Repeatable, Inducible Micro-RNA-Based Technology Tightly Controls Liver Transgene Expression

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Inducible systems for gene expression emerge as a new class of artificial vectors offering temporal and spatial exogenous control of gene expression. However, most inducible systems are less efficient in vivo and lack the target-organ specificity. In the present study, we have developed and optimized an oligonucleotide-based inducible system for the in vivo control of transgenes in the liver. We generated a set of simple, inducible plasmid-vectors based on the addition of four units of liver-specific miR-122 target sites to the 3′ untranslated region of the gene of interest. Once the vector was delivered into hepatocytes this modification induced a dramatic reduction of gene expression that could be restored by the infusion of an antagonist for miR-122. The efficiency of the system was tested in vivo, and displayed low background and strong increase in gene expression upon induction. Moreover, gene expression was repeatedly induced even several months after the first induction showing no toxic effect in vivo. By combining tissue-specific control elements with antagonist treatment we generated, optimized and validated a robust inducible system that could be used successfully for in vivo experimental models requiring tight and cyclic control of gene expression.

Molecular Therapy—Nucleic Acids (2014) 3, e172; doi:10.1038/mtna.2014.25; published online 1 July 2014

Subject Category: siRNAs, shRNAs, and miRNAs Gene vectors

Introduction

Inducible systems for gene expression are a new class of artificial vectors that offer the advantage of temporal and spatial exogenous control of gene expression. The ability to exogenously control gene expression, in vivo, is highly important in biomedical research.1–4 There are at least three areas where gene inducible systems are crucial. Firstly, gene regulation technologies contribute in the efforts to understand the role of specific gene products in fundamental biological processes in both normal development and disease states.1 Secondly, such technologies are important in bioprocess engineering where they offer the advantage of fine-tuning expression of protein or RNA pharmaceuticals, including conditional control for best production performance.5 Thirdly, gene inducible systems have the potential to become in the future, a new form of treatment.6–8

There are several inducible systems for gene expression largely in use today, such as the Cre-lox system.9 This system allows very tight gene expression control, and is used when only a single transition from on-off or off-on state is desired. Once the inducer, the Cre-recombinase is present in the cell, it will alter irreversibly the DNA sequence that contains the loxP sites, and thus, permanently changes gene expression.

Another highly used inducible system is based on the tetracycline-responsive element.2,10 Compared to Cre-lox, the tetracycline-inducible system can be designed in such a way that it can be reversibly turned on or off when desired.

However, some of the disadvantages of the tet-system are its large size and the gene expression leakiness in vivo. Thus, even when gene expression is supposed to be in the off state, there is still a considerable level of protein production. Hence, although the existing systems can already be used in several applications, a versatile, inducible system that has tissue or organ specificity, low or no leakiness, and can be used repeatedly, is required.

MicroRNAs (miRs) are small intracellular molecules, on average 22 nucleotides long, that play an important role in endogenous gene regulation.11–13 They act mainly as post-transcriptional regulators by silencing genes.11,12,14 It has been shown that every tissue has a miR signature.15–17 Depending on the intracellular miR abundance and the level of complementarity between the miRs and their target sites, the gene silencing can vary up to a few hundred-fold. Intelligent vectors have been designed so that endogenous miRs suppress transgene expression in hematopoietic lineages thereby reducing immunosuppression and enabling stable gene transfer.18–20

Such systems, containing one or more complementary sites to endogenous miRs in their 3′-UTRs18,21 were successfully employed for gene silencing given that the levels of endogenous miRs are high. For instance, miR-122 is abundant in hepatocytes (between 50 and 70,000 units/cell) and in malignant transformed hepatoma cell lines, such as Huh7 and controls hundreds of genes via translational suppression.

