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Original

Construction of an All-in-one Double-conditional shRNA Expression Vector

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Abstract: Gene silencing by RNA interference (RNAi) is widely used for assessing gene function. An important advance in the RNAi field was the discovery that plasmid-based RNAi can substitute for synthetic small interfering RNA *in vitro* and *in vivo*. However, constitutive and ubiquitous knockdown of gene expression by RNAi in mice can limit the scope of experiments because this process can lead to embryonic lethality, or result in compensatory overexpression of other genes such that no phenotypic abnormalities occur. Either way, analyses of the physiological roles of the gene of interest in adult mice are not possible. To overcome these limitations, we previously constructed a double-conditional short-hairpin RNA (shRNA) expression vector that can regulate shRNA expression in a spatio-temporal manner with a tetracycline-inducible floxed stuffer sequence selectively excised by application of Cre recombinase. In this study, we aimed to modify this vector to create an all-in-one vector that produces double-conditional transgenic mice through a single round of gene transfer to fertilized eggs. We added a coding region for nuclear localizing Cre (NCre) recombinase with a multi-cloning site for a cell-specific promoter into the double-conditional short-hairpin RNA (shRNA) expression vector that we previously constructed. Using Escherichia coli, we confirmed successful construction of the vector. First, we confirmed isopropyl-β-D-thiogalactopyranoside-induced expression of NCre recombinase through the lac operon as a specific promoter by western blotting. Second, we confirmed functional recombination of the floxed sequence of loxP-like TATA-lox by analysing restriction enzyme-digested fragments. This all-in-one double-conditional shRNA expression vector will be useful for reversible *in vitro* and *in vivo* knockdown of target gene expression, in target cells via promoter-specific expression of NCre, and at specific times by tetracycline application.

Key words: RNA interference, short hairpin RNA, double-conditional vector, tetracycline-inducible system, Cre-loxP system

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Introduction

Silencing of gene expression by RNA interference (RNAi) has become a widely used tool for assessing gene function in a rapid and easy manner[1]. An important advance in the RNAi field was the discovery that plasmid-based RNAi, including that which uses short-hairpin RNA (shRNA), can substitute for transient RNAi caused by synthetic small interfering RNA (siRNA) both *in vitro* and *in vivo*[2]. Several vectors have been designed to direct the constitutive synthesis of shRNA. However, expression of shRNA through polymerase III promoters such as U6 appears to be ubiquitous in all cells and non-regulated. Production of transgenic mice by such expression of shRNA has often resulted in early embryonic death due to inhibition of gene expression[3]. However, it can also result in mice that show no abnormalities in their general phenotype[3]. The latter occurs because of induction of one or more compensatory genes that act as counterparts to the target gene, meaning that whole-body homeostasis is maintained during growth and development. In both cases, an analysis of the roles of the gene of interest in adult transgenic mice is not possible. For such analysis, *in vivo* knockdown of the target gene both within a specific cell (spatio) and at a specific time (temporal) is required[4]. However, conventional methods for establishing these genetically modified animals with spatio-temporal (double-conditional) gene knockdown involve complicated and time-consuming procedures[5].

To overcome these limitations of RNAi, we previously constructed a double-conditional shRNA expression vector[6] from the commercially available pSingle-tTS-shRNA vector (Takarabio-Clontech, Inc., Shiga, Japan[7]. This vector consists of two functional units: a tetracycline (Tet)-induced shRNA expression unit[8] for temporal control, and a recombination unit sequence that is floxed by two loxP-like TATA boxes (TATA-lox sequences)[9] that can be excised by Cre recombinase[10] for cell-specific control.

In this study, we attempted to improve on this previously generated double-conditional shRNA expression vector[6] by constructing an all-in-one vector—one that contains all essential components within a single vector, by integrating a promoter-specific nuclear localizing Cre (NCre) expression unit. We confirmed the inducible expression of NCre based on regulation of the lac promoter (P_{lac}) that can be specifically activated by application of isopropyl-β-D-thiogalactopyranoside (IPTG)[11] and also the functional recombination between floxed TATA-lox sequences in *Escherichia coli*.

