A tightly regulated and reversibly inducible siRNA expression system for conditional RNAi-mediated gene silencing in mammalian cells

Ren-Huang Wu,[1] Tsung-Lin Cheng,[2] San-Ren Lo,[1] Hui-Chun Hsu,[1] Chuan-Fu Hung,[1] Chiao-Fang Teng,[1] Ming-Ping Wu,[3,4] Wen-Hui Tsai,[3,5] Wen-Tsan Chang[1,2]*

[1]*Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC*

[2]*Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC*

[3]*Institute of Clinical Medicine, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC*

[4]*Department of Obstetrics and Gynecology, Chi Mei Foundation Medical Center, Tainan 710, Taiwan, ROC*

[5]*Department of Pediatrics, Chi Mei Foundation Medical Center, Tainan 710, Taiwan, ROC*

*Correspondence to:
Wen-Tsan Chang, Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC. E-mail: wtchang@mail.ncku.edu.tw

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Abstract

Background RNA interference (RNAi) is a powerful and widely used gene silencing strategy for studying gene function in mammalian cells. Transient or constitutive expression of either small interfering RNA (siRNA) or short hairpin RNA (shRNA) results in temporal or persistent inhibition of gene expression, respectively. A tightly regulated and reversibly inducible RNAi-mediated gene silencing approach could conditionally control gene expression in a temporal or spatial manner that provides an extremely useful tool for studying gene function involved in cell growth, survival and development.

Material and methods In this study, we have developed a lactose analog isopropyl thiogalactose (IPTG)-responsive *lac* repressor-operator-controlled RNA polymerase III (Pol III)-dependent human RNase P RNA (H1) promoter-driven inducible siRNA expression system. To demonstrate its tight regulation, efficient induction and reversible inhibition, we have used this system to conditionally control the expression of firefly luciferase and human tumor suppressor protein p53 in both transient transfection cells and established stable clones.

Results The results showed that this inducible siRNA expression system could efficiently induce conditional inhibition of these two genes in a dose- and time-dependent manner by administration of the inducing agent IPTG as well as being fully reverted after withdrawal of IPTG. In particular, this system could conditionally inhibit the expression of both the genes in not only established stable clones but also transient transfection cells, which should greatly increase its usefulness and convenience.

Conclusions The results presented in this study clearly indicate that this inducible siRNA expression system could efficiently, conditionally and reversibly inhibit gene expression with only very low or undetectable background silencing effects under non-inducing condition. Thus, this inducible siRNA expression system provides an ideal genetic switcher allowing the inducible and reversible control of specific gene activity in mammalian cells.

Keywords conditional RNAi system; inducible siRNA expression system; human H1 promoter; *lac* repressor-operator; IPTG; p53

Introduction

RNA interference (RNAi) is an evolutionarily conserved mechanism of post-transcriptional gene silencing induced by double-stranded RNAs (dsRNAs)

including small interfering RNA (siRNA) or short hairpin RNA (shRNA) [1,2]. As compared with other gene inhibitory molecules, such as antisense RNAs and oligonucleotides (ODNs), ribozymes and DNAzymes, dsRNAs have apparently become the most powerful and widely used gene silencing reagents for inhibiting gene activity in mammalian cells [3]. There are practically two strategies in producing active dsRNAs in mammalian cells, exogenous delivery of synthetic siRNAs or shRNAs and endogenous vector-expressed shRNAs or siRNAs formed by annealing two complementary sense and antisense RNAs [4–14]. Among these approaches, the inhibition effect induced by applying synthetic siRNAs or shRNAs is transient short-term and reactivation of the target gene activity normally occurs after a few days [3,6,7]. In contrast, both siRNAs and shRNAs transcribed from endogenous expression cassettes introduced by either naked plasmids or virus vehicles can induce stable long-term and heritable target gene silencing [3,8,15]. Endogenous expression of both siRNAs and shRNAs can be efficiently transcribed from either RNA polymerase (Pol) II- or Pol III-dependent promoters [16]. Currently, the Pol III-dependent RNase P RNA (H1) and U6 small nuclear RNA (U6) promoters from human and mouse have been used most frequently to drive the transient or constitutive expression of shRNAs or siRNAs [8–14]. Thus, the RNAi technique has currently become the most popular strategy for manipulating specific gene expression in mammalian cells.

The constitutive and ubiquitous siRNA and shRNA expression vectors suffer from a limitation in studying gene function involved in cell survival, growth and development. This limitation has prompted the construction of inducible gene silencing systems based on a conditional RNAi mechanism. Conditional RNAi-mediated gene silencing can be established by inducible siRNA or shRNA expression from a controllable RNA Pol III- or Pol II-dependent promoter that allows external regulation of its activity. Currently, two different conditional RNAi strategies, reversible and irreversible conditional systems, have been established [17]. The irreversible systems are based on Cre/*loxP* site-specific recombination-mediated conditional activation or inactivation of RNAi activity [18–22]. In contrast, the reversible systems are constructed by using steric hindrance, transactivation or epigenetic repression mechanisms that are regulated by specific chemical compounds such as doxycycline (tetracycline) and isopropyl thiogalactose (IPTG) [23–35]. These inducible RNAi-based gene silencing strategies have broadened the utilities of RNAi technology in studying gene function. However, none of these conditional RNAi systems have the optimal combination of elements and functions for tight regulation, efficient induction and complete reversibility of shRNA or siRNA expression in mammalian cells. For instance, several distinct Cre/*loxP* site-specific recombination-mediated inducible shRNA expression approaches are functional in either switching on or off control, but lack reversible control of target gene expression. In addition, several different schemes for both the tet-responsive Tet-repressor-operator- and IPTG-responsive *lac* repressor-operator-mediated inducible shRNA expression suffer from the low levels of background transcription in the non-inducing condition. Therefore, construction of an inducible shRNA or siRNA expression system that fulfills these functional criteria is warranted.

The *lac* (lactose) operon is probably the best analyzed transcriptional regulation system and has been widely applied for conditional regulation of gene expression in many model systems under control of the non-hydrolyzable lactose analog IPTG [36]. The regulatory components of this system comprise the *lac* repressor LacI and its DNA-binding sequences, the *lac* operators (*lacOs*), which include one major binding site $lacO_1$ and two minor binding sites $lacO_2$ and $lacO_3$. In the non-inducing condition, the LacI inhibits transcription by binding as a homotetramer to the *lacOs* located downstream of the *lac* promoter. In contrast, in the inducing condition, the binding of LacI to the *lacOs* is relieved, resulting from a conformational change in the LacI and allowing RNA polymerase to initiate transcription [37–39]. Previous studies have shown that a completely symmetric oligonucleotide sequence, containing an inverted repeat of a 15-bp segment from the left half of the natural $lacO_1$ sequence, binds the LacI tenfold more tightly than does the natural $lacO_1$ [40]. In addition, the toxicity, uptake capability and metabolic rate of *lac* inducers, as well as the *in vivo* clearance of inducers, have been intensively analyzed in mammalian cells and whole animals [41,42]. Thus, direct control of mammalian promoters by the IPTG-responsive *lac* repressor-operator system provides tight, inducible regulation and the promise of conditional control of the gene expression [43,44].

To overcome the leakiness associated with the current inducible shRNA expression vectors and further construct a fully reversible conditional RNAi-mediated gene silencing technique, we have developed an inducible siRNA expression system that could conditionally regulate specific gene expression by exogenous administration of an inducing agent. This system was established by using the IPTG-responsive *lac* repressor-operator-engineered human H1 promoter derivatives to drive expression of both the complementary sense- and antisense-RNA transcripts separately. The major component of this system composed of two functional equivalent inducible siRNA expression vectors, $p(H1O_1)_2$ and $p(H1O_{212})_2$, which contained two copies of the symmetric *lac* operators, $lacO_{1S}$ and $lacO_{2S}$, engineered human H1 promoter derivatives, $H1O_1$ and $H1O_{212}$, respectively. To demonstrate the tight regulation, efficient induction and reversible inhibition, we have used this system to conditionally control the expression of firefly luciferase and human tumor suppressor protein p53. The results clearly showed that the expression of both the firefly luciferase and p53 could be conditionally regulated by the inducible siLuc and sip53 expression vectors

in both transient transfection cells and established stable clones. The inhibition patterns clearly exhibited a dose- and time-dependent manner by administration of the inducing agent IPTG as well as could be fully reverted after withdrawal of IPTG. In particular, this inducible siRNA expression system overcame the leaky defect observed in the current inducible shRNA expression vectors and exhibited only very low or undetectable background silencing effects in the non-inducing condition. Development of a tightly regulated, efficiently inductive and reversibly inducible siRNA expression system by simply modifying the existing inducible shRNA expression vectors provides a simple and easy way to use RNAi-mediated gene silencing techniques to study gene function in a temporal or spatial manner.

Materials and methods

Cell culture

BHK (baby hamster kidney fibroblast cell line) and HEK293 (human embryonic kidney epithelial cell line) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel) and 1% antibiotic/antimycotic solution (GIBCO BRL) at 37 °C in a humidified incubator with 5% CO_2. The stable *lac* repressor protein LacI and inducible sip53 expression clones were grown and maintained in growth medium containing 300 μg/ml of hygromycin B and 5 μg/ml of blasticidin S, respectively. The cell lines and clones were routinely subcultured two to three times a week after treatment with 0.1% trypsin (Biowhittaker Acambrex, Walkersville, MD, USA).

