

**SHORT REPORT****Establishment of a positive-readout reporter system for siRNAs**

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**ABSTRACT**

The use of small interfering RNA molecules for therapeutic applications requires development of improved delivery systems, a process that would be facilitated by a non-invasive positive-readout mouse model for studying siRNA pharmacodynamics. Positive readout would yield better signal/noise ratios than existing negative-readout systems. We have engineered a positive-readout luciferase reporter system, activated by successful delivery of siRNA targeting the lac repressor. Co-transfection of a plasmid expressing lac repressor and a plasmid expressing firefly luciferase under the control of an RSV promoter, containing two lac operator sites, resulted in 5.7-fold lower luciferase activity than luciferase-encoding plasmid alone. Inhibition was reversed following addition of synthetic inducer, IPTG, which elevated luciferase expression to normal levels and confirmed functionality of the lac operon. Delivery of 1nM siRNA targeting lac repressor to repressor/reporter co-transfected cells was sufficient to fully restore luciferase expression to levels observed in the absence of repressor. Maximum expression was observed after 48hr, with a rapid decrease thereafter due to the short half life of luciferase. The luciferase positive-readout reporter system is therefore a dynamic indicator of successful RNAi delivery *in vitro* and could be adapted to generate a transgenic mouse capable of reporting RNAi activity non-invasively *in vivo*.

**KEYWORDS:** siRNA, inducible system, lac operon, RNAi, gene silencing, reporter system**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 (Fire et al, 1998; Tuschl et al, 1999; Elbashir et al, 2001). Therapeutic strategies eliciting RNAi involve targeting exogenous genes from pathogens or endogenous genes playing a role in the disease process. There are two basic strategies; a drug approach where siRNA is administered in its final form, or a gene therapy approach where precursor hairpin siRNAs are expressed from viral (or non-viral) vectors providing longer-term expression. The

first strategy is simpler and avoids problems associated with gene delivery, such as antibody-mediated vector neutralization, or restrictive effects of the intact nuclear membrane in non-mitosing cells.

However, effective delivery of therapeutic siRNA to disease target cells remains a major challenge. Although, local delivery can yield promising results, for example in the treatment of wet age-related macular degeneration involving local intravitreal injections with Bevasiranib (Acuity Pharmaceuticals), which is in Phase III clinical trials; delivery of siRNA to reach disseminated or body-wide targets faces particular challenges. These include inefficient cellular up-take, inadequate tissue

biodistribution, limited bioavailability and poor long-term stability of siRNA molecules in the blood stream and cytosol. A wide range of strategies have been evaluated for delivery of siRNA to non-local sites, including hydrodynamic injection (Lewis and Wolff, 2005; Lewis and Wolff, 2007), or covalent conjugation of siRNA molecules to; cholesterol (Soutschek et al, 2004), targeting peptides (Moschos et al, 2007) or antibodies leading to receptor mediated endocytosis (Song et al, 2005). Delivery can also be mediated by siRNA-binding vectors including lipids (Hassani et al, 2005; Bollerot et al, 2006; Santel et al, 2006), cationic polymers (Kataoka et al, 2005; Leng et al, 2007; Oishi et al, 2007) and a variety of other carriers (Minakuchi et al, 2004; Morrissey et al, 2005; Takeshita et al, 2005; Kim et al, 2006; Zimmermann et al, 2006). Despite the elegance of some of these approaches, critical assessment of utility is frequently confounded by the absence of effective systems for evaluation of successful siRNA delivery and activity.

Methods to determinate *in vivo* biodistribution of siRNA molecules include fluorescence (Dunne et al, 2003; Larson et al, 2007), radioactivity (van de Water et al, 2006; Zimmermann et al, 2006), or complexation with magnetic nanoparticles (Medarova et al, 2007). However, these techniques have limitations, notably the label may alter the properties of the vector, affecting the pharmacokinetics (PK), and catabolism of siRNA (and possible anabolism of the labelled moiety), that may impose a fairly short time window for useful interpretation of results. Furthermore, the use of radioactivity is becoming less popular, while the application of magnetic nanoparticles needs expensive and specialist equipment.

In addition, anatomical biodistribution of siRNA is not a good indicator of biological activity, since siRNA action is crucially dependent on transfer through the cell membrane. Techniques to assess siRNA activity (or 'pharmacodynamics') are dependent on the nature of the molecular target. Where siRNA targets mRNA encoding specific enzymes, pharmacodynamics can be assessed by measuring inhibition of enzyme activity in isolated organs, tissues or cells. Unfortunately, such assays are highly invasive, preventing time-resolved repeated 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. To address this transgenic reporter mice or disease models that ubiquitously or selectively express reporter genes such as GFP or luciferase, can be employed to test the silencing effect of siRNA delivery (Palliser et al, 2006). However, such systems are not ideal since models generating selective expression of the target mRNA in a specific organ will skew the data to give a desired outcome; whilst ubiquitously expressing 'negative-readout' models suffer from high levels of background with intrinsic poor signal to noise ratio.

