Original

Thy1 promoter activity in the Rosa26 locus in mice: lessons from Dre-rox conditional expression system

Saaki TAMURA1), Yukiko YASUOKA2), Hiromi MIURA1,3), Gou TAKAHASHI1,4), Masahiro SATO5) and Masato OHTSUKA1,3,6)

1)Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
2)Department of Physiology, Kitasato University, School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0374, Japan
3)Center for Matrix Biology and Medicine, Graduate School of Medicine, Tokai University, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
4)Department of Bioproduction, Tokyo University of Agriculture, 196 Yasaka, Abashiri, Hokkaido, 099-2493, Japan
5)Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima, Kagoshima 890-8544, Japan
6)The Institute of Medical Sciences, Tokai University, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan

Abstract: The pronuclear injection (PI)-based targeted transgenesis (PITT) method allows the generation of targeted transgenic (Tg) mice wherein a single copy of a transgene is integrated into the Rosa26 locus following PI. The Rosa26 locus allows unbiased ubiquitous expression of integrated transgenes; however, it remains little known whether tissue-specific promoters retain their functional properties when placed at the Rosa26 locus. We evaluated tissue-specific activity and reproducibility of exogenous tissue-specific promoters targeted to the Rosa26 locus by generating Thy1-Dre/Dre reporter mice using PITT and assessed spatial expression patterns of the transgenes. The Thy1 promoter targeted to the Rosa26 locus appeared active in virtually all Purkinje cells in the cerebellum and hippocampus. However, mosaic expression of the transgene under the Thy1 promoter was observed in many other organs. This phenomenon was consistent in all the Tg lines generated by PITT, indicating a high degree of reproducibility for this experiment.

Keywords: Rosa26, Thy1 promoter, tissue-specific promoter, transgenic mouse

Introduction

Transgenic (Tg) mice are widely used for analyses of in vivo gene function and as models for human diseases. The production of Tg mice has long relied on direct microinjection (MI) of a DNA fragment into the pronuclei of fertilized eggs. This generates random integration of the transgene (or genes) with multicopy configuration into host chromosomes, often leading to unreliable and unstable expression of the transgene [13]. Researchers must thus select appropriate Tg lines with desired transgene expression patterns that faithfully recapitulate endogenous gene expression. Unfortunately, this selection procedure requires considerable time, effort, and cost. Embryonic stem cell-mediated transgenesis has been developed as an alternative strategy to generate Tg mouse lines, but this procedure is costly and laborious [13].

We developed a novel targeted transgenesis method through MI called pronuclear injection-based target transgenesis (PITT) to overcome drawbacks associated with conventional transgenesis [12]. Later, we developed a refined technique called improved PITT (i-PITT) to allow more efficient targeted insertion of a transgene into a Rosa26 locus using site-specific recombinases and/or
integrate systems [14]. This enabled stable transgene expression in various cell types because of the open chromatin configuration offered by Rosa26 locus. We found that in Tg mice obtained using Pitt/i-Pitt, stable and reliable expression of transgenes were observed throughout the body [12, 15]. Thus, this locus is an ideal site for the insertion of a transgene harboring a ubiquitous promoter.

Compared to the cases wherein transgenes carrying ubiquitous promoters were inserted into the Rosa26 locus, little is known about the insertion of transgenes carrying tissue-specific promoters at this same locus. Since the Rosa26 locus is one of the most frequently used loci for transgene integration and a large number of tools have been specifically developed to modify this locus, an increased depth of knowledge about tissue-specific promoter activities therein would further increase its use and viability. The Thy1 (thymocyte differentiation antigen 1; also called CD90) promoter is often used to express transgenes in neuronal cells and is considered neuron-specific [1, 3, 4]. To our knowledge, studies exploring the Thy1 promoter activities at the Rosa26 locus are yet to be reported.

