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The RNA-Binding Protein RBPMS2 Regulates Development of Gastrointestinal Smooth Muscle

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BACKGROUND & AIMS: Gastrointestinal development requires regulated differentiation of visceral smooth muscle cells (SMCs) and their contractile activities; alterations in these processes might lead to gastrointestinal neuromuscular disorders. Gastrointestinal SMC development and remodeling involves post-transcriptional modification of messenger RNA. We investigated the function of the RNA-binding protein for multiple splicing 2 (RBPMS2) during normal development of visceral smooth muscle in chicken and expression of its transcript in human pathophysiological conditions. METHODS: We used avian replication-competent retroviral misexpression approaches to analyze the function of RBPMS2 in vivo and in primary cultures of chicken SMCs. We analyzed levels of RBPMS2 transcripts in colon samples from pediatric patients with Hirschsprung’s disease and patients with chronic pseudo-obstruction syndrome (CPIO) with megacystis. RESULTS: RBPMS2 was expressed strongly during the early stage of visceral SMC development and quickly down-regulated in differentiated and mature SMCs. Misexpression of RBPMS2 in differentiated visceral SMCs induced their dedifferentiation and reduced their contractility by up-regulating expression of Noggin, which reduced activity of bone morphogenetic protein. Visceral smooth muscles from pediatric patients with CIP0 expressed high levels of RBPMS2 transcripts, compared with smooth muscle from patients without this disorder. CONCLUSIONS: Expression of RBPMS2 is present in visceral SMC precursors. Sustained expression of RBPMS2 inhibits the expression of markers of SMC differentiation by inhibiting bone morphogenetic protein activity, and stimulates SMC proliferation. RBPMS2 transcripts are up-regulated in patients with CIP0; alterations in RBPMS2 function might be involved in digestive motility disorders, particularly those characterized by the presence of muscular lesions (visceral myopathies).

Keywords: Developmental Biology; RNA-Binding Protein; Gastrointestinal Tract; Smooth Muscle Contraction.

Motility of the digestive tract is ensured by the contraction of visceral smooth muscles under the control of the autonomous enteric nervous system (ENS) and the interstitial cells of Cajal (ICC).1–3 Dysfunction of just one of these cell types can lead to the development of gastrointestinal neuromuscular disorders in infants and adults.4,5 Many studies have investigated the role of ENS damage in these diseases and shown that ENS absence caused by precocious differentiation or blockage of its migration triggers Hirschsprung disease (HSCR).2,4 Alteration of ICC number, which perturbs the ICC network, also has been observed in different digestive motility disorders.6,7 Until now, dysfunction of the downstream effector (visceral smooth muscle) rarely was investigated in gastrointestinal neuromuscular disorders.4,5,7 Specifically, muscular lesions, classified as visceral myopathies, have been observed mainly in patients with chronic intestinal pseudo-obstruction (CPIO). However, the systematic analysis of muscular alterations has been overlooked mainly because of the poor knowledge of the molecular mechanisms involved in visceral smooth muscle cell (SMC) homeostasis. The limited number of valuable SMC markers is also an obstacle to the clinical investigation of visceral smooth muscle integrity in these motility disorders.

More is known about the molecular control of visceral SMCs during embryonic development. Visceral SMCs originate from the splanchnopleural mesoderm that forms the primitive visceral mesenchyme via activation of the hedgehog/bone morphogenetic protein (BMP) pathway.2 Differentiation of visceral mesenchymal cells into visceral SMCs can be visualized first through their elongation and clustering and later by the expression of SMC-specific lineage markers, such as α-smooth muscle actin (αSMA), smooth muscle protein-22 (SM22), calponin, smoothelin, and smooth muscle myosin heavy chain (SM-MHC), which precedes contractile function.8 Only a few studies have investigated the molecular mechanisms controlling visceral mesenchyme differentiation into SMCs.

Abbreviations used in this paper: αSMA, α-smooth muscle actin; BMP, bone morphogenetic protein; CIP0, chronic intestinal pseudo-obstruction; DMEM, Dulbecco’s modified Eagle medium; E, embryonic day; ENS, enteric nervous system; FHL2, four and a half LIM domains 2; GFP, green fluorescent protein; HSCR, Hirschsprung disease; ICC, interstitial cells of Cajal; miRNA, messenger RNA; P-AKT, phosphorylated AKT; PH3, phospho-histone H3-Ser10; PSMAD, phosphorylated SMAD; QPCR, quantitative polymerase chain reaction; RBPMS2, RNA-binding protein for multiple splicing 2; RBP, RNA-binding protein; RCAS, Replication-Competent Avian leucosis Sarcoma virus; RRM, RNA recognition motif; SHH, sonic hedgehog; SMC, smooth muscle cell; SM-MHC, smooth muscle myosin heavy chain; SM22, smooth muscle protein-22; SRF, serum response factor.
Endodermal hedgehog signaling activation promotes visceral mesenchyme growth through expression of the patched receptor and of Bmp4 that drives its differentiation into SMCs. Aberrant modulation of BMP activity induces visceral smooth muscle disorganization. The fibroblast growth factor signaling pathway also is activated in visceral mesenchymal cells and is specifically down-regulated during visceral SMC differentiation, which is inhibited by sustained activation of the fibroblast growth factor pathway in vivo. Although a substantial body of works has investigated visceral SMC development, the specific molecular mechanisms controlling its differentiation remain to be clarified.

