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Smoothelin-A Is Essential for Functional Intestinal Smooth Muscle Contractility in Mice

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Background & Aims: In patients with chronic intestinal pseudo-obstruction, intestinal motility is disturbed by either nervous or myogenic aberrations. The cause of the myogenic form is unknown, but it is likely to originate in the contractile apparatus of the smooth muscle cells. Smoothelins are actin-binding proteins that are expressed abundantly in visceral (smoothelin-A) and vascular (smoothelin-B) smooth muscle. Experimental data indicate a role for smoothelins in smooth muscle contraction. A smoothelin-deficient mouse model may help to establish the role of smoothelin-A in intestinal contraction and provide a model for myogenic chronic intestinal pseudo-obstruction. **Methods:** We used gene targeting to investigate the function of smoothelin-A in intestinal tissues. By deletion of exons 18, 19, and 20 from the smoothelin gene, the expression of both smoothelin isoforms was disrupted. The effects of the deficiency were evaluated by pathologic and physiologic analyses. **Results:** In smoothelin-A/B knockout mice, the intestine was fragile and less flexible compared with wild-type littermates. The circular and longitudinal muscle layers of the intestine were hypertrophic. Deficiency of smoothelin-A led to irregular slow wave patterns and impaired contraction of intestinal smooth muscle, leading to hampered transport in vivo. This caused obstructions that provoked intestinal diverticulosis and occasionally intestinal rupture. **Conclusions:** Smoothelin-A is essential for functional contractility of intestinal smooth muscle. Hampered intestinal transit in smoothelin-A/B knockout mice causes obstruction, starvation, and, ultimately, premature death. The pathology of mice lacking smoothelin-A is reminiscent of that seen in patients with chronic intestinal pseudo-obstruction.

nated contractions of the circular and longitudinal smooth muscle layers are responsible for peristalsis of the gastrointestinal tract. Contractions of SMCs are slower than those of skeletal and cardiac myocytes, but are more sustained. Hence, the composition of the contractile apparatus of SMCs differs from that of the striated muscle cells. In both cell types, actin–myosin interactions are at the basis of contraction. The contraction of striated muscle is well understood and accessory proteins, such as troponins, are known to be part of the organization of the contractile apparatus and determine the mode of contraction.^{2,3} The architecture and composition of contractile elements in SMCs, however, still is not understood fully. The contractile apparatus of SMCs is connected with the cytoskeleton via dense bodies. It consists of an actin–myosin axis complemented with structural muscle proteins, including α -actinin and tropomyosin, and more smooth muscle–specific proteins such as calponin, caldesmon, and smoothelin.^{4,5}

Based on their expression pattern, smoothelins have been described as proteins specific for fully differentiated smooth muscle. The 2 major isoforms are smoothelin-A in visceral tissues such as the digestive tract, bladder, and prostate, and smoothelin-B in vascular tissues.^{6,7} Both are found only in actively contracting smooth muscle tissues. Under pathologic conditions with impaired function of smooth muscle, such as aneurysms and restenosis, expression of smoothelins rapidly decreases.^{8,9} In cultured SMCs, smoothelins colocalize with smooth muscle α -actin (α -SMA) stress fibers.^{8,10} Recently, we

The principal function of smooth muscle cells (SMCs) in the intestinal tract is to enable propulsion and mixing of food,¹ which improves the digestion of complex molecules and the absorption of nutrients. Coordi-

Abbreviations used in this paper: α -SMA, smooth muscle α -actin; CIP, chronic intestinal pseudo-obstruction; ICC, interstitial cell of Cajal; SMC, smooth muscle cell; *Smtn*, smoothelin gene.

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showed in vitro that smoothelins can bind physically to α -SMA under normal physiologic conditions.¹¹ These findings point toward a direct role of smoothelin in contraction.

If SMCs are brought into culture, smoothelin expression is down-regulated rapidly,^{6,10} concomitant with their modulation toward a synthetic phenotype. This hampers in vitro investigations of the function of smoothelin in smooth muscle contraction. Therefore, assessment of the function of smoothelins in intestinal contractility requires an in vivo approach. Here, we report the interruption of the smoothelin gene in mice, leading to elimination of both smoothelin-A and smoothelin-B. The smoothelin knockout mice (*Smtn-A/B*^{-/-}) show dysfunction of intestinal motility and contractility and die prematurely. The observed phenotype displays pathologies reminiscent of intestinal diverticulosis, chronic intestinal pseudo-obstruction (CIP), and hollow visceral myopathy in humans.

