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**Short Communication**

# Temporal Control of Cre Recombinase-mediated *in Vitro* DNA Recombination by Tet-on Gene Expression System

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**Abstract** Conditional gene expression and gene deletion are important experimental approaches for examining the functions of particular gene products in mouse models. These strategies exploiting Cre-mediated site-specific DNA recombination have been incorporated into transgenic and gene-targeting procedures to allow *in vivo* manipulation of DNA in embryonic stem cells (ES cells) or living animals. The Cre/*lox* P system has become widely used in conditional gene targeting, conditional gene repair and activation, inducible chromosome translocation, and chromosome engineering. In this project, we have employed the universal transgenic system and the liver-specific promoter system for tightly temporal and liver-specific control of Cre gene expression in mice that (1) integrates the advantages of the Tet-on gene expression system and Cre/*lox* P site-mediated gene activation, and (2) simplifies the scheme of animal crosses through a combination of two control elements in a single transgene. A liver-specific *apoE* promoter was inserted into the promoter cloning site upstream of the rtTA cassette of pCore construct to generate the transgene construct pApoErtTA-tetO-Cre, followed by demonstrating stringent regulation of doxycycline (Dox)-induced Cre-mediated recombination in the *lox* P-flanked transcription STOP cassette-modified BEL-7402 cells. That is to say, in the absence of Dox, the *Cre* gene is not expressed and will not induce site-specific recombination between two *lox* P sites, whereas on exposure to Dox, the *Cre* gene will be expressed and the recombination will occur. Together, these data indicate that the Tet-on gene expression system is able to successfully and stringently control *Cre* expression *in vitro*, which lays a solid foundation for efficient and spatio-temporal *Cre* gene activation in transgenic mice.

**Key words** conditional gene regulation; Cre recombinase; human apolipoprotein E gene (*apoE*) promoter; Tet-on gene expression system; universal transgenic system

Exploration of gene functions and development of mouse models are routinely based on genomic manipulation via homologous recombination in embryonic stem cells (ES cells). Such studies are, however, compromised if the ablated gene is essential for mouse development [1–3]. To overcome these difficulties, a number of strategies for conditional gene inactivation or activation have been developed during past years [1–3].

The Cre/*lox* P system has already been widely used in the different research fields [4,5]. Temporal regulation of Cre expression has been attempted by linking the *Cre* gene to a number of inducible promoters [1–5]. In one study, the *Mx1-Cre* transgene was transiently activated by interferon [6]. However, the excessive amount of interferon used in such induction may lead to biological side effects, which can complicate the analysis of gene functions. Moreover, temporal tissue or cell type specific expression has also been attempted by fusing Cre to a mutated steroid ligand-binding domain of the receptor under the control of tissue or cell type specific promoters. The chimeric protein becomes active upon interaction with the synthetic

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ligand tamoxifen or RU486 [1–3]. Both tamoxifen and RU486 are, however, prone to potential side effects resulting from interference with the endogenous ligands estrogen and progesterone. In addition, spatial and temporal control of DNA recombination can also be achieved by local administration of viruses expressing *Cre*. The major obstacles of this approach are toxicity and immunogenicity associated with viral infection [1–4].

Combining the Cre/*lox* P system with tetracycline-mediated regulation of gene expression is a promising but insufficiently explored alternative to the approaches just described [5,7]. Tetracycline-inducible systems (Tet-off system or Tet-on system) have been the most widely employed inducible regulation systems [8,9]. Based on the characteristics of two systems that work through opposite mechanisms, if a gene is to be kept inactive most of the time and turned on only occasionally, the Tet-on system seems more appropriate. Previously, only partial control of Cre-mediated recombination by the Tet-off system has been achieved [7]. As this system required continuous presence of an inducer to prevent *Cre* expression, long-term maintenance of the necessary inducer concentrations during development could be technically challenging and increase the likelihood of side effects. Furthermore, significant leakiness of the system rendered described mice of very little use. To improve the usefulness of the tetracycline-regulated Cre-*lox* P system, Utomo *et al.* [10] has established a universal transgenic system that combines two layers of regulation, provided by a tissue or cell type specific promoter and the Tet-on system. This system allows accurate regulation of gene recombination in a target cell lineage at any given time and location after a single cross with *lox* P-containing mice. Importantly, only brief exposure to doxycycline (Dox), either systemic or local, is required for induction of Cre-mediated DNA deletion. In our project, we plan to integrate the universal transgenic system [10] and the liver-specific promoter system to realize the spatio-temporal expression of the *Cre* gene in transgenic mice, which will be widely used in conditional gene targeting and conditional gene expression.

