Plgrkt-deficient female mice caused by a 1-bp deletion of exon4 can maintain colonies through sufficient lactation

Plgrkt-deficient female mice caused by a 1-bp deletion of exon4 can maintain colonies through sufficient lactation

Running Head: Intact lactation in *Plgrkt*-deficient female mice

Takayuki Iwaki*, Yuki Tomonari, and Kazuo Umemura

Department of Pharmacology, Hamamatsu University School of Medicine, Hamamatsu, 431-3192 Japan.

*Correspondence to: Dr Takayuki Iwaki, the Department of Pharmacology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashiku, Hamamatsu, 431-3192, Japan

E-mail tiwaki@hama-med.ac.jp

Abstract

Plasminogen (Pg) activation on the cell surfaces is an important for various (patho)physiologic condition. Plg-R_{KT} is a cell membrane protein that binds to Pg and promotes the activation on the cell surface by Pg activators. To evaluate the role of Plg-R_{KT} in atherosclerosis, *Plgrkt* gene in *Ldlr*$^{-/-}$*/Apobec1*$^{-/-}$ was modified using *in vivo* CRISPR/Cas9. Synthetic RNA for *Plgrkt* and Cas9 complex was electroporated into the fertilized eggs in the oviducts. *Plgrkt* deficient mice caused by a 1-bp deletion was established. In contrast to *Plgrkt*$^{-/-}$ mice developed by conventional method, this newly developed mice did not represent lactation failure and could maintain the pups until weanling. The major obvious difference between these lines is the area of gene modification. The conventionally developed mouse possesses about 10 kb deletion of *Plgrkt*, which might relate to the lactation failure. Lactation failure is one of the lethal phenotypes in mammals, and analyses of causative genes are especially important for dairy industries. Further genome-wide analyses with both *Plgrkt*$^{-/-}$ mice will be helpful to find out the uncertain causative genes for lactation failure.

Key words: Plg-R_{KT} deficiency, lactation failure, genome-editing

Introduction

Plasminogen (Pg) is a 92 kDa single-chain glycoprotein that is activated by Pg activators (PA) (Mayer, 1990) *via* cleavage of a single peptide bond at Arg^{561}-Val^{562} leading to the disulfide-linked two-chain plasmin (Pm). The 561 amino acid N-terminal heavy chain contains five kringle domains, four of which bind to Lys-residues in fibrin (Castellino and McCance, 1997), and other proteins, *e.g.*, Pg receptors, in prokaryotes (Bhattacharya *et al.*, 2012) and eukaryotes (Miles *et al.*, 2005; Urano *et al.*, 2018). Pg deficient female mice are fertile (Ploplis *et al.*, 1995); however, their lactation is severely impaired, which leads to the premature expiration of pups (Lund *et al.*, 2000; Green *et al.*, 2006).

Plg-R_{KT} is widely expressed in various cells and is encoded by the *Plgrkt* gene. This gene spans 13.3 kb, and is consisted of 7 exons. Recently, a *Plgrkt*$^{-/-}$ mouse line was developed *via* conventional homologous recombination (Miles *et al.*, 2017). In this line, exons 4–7 of *Plgrkt* were targeted and the neomycin cassette replaced 10.42 kb of the locus. The *Plgrkt*$^{-/-}$ mice were vital and fertile, however, the pups from *Plgrkt*$^{-/-}$ mothers were expired within 2 days due to lactation defect (Miles *et al.*, 2018).

We are using *Ldlr*$^{-/-}$*/Apobec1*$^{-/-}$ (hereafter referred to as *L*$^{-/-}$*/A*$^{-/-}$) mice as a model of the most frequent hypercholesterolemia in humans to investigate roles of coagulation/fibrinolytic factors in atherosclerosis (Iwaki *et al.*, 2006; Miyajima *et al.*, 2018). To analyze Plg-R_{KT} functions in atherosclerosis, we planned to generate *L*$^{-/-}$*/A*$^{-/-}$*/Plgrkt*$^{-/-}$ mice. We are now using the

improved-Genome editing *via* Oviductal Nucleic Acids Delivery (*i*-GONAD) (Ohtsuka *et al.*, 2018), which is based on clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system in oviducts. We are now evaluating the function of Plg-R_{KT} in atherosclerosis by using *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ mice and will present the results elsewhere. However, we rapidly need to report a finding herein.