Materials and Methods

Generation of *Smtn-A/B*^{-/-} and *Smtn-B*^{-/-} Mice

To generate *Smtn-A/B*^{-/-} mice, we replaced part of exon 18 and exon 19–20 with a neomycin resistance gene under the control of the thymidine kinase promoter in reverse orientation (Figure 1A). The targeting vector contained the thymidine kinase gene for negative selection. After electroporation of the *Pvu*II-linearized construct into mouse L129/Sv embryonic stem cells, we selected neomycin-resistant clones with G418 (Invitrogen, Carlsbad, CA) and 1-[2-deoxy]2-fluoro- β -D-arabinofuransyl (Invitrogen). DNA from resistant clones was screened by Southern blotting after *Pst*I restriction digestion, using the 3' probe indicated in Figure 1A. Embryonic stem cells from 2 independent targeted clones were injected into C57Bl/6 blastocysts and implanted into pseudopregnant C57Bl/6 females. Mating of the resulting chimeric males to C57Bl/6 females led to germline transmission of the targeted allele as detected by Southern blotting (Figure 1B). Because the mice had a mixed background (L129/Sv and C57Bl/6), we used littermates as controls. In the food transit experiment we used age- and sex-matched controls. The generation of smoothelin-B knockout mice (*Smtn-B*^{-/-}) is described elsewhere (Rensen et al, unpublished data). In these mice, exons 3–6 of the smoothelin gene are removed and smoothelin-B synthesis is absent; however, smoothelin-A synthesis is not affected. All animal studies were performed according to protocols approved by the Committee on Animal Experimentation of the University of Maastricht.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from various tissues with Tri reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands). Reverse transcription was performed using 1 μ g of RNA in the RevertAid First-Strand complementary DNA synthesis kit (Fermentas, St. Leon-Rot, Germany). Expression of smoothelin messenger RNA in *Smtn-A/B*^{-/-} mice was investigated by reverse-transcription polymerase chain reaction using smoothelin-B-specific primers 1F 5'-CCAGGGGGCAGTATGAA-GAC-3' and 1R 5'-CGCAGGTGGTTGTAGAGCGA-3' and common smoothelin primers 2F 5'-GAGGAGCGCAAGCT-GATCA-3' and 2R 5'-CTGCTGGTGCTGAGAAGGGT-3'. Reverse-transcription polymerase chain reaction products were cloned and sequenced.

Western Blot

Intestinal tissue homogenates ($n = 5$) were prepared in buffer (.25 mol/L sucrose, .01 mol/L Tris-HCl pH 7.4, 2 mmol/L ethylenediaminetetraacetic acid) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentration was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) and 15 μ g was loaded onto a 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Proteins were blotted on a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences, Roosendaal, The Netherlands) and blocked overnight in phosphate-buffered saline containing .2% Tween-20 and 5% Marvel at 4°C. α -SMA was detected using the monoclonal antibody 1A4 (DAKO, Glostrup, Denmark) and a secondary rabbit anti-mouse antibody conjugated with horseradish peroxidase (DAKO). Smooth muscle myosin heavy chain was detected with the rabbit polyclonal immunoglobulin G bt-562 (Camprio Scientific, Veenendaal, The Netherlands) followed by donkey-anti-rabbit horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Bands were visualized by enhanced chemiluminescence. Subsequently, signals were digitized and analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Histology, Immunohistochemistry, and Electron Microscopy

Organs from mice aged from 0 days to 6 months were fixed overnight in 3.7% formaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned, and stained with H&E. Sirius Red staining was performed for the detection of collagen.

Samples of intestine (and several other tissues) were snap-frozen in liquid nitrogen–precooled isopentane and embedded in OCT Tissue Tek compound (Sakura, Chicago, IL). Cryostat sections were stained with biotinylated mouse monoclonal R4A specific for smoothelin.¹² The ABC-peroxidase kit (Vector Laboratories, Inc, Burlingame, CA) was used for detection, followed by diaminobenzidine tetrahydrochloride staining and hematoxylin counterstaining. Interstitial cells of Cajal (ICCs) were identified by antibody against c-kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).¹³

