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In-Depth Characterization of a Mifepristone-Regulated Expression System for AAV5-Mediated Gene Therapy in the Liver

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Gene therapy is being developed for the treatment of inherited diseases, whereby a therapeutic gene is continuously expressed in patients after delivery via viral vectors such as adeno-associated virus (AAV). Depending on the transgene, there could be a limited therapeutic window, and regulating timing and levels of transgene expression is advantageous. To control transgene transcription, the regulatory system GeneSwitch (GS) was evaluated in detail both *in vitro* and *in vivo*. The classical two-plasmid mifepristone (MFP)-inducible GS system was put into one plasmid or a single AAV5 vector. Our data demonstrate the inducibility of multiple transgenes and the importance of promoter and regulatory elements within the GS system. Mice injected with AAV5 containing the GS system transiently expressed mRNA and protein after MFP induction. The inducer MFP could be measured in plasma and liver tissue, and assessment of MFP and its metabolites showed rapid clearance from murine plasma. In a head-to-head comparison, our single vector outclassed the classical two-vector GS system. Finally, we show repeated inducibility of the transgene that also translated into a dynamic phenotypic change in mice. Taken together, this in-depth analysis of the GS system shows its applicability for regulated gene therapy.

INTRODUCTION

Several metabolic and inherited diseases are clinically targeted using gene therapeutic approaches,[1,2] which offer the prospect of a long-term causal correction of diseases that currently only have supportive treatments. One of the main gene therapy challenges is reaching sufficient concentrations of the therapeutic gene to correct disease symptoms, and for approaches based on growth factors or master regulators, fine-tuning of the protein expression is essential. Inducible protein expression also may improve the safety of approaches that have limited therapeutic windows or that require temporal protein expression.

An efficient inducible system should be able to switch a gene on by delivering a clinically approved and safe small molecule and should switch off upon withdrawal of the drug, without any background expression. The inducer drug should be easy to deliver and have rapid on-off dynamics. Several regulatory systems have been engineered to control gene transcription; for example, Tet-on or -off and GeneSwitch (GS). Both systems are based on the binding of a transactivator protein to a specific DNA sequence in the promoter of the transgene and on ligand-dependent activation of the system. In the presence of a small-molecule-inducer, the transactivator protein is activated, which subsequently binds to sequences in the promoter of the transgene, resulting in transcription.

The well-known Tet-on or -off system gene transcription is modulated through the tetracycline-dependent transactivator (rtTA) protein, which is sensitive to doxycycline. Although the Tet system has been optimized for usage at doxycycline concentrations acceptable in the clinic,[3] long-term use of antibiotics is not preferred. Moreover the rtTA is potentially immunogenic,[4] because it contains domains of bacterial origin. This would limit the clinical use of the Tet system to immune privileged areas such as the brain.

The GS system addresses several limitations of the Tet-on or -off system, because it is of mainly human origin and activated by the synthetic steroidal anti-progesterone drug, mifepristone (MFP), a clinically approved drug. MFP acts as an agonist to activate gene transcription by binding to a subunit of the GS protein; this interaction has been described by others previously.[5–8] GS can induce regulated gene expression in rat brain at an MFP concentration that is in range of clinical use.[9] The GS system has been successfully used in an animal model for Parkinson's disease, where glial-cell-derived neurotrophic factor (GDNF) was regulated in rat brain and had neuroprotective and neurorestorative effects.[10,11] Moreover, the GS system was able to modulate interferon (IFN) in the periphery.[12–14] However, it was suggested that the CMV promoter elicited an immune response.

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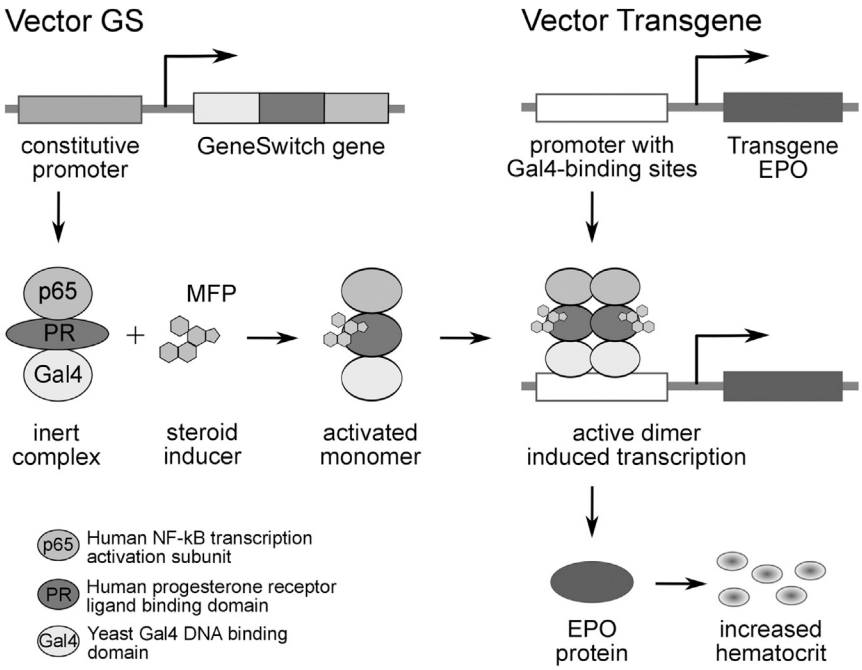


Figure 1. Schematic for the Regulation of Transgene Expression by the Mifepristone-Inducible GeneSwitch System

Within the GeneSwitch (GS) system, there are two protein expression cassettes, one for the inducible transgene of interest and another for GS protein. The regulatory vector (Vector GS) contains the GS gene and a promoter that is driving constant expression of the GS protein. In this example, the promoter is a liver-specific promoter, which is only active in hepatic tissue. GS protein is a fusion protein of three domains: a human NF-κB transcription activation subunit named p65, the human progesterone receptor ligand binding domain (PR), and the yeast Gal4 DNA binding domain (Gal4). After binding of the steroid inducer mifepristone (MFP) to PR, GS protein gets activated and changes conformation. An active dimer is formed that can bind via Gal4 to the four Gal4 binding sites within the inducible promoter of the transgene vector. Then, the p65 subunit of GS protein facilitates the start of transcription of the regulated transgene (here, EPO). EPO protein, on its turn, stimulates production of erythrocytes, resulting in increased hematocrit levels.

The GS system consists of two expression cassettes. One constitutively expresses the GS protein, the chimeric transactivator protein. The other cassette contains a transgene transcribed from an inducible promoter. GS is a fusion protein of two human domains and a 97-amino-acid yeast-derived sequence. The N-terminal domain is the DNA-binding domain of yeast transcription factor Gal4 that binds to a 17-nt-long sequence in the regulatable promoter of the transgene. GS binds to this sequence after dimerization, which is triggered by a conformational change of the ligand binding domain (LBD) in the presence of MFP. The LBD is a domain from the human progesterone receptor; hence, MFP is an inducer. The C-terminal part of GS is the activation domain of nuclear factor kappa B (NFκB) p65 and is responsible for the initiation of transgene transcription (Figure 1).

In the present study, we performed an in-depth optimization of the GS system for adeno-associated virus (AAV) gene therapy in the liver. Different promoters and regulatory elements were tested. A detailed head-to-head comparison of a single-vector system and a two-vector system was performed both *in vitro* and *in vivo*. Moreover, the kinetics of the GS system transgene induction were investigated systematically. The inducer MFP and its metabolites were measured *in vivo* to gain insight in plasma kinetics and liver tissue concentrations. Insulin-like growth factor (IGF) and erythropoietin (EPO) were chosen as transgenes due to the easy plasma readout and having a suitable size to fit into AAV vectors in combination with the GS system. By using several transgenes, proof of concept and wide applicability of the single GS system for AAV gene therapy in the liver were explored. Besides expression of the transgenes IGF and EPO upon MFP treatment, we have demonstrated that the single GS-EPO vector induction translates into a reversible increase in hematocrit levels, a hallmark phenotypic effect of EPO expression.

RESULTS

Mifepristone Induces a Dose-Dependent Regulation of Luciferase Expression by a Single-Vector GS System

In our initial experiments to develop regulated gene therapy, the expression of the GS protein was placed under the control of the cytomegalovirus (CMV) promoter, and firefly luciferase (FL) was used as a readout reporter transgene for MFP inducibility. The two expression cassettes containing GS and FL were cloned in a single vector in the head-to-tail configuration (CMV-GS-FL), as shown in Figure 2A.

The plasmid CMV-GS-FL and a control plasmid expressing GFP from a CMV promoter (CMV-GFP) were transfected into HEK293T cells. Cells introduced with CMV-GFP showed GFP expression, indicating successful transfection (data not shown). Exposure of cells transfected with CMV-GS-FL to MFP caused a concentration-dependent increase of luminescence, indicating MFP-dependent GS activation (Figure 2B).