Materials and methods

Bacterial strains and culture conditions

E. coli strains DH5α (Nippon Gene Co, Tokyo, Japan) and SHuffle Express (SE) (New England Biolabs [NEB] Inc., Ipswich, MA, USA) were grown at 37°C on LB agar plates containing 50 μg/ml ampicillin and 30°C in 2 × YT medium containing 50 μg/ml ampicillin, respectively.

Construction of plasmids

First, plasmids containing NCre and P_{lac} were amplified from pCre-Bos (kindly donated by Dr. Satoru Arata, Showa University) and pGEX-3X (GE Healthcare UK Ltd., Buckinghamshire,

UK) as templates by PCR, using Phusion DNA polymerase (NEB). The primer set for NCre with multiple cloning sites (MCS) was:

・Forward primer 1 (F1)
: 5'- CCA ATGCAT (Nsi I) GTTTAAACGTACGTCCGGATGATCA (Bcl I) TCCACCAT GCCCAAGAAGAAGAGG -3'

・Reverse primer 1 (R1)
: 5'- GGG CCTAGG (Avr II) CGTTAATGGCTAATCGCCATCTTCCAGCAGG -3'

The primer set for P_{lac} was:

・Forward primer 2 (F2)
: 5'- CCA ATGCAT (Nsi I) TAGGCACCCCAGGCTTTACAC -3'

・Reverse primer 2 (R2)
: 5'- GGA AGATCT (Bgl II) GTTTCCTGTGTGAAATTGTTATCC -3'

Underlined sections in the primers are the recognition sites of the restriction enzymes shown in parentheses.

The PCR products amplified from the coding region of NCre were ligated using the DNA ligation kit Mighty Mix (Takarabio Inc., Shiga, Japan) into the pSingle-tTS-2lox- shRNA vector after digestion with Nsi I / Avr II (NEB). The ligation mixture was transformed into DH5α competent cells. Plasmid DNA from these cells was then purified using the GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA), and this was termed the pSingle-tTS-2lox-MCS-NCre shRNA vector. Next, the PCR product of P_{lac} was digested with Nsi I / Bgl II (NEB), for insertion into the Nsi I / Bcl II (NEB) site of the MCS for the NCre coding region of the pSingle-tTS-2lox-MCS-NCre shRNA vector. This yielded the pSingle-tTS-2lox-P_{lac}-NCre shRNA vector (p2lox-P_{lac}-NCre), which was transformed into DH5α competent cells. The inserted P_{lac} and / or NCre regions in the p2lox-P_{lac}-NCre plasmid were amplified by PCR. The P_{lac} + NCre region was amplified using the F2 + R1 primers; the P_{lac}-only region was amplified using the F2 + R2 primers, and NCre-only region was amplified using the F1 + R1 primers. These regions were separated by electrophoresis on a 1% agarose gel. In addition, the amplified P_{lac}-NCre region was sequenced by Macrogen Japan Corporation (Tokyo, Japan) to confirm correct insertion of the P_{lac} and NCre regions.

SDS-PAGE and western blotting to detect IPTG-induced NCre expression

To detect NCre expression regulated by IPTG in *E. coli*, SE cells transformed with the p2lox-P_{lac}-NCre plasmid were incubated for 4 hours in 2 × YT medium with or without IPTG (100 μM). Total protein extracted from each clone was separated by 10% SDS-PAGE and stained with Coomassie brilliant blue (CBB). This gel was then transferred electrophoretically to an Immun-Blot PVDF membrane for protein blotting (Bio-Rad, Hercules, CA, USA), and probed with anti-Cre antibody (Sigma-Aldrich) or anti-β-galactosidase (Promega, Madison, WI, USA). After incubation with the primary antibody, the membrane was incubated with peroxidase-labeled antibody against mouse IgG (H + L) (human serum adsorbed) (Kirkegaard & Perry Labs Inc., Gaithersburg, MD, USA). ImmunoStar Basic (Wako, Tokyo, Japan) was

applied to the membrane following the protocols supplied by the manufacturer, and then the luminescence signal was analyzed using the C-DiGit (LI-COR, Lincoln, NE, USA) charge-coupled device imager.