Materials and Methods

Genome editing of *Plgrkt* gene

The 23 bp sequence (5'-GCGTAACCATGAACTCCTGTTGG, underlined part is the PAM for Cas9) in the exon 4 of *Plgrkt* that contains the initial codon of methionine was elected by using CHOP-CHOP (http://chopchop.cbu.uib.no/), and this sequence was incorporated into the synthetic specific CRISPR RNA (crRNA) for *Plgrkt*. This crRNA and trans-activating crRNA (tracrRNA) were purchased as Alt-R™ CRISPR guide RNAs from Integrated DNA Technologies (IDT, Skokie, IL, USA) with Cas9 protein (Alt-R™ S.p. CAS9 Nuclease 3NLS). The crRNA and the tracrRNA were annealed, and then the annealed complex was mixed with Cas9 protein so that the final concentrations of components were 30 μM (for crRNA/ tracrRNA) and 1 mg/mL (for Cas9 protein). The mixture (1.0 - 1.5 μL) was injected into an oviduct of each plugged *L*$^{-/-}$/*A*$^{-/-}$ female mouse followed by electroporation with an electroporator (NEPA21, NEPA GENE, Ichikawa, Chiba, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of

Hamamatsu University School of Medicine.

Sequencing of *Plgrkt* gene from the genomic DNA and total RNA

Genomic DNA from an ear punch of each offspring after *i*-GONAD was amplified by PCR with Tks Gflex DNA polymerase (Takara BIO, Kusatsu, Japan) and two primers: Plgrkt.comF (5'-ATCCCAGTTGATAGGAGTCCAG) and Plgrkt.comR (5'-ACCTAGTGGTATAGAAATTTTGGCTCAAG), and each amplicon (381 bp) was directly sequenced. In the case of overlapped signals around the PAM was detected, the individual amplicon was subcloned into a cloning vector, and then several clones were sequenced. After confirming successful genome editing of *Plgrkt*, total RNA from aortic lymph nodes of the mutant mouse was extracted using TRIzol reagent (Thermo Fisher Scientific, Tokyo, Japan) to verify the RNA also contained the corresponding mutation. The RNA was subjected to reverse transcription (RT) with PrimeScript II RTase (Takara), and the RT product was amplified by a PCR with Tks Gflex DNA polymerase and two primers: Plgrkt.E4F (5'- ATGGGGTTTATATTCTCGAAATCTATGAACG) and Plgrkt.E7R (5'- TCATTTGTCTGAGAAGAGTTTACTCTGCTCC). The amplicon (444 bp) was also directly sequenced.

Genotyping of each mouse by single PCR with 4 primers

After confirming a nucleotide deletion in exon 4, a wild type (Wt) specific forward primer:

Plgrkt.wt.armsF (5’-TGAACGAAAACATGAAAAACCAACAGGAG, underlined part is Wt specific) and a mutant (Mut) specific reverse primer: Plgrkt.mut.armsR (5’-CATGCGTAACCATGAACTCTGT, underlined part is Mut specific) were designed. The Wt allele was confirmed with Plgrkt.wt.armsF and Plgrkt.comR Wt (268 bp), and the Mut allele was confirmed with Plgrkt.comF and Plgrkt.mut.armsR (157 bp). In order to perform a single PCR with these 4 primers, HiDi DNA polymerase (myPOLS Biotec, Konstanz, Germany) was used.