Induction Rate of the Single-Vector GS System Is Promoter Dependent

For many clinical indications, it is preferred to restrict therapeutic gene expression to a single organ by using tissue-specific promoters. To investigate the influence of different promoters on the GS system for liver application, the universal CMV promoter was compared with the liver-specific human alpha1-antitrypsin (AAT) promoter. Rat insulin-like growth factor-1 (IGF1) was used as a transgene, as it is an

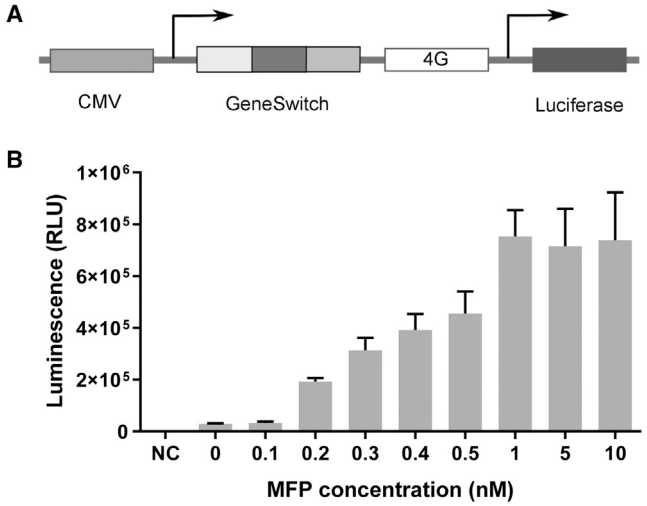


Figure 2. Mifepristone Dose-Dependent Expression of Luciferase by a Single-Vector GS System

(A) Schematic of the two expression cassettes of the GS system within a single vector. The cassettes are put into a tail-to-head configuration, where transcription is in the same direction. GS protein expression is driven by a CMV promoter, and the inducible promoter of luciferase contains four Gal4 binding sites (4G). (B) HEK293T cells were transfected with the CMV-GS-Luc plasmid shown in (A) or a control plasmid (NC). The next day, cells were incubated with different concentrations of MFP ranging from 0 to 10 nM for 48 h. Firefly luciferase activity was measured from three independent samples and averages are shown.

endocrine hormone mainly produced by hepatocytes. Plasmids with GS-IGF1 were cloned under control of the AAT promoter and the CMV promoter, respectively, in front of GS (Figure 3A). The expression cassettes of GS and IGF1 were in opposite transcription directions based on results from Szymanski et al.[12] and in order to avoid close proximity of the promoters to the AAV inverted terminal repeats (ITRs).

MFP induced expression of IGF1 by the hepatocyte cell line Huh7 transfected with the AAT- or CMV-driven plasmids. The AAT promoter construct resulted in much higher IGF1 protein induction than the CMV promoter did, 12 and 1.6 times over baseline, respectively (Figure 3B). In the non-induced state, cells with the AAT-promoter plasmid secreted only 6 ng/mL IGF1, in contrast to 28 ng/mL IGF1 with the CMV promoter, revealing low basal expression of the AAT promoter compared to the high background expression of the CMV promoter. The difference in IGF1 basal expression levels between the AAT and CMV promoters was unexpected, as both promoters are strong and have been widely used to drive gene expression in hepatocellular cells. Additionally, the MFP-induced IGF1 expression was higher with the AAT promoter (IGF1, 76 ng/mL) than with the CMV promoter (IGF1, 45 ng/mL). Consequently, the AAT promoter resulted in low background expression and a high dynamic range of MFP-dependent induction. Because of the superior characteristics of the AAT promoter, it was selected for further testing *in vivo*.

In Vitro Expression Kinetics of the Single-Vector GS System

We next investigated the expression kinetics of the GS system *in vitro*, using EPO as a reporter, to gain more insight into the GS system. A single vector (GS-EPO) was constructed containing the AAT-promoter in front of GS and an EPO expression cassette with the inducible promoter in the opposite direction (Figure 4A).

Huh7 cells were transfected with GS-EPO; incubated with 0, 1, or 10 nM MFP; and harvested 6, 16, 24, or 48 hours after induction. EPO mRNA levels were measured by qRT-PCR, and ELISA was performed to determine EPO protein concentrations in the culture supernatant. Both EPO mRNA and protein levels increased in the presence of MFP (Figures 4B and 4C). EPO mRNA levels increased to reach a maximum at 16 h, followed by protein expression that reached its maximum at 24 h to 48 h. GS protein kinetics were monitored by western blot using an antibody against its p65 domain and quantified relative to endogenous NFκB p65 signal. In the absence of MFP, the amount of GS protein increases from 6 h to 48 h, due to constitutive production of GS from the AAT promoter (Figure 4D). After the addition of 10 nM MFP, the signal for GS protein on the western blot was constant. However, in comparison to non-induced cells GS protein declined over time to two thirds the amount at 24 h and half the amount at 48 h post-administration of MFP. This difference could be either due to lack of antibody recognition, as activated GS changes conformation to form a dimer, or due to the degradation of GS after activation. Hence, induction by MFP results in transgene mRNA expression followed by protein expression, after which all components including the GS protein reach an equilibrium from 24 h to at least 48 h.

In Vitro Induction and Comparison of the Single- and Two-Vector GS Systems

The original GS system is based on two plasmids, but we have put all GS components into a single plasmid and kept within the maximal packaging capacity of the AAV vector. We compared the characteristics of the single- and two-vector GS system *in vitro* and *in vivo* using four constructs (Figure 5A). In addition to the single-vector GS-EPO, described in the previous section, two combinations of the two-vector system were designed: a two-vector system containing GS on one plasmid and the EPO transgene together with the regulated promoter having either four or eight Gal4 binding sites (GS-EPO, 4G-EPO, or 8G-EPO) on the second plasmid. GS-EPO, GS together with 4G-EPO, and GS with 8G-EPO were investigated for inducibility and the effect of the number of GS protein binding sites on (basal) expression levels.

The hepatocyte cell lines Huh7 and Hepa1-6 were transfected with these plasmids, and after 2 days of incubation with MFP, EPO was measured in the culture supernatant. In both cell lines, the addition of MFP induced the expression of EPO from the single- and two-vector systems (Figures 5B and 5C). In Huh7 cells, induction rates for all three conditions tested were just above 6 times (6.2 times for GS and 4G-EPO, 6.4 times for GS and 8G-EPO, and 6.3 times for GS-EPO), indicating that, in these cells, the single- and two-vector GS systems perform similarly (Figure 5B). The measured induction levels were

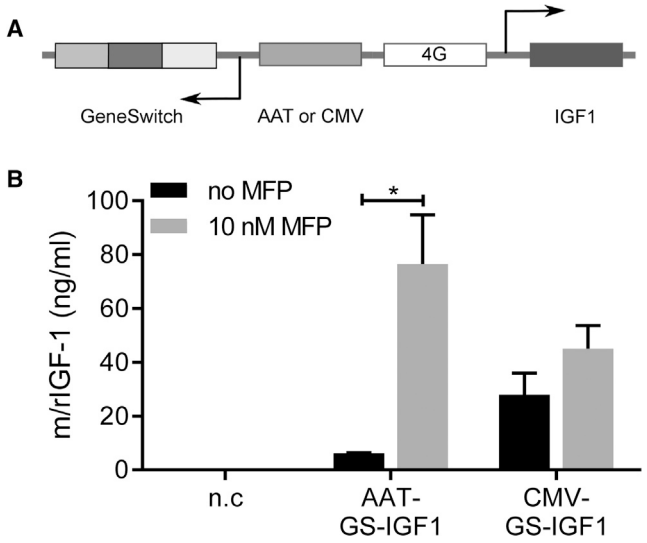


Figure 3. Single-Vector GS System Promoter Optimization and Applicability to Different Transgenes

(A) Configuration of the head-to-head single-vector GS system in which the transgene is insulin-like growth factor 1(IGF1) and the promoter in the GS expression cassette is either CMV or AAT. (B) Huh7 cells were mock transfected (negative control, n.c) or transfected with AAT-GS-IGF1 or CMV-GS-IGF1. The next day, culture medium was replenished by medium without (no MFP, black bars) or with (gray bars) 10 nM MFP. After 48 h, IGF1 secretion into the culture supernatant was measured by ELISA. Data were evaluated using Student's t test for treatment with MFP versus without MFP. The significant difference of $p < 0.05$ between the AAT-GS-IGF1 groups is indicated with an asterisk. There was only a positive trend for the difference between no MFP and 10 nM MFP of the CMV-promoter-containing construct.

higher and more distinctive in Hepa1-6 cells than in Huh7 cells (Figures 5B and 5C), mostly because in Huh7 cells, the constructs have to overcome endogenous EPO expression. In Hepa1-6 cells, the single-vector system outperformed the two-vector system. Whereas the induction ratio in cells transfected with GS and 4G-EPO was 4 times and 14 times using GS and 8G-EPO, with the GS-EPO construct, this was more than 500-fold (Figure 5C). These induction differences were, in large part, due to the different levels of background expression of the GS systems tested. With the single vector, GS-EPO expression of EPO was not detectable without MFP. This was in contrast to the two-vector systems that triggered EPO expression in the absence of MFP. A higher leakiness was observed with the 4G-EPO plasmid, compared to the 8G-EPO plasmid, but only in Hepa1-6 cells and not in Huh7 cells. These results showed an impact of the number of Gal4 binding sites on the activity of the inducible promoter and indicated that an increased number of those sites could result in less background expression.