Construction of lac operator-repressor-based inducible shRNA and siRNA expression vectors

Plasmid vectors were constructed by using standard molecular cloning techniques. The constitutive shRNA and siRNA expression vectors, pHsH1 (Figure 1C) and $p(H1)_2$ (Figure 1D), have been described previously [45]. The inducible shRNA expression vectors, pH1O_1, pH1O_{12}, pH1O_{212}, and pH1O_{22} (Figure 1C), containing one or two copies of *lac* operator sequences, $lacO_{1S}$ (Figure 1E) located downstream of the TATA box and/or $lacO_{2S}$ (Figure 1E) located down- or upstream of the promoter as indicated, were constructed by inserting the $lacO_{1S}$ and $lacO_{2S}$ sequences into the pHsH1 vector by a polymerase chain reaction (PCR)-based cloning method. The inducible siRNA expression vectors, $p(H1O_1)_2$ and $p(H1O_{212})_2$ (Figure 1D), containing two tandem repeats of the H1O_1 and H1O_{212} expression cassette, were constructed by inserting the H1O_1 and H1O_{212} DNA fragments from pH1O_1 and pH1O_{212} into the pH1O_1 and pH1O_{212} vectors, respectively.

Construction of constitutive and inducible shRNA and siRNA expression vectors

A general strategy for constructing shRNA and siRNA expression vectors involved ligating an annealed oligonucleotide duplex into the *Cla*I/*Hin*dIII restriction site of the constitutive and inducible shRNA or siRNA expression vectors. Oligonucleotides formed from a short synthetic DNA fragment targeting the firefly luciferase or p53 genes were purchased from commercial suppliers. Sequences of the synthetic DNA oligonucleotides used in this study have been described previously [45].

Construction of firefly luciferase and p53 expression vectors

Construction of the firefly luciferase expression vector pCMV-FL/RL and tumor suppressor protein p53 expression vector pCMV-p53/EGFP has been described previously (Figure 1A) [45]. The dual-luciferase reporter plasmid pCMV-FL/RL contained the firefly luciferase (*Fluc*+) and *Renilla* luciferase (*Rluc*+) expression cassettes, and pCMV-p53/EGFP contained the p53 (*p53*) and enhanced green fluorescent protein (*EGFP*) expression cassettes. The *Rluc*+ and *EGFP* expression cassettes served as reference protein expression systems for either transfection efficiency or loading control.

Transfection and luciferase assay

Twenty-four hours before transfection, cells were seeded in 6-well culture plates at 1×10^5 cells per well. The cells were cotransfected with 0.5 μg of the RNAi targeting vectors and 1.5 μg of the constitutive or inducible shRNA or siRNA expression vectors, or transfected with 2 μg of the inducible shRNA or siRNA expression vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfected cells were treated with 5 mM IPTG or indicated concentrations of IPTG for 48 h or indicated time courses. The luciferase-expressed cells were harvested and aliquots of the cell lysates containing equal amounts of protein were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) as described by the manufacturer. The EGFP- and p53-expressed cells were examined by inverted fluorescent microscopy or quantified by Western blot analyses. The total protein in the cell lysates was determined by the BCA assay (Pierce, Rockford, IL, USA) as recommended by the manufacturer.

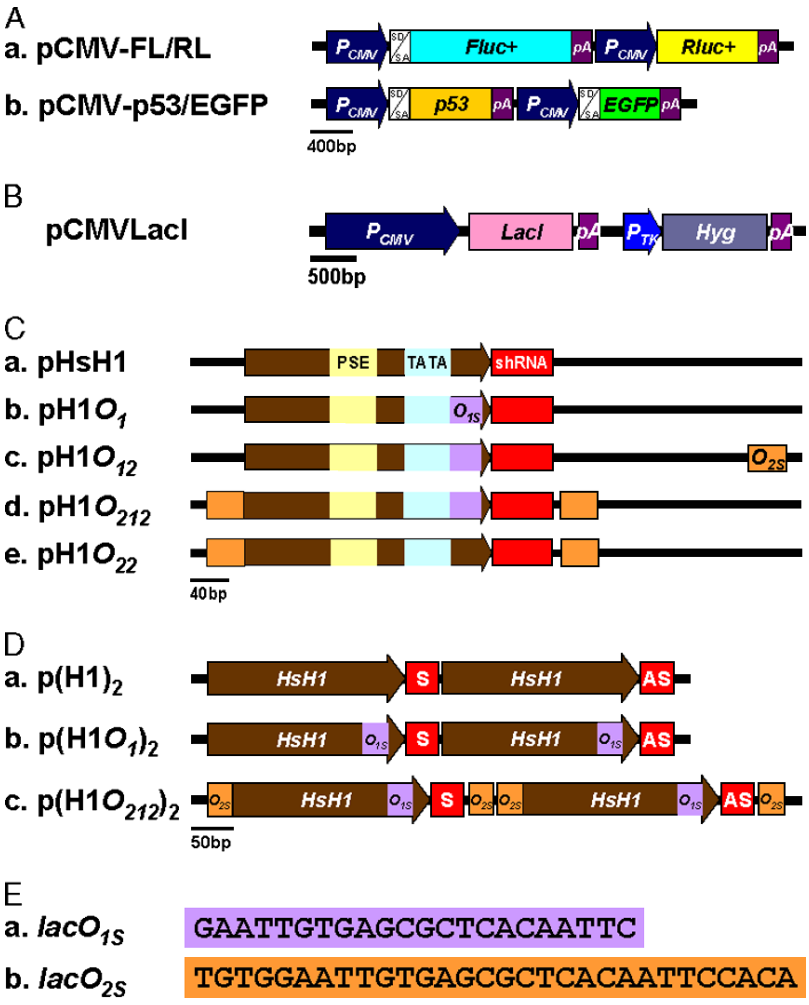

Figure 1. RNAi target vectors and inducible shRNA and siRNA expression vectors. (A) Constructs of the RNAi target vectors. The dual-luciferase reporter vector pCMV-FL/RL (a) contained both the firefly luciferase (*Fluc*+) and *Renilla* luciferase (*Rluc*+) expression cassettes, and the human tumor suppressor protein p53 expression vector pCMV-p53/EGFP (b) contained the *p53* and enhanced green fluorescent protein (*EGFP*) expression cassettes. The *Rluc*+ and *EGFP* expression cassettes serve as reference protein expression systems for the RNAi target vectors, pCMV-FL/RL and pCMV-p53/EGFP, respectively. (B) Construct of the *lac* repressor protein LacI expression vector. The LacI expression vector pCMVLacI contained the *LacI* expression cassette and in particular the hygromycin B resistance gene (*Hyg*) expression cassette that functions as a dominant selectable marker. (C) Constructs of the inducible shRNA expression vectors. The human H1 promoter-driven constitutive shRNA expression vector pHsH1 (a) and IPTG responsive *lac* repressor-operator-controlled human H1 promoter derivative-driven inducible shRNA expression vectors, including pH1O_1 (b), pH1O_{12} (c), pH1O_{212} (d), and pH1O_{22} (e), which contain either one or two copies of symmetric *lac* operator sequences, *lac*O_{1S} (O_{1S}) located downstream of the TATA box and *lac*O_{2S} (O_{2S}) located down- or upstream of the promoter as indicated. (D) Constructs of the inducible siRNA expression vectors. The human H1 promoter-driven constitutive siRNA expression vector p$(H1)_2$ (a) and IPTG-responsive *lac* repressor-operator-controlled human H1 promoter derivative-driven inducible siRNA expression vectors, including p$(H1O_1)_2$ (b) and p$(H1O_{212})_2$ (c), which contain two tandem copies of the H1, H1O_1 or H1O_{212} expression cassette to drive independently the expression of sense and antisense RNAs. (E) Nucleotide sequence of the *lac* operators. The *lac*O_{1S} and *lac*O_{2S} are two completely symmetric *lac* operator variants 22 nucleotides (nt) and 30 nt in length, respectively

Western blot analysis of LacI, EGFP, p53 and β-actin

At 48 h or indicated time courses after transfection of established stable clones, cells were directly lysed on 6-well culture plates in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% NP-40) containing protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). Total protein extracts were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA), and incubated with anti-LacI monoclonal antibody (clone 9A5; Upstate Cell Signaling Solutions, Lake Placid, NY, USA), anti-GFP (B-2), anti-p53 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin monoclonal antibody (Sigma Chemicals, St. Louis, MO, USA), followed by incubation with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The bands were detected by using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and analyzed by using the image processing program ImageJ [46].

Immunohistochemical staining analysis of LacI

Cells that stably expressed the LacI protein grown on glass coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunohistochemical staining was performed according to standard procedures. LacI was detected with anti-LacI monoclonal antibody (clone 9A5; Upstate Cell Signaling Solutions). Secondary antibody used was biotinylated anti-mouse and then incubated with peroxidase-conjugated streptoavidin (DAKO, Carpinteria, CA, USA). Peroxidase staining of red color was developed by aminoethyl carbazole substrate (Zymed Laboratories, San Francisco, CA, USA).

