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Homologous Recombination of Mouse *ZAKI-4* Gene to Disrupt its Expression

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Abstract: *ZAKI-4* inhibits the activity of calcineurin, a Ca^{2+}-dependent protein phosphatase. From *ZAKI-4* gene, two isoforms, α and β are generated by an alternative splicing. In adult mice *ZAKI-4* α mRNA was mainly expressed in brain whereas *ZAKI-4* β mRNA was ubiquitously. To elucidate the specific function of ZAKI-4 isoforms, we plan to establish *ZAKI-4* β knock out mice by homologous recombination. For this purpose, mouse embryonic stem cells were electroporated with a targeting vector in which *ZAKI-4* β sequence was disrupted by cDNA coding neomycin resistance. Six independent clones out of 466 antibiotics-resistant colonies underwent homologous recombination at the *ZAKI-4* β locus. These clones will be used to establish the knock out mice.

Key words: ZAKI-4, homologous recombination, gene targeting, calcineurin

ZAKI-4 has been identified as a thyroid hormone-responsive gene from cultured human skin fibroblasts.[1)] Recent studies revealed that ZAKI-4 belongs to a novel family of proteins which inhibit calcineurin activity.[2-4)] Two mRNAs, α and β are generated by an alternative splicing from *ZAKI-4* gene. *ZAKI-4* α mRNA encodes a protein with 192 amino acids whereas *ZAKI-4* β mRNA encodes with 243 amino acids. The sequences of the two isoforms differ at their N-terminal regions but identical at their C-terminal regions. *ZAKI-4* α and β mRNAs showed a distinctive tissue distribution in adult mice. The expression of *ZAKI-4* α mRNA was restricted to the brain whereas *ZAKI-4* β mRNA was expressed not only in the brain but also in other tissues such as heart and skeletal muscles. This suggests that *ZAKI-4* α and β isoforms play distinct roles. To elucidate the specific function of each isoform, we plane to stablish mice lacking their expression. In this study we selected *ZAKI-4* β as the target to knock out the gene. To construct a targeting vector, cDNA sequences for neomycin resistance and diphtheria toxin were inserted to replace a β-specific exon in *ZAKI-4* gene. A promoterless β-galactosidase gene was also inserted into the transcription initiation site of *ZAKI-4* β to monitor *ZAKI-4* β gene expression in mice. After electroporation of mouse embryonic stem (ES) cells with the targeting DNA construct, we obtained 6 ES clones that have the recombinant sequence.

Materials and Methods

1. A targeting vector to disrupt ZAKI-4 β expression

The targeting vector was constructed in pBluescript SK (+) plasmid (Stratagene, La Jolla, CA, USA.), in which a 6.0 kb of 5′-homologous sequence, an MC1 promoter-driven neomycin phosphotransferase (*neo*^{r}) cassette, a 1.5 kb of 3′-homologous sequence, and an MC1 promoter-driven diphtheria toxin A fragment (DT-A) gene were inserted. To monitor the expression of the mutant gene, a bacterial β-galactosidase gene (*lac Z*) which had no promoter was inserted into the transcription initiation site of *ZAKI-4* β in the targeting vector (Fig. 1).

2. Culture of embryonic stem (ES) cells

A flowchart of the cell culture is shown in Fig. 2. Mouse embryonic fibroblasts (Oriental Yeast Co. Ltd., Tokyo, Japan) were cultured on gelatin coated dishes in Dulbecco-modified Eagle's medium (DMEM) (Immuno-biological Laboratories Co., Ltd, Gunma, Japan) with 10% fetal bovine serum (FBS)