In this work, we have designed and tested a new inducible genetic system based on the presence of miR-122 target
sites in the 3′-untranslated region (3′-UTR) of different vectors. These vectors are silenced in the liver by endogenous miR-122 and activated once the endogenous miRs are blocked by the use of an antagomir.21–23

Results
AmiR122 selectively upregulates transcripts with miR-122 target sites
We first designed a DsRed expressing vector containing four complementary target sites for miR-122 (Table 1). Next, we tested the specific regulatory effect of this miR by comparing the DsRed expression in cells expressing, or lacking, miR-122. Downregulation was only present in Huh7 cells when using the plasmid containing miR-122 target sites (Figure 1a,b). Moreover, when adding the AmiR122, we could restore the expression of the gene containing the miR-122 target sites. The expression was unimpaired by the presence of a control antagomir, AmiR142 (Supplementary Figure S1a), and in the control 293T cells lacking miR-122 (Figure 1a).

AmiR122 regulates gene expression dose-dependently in vivo
The next step was to generate a vector that could easily be used in vivo. Viral promoters, such as the CMV promoter, show a high initial expression but short-lasting gene expression when used in vivo. However, endogenous promoters, such as, ubiquitin C promoter induce a high and long-lasting level of gene-expression in the liver.24 Consequently, we cloned the ubiquitin C promoter into the peGFPLuc vector25 by replacing the CMV promoter. It has been reported that adding four target sites of miR-142-3p at the 3′-UTR region of an exogenous gene in a lentiviral vector contributed to the in vivo-maintained expression of an exogenous gene18 by impairing its expression in hematopoietic cells, thereby preventing an immune response. Thus, apart from the four target sites for miR-122, we added four target sites of miR-142-3p at the 3′UTR region of the new vector, creating the pUbC.Luc.122TS.142TS and pUbC.Luc.142TS plasmids (Table 1). When testing this vector in the myeloid U937 cell line which expresses elevated levels of miR-142, there was more than an 80% decrease in gene expression from the vector containing the miR-142-3p target sites (Supplementary Figure S1b).

Making use of the highly efficient delivery method of hydrodynamic injections, we cojected the vector encoding the eGFPLuc fusion construct carrying the miR-122 and 142-3p target sites (pUbC.Luc.122TS.142TS) with the increasing doses of AmiR122. The pLacZ plasmid (expressing β-galactosidase) was also cojected to normalize the measured luciferase activity for differences in plasmid delivery.

We used different doses of AmiR122 ranging from 0.1 to 20 nmol/injection (Figure 2). Increasing the dose of AmiR blocked endogenous miR-122 and, thus, induced expression from the pUbC.Luc.122TS.142TS plasmid. Interestingly, the use of only 0.1 nmol of AmiR122 induced a 10-fold increase

Table 1 Plasmid characteristics and antagomir sequences.

| Name               | Promoter  | Reporter gene/sequence | miR TS at 3′-UTR |
|--------------------|-----------|-------------------------|------------------|
| pDsRed             | CMV       | dsRed                  |                  |
| pDsRed.122TS;      | CMV;      | dsRed; eGFPLuc         | 122              |
| peGFPLuc           | CMV       | Luciferase             |                  |
| pUbC.Luc           | UbiquitinC| eGFPLuciferase         |                  |
| pUbC.Luc.142TS     | UbiquitinC| eGFPLuciferase         | 142-3p           |
| pUbC.Luc.122TS.142TS | UbiquitinC| eGFPLuciferase         | 122, 142-3p      |
| pLive.Luc          | Albumin   | eGFPLuciferase         |                  |
| pLive.Luc.122TS    | Albumin   | eGFPLuciferase         | 122              |
| AmiR122            | 5′-TcAttGTCAcaC1C3′ |                  |                  |
| AmiR142            | 5′-AgTagAaaCaCiCIc3′ |                  |                  |

For the antagomirs, uppercases denote LNA bases and lowercases DNA bases.
One subgroup was hydrodynamically injected with a dose of 20 nmol of AmiR122 (in physiological salt solution) while the control group was hydrodynamically injected with only physiological salt solution. Before the injections, the mice with the plasmid containing miR-122 target sites had no, or very low, expression. After injecting the AmiR122, the luciferase expression dramatically increased until day 4, reaching a level of $>10^4$ times higher than seen in control mice treated with physiological salt solution only (Figure 3c; Supplementary Figure S2). The effect slowly decreased over the following days. The saline-treated animals showed a small, 10 times boost in luciferase expression that was detectable only for 4 days.