Measurement of Mifepristone Concentrations in Biological Matrices such as Plasma and Liver Tissue

Because the GS system is regulated by MFP, it is important to know the MFP concentrations necessary for gene induction in plasma and in liver. MFP is metabolized and then excreted from the body; hence, its pharmacokinetics are important for the timing of GS activation.

In rats, maximal MFP plasma levels are reached 1 to 2 h after MFP administration.[15] Mice were injected intraperitoneally during 4 consecutive days with 20 mg/kg MFP and sacrificed 2 h after the last injection. MFP was extracted from plasma and liver tissue and measured quantitatively by high-performance liquid chromatography (HPLC) followed by quadrupole time-of-flight mass spectrometry (QTOF-MS). In Figure 6A, positive and negative chromatograms are shown. A peak with a retention time consistent with MFP (2.73 min) was exclusively present in the plasma sample from the MFP-injected mice. Applying the same method, MFP levels could also be measured in liver tissue (Figure 6B). The average concentration of MFP from six mice was 677 ng/mL in the plasma and 3,835 ng/g in the liver. Hence, MFP seems to be a suitable inducer for liver-directed gene therapy.

MFP can be C-hydroxylated, mono-demethylated, and di-demethylated in the liver. These MFP metabolites retain binding affinity to the human progesterone receptor and could induce the GS system.[16,17] To follow MFP and MFP metabolite concentrations in plasma in time, mice were injected on 3 consecutive days with MFP, and plasma was recovered at 1 h, 4 h, 8 h, 16 h, and 24 h after the last MFP injection. Each MFP metabolite has a different retention time, and, to compare MFP to its metabolite concentrations, deuterated MFP was used as an internal standard. MFP and mono- and di-demethylated MFP levels peaked at 1 h after the last MFP injection in the plasma samples, while hydroxylated MFP reached a maximum at 4 h (Figure 6C). Mono-demethylated MFP was more prevalent than di-demethylated MFP in plasma, as the demethylations occur successively. At 16 h post-administration of MFP, the concentration of each compound reached the detection limit of 10 ng/mL, and at 24 h post-injection, no MFP or metabolite could be detected. The half-lives were calculated to be around 3.5 h for MFP and hydroxylated MFP, 1 h for mono-demethylated MFP, and 2.5 h for di-demethylated MFP. Although the half-life of MFP measured in mice was slightly longer than the 2 h reported previously in rats,[15] MFP and its metabolites are rapidly cleared in rodents.

In Vivo Expression Kinetics of the Single-Vector GS System

For testing the kinetic expression of the components of the GS system in mice, the single-vector construct GS-IGF1 and constitutive expression construct IGF1, both with the AAT promoter, were encapsulated into AAV5 (AAV5-GS-IGF1 and AAV5-IGF1). IGF1 was chosen as a transgene because protein-bound IGF1 has a longer half-life than EPO,[18] and the rat IGF1 sequence was used to distinguish between endogenous murine IGF1 and vector-expressed IGF1.

The AAV5 vectors were injected intravenously at a dose of 10e−13 genome copies per animal. One group of mice received the constitutive expression vector AAV5-IGF1 (Figure 7A). The other groups were transduced with AAV5-GS-IGF1 and, 4 weeks later, injected with MFP for 3 consecutive days. Blood was drawn at 1 h, 4 h, 8 h,

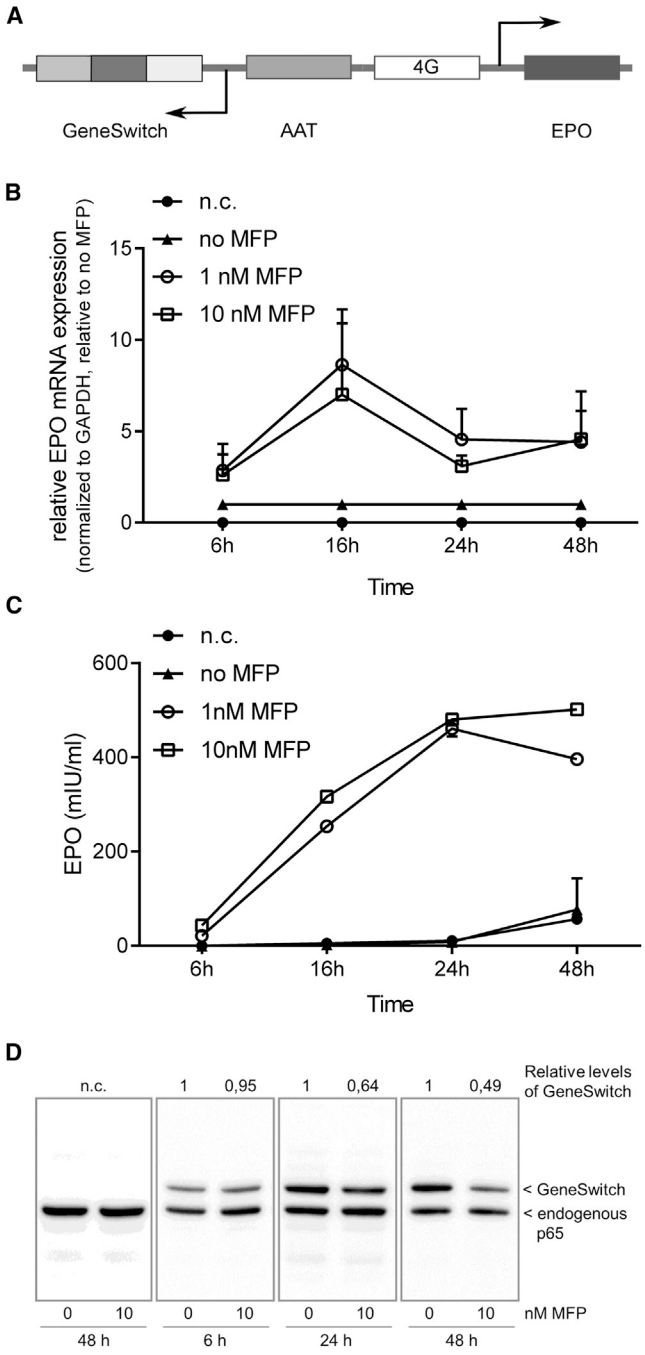


Figure 4. *In Vitro* Expression Kinetics of EPO mRNA, Protein, and GS Protein after MFP Induction of the Single-Vector GS System

(A) Configuration of head-to-head single-vector GS-EPO in which the transgene is erythropoietin (EPO) and with the AAT promoter in the GS expression cassette. (B) EPO mRNA kinetics. Huh7 cells were transfected with single-vector GS-EPO or no plasmid (n.c.). The next day they were incubated with 0 to 10 nM MFP for 6 to 48 h. Then, EPO mRNA expression in the cells was determined by qRT-PCR. Data were analyzed with a two-way ANOVA, and the most significant induction rate was at 16 h after 1 nM and 10 nM MFP ($p < 0.0001$). At 24 and 48 h, induction was also significant ($p < 0.05$). Full statistical analysis can be found in Table S1. (C) EPO protein kinetics. EPO protein expression and secretion into the culture medium were measured by ELISA. Two-way ANOVA at 6 h revealed $p < 0.05$ for no MFP versus 1 nm MFP and $p < 0.0001$ for no MFP versus 10 nm MFP. At 16, 24, and 48 h, $p < 0.0001$ for no MFP versus both 1 nM and 10 nM MFP. (D) Detection of GS protein. The presence of GS protein in cells was assessed by SDS-PAGE followed by western blotting using a p65 antibody. Arrows indicate endogenous p65 and GS protein. Relative GS protein levels were quantified by normalizing the GS protein signal to endogenous p65 signal and set relative to 0 nM MFP of each time point. The values are indicated above each lane.

16 h, and 24 h after each MFP injection (Figure 7A). Following the third MFP injection, the mice were sacrificed, and vector DNA and rat IGF1 mRNA levels were quantitated in the liver. The average transduction efficiency of the AAV5 vector was similar for the groups; however, there was high variance within each group (Figures 7A and 7B). Four animals that seemed to have been mis-injected and had very low liver vector DNA levels (fewer than 10e−6 genome copies/μg DNA) were excluded from the subsequent mRNA and protein analyses.

