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Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX–rich basement membrane zone

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Collagen XIX is an extremely rare extracellular matrix component that localizes to basement membrane zones and is transiently expressed by differentiating muscle cells. Characterization of mice harboring null and structural mutations of the collagen XIX (*Col19a1*) gene has revealed the critical contribution of this matrix protein to muscle physiology and differentiation. The phenotype includes smooth muscle motor dysfunction and hypertensive sphincter resulting from impaired swallowing-induced, nitric oxide–dependent relaxation of the sphincteric muscle. Muscle dysfunction was correlated with a disorganized matrix and a normal complement of enteric neurons and interstitial cells of Cajal. Mice without collagen XIX exhibit an additional defect, namely impaired smooth-to-skeletal muscle cell conversion in the abdominal segment of the esophagus. This developmental abnormality was accounted for by failed activation of myogenic regulatory factors that normally drive esophageal muscle transdifferentiation. Therefore, these findings identify collagen XIX as the first structural determinant of sphincteric muscle function, and as the first extrinsic factor of skeletal myogenesis in the murine esophagus.

Introduction

The ECM consists of a highly heterogeneous mixture of interacting proteins that form a complex array of supramolecular structures, and that bind cell surface receptors and soluble signaling molecules (Ramirez and Rifkin, 2003). The collagens represent the largest family of structural ECM components with 27 designated trimers that are broadly divided into fibrillar and nonfibrillar collagen types (Myllyharju and Kivirikko, 2004). The latter group includes collagens that are components of or associated with basement membranes (BMs), highly specialized macroaggregates that subserve the dual function of organizing multicellular structures and of instructing tissue differentiation, maintenance, and remodeling (Timpl, 1996; Ortega and Werb, 2002). Naturally occurring human mutations and genetically engineered mice have implied specialized roles of this particular class of nonfibrillar collagens at distinct anatomical locations. Illustrative examples of these tissue-restricted pathologies include glomerulonephrites (minor collagens IV), skeletal myopathies (collagens VI and XV), skin blistering (collagens VII and XVII), and vitreoretinal degeneration (collagen XVIII) (Christiano et al., 1993; Zhou et al., 1993; Mochizuki et al., 1994; McGrath et al., 1995; Jobsis et al., 1996; Eklund et al., 2001; Vanegas et al., 2001; Fukai et al., 2002; Marneros et al., 2004).

Collagen XIX is the latest example of a nonfibrillar collagen type that localizes to BM zones and that may potentially have an anatomically restricted function. Collagen XIX is deposited at extremely low amounts ($\sim 10^{-6}$% of dry tissue

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Abbreviations used in this paper: BM, basement membrane; EFS, electrical field stimulation; ES, embryonic stem; ICC-IM, intramuscular interstitial cells of Cajal; LES, lower esophageal sphincter; MRF, myogenic regulatory factor; NANC, nonadrenergic noncholinergic; NC, noncollagenous; NO, nitric oxide; NOS, nitric oxide synthase; SMC, smooth muscle cell.

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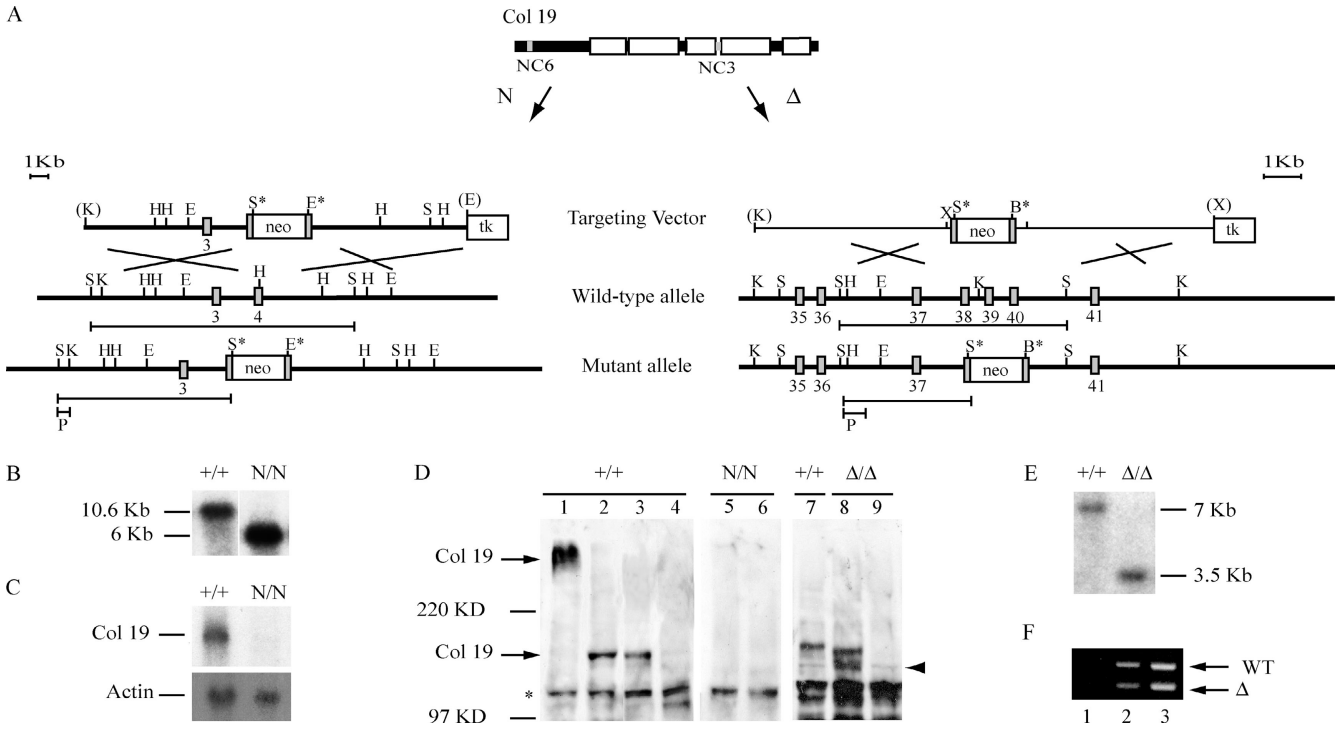

Figure 1. ***Col19a1* gene targeting.** (A) Schematic representation of the α1(XIX) collagen chain where the gray bars correspond to exon 4 and exons 38–40 sequences in the NC6 and NC3 domains, respectively. Targeting strategies are shown on the left for the N19 allele (N) and on the right for the Δ19 allele (Δ). (B) Southern hybridization of SacI-digested wild-type (+/+) and mutant (N/N) DNA to the upstream probe (P). (C) Northern hybridizations of wild-type (+/+) and mutant (N/N) RNA to *Col19a1* and *actin* probes. (D) Collagen XIX immunoblot of wild-type (+/+) and mutant tissues from nullizygous (N/N) mice or mice producing internally deleted α1(XIX) chains (Δ/Δ). Samples were electrophoresed under reducing (2 and 6) and nonreducing (1 and 5) conditions; collagenase- and mock-treated samples are in lanes 4 and 3, respectively. Migration of protein markers are shown on the left; asterisk indicates an unspecific collagenase-resistant product, whereas the arrowhead points to a probable proteolytic product of collagen XIX. Parallel Coomassie blue staining documented equal protein loading in each lane (not depicted). (E) Southern hybridization of SacI-digested wild-type (+/+) and mutant (Δ/Δ) DNA to the downstream probe (P). (F) RT-PCR amplification of collagen XIX transcripts from heterozygous Δ19 (Δ) mice for 25, 30, and 35 cycles (lanes 1, 2, and 3, respectively).

