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ORIGINAL RESEARCH

Loss of MYO5B in Mice Recapitulates Microvillus Inclusion Disease and Reveals an Apical Trafficking Pathway Distinct to Neonatal Duodenum

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SUMMARY

Three mouse models of MYO5B recapitulate the features of microvillus inclusion disease in neonatal mice, including the formation of inclusions and loss of apical transporters in the duodenum. The studies show the presence of a novel apical membrane internalization pathway in neonatal duodenal enterocytes.

BACKGROUND & AIMS: Inactivating mutations in myosin Vb (MYO5B) cause severe neonatal diarrhea in microvillus inclusion disease. Loss of active MYO5B causes the formation of pathognomonic inclusions and aberrations in brush-border enzymes.

METHODS: We developed 3 mouse models of germline, constitutively intestinal targeted, and inducible intestinal targeted deletion of MYO5B. The mice were evaluated for enterocyte cellular morphology.

RESULTS: Germline MYO5B knockout mice showed early diarrhea and failure to thrive with evident microvillus inclusions and loss of apical transporters in the duodenum. IgG was present within inclusions. Apical transporters were lost and inclusions were present in the duodenum, but were nearly absent in the ileum. VillinCre;MYO5B$^{F/F}$ mice showed similar pathology and morphologic changes in duodenal enterocytes. In contrast, when MYO5B KO was induced with tamoxifen treatment at 8 weeks of age, VillinCre^{ERT2};MYO5B$^{F/F}$ mice developed severe diarrhea with loss of duodenal brush-border enzymes, but few inclusions were observed in enterocytes. However, if tamoxifen was administered to 2-day-old VillinCre^{ERT2};MYO5B$^{F/F}$ mice, prominent microvillus inclusions were observed.

CONCLUSIONS: The microvillus inclusions that develop after MYO5B loss show the presence of an unrecognized apical membrane trafficking pathway in neonatal duodenal enterocytes. However, the diarrheal pathology after MYO5B loss is caused by deficits in transporter presentation at the apical membrane in duodenal enterocytes. *(Cell Mol Gastroenterol Hepatol 2016;2:131–157; http://dx.doi.org/10.1016/j.jcmgh.2015.11.009)*

Keywords: Enterocyte Trafficking; Brush Border; Rab11a; Rab8a; Syntaxin 3; NHE3; MYO5B.

Microvillus inclusion disease (MVID) is caused by inactivating mutations in myosin Vb (MYO5B).[1–3] Neonates with MVID have severe life-threatening diarrhea usually beginning in the first week of life.[4,5] Recent studies using in vitro investigations in correlation with human patient pathologic tissues have shown insights into the possible pathophysiology of MVID.[6–9] Our investigations have shown that MYO5B interactions with Rab8a are responsible for deficits in microvillar structure and trafficking, whereas interactions between MYO5B and Rab11a are responsible for microvillus inclusion formation.[9] Importantly, we were able to show that microvillus inclusions developed as a result of apical macropinocytosis.[9] Although in vitro studies using MYO5B knockdown and rescue in CaCo2 and CaCo2-BBE cells have identified important mechanisms that putatively are involved in MVID pathogenesis,[3,7–9] important questions remain about the significance of microvillus inclusions to the diarrhea phenotype and possible implications for treatment.

A recent publication reported a gene trap MYO5B knockout mouse that showed duodenal microvillus inclusions, but the pups died within 12 hours of birth, limiting the pathology analysis.[10] In addition, an inducible intestinal MYO5B knockout mouse recently was reported to develop microvillus inclusions within 4 days of MYO5B loss.[11] However, the sole reliance on adult mice to study a neonatal disease may hamper direct interpretation of the neonatal mechanisms involved in MVID. We now have developed germline, intestinally targeted, and inducible intestinal MYO5B knockout mice. Both germline MYO5B knockout mice and VillinCre;MYO5B$^{F/F}$ mice showed numerous microvillus inclusions in the proximal intestine,

Abbreviations used in this paper: ATPase, adenosine triphosphatase; DPPIV, dipeptidyl peptidase-4; E, embryonic day; KO, knockout; MYO5B, myosin Vb; MVID, microvillus inclusion disease; NHE3, sodium-hydrogen exchanger 3 protein; PCR, polymerase chain reaction; pERM, phosphorylated ezrin-radixin-moesin; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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but showed few inclusions in the distal small bowel. Inclusions contained IgG, suggesting their derivation from apical macropinocytosis involved with internalization of IgG from maternal milk. However, although 8-week-old Villin-Cre^{ERT2};MYO5B$^{F/F}$ mice induced with a single dose of tamoxifen developed severe diarrhea within 48 hours, they showed few microvillus inclusions. Nevertheless, if 2-day-old VillinCre^{ERT2};MYO5B$^{F/F}$ mice received tamoxifen, they developed prominent microvillus inclusions within 3 days. Diarrhea in all 3 mouse models was well correlated with losses in apical transporters in the proximal small intestine. These results show that microvillus inclusions in MYO5B knockout mice are not directly pathologic, but rather show the presence of a specialized apical membrane processing pathway in duodenal enterocytes during the neonatal period. The predominance of pathology in the proximal small intestine suggests that interventions promoting distal intestinal adaptation may represent an important strategy in the treatment of MVID neonates.

Materials and Methods

All authors had access to the study data and reviewed and approved the final manuscript.

