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TITLE:
Development of a Positive-readout Mouse Model of siRNA Pharmacodynamics

ABSTRACT:
Development of RNAi-based therapeutics has the potential to revolutionize treatment options for a range of human diseases. However, as with gene therapy, a major barrier to progress is the lack of methods to achieve and measure efficient delivery for systemic administration. We have developed a positive-readout pharmacodynamic transgenic reporter mouse model allowing noninvasive real-time assessment of siRNA activity. The model combines a luciferase reporter gene under the control of regulatory elements from the lac operon of Escherichia coli. Introduction of siRNA targeting lac repressor results in increased luciferase expression in cells where siRNA is biologically active. Five founder luciferase-expressing and three founder Lac-expressing lines were generated and characterized. Mating of ubiquitously expressing luciferase and lac lines generated progeny in which luciferase expression was significantly reduced compared with the parental line. Administration of isopropyl β-D-1-thiogalactopyranoside either in drinking water or given intraperitoneally increased luciferase expression in eight of the mice examined, which fell rapidly when withdrawn. Intraperitoneal administration of siRNA targeting lac in combination with Lipofectamine 2000 resulted in increased luciferase expression in the liver while control nontargeting siRNA had no effect. We believe a sensitive positive readout pharmacodynamics reporter model will be of use to the research community in RNAi-based vector development.

Introduction:
RNA interference (RNAi) is a powerful approach for suppressing expression of specific genes in mammalian cells, either as a basic research tool to elucidate gene function, or in a clinical setting for therapeutic application.1,2,3 Therapeutic strategies eliciting RNAi involve targeting exogenous genes from pathogens or endogenous genes playing a role in the disease process. Since the landmark discovery of RNAi in 1998, the number of in vivo studies involving siRNA has greatly increased with thousands of studies now reported and >30 clinical trials involving siRNA- or shRNA-based drugs having been opened.4,5
Although local delivery of siRNA may be very effective in some settings 6,7,8,9, for many diseases, delivery to disseminated or body-wide targets is required. There have been several steps towards enabling systemic delivery of siRNA, including both improved chemistry to increase serum stability and reduced immunostimulation,4,10 coupled with delivery strategies such as hydrodynamic injection,11,12 liposomes and lipid-based nanoparticles,13,14,15 polyplexes,16,17 ligand-siRNA conjugates,18,19,20 and inorganic particles.21,22 However, there remain major challenges in studying the performance of siRNA given intravenously. Biodistribution has been studied by fluorescence,23,24 radioactivity,25,26 or complexation with magnetic nanoparticles,27 although tracing techniques may intrinsically alter pharmacokinetics of the labeled molecules and the time window of usefulness for such studies is limited by siRNA catabolism.
In addition, biodistribution of siRNA is not an indicator of biological activity. Techniques to assess siRNA activity (or “pharmacodynamics”) are dependent on the nature of the molecular target, for example, where siRNA targets mRNA encoding specific enzymes, pharmacodynamics can be assessed by measuring inhibition of enzyme activity. Unfortunately, such assays are highly invasive, preventing time-resolved assessment in the same animals, and it can be very easy for researchers to miss spatially off-target effects. In principle, every target-expressing cell type in the body must be evaluated to assess whether siRNA activity is truly restricted to the intended target. One way to address this limitation on study of pharmacodynamics involves the use of transgenic reporter mice or disease models that ubiquitously or selectively express reporter genes such as GFP or luciferase, to test the ability of siRNA to silence expression at the target site.28,29 However, such systems are not ideal since models generating selective expression of the target reporter gene mRNA in a specific organ will skew the data to give a desired outcome; while ubiquitously expressing “negative-readout” models suffer from high levels of background from nontarget tissues, giving an intrinsic poor signal-to-noise ratio.
There is therefore a pressing need for an animal model that sensitively and noninvasively reveals successful siRNA delivery without the need for labeling or tissue sampling. We have previously developed an inducible in vitro system using the lac repressor in which siRNA activity results in a

