Towards RNAi based therapy of liver diseases : diversity and complexity of shRNA and miRNA processing and functions

Maczuga, P.

CHAPTER 6

Regulated knockdown of Apolipoprotein B100 and Huntingtin by the GeneSwitch mifiprestone-inducible system

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Abstract

RNA interference (RNAi) emerges as a potent approach for inhibition of disease-related genes. However overexpression of RNAi inducers may cause cytotoxicity because of saturation of the endogenous microRNA (miRNA) processing pathway. Expression of short hairpin RNA (shRNA) or artificial miRNA from constitutive or tissue specific polymerase II (pol II) promoters has been shown to reduce RNAi toxicity *in vivo* and *in vitro* and controlling and limiting expression by using inducible pol II promoters would allow time- and dose-dependent expression of therapeutic shRNA or miRNA when the inducer is administered. In this study we evaluated the GeneSwitch (GS) system for inducible expression of artificial miRNA targeting Apolipoprotein B100 (miApoB). Highly effective luciferase reporter inhibition by GS-miApoB was observed after induction with mifiprestone (MFP) and the effect was dependent on the amount of the GeneSwitch transactivator (TA). Deletion analysis of the core elements of the GS pol II promoter, the TATA box (TATA) and initiator sequence (INR) revealed that both are important for the functioning of the promoter in response to the inducer and any deletions result in loss of function. When TA and GS-miApoB encoding cassettes were placed in 4 different orientations in a single backbone, variant #3, where the transcription occurs in the opposite directions, showed the best luciferase reporter knockdown when MFP induced and least basal activity when non-induced. Quantification of small interfering RNA (siRNA) processed from the different GS variants revealed that the variant #3 expressed the highest amount of molecules. Finally, we showed that 4 miRNAs targeting the Huntingtin gene (Htt) cloned into the GS variant #3 demonstrated increased GFP and siRNA expression when induced with MFP. Our results demonstrate that the GeneSwitch system is capable of driving expression of artificial miRNA in a controlled manner *in vitro* when placed on a single backbone without compromising its properties. Controlled expression of therapeutic miRNA with the GeneSwitch system may constitute additional safety measure to limit toxicity of RNAi based gene therapy.

Introduction

One of the goals of the RNAi gene therapy is the delivery of inhibitory molecules to the targeted tissue to achieve stable and therapeutic inhibition of disease related genes. However the ability to express high levels of shRNA/miRNA for extended periods of time creates the need for drug-regulated gene expression systems. shRNA have been shown to induce acute toxicity when over-expressed *in vivo*, which leads to fatality in mice [1,2], particularly when potent polymerase III (pol III) promoters are used to drive expression of shRNA, resulting in saturation of the endogenous RNAi machinery leading to the global deregulation of gene expression. The toxicity may be circumvented by expressing shRNA from the weaker pol II promoters which can be tissue specific or inducible [3-7], but the exact rules for successful expression of shRNA from pol II promoters are far from understood. As an alternative to shRNA, artificial miRNA scaffolds can be used as miRNA are naturally expressed from pol II promoters [8]. Inducible expression of artificial miRNA can provide an additional safety level where expression can be quantitatively and temporally controlled.

Several inducible gene expression systems have been developed including tetracycline, rapamycine, IPTG, ecdysone, and MFP dependent technologies. [9-12]. Generally they consist of two elements: the first is a chimeric protein capable of modulating gene expression in a drug-dependent manner and the second is an inducible promoter which is often a minimal pol II promoter linked to sequences recognized by the chimeric protein. Additionally, two mechanisms of gene regulation can be distinguished, called On- and Off-switch. In the first mechanism, the chimeric transactivator protein binds specifically to its DNA recognition sequence within the inducible promoter in the presence of the inducer, which in consequence activates gene expression. In the second mechanism the chimeric protein-repressor is constantly bound to its recognition sequences and upon induction dissociates allowing transcription. Any inducible gene expression system should fulfill several requirements for safe and long term gene therapy application including a low basal expression in the absence of inducer and a high induction ratio. Another important safety requirement is that the chimeric proteins used in inducible systems, in active or inactive form, do not interfere with endogenous gene expression, because chimeric proteins are formed from potent transcription factors that can lead to unspecific activation of endogenous promoters and thus interfere with cellular processes [13]. The safety aspects should also be determined for all inducible system components within the context of immune system activation.

The GeneSwitch regulated expression is one of the best characterized systems for inducible gene expression [11,12,14-16]. It exploits a hybrid transactivator (TA) containing a DNA binding domain from the yeast Gal4 protein (Gal4 BD), a truncated ligand binding domain from human progesterone receptor (PR) and a

p65 transcriptional activation domain of the human NF-kappa B transcription factor (hp65). The second component is the GeneSwitch inducible promoter, which consists of minimal pol II promoter with upstream activation sequences (Gal4) that can be recognized by Gal4 BD domains of the TA protein. The system is induced by MFP, which is an anti progestin that binds to the PR domain of the TA protein. Upon induction the TA changes its confirmation and as a dimer specifically binds to the Gal4 sites to activate gene transcription. The major advantage of the system is that it lacks virally- or bacterially-derived components thus limiting a potential immune reaction to TA expression. Another advantage of the system is its inducer, which is a marketed drug with well characterized pharmacokinetics whereas the induction occurs at sub-therapeutic concentrations [17]. On the other hand, MFP has been shown to interfere with biological pathways which increase toxicity risk for applications where prolonged exposure to the inducer may be necessary [18]. Additionally some of the interactions of TA domains have been already described. Expression of TA for one month resulted in transcriptional changes in the liver [13]. However they were not associated with any biochemical or morphological changes, this aspect needs more attention.

Unlike the doxycycline and tetracycline (Tet) inducible systems, GeneSwitch has not been validated for RNAi induction. [19-24]. The former systems are derived from the bacterial tetracycline inducible system where tetracycline repressor protein (TetR) controls expression of the TetA protein that pumps tetracycline antibiotic out of the cell by binding to tetracycline responsive elements (TRE) upstream of the TetA promoter. Addition of tetracycline (or doxycyline) leads to a change in the TetR confirmation which dissociates from the promoter region and allows expression of TetA. Tetracycline-inducible systems such as TetOn, TetOff or Trex have been shown to be able to control shRNA or artificial miRNA expression *in vitro* and *in vivo* [19-24]. Regulated TetOff expression of shRNA targeting Polo-kinase 1 (Plk1) resulted in 97% mRNA knockdown *in vitro* [22]. Subsequently the Plk1 protein was inhibited up to 86% which correlated with a significant inhibition of tumor cell growth [22]. Additionally, a conditionally replicating Human immunodeficiency virus (HIV)-based vector that stably expresses an inducible antiviral shRNA against HIV-1 was proposed as vaccine for HIV infections [23]. Upon doxycyline induction, virus was shown to spread efficiently to all HIV-susceptible cells. Subsequent doxycycline withdrawal the constitutive shRNA expression cassette was active resulting in viral inhibition [23]. Proof-of-concept has also been established in transgenic mice enabling inducible and reversible expression of shRNA against Replication protein A, subunit 3 (Rpa3) which resulted in a block of cell proliferation and rapid atrophy of the intestinal epithelium which led to weight loss and lethality within 8–11 day after shRNA induction [24]. However, the use of TetOn and TetOff systems in preclinical and clinical studies is limited because they are dependent on bacterially derived –potentially immunogenic

proteins [25-27]. Other systems validated for shRNA or artificial miRNA expressions are the IPTG and ecdysone inducible systems [28,29]. The first one was shown to tightly regulate and induce shRNA targeting luciferase and tumor suppressor protein p53 in both transiently transfected cells and established stable clones [29]. Knockdown of the p53 protein *in vitro* was also demonstrated with the ecdysone-inducible system [30], where dose- and time-dependent inhibition of p53 was associated with changes in cell morphology and concomitant reduction of p21, downstream target of p53. Withdrawal of the inducer completely reversed the phenotype.