Results

Strategy and experimental design of the IPTG-responsive lac repressor-operator-controlled human H1 promoter-driven inducible shRNA expression system

The effectors of RNAi-mediated gene silencing including shRNAs and siRNAs can be either chemically synthesized and exogenously introduced into cells or endogenously expressed from transfected plasmids or infected viruses in nuclei. In addition, previous studies have demonstrated that both shRNAs and siRNAs can efficiently induce gene silencing, whereas neither the sense- nor antisense-RNA transcripts alone had an inhibition effect on gene expression [45]. These studies clearly indicated that the control of dsRNA production, either shRNAs or siRNAs, can manipulate the induction of RNAi-mediated gene silencing. Thus, to establish the conditional RNAi system, an exogenously controllable expression cassette can be used for reversibly inducible shRNA or siRNA transcription.

Among the widely used RNA Pol II- and RNA Pol III-dependent small RNA expression cassettes, the human H1 promoter has been used increasingly for constitutive and ubiquitous shRNA and siRNA expression, resulting in persistent and stable gene silencing activity. In addition, this promoter has not only been analyzed in great detail but also applied for conditional expression of genes [23,27,31,34]. The controllable IPTG-responsive *lac* repressor-operator system, consisting of a *lac* repressor expression cassette and a *lac* operator-engineered promoter-driven target gene expression cassette, is functional *in vitro* and *in vivo* in mammalian systems [43,44]. Induction of the repressed IPTG-responsive *lac* repressor-operator-controlled promoters is easily achieved by administration of a gratuitous synthetic inducing agent IPTG, which efficiently binds to the repressor and causes a conformational change that effectively reduces the affinity of the repressor for the operator [41,47].

To develop a tightly regulated and reversibly inducible RNAi-mediated gene silencing system, we have attempted to construct an IPTG-responsive *lac* repressor-operator-controlled RNA Pol III-dependent promoter-driven inducible shRNA expression cassette based on the LacI tightly bound symmetric *lac* operator-engineered human H1 promoter derivatives. By engineering one copy of the symmetric *lac* operator $lacO_{1S}$ (Figure 1E) downstream of the TATA box and/or one or two copies of the symmetric *lac* operator $lacO_{2S}$ (Figure 1E) flanking the promoter, we have constructed a series of different inducible shRNA expression vectors, including pH1O_1, pH1O_{12}, pH1O_{212}, and pH1O_{22} (Figure 1C), according to the locations of the LacI major binding site $lacO_1$ and the minor binding site $lacO_2$ in the *lac* promoter [36–39]. In addition, we have also used a constitutively active *lac* repressor expression vector pCMVLacI that not only contains the *LacI* gene expression cassette driven by the cytomegalovirus (CMV) promoter (P_{CMV}), but also includes a ubiquitously selectable marker hygromycin B resistance gene (*Hyg*) expression cassette driven by the HSV-thymidine kinase (TK) promoter (P_{TK}) (Figure 1B).

Evaluation of the IPTG-responsive lac repressor-operator-controlled human H1 promoter-driven inducible shRNA expression vectors

To test the IPTG-responsive *lac* repressor-operator-controlled conditional RNAi system, we have established two different cell lines, BHK and HEK293, which stably expressed the *lac* repressor LacI. Both the BHK and HEK293 cells were first transfected with the LacI expression vector pCMVLacI, then selected with antibiotic hygromycin B for the drug-resistant colonies, and further screened for stable clones that constitutively expressed LacI by Western blot analysis. In addition, intracellular localization of the LacI in the isolated stable clones was examined by immunohistochemical staining. As the results in Figure 2 show, including BHK-LacI-5, 11, 25 and 30 (Figure 2A), as well as HEK293-LacI-4, 11, 13 and 16 (Figure 2B), a total of eight clones exhibited stable expression of LacI at different levels, whereas the control clones, such as BHK-31 and 35 (Figure 2A), as well as HEK293-3 and 5 (Figure 2B), displayed no detectable LacI expression. Among these eight clones, the BHK-LacI-5 and 30, as well as HEK293-LacI-4 and 16 clones, particularly exhibited high levels of LacI expression. In addition, immunohistochemical staining of both the BHK-LacI-5 (Figure 2A) and HEK293-LacI-4 (Figure 2B) clones revealed that the LacI was localized in the nucleus.

To evaluate the performance and efficiency of these inducible shRNA expression vectors, we first applied these four vectors to conditionally control the expression of firefly luciferase. Both the LacI expression stable clones, BHK-LacI-5 and 30, were cotransfected with the firefly luciferase expression vector pCMV-FL/RL and the inducible shLuc expression vector, pH1O_1-,

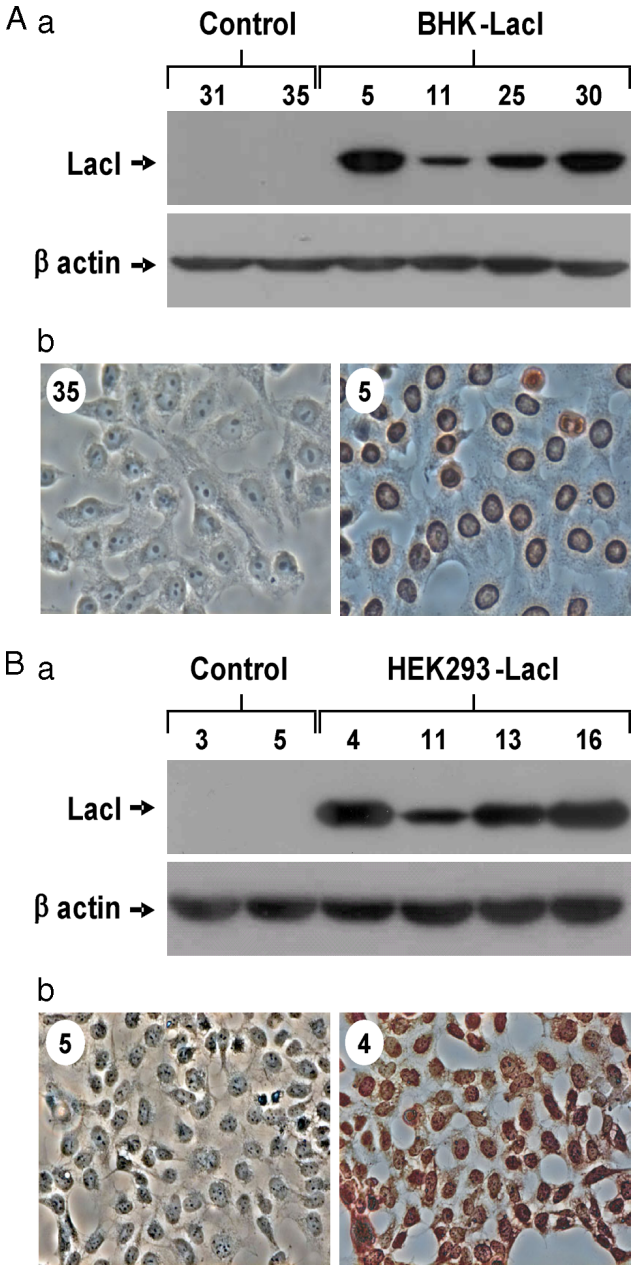

Figure 2. Analyses of the *lac* repressor protein LacI expression in mammalian cells. The pCMVLacI-induced LacI expression in BHK (A) and HEK293 (B) cells by Western blot (a) and immunohistochemical staining (b) analyses. BHK and HEK293 cells were first transfected with 2 μg of the LacI expression vector pCMVLacI by Lipofectamine 2000. After 48 h incubation, the transfected cells were treated with 300 μg/ml of hygromycin B and then selected for the drug-resistant colonies. The drug-resistant transfectants were further screened for expression of the LacI by Western blotting analysis and were also examined for intracellular localization of LacI by immunohistochemical staining analysis. The levels of *β*-actin serve as reference protein for loading control

pH1O_{12}-, pH1O_{212}- or pH1O_{22}-shLuc, or constitutive shLuc expression vector pHsH1-shLuc, and then treated with or without inducing agent (5 mM IPTG). After 48 h incubation, the expression activities of both the firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. As the results in Figure 3 show, in both the BHK-LacI-5 and 30 cells, all four constructs, pH1O_1-, pH1O_{12}-, pH1O_{212}- and pH1O_{22}-shLuc, exhibited significant inhibition effects at different levels on pCMV-FL/RL-induced firefly luciferase expression in the absence of IPTG. In particular, as compared with the pHsH1-shLuc construct, the pH1O_{22}-shLuc construct displayed a strong inhibition effect on pCMV-FL/RL-induced firefly luciferase expression. In the presence of IPTG, as compared with the pHsH1-shLuc construct, all four constructs, pH1O_1-, pH1O_{12}-, pH1O_{212}- and pH1O_{22}-shLuc, exhibited high inhibition effects at similar levels on pCMV-FL/RL-induced firefly luciferase expression. These results clearly indicated that the three vectors, pH1O_1, pH1O_{12} and pH1O_{212}, have varying levels of inhibition effects in the non-inducing condition but possess high levels of inhibition effects in the inducing condition, whereas both the pHsH1 and pH1O_{22} vectors have high levels of inhibition effects in both the inducing and non-inducing conditions.

