Brief Communication

Inducible H1 Promoter-Driven Lentiviral siRNA Expression by Stuffer Reporter Deletion

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ABSTRACT

Introduction of 19–23-bp small interfering RNA (siRNA) into mammalian cells has become a standard procedure to downregulate mRNA with high efficacy. siRNAs can be introduced into cells either as synthetic duplexes or as hairpin structures produced by Pol III promoter-driven vectors. Pol III promoter-expressed small hairpin RNAs (shRNAs) offer a great possibility for the production of endogenous siRNA, which can be used for stable siRNA production *in vivo*. A major drawback of this strategy is the incapability of detecting rapidly occurring cellular responses. Here, we present a lentiviral shRNA-producing vector system, which can be induced by CRE recombinase enzyme to overcome these limitations. Following the addition of CRE, the pLIND (LentiINDucible) will activate siRNA production by deleting EGFP and a stop cassette between the promoter and siRNA oligo. Target gene downregulation capacity was comparable to that of a noninducible siRNA system.

INTRODUCTION

RNA INTERFERENCE (RNAi) has emerged as a novel pathway for the study of loss of function of a vast number of genes in various model systems. The phenomenon, which was first described in plants, later was shown to be operational also in mammalian cells. Thus, by introducing 21-nt small interfering RNA (siRNA) into the cell, it is possible to efficiently downregulate complementary mRNA (Elbashir et al., 2001). After introduction of siRNA into cells, it is thought that the cytoplasmic RNase III, Dicer, will bind to double-stranded (ds) oligonucleotides and separate them. The single-stranded (ss) oligo will then bind to the RNA-induced silencing complex (RISC), a large enzyme complex structure that consists of many different proteins, for example, helicases and nucleases. The RISC-ssRNA complex will fi-

nally find its complementary mRNA strand and cause its degradation. Because the siRNA oligonucleotide duplexes are introduced into cells by transfection and no selection is made, the effect of RNAi will be transient. The silencing will occur 24 hours after transfection and lasts up to 5 days depending on the protein stability.

Depending on the aim, transient silencing may be beneficial or disadvantageous. Brummelkamp et al. and other groups developed siRNA technology further by introducing short hairpin RNA (shRNA)-producing plasmids into the cells, sometimes referred to as second-generation siRNA (Brummelkamp et al., 2002; Paddison et al., 2002; Sui et al., 2002; Yu et al., 2002). These plasmids contain human or mouse Pol III H1 or U6 promoter followed by a sense-loop-antisense siRNA oligo. Promoters in these vectors were shown to produce siRNA with similar silencing efficiency as when introducing

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synthetic siRNAs, but with sustained inhibition, after selection of stable transfectants. Thus, this developmental progress enabled the creation of stably transfected cell lines, leading to the sustained knockdown of the target gene by >90% of its expression under optimal conditions. The technique was not suitable for studying genes involved in maintaining survival or for studying immediate responses following downregulation. Inducible shRNA-producing plasmids are the third-generation siRNAs. These were first created with tetracycline-based methods (Chen et al., 2003; Czuderna et al., 2003) and, recently, using the CRE-LoxP system (Fritsch et al., 2004; Kasim et al., 2004; Tiscornia et al., 2004; Ventura et al., 2004). None of these vector systems had the optimal combination of elements and functions for tightly regulated and efficient induction of shRNA in mouse and human cells.

Here, we introduce a novel inducible lentiviral siRNA-producing vector system. The system uses human H1 promoter, is selectable against enhanced green fluorescent protein (EGFP), and can be induced by NLS-CRE/IRES2/DsRed2 plasmid. The vector design shows the power and the importance of the placement of promoter, LoxP, and the siRNA-producing cassette. The strength of this system is its robust hairpin production, selection ability against EGFP, and specificity compared with earlier tetracycline-induced systems (Gossen et al., 1995). Furthermore, the human H1 promoter makes it possible to use this method in both human and mouse cells, whereas mouse U6 promoter-driven vectors are restricted to mouse cells (Hasuwa et al., 2002).