Besides transcription, which represents the first step of gene expression, many post-transcriptional events regulate the late fate of messenger RNAs (mRNAs) in eukaryotic cells and determine their spatiotemporal pattern of expression. RNA-protein complexes control multiple steps of this process, including mRNA cellular localization, splicing, translational regulation, or mRNA degradation. These complexes contain specific RNA-binding proteins (RBPs) that play important roles during development. The RNA recognition motif (RRM) proteins, a large RBP family involved in regulating RNA metabolism, are expressed in a tissue-specific manner, suggesting that they may participate in distinct developmental processes. Moreover, posttranslational RNA events are involved in the control of differentiation and remodeling of smooth muscle tissues, such as heart and vessels, suggesting that visceral smooth muscle development and plasticity could be regulated similarly. RNA-binding protein for multiple splicing 2 (RBPMS2), a member of the RRM family, is expressed in vertebrate heart and gastrointestinal tract. By using a microarray approach to identify modulators of visceral SMC development, we found that RBPMS2 is expressed during the early stages of chick SMC development (unpublished data).

Here, we investigated RBPMS2 function and mechanism of action during visceral SMC development and assessed its transcript expression in colon from patients with digestive motility disorders. We show that RBPMS2 is an early marker of visceral SMC precursors and that RBPMS2 misexpression in differentiated SMCs increases proliferation and hinders their contractile function. The RBPMS2-dependent contractile differentiation defect is mediated by inhibition of BMP signaling through up-regulation of Noggin, its major inhibitor. These results indicate that ectopic RBPMS2 expression in vivo and in primary cultured SMCs phenocopies the alteration of the contractile function observed in patients with visceral myopathy.

Materials and Methods
Chick Embryonic Gastrointestinal Tissues
Fertilized White Leghorn eggs from Haas Farm (Kaltenhouse, France) were incubated at 38°C in humidified incubators. Gastrointestinal tissues from chick embryos were dissected as described.

Human Colon Samples
Tissue samples from pediatric patients (1 month to 1 year) were from the collection of the Lapeyronie Hospital (Montpellier, France) as previously described. Control samples were right colon specimens from 3 neonates who underwent ileocolic resection for congenital cystic duplication of the terminal ileum. Full-thickness, large-bowel specimens were obtained after rectosigmoidectomy from 3 patients diagnosed with HSCR or 3 patients diagnosed with slow-transit constipation without HSCR and with megacystis (CIPO patients).

Avian Retroviral Misexpression System
Myc-tagged chick full-length RBPMS2 was cloned into the Replication-Competent Avian Leucosis Sarcoma virus strain A (RCAS[A]) vector to produce replication-competent retroviruses that express Myc-RBPMS2. The RCAS(A)-Noggin and RCAS stain B with green fluorescence protein coding region (RCAS[B]-GFP) retroviral constructs were described previously. Retroviral constructs were transfected into the DF-1 chicken fibroblast cell line (ATCC-LGC) to produce retroviruses. Retroviruses were injected into the splanchnopleural mesoderm of stage-10 chicken embryos to target the stomach mesenchyme. Eggs then were placed at 38°C until harvested.

Primary Cultured SMCs and Analysis
Primary cultures from embryonic day 15 (E15) gizzard muscle were prepared as described. Briefly, the tunica mucosalis carefully was separated from the serosa and tunica mucosa before collagenase dissociation. Isolated cells were cultured in Dulbecco's modified Eagle medium (DMEM) in the presence of 0.2% bovine serum albumin and 5 µg/mL insulin in Matrigel-coated plates (VWR, Fontenay-sous-Bois cedex, France) to maintain the cell differentiation status (>95% of isolated cells were Desmin- and αSMA-positive, data not shown). Differentiated SMCs then were infected with different retroviruses and maintained in culture for 1–7 days. In our conditions, avian retroviruses have a high tropism to infect SMCs and low tropism for enteric neurons (Supplementary Figure 1). SMC contractility was monitored with a Nikon inverted microscope and cells were imaged before and after treatment with 10⁻³ mol/L carbachol (Sigma, France), a muscarinic agonist, as previously published. In rescue experiments, differentiated SMCs infected with retroviruses during 3 days were treated with 20 ng/mL of purified BMP4 (Humanzyme, Chicago, IL) for 4 days.

Isometric Tension Measurement During Contractile Activity
E15 gizzards were cut at the level of the ventral and dorsal tendons to avoid damaging the muscular bundles. The right part of the organ connected to the duodenum was used for the measurements (control, n = 23; RBPMS2 misexpression, n = 21). Organs were mounted between 2 stainless steel hooks and placed in an organ bath filled with Tyrode-HEPES solution with 2.5 mmol/L CaCl₂ continuously bubbled with 95% O₂/5% CO₂ and maintained at 37°C. Changes in isometric tension were recorded using an IT1-25 force transducer and an IOX computerized system (EMKA Technologies, Paris, France). Gizzards initially were stretched at a resting tension of 0.5 g and, after a 60-minute equilibration period, contraction was induced with cumulative doses of carbachol (10⁻⁶ to 10⁻³ mol/L). Effects were evaluated by measuring the maximum tension, and data were expressed as changes relative to the basal tension (contraction in g) for 10⁻³ mol/L carbachol concentration.
smooth musculature (Supplementary Figure 2). By using paraffin sections we showed that, at E6, RBPMS2 and αSMA expression overlapped in the undifferentiated visceral mesenchyme (Figure 1B, upper panels). However, after visceral smooth muscle differentiation (E9), RBPMS2 expression rapidly decreased in the smooth muscle layer, whereas calponin (which is a marker of differentiated SMCs) expression increased (Figure 1B, lower panels). In summary, RBPMS2 expression was highest in undifferentiated visceral mesenchymal cells and progressively decreased with muscle differentiation, thus identifying it as an early marker of visceral smooth muscle precursor cells.