Restriction fragment analysis to detect the recombination between floxed TATA-lox sequences

To assess whether the novel vector could functionally regulate floxed TATA-lox sequence recombination by IPTG-induced NCre expression, SE clones with the novel vector were grown in 2 × YT medium with or without IPTG for 16 hours at 30°C. IPTG-induced genetic recombination between the TATA-lox sequences was confirmed by digested fragment analysis using restriction enzyme PvuII (NEB) and electrophoresis on a 1% agarose gel.

Ethics approval

Experimental protocols were approved by the School of Medicine, Showa University Ethical Committee (#1607).

Results

Construction of IPTG-inducible conditional NCre expression vector with P_{lac}

The strategic schema that we used for constructing the all-in-one vector having loxP-like floxed sequence of TATA-lox is shown in Fig. 1 (A) : the overall architecture of novel vector map including P_{lac}, (B) : the multi-cloning sites for the cell-specific promoter of NCre, and (C) : the loxP-like TATA-lox sequence aligned with the original loxP and the partial sequence of the modified U6 promoter ($P_{TightU6}$) including TATA-box.

We confirmed insertion of the P_{lac} and NCre coding regions in the p2lox-P_{lac}-NCre vector by PCR amplification of three fragments (P_{lac} + NCre [1,877 bp], P_{lac} only [803 bp], and NCre only [1,111bp]), which were separated using 1% agarose gel electrophoresis [Fig. 1 (D)]. The faint band at about 1,100bp from the P_{lac} + NCre sample and the smaller bands from the P_{lac}-only and NCre-only samples were thought to be artifacts—non-specific PCR product and primer dimers, respectively. We also confirmed, by DNA sequencing, that the vector contained the P_{lac} sequence followed by the NCre sequence [Fig. 1 (E)].

Evaluation of conditional NCre expression through P_{lac} by IPTG

To evaluate the ability of IPTG-induced expression of NCre in the constructed vector, cellular lysates from SE clones were examined (after 4 hours of incubation with or without IPTG) by SDS-PAGE and CBB staining. We did not observe any detectable enhanced CBB stained bands at about 38 kDa due to NCre (calculated molecular weight, 39.4 kDa) while the induction of endogenous β-galactosidase (116 kDa) by IPTG was observed, which served as an internal control of lac operon activation due to the presence of P_{lac} as shown in Fig. 2A. But we could confirm IPTG-induced expression of NCre when using anti-Cre and anti-β-galactosidase antibodies, as bands at about 38 kDa for NCre and about 125 kDa for β-galactosidase were visible on western blotting as shown in Fig. 2B-1 and Fig. 2B-2, respectively.

