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Murine peripherin gene sequences direct Cre recombinase expression to peripheral neurons in transgenic mice

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Abstract Spatially and temporally regulated somatic mutations can be achieved by using the Cre/loxP recombination system of bacteriophage P1. To develop a cell type-specific system of gene targeting in the peripheral nervous system, we generated the transgenic mouse lines expressing Cre recombinase under the control of the mouse peripherin gene promoter. The activity of the Cre recombinase during embryonic development was examined by mating the peripherin-Cre transgenic mice to the knock-in Cre-mediated recombination reporter strain, R26R. Analysis of F1 embryos from this cross showed specific excision of loxP-flanked sequences in the dorsal root ganglia, trigeminal ganglia, and olfactory epithelium, in a pattern very similar to the expression of the endogenous mouse peripherin gene, and the previously reported peripherin-*lacZ* transgenic mice. Thus, the peripherin-Cre mouse described here will provide a valuable tool for Cre-loxP-mediated conditional expression in the peripheral nervous system.

Key words: ROSA26; Dorsal root ganglion; Sensory neuron; Cre; loxP; Peripherin

1. Introduction

Exploration of gene functions and development of mouse models for human diseases are routinely based on genomic manipulation via homologous recombination in embryonic stem cells. Conventional gene knock-outs generate inherited mutations in all cells, which may lead to embryonic lethality and therefore hamper the ability to analyze gene function or dysfunction during the postnatal period [1,2].

The Cre/loxP site-specific recombination system derived from bacteriophage P1 provides a convenient tool for spatially and temporally regulated somatic mutations [3,4] and efficiently promotes recombination between two 34 bp loxP recognition sequences. The sequences can be inserted at targeted genomic sites of embryonic stem cells through conventional ES cell techniques and homologous recombination [5]. Two loxP sequences flanking the interested genomic region allow the loxP-flanked gene region to be deleted only in cells expressing Cre recombinase. Therefore, spatial and/or temporal excision will depend on the promoter activity driving the Cre recombinase expression.

Peripherin is a neuron-specific type III intermediate filament protein expressed uniquely in the peripheral nervous system [6]. This peripheral-specific neuronal expression makes the peripherin gene promoter an ideal candidate to drive expression of the Cre recombinase to just these neurons. The long-term goal is to use the Per-Cre mouse in conjunction with loxP-inserted mice, to create conditionally expressed knock-out mice and examine the role of peripheral neuron proteins in mouse models of pain, morphine analgesia and inflammation. The loxP-inserted genes will be discussed in future publications. Here we describe the newly developed transgenic mouse line carrying the Cre gene under control of the mouse peripherin gene promoter [7]. To test and monitor the efficiency and tissue specificity of Cre-mediated recombination, we bred the peripherin-Cre (PCRE) transgenic mice with the R26R mouse that harbors a Cre reporter gene construct. The R26R mouse has a ROSA26 gene that has been modified to harbor a loxP-flanked Neo^R cassette which when excised allows the ROSA26 gene promoter to drive expression of the *Escherichia coli lacZ* gene [8]. In this transgenic Cre strategy, the expression of Cre is induced as soon as the transgene is turned on. In the case of the peripherin gene promoter, the turn on is around E10 in the mouse embryo. The deletion induced by Cre will be irreversible, resulting in the removal of all loxP-flanked sequences from this early time in development. Analysis of the F1 embryos by X-gal staining suggests that four out of five peripherin-Cre transgenic mouse lines target Cre recombinase expression to the dorsal root ganglia, trigeminal ganglia and olfactory epithelium in a spatial and temporal pattern nearly identical to that of the endogenous mouse peripherin gene.