**Sustained RBPMS2 Expression Alters Gastrointestinal Development, SMC Differentiation, and Contractile Function**

To investigate RBPMS2 function, we maintained RBPMS2 expression throughout visceral muscle development and differentiation by using an avian replication-competent retroviral misexpression system that allows in vivo targeting of specific genes in the stomach mesenchyme.11,16 Sustained RBPMS2 expression resulted in a dramatic alteration of the stomach morphology (Figure 2A). Specifically, the proventriculus, which is the glandular part of the chick stomach (Figure 2A, white arrowheads), was hypertrophied, whereas the gizzard (the muscular part, Figure 2A, white arrows) was denser and malformed in comparison with controls that overexpressed GFP alone (Figure 2A). However, sustained RBPMS2 expression did not affect stomach development and patterning, as revealed by the normal expression of the mesenchymal marker BARX1 and of the endodermal marker sonic hedgehog (SHH) in RBPMS2 misexpressing stomachs (Figure 2B).

Similarly, the determination of visceral SMCs was not affected because positive αSMA cells still were observed in the smooth muscle layer (Figure 2C); however, SMC differentiation was hindered as indicated by the reduction of calponin expression in RBPMS2 misexpressing stomachs in comparison with GFP controls (Figure 2C, Supplementary Figure 3). HuC/D-positive neurons were organized into well-defined plexuses, suggesting that RBPMS2 misexpression did not induce detectable changes in ENS migration and differentiation (Figure 2C). Finally, the SMC proliferation rate was 1.55-fold higher in RBPMS2 misexpressing stomachs than in GFP controls (Figure 2C, Supplementary Figure 4), as indicated by the expression of phosphorylated histone 3-Ser10 (PH3), a standard marker of G2/M transition. Similarly, sustained RBPMS2 expression in the developing colon mesenchyme did not affect SMC determination, whereas it inhibited calponin expression (Supplementary Figure 5). Altogether, these results indicate that a tight regulation of RBPMS2 expression is important for the normal development and differentiation of gastrointestinal smooth muscle. In addition, we investigated the contractile function at the organ level in control and RBPMS2-overexpressing gizzards (E15) by recording the smooth muscle contraction in ex vivo organ experiments (Figure 2D). Addition of carbachol triggered...
the contraction of gizzard muscles in a dose-dependent manner with a maximal tension reached at $10^{-4}$ mol/L. The contraction was weaker in gizzards overexpressing RBPMS2 (0.24 ± 0.02 g; n = 21) than in control gizzards (0.45 ± 0.04 g; n = 23; P < .001).

To determine how RBPMS2 regulates visceral SMC differentiation, we established primary cultures on Matrigel of visceral differentiated SMCs from E15 gizzard muscles in serum-free medium supplemented with insulin. In this condition, cultured differentiated SMCs can display phenotypic modulation upon exogenous stimulation. Control primary cultured SMCs were spindle-shaped and homogeneously expressed αSMA and calponin, 2 SMC contractile markers, in highly organized filament bundles (Figure 3A). SMCs then were infected with replication-competent retroviruses (RCAS-RBPMS2 construct or RCAS-empty [control]) for 3 days. Although in control cells the expression of αSMA and calponin remained unchanged (Figure 3A, Supplementary Figure 6), in SMCs infected with Myc-tagged RBPMS2 their expression was lost (Figure 3A, Supplementary Figure 7). However, Myc-positive cells still expressed the mesenchymal marker Desmin (Figure 3A), confirming that they were mesenchymal-derived cells. Quantitative reverse-transcription polymerase chain reaction (QPCR) using RNA from cells infected with RCAS-RBPMS2 or RCAS-empty for 3 days confirmed the down-regulation of calponin, αSMA, and SM22 expression (Figure 3B). Analysis of the effect of RBPMS2 overexpression on serum response factor (SRF) and its coactivator Myocardin, which control numerous steps of SMC differentiation, indicated that Myocardin mRNA was upregulated (2-fold), whereas SRF expression was not significantly affected (Figure 3B). Similarly, four and a half LIM domains 2 (FHL2) mRNA, which inhibits the induction of smooth muscle contractile genes regulated by SRF, was up-regulated (Figure 3B, Supplementary Figure 8). To analyze the impact of ectopic RBPMS2 expression on Myocardin protein level, we co-transfected primary cultured SMCs with a plasmid-encoding myocardin and either RBPMS2 or empty (control) retroviral constructs. In SMCs overexpressing RBPMS2, the myocardin protein level was increased in comparison with control (Supplementary Figure 9). Because myocardin accumulates in response to proteasome inhibition by MG132, we transfected primary cultured SMCs with a plasmid encoding for myocardin with or without 5 μmol/L MG132. Ectopic myocardin expression increased calponin expression (Supplementary Figure 9). Moreover, in accordance with published work, proteasome inhibition by MG132 induced accumulation of myocardin, but reduced
calponin expression (Supplementary Figure 9). Altogether, these findings indicate that ectopic RBPMS2 expression in visceral SMCs induces accumulation of myocardin, which is critical for its function. After 7 days, αSMA expression was recovered in RBPMS2-overexpressing SMCs, whereas loss of calponin expression spread to adjacent nontransfected (Myc-negative) cells (Figure 3C, white arrows). As before, RBPMS2-overexpressing cells were desmin-positive (Figure 3C). Moreover, the number of PH3-positive cells was 6-fold higher in cells that overexpressed RBPMS2 than in controls (Figure 3D), suggesting a global change in the proliferative status of SMCs upon RBPMS2 deregulated expression.