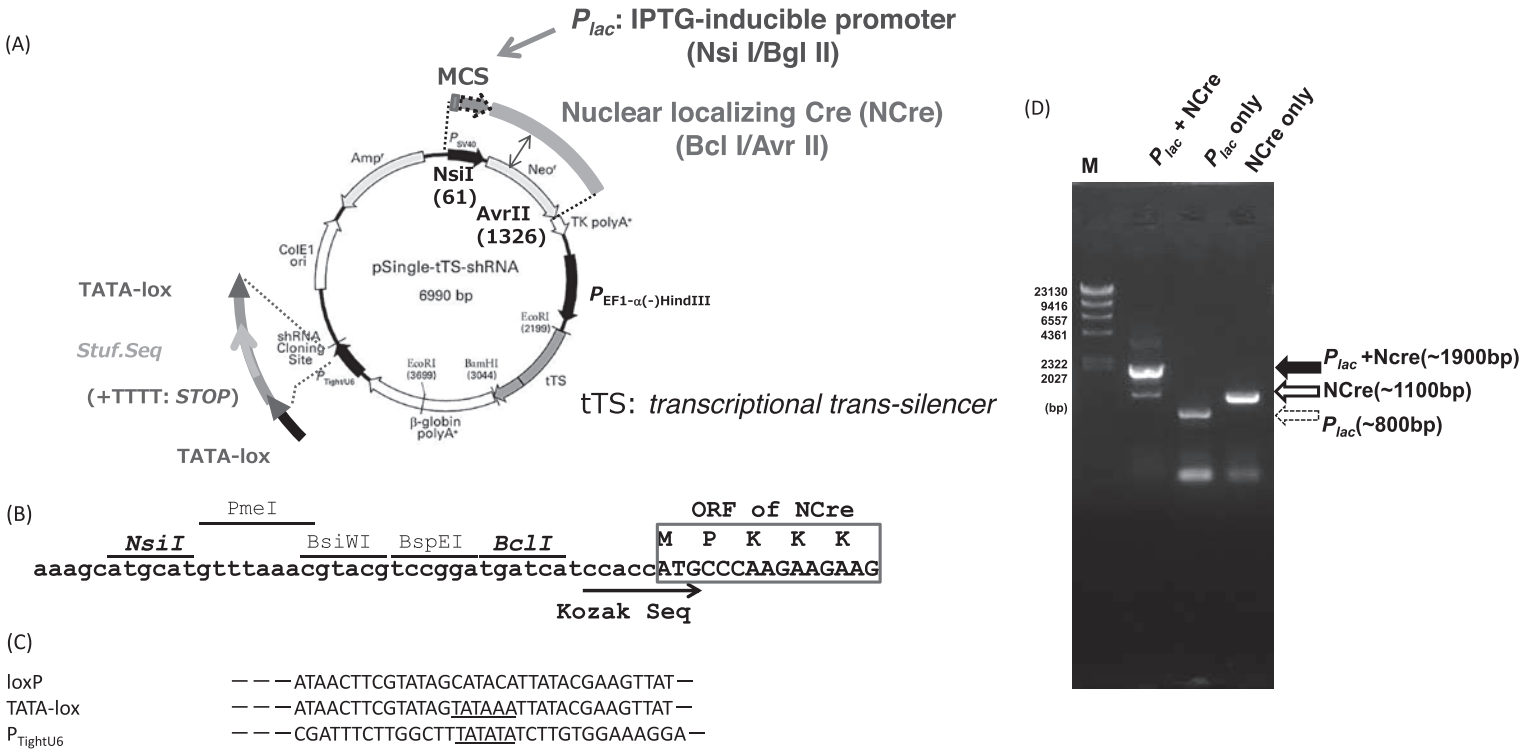

Fig. 1. IPTG-inducible conditional expression vector with P_{lac} and NCre

(A) Vector map of P_{lac} and NCre in the pSingle-tTS-2lox-shRNA vector. This novel all-in-one vector was constructed by modifying a pSingle-tTS-shRNA vector (Takarabio-Clontech Lab), shown in the centre of the circle.

(B) Multiple cloning sites for the cell-specific promoter of NCre. Nsi I / Bcl I sites (shown in bold) in this region were used to clone P_{lac}.

(C) Aligned sequences of loxP (first lane), TATA-lox (second lane) and the partial sequence of the *s*-containing TATA-box (underlined).

(D) PCR amplification of P_{lac} and / or NCre coding regions in the all-in-one vector. Agarose gel (1%) electrophoresis was used to verify the presence of Plac and NCre. Arrows indicate the PCR products of P_{lac} at about 800 bp, NCre at about 1,100 bp, and P_{lac} + NCre at about 1,900 bp. M is the λ / Hind Ⅲ DNA size marker.