positive readout of luciferase expression.30 Based on the well-characterized E. colilac operon,31 in the absence of lactose the lac repressor (lacI) binds as a homotetramer to the lac operators (lacO) located within the promoter region, blocking transcription of the downstream gene (Figure 1a). Lactose (or its synthetic analogue isopropyl β-D-1-thiogalactopyranoside (IPTG)) causes a conformational change in the repressor causing it to vacate the operators allowing RNA polymerases to gain access to the promoter and initiate transcription. When the repressor is removed from the operator, transcription from the lac operon resumes. Introduction of siRNA targeting the lac repressor mRNA will reduce levels of repressor protein allowing expression of a reporter gene, in our case luciferase, downstream of a lacO-containing promoter, thus giving a positive readout of RNAi activity. Scrable et al. independently developed a system in which the bacterial lac expression system was “mammalianized” via appropriate codon usage, to generate a working transgenic mouse model in which luciferase expression was regulated by the lac repressor but could be induced by IPTG administration in the drinking water.32,33,34 Using the Scrable constructs, we have recreated the mouse model here, and in doing so, we have created and tested the first transgenic mouse model in which every cell of the animal can potentially reveal every site of siRNA biological activity. The advantage of this system is it provides noninvasive readouts that allow time-based measurement of body-wide effects of siRNA and therefore provides a platform for comparing and developing siRNA vector systems.

Generation of transgenic mice ::: Results:
The strategy adopted to develop a lac-regulated luciferase expression model was based on that previously performed by Scrable et al.33,34 Briefly, transgenic lines comprising HDLacOLuc and LacIR were generated and then subsequently crossed to produce double transgenic (HDLacOLuc x LacIR) offspring.
In total, five HDLacOLuc founder mice were generated (Table 1), which were then crossed with wild-type C57BL/6J mice to produce F1 pups and establish each line. Four of the five founder mice (424, 673, 676, and 677) produced pups that were genotyped and examined for luciferase expression by noninvasive imaging. Whole body imaging showed virtually no luciferase expression in the F1 pups from founders 673, 676, and 677, together with a wild-type control mouse (Figure 2a and Table 1). On dissection, small amounts of expression could be detected in the fallopian tubes and ovaries of mouse 676-5, and the testes and large intestine of mouse 677-3 (data not shown). In contrast, the F1 offspring of founder mouse 424 (individuals 660, 661, and 662) showed extensive luciferase expression (Figure 2a). Expression levels remained undiminished over time with no loss of signal observed after 3 months. Luciferase expression was confirmed as truly ubiquitous following culling and dissection of three individuals, with the organs being reimaged outside of the carcass. Expression appeared to be especially high in the liver, fallopian tubes and ovaries of all three animals (Figure 2b). HDLacOLuc-424 F1 pups were then mated with wild-type C57BL/6J mice and ubiquitous luciferase expression confirmed in the F2 generation (data not shown).
In addition, three LacIR founder mice were generated (Table 1), two of which (339 and 866) generated offspring when mated with wild-type C57BL/6J mice. Lac repressor expression was confirmed by western blot as a 38 kDa band that was present in all organs examined of a transgenic LacIR-339 F1 pup (Figure 2c); however, expression was highly variable between organs. While expression was high in muscle and spleen, and moderate in the brain and heart, it was low in the lungs, kidney, and liver and could only be detected following an increase in the amount of sample loaded onto the gel. In contrast, all the organs of a transgenic LacIR-866 F1 pup showed high expression (Figure 2c). No expression was detected in a wild-type control mouse. Transgenic F1 mice from founders LacIR-339 and LacIR-866 were subsequently mated with C57BL/6J wild-type mice to produce an F2 generation.
Having established that the HDLacOLuc-424 line had stable ubiquitous luciferase expression, individuals from the F2 generation were mated with individuals from the F2 generation of the LacIR-339 and LacIR-866 lines in order to generate double transgenic progeny with a working reporter system.
In total, 17 mice bred from the HDLacOLuc-424 x LacIR-339 cross and 11 mice from the HDLacOLuc-424 x LacIR-866 cross were examined for luciferase expression (Figure 3a, Supplementary Table S1). Overall a broad range of total body-wide luciferase expression ranging from 1.79 × 105 relative light units (RLU) to 2.15 × 108 RLU was observed. Total luciferase expression in individuals from the HDLacOLuc-424 x LacIR-339 cross was not significantly lower than in HDLacOLuc-424 offspring (Figure 3b). However, some HDLacOLuc-424 x LacIR-339 mice such as 1005 and 4001 had very low levels of expression (Figure 3a, Supplementary Table S1). In