Construction and evaluation of the IPTG-responsive lac repressor-operator-controlled human H1 promoter-driven inducible siRNA expression system

Since a highly effective shRNA could induce strong RNAi-mediated gene silencing even in the presence of a very small number of shRNAs, the leakiness of an inducible shRNA expression system could produce a significant number of shRNAs that may trigger inhibition of target gene expression at different levels. In addition, previous studies have demonstrated that RNAi-mediated gene silencing could only be induced by the presence of dsRNAs but could not be triggered by the existence of either sense- or antisense RNAs alone [45]. Thus, reduction of the dsRNA production or restriction of the sense- and antisense RNAs coexistence could decrease or prevent induction of RNAi-mediated gene silencing. To overcome the leakiness associated with these inducible shRNA expression vectors, we have modified and redesigned a tightly regulated and reversibly inducible siRNA expression system that exhibited very low or undetectable background inhibition effects. By using both the IPTG-responsive *lac* repressor-operator-controlled H1O_1 and H1O_{212} expression cassettes, we have reconstructed two inducible siRNA expression vectors, p(H1O_1)$_2$ and p(H1O_{212})$_2$ (Figure 1C), which contained two tandem repeated H1O_1 or H1O_{212} expression cassettes to drive independently the expression of sense- and antisense-RNA transcripts. In the presence of a large number of *lac* repressor LacI, at least one or both expression cassettes can be bound and repressed by LacI, resulting in very low or undetectable basal transcriptional activity.

To examine the inhibition efficacy of this inducible siRNA expression system, we have first used these two inducible siRNA expression vectors to inhibit the expression of firefly luciferase. BHK cells were

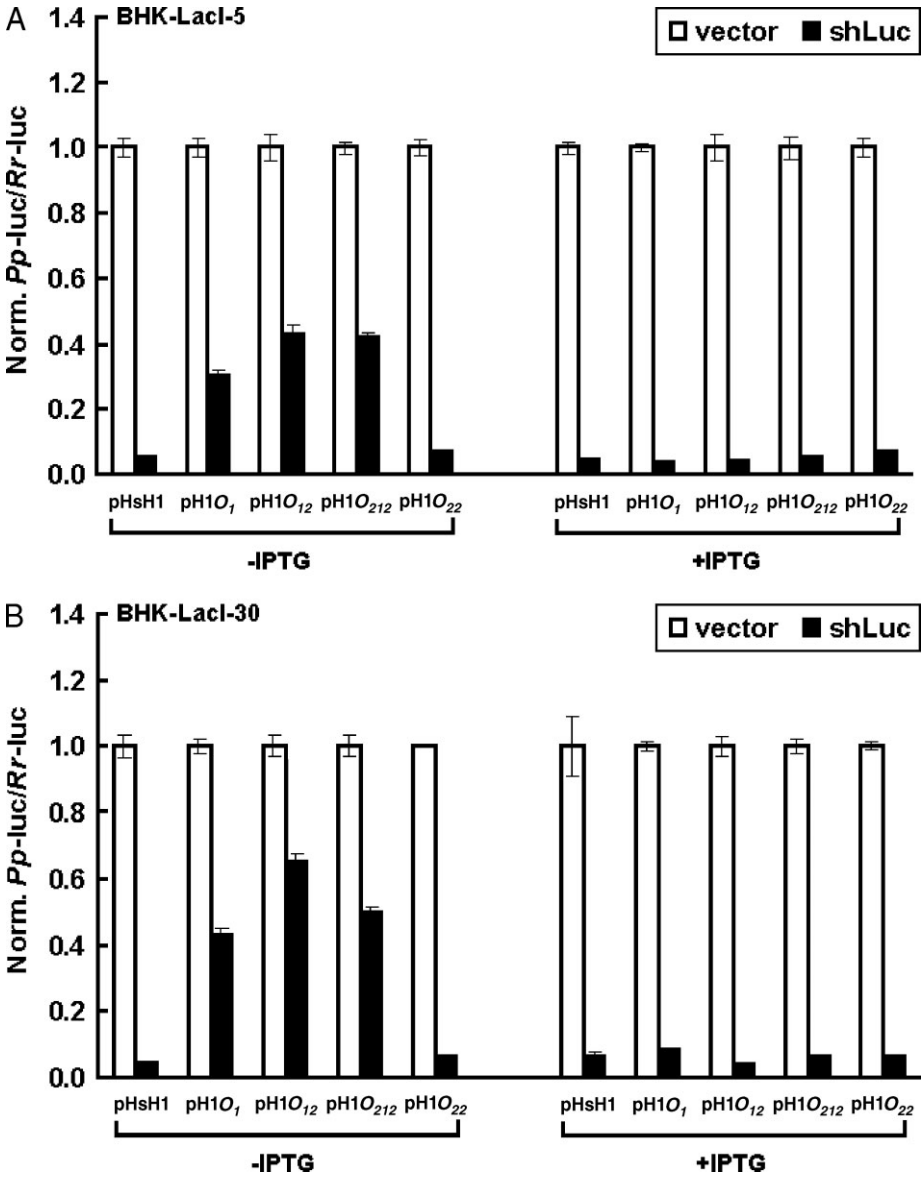

Figure 3. Inhibition effects of the inducible shLuc expression vectors on firefly luciferase expression. Stable LacI expression clones, BHK-LacI-5 (A) and BHK-LacI-30 (B), were cotransfected with 0.5 μg of the firefly luciferase expression vector pCMV-FL/RL and 1.5 μg of the inducible shLuc expression vector, pH1O_1-, pH1O_{12}-, pH1O_{212}- or pH1O_{22}-shLuc, or constitutive shLuc expression vector pHsH1-shLuc by Lipofectamine 2000, and treated with or without 5 mM IPTG as indicated. At 48 h post-transfection, the expression activities of firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. The firefly luciferase/*Renilla* luciferase (*Pp*-luc/*Rr*-luc) ratio was normalized and calculated against the control vector as indicated. The plotted data were averaged from three independent experiments and the bars indicate standard deviation

cotransfected with the firefly luciferase expression vector pCMV-FL/RL and the inducible siLuc expression vector, p(H1O_1)$_2$- or p(H1O_{212})$_2$-siLuc, or constitutive siLuc expression vector p(HsH1)$_2$-siLuc. At 48 h post-transfection, the expression activities of both the firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. As shown in Figure 4A, all three siLuc expression constructs, including p(HsH1)$_2$-, p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc, exhibited strong inhibition effects at similar levels on pCMV-FL/RL-induced firefly luciferase expression. To further examine the tight regulation and inductive efficiency of both the inducible siRNA expression vectors, we compared the inhibition effects between inducible shLuc and siLuc expression vectors on firefly luciferase expression in both inducing and non-inducing conditions. BHK-LacI-5 cells were cotransfected with the pCMV-FL/RL and the pH1O_1- or pH1O_{212}-shLuc, p(H1O_1)$_2$- or p(H1O_{212})$_2$-siLuc, and then treated with or without 5 mM IPTG. After 48 h incubation, the expression activities of both the firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. As the results in Figure 4B show, in the absence of IPTG, both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc constructs exhibited no or very low inhibition effect on pCMV-FL/RL-induced firefly luciferase expression, whereas both the pH1O_1- and pH1O_{212}-shLuc constructs displayed

significant inhibition effects on pCMV-FL/RL-induced firefly luciferase expression. In the presence of IPTG, all four constructs, p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc, as well as pH1O_1- and pH1O_{212}-shLuc, exhibited strong inhibition effects at similar levels on pCMV-FL/RL-induced firefly luciferase expression. These results clearly indicated that both the inducible siRNA expression vectors, p(H1O_1)$_2$ and p(H1O_{212})$_2$, are not only tightly regulated but also efficiently inductive.

To analyze in detail the induction kinetics of this inducible siRNA expression system, we have further examined the dose- and time-dependent conditional inhibition effects of both the inducible siLuc expression constructs on firefly luciferase expression. BHK-LacI-5 cells were cotransfected with the pCMV-FL/RL and the p(H1O_1)$_2$- or p(H1O_{212})$_2$-siLuc, and then treated with or without various concentrations of IPTG for 48 h or 5 mM IPTG for various incubation periods. At the end of each incubation time, the expression activities of both the firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. As the results in Figure 5 show, both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc constructs exhibited dose- and time-dependent conditional inhibition effects on pCMV-FL/RL-induced firefly luciferase expression in the presence of IPTG. In the dose effect experiment, the highest inhibition of firefly luciferase expression was seen in the presence of 0.1 mM IPTG for 48 h in both p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc-transfected cells (Figure 5A). The IPTG concentration eliciting half the maximal inhibition (EC_{50}) of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc constructs in BHK-LacI-5 cells was approximately 0.03 mM. In the time course experiment, the strongest inhibition of firefly luciferase expression was observed in the presence of 5 mM IPTG for 36 h and thereafter in both p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc-transfected cells (Figure 5B). The incubation time achieving half the maximal inhibition ($t_{1/2}$) of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc constructs in BHK-LacI-5 cells was approximately 13.2 and 12.9 h, respectively.