2. Materials and methods

2.1. Construction of peripherin-Cre transgene

Intragenic sequences of the murine peripherin gene including a portion of the first exon, the complete first intron, second exon, second intron and third exon, and the first half of the third intron (P2: +357 to +1460) were isolated as an 1.1 kb fragment after *Sma*I digestion of pGNP1P2 plasmid [7]. pPEC plasmid contains the mouse 5′-flanking region of the peripherin gene which corresponds to the mouse peripherin promoter (P1), Cre recombinase and the MT-1 polyadenylation. The 1.1 kb *Sma*I fragment (P1) from pGNP1P2 was subcloned into the *Hin*dIII site of pPEC, following Klenow fill-in. The resultant clone, pPCRE, corresponds to the mouse peripherin

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promoter (P1) fused to the Cre recombinase transgene with the 1.1 kb of contiguous transcribed sequence of the mouse peripherin gene (Fig. 1A).

2.2. Generation of transgenic lines and screening of transgenic mice

The injection ready Per-Cre linear DNA fragment was prepared by digestion of the pPCRE vector with the restriction enzyme *Apa*I, that removes all vector sequences. Generation of transgenic mice was performed as described in Donovan [9] and founder mice were identified with Cre primers as in Le and Sauer [10]. Expression from those founders that demonstrate germ line transmission of the transgene is described in this work. All animal housing and treatment complies with NIH/AAALAC guidelines.

2.3. Analysis of the transgene expression

To test the efficiency of Cre-mediated site-specific recombination, we used R26R reporter mice (Jackson Laboratories), a gene targeted mouse line that directs expression of the *E. coli lacZ*, upon Cre-mediated excision of the loxP-flanked Neo^R cassette located upstream of the *lacZ* sequences [8]. β-Galactosidase expression was assayed in embryos from crosses between transgenic mice and homozygous R26R reporter mice. Per-Cre+ embryos were identified using PCR amplification analysis of Cre transgenes. The day of appearance of the vaginal plug was designated E0. The histochemical staining procedure for β-galactosidase activity was used to detect *lacZ* expression in whole embryos [11,12]. To analyze the spatial and temporal pattern of transgene expression in the various lines, embryos were removed from pregnant females at various days of gestation and analyzed for *lacZ* activity. Embryos were dissected in sterile phosphate-buffered saline pH 7.4 (PBS) and fixed in 0.2% glutaraldehyde, 5 mM EDTA, 2 mM $MgCl_2$ in PBS for one or several hours at 4°C depending on their size. After three washes in washing buffer (1.5 mM $MgCl_2$, 0.01% deoxycholate, 0.01% NP40 in PBS), staining was carried out overnight at 37°C in PBS containing 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 1 mM $MgCl_2$ and 1 mg/ml X-gal. Embryos were rinsed in PBS, dehydrated in ethanol and cleared in xylene before photography. The stained embryos were embedded in paraffin wax and sectioned at 10 μm. Sections were dewaxed, rehydrated and immunostained with a rabbit polyclonal antibody against peripherin (1/200; Chemicon) and with a goat anti-rabbit Texas red secondary antibody (Dako). Stained structures were identified according to published procedures [13,14].

3. Results

In order to develop a mouse model of cell type-specific gene targeting in the peripheral nervous system, we fused the mouse peripherin gene promoter to the Cre recombinase cDNA. The construct also contains a polyadenylation signal and 1.1 kb of contiguous transcribed sequence of the mouse peripherin gene (Fig. 1A). Laurence Leconte et al. described a nearly identical construct fused to lacZ with a similar pattern of *lacZ* expression specific to the peripheral nervous system [7]. Seven founder transgenic mice carrying the peripherin-Cre transgene were selected based on the PCR genotyping of the Cre recombinase transgene. Five of these demonstrated germline transmission of the transgene and were characterized further (Fig. 1B). Peripherin-Cre transgenic lines were crossed with homologous R26R Cre reporter mice. The progeny of this cross that are heterozygous for the ROSA26 knock-in allele, and inherit the Cre transgene, undergo Cre-mediated excision of the Neo^R cassette restoring *lacZ* expression specifically in the Cre expressing cells, allowing production of the β-galactosidase protein [8]. Of the five lines demonstrating germline transmission, four (PCRE35, PCRE25, PCRE59, PCRE11) yield detectable β-galactosidase activity, reflecting Cre recombinase activity (Table 1).

