> (pDF54) or a constitutive hCMV promoter (pDF60). (B) Fluorescence micrographs of CHO-K1, HT-1080, NHDF and MCF-7 either co-transduced with pDF51/pDF54 and cultivated in the presence (+) and absence (-) of erythromycin (EM) or transduced with pDF60 (1000 genomic particles/cell for each vector). Abbreviations: EM, erythromycin; ET1, erythromycin-dependent transactivator; EYFP, enhanced yellow fluorescent protein; ITR, inverted terminal repeat; pA<sub>hgh</sub>, human growth hormone polyadenylation signal; pA<sub>SV40</sub>, simian virus 40 polyadenylation signal; P<sub>ETR</sub>, erythromycin-responsive promoter; P<sub>hCMV</sub>, human immediate early cytomegalovirus promoter; Selected restriction sites: A, AccI; As, AscI; Bg, BglII; Bs, BstBI; Bsa, BsaBI; C, ClaI; E, EcoRI; H, HindIII; Hi, HincII; M, MluI; N, NdeI; Nh, NheI; Nr, NruI; P, PacI; Pm, Pmel; Pml, PmII; S, SalI; Sa, SacII Sp, SpeI; Sph, SpI; Sw, Swal; X, XbaI.
Table 1. Plasmids used and designed in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description and Cloning Strategy</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>pAAV-MCS</td>
<td>AAV transfer vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pAAV-lacZ</td>
<td>AAV vector expressing lacZ</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pDG</td>
<td>Helper construct encoding AAV Rep/Cap as well as Adeno virus E2A, E4 and VA.</td>
<td>(Grimm et al., 2003)</td>
</tr>
<tr>
<td>pCF18</td>
<td>Plasmid containing ECFP driven by a tetracycline responsive promoter and EYFP driven by a pristinamycin responsive promoter</td>
<td>(Fux and Fussenegger, 2003)</td>
</tr>
<tr>
<td>pCF19</td>
<td>Plasmid containing SEAP cassette</td>
<td>(Fux et al., 2004)</td>
</tr>
<tr>
<td>pCF125</td>
<td>Plasmid expressing ECFP and ET1 from a bidirectional promoter</td>
<td>(Fux et al., 2003)</td>
</tr>
<tr>
<td>pSS134</td>
<td>Plasmid containing SEAP cassette</td>
<td>unpublished</td>
</tr>
<tr>
<td>pWW43</td>
<td>Plasmid expressing E-KRAB</td>
<td>(Weber et al., 2002a)</td>
</tr>
<tr>
<td>pWW76</td>
<td>Plasmid containing tricistronic expression configuration driven by P_{ETRON}</td>
<td>(Weber et al., 2002a)</td>
</tr>
<tr>
<td>pWW78</td>
<td>pTRIDENT1-based tricistronic expression vector for macrolide-responsive auto-regulated expression of up to two desired transgenes.</td>
<td>unpublished</td>
</tr>
<tr>
<td>pBP141</td>
<td>Vector expressing SEAP and ET1 under tetracycline-responsive promoter: P_{CMV*-1-SEAP-IRESPV-ET-pA}</td>
<td>(Fussenegger et al., 1998)</td>
</tr>
<tr>
<td>pMF123</td>
<td>Plasmid encoding tricistronic expression cassette driven by a constitutive SV40 promoter.</td>
<td>(Mitta et al., 2002)</td>
</tr>
<tr>
<td>pMF351</td>
<td>Lentiviral vector encoding EYFP driven by a constitutive hCMV promoter</td>
<td></td>
</tr>
<tr>
<td>pDF37</td>
<td>AAV2 vector containing a tricistronic P_{ETR} driven expression unit. The entire expression unit from pWW73 was excised using SspI/XbaI, polished by Klenow and cloned into pAAV-lacZ which was NotI digested and Klenow polished before, thus resulting in pDF37 (ITR-P_{ETR-IRESPV-IRESP_{CMV-PA_{SV40}-itr}}).</td>
<td>this work</td>
</tr>
<tr>
<td>pDF51</td>
<td>AAV2 vector containing a constitutive hCMV driven ET1 cassette. ET1 was excised from pWW078 using EcoRI/HindIII and cloned into the corresponding sites of pAAV-MCS, thus resulting in pDF51 (ITR-P_{ETR-\text{Intron}<em>{p_globin}-ET1-pA</em>{high-itr}}).</td>
<td>this work</td>
</tr>
<tr>
<td>pDF54</td>
<td>AAV2 vector encoding EYFP driven by the erythromycin responsive P_{ETR} promoter. P_{ETR} was excised from pDF55 using AccI/HindIII and cloned into the corresponding sites of pDF60, thus resulting in pDF54 (ITR-P_{ETR-EYFP-pA_{SV40-itr}}).</td>
<td>this work</td>
</tr>
<tr>
<td>pDF55</td>
<td>AAV2 vector encoding divergent expression units for ECFP driven by P_{ETR} and ET1 driven by a HSP70 minimal promoter. The entire expression cassette was excised from pCF125 using EcoRV/XbaI and cloned into the HindII/SpeI sites of pDF60, thus resulting in pDF55 (ITR-p_{A_{E}}-ECFP\rightarrow P_{ETR-ETR-P_{HSP70min}}\rightarrow ET1-pA_{SV40-itr}).</td>
<td>this work</td>
</tr>
<tr>
<td>pDF56</td>
<td>AAV2 vector encoding ET1 driven by a constitutive SV40 promoter. ET1 was</td>
<td>this work</td>
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</table>
excised from pWW78 using EcoRI/XbaI and cloned into the EcoRI/SpeI sites of pDF63, thus resulting in pDF56 (ITR-P_{SV40-ETR1}-pA_{SV40-ITR}).

**pDF60** AAV2 vector encoding EYFP driven by a constitutive hCMV promoter. P_{hCMV}-EYFP was excised from pMF351 using XbaI/PacI and cloned into Nhel/PacI sites of pDF63, thus resulting in pDF60 (ITR-P_{hCMV}-EYFP-pA_{SV40-ITR}).

**pDF61** AAV2 vector encoding SEAP driven by an erythromycin responsive P_{ETR} promoter. SEAP was excised from pCF019 using Nhel/ClaI and cloned into the Nhel/BsrBI sites of pDF54, thus resulting in pDF61 (ITR-P_{ETR-SEAP-pA_{SV40-ITR}}).

**pDF63** AAV2 vector containing an SV40 promoter followed by an IRES_{PV} and an IRES_{EMCV} element. P_{SV40-IRESPV-IRES_{EMCV}} was excised from pMF123 using SspI/BglII, polished with Klenow and cloned into the polished NcoI/SpeI sites of pAAV-lacZ, thus resulting in pDF63 (ITR-P_{SV40-IRES_{PV}-IRES_{EMCV}-pA_{SV40-ITR}}).

**pDF74** AAV2 vector containing tricistronic expression cassette driven by a P_{ETR-ON8} promoter. The P_{ETR-ON8} promoter was excised from pWW76 using Nhel/EcoRI and cloned into the corresponding sites of pDF63, thus resulting in pDF74 (ITR-P_{ETR-ON8-IRESPV-IRES_{EMCV}-pA_{SV40-ITR}}).

**pDF75** AAV2 vector containing dicistronic expression unit consisting of SEAP followed by an IRES_{PV} element followed by ET1 driven by P_{ETR}. Dicistronic expression cassette was excised from pBP141 using XbaI/PacI and cloned into the Nhel/PacI sites of pDF54, thus resulting in pDF75 (ITR-P_{ETR-SEAP-IRES_{PV}-ET1-pA_{SV40-ITR}}).

**pDF76** AAV2 vector encoding SEAP driven by a P_{ETR-ON8} promoter. This vector was excised from pSS134 using EcoRI/HindIII and cloned into pDF37. This vector was digested using EcoRI/XsaI and the SEAP containing insert was cloned into the corresponding sites of pDF74, thus resulting in pDF76 (ITR-P_{ETR-ON8-SEAP-IRES_{EMCV}-pA_{SV40-ITR}}).

**pDF77** AAV2 vector encoding SEAP under the control of an erythromycin responsive P_{ETR} promoter (additional upstream ATG deleted). P_{ETR} was excised from pDF54 using AccI/EcoRI and cloned into the ClaI/EcoRI sites of pDF61, thus resulting in pDF77 (ITR-P_{ETR-SEAP-pA_{SV40-ITR}}).

**pDF89** AAV2 vector encoding divergent expression units for EYFP driven by P_{ETR} and ET1 driven by a HSP70 minimal promoter. The EYFP cassette was excised from pCF18 using AccI/EcoRV and cloned into the NraI/ClaI sites of pDF55, thus resulting in pDF89 (ITR-P_{AETR-EYFP-pA_{ETR-ET1-pA_{SV40-ITR}}}).

**pDF98** Plasmid containing hEF1α promoter flanked by multiple cloning sites unpublished.

**pDF109** AAV2 vector encoding SEAP driven by a constitutive hCMV promoter. SEAP was excised from pDF61 using EcoRI/SpeI and cloned into the EcoRI/XbaI sites of pAAV-MCS, thus resulting in pDF109 (ITR-P_{hCMV-Intron_p-ghb-m-SEAP-pA_{ghb-ITR}}).

**pDF124** AAV2 vector encoding dicistronic expression unit consisting of EYFP followed by an IRES_{EMCV} element followed by ET1. The IRES-ET1 containing insert was excised from pDF75 using HindIII, polished with Pfu polymerase, digested using BseXI and cloned into the SwaI/BstXI sites of pDF54, thus resulting in pDF124 (ITR-P_{ETR-EYFP-IRES_{EMCV}-ET1-pA_{SV40-ITR}}).

**pDF126** AAV2 vector encoding E-KRAB under the control of a constitutive hCMV promoter. The E-KRAB containing insert was excised from pWW043 using unpublished.
Chapter 2

EcoRI/HpaI and cloned into the EcoRI/HindII sites of pAAV-MCS, thus resulting in pDF126 (ITR-P<sub>hCMV</sub>-Intron<sub>γ</sub>-globin-KRAB-pA<sub>hgh</sub>-ETR).

pDF141 AAV2 vector encoding self-regulated expression cassette consisting of ET1 this work driven by a constitutive SV40 promoter and EYFP driven by P<sub>ETR</sub>. The entire ET1 expression cassette of pDF56 was excised using ClaI/PmlI and cloned into pDF54 which was digested by HindIII and polished by Pfu before, thus resulting in pDF141 (ITR-P<sub>SV40</sub>-ET1-pA<sub>SV40</sub>-P<sub>ETR</sub>-EYFP-pA<sub>SV40</sub>-ITR).

pDF143 AAV2 vector encoding self-regulated expression cassette consisting of ET1 this work driven by a constitutive SV40 promoter and SEAP driven by P<sub>ETR</sub>. The SEAP containing insert was excised from pDF77 using KpnI/SpeI and cloned into the corresponding sites of pDF141, thus resulting in pDF143 (ITR-P<sub>SV40</sub>-ET1-pA<sub>SV40</sub>-P<sub>ETR</sub>-SEAP-pA<sub>SV40</sub>-ITR).

pDF199 AAV2 vector encoding SEAP under the control of a constitutive SV40 this work promoter followed by 2 binding sites for the transrepressor E-KRAB. The 4<sup>th</sup>ETR binding site containing fragment was excised from pWW55 using BstBI/Ndel and cloned into the corresponding sites of pDF76. Two of the binding sites were deleted by recombination during the cloning procedure, thus resulting in pDF199 (ITR-P<sub>ETR</sub>ON2-SEAP-IRES<sub>EMCV</sub>-pA<sub>SV40</sub>-ITR).

pDF200 AAV2 vector encoding SEAP under the control of a constitutive SV40 this work promoter followed by 4 binding sites for the transrepressor E-KRAB. The 4<sup>th</sup>ETR binding site-containing fragment was excised from pWW55 using BstBI/Ndel and cloned into the corresponding sites of pDF76, thus resulting in pDF200 (ITR-P<sub>ETR</sub>ON4-SEAP-IRES<sub>EMCV</sub>-pA<sub>SV40</sub>-ITR).

pDF207 AAV2 vector encoding EYFP under the control of a constitutive SV40 this work promoter followed by 8 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/PacI and cloned into the corresponding sites of pDF76, thus resulting in pDF207 (ITR-P<sub>ETR</sub>ON8-EYFP-pA<sub>SV40</sub>-ITR).

pDF208 AAV2 vector encoding EYFP under the control of a constitutive SV40 this work promoter followed by 4 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/PacI and cloned into the corresponding sites of pDF200, thus resulting in pDF208 (ITR-P<sub>ETR</sub>ON4-EYFP-pA<sub>SV40</sub>-ITR).

pDF209 AAV2 vector encoding EYFP under the control of a constitutive SV40 this work promoter followed by 2 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/PacI and cloned into the corresponding sites of pDF199, thus resulting in pDF209 (ITR-P<sub>ETR</sub>ON2-EYFP-pA<sub>SV40</sub>-ITR).

Abbreviations: AAV2, adeno-associated virus type 2; ECFP, enhanced cyan fluorescent protein (720bp); EM, erythromycin; ET1, erythromycin transactivator (972bp); EYFP, enhanced yellow fluorescent protein (720bp); IRES<sub>EMCV</sub>, internal ribosome entry site of encephalomyocarditisviral origin (502bp); IRES<sub>p450</sub>, internal ribosome entry site of polioviral origin (635bp); ITR, inverted terminal repeat (141bp); KRAB, kruppel-associated box (450bp); pA<sub>γ</sub>, artificial polyadenylation signal (91bp); pA<sub>hgh</sub>, human growth hormone polyadenylation signal (478bp); pA<sub>sv40</sub>, simian virus 40 polyadenylation signal (145bp); P<sub>α&beta;347en</sub>, human elongation factor 1α promoter (1185bp); P<sub>ETR</sub>, erythromycin responsive promoter (200bp); P<sub>ETR</sub>ON, macrolide inducible promoter (530bp); P<sub>hCMV</sub>, human immediate early cytomegalovirus promoter (663bp); P<sub>HSV</sub>70<sub>min</sub>, heat shock protein 70 minimal promoter (350bp); P<sub>sv40</sub>, simian virus 40 promoter (308bp); SEAP, human placental secreted alkaline phosphatase (1560bp).
Following transduction of pDF124-derived AAV particles, active PETR produces a single transcript from which EYFP is translated in a classic cap-dependent manner whereas ET1 production depends on cap-independent IRES_{EMCV}-mediated translation initiation. Undetectable PETR-mediated transcripts result in few initial ET1 proteins, which, in the absence of erythromycin trigger auto-regulated high-level expression of this transactivator along with the cocistronically encoded EYFP. In the presence of erythromycin, ET1 originating from basal PETR activity is inactivated, which interrupts the auto-regulated feed-forward transcription and results in repression of EYFP production. After transduction of pDF89-derived AAV particles, leaky ET1 transcript initiate an auto-regulated expression circuit resulting in simultaneous expression of ET1 and EYFP until ET1-ETR binding is abolished by erythromycin. All three configurations yielded AAV particles, which displayed good regulation performance upon transduction of HT-1080 and MCF-7 and cultivation in the absence or in the presence of erythromycin (Figure 2B). While using equal genomic particle numbers the transduction efficiency varied between pDF124, pDF89 and pDF141. Transduction rates of the bi-directional (pDF89) and the dicistronic expression units (pDF124) were significantly lower compared to the two-promoter set-up (pDF141) (Fig. 2B) which made FACS-based quantitative analysis was rather difficult (Figure 2C). However qualitative fluorescence microscopy suggested excellent regulation performance in individual transduced cells (Fig. 2B).