As a control for possible nonspecific effects due to either the hydrodynamic injection itself, or to indirect effects of the AmiR122 administration, we infused two groups of animals pretreated with the control-plasmid lacking the miR-122 target sites, 32 days after initial plasmid administration. We could identify a clear but transient effect of the hydrodynamic injection itself, namely a boost of gene expression from the preinjected plasmids (Figure 3d), similar to what has been reported before.\textsuperscript{28} In addition, there was a clear, 1.5 orders of magnitude, increase in Luc expression at day 4, in the group injected with AmiR122, but apart from that, both groups showed similar expression profiles.

Once an inducible vector is present in a tissue, it would be very useful if the expression could be induced at different time points and more than once. Consequently, after inducing the expression 32 days after plasmid inoculation, a second test of inducibility was initiated 3 months later, 124 days after the plasmid injection (Figure 3e). Even if the inducibility was approximately one order of magnitude lower than after 32 days, likely secondary to the general decrease in gene expression and to the hepatocytes turn over,\textsuperscript{29} an almost $10^3$-fold boost in gene expression was observed from the AmiR122-treated animals as compared to the control group.

Liver-specific promoter, enhancer and untranslated regions suppress “unspecific” hydrodynamics-induced gene expression

Apart from the promoter used, several other regulatory elements are important when designing highly and stable expressing plasmids. Using several regulatory endogenous elements, Wolff’s laboratory designed a new generation of plasmids, named pLive that have the ability to prolong the transgene expression, at high levels for more than 1 year.\textsuperscript{20,21} We transferred the eGFPLuc fusion gene from the UbC-plasmids into the pLive vector, obtaining a plasmid designated pLive.Luc (Table 1). Into this plasmid, we also added 4 miR-122 target sites to the 3’UTR region, obtaining pLive.Luc.122TS vector. Both these plasmids were tested in mice to determine the level of downregulation induced by miR-122. We have not generated any pLive plasmid containing miR-142-3p target sites, since there was no significant difference for the in vivo long-term expression among plasmids that contained, or lacked, miR-142-3p target sites (Supplementary Figure S3). The downregulation of pLive.Luc.122TS was at similar levels (40–100 times) as seen when using the UbC-based plasmids (Figure 4a).

Next, we tested the capacity of the AmiR122 to induce the expression from pLive.Luc.122TS plasmid. Animals were either coinjected with plasmid and AmiR122, or injected...
with plasmid alone. As expected, the AmiR122 induced an increased luciferase expression, with levels resembling those found in control animals injected with pLive.Luc, lacking the miR-122 target sites (Figure 4b). Similar to what was found for the UbC plasmid, the effect of AmiR122 lasted for more than 4 days.