There is therefore a need for an animal model that reveals successful siRNA delivery, effectively a pharmacodynamic reporter model. Ideally this would involve the use of non-invasive readouts that allow time-based

measurement of body-wide effects of siRNA. In order to facilitate this aim we have developed a positive-readout *in vitro* model in which RNAi activity is reported by luciferase expression. It is based on the well-characterized *E. coli lac* operon (Jacob and Monod, 1961), where 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 *lac* operon. Lactose 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 (Figure 1a), thus giving a positive readout of RNAi activity.

An inducible expression system based on the *lac* operon has recently been developed in mice (Scrabble and Stambrook, 1997; Cronin et al, 2001; Ryan and Scrabble, 2004). We demonstrate here that an inducible system based on the *lac* operon can be used to monitor RNAi activity and has the potential to be developed for use as a pharmacodynamic indicator of siRNA activity.

## MATERIALS AND METHODS

### Cell culture

SiHa human cervical squamous cell carcinoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma, Gillingham, UK), 1.0mM sodium pyruvate, 0.1mM non-essential amino acids, 50U/ml penicillin and 50µg/ml streptomycin. PC-3 human prostate adenocarcinoma cells were maintained in DMEM, 4.5g/l glucose, 2mM glutamine (PAA Laboratories GmbH, UK), supplemented with 10% (v/v) FCS.

### Design of siRNA against LacI repressor

The following siRNAs were designed using the Custom siRNA Design Tool provided by Dharmacon Inc (<http://www.dharmacon.com/DesignCenter/DesignCenterPage.asp>), an algorithm based on the guidelines of Reynolds et al (Reynolds et al, 2004), and were synthesized by MWG, London, UK. BLAST analysis was performed to eliminate sequences with off-target homologies.

#### *siRNA targeting lacI repressor mRNA (silacI):*

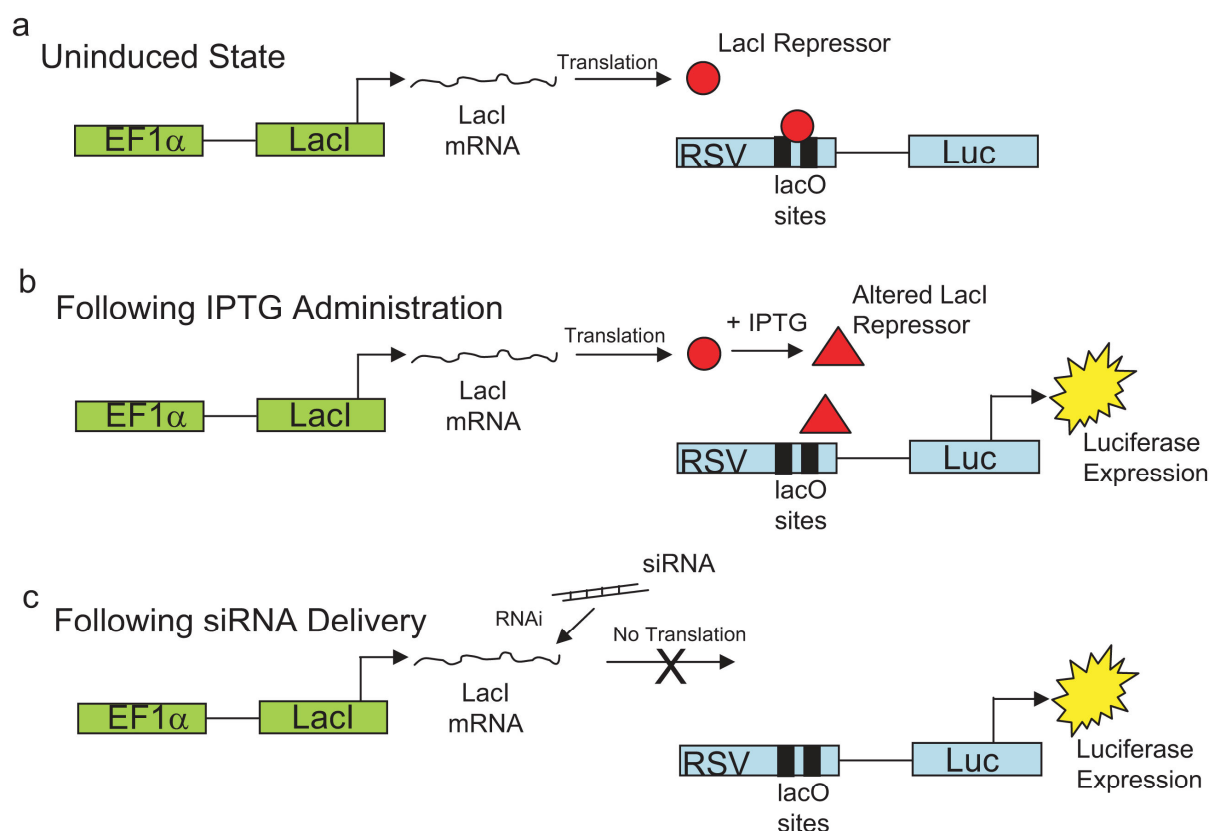
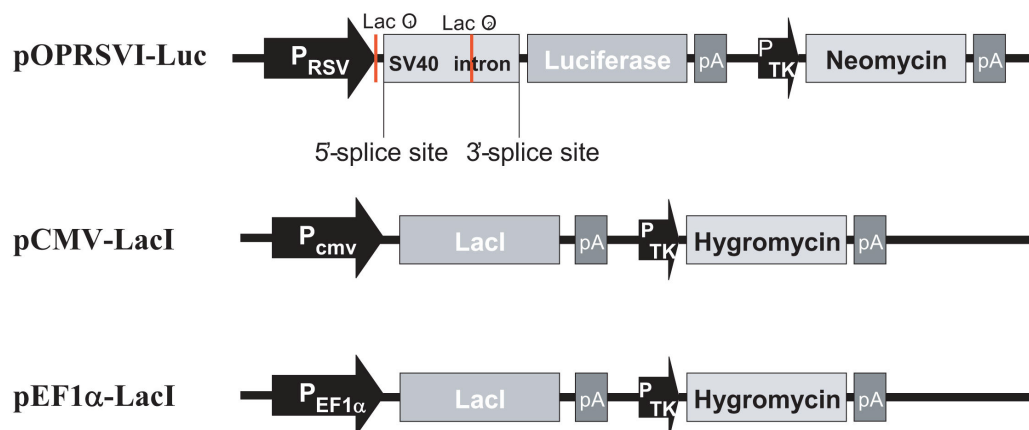
sense: 5'-AUAUCUCACUCGCAAUCAAdTdT-3',  
antisense: 5'UUGAUUGCGAGUGAGAUAUdTdT-3'

