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Original Article

Generation of transgenic mouse line with prostate-specific expression of codon-improved Cre recombinase

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ABSTRACT

Background: Genetically engineered mouse models are useful tools to decipher molecular mechanisms of diseases. As for prostates, a rat probasin promoter has been widely used to drive prostate-specific gene expression. To optimize its codon usage to that of mammals, we used codon-improved Cre recombinase (iCre) for prostate-specific Cre-*loxP* recombination.

Materials and methods: We generated transgenic mice that express iCre driven by conventional probasin promoter in a prostate-specific manner (PB-iCre). Linearized *PB-iCre* transgene deoxyribonucleic acids (DNAs) were microinjected into pronuclei of fertilized mouse embryos. The integration of the transgene was confirmed by Southern blot analysis. A line of transgenic mice expressing a sufficient amount of *iCre* mRNA in its prostate was selected. To test recombinase activity of PB-iCre *in vivo*, its offspring was crossbred with $Pten^{flox/flox}$ mice in which murine prostate adenocarcinoma is reported to occur upon excision of *loxP*-flanked regions.

Results: Eight founder animals were obtained, all of which showed germ line integration of *PB-iCre* transgene by Southern blot analysis. Among them, the prostate from only one line (line 58) expressed a sufficient amount of *iCre* mRNA. This line was crossbred with $Pten^{flox/flox}$ mice to generate PB-iCre58/$Pten^{flox/flox}$. As a result, 12-week-old PB-iCre58/$Pten^{flox/flox}$ mice presented with prostate adenocarcinoma that was histologically similar to human cribriform prostate cancer of Gleason grade 4.

Conclusions: We have successfully established a transgenic mouse line that expresses *iCre* in a prostate-specific manner.

1. Introduction

Genetically engineered mouse models provide us with useful tools to decipher molecular mechanisms of diseases even though there is a question as to whether data obtained from mouse models are directly applicable to human. In particular, conditional gene knockout techniques allowed us to investigate a function of the gene in a specific organ. Regarding prostates, a rat probasin (PB) promoter has been widely used to drive prostate-specific gene expression. PB protein was isolated from rat prostate and is a member of the lipocalin superfamily[1] as well as one of androgen receptor (AR) targets.[2] Similar to other AR target genes, PB promoter harbors AR-binding sites (ARBSs) to which AR binds to activate transcription. There are two ARBSs in wild-type PB promoter, and AR's binding to both ARBSs is necessary for maximal gene induction. Thus, these two ARBSs are collectively called androgen response region (ARR).[3] To date, this PB promoter has been used and modified to drive prostate-specific transgene expression.[2,3] In an effort to drive transgene expression with the highest efficiency, several PB promoters differing in size and the number of ARBS were developed, and their ability for transgene expression was tested.[3] In the end, ARR_2PB promoter that harbors two ARRs, in other words four ARBSs, was selected to drive transgene expression with the highest efficiency.[3] Using this ARR_2PB promoter, Wu et al generated a transgenic mouse expressing prostate-specific Cre and named it PB-Cre4.[4] Subsequently, PB-Cre4 was used for conditional knockout of *Pten* gene in murine prostates.[5]

Meanwhile, widely used Cre recombinase including PB-Cre4 was derived from prokaryotic bacteriophage. To optimize its codon usage to that of mammals, codon-improved Cre recombinase

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(iCre) was generated.[6] Given that prostate-specific Cre transgenic mouse model was not available in Japan and also in the aim of further improving the existing model, we generated our original PB-iCre transgenic mouse using iCre.