For tissue-specific transgene expression in mice, Cre-loxP system derived from bacteriophage P1 has been widely used. Cre enzyme catalyzes recombination between two loxP sites. Numerous Cre mouse-lines have been generated that express the Cre enzyme from a large variety of tissue-specific promoters [5, 9]. In the mouse that contains “tissue-specific Cre expression cassette” together with “a transgene having a loxP-flanked ‘stop’ sequence between the promoter and a gene of interest”, Cre recombines removes the stop sequence and as a result the transgene becomes to be expressed only in the cells where Cre is active. Dre, derived from bacteriophage D6, is another site-specific recombinase capable of catalyzing recombination between two rox sites, but does not cross-react with the Cre-loxP system [2]. Although Dre appears to display comparable recombination efficiency to Cre, only a few studies in which Dre is used for tissue-specific transgene expression have been reported so far.

We generated Tg mice with a Thy1-Dre transgene during the development of the Pitt method wherein Dre expression is controlled by the Thy1 promoter and placed at the Rosa26 locus [16]. Until now, however, we had not examined expression patterns thereof. In this study, we generated double-Tg mice by mating Thy1-Dre mice with fluorescent Dre reporter mice also created by the Pitt method [17] to assess Thy1 promoter activity at the Rosa26 locus and evaluated the expression of the reporter gene in different organs, including the brain, pancreas, intestine, kidney, heart, liver, and retina.

Materials and Methods

Mice
Thy1-Dre founder mice were previously obtained using the Pitt method by injection of the Thy1-Dre expression vector (pBFD) together with iCre mRNA into fertilized eggs from TOKMO-1 mice [16]. These founders were then mated with FLPe Tg mice (RIKEN BRC; #RBRC01835) [8] to eliminate the unnecessary region containing the vector sequence. The Thy1 promoter region used in this study was derived from Thy1-Brainbow-1.1 M (a gift from Joshua Sanes [Addgene plasmid #18726]) [11]. Thy1-Dre mice are of a genetically mixed background derived from the C57BL6/J, DBA2, and 129/Ola mouse lines. A Dre reporter mouse strain was previously generated by the Pitt method by injection of the donor vector (pAXV) into the TOKMO-1 mouse zygote [17]. The mouse contains a Dre reporter cassette (CAG promoter::rox-EGFP::poly(A)::CAT (chlortomycenic acetyltransferase)::poly(A)::rox::tdTomato::poly(A)) at the Rosa26 locus. The genetic background of the Dre reporter mouse strain also derived from C57BL6/J, DBA2, and 129/Ola lines. The Rosa-Dre mouse was generated using i-Pitt by injection of the vector (pBGX) containing the Dre coding sequence into the Tg mice obtained using Pitt/i-Pitt by injection of the donor vector (pAXV) into the TOKMO-3 mouse zygote [14]. In this mouse, Dre expression is driven by the ubiquitous Rosa26 promoter. The genetic background of Rosa-Dre mice is C57BL6/N.

Double-Tg mice containing both the Dre reporter allele and the Thy1-Dre allele delivered pups with a deletion allele for the Dre reporter (showing only tdTomato fluorescence). We also used this strain for analyses as tdTomato reporter mice. These mice are maintained as frozen sperm and will be available upon request.

Mice were maintained at the animal facility in Tokai University School of Medicine. They were provided ad libitum food and water under a 12:12 light and darkness cycle. All animal experiments were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (permit numbers #104001, #115017, #121007, #132013, #143037, and #154014) at Tokai University.

Genotyping
Genotypes of the mouse strains were determined by PCR using genomic DNA isolated from ear biopsies as templates. The presence of the Thy1-Dre allele was checked with the M274/M845 primer set (Supplementary Table 1). The FLPe transgene was amplified with M580 and M587. Presence or absence of the extra sequence was determined using the M070/M582 or M273/M844 primer sets, respectively. The presence of the Dre...
reporter allele was confirmed by PCR using the M124/M274 primer set. The Rosa-Dre allele was detected using the M645/M646 primer set.

**Tissue collection, processing, and fluorescence observation**

The preparation of frozen sections was performed as described previously [15]. Frozen 8 µm sections were imaged for fluorescence after staining with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence observation was performed as previously described [15].