In the current study we evaluated the GeneSwitch system for inducible expression of an artificial miRNA that efficiently targets ApoB. In order to facilitate usage of the GeneSwitch system *in vivo*, its structural elements were incorporated in a single backbone in 4 different variants, which were later evaluated for knockdown efficiency, basal expression and inducibility. Variant #3, where the two elements are transcribed in an antisense orientation, was identified as the best configuration as it demonstrated lowest basal expression when non-induced and highest activation rate in the induced state. Finally to check the system robustness, inducible expression of four additional miRNAs targeting the Htt gene was shown for variant #3. Our data demonstrate the feasibility of the GeneSwitch inducible miRNA expression system that should be considered for future therapeutic RNAi applications.

Results

Knockdown efficacy of GS-miApoB depends on TA concentration and duration of the induction

We have previously identified and tested *in vivo* a highly active artificial miRNA targeting Apolipoprotein B100 (miApoB) [31]. However, the knockdown effect decreased over time and some signs of mild toxicity have been observed. Therefore, as a next step we developed a regulated miApoB expression based on the MFP-inducible GeneSwitch system. The two components of the GeneSwitch system were a chimeric TA that activates transcription exclusively in presence of MFP and the miApoB expressed from the GeneSwitch promoter (GS-miApoB) in response to TA binding (Fig. 1a). As a negative control, a scrambled miRNA not targeting any gene in the mammalian genome was placed under the control of the GeneSwitch (GS-miScr) or CMV promoters (CMV-miScr). Constitutive expressed miApoB (CMV-miApoB) was used as positive control. All constructs expressed green fluorescent protein version emerald (EmGFP), which allowed monitoring of the transfection efficacy and MFP induction (Fig. 1a). The inhibitory effect of GS-miApoB was evaluated by co-transfecting human embryonic kidney 293T (HEK293T) cells with a reporter expressing renilla luciferase that contained the ApoB target sequence in its 3'UTR (Luc-ApoB) and increasing amounts of the TA-encoding plasmid (Fig. 1b). By adding

10 nM MFP 24 h post transfection, transcription from GS-miApoB was induced and luciferase luminescence was measured 48 h later. As a negative control CMV-miScr or GS-miScr were used where the renilla/firefly luciferase ratio was set at 100% and the relative inhibition of the Luc-ApoB reporter was calculated.

In the absence of TA, basal expression resulted in ~60% knockdown of Luc-ApoB both in MFP induced and non-induced state (Fig 1b). When increasing amounts of the TA plasmid were added, relative luciferase inhibition in non-induced cells was between 60% and 85% and upon MFP induction even stronger knockdown was measured (Fig. 1b). Addition of 10 ng TA to GS-miApoB resulted in lowest basal expression and highest induction rate therefore it was chosen for other experiments.

Because GeneSwitch inducible expression peaks between 24 and 72 h after MFP induction, the maximal inhibitory effect was determined over time. HEK293T cells were co-transfected with 50 ng GS-miApoB, 10 ng Luc-ApoB and 10 ng TA plasmids (Fig. 1c). Twenty four hours after transfection cells were induced with MFP and after another 24, 48, and 72 h samples were harvested and luciferase inhibition was measured. Maximal Luc-ApoB knockdown was observed 48 h after MFP induction and it was ~80% in induced cell compared to ~50% in non-induced cells. After 72 h the Luc-ApoB knockdown was weaker and was 51% and 22% in MFP induced and non-induced cells, respectively (Fig. 1c).

GeneSwitch promoter analysis reveals that TATA box and INR are essential components and play important role in MFP induction

Initially we confirmed that the GeneSwitch system is capable of driving miApoB expression and established the optimal TA concentration and MFP induction time. Next, the inducible promoter was optimized to reduce the miApoB basal expression levels since without MFP induction, a ~50% knockdown of Luc-ApoB was already observed. Based on manual sequence identification, two core components of the GeneSwitch promoter, a TATA box and an initiator element (INR) were identified. In order to evaluate their function for basal expression and/or maximal induction of GS-miApoB, mutants lacking TATA (ΔTATA-miApoB), INR (ΔINR-miApoB) or both TATA and INR (ΔTATAΔINR-miApoB) were designed (Fig. 2a). HEK293T cells were co-transfected with 50 ng GS-miApoB, ΔTATA-miApoB, ΔINR-miApoB or ΔTATAΔINR-miApoB together with 10 ng of Luc-ApoB reporter and 10 ng TA plasmid. By adding 10 nM MFP transcription of miApoB was induced and Luc-ApoB knockdown was measured 48 h post induction with reference to CMV-miScr and GS-miScr controls. Upon induction luciferase knockdown by GS-miApoB was 64% while it was 31%, 34% and 36% for ΔTATA-miApoB, ΔINR-miApoB and ΔTATAΔINR-miApoB mutants (Fig. 2b). Moreover, none of the deletion constructs was MFP induced above basal levels indicating that the TATA and INR elements are essential for transcriptional activity of the GeneSwitch promoter. Additionally no GFP expression from the deletion mutants was observed (data not shown).

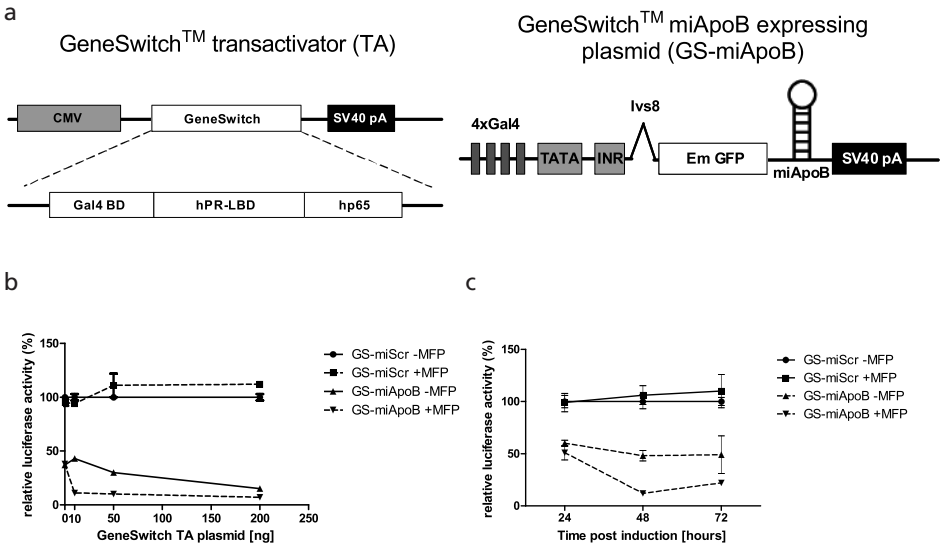


Figure 1. Structure and knockdown efficacy of miRNA targeting ApoB (miApoB) expressed from the GeneSwitch system. (a) Schematic representation of the GeneSwitch system for inducible expression of artificial miRNA. The system consists of two structural components: the transactivator protein (TA) expressed from the CMV promoter and miRNA expressing construct (GS-miApoB). Gal4 BD - Yeast Gal4 DNA binding domain, hPR-LBD - Human progesterone receptor ligand binding domain, p65 - Human NF-kB activation domain subunit, Gal4- Gal4 BD binding site, TATA- TATA box, INR- initiator sequence, EmGFP- Emerald Green Fluorescent Protein (b) Knockdown of Luc-ApoB reporter by GS-miApoB. Cells were transfected with 10 ng Luc-ApoB reporter, 50 ng GS-miApoB expressing plasmid and 10, 50, 100 or 250 ng of GeneSwitch TA expressing plasmid. Twenty four hours after transfection medium was changed and samples were induced with 10 nM MFP (+MFP) or were not induced (-MFP). Renilla and firefly luciferase were measured 48 h post-MFP induction. Renilla luciferase expression was normalized to firefly luciferase expression. CMV-miApoB was used as a positive control, CMV-miScr and GS-miScr served as negative controls and were set at 100%. Data are represented as mean values ±SD from a representative experiment from three independent experiments. (c) Knockdown of the Luc-ApoB reporter, by GS-miApoB at 24, 48 and 72 h post-MFP induction. Cells were transfected with 10 ng Luc-ApoB reporter, 50 ng GS-miApoB expressing plasmid and 50 ng of GeneSwitch TA expressing plasmid. Experimental conditions are as described in Fig. 1b.