In order to characterize PCRE expression during development, timed pregnancy embryos resulting from PCRE× R26R/R26R matings were analyzed at different developmental stages. Endogenous peripherin expression appears early in the mouse embryos starting between embryonic day 9 (E9) and E10 [15]. By E14, peripherin is detectable in subsets of neurons in virtually all of the various nervous system structures where the gene is expressed in the adult. It is difficult to isolate some of these structures from adult animals, due to the diffuse nature of the peripheral nervous system, and the opacity of the adult tissues innervated by these neurons. Therefore, E14

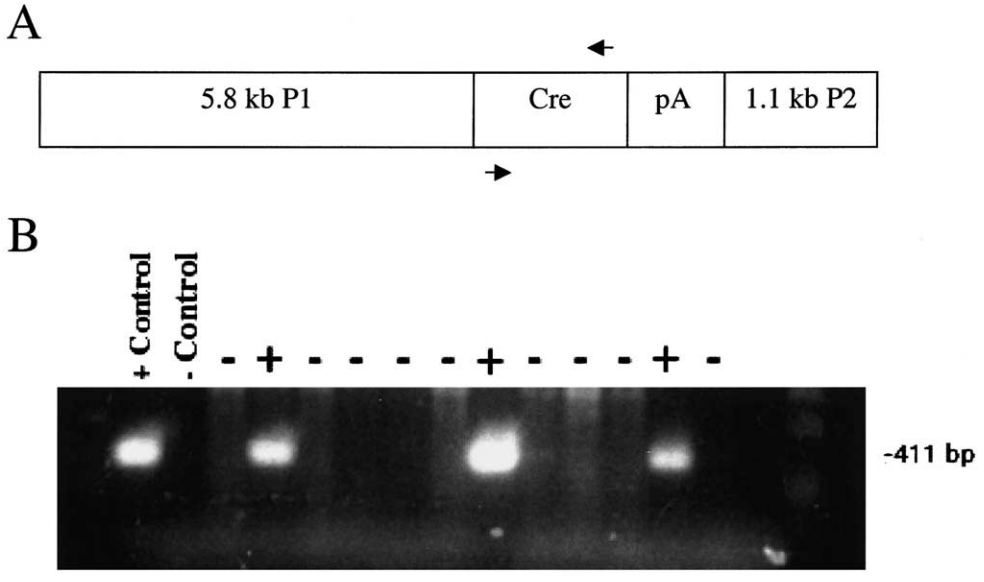

Fig. 1. Peripherin-Cre transgenic mouse production. A: Physical map of the peripherin-Cre construct used to generate peripherin-Cre transgenic mouse. The construct contains 5.8 kb of the 5′-upstream promoter region of the mouse peripherin (P1) gene linked to the Cre recombinase gene (Cre) with an addition of 1.1 kb of intragenic sequences of the mouse peripherin gene (P2). This 1.1 kb fragment (P2) includes part of the first exon, first intron, second exon, second intron and part of the third exon. B: PCR genotyping, founder line determination using Cre primers (arrows) and tail DNA extracted from founder mice. Representative samples depicting the presence of a single 411 bp fragment of the Cre recombinase indicate a positive (+) genotype.