In the present study, the transgene vector was generated, followed by *in vitro* analysis of Dox-induced Cre-mediated DNA recombination in cultured cells.

## Materials and Methods

### Major chemicals and solvents

The human hepatoma cell line BEL-7402 was from the Cell Bank, Center of Experimental Animals, Sun Yat-Sen University. The *lox* P-flanked transcription STOP cassette-modified BEL-7402 cells (*lox* P-modified BEL-7402 cells) were derived by stably transfecting BEL-7402 cells with the plasmid of pApoE-SCS-EGFP (data not shown). pApoE-SCS-EGFP contains the STOP cassette sequence (SCS) from pBS302 [11] positioned downstream of the human apolipoprotein E gene promoter (*apoE* promoter) and upstream of the enhanced green fluorescent protein gene (*EGFP*; pEGFP-C1, Clontech). The transcription STOP cassette consists of a C-terminal sequence of yeast *His*3 gene, an SV40 polyadenylation signal (polyA) and a 54 splice donor site [11]. In the absence of Cre, *EGFP* expression is prevented by the intervening transcriptional STOP sequence flanked by *lox* P sites [11], while in the presence of Cre, Cre-mediated DNA recombination results in removal of the STOP sequence followed by activating EGFP expression in the *lox* P-modified BEL-7402 cells. The general activation strategy is fully demonstrated in **Fig. 1(B)**.

Lipofectamine™ 2000, RPMI 1640 medium, fetal bovine serum (FBS), 0.25% trypsin solution, HEPES, dimethyl sulfoxide (DMSO), culture flasks and plates were supplied by Gibco BRL (Life Technologies Inc., Grand Island, NY, USA). Other chemicals and materials were of analytical grade from commercial sources.

### Source of plasmids

The pLiv.7 vector [12] containing a 3.0 kb *apoE* promoter for the expression of liver-specific genes was kindly provided by Dr. C. Y. FAN (Department of Pathology and Otolaryngology, University of Arkansas for Medical Sciences, USA). The pCore transgenic construct [10] contains two expression modules: (1) Cre recombinase encoding sequence under the regulation of the rtTA-responsive hybrid promoter consisting of tetO heptad repeat and hCMV minimal promoter, and (2) the rtTA cassette containing rtTA encoding sequence and SV40 polyadenylation site. The *Eco*RI site upstream of the rtTA cassette allows cloning of virtually any promoter of acceptable length to direct rtTA expression. In the current work, the 3.0 kb *apoE* promoter was inserted to generate apoErtTA-tetO-Cre transgene. The 4 kb *p53* intron separates the modules to reduce potential transcriptional interference. The vector of pBS185 [11] containing the Cre coding sequence under the control of the *CMV* promoter was generously provided by Dr. B. SAUER (Stowers Institute for Medical Research, Kansas City, Missouri, USA).