Figure 3 In vivo luciferase expression profiles from pUbC.Luc plasmids containing or lacking target sites for miR-122, with, or without, injection of AmiR122. NMRI mice were hydrodynamically injected with 5 µg of plasmids with complementary target sites for both miR-122 and miR-142-3p (pUbC.Luc.122TS.142TS) or for mir-142-3p only (pUbC.Luc.142TS), and followed over time. Each value represents the average of the treated group (+SD). Plotted values are above the background signal of the IVIS machine of $1 \times 10^6$ photons/sec/cm²/sr. (a) Luciferase expression in mice injected with plasmids containing target sites for miR-122 and/or miR-142-3p and followed over time ($n \geq 4$). (b) Luciferase expression in mice co-injected with AmiR122 and followed over several days ($n \geq 3$). (c) Effect of AmiR122 administration on plasmid containing miR-122 target sites. A number of eight animals were preinjected with pUbC.Luc.122TS.142TS. After 1 month, four animals/group were hydrodynamically injected with either 20 nmol of AmiR122 in physiological salt solution (PSS) or with PSS only ($n = 4$). (d) Effect of AmiR122 administration on plasmid without miR-122 target sites. A number of eight animals were preinjected with pUbC.Luc.142TS. After 1 month, four animals/group were hydrodynamically injected with either 20 nmol of AmiR122 in PSS or with PSS only ($n \geq 3$). (e) The effect of a second AmiR administration 124 days from the date when the plasmid with miR-122 target sites was hydrodynamically injected, and 90 days after the first dose of AmiR122. A second dose of 20 nmol of antagonim in PSS or PSS only was hydrodynamically delivered ($n \geq 3$).
In order to assay if the pLive.Luc.122TS could be induced at a later time point, we then treated the two groups of animals 6 months after plasmid administration using the same strategy as for the pUbC.Luc-based vectors (Figure 3c, d). Importantly, compared to the pUbC.Luc treatment, when using the pLive.Luc vector there was no unspecific inducible effect neither from AmiR122 nor upon saline injection (Figure 4c). When the same treatment was applied on animals preinjected with the pLive.Luc.122TS vector, there was a boost in expression only from the group receiving the antagomir (Figure 4d). The expression from this group was maximal at day 3, reaching the same level as seen in animals receiving the control plasmid, pLive.Luc. Furthermore, to verify the specific effect of AmiR122 in inducing the pLive.Luc.122TS expression, the unrelated AmiR142 was also tested. As seen in (Figure 5a), it was only the AmiR122 that induced expression, thus demonstrating the specificity of the antagonir treatment. This effect was specific due to a decrease in miR-122 as demonstrated by a significant upregulation of miR-122 target proteins such as AldoA and Gys1 (Figure 5b).

To identify possible acute liver toxicity of the antagomir treatment, we measured basic toxicity indicators, such as creatine phosphokinase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) 3 days after oligonucleotide administration (Supplementary Figure S4). No indication of acute toxicities either in the AmiR122- or AmiR142-treated groups was observed.

Discussion

We describe a new, inducible system for the control of gene expression in hepatocytes both in vitro and in vivo. One of the most appealing features of this system is its simplicity: it is based on the addition of 4 units of miR-122 target sites, an ~120bp sequence, to the 3’UTR region of the gene of interest. Compared to the most known inducible vectors, 2,9,10,32–34 this design is by far the simplest. Furthermore, one major advantage over the tetracycline-based inducible vectors is the absence of transactivator.

The efficiency of the system was tested in vivo and displayed up to between 10^4- and 10^5-fold increase in gene expression.
expression, upon induction. Using the first generation of vectors, this level of inducibility was partially due to the unspecific effect of the hydrodynamic injection per se, similar to what has been reported before. This phenomenon was not only due to the blocking of miR-122 acting on the specific target sites in the reporter-mRNA, instead, the lower and somewhat delayed effect seen on the expression from plasmids lacking the specific target sites indicates that this outcome was in part also dependent on other, indirect, effects. Thus, blocking of miR-122 seems also to affect other molecules involved in expression of the ubiquitin-C driven reporter construct. Importantly, using the second generation vectors based on the pLive design, we could avoid these unwanted effects. Compared to the described inducible vectors, this very high range of inducibility in vivo is only achieved by the Cre-lox system. However, our system in contrast to the Cre-lox system is reversible. Another advantage of the miR-based inducible system is that gene expression can be induced repeatedly, even several months after the first induction. In addition, when testing the toxicity of the AmiR122, our new antagonomir-based system shows no apparent toxic effect in vivo.

The inducer of the described inducible system, AmiR122, is a short 15-mer LNA-DNA oligonucleotide, and is already in clinical phase II by Santaris Pharma A/S, under the name Miravirsen (SPC3649) for the treatment of chronic hepatitis C virus infection. Even though in this study we have only used hydrodynamic infusion as a delivery method, the AmiR122 can be delivered using intravenous administration (as in the mentioned human trial).