Figure 1. *Smtm-A/B^{-/-}* mice have a greatly reduced lifespan. (A) Targeting strategy for the generation of *Smtm-A/B^{-/-}* mice. The top line in A shows the structure of the *Smtm* gene. ■, smoothelin-B exons; □, exons common to smoothelin-A and smoothelin-B; □, targeted exons. The middle line in A shows the *Smtm-A/B^{-/-}* targeting vector containing neomycin resistance (NEO) and herpes simplex virus thymidine kinase (TK) genes, both transcribed in the reverse direction to that of the *Smtm* gene, as indicated by arrows. The bottom line shows the structure of the targeted *Smtm* allele. The distance between the *Pst*I sites in the wild-type (top) and mutant (bottom) locus, and the location of the probe used in Southern blot analyses after *Pst*I digestion, is shown. (B) Southern blot analyses of genomic DNA from wild-type and targeted embryonic stem cells after *Pst*I digestion with the probe indicated in A. The wild-type *Pst*I fragment (WT) has a size of 4.9 kb, whereas the mutant *Pst*I fragment (T) is 3.0 kb. (C) Reverse-transcription polymerase chain reaction analysis of RNA isolated from the colon shows that *Smtm-A/B^{-/-}* mice express shorter smoothelin transcripts with exons 16 or 17 spliced to exon 21 (see Methods section for details). (D) Immunohistochemical stainings of jejunum show the loss of smoothelin expression in *Smtm-A/B^{-/-}* mice. The double-headed arrows indicate the thickness of the muscle layers (see also Figure 4). (E) α -SMA concentration is significantly lower in *Smtm-A/B^{-/-}* mice, whereas smooth muscle myosin heavy chain concentration does not differ ($n = 5$). (F) Kaplan-Meier survival curve of *Smtm-A/B^{+/+}*, *Smtm-A/B^{+/-}*, and *Smtm-A/B^{-/-}* mice showing that 50% of *Smtm-A/B^{-/-}* mice die within 3 weeks after birth ($n = 263$).

Full-thickness biopsy specimens from ileum were obtained from a patient with CIP. Age-matched control specimens were obtained from patients with diseases unrelated to gastrointestinal motility disorders. The use of human tissues was approved by the Medical Institutional Ethics Committee of the Faculty of Medicine, University of Luebeck, Germany. Tissues were processed as described by Vanderwinden et al.¹⁴

For electron microscopy, tissues were fixed in 3% phosphate-buffered glutaraldehyde for 24 hours and postfixed for 1 hour in 1% osmium tetroxide. Tissues then were dehydrated through a graded ethanol series and routinely embedded in Epon (SPI Supplies, West Chester, PA). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a Philips CM100 electron microscope (Philips, Eindhoven, The Netherlands).

Whole-Gut Transit Time Test

To determine the intestinal function, a whole-gut transit time test was used as previously described.¹⁵ We injected 100 μ L of 6% carmine (Sigma-Aldrich C1022) in phosphate-buffered saline (pH 7.0) into the stomachs of 6 *Smtm-A/B^{+/+}*, *Smtm-A/B^{+/-}*, and *Smtm-A/B^{-/-}*, and 5 *Smtm-B^{+/+}* and *Smtm-B^{-/-}* mice, and monitored their feces for the first appearance of red dye.

Contrast Radiography

After sedation with .15 μ L Nembutal (Ceva Sante Animale BV, Maassluis, The Netherlands) by intraperitoneal injection, .3 mL of barium sulfate suspension (polibar 1 g/mL, E-Z-EM Inc, Lake Success, NY; diluted 1:4 with water) was

injected into the stomachs of 2 *Smtn-A/B*^{-/-} and 2 *Smtn-A/B*^{+/+} mice (12 weeks old). Progression through the gastrointestinal tract was followed by continuous radiographic examination (60 kV, 1.6 mA). Digital radiographic images were taken every 10 minutes with a Philips Diagnost 1997 device (Philips Medical Systems, Best, The Netherlands).

Intestinal Contractility

Intestinal contractility was studied on 4–6-week-old mice ($n = 6$ for each group). Before anesthetizing mice with diethyl ether, we fasted them for 24 hours with free access to water. The small intestine of *Smtn-A/B*^{-/-} and *Smtn-A/B*^{+/+} littermates (and of *Smtn-B*^{-/-} and *Smtn-B*^{+/+} littermates) was removed and put in ice-cold aerated Krebs–Ringer solution. A 10-cm segment of the jejunum was opened along the longitudinal axis and the mucosa was removed. Longitudinal muscle strips of 6.0 mm of the jejunum were mounted in organ baths (5 mL) filled with Krebs–Ringer solution, maintained at 37°C, and aerated with a mixture of 5% CO₂ and 95% O₂. The muscle strips were positioned between 2 platinum ring electrodes (distance, 10 mm; diameter of rings, 3 mm) that were mounted on a Plexiglas (or Perspex) rod. The lower end of the muscle strip was fixed and the other end of the muscle strip was connected to a strain gauge transducer (Scaime, Annemasse, France) for continuous recording of isometric tension. After an equilibration period of 30 minutes during which the strips were washed every 5 minutes, the muscle strips were contracted with .1 μ mol/L carbachol. After washout of carbachol, the strips were stretched (increments of 5 mN). After stabilization of the basal tone, muscle strips again were contracted with .1 μ mol/L carbachol. This procedure was repeated until the contraction to .1 μ mol/L carbachol was maximal. This point was taken as the point of the optimal length–tension relation.¹⁶ Muscle strips then were allowed to equilibrate for 60 minutes before starting the experiment. During the equilibration period, the muscle strips were washed every 15 minutes with Krebs–Ringer solution. The contractile effect of electrical field stimulation (.5–8 Hz, 40 V, pulse width, 1 ms; pulse train, 10 s), enteric nerves, carbachol (1 nmol/L to 1 μ mol/L), prostaglandin F₂ α (1 nmol/L to 10 μ mol/L), substance-P (.1–100 nmol/L), serotonin (1 nmol/L to 10 μ mol/L), and KCl (50 mmol/L) was investigated. To block inhibitory responses to nitric oxide, contractions were studied in the presence of L-nitroarginine, a blocker of nitric oxide synthase. An adjacent jejunal segment was fixed in 4% formaldehyde for histologic examination and determination of the cross-sectional area. Contractions were normalized to the cross-sectional area of the longitudinal jejunal muscle layer.