### Plasmid construction

For the liver-specific expression of the *Cre* gene *in vivo*,

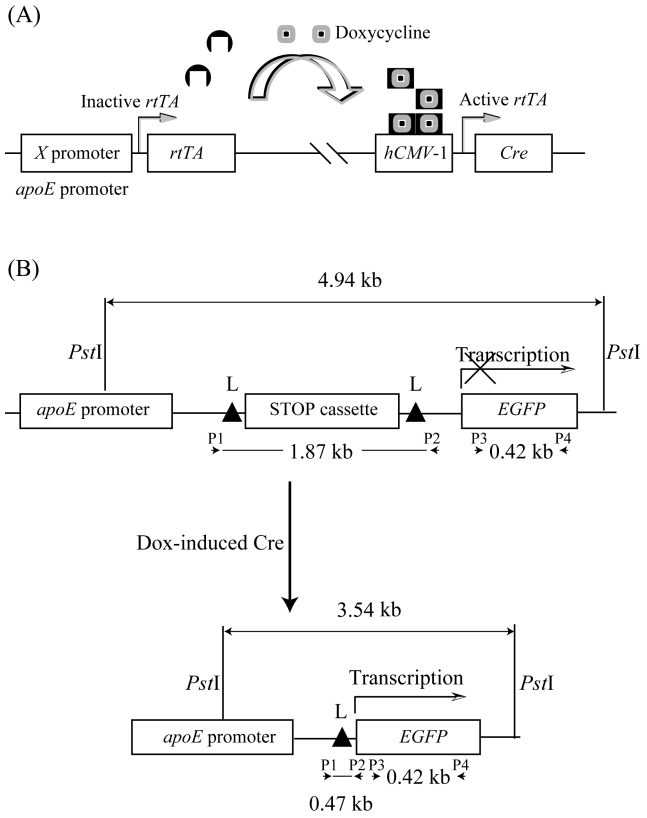


**Fig. 1 Experimental design**

(A) Design of conditional Cre-mediated recombination. A liver-specific apolipoprotein E gene (*apoE*) promoter in the current studies is used to direct the expression of rtTA to the murine liver. Without doxycycline (Dox), rtTA is inert and unable to activate *Cre* transcription, while in the presence of Dox, rtTA binds to the tetO-hCMV promoter leading to *Cre* expression. The construct map is not drawn to the scale. (B) *In vitro* assay for Dox-controlled Cre-mediated DNA recombination. In the absence of Cre, *EGFP* expression is prevented by the intervening transcriptional STOP cassette sequence (SCS) flanked by *lox* P sites [11,15]. Cre-mediated DNA recombination results in removal of the STOP sequence followed by *EGFP* expression. The SCS consists of a C-terminal sequence of yeast His3 gene, an SV40 polyadenylation signal (polyA) and a 54 splice donor site [15]. The primers (P1 and P2) represented by small arrows were used in PCR analysis of recombination events. P1 and P2 recognize sequences of human *apoE* gene 1st intron and *EGFP*, respectively. Amplification with primers EGFP-P3 (5′-ACCTACGGCGTGCAGTGCTTC-3′) and EGFP-P4 (5′-ACTGGGTGCTCAGGTAGTGGTTG-3′) resulted in 420 bp amplified product of EGFP coding sequence of the transgene. Primers P3/P4 were used as an internal control for PCR efficiency. The construct map is not drawn to the scale. L, *lox* P site.

a liver-specific *apoE* promoter was inserted into the promoter cloning site upstream of the *rtTA* cassette (e.g., *Eco*RI site) of pCore construct [10] to generate a transgene construct of pApoErtTA-tetO-Cre in order to target the *rtTA* expression to murine liver *in vivo* because in the Tet-on regulated transgenic mice, tissue specificity of *Cre* gene expression is conferred by the promoter driving *rtTA* expression [1–3].

The 3.9 kb *Sac*II/*Mun*I fragment, containing an *apoE* promoter (about 3.0 kb) and an *apoE* intron (0.9 kb), from the vector of pLiv.7 was blunted (*Sac*II at 5′ end and *Mun*I at 3′ end) using a Klenow fragment, and subsequently cloned into the blunted and dephosphorylated *Eco*RI site of pCore construct to produce the vector pApoErtTA-tetO-Cre. The orientation of the inserted DNA in the frame was identified using *Sal*I single enzyme digestion.

## Analysis of Dox-induced Cre-mediated DNA recombination in cultured cells

*Cell transfection* Before establishing transgenic mice, the *lox* P-modified BEL-7402 cells were transiently transfected with plasmids to further test the Tet-on system for validity and tightness, and the function of pApoErtTA-tetO-Cre at the cellular level. The cell culture and transient transfection were performed as previously reported [13].

The *lox* P-modified BEL-7402 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 2 mM *L*-glutamine (Sigma, St. Louis, USA), 100 U/ml penicillin (Sigma), and 100 mg/ml streptomycin (Sigma), refreshed 2–3 times per week and passed when being confluent. The cells were kept at 5% $CO_2$ in a humidified 37 °C incubator. The day before transfection, *lox* P-modified BEL-7402 cells were added into six-well culture plates, and transiently transfected with the corresponding plasmid (for details, see legend of **Fig. 3**) according to the liposome-mediated transfection method using Lipofectamine™ 2000 once the culture reached 70%–80% confluency. The transfecting solution was replaced with the fresh complete medium 6–10 h after transfection. In the pApoErtTA-tetO-Cre+Dox transfecting group, the Tet-on system was activated for 48 h by the addition of Dox (1 mg/ml; Clontech) after discarding transfecting solution. The transfection experiment was repeated three times with 10 μg of each corresponding plasmid being employed, respectively. In addition, all plasmids used for transfection were purified using EndoFree plasmid maxi kit (Qiagen, Hilden, Germany) and non-linearized.