Rat IGF1 mRNA levels were quantified using rat-specific IGF1 primers and compared to those of the AAV5-GS-IGF group that did not receive MFP. AAV5-IGF1-injected animals constitutively express IGF1 at around 60 times over the non-induced AAV5-GS-IGF1-transduced animals. After induction of the AAV5-GS-IGF1 groups, IGF1 mRNA concentrations increased up to 4 h after administration of MFP and, subsequently, gradually decreased to non-induced levels at 24 h post-administration of MFP. Concordantly, a time-dependent increase in IGF1 mRNA levels after induction with MFP using the GS system was detected *in vivo* (Figure 7C).

Finally, IGF1 protein levels were determined in murine plasma upon MFP induction on 3 consecutive days. The plasma concentrations of IGF1 varied between 100 and 400 ng/mL for all groups over these 3 days (Figure S1). Higher steady-state IGF1 levels were detected in mice injected with the constitutive AAV5-IGF1 vector compared to the uninduced AAV5-GS-IGF1-injected group. IGF1 protein induction was determined at 1, 4, 8, 16, and 24 h after the last induction. A clear time-dependent increase was observed in all animals (Figure 7D), demonstrating a tight regulation of the transgene expression over time. Taken together, these results indicate MFP and time-dependent regulated transgene mRNA and protein expression by the GS system *in vivo*.

In Vivo Induction and Comparison of the Single- and Two-Vector GS System

After evaluating the kinetics of MFP and the GS system in mice, we compared the characteristics of the single- and the two-vector GS systems encapsulated in AAV5. Instead of IGF1, EPO was used as the reporter gene, because it can be easily measured in blood and increases hematocrit, providing a simple measurable functional effect.

Mice were transduced with 10e−12 genome copies of the constitutive expressing AAV5-EPO or the single-vector virus AAV5-GS-EPO.

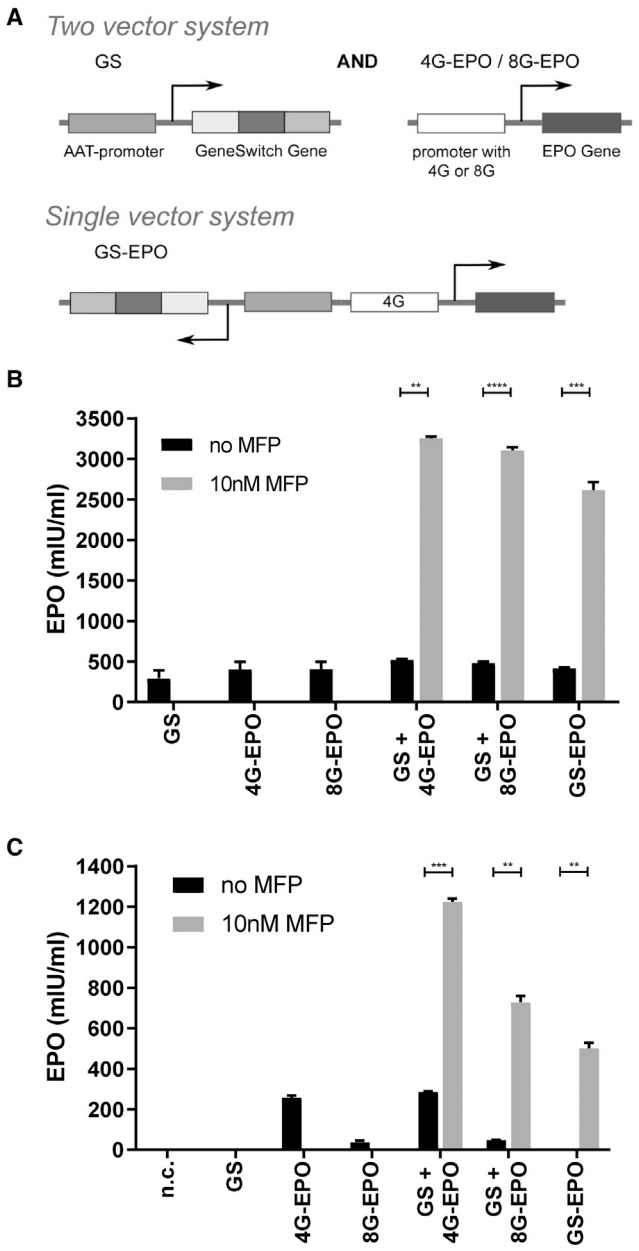


Figure 5. Mifepristone-Inducible Expression of EPO by Single- and Two-Vector GS System *In Vitro*

(A) Within the two-vector system, the GS protein expression cassette is in one vector (GS), and the transgene expression cassette with EPO is in the second vector. The latter contains either four Gal4 (4G-EPO) or eight Gal4 (8G-EPO) binding sites in their inducible promoter. The single-vector system comprises both the GS protein and EPO expression cassettes in one vector (GS-EPO), here in tail-to-tail orientation with transcription in opposing directions. (B) The different plasmids illustrated in (A) were transfected into Huh7 cells. The next day, they were incubated with 0 or 10 nM MFP for 48 h. Then, an ELISA was performed on culture supernatant to measure the concentration of secreted EPO. (C) Same as in (B), except in Hepa1-6 cells. Mock-transfected cells are indicated with negative control (n.c.). In the GS-EPO-treated cells, the background levels of EPO without MFP were too low to visualize in the graph. Data were evaluated using Student's t test to compare treatment with MFP versus without MFP. MFP treatment significantly induced EPO expression in both cell lines. **p < 0.01; ***p < 0.001; ****p < 0.0001.

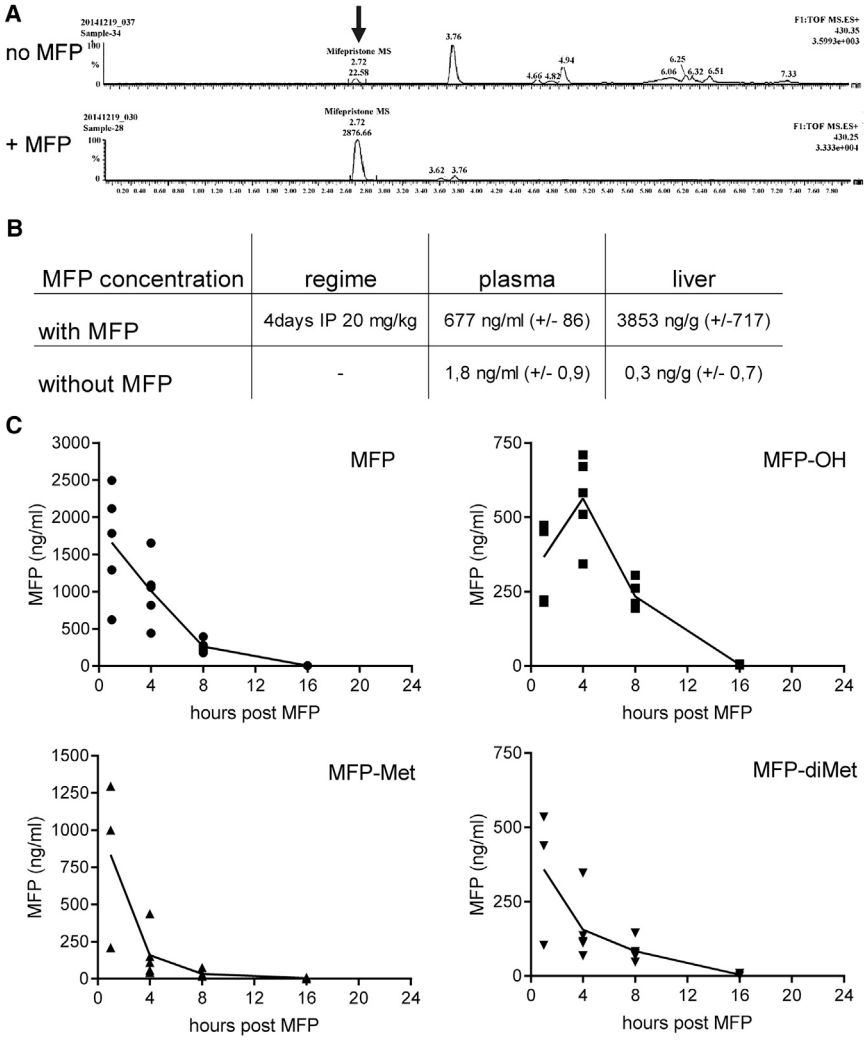
In addition, two groups of mice were injected intravenously with AAV5-GS in combination with either AAV5-4G-EPO or AAV5-8G-EPO, each virus at 10e−12 genome copies per animal (Figure 8A). To investigate repeated inducibility of the GS system, the mice received two rounds of MFP at 4 and 8 weeks post-transduction. Blood was taken regularly from the mice to measure EPO plasma concentrations and hematocrit (Figure 8B).