Intracellular Recordings

A standard microelectrode technique was used to record slow waves from the SMCs of the small intestine. Pieces of duodenum (2 cm beyond the pyloric sphincter) from the same mice as used for the intestinal contractility studies were

used. The segments were opened along the mesenteric border and the mucosa and submucosa were removed. A muscle strip (15 $\times$ 6 mm) then was pinned, serosal side down, to the Sylgard floor (Dow Corning Europe, La Hulper, Belgium) of a recording chamber that was placed on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan). The tissue was superfused continuously (10 mL/min; temperature, 36.5°C–37°C) with oxygenated Krebs–Ringer solution.

Throughout the experiment, the L-type Ca²⁺ blocker nifedipine (1 μ mol/L) (Sigma Chemical, St. Louis, MO) was present in the superfusion solution to reduce contraction of the intestinal smooth muscle.^{17,18} Intracellular recordings of the SMCs were made with borosilicate glass microelectrodes (1-mm outer diameter; Clark Electromedical Instruments, Reading, UK) pulled on a P-97 Brown–Flaming micropipette puller (Sutter Instrument Co., Novato, CA). The electrodes were back-filled with 1 mol/L KCl (resistance, 50–70 M Ω). The electrode was positioned by a micromanipulator (Narishige MO388; Narishige Scientific Instrument Lab, Tokyo, Japan). Passive electrical events were measured with an Axoclamp 2A current-voltage amplifier (headstage HS-2 L, gain 0.1) connected to a Labmaster TL-1 DMA interface (Axon Instruments, Foster City, CA). The amplifier bridge circuit was balanced for each electrode before impalement, and capacitance was compensated for during injection of rectangular electrical current pulses (–.2 nA, 7 ms) through the microelectrode. After amplification and low-pass filtering (3 kHz), the signal was digitized at a sample rate of 5 kHz and stored on a computer using the pClamp 6.0.2 software (Axon Instruments).

Morphometric Analyses

To determine the cross-sectional area of the longitudinal and circular muscle layer of the jejunum, cross-sections were stained with H&E. Images were captured using a Zeiss Axioscope (Zeiss, Göttingen, Germany) and a standard CCD camera (Stemmer Imaging, Puchheim, Germany), and analyzed with Leica QWin image analysis software (Leica Microsystems, Cambridge, UK). To determine the number of nuclei of the longitudinal and circular muscle layers of these sections, 2 opposite angular segments (15°) of the intestinal wall were selected and counted manually.

Statistical Analyses

Statistical significance was calculated by repeated-measures 1-way ANOVA followed by Bonferroni's multiple comparison test, or 2-tailed (paired) Student *t* tests using Graphpad Prism software (version 4.0; GraphPad Software, Inc, San Diego, CA). Groups were considered significantly different when the *P* value was less than .05. Values are expressed as mean $\pm$ standard error of the mean.

Results

Mouse Model

Smoothelin-A/B^{-/-} mice were generated by removal of exons 18–20 (Figure 1A and B). These exons

Figure 2. Whole-gut transit time of *Smtm-A/B^{+/+}*, *Smtm-A/B^{+/-}*, *Smtm-A/B^{-/-}*, *Smtm-B^{+/+}*, and *Smtm-B^{-/-}* mice. (A) Whole-gut transit time of *Smtm-A/B^{-/-}* mice is doubled compared with *Smtm-A/B^{+/+}*, *Smtm-A/B^{+/-}*, *Smtm-B^{+/+}*, and *Smtm-A/B^{-/-}* mice. ($P < .05$ in repeated-measures ANOVA followed by Bonferroni's multiple comparison tests.) (B) Representative picture of contrast radiography of *Smtm-A/B^{+/+}* (left) and *Smtm-A/B^{-/-}* (right). Picture was taken 20 minutes after injecting the barium sulfate solution into the stomach. S, stomachs; d, duodenum. *Smtm-A/B^{-/-}* mice show a greatly dilated proximal duodenum and a slower progression of food transport into the jejunum.