*EGFP assay* 48–96 h after transfection cells were screened for *EGFP* expression under fluorescence microscopy (excitation 450–490 nm).

*DNA recombination analysis by polymerase chain reaction (PCR) and Southern blot analysis* To detect the post-Cre-mediated DNA recombination product, the primers P1 (5′-GGAATTTTCTATGGAGGCCG-3′) and P2 (5′-GAACTTCAGGGTCAGCTTG-3′) were used to detect DNA recombination mediated by Cre. PCR reactions using

these primers involved 30 cycles of denaturation (45 s at 94 °C), primer annealing (45 s at 62 °C), and primer extension (2 min at 72 °C).

To further confirm the post-Cre-mediated DNA recombination product, Southern blots were performed by standard techniques [14] and following the manufacturer's instructions of North2South® direct HRP labeling and detection kit (Pierce, Rockford, IL, USA). Briefly, genomic DNA was digested overnight with *Pst*I, fractionated by electrophoresis through 0.8% agarose gel in tris-borate-EDTA (TBE) buffer (90 mM tris-borate, 2 mM EDTA, pH 8.0), transferred onto a positively charged nylon membrane (Schleicher & Schuell, Keene, NH, USA), which was not fixed with UV crosslinking, by alkaline transfer, and subjected to prehybridization and hybridization with the probe of about 740 bp HRP-labeled EGFP fragment synthesized according to the protocol of the probe labeling in kit. After stringency washes, the membranes were subjected to chemiluminescence analysis. The chemiluminescence-treated membranes were then exposed to X-ray film (X-Omat AR-5, Eastman Kodak Company, Rochester, NY, USA), usually for 1–10 min at room temperature.

PCR amplification and Southern blot analysis were performed on genomic DNA prepared with standard protocols [14] from the *lox* P-modified BEL-7402 transfected with or without plasmid.

## Results and Discussion

### Construction of the Cre expressing vector pApoErtTA-tetO-Cre

The human *apoE* regulatory region (3.9 kb) (**Fig. 2**), excised from pLiv.7 with *Sac*II and *Mun*I and blunted at 3′ and 5′ ends, was cloned into the blunted and dephosphorylated *Eco*RI site of pCore construct (9 kb) (**Fig. 2**), designated pApoErtTA-tetO-Cre (12.9 kb), as verified by enzyme digestion (**Fig. 2**). The orientation of the *apoE* promoter insert in the frame was identified by *Sal*I. The positive recombinant gave 8.23 kb and 4.67 kb bands (**Fig. 2**), while the inverted recombinant generated two fragments of 12.13 kb and 0.77 kb (data not shown) after digestion by *Sal*I. In addition, the complete sequence of the dormant transgene was further verified by sequencing (data not shown).

### Tight regulation of Dox on DNA deletion

As shown in **Fig. 1(B,C)**, the strategies of conditional expression of Cre protein with Tet-on system and of EGFP mediated by the Cre/*lox* P switching expression system are presented, respectively.

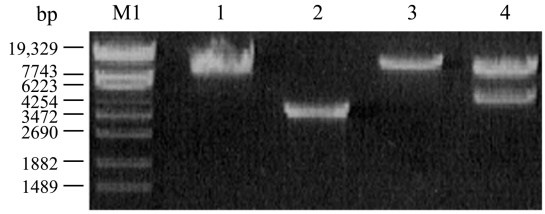


**Fig. 2 Identification of the resulting vector pApoErtTA-tetO-Cre by enzyme digestion analysis**

M1, λ-*Eco*T14I DNA marker (TaKaRa); 1, vector DNA for ligation; 2, insert DNA for ligation; 3, pApoErtTA-tetO-Cre-*Xho*I; 4, pApoErtTA-tetO-Cre-*Sal*I.