weight) in the BM zones of vascular, neural, and mesenchymal tissues (Myers et al., 1997). Collagen XIX forms higher order aggregates that may conceivably modulate cell–matrix interactions, cell–cell communications, and/or local concentrations of signaling molecules (Myers et al., 2003). Embryonic expression of the collagen XIX (*Col19a1*) gene is transient and confined almost exclusively to differentiating muscles (Sumiyoshi et al., 2001). Onset of *Col19a1* expression in myotomes and myotome derivatives occurs soon after activation of the myogenic regulatory factor (MRF) gene *Myf5,* and declines concomitantly to the accumulation of *myogenin* transcripts (Sumiyoshi et al., 2001).

The above findings are consistent with the notion that collagen XIX may be involved in muscle differentiation and function (Sumiyoshi et al., 2001). Unfortunately, experimental validation of this postulate has been hampered by the paucity of collagen XIX in tissues. Therefore, the present work was undertaken to overcome this problem using genetic means. Toward this end, we created a null mutation (N19) and a structural mutation (Δ19) of the α1(XIX) collagen chain by targeting different regions of the *Col19a1* gene in mouse embryonic stem (ES) cells. Characterization of the resulting mouse phenotypes demonstrated that collagen XIX plays a dual role in muscle physiology and differentiation. Specifically, we found that proper assembly of the collagen XIX–rich BM zone is a prerequisite for nitric oxide (NO)–dependent relaxation of the lower esophageal sphincter (LES) muscle, and that collagen XIX deposition into the matrix of the developing esophagus is an extrinsic determinant of skeletal myogenesis in this organ.

Results

Generation of collagen XIX mutant mouse strains

The mouse α1(XIX) chain is 1,136 residues long and consists of a discontinuous collagenous region flanked by a cysteine-rich noncollagenous (NC) amino terminal (NC6) and a 19-residue carboxyl peptide (NC1) (Fig. 1 A; Sumiyoshi et al., 1997). Recent ultrastructural analyses have shown that the NC interruptions impart flexibility to the otherwise rigid triple helical (collagenous) domain; they have also documented that interactions amongst globular NC6 domains are responsible for the formation of collagen XIX oligomers (Myers et al., 2003). Therefore, two different mutations were engineered in the mouse in order to compare and contrast the phenotypic consequences of assembling BM zones devoid of collagen XIX or containing structurally abnormal collagen XIX trimers.

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The null mutation (N19) was generated by inserting the PGK-*neo* cassette in place of exon 4 (Fig. 1 A, left). Exon 4 codes for 32 internal amino acids of the 268-residue NC6 peptide and includes split codons for the first and last residues (Sumiyoshi et al., 1997). After homologous recombination in ES cells, chimeric animals from two correctly targeted ES clones were generated and germ line transmission of the mutant allele was followed by Southern blot analysis (Fig. 1 B). Northern hybridizations failed to detect *Col19a1* transcripts in homozygous mutant tissues (Fig. 1 C). Moreover, sequencing of RT-PCR–amplified products across and downstream of the targeted genomic region excluded the existence of shorter, in-frame *Col19a1* transcripts (unpublished data). Immunoblots of partially purified collagen preparations from homozygous mutant and wild-type tissues corroborated the mRNA data by documenting absence of the expected 165-kD collagenase-sensitive product in the former compared with the latter specimen (Fig. 1 D). Unfortunately, the same antibodies proved unsuitable to confirm loss of collagen XIX in tissues. This last point notwithstanding, we concluded that the N19 allele does indeed represent a null mutation.

The structural mutation (Δ19) was created by inserting the PGK-*neo* cassette in place of exons 38–40 (Fig. 1 A, right). Exons 38–40 code for the 20-residue NC3 interruption of the helical domain and for one and six collagenous tripeptides located amino- and carboxyl-terminal of it, respectively (Sumiyoshi et al., 1997). Chimeric animals were generated from two independently derived clones and the progeny was genotyped by Southern blot analysis using a diagnostic restriction enzyme cleavage site (Fig. 1 E). The deletion of exons 39–40 maintains the frame of the *Col19a1* transcript, and thus it is predicted to yield an internally deleted α1(XIX) chain that should participate in homotrimer formation. Sequencing of RT-PCR–amplified products confirmed that the mutant transcript is in frame (unpublished data), whereas immunoblots identified a collagenase-sensitive product in the mutant tissue slightly smaller than the wild-type 165-kD species (Fig. 1 D). Finally, PCR amplification estimated that the mutant and wild-type *Col19a1* alleles are expressed at comparable levels in the heterozygous Δ19 mouse (Fig. 1 F). Therefore, the Δ19 allele represents a structural mutation that eliminates one of the flexible points in the triple helix. Characterization of the two collagen XIX mutations initially focused on the more severe phenotype of the nullizygous mouse.

Collagen XIX null mice display altered esophageal morphology

Heterozygous N19 mice were born at the expected Mendelian frequency; they were morphologically normal, viable, and fertile. Homozygous N19 mice were born at the expected frequency as well, but the vast majority of them (~95%) died within the first 3 wk of postnatal life, showing signs of malnourishment. Postmortem inspection of newborn homozygous mutants did not detect gross anatomical abnormalities, except for the smaller size of the internal organs. On the other hand, necroscopy of the few *Col19a1*$^{-/-}$ mice that survived past weaning stage revealed a dilated