(E)

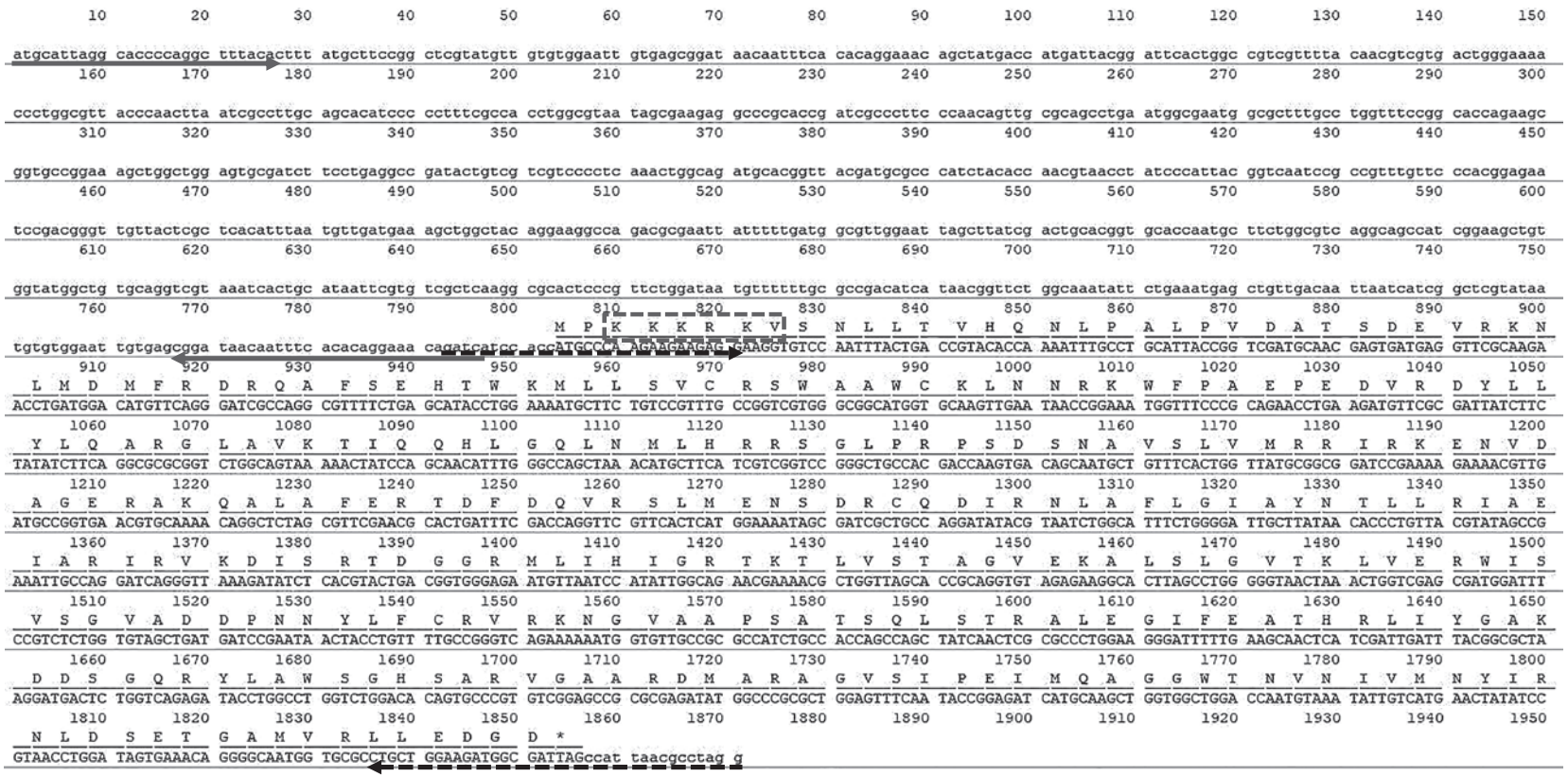

Fig. 1. IPTG-inducible conditional expression vector with P_{lac} and NCre

(E) Full sequence of P_{lac} and NCre coding region in the all-in-one vector. The primer sequences for the coding region of NCre (upper case) are shown in dotted lines and for the coding region of P_{lac} (lower case) are shown in solid lines with the restriction enzyme cutting sites of Nsi I (atgcat) and Avr II (cctagg) to be used for insertion. The nuclear localization signal peptide sequence of NCre is indicated by the dashed box.