After the second round of MFP injections, the mice were sacrificed, and vector DNA was measured with EPO- or GS-specific primers (Figure 8A). The liver-specific transduction of the different AAV5-delivered constructs was similar for all groups (Figure 8C).

AAV5-EPO caused continuous transgene expression, resulting in plasma EPO levels of approximately 600 milli-international units [mIUs]/mL (Figure 8Di). This caused a high hematocrit, and after a month, it was decided that the mice would be sacrificed (Figure 8E). The groups injected with either the single- or the two-vector GS system all had increased EPO plasma levels after induction with MFP. The EPO levels dropped to background at 4 days after the last MFP injection (data not shown) and remained at basal levels till the week before the next MFP-induction round (Figures 8Dii–8Div). The induction ratio of the single vector was more than 200 times, while the ratios with the two-vector system with 4G-EPO or 8G-EPO were 60 and 20 times, respectively. The difference of the induction ratios was mainly a consequence of the low background expression levels and not the amplitude of expression observed with the single-vector system. Although the two-vector system GS and 4G-EPO showed high levels of EPO after induction, they revealed considerably more basal expression than the single-vector system. As had been observed *in vitro*, the addition of four additional Gal4 binding sites, to a total of eight, reduced background levels as well as the induced expression levels, resulting in a lower induction ratio. The induced EPO production in mice injected with the single-vector system had functional effects and increased red blood cell volume (Figure 8D). Hematocrit levels were above normal in mice injected with either of the two-vector systems in the absence of MFP, due to background EPO expression. In summary, *in vivo*, the single-vector GS system had low background expression and a high induction rate, two requirements that are essential for developing regulated AAV gene therapy. Mice injected with the single-vector system had a normal hematocrit in the non-induced state, and hemoglobin levels increased upon MFP injection, returning to baseline in the absence of the inducer (Figure 8F). Hence, the GS system can be adapted to cause background-free repeated induction of a therapeutic transgene, resulting in an inducible phenotype.

DISCUSSION

Gene therapy offers the potential of a long-term solution for many chronic diseases whereby the transgene is continuously



MFP concentration	regime	plasma	liver
with MFP	4days IP 20 mg/kg	677 ng/ml (+/- 86)	3853 ng/g (+/-717)
without MFP	-	1,8 ng/ml (+/- 0,9)	0,3 ng/g (+/- 0,7)

Figure 6. Assay Development for Detection of Mifepristone and Its Metabolites in Biological Matrices

(A) Mice received intraperitoneal MFP injections (20 mg/kg) for 4 consecutive days. Then, plasma and liver samples were taken 2 h after the last MFP injection and measured by UPLC-QTOF-MS. Mass spectrometry chromatograms for mifepristone at 2.73 min in murine plasma samples from mice without (no MFP) or with (+ MFP) MFP injections are shown in (A). The table in (B) indicates the average MFP concentration in plasma and liver samples of six mice. The standard deviation is in brackets. (C) Mice were treated with MFP for 3 consecutive days, and blood samples on the third day were taken at the indicated hours post-injection, followed by measurement on UPLC MSMS to determine concentrations of MFP (top left) and three MFP metabolites: C-hydroxylated MFP (top right), mono-demethylated MFP (bottom left), and di-demethylated MFP (bottom right).

expressed following a single-vector administration. However, in some cases, it would be desirable to regulate transgene expression within a well-defined therapeutic window in time or to regulate the rate of expression of a therapeutic transgene. The present report describes a series of *in vitro* and *in vivo* proof-of-concept studies to explore the MFP-inducible GS system in regard of timing and transgene expression levels and carrying out an in-depth characterization of the regulated gene expression system. Our data support the rationale that the GS system could be applied in future clinical development programs.

The classical GS system consists of two vectors with separate GS and transgene expression cassettes. We chose to develop a single-vector system, because it circumvents the hurdle of delivery of both cassettes at a predefined ratio in the same target cell *in vivo*. Furthermore, a single product would simplify AAV manufacturing. Based on the relatively large size of GS (2.5 kb), the additional cargo size is limited to approximately 2 kb to not exceed the AAV packaging limit of approximately 4.7 kb. With suitably sized transgenes such as luciferase, EPO, and IGF, we were able to show inducibility of the single-vector system for all three genes. Albeit in a different expression cassette orientation, others have used a single-vector AAV with transgenes GFP, GDNF, and IFN.[9,10,12] Collectively, this demonstrates wide applicability of the single-vector GS system to multiple transgenes. Besides transgene size, other discriminating points in the development of a single vector are the orientation of both expression cassettes and the promoter for the GS protein, as described by Szymanski et al.[12] In addition, we showed that the promoter can affect the inducibility of the system, as the use of the AAT promoter improved induction ratios, compared to those of the CMV promoter, because it has lower non-induced expression and higher transgene expression after MFP addition. The use of the liver-specific AAT promoter adds additional value to the GS system, because expression will be limited to hepatocytes and, hence, increase the safety of the approach.

The GS system is designed to be silent in a non-induced state and active after the addition of MFP. The advantage of a positively induced system is that it requires the administration of the inducer MFP only when necessary, increasing the safety profile of the regulated expression system. The need-on-demand principle for MFP would allow discontinuation of therapy in the case of adverse effects, or when treatment is no longer required, by simply stopping administration of the inducer drug. In mice, we observed a rapid (16–24 h) clearance of MFP and metabolites after the last injection with the inducer. Moreover, transgene levels increased at first and then decreased, showing that the GS system can be switched off by the removal of MFP. It should be noted that the half-life of MFP in

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Group	Virus name	MFP	sampling post MFP	transduction efficiency average (gc/ug DNA)
1	AAV5-IGF	no	n.a.	2,48E+07
2	AAV5-GS-IGF	no	n.a.	1,04E+07
3	AAV5-GS-IGF	yes	1h	5,95E+07
4	AAV5-GS-IGF	yes	4h	7,53E+07
5	AAV5-GS-IGF	yes	8h	7,50E+07
6	AAV5-GS-IGF	yes	16h	3,27E+07
7	AAV5-GS-IGF	yes	24h	1,41E+07

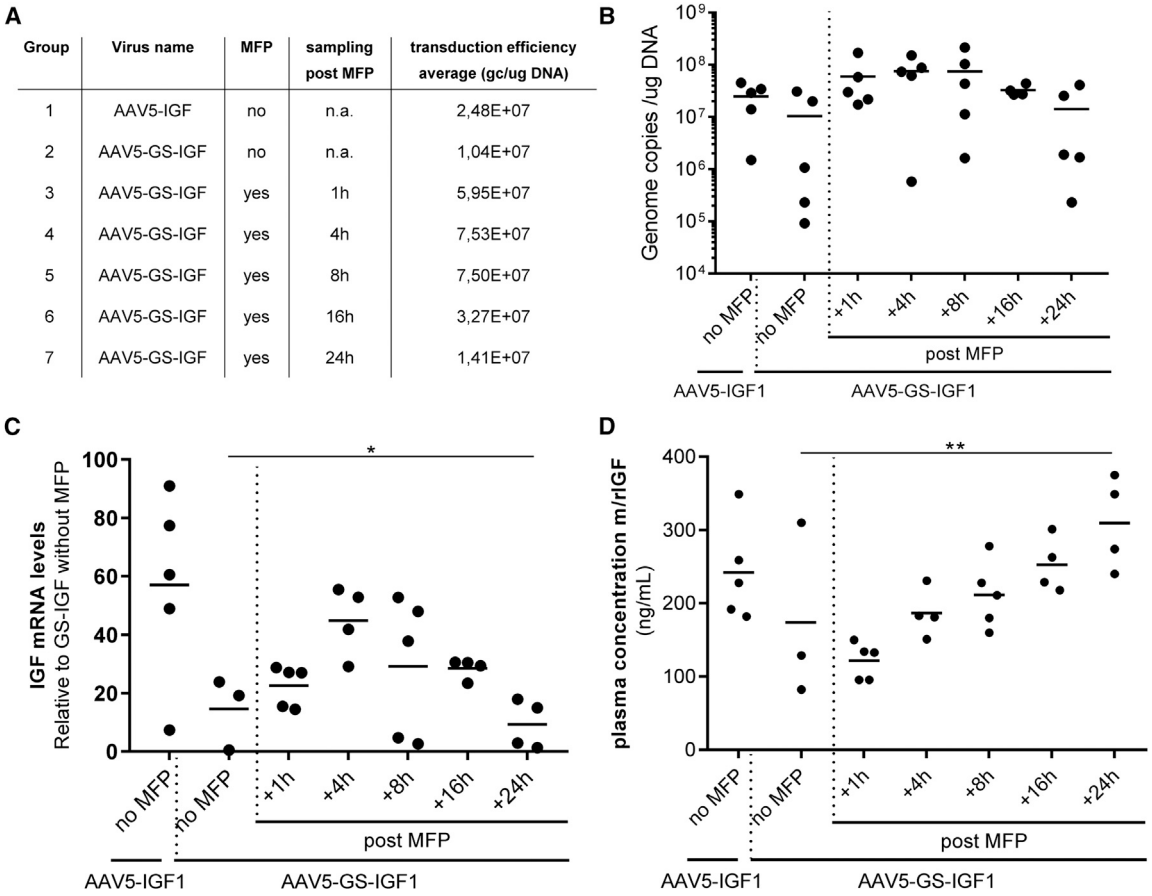

Figure 7. *In Vivo* Expression Kinetics of IGF mRNA and Protein after MFP Induction in Murine Liver by the Single-Vector GS System