This inducible system was optimized for controlling transgenes expression in the liver. There are several possible applications having the liver as the main target organ. Some examples include hepatitis viral infections and liver tumors that are extremely difficult to treat. Using vectors that are under the control of miR-122 to express therapeutic proteins, one can combine gene therapy with oligonucleotide-based therapy to fine-tune protein expression. In case of hepatitis C virus infection, the inclusion of other transgenes that can protect the hepatocytes or eliminate the virus particles could in future potentially be regarded as an alternative to the current inefficient treatments. Another potential therapeutic strategy could be against cancer. MiR122 is considerably down-regulated in particular types of hepatocarcinomas. Using vectors similar to the one described here, but expressing cell-toxic transgenes under the control of a hepatocyte-specific promoter, one could induce apoptosis selectively in the hepatic tumoral cells and not in normal hepatocytes. In this scenario, it might be necessary to fine-tune the system using antagonomirs if the tumor cells resisted the toxicity, owing to the remaining low levels of miR122. However, here the balance needs to be carefully monitored to avoid as much as possible toxicity in normal hepatocytes. Another reason why the duration of the antagonomir activity should be carefully adjusted is because there is an increase evidence showing modified signaling pathways such as the interferon pathway that are potentially involved in several diseases.

The specificity of our miR-based inducible system was tested in vitro and also in vivo. The presence of the miR target sites restricts the downregulation to those cells that have high levels of the corresponding endogenous miRs. In order to limit the expression only to selected cell populations, tissue-specific promoters can be used. Thus, to reduce the background expression, it is important to use a tissue-specific promoter that is active only in those cells for which the miR target sites in the vector are designed. In our case, in order to have a selective, inducible system, the preferred vector contains a liver-specific promoter and in combination with miR-122 target sites in the 3′UTR.

In this work, we have used the hydrodynamic technique to deliver plasmids with, or without, the corresponding inducer, AmiR122. Unspecific effects of the hydrodynamic technique consisting of the induction of endogenous and exogenous gene expression, were shown before and we have also observed this phenomenon in our studies. This side effect can be avoided if the inducer can be non-hydrodynamically injected or, as we demonstrate, by the use of tissue-specific control elements, which are insensitive to hydrodynamic
changes. To our knowledge, this has not been reported before. Combining the same tissue-specific elements and the minicircle (MC) technology may further improve the outcome, since MC vectors have shown robust long-term expression in hepatocytes. The use of chemical-based carriers, such as cell-penetrating peptides for oligonucleotide delivery or encapsulated liposomes may also obviate the need for hydrodynamic delivery of the AmiR. Furthermore, the technique is not limited to plasmids, since it should also be possible to use viral vectors with hepatic tropism for the delivery.

By combining tissue-specific control elements with antagonim treatment, it was possible to generate a versatile, inducible system, which allows in vivo repeated and tight induction of gene expression in the liver, even after long-time periods.

Materials and methods

Plasmids and oligonucleotides. The plasmid pDsRed, encodes the dsRed fluorescent protein (Table 1). pDsRed.122TS is an additional element of pDsRed plasmid also contains 4 miR-122 target sites placed in the 3′ UTR. pLacZ is a β-galactosidase expressing plasmid kindly provided by Pontus Blomberg (Vecura, Karolinska University Hospital Huddinge, Sweden). The plasmid pEGFPLuc (Clontech, BD Bioscience, San Jose, CA, USA) expresses the enhanced green fluorescent protein in fusion with firefly Luciferase and modified as described previously. This vector contains the human cyto-megalovirus (CMV) immediate-early enhancer and promoter; it lacks modifications in the 3′ UTR and was used as an internal control by cotransfection into Huh7 cells. In the pUbC. Luc, the CMV promoter and enhancer were replaced with the ubiquitin C (UbC) promoter and enhancer. pUbC.Luc.142TS is the same plasmid with the addition of four target sites of miR-142-3p in the 3′ UTR, while pUbC.Luc.122TS.142TS contains an additional four target sites for miR-122 in the 3′ UTR. pLive.Luc has the same eGFPLuc-fusion as pUbC.Luc, but it lacks modifications in the 3′ UTR and was used as an internal control by cotransfection into Huh7 cells. In the pUbC. Luc, the CMV promoter and enhancer were replaced with the ubiquitin C (UbC) promoter and enhancer. pUbC.Luc.142TS is the same plasmid with the addition of four target sites of miR-142-3p in the 3′ UTR, while pUbC.Luc.122TS.142TS contains an additional four target sites for miR-122 in the 3′ UTR. The plasmid pUbC.122 TS.142 TS.15mer contains 15mer DNA-LNA mixmers complementary to the endogenous miR-122. As a control oligonucleotide, we used a 15mer DNA-LNA mixmer complementary to the endogenous miR-142-3p (AmiR142) (Eurogentech S.A.).