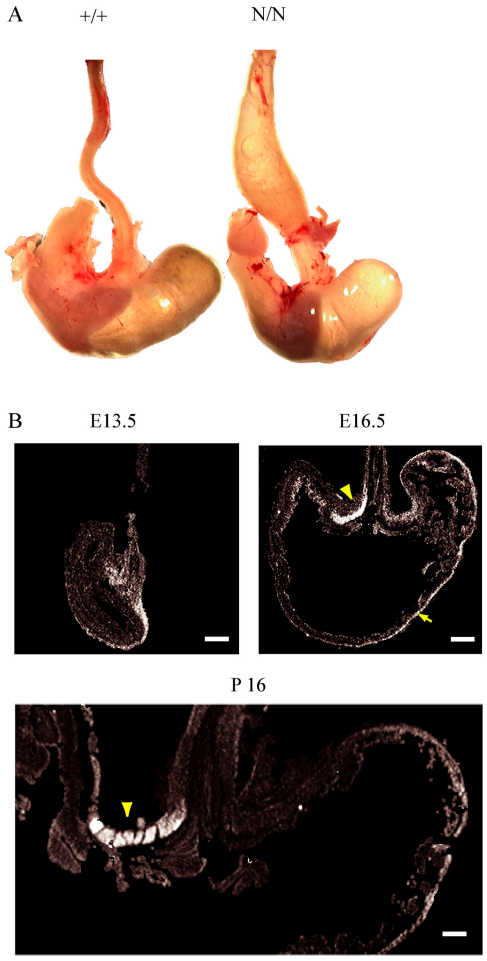

Figure 2. **Analysis of gastroesophageal region.** (A) Representative megaesophagus of a 3-mo-old *Col19a1* null mouse (N/N); note the esophageal enlargement that begins immediately above the diaphragm. (B) In situ hybridizations of the gastroesophageal region from E13.5, E16.5, and P16 wild-type mice to the antisense *Col19a1* probe. Sense probe yielded no signal above background (not depicted). Arrowhead and arrow indicate the internal LES and the proximal stomach, respectively. Bars, 0.5 mm.

esophagus (megaesophagus) with retention of ingesta, immediately above the diaphragm level (Fig. 2 A). Based on these observations, we reevaluated the pattern of *Col19a1* expression in the embryonic digestive system, and found it to coincide with the formation and growth of the gastroesophageal junction. Specifically, in situ hybridizations revealed high *Col19a1* expression in the lower-third portion of the embryonic esophagus destined to become the abdominal segment; thereafter *Col19a1* activity becomes gradually restricted to the mature LES, while decreasing in the muscle layer of the proximal stomach (Fig. 2 B).

Abnormal LES physiology in collagen XIX null mice

Megaesophagus is a distinguishing feature of severe human achalasia, an esophageal motility disorder characterized by elevated basal tone and impaired swallowing-induced relaxation of the LES (Goyal, 2001). This phenotypic trait, coincident with high and persistent *Col19a1* expression in the LES,

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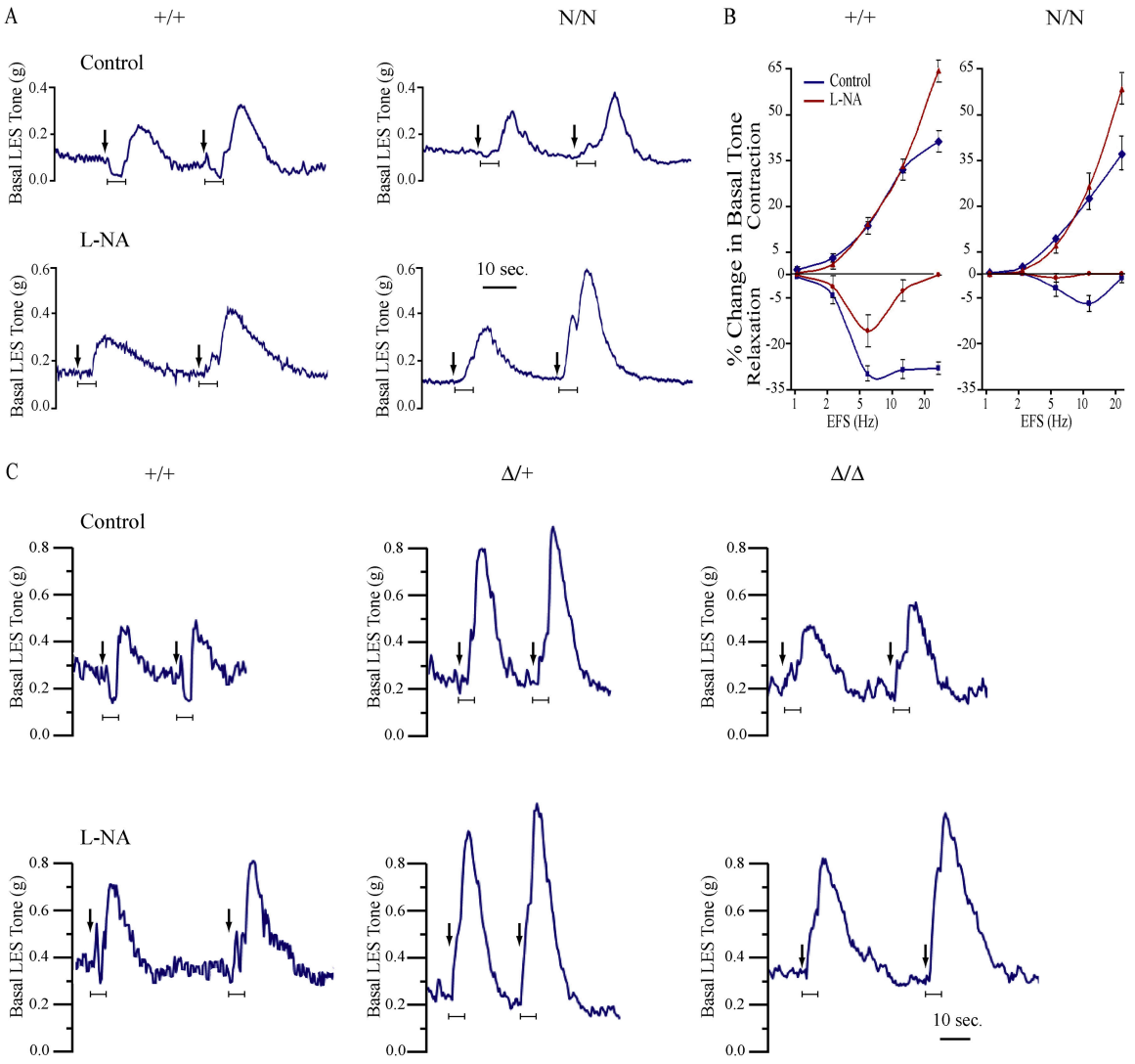

Figure 3. **LES in vitro physiology.** (A) Typical tracings to illustrate LES responses to 10- and 20-Hz EFS (20 V, 0.5-ms pulse duration, 4-s train) in control (top) and L-NA–treated (bottom) samples from 3-mo-old wild-type (+/+) and collagen XIX null (N/N) animals. (B) Summary of quantitative relaxation and contraction data of wild-type (+/+) and collagen XIX null (N/N) animals ($n = 6$) in the presence and absence of L-NA. (C) Typical tracings to illustrate the same responses as in A using heterozygous (Δ/+) and homozygous Δ19 (Δ/Δ) mice.

prompted us to examine whether early demise of homozygous mutants may be in part accounted for by sphincteric muscle dysfunction. Accordingly, the physiology of the collagen XIX–deficient LES muscle was assessed in vitro by monitoring basal muscle tone in response to electrical field stimulation (EFS), and in vivo by examining swallowing-induced LES relaxation using intraluminal esophageal manometry.