2. Materials and Methods

2.1. Construction of plasmids and generation of transgenic mouse

Codon-improved Cre recombinase, originally developed by Sprengel R et al,[6] was kindly provided by Dr. Manabu Abe (Niigata University). The iCre expression cassette was amplified by polymerase chain reaction (PCR) using the following primer set: 5′-ATGGTGCCCAAGAAGAAAGG-3′ and 5′-TCAGTCCCCATCCTCGAGC-3′. The amplified iCre coding sequence was cloned into pCRII-TOPO vector (Invitrogen, Walthman, MA, USA), followed by excision at *EcoRI* sites present in the vector. The *EcoRI-EcoRI* fragment containing 1-kb iCre coding sequence was subcloned into pBST-N[7] digested with *EcoRI*, giving iCre-pBST-N. A bacterial artificial chromosome (BAC) clone 402J5 containing rat PB promoter was purchased from BACPAC Resources Center (CHORI, Oakland, CA, USA). BAC clone DNAs were purified using QIAGEN Large construct kit

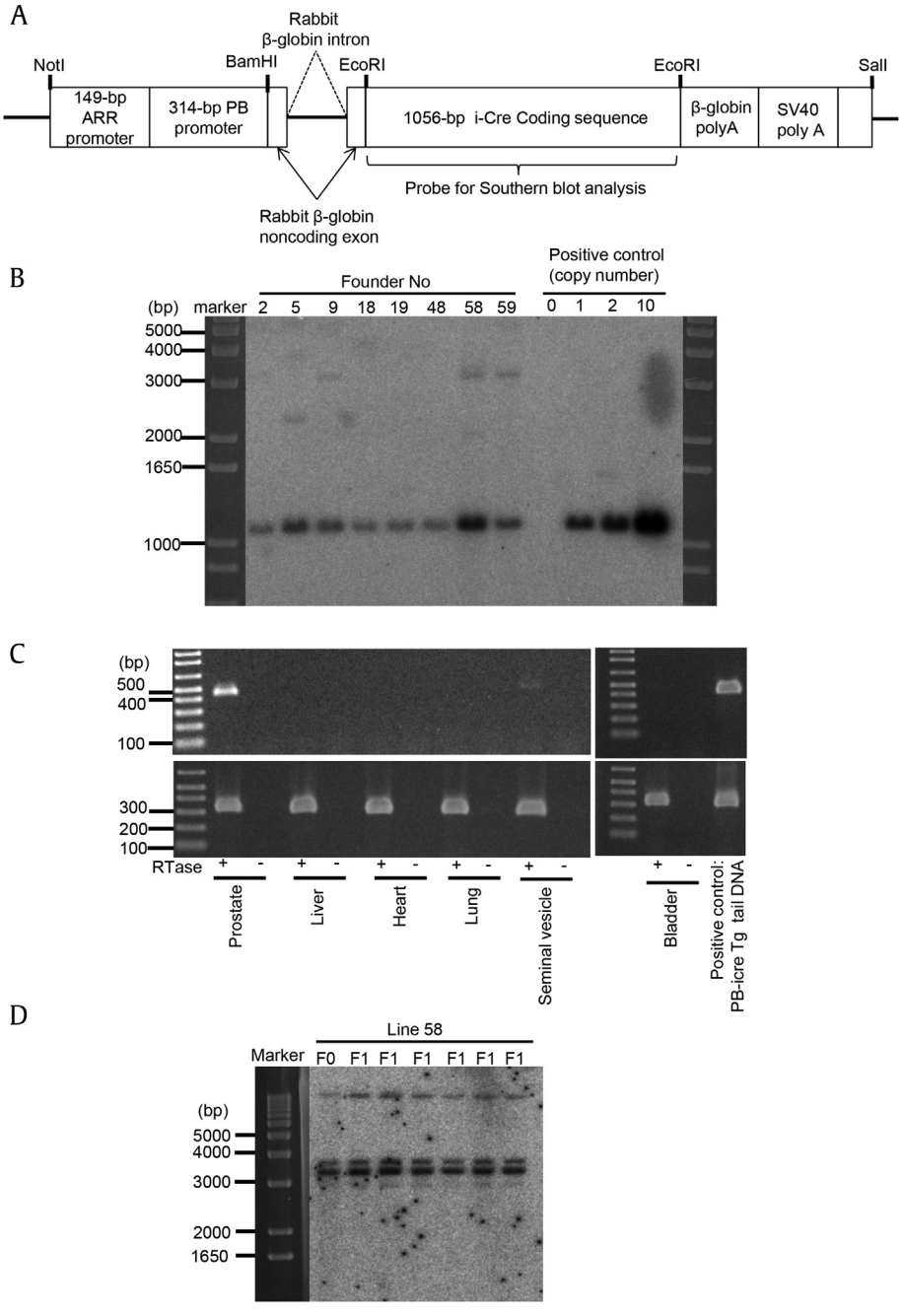

Fig. 1. Generation of PB-iCre transgenic mice. (A) Plasmid construction of ARR_2PB-iCre-pBST-N is represented. ARR_2PB-iCre-pBST-N was linearized with *NotI* and *SalI* for microinjection. (B) Southern blot analysis of genomic DNA derived from eight different transgenic founders is shown. Genomic DNA was digested with *EcoRI* for copy number evaluation. ARR_2-PB-iCre-pBST-N digested with *EcoRI* was diluted in accordance with copy number and used for positive controls. (C) RT-PCR analysis of prostate mRNAs is shown. Upper panel shows prostate-specific *iCre* mRNA expression in 5-week-old F1 PB-iCre58 (expected band size is 423 bp). Lower panel shows *GAPDH* mRNA expression as a control (expected band size is 302 bp). (D) Southern blot analysis of genomic DNA derived from PB-iCre58 founder and six pups of F1 generation is shown. Genomic DNA was digested with *BamHI*. Intergenerational segregation of transgene was not detected. RT-PCR, reverse transcription polymerase chain reaction ; ARR, androgen response region; PB, probasin.