Cell lines and transfections. The Huh7, human hepatoma cell line expressing high levels of miR-122 and the human leuemic monocyte lymphoma cell line, U937, expressing high levels of miR-142-3p were used for in vitro assays. The 293T cell line does not express miR-122 and was used as a control. Transfections of AmiR122 and plasmids in Huh7 and 293T cell lines were done using Lipofectamine 2000, (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. The U937 cells were nucleo-transfected using Amaxa’s protocol (Lonza, Lonza Cologene AG, Germany). Gene expression was assayed after 48 hours.

Real-time PCR quantification of different mRNAs. cDNA was synthesized using 0.2 μg of Trizol-extracted liver total RNA in 20 μl reaction mixture, containing oligo(dT)12–18 primers and Superscript II reverse transcriptase (GIBCO Invitrogen), according to the manufacturer’s instructions. Real-time quantitative RT-PCR Taqman assays were performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were done in quadruplicates with the use of a predeveloped gene expression assay mix (Applied Biosystems) containing primers and a probe for the mRNA of interest. Additional reactions for each experiment included predeveloped gene expression assay mix for HPRT1 for normalizing the RNA input. All data were analyzed with 7300 System SDS Software v 1.4.

Animal experiments. Hydrodynamic tail vein injections of plasmids in physiological 0.9% NaCl solution were carried out as previously described. Briefly, 8% v/v was introduced by tail vein injection over a period of 5 seconds to inbred NMRI or Balb/c adult mice. Mice were anesthetized with 4% isoflurane and injected intraperitoneally with 150 mg/kg D-luciferin in 500 μl sterile PBS. Anesthetized animals were imaged for 1 second up to 5 minutes, depending on the intensity of the luminescence signal, using an intensified CCD camera (IVIS Imaging System, Xenogen, Alameda, CA). During imaging, the mice were kept under anesthesia using 2.3% isoflurane. Images are comprised of pseudocolored images representing intensity of emitted light (red most intense and blue least intense) superimposed on grayscale reference images for orientation. Data analysis was performed using a Living Image 3.2. software (Caliper LifeSciences, Xenogen) . All animal experiments were approved by the local ethical committee in Stockholm, Sweden.

Luciferase-based assays. For most animal experiments, we used an in vivo luminescence-based assay (IVIS 100, Xenogen). For titration of the AmiR122 dose, liver extracts were measured for level of luciferase expression (Luciferase assay Kit A, Bioheima AB) and related to the expression of β-galactosidase to compensate for different transfection efficiency. The β-galactosidase was measured using Luminescence β-Gal kit (Clontech) following the manufacturer’s protocol. These samples where analyzed using the GloMax instrument (Promega, Sweden).

Statistical analysis. Pair-wise comparisons among treatments were made using a Student’s t-test. Comparisons among multiple treatments were made using one-way analysis of variance, followed by the SNK test. A P value of <0.05 was considered as significant difference.

Supplementary material

Figure S1. Effects of miR-122 and miR-142-3p on plasmid expression in transfected cells.

Figure S2. Effect of AmiR122 administration on expression from plasmids containing miR-122 target sites.

Figure S3. In vivo luciferase expression profiles from pUbC.Luc plasmids containing, or lacking, target sites for miR-142-3p.

Figure S4. Toxicity assay for mice treated with either AmiR142 or AmiR122.
Acknowledgments. This work was supported by grants from the Swedish Research Council, the Swedish Cancer Society, The Swedish Childhood Cancer Foundation, Crown Princess Margareta’s Foundation for the Visually Impaired, King Gustaf V Jubilee Foundation, Stockholm Cancer Society, the Swedish County Council (ALFproject), Aroseniusfonden and Karolinska Institutet. The authors declare that no conflict of interest exists.

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