Western bloating of whole cell extraction

The cells in aortic lymph nodes were lysed using Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific) according to the manufactures’ instructions. After adjustment of the total protein concentration, the samples were electrophoresed with 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Hybond P) (Cytvia, Tokyo, Japan), and the membrane was blocked with EzBlock Chemi (ATTO Corporation, Tokyo, Japan) for 30 min at room temperature. The membrane was exposed to a rabbit anti-human Plg-R_{KT} polyclonal antibody (Product#HPA011144, ATLAS ANTIBODIES, Bromma, Sweden) and a goat anti-rabbit IgG-HRP conjugate (Cell Signaling Technology Japan, Japan), and then it was visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Japan) according to manufacturers’ instructions.

Histological analysis of mammalian glands

The Mammary gland tissues from various genotypes were collected at 2 and 4 weeks after delivery. They were fixed with periodate-lysine-paraformaldehyde (PLP) for 16 h at 4 °C. After the fixation, they were processed and embedded into paraffin. A section was obtained at 4 μm thickness and stained with hematoxylin II and eosin Y (H & E).

Results

The efficiency of genome editing by *i*-GONAD was very high, and some mutations were observed in about half of the delivered pups from $L^{-/-}/A^{-/-}$ mothers. Among them, a strain containing a 1-bp deletion in exon 4 was selected as a new line of $L^{-/-}/A^{-/-}/Plgrkt^{-/-}$ mouse. The founder mouse was back-crossed into $L^{-/-}/A^{-/-}$ mice, and the resulted $L^{-/-}/A^{-/-}/Plgrk^{+/-}$ mice were backcrossed into $L^{-/-}/A^{-/-}$ mice at least three times to eliminate unexpected off-target effects, and then the colonies for $L^{-/-}/A^{-/-}/Plgrkt^{-/-}$ mice were expanded. Aortic lymph nodes from $L^{-/-}/A^{-/-}/Plgrkt^{-/-}$ mouse confirmed by genomic DNA were used to extract total RNA, and RT-PCR for *Plgrkt* was performed. This amplicon was subjected to a direct Sanger sequence, and 1-bp deletion with premature stop codon was identified (Fig.1a). HiDi DNA polymerase possesses a unique property that enables to the identification of a mismatch of 3’ end in primers. Thus, we used it with 4 primers to identify the genotypes of pups in a single PCR, and each genotype was successfully confirmed (Fig.1b). The whole-cell lysates of aortic lymph nodes from various genotypes were subjected to western blotting

against Plg-R_{KT}, and no obvious signals were detected from *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ mouse (Fig.1c). Taken together, this line was truly *Plgrkt* deficient in *L*$^{-/-}$/*A*$^{-/-}$ background.

To eliminate any effects by the phenotypes manifested by *L*$^{-/-}$/*A*$^{-/-}$ background, a *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ mouse was backcrossed to C57Bl/6 mice several times to establish *Plgrkt*$^{-/-}$ mice. This newly developed *Plgrkt*$^{-/-}$ female mice also maintained the pups until weaning. In addition, the average litter size and survival rate of pups until weaning were not different regardless of the genotype of *Plgrkt* in Wt and *L*$^{-/-}$/*A*$^{-/-}$ (Table.S1). H&E stains demonstrated that mammary tissues in all genotypes were well organized two weeks after delivery. Fatty-droplets were observed in the secreting cells and the ducts, which indicated that proper milk production occurred in all genotypes. The structure of them returned to an almost non-pregnant state at four weeks (Fig.S1).

Discussion

Plg-R_{KT} is considered to paly various pathophysiological roles *in vivo*, and conventionally developed *Plgrkt*$^{-/-}$ mice were utilized to prove its roles in inflammatory macrophage recruitment (Miles *et al.*, 2017), cutaneous wound healing (Ny *et al.*, 2020), platelet activation (Whyte *et al.*, 2021), and adipose tissue development (Samad *et al.*, 2021). It has been considered that Pg activation in the mammary grand tissues is important to proper lactation due to the phenotypes manifested by conventional *Plg*$^{-/-}$ (Ploplis *et al.*, 1995) and *Plgrkt*$^{-/-}$ (Miles *et al.*, 2018) mice. Surprisingly, *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ female mice not only delivered as expected but also successfully

maintained the pups until weaning. We thought that hypercholesterolemia could rescue the lactation failure in *Plgrkt* deficient state, then, *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ mice were backcrossed to C57Bl/6J mice to generate new *Plgrkt*$^{-/-}$ mice. The lactation in the new *Plgrkt*$^{-/-}$ mice was also not problematic.