To investigate the effects of RBPMS2 overexpression on visceral SMC contractility, primary cultured SMCs were stimulated with carbachol. Many control cells (RCAS-empty) became round and detached from the disc surface, showing their effective capacity to contract (Figure 3E). Conversely, RBPMS2-overexpressing SMCs were more broad-shaped than control cells and, after addition of carbachol, only few became round and detached from the disc surface (Figure 3E). Recently, intestinal SMC dedifferentiation and inability to contract upon carbachol stimulation were correlated with decreased expression of cholinergic receptor muscarinic 3 mRNA and of carbachol-induced phosphorylated AKT (P-AKT).
Similarly, we found that cholinergic receptor muscarinic 3 mRNA expression was decreased by 56% in RBPMS2-overexpressing SMCs compared with controls (Supplementary Figure 10). Moreover, addition of 10^-4 mol/L carbachol for 14 minutes resulted in an increase of P-AKT to 264% in control SMCs, although the total level of AKT was unchanged. Conversely, no detectable change in P-AKT level was observed in RBPMS2-overexpressing SMCs (Figure 3E). These experiments show that RBPMS2 sustained expression in differentiated SMCs hinders their ability to contract and favor their proliferation, leading to their dedifferentiation.

**RBPMS2 Inhibits BMP Signaling Through Induction of Noggin Expression**

To identify the underlying molecular mechanism(s) of RBPMS2 action, we analyzed the gene expression profiles of primary cultured SMCs infected with RCAS-RBPMS2 or RCAS-empty retroviruses for 3 days (Figure 4A) by microarray analysis.

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**Figure 4.** RBPMS2 inhibits BMP signaling through Noggin up-regulation. (A) Identification of RBPMS2 target genes by microarray using RNA from primary cultured SMCs infected with RCAS-empty or RCAS-RBPMS2 for 3 days. Highest signals are in red, and lowest signals are in green. (B) Analysis of Noggin expression by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-RBPMS2 retroviruses for 3 days. Data were normalized to ubiquitin expression and shown as mean ± standard deviation. In situ hybridization analysis in whole-mount (upper panels) and serial longitudinal sections (lower panels) of E7 stomachs infected with RCAS-GFP (control), or (C) RCAS-GFP and RCAS-RBPMS2 or (D) RCAS-Noggin retroviruses. Infection was confirmed by observation of GFP expression or detection of Env expression. (E) Immunoprecipitation assays to identify mRNAs that interact with RBPMS2. Schematic representation of the approach (left panel) and QPCR to detect RBPMS2-associated mRNAs using specific primers (right panel). The mean expression levels ± standard error of the mean of Noggin and ubiquitin were measured in each condition. (F) Immunoblot analysis of PSMAD1 (55 kilodaltons) and Calponin (34 kilodaltons) expression in primary cultured SMCs infected with RCAS-empty or RCAS-RBPMS2 for 3 days. Anti-Myc antibodies detected the expression of Myc-tagged RBPMS2 (26 kilodaltons). Loading was verified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (37 kilodaltons).
RBPMS2 overexpression in differentiated SMCs induced the down-regulation of calponin and also of markers of SMC differentiation, such as caldesmon and SM22. Conversely, Noggin, the BMP signaling pathway inhibitor, was 360-fold up-regulated (Figure 4A), whereas BMP transcriptional targets, including PITX2, ID2, and ID4, were down-regulated, suggesting a major inhibition of BMP activity (data not shown). Noggin up-regulation was confirmed by QPCR using primary SMCs harvested after 3 days of RCAS-RBPMS2 infection (Figure 4B).

Because Noggin expression pattern has not been described in detail, we performed in situ hybridization and showed that Noggin was expressed in gut mesenchymal derivatives as early as RBPMS2 (Supplementary Figure 11). Moreover, because misexpression of Noggin in stomach induces a hypertrophic phenotype that is highly reminiscent of the defects observed upon RBPMS2 misexpression, we monitored Noggin expression by in situ hybridization after RBPMS2 misexpression in the gastrointestinal mesenchyme as before. Noggin was strongly up-regulated in stomach and lung in comparison with controls (Figure 4C). Conversely, misexpression of Noggin had a moderate impact on the spatiotemporal expression of RBPMS2 (Figure 4D). Altogether, these results show that RBPMS2 induces Noggin expression and accumulation in vivo and in primary cultured SMCs.