Incorporation of GS-miApoB and TA elements in a single backbone results in high MFP inducibility and high basal expression levels

Inducible systems where all components can be integrated in a single backbone are desirable for therapeutic RNAi applications. Therefore the TA and GS-miApoB elements were placed adjacent to each other in a single vector backbone (Fig. 3a). The TA expression cassette was cloned upstream to GS-miApoB resulting in sense orientation of the two elements (Fig. 3a). HEK293T cells were co-transfected with 50 ng of the GS-miApoB single backbone variant with 10 ng of Luc-ApoB. Twenty four hours post transfection cells were induced and after another 48 h GFP induction was monitored. High GFP expression was observed for both GS-miScr and GS-miApoB only upon MFP

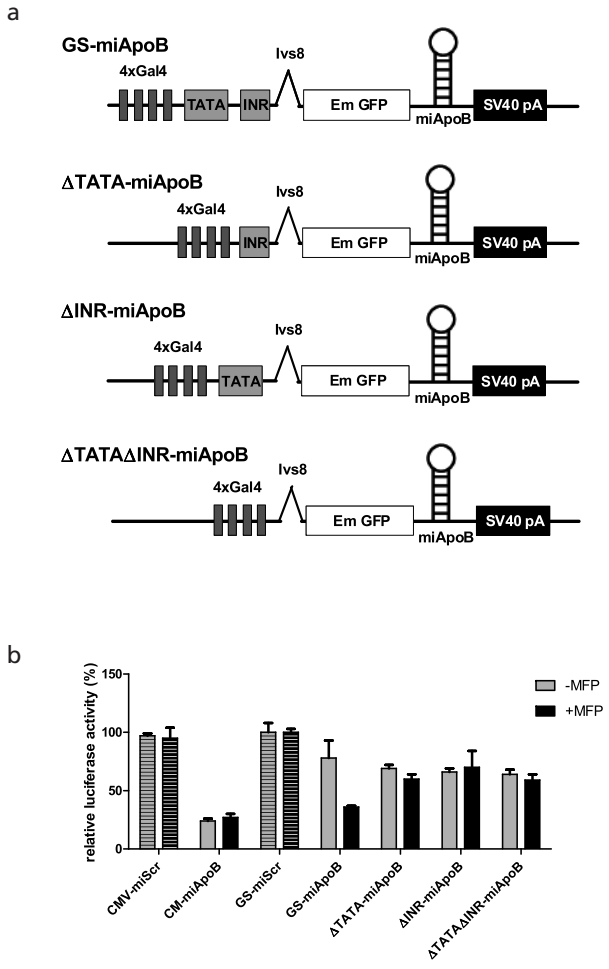


Figure 2. Structure and knockdown efficacy of miApoB expressed from a single backbone GS-miApoB. (a) Schematic representation of GS-miApoB for inducible expression of artificial miApoB, where all structural components were placed in a single backbone. The names of the structural components are indicted in Fig. 1. Arrows represent the direction of transcription (b) Representative image of GFP fluorescence 48 h without (-MFP) or after induction with 10 nM MFP (+MFP). Cells were transfected with 10 ng Luc-ApoB reporter and 50 ng GS-miApoB expressing plasmid. Pictures were taken using the same exposure and magnification settings. (c) Changes in the number of GFP positive cells after MFP induction. Experimental setup was as described in (b) 1000 events were counted with flow cytometry and fold change of GFP expression was calculated for each construct by dividing the amount of cells GFP positive after MFP induction by the number of GFP positive cells without MFP induction. (d) Knockdown of Luc-ApoB reporter, by the single backbone GS-miApoB system. Cells were transfected with 10 ng Luc-ApoB reporter and 50 ng GS-miApoB expressing plasmid. Twenty four hours after transfection medium was changed and samples were either induced with 10 nM MFP (+MFP) or were not induced (-MFP). Renilla and firefly luciferase were measured 48 h post-induction. Renilla luciferase expression was normalized to firefly luciferase expression. CMV-miApoB was used as a positive control, CMV-miScr and GS-miScr served as negative controls and were set at 100%. Bars with stripes represent negative controls. Data are represented as mean values ±SD from a representative experiment from three independent experiments.

induction (Fig. 3b). Quantification of GFP positive cells with flow cytometry revealed that there were ~4 fold more GFP positive cells for GS-miScr and GS-miApoB when induced with MFP compared to non-induced cells (Fig. 3c). To determine whether the induction observed on GFP correlates with Luc-ApoB knockdown by the GS-miApoB single backbone variant, luciferase assay was performed. Surprisingly for the single backbone GS-miApoB variant MFP induction did not further decrease Luc-ApoB expression (Fig. 3d). In both non-induced and induced cells Luc-ApoB was inhibited