An apparent difference between these *Plgrkt*$^{-/-}$ lines is the area of gene modification. The conventionally developed mouse possesses about 10 kb deletion of *Plgrkt*. Although there is no information except *Plgrkt* itself in the deleted locus, the area might code unrevealed functions. According to the human SNP database for the *PLGRKT* gene (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=55848), seven nonsense mutations and five frameshift mutations are reported in the two exons corresponding to those in the murine *Plgrkt* gene in this study (Table S2). Although the frequency of homozygous and/or compound heterozygous of these mutations in humans are unknown, no notable phenotypes have been reported as *PLGRKT* deficiencies. Therefore, these mutations in humans might unlikely associate with not only lactation but also other putative phenotypes. In this sense, *Plgrkt* deficiency caused by making stop codon through one base pair deletion might possess no remarkable phenotypes.

Lactation failure is one of the lethal phenotypes in mammals, and analyses of causative genes are especially important for dairy industries. Further genome-wide analyses with both *Plgrkt*$^{-/-}$ mice will be helpful to find out the uncertain causative genes for lactation failure. The new *Plgrkt*$^{-/-}$ mice were already deposited to the National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN, https:// www.nibiohn.go.jp/en/), and will be available soon.

Author contributions

Y. Tomonari performed the experiments. T. Iwaki analyzed results and made the figures. T. Iwaki and K. Umemura designed the research and wrote the paper.

Acknowledgements

We acknowledge Ms. Kimiko Hara and Mr. Yasuhiro Kinpara of the department of pharmacology at the Hamamatsu University School of Medicine for their expert maintenance of the mouse colonies. This work was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI 21K08772.

Disclosure of Conflict of Interests

The authors declare that there is no conflict of interest regarding the contents of the paper.

References

Bhattacharya S, Ploplis VA and Castellino FJ (2012) Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. *J Biomed Biotechnol* **2012**, 482096.

Castellino FJ and McCance SG (1997) The kringle domains of human plasminogen. *Ciba Found Symp* **212,** 46-60; discussion 60-45.

Green KA, Nielsen BS, Castellino FJ, Romer J and Lund LR (2006) Lack of plasminogen leads to milk stasis and premature mammary gland involution during lactation. *Dev Biol* **299,** 164-175.

Iwaki T, Sandoval-Cooper MJ, Brechmann M, Ploplis VA and Castellino FJ (2006) A fibrinogen deficiency accelerates the initiation of LDL cholesterol-driven atherosclerosis via thrombin

generation and platelet activation in genetically predisposed mice. *Blood* **107,** 3883-3891.

Lund LR, Bjorn SF, Sternlicht MD, Nielsen BS, Solberg H, Usher PA, Osterby R, Christensen IJ, Stephens RW, Bugge TH, Dano K and Werb Z (2000) Lactational competence and involution of the mouse mammary gland require plasminogen. *Development* **127,** 4481-4492.

Mayer M (1990) Biochemical and biological aspects of the plasminogen activation system. *Clin Biochem* **23,** 197-211.

Miles LA, Baik N, Bai H, Makarenkova HP, Kiosses WB, Krajewski S, Castellino FJ, Valenzuela A, Varki NM, Mueller BM and Parmer RJ (2018) The plasminogen receptor, Plg-RKT, is essential for mammary lobuloalveolar development and lactation. *J Thromb Haemost* **16,** 919-932.

Miles LA, Baik N, Lighvani S, Khaldoyanidi S, Varki NM, Bai H, Mueller BM and Parmer RJ (2017) Deficiency of plasminogen receptor, Plg-RKT, causes defects in plasminogen binding and inflammatory macrophage recruitment in vivo. *J Thromb Haemost* **15,** 155-162.

Miles LA, Hawley SB, Baik N, Andronicos NM, Castellino FJ and Parmer RJ (2005) Plasminogen receptors: the sine qua non of cell surface plasminogen activation. *Front Biosci* **10,** 1754-1762.