code for the calponin homology domain that is involved in actin binding.¹¹ The deletion resulted in smaller smoothelin transcripts with an intact reading frame, corresponding to variants with exons 16 or 17 spliced to exon 21 (Figure 1C). However, smoothelin protein could not be detected in smooth muscle tissues of *Smtm-A/B^{-/-}* mice using an antibody against an epitope upstream of the deletion (Figure 1D), indicating that the targeting resulted in a null-mutation. Western blot analyses showed that α -SMA was down-regulated in intestines of *Smtm-A/B^{-/-}* mice and smooth muscle myosin heavy chain concentration was similar (Figure 1E).

Smtm-A/B^{-/-} pups were born at the expected Mendelian ratio (*Smtm-A/B^{+/+}* 30%; *Smtm-A/B^{+/-}* 46%; *Smtm-A/B^{-/-}* 24%, $n = 415$). At birth, *Smtm-A/B^{-/-}* mice had a size and weight comparable with their wild-type littermates, but their postnatal growth was retarded overtly and surviving animals reached only approximately 80% of normal body weight. In the *Smtm-A/B^{-/-}* mice, a remarkable absence of visceral and subcutaneous fat was observed. About 50% of *Smtm-A/B^{-/-}* mice died before weaning at 3 weeks of age (Figure 1F). *Smtm-A/B^{-/-}* mice that developed into adulthood were infertile. In contrast, *Smtm-A/B^{+/-}* (and also *Smtm-B^{-/-}* mice) had no overt abnormalities, were fertile, and had a normal life span.

Whole-Gut Transit Time, Contractility, and Slow Wave Recordings

Motility of the intestine was determined by functional assays. Carmine, a red dye, was injected into the

stomach and stool was monitored. Despite a comparable length of the intestinal tract, the whole-gut transit time of *Smtm-A/B^{-/-}* mice was about twice as long as that of wild-type littermates (Figure 2A). *Smtm-B^{-/-}* mice showed no increase in whole-gut transit time (Figure 2A). Contrast radiography after loading barium sulfate into the stomach of *Smtm-A/B^{-/-}* mice confirmed the slower food transport in *Smtm-A/B^{-/-}* mice and showed a greatly dilated proximal duodenum (Figure 2B), which was confirmed by macroscopic and microscopic observations. Twenty minutes after injection of barium sulfate, hardly any barium had passed the duodenum in *Smtm-A/B^{-/-}* mice, whereas in *Smtm-A/B^{+/+}* mice barium had proceeded several centimeters into the jejunum.

Isolated jejunal smooth muscle strips showed spontaneous spike activity in wild-type *Smtm-A/B^{-/-}* and *Smtm-B^{-/-}* mice. However, the amplitude and frequency of these spontaneous spikes were significantly lower in muscle strips of *Smtm-A/B^{-/-}* mice (amplitude: *Smtm-A/B^{+/+}* 8.57 ± 1.23 , *Smtm-A/B^{-/-}* $2.44 \pm .39$; frequency: *Smtm-A/B^{+/+}* 43.09 ± 1.04 , *Smtm-A/B^{-/-}* 31.30 ± 1.70). When subjected to several contractile agonists, the forces generated by these strips were in *Smtm-A/B^{-/-}* mice at least 4 times smaller than in control mice (Figure 3A). Electrical stimulation of excitatory enteric nerves showed at least a 5-fold stronger contraction in wild-type mice compared with *Smtm-A/B^{-/-}* mice (Figure 3A). Also, receptor-independent contractions to KCl and receptor-dependent contractions to carbachol, prostaglandin F₂ α , substance P, and serotonin were significantly lower for

Figure 3. Intestinal smooth muscle of *Smtm-A/B*^{-/-} mice shows reduced contractility and irregular slow wave patterns. (A) Maximal contractile responses to KCl (K⁺), prostaglandin F₂α (PGF₂α), serotonin (5-HT), carbachol (Carb), substance P (SubP), and electrical field stimulation are reduced strongly in jejunal longitudinal muscle strips from *Smtm-A/B*^{-/-} mice. □, *Smtm-A/B*^{+/+}; ■, *Smtm-A/B*^{-/-}. (B) Representative tracing of a slow wave measured in a small intestinal smooth muscle cell of an *Smtm-A/B*^{+/+} mouse. (C, D) Two examples of slow waves measured in small intestinal smooth muscle cells of *Smtm-A/B*^{-/-} mice. The slow waves show lower and irregular amplitudes and a remarkable variability in frequency.

Smtm-A/B^{-/-} mice (Figure 3A). In the same experimental set-up, contractility of jejunal strips of *Smtm-B*^{-/-} mice was comparable with *Smtm-A/B*^{+/+} mice.