contrast, the total luciferase expression in individuals from the HDLacOLuc-424 x LacIR-866 cross was significantly lower than in the HDLacOLuc-424 line (Figure 3b). Given that the HDLacOLuc-424 x LacIR-339 and HDLacOLuc-424 x LacIR-866 mice share the same genetic background for luciferase, the reduction in luciferase expression is likely to be attributable to the increased expression of the lac repressor in the 866 descendant mice (Figure 2c).

Induction of luciferase expression by IPTG ::: Results:
The double transgenic mice were tested for induction of luciferase expression using IPTG. Although not all mice responded, eight of the mice showed a clear increase when administered IPTG either in the drinking water or given intraperitoneally (i.p.) (representative examples are shown in Figure 4). Unsurprisingly, mice with a high initial luciferase expression level (>1 × 108 RLU) failed to show any increase on IPTG treatment; however, some mice with low levels of expression (<3 × 107) also failed to be induced. Of the eight mice that were successfully induced, increased luciferase expression was observed in individuals from both LacIR lines (339 and 866). The greatest induction was observed in mouse 220 (HDLacOLuc-424 x LacIR-866), which showed a fivefold increase in luciferase expression above baseline (from 9.13 × 106 RLU to 4.56 × 107 RLU) 72 hours after IPTG administration in the drinking water. Importantly, luciferase expression fell sharply following withdrawal of IPTG treatment, with levels returning to the background state for the animal within 48 hours. Similarly, if IPTG was given as an i.p. injection, rapid luciferase induction was observed within 24 hours (Figure 4).

Design and testing of siRNA targeting mammalianized lac repressor ::: Results:
We have previously demonstrated that siRNA targeting the bacterial lac repressor, when codelivered with the reporter/repressor plasmid constructs restores luciferase activity to levels observed in the absence of repressor and comparable with IPTG-mediated induction.30 As, in this study, a mammalianized version of the lac repressor was used to allow long-term expression in the transgenic mice,33 three new siRNA sequences were designed and tested. Following the transient transfection of PC-3 cells with pLacIR and various siRNAs, all siLac sequences mediated a substantial reduction in Lac expression (Figure 5a); siLac#3 demonstrated the greatest effect with protein expression undetectable even at the lowest (1 nmol/l) siRNA dose. Cells that received non-Lac targeting siRNA (including siRNA targeting the bacterial version of the repressor) showed no reduction in lac expression. Importantly, siLac#3 had no effect on the level of luciferase expression when codelivered with pHDLacOLuc (Figure 5b), confirming this sequence has no off-target effect on luciferase mRNA. Having identified a potent sequence, a second siRNA containing serum stabilizing modifications (siSTABLE-siLac#3) was synthesized in preparation for in vivo delivery applications. Cotransfection of siSTABLE-siLac#3 with pLacIR reduced repressor expression to levels comparable with the unmodified siLac sequence (Figure 5c); a control siSTABLE sequence having little effect.
siLac#3 and siSTABLE-Lac#3 were then assessed for their ability to restore luciferase expression using a transient cotransfection assay involving delivery of up to four components, including the reporter (HDLacOLuc) and repressor (LacIR) plasmids. As a control GL4.74, a Renilla luciferase expression plasmid to which siLac#3 or siSTABLE-Lac#3 have no target, was also delivered in order that the transfection efficiency of firefly luciferase expression could be normalized. The transient transfection of NIH3T3 cells with HDLacOLuc, GL4.74, and LacIR demonstrated reduced firefly luciferase activity in comparison with cells that received no repressor plasmid (Figure 5d). However, the reduction in luciferase expression was only small since HDLacOLuc was delivered in tenfold excess compared with LacIR to limit transcriptional interference from the β-actin promoter (driving LacIR) over the weaker HD promoter (driving luciferase) and thus reducing expression in a transient assay35 (data not shown). As expected, the lac repressor–mediated fall in luciferase expression was fully restored on induction with 5 mmol/l IPTG. Importantly, the codelivery of siRNA targeting the repressor (siLac#3 or siSTABLE-Lac#3) also fully restored luciferase expression, while control siRNAs (siHBsAg and siSTABLE-Cont.) had no effect. These results confirm that siLac#3 and siSTABLE-Lac#3 are capable of downregulating lac repressor in cells bearing the dual expression system mediating a rise in reporter gene expression. As a result, a positive readout is generated from RNAi activity.

