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Conditional (intestinal-specific) knockout of the riboflavin transporter-3 (RFVT-3) impairs riboflavin absorption

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The water-soluble vitamin riboflavin (RF) is required for normal cellular metabolism, proliferation, and growth. In the biologically active forms (flavin mononucleotide and flavin adenine dinucleotide), RF plays a key metabolic role in the transfer of electrons in biological oxidation-reduction reactions involving carbohydrate, lipid, amino acid, and certain water-soluble vitamins (pyridoxine and folate) (3, 32). Studies have also shown a role for RF in protein folding within the endoplasmic reticulum (38). More recent investigations have demonstrated anti-oxidant and anti-inflammatory properties for RF (13, 20, 33, 35), and further showed a role for the vitamin in normal immune function (23, 34). RF deficiency leads to a variety of abnormalities, including degenerative changes of the nervous system, anemia, skin lesions, cataract, and growth retardation (28, 32); RF deficiency also leads to an increase in the susceptibility to cancer (24). Deficiency/suboptimal levels of this micronutrient occur in chronic alcoholism, diabetes mellitus, inflammatory bowel disease, and Brown-Vialetto Van Laere and Fazio Londe syndromes [the latter are neurological disorders caused by mutations in RFVT-2 and -3 (1, 2, 4, 5, 9, 10, 14, 16, 17, 19, 29)].

Mammals cannot synthesize RF endogenously. Therefore, they must obtain the vitamin from exogenous sources via intestinal absorption. Two sources of RF are available to the intestine: a dietary source and a bacterial source [the latter is in regard to the vitamin produced by the microbiota of the large intestine (12, 15, 40)]. Previous studies from our laboratory and others have shown that small and large intestinal RF uptake is specific and carrier-mediated in nature (30–32). The recently cloned RF transporters-2 and -3 [RFVT-2 and -3; products of the SLC52A2 and SLC52A3 genes, respectively (6, 41, 42)] are both expressed in the gut, with expression of RFVT-3 being significantly higher than that of RFVT-2 (36, 42); the former is also more efficient in transporting RF than the latter (36). Also, live cell confocal imaging studies of polarized intestinal epithelial cells have shown that RFVT-3 is predominantly expressed at the apical membrane domain while RFVT-2 is mostly expressed at the basolateral membrane domain of these cells (36).

To date, however, the relative contribution of the RFVT-3 system to total carrier-mediated RF uptake in the native intestine in vivo is not well defined. Our recent in vitro study using the intestinal Caco-2 cell line and SLC52A3 gene-specific small-interfering RNA approach showed an important role for RFVT-3 in RF uptake (36). The aim of this study was to define the role of RFVT-3 in RF uptake in native intestine in vivo, and for that we have generated a conditional (intestinal-specific) RFVT-3 cKO mouse model. Our results showed that the RFVT-3 system is indeed the main transporter involved in RF uptake in the native small and large intestine, and that this dysfunction impairs normal RF body homeostasis.

MATERIALS AND METHODS

Materials

[1]HRF and [3]Hbiotin (specific activity 21.2 and 60 Ci/mmole, respectively; radiochemical purity >97% for both) were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Anti-RFVT-3 polyclonal and anti-β-actin monoclonal antibodies were obtained from Gene Tex (Irvine, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-rabbit IRDye-800 and anti-mouse IRDye-680 antibodies were purchased from LI-COR Bioscience (Lincoln, NE). All chemi-
cals and reagents were purchased from commercial vendors and were of analytical/molecular biology grade.

**Generation of Conditional (Intestinal-Specific) RFVT-3 cKO Mice**

An 11.88-kb region of the *Slc52a3* gene was subcloned from a C57BL/6 BAC clone (RP23:424F10) using a homologous recombination-based technique (ingenious Targeting Laboratory, Ronkonkoma, NY). The region was designed such that the 5’-homology arm extends 6.55 kb to a single LoxP site. The 3’-homology arm extends 2.61 kb 3’ to a LoxP/FRT-flanked Neo cassette. A single LoxP site was inserted upstream of exon 2 in intron 1–2, and the LoxP/FRT-flanked Neo cassette was inserted downstream of exon 3 in intron 3–4. The size of the target region is 2.72 kb containing exons 2 and 3 (Fig. 1A).