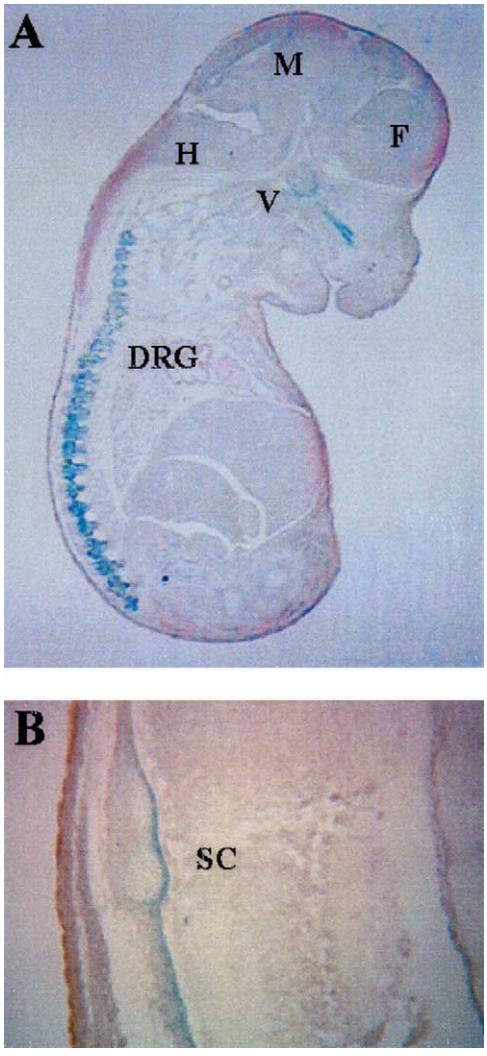

Fig. 2. Histochemical analysis of the β-galactosidase activity in an E14 embryo. A: Sagittal section of a PCRE line 35×R26R cross embryo. β-Galactosidase expression is detected in trigeminal ganglia (V) and dorsal root ganglia (DRG) with no ectopic expression in the central nervous system (F, forebrain: H, hindbrain; M, midbrain). B: Sagittal section of a line 35×R52R cross embryo at the level of the sympathetic chain (SC) showing lacZ expression.

was chosen as the time point to analyze transgene expression in all five transgenic lines both in whole-mount embryos and paraffin sections (Figs. 2 and 3).

At E14, the *lacZ* staining observed in the dorsal root ganglia (DRG), trigeminal ganglia (and cranial nerve V) and olfactory epithelium was consistent for all four lines (35, 25, 59, 11) (Table 1, Figs. 2 and 3). Two lines, 35 and 59, were analyzed in more detail at earlier stages, E10 and E11, while line 35 was also analyzed at E16. E16 embryos from line 35 had an identical *lacZ* staining pattern as the E14 embryos, except that the sympathetic ganglia neurons were also stained positive (Table 1). Despite a nearly identical *lacZ* staining pattern reported earlier for a peripherin-*lacZ* transgene construct [7], *lacZ* staining was not detected at E10 and E11 in both line 35 and line 59 (line 59 data not shown), suggesting a slightly different temporal expression for the PCRE constructs (Table 1). Embryos that were genotypically negative for the PCRE transgene did not show any staining at any of the stages observed. The spatial expression pattern of the PCRE transgene is consistent with the X-gal staining pattern from the peripherin-*lacZ* transgenic lines where *lacZ* expression is driven by nearly identical peripherin gene sequences [7], as well as immunohistochemical analyses of the peripherin protein in both mouse [15] and rat embryos [16]. In dorsal root ganglia, all neuronal classes known to be dependent of TrkA, B or C and having distinct soma diameter distributions were equivalently stained by lacZ (data not shown). The strong *lacZ* staining in DRG and trigeminal ganglia indicates an efficient Cre recombinase activity restricted to peripheral sensory neurons.

4. Discussion

The goal of this work was to create a mouse with Cre expression that could be used to conditionally knock-out the expression of loxP-modified genes in just the peripheral nervous system. We describe a peripherin-Cre transgenic mouse that places the Cre recombinase under the transcriptional control of the murine peripherin gene promoter. Peripherin is a neuron-specific type III intermediate filament protein expressed in well-defined populations of neurons. Its expression initiates soon after neurons become post-mitotic [6]. Ontogeny studies of peripherin gene expression in mice indicate that peripherin is found developmentally only in regions where it is later found in the adult [15]. The peripherin gene promoter was chosen for this construct due to earlier studies in transgenic mice that identified the peripherin promoter sequences required for cell type-specific peripherin gene expression [7].