Effect of siRNA targeting the lac repressor in the double transgenic mouse model ::: Results:
Finally, we tested the effect of delivering siRNA targeting the lac repressor on luciferase expression in the mouse model. Individual mice that had shown evidence of IPTG induction were injected i.p. with a complex of siSTABLE-Lac#3 and Lipofectamine 2000. A representative

example is shown in Figure 6. Following administration an increase in luciferase expression was observed in peritoneal regions, most likely including the surface of the liver and possibly other organs. To enable comparison in the same individual mice, after 7 weeks (to ensure restoration of basal expression levels had occurred), control siRNA (siNT) was administered at the same dose. No increase in luciferase expression was detected (Figure 6). We also tested intramuscular administration of naked siRNA; however, as expected in the absence of electroporation, no increase in luciferase expression was observed (data not shown).

Discussion:

Scrable et al. previously developed the luciferase-expressing lac repressor model in order to visualize the dynamics of gene expression in real-time in the living mouse.32,33,34 We have recapitulated the model here to provide a positive readout of siRNA activity. Originally, Scrable et al. reported that upon IPTG induction body-wide luciferase expression fell back to 34% of the induced levels after 48 hours and returned to initial background levels after 7 days. The mice generated in this study appeared to have more rapid kinetics since luciferase expression returned to background within 24–48 hours of IPTG removal. As a result, they appear to be more responsive than the animals from the first study possibly as a result of greater repressor protein expression.

Luciferase expression was variable among the single transgenic offspring of the HDLacOLuc-424 founder mouse. However, significant repression of luciferase expression was observed in the double transgenic progeny mice resulting from a cross with the LacIR-866 line in which lac repressor expression was robust and ubiquitous. Luciferase expression levels within the individual double transgenic mice were stable for over 12 months in some cases, with changes (increased signal) only observed due to hair loss in more elderly mice. Despite the significant degree of luciferase repression in the HDLacOLuc x LacIR mice, only 2 of the 28 individuals examined (1005 and 255) showed levels of luciferase expression that were as low as wild-type or LacIR mice. Consequently, in our hands from these particular matings, the system appeared to be slightly leaky and complete repression was rarely obtained. There is also a high degree of animal-to-animal variability in the luciferase expression in the double transgenics that needs to be addressed before widespread practical application as a test model. Further in-breeding may help to reduce the variability between individuals. We believe the situation of incomplete repression could be improved through the use of additional regulatory factors such as the genetic switch proposed by Deans et al.36 These authors have developed a cassette comprising a short hairpin RNA construct that targets the reporter gene but whose expression is under the control of another repressor (Tet) which in turn is regulated by lac operator sites located within its promoter. Consequently, in the off state, the lac repressor prevents expression of the reporter gene and represses the expression of the Tet repressor. At the same time, expression of the hairpin RNA can occur that further reduces any residual reporter gene expression. Induction can be achieved with IPTG allowing reporter gene expression through the combined release of lac from the operator sites located within the reporter gene promoter and also the promoter of the TetR resulting in TetR expression and repression of the short hairpin RNA expression. With reference to our system, one would envisage that siRNA targeting the LacR would function in the same manner as IPTG (thus giving rise to luciferase expression) but in a system less prone to leakiness. Nevertheless, induction of luciferase in our current model was rapid when mice were given IPTG either via the oral or i.p. route. The degree of luciferase induction was variable between individuals but fell rapidly when IPTG was removed, suggesting re-expression of the repressor protein and silencing of the reporter; an important determinant of a sensitive readout model.