Mechanical responses to nonadrenergic noncholinergic (NANC) nerve stimulation with EFS of sphincteric muscle strips from wild-type and nullizygous adult mice were measured in the absence and in the presence of L-NA, an inhibitor of NO synthase (NOS; Mashimo et al., 1996). As expected, we found that EFS elicited frequency-dependent relaxation of wild-type LES strips followed by pronounced rebound contraction, and that muscle relaxation was significantly reduced by L-NA treatment (Fig. 3, A and B). In marked contrast, mutant muscle strips failed to produce significant relaxation in response to EFS; furthermore, L-NA virtually eliminated any residual relaxation (Fig. 3, A and B). Comparable results were obtained in intact animals. Intraluminal pressure recorded at the LES level of adult *Col19a1* null mice in fact showed significantly higher basal tone (three- to eightfold) than wild-type animals (Fig. 4 A). It also documented severely impaired or absent relaxation upon swallowing; even when present, relaxation was abnormally brief (Fig. 4 A). The results of the manometric tests were remarkably similar to those reported for achalasic patients (Richter, 2001). Altogether, the in vitro and in vivo experiments indicated that NO-dependent neurotransmission is perturbed in the collagen XIX–deficient LES.

Nitrergic nerves and ICC-IM are present in the mutant LES

It has been proposed that c-Kit–positive intramuscular interstitial cells of Cajal (ICC-IM) transduce inhibitory signals from nerve terminals to sphincteric smooth muscle cells

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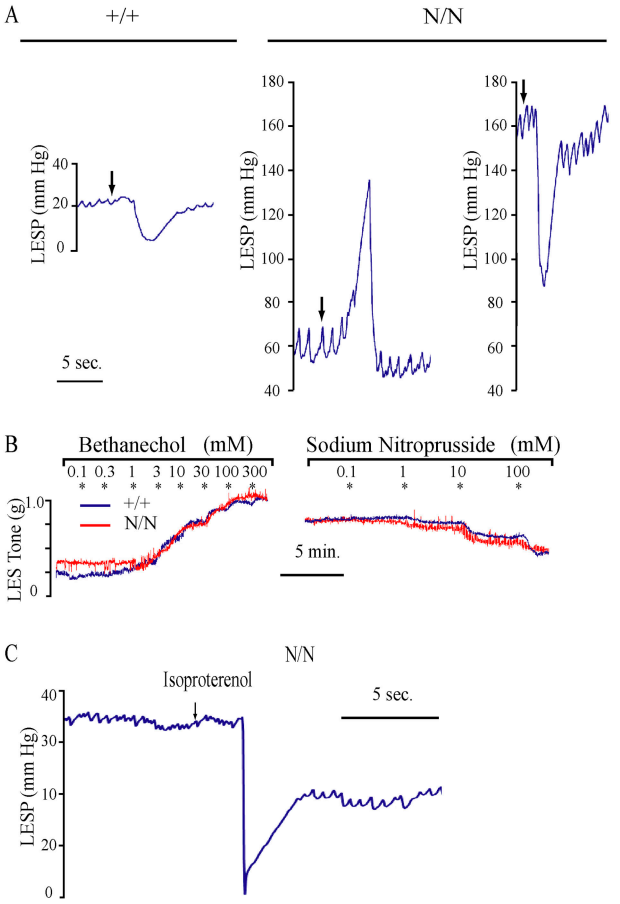

Figure 4. **LES in vivo physiology.** (A) Typical tracings to illustrate swallow-induced changes in LES pressure of 3-mo-old wild-type (+/+) and collagen XIX null (N/N) mice. Contrast the normal basal tone and LES relaxation in wild-type mice with the higher basal tone with frank contraction (one example in the middle tracing) or extremely high basal tone with incomplete relaxation (another example in the right tracing) in mutant mice. (B) Measurements of LES muscle tension in the presence of cumulative concentrations of bethanechol (left) and sodium nitroprusside (right) in tissue strips from 3-mo-old wild-type (blue) and null (red) mice. (C) Isoproterenol hydrochloride (0.4 μg/Kg; i.v.) treatment of a 3-mo-old *Col19a1*$^{-/-}$ mouse.

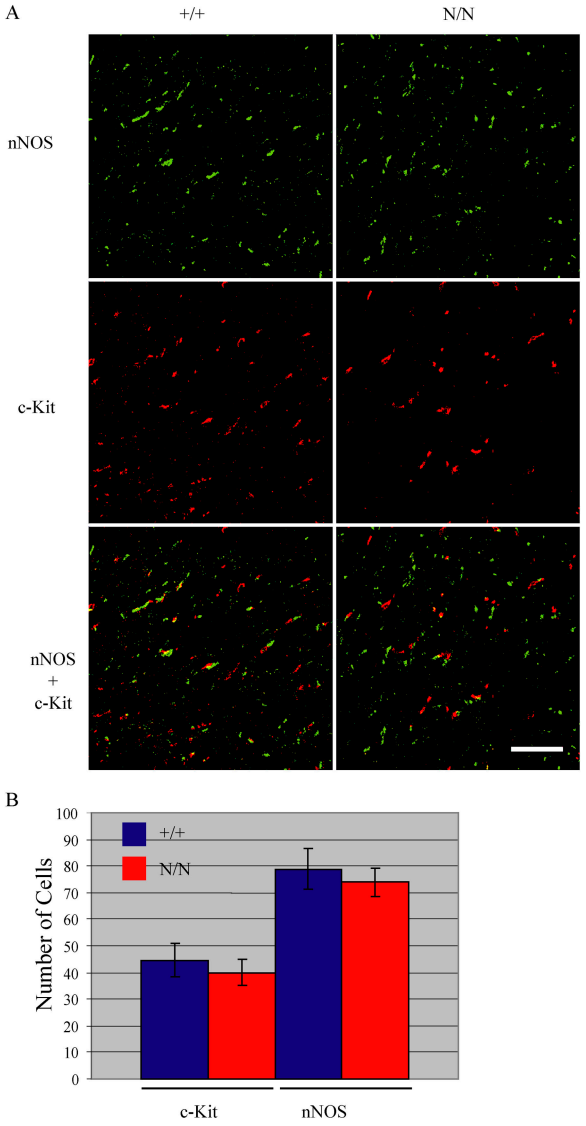

Figure 5. **Cellular analyses of mutant LES.** (A) Confocal micrographs of wild-type (+/+) and collagen XIX null (N/N) LES muscles stained for nNOS and c-Kit with the superimposed images shown at the bottom. Bar, 50 μm. (B) Numbers of c-Kit– and nNOS-positive cells in 0.235-mm^2 fields from wild-type and mutant tissues (n = 4). An average of 44.5 and 40 c-Kit–positive cells and 79 and 74 nNOS-positive cells were counted in wild-type and mutant tissues, respectively. Bars indicate SEM.