#### *siRNA targeting GFP (siGFP):*

sense: 5'-GCAAGCUGACCCUGAAGUUCAU-3',  
antisense: 5'-GAACUUCAGGGUCAGCUUGCCG-3'

### Construction of *lac* operator-repressor plasmid expression vectors

The *lac* operator-repressor system employed in this study comprises two components based on the LacSwitch II

**A:****B:**

**Figure 1. A.** Utilization of the *lac*-operon to generate a positive-readout reporter model for the detection of siRNA. **a**) In the uninduced state *lac* repressor (*lacI*) is transcribed and translated into protein (Lacl), which binds *lac* operators (*lacO*) located within the promoter and an SV40 intron upstream of the luciferase gene, suppressing reporter gene expression. **b**) IPTG alters the conformation of Lacl preventing it from binding to *lacO*, allowing RSV driven luciferase expression. **c**) siRNA specifically targeting *lacI* mRNA causes repressor protein levels to fall permitting RSV driven luciferase expression. **B.** Plasmid vectors employed in the inducible luciferase expression system. pOPRSVI-Luc was created by excision of the luciferase reporter gene from pGL-3 Basic by *Xba*I and *Xho*I restriction digestion and ligated into the multiple cloning site of pOPRSVI/MCS. pOPRSVI-Luc contains two *lac* operator binding sites (*lacO*<sub>1</sub> and *lacO*<sub>2</sub>) within the RSV promoter and the SV40 intron. pCMV-LacI (a component of the LacSwitch Inducible System) expresses *lac* repressor from a CMV promoter; pEF1 $\alpha$ LacI was created by replacing the CMV promoter with the human elongation factor 1 alpha (EF1 $\alpha$ ) promoter excised from pEF1 $\alpha$ Lux.G by *Afl*III and *Bal*II digestion.

Inducible Mammalian Expression System (Stratagene, La Jolla, CA, USA). Component one, the pCMV-LacI eukaryotic *lac* repressor expression vector, was modified by replacing the CMV promoter with the human elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoter excised from pEF1 $\alpha$ LUX.G (a gift from Dr Deborah Gill, University of Oxford) by *Afl*III and *Bal*III restriction digest. Component two, the pOPRSVI/MCS operator vector, comprises an RSV promoter into which are embedded two *lac* operator binding sites upstream of a multiple cloning site. The firefly luciferase gene was excised from pGL3-Basic (Promega, Southampton, UK) using *Xba*I and *Xho*I restriction enzymes and inserted into the MCS of pOPRSVI/MCS to generate the pOPRSVI-Luc reporter vector.

