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

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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 therapeutic 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 [1-3]. Among these, vectors based on the replication-defective adeno-associated virus type 2 have attracted special attention as tools for clinical gene transfer. Different character-
istics, 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 [4-10].

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 [11,12], numerous other control modalities have followed including those responsive to streptogramin [13], macrolide [14] and aminocoumarines [15], immunosuppressive agents (rapamycin) [16], hormones [17-19], or susceptible to temperature [20,21], quorum sensing molecules [22,23] and gaseous acetaldehyde [24]. 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 [25-27], (ii) the regulated expression of transgenes based on the tetracycline-responsive TET ON and TET OFF systems [28-31] and, to lesser extent, (iii) the regulated expression of transgenes by rapamycin-controlled transgene expression [32,33].

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 [34,35] and improved purification and concentration protocols [36,37], 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) [14]. E.REX systems exist in two different configurations: (i) the E 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 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 [14]. (ii) The E ON technology consists of a macrolide-dependent transrepressor (E-KRAB (ET4), [14]), a fusion of the E. coli MphR(A) repressor protein [E] and the KRAB (KruEpplen-associated box) transsilencing domain of the human kox-1 gene), which binds and represses chimeric promoters (P ETR 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 ON, these control arrangements are known as ON-type or E ON systems [14].

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 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-Pβ cmv-intronβ-globin-ET1-pAHIGH-ITR) harboring an ITR (inverted terminal repeats)-flanked P β cmv (human cytomegalovirus immediate early promoter) driven and pAhigh- (human growth hormone-derived polyadenylation site) terminated ET1 (macrolide-dependent transactivator) expression unit containing a β-globin intron (intronβ-globin) reported to increase transcript processing and overall ET1 production levels and pDF54 (ITR-P ETR EYFP-pA SV40-ITR) containing an ITR-flanked expression unit encoding P ETR (macrolide-responsive promoter) driven and pA 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; [38]) 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) cultured in the presence (+EM) or absence (-EM) of the macrolide antibiotic erythromycin (EM). Fluorescence micrographs of transduced populations maintained for 48 h in erythromycin-free medium revealed
bright green fluorescence in all cell types. Transduced populations cultivated for two days in the presence of 1 μg/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 cytomegalovirus 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 48 h 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-pA-EYFP→P<hcmvm>intron-ETR-P<hsps70min>→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<hsps70min> (minimal version of the Drosophila melanogaster heat-shock protein 70 promoter) driving ET1 in the opposite direction.

Following transduction of pDF124-derived AAV particles, active P<etr> produces a single transcript from which EYFP is translated in a classic cap-dependent manner whereas ET1 production depends on cap-independent IRESEMCV-mediated translation initiation. Undetectable P<etr>-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 co-translationally encoded EYFP. In the presence of erythromycin, ET1 originating from basal P<etr> 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 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 P<etr>-driven SEAP (human placental secreted alkaline phosphatase) (ITR-P<etr>-SEAP-pA<sv40>-ITR) (Figure 3A). Co-transduction of HT-1080 and MCF-7 with pDF77- and pDF51- (ITR-P<hcmv>-ET1-pA<sv40>-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). Furthermore, we have designed and evaluated pDF143 (ITR-P<sv40>-ET1-pA<sv40>-P<etr>-SEAP-pA<sv40>-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>-intronβ-globin-SEAP-pA<high>-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 EON-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 [39]. We have constructed binary EON-controlled AAV-based expression systems consisting of pDF126 (ITR-P<hcmv>-Intron-β-globin-EKRAB-pA<high>-ITR), and pDF207 (ITR-P<etr>-ON8-EYFP-
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_{ICMV} promoter (pDF51) and EYFP driven by P_{ETR} (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_{hgh}, human growth hormone polyadenylation signal; pA_{SV40}, simian virus 40 polyadenylation signal; P_{ETR}, erythromycin-responsive promoter; P_{ICMV}, human immediate early cytomegalovirus promoter; Selected restriction sites: A, Accl; As, Ascl; Bg, BglII; Bs, BstBI; Bsa, BsaBI; C, Clal; E, EcoRI; H, HindIII; Hi, Hincll; M, MluI; N, NdeI; Nh, Nhel; Nr, NruI; P, Pael; Pm, Pmel; Pml, PmlII; S, Sall; Sa, Sacll Sp, Spel; Sph, Sphl; Sw, Swal; X, XbaI.
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; pAI, synthetic polyadenylation signal; pASV40, simian virus 40 polyadenylation signal; PETR, erythromycin-responsive promoter; PHSP70min, minimal version of the Drosophila melanogaster heat-shock protein 70 promoter; PSV40, simian virus 40 promoter.
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 PETR, pDF143, a self-regulated expression unit expressing ET1 from PSV40 and driving SEAP from PETR, and pDF109, harboring a constitutive SEAP expression unit driven by PhCMV. (B) SEAP expression levels profiled 48 h 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. [52]. Abbreviations: EM, erythromycin; ET1, erythromycin transactivator; ITR, inverted terminal repeat; pASV40, simian virus 40 polyadenylation signal; PhEF1α, human elongation factor 1α promoter; PETR, erythromycin-responsive promoter; SEAP, human placental secreted alkaline phosphatase; PSV40, simian virus 40 promoter.
pSv40-ITR), pDF208 (ITR-PetrON4-EYFP-pSv40-ITR) or pDF209 (ITR-PetrON2-EYFP-pSv40-ITR), which harbor EYFP under control of different E-KRAB-specific macrolide-inducible promoters characterized by different ETR dose-response characteristics of one-vector and binary combinations, basal and maximum expression levels were a function of the number of ETR repeats associated with Psv40 (Figures 4A and 4C).