**Generation of the Homozygous LoxP+/+ Mice**

Identification of somatic Neo-deleted mice. Targeted iTL BA1 (129/SvEv × C57BL/6N) hybrid embryonic stem cells were micro-injected in C57BL/6 blastocytes. Resulting chimeras with a high- percentage agouti coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. The Neo deletion was confirmed by PCR using forward primer 5’-GTTTGGATATGCTTCCAGATG-3’ and reverse primer 5’-CCAGGTGGGTCTGTGTTTGTAG-3’. Subsequently, a primer set (forward 5’-CAGTGTATGTTAGTAGGTCG-3’ and reverse 5’-CATGCTGCGAGATGTCGAGG-3’) was used to screen mice for presence of the FLP transgene. Additionally, a PCR was performed to detect the presence of the distal LoxP site using a forward primer 5’-TACTCTGTACACAGGGAGGAGGAGCCCTC-3’ and a reverse primer 5’-CAGTGTATGTTAGTAGGTCG-3’. The 3’-homology arm integration was confirmed by PCR using forward primer 5’-TCACCAGGCTCTTGTGTTAAG-3’ and reverse primer 5’-GCATAAGCTTTGATGCTTCCAGGAC-3’.

Identification of germline Neo-deleted mice. The confirmed somatic Neo-deleted male mice were mated with C57BL/6N wild-type female mice to generate germline Neo-deleted mice (*Slc52a3* (LoxP+/+)) for germline Neo deletion and FLP absence.

Identification of homozygous germline neo-deleted mice. The confirmed germline Neo-deleted heterozygote mice (*Slc52a3* (LoxP+/−)) were mated to generate homozygous (Slc52a3) germline Neo-deleted mice. Resulting pups were genotyped for Neo deletion and confirmed heterozygote mice (Slc52a3 (LoxP+/−)) for germline Neo deletion and FLP absence.

**Quantitative RT-PCR**

One microgram of total RNA was treated with DNase I, and cDNA was synthesized using an i-Script kit (Bio-Rad). RT-qPCR was performed using the CFX96 real-time i-Cycler (Bio-Rad) and SYBR green PCR kit (Bio-Rad). Primers used were specific for mouse RFVT-3 (forward 5’-GGATCAGTGGAGCCAGTGG-3’ and reverse 5’-GACCTTTAAGCCAGGATG-3’) and mouse RFVT-2 (forward 5’-TCTGGCCTCACACCA-3’ and reverse 5’-GACCAACAGCCTGCA-3’). Additionally, RT-qPCR for stress-response genes glutathione peroxidase (GPX1, 5’-TCTTTACCTTCCTGGGAA-3’ and reverse 5’-GGACACGAGGTTTCTAGT-3’), superoxide dismutase (SOD1, forward 5’-GATGACTTGGGAAAGGTGGG-3’ and reverse 5’-CTTGGCAATCCCATCCT-3’), and Flavin-containing monoxygenase 2 (FMOD2, forward 5’-CAGCTTAGCCATTGCT-3’ and reverse 5’-TGTATTCGCCGCTATAGG-3’) was performed. The housekeeping gene used in all amplifications was mouse β-actin (forward 5’-ATCTCTTCTCTCTTCTGGA-3’ and reverse 5’-TCTATGGAGTGCACAGGA-3’). The relative expression was quantified by normalizing Ct values with corresponding β-actin as described before (21). The negative control reactions with respective gene-specific primers, PCR mix, and without RT product were used in every experiment.