#### Transfection of lac-operator-repressor system

To confirm the inducible nature of the lac-operator-repressor expression system,  $5 \times 10^4$  SiHa cells were transfected in triplicate with 0.2 $\mu$ g pOPRSVI-Luc alone or together with either 0.2  $\mu$ g pEF1 $\alpha$ -LacI or various amounts (ranging from 0.02 to 0.2 $\mu$ g) of pCMV-LacI, using DOTAP liposomal transfection agent (Sigma-Aldrich, Gillingham, UK) (w:w ratio of 5:1) in serum free medium according to the manufacturer's instructions. Serum containing medium was added to cells after 4hr. After 39hr selected cells were treated with 5mM IPTG (EF1 $\alpha$ -LacI) or 25mM IPTG (pCMV-LacI). Forty eight hours after transfection cells were harvested and luciferase expression measured using Luciferase Assay Reagent (Promega, Southampton, UK) and a luminometer (Berthold, Harpenden, UK). Expression in each sample was normalized for total protein content.

#### Confirmation of lac repressor silencing by western blotting

To examine silencing from siRNA designed to target the lac repressor,  $5 \times 10^5$  PC-3 cells were transfected with 2 $\mu$ g pEF1 $\alpha$ -LacI using Lipofectamine (Invitrogen, Paisley, UK) according to the manufacturer's instructions. After 2hr 100nM siRNA targeting siLac or siGFP complexed with Oligofectamine (Invitrogen, Paisley, UK) was added to cells. Serum containing medium was added to cells 4hr later. Forty eight hours after transfection, total cellular protein was extracted using M-PER solution (Pierce Biotechnology, Rockford, IL, USA) containing protease inhibitor cocktail (Roche, Welwyn Garden City, UK). Protein concentration was determined by advanced protein assay reagent (Cytoskeleton, Denver, CO, USA); 30 $\mu$ g of protein was loaded on a 12% (w/v) SDS-PAGE gel. The proteins were transferred to a PVDF membrane and blocked in 5% (w/v) dried milk in Tris buffered saline pH 7.4, 0.1% (v/v) Tween 20 (TBST) at room temperature for 90min. The blot was then washed three times with TBST and incubated with mouse anti-LacI clone 9A5 primary antibody (Millipore UK Ltd, Watford, UK) diluted 1 in 1000 at room temperature for 2hr. The blot was then washed three times with TBST and incubated with goat anti-mouse IgG-HRP secondary antibody (Dako, Ely, UK) diluted 1 in 5000 at room temperature for 1hr. The blot was washed and the bands visualized by chemiluminescence using LumiGLO (KPL, Gaithersburg, MD, USA) on a ChemImager (Alpha Innotech Corp, San Leandro, CA,

USA), and analysed using Fluorchem 8000 software. Each blot was stripped and re-probed with sc-5286 anti- $\alpha$ -tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 as a protein loading control.

#### Restoration of luciferase activity following addition of siRNA against LacI repressor

To assess the effect of siLacI on the inducible reporter system,  $4 \times 10^4$  PC-3 cells were transfected with i) 200ng pOPRSVILuc, ii) 200ng pOPRSVILuc and 200ng pEF1 $\alpha$ LacI, or iii) 200ng pOPRSVILuc and 200ng pEF1 $\alpha$ LacI plus various doses of siRNA (siLacI or siGFP) ranging from 1 to 50nM final concentration, using Lipofectamine (Invitrogen, Paisley, UK). Serum containing medium was added to cells after 4hr. 5mM IPTG was added to various control wells 24hr prior to measuring luciferase activity which was measured 24, 48 or 72hr post transfection.