Miyajima C, Iwaki T, Umemura K, Ploplis VA and Castellino FJ (2018) Characterization of Atherosclerosis Formation in a Murine Model of Type IIa Human Familial Hypercholesterolemia. *Biomed Res Int* **2018,** 1878964.

Ny L, Parmer RJ, Shen Y, Holmberg S, Baik N, Bäckman A, Broden J, Wilczynska M, Ny T and Miles LA (2020) The plasminogen receptor, Plg-R(KT), plays a role in inflammation and fibrinolysis during cutaneous wound healing in mice. *Cell Death Dis* **11,** 1054.

Ohtsuka M, Sato M, Miura H, Takabayashi S, Matsuyama M, Koyano T, Arifin N, Nakamura S, Wada K and Gurumurthy CB (2018) i-GONAD: a robust method for in situ germline genome engineering using CRISPR nucleases. *Genome Biol* **19,** 25.

Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L, Plow EF and Collen D (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation* **92,** 2585-2593.

Samad F, Bai H, Baik N, Haider P, Zhang Y, Rega-Kaun G, Kaun C, Prager M, Wojta J, Bui Q, Chakrabarty S, Wang J, Parmer RJ and Miles LA (2021) The plasminogen receptor Plg-R(KT) regulates adipose function and metabolic homeostasis. *J Thromb Haemost* in press (doi: 10.1111/jth.15622).

Urano T, Castellino FJ and Suzuki Y (2018) Regulation of plasminogen activation on cell surfaces and fibrin. *J Thromb Haemost* **16**, 1487-1497.

Whyte CS, Morrow GB, Baik N, Booth NA, Jalal MM, Parmer RJ, Miles LA and Mutch NJ (2021) Exposure of plasminogen and a novel plasminogen receptor, Plg-RKT, on activated human and murine platelets. *Blood* **137,** 248-257.