The specificity of Cre activity was determined by using a knock-in Cre reporter mouse, R26R [8]. Cre-mediated recombination can be monitored with single-cell resolution by using reporter mouse strains harboring a floxed cassette which, when deleted, allows the expression of a reporter protein, such as β-galactosidase [5,17]. The Cre reporter mouse strain utilized for this work, R26R Cre reporter strain, takes advan-

Table 1
Comparison of tissue specificity of PCRE transgene expression in five PCRE founder lines during embryo development

Line	Dorsal root ganglia	Trigeminal ganglia	Olfactory epithelium	Sympathetic chain	Brainstem nuclei	Enteric neurons
11 (E13)	+/−	+	+	−	+	−
25 (E13)	+	+	+	−	−	−
59 (E13)	+	+	+	−	−	−
35 (E13)	+	+	+	−	−	−
35 (E10)	−	−	−	−	−	−
35 (E11)	−	−	−	−	−	−
35 (E16)	+	+	+	+	−	−

PCRE transgene expression was tested by detecting β-galactosidase activity in the tissues of mice embryos carrying ROSA26 *lacZ* reporter gene and peripherin-Cre transgene. β-Galactosidase activity was evaluated qualitatively. Its presence and absence are indicated as + and −, respectively.

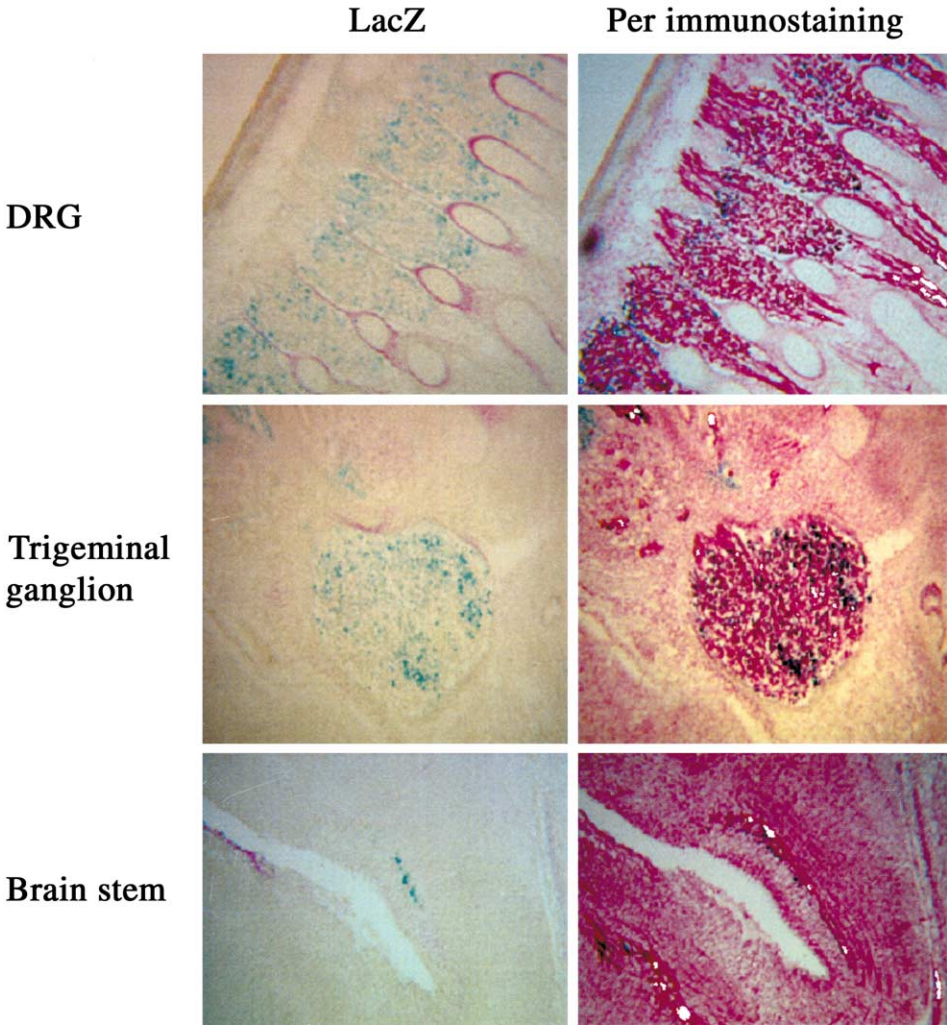

Fig. 3. Peripherin and β-galactosidase expression in an E14 transgenic embryo. Sagittal sections of a peripherin-Cre line 35×R26R cross embryo were stained for lacZ expression (left pictures). The adjacent sections were immunohistochemically stained for peripherin using a polyclonal antibody and fast red substrate system (Dako). β-Galactosidase was detected in DRG, trigeminal ganglia and brainstem (mesencephalic nuclei of trigeminal nerve).

