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

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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 pro-basin 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 crossed with Ptenfllox/lox 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 crossed with Ptenfllox/lox mice to generate PB-iCre58/Ptenfllox/lox. As a result, 12-week-old PB-iCre58/Ptenfllox/lox 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.

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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 response region (ARR). 3 To date, this PB promoter has been used and modified to drive prostate-specific transgene expression. 4, 5

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. 5 In the end, ARR2PB promoter that harbors two ARRs, was selected to drive transgene expression with the highest efficiency. 5 Using this ARR2PB promoter, Wu et al. generated a transgenic mouse expressing prostate-specific Cre and named it PB-Cre4. 3 Subsequently, PB-Cre4 was used for conditional knockout of Pten gene in murine prostates. 3

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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. 5 In the end, ARR2PB promoter that harbors two ARRs, in other words four ARBSs, was selected to drive transgene expression with the highest efficiency. 5 Using this ARR2PB promoter, Wu et al. generated a transgenic mouse expressing prostate-specific Cre and named it PB-Cre4. 3 Subsequently, PB-Cre4 was used for conditional knockout of Pten gene in murine prostates. 3

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
(iCre) was generated. 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., 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’-TCAGTCCCCCATCTGAGC-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-N7 digested with EcoRI, giving iCre-pBST-N. A bacterial artificial chromosome (BAC) clone 402JS containing rat PB promoter was purchased from BACPAC Resources Center (CHORI, Oakland, CA, USA). BAC clone DNAs were purified using QiAGEN Large construct kit.

![Diagram of plasmid construction and analysis](image-url)

**Fig. 1.** Generation of PB-iCre transgenic mice. (A) Plasmid construction of ARR2PB-iCre-pBST-N is represented. ARR2PB-iCre-pBST-N was linearized with NotI and SalI for micro-injection. (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. ARR2-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.
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'-CGCTCTAGATGTGTACATTTAATAACATCTAC-3' and 5'-CGGCGGATCCCTGTAGGGTATTCTGACCTC-3'; 149-bp ARR: 5'-GTAGGGCGGCGGATGATACCATGGTCTTCTAGTCTTTTTC-3' and 5'-CGCTTCTAGAATCATTGCTCATTGCTAGTACATTTAATAACATCTAC-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 ARR2PB-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. ARR2PB-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'-CTGCTTCTAGAATCATTGCTCATTGCTAGTACATTTAATAACATCTAC-3' and 5'-TCAGCATTCTCCCACCATCG-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 ARR2PB-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^lox/lox^ mice (RIKEN BioResource Center, Ibaraki, Japan) were crossed 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'-ACTCCCCACCACAAACACG-3' and 5'-GTTTAAAGGTATGTGATCTACGCAC-3' and 5'-TGCTGAAAGACACGCTAGACTC-3'.

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'-GTTTAAAGGTATGTGATCTACGCAC-3' and 5'-TGCTGAAAGACACGCTAGACTC-3' and 5'-GCCTGTCGAAGCTGAACAAC-3' and 5'-TCAGCATTCTCCCACCATCG-3' and 5'-TCAGCATTCTCCCACCATCG-3'. The primer set for GAPDH was 5'-ATGGTGAGCTGGGTGGAAGC-3' and 5'-TCAGCATTCTCCCACCATCG-3'.

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. 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^lox/lox^ mice

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

Recombination of floxed alleles was first validated by PCR using transgenes excised from 12-week-old PB-iCre58/Pten^lox/lox^, PB-iCre58/Pten^lox/lox^, or control Pten^lox/lox^ mice. Genomic PCR products of transgene from PB-iCre58/Pten^lox/lox^ and PB-iCre58/Pten^lox/lox^ mice yielded recombination-specific 220-bp band (Fig. 2A).

In line with the earlier report, hematoyxin/eosin staining of prostate from 12-week-old PB-iCre58/Pten^lox/lox^ mouse 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^lox/lox^ was 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^lox/lox^ demonstrated similar pathological findings among all lobes.

Furthermore, immunohistochemistry showed increased signal of p-S6 (Fig. 2C) and Ki-67 (Fig. 2D) in prostates of PB-iCre58/Pten^lox/lox^ presumably because of augmented phosphatidyl inositol 3-
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 successive 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. In comparison to other models, the forte of PB-iCre58/Ptenfl/fl 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.

![Prostate-specific Pten deletion using PB-iCre transgenic mice.](image-url)
development and considered as key drivers of cancer progression. Therefore, PB-iCre58/Pten\textsuperscript{fl/fl} 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-Cre\textsuperscript{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\textsuperscript{fl/fl} mice which were similar to Gleason-grade-4 human prostate cancer, we believe that our model is compatible with conventional PB-Cre\textsuperscript{4}.

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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