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Note

Complete Deletion of *Slc52a2* Causes Embryonic Lethality in Mice

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Riboflavin (vitamin B2) plays an important role in cellular growth and function. Riboflavin transporter 2 (RFVT2) is widely expressed in several tissues, especially in the brain and salivary glands, and plays an important role in the tissue disruption of riboflavin. During the last 10 years, mutations in *SLC52A2* have been documented in patients with a rare neurological disorder known as Brown–Vialetto–Van Laere syndrome. However, no suitable animal model of this disease has been reported. Here, we aimed to clarify the physiological role of RFVT2 using *Slc52a2*-mutant mice. The appearance, body weight, and plasma riboflavin concentration of *Slc52a2* heterozygous mutant (*Slc52a2*+/−) mice were similar to those of wild-type (WT) mice. However, intercrossing between *Slc52a2*+/− mice failed to generate *Slc52a2* homozygous mutant (*Slc52a2*−/−) mice. This suggested that *Slc52a2* gene deficiency results in early embryonic lethality. Our findings suggested that RFVT2 is essential for growth and development, and its deletion may influence embryonic survival.

Key words riboflavin transporter 2; mouse model; embryonic lethality

INTRODUCTION

Riboflavin (vitamin B2) is an indispensable nutrient for cellular growth and function.[1] The active coenzymes, flavin mononucleotide (FMN) and FAD, which are made from riboflavin. Riboflavin deficiency leads to growth impairment, which is causally related to the role of riboflavin in generation of energy from mitochondrial metabolism.[2] Human riboflavin transporters RFVT1–3/*SLC52A1–3* have been identified.[3] RFVT2 predicted to have 10 membrane-spanning domains.[4] The RFVT2-mediated uptake of riboflavin has been shown to be Na^+-, Cl^--, and pH-independent.[4,5] *RFVT2* mRNA is ubiquitously expressed.[4] It has been suggested that RFVT2 is essential for tissue distribution of water-soluble riboflavin.[5]

Since 2010, several mutations in the *SLC52A3* and *SLC52A2* genes have been shown to be linked to Brown–Vialetto–Van Laere syndrome (BVVLS).[6] BVVLS patients with *SLC52A3* mutations have a higher frequency of facial weakness and lower blood riboflavin levels.[7] However, abnormal gait and/or ataxia and optic nerve atrophy appear to be more prevalent features of patients with *SLC52A2* mutations.[7] In addition, improvements in motor abilities, respiratory function and/or cranial nerve deficits upon riboflavin supplementation are observed in 70% patients, with the remaining patients showing stabilization of the current disease stage. The responses to riboflavin supplementation are similar in patients with *SLC52A2* and *SLC52A3* mutations. It has been suggested that immediate and continuous riboflavin administration may prevent neurological changes.[7] In previous studies, we have shown that *Slc52a3*-knockout mice exhibit phenotypes similar to those seen in patients with *SLC52A3* mutations, which are associated with riboflavin deficiency.[8] An analysis of skin fibroblasts from patients with *SLC52A2* mutations revealed a significant reduction in electron transport chain complex I and II activity.[9] However, the pathophysiological mechanism of these symptoms is unclear.

In this study, we aimed to clarify the significance of Rfvt2 *in vivo* using *Slc52a2*-mutant mice. The appearance, body weight, and plasma riboflavin concentration of *Slc52a2* heterozygous mutant (*Slc52a2*+/−) mice were not different from those of wild-type (WT) mice. However, intercrossing between *Slc52a2*+/− mice failed to generate *Slc52a2* homozygous mutant (*Slc52a2*−/−) mice. These results suggested that Rfvt2 deficiency causes embryonic lethality in mice.

MATERIALS AND METHODS

Animals All animal studies were conducted in accordance with the *Guidelines for Animal Experiments of Kyoto University.* Embryos with an *Slc52a2* mutation (C57BL/6-*Slc52a2*$^{tm1(KOMP)Vlcg}$) were purchased from the Knockout Mouse Project (KOMP) Repository.[10] The targeting vector is described in Fig. 1A. To determine mouse genotypes, genomic DNA was isolated from tail biopsies using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.), and PCR analysis was performed using the TaKaRa Ex Taq® Hot Start Version reaction mix (TaKaRa Bio, Shiga, Japan). The primer sets were as follows: a forward primer, 5′-CCA GAC CCT AAG GCC CAT CAG-3′, and a reverse primer, 5′-CAG CAC GCC ATT GGT CAG AG-3′, for detecting the wild-type alleles and a forward primer, 5′-GGT AAA CTG GCT CGG ATT AGG G-3′, and a reverse primer, 5′-TTG ACT GTA GCG GCT GAT GTT G-3′, for detecting mutant alleles. PCR cycling conditions were as follows: 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. Heterozygous mice (8 weeks old) were mated overnight and vaginal plugs were examined the following morning. Plug detection was considered to correspond to day 0.5 of pregnancy. Embryos at E10.5, pups at postnatal day 0, and adult mice older than 8 weeks were used for subsequent experiments. The mice were housed