**In situ hybridization**

Anesthetized adult mice were perfused with phosphate-buffered saline, followed by 4% paraformaldehyde. Brain samples were fixed overnight in 4% paraformaldehyde at 4°C and then washed in phosphate-buffered saline, dehydrated, and embedded in paraffin for sectioning into 3 µm slices. Dre mRNA expression patterns in the hippocampus, cerebellum, and choroid plexus were analyzed using the hybridization probe for the Dre gene. A DNA fragment containing a part of the Dre gene was amplified by PCR with primers M645 and M1096 (Supplementary Table 1), followed by synthesis of the RNA probe using the DIG RNA Labeling Kit (Roche Diagnostics). In situ hybridization was performed as previously described [21].

**Results**

**Thy1-Dre mice**

We previously developed Tg founder mice wherein the Thy1-Dre transgene had been inserted into the Rosa26 locus (Fig. 1A) [16]. Each of the resulting three

![Fig. 1. Schematic diagram of the transgenes contained in each Tg mouse. All transgenes were inserted into the Rosa26 locus. Arrowheads indicate primers. (A) Removal of extra sequence by FLP recombinase to generate the Thy1-Dre allele. DSR, downstream region. (B) Gene switching from eGFP to tdTomato expression in Dre-rox system. Dre reporter allele was designed to express green (eGFP) fluorescence; when Dre recombinase is expressed, the region between the rox sites is removed, and the fluorescence switches from green to red (tdTomato). Tg mice with the recombinant allele were used as tdTomato reporter mice. (C) Transgene architecture for Rosa-Dre mice with the Dre gene expressed under the endogenous Rosa26 promoter.](image-url)
founders was mated with a FLPe Tg mouse [8] to remove the sequence (extra sequence in Fig. 1A) not required for the present experiment. The resultant three Tg lines, which are hereinafter referred to as Thy1-Dre mice, were then used for further analyses.

**Confirmation of successful Dre-rox recombination by alteration in fluorescent marker expression**

A fluorescent Dre reporter strain was previously generated using PITT (Fig. 1B) [17]. However, we had yet to confirm its detection capability for the Dre-rox recombination. We thereby injected a Dre expression plasmid into the pronuclei of fertilized eggs obtained from the fluorescent Dre reporter mice to examine whether fluorescence could be changed from green (eGFP) to red (tdTomato) after Dre-rox recombination. The embryos (morulae to early blastocysts) derived from the Dre reporter mice exhibited eGFP, but not tdTomato, expression in the absence of Dre expression (-Dre in Fig. 2A). However, Dre-positive embryos (+Dre in Fig. 2A) did exhibit tdTomato expression when the Dre expression vector was injected, indicating successful Dre-rox recombination in these embryos.

The detection capability of the Dre-rox recombination was further tested by crossing the Dre reporter strain with rosa-Dre mice that express Dre mRNA through the ubiquitous Rosa26 promoter (Fig. 1C) [14]. Offspring having both transgenes exhibited tdTomato expression (2 and 5 in Fig. 2B), whereas eGFP fluorescence was observed in the offspring with a Dre reporter transgene alone (1, 3, and 4 in Fig. 2B) as seen in the ears and livers in adult mice (left and upper right panels in Fig. 2B). Recombination outcomes were also confirmed by PCR (lower right panel in Fig. 2B). Results indicated that the produced Dre reporter strain performed favorably.

**Thy1 promoter at the Rosa26 locus is active in the brain**

We assessed Thy1 promoter-directed transgene expression patterns in the brain by crossing each of the three Thy1-Dre mouse lines with the Dre reporter strain. Processed brain tissue sections were examined for the presence of fluorescence using fluorescence microscopy. Purkinje cells in the cerebellums of double-Tg mice (Thy1-Dre/Dre reporter) exhibited near-uniform red fluorescence throughout all three lines (Fig. 3A). Conversely, a single Tg offspring having only the Dre reporter cassette only exhibited green fluorescence in Purkinje cells. No fluorescence was detected in wild-type (WT) mice. These data indicate that the Thy1 promoter located at the Rosa26 locus is active in virtually all Purkinje cells and/or ancestral cells. However, it was difficult to detect fluorescence in other brain cells in the frozen sections.