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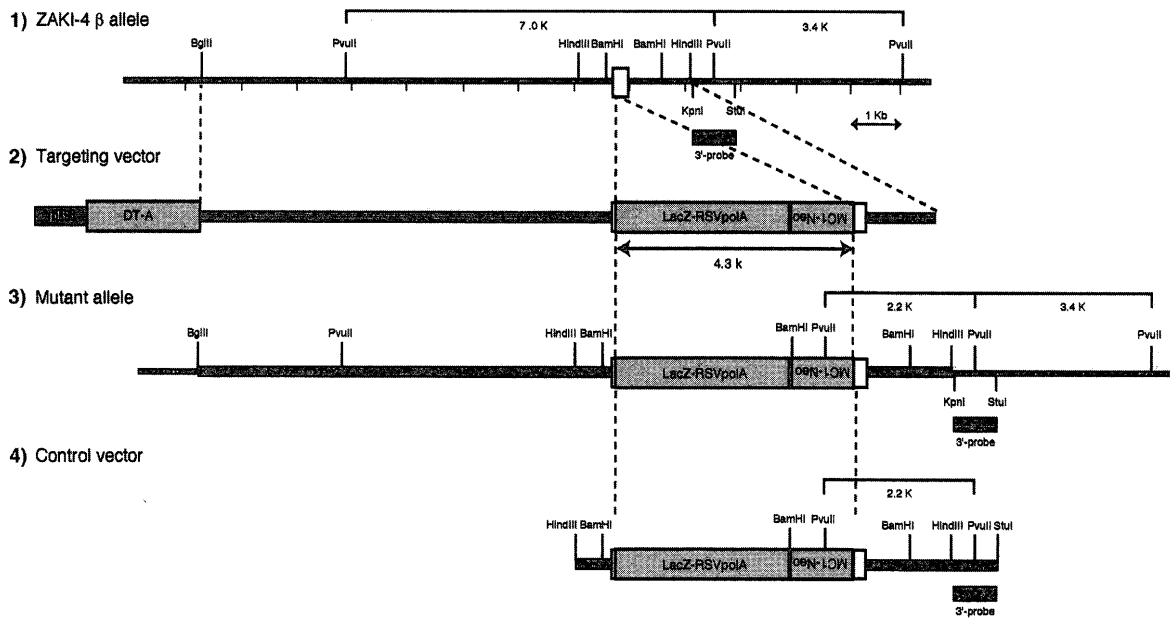

Fig. 1 Targeting construct and partial restriction map of the *ZAKI-4 β* gene locus before and after targeting event.
(1) Restriction mapping around the wild type mouse *ZAKI-4 β* exon. Only the restriction sites relevant for the construction of a targeting vector and the analysis of the recombination event are shown. (2) Schematic view of the targeting vector. The vector contains a 4.3 kb of *lacZ-neo*^{r} cassette in the β exon and a 1.1 kb diphtheria toxin A fragment gene (DA-T) at th 5′-end. MC1 promoter derived *neo*^{r} gene was inserted in the opposite orientation. (3) Schematic view of the mutant allele. The probe used in southern Southern blot analysis is shown. (4) shows a control vector for the Southern blot analysis. Since this vector contains 3′-region of the recombination site, same the size of band (2.2 kb) as mutant allele is detected by Southern blot analysis.

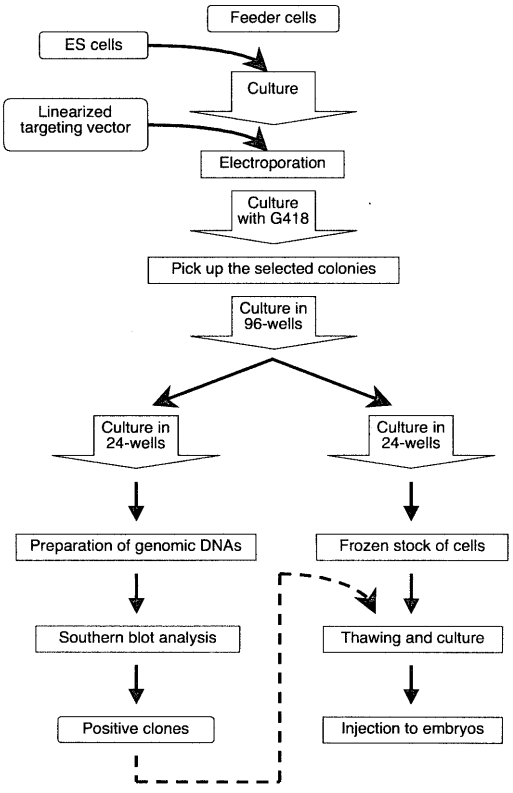

Fig. 2 Flow chart of the experiment.
ES cells were always cultured on the feeder cells. Five sets of electroporation were performed (in a set of electroporation, 20 μg of the vector was electroporated into the 1.2×10^7 cells of ES cells.) and cultured on 25 dishes with 6 cm diameter in the presence of G-418. In 466 clones selected with G-418, only 6 clones were found to have undergone to homologous recombination.