**Engineered AAV-derived particles transducing tightly regulated production of secreted proteins**

Tight regulation of therapeutic transgenes remains a major challenge for current gene therapy initiatives. In order to assess whether AAV-based transduction systems enable delivery of tightly regulated expression of a human model glycoprotein, we constructed pDF77 harboring a PETR-driven SEAP (human placental secreted alkaline phosphatase) (ITR-PETR-SEAP-pASV40-ITR) (Figure 3A). Co-transduction of HT-1080 and MCF-7 with pDF77- and pDF51- (ITR-P_{hCMV}-ET1-pASV40-ITR) derived AAV particles provided excellent regulation performance characterized by high-level SEAP production in the absence of erythromycin and repressed SEAP levels in the presence of erythromycin (Figure 3B).
Figure 2. Self- and auto-regulated AAV type 2-based expression of fluorescent proteins. (A) Schematic representation of an auto-regulated (pDF124), a bidirectional (pDF89) and a self-regulated (pDF141) AAV type 2-based expression unit. (B) Fluorescence micrographs of human fibrosarcoma cells (HT-1080) and a human breast cancer cell line (MCF-7) transduced with pDF124-, pDF89- and pDF141-derived AAV particles (2000 genomic particles/cell) cultivated in the presence (+) and absence (-) of EM. (C) FACS-mediated quantification of EYFP in HT-1080 transduced with equal amounts of viral particles (2000 genomic particles/cell) and cultivated in the presence (+EM) and absence (-EM) of erythromycin. Abbreviations: EM, erythromycin; ET1, erythromycin-dependent transactivator; ETR, ET1-specific operator; EYFP, enhanced yellow fluorescent protein; IRES, internal ribosome entry site; ITR, inverted terminal repeat; pA, synthetic polyadenylation signal; pA<sub>SV40</sub>, simian virus 40 polyadenylation signal; P<sub>ETR</sub>, erythromycin-responsive promoter; P<sub>HSP70min</sub>, minimal version of the Drosophila melanogaster heat-shock protein 70 promoter; P<sub>SV40</sub>, simian virus 40 promoter.
Furthermore, we have designed and evaluated pDF143 (ITR-P_{SV40}-ET1-pASV40-P_{ETR}-SEAP-pASV40-ITR), a one-vector expression configuration which is isogenic to pDF141 but encoding SEAP instead of EYFP. pDF143 exhibited similar SEAP regulation profiles in HT-1080 and MCF-7 compared to co-transduction of pDF77-/pDF51-derived AAV particles suggesting that there is no interference between ET1-driving P_{SV40} and ET1-specific P_{ETR} (Figure 3A and 3B). Overall, maximum expression levels for the one-vector configuration were even higher than for the binary setting likely because for the binary system two different particles have to transduce a single cell which is a more rare event. Transduction of HT-1080 and MCF-7 with a constitutive control vector, pDF109 (ITR-P_{hCMV-intron}β-globin-SEAP-pA_{hgh}-ITR) showed increased SEAP production compared to fully induced pDF143-derived particles, which might be associated with either higher promoter strength or the β-globin intron.

**AAVs engineered for E_{ON}-controlled transgene expression**

Various therapeutic initiatives require transgene control systems, which trigger heterologous target genes after administration of the regulating agent and remain repressed in the absence of the inducer (Weber and Fussenegger, 2006). We have constructed binary E_{ON}-controlled AAV-based expression systems consisting of pDF126 (ITR-P_{hCMV-Intron}β-globin-EKRAB-pA_{hgh}-ITR), and pDF207 (ITR-P_{ETRON8}-EYFP-pASV40-ITR), pDF208 (ITR-P_{ETRON4}-EYFP-pASV40-ITR) or pDF209 (ITR-P_{ETRON2}-EYFP-pASV40-ITR), which harbor EYFP under control of different E-KRAB-specific macrolide-inducible promoters characterized by different ETR tandem repeats placed 3’ of the constitutive P_{SV40} promoter (Weber et al., 2002a). Functionality of the ON-type system was validated by co-transduction of pDF126/pDF209-derived into HT-1080 and MCF-7 and profiling of EYFP expression after 48h cultivation in the presence or absence of erythromycin (Figure 4B). FACS-based comparison of HT-1080 co-transduced with pDF126-/pDF207-, pDF126-/pDF208- and pDF126-/pDF209-derived AAV particles and cultivated for 48h in the presence and absence of erythromycin revealed that although overall induction factors were almost identical among different particle combinations, basal and maximum expression levels were a function of the number of ETR repeats associated with P_{SV40} (Figures 4A and 4C).
Figure 3. AAV type 2-based regulated expression of secreted proteins. (A) Schematic representation of pDF77, harboring a SEAP expression cassette under the control of P_ETR, pDF143, a self-regulated expression unit expressing ET1 from P_SV40 and driving SEAP from P_ETR and pDF109, harboring a constitutive SEAP expression unit driven by P_hCMV (B) SEAP expression levels profiled 48h after (co-)transduction of transgenic AAV particles (1000 genomic particles/cell for pDF51/77, 1000 genomic particles/cell for pDF143 and 1000 genomic particles/cell for pDF109) derived from indicated vectors and cultivated in the presence (+) or absence (-) of EM. SEAP expression is shown in units/liter (U/l) as defined by Schlatter et al. (Schlatter et al., 2002). Abbreviations: EM, erythromycin; ET1, erythromycin transactivator; ITR, inverted terminal repeat; P_SV40, simian virus 40 polyadenylation signal; P_hEF1α, human elongation factor 1α promoter; P_ETR, erythromycin-responsive promoter; SEAP, human placental secreted alkaline phosphatase; P_SV40, simian virus 40 promoter.
Figure 4. AAV type 2-based erythromycin-inducible EYFP expression. (A) Schematic representation of pDF126 harboring a P_{lCMV}-driven E-KRAB expression unit and pDF206/207/208 encoding EYFP transcribed by P_{ETRON} promoter derivatives containing 8 (pDF207), 4 (pDF208) and 2 (pDF209) E-KRAB-specific ETR operator modules. (B) Fluorescence micrographs of HT-1080 and MCF-7 co-transduced with pDF209/pDF126-derived AAV particles (1000 genomic particles/cell for pDF207/208/209 and 4000 genomic particles/cell for pDF126) and cultivated in the presence (+EM) and absence (-EM) of erythromycin (C) FACS-mediated quantification of EYFP expression in HT-1080. EYFP-specific FACS diagrams of HT-1080 co-transduced (1000 genomic particles/cell) by pDF126/pDF207, pDF126/pDF208 and pDF126/pDF209 were cultivated for 48h in the presence (+) and absence (-) of EM. Abbreviations: EM, erythromycin; EYFP, enhanced yellow fluorescent protein; ITR, inverted terminal repeat; KRAB, kruppel associated box; P_{ETRON}, erythromycin-inducible promoter.
Reversibility and adjustability of AAV-transduced macrolide-controlled transgene expression

Reversibility and adjustability are key characteristics for future clinical implementation of human-compatible transgene control modalities. In order to assess the reversibility of macrolide-responsive transgene expression in an AAV expression configuration we transduced HT-1080 and MCF-7 with pDF143-derived AAV particles and cultivated (i) in the presence (++) or absence (---) of EM over 9 days, (ii) in the presence (+++) or absence (--+) of EM over 6 days and then incubated in reversed EM conditions for the remaining three days and (iii) in medium whose EM status was alternated every three days from +EM to −EM to +EM (++) or from −EM to +EM to −EM (−−). SEAP accumulation was always measured prior to any EM status switch.

HT-1080 and MCF-7 showed excellent switching characteristics upon application of removal of erythromycin (Figure 5A and 5B).

In order to assess the dose-response characteristics of binary and one-vector-based macrolide-responsive AAV transduction systems HT-1080 and MCF-7 were transduced with pDF143- or co-transduced with pDF77+/pDF51-derived AAV particles and cultivated for 48h in the presence of different EM concentrations before SEAP production was quantified. Whereas SEAP expression was completely repressed between 20 and 100ng/ml for both configurations gradual decrease in antibiotic treatment resulted in dose-dependent increase of transgene expression until maximum expression levels were reached in the absence of EM (Figure 5C and 5D). The differences in the dose-response characteristics of one-vector and binary configurations may result from different ET1-PETR stoichiometries associated with those technologies.

Transgenic AAV-derived particles mediate E_{OFF}-controlled transgene expression in mice

For in-vivo validation of AAV-based macrolide-responsive transgene transduction we injected $5\times 10^{10}$ pDF143-derived genomic AAV particles intramuscularly into mice and treated them optionally with intraperitoneal erythromycin injections (2mg/mouse every 24h). Profiling of serum SEAP levels of treated mice showed significant production of this glycoprotein in mice, which had not received EM doses whereas SEAP production was repressed whenever EM was administered (Figure 6).
Figure 5. Reversibility and adjustability of macrolide-responsive SEAP expression in HT-1080 and MCF-7 transduced by transgenic AAV type 2-derived particles. (A) HT-1080 and (B) MCF-7 were transduced with pDF143-derived AAV particles (2000 genomic particles/cell) and six equal populations (1-6) were cultivated in media containing different antibiotic concentrations. Cells were (i) cultivated in the presence (+++) or absence (---) of EM over 9 days, (ii) cultivated in the presence (++) or absence (+−) of EM over 6 days and then incubated in reversed EM conditions for the remaining three days or (iii) cells were cultivated in medium whose EM status was alternated every three days +EM to −EM to +EM (+−−) or from −EM to +EM to −EM (−+) and SEAP expression was quantified. Adjustability of SEAP expression of pDF143 (1000 genomic particles/cell) (C) and pDF51/77 (500 genomic particles/cell) (D) in HT-1080 and MCF-7 cultivated for 48h in the presence of increasing EM concentrations. SEAP expression is shown in units/liter (U/l) as defined by Schlatter et al. (Schlatter et al., 2002). Abbreviations: EM, erythromycin; SEAP, human placental secreted alkaline phosphatase.
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Figure 6. Macrolide-triggered SEAP expression in mice injected with pDF143 (ITR-\(P_{SV40}\)-ET1-\(pA_{SV40}\)-\(P_{ETR}\)-SEAP-\(pA_{SV40}\)-ITR)-derived AAV type 2 particles. pDF143-derived AAV particles were administered intramuscularly and SEAP levels in the serum were measured at two different time points for one group in the absence of erythromycin, and for the other group with EM injections every 24h. SEAP expression is shown in milliunits/liter (mU/l) as defined by Schlatter et al. (Schlatter et al., 2002). Abbreviations: EM, erythromycin; SEAP, human placental secreted alkaline phosphatase.

Discussion

In recent years, gene delivery systems based on adeno-associated viral vectors have become one of the most promising tools for delivering transgenes in vivo. After major setbacks in trials with retroviral as well as adenoviral vectors (Check, 2002; Lehrman, 1999), AAVs present a strong alternative for the safe and efficient delivery of transgenes. We have pioneered the delivery and regulation of transgenes by integrating the recently developed erythromycin-controllable E.REX system into AAV type 2-derived backbones. Considering that erythromycin is a clinically licensed drug and that AAV vectors have an excellent safety profile record, such a system is suitable for in vivo applications and is promising for clinical initiatives. Maximum erythromycin doses of up to 4g/day in adults are in accordance with FDA and AHFS (American Society of Health-System Pharmacists) guidlines. (WWW.FDA.gov; WWW.AHFSdruginformation.com). These doses are within the range of
the amounts we have tested in mice. We are therefore confident that the AAV-encoded and erythromycin-controlled transgenes will be compatible with future clinical applications.

Research, in which AAV vectors deliver adjustable transgenes, has been conducted mainly with tetracycline responsive systems, which trigger or repress expression upon the addition of tetracycline (Chattaro et al., 2003; Gafni et al., 2004; Haberman et al., 1998; Jiang et al., 2004; Stieger et al., 2006).

We have designed a range of AAV type 2-based vectors encoding different configurations of the $E_{\text{OFF}}$ expression system to evaluate optimal arrangements for further in vivo studies. A binary set-up, where the transactivator is delivered on one vector and the gene of interest is driven by $P_{\text{ETR}}$ on another vector, worked well in vitro. The expression levels, vector titers and regulation performance of the tested configurations were excellent, indicating an efficient co-transduction of the two types of transgenic AAV particles. Regardless of whether intracellular or secreted transgenes were expressed, the regulation factors of all the tested cell lines were between 10 and 400. Although transgene expression mediated by AAVs engineered for macrolide-responsive transgenes expression were typically lower after induction compared to isogenic AAV derivatives containing constitutive promoters the overall production levels were in the same order of magnitude in vitro. Isogenic $E_{\text{ON}}$ systems also enabled excellent regulation performance but overall induction factors were typically lower, since cells exclusively transduced with the transgene-encoding AAV particle exhibit constitutive expression. Therefore, to achieve optimized $E_{\text{ON}}$-controlled transgene expression from transgenic AAVs arranged in a binary vector set, the transrepressor-encoding AAV has to be administered in excess compared to the transgene-encoding counterpart or cell lines have to be used, which are particular susceptible to AAV-based transduction.

Generally, even though binary systems have the disadvantage that two vectors must enter the same cell to obtain regulated expression, this design is advantageous for some applications, namely when delivering large transgenes. Although binary systems are powerful tools, in a therapeutic setting it may be desirable to combine the regulation modules on a single vector. The advantages of such a setting are: (i) only one virus must enter the target cell to obtain regulated expression of the transgene, (ii) exact virus titration is easier and (iii) lower virus doses are required for comparable transgene expression.

We have pioneered both self-regulated and auto-regulated macrolide-responsive recombinant adeno-associated viral vectors for tightly controlled transgene expression. Although auto-regulated expression units are more compact than self-regulated configurations we had difficulty achieving reasonably high transduction efficiencies and expression levels.
Since qRT-PCR-based analysis of viral particles revealed identical number of encapsidated genomes among our AAV portfolio, the lower transduction rates of auto-regulated AAVs may result from their genetic configuration rather than virus assembly. In contrast, self-regulated expression units, consisting of a strong constitutive promoter driving the expression of the transactivator as well as a macrolide-responsive promoter driving the desired transgene on the same vector, showed excellent performance in a variety of cell types. The vector design using compact promoter and polyadenylation elements in a self-regulated configuration allows the integration of a transgene with a size of up to 2500bp which is sufficient for various clinically important transgenes (for example, erythropoietin or vascular endothelial growth factor). We did not observe promoter interference in the tested cell types, although the strong constitutive $P_{SV40}$ promoter driving ET1 and the erythromycin-responsive promoter were on the same construct. We were successful in expressing intracellular as well as secreted transgenes in a highly controlled manner and showed precise titration of transgene expression upon addition of varying erythromycin concentrations as well as reversibility upon the addition or removal of the regulating agent. After switching from a fully repressed to a fully induced state or vice versa a short lag phase was observed before the new expression state was reached. This behaviour might be due to trace amounts of antibiotic remaining in the culture (by switching from $+EM$ to $–EM$) or due to the degradation kinetics of transcripts and transactivators (from $–EM$ to $+EM$).

One-vector configurations would be suitable for in vivo studies and would enable efficient targeting of a wide variety of cells by systemic administration or by local injection of the viral particles encoding the therapeutic transgene. We exemplified the functionality of the system in vivo by administrating pDF143-derived AAV particles intramuscularly to mice and analyzing transgene expression in the presence and absence of antibiotic for several days.

Most current studies on regulated transgene expression are carried out using retroviral vectors (Markusic et al., 2005; Mitta et al., 2005; Mitta et al., 2004; Pluta et al., 2005; Vigna et al., 2005). Although these systems are powerful and allow the efficient long-term expression of transgenes, they have several drawbacks, such as (i) the necessity of retroviral vectors for integration into transcriptionally active regions, which may interfere with desired transgene control, (ii) random integration, possibly triggering oncogene activation and (iii) silencing of the integrated transgene upon integration into the host chromosome. Adeno-associated viral vectors on the contrary, remain mainly episomal after entering the target cell and, therefore, do not have aforementioned drawbacks, thus providing a powerful alternative for upcoming gene-therapy studies.
Conclusions
We have designed an array of novel AAV type 2-based expression vectors, which enable safe and efficient transduction of mammalian cells for macrolide-adjustable transgene expression. We have pioneered binary ON- and OFF-type systems as well as compact bidirectional, auto-regulated and self-regulated one vector expression configurations for regulation of intracellular as well as secreted proteins in mammalian cells. We have also engineered a compact self-regulated AAV which demonstrated efficient transduction, expression and regulation of a reporter gene in mice.

Methods

Plasmid construction. Table 1 lists all the plasmids used in this study as well as detailed information on their construction and the length of the genetic elements.

Cell culture and transfection. Human embryonic kidney cells, transgenic for the adenovirus type 5-derived E1 region as well as for the simian virus 40 (SV40) large T-antigen (HEK293-T; (Mitta et al., 2002)), human fibrosarcoma cells (HT-1080; ATCC CCL-121), human breast cancer cells (MCF-7, ATCC HTB-22) and normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany; cat. no. C-12300, lot no. 1070402) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany; cat. no. 3302-P231902, lot no. P231902) and 1% penicillin/streptomycin solution (Sigma Chemicals, St. Louis, MO, USA). Chinese hamster ovary cells (CHO-K1; ATCC CCL-61) were cultivated in ChoMaster® HTS medium (Cell Culture Technologies GmbH, Gravesano, Switzerland) supplemented with 5% FCS (PAN Biotech GmbH) and 1% penicillin/streptomycin solution. All cell lines were cultivated at 37°C in a 5% CO₂-containing humid atmosphere.

