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Genetic mutation of *Frem3* does not cause Fraser syndrome in mice

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Abstract: QBRICK, FRAS1, and FREM2 compose a family of extracellular matrix proteins characterized by twelve consecutive CSPG repeats and single or multiple Calx-β motifs. Dysfunction of these proteins have been associated with Fraser syndrome, which is characterized by malformation of skin, eyes, digits, and kidneys. FREM3 is another member of the 12-CSPG protein family. However, it remains unknown whether genetic dysfunction of FREM3 also causes Fraser syndrome or another developmental disorder. Here we investigated a *Frem3* mutant mouse line generated by CRISPR/Cas9-mediated genome editing. The FREM3 mutant homozygotes were born at the expected Mendelian ratio and did not possess any defects characteristic of Fraser syndrome. These results indicate that the dysfunction of FREM3 is not associated with Fraser syndrome.
Key words: Bifid Nose Renal Agenesis and Anorectal malformations (BNAR), CRISPR/Cas9, Fraser syndrome, FREM3, Manitoba-oculo-tricho-anal syndrome (MOTA).

Introduction

Fraser syndrome is a recessive multi-organ disorder characterized by cryptophthalmos, syndactyly, renal agenesis, and other morphogenetic defects [10]. The phenotypic similarities between Fraser syndrome patients and mouse "blebbing" mutants suggested that the blebbing mutant mice represented an animal model of Fraser syndrome [2]. In these blebbing mutants, *Grip1*, *Qbrick* (also known as *Frem1*), *Fras1*, and *Frem2* were found to be disrupted [3, 8, 12, 13]. In Fraser syndrome patients, *GRIP1*, *FRAS1*, and *FREM2* mutations were also identified [3, 8, 14], whereas *FREM1* (encoding human QBRICK) mutations were recently found in Manitoba-oculo-tricho-anal (MOTA) syndrome and Bifid Nose Renal Agenesis and Anorectal malformations (BNAR), both of which phenotypically resemble those with Fraser syndrome [1, 11].

FRAS1, FREM2, and QBRICK, are all extracellular matrix proteins and localize to the basement membrane (BM) zone [5, 15], whereas GRIP1 is an intracellular adaptor protein necessary for FRAS1 secretion [13]. In mammals, there is another member the of 12-CSPG protein family, named FREM3. FREM3 consists of an NV domain, twelve CSPG repeats, and three Calx-β motifs. FREM3 is also localized to the BM zone [4, 9]. The expression pattern of FREM3 is distinct from other three 12 CSPG proteins; while FRAS1, FREM1, and FREM2 are similar in their tissue expression pattern and abundantly expressed in embryonic tissues, FREM3 expression is low during embryonic period but high in several adult tissues such as skin, salivary gland, and eye [4]. Despite structural resemblance between FREM3 and the other 12-CSPG proteins, no genetic disorder in which FREM3 is ablated has been reported to date. In this study, to address this question, we generated *Frem3* mutant mice by CRISPR/Cas9-based genome editing and investigated their phenotype.

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Materials and Methods

Animals

B6D2F1 mice were purchased from Japan SLC (Shizuoka, Japan). All mouse experiments were performed in compliance with the institutional guidelines and were approved by the Animal Care Committee of Osaka University.

Plasmids

A 300 bp genomic DNA fragment of *Frem3* was amplified with the primer pair 5'-ACTCCAGAATTCCATCGGACTATGGCTGGA-3' and 5'-CGGATCCCCGCGCTGCACTCGAATCACTA-3' and cloned into pEGxxFP vector [6] by using EcoRI and BamHI sites to generate Frem3/pEGxxFP plasmid.

