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# *L7/Pcp-2*-specific expression of Cre recombinase using knock-in approach

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**Abstract**

We report a knock-in mouse expressing Cre recombinase from the translational initiation site (ATG) of the endogenous *L7/Pcp-2* gene. The resulting Cre expression matches the pattern of *L7/Pcp-2* expression that is restricted to cerebellar Purkinje cells and retinal cells. Moreover, the Cre mouse showed no significant behavioral abnormality. Thus, our novel Cre mouse can be used for generation of Purkinje cells and retinal cell-specific gene expression and/or knockout in mouse using the Cre/*lox*P system.


*Keywords:* Tissue-specific recombination; Cre recombinase; *L7/Pcp-2*; Knock-in; Purkinje cell; Cerebellum; Bipolar cell; Retina

The ability to study gene function in complex biological system, such as the nervous system, has been vastly improved by the Cre/*lox*P system for conditional genomic manipulation in mice [1]. The Cre recombinase binds to *lox*P target sequences on a chromosome and either deletes or inverts the intervening DNA depending on the individual orientation of the *lox*P sites. Producing a tissue-restricted pattern of Cre will thus give rise to cell type-specific genomic alterations. Tissue-specific conditional knockouts circumvent problems associated with conventional knockouts, in which the altered gene will be present from conception in all cells of the animal. However, cell type specificity for gene inactivation remains a difficult challenge. Particularly in the central nervous system, few genetic promoters are known that can direct gene expression in small but defined neuronal subpopulation. Cerebellar Purkinje cells are an exception to the problem described above. They are the principal neurons of the cerebellar cortex and its exclusive efferents and are thought to be involved in motor coordination and motor learning but they may also participate in cognitive functions [2,3]. There are several genes that are predominantly expressed in Purkinje cells. These include the *L7/Pcp-2* gene [4,5]. L7/Pcp-2 is a protein of unknown function expressed exclusively and abundantly in Purkinje cells and retinal bipolar neurons [4–6]. The regulatory region of *L7/Pcp-2* gene that could drive exogenous genes [7] was used to direct Cre expression [8]. Although Purkinje neuron-specific expression was successful, non-specific expression was observed in many parts of the brain and other tissues [8]. Important regulatory elements for specific expression are often missing in cloned promoter DNA fragments. Furthermore, the influence of integrated locus acts on the expression of transgene and non-specific expression is often observed. In order to clear these distresses, we adopted knock-in strategy, allowing the Cre gene expression strictly under endogenous control element(s) of *L7/Pcp-2* gene, which can be applied. Since *L7/Pcp-2*

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null mutant mice have no abnormality detectable [9,10], the Cre gene inserted into the translational initiation site of the *L7/Pcp-2* gene can be expressed without influencing the function of the cerebellum and the retina of the whole animal.

## Materials and methods

*Cre mouse production.* A 129/SvJ mouse liver genomic DNA Lambda DASHII liberally (Stratagene) was screened with a probe corresponding to the ICR *L7/Pcp-2* genomic DNA fragment. Two clones covering the locus of the *L7/Pcp-2* gene as well as 5′ upstream (14 kb), L7-1, and 3′ downstream (16 kb), L7-2, were isolated and mapped by restriction digestion and DNA sequence (data not shown). A knock-in-type targeting vector (Fig. 1A) was made by inserting the Cre recombinase gene with nuclear localizing signal and 1.7 kb the pgk-neo-polyA gene into the *Pvu*I site of the second exon. The targeting vector contained *Sph*I–*Pvu*I 7 kb of homologous DNA upstream (left arm) of the *Pvu*I site and *Pvu*I–*Xho*I 5 kb of homologous DNA downstream (right arm) of the *Pvu*I site. The MC1 DTA gene [11] was attached to the 3′ end of the vector for negative selection. The linearized targeting vector was electroporated at 0.4 kV, 250 μF (Gene Pulser 2, Bio-Rad) into embryonic stem (ES) cells (RW4, Genome-system). The cells maintained on sub-confluent embryonic fibroblasts and neomycin-resistant ES cells colonies were screened as described [12]. The ES cells harboring homologous recombination were determined by Southern blotting using 3′ probe *Xho*I–*Eco*RV 0.6 kb depicted in Fig. 1A. The targeted ES cells were aggregated with BDF1 eight-cell embryos to generate chimeric mice which were crossed to C57BL/6 for production of targeted offspring as described [13].

