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**Author:** Martier, R.M.
**Title:** Therapeutic RNAi-based gene therapy for neurodegenerative disorders: slowing down the ticking clock
**Issue Date:** 2020-05-27
Chapter 6

In-depth characterization of a Mifepristone regulated expression system for AAV5-mediated gene therapy in the liver

Jolanda M. Liefhebber¹, Raygene Martier¹,², Tom Van der Zon¹, Sonay Keskin¹, Angelina Huseinovic¹,³, Jacek Lubelski¹, Bas Blits¹, Harald Petry¹, Pavlina Konstantinova¹

¹ Department of Research & Development, uniQure N.V., Amsterdam, The Netherlands;
² Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands;
³ Amsterdam UMC, The Netherlands

Mol. Ther. Methods & Clinical Development. (2019); 13: 512-525
Abstract
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 was evaluated in detail both \textit{in vitro} and \textit{in vivo}. The classical two plasmid mifepristone (MFP)-inducible GeneSwitch system was put into one plasmid or a single AAV5 vector. Our data demonstrate inducibility of multiple transgenes and the importance of promoter and regulatory elements within the GeneSwitch system. Mice injected with AAV5 containing the GeneSwitch 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 GeneSwitch 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 GeneSwitch system shows its applicability for regulated gene therapy.

Keywords
Regulated gene expression, mifepristone inducible GeneSwitch system, AAV5 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/off and GeneSwitch. Both systems are based on 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-inducing compound the transactivator protein is activated and can subsequently bind to sequences in the promoter of the transgene, which is then being transcribed.

The well-known Tet-on/off system gene transcription is modulated through the transactivator-protein (rtTA) 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 GeneSwitch system, addresses several limitations of the Tet-on/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 GeneSwitch protein, this interaction has been described by others previously.\(^5-8\) GeneSwitch, can induce regulated gene expression in rat brain at an MFP concentration that is in range of clinical use\(^9\). The GeneSwitch 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 GeneSwitch system was able to modulate interferon (IFN) in the periphery, however in that study a CMV promoter was used which could elicit an immune response due to expression in antigen presenting cells\(^12-14\). Besides, in the periphery IFN has been modulated, however in that study GeneSwitch was behind a CMV promoter which could elicit an immune response due to expression in antigen presenting cells\(^12-14\).

The GeneSwitch system consists of two expression cassettes: one constitutively expressing the GeneSwitch protein, the chimeric trans-activator protein. The other cassette
contains a transgene transcribed from an inducible promoter. GeneSwitch 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 nucleotide long sequence in the regulatable promoter of the transgene. GeneSwitch 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 that MFP is an inducer. The C-terminal part of GeneSwitch is the activation domain of NFκappaB p65 and is responsible for the initiation of transgene transcription (Figure 1).

In the current study we performed an in-depth optimization of the GeneSwitch system for AAV gene therapy in the liver. Different promoters and regulatory elements were tested. A detailed head-to-head comparison of a single- and two-vector system were performed both in vitro and in vivo. Moreover, the kinetics of the GeneSwitch system transgene induction was 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 read out and having a suitable size to fit into AAV vectors in combination with the GeneSwitch system. By using several transgenes, proof of concept and wide applicability of the single GeneSwitch system for AAV gene therapy in the liver was explored. Besides expression of the transgenes IGF and EPO upon MFP treatment, we have demonstrated that the single GeneSwitch-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 GeneSwitch system

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

This 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 GeneSwitch activation (Figure 2b).
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Figure 1. Schematic for the regulation of transgene expression by mifepristone inducible GeneSwitch system. Within the GeneSwitch system there are two protein expression cassettes, one for the inducible transgene of interest and another for GeneSwitch protein. The regulatory vector (Vector GS) contains the GeneSwitch gene and a promoter that is driving constant expression of GeneSwitch protein. In this example the promoter is a liver specific promoter, which is only active in hepatic tissue. GeneSwitch protein is a fusion protein of three domains: human NF-kB transcription activation subunit named p65, human progesterone receptor ligand binding domain (PR) and yeast Gal4 DNA binding domain (Gal4). After binding of the steroid inducer mifepristone (MFP) to the PR domain, GeneSwitch protein gets activated and changes conformation. An active dimer is formed that can bind via the Gal4 domain to the four Gal4 binding sites within the inducible promoter of the transgene vector. Then the p65 subunit of GeneSwitch 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.