Figure 1

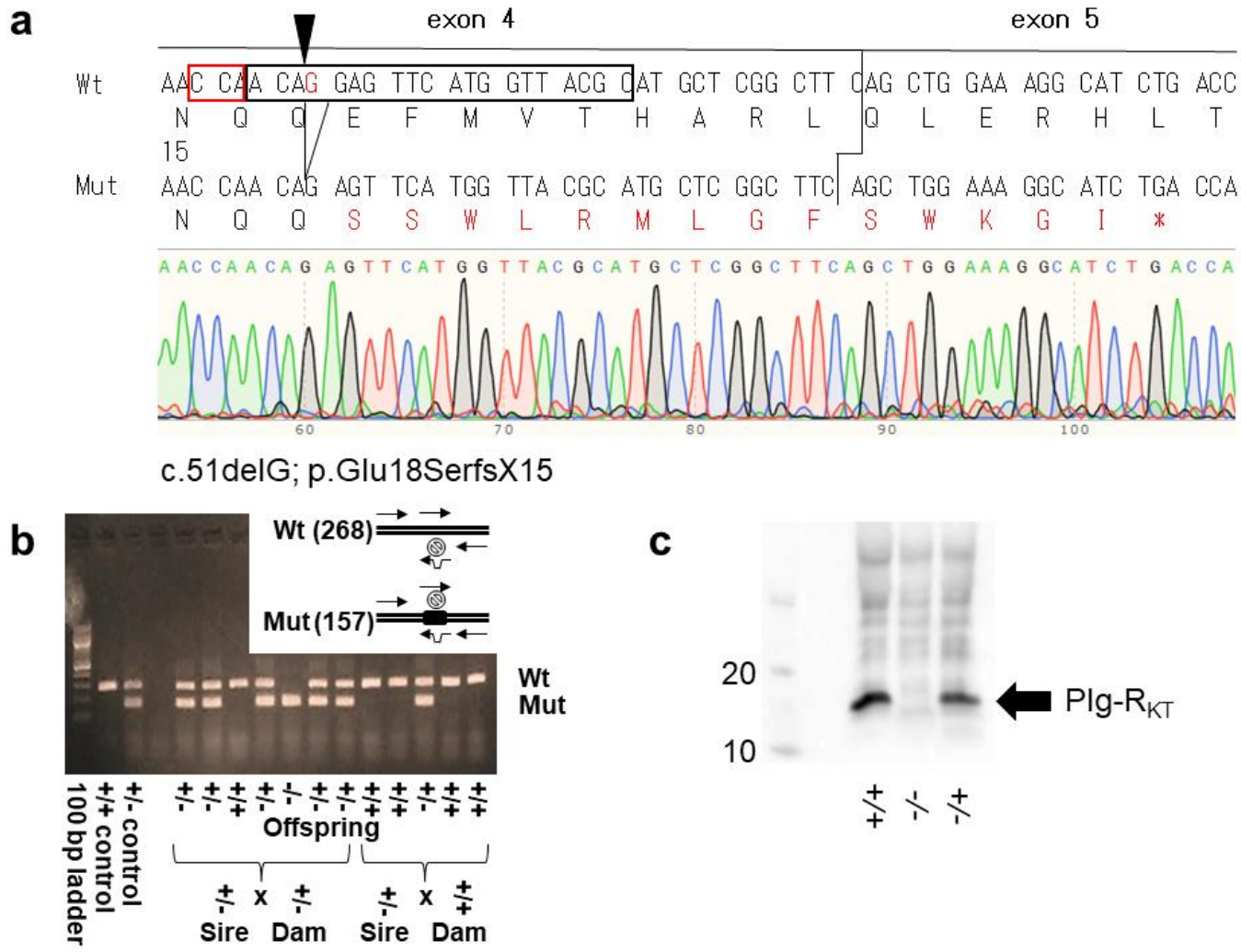

Figure 1. (a) cDNA sequence of Wt and Mut. The deletion of 1-bp guanine in the Mut allele caused a frameshift and premature stop codon. (b) A single PCR with 4 primers identified the genotyping of all combinations of *Plgrkt* genotypes. (c) The western blotting revealed that Plg-R_{KT} was absent in *L*$^{-/-}$/*A*$^{-/-}$/*Plgrkt*$^{-/-}$ mice.