Production of adeno-associated viral particles. AAV particles were produced by cotransfection of the helper plasmid pDG (Grimm et al., 2003) and the transgene-encoding AAV vector into HEK293-T using optimized calcium phosphate transfection protocols. In brief, 2x10⁶ HEK293-T were seeded per culture dish (diameter 10cm) and cultivated overnight. For each plate 15µg of helper plasmid were mixed with 5µg AAV vector and CaCl₂ was added to a final concentration of 0.25M. The mixture was added slowly to an equal
volume of HEPES-buffered saline solution (HeBS; 50mM HEPES, 280mM NaCl, 1.5mM Na₂HPO₄, pH 7.1), vortexed briefly, and incubated for 2min. before adding the DNA-calcium phosphate precipitate solution to the monolayer culture. Precipitates were removed after 6h, cells were supplemented with 1% FCS-supplemented DMEM and incubated for 60h. The supernatant was discarded, the cells were detached using a cell scraper and resuspended in 2ml PBS (138mM NaCl, 8.1mM Na₂HPO₄, 1.47mM KH₂PO₄, 2.67mM KCl; Invitrogen, cat. no. 21600-069, lot. no. 1255481) per plate. Each cell suspension was transferred to a Falcon tube and pelleted by centrifugation for 3min. at 280xg. Following another washing step using 5ml PBS, the cells were resuspended in 2ml PBS and the intracellular viral particles were released after cell lysis induced by three consecutive freeze-thaw cycles consisting of shuttling the tubes between liquid nitrogen and a 37°C water bath (tubes were vortexed vigorously after each thawing step). The cell debris was eliminated by centrifugation for 5min. at 10’000xg and the supernatant containing the crude virus stock was collected, supplemented with 50U/ml of Benzonase (Sigma, cat. no. E1014) and incubated for 30min at 37°C. Iodixanol density-gradient purification of viral particles was performed using a protocol adapted from Zolotukhin et al. (Zolotukhin et al., 1999). In brief, crude viral stocks were diluted in PBS to a final volume of 12ml. Iodixanol step gradients were prepared in an ultracentrifuge tube by sequential underlaying of the crude viral preparation with 5ml of a 15% (plus 1M NaCl in the first layer), 5ml of a 25%, 4ml of a 40% and 4ml of a 60% iodixanol-containing PBS. For better distinction of the gradient layers 2.5µl/ml of a 0.5% Phenol Red (Sigma, cat. no. P0290) stock solution was added to the 60% and the 25% layers. Step gradients were centrifuged for 3.5h at 150’000xg at 18°C. The clear 40% fraction was harvested after puncturing the ultracentrifuge tube on the side with a syringe equipped with a 16G needle. A heparin affinity column (HiTrap Heparin HP, Amersham Biosciences, Sweden; cat. no. 17-0406-01) was equilibrated with 10 bed volumes of binding buffer (10mM Na₂PO₄, pH 7) and run at a flow rate of 1ml/min. The AAV particle-containing 40% fraction harvested from the step gradient was loaded onto the heparin affinity column. After washing the column with 10 bed volumes of binding buffer the viral particles were eluted with 5 bed volumes of elution buffer (10mM Na₂PO₄, 1M NaCl, pH 7) and then concentrated using a spin column with a molecular weight cut-off (MWCO) of 30 kDa (Vivaspin, Vivascience, Germany; cat. no. VS1521).
Virus titration by quantitative real-time PCR. Crude viral preparations were treated as described in (Clark et al., 1999). Primers and the Taqman probe were designed to anneal to (i) the SV40 polyadenylation signal (pA) (forward primer, 5'-AGCAATAGCATCAAAAATTTCACAA-3', reverse primer, 5'-GACATGATAAGATACATTGAGTTTGG-3', Taqman FAM/TAMRA probe, 5'-AGCATTTTTTTCACTGCATTGTGTTGTTTGG-3'), (ii) the SEAP open reading frame (forward primer, 5'-AGGCCCGGGACAGGAA-3', reverse primer, 5'-GCCGTCCTTGAGCAGACATGC-3') or (iii) the erythromycin-dependent transactivator open reading frame (forward primer, 5'-AGCAGGCCCTCGATGGTA-3', reverse primer, 5'-AGCAGGCCCTCGATGGTA-3'). Absolute quantification was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Warrington, UK, cat. no. 4324018) or Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK, cat. no. 4367659) on AB Prism 7500 RT-PCR quantitative PCR hardware according to the manufacturer’s instructions (Applied Biosystems, Weiterstadt, Germany). The reference standard consisted of a four log-spanning dilution of pDF60 harboring a single SV40 pA sequence, pDF51 harboring the ET1 transactivator or pDF109 harboring the SEAP open reading frame.

Quantification of reporter gene expression. Enhanced yellow fluorescent protein (EYFP) was visualized using a Leica DM-RB fluorescence microscope (Leica Inc., Heerbrugg, Switzerland) equipped with an XF114 filter (Omega Optical Inc., Brattleboro, VT, USA). Fluorescence was quantified 48h after transduction using a fluorescence-activated cell sorter (Coulter FC500, Beckman Coulter Inc., FL, USA) with CXP software (Beckman Coulter) installed. SEAP production was quantified in cell-culture supernatants 48h after transduction and in mouse serum 3 days to 7 days after injection as described in (Schlatter et al., 2002).

Chemicals used for transgene regulation. For all in-vitro experiments, erythromycin (Fluka, Buchs, Switzerland cat. no. E-5389) was prepared as a stock solution of 1mg/ml in ethanol and used at a final concentration of 1μg/ml. For in-vivo studies, 200μl of a 10mg/ml erythromycin solution (10% ethanol, 90% PBS) were daily intraperitoneally injected into each animal.

In vivo studies. Female OF1 (oncins france souche 1) mice were obtained from Charles River Laboratories (Lyon, France). Mice were treated with intramuscular injections 5x10^{10} vector genomes/mouse. Erythromycin was administered intraperitoneally 1h after injection of the transgenic AAV particles and repeated every 24h. Blood samples were
collected retroorbitally and serum was produced using microtainer SST tubes (Beckton Dickinson, Plymouth, UK). All animal experiments were approved by the French Ministry of Agriculture and Fishery and performed by M.D.-E. at the Institut Universitaire de Technologie, IUTA, F-69622 Villeurbanne Cedex, France.

Authors’ contributions

DAF designed and performed all experiments except the animal studies which were conducted by MDE. MF participated in the conception and design of the study and helped to draft the manuscript.

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References


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CHAPTER 3

InXy and SeXy, Compact Heterologous Reporter Proteins
For Mammalian Cells

David A. Fluri, Jens M. Kelm, Guillaume Lesage, Marie Daoud-El Baba
and Martin Fussenegger (2007).
Biotechnology and Bioengineering, 98(3): 655-667
Abstract

Mammalian reporter proteins are essential for gene-function analysis, drug screening initiatives and as model product proteins for biopharmaceutical manufacturing. *Bacillus subtilis* can maintain its metabolism by secreting Xylanase A (XynA), which converts xylan into shorter xylose oligosaccharides. XynA is a family 11 xylanase monospecific for D-xylose containing substrates. Mammalian cells transgenic for constitutive expression of wild-type *xynA* showed substantial secretion of this prokaryotic enzyme. Deletion analysis confirmed that a prokaryotic signal sequence encoded within the first 82 nucleotides was compatible with the secretory pathway of mammalian cells. Codon optimization combined with elimination of the prokaryotic signal sequence resulted in an exclusively intracellular mammalian Xylanase A variant (InXy) while replacement by an immunoglobulin-derived secretion signal created an optimal secreted Xylanase A derivative (SeXy). A variety of chromogenic and fluorescence-based assays adapted for use with mammalian cells detected InXy and SeXy with high sensitivity and showed that both reporter proteins resisted repeated freeze/thaw cycles, remained active over wide temperature and pH ranges, were extremely stable in human serum stored at room temperature and could independently be quantified in samples also containing other prominent reporter proteins such as the human placental alkaline phosphatase (SEAP) and the *Bacillus stearothermophilus*-derived secreted α-amylosy (SAMY). Glycoprofiling revealed that SeXy produced in mammalian cells was N-glycosylated at four different sites, mutation of which resulted in impaired secretion. SeXy was successfully expressed in a variety of mammalian cell lines and primary cells following transient transfection and transduction with adeno-associated virus particles (AAV) engineered for constitutive SeXy expression. Intramuscular injection of transgenic AAVs into mice showed significant SeXy levels in the bloodstream. InXy and SeXy are highly sensitive, compact and robust reporter proteins, fully compatible with pre-existing marker genes and can be assayed in high-throughput formats using very small sample volumes.

Abbreviations:

AAV, adeno-associated virus; BHK, baby hamster kidney cells; CHO-K1, chinese hamster ovary cells; Cy3, cyanine 3; Cy5, cyanine 5; HEK293-T, human embryonic kidney cells transgenic for simian virus 40 large T antigen; His-Tag, hexa-histidine tag; HT-1080,
human sarcoma cell line; InXy, intracellular xylanase A; NHDF, normal human dermal fibroblasts; SAMY, Bacillus stearothermophilus-derived secreted α-amylase; SEAP, human placental secreted alkaline phosphatase; SeXy, secreted xylanase A; SS_{Igk}, signal sequence derived from the murine Igκ-chain V-12-C region; SS_{IL-2}, signal sequence derived from the human interleukin 2 gene; XynA, Bacillus subtilis xylanase A

**Introduction**

Secreted mammalian reporter proteins have been used extensively for functional genomic research (Kramer and Fussenegger, 2005), as marker genes in vivo (Mitta et al., 2005), for prototype gene therapy scenarios (Liu et al., 2004; Weber et al., 2004) as well as for high-throughput screening initiatives (Durocher et al., 2000; Lee et al., 2004). A variety of intracellular reporter proteins are available for use in mammalian cells such as (i) specific enzymes (β-galactosidase, thymidine kinase or chloramphenicol acetyltransferase) (An et al., 1982; Gorman et al., 1982; Mannhaupt et al., 1988; Searle et al., 1985), (ii) fluorescent reporter proteins represented by the pioneering green fluorescent protein (e.g. GFP, YFP or RFP) (Hadjantonakis et al., 2003; Yang et al., 2000), or (iii) bioluminescent proteins of the luciferase type (de Wet et al., 1985; Lorenz et al., 1991). While secreted reporter proteins are (i) easily accessible in the cell culture supernatant, (ii) do not require complex cell lysis and sample preparations, (iii) enable simple profiling of expression kinetics using a single culture and (iv) could serve as model product proteins for generic biopharmaceutical manufacturing scenarios, only a few secreted reporter genes are available including the human placental secreted alkaline phosphatase (SEAP) (Berger et al., 1988), secreted luciferase (Markova et al., 2004) and the Bacillus stearothermophilus-derived secreted α-amylase (Schlatter et al., 2002). SEAP and SAMY reporter proteins have functional endogenous homologs for which phosphatase and α-amylase activities must be inactivated before the heterologous marker protein can be reliably quantified (Berger et al., 1988).

*Bacillus subtilis* is able to metabolize the carbohydrate xylan, which is found associated with cellulose in plant cell walls (Roncero, 1983). Xylan is a polymer consisting of a β-1,4-linked xylose backbone with branches formed by xylose, other pentoses, hexoses, and uronic acid. Xylanases have been expressed and used as reporter proteins in bacteria and plants (Kamionka et al., 2004; Vickers et al., 2003). Bacterial family 10 xylanases (displaying activity on both, cellulose and hemi-cellulose) as well as true cellulases have been previously expressed in mammalian cells in order to improve nutritional performance of transgenic
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animals (Fontes et al., 1999; Soole et al., 1993; Zhang et al., 1997; Zhang et al., 1999). We have engineered B. subtilis xylanase A, a family 11 xylanase, which, in contrast to family 10 xylanases, exclusively catabolizes D-xylose-containing substrates for optimal intracellular production as well as for high-level secretion in mammalian cells. The modified xylanases produced by different mammalian cell lines and primary cells were extremely stable and could be quantified using a variety of chromogenic and fluorescent assays. Even after intramuscular transduction using adeno-associated virus particles, secreted xylanase could be detected in the bloodstream of mice.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description and Cloning Strategy</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pEF4-MycHis</td>
<td>Mammalian expression vector containing $P_{hEF1\alpha}$</td>
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<tr>
<td>pSEAP2</td>
<td>Expression plasmid encoding SEAP</td>
<td>Clontech</td>
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<td>pSS158</td>
<td>Expression plasmid encoding SAMY</td>
<td>Schlatter et al. 2002</td>
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<tr>
<td>pDF38</td>
<td>AAV expression vector harboring $P_{lUCMV}$</td>
<td>This work</td>
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<td>pDF149</td>
<td>Expression plasmid encoding full length B. subtilis xylanase A ($XynA$) driven by $P_{hEF1\alpha}$. $XynA$ was amplified from genomic B. subtilis DNA (strain 168) using ODF42/ODF43 and digested with BamHI/XbaI before cloning the fragment into the corresponding sites of pEF4-MycHis in-frame with C-terminal Myc- and His-tags resulting in pDF149 ($P_{hEF1\alpha}XynA-pA_{BGH}$).</td>
<td>This work</td>
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<td>pDF163</td>
<td>Expression plasmid encoding a XynA variant ($XynA_M$), harboring several codon modifications at its N-terminus for optimized translation in mammalian cells, driven by $P_{hEF1\alpha}$. $XynA_M$ was amplified from pDF149 using ODF53/ODF43 digested with EcoRI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF163 ($P_{hEF1\alpha}xynA_M-pA_{BGH}$).</td>
<td>This work</td>
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<td>pDF164</td>
<td>Expression plasmid encoding $XynA_M$ fused to Igκ secretion signal (METDTLLLWLLLWVPGSTGD, Coloma et al., 1992), derived from the murine Igκ-chain V-J2-C region, driven by $P_{hEF1\alpha}$, $SS_{Ig\kappa}-xynA_M$ was amplified from pDF163 using ODF41/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF164 ($P_{hEF1\alpha}SS_{Ig\kappa}xynA_M-pA_{BGH}$).</td>
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<td>Plasmid</td>
<td>Description and Cloning Strategy</td>
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<td>pDF184</td>
<td>Expression plasmid encoding N-terminally truncated form of XynAM (xynAM1-27) driven by PheF1α. XynAM1-27 was amplified from pDF163 using ODF56/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF184 (pheF1α-xynAM1-27-pA BGH)</td>
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<td>pDF186</td>
<td>Expression plasmid encoding N-terminally truncated form of XynAM (xynAM1-87) driven by PheF1α. XynAM1-87 was amplified from pDF163 using ODF58/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF186 (pheF1α-xynAM1-87-pA BGH)</td>
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<td>pDF187</td>
<td>Expression plasmid encoding N-terminally truncated form of XynAM (xynAM1-115) driven by PheF1α. XynAM1-115 was amplified from pDF163 using ODF59/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF187 (pheF1α-xynAM1-115-pA BGH)</td>
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<td>pDF188</td>
<td>Expression plasmid encoding N-terminally truncated form of XynAM (xynAM1-139) driven by PheF1α. XynAM1-139 was amplified from pDF163 using ODF60/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF188 (pheF1α-xynAM1-139-pA BGH)</td>
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<tr>
<td>pDF189</td>
<td>AAV2 vector encoding intracellular xylanase (InXy) fused to human IL-2 secretion signal (MYRMQLSCIALSLALVTNS, Sasada et al. 1998), driven by PheCMV. SS IL-2-InXy was excised from pDF190 with BamHI/Pmel and cloned without Myc- and His tags into the BamHI/EcoRV sites of pDF38 resulting in pDF189 (ITR-PheCMV-Intronβ-globin-SS IL-2-InXy-pA HGH-ITR)</td>
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<tr>
<td>pDF190</td>
<td>Expression plasmid encoding InXy fused to a human interleukin 2 (IL-2) secretion signal, driven by PheF1α. SS IL-2-InXy was amplified from pDF186 using ODF71/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF190 (pheF1α-SS IL-2-InXy-pA BGH)</td>
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<tr>
<td>pDF191</td>
<td>Expression plasmid encoding SeXy, a minimal intracellular version of XynA (InXy) fused to Igk secretion signal, driven by PheF1α. SS Igk-InXy (SeXy) was amplified from pDF186 using ODF72/ODF43 digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF191 (pheF1α-SeXy-pA BGH)</td>
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<tr>
<td>pDF193</td>
<td>AAV2 vector encoding SeXy driven by PheCMV. SeXy was excised from pDF191 with BamHI/Pmel and cloned into the BamHI/EcoRV sites of pDF38 resulting in pDF193 (ITR-PheCMV-Intronβ-globin-SeXy-pA HGH-ITR)</td>
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<tr>
<td>Plasmid</td>
<td>Description and Cloning Strategy</td>
<td>Reference or source</td>
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<td>pDF213</td>
<td>Expression vector encoding mutated SeXy (N30A). Mutated SeXy was amplified from pDF191 in a two-step fusion PCR reaction. (i) Two overlapping fragments were generated by PCR amplification using ODF74/ODF43 and ODF75/ODF72 on pDF191. (ii) The two fragments were purified and used as a template for the amplification of mutated SeXy, using ODF72/ODF43. The final product was digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF213 (P&lt;sub&gt;hEF1α&lt;/sub&gt;-SeXy&lt;sub&gt;N30A&lt;/sub&gt;-pA&lt;sub&gt;BGH&lt;/sub&gt;).</td>
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<tr>
<td>pDF214</td>
<td>Expression vector encoding mutated SeXy (N42A, N47A, N51A). Mutated SeXy was amplified from pDF191 in a two-step fusion PCR reaction. (i) Two overlapping fragments were generated by PCR amplification using ODF76/ODF43 and ODF77/ODF72 on pDF191. (ii) The two fragments were purified and used as a template for the amplification of mutated SeXy, using ODF72/ODF43. The final product was digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF214 (P&lt;sub&gt;hEF1α&lt;/sub&gt;-SeXy&lt;sub&gt;N42A,N47A,N51A&lt;/sub&gt;-pA&lt;sub&gt;BGH&lt;/sub&gt;).</td>
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<td>pDF215</td>
<td>Expression vector encoding mutated SeXy (N163A). Mutated SeXy was amplified from pDF191 in a two-step fusion PCR reaction. (i) Two overlapping fragments were generated by PCR amplification using ODF78/ODF43 and ODF79/ODF72 on pDF191. (ii) The two fragments were purified and used as a template for the amplification of mutated SeXy, using ODF72/ODF43. The final product was digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF215 (P&lt;sub&gt;hEF1α&lt;/sub&gt;-SeXy&lt;sub&gt;N163A&lt;/sub&gt;-pA&lt;sub&gt;BGH&lt;/sub&gt;).</td>
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<td>pDF216</td>
<td>Expression vector encoding mutated SeXy (N203A). Mutated SeXy was amplified from pDF191 in a two-step fusion PCR reaction. (i) Two overlapping fragments were generated by PCR amplification using ODF80/ODC10 and ODF81/ODF72 on pDF191. (ii) The two fragments were purified and used as a template for the amplification of mutated SeXy, using ODF72/ODC10. The final product was digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF216 (P&lt;sub&gt;hEF1α&lt;/sub&gt;-SeXy&lt;sub&gt;N203A&lt;/sub&gt;-pA&lt;sub&gt;BGH&lt;/sub&gt;).</td>
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<td>pDF218</td>
<td>Expression vector encoding mutated SeXy (N42A,N47A,N51A,N203A). Mutated SeXy was amplified from pDF214 in a two-step fusion PCR reaction. (i) Two overlapping fragments were generated by PCR amplification using ODF80/ODC10 and ODF81/ODF72 on pDF214. (ii) The two fragments were purified and used as a template for the amplification of mutated SeXy, using ODF72/ODC10. The final product was digested with BamHI/XbaI and cloned into the corresponding sites of pEF4-MycHis resulting in pDF218 (P&lt;sub&gt;hEF1α&lt;/sub&gt;-SeXy&lt;sub&gt;N42A,N47A,N51A,N203A&lt;/sub&gt;-pA&lt;sub&gt;BGH&lt;/sub&gt;).</td>
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Table 1. Plasmids used and designed in this study. Abbreviations: AAV2, adeno-associated virus type 2; InXy, intracellular xylanase; His-tag, hexa-histidine tag; ITR, inverted terminal repeat; Myc-tag, myc proto-oncogene epitope tag; pA_{BGH}, bovine growth hormone polyadenylation signal; pA_{SV40}, simian virus 40 polyadenylation signal; pA_{HGH}, human growth hormone polyadenylation signal; P_{hEF1α}, human elongation factor 1α promoter; P_{hCMV}, human immediate early cytomegalovirus promoter; SAMY, Bacillus stearothermophilus-derived secreted α-amylase; SeXy, secreted xylanase; SEAP, human placental secreted alkaline phosphatase; SS_{Igκ} signal sequence derived from the murine Igk-chain V-12-C region; SS_{IL-2}, signal sequence derived from the human interleukin 2 gene.
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**Table 2.** Oligonucleotides used in this study.
Materials and Methods