## RESULTS

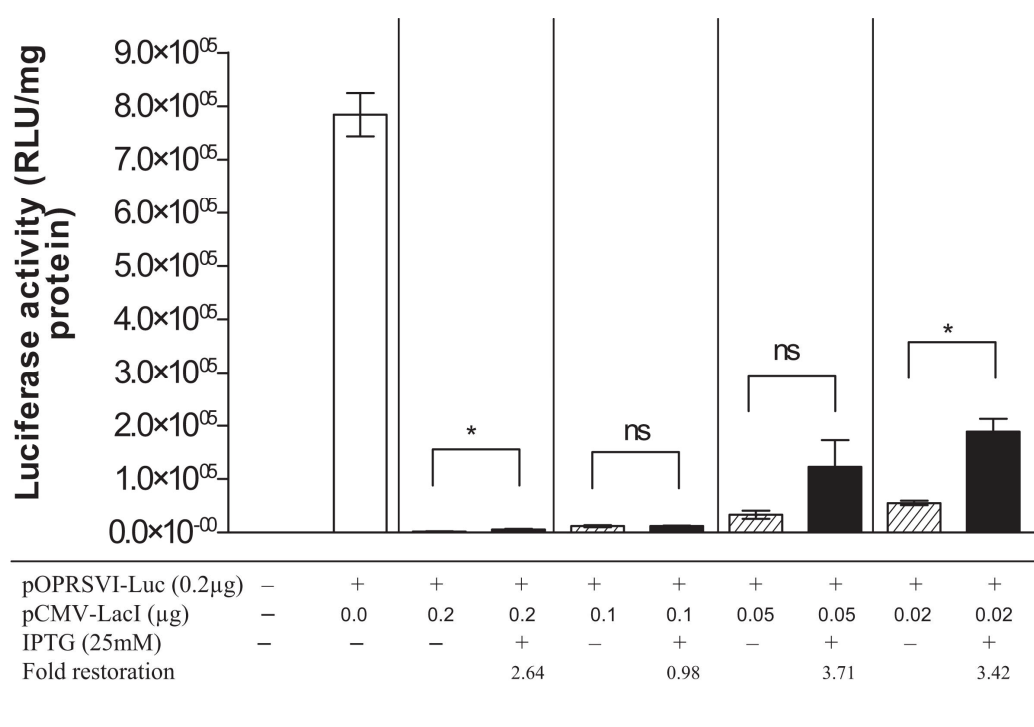
#### Generation of an inducible luciferase reporter system

To generate a functional inducible expression system, permissive to regulation by siRNA (Figure 1a), the amount of repressor protein generated inside cells is crucial. Too high and the siRNA will be ineffective, while too low and the system will be leaky with high background luciferase activity. Consequently both the optimal ratio of repressor plasmid to operator plasmid, and selection of a promoter of appropriate strength to drive *lacI*, must be determined.

Components of the LacSwitch II Inducible Mammalian Expression System (Stratagene, La Jolla, CA, USA) comprising the LacI-expression vector (pCMV-LacI) and *lac*-operator containing vector (pOPRSVI/MCS) were utilized for this study. Firefly luciferase was excised from pGL-3 Basic (Promega, Southampton, UK) and inserted into the multiple cloning site of pOPRSVI/MCS, such that it was under the control of the respiratory syncytial virus (RSV) promoter to generate pOPRSVI/Luc (Figure 1b).

Luciferase activity from pOPRSVI-Luc transfected SiHa cells was down-regulated in a dose-responsive manner when co-transfected with pCMV-LacI (Figure 2). Addition of the synthetic inducer isopropyl  $\beta$ -D-thiogalacto-pyranoside (IPTG) causes a conformational change in the repressor by decreasing its affinity for the operator. However 25mM IPTG had little effect on the level of luciferase expression when pCMV-LacI was delivered at doses greater than 20 ng. In fact even when pCMV-LacI was present at one tenth of the amount of pOPRSVI/Luc, the addition of IPTG restored only 25% of the luciferase activity generated in the absence of repressor. This would suggest that either the repressor protein is over-expressed when under the control of the CMV promoter for optimal inducibility, or the presence of the CMV promoter may affect expression from the RSV promoter in pOPRSVILuc (Farr, 1991).

We therefore generated a second repressor construct in which *lacI* was under the control of a weaker promoter, namely human elongation factor alpha (pEF1 $\alpha$ -LacI) (Figure 1b). Western blot analysis of transfected CaSki



**Figure 2.** Testing the inducible nature of the lac-operator-repressor system.  $5 \times 10^4$  SiHa cells were transfected in triplicate with either 0.2  $\mu$ g pOPRSVI-Luc alone or co-transfected with various amounts of pCMV-LacI as indicated. After 39 h selected cells were treated with 25 mM IPTG and incubated for a further 9 h prior to luciferase analysis. Statistical analysis was performed using the Kruskal-Wallis test and Mann Whitney tests due to heterogeneity of variances (\*,  $p < 0.05$ ; ns = not significant).

cells confirmed lower Lac expression from the pEF1 $\alpha$ LacI construct than CMVLacI (data not shown). Functional repression from pEF1 $\alpha$ LacI was shown following co-transfection of SiHa cells with an equal amount of pORSVI-Luc resulted in 5.7-fold lower luciferase expression compared with cells transfected with pORSVI-Luc alone (Figure 3). However the addition of only 5mM IPTG to co-transfected cells was sufficient to fully restore luciferase activity to the level observed in non-repressed cells. Co-transfection of cells with a two-fold excess of pEF1 $\alpha$ LacI did not result in any greater repression (data not shown).