Application of uncomplexed naked siSTABLE-Lac#3 into thigh muscle failed to induce a luciferase signal presumably since the siRNA does not enter cells effectively in the absence of an entry mechanism such as electroporation. In contrast, Lipofectamine 2000 complexation of serum stabilized siRNA at a dose of 1 mg/kg administered by the i.p. route resulted in a threefold increase in luciferase expression (compared with negative control siRNA) in peritoneal organs confirming the potential of the model to identify siRNA activity. No expression was observed when the dose was reduced to 0.3 mg/kg (data not shown). As Lipofectamine 2000 is not a particularly sophisticated in vivo delivery vector, it is likely that more effective vehicles will yield greater responses. Furthermore, the limit of detection may be reduced when induction is obtained in organs at greater depth from the skin. One application of the model would be to compare the commercially available delivery systems to determine those that are optimal for each researcher's particular requirement.

Shen and colleagues at Abbott laboratories have developed a positive-readout cellular model based on the Tet repressor system in which Tet repressor protein binds to Tet operator sites located within the luciferase promoter.37 Use of specific siRNAs to target the tet repressor results in an increase in luciferase expression and thus a positive readout of siRNA activity. By encoding this system in cancer cell lines, they were able to implant tumor xenografts into nude mice and rats and compare a range of delivery vectors for tumor targeting. They concluded that 2.5 mg siRNA/kg formulated in DODAP-based liposomes (Avanti Polar Lipids) or SNALP-based liposomes26 were the only systems capable of delivery to liver tumors following intravenous injection. While this is a very valuable assay system it cannot provide information regarding activity of the siRNA in the rodent cells (i.e., off target in terms of cellular targeting). This can only be achieved through use of transgenic models in which every cell expresses the reporter system such as the one described here. While our mice would be refractory to developing human tumor xenografts, it would be possible to encode the repressor system into a mouse tumor line that could then provide a model of tumor targeting. Alternatively, we envisage that orthotopic tumor models could be developed where targeting to specific organs or metastases might provide a more realistic model.

Kleinman et al. demonstrated that not all instances of downregulation of gene expression following siRNA administration occur via the RNAi pathway.38 Instead, in some instances, silencing occurs via the nonspecific mechanism of TLR3 activation and is in fact an siRNA-class effect. The authors demonstrated that inhibition of blinding choroidal neovascularization from a model of age-related macular degeneration in mice was as effective using control nonspecific siRNA as by administration of siRNA targeting VEGFa or VEGFR1 and that the effects could occur via cell surface toll-like receptor-3 (TLR3), its adaptor TRIF and induction of interferon-γ and interleukin-12. The use of a positive-readout reporter system would help to address some of these issues of RNAi versus TLR3 activation. For example, injection of a cocktail of siRNA targeting VEGFa or control together with siLacor control into the eyes of our reporter mice given laser-injury induced choroidal neovascularization (a model predictive of efficacy in humans) could help show if suppression of choroidal neovascularization effects were mediated by RNAi where luciferase induction would be observed or by TLR3 where no luciferase signal would be seen.

