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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR TREATMENT OF CHRONIC INTESTINAL PSEUDO-OBSTRUCTION

(57) Abstract: The present invention relates to methods and compositions for the treatment of chronic intestinal pseudo-obstruction.

METHODS AND PHARMACEUTICAL COMPOSITIONS FOR TREATMENT OF CHRONIC INTESTINAL PSEUDO-OBSTRUCTION

FIELD OF THE INVENTION:

The present invention relates to methods and compositions for the treatment of chronic intestinal pseudo-obstruction.

BACKGROUND OF THE INVENTION:

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 (4, 6). Until now, dysfunction of the downstream effector (visceral smooth muscle) rarely was investigated in gastrointestinal neuromuscular disorders (4, 6, and 7). Specifically, muscular lesions, classified as visceral myopathies, have been observed mainly in patients with chronic intestinal pseudo-obstruction (CIPO). 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. Consequently, it is crucial to develop new drugs that will be suitable for the treatment of chronic intestinal pseudo-obstruction (CIPO). In this way, it has been suggested that characterisation of new therapeutic targets in chronic intestinal pseudo-obstruction (CIPO) may be highly desirable.

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 (SMMHC), which precedes contractile function (8). Only a few studies have investigated the molecular mechanisms controlling visceral mesenchyme differentiation into SMCs. Endodermal hedgehog signaling activation promotes visceral mesenchyme growth through expression of the *Patched* receptor and of *Bmp4* that drives its differentiation into SMCs (9). Aberrant modulation of BMP activity induces visceral smooth muscle disorganization (10). 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 (11). 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 final 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 (12). 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 (13). Moreover, postregulatory RNA events are involved in the control of differentiation and remodeling of smooth muscle tissues, such as heart and vessels(14), suggesting that visceral smooth muscle development and plasticity could be regulated similarly. Moreover, *RNA-binding protein for multiple splicing 2* (*RBPMS2*), a member of the RRM family, is expressed in vertebrate heart and gastrointestinal tract (13, 15).

SUMMARY OF THE INVENTION:

The present invention relates to a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors for use in the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof.

The present invention also relates to a method of identifying a subject having a chronic intestinal pseudo-obstruction (CIPO) which comprises the step of analyzing a biological sample from said subject for determining the RBPMS2 expression level.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors 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. The inventors used avian replication-competent retroviral misexpression approaches to analyze the function of RBPMS2 in vivo and in primary cultures of chicken SMCs. The inventors analyzed the expression of *RBPMS2* transcripts in colon samples from pediatric patients with Hirschsprung's disease and patients with chronic pseudo obstruction syndrome (CIPO) with megacystis. The inventors surprisingly demonstrated that *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, reduced their contractility by up-regulating expression of *Noggin*, which reduced activity of bone morphogenetic protein signaling pathway, and increased their proliferation. Visceral smooth muscles from pediatric patients with CIPO expressed high levels of *RBPMS2* transcripts, compared with smooth muscle from patients without this disorder. Alterations in RBPMS2 function is involved in digestive motility disorders, particularly those characterized by the presence of muscular lesions (visceral myopathies).

Therapeutic methods and uses

Accordingly, the present invention relates to a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors for use in the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof.

As used herein, the term "subject" denotes a mammal. In a preferred embodiment of the invention, a subject according to the invention refers to any subject (preferably human) afflicted with chronic intestinal pseudo-obstruction (CIPO) or digestive motility disorders, particularly those characterized by the presence of muscular lesions (visceral myopathies).

The method of the invention may be performed for any type of chronic intestinal pseudo-obstruction (CIPO). The term "Chronic intestinal pseudo-obstruction" or "CIPO" as used herein refers to digestive motility disorders (visceral myopathies) that occurs in the

gastrointestinal (GI or digestive) tract. Chronic intestinal pseudo-obstruction (CIPO) refers to a rare and highly morbid syndrome characterized by impaired gastrointestinal propulsion together with symptoms and signs of bowel obstruction in the absence of any lesions occluding the gut lumen.