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 for 48 h in the presence and 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 48 h 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 Psv40 (Figures 4A and 4C).

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 48 h in the presence of different EM concentrations before SEAP production was quantified. Whereas SEAP expression was completely repressed between 20 and 100 ng/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 ETR-Petr stoichiometries associated with those technologies.
Figure 4
AAV type 2-based erythromycin-inducible EYFP expression. (A) Schematic representation of pDF126 harboring a P\textsubscript{CMV}\textsuperscript{driven} E-KRAB expression unit and pDF206/207/208 encoding EYFP transcribed by P\textsubscript{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 (4000 and 1000 genomic particles/cell) by pDF126/pDF207, pDF126/pDF208 and pDF126/pDF209 were cultivated for 48 h in the presence (+) and absence (-) of EM. Abbreviations: EM, erythromycin; EYFP, enhanced yellow fluorescent protein; ITR, inverted terminal repeat; KRAB, kruppel associated box; P\textsubscript{ETRON}, erythromycin-inducible promoter.
vitro. Isogenic EON 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 EON-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.

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 pDFI43-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 pDFI143 (1000 genomic particles/cell) (C) and pDFS1/77 (500 genomic particles/cell) (D) in HT-1080 and MCF-7 cultivated for 48 h in the presence of increasing EM concentrations. SEAP expression is shown in units/liter (U/l) as defined by Schlatter et al. [52]. Abbreviations: EM, erythromycin; SEAP, human placental secreted alkaline phosphatase. Abbreviations: EM, erythromycin; SEAP, human placental secreted alkaline phosphatase.
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 2500 bp 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 [46-50]. 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.

**Conclusion**

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 config-
urations 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; [51]), 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, Aidgenbach, 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 co-transfection of the helper plasmid pDG [38] and the transgene-encoding AAV vector into HEK293-T using optimized calcium phosphate transfection protocols. In brief, 2 × 10⁶ HEK293-T were seeded per culture dish (diameter 10 cm) and cultivated overnight. For each plate 15 μg of helper plasmid and 30 kDa (Vivaspin, Vivasience, Germany; cat. no. VS1521).

**Virus titration by quantitative real-time PCR**

Crude viral preparations were treated as described in [36]. Primers and the Taqman probe were designed to anneal to (i) the SV40 polyadenylation signal (pa) (forward primer, 5'-AGCAATAGCAGCAGAAATACTTACAAA-3', reverse primer, 5'-GACATGATAAGATCAGAGCTGTTTTCG-3', Taqman FAM/TAMRA probe, 5'-GACATGATAAGATCAGAGCTGTTTTCG-3'), (ii) the SEAP open reading frame (forward primer, 5'-AGCAATTTTTTTCCATGTGTTTATGTTG-3'), (iii) the erythromycin-dependent transactivator open reading frame (forward primer, 5'-GGCGTCCITGAGACATAGC-3') or (iii) the erythromycin-dependent transactivator open reading frame (forward primer, 5'-GGCGTCCITGAGACATAGC-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. 17-0406-01) was equilibrated with 10 bed volumes of binding buffer (10 mM Na₂PO₄, pH 7) and run at a flow rate of 1 ml/min. The AAV particle-containing 40% fraction 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. 1070402) was equilibrated with 10 bed volumes of binding buffer (10 mM Na₂PO₄, pH 7) and run at a flow rate of 1 ml/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 (10 mM Na₂PO₄, 1 M NaCl, pH 7) and then concentrated using a spin column with a molecular weight cut-off (MWCO) of 30 kDa (Vivaspin, Vivasience, Germany; cat. no. VS1521).