**Induction rate of the single vector GeneSwitch 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 GeneSwitch 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 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 GeneSwitch (Figure 3a). The expression cassettes of GeneSwitch 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.

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, 1.6 and 12 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 (76 ng/ml IGF1) than with the CMV promoter (45 ng/ml IGF1). Consequently, the AAT promoter resulted in low

Figure 2. Mifepristone dose dependent expression of luciferase by a single-vector GeneSwitch system. A. Schematic of the two expression cassettes of the GeneSwitch system within a single vector. The cassettes are put into a tail to head configuration, where transcription is in the same direction. GeneSwitch 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 (N.C.). The next day cells were incubated with different concentrations of MFP ranging from 0 to 10nM for 48h. Firefly luciferase activity was measured from three independent samples and averages are shown.
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 GeneSwitch system**

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

Huh7 cells were transfected with GS-EPO, incubated with 0, 1 or 10nM MFP and harvested 6, 16, 24 or 48 hours after induction. EPO mRNA levels were measured by RT-qPCR 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 (Figure 4b and 4c). EPO mRNA levels increased to reach a maximum at 16h, followed by

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Figure 3. Single-vector GeneSwitch system promoter optimization and applicability to different transgenes. A. Configuration of head-to-head single vector GeneSwitch system in which the transgene is insulin like growth factor 1 (IGF1) and the promoter in the GeneSwitch 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 10nM MFP (gray bars). After 48h, IGF1 secretion into the culture supernatant was measured by ELISA. Data were evaluated using student’s t test for the treatment with MFP versus without MFP. The significant difference of p < 0.05 between the AAT-GS-IGF1 groups is indicated with (*). There was only a positive trend for the difference between no MFP and 10 nM MFP of the CMV promotor containing construct.
Figure 4. In vitro expression kinetics of EPO mRNA, protein and GeneSwitch protein after MFP induction of the single-vector GeneSwitch 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 GeneSwitch 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 10nM MFP for 6 to 48h hours. Then EPO mRNA expression in the cells was determined by reverse transcription qPCR. Data were analyzed with a two-way ANOVA and the most significant induction rate was at 16h after 1nM and 10 nM MFP (p < 0.0001). At 24 and 48h induction was also significant (p < 0.05). Full statistical analysis can be found in supplemental table 1. C. EPO protein kinetics. EPO protein expression and secretion into the culture medium was measured by ELISA. Two-way ANOVA at 6h 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 48h, p values were < 0.0001 for no MFP versus both 1 nM and 10 nM MFP. D. Detection of GeneSwitch protein. Presence of GeneSwitch protein in cells was assessed by SDS-PAGE followed by western blotting using an p65-antibody. Arrows indicate endogenous p65 and GeneSwitch protein. Relative GeneSwitch protein levels were quantified by normalizing the GeneSwitch protein signal to endogenous p65 signal and set relative to 0nM MFP of each timepoint. The values are indicated above each lane.

protein expression that reached its maximum at 24h to 48h. GeneSwitch protein kinetics were monitored by western blot using an antibody against its p65-domain and quantified relative to endogenous NF-kappaB p65 signal. In the absence of MFP, the amount of GeneSwitch protein increases from 6h to 48h, due to constitutive production of GeneSwitch from the AAT-promoter (Figure 4d). After addition of 10nM MFP the signal for GeneSwitch protein on the western blot was constant. However, in comparison to non-induced cells GeneSwitch protein declined over time, to two-thirds the amount at 24h and half the amount at 48h post MFP. This difference could either be due to lack of antibody recognition, as activated GeneSwitch changes conformation to form a dimer, or due to degradation of GeneSwitch after activation. Hence, induction by MFP results in
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transgene mRNA expression followed by protein expression, after which all components including the GeneSwitch protein reach an equilibrium from 24 hours to at least 48h.