A double-stranded DNA fragment was generated by annealing the following pairs of oligonucleotides: 5-caccCCTCCCTGGGGAGCGCTTAA-3' and 5-aaacTTAAGCGCTCCCCAGGGAGG-3' for gS01; 5'-caccACCACCATTAAGCGCTCCCC-3' and 5'-aaacGGGGAGCGCTTAATGGTGGT-3' for gAS03; and 5'-caccCCCTGGGGAGCGCTTAATGG-3' and 5'-aaacCCATTAAGCGCTCCCCAGGG-3' for gS04. These DNA fragments were cloned into pX330 vector [6] by using BbsI cloning sites.

EGFP reconstitution assay

HEK293T cells were transfected with pEGxxFP and pX330 plasmids by the calcium phosphate method. *Cetn1* is used as a positive control [6]. EGFP fluorescence 48 h after transfection was observed.

Generation of *Frem3* mutant mice

Frem3 mutant mice were generated by the CRISPR/Cas9 system as described previously [6]. Superovulated B6D2F1 female mice were mated with B6D2F1 males, and fertilized eggs were collected from their oviducts. The pronuclear stage eggs were microinjected with 5 ng/μl of pX330 plasmid, cultivated in KSOM overnight, and then transferred into the oviducts of pseudopregnant ICR females. The *Frem3* mutant mouse strain used in this study was deposited under the name B6D2-Frem3<em1Osb>, and available through either the Riken BioResource Center (Riken BRC; Tsukuba, Japan) or the Center for Animal Resources and Development, Kumamoto University (CARD; Kumamoto, Japan). The stock ID number of *Frem3* mutant mouse strain is 09954 (Riken BRC) or 2509 (CARD), respectively.

Genotyping

The genotypes of *Frem3*^{em1} mice were determined by genomic PCR using the primer pair 5'-ACTCCAGAATTCCATCGGACTATGGCTGGA-3' and 5'-CGGATCCCCGCGCTGCACTCGAATCACTA-3'. WT and *Frem3*^{em1} genomic DNA both gave PCR products of approximately 300 bp. The PCR products were digested with AfeI. Although the PCR product derived from the WT allele was digested into bands of 168 and 132 bp, the PCR product derived from the *Frem3*^{em1} allele remained undigested because the *Frem3*^{em1} mutation abolished the AfeI site. This size difference of AfeI digests is visible by 2% agarose gel electrophoresis.

RT-PCR

Six-month-old skin total RNA was isolated by using RNeasy Mini (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg of total RNA by using SuperScript III (Invitrogen, Carlsbad, CA) with oligo dT primer. RT-PCR was performed using KOD Fx Neo (Toyobo, Osaka, Japan). Primer sets used were as follows: 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and 5'-ATGGAGCCACCGATCCACA-3' for *Actb*; 5'-CAAATCTAGAGGATCCGGCATCCTCTAGATATATCAACTCC-3' and 5'-ATTCCTGCAGGGATCCCACACACTCCTCTTCACAGTTGGTG-3' for *Frem3*. The numbers of thermal cycling were 30 and 40 cycles for *Actb* and *Frem3*, respectively.

Histology

Dissected *Frem3*$^{em1/em1}$ adult kidney was fixed in 4% paraformaldehyde/PBS at 4°C overnight and then embedded in paraffin. Paraffin-embedded tissue was sectioned at the thickness of 10 μm. Deparaffinized sections were stained with Mayer's hematoxylin and eosin. Sections were observed with Olympus IX70 fluorescence microscope (Tokyo, Japan).

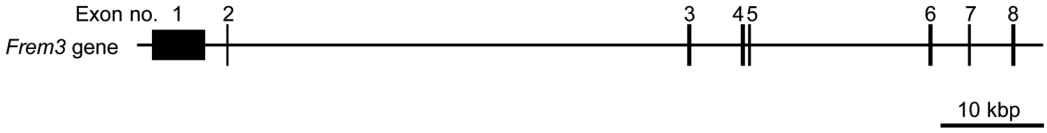
Results and Discussion

Generation of *Frem3* mutant mice by CRISPR/Cas9-based genome editing

A targeted mutation of *Frem3* was designed to occur in exon1 based on the CRISPR/Cas9 system (Fig. 1A) [6]. To introduce a frameshift mutation by double strand break-mediated repair into *Frem3*, three single guide RNAs (sgRNAs), i.e., sgS01, sgAS03, and sgS04 were designed. Each of these sgRNAs recognizes a 20-base-pair sequence immediately downstream of the start codon located within exon 1 of *Frem3* (Figs. 1A–C).