We then examined the expression of Dre mRNA in the Purkinje cells of the cerebellum and other brain regions, such as the hippocampus and choroid plexus, of Thy1-Dre mice using an in situ hybridization experiment carried out on paraffin sections of the adult brain. Dre mRNA was detected in the cells of these tissues, indicating that the Thy1 promoter in the Rosa26 locus was active in those regions of the adult brain (Fig. 3B).
Thy1 promoter targeted to the Rosa26 locus is active in many organs, but its mode is mosaic

Other tissues, including the pancreas, intestine, kidney, heart, liver, and retina, were examined for the presence of fluorescence. In all of the three double-Tg mouse lines (Thy1-Dre/Dre reporter) tested, red fluorescence presented mosaically in some cell clusters (Fig. 4), suggesting that the Thy1 promoter located in the Rosa26 locus might have been active in the ancestral cells from which these cell clusters were originated. The degree of mosaicism was more or less consistent throughout the three lines tested. Notably, some sets of cells from the intestine, kidney, and liver did not present with fluorescence in either the double-Tg mice (Thy1-Dre/Dre reporter) or the original Dre reporter mouse strains. Conversely, no biased expression of red fluorescence was observed in cells from the organs of the tdTomato reporter mice (Fig. 4).

Discussion

Thy1 promoter activities at the Rosa26 locus were examined using Tg mice generated by PITT/i-PITT. The Thy1 promoter is frequently utilized in Tg mice generated by traditional random integration transgenesis [10–13]. These Tg mice are mainly used for analyses of gene functions in the brain, where the Thy1 promoter is predominantly active. In general, however, transgene expression patterns vary and can be biased in Tg offspring produced by random integration-based transgenesis techniques [6, 13]. This study examined the activity of the Thy1 promoter inserted at the Rosa26 locus as a single-copy configuration. The Tg mice obtained in this study exhibited transgene expression in brain cells and those of other tissues throughout all mouse lines examined. The present results recapitulate those reported by other researchers experimenting on Thy1 promoter-directed Tg mice generated by random transgenesis [7].

Our models exhibited red fluorescence in a mosaic expression pattern especially in organs outside the brain. To the best of our knowledge, previous reports are yet to discuss Thy1 promoter-directed transgene expression in organs other than the brain in detail. The observed mosaic expression pattern might have been caused by the specific properties of the Rosa26 locus into which the transgene was inserted. The Rosa26 genomic region possesses an open chromatin configuration that enables the ubiquitous expression of transgenes inserted therein. This may have elicited a certain level of leaky expression of the transgene. If this were the case, this leaky transgene expression might depend on the promoter used; the tissue-specific Nephrin promoter only functioned in the target cells when it was inserted at the Rosa26 locus [20]. Another possibility is that the extra Thy1 promoter-directed transgene expression resulted from recombination occurring in ancestral cells that endogenously expressed the Thy1 gene, with cells exhibiting red fluorescence not
necessarily expressing Dre mRNA at the time of observation. This possibility should be addressed in the future through in situ hybridization to detect Dre (and/or Thy1) mRNA expression as was performed in the brain tissue in this experiment. Finally, it is possible that the Thy1 promoter itself does not demonstrate strict tissue specificity despite its frequent use as a neuron-specific promoter [1, 3, 4]. Of note, the Thy1 gene was initially identified as being expressed in thymocytes [10, 19]. Importantly, each of the possibilities mentioned above is not exclusive to any other.