**In vitro induction and comparison of the single- and two-vector GeneSwitch system**

The original GeneSwitch system is based on two plasmids, but we have put all GeneSwitch 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 GeneSwitch 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 GeneSwitch on one plasmid (GS) 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 GeneSwitch protein binding sites on (basal) expression levels.

The hepatocyte cell lines Huh7 and Hepa1-6 were transfected with these plasmids and after two days of incubation with MFP, EPO was measured in the culture supernatant. In both cell lines, addition of MFP induced the expression of EPO from the single and two vector systems (Figure 5b and c). 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 GeneSwitch systems perform similarly (Figure 5b). The measured induction levels were higher and more distinctive in Hepa1-6 cells than in Huh7 cells (Figure 5b and c), mostly because in Huh7 cells the constructs have to overcome endogenous EPO expression. In Hepa1-6 cells the single vector 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 GeneSwitch 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 which 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 GeneSwitch 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 GeneSwitch activation.

In rats, maximal MFP plasma levels are reached one to two hours after MFP administration\textsuperscript{15}. Mice were injected intraperitoneally during four consecutive days with 20mg/kg MFP and sacrificed 2h after the last injection. MFP was extracted from plasma and liver tissue and measured quantitatively by HPLC followed by quadrupole time of flight-mass spectrometry. In Figure 6a a positive and negative chromatogram 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 (Table in Figure 6b). The average concentration of MFP from six mice was 677 ng/ml in the plasma and 3835 ng/g in the liver. Hence, MFP seems to be a suitable inducer for liver-directed gene therapy.

MFP can be C-hydroxylated, mono- and di-demethylated and in the liver. These MFP metabolites retain binding affinity to the human progesterone receptor and could induce the GeneSwitch system\textsuperscript{16,17}. To follow MFP and MFP metabolite concentrations in plasma in time, mice were injected three consecutive days with MFP and plasma was recovered at 1h, 4h, 8h, 16h and 24h post 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, mono- and di-demethylated MFP levels peaked at 1h post last MFP injection in the plasma samples, while hydroxylated MFP reached a maximum at 4h (Figure 6c). Mono-demethylated MFP was more prevalent than di-demethylated MFP in plasma, as the demethylations occur successively. At 16h post MFP the concentration of each compound reached the detection limit of 10ng/ml and at 24h post injection no MFP or metabolite could be detected. The half-lives were calculated to be around 3.5h for MFP and hydroxylated MFP, 1h for mono-demethylated MFP and 2.5h for di-demethylated MFP. Although the half-life of MFP measured in mice was slightly longer than the 2 hours reported previously in rats\textsuperscript{15}, MFP and its metabolites are rapidly cleared in rodents.

**In vivo expression kinetics of the single vector GeneSwitch system**

For testing kinetic expression of the components of the GeneSwitch 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 transgene, because protein-bound IGF1 has a longer half-life than EPO\textsuperscript{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 10e13 genome copies (gc) 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 three consecutive days. Blood was drawn at 1h, 4h, 8h, 16h or
**Figure 5. Mifepristone inducible expression of EPO by single- and two-vector GeneSwitch system in vitro.**

A. Within the two-vector system the GeneSwitch 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 GeneSwitch protein and erythropoietin (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 10nM MFP for 48h. Then ELISA was performed on culture supernatant, to measure the concentration of secreted EPO. C. Same as 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 with MFP versus without MFP. MFP treatment significantly induced EPO expression in both cell lines. (**p < 0.01; ***p < 0.001; ****p < 0.0001)
24h 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 (Figure 7a and b). Four animals seemed to have been miss-injected and had very low liver vector DNA levels (less than 10e6 gc/ug DNA), were excluded from the subsequent mRNA and protein analyses.