(QIAGEN, Hilden, Germany). PB promoter region encompassing −286 bp to +28 bp (314-bp fragment) and ARR encompassing −244 bp to −96 bp (149-bp fragment) were amplified from purified BAC clone DNAs by PCR. Primer sets used for PCR were as follows: 314-bp PB promoter: 5′-CGCTCTAGA-TAGTCATCATGTTTAAACATCTACC-3′ and 5′-CGCGGATCCCTGTAGG-TATCTGGACCTC-3′; 149-bp ARR: 5′-GTAGCGGCCGCATGATAGCAT CTTGTTCTTAGTCTTTTTC-3′ and 5′-CGCTCTAGAACATTGTGTCATCTAG TATACAAATAGG-3′. Resultant 314-bp PB promoter fragment was ligated into *XbaI/BamHI* site of iCre-pBst-N to give PB-iCre-pBst-N. Subsequently, 149-bp ARR was inserted upstream of PB-iCre-pBst-N to generate ARR_2PB-iCre-pBST-N harboring 2 ARRs (Fig. 1A).

C57BL/6N female mice were superovulated, and collected oocytes were fertilized *in vitro* with C57BL/6N-derived sperms. ARR_2PB-iCre-pBST-N DNA was linearized with *NotI* and *SalI* (Fig. 1A) and microinjected into pronuclei of fertilized mouse embryos (C57BL/6N; Clea Japan, Inc., Tokyo). Ninety-four survived embryos were transferred into oviducts of 0.5-day postcoitum recipients.

Mice carrying transgene were first identified by PCR using the following primers: 5′-CCTGGTGCAAGCTGAACAAC-3′ and 5′-TCAG-CATTCTCCCACCATCG-3′. Approximately 150 C57BL/6N mice were used to establish a transgenic line and for further crossbreeding. Mice not harboring transgene or not expressing a sufficient amount of iCre mRNA were euthanized. Animals are handled in accordance with Institutional Animal Care and Use Committee of the University of Tokyo (Permit Number: M-P12-53).

2.2. Southern blot analysis

Mice harboring transgene confirmed by PCR were further subjected to Southern blot analysis of genomic DNA. Approximately 1-kb iCre coding sequence excised from ARR_2PB-iCre-pBST-N with *EcoRI* was used for the probe of Southern blot analysis (Fig. 1A). Five micrograms of genomic DNA extracted from tails was digested with either *EcoRI* (2 recognition sites within a transgene) for copy number evaluation or *BamHI* (1 recognition site within a transgene) to detect intergenerational segregation of transgene (Fig. 1A). Digested DNAs were electrophoresed in agarose gels, transferred to Nylon membranes (Biodyne PLUS; Pall Corporation, Pensacola, FL, USA) and hybridized with the iCre probe in accordance with standard protocols.