MATERIALS AND METHODS

Construct design

pLL3.7 vector was digested with EcoRI and PciI. Vector was dephosphorylated with shrimp alkaline phosphatase (SAP) (USB Corporation, Cleveland, OH) and ligated with synthesized and phosphorylated MfeI-LoxP-AscI-AAAAA-XhoI-oligo (AAT TGA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT GGC GCG CCA AAA ACT CGA GA) to EcoRI and PciI sites using T4 DNA ligase (Promega, Madison, WI). pSUPER vector was modified by cutting with BglII and ClaI, dephosphorylating with SAP, and ligating with phosphorylated and synthesized BglII-LoxP-TTTTT-NotI-TTTTT-ClaI-oligo (5'-GAT CTA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT TTT TTG CGG CCG CTT TTT AT-3'). Pol III H1 promoter-LoxP-terminator fragment was cleaved from modified pSUPER plasmid by using NotI and XbaI. This NotI-XbaI fragment was ligated to modified and dephosphorylated pLL3.7 vector.

To mimic the natural initiation site of human gene for H1 RNA, we designed three cytosine nucleotides before

the initiation codon of shRNA (Baer et al., 1990). The phosphorylated siRNA oligos with hairpin sequences targeted against Bruton's tyrosine kinase (Btk) Btk1 (5'-CGC GCC CAG CCA AAG TCA TGA TGA ATC TTC AAG AGA GAT TCA TCA TGA CTT TGG CTT TTT G-3'), Btk2 (5'-CGC GCC CAA CTG TTT GGT AAA CGA TCA TTC AAG AGA TGA TCG TTT ACC AAA CAG TTT TTT G-3'), and Btk3 (5'-CGC GCC CAG TTC AGC AGC AAA TCT GAC TTC AAG AGA GTC AGA TTT GCT GCT GAA CTT TTT G-3'), were inserted to XhoI and AscI sites. The vectors were verified by sequencing downstream from the H1 promoter and upstream from WRE (Fig. 1). The 5' and 3' primer sequences were 5'-CCT GCA ATA TTT GCA TGT CG-3' and 5'-AGA GAC AGC AAC CAG GAT TT-3', respectively.

The pNLS-CRE/IRES2/DsRed2 vector was created by cutting NLS-CRE from the pMSCVpuroCRE vector with BglII enzyme. The BglII fragment was ligated to the pIRES2-DsRed2 vector (BD Biosciences, Clontech, Palo Alto, CA) and verified with control digestions.

Cell culture and reagents

Cos-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Transfections were done with equal mixture of two or three (1:1 or 1:1:1) plasmids, pSGT/Btk, pNLS-CRE/IRES2/DsRed2 (only when stated), and pLIND using FuGene6 (Roche Diagnostics, Mannheim, Germany) and following the manufacturer's instructions. The total amount of DNA per well was 1 μ g, of which 0.11 μ g constituted the contribution for each of the three pLIND siRNA constructs. Cells were processed 60 hours after transfection for both confocal and protein analysis.

Western blot analysis

The cells were harvested and washed with phosphate-buffered saline (PBS) and finally lysed in RIPA buffer on ice for 20 minutes as described earlier (Heinonen et al., 2002). The cell lysate was resuspended in sample buffer and loaded into Criterion-XT Bis-Tris precasted gels (Bio-Rad, Hercules, CA). Equal amount of proteins were loaded, and the gels were run with XT MOPS running buffer (Bio-Rad) for 1 hour with constant voltage of 200 V. Blotting to 0.2 μ m nitrocellulose membrane (Bio-Rad) was done using the Criterion blotting unit for 30 minutes at 100 V. The membrane was blocked with 5% milk for 2 hours. The dilutions for primary antibodies were: rabbit anti-Btk (1:5,000), mouse anti- β -actin (1:200,000) (Sigma-Aldrich Chemie, Steinheim, Germany), and mouse anti-CRE (1:7,000) (Chemicon International, Temecula, CA). Membrane was incubated overnight with primary antibodies and for 2 hours with secondary antibodies. Secondary antibodies used were

FIG. 1. Schematic representation of pLIND and pNLS-CRE/IRES2/DsRed2 expression constructs and their function. **(A)** The pLIND plasmid alone allows the EGFP stuffer reporter cassette to be expressed under the CMV promoter. The shRNA remains unexpressed, as the H1 promoter is not functional due to the presence of a termination sequence (five thymidines) after the first LoxP site and two clusters of four repetitive thymidines inside the CMV promoter. **(B)** After the expression of pNLS-CRE/IRES2/DsRed2 plasmid, the CRE will cause LoxP recombination, which will remove the EGFP stuffer cassette, and the shRNA will be expressed. LTR, long terminal repeat; Psi, extended viral packaging signal; FLAP, HIV-1 flap element; WRE, woodchuck hepatitis virus posttranscriptional regulatory element; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; DsRed2, *Discosoma* sp. red fluorescent protein 2.