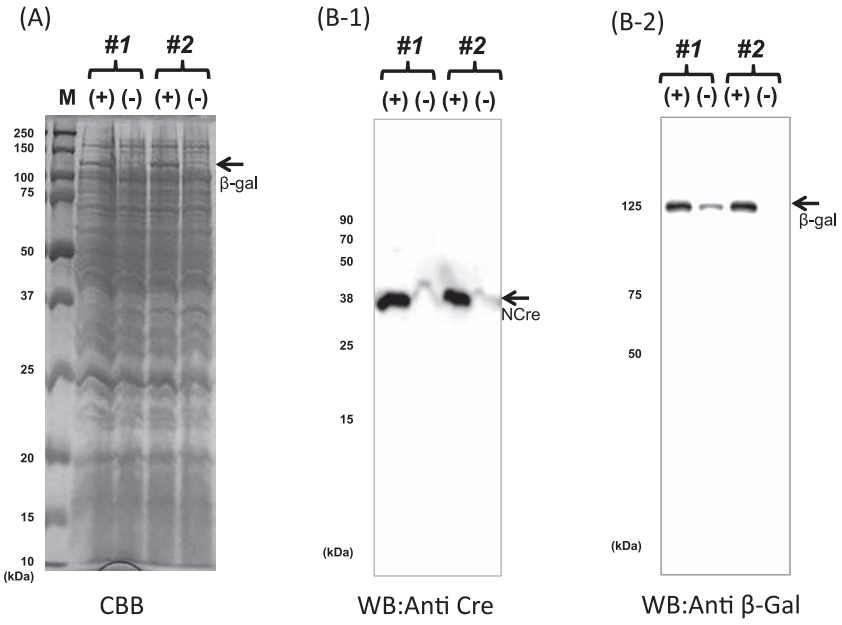

Fig. 2. IPTG-induced conditional expression of NCre

(A) Total protein staining with CBB following SDS-PAGE. Two clones were used, thus results are duplicated. The arrow indicates the expressed β-galactosidase fragment at about 116 kDa.

(B) Western immunoblot analyses with anti-Cre antibodies used as the primary antibodies for NCre expression (B-1) and anti-β-galactosidase antibodies used as the primary antibodies for endogenous β-galactosidase expression (B-2). The (−) and (+) indicate use of cells that were not induced with IPTG, and those that were induced with IPTG, respectively.

Evaluation of IPTG-induced recombination between two TATA-lox sequences

To evaluate whether the constructed vector could functionally regulate TATA-lox sequence recombination by IPTG-induced NCre expression in SE cells, we incubated cells with the p2lox-P_{lac}-NCre vector with and without IPTG. IPTG-treated SE cells resulted in a band at about 4,500 bp that appeared to be a result of Cre-mediated genetic recombination, but this band was absent when SE cells were not treated with IPTG (two lanes in Fig. 3A labeled before PvuⅡ cut). Next, we tried to confirm the recombination more precisely by restriction fragment analysis, using PvuⅡ (two lanes in Fig. 3A labeled after PvuⅡ cut). A fragment of about 4.3 kbp (calculated as 4,291 bp) resulted from IPTG-treated SE cells that appeared to be a result of the loss of one of the PvuⅡ sites between the two TATA-lox sequences by the IPTG-induced expression of NCre, but this band was absent when SE cells were not treated with IPTG, and three fragments of about 7 kbp (calculated as 7,000 bp), 2.3 kbp (calculated as 2,313 bp) and 0.9 kbp (calculated as 932 bp) still appeared in both cases. This result is schematically described in Fig. 3B. This functional recombination occurred in the SE strain of *E. coli*, but it did not occur in other strains that we tested—DH5α, BL21-CodonPlus (DL3)-RP (Agilent Technologies Co., SantaClara, CA, USA), NEB 5-alpha F'-I^q (NEB), and NEB Stable (NEB) (data not shown).