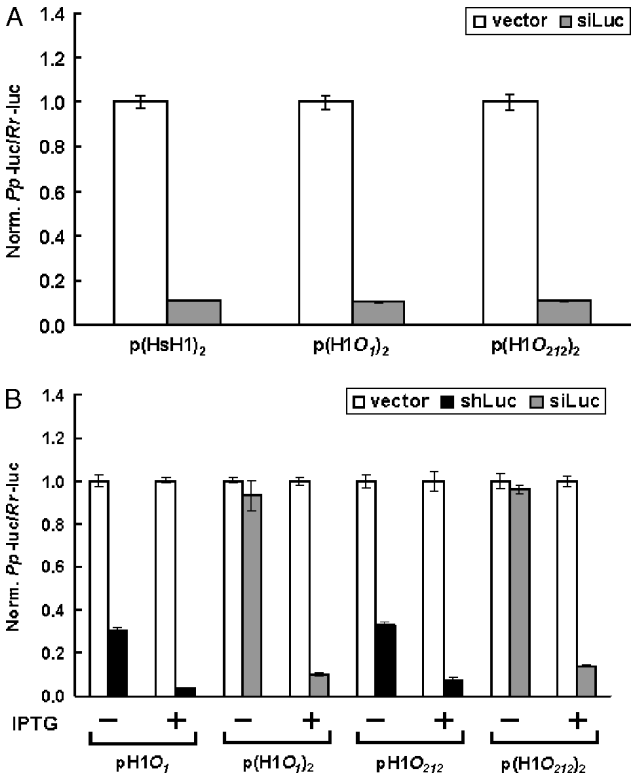

Figure 4. Inhibition effects of the inducible siLuc expression vectors on firefly luciferase expression. (A) Inhibition effects of the inducible siLuc expression vectors on firefly luciferase expression in the non-repressed condition. BHK cells were cotransfected with 0.5 μg of the firefly luciferase expression vector pCMV-FL/RL and 1.5 μg of the inducible siLuc expression vector, p(H1O_1)$_2$- or p(H1O_{212})$_2$-siLuc, or the constitutive siLuc expression vector p(HsH1)$_2$-siLuc by Lipofectamine 2000 as indicated. (B) Inhibition effects of the inducible shLuc and siLuc expression vectors on firefly luciferase expression. BHK-LacI-5 cells were cotransfected with 0.5 μg of the firefly luciferase expression vector pCMV-FL/RL and 1.5 μg of the inducible shLuc expression vector, pH1O_1-shLuc or pH1O_{212}-shLuc, or inducible siLuc expression vector, p(H1O_1)$_2$-siLuc or p(H1O_{212})$_2$-siLuc, by Lipofectamine 2000, and treated with or without 5 mM IPTG as indicated. At 48 h post-transfection, the expression activities of firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. The firefly luciferase/*Renilla* luciferase (*Pp*-luc/*Rr*-luc) ratio was normalized and calculated against the control vector as indicated. The plotted data were averaged from three independent experiments and the bars indicate standard deviation

Application of the inducible siRNA expression system for conditional inhibition of p53 expression in transient cotransfection experiments

To further demonstrate the tight regulation and efficient induction of this inducible siRNA expression system, we have constructed two inducible sip53 expression vectors, p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53, as well as two inducible shp53 expression vectors, pH1O_1- and pH1O_{212}-shp53, for conditional inhibition of p53 expression. Both the BHK-LacI-5 and 30 cells were cotransfected with the p53 expression vector pCMV-p53/EGFP and the inducible shp53 expression vector, pH1O_1- or pH1O_{212}-shp53, or inducible sip53 expression vector, p(H1O_1)$_2$- or p(H1O_{212})$_2$-sip53, and then treated with or without 5 mM IPTG. After 48 h incubation, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. As shown in Figure 6, in the presence of IPTG, all four constructs, pH1O_1- and pH1O_{212}-shp53, as well as p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53, exhibited strong inhibition effects at similar levels on pCMV-p53/EGFP-induced p53 expression. In the absence of IPTG, both the inducible sip53 expression constructs, p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53, displayed no inhibition effect on pCMV-p53/EGFP-induced p53 expression, whereas both the inducible shp53 expression constructs, pH1O_1- and pH1O_{212}-shp53, exhibited significant inhibition effects at similar levels on pCMV-p53/EGFP-induced p53 expression. These

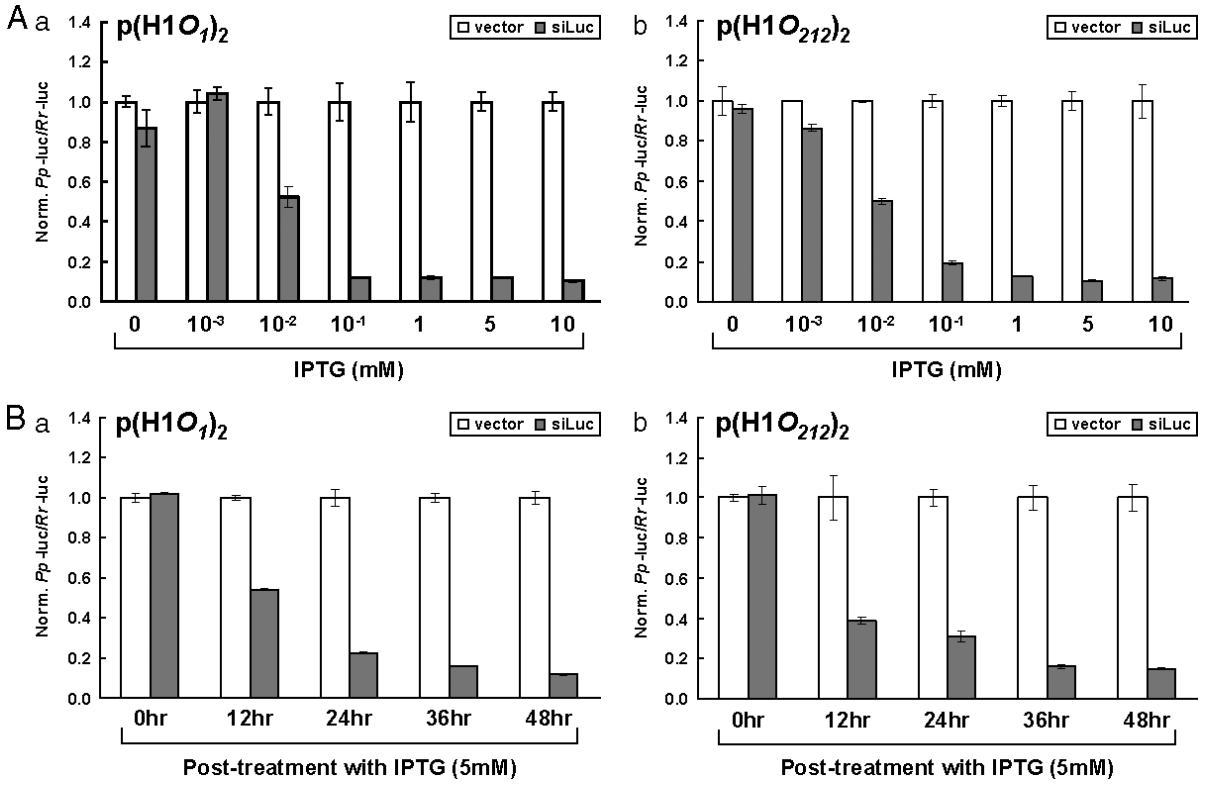

Figure 5. Dose- and time-dependent inhibition effects of the inducible siLuc expression vectors on firefly luciferase expression. BHK-LacI-5 cells were cotransfected with 0.5 μg of the firefly luciferase expression vector pCMV-FL/RL and 1.5 μg of the inducible siLuc expression vector, p(H1O_1)$_2$-siLuc (a) or p(H1O_{212})$_2$-siLuc (b), by Lipofectamine 2000, and treated with or without various concentrations of IPTG for 48 h (A) or 5 mM IPTG for various incubation periods (B) as indicated. At the end of each incubation period, the expression activities of firefly and *Renilla* luciferases in the total protein extracts were measured using the dual-luciferase reporter assay system. The firefly luciferase/*Renilla* luciferase (*Pp*-luc/*Rr*-luc) ratio was normalized and calculated against the control vector as indicated. The plotted data were averaged from three independent experiments and the bars indicate standard deviation

results confirmed again that both the inducible siRNA expression vectors, p(H1O_1)$_2$ and p(H1O_{212})$_2$, are not only tightly regulated but also efficiently inductive, whereas both the inducible shRNA expression vectors, pH1O_1 and pH1O_{212}, are leaky in the non-inducing condition.

To further analyze the induction kinetics of this inducible siRNA expression system, we have examined the dose- and time-dependent conditional inhibition effects of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs on p53 expression. BHK-LacI-5 cells were cotransfected with the pCMV-p53/EGFP and the p(H1O_1)$_2$- or p(H1O_{212})$_2$-sip53, and then treated with or without various concentrations of IPTG for 48 h or 5 mM IPTG for various incubation periods. At the end of each incubation time, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. As the results in Figure 7 show, both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs exhibited dose- and time-dependent conditional inhibition effects on pCMV-p53/EGFP-induced p53 expression in the presence of IPTG. In the dose response experiment, the highest inhibition of p53 expression was seen in the presence of 0.1 mM IPTG for 48 h in both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53-transfected cells (Figure 7A). The IPTG EC_{50} of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs in BHK-LacI-5 cells was approximately 0.06 mM. In the time course experiment, the strongest inhibition of p53 expression was observed in the presence of 5 mM IPTG for 36 h and thereafter in both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53-transfected cells (Figure 7B). The $t_{1/2}$ of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs in BHK-LacI-5 cells was approximately 22.4 and 21.5 h, respectively.