**Western Blot Analysis**

Villus epithelial cells were isolated from the jejunum of RFVT-3 cKO mice and control littermates as described before (25). Cells were lysed in RIPA buffer (Sigma) containing complete protease inhibitor cocktail (Roche, NJ), and the soluble protein fraction was isolated by centrifugation at 12,000 revolutions/min for 10 min. SDS-PAGE of 60 μg of protein was performed on a 4–12% mini gel (Invitrogen). The protein was transferred to an immobilon polyvinylidene difluoride membrane, and the blot was blocked in LI-COR blocking buffer. The blot was washed three times in PBS-Tween 20 and then incubated with anti-rabbit IRDye 800 and anti-mouse IRDye 680 (LI-COR Bioscience) secondary antibodies (1:30,000) for 45 min at room temperature. The specific immunoreactive bands were captured using the Odyssey infrared imaging system (LI-COR Bioscience), and their densities were quantified using the LI-COR software (LI-COR Bioscience).

**Measurement of Blood RF Levels**

Blood levels of RF in the RFVT-3 cKO mice and sex-matched control littermates were determined using a glutathione reductase assay kit (Cayman Chemical, Ann Arbor, MI) (8). Briefly, blood samples were collected from dead mice, and the glutathione reductase activity was determined in erythrocytes as described in the manufacturer’s protocol.
Fig. 1. Generation of conditional (intestinal-specific) RFVT-3 knockout (cKO) mice, genotyping, and validation of RFVT-3 loss at the protein and mRNA expression level. A: schematic depiction of the homologous recombination to introduce the LoxP site upstream of exon 2 and downstream of exon 3 of the Slc52a3 gene. A 5’-LoxP recombination site was inserted in intron 1 of the Slc52a3 gene together with a Neo cassette, and a second 3’-LoxP recombination site was introduced into intron 3. Exons 2 and 3 of the Slc52a3 gene were crossed with transgenic mice expressing the Cre-recombinase in intestinal epithelial cells under the control of villin promoter. B: genomic PCR analysis of mouse ear DNA samples using two primer pairs to show the presence of LoxP sites and Cre transgenes. C: loss of RFVT-3 mRNA expression due to conditional deletion of exons 2 and 3 of Slc52a3 in the small intestine was confirmed by RT-qPCR. D: quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping, liver and kidney of cKO mice, and their sex-matched littermates to determine the expression level of RFVT-3 as described in MATERIALS AND METHODS. Data are means ± SE of 6–9 sets of mice. *P < 0.01. E: expression of RFVT-3 protein in the intestine of the RFVT-3 cKO and control littermates. Western blot analysis was performed using specific anti-RFVT-3 polyclonal antibodies as described in MATERIALS AND METHODS. Data are means ± SE of at least 3 separate sets of mice. *P < 0.01. F: quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping, liver and kidney of cKO mice, and their sex-matched littermates to determine the expression level of RFVT-2 as described in MATERIALS AND METHODS. Data are means ± SE of 6–9 sets of mice.
Phenotype Assessment

Body weight, bone density, and other changes were monitored in the RFVT-3 cKO mice and sex-matched wild-type littermates. Animals were anesthetized and placed in a Kodak Image station (Kodak Molecular Imaging Systems, New Haven, CT) for performing X-ray imaging analysis. Body weight was determined weekly using a Mettler Toledo scale. Estimation of bone density and determination of bone length in X-ray images were performed using Scion Image 4.0.3 (Scion, Frederick, MD).

Histology

The RFVT-3 cKO mice and sex-matched wild-type littermates were killed, and the intestine, heart, liver, and kidneys were immediately removed and fixed in 10% formalin overnight. The tissue samples were sectioned and paraffin embedded. Hematoxylin and eosin (H&E)-stained slides were prepared using standard histological techniques (Long Beach Veterans Medical Center Clinical Histology Laboratory). In another study the oriented histology section of the small bowel wall was semiquantitatively analyzed to measure the average villus height for each animal. Briefly, H&E-stained sections were viewed in a conventional Nikon Eclipse 50i microscope equipped with a ×10 ocular with a micrometer and a ×10 objective. The surface of the bowel mucosa was identified as the junction between crypts and villi, and the height of five representative villi was measured in five separate ×10 fields. The mean and SD were calculated. All groups of data were compared using the Student’s t-test, and the difference of the mean with the SE was calculated. The microscopic analysis was performed by a board-certified anatomic pathologist.