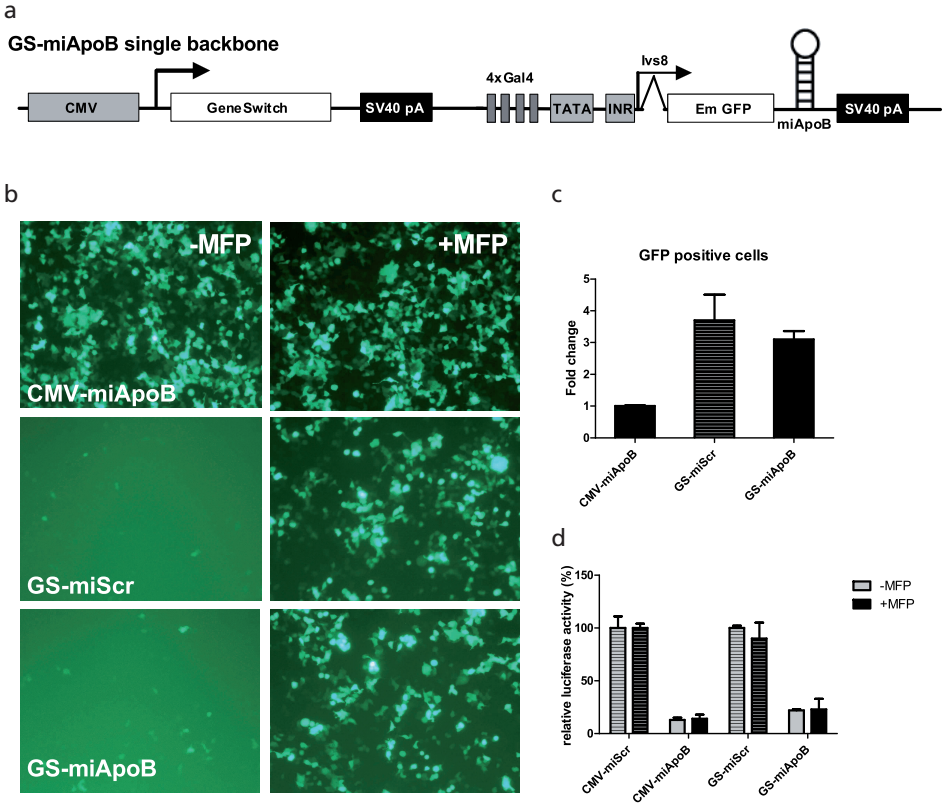


Figure 3. Structure and knockdown efficacy of GS-miApoB expressed from the GeneSwitch system with consecutive deletions in the inducible promoter. (a) Schematic representation of GS-miApoB for inducible expression of artificial miRNA, where several structural parts were deleted. Three different GeneSwitch deletion variants were designed where the TATA box (ΔTATA-miApoB), the INR sequence (ΔINR-miApoB) or both components were deleted (ΔTATAΔINR –miApoB) (b) Knockdown of Luc-ApoB reporter, by GS-miApoB after induction with the different GeneSwitch deletion variants: ΔTATA-miApoB, ΔINR-miApoB or ΔTATAΔINR–miApoB. Cells were transfected with 10 ng Luc-ApoB reporter, 50 ng GS-miApoB, ΔTATA-miApoB, ΔINR-miApoB or ΔTATAΔINR–miApoB and 10 ng of the TA plasmid. Reaction conditions were as described in Fig. 1b. Bars with stripes represent negative controls. Data are represented as mean values ±SD from a representative experiment from three independent experiments.

up to 80% indicating that the basal expression with the single backbone GS-miApoB was greater than with the two plasmids system. Reduction of the GS-miApoB and MFP concentrations did not show an improvement in basal expression which still resulted in ~80% Luc-ApoB knockdown (data no shown).

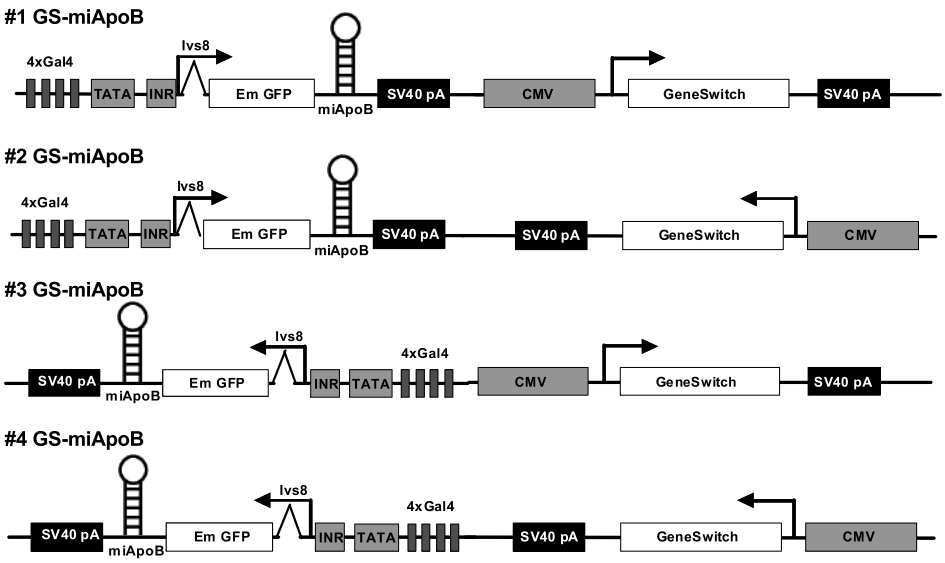
Inducibility and basal expression depends on the positioning and orientation of GS-miApoB and TA elements within a single backbone

It has been shown that the expression and activity of the inducible systems depends on the orientation of expression cassettes in a single backbone [32-34]. To achieve optimal induction and minimal basal expression, the TA and GS-miApoB elements were cloned in different orientations resulting in 4 distinct variants (Fig. 4a). In all variants GS-miApoB was cloned upstream of TA expression cassette. In variant #1 both cassettes were in sense orientation. In variant #2 GS-miApoB and TA cassettes were in a head-to-head orientation. In variant #3 and #4 GS-miApoB and TA cassettes were opposite and antisense to each other, respectively (Fig. 4a). HEK293T cells were co-transfected with 50 ng of variants #1-#4, induced with MFP 24h later and 48 h post induction, Luc-ApoB knockdown was measured (Fig. 4b). As anticipated, the different orientations varied in terms of basal expression and induction. Variants #1 and #2 exhibited mild induction and low basal activity. For variant #1 the Luc-ApoB knockdown was 32% and 38% for non-induced and MFP induced cells, respectively. For variant #2 knockdown was 28% for non-induced and 42% for MFP induced cells respectively. Variants #3 and #4 exhibited lowest basal activity and highest induction rate. For variant #3 basal expression resulted in 25% knockdown of Luc-ApoB, which was stronger upon induction and reached 59%. Similarly variant #4 resulted in 22% and 51% knockdown when non-induced and MFP induced, respectively.

Next, the correlation was determined between the inhibitory effect, induction rate, and the number of processed siApoB molecules from the GS-miApoB variants #1-#4

Figure 4. Structure and knockdown efficacy of GS-miApoB expressed from a single backbone with four different orientations of the GeneSwitch and TA elements. (a) Schematic representation of variants #1 - #4 of the single backbone GS-miApoB, with different orientations of the GeneSwitch and TA elements In variant #1 both cassettes were in sense orientation. In variant #2 GS-miApoB and TA cassettes were in head-to-head orientations. In variant #3 and #4 GS-miApoB and TA cassettes were opposite and antisense to each other, respectively. The names of the structural components are indicted in Fig. 1a. Arrows represent direction of transcription. (b) Knockdown of the Luc-ApoB reporter, by the single backbone GS-miApoB system variants #1 to #4. Cells were transfected with 10 ng Luc-ApoB reporter and 50 ng GS-miApoB variants #1-#4. Reaction conditions are as described in Fig. 1b. Bars with stripes represent negative controls. Data are represented as mean values ±SD from three experiments analyzed with the factor correction method. (c) siApoB expression in HEK293T cells from the single backbone GS-miApoB variants #1-#4. RNA was isolated 48 hours post-MFP induction and siApoB-specific small RNA TaqMan was performed. siRNA copy number per cell was calculated based on the amplification plot of a dilution series of synthetic siApoB RNA standard (IDT, Coralville, IA). The trend line value of the standard line was used to calculate the siApoB copy number cell, assuming 15 pg RNA per cell [39].