Mice were transduced with AAV5 expressing rat IGF constitutively (AAV5-IGF, group 1) or regulated through the GS system (AAV5-GS-IGF, groups 2 to 7). Four weeks later, groups 3 to 7 were given MFP on 3 consecutive days, and blood was taken at the indicated hours after each injection (+1h, +4h, etc). Group 1 and group 2 did not receive MFP (no MFP). On the third day, the animals were sacrificed, and the livers were dissected. One animal in the 16 h-group died before MFP injections for unknown reasons. (A) Table indicates with which AAV5 the groups were injected, whether MFP treatment was administered, and time post-MFP treatment for blood sampling. The average transduction efficiency of vector DNA was determined in liver tissue 5 weeks after virus injection by qPCR. (B) Transduction efficiency of vector DNA of each mouse from each group. The small lines indicate the average per group. Four animals seemed to be mis-injected and had fewer than 10e−6 genome copies per microgram of DNA; they were excluded from the subsequent mRNA and protein analyses. (C) Liver samples were analyzed for rat IGF mRNA levels by reverse transcription and qPCR. Levels were normalized to GAPDH and put relative to mRNA levels in mice transduced with GS-IGF without induction (group 2). A one-way ANOVA revealed a significant change in IGF mRNA levels over time post-MFP induction. Full statistical analysis can be found in Table S1. (D) IGF plasma concentrations were determined in the blood samples after 3 days of MFP injections by ELISA against mouse and rat IGF. There was a significant increase in plasma concentration post-MFP induction (one-way ANOVA).

humans is around 25 to 30 h; therefore, it is expected that the induced transgene expression in humans would last longer than in rodents.[19]

One requirement for a regulated expression planned for clinical use would be to turn it on and off repeatedly. Previous data showed effective usage of the GS system in a murine model of experimental allergic encephalomyelitis, with one plasmid containing the inducible system that regulated IFN expression.[20] This single-vector system was packaged into AAV1 and injected intramuscularly into mice, and IFN was induced over nearly a year.[20] We carried out a similar *in vivo* study using AAV5 to target hepatocytes in the liver. The GS system was induced twice with MFP, resulting both times in high EPO plasma levels with a meaningful impact on hematocrit levels that increased temporarily. Both studies demonstrate the broad and robust versatility of the GS system.

A critical feature in optimizing a regulation system for gene expression is to avoid background expression of the system. Our study has identified several factors that are involved in non-induced transgene expression: promoter choice, regulatory elements, and the application as a single- or two-vector system. The use of the liver-specific AAT promoter in conjunction with GS resulted in a higher induction ratio, mainly due to low basal transgene expression in the absence of MFP. Increasing the number of Gal4 binding sites from four to eight to more tightly regulate transcription also resulted in reduced basal expression levels. However, while this approach reduced

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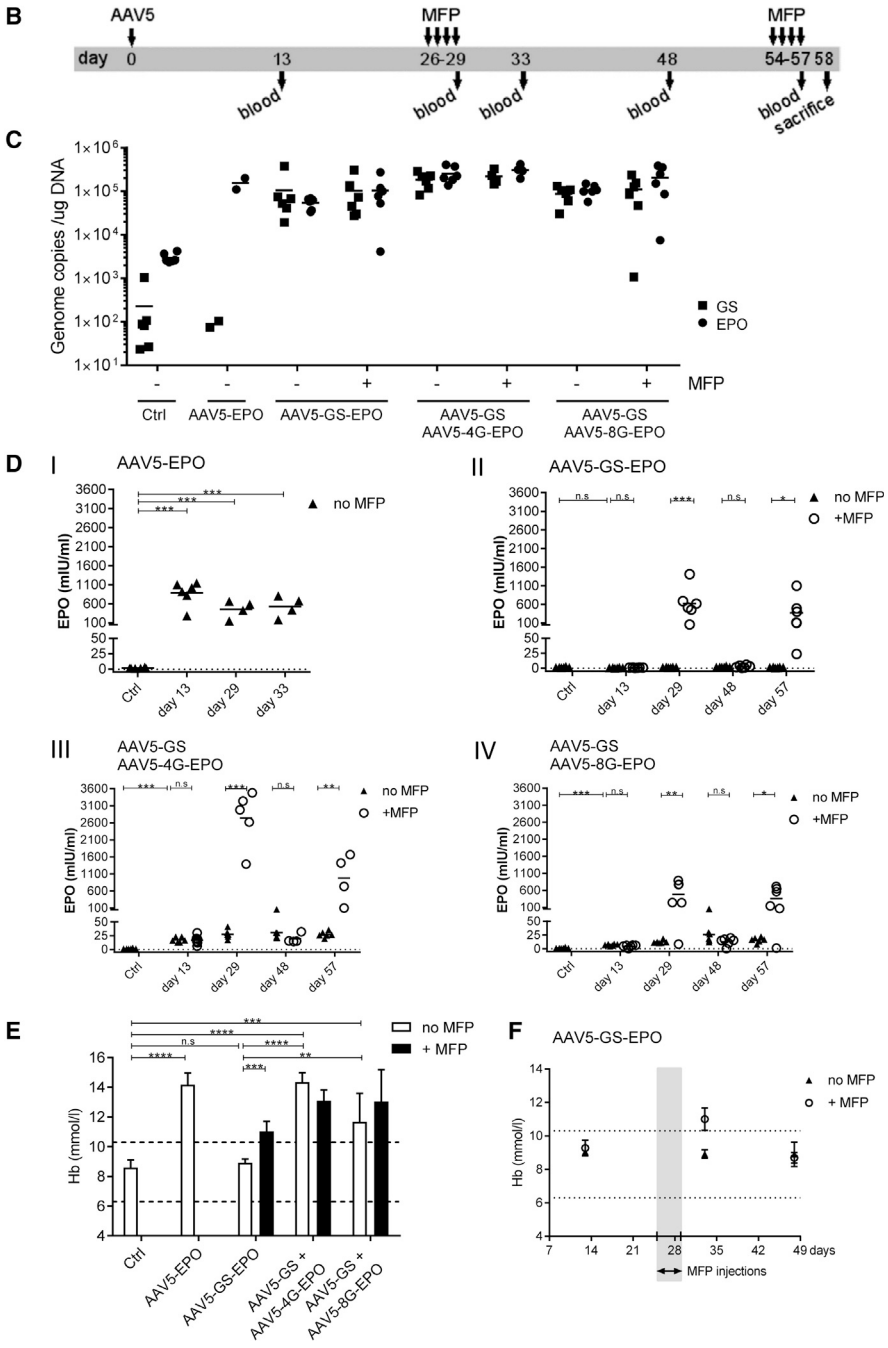
Group	name virus 1	name virus 2	gc injected per animal virus 1	gc injected per animal virus 2	MFP	transduction efficiency (gc/ug DNA) virus 1	transduction efficiency (gc/ug DNA) virus 2
1	- (Ctrl)	-	-	-	no	-	-
2	AAV5-EPO	-	1,0E+12	-	no	1,5E+05	-
3	AAV5-GS-EPO	-	1,0E+12	-	no	8,0E+04	-
4	AAV5-GS-EPO	-	1,0E+12	-	yes	1,0E+05	-
5	AAV5-GS	AAV5-4G-EPO	1,0E+12	1,0E+12	no	1,8E+05	2,5E+05
6	AAV5-GS	AAV5-4G-EPO	1,0E+12	1,0E+12	yes	2,2E+05	3,0E+05
7	AAV5-GS	AAV5-8G-EPO	1,0E+12	1,0E+12	no	8,7E+04	1,1E+05
8	AAV5-GS	AAV5-8G-EPO	1,0E+12	1,0E+12	yes	1,1E+05	2,1E+05



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background expression, both *in vitro* and *in vivo*, it also lowered maximal expression, resulting in a lower induction ratio in mice. The lowest background expression levels were observed using a single-vector system: mice transduced with the single vector GS-EPO and not injected with MFP had EPO plasma levels that were similar to those of the control group. Therefore, the single vector with the AAT-promoter meets the safety criteria of reducing basal expression levels of the transgene.