Our current model is limited by high interanimal variation in the baseline luciferase expression and the IPTG induction. However, when testing siRNA activity, it is possible to use the same individual to compare luciferase induction achieved with siLac to the level observed after subsequently delivering nontargeting siRNA. Internally controlling studies in this manner has circumvented the issue of variability sufficiently to allow us to demonstrate that positive readout from siRNA activity can be achieved. Although further development is required for widespread practical application, we believe a dynamic, rapidly responsive positive-readout reporter model for siRNA pharmacodynamics has great potential for improving siRNA vector design and testing accelerating the development of a new wave of genetic medicines to treat a range of disorders.

Materials and methods:

Plasmid DNA. The regulatable HDLacOLuc construct expresses firefly luciferase driven by the promoter from the human Huntington's Disease (HD) locus into which were embedded two synthetic lac operators. The HD promoter fragment was obtained by NcoI digestion of the qp25 construct (David Housman, HDF CHDI Reagent Resource Bank) containing the huntingtin promoter and exon 1 to yield an 800 bp product that was inserted into the NcoI site of the pGL3-Basic vector (Promega, Madison, WI). The two synthetic lac operators (ATTGTGAGCGCTCACAAT) were inserted at locations (-69 and -198) flanking the transcription start site, mimicking the spacing found in the lac operon of E. coli using splice overlap extension by PCR and a unique Blp1 site in the HD promoter.34 The LacIR construct contains the lac repressor transgene that resembles a typical mammalian gene in terms of codon usage under the control of the human β-actin promoter32,33 (Supplementary Figure S1). pGL4.74 expresses Renilla luciferase under the control of HSV-TK promoter (Promega, Southampton, UK).

siRNA. siRNAs targeting the mammalianized version of the lac repressor were designed using the Dharmacon design software. Sequences siLac#1 (mRNA target sequence NNAGAAAGAAGUGGAGUUGAA), siLac#2 (mRNA target sequence NNGCCAAUAGCUGAAAGAGAA), and siLac#3 (mRNA target sequence NNGAAGACAGCUCAUGUUAUA) were synthesized by Thermo Fisher Scientific (Cramlington, UK). A chemically stabilized version of siLac#3 (siSTABLE-Lac#3) (converted to the 2′-hydroxyl version) together with a nontargeting siRNA control (D-001700-01-20; Thermo Scientific Dharmacon) were also synthesized. siRNA targeting GFP (siGFP) (mRNA target sequence

CGGCAAGCUGACCCUGAAGUUCAU), luciferase (siLUC) (mRNA target sequence AACUUACGCUGAGUACUUCGA), bacterial LacI (mRNA target sequence AAAUAUCUCACUCGCAAUCAA), and siRNAs targeting Hepatitis B surface antigen (siHBsAg#1, mRNA target sequence AACAUCACAUCAGGAUUCCUA, siHBsAg#2, mRNA target sequence AAUCACUCACCAACCUCUUGU and siHBsAg#3, mRNA target sequence CUAUAUGGAUGAUGUGGUA) were synthesized by Qiagen (Crawley, UK).

Cells. NIH/3T3 murine fibroblasts (CRL-1658) obtained from ATCC (Manassas, VA) were maintained in DMEM containing 10% fetal calf serum. PC-3 human prostate carcinoma cells were maintained in Ham's F12 media containing 10% fetal calf serum.