Because RBPMS2 can bind to RNAs via its RRM domain, we investigated whether Noggin up-regulation in primary SMCs and in vivo upon RBPMS2 misexpression could be caused by interaction between RBPMS2 and Noggin. First, Myc-tagged RBPMS2 from infected DF-1 cells was immunoprecipitated with anti-Myc antibodies bound to protein A Sepharose beads in the presence of cells was immunoprecipitated with anti-Myc antibodies Noggin primary SMCs and in vivo upon major inhibition of BMP activity (data not shown). Noggin up-regulation was confirmed by QPCR using primary SMCs harvested after 3 days of RCAS-RBPMS2 infection (Figure 4B).

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These results show that RBPMS2 positively regulates Noggin expression, leading to inhibition of BMP activity.

**Noggin Downstream of RBPMS2 Alters SMC Differentiation**

To evaluate whether Noggin is an essential relay of RBPMS2, we focused on Noggin function during visceral SMC development and differentiation. We thus misexpressed Noggin in stomach throughout visceral muscle development and analyzed the differentiation of visceral SMCs. Noggin misexpression inhibited calponin expression in comparison with GFP controls, showing at an alteration of visceral SMC differentiation without significant changes of the proliferative rate in vivo (Supplementary Figure 4). Moreover, immunofluorescence analysis of primary cultured SMCs after 3 days of infection with RCAS-Noggin retroviruses showed that αSMA was expressed uniformly, whereas calponin expression was strongly decreased (Figure 5A). QPCR analysis of these cells showed that calponin and SM22 were down-regulated, whereas αSMA, myocardin, and FHL2 were induced (Figure 5B, Supplementary Figure 8). Noggin overexpression also increased myocardin protein level (Supplementary Figure 9), as previously observed with RBPMS2 overexpression. Conversely, Noggin overexpression did not affect the proliferation rate of primary SMCs, as indicated by the absence of significant variations in the number of PH3-positive RCAS-Noggin cells in comparison with control cells (RCAS-empty) (Figure 5C, 7 days of infection). These experiments show a common repressive action of RBPMS2 and Noggin on SMC differentiation, but divergent effects on the proliferative rate of primary cultured SMCs.

Noggin inhibits BMP signaling by interfering with homodimerization or heterodimerization of BMP ligands, thus blocking their interaction with receptors and preventing their activation.25 Because our results suggest that the RBPMS2 effect on calponin expression in SMCs is mediated through inhibition of the BMP pathway via Noggin induction, we assessed the impact of RBPMS2 misexpression on SMC differentiation in the presence or absence of BMP4, the most strongly expressed BMP ligand in the gastrointestinal musculature.2,16,19 As previously observed in vascular SMC cultures, addition of BMP4 for 4 days increased differentiation of primary SMCs (data not shown).19 Addition of 20 ng/mL BMP4 to RCAS-RBPMS2-infected SMCs restored calponin expression in the uninfected neighboring cells, but not in the infected cells (Figure 5D, right panel, and Figure 5E for quantification). Indeed, RBPMS2 overexpression in SMCs decreases calponin expression in infected (Myc-positive) and uninfected (Myc-negative) neighboring cells (Figures 3C and 5D, left panel), suggesting that RBPMS2 acts both in an autocrine and paracrine manner. QPCR analysis confirmed that BMP4 addition to cells infected with RCAS-RBPMS2 restored expression of both calponin and SM22 (Figure 5F).

These experiments show that Noggin hinders SMC differentiation downstream of RBPMS2.

**RBPMS2 Transcripts Are Highly Expressed in Visceral Smooth Muscles of Patients With CIPO**

Intestinal motility disorders in infants comprise many heterogeneous diseases that are classified as gastrointestinal neuromuscular disorders and have clinical
symptoms ranging from simple constipation to intestinal occlusion.\textsuperscript{4,5} Recently, specific smooth muscle defects were shown to be involved in the pathogenesis of pediatric digestive motility disorders.\textsuperscript{7} Because our findings suggest that RBPMS2 might be involved in visceral SMC development and differentiation, we analyzed the expression of RBPMS2 transcripts in colon biopsy specimens from pediatric patients with a history of chronic constipation associated with megacystis (CIPO), or aganglionosis (HSCR) and from neonates without digestive motility disorders (controls). Histologic analysis revealed the presence of regular circular and longitudinal smooth muscle layer in each case. Vacuolization of smooth muscles was observed in the circular smooth muscle of CIPO patients, a finding characteristic of visceral myopathy\textsuperscript{4} (Figure 6, upper panels, inset, and arrowheads). In situ hybridization showed that RBPMS2 transcripts were strongly expressed only in circular smooth muscle of colon biopsy specimens from patients with CIPO, whereas its expression was significantly lower or absent in controls and in patients with HSCR (Figure 6, lower panels). Conversely, RBPMS2 transcripts were expressed in ENS of colon sections from control neonates and patients with CIPO.
but not in patients with HSCR (owing to congenital absence of ganglia) (Figure 6).

These results suggest that visceral myopathies are associated with abnormal *RBPMS2* transcript expression in visceral smooth muscles.