Discussion

In this study, we constructed an all-in-one of double-conditional shRNA expression vector by

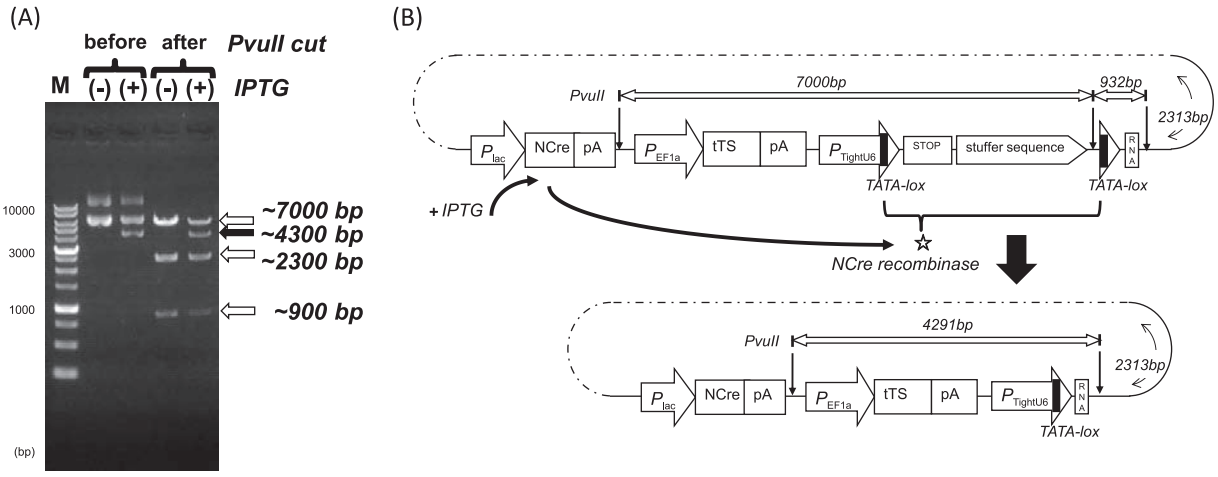

Fig. 3. IPTG-induced floxed TATA-lox sequence recombination in the p2lox-P_{lac}-NCre vector in SE *E. coli* cells. Fragments before and after cutting with Pvu II are shown—ie, the non-cut circular plasmid DNA and the Pvu II-cut linearized fragments from the plasmid DNA. Fragments before and after cutting with Pvu II are also shown following 16-hour incubation of cells in the absence (−) and presence (+) of IPTG.

(A) Agarose gel electrophoresis was used for the restriction fragment analysis. In the last two lanes on the right, the black arrow indicates the fragment with IPTG induction at about 4,300 bp. The white arrows indicate fragments of about 7,000 bp, 2,300 bp and 900 bp, with and without IPTG induction. M is the 1 kb ladder DNA size marker.

(B) Schematic representation of IPTG-induced NCre-mediated recombination in the p2lox-P_{lac}-NCre vector. The Pvu II restriction site is indicated by small black vertical lines. The expected Pvu II restriction fragment sizes are indicated before (upper) and after (lower) the NCre (☆ and bold arrow)-mediated recombination by IPTG application (+ IPTG).

adding the NCre expression cassette to the pSingle-tTS-2lox-shRNA vector that we previously constructed[6]. To do so, the site-specific promoter integrated ahead of NCre was replaced between the P_{sv40} and the Neomycin-resistant region of the original pSingle-tTS-shRNA vector to control the expression of NCre using IPTG in *E. coli*. This represents promotor-specific control of NCre expression which can regulate NCre expression in a cell-specific manner. This research has created an experimental foundation for producing transgenic mice with this double-conditional shRNA expression vector—an all-in-one vector. With this type of vector, only a single round of gene transfer into the host cells or fertilized eggs is needed because this novel vector contains both the Tet-inducible floxed TATA-lox sequence and the cell-specific Cre expression systems. For this reason, it is not necessary to cross genetically engineered mice expressing the NCre recombinase in a cell-specific manner with mice that have been engineered to have Tet-inducible shRNA expression in a Cre-mediated floxed manner. We expect that this will enable the generation of genetically engineered mice in a shorter period and with less effort.