**Vector construction.** A detailed description of plasmid construction and oligonucleotides used is provided in Tables 1 and 2.

**Cell culture, transfection and transduction.** Human embryonic kidney cells, transgenic for the adenovirus type 5-derived E1 region as well as for the simian virus 40 (SV40) large T-antigen (HEK293-T; (Mitta et al., 2002)), human fibrosarcoma cells (HT-1080; ATCC CCL-121) and normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany; cat. no. C-12300, lot 1070402) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany; cat. no. 3302-P231902, lot no. P231902) and 1% penicillin/streptomycin solution (Sigma Chemicals, St. Louis, MO). Chinese hamster ovary cells (CHO-K1; ATCC CCL-61) were cultivated in ChoMaster® HTS medium (Cell Culture Technologies GmbH, Gravesano, Switzerland) supplemented with 5% FCS and 1% penicillin/streptomycin solution. CHO-K1 cell lines stably expressing secreted xylanase were constructed by transfection of pDF164 into CHO-K1 cells and selection in ChoMaster® HTS medium supplemented with 5% FCS, 1% penicillin/streptomycin and 100 mg/ml zeocin (Invitrogen). All cell lines were cultivated at 37°C in a 5% CO₂-containing humid atmosphere. Transient transfections were performed using standard CaPO₄-based protocols or FuGENE® (Roche Diagnostics AG, Basel, Switzerland) transfection reagent according to the manufacturers protocol.

For *in-vitro* experiments an MOI of 100 genomic particles/cell was used.

**Production of adeno-associated virus (AAV) particles.** AAV particles were produced by co-transfection of the helper plasmid pDG (Grimm et al., 2003) and the transgene-encoding AAV vector into HEK293-T using an optimized calcium phosphate-based transfection protocol. In brief, 30x 2x10⁶ HEK293-T cells were seeded into 30 culture dishes (diameter 10cm) and cultivated overnight. Per 10cm dish 15µg of pDG were mixed with 5µg AAV vector and 0.25M CaCl₂ was added to reach a final volume of 400µl. The mixture was added slowly to an equal volume (400µl) of HEPES-buffered saline solution (HeBS; 50mM HEPES, 280mM NaCl, 1.5mM Na₂HPO₄, pH 7.1), vortexed briefly and incubated for 2min before adding the DNA-CaPO₄ precipitate solution to the monolayer culture. The precipitate was removed after 6h, and the cells were incubated in 10% FCS-supplemented DMEM for 60h. For AAV particle recovery, the culture supernatant was discarded, the cells were detached using a cell scraper and resuspended in 1ml PBS (137.9mM NaCl, 8.1mM
Na₂HPO₄, 1.47mM KH₂PO₄, 2.67mM KCl; Invitrogen, Carlsbad, CA) per 10cm dish. The combined cell suspensions were then transferred to a 50ml Falcon tube and pelleted by centrifugation for 3min at 1200xg. After another washing step using 30ml PBS, the cells were resuspended in 20ml PBS and the intracellular viral particles were released following cell lysis, then induced by three consecutive freeze-thaw cycles consisting of shuttling the tube between liquid nitrogen and a 37°C water bath (tube was vortexed vigorously after each thawing step). The cell debris was eliminated by centrifugation for 5min at 10,000xg and the supernatant containing the crude viral particle stock was collected, supplemented with 50U/ml of Benzonase (Sigma, cat. no. E1014) and incubated for 30min at 37°C. Iodixanol density gradient purification of viral particles was performed according to Zolotukhin et al. (Zolotukhin et al., 1999). Step gradients were centrifuged for 3.5h at 150,000xg (18°C). The clear 40% iodixanol layer was harvested by puncturing the side of the tube with a 16G needle attached to a syringe. A heparin affinity column (HiTrap Heparin HP, Amersham Biosciences, Sweden; cat. no. 17-0407-01) was equilibrated with 10 bed volumes of binding buffer (10mM Na₂PO₄, pH 7) at a flow rate of 4ml/min. The 40% iodixanol layer of the gradient containing the viral particles was loaded onto the column. After washing with 10 bed volumes of binding buffer the viral particles were eluted with 5 bed volumes of elution buffer (10mM Na₂PO₄, 1M NaCl, pH 7) and were then concentrated using a spin column with a molecular weight cut-off (MWCO) of 30 kDa (Vivaspin, Vivascience, Germany; cat. no. VS1521).

**Xylanase assays. DNS (3,5-dinitrosalicylic acid) assay:** 50µl of xylanase A-containing cell culture supernatant were centrifuged at 16,000xg for 1min to remove cell debris and 30µl were then transferred to 90µl of substrate solution (100mM sodium acetate, pH5.5, 1% birchwood xylan [Sigma, cat. no. X0502, lot no. 044K0169]) and incubated for 30min at 37°C. Xylanase A-mediated xylan degradation was stopped by addition of 120µl DNS solution (dissolve 1g 3,5-dinitrosalicylic acid [Sigma, cat. no. D0550, lot no. 80K3490] and 30g sodium potassium tartrate in 20ml 2N NaOH and adjust to 100ml using dH₂O). Boiling of the reaction mixture for 5min results in DNS-mediated covalent chromogenic modification of glucose liberated by xylanase A-driven xylan breakdown, which can be quantified after addition of 760µl H₂O by scoring absorbance at 540nm and comparing the value to a calibration curve produced with pure glucose (Fluka, cat. no. 49139, lot R00354). Xylanase units were calculated according to the following equation:
Enzymatic activity (U*l⁻¹): EA = µmol*min⁻¹*l⁻¹ glucose produced

**PAHBAH (p-hydroxybenzoic acid hydrazide) assay:** 50µl of xylanase A-containing cell culture supernatant were centrifuged at 16,000xg for 1min to remove cell debris and 30µl were then transferred to 90µl substrate solution (100mM sodium acetate, pH 5.5, 1% birchwood xylan [Sigma, cat. no. X0502, lot no. 044K0169]) and incubated for 30min at 37°C. Xylanase A-mediated xylan degradation was stopped by addition of 1ml PAHBAH reagent (1% p-hydroxybenzoic acid hydrazide [ABCR, cat. no. AV12702] in 0.5M NaOH). Boiling of the reaction mixture for 5 min results in PAHBAH-mediated covalent chromogenic modification of glucose liberated by xylanase A-driven xylan breakdown, which can be quantified by scoring absorbance at 405nm and comparison to a calibration curve generated using pure glucose (Fluka). Xylanase units were calculated using the equation indicated above.

**Remazol brilliant blue xylan (RBB-xylan) assay:** 50µl of xylanase A-containing cell culture supernatant were centrifuged at 16,000xg for 1min to remove cell debris. 30µl were then transferred to 90µl RBB-xylan solution and incubated for 60 min at 37°C. Xylanase-mediated RBB release from RBB-xylan was stopped by addition of two volumes of 100% ethanol and the reaction mixture was centrifuged for 2min at 16,000g. 200µl of the supernatant were transferred to a 96-well plate, absorbance was measured at 600nm and compared to readings taken from standardized solutions containing defined xylanase (Sigma, cat. no. X3876) concentrations.

**Fluorescence-based xylanase assay:** The EnzCheck® xylanase assay (Molecular Probes, Eugene, OR, USA; cat. no. E33650, lot no. 35416A) was performed according to the manufacturer’s protocol. In brief, 20µl of cell culture supernatant or cleared cell lysate were centrifuged at 16,000g for 1min to remove debris. 15µl were transferred to a 384-well plate and incubated for 30min at 37°C after addition of a substrate mix (fluorescence substrate [3.3% v/v] in 100mM sodium acetate). Fluorescence intensity was measured at an excitation wavelength of 340nm and an emission wavelength of 450nm on a Genios Pro multiwell plate reader (Tecan AG, Männedorf, Switzerland). Xylanase from *Trichoderma viride* (Sigma, cat. no. X3876, lot no. 036K4147) was used as an internal standard.

**Glycosylation profiling and immunofluorescence.** Cell extracts were prepared by detaching HEK293-T cells from 6-well plates using a cell scraper, collecting them by centrifugation at 280xg and washing the cells once with 2ml PBS. The cells were then
resuspended in 400µl PBS and lysed by three freeze/thaw cycles consisting of shuttling the sample twice between a 37°C water bath and liquid nitrogen before clearing the lysate by centrifugation at 16,000xg and 4°C for 5min. Preparation of secreted proteins was performed by harvesting cell culture supernatants, followed by centrifugation for 2min at 16,000g to remove cells and cell debris and subsequent transfer of cleared supernatants to fresh tubes.

For glycosylation analysis, 90µl of cell culture supernatants were treated with PNGase F (New England Biolabs, Beverly, MA; cat. no. P0704S, lot no. 30) as indicated in the manufacturer’s protocol. 40µl of the de-glycosylated samples were mixed with 10ml 5x SDS loading buffer (50% glycerol, 10%SDS, 250mM Tris, pH6.8 containing 10% (v/v) β-mercaptoethanol) and boiled for 5min before loading on a 9% SDS-polyacrylamide gel. Proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA; cat. no. IPVH20200, lot no. K3AN0841E). After blocking in TBS (50mM Tris, 150mM NaCl, pH7.5) containing 3% non-fat dry milk, the membrane was probed with a monoclonal antibody specific for the hexa-histidine tag (HIS, Novagen, Madison, WI, USA; cat. no. 70796-3 lot N56036) or mouse serum (diluted 1/50) and visualized using an anti-mouse IgG coupled to a horseradish peroxidase (ECL™ Anti Mouse IgGxHRP, Amersham, Buckinghamshire, UK) and a chemiluminescence-based assay (ECL™ plus, Amersham) according to the manufacturer’s protocol.

**Immunofluorescence-based analysis.** An 8-chamber culture slide (NUNC™, Roskilde, Denmark) was inoculated with 5000 HT-1080 cells per well. After 2 days in culture the cells were fixed with 4% paraformaldehyde (Sigma, cat. no. 76240) in phosphate-buffered saline (PBS; Invitrogen) for 15 min, and subsequently washed twice for 5min in PBS. Cell monolayers were then permeabilized for 15min using 0.5% Triton X-100 in PBS containing 1%BSA and then washed twice for 5min with PBS. Cells were then blocked for 2h in PBS containing 1% BSA and 5% goat serum (Sigma, cat. no. G9023). Primary antibodies specific for the endoplasmic reticulum marker protein GRP78/BiP (rat polyclonal; Sigma, cat. no. G8918, lot 073K4875) and hexa-histidine tag (mouse monoclonal; Novagen, Madison, WI, USA; cat. no. 70796-3 lot N56036) were diluted in 1% BSA and 5% goat serum-containing PBS. Primary antibodies were incubated for 4h at 4°C and subsequently washed three times in PBS. Cy3-coupled anti-mouse (Jackson Immunochemicals, West Grove, PA; cat. no. 115-165-146) and Cy5-coupled anti-rabbit (Jackson Immunochemicals, West Grove, PA; cat. no. 111-175-144) secondary antibodies were incubated for 4 hours at room temperature. Finally, the cells were washed three times with PBS and embedded using Tris-buffered glycerol (a 3:7
mixture of 0.1 M Tris-HCl [pH 9.5] and glycerol supplemented with 50 mg/ml n-propyl-gallat).