(Life Technologies, Inc., Rockville, MD, USA). They were used as feeder cells for embryonic stem cells after mitomycin C treatment. The TT2 cell line of mouse ES cells was used because this cell line was derived from F1 embryo from C57BL/6 and CBA mice.[5] The ES cells were cultured on the feeder cells in DMEM, supplemented with 20% FBS, 10^3 U/ml leukemia inhibitory factor (Chemicon International Inc., CA, USA), 100 μM β-mercaptoethanol (Katayama Chemical, Osaka, Japan), nucleotides mix (30 μM of each of adenosine, guanosine, cytidine and uridine, 10 μM thymidine) and non-essential amino acid mix (8.9 μg/ml L-alanine, 15.0 μg/ml L-asparagine, 13.3 μg/ml L-aspartic acid, 14.7 μg/ml L-glutamic acid, 11.5 μg/ml L-proline, 10.5 μg/ml L-serine, 7.5 mg/ml glycine) (ICN Biomedical Inc., Costa Mesa, CA, USA). The medium was changed every 24 hrs. The 1.2×10^7 ES cells were transfected with 20 μg of the targeting vector linearized by *Sac* II digestion by electroporation using a cuvette of 0.5-cm path length with Bio-Rad Gene Pulser (250 V, 500 μF) (Bio-Rad Laboratory, Inc., Hercules, CA, USA). Following the 5 sets of electroporation, ES cells were seeded at 2.4×10^6 per 6-cm dish and cultured in the presence of 400 μg/ml of G-418 (Sigma, St. Louis, MO, USA) for 1 week.

3. Southern blotting

Aliquots of 7.5 μg of genomic DNA extracted from the ES cells were digested with *Pvu* II and loaded on 0.7% agarose gel electrophoresis using the Mupid electrophoresis sys-

tem (Advance Co., Ltd., Tokyo, Japan). Then, they were transferred onto a GeneScreen Plus membrane (NEN™ Life science Products, Inc., Boston, MA, USA). *Kpn* I/*Stu* I fragment from pGEM-mZ17[6] were labeled with [^{32}P]dCTP (111 TBq/mmol, NEN™ Life Science Products, Inc.) using Random Primed Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany) and used as a probe for hybridization. Hybridization and subsequent washing were carried out as described previously.[7] Signals on Southern blot were detected by Molecular Imager System (GS-363, Bio-Rad Laboratory, Inc.).

Results and Discussion

Four hundred sixty six colonies of ES cells were obtained after selection with G-418. They were regrown in 24-well plates in duplicate; one was used for DNA extraction and the other was stored at –80°C. Fig. 3 shows one of the representative results obtained from Southern blotting. Because there is a *Pvu* II site in the *neo*^{r} cassette, the homologous recombinant must generate a 2.2 kb whereas a longer band 7.0 and 3.4 kb must be detected in the cells without the homologous recombination. Homologous recombination should involve only one allele. The recombinant cells should present 7.0, 3.4 and 2.2 kb. Note that lanes 2, 3 and 5 are the examples of the recombinant clones (Fig. 3). In total, six ES cell clones were selected. These ES clones were thawed, regrown and stored for injection into blastocysts to establish *ZAKI-4* β knock out mice. Our result shows that more than 98% (424/466) of ES cell clones carry the *neo*^{r} gene by random incorporation of the targeting sequence. In previous report 3 homologous recombinants out of 330 antibiotics-resistant colonies were isolated using the same ES cell line.[8] The ratio of the homologous recombinant to antibiotics-resistant colonies in this transfection was higher than the previous report.[8] In this study six ES cell lines were established to generate chimeric mice which were necessary to produce *ZAKI-4* β knock out mice.

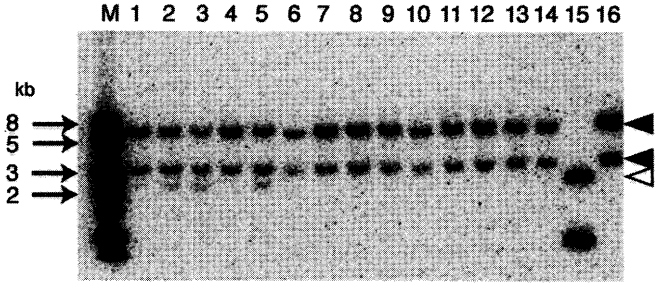

Fig. 3 Southern blot analysis.
Blots were probed with the 500-bp *Kpn* I/*Stu* I fragment that is located just 3′- of the recombination site (Fig. 1). The filled arrowheads indicate the location of the normal allele, the open arrowhead shows the mutated gene (lanes 2, 3 and 5). Note that in lanes 2, 3 and 5, 2.2 kb of the mutant allele was detected. M, 1 kb ladder marker; lane 1 ~ 14, genomic DNA from antibiotics-selected ES cell clones which has been digested with Pvu II; lane 15, control vector digested with *Pvu* II; lane 16, genomic DNA from ES cells which were not transfected with targeting vector.

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