a


#1 GS-miApoB
4xGal4
TATA
INR
Ivs8
Em GFP
miApoB
SV40 pA
CMV
GeneSwitch
SV40 pA
#2 GS-miApoB
4xGal4
TATA
INR
Ivs8
Em GFP
miApoB
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SV40 pA
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CMV
#3 GS-miApoB
SV40 pA
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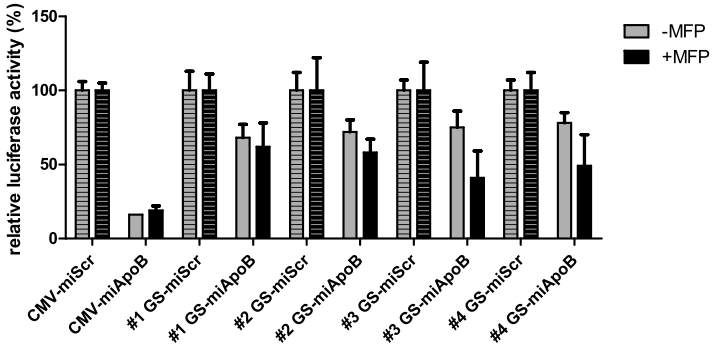
b


relative luciferase activity (%)
150
100
50
0
-MFP
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CMV-miScr
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#1 GS-miApoB
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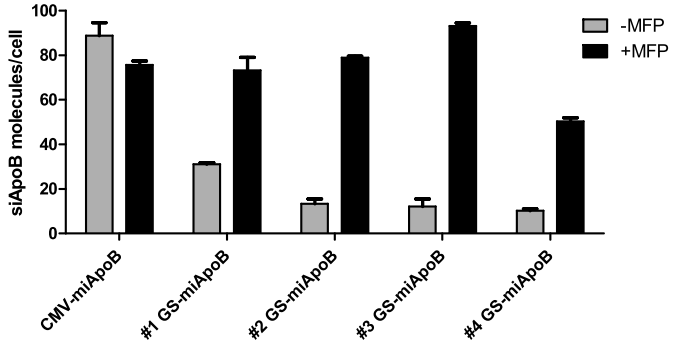
c


siApoB molecules/cell
100
80
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#2 GS-miApoB
#3 GS-miApoB
#4 GS-miApoB

(Fig. 4c). Small RNA specific Taqman assay was designed for detecting the guide strand of miApoB and the amount of molecules per cell was calculated based on synthetic standard line. All variants showed a clear increase of the amount of siApoB molecules per cell after MFP induction. Variant #1 expressed the highest amount of siApoB molecules compared to other variants. Variants #3 and #4 exhibited lowest basal expression and highest induction rate which is in line with Luc-ApoB knockdown data.

Variant #3 where the GS-miApoB and TA elements were positioned opposite to each other showed the best configuration for inducible expression of artificial miRNA targeting ApoB. To check if this is a general rule, we tested the inducibility of four additional efficient miRNAs targeting the Htt gene cloned in GS variant #3. miH10, miH12, miS7 and miS15 are targeting different regions of Htt and have been already validated as potent huntingtin inhibitors (data not shown). HEK293T cells were co-transfected with 50 ng of GS-miH10, GS-mH12, GS-miS7 or GS-miS15 variants #3 and 48 h post MFP induction total RNA was isolated (Fig. 4b). The induction was measured by quantifying the siRNA molecules expressed with and without MFP induction using specific small RNA Taqman assay. The results show between 4 and 7 times siRNA induction proving the robustness of GS variant #3 for inducible miRNA expression (Fig. 5a).

Discussion

Recent developments towards clinical applications of RNAi made it apparent that regulable and temporal expression may be necessary to circumvent toxicity resulting from aspecific off-targeting effects or oversaturation of the endogenous miRNA pathway. Inducible systems have been widely used for control of gene expression. These systems are composed of a chimeric transcription factor, inducer and a promoter that reacts upon transcription factor binding. The GeneSwitch inducible system is one of the promising candidates for RNAi gene therapy since it lacks bacterially derived components and the MFP inducer is an already approved drug with known pharmacokinetics. Although the GeneSwitch system can effectively regulate gene expression *in vivo* and *in vitro* using plasmid or viral delivery, to date there are no reports of its application for RNAi induction. We addressed this question by optimizing miApoB expression from the GeneSwitch system initially using a two-plasmid system and later by combining the GeneSwitch elements in four different orientations in a single backbone.

The transactivator and promoter elements are essential parts of each inducible system that characterize its parameters: basal expression, fold induction, and maximum expression levels. Therefore, we investigated the optimal conditions for minimal basal activity without induction with MFP and the maximal inducibility after addition of inducer. By increasing the amount of TA plasmid the basal expression of the inducible promoter also increased suggesting that even in non-active state TA is

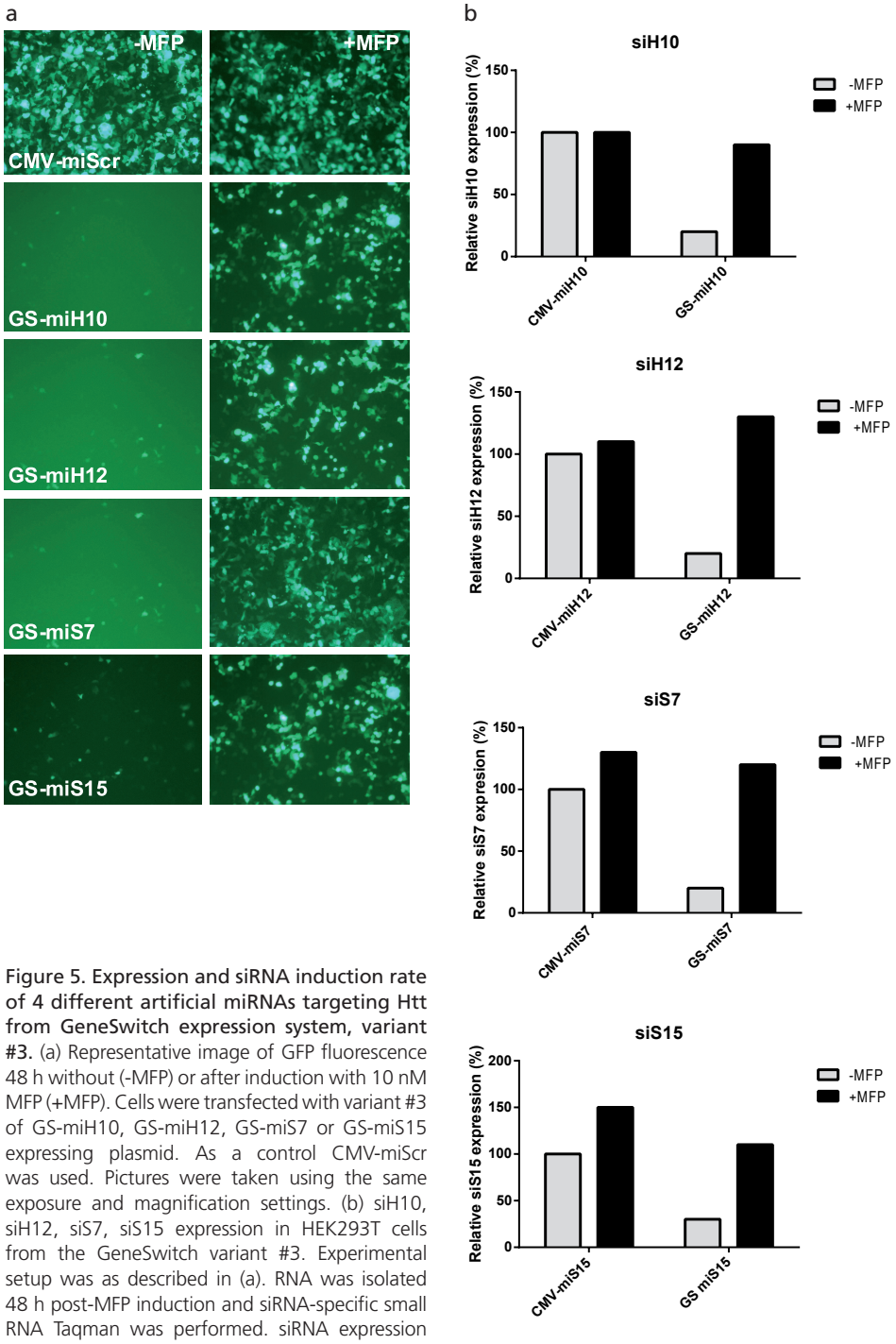

Figure 5. Expression and siRNA induction rate of 4 different artificial miRNAs targeting Htt from GeneSwitch expression system, variant #3. (a) Representative image of GFP fluorescence 48 h without (-MFP) or after induction with 10 nM MFP (+MFP). Cells were transfected with variant #3 of GS-miH10, GS-miH12, GS-miS7 or GS-miS15 expressing plasmid. As a control CMV-miScr was used. Pictures were taken using the same exposure and magnification settings. (b) siH10, siH12, siS7, siS15 expression in HEK293T cells from the GeneSwitch variant #3. Experimental setup was as described in (a). RNA was isolated 48 h post-MFP induction and siRNA-specific small RNA Taqman was performed. siRNA expression levels were normalized to beta-actin as an internal control, and the relative gene expression $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen was used for analysis of PCR data [40].