The slow waves measured in the SMCs of *Smtm-A/B*^{-/-} mice were very irregular and variable, whereas in *Smtm-A/B*^{+/+} littermates, regular slow waves were observed in all cells tested (Figure 3B–D). The amplitude of the slow waves in *Smtm-A/B*^{-/-} mice was significantly smaller (10 ± 1 mV) compared with *Smtm-A/B*^{+/+} mice (24 ± 1 mV), and the frequency of the slow waves was diminished significantly in *Smtm-A/B*^{-/-} vs *Smtm-A/B*^{+/+} mice (33.7 ± 2.0 cycles/min vs 39.8 ± .6 cycles/min). In addition, there was a much higher variability in frequency in *Smtm-A/B*^{-/-} (0–53 cycles/min) compared with *Smtm-A/B*^{+/+} littermates (35–46 cycles/min). In the same experimental set-up, the slow waves measured in the SMCs of *Smtm-B*^{-/-} mice did not differ from wild-type mice.

Pathology of the Intestine

To ascertain whether the intestinal contractile dysfunction of *Smtm-A/B*^{-/-} mice led to development of

intestinal pathologies, we examined the morphology and histology of the digestive tract. No changes in the structure of the stomach were observed, although the structure of the intestine was compromised in *Smtm-A/B*^{-/-} mice. The intestinal wall of *Smtm-A/B*^{-/-} mice was stiff and fragile. The duodenum often was dilated (see also Figure 2B) and the mass of the jejunal smooth muscle was increased significantly compared with wild-type littermates (Table 1). The cross-sectional area of both longitudinal and circular muscle layers was approximately 4-fold greater in *Smtm-A/B*^{-/-} mice than in wild-type mice (Figure 4A and B), which could be attributed largely to SMC hypertrophy (Table 1). Nuclei and cytoplasm of the intestinal SMCs were larger in smoothelin-deficient mice (Figures 4C, D, and 5). Extracellular matrix volume did not increase significantly as deduced from Sirius red staining and the distribution and appearance of the ICCs were normal (data not shown). Electron microscopy showed subtle differences in the ultrastructural organization of the intestinal SMCs. In *Smtm-A/B*^{-/-} mice, the distribution of mitochondria and dense bodies appeared to be organized less strictly (Figure 5A and B). In addition, the micropinocytotic vesicles that are characteristic of smooth muscle were common in *Smtm-A/B*^{+/+} mice but almost absent in *Smtm-A/B*^{-/-} mice (Figure 5C and D). Hypertrophy was less common and less extensive in the more distal part of the gut. Numerous diverticula were observed in all parts of the intestine with the exception of the cecum (Figure 4E and F). The diverticula were located predominantly on the mesenteric side. In 50% of the *Smtm-A/B*^{-/-} mice, the diverticula already were present 24 hours after birth. The diverticula increased in number and size as the mice aged (Figure 4F). Inflammation of the diverticula and the mesentery was common, and perforations leading to sepsis and death were observed (Figure 4G). In addition, the intestinal villi in the affected areas were irregular and atrophic, which is probably a consequence of the dis-

Table 1. *Smtm-A/B*^{-/-} Mice Show Jejunal Smooth Muscle Hypertrophy

	<i>Smtm-A/B</i> ^{+/+} (n = 5)	<i>Smtm-A/B</i> ^{-/-} (n = 4)	P value
Intestinal CSA ^a	4.533 ± 1.246	7.582 ± 1.780	<.05
Intestinal muscle CSA ^a	.376 ± .130	1.684 ± .381	<.001
Muscle area/intestinal area	9% ± 3%	23% ± 3%	<.001
Nuclei longitudinal muscle ^b	71 ± 12	81 ± 20	NS
Nuclei circular muscle ^b	51 ± 13	79 ± 15	<.05

CSA, cross-sectional area; NS, not significant.

^aValues shown in mm².

^bNumber of nuclei per 10% of the intestinal circumference.

Figure 4. *Smtm-A/B^{-/-}* mice develop lethal pathologies in the digestive tract. Cross-sections of jejunum of 8-week-old (A) *Smtm-A/B^{+/+}* and (B) *Smtm-A/B^{-/-}* littermates showing irregular villi and profound hypertrophy of both circular and longitudinal smooth muscle layers in the *Smtm-A/B^{-/-}* mouse. H&E staining of jejunum of (C) 8-week-old *Smtm-A/B^{+/+}* and (D) *Smtm-A/B^{-/-}* littermates showing hypertrophy of SMCs in the *Smtm-A/B^{-/-}* mouse. (E) A small diverticulum (dv) of the jejunum of a 10-day-old *Smtm-A/B^{-/-}* mouse still surrounded by smooth muscle. (F) Cross-section of a colon of a 21-week-old *Smtm-A/B^{-/-}* mouse showing typical severe diverticulosis. Little or no tunica muscularis is present on the side where the diverticula are observed, whereas the opposing side shows smooth muscle hypertrophy. (G) Perforation (indicated by the arrow) of a colonic diverticulum of a 10-day-old *Smtm-A/B^{-/-}* mouse. Leakage of intestinal material has caused the development of a large abscess in the abdominal cavity.