It is noteworthy that red fluorescence expression in the organs was almost identical across all three double-Tg lines used in this study (Figs. 3 and 4), suggesting highly reproducible Thy1 promoter activity in lines generated by PiTT. This is an advantage for targeted transgenesis and is an improvement over laborious and time-consuming traditional methods for obtaining Tg lines [6]. Our results suggest promise for targeted transgenesis-based methods for acquisition of Tg mice, without subsequent selection among generated mice for suitable Tg lines.

Of note, it will be of interest to compare the expression patterns of the reporter genes across different tissues of present mice and those of previously generated double-Tg mice (Tg(Thy1-cre)1Vln/Cre reporter) (Supplementary Fig. 1) [7]. Reporter gene expression in both mouse lines is caused by site-specific recombination (by Dre or Cre). The information on Thy1-Cre mice found on the Cre portal of the Mouse Genome Informatics [MGI] website indicates a lack of obvious differences in expression patterns thereof throughout tissues and organs in both Tg mice. We surmise that Thy1 promoter activities at the Rosa26 locus are similar to those observed in the widely used Thy1-Cre Tg mouse generated by random

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**Fig. 4.** Fluorescence expression in the pancreas, intestine, kidney, heart, liver, and retina tissues from the double-Tg (Thy1-Dre/Dre reporter), Dre reporter, tdTomato reporter, and WT mouse lines.
integration-based transgenesis [7]. This, in turn, suggests that our mice could be utilized as an alternative to these pre-existing Thy1-Cre Tg mice.

Certain sets of cells in the intestine, liver, and kidney did not fluoresce in Dre reporter mice despite the use of the ubiquitous CAG promoter for reporter gene expression in those mice. Notably, in fluorescent mice containing the CAG-reporter transgene previously established using the PITT method, reporter expression was ubiquitous and stable throughout these tissues [12, 15]. Lack of fluorescence in certain sets of cells might be attributed to the presence of bacterial sequences within the CAT [or other] gene cassette, and the fact that red fluorescence was ubiquitously and stably observed after the removal of a stopper region containing the CAT sequence strongly supports this possibility. Additionally, even in CAG-reporter mice, transgene expression was uneven in organs where the vector-derived sequence was placed in proximity to the CAG-reporter-expression cassette [12]. It may thus be reasonable to consider that the presence of a bacterial sequence may hamper the activity of a promoter included in the integrated transgene.

We detected Dre mRNA expression in hippocampus and choroid plexus by in situ hybridization experiment, since tdTomato fluorescence was difficult to detect in these cells in the frozen sections. One of the possible reasons why fluorescence was hard to detect was absence or low levels of Dre-roc recombination in these cells. However, we rather thought that this resulted from low expression of tdTomato transcript from CAG promoter and the amount of tdTomato protein produced was below the detectable level in these brain cells, at least with this transgene configuration. Actually, we previously noticed that it was difficult to see obvious fluorescence in neuronal cells of the fluorescent Tg mice with similar transgene configuration (data not shown) [12, 15]. A high sensitive method such as immunohistochemical analysis using anti-tdTomato antibody may dissolve this problem.

In this study, we used a Dre-roc site-specific recombination system for conditional gene expression because the PITT method was difficult to apply to Cre-loxP-based conditional gene expression necessitating the insertion of a floxed cassette; targeted gene insertion was achieved by the Cre-loxP system when we first established the PITT method. Although our refined PITT method, i-PITT, enables floxed cassette insertion, our current study indicates that the use of the Dre-roc system could be an alternative to conditional gene expression as it appears comparable to the Cre-loxP system in terms of recombination efficiency. Notably, several recently published reports have also demonstrated the utility of the Dre-roc system in mice [2, 9, 18].

Acknowledgements

We thank Support Center for Medical Research and Education, Tokai University, for technical support. We would also like to thank Enago (www.enago.jp) for the English language review. This work was supported in part by the Grant-in-Aid for Scientific Research (25290035) from Japan Society for the Promotion of Sciences (JSPS) and A-STEP (AS231Z00219E) from the Japan Science and Technology (JST) to M.O., and the Tokai University School of Medicine Research Aid (2014) to S.T.

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