As used herein, the term "RBPMS2" has its general meaning in the art and refers to RNA-Binding Protein for Multiple Splicing-2 (SEQ ID NO:1) (Notarnicola et al., 2012). The term "RBPMS2" refers to the RNA-Binding Protein RBPMS2, an early marker of SMC precursor cells and that ectopic expression of RBPMS2 in differentiated SMCs conducts to their dedifferentiation and triggers their proliferation.

The term "expression" when used in the context of expression of a gene or nucleic acid refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include messenger RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins (e.g., RBPMS2) modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, myristilation, and glycosylation.

An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene.

The term "RBPMS2 antagonist" refers to a compound that selectively blocks or inactivates the RBPMS2. The term "RBPMS2 antagonist" also refers to a compound that selectively blocks the binding of RBPMS2 to RNAs via its RRM domain. The term "RBPMS2 antagonist" also refers to a compound that selectively blocks RBPMS2 binding to Noggin, inducing Noggin up-regulation and then inhibiting BMP signalling. The term "RBPMS2 antagonist" also refers to a compound that is a RBPMS2 dimerization inhibitor. In one embodiment, the RBPMS2 antagonist of the invention is a RBPMS2 dimerization inhibitor. As used herein, the term "selectively blocks or inactivates" refers to a compound that preferentially binds to and blocks or inactivates RBPMS2 with a greater affinity and potency, respectively, than its interaction with the other sub-types or isoforms of the RBPMS family. Compounds that prefer RBPMS2, but that may also block or inactivate other nuclear

receptor sub-types, as partial or full antagonists, are contemplated. Typically, a RBPMS2 antagonist is a small organic molecule, a peptide, a polypeptide, an aptamer or an intra-antibody.

As used herein, the term "RBPMS2 dimerization inhibitor" refers to a compound that selectively prevents or blocks RBPMS2 dimerization. The term "RBPMS2 dimerization inhibitor" refers to a compound that targets the residue Leucine 49 of RBPMS2 protein and blocks RBPMS2 dimerization. The term "RBPMS2 dimerization inhibitor" also refers to a compound that targets the RRM-homodimeriztion motif (residues 47-50 of the SEQ ID NO:1). Typically, a RBPMS2 dimerization inhibitor is a small organic molecule, a peptide, a polypeptide, an aptamer or an intra-antibody.

In another embodiment, the RBPMS2 antagonist of the invention is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996). Then after raising aptamers directed against RBPMS2 of the invention as above described, the skilled man in the art can easily select those inhibiting RBPMS2 dimerization or inhibiting RBPMS2.

In one embodiment, the compound of the invention is an inhibitor of RBPMS2 expression.

Inhibitors of RBPMS2 expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of RBPMS2 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of RBPMS2 proteins, and thus activity, in a

cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding RBPMS2 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically alleviating gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of RBPMS2 expression for use in the present invention. RBPMS2 gene expression can be reduced by contacting the subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that RBPMS2 expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of RBPMS2 expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RBPMS2 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of RBPMS2 expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively,

anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing RBPMS2. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging

cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Cliffton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold

particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a particular embodiment, the compound of the invention may be administered sequentially or concomitantly with one or more muscular differentiation factors.

The term "muscular differentiation factor" as used herein refers to any muscular differentiation factor known to one of skill in the art to be effective to induce smooth muscle cell differentiation. For instance, the muscular differentiation factors include but are not limited to bone morphogenetic protein such as BMP4, SRF and its co-factor Myocardin, α-smooth muscle actin (αSMA), smooth muscle protein-22 (SM22), calponin, smoothelin, and smooth muscle myosin heavy chain (SMMHC).

In one embodiment, the present invention relates to a method of treating chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof, comprising the step of administering to said subject a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors.

Pharmaceutical composition

The compound of the invention may be used or prepared in a pharmaceutical composition.