turbed motility and may be due to inflammation of the affected areas. The absence of fat deposition in *Smtm-A/B^{-/-}* mice may be the consequence of diminished absorption as a result of the deteriorated structure of the villi. Inspection of the intestine of *Smtm-B^{-/-}* mice did

not show any abnormalities. Also, these mice had normal fat disposition, indicating sufficient food adsorption from the intestine. Furthermore, there are no morphologic changes in the intestinal vasculature of both *Smtm-A/B^{-/-}* and *Smtm-B^{-/-}* mice at this age.

Smoothelin Expression in Intestine of CIP Patients

Because the phenotype of the *Smtm-A/B^{-/-}* mice shares many aspects of the pathology seen in patients with CIP, we investigated the expression of smoothelin in intestines of those patients. In controls, smoothelin immunoreactivity stained all muscle layers uniformly, whereas, despite a normal morphology, lack of smoothelin was observed in the ileal circular muscle layer of a patient with CIP (Figure 6).

Discussion

Digestive motility is a highly coordinated process that enables mixing, propulsion, and absorption of food by the digestive tract. Disturbances in intestinal motility can occur at different levels as a result of aberrations in the enteric nervous system, ICCs, and/or the contractility of smooth muscle cells. They all lead to impaired gastrointestinal propulsion. When no obvious lesions are

Figure 5. Differences in size and ultrastructure of intestinal SMCs in *Smtm-A/B^{-/-}* vs *Smtm-A/B^{+/+}* mice. Electron micrograph images of (A, C) *Smtm-A/B^{+/+}* and (B, D) *Smtm-A/B^{-/-}* mice show differences in cell size, thickness of the intercellular basal lamina, and the distribution of the mitochondria (A, B; 2200X). At larger magnification (C, D; 21,000X) micropinocytotic vesicles (arrow) are apparent in *Smtm-A/B^{+/+}* SMCs.

Figure 6. Absence of smoothelin in parts of the intestinal muscle layer of CIP patients. (A) Immunohistochemical staining of ileum obtained from a patient with CIP shows the absence of smoothelin in the circular smooth muscle layer (CM), except for the innermost border as indicated by arrows. (B) H&E staining of an adjacent section from the same specimen showing normal morphology. (C) Immunohistochemical staining of control ileum shows a homogeneous distribution of smoothelin in both circular (CM) and longitudinal (LM) smooth muscle layers.

obstructing intestinal transit, impaired food progress often is referred to as *chronic intestinal pseudo-obstruction*.^{19,20} Proper diagnosis and treatment of this rare and disabling disorder remain difficult, largely because the underlying pathology is understood poorly. Most attention has been paid to nervous system function in CIP, although smooth muscle myopathies appear to be responsible for the disease in approximately one third of the patients with congenital CIP, and in all cases of hollow visceral myopathy.^{21,22} For the neuronal-based CIP, a few relevant mouse models recently have become available.²³ The myopathy-related CIP has obtained less attention and, to the best of our knowledge, no animal model is available. We describe a mouse model that displays clinical manifestations similar to CIP/hollow visceral myopathy, caused by a mutation in the smooth-

elin gene. Moreover, we report on the absence of smoothelin in apparently unaffected intestine in CIP patients, providing evidence that smooth muscle dysfunction as a result of smoothelin deficiency might contribute to the development of this disease in humans.

Smoothelin-A is a marker protein for visceral SMCs.^{6,10} Analysis of intestinal muscle strips showed that inactivation of the smoothelin gene results in a decrease in contractile potential of intestinal SMCs. *Smtn-A/B*^{-/-}-derived muscle strips displayed a 4 times lower contractile force per unit muscle mass as control mice, independent of the used agonists and concentration. In contrast, contractility of intestinal smooth muscle strips of *Smtn-B*^{-/-} mice was similar to wild-type mice (data not shown). These results show that smoothelin-A is the functional smoothelin isoform in intestinal tissues and that this protein is a key component in the contractile machinery of intestinal smooth muscle. The exact role of smoothelin-A in smooth muscle contraction remains to be elucidated. However, the reduced response to nonreceptor-mediated and receptor-mediated excitation indicates that smoothelin-A must be associated closely to the primary components of the contractile apparatus. In addition, the recently shown direct physical interaction of smoothelin with α -SMA¹¹ and the observed down-regulation of α -SMA in intestines of *Smtn-A/B*^{-/-} mice indicate a cooperation of these 2 proteins.¹¹