2.3. Crossbreeding of PB-iCre mice with floxed strains

To confirm recombination of floxed alleles, *Pten*$^{flox/flox}$ mice[8] (RIKEN BioResource Center, Ibaraki, Japan) were crossbred with PB-iCre mice. Successful recombination of floxed alleles by iCre was confirmed by PCR using the following primers designed to detect recombination-specific bands: 5′-ACTCCCACCAATGAACAAAC-3′ and 5′-GGTTTAAGATTGTATGTGATCATCT-3′.[8]

2.4. RNA extraction and RT-PCR analysis

Total RNA was isolated from murine tissues (prostate, liver, heart, liver, seminal vesicle, and bladder) with TRIzol reagent (Invitrogen). Extracted RNA was converted to cDNA using RNA PCR Kit AMV Ver.3.0 (Takara, Shiga, Japan). The equal amount of cDNA was PCR amplified with TaKaRa Ex Taq. The primer set for *iCre* was 5′-CCTGGTGCAAGCTGAACAAC-3′ and 5′-TCAGCATTCTCCCACCATCG-3′. The primer set for *GAPDH* was 5′-ATGGTGAAGGTCGGTGTGAACG-3′ and 5′-TGGTGAAGACAC CAGTAGACTC-3′.

2.5. Histological analysis

For histological analysis, excised prostates were fixed with 4% formaldehyde; then, paraffin-embedded sections were made in accordance with standard protocols. Antibodies used for immunohistochemistry were anti-phospho-S6 ribosomal protein Ser235/236 [1:200; 2211 from Cell Signaling Technology (CST), Danvers, MA, USA] and Ki-67 (1:100; CST 12202) as primary antibodies and biotinylated goat anti-Rabbit IgG (1:500; Jackson ImmunoResearch, PA, USA) as a secondary antibody. After avidin−biotin amplification (ABC Kit; Vector Laboratories, Burlingame, CA, USA), staining was developed with 3′-diaminobenzidine substrate.

3. Results

3.1. Generation of PB-iCre mice

Fig. 1A is a construction of a plasmid harboring a transgene. Eight founder animals harboring transgene were initially identified by PCR. Germ line integration of transgene was further confirmed by Southern blot analysis. All lines appeared to carry *iCre* transgene (Fig. 1B). Among them, only line 58 produced detectable amount of *iCre* mRNA confirmed by reverse transcription polymerase chain reaction (RT-PCR) of prostate from 5-week-old F1 offspring (Fig. 1C). Regarding tissue specificity, trace amounts of *iCre* mRNA was detected in seminal vesicle of line 58 (Fig. 1C), which was consistent with the earlier report.[3] Southern blot analysis revealed multiple transgene integrations into genome of line 58 (Figs. 1B, 1D). Intergenerational segregation of transgene in line 58 was not detected by comparing band patterns of F0 founder and F1 offspring in Southern blot analysis (Fig. 1D). Also, line 58 did not show any phenotypic abnormality as compared with wild-type C57BL/6N mice. Therefore, we decided to use line 58 for subsequent analysis, which is hereafter called PB-iCre58. In addition, these transgenic mice presented with no apparent physical abnormalities or infertility.

3.2. In vivo confirmation of iCre-induced recombination using *Pten*$^{flox/flox}$ mice

To confirm prostate-specific Cre recombinase activity, PB-iCre58 mice were crossbred with *Pten*$^{flox/flox}$ mice.[8] Of note, we chose not to use *lacZ* reporter system because of confounding endogenous β-galactosidase activity, especially in secretory epithelial cells.[9] Instead, we directly crossbred PB-iCre58 with *Pten*$^{flox/flox}$ because conditional *Pten* knockout has been reported to lead to prostatic adenocarcinoma.[5,10]

Recombination of floxed alleles was first validated by PCR using prostates excised from 12-week-old PB-iCre58/*Pten*$^{flox/flox}$, PB-iCre58/*Pten*$^{flox/+}$, or control *Pten*$^{flox/flox}$ mice. Genomic PCR products of prostates from PB-iCre58/*Pten*$^{flox/flox}$ and PB-iCre58/*Pten*$^{flox/+}$ mice yielded recombination-specific 220-bp band (Fig. 2A).[10]