(SMCs; Ward et al., 1998). Therefore, antibodies against c-Kit and nNOS were used to assess whether or not loss of nitrergic nerves and/or ICC-IM may account for sphincteric muscle dysfunction in *Col19a1*$^{-/-}$ mice. Immunofluorescence staining of wild-type and homozygous mutant LES muscle showed more nNOS-positive cells than c-Kit–positive ICC-IM in both samples; moreover, the two cell types were often seen in close association (Fig. 5 A). Quantitative analysis of the confocal images demonstrated the presence of statistically comparable numbers of each cell type in the wild-type and mutant tissues (Fig. 5 B). No remarkable differences were also observed with additional neurospecific markers, such as vasoactive intestinal peptide and choline acetyltransferase (unpublished data).

Altered matrix in collagen XIX–deficient LES tissue

Loss of collagen XIX could in principle alter the architecture of the smooth muscle matrix or the intrinsic ability of SMC to relax upon nerve stimulation. The latter possibility was assessed in the collagen XIX–deficient LES muscle by in vitro and in vivo assays. An exogenously applied NO donor caused relaxation of mutant LES muscle strips precontracted with bethanechol (Fig. 4 B). Similarly, administration of a β-adrenoreceptor agonist to *Col19a1*$^{-/-}$ mice caused normal LES relaxation (Fig. 4 C). Therefore, these results demonstrated integrity of the SMC membrane and of the intracellular signal transduction machinery responsible for LES relaxation in *Col19a1* null mice. Along these lines, immunofluoresence staining of connexin 43, smooth muscle actin, α-actinin, and vinculin failed to identify variations between wild-type and mutant SMC (unpublished data).

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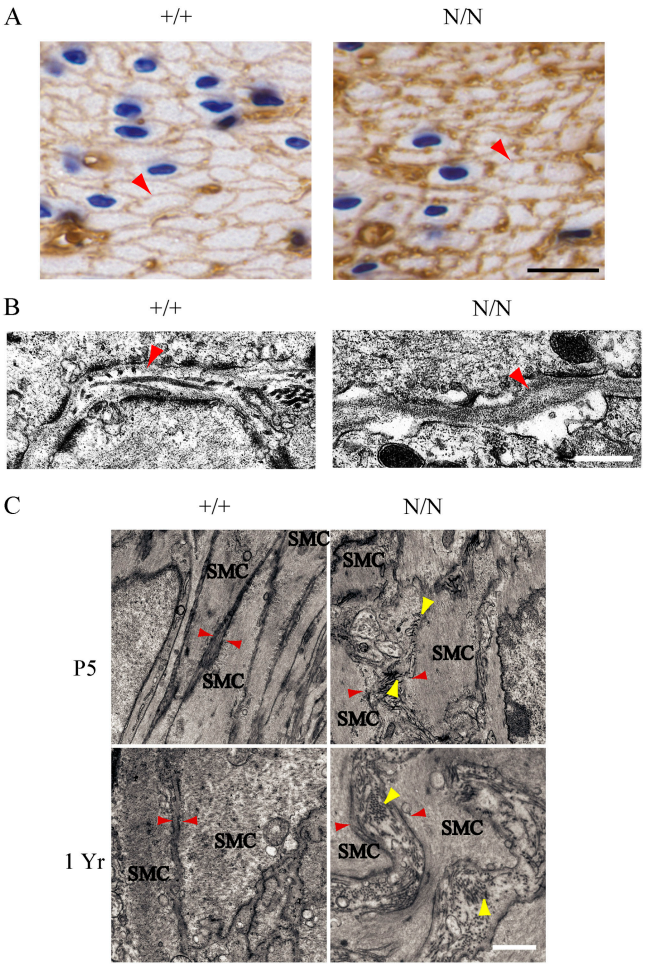

Figure 6. **BM in the mutant LES.** (A) Light microscopy of representative LES tissues from 7-mo-old wild-type (+/+) and N19/N19 (N/N) mice immunostained with antibodies against nidogen-1, showing positive staining of the BM around SMC (arrowhead). Bar, 0.01 mm. (B) Electron microscopy of 7-mo-old wild-type (+/+) and N19/N19 (N/N) LES documenting the thicker BM in the mutant sample (arrowhead). Bar, 0.5 μm. (C) Electron microscopy of smooth muscle in the medial portion of the gastroesophageal junction of P5 and 1-yr-old wild-type and collagen XIX null (N/N) mice. Intercellular spacing in neonatal mice ranges from 30 to 316 nm in wild-type vs. 55 to 708 nm in mutant mice, whereas in adult animals ranges from 94 nm to 1.0 μm in wild-type vs. 99 nm to 3.3 μm in mutant mice. Red and yellow arrowheads point to spacing between smooth muscle cells (SMC) and collagen fibrils, respectively. Bar, 1 μm.

Immunostaining of collagen XIX–deficient LES tissue with antibodies against nidogen-1 documented a largely preserved BM, which, however, stained more intensively than the wild-type counterpart (Fig. 6 A). Electron microscopy confirmed this result by showing a thicker BM around the mutant SMC (Fig. 6 B). It also revealed that intercellular spacing of the mutant smooth muscle is appreciably greater than the wild-type control (Fig. 6 C). Additional abnormalities include convoluted SMC profiles, excessive extracellular accumulation of collagen fibrils, and highly irregular intercellular space (Fig. 6 C). Consistent with progressive degeneration of matrix organization, these morphological abnormalities were more pronounced in adult than newborn mutant mice (Fig. 6 C). Collectively, these analyses suggested a specialized and highly restricted role of collagen XIX in organizing the BM zone of the LES.

Impaired muscle transdifferentiation in the collagen XIX null esophagus

Necroscopic examination of the esophagus of adult *Col19a1*$^{-/-}$ mice revealed the presence of another phenotypic manifestation indicative of a morphogenetic defect. The muscle layer of the murine esophagus undergoes a transdifferentiation process from smooth to skeletal muscle that begins at about embryonic day 15.5 (E15.5) and that ends approximately at postnatal day 21 (P21; Patapoutian et al., 1995; Kablar et al., 2000). This poorly understood developmental program is accompanied by rostrocaudal expression of MRF genes, and varies in timing and extent depending on the mouse strain (Patapoutian et al., 1995; Kablar et al., 2000; unpublished data).

Progression of muscle transdifferentiation in the wild-type 129/Sv mouse was monitored by following the expression of *myogenin*, an MRF that instructs skeletal muscle differentiation (Molkentin and Olson, 1996). In situ hybridizations at different prenatal and postnatal stages of esophageal development revealed that the front of *myogenin* expression reaches diaphragm level at birth, and gradually progresses into the abdominal segment of the esophagus during the first week of postnatal life (Fig. 7 A). The same analysis documented that the postnatal front of *myogenin* expression in the collagen XIX–deficient esophagus remains at the same level as at birth (Fig. 7 B). Immunostaining of the wild-type and mutant specimens for skeletal and smooth muscle–specific proteins demonstrated that loss of MRF gene expression translates into failed muscle transdifferentiation in the entire abdominal segment of the *Col19a1*$^{-/-}$ esophagus (Fig. 7 C). These results conclusively established a causal relationship between extracellular deposition of collagen XIX and developmentally programmed activation of MRF-driven smooth muscle transdifferentiation.