Rat IGF1 mRNA levels were quantified using rat specific IGF1 primers and compared to the AAV5-GS-IGF group that did not receive MFP. AAV5-IGF1 injected animals constitutively express IGF1 and 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 hours after administration MFP, and subsequently gradually decreased to non-induced levels at 24h post MFP. Concordantly, a time dependent increase in IGF1 mRNA levels after induction with MFP using the GeneSwitch system was detected in vivo (Figure 7c).

Finally, IGF1 protein levels were determined in murine plasma upon MFP induction at three consecutive days. The plasma concentrations of IGF1 varied between 100 and 400 ng/ml for all groups over these three days (Supplemental Figure 1). 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 24h after the last induction. A clear time-dependent increase was observed in all animals (Fig7D), 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 GeneSwitch system in vivo.

**In vivo induction and comparison of the single- and two-vector GeneSwitch system**

After evaluating the kinetics of MFP and the GeneSwitch system in mice, we compared the characteristics of the single and the two vector GeneSwitch system encapsulated in AAV5. Instead of IGF1, EPO was 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 10e12 gc of the constitutive expressing AAV5-EPO or the single vector virus AAV5-GS-EPO. In addition, two groups of mice were injected intravenously with AAV5-GS either in combination with AAV5-4G-EPO or AAV5-8G-EPO, each virus at 10e12 gc per animal (Table in Figure 8a). To investigate repeated inducibility of the GeneSwitch 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 GeneSwitch specific primers (Table in 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 mIU/ml (Figure 8d, panel i). This caused a high hematocrit and after a month it was decided to sacrifice the mice. (Figure 8e). The groups injected with either the single or the two vector GeneSwitch system, all had increased EPO plasma levels after induction with MFP. The EPO levels dropped to background at four days post last MFP injection (data not shown) and remained at basal levels till the week before the next MFP-induction round (Figure 8d, panels ii, iii, iv). The induction ratio of the single vector was more than 200 times, while the ratios with 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 were 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 8e). 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 GeneSwitch 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 GeneSwitch 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 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 GeneSwitch 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 GeneSwitch system could be applied in future clinical development programs.

The classical GeneSwitch system consists of two vectors with separate GeneSwitch and transgene expression cassettes. We chose to develop a single vector system, because
Figure 6. Assay development for detection of mifepristone and its metabolites in biological matrices. A. Mice received intraperitoneal MFP injections (20mg/kg) for four consecutive days. Then plasma and liver samples were taken 2 hours 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 injections (+MFP) are shown in A. The table in B. contains the average MFP concentration in plasma and liver samples of six mice. The standard deviation is
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C. Mice were treated with MFP for three consecutive days, blood samples on the third day were taken at indicated hours post injection, followed by measurement on UPLC MSMS to determine concentrations of MFP (i) and three MFP metabolites C-hydroxylated MFP (ii), mono-demethylated MFP (iii) and di-demethylated MFP (iv).

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 relative large size of GeneSwitch (2,5kb), the additional cargo size is limited to approximately 2 kb to not exceed the AAV packaging limit of approximately 4,7kb. With suitable 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 IFN9,10,12. Collectively, this demonstrates wide applicability of the single vector GeneSwitch 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 GeneSwitch protein, as described by Szymanski et al12. 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 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, 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 case of adverse effects or when treatment is no longer required, by simply stop taking the inducer drug. In mice we observed a rapid (16-24 hour) clearance of MFP and metabolites after the last injection with the inducer. Moreover, transgene levels increased at first and then decreased, showing that the GeneSwitch system can be switched off by the removal of MFP. It should be noted that the half-life of MFP in humans is around 25 to 30 hours, and it therefore is expected that the induced transgene expression in humans would last longer than in rodents19.