Following another washing step using 5 ml PBS, the cells were resuspended in 2 ml PBS and the intracellular viral particles were released after cell lysis induced by three consecutive freeze-thaw cycles consisting of shutting 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 5 min. at 10'000 × g and the supernatant containing the crude virus stock was collected, supplemented with 50 U/ml of Benzonase (Sigma, cat. no. E1014) and incubated for 30 min at 37°C. Iodixanol density-gradient purification of viral particles was performed using a protocol adapted from Zolotukhin et al. [37]. In brief, crude viral stocks were diluted in PBS to a final volume of 12 ml. Iodixanol step gradients were prepared in an ultracentrifuge tube by sequential underlaying of the crude preparation with 5 ml of a 15% (plus 1 M NaCl in the first layer), 5 ml of a 25%, 4 ml of a 40% and 4 ml of a 60% iodixanol-containing PBS. For better distinction of the gradient layers 2.5 μl/ml of a 0.5% Phenol Red (Sigma, cat. no. P2090) stock solution was added to the 60% and the 25% layers. Step gradients were centrifuged for 3.5 h at 150'000 × g 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. 107-0406-01) was equilibrated with 10 bed volumes of binding buffer (10 mM Na₂PO₄, pH 7) and run at a flow rate of 1 ml/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 (10 mM Na₂PO₄, 1 M NaCl, pH 7) and then concentrated using a spin column with a molecular weight cut-off (MWCO) of 30 kDa (Vivaspin, Vivasience, Germany; cat. no. VS1521).
| Plasmid                        | Description and Cloning Strategy                                                                 | Reference or source |
|-------------------------------|---------------------------------------------------------------------------------------------------|---------------------|
| pAAV-MCS                      | AA V transfer vector                                                                              | Stratagene          |
| pAAV-lacZ                     | AA V vector expressing lacZ                                                                        | Stratagene          |
| pDG                           | Helper construct encoding AAV Rep/Cap as well as Adeno virus E2A, E4 and VA.                      | [38]                |
| pCF18                         | Plasmid containing ECFP driven by a tetracycline responsive promoter and EYFP driven by a pristinamycin responsive promoter | [53]                |
| pCF19                         | Plasmid containing SEAP cassette                                                                   | [54]                |
| pCF125                        | Plasmid expressing ECFP and ET1 from a bidirectional promoter                                      | [21]                |
| pSS134                        | Plasmid containing SEAP cassette                                                                   | unpublished         |
| pWW43                         | Plasmid expressing E-KRAB [14]                                                                    | [55]                |
| pWW78                         | pTRIDENT1-based tricistronic expression vector for macrolide-responsive auto-regulated expression of up to two desired transgenes. |                    |
| pAAV-MCS                      | AA V transfer vector                                                                              |                    |
| pAAV-lacZ                     | AA V vector expressing lacZ                                                                        |                    |
| pDG                           | Helper construct encoding AAV Rep/Cap as well as Adeno virus E2A, E4 and VA.                      |                    |
| pCF18                         | Plasmid containing ECFP driven by a tetracycline responsive promoter and EYFP driven by a pristinamycin responsive promoter |                    |
| pCF19                         | Plasmid containing SEAP cassette                                                                   |                    |
| pCF125                        | Plasmid expressing ECFP and ET1 from a bidirectional promoter                                      |                    |
| pSS134                        | Plasmid containing SEAP cassette                                                                   |                    |
| pWW43                         | Plasmid expressing E-KRAB [14]                                                                    |                    |
| pWW78                         | pTRIDENT1-based tricistronic expression vector for macrolide-responsive auto-regulated expression of up to two desired transgenes. |                    |
| pBF141                        | Vector expressing SEAP and ET1 under tetracycline-responsive promoter: PhCMV*-1-SEAP-IRESPV-ET-pA | unpublished         |