To evaluate the effectiveness of Dox control of Cre-mediated DNA deletion *in vitro* by screening *EGFP* expression under fluorescence microscopy, *lox* P-flanked STOP cassette-modified BEL-7402 cells, stably carrying the dormant transgene pApoE-SCS-EGFP, were developed (data not shown). To address this end, pApoErtTA-tetO-Cre and pBS185 (**Fig. 3**) were transfected into the *lox* P-modified BEL-7402 cells, and thereafter cells grew without or with Dox (1 mg/ml).

In the negative control group, cells had almost no background under fluorescence microscopy [**Fig. 3(A)**, a] and phase contrast microscopy (data not shown). Cre expressed from pBS185 successfully removed STOP sequences from the EGFP reporter plasmid (e.g, pApoE-SCS-EGFP) as measured by EGFP fluorescence [**Fig. 3 (A)**, b]. In the absence of Dox, EGFP expression could not be detected in the cells transfected with pApoErtTA-tetO-Cre [**Fig. 3(A)**, c] as expected, due to no activation of rtTA by Dox, followed by no induction of Cre expression via active rtTA, which did not remove the strong transcriptional STOP sequence inserted between the promoter and the coding sequences by Cre-mediated recombination so as not to lead to expression of EGFP. In contrast, after exposure to Dox, EGFP expression was detected in the *lox* P-modified BEL-7402 cells transfected with pApoErtTA-tetO-Cre [**Fig. 3(A)**, d]. In addition, no background was also observed under fluorescence microscopy and phase contrast microscopy when the *lox* P-modified BEL-7402 cells were transiently transfected with pCore construct without *apoE* promoter (data not shown).

Finally, Cre-mediated DNA recombination was directly identified by PCR analysis [**Fig. 3(B)**]. In cells transfected with salmon sperm DNA alone and pApoErtTA-tetO-Cre, a 1870 bp non-recombined fragment was observed, while