capable of binding to Gal4 elements in the GeneSwitch promoter. Another important aspect of the inducible systems is their temporal control of gene expression. The effect of the induction is dose dependent and increases until it reaches maximal expression. Generally after 24 h the expression using GeneSwitch system reaches maximum level *in vitro* [11,35], however in our case 24 hours time was not sufficient to observe increase in luciferase inhibition. The maximal knockdown of luciferase was observed after 48 hours and after 72 hours the inhibition of luciferase was weaker.

In this study, we also carried out deletion analysis of the GeneSwitch promoter to identify regions that control the responsiveness and the spatiotemporal gene expression. Manual analysis of the GeneSwitch promoter identified common elements of pol II promoters: a TATA box and an INR sequence [36,37]. Deletion analysis showed that both TATA box and INR are essential components of the GeneSwitch promoter and any perturbations resulted in a functional loss probably due to inefficient transcription. An interesting alternative to the promoter modification is to incorporate more Gal4 sequences upstream to the promoter, which can improve promoter inducibility. This was already shown for mIFNb expression with the GeneSwitch inducible system [14]. Although we did not optimize the TA expression cassette itself an interesting option is to use tissue specific promoters for expression of the TA protein. This would lower the extent of unregulated expression associated with the constitutive TA expression from the CMV promoter. Alternatively TA can be expressed from the promoter that, like the transgene, is responsive to MFP. In this auto-inducible system, the TA can induce both the transgene and its own expression.

A vector based regulated expression for GeneSwitch system has already been validated *in vitro* and *in vivo* using mouse interferon beta (mIFNb) [14]. Similarly to our studies four variants of a single backbone expression system with different orientations were tested and all 4 variants resulted in mIFNb *in vitro* induction raging from 360 – 1480 fold. In two variants (pGT23 and pGT24) the mIFNb cassette was cloned upstream of TA expression cassette in sense and opposite orientations respectively. In two other (pGT25 and pGT25) the TA expression cassette was upstream of mIFNb and was cloned in sense and head-to-head orientations respectively. This means that pGT23 is equivalent to our variant #1, pGT24 is variant #3 and pGT25 is GS-miApoB. pGT26 does not have equivalent in our study. The variant which exhibited the greatest induction was pGT23 followed by pGT26, pGT24 and pGT25. Interestingly, all single backbone variants outperformed the two-plasmid system in the inducible rate and the level of mIFNb expression but all construct, when induced, expressed less mIFNb than the constitutive CMV promoter. Surprisingly, *in vivo* measurement of IP-10, which is a validated biomarker for mIFNb showed a different trend and the variant that resulted in the best IP-10 induction was pGT26 followed by variants pGT25, pGT24 and pGT23. *In vivo* all variants were as active as the two-plasmid system in

IP-10 induction and they were also outperforming CMV expressed mIFNb. Despite the similar approach to optimize the GeneSwitch system, a direct comparison to our results is not feasible because the luciferase knockdown is caused by an increase of the siRNA amount which in consequence inhibits the protein expression. Additionally the exact mechanism of the GeneSwitch system induction has not been investigated thus a high protein expression may be a combination of an increased transcription and increased translation or both. By contrast to reported induction of mIFNb, our results show much lower induction of expressed siRNA: 4-6 fold increase in MFP induced cells compared to the non-induced cells.

Inducible expression is an interesting approach to avoid potential problems that can be associated with therapeutic RNAi applications. It has been reported that shRNA can lead to severe toxicity when high doses are used [1,2] and that by lowering siRNA expression -either using pol II promoters or by incorporation of a siRNA sequence in an artificial miRNA scaffold [9]- toxic side effects can be abolished. In our previous study, we have demonstrated a more sustained ApoB target knockdown by miApoB over the shApoB in long-term *in vivo* experiments [31]. Nonetheless, the inhibitory effect of miApoB expressed with a liver-specific promoter and shApoB was lost after 27 weeks. Additionally it has been shown that processing of shRNA and miRNA may lead to unanticipated silencing of other genes (off-targeting) which also can cause toxicity. Because of the complexity of RNAi processing *in vivo*, these interactions are difficult to predict and evaluate. Still RNAi toxicity has been shown to be dose dependent therefore limiting the expression of siRNA molecules with inducible systems may reduce potential risk. Although the present study provides a proof of concept for controlling siRNA expression *in vitro*, the reduction of the toxicity of GeneSwitch expressed miRNA *in vitro* and *in vivo* needs to be further investigated.

An important aspect is the robustness of the GeneSwitch system, which should be capable of expressing any miRNA. We have shown that variant #3 was also capable of driving expression of 4 miRNAs targeting Htt. Unequivocal induction of both GFP and miRNA expression was observed. Additionally gene knockdown *in vivo,* using the GeneSwitch system, needs to be investigated. The plasmids described in this study could be used in the context of viral vectors such as AAV for a long-term expression *in vivo*. Beside Htt knockdown for Huntington disease and ApoB for FH, other potential applications that can be treated with GeneSwitch inducible RNAi include cancer and viral infections (HIV, Hepatitis B virus and Hepatitis C virus).

In conclusion, we have tested the GeneSwitch system and constructed a single backbone to express the artificial miRNA targeting ApoB and Htt. This vector enabled regulation of the miRNA expression and proved efficient in Luc-ApoB knockdown *in vitro*. Combination of the AAV delivery and the inducible expression of an artificial miRNA would allow a long-term therapeutics effect simultaneously limiting possible

side effects. This will provide an additional safety level for future clinical applications compared to the ubiquitously expressed shRNA/miRNA.

Materials and methods

DNA constructs

GeneSwtich miApoB (GS-miApoB) construct was made by amplification of a 1384 bp fragment of pCDNA6.2 construct containing EmGFP and miApoB using pr967 and pr968 primers and ligating into the NcoI and KpnI sites of the pIF1683 (Inovio, San Diego, CA). The pIF1683 contains minimal pol II promoter with 4 upstream activation elements (Gal4). The construction of pCDNA6.2 containing miRNA targeting ApoB has been described previously [36]. Similarly negative scrambled control miRNA (GS-miScr) was made by PCR amplification o pcDNA6.2-GW/EmGFP-miR-neg (Invitrogen, Carlsbad, CA) using the same primer set. The GeneSwitch TA plasmid was constructed by amplification of 2200 bp fragment of pGS1984 (Inovio, San Diego, CA) using primers pr965 and pr966 and ligating into the XbaI and RsrII sites of the pTRCGW vector (Inovio, San Diego, CA). pGS1984 contains TA under the control of the beta-actin promoter. The end construct expressed TA protein form constitutive CMV promoter. All miRNA and primer sequences are listed in Table S1.