The imaging system consisted of an inverted fluorescence microscope (Leica DMIRB/E, Glattbrugg, Switzerland) equipped with a Leica 63x oil immersion objective, a confocal scanner (Leica TCS SP1) featuring an argon and helium-neon laser and a Silicon Graphics Workstation (SGI, Schlieren, Switzerland) with Imaris 3D multi-channel image processing software installed (Bitplane, AG, Zurich, Switzerland) (Messerli et al., 1993).

Animal studies. Female OF1 (oncins france souche 1) mice were obtained from Iffa-Credo (Lyon, France). Mice were treated with intramuscular injections of 200µl PBS containing pDF193-derived AAV particles adjusted to 1x10^{10} particles/ml. Blood samples were collected retroorbitally at day 6, 19 and 34 and serum was prepared using microtainer SST tubes (Beckton Dickinson, Plymouth, UK). All experiments involving animals were conducted according to European Community legislation (86/609/EEC), and have been approved by the French Republic (No. 69266310) and performed by M.D.-E. at the Institut Universitaire de Technologie, IUTA, F-69622 Villeurbanne Cedex, France.

Prediction of N-glycosylation sites. All predictions of possible glycosylation sites were performed by using NetNGlyc software (www.cbs.dtu.dk/services/NetNGlyc).

Results

Cloning and expression of *Bacillus subtilis* xylanase A in mammalian cells

PCR-mediated amplification of full-length genomic *Bacillus subtilis* xylanase A (*xynA*) and constitutive production in a transfected human embryonic kidney cell line (HEK293-T) of an entirely eukaryotic expression platform (pDF149; P_{hEF1α}-xynA-pA_{BGH}) showed that Xylanase A is efficiently secreted (Figure 1A). Computational analysis of the bacterial coding sequence revealed several codons in the N-terminus, which were predicted to be suboptimal for efficient translation in mammalian cells. We have therefore designed a modified mammalian cell-compatible *xynA* derivative (*xynAM*) harboring a codon-optimized 5’-terminus (pDF163; P_{hEF1α}-xynAM-pA_{BGH}). Comparative expression profiling using the isogenic vectors pDF149 (P_{hEF1α}-xynA-pA_{BGH}) and pDF163 (P_{hEF1α}-xynAM-pA_{BGH}) showed an almost two-fold higher production level of XynAM compared to the native prokaryotic XynA (Figure 1A).
In order to characterize the sequence motif mediating Xylanase A secretion in mammalian cells, we have designed a variety of N-terminal xynA deletion mutants and expressed them in HEK293-T using an expression configuration isogenic to pDF163. The observation (i) that pDF184 (P_{hEF1α}-xynA_{M1-pA_{BGH}}) mediated Xylanase A secretion levels identical to pDF163 (P_{hEF1α}-xynA_{M-pA_{BGH}}), (ii) that pDF187 (P_{hEF1α}-xynA_{M1-115-pA_{BGH}}) as well as pDF188 (P_{hEF1α}-xynA_{M1-139-pA_{BGH}}) showed no Xylanase A production at all and (iii) that Xylanase A mutants expressed from pDF186 (P_{hEF1α}-xynA_{M1-82-pA_{BGH}}) were produced but no longer secreted, suggests that the mammalian cell-compatible prokaryotic secretion signal is encoded within the first 27 amino acids of XynA’s N-terminus (Figure 1A). pDF186-encoded XynA_{M1-82} is therefore an ideal intracellular mammalian reporter protein referred to as InXy (intracellular xylanase A).

In order to modify XynA for high-level secretion in mammalian cells we have fused InXy N-terminally to two strong mammalian secretion signals (SS) derived from human interleukin 2 (IL-2; SS_{IL-2}) (pDF190; P_{hEF1α}-SS_{IL-2}-InXy-pA_{BGH}) or from the murine immunoglobulin Igk-chain V-J2-C region (SS_{Igk}) (pDF164; P_{hEF1α}-SS_{Igk}-xynA_{M-pA_{BGH}}; pDF191; P_{hEF1α}-SS_{Igk}-InXy-pA_{BGH}). Comparative production profiling in transiently transfected HEK293-T cells showed that SS_{Igk}-containing XynA variants were more efficiently secreted than the SS_{IL-2}-InXy fusion protein (pDF190; P_{hEF1α}-SS_{IL-2}-InXy-pA_{BGH}) and the codon- optimized full-length prokaryotic variant (pDF163; P_{hEF1α}-xynA_{M-pA_{BGH}}) (Figure 1B). Although the secretion performance of pDF164 (P_{hEF1α}-SS_{Igk}-xynA_{M-pA_{BGH}}) and pDF191 (P_{hEF1α}-SS_{Igk}-InXy-pA_{BGH}) was equivalent, SS_{Igk}-InXy was smaller and therefore considered the ideal secreted xylanase (SeXy), which was used for further studies.

Transient transfection of SeXy-encoding expression vectors into different cell types showed comparable expression and secretion levels (i) in the human cell lines HEK293-T and HT-1080, (ii) in the biotechnologically relevant rodent cell lines CHO-K1 and BHK-21 as well as to a lower extent (iii) in human primary fibroblasts (NHDF) (Figure 1C). Immunofluorescence analysis of HEK293-T transiently transfected for InXy (pDF186; P_{hEF1α}-InXy-pA_{BGH}) and SeXy (pDF191; P_{hEF1α}-SeXy-pA_{BGH}) production showed that SeXy co-localized with the ER marker protein GRP78/BiP indicating that it is secreted via the classical mammalian secretory pathway, whereas InXy was found exclusively in the cytosol (Figure 2).
Enzymatic assays

We have refined different assays originally established for quantification of fungal and bacterial xylanases and evaluated their sensitivity for detection of SeXy production in the culture supernatants of mammalian cells. Two assays (DNS, 3,5-dinitrosalicylic acid; PAHBAH, p-hydroxybenzoic acid hydrazide) capitalize on chromogenic modification of a substrate by reducing sugar liberated by xylanase-mediated degradation of purified birchwood xylan (Lever, 1972) (Bailey and Poutanen, 1989), the remazol brilliant blue (RBB) assay is based on xylanase-driven release of RBB from modified RBB-xylan (Biely et al., 1985) and the fluorescence assay which takes advantage of xylanase-based conversion of a synthetic substrate into a fluorescent product (EnzChek ultra xylanase assay, Molecular Probes). Key performance parameters including detection limit and background activity of all xylanase assays were determined by analysis of serially diluted HEK293-T culture supernatants 48h after transient production of pDF191-encoded SeXy.

The DNS method was the least sensitive of the four xylanase assays (detection limit at 20mU/ml) and also displayed elevated levels of background signal originating from the residual glucose in the culture medium or endogenous glycoproteins produced by the cells. The PAHBAH assay showed increased sensitivity (detection limit at 10mU/ml) but featured the same elevated background levels as the DNS method. RBB-Xylan is a convenient substrate with very low background activity but only average sensitivity (detection limit at 5mU/ml). The most powerful assay in terms of detection limits, ease of handling and low background activity is the EnzCheck® Ultra Xylanase Assay Kit (Molecular Probes) allowing fast detection of xylanase activity as low as 0.5 mU/ml at negligible background levels (Table 3).
Figure 1. Quantification of xylanase A activity in HEK293-T cells. (A) Culture supernatants (black) as well as cell lysates (grey) of HEK293-T cells transiently transfected with pDF149 (P_{hEF1α}-xynA-pA_{BGH}), pDF163 (P_{hEF1α}-xynA_{M}-pA_{BGH}), pDF184 (P_{hEF1α}-xynA_{MD1-27}-pA_{BGH}), pDF186 (P_{hEF1α}-xynA_{MD1-82}-pA_{BGH}), pDF187 (P_{hEF1α}-xynA_{MD1-115}-pA_{BGH}) and pDF188 (P_{hEF1α}-xynA_{MD1-139}-pA_{BGH}) were assayed for xylanase production. (B) XylanaseA production was determined in cell culture supernatants transiently transfected with pDF164 (P_{hEF1α}-SS_{IL2}-xynA_{M}-pA_{BGH}), pDF186 (P_{hEF1α}-InXy-pA_{BGH}), pDF190 (P_{hEF1α}-SS_{IL2}-InXy-pA_{BGH}), pDF191 (P_{hEF1α}-SeXy-pA_{BGH}) and pDF163 (P_{hEF1α}-xynA_{M}-pA_{BGH}). (C) Xylanase A production was determined in different cell types transiently transfected with pDF191 (P_{hEF1α}-SeXy-pA_{BGH}).
Figure 2. Subcellular localization of xylanase A in HEK293-T cells. HEK293-T cells were transfected either with pDF191 (PheF1α-SeXy-pAECG) and pDF186 (PheF1α-InXy-pAECG) or no vector (control). Intracellular localization was visualized by immunofluorescence microscopy using SeXy- and 78kDa glucose regulated protein (GRP78/BiP)-specific primary antibodies as well as secondary Cy3-coupled anti-mouse and Cy5-coupled anti-rabbit antibodies. The third and fourth column show an overlay of the Cy3 and the Cy5 channels to visualize xylanase localization relative to the endoplasmic reticulum (ER) and a differential interference contrast (DIC) image showing all cells in range.
Table 3. Comparison of different assays used to quantify xylanase A activity from cell culture supernatants.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Detection limit [mU/ml]</th>
<th>Assay time [min]</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNS</td>
<td>20</td>
<td>60</td>
<td>High (&gt;50mU/ml)</td>
</tr>
<tr>
<td>PAHBAH</td>
<td>10</td>
<td>60</td>
<td>High (&gt;50mU/ml)</td>
</tr>
<tr>
<td>RBB-Xylan</td>
<td>5</td>
<td>80</td>
<td>Medium (10mU/ml)</td>
</tr>
<tr>
<td>EnzCheck®</td>
<td>0.5</td>
<td>40</td>
<td>Low (1mU/ml)</td>
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</table>

**Stability of SeXy and InXy**

Stability is a key characteristic of reporter proteins as it enables precise detection of actual protein levels without considering protein degradation or a considerable loss of activity during the assay procedure. We have assayed the stability of SeXy in human serum by mixing cell culture supernatants of pDF191-transfected HEK293-T cells with human AB serum and incubated the samples at 37°C for 15 days. Samples were removed every three days and frozen at -20°C. The stability of SeXy in serum was excellent with only a minor loss of activity over the investigated time period (Figure 3A). SeXy’s half-life ($t_{1/2}$) of 1500h compared favorably with the best-in-class reporter protein the human placental secreted alkaline phosphatase (SEAP, $t_{1/2}$ 502h; (Schlatter et al., 2002)) as well as the low molecular weight urokinase-type plasminogen activator (u-PA$_{LMW}$, $t_{1/2}$ 61h; (Schlatter et al., 2002)) and was of the same order of magnitude as the Bacillus stearothermophilus-derived secreted α-amylase (SAMY, $t_{1/2}$ 1505h; (Schlatter et al., 2002)) (Figure 3A).

Besides stability at room temperature in complex solutions such as human serum, stable activity over a broad pH range, the robustness in multiple freeze/thaw cycles and resistance to increased temperatures qualify perfect reporter proteins. SeXy and InXy, incubated for 48h in buffers adjusted to different pH, retained its standard activity within a pH range from 3.5 to 9 (Figure 3B). Although not derived from an extremophilic organism as SAMY, SeXy and InXy retained their activity over a broad temperature range with a peak activity around 45°C (Figure 3C). Multiple freeze/thaw (-20°C/+20°C) cycles were tolerated by SeXy and InXy without showing any decrease in activity (Figure 3D). The observation that InXy resists freeze/thaw cycles significantly simplifies assay protocols as InXy-producing cells could simply be frozen for storage and thawed for immediate analysis as InXy is completely liberated from disrupted cells (Figure 3D).
Figure 3. Quantification of SeXy and InXy activity in culture supernatants of HEK293-T transiently transfected with pDF191 (PMel-SeXy-pA<sub>BGH</sub>) and pDF186 (PMel-InXy-pA<sub>BGH</sub>) treated under different conditions. (A) Stability of native (●) and peptide, N-glycosidase F (PNGase F)-treated (■) SeXy incubated in human serum. (B) Activity of SeXy (black) and InXy (grey) after incubation at different pH values for 48h at 37°C. (C) SeXy (black) and InXy (grey) activity at different incubation temperatures. (D) A change in enzyme activity was determined after up to six consecutive freeze/thaw cycles with an assay of SeXy (black) and InXy (grey) activity after each round.
Compatibility of SeXy with other secreted reporter proteins and stable expression of SeXy in transgenic CHO cells

Combinatorial expression analysis and elucidation of complex regulatory cascades require the simultaneous use of different compatible reporter proteins whose activity can be independently assayed in the same cell culture supernatant (Fux and Fussenegger, 2003). In order to assess the enzymatic compatibility of SeXy with other established reporter proteins such as SEAP and SAMY we cotransfected HEK293-T cells with pSEAP2-control (P<sub>SV40</sub>-SEAP-pA<sub>SV40</sub>-E<sub>SV40</sub>), pSS158 (P<sub>hCMV</sub>-SAMY-pA<sub>BGH</sub>), and pDF191 (P<sub>SV40</sub>-SeXy-pA<sub>BGH</sub>) and followed expression of the three secreted reporter proteins for 48h after transfection. All three reporter proteins showed a comparable increase in reporter activity in the supernatant over time, suggesting that they would be compatible in simultaneous analysis (Figure 4A).

SeXy was stably expressed in a clonal CHO-K1 cell line and proliferation rate as well as specific reporter productivity were monitored over three days. Transgenic CHO-K1 cells showed wild type-like growth characteristics and sustained SeXy production (Figure 4B).

Glycosylation analysis of SeXy

Most of today’s blockbuster protein pharmaceuticals are glycoproteins. In order for a reporter protein to capture product parameters relevant for biopharmaceutical manufacturing it needs to be glycosylated. In order to characterize SeXy’s glycoprofile after production in mammalian cells, we incubated pDF191 (P<sub>SV40</sub>-SeXy-pA<sub>BGH</sub>)-transfected HEK293-T cells with tunicamycin (inhibiting N- as well as O-glycosylation), or treated SeXy-containing culture supernatants with PNGase F (exclusive elimination of N-glycans).

Western blot analysis of SeXy isoforms revealed that untreated cell culture supernatants showed distinct SeXy-specific bands suggesting that SeXy is produced in several N-glycosylated isoforms. Cultivation of SeXy-producing cells in tunicamycin-containing cell culture medium or treating the supernatants with PNGase F produced a single band of 22kDa which is expected to represent the unglycosylated form of SeXy (Figure 5). Software-based analysis of SeXy suggested seven N-glycosylation sites which we have eliminated by designing the following mutants: pDF213 (P<sub>hEF1a</sub>-SeXy<sub>N30A</sub>-pA<sub>BGH</sub>), pDF214 pDF234 (P<sub>hEF1a</sub>-SeXy<sub>N42A</sub>-pA<sub>BGH</sub>), pDF235 (P<sub>hEF1a</sub>-SeXy<sub>N47A</sub>-pA<sub>BGH</sub>), pDF236 (P<sub>hEF1a</sub>-SeXy<sub>N51A</sub>-pA<sub>BGH</sub>), pDF215 (P<sub>hEF1a</sub>-SeXy<sub>N163A</sub>-pA<sub>BGH</sub>) and pDF216 (P<sub>hEF1a</sub>-SeXy<sub>N203A</sub>-pA<sub>BGH</sub>). Whereas pDF213- and pDF236 -encoded SeXy yielded the same glycosylation patterns as wild-type SeXy (Figure 6), all other SeXy mutants showed a distinct but different glycoprofile,
suggesting that Asn 42, 47, 163 and 203 are indeed glycosylated (Figure 6). We were able to detect all glycoforms in the supernatant except for SeXY_{N42A,N47A,N51A,N163A,N203A} (pDF220) which has all of its glycosylation sites eliminated and was found exclusively in the cytosol. This observation indicated that glycosylation is essential for secretion of SeXy.

**Figure 4.** (A) Simultaneous expression and quantification of three different secreted mammalian reporter proteins in the same culture supernatant. pDF191 (p_{hEF1α}-SeXy-pAV), pSS158 (p_{hCMV}-SAMY-pAV) and pSEAP2-control (p_{SV40-SEAP-pAV}) were co-transfected at equimolar ratios and reporter proteins were quantified in the same supernatants. (B) Analysis of proliferation and specific production rates of a CHO-K1 cell clone stably expression SeXy.
SeXy expression by different cell lines and in mice after transduction with transgenic adeno-associated virus particles

In order to validate SeXy as a universal reporter protein for in-vitro as well as in-vivo use, we have engineered adeno-associated virus (AAV) particles for transduction of a variety of mammalian cell lines (HEK293-T; HT-1080; CHO-K1), primary cells (normal human dermal fibroblasts [NHDF]) as well as mice with constitutive SeXy expression. Transduction of HEK293-T, HT-1080, CHO-K1 and NHDF with pDF193- (ITR-P_{hCMV-Intron_{β-globin-SeXy-pAHIGH-ITR}}- and PDF189- (ITR-P_{hCMV-Intron_{β-globin-SSIL-2-InXy-pAHIGH-ITR}}) derived AAV particles induced SeXy production in all cell types after 48h (Figure 7).