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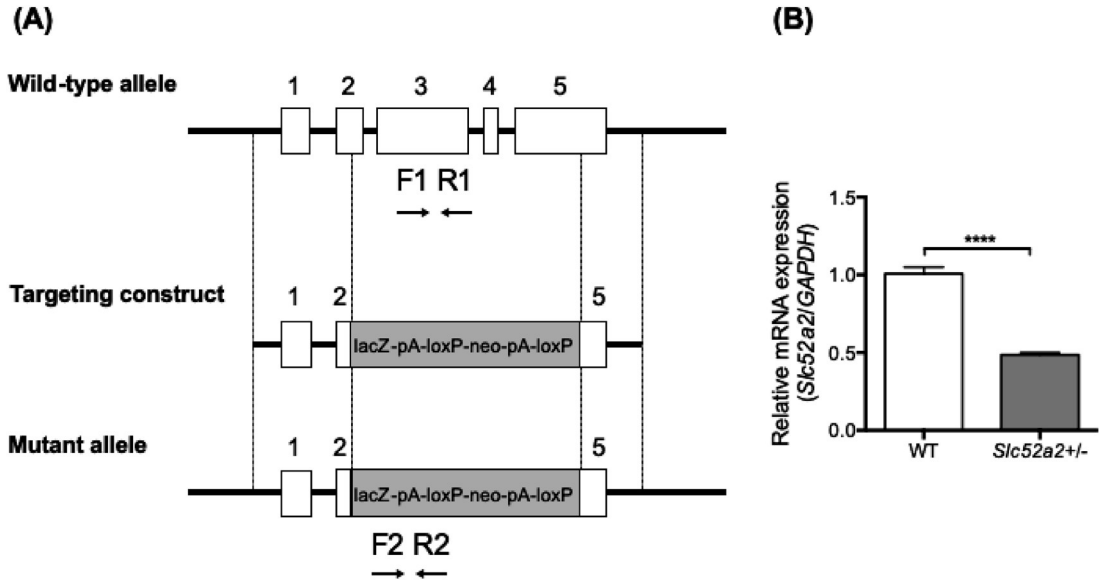

Fig. 1. Targeted Disruption of *Slc52a2*

(A) Diagram of *Slc52a2* mutant construct. Exons are indicated by white closed boxes. Primers used for PCR are depicted as arrowheads. (B) *Slc52a2* mRNA expression in brains from 8-week-old WT and *Slc52a2*+/− mice. Each bar represents the mean ± standard error of the mean (S.E.M.). (WT, $n=6$; *Slc52a2*+/−, $n=22$ from three litters). ****$p<0.0001$, compared to WT.

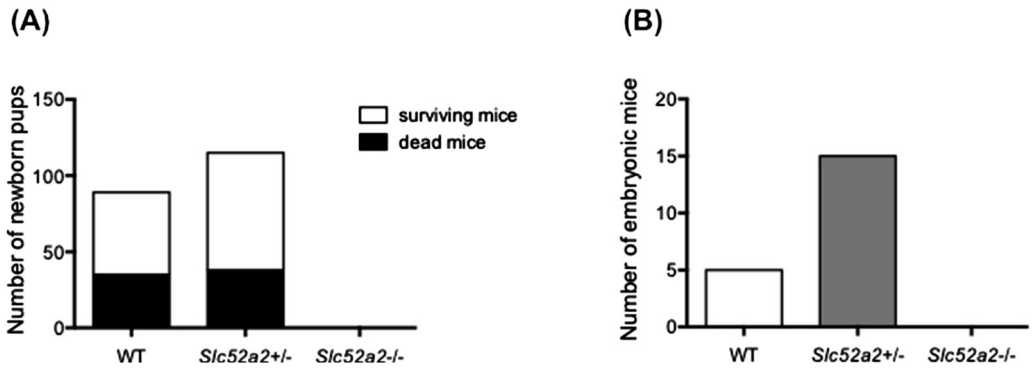

Fig. 2. Genotypic Analysis of *Slc52a2*-Mutant Mice

(A) Genotype distributions after birth ($n=204$ from 72 litters). The white bar represents the number of surviving mice and the black bar represents the number of mice that died immediately after birth. (B) Genotype distributions in fetal mice at E10.5 ($n=20$ from three litters).

under a 12-h light/dark cycle in a temperature-controlled environment, and were given water *ad libitum* and a standard chow diet (F-2; Funabashi Farm, Funabashi, Japan) before being used in experiments. All protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (Permission No. MedKyo20121).