Statistical Analysis

All data (uptake, RT-qPCR, and Western blot analysis) presented are means ± SE of at least three independent experiments with at least three different sets of animals. Uptake of [3H]RF (and [3H]biotin) by the carrier-mediated process was determined by subtracting uptake in the presence of 1 mM of unlabeled RF (or biotin) from that in its absence. The latter finding confirms specificity of the RFVT-3 cKO phenotype. The RFVT-3 cKO animals was significantly (P < 0.01) lower than that of age- and sex-matched wild-type littermates (Fig. 2C). In addition, X-ray image analysis of RFVT-3 cKO mice compared with wild-type littermates showed a significant (P < 0.001) decrease in bone density (femur and tibia; Fig. 2D) and a significantly (P < 0.001) shorter femur, tibial, and pelvic bones (Fig. 2E). However, no histological abnormalities were found in the heart, liver, kidney, jejunum, ileum, cecum, and proximal and distal colon of the RFVT-3 cKO mice (data not shown). In addition, no significant changes in the length of the jejunal crypts and villi of the cKO mice compared with control littermates were seen (data not shown). Finally, a significantly (P < 0.01) lower RF blood level (erythrocyte glutathione reductase activity was measured to reflect the level of RF in blood samples; see MATERIALS and METHODS) was found in the RFVT-3 cKO mice compared with their sex-matched littermate controls (Fig. 2F).

Effect of Loss of RFVT-3 on Intestinal RF Uptake

We used both an in vivo (gut loops) and an in vitro (isolated intestinal epithelial cells) preparation to examine the effect of knocking out the intestinal RFVT-3 on gut RF uptake. Results were compared with RF uptake by the same preparation from sex-matched littermate controls. The results showed RF (0.24 μM) uptake to be severely (P < 0.01) inhibited in jejunal loops of RFVT-3 cKO mice compared with control littermates (Fig. 3A). F0 such inhibition was observed in the uptake of the unrelated vitamin biotin (83 nM) by jejunal loop of RFVT-3 cKO mice compared with uptake by their control littermates (Fig. 3B). The latter finding confirms specificity of the RFVT-3 cKO model. Similarly, RF uptake by epithelial cells freshly isolated from the jejunum of RFVT-3 cKO mice was found to be significantly (P < 0.01) suppressed compared with uptake by cells from control littermates (Fig. 3C) [the residual RF uptake observed in this preparation from the RFVT-3 cKO mice is most likely due to uptake across the exposed basolateral membrane where RFVT-2 is expressed (36)].

Previous studies have shown that the normal microflora of the large intestine synthesizes a considerable amount of RF (12, 15, 40) and that colonicocytes have an efficient carrier-

specific deletion of the Slec52a3 gene at both the mRNA (PCR) in the gut [and its normal expression in other tissues (liver and kidney)] (Fig. 1, C and D) and the protein (Western blotting) levels (Fig. 1E). Finally, we did not observe any change in the level of expression of the other RF transporter that is also expressed in the gut, i.e., the RFVT-2, in the intestine of the RFVT-3 cKO mice (Fig. 1F). These findings confirm the intestinal-specific nature of the RFVT-3 cKO and shows that such a knockout does not lead to an induction in the level of expression of RFVT-2 in the gut.
mediated mechanism for RF uptake (37). Therefore, we also examined carrier-mediated RF (0.24 µM) uptake in vivo in the colon of the RFVT-3 cKO mice and compared it with uptake with control littermates. The results again showed a significant (P < 0.01) inhibition in carrier-mediated RF uptake in the colon of RFVT-3 cKO mice compared with sex-matched control littermates (Fig. 4A). The residual expression of RFVT-3 in the colon (Fig. 4B) is likely due to the fact that the villin expression decreases in the distal portion of the colon (22), which leads to a decrease in Cre expression and thus less