The observed disturbance of the slow waves in SMCs of *Smtn-A/B*^{-/-} mice is intriguing and correlates well with the disturbed spontaneous activity of smooth muscle strips. Slow waves are generated by ICCs. ICCs do not contain smoothelin and both the number and distribution of these cells is normal in *Smtn-A/B*^{-/-} mice. Therefore, the loss of regularity must be caused by changes in the interaction between ICCs and SMCs or in the characteristics of SMCs (as found by electron microscopy), which interfere with proper communication between these cells. Alternatively, changes in the spatial structure of the smooth muscle layers may break-up the ICC network. SMCs then will receive noncoordinated signals from several ICCs. The latter is in line with the analysis of the *Smtn-A/B*^{-/-} slow waves, which can be considered to be the result of 2 or more independent slow waves (according to Fourier analysis, data not shown). Regardless, the lack of smoothelin results in structural changes in the smooth muscle that interfere with the slow wave generation, which in turn diminishes the regulation of peristalsis.

The lack of smoothelin-A and consequent reduced contractility in *Smtn-A/B*^{-/-} mice has significant consequences for the intestinal performance. The retarded passage of the carmine bolus showed that whole-gut

transit time was approximately doubled. This was confirmed by contrast radiography that showed the transit in the intestine of *Smtn-A/B*^{-/-} mice to be much slower compared with *Smtn-A/B*^{+/+} littermates. Furthermore, the radiographs showed that the duodenum of *Smtn-A/B*^{-/-} mice is dilated, which may be the result of chronic pseudo-obstruction in the small intestine. This deduction is supported by the hypertrophy of the smooth muscle in the intestinal wall, the deterioration of the intestinal mucosa and villi, and the numerous diverticula found in the intestine of *Smtn-A/B*^{-/-} mice. The 4-fold increased muscular cross-sectional area in *Smtn-A/B*^{-/-} mice is likely to be an adaptive response to increased workload (as the result of the diminished contractility) of this pulsatively contracting muscle. Similar hypertrophy has been described for segments of the gut upstream of an obstruction²⁴ and also has been documented extensively for other muscle types under increased workload such as the cardiac muscle.^{25–27} The diverticula, which already are found in very young mice and increase in number and size with age, point toward an increasing transverse pressure on the wall caused by the retarded food transport. The increased wall thickness decreases the flexibility of the intestinal wall. As a result, the pressure provokes herniation of mucosa and submucosa through the intestinal muscular wall at the mesenteric side, which is weaker because of the entering of mesenteric blood vessels and nerves.²⁸ Thus, diverticulosis in *Smtn-A/B*^{-/-} mice most likely results from a loss of intestinal SMC contractile performance in combination with increased wall rigidity. *Smtn-A/B*^{-/-} mice with sick and apathetic behavior were shown to have extensive diverticulosis. Human intestinal diverticulosis is a rather common disease, affecting 30%–50% of people over the age of 60 and causing strong and recurrent abdominal pain.²⁹ The premature death of *Smtn-A/B*^{-/-} mice is most likely due to intestinal perforation or starvation caused by intestinal dysfunction and diverticulosis.

The present study focused on intestinal problems as a consequence of interruption of the smoothelin gene because the intestine always was affected. Investigations of other parts of the gastrointestinal tract (esophagus, stomach) did not show aberrations. However, reduced function was found for other organs such as the urogenital system. In some animals, these pathologies occurred simultaneously, in other animals 1 or 2 of the organ systems were affected. We have no explanation for the variety in onset and type of pathology or for the fact that in the group of *Smtn-A/B*^{-/-} mice with intestinal malfunction some mice died on the first day after birth whereas others lived for months. However, similar variations are found in CIP and hollow visceral myopathy.

Back-crossing on other mouse strains had no effect on this variation, which makes genomic diversity as the cause of the observed differences less likely. The physiologic part of this study was performed with mice that survived for more than 5 weeks. As can be deduced from Figure 1F, this was a small group and therefore the results were obtained from mice that had a late onset of intestinal dysfunction.

In conclusion, we have shown that smoothelin-A deficiency causes a major decrease in the contractile performance of intestinal smooth muscle. By showing that the reduction in contractility occurs independently of the signal transduction pathway activated, we have identified smoothelin-A as a structural protein that is crucial for normal intestinal smooth muscle contractility. Mutations in the *Smtn* gene or alterations in smoothelin-A levels may play an important role in the development of smooth muscle myopathies such as CIP or hollow visceral myopathy and intestinal diverticulosis.

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