Figure S1

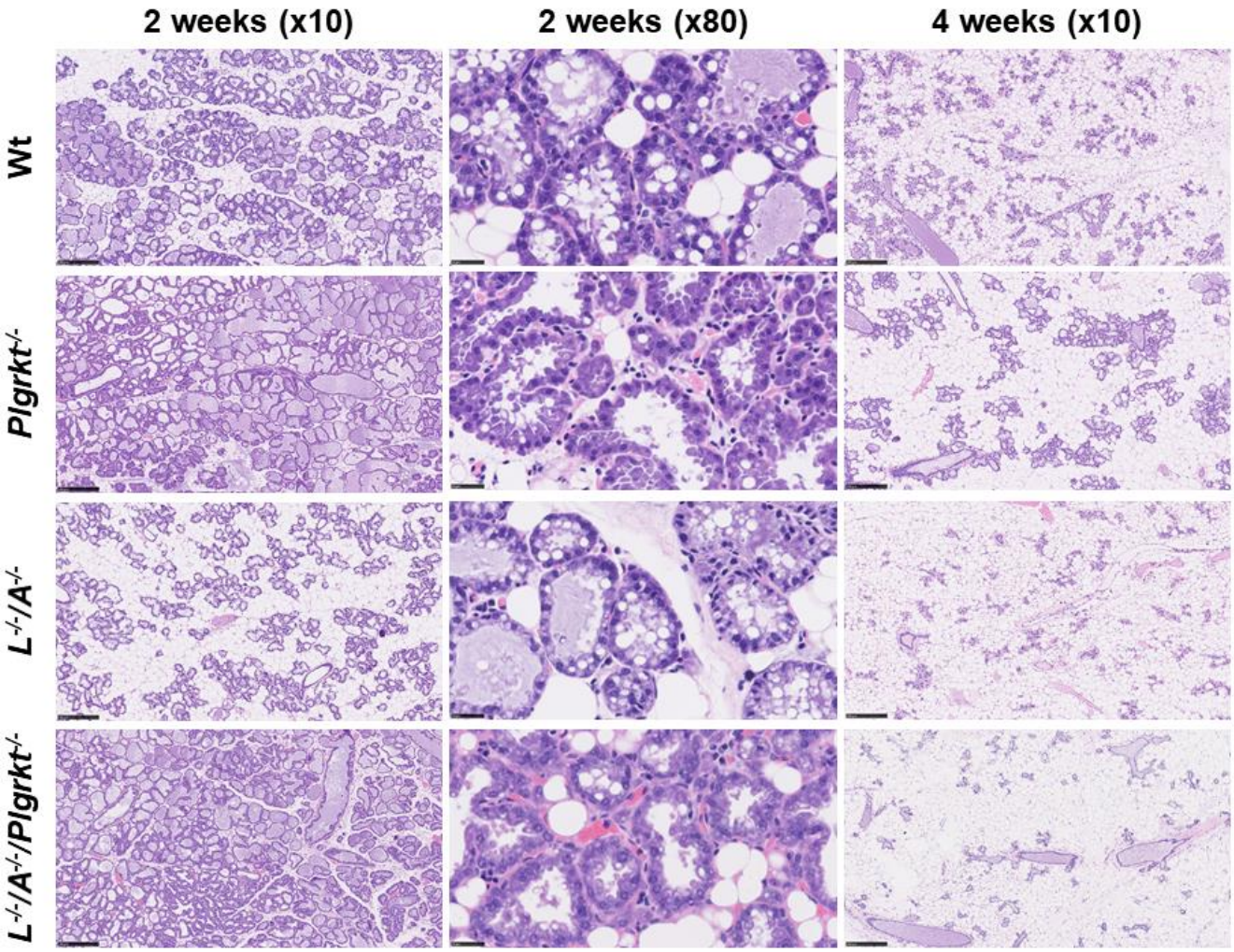

Figure S1. H&E stains of mammary tissues at 2 or 4 weeks after deliveries. The images were captured using NanoZoomer 2.0HT (Hamamatsu Photonics, Hamamatsu, Japan) with a single objective lens (UPlanSApo 20x/0.75, Olympus, Tokyo, Japan). The lower and higher magnification was computationally converted. The scale bars at x10 and x80 were 250 μm and 25 μm, respectively.

Table S1

	Plgrkt		Breeder	Births	Delivered	Litter	Dead	Survival	*Plgrkt*		
	Sire	Dam	sets		pups	size	pups	rate (%)	+/+	+/-	-/-
Wt	+/-	+/-	21	66	284	4.3	55	80.6	66	119	44
	-/-	-/-	16	20	122	6.1	38	68.9	0	0	84
$L^{-/-}/A^{-/-}$	+/-	+/-	18	64	352	5.5	89	74.7	61	143	59
	-/-	-/-	63	214	1019	4.8	232	77.2	0	0	787

Table S1. The genotypes of Sire and Dam, the results of breeding, and the distributions of offspring.

Table S2.

	dbSNP ID	Description
Nonsense	rs771249723	c.C17G; p.Ser6X
	rs764478425	c.C73T; p.Arg25X
	rs1163338012	c.C79T; p.Gln27X
	rs555291353	c.C91T; p.Gln31X
	rs778931477	c.C103T; p.Gln35X
	rs772479229	c.C136T; p.Gln46X
	rs143233000	c.G146A; p.Trp49X
Frameshift	rs1425219044	c.32-35delAAAG; p.Glu11Alafs2X
	rs756279325	c.74-75delGA; p.Arg25Profs12X
	rs1174631238	c.140delT; p.Ile47Metfs22X
	rs1240322981	c.176delC; p.Thr59Ilefs10X
	rs752488781	c.188delT; p.Ala64Glnfs5X

Table S2. The nonsense mutations and frameshift mutations of the *PLGRKT* gene in humans.