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 GeneSwitch system in a murine model of experimental allergic encephalomyelitis, with one plasmid containing the inducible system that regulated interferon expression20. This single vector system was packaged into AAV1 and injected intramuscularly into mice and interferon was induced over nearly a year20. We carried out a similar in vivo study using AAV5 to target hepatocytes in the liver. The Gene switch system was induced twice with MFP resulting both times in high EPO plasma levels with a meaningful impact on hematocrit levels.
Figure 7. *In vivo* expression kinetics of IGF mRNA and protein after MFP induction in murine liver by the single-vector GeneSwitch system. Mice were transduced with AAV5 expressing rat IGF constitutively (AAV5-IGF, group 1) or regulated through the GeneSwitch system (AAV5-GS-IGF, group 2 to 7). Four weeks later, groups 3 to 7 were given MFP on three 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 dissected. One animal of the 16h group died before MFP injections for unknown reasons. A. Table indicates with which AAV5 the groups were injected, treatment of MFP and time post MFP 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 miss-injected and had below 10^6 genome copies per ug 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 supplemental table 1. D. IGF plasma concentrations were determined in the blood samples after three 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).
that increased temporarily. Both studies demonstrate the broad and robust versatility of the Gene Switch 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 GeneSwitch 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, whilst this approach reduced background expression, both \textit{in vitro} and \textit{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 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 GeneSwitch system. \textit{In vivo} with the single vector AAV5-GS-EPO a more than 200 times increased transgene expression was observed compared to the basal state in the presence of MFP, indicating a two-log range regulated EPO expression. To compare the induction rate of GeneSwitch to other inducible systems \textit{in vivo}, multiple variables need to be taken into account, including the promoter, vector architecture, the transgene, a single or two-vector, the target cell and the inducer. We preferred the AAT-promoter over the CMV-promoter, with a 7,5-times induction rate difference and with the additional benefit of restricting transgene expression to the liver. It has been reported that the transthyretin promoter can drive GeneSwitch protein to regulate IL12 expression. Mice injected with the inducible adenoviral vector showed two to three log increased IL12 levels, depending on viral load and MFP dose\textsuperscript{21}. Other tissue specific promoters that were used in combination with the GeneSwitch system were selective for brain and muscle and had 24 and 500 times induction ratios respectively\textsuperscript{10,22}. Hence, the performance of the AAT-promoter as part of the GeneSwitch system is comparable to the previously reported results.

The single vector architecture of the GeneSwitch system as well as the type of transgene, can make a difference for inducibility\textsuperscript{12}. The orientation of the expression cassettes determined the induction rates for SEAP between 4 to 900 times, while the rates from vectors with EPO were between 19 and 34 times, which is significantly lower than 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 transgene\textsuperscript{23}. Injection of the vector into mice, gave similarly to the AAV5-GS-EPO vector, a dynamic range of 250 times. In contrast, an \textit{in vivo} study using the Tet-on
Figure 8. Mifepristone inducible expression of EPO in murine liver by the single- and two-vector GeneSwitch system. A. Table indicating the groups, the name and concentration of the AAV5 injected and the MFP treatment. The average vector DNA level of each vector per group determined in murine liver tissue using qPCR. B. Illustration of experimental set up. 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 GeneSwitch sequences (GS, squares) and EPO sequences (EPO, circles). D. EPO concentrations in the plasma of mice injected with different AAV5 vectors. In panel i animals were transduced with AAV5-EPO and EPO plasma concentration was measured at day 13, 29 and 33. The mice of panel ii received the single vector AAV5-GS-EPO. In panel 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 day 13, 29, 48 and 57. Animals subjected to two rounds of four days of MFP injections are indicated with circles, the non-MFP injected animals with triangles. Each panel 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 they are not significantly different. E. All lymphocytes were subjected to hematocrit measurement.
In-depth characterization of a Mifepristone regulated expression system