Real-Time PCR Total RNA was isolated from brains dissected at 8 weeks of age, using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and was then reverse transcribed. TaqMan Gene Expression assays were obtained from Life Technologies (*Slc52a2*, Mm01205717_g1; Carlsbad, CA, U.S.A.). Real-time PCR was performed to determine the mRNA expression level of *Slc52a2* as described previously.[4)]

Measurement of Riboflavin We collected samples of blood and tissue from 16-week-old mice. The concentrations of riboflavin in blood and tissue samples were measured by HPLC (LC-10ADVP; Shimadzu, Kyoto, Japan) according to a previously reported method.[11)]

Statistical Analysis Statistics were performed using GraphPad Prism (version 7; GraphPad Software, Inc., La Jolla, CA, U.S.A.). All values are expressed as the mean ± standard error of the mean (S.E.M.), and the differences were analyzed for significance using an unpaired Welch's *t*-test. Multiple comparisons were performed using Bonferroni's two-tailed test, after a one-way ANOVA. The significance was shown based on the *p*-value (****$p<0.0001$).

RESULTS

Targeted Disruption of the *Slc52a2* Gene The mouse *Slc52a2* gene was deleted from exon 2 to 5, and was integrated with a trapping cassette. PCR analysis confirmed the targeted *Slc52a2* allele in genomic DNA isolated from tail biopsies of the offspring (Fig. 1A). Furthermore, real-time PCR analysis demonstrated that *Slc52a2* mRNA levels in the brain were significantly lower in *Slc52a2*+/− mice than in WT mice (Fig. 1B).

Genotyping of newborn pups from *Slc52a2*+/− parents revealed that intercrossing between heterozygotes only produced *Slc52a2*+/− and WT mice, and failed to generate *Slc52a2*−/− mutant mice (Fig. 2A). The same result was observed in embryos at E10.5 (Fig. 2B).

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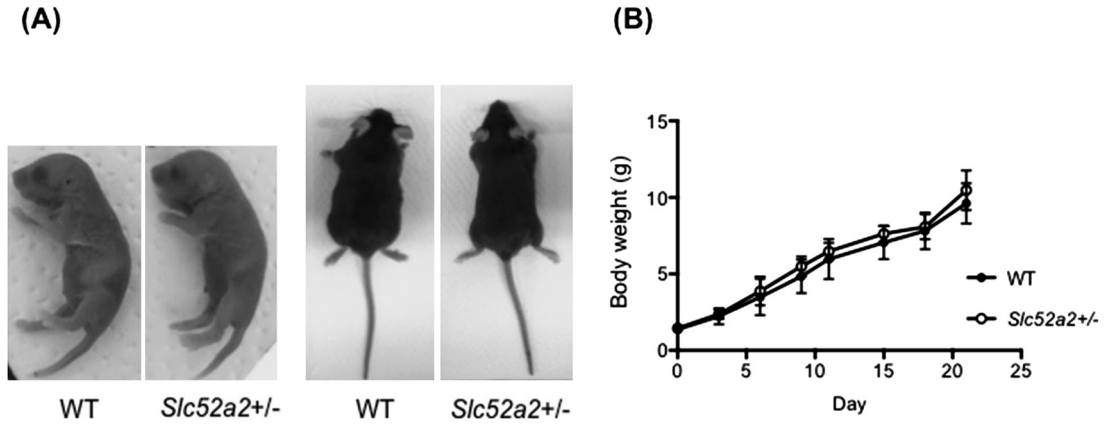

Fig. 3. Phenotypic Analysis of *Slc52a2*+/− Mice

(A) Gross appearance of whole bodies of WT and *Slc52a2*+/− mice at postnatal day 0 and 10 weeks. (B) Changes in body weight of WT and *Slc52a2*+/− mice up to 3 weeks after birth. (WT, $n=5$; *Slc52a2*+/−, $n=7$).