The Δ19 mutation perturbs only LES function

The milder phenotype of the Δ19 mutation afforded the opportunity to further explore the role of collagen XIX in skeletal myogenesis and sphincteric muscle relaxation. Heterozygous and homozygous Δ19 mice were born at the expected frequency and were apparently unaffected by the deposition into the ECM of abnormal α1(XIX) homotrimers. However, a significant number of adult (8–12-mo-old) heterozygous and homozygous mutant mice were noted to display evident signs of compromised fitness, such as weight loss, lethargy, and patchy hair loss. Therefore, adult Δ19 heterozygotes and homozygotes were analyzed for possible manifestations in the gastroesophageal system.

Immunohistological examination of gastroesophageal tissues from four adult Δ19/+ mice and four Δ19/Δ19 littermates revealed normal transdifferentiation of the muscle layer in the abdominal segment of the esophagus (Fig. 6 B). By contrast, the EFS assay documented reduced or absent relaxation of LES muscle strips in half of the eight heterozygous and eight homozygous Δ19 specimens examined (Fig. 3 C). Moreover, LES samples from randomly chosen

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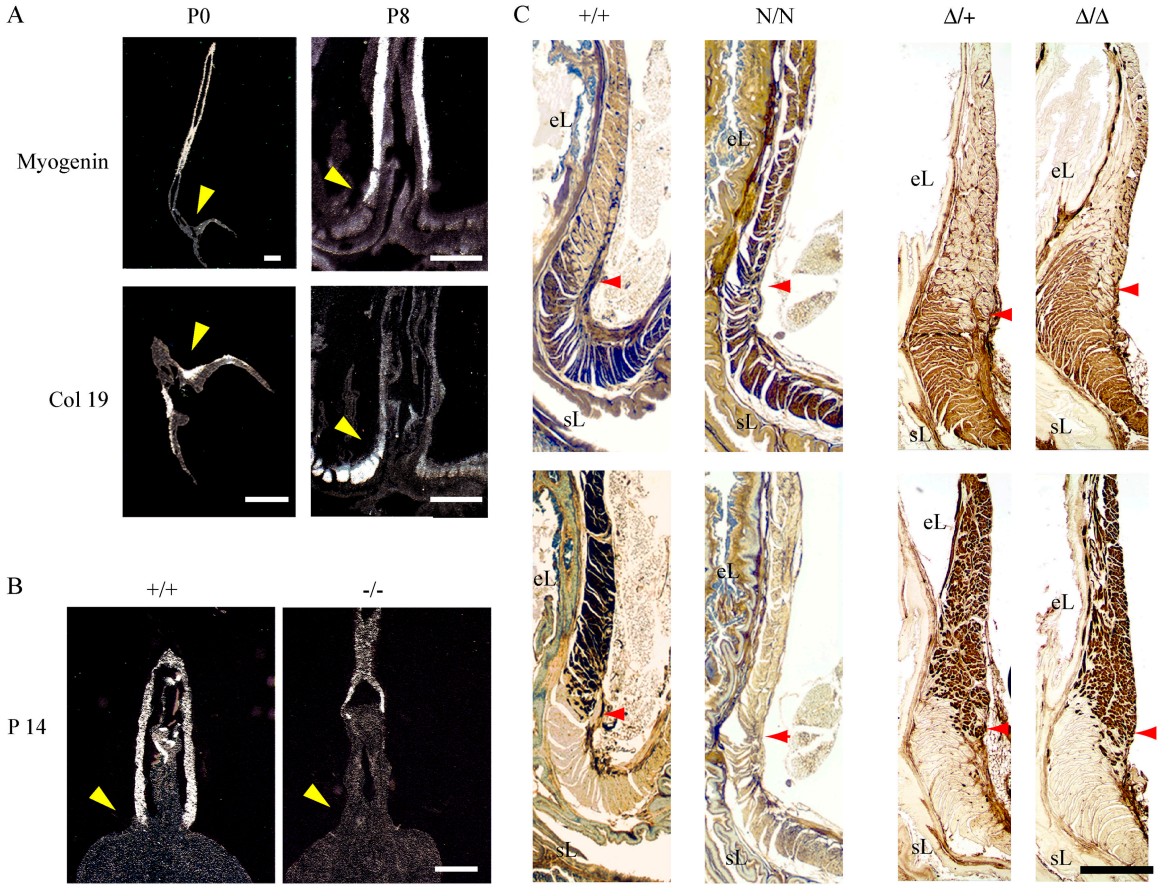

Figure 7. **Esophageal muscle transdifferentiation.** (A) In situ hybridizations of gastroesophageal specimens from P0 and P8 wild-type mice to *myogenin* and *Col19a1* antisense probes. (B) In situ hybridizations of P14 wild-type (+/+) and collagen XIX null (−/−) gastroesophageal specimens to *myogenin* antisense probe. In both panels, sense probes yielded no signals above background (not depicted). (C) Immunostaining of the abdominal segments of esophagi from 18-mo-old wild-type (+/+) or collagen XIX null (N/N) mice, and from 1-yr-old mice heterozygous (Δ/+) or homozygous (Δ/Δ) for the collagen XIX deletion using antibodies against smooth muscle actin (top) or skeletal muscle myosin (bottom). The lumina of the esophagus and stomach are indicated by eL and sL, respectively. In A and B, the yellow arrowhead points to the LES; in C, the red arrowhead indicates the normal transition point between skeletal and smooth muscle. Bars, 0.5 mm.

Δ19/+ or Δ19/Δ19 mice often displayed altered nidogen-1 immunostaining (unpublished data). Therefore, we concluded that the structural and compositional integrity of the BM zone are both prerequisites for proper LES function, and that only the latter is required for the developmentally regulated process of skeletal myogenesis.

Discussion

Compositional diversification of BMs at distinct anatomical locations is a major determinant of the functional specificity of these highly specialized and widely distributed ECM structures (Timpl, 1996). A growing body of genetic evidence indicates that the functional integrity of individual BM zones also requires the deposition of collagen molecules that connect them to the underlying connective tissue stroma (Ortega and Werb, 2002). Illustrative examples include the role of BM-associated collagens XV and XVIII in providing integrity to skeletal muscle, microvessels, and vitreous (Eklund et al., 2001; Fukai et al., 2002). The present paper adds collagen XIX to the list of BM-associated collagens by implicating this molecule in the organization of the pericellular matrix of the sphincteric smooth muscle. It has also unraveled an unexpected role of this rare collagen type in esophageal morphogenesis.