**Discussion**

In this work, we investigated the expression and function of the RNA-binding protein *RBPMS2* in the developing chick gastrointestinal tract. *RBPMS2* expression in the chick gastrointestinal mesenchymal layer is regulated temporally because it is high at E4–E6 and then progressively is reduced at a later stage. This dynamic expression pattern corresponds to the progression of visceral undifferentiated mesenchymal cells into differentiated SMCs. Only few genes have such a dynamic expression pattern in visceral SMC precursors. αSMA is expressed as early as *RBPMS2*, but then is maintained also in differentiating SMCs, when *calponin*, *SM-MHC*, and *myocardin* also are expressed. These data identify *RBPMS2* as a marker of undifferentiated visceral SMCs.

We then show that sustained *RBPMS2* expression hinders visceral SMC differentiation in vivo and in SMC primary cultures through up-regulation of *Noggin* expression that leads to inhibition of the BMP signaling pathway. Previous studies showed that exogenous stimulation of BMP activity could prevent dedifferentiation of vascular SMCs in cell culture, suggesting that BMP activation is essential for modulating the vascular SMC phenotype.\(^{19}\) Our findings confirm this observation in vivo and suggest that *Noggin* is a key regulator during visceral SMC development and also may be involved in the phenotypic regulation of SMCs in culture (Figure 7).

We also found differences between the action of *RBPMS2* and *Noggin* mainly in SMC primary cultures. Indeed, *RBPMS2*, but not *Noggin*, overexpression induced SMC proliferation and transient repression of αSMA expression. Therefore, in addition to the Noggin-BMP axis, *RBPMS2* might regulate other pathways that contribute to the dedifferentiation and increased proliferation of visceral SMCs, thus dissociating the effect on proliferation from the effect on differentiation.

We show that *RBPMS2* and *Noggin* overexpression in differentiated SMCs hinders their differentiation associated with myocardin up-regulation. Indeed, recently, Yin et al.\(^{22}\) reported that myocardin accumulation inhibits its

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**Figure 6.** *RBPMS2* transcript expression is abnormally high in smooth muscles from colon specimens of patients with CIPO. H&E staining of colon sections from pediatric patients with CIPO, HSCR, and from neonates without digestive motility disorders (controls) (upper panels). The visceral musculature is composed of circular smooth muscles (CM) and longitudinal smooth muscles (LM). High-magnification inset of the CIPO circular muscle layer shows extensive vacuolization (arrowheads). In situ hybridization using an anti-human *RBPMS2* riboprobe of consecutive sections from the same specimens (lower panels). Arrows and red arrows indicate staining in ganglia cells and in the circular smooth muscle layer, respectively.

**Figure 7.** Model of the gastrointestinal SMC development regulated by the *RBPMS2* and BMP pathways. *RBPMS2* regulates the early stage of SMC differentiation through the control of the expression of the BMP inhibitor *Noggin* and the inhibition of contractile proteins.
own function and that proteosomal degradation of myocardin is required for its full transcriptional activity. Similarly, we show that myocardin accumulation results in a reduction of calponin expression (a marker of differentiated SMCs). In addition, RBPM2 and Noggin misexpression led to up-regulation of the SRF target gene FHHL2, which interacts with SRF and inhibits induction of smooth muscle contractile genes by SRF.\(^\text{21}\) Altogether, these results support the notion that activation of the RBPM2/Noggin pathway inhibits SMC contractile genes through functional alteration of the myocardin/SRF pathway.

In conclusion, we show that, in chick embryos, RBPM2 is expressed during the early stages of visceral SMC development and that its expression is progressively lost during differentiation of visceral smooth muscles. Ectopic expression of RBPM2 in primary culture of differentiated SMCs triggers an increase of their proliferative rate and hinders their contractile function, which also was observed at the organ level. Our findings show that regulated expression of RBPM2 is important for the correct development and differentiation of visceral SMCs. We then found that RBPM2 transcript expression was significantly higher in circular smooth muscle cells from colon specimens of pediatric patients with CIPO (digestive dysmotility syndrome in the absence of physical obstruction of the bowel),\(^\text{4–7,26}\) whereas its expression was very low or absent in specimens from patients without digestive motility disorders or with HSCR (a developmental ENS disorder). Some investigators have reported abnormal architecture of the tunica muscularis of colon specimens from patients with CIPO, suggesting a potential primary alteration of the visceral smooth muscles.\(^\text{6,7}\) In summary, we identified RBPM2 as a new marker of visceral SMC remodeling that could be useful for the characterization of smooth muscle alteration in visceral myopathies. Future studies are needed to investigate the potential function of RBPM2 in CIPO etiopathogenesis, particularly the alteration in the tunica muscularis.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of the article.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Western Blotting

For protein immunoblotting, protein extracts (10 μg) were separated on 10% polyacrylamide gels (Bio-Rad Laboratories, France) and then blotted on nitrocellulose membranes. Membranes were incubated with primary antibodies (anti-Myc from Ozyme [Montigny-Le-Bretonneux, France], anti-avian calponin from Sigma-Aldrich, anti-phosphoSMAD1 from Cell Signaling [Danvers, MA], anti-myocardin from Santa Cruz Biotechnology [Santa Cruz, CA], anti-glyceraldehyde-3-phosphate dehydrogenase from Sigma-Aldrich, and anti-total AKT and anti-P-AKT from Cell Signaling) overnight and then with the relevant horseradish peroxidase-conjugated secondary antibodies. Detection was performed by chemiluminescence on Kodak films. Glyceraldehyde-3-phosphate dehydrogenase expression was used to confirm equal loading.