The GeneSwitch inducible promoter mutation variants variant were designed as followed. Promoter fragments of 200 bp with desired mutation (ΔTATA, ΔINR, ΔTATAΔINR) were synthesized and cloned into pIDTsmart vector resulting in pIDTsmartΔTATA, pIDTsmartΔINR, pIDTsmartΔTATAΔINR vectors (IDT, Coralville, IA). Then pIDTsmartΔTATA smart was digested with EcoRI and XhoI ligated into pIF1683 plasmid digested with the same restriction sites resulting in ΔTATA-miApoB construct. ΔTATAΔINR-miApoB was constructed analogously to ΔTATA-miApoB construct. ΔINR-miApoB was made by with EcoRI and BglI digestion of pIDTsmartΔINR and ligation into the same restriction sites of pIF1683.

The GeneSwitch single backbone variant was designed as followed. The GS fragment (1223 bp) was cut from pIF1683 plasmid by SbfI and Acc65I digestion and ligated into the *PstI* and *Acc65I* digested pKS+ (Stratagene, La Jolla, CA) resulting in pKS-GS plasmid. Next the TA expressing plasmid was digested with SspI and FspI and blunt ligated into BamHI sites of pKS-GS resulting in pKS-GS-TA plasmid. To replace beta-actin for CMV promoter in the TA expression cassette, the GS-TA fragment (2539 bp) was obtained by NheI digestion of pKS-GS-TA and ligated into SpeI digested pTRCGW. The construct was named as pTRC-GS-TA. Finally, the 1384 bp fragment of pCDNA6.2 construct containing EmGFP and miApoB was PCR amplified using primers miRfw and miRrv and ligated into the SacII and NotI sites of the pTRC-GS-TA resulting in a single backbone GS-miApoB construct. GeneSwitch orientations #1-#4 were designed as followed. The TA expression cassette was digested with KpnI and ligated into GS-miApoB construct,

which was digested with the same enzyme. The insert was ligated in sense or antisense orientations resulting in variant #1 and variant #3. To create variants #2 and #4 the GS-miApoB expression cassette in variants #1 and #3 was inverted by SbfI digestion and relegation. The presence of miApoB was verified by sequencing.

To create vectors expressing miRNA targeting Htt, 278 bp fragments of pCDNA6.2 constructs containing miH10, miH12, miS7 and S15 were PCR amplified with primers prGS3fw and prGS3rv and the inserts were blunt ligated into DraV and EcorV digested variant #3 construct. Construction of the Luc-ApoB reporter vector has been described before [38] Generation of this reporter vector has been described previously. All the sequences of the oligonucleotides used in this study are listed in Table S1.

Cell culture and transfections

The human embryonic kidney (HEK293T) cell line were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) containing 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin, at 37°C and 5% CO_2. For luciferase assay or siRNA expression analysis, cells were seeded in 96- or 24-well plates at a density of 3×10^4 or 1.2×10^5 cells per well, respectively, in DMEM one day prior transfection. Transfections were performed with Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Cells were induced with 10 nM mifiprestone (Sigma) which was prepared the day of the induction from the 1mM stock.

Luciferase assay

HEK293T cells were transfected with 10 ng Luc-ApoB reporter, 50 ng GS-miApoB expressing plasmid and 10, 50, 100 or 250 ng of TA expressing plasmid or with 10 ng Luc-ApoB reporter or 50 ng of GeneSwitch variants #1- #4. Twenty four hours after transfection medium was changed and samples were induced with 10 nM Mifiprestone (+MFP) or were not induced (-MFP). Renilla and firefly luciferase were measured at indicated time points post-MFP induction. Renilla luciferase expression was normalized to firefly luciferase expression. CMV-miApoB was used as a positive control, CMV-miScr and GS-miScr served as negative controls and were set at 100%. Transfected cells were assayed at 48 h post-transfection in 20 µl 1x passive lysis buffer (Promega, Madison, WI) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Flow cytometry

Cells were transfected with 10 ng Luc-ApoB reporter and 50 ng of GS-miApoB single backbone variant. Twenty four hours after transfection medium was changed and samples were induced with 10 nM Mifiprestone (+MFP) or were not induced (-MFP). Cells were fixed and analyzed on FACSCalibur flow cytometer instrument (BD Biosciences, San Jose, CA). 1000 events were counted and fold change of GFP

expression was calculated for each construct by dividing the amount of cells GFP positive after MFP induction by the number of GFP positive cells without MFP induction.

siRNA and miRNA detection by small RNA Taqman assay

RT reactions for siApoB, siH10, siH12, siS7 and siS15 expression were performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using 10 ng RNA and 3 µl custom-made specific RT-stem-loop primers (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The TaqMan assay was done in 20 µl using 1,33 µl cDNA, 1 µl custom-made siRNA-specific primer with FAM-labeled fluorogenic probe (Applied Biosystems) and 10 µl TaqMan 2× Universal PCR Master Mix (Applied Biosystems). Amplification of the beta actin gene was used as a RNA quality and loading control. For siApoB copy number per cell was calculated based on the amplification plot of a dilution series of synthetic siRNA standards (IDT, Coralville, IA). Tenfold dilutions (100 pg – 1 fg) of the guide strand of the synthetic siRNA oligo were used to create a standard line. The copy number of each dilution was calculated according to the formula 1 mol = 6.02×10^{23} molecules. The trend line value of the standard line was used to calculate the siRNA copy number per cell, assuming 15 pg RNA per cell. siH10, siH12, siS7 and siS15 expression levels were normalized to beta actin as an internal control, and the relative expression $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen was used for analysis of the induction [40].

Supplementary material

Table S1. Oligonucleotides used in this study

Name	Sequence (5'-3')
pr965	CGTCTAGAGCATTGGAACGCGGATTCCC
pr966	TTCGGTCCGTCGAGCCCTACAGGTTGTC
pr967	TTCCATGGGCAGGCTTTAAAACCATGGTGAGCAAGGGCGAGGAGCTGTC
pr968	TTGGTACCATGCATCCTGCAGGCTAGAGCCCCAGCTGCGCAGATCCATCAGAGATTTTGAGAC
miRfw	ACTCCGCGGGCAGGCTTTAAAACCATGGTGAG
miRrv	ACTGCGGCCGCGTACAAGAAGCTGGGTCTAGAT
prGS3fw	GGCATGGACGAGCTGTACAA
prGS3rv	CTCTAGATCAACCACTTTGT