Structural role of collagen XIX in LES physiology

Activation of inhibitory NANC nerves is critical for LES relaxation upon swallowing (Goyal and Hirano, 1996). Although NO is widely recognized as the major inhibitory neurotransmitter of NANC nerves, the mechanism and factors responsible for conveying NO signals to SMC remain ill-defined. Conflicting models postulate that the highly labile NO either diffuses freely in the extracellular space between nerve varicosities and SMC or is transduced to the target muscle cells by ICC-IM (Ward et al., 1998; Sivarao et al., 2001). The achalasia-like manifestations of mice lacking collagen XIX or producing structurally abnormal collagen XIX trimers imply that NO-dependent smooth muscle relaxation requires a properly organized LES matrix as well. That LES muscle relaxation is impaired in spite of a seemingly normal complement of ICC-IM and nitrergic nerves and of functionally viable SMC further supports this conclusion.

The hypertensive and nonrelaxing LES of the *Col19a1* mutant mice resembles the clinical manifestations of human

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patients with achalasia (Goyal, 2001; Richter, 2001). The etiology of this primary esophageal motor disorder is heterogeneous and may include genetic, infectious, autoimmune, and degenerative factors. Loss of nitrergic nerves in achalasia is widely believed to cause hypertensive LES due to unopposed cholinergic excitation, a notion indirectly supported by the basal LES hypertension in *nNOS*$^{-/-}$ mice (Goyal, 2001; Richter, 2001; Sivarao et al., 2001). Similarly, impaired LES relaxation to swallowing in *nNOS*$^{-/-}$ mice underscores the prominent contribution of nitrergic neurotransmission to inhibitory neurotransmission (Sivarao et al., 2001). The manometric data from *Col19a1*$^{-/-}$ and *nNOS*$^{-/-}$ mice are virtually identical and as such, they emphasize functional equivalency between NO release from nitrergic varicosities and ECM organization. By contrast, c-Kit mutant (*W/W*^{v}) mice, which lack ICC-IM, have a hypotensive LES with normal NANC relaxation (Sivarao et al., 2001). Hence, the data from the *Col19a1* and *nNOS* null mice concur in strongly suggesting that ICC-IM play a lesser role than previously suggested in sphincteric muscle relaxation.

The structural role of the collagen XIX–rich BM zone may extend beyond supporting NO-dependent relaxation of the LES to organizing the neuromuscular junction of the sphincteric muscle. In this respect, an analogy could be drawn with perlecan in clustering acetylcholinesterase to the synaptic basal lamina of the neuromuscular junction (Arikawa-Hirasawa et al., 2002). Our postulate is based on the intriguing observation that the NOS inhibitor L-NA affects the relaxation of mutant muscles relatively less than wild-type muscles (Fig. 3). Irrespective of the underlying mechanism, our work conclusively proves that collagen XIX is a new contributing factor to sphincteric muscle physiology.

Instructive role of collagen XIX in esophageal development

Collagenous and elastic macroaggregates have been traditionally viewed as the main structural determinants of connective tissue architecture. However, there is emerging evidence that they also participate in modulating a variety of cellular activities and signaling events (Ortega and Werb, 2002; Ramirez and Rifkin, 2003). For example, proteolytic products of collagens XV and XVIII—also known as restin and endostatin—have been reported to control programs as diverse as angiogenesis, neuronal cell migration, and epithelial cell morphogenesis (O'Reilly et al., 1997; Sasaki et al., 1998; Ackley et al., 2001; Karihaloo et al., 2001). Moreover, failed regression of hyaloid vessels in the eyes of collagen XVIII-deficient mice has been interpreted to imply that this BM-stabilizing molecule promotes programmed cell death and macrophage activation during tissue remodeling (Fukai et al., 2002). Failed muscle transdifferentiation in *Col19a1*$^{-/-}$ mice similarly implicates this collagen type in modulating a specific morphogenetic process.

Developmentally programmed cell transdifferentiation is a rare phenomenon in vertebrates that has been described for a few contractile cell types, including the mouse esophagus and the chick iris (Volpe et al., 1993; Patapoutian et al., 1995; Link and Nishi, 1998a; Kablar et al., 2000). Transdifferentiation in both organ systems involves the conversion of smooth muscle to skeletal muscle. Co-culture experiments have suggested that activin and follistatin coordinate muscle transdifferentiation in the chick iris (Link and Nishi, 1998b). That normal development of striated muscles and nicotinic receptor clusters take place in *Mash1*$^{-/-}$ mice, which lack enteric neurons, has indicated that skeletal myogenesis in the mouse esophagus occurs independently of innervation (Sang et al., 1999).

The biological mechanism responsible for skeletal myogenesis in the mature muscle layer of the mouse esophagus is controversial. In the original description of the phenomenon, Patapoutian et al. (1995) reported that smooth-to-skeletal muscle conversion is preceded by *MyoD* and *myogenin* expression. Kablar et al. (2000) subsequently used transgenic and knock-in mice to document that initiation and progression of muscle transdifferentiation depend on *Myf5* expression. The apparent discrepancy between these two reports may reflect the fact that each followed muscle transdifferentiation in different esophageal segments (i.e., abdominal vs. thoracic/cervical). Others have argued that smooth and skeletal muscles originate from distinct precursor cells already present at early embryonic stages (Zhao and Dhoot, 2000a,b; Rishniw et al., 2003). However, this argument is not supported by evidence of significant SMC apoptosis during esophageal development (Patapoutian et al., 1995; Kablar et al., 2000).

Our findings implicate the collagen XIX–rich matrix as the first extrinsic factor to guide skeletal myogenesis in the developing mouse esophagus. Interestingly, the same morphogenetic defect was not observed in the C57/Bl/6J genetic background, implying that collagen XIX action is modulated by modifier gene products. On the other hand, association of an achalasia-like phenotype with the same physiological and morphological manifestations in both C57/Bl/6J and 129T2/SvEmsJ mutant mice indicated that distinct mechanisms are responsible for the genesis of LES dysfunction and failed muscle transdifferentiation. Along these lines, normal esophageal muscle transdifferentiation in mice producing abnormal collagen XIX trimers demonstrated that a specific peptide sequence (rather than the whole molecule) is involved in muscle transdifferentiation. One attractive mechanism is that the collagen XIX–rich matrix may control the distribution and/or activity of growth factors that ultimately trigger MRF gene expression in the abdominal esophagus. The elegant work of Myers et al. (2003) supports this hypothesis. These investigators have shown that collagen XIX forms higher order aggregates in which individual molecules extend radially from a globular core of interacting NC6 domains. Therefore, they have argued that this configuration, together with the NC6 heparin-binding site, may contribute to localize and concentrate signaling molecules within the BM zone. Such a model is analogous to the recently reported involvement of extracellular microfibrils in limb patterning and lung morphogenesis through the modulation of TGFβ/BMP signaling (Arteaga-Solis et al., 2001; Neptune et al., 2003). Alternatively, the flexibility of the collagen XIX monomers and the presence of a Tsp-N module in NC6 may regulate skeletal myogenesis by mediating critical cell–matrix and/or cell–cell interactions (Myers et al., 2003).