Figure 5. Western blot analysis of SeXy derived from pDF191 (P_{hEF1a-SeXy-pA_BGH})-transfected HEK293-T cells cultivated in the presence and absence of 2µg/ml tunicamycin and optionally treated with Peptide N-Glycosidase F (PNGase F).

Intramuscular injection of mice with pDF193-derived AAV, showed significant SeXy levels in their serum after 6 days (2U±0.3U/l). Mouse sera analyzed 19 and 34 days after injection of transgenic AAVs showed no further SeXy production but contained SeXy-specific antibodies when used to probe Western blot membranes with bound SeXy from
supernatants of pDF191-transfected HEK293-T (Figure 8). The Western blot signals were identical to those shown in Figures 5 and 6 using HIS tag-specific antibodies, which confirms the specificity of the mouse antibodies for a highly glycosylated SeXy protein. These SeXy-specific mouse antibodies, which suggest that SeXy is immunogenic, may clear the reporter protein from the bloodstream.

**Figure 6.** Western blot analysis of SeXy mutants harboring specific mutations at predicted N-glycosylation sites. pDF191 (P_{hEF1α}-SeXy-pA_{BGH}), pDF213 (P_{hEF1α}-SeXyN30A-pA_{BGH}), pDF234 (P_{hEF1α}-SeXyN42A-pA_{BGH}), pDF235 (P_{hEF1α}-SeXyN47A-pA_{BGH}), pDF236 (P_{hEF1α}-SeXyN51A-pA_{BGH}), pDF215 (P_{hEF1α}-SeXyN163A-pA_{BGH}), pDF216 (P_{hEF1α}-SeXyN203A-pA_{BGH}) and pDF220 (P_{hEF1α}-SeXyN42A,N47A,N51A,N163A,N203A-pA_{BGH}) were transiently transfected into HEK293-T and supernatants as well as cell pellets (after three times freeze/thaw) were used for Western Blot-based analysis of SeXy glycoprofiles.
Figure 7. Quantification of SeXy in cell culture supernatants of different mammalian cell lines and primary cells transduced with transgenic AAV particles. pDF193 (ITR-P$_{hCMV}$-Intron-β-globin-SeXy-pA$_{HGH}$-ITR) as well as pDF189 (ITR-P$_{hCMV}$-Intron-β-globin-SS$_{IL2}$-xynA$_{MF}$-pA$_{HGH}$-ITR)-derived AAV particles were used to transduce HEK293-T, HT-1080, CHO-K1 and NHDF monolayer cultures. Expression was quantified in each supernatant 48h after transduction.

Figure 8. Western blot analysis of SeXy produced by pDF193-transduced HEK293-T using serum of mice intramuscularly injected with SeXy-producing AAV particles. Mouse serum was probed for the presence of circulating antibodies against SeXy after 19 and after 34 days. A membrane containing bound SeXy was probed with diluted mouse serum and bands were visualized using an horseradish peroxidase (HRP)-coupled anti-mouse antibody.
Discussion

*Bacillus subtilis* -derived xylanase A versions engineered for intracellular (InXy) and secreted (SeXy) production in mammalian cells share several characteristics of an ideal mammalian reporter protein and have considerable advantages over currently used secreted reporter systems: (i) with a size of only 642bp (InXy) and 702bp (SeXy) the xylanase A variants are amongst the most compact (secreted) reporter genes which favors their use in space-limited expression scenarios such as viral transduction systems. (ii) The lack of structural and functional homologs in mammalian cells enables interference-free quantification of InXy and SeXy in the absence of endogenous background signals and without the need for complex sample preparations. (iii) Since InXy and SeXy process an unphysiologic substrate their expression is unlikely to elicit any pleiotropic effects. (iv) InXy and SeXy are an isogenic pair of reporter proteins enabling precise assessment of intracellular production and secretion capacities of mammalian cells. (v) Both reporter proteins are compatible with a variety of different assay systems including the highly sensitive fluorescence-based detection technology. (vi) Xylanase A-based reporter proteins are extremely stable in complex solutions and at room temperature over extended periods of time, which enables consistent quantifications any time after sampling without considering complex degradation profiles. (vii) InXy and SeXy activities are highly robust allowing precise quantification over a wide range of temperature and pH changes. (viii) InXy and SeXy are resistant to repeated freeze/thaw cycles facilitating handling and reproducible analysis. Also, a single freeze/thaw cycle releases ready-to-assay InXy from mammalian cells. (ix) Since SeXy is N-glycosylated like most marketed protein pharmaceuticals it could be used for evaluation of generic biopharmaceutical manufacturing scenarios. (x) SeXy is compatible with viral transduction and can be expressed in a variety of mammalian cell types. (xi) InXy and SeXy are functionally compatible with other prominent reporter proteins such as SEAP and SAMY and can be independently quantified in a sample containing both of these proteins. (xii) SeXy circulates in the bloodstream of mice after intramuscular transduction.

Earlier findings exemplified the targeting of proteins harboring prokaryotic secretion signals to the mammalian secretion apparatus (Fontes et al., 1999; Schlatter et al., 2002; Soole et al., 1993) and it was therefore not unexpected that wild type Xylanase A transiently expressed in mammalian cells was secreted to the supernatant. Although secretion of *B. subtilis* Xylanase A was substantial, it could be further increased by optimizing the codons for
optimal translation in mammalian cells and by replacing the microbial signal sequence by mammalian homologues. An immunoglobulin-derived signal sequence promoted the highest secretion level of different Xylanase A variants and was therefore used as the standard secreted xylanase A (SeXy) for use in mammalian cells. Preliminary glycoprofiling suggested SeXy to be N-glycosylated at four different sites. Since most protein pharmaceuticals are N-glycosylated SeXy could be an attractive reporter protein to study prototype biopharmaceutical scenarios and their impact on overall product quality. Glycosylation analysis using SeXy is particularly straightforward as the protein (i) is very stable in cell culture supernatants, (ii) yields characteristic banding patterns with distinct signals for all five glycosylation states and (iii) is C-terminally tagged with a hexa-histidine tag for convenient direct analysis by western blot or purification on nickel resins. InXy and SeXy are easily traceable using different assays, which vary in cost, speed and sensitivity. Although and PAHBAH tests capitalizing on chromogenic detection of reducing sugars released by the reporter proteins from standardized xylan are rapid and low cost, sugars in the cell culture medium or released by cells increase background and compromise assay sensitivity. While the RBB assay proved to be reasonably robust, cheap and sensitive for every-day use, the fluorescence-based InXy and SeXy detection assay is the most sensitive and should be applied for reference studies.

For most assay and protein characteristics InXy and SeXy compare favorably with other secreted reporter genes such as SEAP and SAMY. However, unlike SEAP and SAMY for which functional homologs (phosphatases and amylases, exist in mammalian cells and which may contribute to significant background, InXy and SeXy are functionally heterologous and if xylanase activity is detected in mammalian samples it is exclusively originating from these reporter proteins. Sensitive and robust secreted reporter proteins will become increasingly important as model proteins for biopharmaceutical manufacturing, for gene-function analysis and promoter characterization as well as for drug screening initiatives. InXy and SeXy are expected to meet with those applications and challenges at a high standard and represent a unique addition to the available portfolio of secreted reporter proteins.
Chapter 3

Acknowledgements

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References


CHAPTER 4

A Novel System For Trigger-Controlled Drug Release
From Polymer Capsules

David A. Fluri, Christian Kemmer, Marie Daoud-El Baba and
Martin Fussenegger (2008)
submitted
Abstract

Technologies currently available for the controlled release of protein therapeutics involve either continuous or tissue-specific discharge from implants or engineered extracellular matrix mimetics. For some therapeutic applications the trigger-controlled release of protein cargo from a synthetic implant could be highly desirable. We have designed the CellEase technology, a two-component system consisting of cellulose sulfate (CS) polydimethyl-diallylammonium chloride (pDADMAC) capsules harboring mammalian sensor cells transgenic for trigger-inducible expression of an engineered secreted mammalian cellulase (SecCell). SecCell is a *Bacillus subtilis*-derived (1-4)-β-glucanase, which was modified by replacing the N-terminal part of the bacterial enzyme with a murine Igκ-chain V-12-C region-derived secretion signal. SecCell was engineered for doxycycline- or erythromycin-inducible expression to enable trigger-controlled secretion by mammalian cells. Detailed characterization of SecCell showed that it was glycosylated and efficiently secreted by a variety of mammalian sensor cells such that it could internally rupture CS-pDADMAC capsules within which the cells had been encapsulated. When SecCell was inducibly expressed in sender cells, that were co-encapsulated with producer cell lines expressing therapeutic proteins, the removal of relevant inducer molecules enabled the time-dependent discharge of these therapeutic proteins, the kinetics of which could be modified by varying the concentration of inducer molecules or the amount of encapsulated sender cells. SecCell’s capacity to rupture CS-pDADMAC capsules exclusively internally also enabled the independent trigger-induced release of different proteins from two capsule populations harboring different inducible SecCell sensor cells. CellEase-based protein release was demonstrated in vivo using capsules implanted intraperitoneally into mice that enabled the doxycycline-controlled release of a model glycoprotein and accumulation in the bloodstream of treated animals. Trigger-induced breakdown of tissue-compatible implants which provide a timely controlled release of biologics may foster novel opportunities in human therapy.

Introduction

The controlled release of bioactive molecules from polymer matrices or polymer capsules has been proposed as a promising approach in various therapeutic interventions in order to avoid multiple dosing and to sustain continuous or pulsed release over time. The
entrapment and immuno-isolation of small-molecule drugs, hormones, protein therapeutics or cell lines engineered for production of biologics in the patient’s body have been designed for the treatment of various diseases such as infections (Sanchez et al., 1996; Suzuki et al., 1998), cancer (Lohr et al., 2002; Pelegrin et al., 1998; Read et al., 2001; Sakai et al., 2005), diabetes (Chang, 1997; Stadlbauer et al., 2006; Wang et al., 1997) and different genetic disorders (Hortelano et al., 1996; Ross et al., 2000).

Most controlled release systems currently available have either been chemically designed for sustained auto-catalytic or tissue-specific discharge of the therapeutic cargo, or engineered to release the therapeutic load in response to physical cues such as pH (Balabushevich et al., 2003; Dejugnat and Sukhorukov, 2004), light (Skirtach et al., 2004), ionic strength (Wang et al., 2005) magnetic resonance, (Edelman et al., 1985; Langer et al., 1985) or an electric field (Kiser et al., 1998). Unfortunately, polymers designed for controlled release are often limited in their chemical flexibility, while most physical stimuli are impractical for in vivo applications. Also, the timing of release and overall release kinetics are often difficult to control.

We have designed a versatile two-component controlled release technology (CellEase) which consists of a biocompatible polymer capsule and a transgenic sensor cell line engineered for trigger-inducible expression of a chimeric secreted cellulase which catalyses capsule breakdown thereby enabling the release of biopharmaceuticals produced by co-encapsulated producer cells.

Capitalizing on established cellulose sulfate (CS)-poly-diallyldimethyl ammonium chloride (pDADMAC)-based polymerization chemistry, we have established a high-throughput protocol for the production of cell- or biologics-containing microcapsules (Weber et al., 2006). CS-pDADMAC polymers are known for their high biocompatibility (Pelegrin et al., 1998; Wang et al., 1997), lack of cytotoxicity (Dautzenberg et al., 1999) and their cheap and straightforward production protocols (Merten et al., 1991; Stiegler et al., 2006). Owing to their covalent polymer structure and their chemistry, implanted CS-pDADMAC capsules are inert to metabolic breakdown and survive for several months in vivo (Pelegrin et al., 1998; Wang et al., 1997).

Cellulases, which can cleave the polymer backbone of CS-pDADMAC capsules, are typically absent from mammalian tissues. We have engineered a Bacillus subtilis (1-4)-β-glucanase (cellulase) by the N-terminal fusion to an IgG-type secretion signal sequence with expression placed under control of the TET (Gossen and Bujard, 1992) or E.REX (Weber et al., 2002) systems for trigger-inducible expression and secretion by mammalian cells.
TET/E.REX are prototypic transgene control system which are responsive to clinically licensed antibiotics (tetracycline/doxycycline, erythromycin) and consist of chimeric transactivators (tTA/ET1), designed by fusing bacterial response regulators (TetR/E) to a eukaryotic transactivation domain (VP16), which binds and activates promoters (P_{hCMV*1}/P_{ETR}) containing transactivator-specific operator sites (tetO/O_{ETR}) 5’ of minimal eukaryotic promoters. In the presence of regulating antibiotics the transactivators are released from their cognate promoters and transgene expression is silenced in a dose-dependent manner (Weber and Fussenegger, 2006, 2007). TET and E.REX systems have been shown to be compatible and could be used for independent control of different transgenes in a single mammalian cell or mixed cell populations (Weber et al., 2002).

By co-encapsulating sensor cells, engineered for TET-/E.REX-controlled expression of the secreted mammalian cellulase, with biologics-secreting production cell lines into CS-pDADMAC capsule we could trigger-control capsule rupture and fine-tune release kinetics of protein therapeutics in vitro as well as in mice. The CellEase technology may foster advances in timely delivery of specific therapeutic doses in future gene therapy and tissue engineering protocols.

### Material and Methods

**Vector design.** pAAV-MCS (P_{hCMV-Intron_{b-globin}-pA_{hGH}}) [Stratagene, La Jolla, CA, USA], pEF4-MycHisA (P_{hEF1α-MCS-TMYC-T_{HIS}-pA_{bGH}}) [Invitrogen, Carlsbad, CA, USA], pIRESbleo (P_{hCMV-MCS-Intron_{synthetic-IRESEMCV-Bleo-pA_{bGH}}}) [Clontech, Palo Alto, CA, USA], p Pur (P_{SV40-Puro-pA_{SV40}}) [Clontech], pSEAP2-control (P_{SV40-MCS-SEAP-pA_{SV40}}) [Clontech], pDF51 (P_{hCMV-Intron_{p-globin}-ET1-pA_{bGH}}) (Fluri et al., 2007a), pDF75 (P_{ETR-SEAP-IRESPV-ET1-pA_{SV40}}) (Fluri et al., 2007a), pDF109 (P_{hCMV-Intron_{p-globin}-SEAP-pA_{bGH}}) (Fluri et al., 2007a) pDF191 (P_{hEF1α-SeXy-pA_{bGH}}) (Fluri et al., 2007b), pMF111 (P_{hCMV*-1-SEAP-pA_{SV40}}) (Fussenegger et al., 1997), pMF172 (P_{PIR-SEAP-pA_{SV40}}) (Fussenegger et al., 2000), pSAM200 (P_{SV40-tTA-pA_{SV40}}) (Fussenegger et al., 1997) and pWW35 (P_{SV40-ET1-pA_{SV40}}) (Weber et al., 2002) have been described previously. The cellulase of *Bacillus subtilis* 168 (GenBank accession no.: AY044252) was PCR-amplified from genomic DNA using oligonucleotides ODF67: (5’-CGGGATCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTGCTGGTCCAGGTTCCACTGTCAGGGCACAAAACGCACCAGTAGCC; underlined; start codon in italics) and ODF68: (5’-
GAATTCATCTAGAATTTGGTCTGTCCCAATT; EcoRI and XbaI, underlined), restricted with BamHI/XbaI and cloned into the corresponding sites (BamHI/XbaI) of pEF4-MycHisA (pDF195 [pHEFl α-SecCell-pA bGH]). This fused the cellulase 5’ to the signal sequence derived from the murine Igk-chain V-12-C region (SS Igk) replacing the first 115 nucleotides of the open reading frame and 3’ to the Myc tag (TMYC). SecCell was excised from pDF195 by BamHI/Pmel and cloned into the compatible sites (BamHI/HincII) of pAAV-MCS resulting in pDF196 (P hCMV-Intron p-globin-SecCell-pA bGH). pDF260 (P ETR-SecCell-pA bGH) was assembled by restricting pDF196 with ClaI/BglII and ligating SecCell into the corresponding sites (ClaI/BglII) of pDF85 (P ETR-Intron p-globin-SEAP-pA bGH). pDF85 had been constructed by excising P ETR from pDF75 with BssHII/NruI and cloning the insert into the MluI/BsaBI sites of pDF109. pDF301 (P hCMV*-I-SecCell-pA SV40) was constructed by a multistep cloning procedure including (i) excision of SecCell from pDF196 using EcoRI/XhoI, (ii) subcloning (EcoRI/XhoI) into pMF172 resulting in pDF300 (P PIR-SecCell-pA SV40) and (iii) excising SecCell from pDF300 with SpeI/HindIII and cloning the fragment into the compatible sites (XbaI/HindIII) of pMF111. pDF323 (P hCMV-ET1-Intron synthetic-IRES EMCV-Bleo-pA bGH) was assembled by excising ET1 from pDF51 (EcoRI/BglII) and inserting it (EcoRI/BamHI) into pIRESbleo.