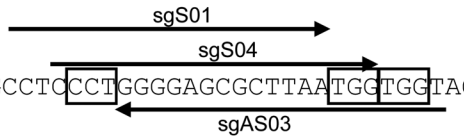
To evaluate the DNA cleavage efficiency of the designed sgRNAs complexed with Cas9 *in vitro*, a reporter plasmid Frem3/pCAG-EGxxFP, which harbors a 300 bp genomic DNA fragment of *Frem3* including the target

A



B

gaattccatcggact**ATG**GCTGGAGATTCTCTGAGCCTTCCTGGGATGTCCCTGCAGCTG
CTAGTGACTCTCACCTGCCTGCTTCTGACTTGTGCACTGAGGGAAAGGGTACCCTTGAGT



GAAAACGTGTCCCACTGTGAGCTTTACCTGCCTCCCTGGGGAGCGCTTAATGGTGGTAGT
CACCAGAACCCTGGCATTCTGATCGCCAACTCCGGGTTCCAAGTGCCACAGGGCCGCTCG
GTTTGGCTTAACCCACTGCGGGATCTAGTGATTCGAGTGCAGCGCGGGGATC

C

Guide name	Target sequence	Off-target 20 mer	Off-target 12 mer
sgS01	CCTCCCTGGGGAGCGCTTAA	0	0
sgAS03	ACCACCATTAAGCGCTCCCC	0	0
sgS04	CCCTGGGGAGCGCTTAATGG	0	2

D

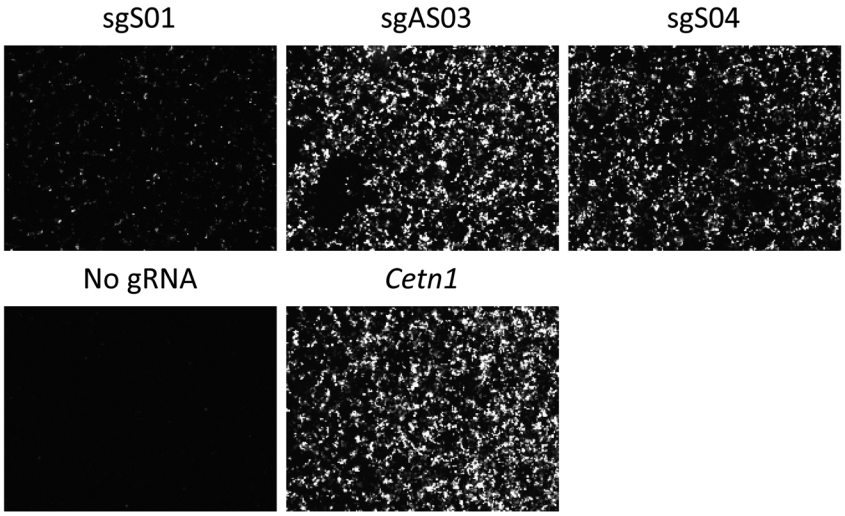


Fig. 1. Genome editing of *Frem3* by CRISPR/Cas9. (A) Genomic structure of the *Frem3* gene. (B) Location of guide RNA target sites in *Frem3* genomic sequence. Boxed areas represent PAMs. Arrows indicate target sequence of guide RNAs. (C) Sequence of guide RNAs and number of off-target sites in the mouse genome. (D) *In vitro* EGFP reconstruction assay. *Cetn1* is used as a positive control.

sites for sgRNAs, was designed [6]. Each pX330 plasmid which expresses both the designed sgRNA and Cas9 protein was co-transfected together with Frem3/pEGxxFP into HEK293T cells, then the target DNA cleavage efficiency of each sgRNA/Cas9 complex was evaluated by reconstituted EGFP fluorescence. Among sgRNAs tested, sgAS03 produced the strongest EGFP fluorescence (Fig. 1D), indicating that the double strand break-dependent repair occurred most efficiently.