goat antimouse-horseradish peroxidase (HRP) and goat antirabbit-HRP at a dilution of 1:2,000 (Dako A/S, Glostrup, Denmark). The membrane was washed extensively and developed using the Supersignal West Pico Chemiluminescent substrate kit (Pierce, Rockford, IL).

RESULTS AND DISCUSSION

Design and cloning of pLIND and pNLS-CRE/IRES2/DsRed2

To generate a lentiviral-inducible shRNA-expressing plasmid, we chose to develop a CRE-LoxP recombinase-based system. This system has been shown to work tightly compared with tetracycline-based methods, which frequently are leaky. We chose the H1 promoter because it has been shown to function more efficiently than the U6 promoter (Boden et al., 2003). Another important fact

was that H1 can be expressed in both human and mouse cells, thereby allowing us to use it more broadly.

We cloned a stuffer reporter sequence of 1425 bp flanked by LoxP sites (34 nt) between the H1 promoter and the transcription initiation site (Fig. 1). After CRE-induced LoxP recombination, the total number of nucleotides between promoter and transcription start site is 42. We have shown that this approach functions in the context of the H1 promoter, despite the fact that it is even more compact than the U6 promoter (100 nt vs. 250 nt.) (Paule and White, 2000; Myslinski et al., 2001). The stuffer reporter consists of five thymidine nucleotides followed by a CMV promoter-driven EGFP and functions as a stop cassette for shRNA expression. The CMV promoter contains two clusters of four repetitive thymidines, which serve as an additional stop sequence to avoid readthrough. The EGFP functions both as a reporter and the control for a silent vector, that is, a vector

that does not express shRNA. Specific synthetic oligo sequences that target Btk mRNA (see Materials and Methods) were inserted into *AscI* and *XhoI* sites. After ligation, the *XhoI* site was destroyed following the irreversible ligation of *SalI* and *XhoI* sites. This was used as a control for successful shRNA-oligo ligation.

For expression of inducer CRE, we cloned the NLS-CRE fusion gene into the pIRES2/DsRed2 plasmid, creating pNLS-CRE/IRES2/DsRed2. Expression of DsRed2 (*Discosoma* sp. red fluorescent protein 2) serves as an indirect marker for generation of CRE recombinase. CRE expression induces *LoxP* recombination, which leads to the removal of the EGFP and the stop cassette, thereby activating shRNA production (Fig. 1).

CRE recombination

Owing to the fact that the internal ribosome entry site (IRES) sequence allows expression of more than a single gene while favoring expression of 5' sequences, the DsRed2 should serve as a relevant marker for NLS-CRE. The DsRed2 was monitored and visualized under a fluorescent microscope. Cells transfected with pLIND only showed high EGFP expression as a marker for the silent pLIND vector. When the pNLS-CRE/IRES2/DsRed2 was combined with pLIND, >95% of the reporter activity (EGFP) was lost. The phenotype of CRE-mediated recombination was verified with fluorescence microscopy (Fig. 2) and confirmed with FACS (data not shown).

Efficient downregulation of Btk followed by CRE-mediated induction of pLIND

To test the pLIND vector's capacity to produce siRNA and downregulate its target, we chose to study the Btk gene, which encodes a cytoplasmic tyrosine kinase mutated in X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice. We have previously shown successful downregulation of Btk by synthetic siRNA, where we found that combining three siRNAs against the target gene causes stronger downregulation than when using single siRNAs (Heinonen et al., 2002).

We used the pSUPER_RETRO plasmid as a positive control for shRNA production and target gene knock-down. We have previously cloned shRNA oligos containing the same Btk target sites as in pLIND (unpublished observations). pSUPER_RETRO is a well-established shRNA-producing plasmid used to knock down different genes (Brummelkamp et al., 2002). Similar to pLIND, it also has an H1 promoter, making analysis of promoter efficiency comparable. We used pLIND plasmid only as a negative control, which did not show any signs of leakage of shRNA expression (Figs. 1 and 3). Downregulation of Btk protein measured with densitometry was >90% (Fig. 3). The downregulation induced by pSUPER_RETRO was approximately the same compared with pLIND. The slight difference seen in the downregulated Btk bands (Fig. 3) was probably due to the cotransfection, where not all cells that contained pLIND contained pNLS-CRE/IRES2/DsRed2.