| pMF123                        | Plasmid encoding tricistronic expression cassette driven by a constitutive SV40 promoter.         | [56]                |
| pMF351                        | Lentiviral vector encoding EYFP driven by a constitutive hCMV promoter                            | [51]                |
| pDF37                         | AAV2 vector containing a tricistronic PETR driven expression unit. The entire expression unit from pWW73 was excised using Spil/Ixob, polished by Klenow and cloned into pAAV-lacZ which was NotI digested and Klenow polished before, thus resulting in pDF37 (ITR-PETR-IRESPV-IRESEMCV-pASV40-ITR). | this work |
| pDF51                         | AAV2 vector containing a constitutive hCMV expressed ET1 cassette. ET1 was excised from pWW78 using EcoRI/HindIII and cloned into the corresponding sites of pAAV-MCS, thus resulting in pDF51 (ITR-PETR-IntronEMCV-ET1-pASV40-ITR). | this work |
| pDF54                         | AAV2 vector encoding EYFP driven by the erythromycin responsive PETR promoter. PETR was excised from pDF55 using AclI/NheI and cloned into the corresponding sites of pDF60, thus resulting in pDF54 (ITR-PETR-EYFP-pASV40-ITR). | this work |
| pDF55                         | AAV2 vector encoding divergent expression units for ECFP driven by PETR and ET1 driven by a HSP70 minimal promoter. The entire expression cassette was excised from pCF125 using EcoRV/Ixob and cloned into the HindIII/SpeI sites of pDF60, thus resulting in pDF55 (ITR-PETR-ECFP-pASV40-PETR-ET1-EYFP-pASV40-ITR). | this work |
| pDF56                         | AAV2 vector encoding ET1 driven by a constitutive SV40 promoter. ET1 was excised from pWW78 using EcoRI/Xxob and cloned into the EcoRI/SpeI sites of pDF63, thus resulting in pDF56 (ITR-PETR-ET1-pASV40-ITR). | this work |
| pDF60                         | AAV2 vector encoding EYFP driven by a constitutive hCMV promoter. PETR was excised from pMF351 using Xxob/Pad and cloned into NheI/PAD sites of pDF63, thus resulting in pDF60 (ITR-PETR-IntronEMCV-ET1-pASV40-ITR). | this work |
| pDF61                         | AAV2 vector encoding SEAP driven by an erythromycin responsive PETR promoter. SEAP was excised from pCF19 using NheI/Clal and cloned into the NheI/BstBI sites of pDF54, thus resulting in pDF61 (ITR-PETR-SEAP-pASV40-ITR). | this work |
| pDF63                         | AAV2 vector encoding an SV40 promoter followed by an IRESpsv and a IRESemcv element. PETR was excised from pMF123 using Spil/Igll, polished with Klenow and cloned into the polished NcoI/SpeI sites of pAAV-lacZ, thus resulting in pDF63 (ITR-PETR-IRESPSv-IRESEMCV-pASV40-ITR). | this work |
| pDF74                         | AAV2 vector containing tricistronic expression cassette driven by a PETRON8 promoter. The PETRON8 promoter was excised from pWW76 using NheI/EcoRI and cloned into the corresponding sites of pDF63, thus resulting in pDF74 (ITR-PETRON8-IRESPSv-IRESEMCV-pASV40-ITR). | this work |
| pDF75                         | AAV2 vector encoding dicistronic expression unit consisting of SEAP followed by an IRESpsv element followed by ET1 driven by PETR. Dicistronic expression cassette was excised from pBPI41 using Xxob/Pad and cloned into the NheI/PAD sites of pDF54, thus resulting in pDF75 (ITR-PETR-IRESPSv-ET1-pASV40-ITR). | this work |
| pDF76                         | AAV2 vector encoding SEAP driven by a PETRON8 promoter. SEAP was excised from pSS134 using EcoRII HindIII and cloned into pDF37. This vector was digested using EcoRII/Ascl and the SEAP containing insert was cloned into the corresponding sites of pDF74, thus resulting in pDF76 (ITR-PETRON8-SEAP-IRESPSv-pASV40-ITR). | this work |
| pDF77                         | AAV2 vector encoding SEAP under the control of an erythromycin responsive PETR promoter (additional upstream ATG deleted). PETR was excised from pDF54 using AccI/EcoRI and cloned into the ClaI/EcoRI sites of pDF61, thus resulting in pDF77 (ITR-PETR-SEAP-pASV40-ITR). | this work |
| pDF89                         | AAV2 vector encoding divergent expression units for EYFP driven by PETR and ET1 driven by a HSP70 minimal promoter. The EYFP cassette was excised from pCF18 using AccI/EcoRI and cloned into the NcoI/Clal sites of pDF55, thus resulting in pDF89 (ITR-PETR-EYFP-pETR-IRESPSv-IRESEMCV-pASV40-ITR). | this work |
| 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 EcoRII/Ixob and cloned into the EcoRI/Ixob sites of pAAV-MCS, thus resulting in pDF109 (ITR-PETR-SEAP-pASV40-ITR). | this work |
| pDF124                        | AAV2 vector encoding dicistronic expression unit consisting of EYFP followed by an IRESEMCV element followed by ET1. The IRES-ET1 containing insert was excised from pDF75 using HindIII, polished with Pfu polymerase, digested using BstXI and cloned into the Swal/BstXI sites of pDF54, thus resulting in pDF124 (ITR-PETR-EYFP-IRESEMCV-ET1-pASV40-ITR). | this work |
**Table 1: Plasmids used and designed in this study (Continued)**