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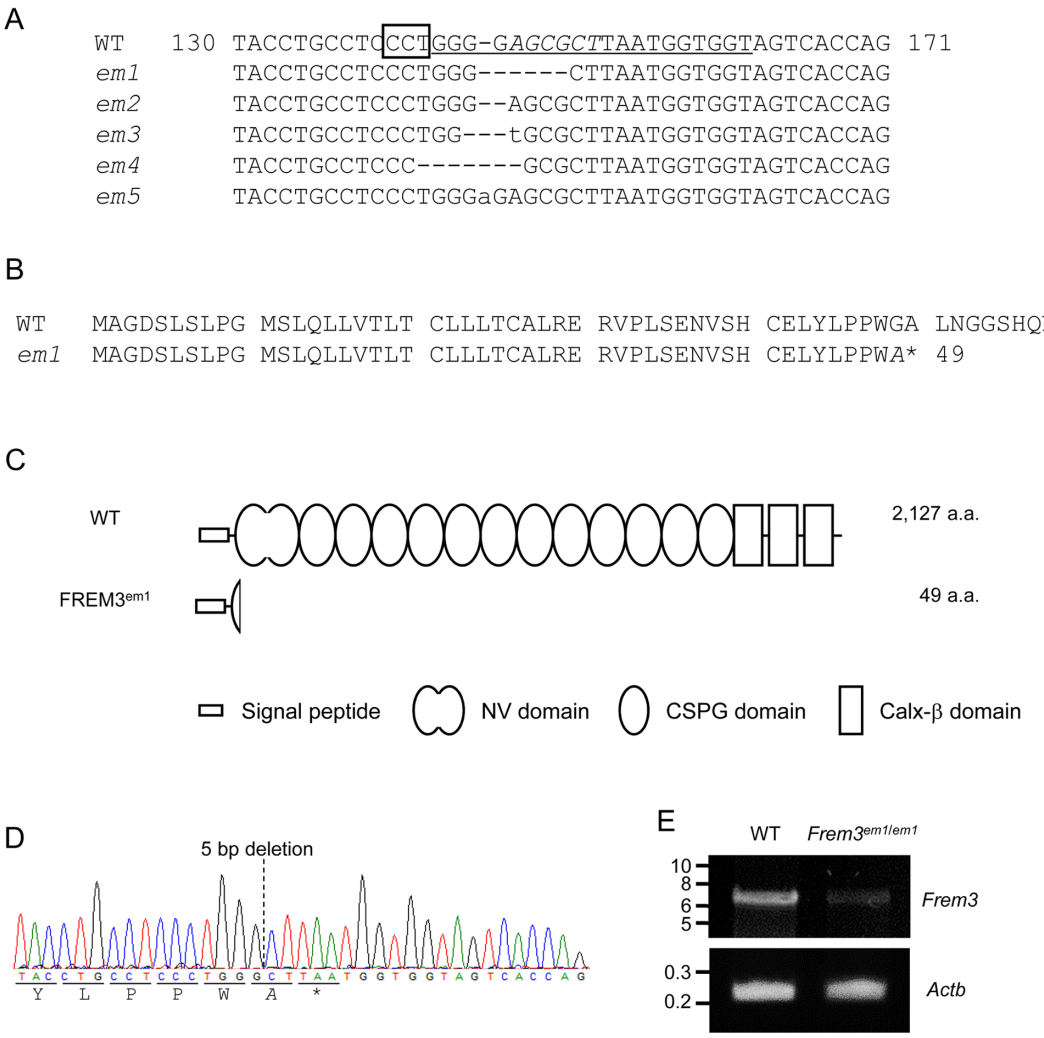
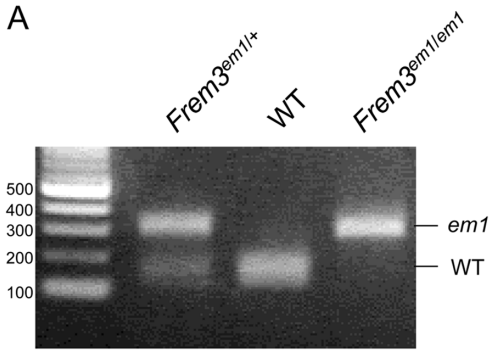