#### Down regulation of Lac repressor protein by siRNA

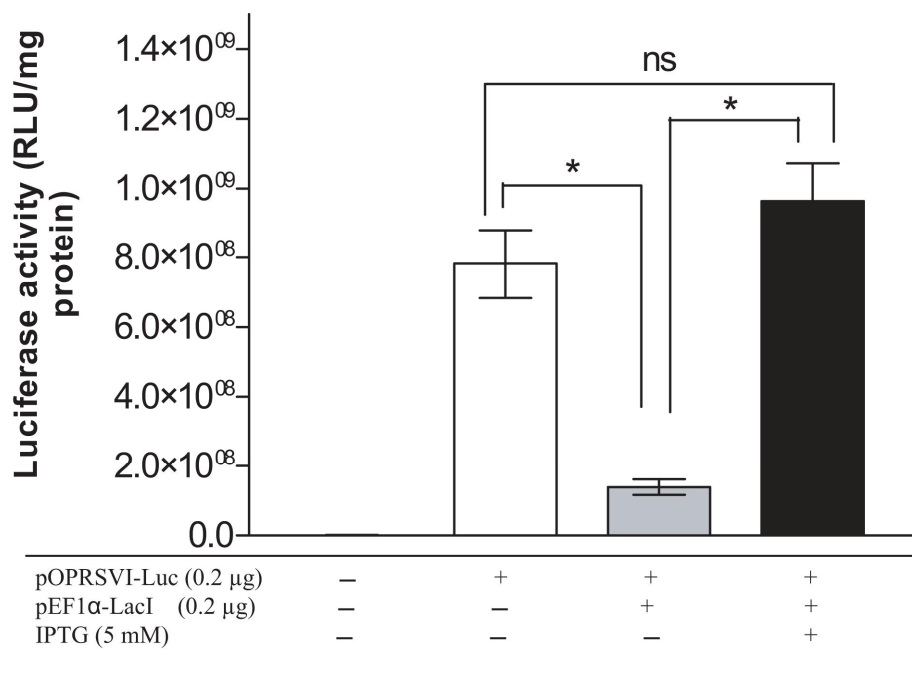
Having successfully generated an inducible luciferase expression system, siRNA targeting the lac repressor mRNA (siLacI) was designed and tested using PC-3 cells transfected with pEF1 $\alpha$ LacI. Two hours following plasmid delivery 100 nM siLacI or non-target siGFP was added to cells. The application of siLacI resulted in undetectable levels of repressor protein by western blotting after 48hr (Figure 4a). In contrast transfection of siGFP had only a minor effect on the level of repressor protein.

#### siLac restores luciferase activity in the inducible system

Having demonstrated the siRNA was functional in down regulating the target LacI repressor protein, we co-administered siLacI to PC-3 cells transfected with the two components of the inducible expression system in an

attempt to induce luciferase activity. siRNA and plasmid DNA were prepared in the same liposomal complex to ensure cells received both nucleic acids. The co-delivery of the repressor plasmid with the reporter plasmid reduced luciferase expression, while addition of 5mM IPTG fully alleviated repression as previously described (Figure 4b). IPTG had no effect on luciferase expression from cells transfected with pOPRSVILuc alone. Delivery of 1nM siLacI was sufficient to fully restore luciferase activity compared with cells transfected with the inducible system alone. In contrast delivery of a siRNA targeting GFP failed to increase luciferase activity at low doses, although non-specific activity was observed at 50nM siGFP. These data confirm that the induced luciferase activity was due to a sequence specific down-regulation of the Lac repressor.

Luciferase has a half life of 3-4hr in mammalian cells, consequently reduced mRNA levels are rapidly converted into lower protein levels and as a result it is a more dynamic indicator than longer lived reporters such as green fluorescent protein. This is important for time based measurements since once siLacI is exhausted the effects can be detected by the rapid disappearance of luciferase signals. Indeed following IPTG induction or siLacI delivery to cells transfected with the inducible system, luciferase expression decreased rapidly between 48 and 72hr (Figure 4c). This decline in luciferase activity suggests that the level of lac repressor inside cells is being re-established and is therefore available to occupy *lacO* sites repressing luciferase activity.



**Figure 3.** Luciferase activity repressed by EF1 $\alpha$ -LacI expression can be fully restored by IPTG induction.  $5 \times 10^4$  SiHa cells were transfected with 0.2  $\mu$ g pOPRSVI-Luc alone or co-transfected with 0.2  $\mu$ g pEF1 $\alpha$ -LacI. After 39hr selected cells were treated with 5mM IPTG for 9hr prior to analysing cells for luciferase activity. Statistical analysis was performed using the Kruskal-Wallis test and Mann Whitney tests due to heterogeneity of variances (\*,  $p < 0.05$ ; ns = not significant).