Evaluation of the inducible siRNA expression system on conditional inhibition of endogenous p53 expression in transient and stable transfection cells

To apply directly this inducible siRNA expression system in conditional control of gene expression, we have first examined the induction kinetics of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs on conditional inhibition of p53 expression in transient transfection cells. HEK293-LacI-4 cells were transfected with the p(H1O_1)$_2$- or p(H1O_{212})$_2$-sip53, and then treated with or without various concentrations of IPTG for 48 h or 5 mM IPTG for various incubation periods. At the end of each

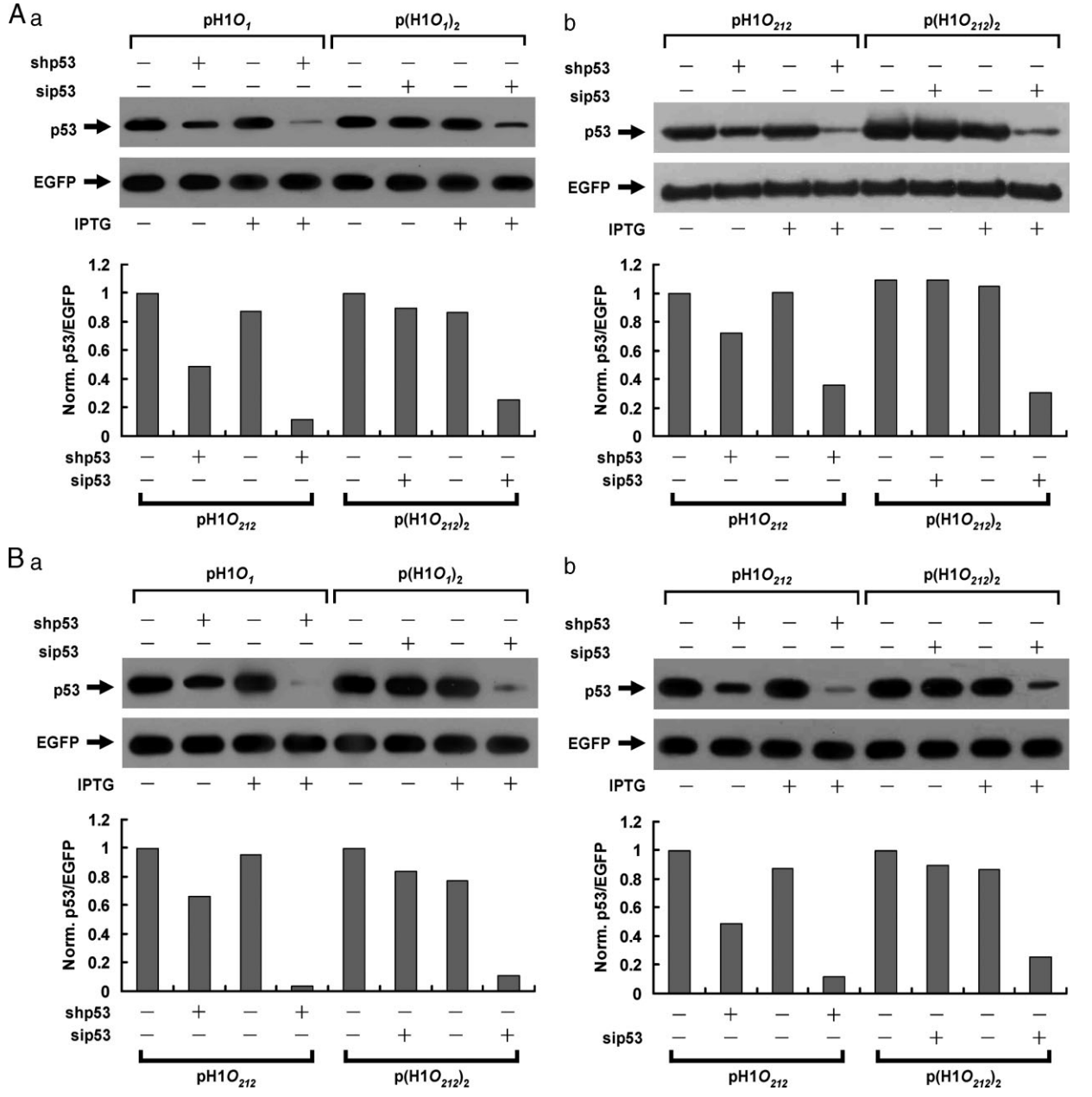

Figure 6. Inhibition effects of the inducible shp53 and sip53 expression vectors on human tumor suppressor protein p53 expression in transient cotransfection experiments. BHK-LacI-5 (A) and BHK-LacI-30 (B) cells were cotransfected with 0.5 μg of the p53 expression vector pCMV-p53/EGFP and 1.5 μg of the inducible shp53 expression vector, pH1O_1-shp53 (a) or pH1O_{212}-shp53 (b), or inducible sip53 expression vector, p(H1O_1)$_2$-sip53 (a) or p(H1O_{212})$_2$-sip53 (b), by Lipofectamine 2000, and treated with or without 5 mM IPTG as indicated. After 48 h incubation, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. The levels of EGFP serve as reference protein for transfection efficiency

incubation time, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. As shown in Figure 8, both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs exhibited dose- and time-dependent conditional inhibition of p53 expression in the presence of IPTG. In the dose response experiment, the highest inhibition of p53 expression was seen in the presence of 0.1 mM IPTG for 48 h in both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53-transfected HEK293-LacI-4 cells (Figure 8A). The IPTG EC_{50} of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs in HEK293-LacI-4 cells was approximately 0.07 and 0.04 mM, respectively. In the time course experiment, the strongest inhibition of p53 expression was observed in the presence of 5 mM IPTG for 36 h in both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53-transfected HEK293-LacI-4 cells (Figure 8B). The $t_{1/2}$ of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53 constructs in HEK293-LacI-4 cells was approximately 23.7 and 23.6 h, respectively.

To apply broadly this inducible siRNA expression system in conditional control of gene expression in a stable manner, we have established two inducible sip53 expression stable clones, p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47. HEK293-LacI-4 cells were first transfected with

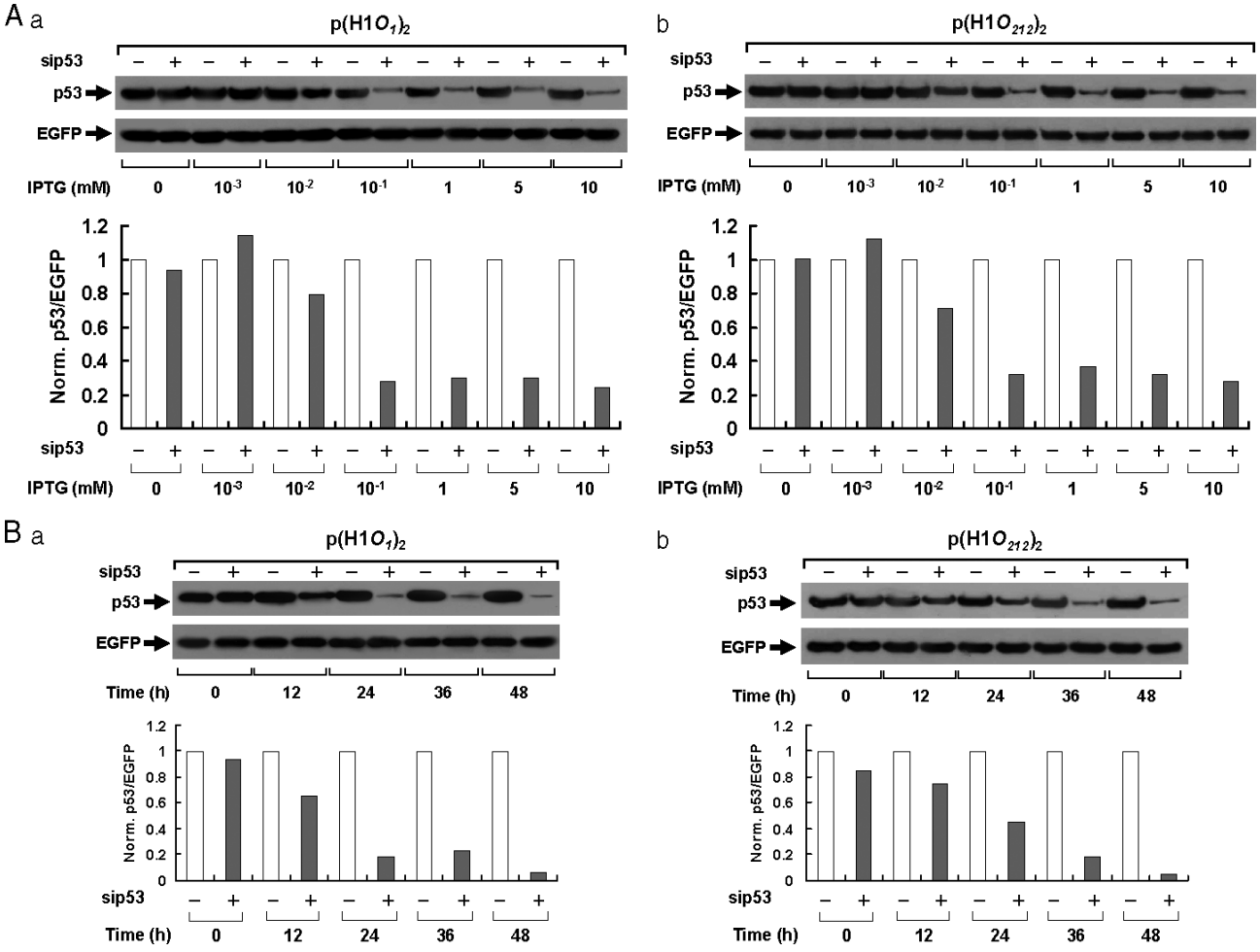

Figure 7. Dose- and time-dependent inhibition effects of the inducible sip53 expression vectors on p53 expression in transient cotransfection experiments. BHK-LacI-5 cells were cotransfected with 0.5 μg of the p53 expression vector pCMV-p53/EGFP and 1.5 μg of the inducible sip53 expression vector, p(H1O_1)$_2$-sip53 (a) or p(H1O_{212})$_2$-sip53 (b), by Lipofectamine 2000, and treated with or without various concentrations of IPTG for 48 h (A) or 5 mM IPTG for various incubation periods (B) as indicated. At the end of each incubation period, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. The levels of EGFP serve as reference protein for transfection efficiency