Transfection assays. To test the efficacy of siRNA sequences targeting the lac repressor, 2 × 105 PC-3 cells were plated out in six-well plates. A 1.6 μg of pLacIR was transfected alone or in combination with various siRNAs (at a final concentration of 1 or 10 nmol/l) using Lipofectamine (Invitrogen, Paisley, UK) in serum-free medium according to the manufacturer's instructions. After 4 hours, serum containing media was added to give a final serum concentration of 10%. Cells were lysed after 48 hours and analyzed for LacIR protein expression by western blot. Assays to determine the efficacy of siSTABLE-Lac#3 were examined after 24 hours rather than 48 hours. To assess off-target effects of siLac#3 on luciferase expression, 4 × 104 PC-3 cells were plated into 24-well plates. A 0.4 μg of HDLacOLuc alone or with siLuc or siLac#3 (10 nmol/l final concentration) in complex with Lipofectamine was added to cells. Cells were lysed after 24 hours and luciferase expression measured using luciferase assay reagent (Promega, Southampton, UK) and a luminometer (Berthold Technologies, Harpenden, UK), readings normalized for protein content.

To assess the efficacy of siRNA in the context of the reporter system, 4 × 104 NIH/3T3 or PC-3 cells were plated out in 48-well plates. A 200 ng HDLacOLuc and 10 ng GL4.74 were added to cells alone, or together with 20 ng LacIR using Lipofectamine. In addition, some cells received 10 nmol/l siRNA delivered within the same Lipofectamine complexes as the plasmid DNA. Cells were transfected in replicates of five and the experiment performed on at least three occasions. After 24 hours, appropriate cells received 5 mmol/l IPTG. After 48 hours, cells were lysed and luciferase expression analyzed. Both firefly and Renilla luciferase were measured using the dual glow reporter system (Promega, Southampton, UK). Readings were normalized for protein content and expressed as a ratio of firefly luciferase expression divided by Renilla luciferase expression. Statistical analysis was performed using the Levene Statistic to confirm homogeneity of variances followed by an analysis of variance using Bonferonni correction with post hoc analysis.

Western blotting. Transfected cells were lysed and total cellular protein extracted using M-PER solution (Pierce Biotechnology, Rockford, IL) containing protease inhibitor cocktail (Roche, Welwyn Garden City, UK). Protein concentration was determined by BCA assay; 20 μg of total protein was loaded onto each well of a 12% SDS-PAGE gel and run at 170 V for 40 minutes. The proteins were transferred to a nitrocellulose membrane and blocked in 5% dried milk in Tris-buffered saline pH 7.4, 0.1% Tween 20 (TBST) at room temperature for 60 minutes. The blot was then washed three times with TBST and incubated with mouse anti-LacI clone 9A5 primary antibody (Millipore UK, Watford, UK) diluted 1 in 1,000 at room temperature for 2 hours. The blot was then washed three times with TBST and incubated with goat antimouse IgG-HRP secondary antibody (Dako, Ely, UK) diluted 1 in 5,000 at room temperature for 1 hour. The blot was washed and the bands visualized by chemiluminescence using ECL western blotting detection reagent (GE Healthcare, Little Chalfont, UK) on a ChemiImager (Alpha Innotech, San Leandro, CA), and analyzed using Fluorchem 8000 software. Each blot was stripped and reprobed with sc-5286 anti-α-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000 as a protein loading control.

Transgenic mouse production. HDLacOLuc transgenic mice were generated by isolating the transgenic construct from pHDLacOluc via BglII/BamHI digestion (Figure 1b) followed by PvuI digestion to leave a 2.7 kb isolated fragment which was purified and diluted to 5 ng/μl in microinjection buffer (10 mmol/l Tris–HCl pH7.4, 0.1 mmol/l EDTA). LacIR transgenic mice were generated by isolating the transgenic construct from pLacIR via KpnI/SacI partial digestion (Figure 1b) to give a 7.5 kb fragment isolated and purified as described above. Three-week-old female B6CBAF1 mice were superovulated and mated. The resulting fertilized oocytes were collected from the oviduct of plugged female mice and cultured until two clear pronuclei were visible. The purified HDLacOLuc or LacIR transgenic constructs were microinjected into one of the pronuclei and injected embryos were cultivated overnight to the two-cell stage. Two-cell embryos were then reimplanted into the oviduct of pseudo pregnant CD1 foster mothers at 0.5 days after coitum. Between 18 and 19 days later, pups were born from the foster mothers. Ear biopsies from these

pups were taken, DNA extracted and PCR performed to identify transgenic founder mice. Founder mice were mated with C57BL/6J mice and the resultant pups genotyped to confirm germline transmission of the transgenes. These F1 transgenics were mated with C57BL/6J mice to produce an F2 generation and establish the line.