## DISCUSSION

By employing the lac operon system we have successfully induced luciferase expression following application of siRNA targeting *lacI* mRNA, and thus generated a positive readout from RNA interference. Correct functioning of the regulatory system was achieved using the relatively weak EF1 $\alpha$  promoter to drive repressor protein expression, with complete restoration of luciferase expression following IPTG administration. In contrast the CMV promoter driven repressor resulted in only 25% restoration on IPTG addition, suggesting LacI overexpression or the presence of the CMV promoter affected expression from the RSV promoter (Farr, 1991). This issue could be resolved with the use of an internal control such as a Renilla luciferase driven from the same RSV promoter. The signal to noise ratio (*i.e.*, the difference in expression when fully induced by IPTG and the uninduced state) increased from 3.7 for CMV to 6.9 for EF1 $\alpha$ .

siLacI potently silenced repressor protein expression when delivered to cells 2hr after DNA delivery, albeit at high concentration (100nM) (Figure 4a). However when siRNA and DNA were delivered in the same complex in order to ensure the same cells were transfected, siLacI concentration as low as 1nM fully restored luciferase activity (Figure 4b). This demonstrates efficacy at the low siRNA concentrations that would be expected in target cells following *in vivo* delivery. It may be possible to further enhance silencing activity and specificity by

engineering the 3' UTR of the lac repressor with custom sites as targets for effective siRNAs.

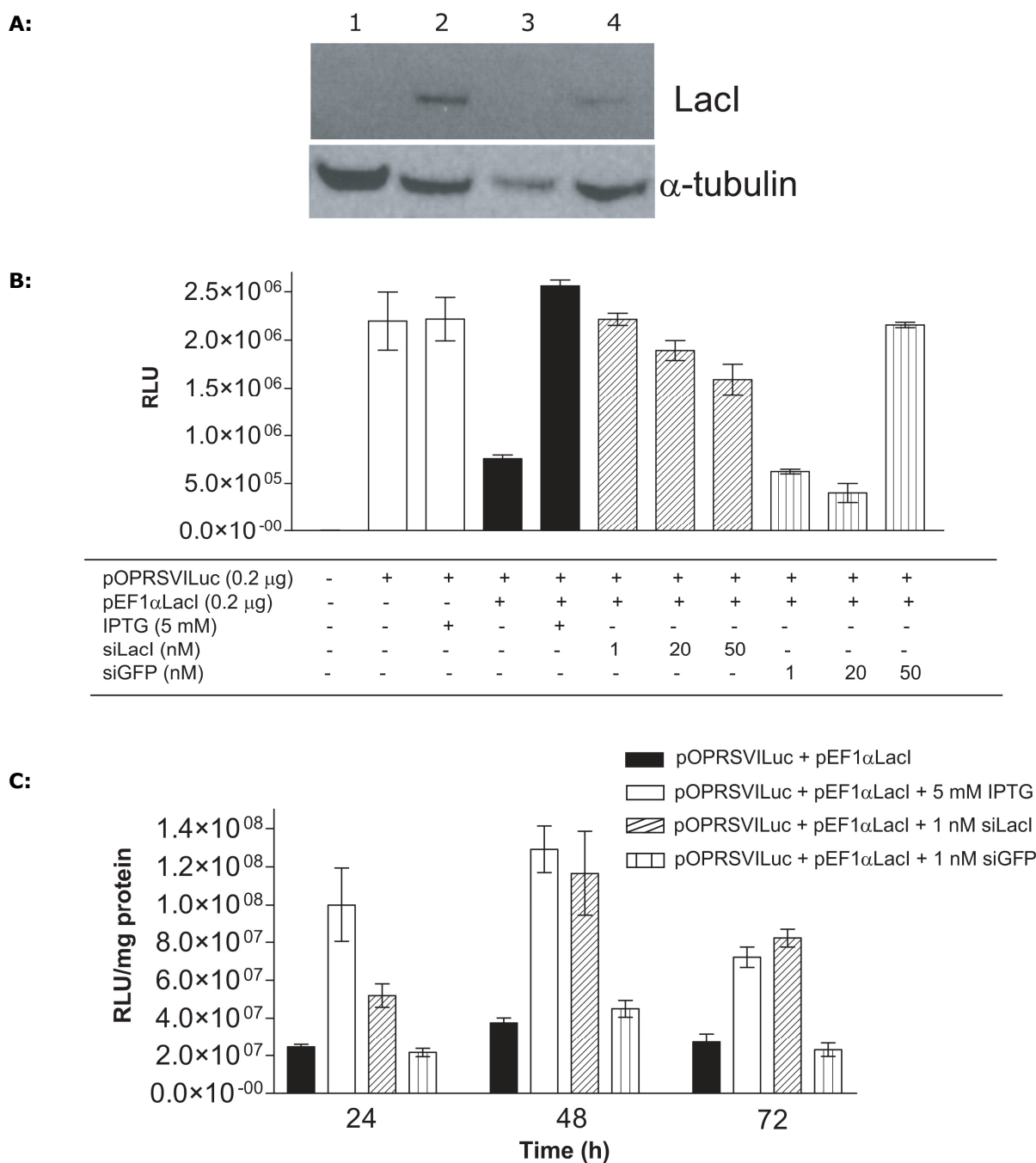
The fact that following a burst of luciferase activity on IPTG administration the luciferase activity fell rapidly over time, falling to around 50% of the level measured at 48hr at 72hr, demonstrates the short half-life of luciferase and its suitability as a dynamic indicator of siRNA activity inside cells.