In line with the earlier report,[5,10] hematoxylin/eosin staining of prostates from 12-week-old PB-iCre58/*Pten*$^{flox/flox}$ mice showed epithelial cells filling the entire lumen, which is very similar to human cribriform prostate cancer that is classified as Gleason grade 4 (Fig. 2B). Meanwhile, PB-iCre58/*Pten*$^{flox/+}$ only presented with hyperplasia, in which monolayer structure of epithelium is retained despite its hyperplastic undulating proliferation into the lumen (Fig. 2B). Anatomically, a murine prostate comprises ventral, dorsal, and anterior lobes. In our model, PB-iCre58/*Pten*$^{flox/flox}$ demonstrated similar pathological findings among all lobes.

Furthermore, immunohistochemistry showed increased signal of p-S6 (Fig. 2C) and Ki67 (Fig. 2D) in prostates of PB-iCre58/*Pten*$^{flox/flox}$ presumably because of augmented phosphatidyl inositol 3-

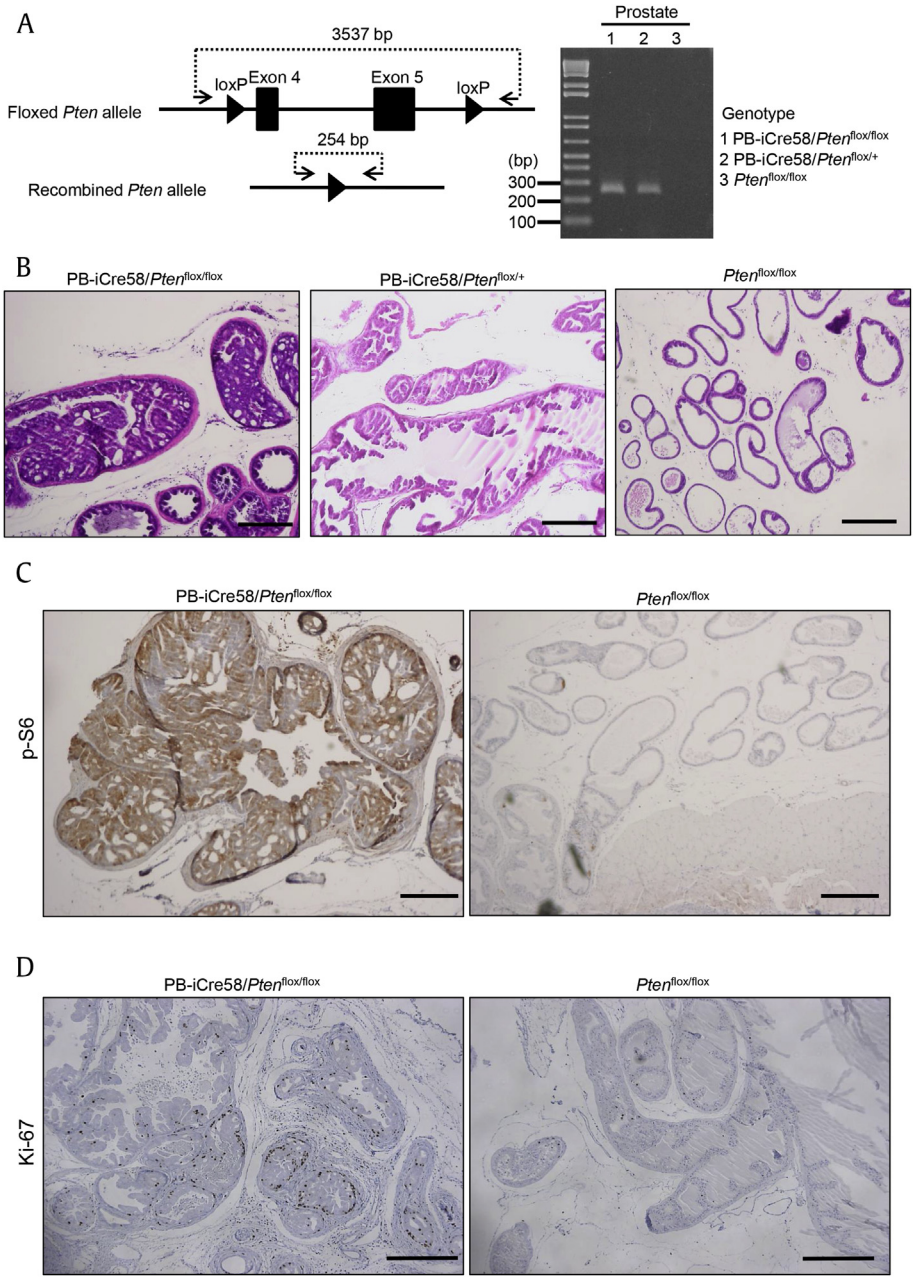

Fig. 2. Prostate-specific *Pten* deletion using PB-iCre transgenic mice. (A) Confirmation of iCre-mediated recombination in prostates of PB-iCre58/*Pten*$^{flox/flox}$ and PB-iCre58/*Pten*$^{flox/+}$ mice is illustrated. A successful recombination yields a 220-bp recombination-specific band. Arrows indicate forward and reverse primers. (B) Hematoxylin/eosin staining images of prostates from 12-week-old PB-iCre58/*Pten*$^{flox/flox}$, PB-iCre58/*Pten*$^{flox/+}$, or control *Pten*$^{flox/flox}$ mice are shown. Scale bars, 300 μm. (C) Immunohistochemistry results of phospho-S6 are shown. Prostates of 12-week-old PB-iCre58/*Pten*$^{flox/flox}$ mice showed significantly increased signal due to Pten ablation. Scale bars, 300 μm. (D) Corresponding to increased proliferation, prostates of 12-week-old PB-iCre58/*Pten*$^{flox/flox}$ mice showed increased nuclear staining of Ki-67. Scale bars, 300 μm.

kinase/mammalian target of rapamycin signaling as a result of *Pten* deletion and consequently increased proliferation. However, unlike human prostate cancer, proliferated cells never appeared to invade the basal membrane and remained as localized disease.

4. Discussion