FIG. 2. Fluorescence microscope monitoring of CRE-inducible shRNA production, followed by EGFP stuffer reporter deletion. Transfection of Cos-7 cells with pLIND alone (a, b, c) or together with pNLS-CRE/IRES2/DsRed2 (d, e, f). Images were taken under FITC-filter (a, d), Cy-3 filter (b, e), and brightfield (c, f).

FIG. 3. Western blot analysis of CRE-inducible, shRNA-mediated knockdown of Btk expression. Transfections were made with pSGT/Btk alone (lane 1), with pLIND (lane 2), with pSUPER_RETRO/Btk (lane 3), and with pLIND/Btk and pNLS-CRE/IRES2/DsRed2.

The capability of pLIND to produce lentiviral titers was studied using the Phoenix-GP cell line containing the pΔR 8.91 and pMDG plasmids. The titers were shown effective and functional by transducing HeLa, Raji (human B lymphocyte, Burkitt's lymphoma), and Nalm-6 (human B cell precursor, leukemia) cell lines (data not shown). To validate the viral supernatant quality, titration assays were made with HeLa cells using three amounts (50 μ l, 10 μ l, and 1 μ l) of viral supernatant per 100,000 HeLa cells. The pLIND-transformed cells were detected with flow cytometry. The estimated viral particle count was pLIND/Btk1 = 1.22×10^6 viral particles per milliliter of supernatant; pLIND/Btk2 = 1.57×10^6 viral particles per milliliter of supernatant; pLIND/Btk 3 = 1.57×10^6 viral particles per milliliter of supernatant.

CRE-LoxP vector systems, developed while this paper was in preparation, used either a modified U6 promoter with the LoxP site inserted into the TATA box (Coumoul et al., 2004; Tiscornia et al., 2004; Ventura et al. 2004) or an approach where a LoxP-STOP-LoxP cassette is planted into the hairpin loop (Fritsch et al., 2004; Kasim et al., 2004). However, neither of these systems was found to be optimal for inducible shRNA production. Problems that have occurred in the modified LoxP approach include the less efficient CRE-induced recombination between modified and wild-type LoxP sites (Lee

and Saito, 1998). The weakness of the latter approach is reduced production of siRNA as a result of the longer shRNA transcript, which is processed less efficiently (Fritsch et al., 2004; Ventura et al., 2004). This may also create problems in specificity, as transcripts >30 nt have been reported to trigger the interferon (IFN) response pathway (Minks et al., 1979). In contrast to H1 promoter-driven vectors, U6 promoter-driven vectors have been reported to give a higher frequency of induction of IFN-stimulated genes (IFGs) (Pebernard and Iggo, 2004). Moreover, it has been reported that H1 promoter-based vectors will cause stronger gene silencing when using the same target genes (Boden et al., 2003). Boden et al. (2003) have shown that adding a 27-nt spacer sequence between promoter and the transcription initiation site will increase the vectors' downregulation efficiency.

The pLIND vector system has many advantages. First, in the absence of CRE, the pLIND is not leaky for shRNA expression. Second, pLIND vector-positive cells can be selected, sorted against EGFP, and expanded before activating siRNA production. Third, the vector seems to have strong siRNA production compared to the available tetracycline-inducible or CRE-LoxP-inducible systems. The high recombination and efficient downregulation capacity of pLIND plasmid can be explained by right positioning of the LoxP site compared with the promoter and transcription initiation site. The 42 nt between the promoter and the transcription initiation site seem to have no effect for shRNA production or recombination in pLIND plasmid.

In conclusion, we have created a novel lentiviral CRE-inducible H1 promoter-driven shRNA expression plasmid. We induced this system using the pNLS-CRE/IRES2/DsRed2 expression plasmid encoding a NLS-CRE and DsRed2 as a marker. The pLIND vector system has been shown to serve as an efficient tool for gene knockdown studies, lacking the problems of poor recombination efficiency and low target knockdown, and could have great potential for a vast number of applications.

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