In Situ Hybridization and Immunodetection

Immunofluorescence and immunohistochemistry experiments with chick and human paraffin-embedded sections were performed as described. For immunodetection, anti-αSMA (Sigma-Aldrich), anti-Myc (Ozyme), anti-avian calponin (Sigma-Aldrich), anti-HuC/D (Invitrogen, France), anti-desmin (Euromedex, Mundolsheim Cedex, France), and anti-phospho-histone H3-Ser10 (Millipore, Molsheim, France) antibodies were used. Nuclei were stained with Hoechst (Invitrogen). H&E staining was performed using standard procedures. In situ hybridization experiments using dissected gut or paraffin sections were performed as described. Anti-sense riboprobes were generated by PCR amplification using specific primer sets and subcloned. The following chick templates were used: αSMA, Env, RBPMS2, BARX1, SHH, and Noggin. Human RBPMS2 complementary DNA was isolated, sequenced, and used to prepare riboprobes for in situ hybridization. Images were acquired using a Nikon AZ100 stereomicroscope and a Carl-Zeiss AxioImager microscope.

Microarray Experiments and QPCR

Total RNAs were extracted from SMC primary cultures with the HighPure RNA Isolation kit (Roche Diagnostic, France) and reverse transcription was performed as described. For microarray experiments, resulting complementary DNAs were biotinylated and hybridized to Affymetrix GeneChip Chicken Genome Arrays (Santa Clara, CA) following the manufacturer’s protocols (IRB, CHRU Montpellier, France). For QPCR, gene expression levels were measured using LightCycler technology (Roche Diagnostics). PCR primers (Supplementary Table 1) were designed using the LightCycler Probe Design software 2.0. Each sample was assayed from 3 independent experiments performed in triplicate. Expression levels were determined with the LightCycler analysis software (version 3.5) relative to standard curves. Data were represented as the mean level of gene expression relative to the expression of the reference gene ubiquitin. Data were analyzed using the Student t test and results were considered significant when the P value was less than .05 (*), P < .01 (**), or P < .001 (***) in Figures 3 and 5 and Supplemental Figures 4, 8, and 10.
Supplementary Figure 1. Efficiency and tropism of avian retroviruses on differentiated SMC primary cultures. (A) Enteric neurons are not targeted by the retroviral misexpression system used in this study. Immunofluorescence analysis of SMC primary cultures prepared from E15 gizzard muscles infected with Myc-tagged RBPMS2 (RCAS-RBPMS2) or RCAS-empty retroviruses for 3 days. RBPMS2 retroviruses targeted exclusively visceral SMCs as indicated by Myc expression. Ectopic RBPMS2 expression had no indirect effect on the formation of the enteric plexus and on neuron differentiation as observed using anti-HuC/D antibodies. Scale bar, 20 μm. (B) Immunofluorescence analysis of primary cultured SMCs infected with high titers of RCAS-RBPMS2 or RCAS-empty retroviruses for 3 days. Nuclei were visualized with Hoechst. Anti-Myc antibodies were used to identify cells infected by RCAS-RBPMS2 retroviruses. Scale bar, 20 μm.

Supplementary Figure 2. αSMA expression in the chick gastrointestinal system. Whole-mount in situ hybridization of dissected E5, E6, and E9 chick gastrointestinal tracts using an anti-sense riboprobe directed against αSMA. αSMA is expressed in the whole mesenchymal gastrointestinal tract with no difference along the anteroposterior axis in the developing and differentiated visceral smooth musculature.
**Supplementary Figure 3.** Sustained RBPMS2 expression strongly inhibits calponin expression. Western blot analysis of protein extracts from RCAS-Myc-RBPMS2 misexpressing stomachs (E7) compared with control organs (RCAS-GFP). A total of 10 μg of whole protein extracts were processed for each condition. Chick calponin was detected with anticalponin antibodies (Sigma-Aldrich). The right panel shows the quantification of the Western blot data.

**Supplementary Figure 4.** Impact of RBPMS2 and Noggin sustained expression on visceral SMC differentiation and proliferation in vivo. (A) Serial transversal sections from E9 chick stomachs infected with RCAS-GFP, RCAS-RBPMS2, or RCAS-Noggin retroviruses were analyzed by immunofluorescence. Differentiated SMCs were identified with anticalponin antibodies and mitotic cells with anti-PH3 antibodies. Sustained RBPMS2 and Noggin expression inhibit calponin expression. (B) Quantification of mitotic cells using the mitotic marker PH3 in E9 chick stomachs infected with RCAS-GFP, RCAS-RBPMS2, or RCAS-Noggin retroviruses by immunofluorescence. Values are the mean ± SEM of 3 independent experiments. Statistical analysis using the Student t test confirmed the significant increase (1.55-fold; *P < .05) of PH3-positive cells upon RBPMS2 misexpression. The 1.37-fold induction after Noggin misexpression was not statistical significant.
Supplementary Figure 5. Sustained RBPMS2 expression alters chick colonic SMC differentiation. (A) E7 colons after retroviral misexpression of RCAS-empty (control) (left panel) or of RCAS-RBPMS2 and RCAS-GFP (right panels). The presence of retroviruses was confirmed by direct observation of GFP expression (right panel; green arrows indicate the colon). (B) Serial cross-sections of E7 colons infected with RCAS-empty (control; upper panels) or RCAS-RBPMS2 retroviruses (lower panels) analyzed by immunofluorescence. Nuclei were visualized with Hoechst. Antibodies directed against αSMA and calponin were used. Anti-GFP antibodies were used to confirm infection of the colon musculature. Sustained expression of RBPMS2 specifically in the colon resulted in no morphologic alterations in comparison with controls. In addition, RBPMS2 misexpression did not affect the determination of colon SMCs because αSMA-positive cells still were observed in the smooth muscle layer. Conversely, it inhibited calponin expression in comparison with control colons, indicating that RBPMS2 misexpression hinders SMC differentiation also in the developing colon.
Supplementary Figure 6. Time-line study of RBPMS2 overexpression in primary cultures of differentiated SMCs. Immunofluorescence analysis of primary SMCs infected with RCAS-empty (left panels) or RCAS-RBPMS2 (right panels) retroviruses for 1 (D1), 2 (D2), and 3 (D3) days. Nuclei were visualized with Hoechst. Antibodies directed against SMC markers: αSMA (upper panels) and calponin (lower panels) were used. Anti-Myc antibodies were used to identify cells infected by RCAS-RBPMS2 retroviruses. RBPMS2 misexpression inhibited first the expression of calponin (after 2 days), and after 3 days αSMA expression also was down-regulated. Scale bar, 20 μm.