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 Hb levels. F. Average hematocrit levels from animals transduced with the single vector AAV5-GS-EPO at day 13, 33 and 48. Triangles represent the mice without MFP and circles is the group injected with MFP. Gray bar indicates when MFP was injected and dotted lines are the borders of normal hematocrit levels. (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).
system, controlling EPO expression in a single vector with both cassettes facing inward the induction rate reported was 15 times above baselines. 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\textsuperscript{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 experiments, rapamycin administration resulted in an induction rate of 200 times\textsuperscript{25,26}. A main advantage of the two-vector rapamycin system is its tight regulation compared to other systems\textsuperscript{27}. We here 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 has not been studied extensively for GeneSwitch, nor for other regulated gene expression systems, and was therefore investigated by us \textit{in vitro} and \textit{in vivo}. \textit{In vivo} we observed that the highest plasma levels of MFP and metabolites were measured at 1 to 4 hours post injection, followed by a peak expression of mRNA at 4 to 8 hours. Transgene protein levels could be measured the same day and the next day; a timeframe confirmed with our two vector system and by others\textsuperscript{21,22}. The rise of transgene mRNA followed by increasing levels of protein correlated with the \textit{in vitro} results. We also managed to detect GeneSwitch protein by western blot in liver tissue, albeit weakly, showing that after MFP induction GeneSwitch protein decreased, confirming our \textit{in vitro} data (data not shown). We’d 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 hours and could not be measured four days post the last MFP injection (data not shown). On the other hand, using GDNF as transgene with a half-life of 37 hours resulted in longer expression, as GDNF was still detectable one week post MFP injections\textsuperscript{10}. Doxycycline is cleared from the body in 15-25 hours, which is shorter than MFP (25-30 hours), but is around twice as long for rapamycin (60 hours). Expression from the GeneSwitch system might therefore, due to MFP inducibility, be switched off relatively quickly.

One of the advantages of using MFP as an inducer in the GeneSwitch system is the fact that it is licensed for clinical use. The GeneSwitch inducer MFP and its safety has been studied only short term and in females. However, the drug is currently investigated for long term use in psychiatric disorders with male and female participants\textsuperscript{28}. Moreover, the MFP concentration inducing expression in our \textit{in vivo} experiments has been safely tested in psychiatric patients\textsuperscript{29–31}. Besides progesterone-antagonizing effects MFP has anti-glucocorticoid properties. Although, the IC\textsubscript{50} of the latter effects is a 100-fold lower, and low-dose MFP is not expected to have an effect on the glucocorticoid receptor. In the current experiments, using a single vector GeneSwitch system MFP induced the transgene at a concentration between 0.2 nM and 1 nM. This is as low as
demonstrated previously\textsuperscript{32}. Alternative regulated systems use rapamycin or tetracyclins as 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\textsuperscript{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 is restricting the expression to the area of choice. In 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 \textit{in vivo}. Rapamycin and doxycyclin have difficulty reaching the brain and CSF levels are 30\% and 25\% of the plasma concentration, respectively\textsuperscript{33,34}. MFP does cross the blood brain barrier, and the GeneSwitch system has been successfully utilized in rat brain\textsuperscript{9}. Additionally, it is possible to induce expression from the GeneSwitch system which is injected in mice muscle or when expressed in lung cells\textsuperscript{22,35}. Hence, the Gene Switch 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\textsuperscript{36}. Preclinical studies in animal models have demonstrated proof of concept for several other types of liver disorders\textsuperscript{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\textsuperscript{36,37}. As more disease indications for the liver are moving closer to the clinic, inducible systems will inevitable be needed in the future to allow a more controlled regulation of transgenes that may induce transgene-specific immune responses or cause other unwanted effect at sustained expression and/or high doses. The data presented in this study supports the applicability of regulatable GeneSwitch system delivered by AAV for future clinical application in the liver.

