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Successful lactation in *Plgrkt*-deficient female mice caused by a 1-bp deletion of exon4

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Research Article

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Abstract

Plasminogen (Pg) activation on the cell surface is important for various (patho)physiologic conditions, and Plg-R_{KT} is a cell membrane protein that binds to Pg and promotes its activation. 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 were established through a 1-bp deletion, and in this research communication we report their lactational ability. In contrast to *Plgrkt*$^{-/-}$ mice developed by a conventional method, these newly developed mice did not suffer lactation failure and could maintain their pups until weaning. 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 a lethal phenotype in mammals, and analyses of causative genes are especially important for dairy industries. Further genome-wide analyses with both *Plgrkt*$^{-/-}$ mice may help to establish causative genes for lactation failure.

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, 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 death 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 consists 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 died within 2 d due to lactation failure (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 method of 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. Here we wish to report our finding that lactation does not fail in these mice.

Materials and methods

Genome editing of Plgrkt gene

We used the *i*-GONAD methodology of Ohtsuka *et al*. (2018). 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

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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 were detected around the PAM, 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′- TCATTTGTCTGAGAAGAGTTTACTCTGCT CC). 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′-TGAACGAAAAC ATGAAAAACCAACAGGAG, 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 resultant $L^{-/-}/A^{-/-}/Plgrk^{+/-}$ 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 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 back-crossed to C57Bl/6 mice several times to establish $Plgrkt^{-/-}$ mice. These newly developed $Plgrkt^{-/-}$ female mice 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 1). H&E stains demonstrated that mammary tissues in all genotypes were well organized two weeks after they had delivered pups (online Supplementary Fig. S1). Fatty-droplets were observed in the secreting cells and the ducts, which indicated that proper milk production had occurred in all genotypes. Mammary structure then returned to an almost non-pregnant state at four weeks.

Discussion

Plg-R_{KT} is considered to play 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 essential for normal 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

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Fig. 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.

Table 1. The genotypes of sire and dam, the results of breeding, and the distribution of offspring as evidence of lactation success

	Plgrkt		Breeder sets	Births	Delivered pups	Litter size	Dead pups	Survival rate (%)	*Plgrkt*		
	Sire	Dam							+/+	+/−	−/−
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

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 (online Supplementary Table S1). Although the frequency of homozygous and/or compound heterozygous of these mutations in humans are unknown, no notable phenotypes have been reported as Plg-RKT deficiencies. Therefore, these mutations in humans are unlikely with lactation failure or other putative phenotypes. In this sense, *Plgrkt* deficiency caused by introducing the stop codon through one base pair deletion might possess no remarkable phenotypes.

In conclusion, *Plgrkt* deficient mice established through a 1-bp deletion lactated successfully. Lactation failure is a lethal phenotype in mammals, and analyses of causative genes are especially important for dairy industries. Further genome-wide analyses with both $Plgrkt^{-/-}$ mice may 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.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000292

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