Although the transient transfection studies demonstrated proof of principle using a *lac* operator-repressor based on bacterial sequences, the system fails in a transgenic mouse due to CpG methylation and silencing (Scrabble and Stambrook, 1997). Instead Cronin et al. have developed a mouse by modifying codon usage and structure such that the lac repressor transgene resembled a typical mammalian gene (Scrabble and Stambrook, 1997; Cronin et al, 2001). Scrabble and co-workers, following mammalianisation of the lac operon codon usage, generated two lines bearing either the repressor protein (driven from a 4.3kb fragment of human  $\beta$ -actin promoter containing a rabbit globin spacer, thus avoiding silencing of strong viral promoters in mice), or a tyrosinase transgene under the control of an inducible promoter. Subsequent crossing of the lines generated double transgenics, in which pigmentation of the mouse was controlled by administration of IPTG in the drinking water (Cronin et al, 2001). More recently, Ryan and Scrabble have successfully regulated a luciferase reporter gene with the *lac* operon system (Ryan and

Scrable, 2004). Luciferase was placed under the control of the Huntingdon promoter, HD being expressed ubiquitously throughout life, in which were embedded two lacO sites. Using the Ivis system (Xenogen) and a sensitive CCD camera to detect positive signals, allowed visualization of the dynamics of gene expression in real-time and in a non-invasive manner. According to Xenogen the camera is capable of detecting light  $<100$  photons/s/cm<sup>2</sup>. Since autoluminescence of mice is about 1000 photons/s, Xenogen estimate *in vivo* sensitivity is around 100 subcutaneous high-expressing transduced cells. Since one centimetre of depth will attenuate the signal 10 fold, sensitivity for deep tissue is around 1000 high

expressing cells. Where issues of sensitivity may be problematic, organs could be dissected and imaged outside the carcass, whilst alternative methods, such as immunohistochemistry for luciferase protein or protein extraction and western blotting could be employed to confirm findings.

We envisage such a system could be employed to report the silencing efficacy of siRNA and its real-time biodistribution. This reporter mouse would be an important tool providing important insights into the development novel siRNA delivery systems and improve the pipeline of molecules available for therapies.



**Figure 4.** Silencing of lac repressor restores luciferase expression. **A.**  $5 \times 10^5$  PC-3 cells were transfected with 2 µg of pEF1α-LacI vector using Lipofectamine. After 2hr 100nM siLacI or siGFP complexed with Oligofectamine were added to cells. Expression of

LacI was analysed 48hr after DNA delivery by western blotting using an anti-LacI antibody diluted 1/1000. Expression of  $\alpha$ -tubulin was determined using an anti- $\alpha$ -tubulin antibody diluted 1/1000 as a loading control. [Key: Lane 1; mock, Lane 2; pEF1 $\alpha$ LacI, Lane 3; pEF1 $\alpha$ LacI + siLacI, Lane 4; pEF1 $\alpha$ LacI + siGFP]. **B.**  $4 \times 10^4$  PC-3 cells were transfected with i) 0.2 $\mu$ g pOPRSVI-Luc alone (white bars), ii) 0.2 $\mu$ g pOPRSVILuc and 0.2 $\mu$ g pEF1 $\alpha$ -LacI (black bars) or iii) 0.2 $\mu$ g pOPRSVILuc, 0.2 $\mu$ g pEF1 $\alpha$ -LacI and various doses of siRNA as indicated (siLacI diagonal striped bars, siGFP vertical striped bars). Control cells received 5mM IPTG after 24hr and luciferase expression was measured after 48hr. **C.**  $4 \times 10^4$  PC-3 cells were co-transfected with 0.2 $\mu$ g pOPRSVI-Luc and 0.2 $\mu$ g pEF1 $\alpha$ -LacI in the presence or absence of siRNA. Selected cells were exposed to 1nM siLacI or 1nM siGFP and luciferase activity measured at the indicated time point after siRNA delivery.

## ACKNOWLEDGMENTS

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## COMPETING INTERESTS

None declared.

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