Material and Methods

Plasmid construction

Erythropoietin (EPO) coding sequence is from M18189.1, Insulin like growth factor 1 (IGF1) coding sequence from M15480.1 (rat) and human growth hormone polyA tail (pA) sequence from NM_022560, these were synthesized and digested from shuttle vectors prepared by BaseClear (Leiden, The Netherlands). The alpha1 anti-trypsin promoter combined with the mouse albumin gene enhancer (AAT-promoter) was taken from a plasmid described before\textsuperscript{38}. plF1683 (Inovio, San Diego, CA) contained minimal pol II
promoter with four upstream activation elements (4xGal4) and IVS8 that was cloned in front of the transgene. The GeneSwitch sequence (GS) was from pGS1694 (Inovio, San Diego, CA). The specific order of elements within the expression cassettes designed in this study will be 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 medium (Gibco), supplemented with 10% fetal bovine serum with or without penicillin/streptomycin at 37°C, 5% CO2. Trypsin 0.25% EDTA (Gibco) was utilized for cell detachment and cells were seeded one day prior to transfection. HEK293 cells were transfected using Lipofectamine 2000, according to 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. The following day culture supernatant was replaced by medium with the appropriate MFP concentration. MFP was diluted from a 1mM MFP-ethanol stock solution into medium.

Luciferase assay
To measure luciferase activity, culture medium was removed and cells were washed ones in PBS, followed by lysis with 1x passive lysis buffer (Promega). Plates were incubated on an orbit-shaker at 450 rpm at room temperature for 20 min. Firefly luciferase activity of 10ul samples was measured with the dual-luciferase reporter assay system (Promega) according to manufacturer’s protocol. An average of luciferase counts was taken from triplicate transfections.

ELISA
For EPO and IGF1 ELISA 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 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 Inc., Chatsworth, CA) according to the manufacturer’s protocol. Total RNA was isolated from liver sections or from Huh7 cells using Trizol (Invitrogen, Carlsbad, CA)
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 DyNAmo cDNA synthesis kit (Thermo Scientific, F-470L) and 500 ng of total RNA.

Real time PCR amplification was performed with 10 time diluted cDNA or 250 ng gDNA, Fast SYBR Green Master Mix (Thermo Scientific, 4385612) and primers specific for EPO (ATATCACCCTCCCAGACACC and CAGGACAGCTTCTGAGAGCA), IGF (TCACAGGGATGCAAGAT and GTCAACATGACGCACC), GeneSwitch (AGCATGCGATATTTGCGAC and AGAGTAGCGACACTCCCAGT), AAT (AGGCCCAACTTGCTACGTGTATAAGT and CAGCGTCCTGTGTCGAAGGT), beta-Actin (ACGGCCAGGGTCATCCTATTGG and CAAGAAGGAAGGCTGGAAAGA) and/or GAPDH (TCCACCCATGGCAAATTCC and GGGATTTCCATTGATGACAAGCT). PCR reaction conditions were: 95°C for 20 sec, followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C. The assays were performed on ABI 7500 Fast (Applied Biosystems, Foster City, CA). EPO and IGF mRNA expression levels were normalized to GAPDH or beta-Actin and the relative gene expression $2^{-\Delta\Delta CT}$ 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 1x 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 Tris-bis, 4-12% gel in a Biorad system with MOPS SDS Running buffer (Life Technologies). To transfer proteins from the gel onto Immun-Blot PVDF Membrane (Biorad) a wet blotting system (Biorad) 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 NF-kappaB p65 (abcam ab7970) in blocking buffer. After washing the membrane, the antibody swine-anti-rabbit with 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 inverted terminal repeats (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 ITRs-expression cassette, the replicon enzyme and the capsid protein. The cells were lysed and crude lysate was treated with Benzonase (50U/ml) (Merck, Darmstadt, Germany) for 1 hour 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 quantitative PCR.

**In vivo**

C57BL6 mice were maintained under a 12-hour light: 12-hour 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 1e12 and IGF expressing viruses at 1e13 vector genome copies per animal. Four weeks after, mice injected with AAV5-GS-IGF were treated with 20mg/kg MFP three days in a row, except for the non-induced group of mice. In the experiment concerning EPO expression, four- and eight-weeks post-transduction several groups of mice were injected intraperitoneally with 20mg/kg MFP for four consecutive days. Blood was taken at the indicated days of the experiment; in general before transduction, before and after induction with MFP. All blood samples were collected in tubes with heparin and after centrifugation at 1500rpm for 15min plasma was stored at -80C and used for ELISA or MFP measurement at a later stage. Hematocrit was analyzed in a HemoCue 201+ (HemoCue AB, Ängelholm, Sweden) with a drop of blood immediately collected on a microcuvette. When the animals were sacrificed their liver was dissected.