Cell culture, transfection and construction of stable cell lines. Human embryonic kidney cells, transgenic for the adenovirus type 5-derived E1 region and the simian virus 40 (SV40) large T-antigen (HEK293-T; (Mitta et al., 2002)), human fibrosarcoma cells (HT1080; ATCC CCL-121), human cervical carcinoma cells (HeLa; ATCC CCL-2), baby hamster kidney cells (BHK-21, ATCC CCL-10) and all HEK293-T derivatives (HEK-ET15, HEK-tTA2, HEK-DF26011, HEK-DF3019) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; PAN Biotech GmbH, Aidenbach, Germany; cat. no. 3302-P251110, lot no. P251110) and 1% penicillin/streptomycin solution (Sigma Chemicals, St. Louis, MO, USA). Chinese hamster ovary cells (CHO-K1; ATCC CCL-61; CHO-B13-24; ATCC CRL-11397) and the CHO-K1 derivative CHO-SEAP 18 (see below) were cultivated in ChoMaster® HTS medium (Cell Culture Technologies GmbH, Gravesano, Switzerland) supplemented with 5% FCS and 1% penicillin/streptomycin solution. HEK-ET15 was created by transfecting pDF323 into HEK293-T and selecting for two weeks using 100µg/ml zeocin (Invitrogen) before clonal selection in 96-well plates. HEK-tTA2 was engineered by co-transfecting pSAM200 and
pIRES-bleo into HEK293-T and selection using 100µg/ml zeocin for two weeks before clonal selection in 96-well plates. HEK-260\textsubscript{11} was created by co-transfecting pDF260 and pPur into HEK-ET1\textsubscript{5} and subsequent selection for two weeks using 1µg/ml puromycin. HEK-301\textsubscript{9} was engineered by co-transfecting pDF301 and pPur into HEK-tTA\textsubscript{3} before selection for two weeks using puromycin (Calbiochem, San Diego, CA, USA). Both HEK-260\textsubscript{11} and HEK-301\textsubscript{9} were screened clonally in 96-well plates for optimal regulation performance. CHO-SEAP\textsubscript{18} was obtained by co-transfecting pPur and pSEAP2-control (Clontech) into CHO-K1 followed by selection for two weeks in medium containing 10µg/ml puromycin and screening for maximum human placental secreted alkaline phosphatase (SEAP) expression.

Capsules containing cells were cultivated in mixed medium consisting of 50% DMEM (Invitrogen) and 50% HTS (Cell Culture Technologies GmbH) supplemented with 7.5% FCS (PAN biotech GmbH).

**Chemicals used for transgene regulation.** For all *in vitro* experiments, erythromycin (Sigma, E-5289) was dissolved in ethanol and used at a final concentration of 1µg/ml. Doxycycline (Sigma, D-9891) was dissolved in PBS and used at a final concentration of 1µg/ml for *in vitro* experiments and at a final concentration of 25mg/kg for *in vivo* experiments.

**Quantification of cellulase, SEAP and antibodies.** Cellulase was quantified using EnzCheck\textsuperscript{®} fluorescent substrate (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s protocol. In brief, 20µl of cell culture supernatant was centrifuged at 16,000xg for one minute to remove cell debris and 15µl of appropriate dilutions in PBS (Invitrogen, cat. no. 21600-069) were transferred to a 384-well plate containing 15µl of sodium acetate buffer (200mM sodium acetate, pH 5.5) per well supplemented with 3µl of substrate solution. Plates were incubated at 37°C for 30 minutes and fluorescence intensity was measured subsequently at 340nm (excitation wavelength) and 450nm (emission wavelength) in a Genios Pro multiwell plate reader (Tecan AG, Maennedorf, Switzerland). Absolute cellulase levels were determined by comparing the fluorescence intensities to readings of standardized solutions containing known concentrations of *Aspergillus niger* cellulase (Sigma, cat. no. 22178). Quantification of human placental secreted alkaline phosphatase (SEAP) in the supernatant of microencapsulated cells or in mouse serum was performed as described previously (Berger et al., 1988). Anti CD18 IgG was quantified using a standard sandwich ELISA. In brief, high protein-binding 96-well plates (Corning, NY, USA) were coated overnight with Fc-specific anti-human IgG (Sigma, cat. no. I2136, lot no. 105K4774) before blocking for 2h with 1%
BSA in PBS (Invitrogen). Diluted culture supernatants were added to individual wells, incubated for 2h at 4°C before washing three times with 200µl PBS containing 0.02% Tween and adding a secondary peroxidase-coupled anti-human IgG (Sigma, cat. no. A0170, lot no. 026K4784). After another three washing steps, chromogenic tetramethyl benzidine (TMB) substrate (Interchim, Montluçon, France) was added and the reaction was stopped after 10 minutes by adding 50µl/well of 1M H₂SO₄ before measuring absorbance at 450nm in a multi-well plate reader (TECAN). Absolute antibody levels were determined by comparing absorbance readings to those for purified IgG antibody standard (gammanorm®, Octapharma GmbH, Langenfeld, Germany).

**Encapsulation.** Cells were encapsulated using the Inotech Encapsulator IE-50R (Inotech AG, Basel, Switzerland) according to the manufacturer’s protocol at the following settings: 0.2mm nozzle, 20ml syringe at a flow rate of 410 units, a nozzle vibration frequency of 1250s⁻¹ and 1150V for bead dispersion. Cellulose sulfate (CS) (Euroferm GmbH, Erlangen, Germany, batch no. FCY-71) was dissolved to 2% (w/v) in PBS and stirred for 16h. The polymerization solution consisted of 1% Poly-diallyl-dimethyl-ammoniumchloride (pDADMAC) (Euroferm) in PBS containing 0.0002% Tween-20. For encapsulation, the cells, proteins or fluorescently labeled molecules were re-suspended at desired concentrations in CS and transferred to a syringe before injecting the solution into the encapsulator which generated CS droplets and sprayed them into the agitated polymerization solution where product-containing CS-pDADMAC capsules formed. After hardening of the CS-pDADMAC capsules, the polymerization solution was exchanged with PBS and the capsules were incubated in tissue culture plates at 37°C and 5% CO₂ in a humidified atmosphere.

**Immunoblotting.** Culture supernatants from transiently transfected HEK293-T were mixed with 5x SDS loading buffer (50% glycerol, 10% SDS, 250mM Tris, pH 6.8 containing 10% (v/v) β-mercaptoethanol) and boiled for five minutes before loading onto a 10% denaturing SDS-PAGE gel. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking in Tris buffered saline (TBS, 50mM Tris, 150mM NaCl, pH7.5) containing 3% skimmed dry milk (Rapilait, Migros, Switzerland), the membranes were incubated with a primary antibody specific for the C-myc tag (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. SC-40, lot no. E2207). Specific bands were visualized using an anti-mouse IgG coupled to horseradish peroxidase (ECL™ anti-mouse IgGxHRP, Amersham, Buckinghamshire, UK; cat. no. NA931V, lot no.
and a chemiluminescence-based assay (ECL™ plus, Amersham) according to the manufacturer’s protocol.

**Microscopy.** Microscopic analysis and time-lapse movies were generated on a LEICA DMI-6000 microscope equipped with appropriate filters for EYFP, FITC and Cy3 detection, and a heated, humidified incubation chamber containing 5% CO₂.

**Animal studies.** Female OF1 (oncins france souche 1) mice were obtained from Charles River Laboratories (Lyon, France). 700µl of PBS solution containing 50% capsules was administered intraperitoneally to mice. A PBS solution containing DOX was injected every 24 hours. Blood samples were collected retroorbitally 3, 6 and 9 days after capsule injection.

All experiments involving mice were performed according to the European Community Council directive (86/609/EEC), approved by the French Ministry of Agriculture and Fishery (Paris, France) and performed by M.D.-E.-B. at the Institut Universitaire de Technologie, IUTA, F-69622 Villeurbanne Cedex, France.

**Results**

**Design and characterization of a secreted mammalian cellulase**

The *Bacillus subtilis* endo (1-4)-β-glucanase, was PCR-amplified from genomic DNA and fused to a strong mammalian secretion signal sequence derived from the murine Igκ-chain V-12-C region. The secreted cellulase (SecCell) was cloned downstream of a constitutive promoter (P<sub>hCMV</sub>) as well as two different inducible promoters which enabled doxycycline- (P<sub>hCMV*-1</sub>) and erythromycin- (P<sub>ETR</sub>) adjustable transgene expression (Fig. 1A). Transient transfection of SecCell into different human (HEK293-T, HeLa, HT-1080) and hamster (CHO-K1 and BHK-21) cell lines resulted in high-level cellulase activity in the culture supernatants unless SecCell production was repressed by doxycycline (DOX) or erythromycin (EM) (Fig. 1B).

The apparent size of the modified cellulase as well as possible glycosylation was assayed by producing SecCell in HEK293-T in the presence and absence of the glycosylation inhibitor tunicamycin. Western blot analysis of culture supernatants showed that the SecCell produced from tunicamycin-free cultures had a molecular weight of 55kDa which was lower when the cells were treated with tunicamycin. This suggested that the cellulase was glycosylated in mammalian cells (Fig. 1C).
Cellulase properties were further explored by assaying enzymatic activity after incubation at different temperatures. Activity was relatively constant between 30 and 55°C but dropped sharply upon incubation above 60°C (Fig. 1D). The stability of the modified cellulase in human serum was also assayed by mixing HEK293-T-produced enzyme with human AB serum before incubating the mixture for a total period of 12 days during which samples were taken every 96 hours (Fig. 1E). The enzymatic half-life was calculated by linear regression of decreasing enzymatic activities to be around 42 days, which is comparable to other secreted proteins of bacterial origin (Fluri et al., 2007b; Schlatter et al., 2002).
Figure 1. Transient expression of modified *B. Subtilis* cellulase in mammalian cells. (A) Schematic representation of vectors used for the CellEase system. (B) Transient expression of cellulase in HEK293-T, HeLa, HT-1080, BHK-21 and CHO-K1 cells. Cellulase was either driven by a constitutive (P*_{hCMV}* ) or antibiotic-responsive promoters (P*_{hCMV*1} and P*_{ETR}*. (C) Western blot analysis of cell culture supernatants derived from HEK293-T populations transiently transfected with (i) pDF196 and cultivated in the presence or absence of the glycosylation inhibitor tunicamycin (2 μg/ml), (ii) pDF301/pSAM200 (+/- DOX [2 μg/ml]) or (iii) mock-transfected to provide a negative control.

Abbreviations: DOX, doxycycline; ET1, erythromycin-dependent transactivator; pA*_{hgh}*, polyadenylation signal of the human growth hormone; pA*_{SV40}*, polyadysylation signal of the simian virus 40; P*_{hCMV*1}*, tetracycline-responsive promoter; P*_{ETR}*, erythromycin-responsive promoter; P*_{hCMV}*, human cytomegalovirus immediate early promoter; P*_{SV40}*, simian virus 40 promoter; SS*_{Igk}*, signal sequence derived from the murine Igk-chain V-12-C region; tTA, tetracycline-dependent transactivator; T*_{myc}*, protein tag encoding a c-myc epitope
Capsule properties and rupture characteristics

Molecular weight cut-off (MWCO): Cellulose sulfate capsules were incubated for 16 hours in the presence of FITC-dextran solutions of different molecular weight. After incubation, capsules were washed thoroughly and then analyzed by fluorescence microscopy. Fluorescence micrographs show strong signals for the two FITC-dextrans of lower molecular weight (10kDa and 20kDa) and no signal for FITC-dextrans of higher molecular weight (40kDa, 70kDa), indicating a molecular weight cut-off between 20 and 40kDa for the 2% capsules (Fig. 2A).

Upon incubation of 2% CS-containing capsules for 30 minutes at 37°C with cellulases of the same target specificity, either Aspergillus niger cellulase (MW 25kDa, below molecular cut-off) or SecCell (50kDa, above molecular cut-off) only the A. niger cellulase was able to trigger capsule rupture at concentrations as low as 1U/ml. The larger-sized SecCell which is above the MWCO and unable to penetrate the capsules failed to induce capsule degradation even at 5U/ml (Fig. 2B). This observation suggests that capsule breakdown can only be initiated from the inside and not from the outside which would require SecCell to be co-encapsulated or produced inside the capsules in order to control capsule breakdown and release of microencapsulated drugs in a robust and reliable manner. Although the underlying mechanistic details of SecCell’s inside-specific capsule breakdown characteristics remain elusive, this quality may enable sequential or independent release of various therapeutic cargos using different stimuli (see below).

CellEase technology – trigger-inducible capsule rupture and protein release

In order to establish and characterize CellEase technology for trigger-inducible release of protein drugs we have co-encapsulated HEK-3019 (5x10^5 cells/ml of CS), transgenic for tetracycline-responsive SecCell expression, and CHO-SEAP_{18} (2.5x10^5 cells/ml of CS), engineered for constitutive SEAP (58 kDa, above MWCO) production into CS-pDADMAC capsules and cultivated them for 6 days in the presence or absence of doxycycline (DOX). Control capsules contained the same number of the parental cell line HEK293-T instead of the SecCell producing HEK-3019 and CHO-SEAP_{18}. Every 24 hours, capsule micrographs were taken and accumulated SEAP activity was assayed in the supernatant.
Figure 2. Membrane properties of CS-pDADMAC capsules. (A) CS capsules were incubated overnight in the presence of FITC-dextran with different molecular weight and then washed and analyzed by fluorescence microscopy. (B) CS-pDADMAC capsules were incubated for 30 min in the presence of increasing concentrations of either a fungal cellulase (MW 25kDa) or SecCell derived from mammalian culture supernatants (MW 55kDa). The percentage of intact capsules was normalized to capsules which had not been exposed to any cellulase.
Capsules cultivated in the absence of DOX (maximum induction of SecCell in HEK-301₉) started to collapse after an initial lag phase of around 36 hours after encapsulation, leading to a strong increase in SEAP activity in the supernatant compared to intact capsules cultivated in the presence of DOX (repression of SecCell in HEK-301₉) (Fig. 3A and 3B). The control capsules containing CHO-SEAP₁₈ cells together with parental HEK293-T behaved in exactly the same manner as the CellEase capsules containing HEK-301₉ in which SecCell was repressed by doxycycline (Fig. 3A and 3B).

The induction of cellulase expression lead to destabilization and rupture of the capsules followed by discharge of their contents. To illustrate the capsule breakdown, two time-lapse microscopy experiments were performed; they revealed (i) the immediate rupture of a cellulose sulfate capsule after addition of cellulase solution (A. Niger cellulase MW. 25kDA) http://www.fussenegger.ethz.ch/people/flurida/movie/movie1 and (ii) that the rupture process was triggered after 36h by encapsulated HEK-301₉. Capsules with SecCell production fully induced were traced by co-encapsulating 150kDa FITC-dextran whereas negative-control capsules containing HEK293-T were not fluorescently labeled http://www.fussenegger.ethz.ch/people/flurida/movie/movie2.