tage of the ubiquitous expression of the ROSA26 gene. The fact that R26R is a knock-in adds to the reliability and reproducibility of the *lacZ* expression pattern obtained and avoids the confounding issues of penetrance and site of insertion effects that often add variability to the results when using a transgenic reporter line [8,18].

The present study confirms earlier work that the peripherin gene 5.8 kb upstream promoter region and 1.1 kb intragenic sequences can direct transgene expression specifically to peripheral neurons [7]. Four of five PCRE transgenic lines have functional levels of Cre recombinase expression in dorsal root ganglia, cranial nerve V and trigeminal ganglia, olfactory epithelium, with only one line (line 35) demonstrating sympathetic chain staining. Dorsal root ganglia staining reveals a relatively uniform and strong Cre activity initiating at E12–13. Interestingly, lacZ expression was found only in a subset of peripherin expressing neurons as compared to transgenic Per-LacZ expression [7]. One can suppose that this subset involves structures which have the highest level of peripherin expression. These data are in good agreement with the tissue specificity described for peripherin expression, but are slightly imperfect when considering the temporal expression of peripherin during development [7,15]. X-gal staining in our tests appeared between E12 and E13, which is about 2–3 days later than the results of peripherin immunohistochemistry and analysis of the peripherin-lacZ transgenic mouse [7,15]. One possible explanation for this might be that the earliest peripherin expressing tissues, such as the cervical ventral roots of the spinal cord and subcardiac regions, do not express the Cre recombinase in the PCRE transgenic lines. X-gal staining of E16 embryos indicates that the transgene is still not expressed at later stages in these regions. An alternative explanation is that there may be a delay in Cre activity, due to a putative need for a certain level of accumulation of the enzyme from a weakly expressing transgene promoter. However, the most likely explanation is that the slight differences in expression patterns between the four transgenic lines and transgene expression vs. peripherin immunohistochemical data probably reflect site of chromosomal insertion or local genomic effects on transgene expression [18]. Despite these slight variations in expression, there is no apparent ectopic expression of Cre recombinase in any of the transgenic lines we examined. Therefore, the present study shows an expression pattern of Cre recombinase that is nearly identical to that of the endog-

enous murine peripherin gene, with a slightly retarded onset of expression during embryogenesis.

These mouse lines allow deletions in loxP harboring genes from specific embryonic tissues and stages where the peripherin-Cre construct is expressed. This approach could be adapted to site- and time-specific gene targeting strategies as described by Metzger and Chambon [19] and Weber et al. [20], if one requires a specific deletion at latter stages of development (i.e. postnatal). Some cell types which express peripherin at low levels (e.g. spinal motoneurons and enteric neurons) were not identified in R26R embryos suggesting that this peripherin-Cre construct is only expressed sufficiently for loxP excision, in cells with a high level of endogenous peripherin gene expression. Gene targeting in these cells may be obtained using future Cre constructs under the control of a larger peripherin gene promoter such as has been used for a brain-specific CAMKII gene BAC [21].

Strong Cre recombinase expression with such high specificity to primary sensory neurons of both the dorsal root ganglion and trigeminal ganglia, is potentially of great value for putative Cre-loxP-mediated gene targeting experiments in peripheral sensory neurons. Since the primary afferent neuron is the primary site of nociception, which carries the nociceptive information from skin to the spinal cord [22,23], targeted Cre expression to these neurons should enable testing of candidate genes for their role in pain perception in a much more specific and accurate way than conventional knock-out models allow.

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