**MFP quantification**

MFP was quantified at Eurofins | PROXY Laboratories the Netherlands according to company’s SOPs. In brief, after addition of internal standard D3-Mifepristone, MFP was extracted from 50ul of plasma or approximately 10 mg of 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 (Quadrupole Time of Flight-Mass Spectrometry). Then MFP concentrations were calculated from the recorded chromatograms.

**Statistical Analysis**

Data were analyzed using either the one-way ANOVA, two-way ANOVA or Student’s t-test with a predefined significance level of $\alpha=0.05$ to determine statistically significances between two groups. The p values are represented by the following number of asterisks:
*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Table S1 shows the full statistical analysis for the one-way ANOVA and two-way ANOVA.

Acknowledgements

We’d like to thank Richard van Logtenstein, Cynthia Brouwers and Stephan Pouw for their technical support during the animal experiments. Kimberley Pietersz and Jolanda Snapper are appreciated for their help in acquiring several of the research tools.

The authors declare no conflict of interest. The researchers are or were employed by uniQure N.V. and the research project was partly funded by the European grant Eurostar project E!7900: ESTAR13113 Regulated gene expression for Huntington’s disease therapy.

Author contributions

Conceptualization: H.P., P.K and J.M.L. Investigation: J.M.L., R.M., T.Z., S.K. and A.H. Recourses: J.Lu. 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.
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Chapter 6

Phenotypic correction of a mouse model for primary hyperoxaluria with adeno-associated virus gene transfer. *Mol. Ther.* 19: 870–875.
Supplemental data

Supplemental figure 1. In vivo expression of IGF protein after MFP induction in murine liver by the single-vector GeneSwitch system. Mice were transduced with AAV5 expressing rat IGF constitutively (AAV5-IGF) or regulated through the GeneSwitch system (AAV5-GS-IGF). Four weeks later, MFP was given three consecutive days (1st, 2nd, 3rd) and blood was taken at the indicated hours after each injection (+1h, +4h, etc). One group of the AAV5-GS-IGF injected mice and the AAV5-IGF1 group did not receive MFP (no MFP), from them blood was collected at a convenient time on day 1, 2 and 3 when the other groups received MFP. The IGF plasma concentrations were determined by ELISA against mouse and rat IGF.
Supplemental Table 1. Statistical analysis using either the one-way or two-way ANOVA. Statistical analysis was performed using Graphpad Prism 8.0.0.

| Figure | Test | Source of Variation | % of total variation | P value | P value summary | F (DFn, DFd) |
|--------|------|---------------------|----------------------|---------|----------------|--------------|
| 4b     | Two-way ANOVA, Dunnett's multiple comparisons test | Interaction: 11.36 | 0.0671 | ns | F (9, 40) = 1,984 |
|        |      | Time: 9.693         | 0.0045 | ** | F (3, 40) = 5.076 |
|        |      | MFP treatment: 49.33| <0.0001 | **** | F (3, 40) = 25.83 |
| 4c     | Two-way ANOVA, Dunnett's multiple comparisons test | Interaction: 14.86 | <0.0001 | **** | Yes |
|        |      | Time: 37.92         | <0.0001 | **** | Yes |
|        |      | MFP treatment: 47.17| <0.0001 | **** | Yes |
| 7c     | One-way ANOVA | NA | NA | 0.0246 | * | F (5, 19) = 3,347 |
| 7d     | One-way ANOVA | NA | NA | 0.0019 | ** | F (5, 19) = 5,907 |
| 8e     | Two-way ANOVA, Bonferroni's multiple comparisons test | Interaction: 5,688 | 0.0098 | ** | Yes |
|        |      | MFP treatment: 0.7851| 0.1548 | ns | No |
|        |      | AAV5 vector: 74.25 | <0.0001 | **** | Yes |