The transgene induction rate provides an estimate of the dynamic range of protein expression using the GS system. We observed an increase in transgene expression of more than 200 times *in vivo* with the single AAV5-GS-EPO vector compared to the basal state in the presence of MFP, therefore indicating a two-log-range-regulated EPO expression. To compare the induction rate of GS to that of other inducible systems *in vivo*, multiple variables need to be taken into account, including the promoter, vector architecture, the transgene, a single- or two-vector system, the target cell, and the inducer. We had a preference for the AAT promoter over the CMV promoter, with an induction rate difference 7.5 times and with the additional benefit of restricting transgene expression to the liver. It has been reported that the transthyretin promoter can drive GS protein to regulate interleukin-12 (IL-12) expression. Mice injected with the inducible adenoviral vector showed two- to three-log increased IL-12 levels, depending on viral load and MFP dose.[21] Other tissue-specific promoters that were used in combination with the GS system were selective for brain and muscle and had induction ratios of 24 and 500 times, respectively.[10,22] Hence, the performance of the AAT promoter as part of the GS system is comparable to the previously reported results.

The single-vector architecture of the GS system, as well as the type of transgene, can make a difference for inducibility.[12] The orientation of the expression cassettes determined the induction rates for secreted embryonic alkaline phosphatase (SEAP) between 4 to 900 times, while the rates from vectors with EPO were between 19 and 34 times, which is significantly lower than that of the AAV5-GS-EPO we have tested. The Tet-on system has been incorporated into a single AAV vector with a liver-specific albumin promoter and expressing luciferase as a transgene.[23] Injection of the vector into mice gave, similarly to the AAV5-GS-EPO vector, a dynamic range of 250 times. In contrast, in an *in vivo* study using the Tet-on system, controlling EPO expression in a single vector with both cassettes facing inward, the induction rate reported was 15 times above baseline. This relatively low increase after administration of doxycycline was mainly due to the high basal expression levels that already were sufficient to increase the hematocrit in mice.[24] In contrast, a two-vector AAV rapamycin system expressing EPO did not elevate hematocrit levels in the absence of the inducer, and, similar to our findings, rapamycin administration resulted in an induction rate of 200 times.[25,26] A main advantage of the two-vector rapamycin system is its tight regulation, compared to other systems.[27] Here, we report that, using a single vector, a similar tight control of gene regulation and predictable repeated induction, leading to an induced phenotype, can be achieved.

The kinetics and orchestration of transgene expression have not been studied extensively for GS or for other regulated gene expression systems and was, therefore, investigated by us *in vitro* and *in vivo*. *In vivo*, we observed that the highest plasma levels of MFP and metabolites were measured at 1 to 4 h post-injection, followed by a peak expression of mRNA at 4 to 8 h. Transgene protein levels could be measured on the same day and the next day—a time frame confirmed with our two-vector system and by others.[21,22] The rise of transgene mRNA followed by increasing levels of protein correlated with the *in vitro* results. We also managed to detect GS protein by western blot in liver tissue, albeit weakly, showing that after MFP induction, GS protein decreased, confirming our *in vitro* data (data not shown). We would expect similar kinetics for other regulated gene expression systems; however, how long it takes before expression can be switched off will depend on the pharmacodynamics of the inducer and the stability of the mRNA and protein. Here, we used EPO as transgene, which has a half-life of around 5 h and could not be measured 4 days after the last MFP injection (data not shown). On the other hand, using GDNF as transgene with a half-life of 37 h resulted in longer expression, as GDNF was still detectable 1 week after MFP injection.[10] Doxycycline is cleared from the body in 15–25 h, which is shorter than the time for MFP (25–30 h) but rapamycin is around twice as

Figure 8. Mifepristone-Tnducible Expression of EPO in Murine Liver by the Single- and Two-Vector GS System

(A) Table indicating the groups, the name and concentration of the AAV5 injected, whether MFP treatment was administered, and the average vector DNA level of each vector per group determined in murine liver tissue using qPCR. (B) Illustration of experimental setup. Arrows above timeline indicate the day of AAV5 transduction and the days of intraperitoneal injections with MFP. Arrows below the timeline specify when blood samples were taken and at day 58, when the animals were sacrificed. (C) Individual values of vector DNA levels in liver tissue of each AAV5, determined in mice 9 weeks post-vector injection using qPCR. Specific primers were used to quantify GS sequences (squares) and EPO sequences (EPO, circles). (D) EPO concentrations in the plasma of mice injected with different AAV5 vectors. (i) Animals were transduced with AAV5-EPO, and EPO plasma concentration was measured at days 13, 29, and 33. (ii) Mice received the single vector AAV5-GS-EPO. In (iii) and (iv), the animals were co-injected with AAV5-GS and AAV5-4G-EPO or AAV5_8G-EPO. EPO plasma levels were measured at days 13, 29, 48, and 57. Animals subjected to two rounds of 4 days of MFP injections are indicated with circles; the non-MFP-injected animals are indicated with triangles. Each subpanel contains the EPO plasma concentration of animals without vector and MFP injections (Ctrl). Data were evaluated using Student's t test to determine the significance between groups treated with and without MFP. Some measurements are also indicated with n.s to show that they are not significantly different. (E) All groups were subjected to hematocrit measurement at day 33. The hematocrit levels between the dotted lines at 6.3 and 10.3 mmol/L are considered normal or healthy. Basal expression levels between groups were analyzed by a two-way ANOVA (see Table S1). A second comparison was done with a t test to determine the effect of MFP on hemoglobin (Hb) levels. (F) Average hematocrit levels from animals transduced with the single vector AAV5-GS-EPO at days 13, 33, and 48. Triangles represent the mice without MFP, and circles represent the mice injected with MFP. Gray bar indicates when MFP was injected, and dotted lines indicate the borders of normal hematocrit levels. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

long (60 h). Therefore, expression from the GS system might, due to MFP inducibility, be switched off relatively quickly.

One of the advantages of using MFP as an inducer in the GS system is the fact that it is licensed for clinical use. The GS inducer MFP and its safety have been studied only short term and in females. However, the drug is currently being investigated for long-term use in psychiatric disorders with male and female participants.[28] Moreover, the MFP concentration inducing expression in our *in vivo* experiments has been safely tested in psychiatric patients.[29–31] Besides progesterone-antagonizing effects, MFP has anti-glucocorticoid properties. However, the half-maximal inhibitory concentration (IC_{50}) of the latter effects is 100-fold lower, therefore the low-dose of MFP is not expected to have an effect on the glucocorticoid receptor. In the present experiments, using a single-vector GS system, MFP induced the transgene at a concentration between 0.2 nM and 1 nM. This is as low as the concentration demonstrated previously.[32] Alternative regulated systems use rapamycin or tetracyclins as the inducer. Rapamycin is an immunosuppressant, and clinical application would require development of non-toxic rapamycin orthologs. Tetracyclins have short-term side effects, and long-term use of tetracyclins could increase antibiotic resistance in microorganisms. Recently, a Tet-on system was optimized to be inducible at low-enough concentrations to have the potential to not invoke resistance.[3]

Gene therapy should be restricted to the organ where the gene product is required. The delivery to the target organ is achieved by the choice of the AAV variant, by the route of administration, and by a promoter that restricts the expression to the area of choice. In the case of a regulated gene expression system, it is also necessary to ensure that the inducer is reaching the target cells. We found that sufficient concentrations of MFP reach the liver *in vivo*. Rapamycin and doxycyclin have difficulty reaching the brain, and cerebrospinal fluid (CSF) levels are 30% and 25% of the plasma concentration, respectively.[33,34] MFP does cross the blood-brain barrier, and the GS system has been successfully utilized in rat brain.[9] Additionally, it is possible to induce expression from the GS system, which is injected in mouse muscle, or when expressed in lung cells.[22,35] Hence, the GS system is applicable to multiple organs and can be controlled by tissue-specific promoters.

The ongoing successes for clinical trials for hemophilia A and hemophilia B have led to increasing enthusiasm for AAV gene therapy targeting liver diseases.[36] Preclinical studies in animal models have demonstrated proof of concept for several other types of liver disorders.[36] Many of these disorders, such as glycogen storage disease type Ia, citrullinemia type I, ornithine transcarbamylase deficiency, phenylketonuria, Wilson disease, methylmalonic acideamia, and Crigler-Najjar syndrome, are currently under investigation by several pharmaceutical companies.[36,37] As more disease indications for the liver are moving closer to the clinic, inducible systems will inevitably be needed in the future to allow a more controlled regulation of transgenes that may induce transgene-specific immune responses or cause other unwanted effects at sustained expression and/or high doses. The data presented in this study support the applicability of a regulatable GS system delivered by AAV for future clinical application in the liver.