Supplementary Figure 7. Immunofluorescence analysis of SMC primary cultures prepared from E15 gizzard muscles and infected with Myc-tagged RBPMS2 (RCAS-RBPMS2) retroviruses for 3 days. In RBPMS2-overexpressing cells, calponin expression is inhibited.
Supplementary Figure 8. Expression of mRNA levels coding for different protein partners of myocardin/SRF that modulate myocardin/SRF function during visceral SMC differentiation analyzed by QPCR in primary cultured SMCs infected with RCAS-empty, RCAS-RBPMS2, or RCAS-Noggin retroviruses for 7 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-RBPMS2 and RCAS-Noggin vs RCAS-empty). We confirmed the up-regulation of Myocardin mRNAs upon RBPMS2 and Noggin overexpression. We then found that Noggin overexpression increased Kruppel-like factor 4 (KLF4) mRNA expression by 1.5-fold. Conversely, Noggin and RBPM52 overexpression did not affect Forkhead box O4 (FOXO4) and C terminus of Hsc70-Interacting Protein (CHIP) expression, whereas it increased by more than 2-fold the level of FHL2 mRNA. ***P < .001.
Supplementary Figure 9. Accumulation of myocardin protein in visceral SMC inhibits its function. (A) Western blot analysis of primary SMC cultures after Myocardin and RBPM52 or Noggin ectopic expression. Primary differentiated SMCs were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and cotransfected with JetPEI (Polyplus, France) with 2.5 μg of a mouse myocardin construct and 2.5 μg of empty or RBPM52 or Noggin retroviral constructs and analyzed after 48 hours. A total of 15 μg of whole protein extracts were processed for each condition. Myocardin (95 kilodaltons) was detected with the H300 antibody. Co-expression of Myocardin and RBPM52 or Noggin results in accumulation of Myocardin (lanes 3 and 4). Right panel shows the quantification of the Western blot data. (B) Western blot analysis of primary SMC cultures after proteasomal inhibition with 5 μmol/L MG132. Primary differentiated SMCs were grown in DMEM supplemented with 10% FBS and transfected, or not, with 5 μg of a mouse myocardin construct and analyzed after 48 hours. MG132 (proteasome inhibitor) was added to the medium for 12 hours after 36 hours of transfection. A total of 15 μg of whole protein extracts were processed for each condition. SMCs cultured in 10% FBS express low level of calponin (lane 1) that is not changed by incubation with MG132 (lane 2). Ectopic expression of Myocardin induces an increase of calponin expression (lane 3). Incubation with MG132 leads to myocardin accumulation, but reduces calponin expression (lane 4). Right panel shows the quantification of the Western blot data.
Supplementary Figure 10. Analysis of cholinergic receptor muscarinic 3 (CHRM3) and calponin mRNA expression by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-RBPMS2 retroviruses for 7 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-RBPMS2 vs RCAS-empty). *P < 0.05. CHRM3 mRNA level was decreased by 56% and calponin mRNA level by 67% in RBPMS2-overexpressing SMCs compared with control SMCs.

Supplementary Figure 11. Noggin expression in the chick gastrointestinal (GI) system. Whole-mount in situ hybridization of dissected E6 and E9 chick GI tracts using an antisense riboprobe directed against Noggin. Early expression of Noggin is observed in the mesenchyme of the GI tract at E6.

Supplementary Figure 12. Western blot analysis with anti-Myc antibodies after immunoprecipitation to assess the interaction between RBPMS2 and Noggin. Myc-tagged RBPMS2 in infected DF-1 cells was immunoprecipitated with anti-Myc antibodies bound to protein A Sepharose beads in the presence of total RNA from E6 chick gut. Fifty percent of beads were analyzed by Western blotting to detect Myc-tagged proteins. In the input and positive experiment lines a 26-kilodalton band corresponding to Myc-tagged RBPMS2 was observed.
### Supplementary Table 1. Gene Specific Chick (c) Primers Used for QPCR

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*CHRM3, cholinergic receptor muscarinic 3.*