Although the precise mechanism underlying the role of collagen XIX in muscle transdifferentiation remains undeter-

mined, our work has yielded a number of interesting observations and plausible predictions. First, impaired muscle transdifferentiation is only seen in the absence of collagen XIX deposition and is associated with failed *myogenin* activation. This finding could be interpreted to indicate that collagen XIX lies upstream of the MRF(s) driving this morphogenetic pathway. Second, early embryonic expression of *Col19a1* is in the region that will eventually become the abdominal segment of the adult esophagus, and that fails to transdifferentiate in collagen XIX null mice. Therefore, it is plausible to argue that SMCs become fated for cell conversion soon after onset of *Col19a1* expression in the lower-third portion of the primitive esophagus. Third, persistency of the smooth muscle phenotype in null mice is confined to the abdominal segment and conversely, *Col19a1* gene activity is absent in the upper two thirds of the wild-type esophagus. A likely explanation of these observations is that distinct factors and different mechanisms drive muscle transdifferentiation along the esophageal axis. Implicitly, this last prediction may reconcile the controversy about the identity of the MRF(s) driving esophageal transdifferentiation (Patapoutian et al., 1995; Kablar et al., 2000). The availability of the *Col19a1* null mouse provides the opportunity to test these predictions and further characterize this poorly understood biological phenomenon.

Materials and methods

Generation of *Co119a1* mutant mice

Targeting vectors were engineered using *Coll9a1* fragments isolated from a 129T2/SvEmsJ mouse genomic library by inserting the PGK-*neo* cassette within the HindIII site of exon 4 (N19) or in place of the 3-kb-long XbaI fragment containing exons 38–40 (Δ19; Sumiyoshi et al., 1997). Maintenance, transfection, and selection of mouse ES cells, as well as generation of chimeric animals were performed as described previously (Andrikopoulos et al., 1995). Electroporated ES cells and mutant mice were genotyped by Southern blot analysis using diagnostic restriction enzyme sites and probes outside and inside of the targeted region. Northern blot hybridization and sequencing of RT-PCR products were used to confirm the identity and relative expression levels of the mutant transcript. RT-PCR analysis of Δ19/+ transcripts was performed after 25, 30, or 35 cycles in order to compare the relative representation of wild-type and mutant products during the linear phase of the amplification. Mutant mice were generated in the Mount Sinai Mouse Genetics Shared Resource Facility (New York, NY) and bred onto the pure 129T2/SvEmsJ genetic background.

Immunoblot analysis

Crude collagen XIX fraction was extracted from adult brains of wild-type and mutant mice as described previously (Sumiyoshi et al., 1997). The final 5 M NaCl precipitate was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% NP-40; ~100 μg of the crude collagen XIX preparation was then separated on 4.5% SDS-PAGE and electroblotted onto a PVDF membrane in the presence of 15 mM sodium-borate buffer. Immunoblots were probed with rabbit pAbs raised against a recombinant fusion protein produced by the pMAL-c2 expression plasmid (New England Biolabs, Inc.) and containing the sequence encoding aa 1004–1036 of collagen XIX. Immunocomplexes were detected using the ECL blotting system (Amersham Biosciences) with secondary goat anti–rabbit IgG conjugated with HRP (Santa Cruz Biotechnology, Inc.). For collagenase digestion, 7.5 U bacterial collagenase type III (Advance Biofactures) was added to 100 μg of crude collagen XIX extract, and was incubated for 2 h at 37°C in 50 mM Tris-HCl, pH 7.2, and 20 mM $CaCl_2$.

Immunohistochemistry, electron microscopy, and in situ hybridizations

Esophagi were removed along with the LES from necropsied animals, fixed in 4% PFA, embedded in paraffin, serially sectioned, and immunostained with alkaline phosphatase conjugated to mAbs against smooth muscle actin or skeletal fast myosin (Sigma-Aldrich). Rabbit pAbs against nidogen-1 were provided by Dr. Ulrike Mayer (University of Manchester, Manchester, UK; Fox et al., 1991). For immunofluorescence staining, frozen LES sections were incubated overnight at 4°C with rabbit anti-nNOS antibody (1:500; BD Biosciences) and goat anti-cKit antibody (1:500; Santa Cruz Biotechnology, Inc.). After removal of unbound antibodies, sections were incubated with FITC-conjugated donkey anti–rabbit IgG and Texas red–conjugated donkey anti–goat IgG (1:200; Jackson ImmunoResearch Laboratories) for 2 h at RT. Slides were examined using a confocal laser scanning microscope (TCS-SP (UV); Leica) equipped with a four-channel spectrophotometer scan head and four lasers (Ar-UV, Argon, Krypton, and HeNe). Sections were illuminated simultaneously with the λ = 488- and λ = 568-nm laser lines and the AOTF was adjusted such that no signal "cross-talk" occurred between channels. Gastroesophageal junctions were collected from 4-mo-old animals, fixed overnight in 4% PFA, processed through paraffin, and sectioned at 5–7-μm thickness. Sections were deparaffinized and treated with 100 μg/ml protease XXIV (P8038; Sigma-Aldrich) for 10 min at 37°C (Willem et al., 2002). Antibodies against nidogen-1 (1:1,000) were applied for 2 h at RT in a humid chamber; the streptavidin-HRP technique (LSAB 2 system; DakoCytomation) was used to localize the antibody and hematoxylin was used as a counterstain. Samples of the same tissues used for immunohistochemistry were processed for electron microscopy by fixation in 2% PFA plus 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 4 h at 4°C. Tissues were rinsed, postfixed for 1 h in 2% osmium tetroxide at RT, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate for 20 min and with lead citrate for 5 min. Photographs were taken on a transmission electron microscope (CM-12; Philips) at 80 kV or using a transmission electron microscope (H-7000; Hitachi) operated at 75 kV. Confocal and electron microscopy were performed at the Mount Sinai Microscopy Shared Resource Facility (New York, NY) and at the Hospital for Special Surgery Analytical Microscopy Core Facility (New York, NY). In situ hybridizations were performed on serial tissue sections using *Col19a1* and *myogenin* probes as described previously (Sumiyoshi et al., 2001).

Physiological tests

Mechanical responses of LES strips to EFS were measured using standard organ bath techniques in the presence or absence of 1 mM Nω-nitro-L arginine (L-NA; Mashimo et al., 1996). LES tone was also measured in the presence of increasing concentrations of bethanechol (0.1–300 μM) followed by sodium nitroprusside (0.1–100 μM) as described previously (Chakder et al., 1997). Intraluminal esophageal manometry was performed as described by Sivarao et al. (2001) using a custom-designed catheter assembly (Dentsleeve; Dentsleeve Pty Ltd.) that consists of silicon tubing made of three individual channels of a 0.3-mm inside and 0.6-mm outside diameter each. Swallows were induced by instilling a 10−20-μl bolus of water on the tongue of the animals. To determine the integrity of the sphincteric muscle, isoprotenerol hydrochloride (0.4 μg/Kg) was injected i.v.

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This work is dedicated to the memory of Rupert Timpl.

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