Here, we generated readily available transgenic mouse model that expresses codon-improved Cre recombinase driven by prostate-specific PB promoter. We confirmed the mRNA expression of *iCre* at the age of 5 weeks in addition to successful recombination of floxed *Pten* alleles at the age of 12 weeks. Southern blot analysis showed no intergenerational segregation of transgene between F0 founder and F1 offspring. The possible advantage of our transgenic mice is higher recombination activity due to the use of codon-improved Cre recombinase. This PB-iCre58 model we generated can be used for conditional gene knockout of arbitrary genes in prostates.

As for other available murine prostate cancer models, Mimeault et al concisely summarized phenotypic features and latency to develop prostate cancer about existing models in their review.[11] In comparison to other models, the forte of PB-iCre58/*Pten*$^{flox/flox}$ is that it develops prostate cancer within a relatively short period of time, as early as 12 weeks in our study, which expedites research. In addition, loss of heterozygosity and mutations of *Pten* gene are reported to occur at an early stage of human prostate cancer

development and considered as key drivers of cancer progression.[12] Therefore, PB-iCre58/*Pten*$^{flox/flox}$ is likely to mimic human prostate cancer, making it an ideal model for preclinical studies such as drug development.

The limitation of this study is that we have not directly compared conventional PB-Cre4[4] and our PB-iCre58 with regard to the degree of recombination by Cre recombinase. However, considering the mRNA expression of *iCre* at the age of 5 weeks and the pathological findings of 12-week-old PB-iCre58/*Pten*$^{flox/flox}$ mice which were similar to Gleason-grade-4 human prostate cancer, we believe that our model is compatible with conventional PB-Cre4.

Genetically engineered mouse models are necessary for preclinical testing of drugs. Our model enables conditional knockout of arbitrary genes in murine prostates, which helps elucidation of the function of each gene in preclinical settings. Hopefully, our model facilitates the research of prostates not only in the field of cancer but also in other diseases or normal development.

5. Conclusion

We have successfully established a transgenic mouse line that expresses iCre in a prostate-specific manner.

Conflicts of interest

All authors have no conflict of interest to declare.

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