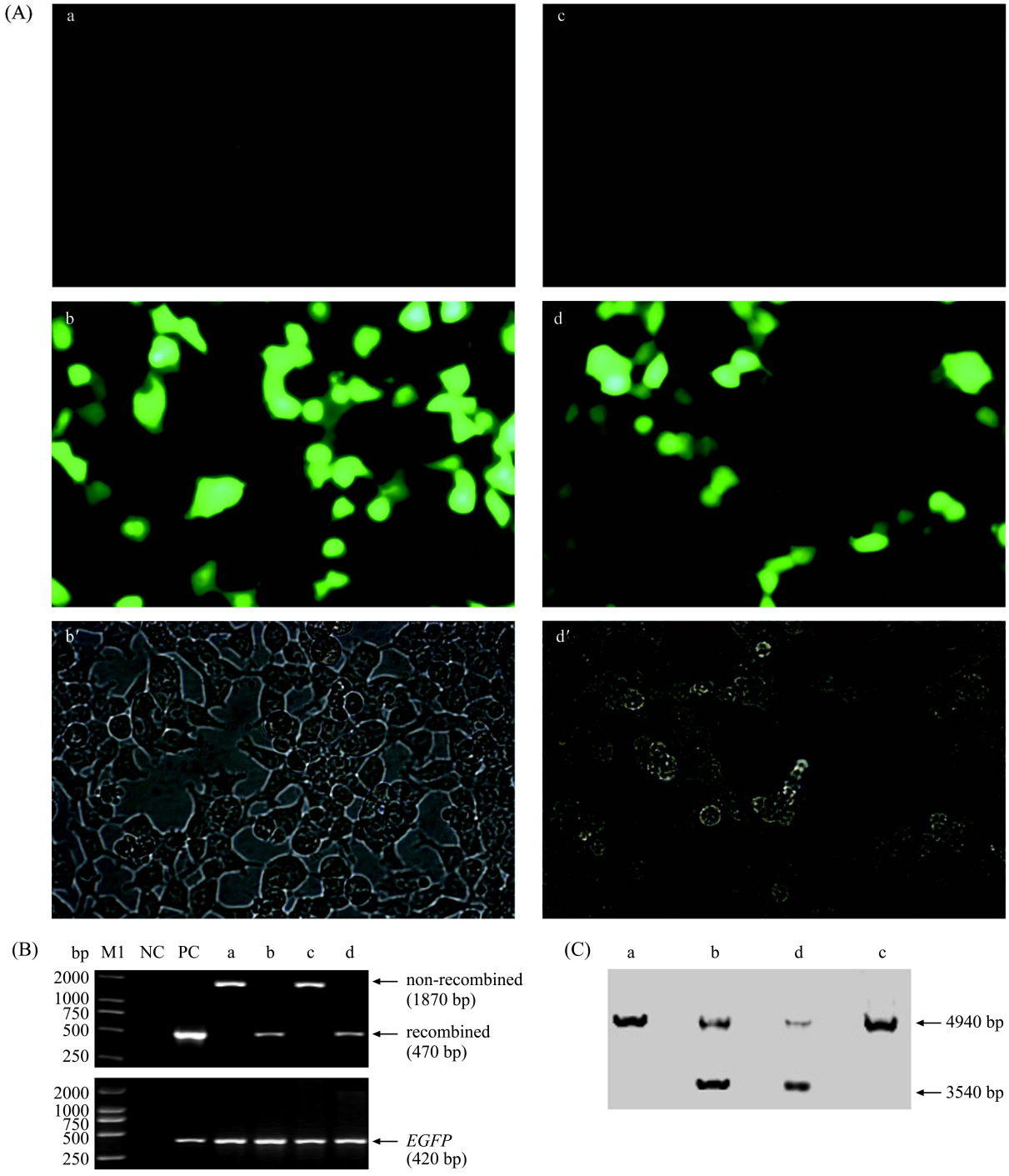


**Fig. 3 *In vitro* conditional expression of EGFP mediated by Dox-induced Cre recombinase**

(A) EGFP assay under fluorescence microscopy. (B) Identification of Cre-mediated DNA recombination product by PCR. A typical analysis of the PCR products by agarose gel electrophoresis from a positive control [pApoE-lox P-EGFP (resulted from removal of SCS from the vector pApoE-SCS-EGFP *in vitro* digested with commercially available Cre recombinase) for recombined DNA and EGFP] (lane PC), genomic DNA from *lox* P-modified BEL-7402 cells transfected with plasmid mentioned below. The arrows indicated the positions of predicted PCR products. The 470 bp and 1870 bp fragments were diagnostic for the recombined DNA and non-recombined DNA, respectively. The 420 bp PCR product was a result of EGFP amplification. (C) Southern blot analysis of recombination in *lox* P-modified BEL-7402 cells. Non-recombined *lox* P-SCS transgene produced a 4.94 kb *Pst*I fragment while the 3.54 kb recombined fragment was only detected in cells containing the Cre transgene. M1, PCR marker (DL2000, TaKaRa); NC, PCR without template DNA input; a, cells transfected with salmon sperm DNA alone; b and b′ (under phase contrast microscopy), cells transfected with pBS185 (positive control) alone; c, cells transfected with pApoErtTA-tetO-Cre alone; d and d′ (under phase contrast microscopy), cells transfected with pApoErtTA-tetO-Cre with the addition of Dox.

cells transfected with pBS185 or pApoErtTA-tetO-Cre followed by exposure to Dox generated a 470 bp recombined fragment [**Fig. 3(B)**]. Note that absence of the 1870 bp fragment does not indicate that recombination occurred in every cell as non-recombined transgenes were detected in Southern blot analysis [**Fig. 3(C)**]. The absence of the non-recombined fragment is due to the fact that PCR kinetics favors amplification of the shorter target sequence.

These data demonstrate tight regulation of Cre-mediated recombination by Dox in the *lox* P-modified BEL-7402 cells.

Additionally, the tissue specificity of expression for the recombinationally activated dormant transgene mediated by the Cre/*lox* P switching expression system in the transgenic mice is a function both of the promoter specificity of the target transgene and of the promoter specificity of the *Cre* transgene. In this project, the liver-specific promoter system was employed to indirectly target *Cre* expression to the physiologically relevant site-hepatocyte of target proteins, such as the viral antigen(s), because the rtTA expression in the transgenic mouse liver was controlled by *apoE* promoter, which in turn determined the site(s) of *Cre* transgene expression *in vivo*.

In summary, we conclude that the Tet-on gene expression system used here is able to successfully and stringently control the expression of Cre recombinase *in vitro*, which lays a solid foundation for efficient and spatiotemporal *Cre* gene activation in transgenic mice, in which utilizing the combined systems, Cre transcription can be activated in the presence of Dox and blocked by its withdrawal.

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