Fig. 2. Effect of the RFVT-3 cKO on mouse phenotype (body weight and bone density and length) and riboflavin (RF) blood level. A: representative image of a cKO mouse (right) and sex-matched wild-type littermate (left) showing ~50% reduction in their body size. B: representative X-ray image of a cKO mouse (right) and sex-matched wild-type littermate (left) showing difference in size and bone length. C: bar graph showing marked difference in body weight of cKO mice compared with control littermates (*P < 0.01). D: bar graph showing significant decrease in bone density in cKO mice (expressed as %relative to wild-type littermates) (**P < 0.001). E: bar graph showing significant decrease in bone length in cKO mice (expressed as %relative to wild-type littermates) (**P < 0.001). F: RF levels in blood of cKO mice and wild-type littermates. *P < 0.01. Data are means ± SE of 6 sets of mice.

Fig. 3. Determination of effect and specificity of the RFVT-3 cKO on RF uptake in vivo and in vitro. A: carrier-mediated RF uptake by in vivo jejunal loop of cKO mice and sex-matched littermates. B: carrier-mediated biotin uptake by in vivo jejunal loop of RFVT-3 cKO mice and control littermates. C: carrier-mediated RF uptake by in vitro freshly isolated intestinal epithelial cells of cKO mice and wild-type littermates. Data are means ± SE of at least 3 separate uptake determinations performed on at least 3 different sets of mice. *P < 0.01.
efficient RFVT-3 gene knockout in that part of the colon (22). No change in level of expression of the RFVT-2 mRNA was found in the colon of RFVT-3 cKO compared with control littermates (2.6 ± 1 and 3.1 ± 0.6 arbitrary units in wild-type littermates and cKO, respectively).

**Level of Expression of Oxidative Stress-Responsive Genes in the Intestine of the RFVT-3 cKO Mice**

Recent studies have shown that RF deficiency leads to oxidative stress in intestinal epithelial cells (18). Therefore, we examined the effect of loss of RFVT-3 expression in the intestine on level of expression of oxidative stress-responsive genes (FMO2, GPX1, and SOD1) in the jejunum; results were compared with those obtained with control littermates. The results showed a significant (P < 0.01 for all) increase in the level of mRNA expression of FMO2, GPX1, and SOD1 in jejunal mucosa of RFVT-3 cKO compared with wild-type littermates (Fig. 5, A–C, respectively). These findings are in line with a state of depletion of RF in intestinal epithelial cells and points to a role for RF in the maintenance of normal intestinal health (18, 26).

**Effect of RF Supplementation of the RFVT-3 cKO Mice on Animal Phenotype**

In this study, we examined the effect of supplementing the RFVT-3 KO mice with pharmacological amounts of RF on the observed growth retardation, changes in bone density and length, and on the ocular surface abnormalities and early death phenotypes. This was done by providing RF (500 μM) in the drinking water to dams during pregnancy and lactation, and to the RFVT-3 cKO mice throughout their life. The results showed that RF supplementation of the RFVT-3 cKO mice led to correction of all the above-described abnormal phenotypes and that the KO animals grew at a similar rate as their control littermates (Fig. 6, A and B). In addition, the level of expression of the oxidative stress-responsive genes (FMO2, GPX1, and SOD1) was found to be similar in the jejunum of the RFVT-3 cKO mice and wild-type littermates (Fig. 6, C–E, respectively).

**DISCUSSION**

The aim of this study was to develop a better understanding of the role of the recently cloned RFVT-3 in the intestinal carrier-mediated absorption process of RF in the native gut in vivo. RF, a member of the B family of water-soluble vitamins, plays critical roles in normal cellular metabolism and function of humans and other mammals. However, it cannot be synthesized endogenously and, thus, must be obtained from exogenous sources via intestinal absorption. To achieve our aim, we developed a RFVT-3 cKO mouse model using the Cre/Lox approach and verified the near absence of the RFVT-3 protein/mRNA in the gut of the cKO animals compared with their sex-matched wild-type littermates. We also showed that ex-
pression of RFVT-3 in other tissues (liver and kidney) is not affected, i.e., similar in the cKO animals and their control littermates. Furthermore, we found that knocking out the RFVT-3 system in the intestine does not lead to induction in the expression of RFVT-2, an RF transporter that is also expressed in the gut.