MATERIALS AND METHODS

Plasmid Construction

The EPO coding sequence is from M18189.1, the IGF1 coding sequence is from M15480.1 (rat), and the human growth hormone poly(A) tail (pA) sequence is from NM_022560; these were synthesized and digested from shuttle vectors prepared by BaseClear (Leiden, the Netherlands). The AAT promoter combined with the mouse albumin gene enhancer (AAT promoter) was taken from a plasmid described previously.[38] pIF1683 (Inovio, San Diego, CA, USA) contained minimal RNA polymerase II promoter with four upstream activation elements (4xGal4) and IVS8, which was cloned in front of the transgene. The GS sequence was from pGS1694 (Inovio, San Diego, CA, USA). The specific order of elements within the expression cassettes designed in this study are summed up here: CMV-GS-luciferase has CMV-GS-pA-4xGal4-IVS8-luciferase-pA, CMV-GS-IGF has pA-IGF-IVS8-4xGal4-CMV-IVS8-GS-pA, AAT-GS-IGF has pA-IGF-IVS8-4xGal4-AAT-IVS8-GS-pA, EPO has AAT-EPO-pA, GS has 4xGal4-AAT-IVS8-GS-pA, 4G-EPO has 4xGal4-IVS8-EPO-pA, 8G-EPO has 4xGal4-4xGal4-IVS8-EPO-pA, and GS-EPO has pA-EPO-4xGal4-AAT-IVS8-GS-pA.

Cell Culture, Transfection, and MFP Induction

Huh7, Hepa1-6, and HEK293 cells were cultured in DMEM (GIBCO), supplemented with 10% fetal bovine serum with or without penicillin and streptomycin at 37°C, 5% CO_2. Trypsin-EDTA (0.25%; GIBCO) was utilized for cell detachment, and cells were seeded 1 day prior to transfection. HEK293 cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol. Lipofectamine 3000 at a ratio of 1:0.75 for P3000:Lipofectamine 3000 was used to transfect Huh7 and Hepa1-6 cells. On the following day, culture supernatant was replaced by medium with the appropriate MFP concentration. MFP was diluted from a 1-mM MFP-ethanol stock solution into medium.

Luciferase Assay

To measure luciferase activity, culture medium was removed, and cells were washed once in PBS, followed by lysis with 1× Passive Lysis Buffer (Promega). Plates were incubated on an orbit shaker at 450 rpm at room temperature for 20 min. FL activity of 10-μL samples was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. An average of luciferase counts was taken from triplicate transfections.

ELISA

For EPO and IGF1 ELISAs, murine plasma or cell culture supernatant was collected. The samples were diluted in specimen diluent buffer for EPO measurements and in calibrator diluent buffer for IGF1 measurements to range within the standard curve values. After that, the manufacturer's protocol was followed (R&D Systems; DEP00 and

MG100). EPO or IGF1 concentrations were calculated using a trend line derived from the standard curve samples in Excel.

RNA and DNA Isolation, cDNA Synthesis, and qPCR

Genomic DNA (gDNA) was isolated from murine liver using the DNeasy Blood & Tissue Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's protocol. Total RNA was isolated from liver sections or from Huh7 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. gDNA was removed by double-stranded DNase treatment using engineered shrimp DNase (Thermo Scientific, EN0771). First-strand cDNA was reverse transcribed using random hexamer primers with the DyNAmo cDNA Synthesis Kit (Thermo Scientific, F-470L) and 500 ng total RNA.

Real-time PCR amplification was performed with 10 times diluted cDNA or 250 ng gDNA, Fast SYBR Green Master Mix (Thermo Scientific, 4385612), and primers specific for EPO (ATATCAC CGTCCCAGACACC and CAGGACAGCTTCTGAGAGCA), IGF (TCACAGGGATGCCAAGAT and GTCAACATGAGCGCACC), GS (AGCATGCGATATTTGCCGAC and AGAGTAGCGACACTC CCAGT), AAT (AGGCCAACTTGTCTACGTTTAGTATG and CAGCGTCCTGTGTCCAAGGT), beta-actin (ACGGCCAGGTCAT CACTATTG and CAAGAAGGAAGGCTGGAAAAGA), and/or GAPDH (TCCACCCATGGCAAATTCC and GGGATTTCCATT GATGACAAGCT). PCR reaction conditions were as follows: 95°C for 20 s, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. The assays were performed on an ABI 7500 Fast System (Applied Biosystems, Foster City, CA, USA). EPO and IGF mRNA expression levels were normalized to GAPDH or beta-actin, and the relative gene expression $2^{-\mathrm{DeltaDeltaCt}}$ method was used for analysis of PCR data. AAV5 titers were determined using a standard curve, made with plasmid containing the AAT promoter and EPO coding sequence.

SDS-PAGE, Transfer, and Western Blot

Cells were washed in PBS and lysed in 1× NuPAGE LDS Sample Buffer (Life Technologies). NuPAGE Reducing Agent was added to the samples prior to heating at 95°C. Proteins were separated on a NuPAGE Novex Bis-Tris 4-12% gel in a Bio-Rad system with MOPS SDS Running Buffer (Life Technologies). To transfer proteins from the gel onto an Immun-Blot PVDF Membrane (Bio-Rad), a wet blotting system (Bio-Rad) was used with NuPAGE transfer buffer containing 10% methanol. The membrane was blocked with 5% semiskimmed milk (Sigma), 0.1% Tween 20 (Calbiochem) in PBS (GIBCO). This was followed by incubation with the primary antibody against NFκB p65 (abcam, ab7970) in blocking buffer. After washing the membrane, the antibody swine-anti-rabbit with horseradish peroxidase (HRP) conjugate (Dako) was added. Subsequent to washing the membrane, HRP was visualized by ECL Lumilight Plus (Roche), and the signal was captured with the ImageQuant LAS4000 (GE Healthcare). Quantification of the protein bands in the images was done with ImageJ Fiji.

AAV5 Vector Production

AAV5 vectors used in this study were produced by a baculovirus-based AAV production system. Briefly, the expression cassettes of interest were cloned into a uniQure transfer plasmid in order to generate an entry plasmid. The presence of the two ITRs was confirmed. The ITR expression cassette was inserted in a recombinant baculovirus vector by homologous recombination in Sf9 cells, and clones were selected by plaque purification. The recombinant baculoviruses containing the ITR expression cassette were further amplified and screened for the best production and stability by PCR and qPCR. To generate AAV5, cells were infected with recombinant baculoviruses expressing the ITR expression cassette, the replicon enzyme, and the capsid protein. The cells were lysed, and crude lysate was treated with Benzonase (50 U/mL) (Merck, Darmstadt, Germany) for 1 h at 37°C. AAV5 was purified on an AVB Sepharose column (GE Healthcare, Little Chalfont, UK) using an AKTA purification system (GE Healthcare), and the final concentration was determined by qPCR.

In Vivo

C57BL6 mice were maintained under a 12-h:12-h light:dark cycle in a clean facility with free access to food and water. Experiments were performed with the approval of the Animal Ethics Committee (DEC) in the Netherlands. Three-month-old animals were intravenously injected with AAV5; EPO-expressing viruses at 1e−12 and IGF-expressing viruses at 1e−13 vector genome copies per animal. Four weeks later, mice injected with AAV5-GS-IGF were treated with 20 mg/kg MFP 3 days in a row, except for the non-induced group of mice. In the experiment concerning EPO expression, 4 and 8 weeks post-transduction, several groups of mice were injected intraperitoneally with 20 mg/kg MFP for 4 consecutive days. Blood was taken on the indicated days of the experiment; in general, before transduction and before and after induction with MFP. All blood samples were collected in tubes with heparin, and after centrifugation at 1,500 rpm for 15 min, plasma was stored at −80°C and used for ELISA or MFP measurement at a later stage. Hematocrit was analyzed using a HemoCue 201+ analyzer (HemoCue, Ängelholm, Sweden), with a drop of blood immediately collected on a microcuvette. When the animals were sacrificed, their livers were dissected.

MFP Quantification

MFP was quantified at Eurofins PROXY Laboratories (Leiden, the Netherlands) according to the company's SOPs. In brief, after the addition of internal standard D3-mifepristone, MFP was extracted from 50 μL plasma or approximately 10 mg liver tissue. The MFP standard curve was prepared in the appropriate matrix. Chromatographic separation of the samples was performed on a UPLC (ultra-performance liquid chromatography) column, followed by positive electrospray ionization at QTOF-MS. Then, MFP concentrations were calculated from the recorded chromatograms.

Statistical Analysis

Data were analyzed using the one-way ANOVA, two-way ANOVA, or Student's t test, with a predefined significance level of $\alpha = 0.05$

to determine statistically significant differences between two groups. The p values are represented by the following number of asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. Table S1 shows the full statistical analysis for the one-way ANOVA and two-way ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.05.002.

AUTHOR CONTRIBUTIONS

Conceptualization: H.P., P.K., and J.M.L.; Investigation: J.M.L., R.M., T.V.d.Z., S.K., and A.H.; Resources: J.L. and B.B.; Supervision, Formal Analysis, Visualization, and Writing – Initial Draft: J.M.L.; Project Administration and Writing – Review and Editing: P.K. and H.P.; Funding Acquisition: P.K.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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