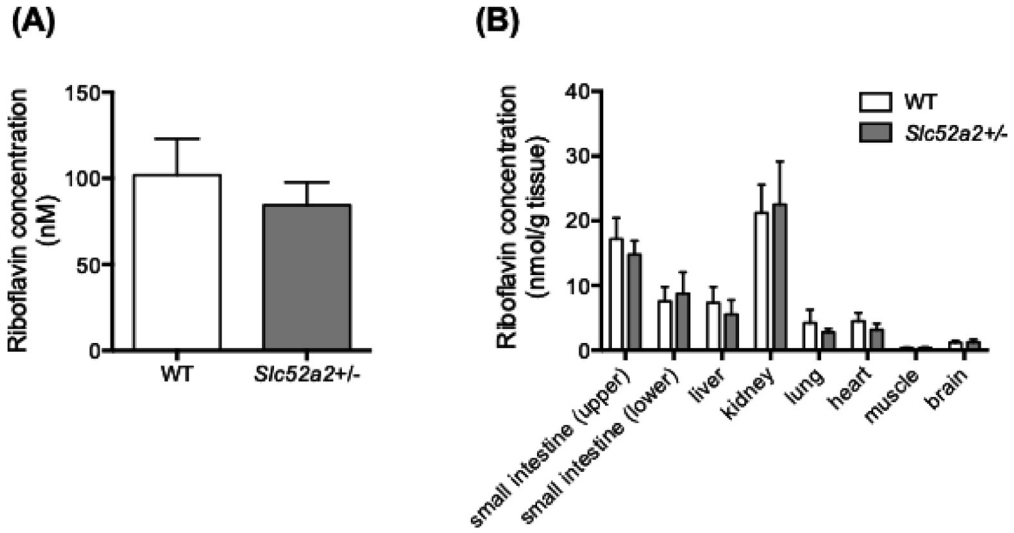

Fig. 4. Riboflavin Levels in Plasma and Tissues from WT and *Slc52a2*+/− Mice

Plasma and tissue samples were obtained from 16-week-old WT and *Slc52a2*+/− mice. Riboflavin levels in the plasma (A) and tissues (B) were measured by HPLC. Each bar represents the mean ± S.E.M. (WT, $n=6$; *Slc52a2*+/−, $n=5$).

Table 1. Gene Mutations in Patients with *SLC52A2*-Mutant BVVLS

Gene mutation	G306R × G306R	G306R × L312P	L312P × L312P	G306R × L339P	G306R × R284D	L312P × L339P	G306R × Y305C	L312P × W31S	L123P × L339P
Number	17	1	1	4	2	2	1	1	1

Modified from Haack *et al.*,[12] Foley *et al.*,[13] and O'Callaghan *et al.*[7] In *in vitro* functional analyses, the mutations with a white background showed a moderate decrease in function, while the mutations with a gray background showed almost complete loss of function.

Riboflavin Homeostasis and Phenotypic Analysis in *Slc52a2*+/− Mice Macroscopically, the appearance of *Slc52a2*+/− pups and adults were not different from WT mice (Fig. 3A), and the body weights of *Slc52a2*+/− and WT mice were similar within 3 weeks of birth (Fig. 3B).

We measured riboflavin concentration in plasma (Fig. 4A) and tissues, including the upper and lower small intestine, liver, kidney, lung, heart, muscle, and brain in 16-week-old WT and *Slc52a2*+/− mice (Fig. 4B). No differences in plasma or tissue riboflavin concentrations were observed between *Slc52a2*+/− and WT mice.

DISCUSSION

In this study, we attempted to produce *Slc52a2*-mutant mice as a pathological model of *SLC52A2*-mutant BVVLS. However, *Slc52a2*−/− mice were not observed among newborn pups or E10.5 embryos, including those that died due to maternal neglect. RFVT2 is widely expressed in tissues throughout the body. Therefore, the complete deletion of *Slc52a2* expression resulted in embryonic lethality in the early stages of embryonic development.

In *in vitro* functional analyses, *SLC52A2* mutations p.G306R and p.L312P show a moderate, but significant, decrease in

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transport activity.[12] These mutations have been detected in 30 BVVLS patients (Table 1). Except for one patient, previous studies have shown that BVVLS patients with *SLC52A2* mutations have one allele that encodes functional RFVT2.[12,13] A previously described patient with mutations in p.L123P and p.L339P is thought to have survived due to the retention of a low level of RFVT2 activity. Taken together, these data suggested that RFVT2 is essential for embryonic cell survival *in vivo*, and complete deletion may lead to embryonic lethality.

Phenotypic analysis showed no difference between WT and *Slc52a2*+/− mice. In addition, the riboflavin concentrations in plasma and tissues were unchanged compared with those in WT mice. These results revealed that *Slc52a2*+/− mice show normal growth, which is consistent with the results reported for *Slc52a3*+/− mice.[8] In clinical reports, parents or sibling with heterozygous mutation are healthy, suggesting an autosomal recessive mode of inheritance.[14] Therefore, the *Slc52a2*+/− mouse phenotype may mimic the phenotype of parents of BVVLS patients.

When the *Slc52a2* gene was completely deleted by homologous recombination with a long-chain sequence, *Slc52a2*−/− mice were not generated. Creating a single-nucleotide polymorphism animal model, in which some Rfvt2 function is retained, may be an alternative method for producing a pathological model of RFVT2-mutant BVVLS.

In conclusion, RFVT2 is an essential transporter for growth and development, and its deletion may influence embryonic survival.

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Conflict of Interest The authors declare no conflict of interest.

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