Double transgenics were established by mating HDLacOluc F2 transgenic mice with LacIR F2 transgenic mice. Offspring were genotyped to identify individuals carrying both transgenes.

Genotyping by PCR. PCR primers (HDLacOluc forward 5′-TCCTATGATTATGTCCGGTTATGTAAA, HDLacOLuc reverse 5′-GACTGGCGACGTAATCCACGATCT, LacIR forward 5′-AACCAGGCCAGCCATGTTTCTGC, and LacIR reverse 5′-AACCAGATGCTCCACACCCAGTCTT) (Sigma Genosys, Welwyn Garden City, UK) were designed to amplify a 350 bp region of HDLacOluc and a 417 bp region of LacIR. Template DNA obtained from ear biopsies was amplified using the thermal cycling conditions; 94 °C for 15 minutes, followed by 35 cycles of 94 °C for 60 seconds, 55 °C for 60 seconds, and 72 °C for 40 seconds and ending with a single completion stage of 72 °C for 5 minutes. The reaction conditions were generated in an Applied Biosystems 7000 Sequence Detection System. The standard amplification reaction contained QuantiTect Probe PCR mastermix (which includes HotStarTaqDNA Polymerase, Tris–HCl (pH8.7), KCl, (NH4) SO4 8 mmol/l MgCl2, dNTPs, and ROX passive reference dye) (Qiagen), to which was added 400 nmol/l forward primer and 400 nmol/l reverse primer. Two microlitres of template DNA from each sample was added. Positive controls were performed using 1–5 ng of plasmid (HDLacOLuc or LacIR) DNA, while negative controls were performed using water instead of template. As a further control, the integrity of the sample DNA was confirmed by PCR using murine Connexin 31 primers (forward primer 5′-CTGGACTCTGACATGTGCACATAC, reverse primer 5′-CTACATGCAGGATGACCAGCATAG).

In vivo imaging. Mice were anesthetized with isofluorane and injected intraperitoneally with 100 µl of sterile filtered 15 mg/ml D-Luciferin (Gold Biotechnology, St Louis, MO) in PBS. After 10 minutes, mice were imaged using the IVIS 100 system (Xenogen, Alameda, CA). Images were captured using Living Image 2.50.2 software. When required after whole body imaging mice were culled, dissected and the organs arranged and reimaged outside the carcass.

Induction of luciferase with IPTG. Mice were imaged and then given either 10 mmol/l IPTG (Stratagene, Leicester, UK) in the drinking water or 100 mmol/l IPTG administered intraperitoneally and reimaged 24, 48, or 72 hours later.

Delivery of siRNA in vivo. Mice that showed evidence of luciferase induction upon IPTG induction were given an intraperitoneal injection of siRNA (siSTABLE-Lac#3) complexed with Lipofectamine 2000 at 1 mg/kg and imaged after 48 hours. Mice that showed an increase in luciferase expression where treated 7 weeks later with nontargeting control siRNA complexed with Lipofectamine 2000 at the same dose. Mice were also given intramuscular injections of 10 µg naked siRNA (siSTABLE-Lac#3 into one thigh and nontargeting control siRNA into the opposite thigh muscle) and reimaged after 48 and 96 hours.

SUPPLEMENTARY MATERIAL

Figure S1. Construction of the LacIR coding sequence.

Table S1. Mean luciferase expression in each mouse.