Fig. 2. Generation of *Frem3* mutant mice. (A) *Frem3* mutations observed in founder mice. sgAS03 and PAM sequences are indicated with underline and box, respectively. AfeI restriction site is shown in italic. (B) N-terminal amino acid sequence of wildtype FREM3 and the em1 mutant protein. Amino acid substitution in the em1 mutant protein is indicated in italic. (C) Schematic representation of wildtype FREM3 and the em1 mutant protein. (D) Direct sequencing result of *Frem3*^{em1} cDNA. No aberrant splicing is observed. (E) RT-PCR of *Frem3* cDNA. Result of *Actb* is also shown as an internal control. Numbers indicate DNA marker size in kbp.

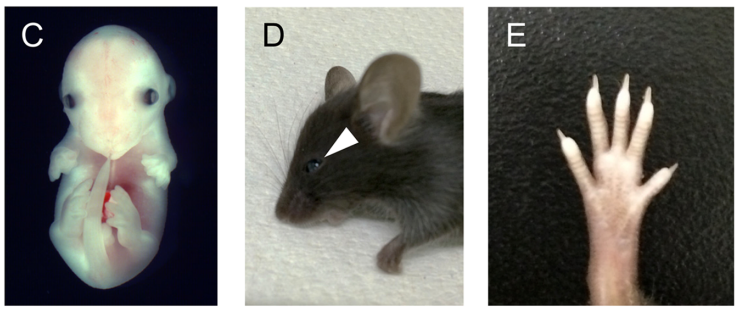
Generation of *Frem3* mutant mice

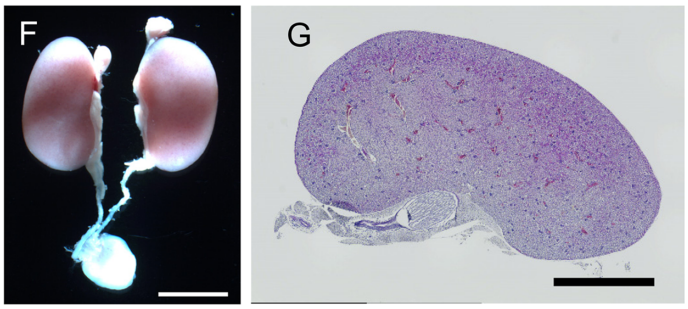
To generate *Frem3* mutant mice, fertilized eggs were injected with the pX330 plasmid that expresses sgAS03 and transplanted into the oviduct of pseudopregnant mice. Genomic DNA sequencing of F0 pups identified various double strand break-mediated mutations around the sgAS03 target site (Fig. 2A). These F0 mice were mated with wild-type B6D2F1 to obtain F1 heterozygous mice. Among several alleles with insertions or deletions (Fig. 2A), we identified an allele named *Frem3*^{em1}, in which a 5-base-pair deletion generates a novel codon encoding Ala49 followed by a termination codon (Figs. 2A and B). Since the signal sequence of FREM3 includes the first 27 amino acids, the mature FREM3 mutant polypeptide from the *Frem3*^{em1} allele would be 22 amino acid residues, with no protein domains (Fig. 2C). The mutated exon cannot be masked by alternative splicing because the 5-base-pair deletion is located within exon 1 in which the initiation codon is also included, as confirmed by direct sequencing of PCR-amplified *Frem3*^{em1} cDNA (Fig. 2D). The transcript level of *Frem3* decreased in *Frem3*$^{em1/em1}$ mice compared with that in wildtype, probably because of nonsense-mediated mRNA decay caused by a frame-shifting 5-base-pair deletion (Fig. 2E). It is reported that illegitimate translation occurs from out-of-frame mutant allele [7]. Even if it occurs in *Frem3*^{em1} mutant allele, the resulting illegitimate translation product lacks N-terminal signal sequence and therefore is never secreted into extracellular space. Collectively these results indicate that *Frem3*^{em1} ablates production of functional FREM3 protein.