Reference list

1. Borel, F, van Logtenstein, R, Koornneef, A, Maczuga, P, Ritsema, T, Petry, H, et al. (2011). In vivo knock-down of multidrug resistance transporters ABCC1 and ABCC2 by AAV-delivered shRNAs and by artificial miRNAs. *J RNAi Gene Silencing*; **7:** 434-442.
2. Grimm, D, Streetz, KL, Jopling, CL, Storm, TA, Pandey, K, Davis, CR, et al. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*; **441:** 537-541.
3. Giering, JC, Grimm, D, Storm, TA and Kay, MA (2008). Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol Ther*; **16:** 1630-1636.
4. Song, J, Pang, S, Lu, Y, Yokoyama, KK, Zheng, JY and Chiu, R (2004). Gene silencing in androgen-responsive prostate cancer cells from the tissue-specific Prostate-specific antigen promoter. *Cancer Res*; **64:** 7661-7663.
5. Song, J, Pang, S, Lu, Y and Chiu, R (2004). Poly(U) and polyadenylation termination signals are interchangeable for terminating the expression of shRNA from a pol II promoter. *Biochem Biophys Res Commun*; **323:** 573-578.
6. Xia, H, Mao, Q, Paulson, HL and Davidson, BL (2002). siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotech*; **20:** 1006-1010.
7. Xia, XG, Zhou, HX, Samper, E, Melov, S. Xu, ZS. (2006). Pol II-expressed shRNA knocks down Sod2 gene expression and causes phenotypes of the gene knockout in mice *PLoS Genet*;**2**: 73-80.
8. Boudreau, RL, Martins, I and Davidson, BL (2009). Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Mol Ther*; **17**: 169-175.
9. Henriksen, JR, Løkke, C, Hammerø, M, Geerts, D, Versteeg, R, Flřgstad, T, et al. (2007). Comparison of RNAi efficiency mediated by tetracycline-responsive H1 and U6 promoter variants in mammalian cell lines. *Nucleic Acids Res*; **35:** e67.
10. Matthess, Y (2005). Conditional inhibition of cancer cell proliferation by tetracycline-responsive, H1 promoter-driven silencing of PLK1. *Oncogene*; **24:** 2973-2980.
11. Nordstrom, JL (2003). The antiprogestin-dependent GeneSwitch system for regulated gene therapy. *Steroids*; **68:** 1085-1094.
12. Xu, ZL, Mizuguchi, H, Mayumi, T and Hayakawa, T (2003). Regulated gene expression from adenovirus vectors: a systematic comparison of various inducible systems. *Gene*; **309:** 145-151.
13. Reboredo, M, Kramer, MG, Smerdou, C, Prieto, J and De Las, RJ (2008). Transcriptomic effects of Tet-on and mifepristone-inducible systems in mouse liver. *Hum Gene Ther*; **19:** 1233-1247.
14. Harkins, RN, Szymanski, P, Petry, H, Brooks, A, Qian, HS, Schaefer, C, et al. (2007). Regulated expression of the interferon-[beta] gene in mice. *Gene Ther*; **15:** 1-11.
15. Bhat, RA, Stauffer, B, Komm, BS and Bodine, PVN (2004). Regulated expression of sFRP-1 protein by the GeneSwitch system. *Protein Expr Purif*; **37:** 327-335.
16. Taylor, J, Rohatgi, P, Spencer, HT, Doyle, D and Azizi, B (2010). Characterization of a molecular switch system that regulates gene expression in mammalian cells through a small molecule. *BMC Biotechnol*; **10:** 15.
17. Burcin, MM, Schiedner, G, Kochanek, S, Tsai, SY and O'Malley, BW (1999). Adenovirus-mediated regulable target gene expression in vivo. *Proc Natl Acad Sci U S A*; **96:** 355-360.
18. Cadepond, F, Ulmann, A and Baulieu, EE (1997). RU486 (mifepristone): mechanisms of action and clinical uses. *Annu Rev Med*; **48:** 129-156.
19. Seibler, J, Kleinridders, A, Kuter-Luks, B, Niehaves, S, Bruning, JC and Schwenk, F (2007). Reversible gene knockdown in mice using a tight, inducible shRNA expression system. *Nucleic Acids Res*; **35:** e54.
20. Aagaard, L, Amarzguioui, M, Sun, G, Santos, LC, Ehsani, A, Prydz, H, et al. (2007). A facile lentiviral vector system for expression of doxycycline-inducible shRNAs: knockdown of the pre-miRNA processing enzyme drosha. *Mol Ther*; **15:** 938-945.
21. Premsrirut, P, Dow, L, Kim, S, Camiolo, M, Malone, C, Miething, C, et al. (2011). A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell*; **145:** 145-158.
22. Kappel, S, Matthess, Y, Zimmer, B, Kaufmann, M and Strebhardt, K (2006). Tumor inhibition by genomically integrated inducible RNAi-cassettes. *Nucleic Acids Res*; **34:** 4527-4536.
23. Westerhout, EM, Vink, M, Haasnoot, PC, Das, AT and Berkhout, B (2006). A conditionally replicating HIV-based vector that stably expresses an antiviral shRNA against HIV-1 replication. *Mol Ther*; **14:** 268-275.
24. McJunkin, K, Mazurek, A, Premsrirut, PK, Zuber, J, Dow, LE, Simon, J, et al. (2011).

Reversible suppression of an essential gene in adult mice using transgenic RNA interference. *Proc Natl Acad Sci U S A*; **108:** 7113-7118.

25. Wiznerowicz, M and Trono, D (2003). Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol*; **77:** 8957-8961.
26. Coumoul, X, Li, W, Wang, RH and Deng, C (2004). Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. *Nucleic Acids Res*; **32:** e85.
27. Konstantinova, P, de Vries, W, Haasnoot, J, ter Brake, O, de Haan, P and Berkhout, B (2006). Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA. *Gene Ther*; **13:** 1403-1413.
28. Gupta, S, Schoer, RA, Egan, JE, Hannon, GJ and Mittal, V (2004). Inducible, reversible, and stable RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*; **101:** 1927-1932.
29. Wu, RH (2007). A tightly regulated and reversibly inducible siRNA expression system for conditional RNAi-mediated gene silencing in mammalian cells. *J Gene Med*; **9:** 620-634.
30. Gupta, S, Schoer, RA, Egan, JE, Hannon, GJ and Mittal, V (2004). Inducible, reversible, and stable RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*; **101:** 1927-1932.
31. Maczuga, P, Lubelski, J, van Logtenstein, R, Borel, F, Blits, B, Fakkert, E, et al. (2013). Embedding siRNA sequences targeting Apolipoprotein B100 in shRNA and miRNA scaffolds results in differential processing and in vivo efficacy. *Mol Ther*; 21: 217-227.
32. Chtarto, A, Bender, HU, Hanemann, CO, Kemp, T, Lehtonen, E, Levivier, M, et al. (2003). Tetracycline-inducible transgene expression mediated by a single AAV vector. *Gene Ther*; **10:** 84-94.
33. Chtarto, A, Yang, X, Bockstael, O, Melas, C, Blum, D, Lehtonen, E, et al. (2007). Controlled delivery of glial cell line-derived neurotrophic factor by a single tetracycline-inducible AAV vector. *Exp Neurol*; **204:** 387-399.
34. Vanrell, L, Di, SM, Blanco, L, Otano, I, Gil-Farina, I, Baldim, V, et al. (2011). Development of a liver-specific Tet-on inducible system for AAV vectors and its application in the treatment of liver cancer. *Mol Ther*; **19:** 1245-1253.
35. Nicholson, L, Singh, GK, Osterwalder, T, Roman, GW, Davis, RL and Keshishian, H (2008). Spatial and temporal control of gene expression in drosophila using the inducible geneSwitch GAL4 system. I. Screen for larval nervous system drivers. *Genetics*; **178:** 215-234.
36. Juven-Gershon, T, Hsu, JY, Theisen, JW and Kadonaga, JT (2008). The RNA polymerase II core promoter - the gateway to transcription. *Curr Opin Cell Biol*; **20:** 253-259.
37. Baumann, M, Pontiller, J and Ernst, W (2010). Structure and basal transcription complex of RNA polymerase II core promoters in the mammalian genome: an overview. *Mol Biotechnol*; **45:** 241-247.
38. Koornneef, A, Maczuga, P, van Lontenstein, R, Borel, F, Blits, B, Ritsema, T, et al. (2011). Apolipoprotein B knockdown by AAV-delivered shRNA lowers plasma cholesterol in mice. *Mol Ther*; **19:** 731-740.
39. Chen, C, Ridzon, DA, Broomer, AJ, Zhou, Z, Lee, DH, Nguyen, JT, et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucl Acids Res*; **33:** e179.
40. Livak, KJ and Schmittgen, TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-[delta][delta] CT method. Methods; 25: 402-408.