Construction and Validation of MYO5B Deletion Mice

Embryonic stem cells harboring a knockout first allele for MYO5B (Figure 1*A*) were obtained from the Knockout Mouse Project (KOMP, Davis, CA). Embryonic stem cells (CSD78985 clone B10) were injected into C57BL/6 mouse blastocysts by the Vanderbilt Stem Cell Shared Resource. Ten chimeric male mice (50%–80% chimeric) were produced and mated with albino C57BL/6 mice to produce chimeric founders. One chimera was responsible for germline transmission to black offspring. The heterozygous mice from this line were mated for 1 year and produced no viable homozygous offspring. The mice therefore were crossed onto CD1 mice and after 3 generations crossed heterozygous mice gave birth to viable homozygous knockout offspring.

To produce floxed allele mice, the MYO5B$^{+/-}$ mice were bred with actin-FLP mice to derive MYO5B$^{flox/+}$ mice (Figure 1*A*). The MYO5B$^{flox/+}$ heterozygotes were crossed to produce homozygous MYO5B$^{flox/flox}$ mice (MYO5B$^{F/F}$). The MYO5B$^{F/F}$ mice then were crossed with both VillinCre and VillinCre^{ERT2} mice to produce VillinCre;MYO5B$^{F/F}$ targeted intestinal MYO5B deletion mice and VillinCre^{ERT2};MYO5B$^{F/F}$ inducible intestinal knockout mice. The care, maintenance, and treatment of animals in these studies followed protocols approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

DNA was isolated from mice tails using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All genotyping was confirmed by specific polymerase chain reaction (PCR) with GoTaq Green Master Mix (Promega, Madison, WI) (Figure 1*B*) using the following PCR primer pairs: for Myo5B: MYO5BKO-F2, 5'-CTTGAGTTTGTTTAGTCTCTTGTCCCCTTTG-3' and MYO5B KO-R3, 5'-CCGCTGACTATGATGGATTGGTTCTTTTC-3'; for LacZ: LacZ-1, 5'-TGCCGCTCATCCGCCACAT-3' and LacZ-2, 5'-CACCGATCGCCCTTCCCAACAGT-3'; for VillinCre: 16775, 5'-GCCTTCTCCTCTAGGCTCGT-3', 16776, 5'-TATAGGGCA-GAGCTGGAGGA-3', and oIMR9074, 5'-AGGCAAATTTTGGTG-TACGG-3'; and for VillinCre^{ERT2}: 5'Cre, 5'-CGCGAACATCTT CAGGTTCT-3' and 3'Cre, 5'-CAAGCCTGGCTCGACGGCC-3'. The following cycling parameters were used for Myo5B: 95°C (3 min), 95°C (30 s), 64°C (30 s), 72°C (45 s), and 72°C (5 min) with steps 2–4 repeated for 35 cycles. For LacZ and VillinCre PCR, the annealing temperature of 62°C was used, and VillinCre^{ERT2} PCR used 58°C.

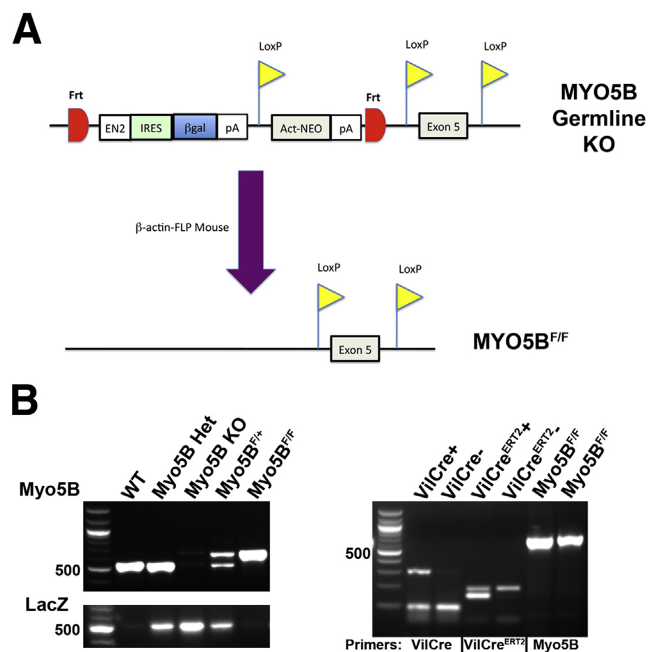

Figure 1. Generation of germline MYO5B KO and MYO5B$^{F/F}$ mice. (*A*) Schematic representation of the knockout first allele for MYO5B from the Knockout Mouse Project is shown. This construct was used to generate germline MYO5B KO mice by early termination of the Myo5B transcription before exon 5. Mice harboring this allele were mated to *β*-actin–FLP mice to also create MYO5B$^{F/F}$ mice in which LoxP sites flank exon 5. (*B*) PCR of genomic DNA shows the different PCR product patterns used to identify the genotype of a mouse. The 100-bp DNA Ladder (New England Biolabs, Ipswich, MA) is shown in the *left lane* of gels to denote PCR product size. The 500-bp marker is indicated.

For validation of Myo5B messenger RNA loss, total RNA was extracted from frozen duodenum samples using TRIzol (Invitrogen, Carlsbad, CA). Total RNA (1 *μ*g) was treated with RQ1 RNase-free DNase (Promega), and then reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR then was performed with Advantage Taq (Clontech, Mountain View, CA) using the following primers: exon 4: sense, 5'-CCTACGAGCAGCTGCCAATCTAC-3' and exon 5: antisense, 5'-GACACCGTCTTGCCTGCTCCAGACTCTC-3'. Cycling was performed at 95°C for 15 seconds and at 68°C for 20 seconds, for 42 cycles.

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