the inducible sip53 expression stable construct, p(H1O_1)$_2$-sip53/CMV-BSD or p(H1O_{212})$_2$-sip53/CMV-BSD. At 48 h post-transfection, the transfected cells were treated with blasticidin S, then selected for the drug-resistant colonies, and further screened for conditional inhibition of p53 expression in the presence of IPTG. Both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones were treated with or without various concentrations of IPTG for 48 h or 5 mM IPTG for various incubation periods. At the end of each incubation time, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. As the results in Figures 9A and 9B show, the IPTG induced conditional inhibition of p53 expression in a dose- and time-dependent manner. In the dose effect experiment, the highest inhibition of p53 expression was achieved with 1 and 0.1 mM of IPTG in both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones, respectively. The IPTG EC_{50} of both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones was approximately 0.37 and 0.03 mM, respectively. In the time course experiment, the strongest inhibition of p53 expression was seen at around 36 and 24 h in both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones, respectively. The $t_{1/2}$ of both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones was approximately 29.8 and 14.8 h, respectively.

To evaluate the reversibility of this inducible siRNA expression system, we have particularly examined the reactivation of p53 expression in the non-induced condition after withdrawal of IPTG. Both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones were first treated with 5 mM IPTG for 48 h, then washed three times to remove IPTG and cultured in normal medium for various incubation periods. At the end of each incubation time, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. As the results in Figure 9C show, the inhibition of p53 expression induced by IPTG was reversed after removal of IPTG from the culture. Reactivation of the p53 expression to the level of the non-inducing condition was seen at around 48 to 72 h in both the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones. The reactivation time achieving half the level of the non-inducing condition ($t_{1/2}$) of both

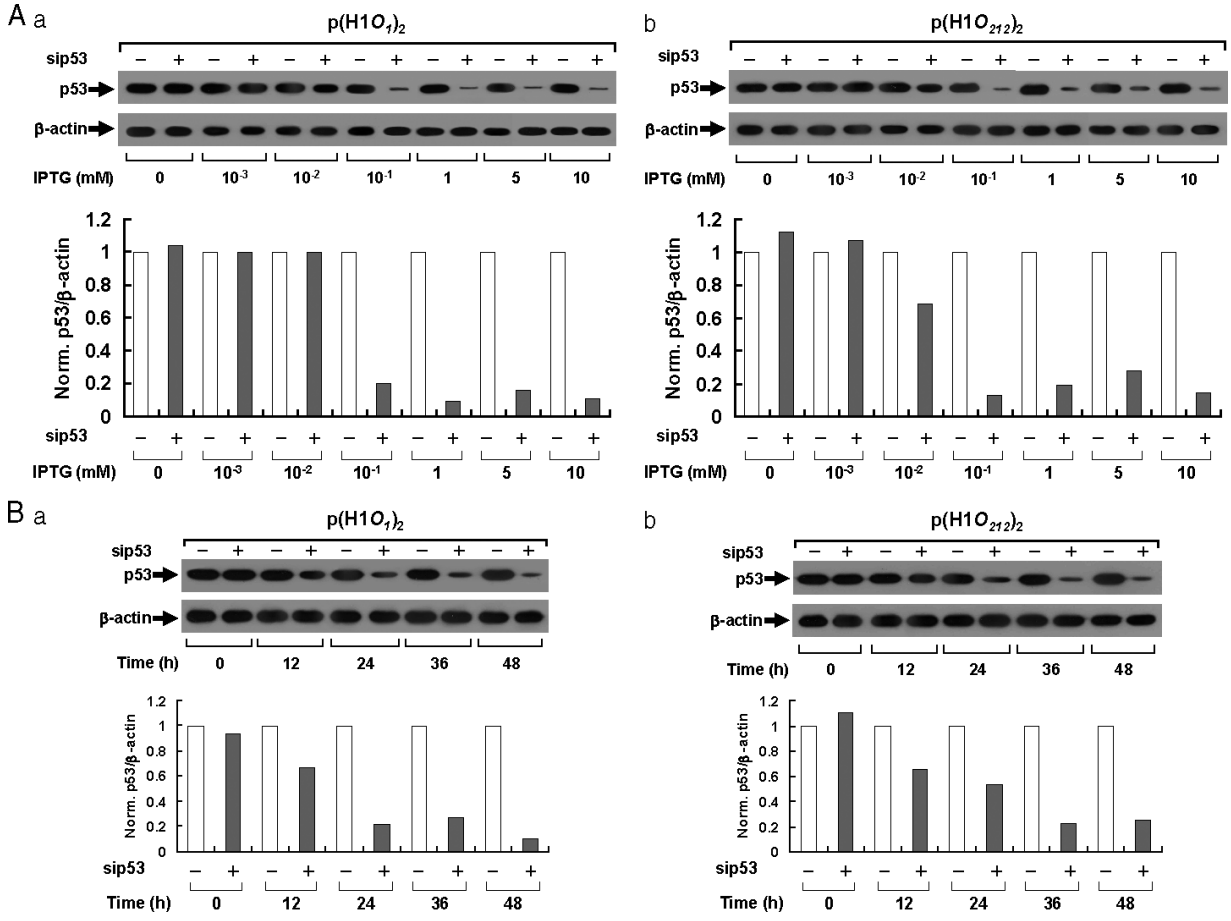

Figure 8. Dose- and time-dependent inhibition effects of the inducible sip53 expression vectors on endogenous p53 expression in transient transfection experiments. HEK293-LacI-4 cells were transfected with 2 μg of the inducible sip53 expression vector, p(H1O_1)$_2$-sip53 (a) or p(H1O_{212})$_2$-sip53 (b), by Lipofectamine 2000, and treated with or without various concentrations of IPTG for 48 h (A) or 5 mM IPTG for various incubation periods (B) as indicated. At the end of each incubation period, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. The levels of β-actin serve as reference protein for loading control

the p(H1O_1)$_2$-sip53-7 and p(H1O_{212})$_2$-sip53-47 clones was approximately 31.2 and 44 h, respectively. These results indicated that the action of this inducible siRNA expression system is not only highly inducible but also fully reversible.

Discussion

In this study, we have established an IPTG-responsive *lac* repressor-operator-controlled RNA Pol III-dependent human H1 promoter-driven inducible siRNA expression system that could conditionally induce target-specific gene silencing under the control of IPTG. In addition, we have further demonstrated that this inducible siRNA expression system could function not only in established stable clones, but also particularly in transient transfection cells. The inhibition effect of this conditional RNAi-mediated gene silencing displayed stringent dose- and time-dependent kinetics after administration of the IPTG, as well as importantly exhibited a very low or undetectable background inhibition in the non-inducing status. Remarkably, the inhibition effect induced by this system was completely reversible after withdrawal of IPTG. Thus, this inducible siRNA expression system provides an ideal genetic switcher allowing the inducible and reversible control of specific gene activity in mammalian cells.

Previous studies have shown that insertion of the Tet operator TetO into the human H1 promoter at different positions upstream of the transcription initiation site has a differential effect on its leakiness in the non-inducing condition or activation in the inducing condition [23,27,31,34]. Examination of the inhibition effects induced by the pH1O_1-, pH1O_{12}-, pH1O_{212}- and pH1O_{22}-shLuc constructs on firefly luciferase expression revealed that only pH1O_{22}-shLuc exhibited strong inhibition in both the non-inducing and inducing conditions (Figure 3), suggesting that constructing a single copy of the symmetric *lac* operator *lac*O_{1S} (Figure 1E) downstream of the TATA box is essential for conditional regulation of the human H1 promoter. In contrast, the pH1O_1-, pH1O_{12}- and pH1O_{212}-shLuc constructs displayed similar inhibition effects on firefly luciferase expression, indicating that constructing additional one or two copies of the symmetric *lac* operator *lac*O_{2S}