Our results showed that all RFVT-3 cKO mice develop distinct phenotypes that include growth retardation (total body weight was markedly decreased compared with control littermates), decreased bone density and length, lethargic behavior, hunched back posture, and ocular surface abnormalities compared with wild-type littermates. In addition, their blood RF level was found to be significantly lower than that of control littermates, pointing to an important role for the RFVT-3 system in the maintenance of normal RF body homeostasis. Finally, almost two-thirds of the RFVT-3 cKO mice died prematurely between the age of 6 and 12 wk. The cause of death is not clear but could not be attributed to histological abnormalities in critical organs of the RFVT-3 cKO mice, since no such abnormalities were observed in the heart, liver, kidney, and the small and large intestine.

Our transport investigations in the native small intestine (jejunum) of the RFVT-3 cKO mice in vivo showed severe inhibition in carrier-mediated RF uptake compared with uptake by the jejunum of the wild-type littermates. In contrast, uptake of the unrelated vitamin biotin was found to be similar in the RFVT-3 cKO mice and to control littermates demonstrating substrate specificity of the knockout. Likewise, RF uptake in freshly isolated native jejunal epithelial cells in vitro was severely inhibited in the RFVT-3 cKO mice compared with control littermates. Similarly, carrier-mediated RF uptake in the native colon in vivo was found to be severely reduced in the RFVT-3 cKO mice compared with uptake in the colon of wild-type littermates. These findings clearly show that RFVT-3 plays an important role in the absorption of both dietary RF (which takes place in the small intestine) and the microbiota-generated RF (which takes place in the large intestine).

Recent studies have shown that RF deficiency leads to oxidative stress in intestinal epithelial cells (18, 26). Thus, we investigated the effect of loss of RFVT-3 expression in the intestine on level of expression of the oxidative stress-responsive genes FMO2, GPX1, and SOD1. Our results showed the expression of all these genes to be markedly increased in the jejunal mucosa of the RFVT-3 cKO mice compared with wild-type littermates. These data support an important role of RF in maintenance of normal intestinal health (18, 26).
Finally, the results of the effect of RF supplementation [RF (500 μM) was added to the drinking water of dams during pregnancy and lactation, and to the cKO mice during their entire life span] of the RFVT-3 cKO mice showing reversal of all observed abnormal phenotypes (growth retardation, bone density and length, ocular surface abnormalities, and early death) as well as the restoration in the level of expression of oxidative stress-responsive genes clearly demonstrate the importance of RF in overall health, growth, and development.

In summary, results of these investigations provide clear evidence for the essential role played by RFVT-3 in carrier-mediated RF uptake in native intestine in vivo, and in the evidence for the essential role played by RFVT-3 in carrier-mediated RF uptake in native intestine in vivo, and in the evidence for the essential role played by RFVT-3 in carrier-mediated RF uptake in native intestine in vivo, and in the maintenance of normal RF body homeostasis. It is hoped that the development of this mouse model, which allows the generation of tissue-specific deletion of RFVT-3, would serve as a catalyst for further investigations into the role of RFVT-3 in intestinal riboflavin transport in the physiology/pathophysiology of other critical tissues.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
V.S.S., N.L., and H.M.S. conception and design of research; V.S.S., N.L., and H.M.S. interpreted results of experiments; V.S.S. and N.L. performed experiments; V.S.S., N.L., and H.M.S. analyzed data; V.S.S. and C.L. edited and revised manuscript; V.S.S., N.L., and H.M.S. edited and revised manuscript; V.S.S., N.L., and H.M.S. approved final version of manuscript.

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