B

Genotype	Number of neonate
WT	31
Frem3$^{em1/+}$	82
Frem3$^{em1/em1}$	43





H

Defects	Occurrence in *Frem3*$^{em1/em1}$
Dystrophic epidermolysis bullosa	0/11
Cryptophthalmos	0/13
Syndactyly	0/13
Renal agenesis/dysgenesis	0/13
Fusion of pulmonary lobes	0/13

Fig. 3. No Fraser syndrome-like defects were observed in *Frem3* mutant mice. (A) Genotyping of WT and (B) Number of pups obtained from matings between *Frem3*$^{em1/+}$ heterozygotes. (C–F) Appearance of an E15.5 embryo (C), adult eyelid (D), adult digit (E), and adult kidney (F). Bar, 5 mm. (G) Hematoxylin-eosin staining of *Frem3*$^{em1/em1}$ kidney section. Bar, 2 mm. (H) Occurrence of defects typically observed in Fraser syndrome model animals in *Frem3*$^{em1/em1}$ mice.

Phenotypic characterization of *Frem3* mutant mice

Heterozygous mating between *Frem3*$^{em1/+}$ gave wildtype, *Frem3*$^{em1/+}$, and *Frem3*$^{em1/em1}$ offspring at the expected Mendelian ratio, indicating no apparent embryonic lethality of *Frem3*$^{em1/em1}$ mice (Figs. 3A and B). *Frem3*$^{em1/em1}$ males and females were both fertile; mating *Frem3*$^{em1/em1}$ females with wildtype males and mating wildtype females with *Frem3*$^{em1/em1}$ males gave 7.4 ± 3.1

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(n=18) and 9.4 ± 1.2 (n=11) pups (average ± SD), respectively. There was no difference in average litter size between these matings (P=0.09 by Student's t-test). To address whether the loss of FREM3 cause any developmental defects reminiscent of Fraser syndrome, MOTA, or BNAR, *Frem3*$^{em1/em1}$ mice were phenotypically investigated, with special focus on dystrophic epidermolysis bullosa, cryptophthalmos, syndactyly, renal agenesis, and lung lobe fusion. However, no defects characteristic of Fraser syndrome, BNAR, or MOTA syndrome were observed in *Frem3*$^{em1/em1}$ mice (Figs. 3C–H).

Frem3$^{em1/em1}$ appeared normal with no apparent Fraser syndrome- or MOTA/BNAR-like developmental defects observed in *Frem3*$^{em1/em1}$ mice. These results indicate that dysfunction of FREM3 is not associated with Fraser syndrome, BNAR, or MOTA. However, there is still a possibility that FREM3 supports QBRICK/FRAS1/FREM2 function. Due to this possibility it might be interesting to investigate whether phenotypic severity is enhanced by the presence of a *Frem3* mutation in *Qbrick*, *Fras1*, or *Frem2* mutant mice.

In summary, we generated *Frem3* mutant mice and found that there are no Fraser-syndrome-like developmental defects.

Author Contributions

DK, and MI designed experiments. DK, MM, and MK performed experiments. DK and MI wrote manuscript.

Conflict of Interest

The author declared no competing interest.

Acknowledgments

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