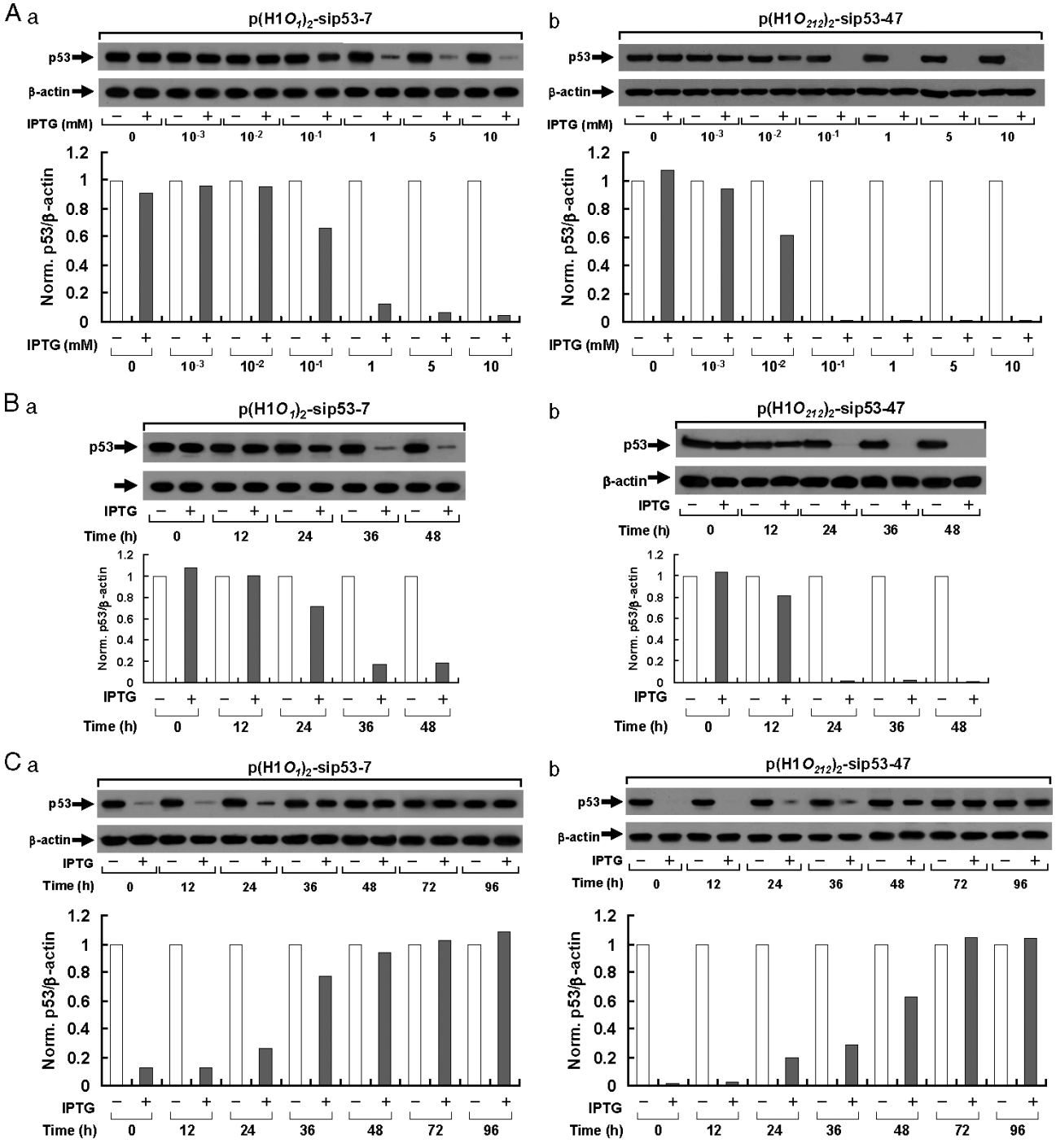

Figure 9. Dose- and time-dependent as well as reversible inhibition effects of the inducible sip53 expression vectors on endogenous p53 expression in established stable clone experiments. HEK293-LacI-4 cells were first transfected with 2 μg of the inducible sip53 expression stable construct, p(H1O_1)$_2$-sip53/CMV-BSD or p(H1O_{212})$_2$-sip53/CMV-BSD, by Lipofectamine 2000. After 48 h post-transfection, the transfected cells were treated with 5 μg/ml of blasticidin S and then selected for the drug-resistant colonies, and the drug-resistant stable clones were further screened for conditional inhibition of p53 expression in the presence of IPTG. The conditional inhibition of p53 expression stable clones, p(H1O_1)$_2$-sip53-7 (a) and p(H1O_{212})$_2$-sip53-47 (b), were treated with or without various concentrations of IPTG for 48 h (A) or 5 mM IPTG for various incubation periods (B) as indicated. Both the p(H1O_1)$_2$-sip53-7 (a) and p(H1O_{212})$_2$-sip53-47 (b) clones were first treated with 5 mM IPTG for 48 h, then washed three times to remove IPTG and further cultured in the absence of IPTG for various incubation periods (C) as indicated. At the end of each incubation period, the expression levels of p53 in the total protein extracts were determined by Western blot analysis. The levels of β-actin serve as reference protein for loading control

(Figure 1E) flanking the *lacO*$_{1S}$-engineered human H1 promoter has no further effect on its function. These results indicated that either insertion of TetO or *lacO*$_{1S}$ downstream of the TATA box had a similar effect on its leakiness in the non-inducing condition and activation in the inducing condition. Therefore,

this IPTG-responsive *lac* repressor-operator-controlled inducible siRNA expression system can be adapted to the tet-responsive Tet repressor-operator-controlled inducible siRNA expression system.

The currently available conditional RNAi systems have often suffered from leakiness of high background silencing effects in the non-inducing condition, inefficiency of gene silencing effects in the inducing condition, or non-reversibility of gene silencing effects after withdrawal of inducing agents. As compared to previously reported conditional RNAi systems established by using Cre/*loxP* site-specific recombination, tet-responsive Tet repressor-operator or IPTG-responsive *lac* repressor-operator-driven inducible shRNA expression strategies, the presently developed inducible siRNA expression system not only efficiently induced RNAi-mediated gene silencing but also reversibly regulated gene activity under the control of IPTG [17–35]. Analysis of the inhibition effects induced by the p(HsH1)$_2$-, p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc constructs on firefly luciferase expression revealed that all three constructs exhibited high inhibition efficiencies at similar levels (Figure 4A), indicating that both the symmetric *lac* operator *lacO*$_{1S}$ and *lacO*$_{2S}$-engineered human H1 promoter-driven inducible siRNA expression vectors, p(H1O_1)$_2$ and p(H1O_{212})$_2$, function normally as wild-type p(HsH1)$_2$ vectors. In addition, evaluation of both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-siLuc- as well as both the p(H1O_1)$_2$- and p(H1O_{212})$_2$-sip53-induced conditional inhibition of firefly luciferase (Figure 5) and p53 (Figures 7–9) expression showed that both the p(H1O_1)$_2$ and p(H1O_{212})$_2$ vectors could inducibly and reversibly mediate gene silencing under the control of IPTG. These results clearly indicated that both the p(H1O_1)$_2$ and p(H1O_{212})$_2$ vectors are functionally equivalent in conditional inhibition of gene expression. To simply and efficiently apply this inducible siRNA expression system for conditional inhibition of gene expression, we have currently focused on the p(H1O_1)$_2$ vector only.

The inhibition activities of conditional RNAi-mediated gene silencing normally exhibit inducing or reverting time delay after administration or withdrawal of inducing agent, respectively. Both the inducing and reverting time delays are dependent on the induction strategy, siRNA expression level, target mRNA abundance and encoded protein stability. Among these four parameters, both the mRNA expression level and protein degradation rate are determined by the specificity of target genes. As compared with the most frequently used strategies for constructing conditional RNAi systems, such as Cre/*loxP* site-specific recombination and tet-responsive Tet repressor-operator, the induction mechanism of IPTG-responsive *lac* repressor-operator-controlled conditional RNAi systems is similar to that of tet-responsive Tet repressor-operator strategy [17–34]. Therefore, the levels of siRNA expression in these two systems are similar due to closely related modifications with different repressor binding sequences in the same promoter. However, the level of siRNA expression in the Cre/*loxP* site-specific recombination strategy is presumably higher than in both the tet-responsive Tet repressor-operator and IPTG-responsive *lac* repressor-operator systems.

The tumor suppressor p53 is a molecular guardian in the regulation of appropriate cellular responses to various stress conditions, such as DNA-damaging agents. Dysfunction of this protein can result in devastating consequences, in particular the development of malignant cancers. It is frequently inactivated via multiple mechanisms during tumorigenesis. The most common inactivation mechanism is mutation at the *TP53* gene that is detected in more than 50% of human cancers. Due to its pivotal role in regulating abnormal cell growth and its frequent inactivation in a large fraction of human tumors, p53 is an appealing target for novel anticancer therapeutic strategies. However, a great deal of our knowledge of the p53 action and signaling mechanisms remains incomplete, so establishing a more unified and coordinated strategy to dissect its physiological function is extremely important. Thus, the construction of a tightly regulated and reversibly inducible sip53 expression system provides a unified and coordinated approach for studying and identifying the targets of p53. This will lead to a better understanding of p53 biological function and its action of mechanism.

In summary, the results presented in this study clearly demonstrated that this inducible siRNA expression system not only tightly regulates the silencing effect induced by RNAi mechanism under the control of IPTG but also provides an extremely valuable tool for studying the genes involved in cell growth, survival and development. Although previous reports have described the usefulness of conditional RNAi-mediated gene silencing, this study further advances its utilities for both basic gene function study and clinical therapeutic application. In addition, development of the IPTG-responsive *lac* repressor-operator-controlled RNA Pol III-dependent human H1 promoter-based conditional RNAi system provides an opportunity for constructing dual conditional RNAi-mediated gene silencing systems under the control of two different inducing agents that can be used to control two particular gene activities simultaneously.

Acknowledgements

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