*Reporter mice production.* 1.7 kb CAG (the cytomegalovirus immediate early enhancer-chicken β-actin hybrid) promoter [14], 1.5 kb STOP sequence derived from pBR302 (Gibco-BRL) and 0.7 kb puromycin-resistant gene derived from pPUR (Clontech) flanked by *lox*P sites, 8 bp *Pme*I linker fragment, and 0.6 kb polyA signal were constructed into *Eco*RV site of BluescriptII SK (−) (Stratagene). This vector is called pCAG-XstopX-polyA. Then 3.5 kb LacZ gene with nuclear localizing signal or 0.7 kb hrGFP gene fragment (Stratagene) was inserted into *Pme*I site of pCAG-XstopX-polyA. Cre-mediated recombination removes the stop sequence, allowing expression of β-galactosidase or GFP in all cell types in which the CAG promoter is active. Reporter founders were produced by pronuclear injection of the linearized transgenes, 8.0 kb pCAG-XstopX-NLLacZ or 5.3 kb pCAG-XstopX-GFP (Fig. 1B) DNA into BDF1 zygotes as described [13]. Integration of transgenes was determined by Southern blotting using LacZ probe and STOP probe depicted in Fig. 1B. The reporter founders were back-crossed into C57BL/6. All animals were cared for according to the Ethical Guidelines established by the Institutional Animal Care and Use Committee at Mie University.

*LacZ assay and immunohistochemistry.* Brains, eyes, and the other organs were removed from deeply anesthetized animals. LacZ staining of the whole mount and sections was done as described [13]. Immunohistochemical staining was performed with a TSA Biotin System (NEN) according to the manufacturer's instructions. The following antibodies were used in this study: anti-Cre (1:40, 10 μg/ml IgG, unpublished material); anti-calbindin (1:400, Chemicon); anti-PKC (Sigma); biotinylated anti-rabbit IgG (1:100, Vectastain); HRP-conjugated anti-mouse IgG (1:400, Chemicon); and Cy3-conjugated anti-mouse IgG (Jackson). Calbindin and protein kinase C (PKC) are marker proteins of cerebellar Purkinje cell and retinal bipolar neuron, respectively.

## Results and discussion

### *Generation of L7-Cre knock-in mice*

We constructed a targeting vector in which Cre recombinase gene and neomycin-resistance genes were introduced in the first ATG region of *L7/Pcp-2* gene exon 2 (Fig. 1A). ES cells were transfected with the linearized targeting vector and were tested for homologous recombination by Southern blot. Of 400 neo-resistant colonies screened, 60 were found to have a correctly targeted allele, giving an overall frequency of 15%. The targeted ES cells were then aggregated with eight-cell embryos. Two knock-in chimeric males created from

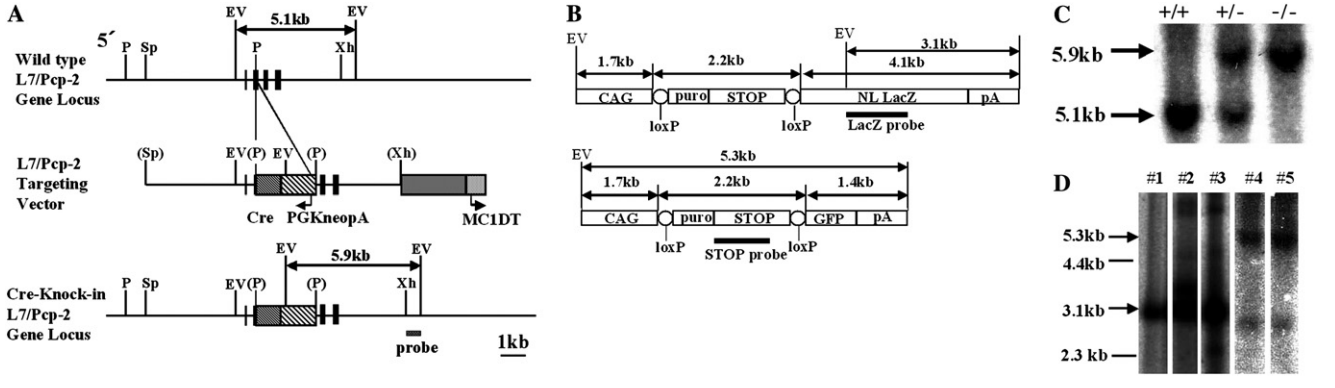


Fig. 1. Generation of L7-Cre knock-in mice by homologous recombination and generation of reporter mice by pronuclear injection of transgene. (A) Restriction maps showing the endogenous 129/SV *L7/Pcp-2* gene, the structure of the targeting vector, and the targeted *L7/Pcp-2* Cre knock-in allele. The black box denotes the region encoding exon of *L7/Pcp-2* gene. (B) Schematic representation of constructs pCAG-XstopXlacZ and pCAG-XstopXGFP for production of reporter mice. (C) Southern blot hybridization using 3′ probe of *Eco*RV-digested genomic DNA from offspring. The wild-type allele gives a 5.1 kb fragment while the targeted allele gives a 5.9 kb fragment. (D) Southern blot hybridization using LacZ probe (#1,#2, and #3) or STOP (#4 and #5) probe of *Eco*RV-digested genomic DNA from heterozygous of reporter lines. Concatamers in head-to-tail orientation of the pCAG-XstopX-NLLacZ and the pCAG-XstopX-GFP transgene give 3.1 kb fragments and 5.3 kb fragments, respectively. *Abbreviations:* P, *Pvu*I; Sp, *Sph*I; Xh, *Xho*I; and EV, *Eco*RV.