| Plasmid | Description |
|---------|-------------|
| pDF126  | AAV2 vector encoding E-KRAB under the control of a constitutive hCMV promoter. The E-KRAB containing insert was excised from pWWV043 using EcoRI/HpaI and cloned into the EcoRI/HindIII sites of pAAV-MCS, thus resulting in pDF126 (ITR-P<sub>CMV</sub>-Intron<sub>ph</sub>-E-KRAB-pA<sub>hCMV</sub>-ITR). |
| pDF141  | AAV2 vector encoding self-regulated expression cassette consisting of ETI driven by a constitutive SV40 promoter and EYFP driven by P<sub>ETR</sub>. The entire ETI expression cassette of pDF56 was excised using CiaI/PmlI and cloned into pDF54 which was digested with HindIII and polished by Pfu before, thus resulting in pDF141 (ITR-P<sub>SV40</sub>-ETI-pA<sub>SV40</sub>-P<sub>ETR</sub>-EYFP-pA<sub>SV40</sub>-ITR). |
| pDF143  | AAV2 vector encoding self-regulated expression cassette consisting of ETI driven by a constitutive SV40 promoter and SEAP driven by P<sub>ETR</sub>. The SEAP containing insert was excised from pDF77 using KpnI/Spel and cloned into the corresponding sites of pDF141, thus resulting in pDF143 (ITR-P<sub>SV40</sub>-ETI-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 promoter followed by 2 binding sites for the transrepressor E-KRAB. The 4*ETR binding site-containing fragment was excised from pWWV55 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-IRE<sub>EMCV</sub>-PA<sub>SV40</sub>-ITR). |
| pDF200  | AAV2 vector encoding SEAP under the control of a constitutive SV40 promoter followed by 4 binding sites for the transrepressor E-KRAB. The 4*ETR binding site-containing fragment was excised from pWWV55 using BstBI/Ndel and cloned into the corresponding sites of pDF76, thus resulting in pDF200 (ITR-P<sub>ETR</sub>ON4-SEAP-IRE<sub>EMCV</sub>-PA<sub>SV40</sub>-ITR). |
| pDF207  | AAV2 vector encoding EYFP under the control of a constitutive SV40 promoter followed by 8 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/Pacl 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 promoter followed by 2 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/Pacl 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 promoter followed by 2 binding sites for the transrepressor E-KRAB. EYFP was excised from pDF34 using EcoRI/Pacl 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; EYFP, enhanced cyan fluorescent protein (720 bp); EM, erythromycin; ETI, erythromycin transactivator (972 bp); EYFP, enhanced yellow fluorescent protein (720 bp); IRES<sub>EMCV</sub>, internal ribosome entry site of encephalomyocarditisviral origin (502 bp); IRES<sub>PhEF1α</sub>, internal ribosome entry site of polioviral origin (635 bp); ITR, inverted terminal repeat (141 bp); KRAB, kruppel-associated box (450 bp); pA<sub>hCMV</sub>, human growth hormone polyadenylation signal (478 bp); pA<sub>SV40</sub>, simian virus 40 polyadenylation signal (145 bp); pA<sub>EM</sub>, human elongation factor 1α promoter (1185 bp); p<sub>ETR</sub>, erythromycin responsive promoter (200 bp); p<sub>ETR</sub>ON, macrolide inducible promoter (350 bp); p<sub>EMCV</sub>, human immediate early cytomegalovirus promoter (663 bp); p<sub>PHSP70min</sub>, heat shock protein 70 minimal promoter (350 bp); p<sub>SV40</sub>, simian virus 40 promoter (308 bp); SEAP, human placental secreted alkaline phosphatase (1560 bp)

**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 1 mg/ml in ethanol and used at a final concentration of 1 μg/ml. For *in vivo* studies, 200 μl of a 10 mg/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 5 × 10<sup>10</sup> vector genomes/mouse. Erythromycin was administered intraperitoneally 1 h after injection of the transgenic AAV particles and repeated every 24 h. Blood samples were collected retroorbitally and serum was produced using microtainer SST tubes (Beckton Dickinson, Plymouth, UK). All animal experiments were performed 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. All authors read and approved the final manuscript.

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