A problem that may arise by putting all systems in the one vector is that the vector may become very long, depending on the size of cell-specific promoter. We carried out preliminary experiments and confirmed that integrating the core region of our all-in-one vector (Fig. 4) is possible with the Transposon plus Insulators and Super PiggyBac Transposase systems (Transposagen Inc., Lexington, KY, USA), which have been reported to be capable of mobilizing 100 kb DNA fragments in mouse

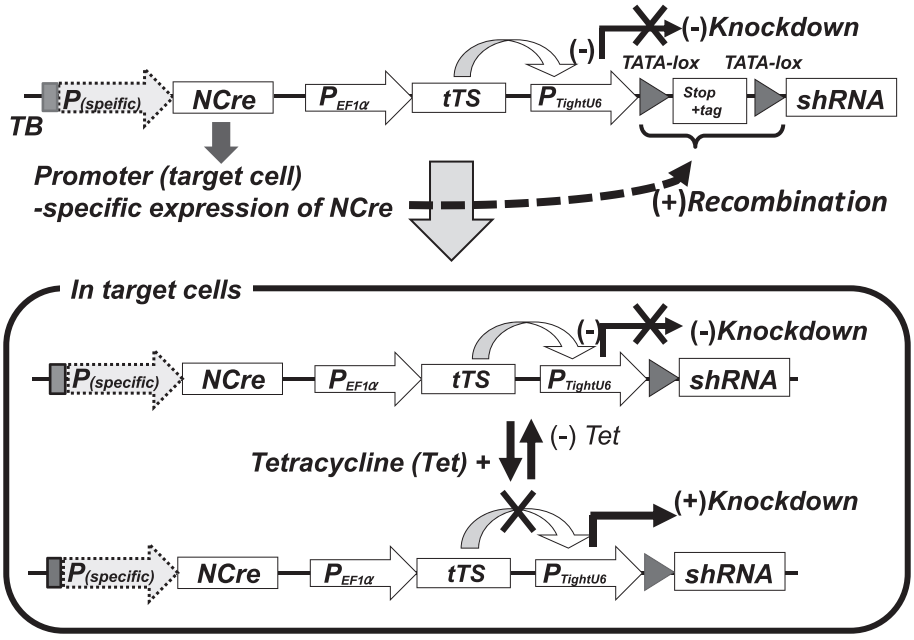

Fig. 4. Schematic representation of *in vitro* or *in vivo* application of the all-in-one double-conditional shRNA vector

The core region of the vector (shown in the upper part of the schematic) is integrated into the mouse genome. In specific differentiated target cells, promoter-specific expression of NCre will excise the stuffer sequence containing the terminator poly T signal sequence between the loxP-like TATA-lox sequences. Before Tet application, the modified Tet-inducible promoter ($P_{TightU6}$) is inactivated (arrow with cross) by the transcriptional trans-silencer (tTS). After Tet application, Tet binds to the tTS, which frees the $P_{TightU6}$ from the tTS, leading to activation of the $P_{TightU6}$ and expression of the shRNA, which causes knockdown of the target gene product (shown in the lowest part of the schematic).

embryonic stem cells[12, 13].

The advantage of this novel system is that it provides a means of reversible knockdown of a gene of interest by Tet application at the target life stage in an experiment. When P_{lac} is replaced with a target cell-specific promoter, NCre will express within the target cells, leading to shRNA-mediated knockdown of the gene of interest in these cells by Tet application (Fig. 4). In addition, for clinical use, replacing P_{lac} with a tumor-specific promoter and an shRNA sequence that targets an anti-apoptotic factor such as Bcl-2 could induce tumor cell-specific apoptosis by Tet application[14].

In conclusion, the novel vector that we constructed enables spatial and temporal control of shRNA expression *in vivo* to facilitate functional genetic regulation in specific cells. It could also be used for cell targeting, to analyze the functional roles of specific genes and the physiological roles of specific cells during precise periods of life.

Conflict of interest disclosure

We have no financial conflict of interest to disclose concerning this study.

Acknowledgments

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