**Adjusting protein release by fine-tuning capsule rupture kinetics**

We also sought to control capsule rupture by applying an external stimulus at a defined point in time, which would lead to the induction of SecCell production and liberation of the therapeutic cargo at a later required time. Four groups of identical capsules, containing HEK-301₉ (5x10⁵ cells/ml of CS) and CHO-SEAP₁₈ (2.5x10⁵ cells/ml of CS), were cultivated with SecCell expression de-repressed sequentially by DOX removal either (i) immediately after (group 1), (ii) 24h after (group 2) or (iii) 48 h after encapsulation and seeding (group 3). Group 4 was cultivated in the continued presence of doxycycline and served as a baseline to illustrate the tightness of the CellEase system. The cumulative SEAP activity of the supernatants of the four groups was monitored for six days (Fig. 4A). An increase in supernatant SEAP activity reflected the induction time frame of the respective capsule population and demonstrated timely controlled induction of capsule rupture.
Figure 3. Capsule rupture induced by DOX-controlled SecCell expression. (A) CHO-SEAP$_{18}$ were co-encapsulated with HEK-301$_{9}$, and capsules were cultivated in the presence (2µg/ml) or absence of DOX for 6 days. Light micrographs of capsule populations were taken at 24h intervals to profile capsule integrity. (B) Cumulative SEAP activity in the supernatant was measured every 24h. Capsule populations containing CHO-SEAP$_{18}$ together with parental HEK293-T served as a negative control. Time-lapse movies showing capsule rupture following addition of 5U/ml A. niger cellulase (http://www.fussenegger.ethz.ch/people/flurida/movie/movie1) or DOX-responsive SecCell induction (http://www.fussenegger.ethz.ch/people/flurida/movie/movie2) can be viewed online.

Another option to control the time of release is the titration of SecCell expression by using different DOX concentrations. Capsules containing HEK-301$_{9}$ (5x10$^5$ cells/ml of CS) and CHO-SEAP$_{18}$ (2.5x10$^5$ cells/ml of CS) were seeded into a 24-well plate and incubated with 0, 0.1, 0.5, 2, and 20ng/ml DOX. Again, the cumulative SEAP activity of the supernatants was scored for 6 days. Capsule rupture and SEAP accumulation in the supernatant took progressively longer as DOX concentrations increased (from 0 ng/ml to 2 ng/ml). For the two highest DOX concentrations (2ng/ml and 20ng/ml), SEAP readings in the supernatant dropped to background levels indicating full repression of SecCell expression (Fig. 4B).
As a third option to control SecCell-mediated protein release from cellulose sulfate/pDADMAC capsules, we have varied the numbers of HEK-3019 responder cells (5x10^4/ml of CS, 5x10^5/ml of CS and 1x10^6/ml of CS) co-encapsulated with a constant amount of CHO-SEAP_{18} (2.5x10^5 cells/ml of CS). SEAP accumulation profiles were again compared in repressed (+ DOX) and induced (-DOX) SecCell expression states for six days (Fig. 4C). Encapsulation of higher numbers of the responder cell line HEK-3019 lead to a quicker response to the stimulus, but also slightly increased the overall leakiness of the CellEase system possibly due to capsule rupture as a result of leaky cellulase expression from the tetracycline-responsive promoter.

**Co-cultivation of different capsule populations**

For various applications such as a single-shot vaccine or temporally spaced, treatments we sought to test whether it would be possible to deliver a mixed capsule population, and to release the cargo at different points in time by inducing release with different stimuli. The capacity of the CellEase system to control the release of different molecules after inducing capsule rupture by two different antibiotics was investigated. We encapsulated (i) HEK-3019 (5x10^5 cells/ml of CS) together with CHO-SEAP_{18} (2.5x10^5 cells/ml of CS) for one capsule population, and (ii) HEK-260_{11} (0.75x10^5 cells/ml of CS) engineered for macrolide-responsive SecCell expression, together with IgG-producing CHO-B13-24 cells (5x10^5 cells/ml of CS) for the other. The two populations were mixed in a 1:1 ratio and incubated under various antibiotic conditions. To distinguish the two capsule populations in the mixture the HEK-3019/CHO-SEAP_{18}-containing capsules were stained with FITC-dextran (150kDa) whereas the HEK-260_{11}/CHO-B13-24-containing capsules were stained with tetramethylrhodamine isothiocyanate (TRITC)(150kDa). The mixed capsule populations were seeded into four different groups which were incubated in the absence or presence of one or both antibiotics (DOX and EM) for five days. Accumulated SEAP and anti CD-18 antibody were measured in the culture supernatants after 5 days (Fig 5A). SEAP and IgG levels indicated that the respective release from the capsules was entirely controlled by the presence or absence of either antibiotic. Exclusive rupture of one capsule population did not appear to influence the other one since SecCell can only degrade capsules internally. Capsule integrity was also analyzed by microscopy 5 days after incubation. Bright-field and fluorescence micrographs showed exclusive rupture of capsule populations harboring cells with induced SecCell and confirmed the integrity of capsules containing HEK-3019 or HEK-260_{11} with repressed SecCell (Fig 5B).
Figure 4. Modulation of release patterns using the CellEase system. (A) CHO-SEAP$\text{}_{18}$ were co-encapsulated with HEK-301$_9$ and capsule rupture was induced at different points in time (0, 24 and 48 hours) after encapsulation. Cumulative SEAP activity in the supernatant was then analyzed every 24h for the next 6 days and compared to readings for capsules cultivated in the presence of doxycycline (DOX) (SecCell production repressed) for the entire period of time. (B) CHO-SEAP$_{18}$, co-encapsulated with HEK-301$_9$ and incubated at different DOX concentrations (● 20ng/ml, △ 2ng/ml, ■ 0.5ng/ml, ▲ 0.1ng/ml and ● 0ng/ml). SEAP accumulation in the supernatant was measured every 24 hours for 6 days. (C) CHO-SEAP$_{18}$ was co-encapsulated with different numbers of cells (5x10$^4$ cells/ml, 5x10$^5$ cells/ml and 1x10$^6$ cells/ml) of HEK-301$_9$ and incubated in the presence and absence of DOX for 6 days. Cumulative SEAP activity was measured in the supernatant every 24h.
Figure 5. Co-cultivation of two different capsule populations. SEAP-producing CHO-SEAP₁₂ were co-encapsulated with HEK30₁₀ and a high molecular weight FITC-Dextran (150 kDa), whereas antibody-producing CHO-B₁₃-2₄ were co-encapsulated with HEK-2₆₀₁₁ and a high molecular weight TRITC-Dextran (150 kDa). The two capsule populations were subsequently mixed at a ratio of 1:1 and cultivated under different antibiotic conditions for 5 days. (A) SEAP and IgG levels in the culture supernatant were analyzed for the different conditions 120 hours after seeding the capsules. (B) Integrity of the two capsule populations was shown by bright field (upper row) and fluorescence micrographs (lower two rows) taken 120 hours after capsule seeding. Abbreviations: BF, bright field; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.
In vivo release of a model glycoprotein upon withdrawal of doxycycline

Capsules containing HEK-301\textsubscript{9} (5x10\textsuperscript{5} cells/ml of CS), transgenic for doxycycline-responsive SecCell expression, and 100\textmu l SEAP (2U/ml) concentrated from serum free CHO-SEAP\textsubscript{18} supernatants were injected intraperitoneally into two groups of mice. One group was given daily doxycycline injections (+DOX) to maintain CellEase in a repressed state, whereas the second group was left untreated to induce capsule rupture (Fig. 6A). Parallel to the in vivo experiment, the same capsule populations were cultivated in the presence and absence of DOX in petri dishes and release profiles were compared to the in vivo situation (Fig. 6B).

Figure 6. Antibiotic-controlled release of capsule cargo in mice. (A) SEAP activity in mouse serum 3, 6 and 9 days after intraperitoneal injection of capsules containing concentrated SEAP and HEK-301\textsubscript{9} and intraperitoneal administration of doxycycline (+DOX) or PBS (-DOX). (B) At the same time, capsule populations were cultured in vitro and SEAP expression levels in the supernatant were analyzed every 24 hours. Cell culture medium was exchanged and capsules were washed daily after sample removal.
Discussion

Therapeutic interventions using novel drug carriers are being continuously designed to overcome limitations in traditional drug delivery. To avoid multiple dosing and to sustain a therapeutic level of the active drug over a prolonged period of time, different approaches have been established: (i) drug-containing implants (Langer, 1990; Richards Grayson et al., 2003; Saito et al., 2001), (ii) micro-pumps (Ryu et al., 2007; Webb et al., 2002), (iii) the encapsulation of cell lines or primary cells continuously producing biologics (Pelegrin et al., 1998; Regulier et al., 1998; Sailaja et al., 2002; Wang et al., 1997), (iv) biodegradable polymers containing embedded therapeutic substances (Jain, 2000; Mathiowitz et al., 1997) or (v) implants of drug-containing capsules designed to rupture in response to biological, chemical or physical stimuli (Angelatos et al., 2005; De Geest et al., 2005; Itoh et al., 2006; Kiser et al., 1998).

Active bacterial cellulases can be efficiently expressed in mammalian cells without showing significant cytotoxicity (Soole et al., 1993; Zhang et al., 1997; Zhang et al., 1999). Enzyme-induced breakdown of physiologically inert polymer capsules triggering the release of the encapsulated material represents a powerful tool to precisely adjust time and delivery kinetics of biologics to therapeutic requirements. We have successfully combined encapsulation technology adapted for cellulose sulfate/pDADMAC polymers, which have a track record in production scalability (Dautzenberg et al., 1999; Stadlbauer et al., 2006) biocompatibility (Dautzenberg et al., 1999) and metabolic stability (Lohr et al., 2001; Wang et al., 1997), with tight expression control of a mammalian designer cellulase using two prototype transcription control systems (TET and E.REX (Gossen and Bujard, 1992; Weber et al., 2002). This CellEase technology enabled precise and controllable rupture of the cellulose-containing polymer shell and timely release of therapeutic content.

By varying the relative cellulose-sulfate/pDADMAC concentrations and production parameters capsule porosity could in principle be tuned for selective retention of specific biologics while sustaining cell growth and viability by a free flow of nutrients and waste product across the capsule membrane. We have chosen to use a molecular weight cut-off between 25 and 40kDa which enables retention of most protein therapeutics including IgGs. CellEase capsules could either accommodate biologics or cells producing desired protein therapeutics. Microencapsulation of therapeutic proteins requires separate production and downstream processing efforts making drug delivery as expensive as classic injection-based therapies. In-situ production of biologics by microencapsulated cells alleviates classical
biopharmaceutical manufacturing which could make therapy more efficient and affordable. Although the fate of the sensor and producer cells released into the body might be considered problematic, our studies using xenotypic cell lines in mice did not reveal any immediate adverse effects in the animal. Alternatively, the use of autologous cells may completely eliminate concerns about side effects of heterologous cell material. The recently developed protein-transducing nanoparticles also enable simultaneous production and encapsulation by packaging of therapeutic proteins into lentivirus-derived nucleic acid-free nanoparticles (Link et al., 2006). However, in comparison to CellEase technology, protein-transducing nanoparticles release their therapeutic cargo in an uncontrolled manner directly upon contact into any cells, which, unlike CellEase, lacks release control and limits therapeutic impact to intracellular targets (Link et al., 2006).

CellEase technology is relatively straightforward, robust and advantageous for the following reasons: (i) CS-pDADMAC capsules can be produced at low cost and large scale using multi-nozzle devices for production of clinical-grade capsules (Lohr et al., 1999; Salmons et al., 2003) (ii) Capsule parameters such as molecular weight cut-off and cellulose content could easily be varied to modify growth and production characteristics inside capsules and adjust release kinetics. (iii) The availability of an engineered ready-to-use sensor cell line transgenic for trigger-controlled SecCell production increases flexibility as this cell line can be co-encapsulated with any established constitutive producer cell line or primary cell via co-encapsulation. We have shown that the relative number of encapsulated sensor and producer cells can be used to adjust drug-release kinetics. For one-cell line solutions producer cells could also be engineered for regulated SecCell expression. (iv) Having used the TET and E.REX systems to trigger SecCell expression, capsule rupture and biologics release, we assume that CellEase is compatible with any transcription control system (Weber and Fussenegger, 2007). (v) Release kinetics can be modified by timing and dosing of the SecCell trigger molecules. (vi) The unique characteristic of SecCell to exclusively breakdown CS-pDADMAC capsules internally and leave the outer surface of the capsule intact makes CellEase technology suitable for the administration of mixed capsule populations, which differ, for instance, in the type and number of sensor cells, thereby allowing the controlled release of different molecules at different points in time and with different release kinetics without having to repeat capsule administration. (vii) Although we have used antibiotics as a heterologous inducer in vivo it may also become possible to wire CellEase to endogenous signals, which are increased during a disease or infection state (e.g., hormone or interleukins),
to trigger release of therapeutic biologics and provide a rapid and self-sufficient first-line protection.

By providing in-situ production of biologics and being robust, precise, versatile and functional \textit{in vivo}, CellEase technology has the potential to make future drug delivery more efficient and affordable.

**Conclusion**

In this work we present a new approach for the trigger-induced release of biologics from biocompatible CS-pDADMAC capsules. Capsule rupture and the accompanying cargo release was controlled by the inducible expression of a modified bacterial cellulase (SecCell) from mammalian sensor cells. We have proved the functionality of the system by engineering sensor cells that express SecCell under the control of either doxycycline or erythromycin responsive promoters. After induction, with the corresponding antibiotic, SecCell was expressed and secreted from the sensor cells and caused the destabilization of the capsule from the inside, rupturing the capsule and releasing its contents. Model glycoproteins, either expressed by co-encapsulated cells or co-encapsulated purified factors, were able to be released from capsules upon induction \textit{in vitro} as well as \textit{in vivo}. This technology may open new possibilities for trigger-induced release of therapeutic cargos. And by combining CellEase with available gene-regulation systems one can create the tools to utilize a broad variety of cues to control the release of biologics in therapeutic settings.

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References


Conclusion
Conclusion

Modern Biotechnology has made enormous progress in recent years and become an important field of investment for many big pharmaceutical companies. Established disciplines of this new field includes the recombinant production of growth hormones, cytokines and monoclonal antibodies. Research into these areas has produced novel and innovative technologies such as gene- and cell-therapies some of which have even entered clinical trials over the few last years. This work details the development of new tools that may help to overcome certain problems accompanying the production, delivery, expression, and controlled delivery of biologics.

New recombinant adeno-associated virus type 2 (rAAV2) based gene therapy vectors were designed and allow for tight regulation of transgene expression by a clinically licensed antibiotic after infection of target cells. Many different configurations of vector systems were characterized such as a binary ON-and OFF-type, an auto-regulated, a self-regulated and a bi-directional setup. Due to the safety and efficiency of rAAV2, these new vectors are valuable tools for transgene delivery in vitro as well as in vivo and further down the road may be promising tools for the delivery and regulation of therapeutic transgenes in clinical settings.

Reporter proteins are valuable tools to monitor many essential parameters in biotechnological applications. Complex questions in metabolic engineering, process optimization, gene- and cell-therapies and basic research can be answered by an array of available reporter proteins specialized for such tasks. In this work a pair of novel heterologous reporter proteins were designed, for both intracellular and secreted applications, for use in mammalian cells. These new reporters are sensitive, compact, stable, easy to assay and compatible with existing reporter proteins. Future applications may benefit from these new reporter proteins where size and stability of delivered reporter-transgenes are critical or direct assays need to be made without pretreatment of the samples.

The delivery of therapeutics has become key in the advent of new, complex therapies such as protein drugs, gene-therapy and cell-therapies. Owing to the complexity, low stability and immunogeneicity of these new drugs, alternatives to traditional routes of delivery have to be developed. A versatile system for the delivery of biologics is presented based on the rupture of bio-compatible polymer capsules upon the application of an external cue. This system potentially allows for the release of varying cargos including protein therapeutics, viral particles, liposomes or entire cells. The sensor cell line, which captures the applied signal and induces capsule rupture, can be engineered to be responsive to different stimuli.
such as various small molecules, hormones, gaseous molecules or even physical cues such as temperature fluctuations. The combination of bio-compatible encapsulation protocols together with the drug release “on-demand” may foster applications in future initiatives for controlled drug delivery.

The optimization of the development and deployment of efficient new biologics is an active field of academic and industrial research and we hope to have contributed with this work in the development of more efficient future therapies.
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Publications


Presentations

David A. Fluri and Martin Fussenegger (2004). Regulated Gene Expression Using Adeno-Associated Viral Vectors. Poster presentation at the 5th symposium of the department of biology ETH Zurich, Davos, Switzerland

David A. Fluri, Guillaume Lesage and Martin Fussenegger (2006) Secreted Xylanase - A Novel Mammalian Reporter Gene. Poster presentation at the 4th European biotech workshop, Ittingen, Switzerland