In one embodiment, the invention relates to a pharmaceutical composition comprising the compound of the invention and a pharmaceutical acceptable carrier for use in the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof.

Typically, the compound of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable

carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The compound of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from

inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the

condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

Pharmaceutical compositions of the invention may include one or more muscular differentiation factors.

In one embodiment, said additional muscular differentiation factors may be contained in the same composition or administrated separately for simultaneous, separate or sequential use in the treatment of chronic intestinal pseudo-obstruction (CIPO).

Screening method

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof, wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that are antagonists or inhibitors of expression of RBPMS2.

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof, wherein the method comprises the steps of:

(i) providing a RBPMS2, providing a cell, tissue sample or organism expressing the RBPMS2,

(ii) providing a candidate compound such as small organic molecule, peptide, polypeptide, aptamer or intra-antibodies,

(iii) measuring the activity of the RBPMS2,

(iv) and selecting positively candidate compounds that blocks RBPMS2 dimerization, inhibits RBPMS2 activity by blocking the binding of RBPMS2 to RNAs via its RNA recognition motif (RRM) domain or blocks RBPMS2 binding to Noggin inducing Noggin up-regulation and then inhibiting BMP signalling.

Methods for measuring the activity of the RBPMS2 are well known in the art. For example, measuring the RBPMS2 activity involves measuring RBPMS2 dimerization level on the RBPMS2 cloned and transfected in a stable manner into a CHO cell line, human embryonic kidney (HEK) cell line or human CIPO cell line, measuring Noggin expression level, measuring the expression level of contractile proteins, determining the hypertrophic phenotype or measuring SMC contractility level in the presence or absence of the candidate compound.

Tests and assays for screening and determining whether a candidate compound is a RBPMS2 antagonist or RBPMS2 expression inhibitor are well known in the art. In vitro and in vivo assays may be used to assess the potency and selectivity of the candidate compounds to reduce RBPMS2 activity.

Activities of the candidate compounds, their ability to bind RBPMS2 and their ability to inhibit RBPMS2 activity may be tested using isolated smooth muscle cells (SMC) expressing RBPMS2, CHO cell line, human embryonic kidney cell line (HEK) or human CIPO cell line cloned and transfected in a stable manner by the human RBPMS2.

Cells and smooth muscle cells expressing another RNA-binding protein than RBPMS2 may be used to assess selectivity of the candidate compounds.

In one embodiment, the present invention relates to a method of screening a candidate compound for use as a drug for the treatment of chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof, wherein the method comprises the steps of:

i) providing a polypeptide comprising amino acid residues 47-50 of the SEQ ID NO:1,

ii) providing a candidate compound such as small organic molecule, intra-antibodies, peptide or polypeptide,

iv) measuring the binding of the candidate compound to the polypeptide of step i) using appropriate biophysical techniques,

v) and positively selecting candidate compounds that bind to the polypeptide of step i).

Typically, the candidate compound bind to the amino acid residues 47-50 of the SEQ ID NO:1 of the polypeptide and blocks polypeptides dimerization.

Methods for measuring the binding of the candidate agent to the polypeptide comprising amino acid residues 47-50 of the SEQ ID NO:1 are well known in the art. For example, measuring the binding of the candidate agent to said polypeptide may be performed by biophysical techniques such as binding tests and crystallography.

Diagnostics methods

A further aspect of the invention relates to a method of identifying a subject having a chronic intestinal pseudo-obstruction (CIPO) which comprises the step of analyzing a biological sample from said subject for:

(i) determining the RBPMS2 expression level,

(ii) comparing the RBPMS2 expression level in the sample with a reference value,

(iii) detecting differential in the RBPMS2 expression level between the sample and the reference value is indicative of a subject having a chronic intestinal pseudo-obstruction (CIPO).

Analyzing the RBPMS2 expression level may be assessed by any of a wide variety of well-known methods for detecting expression of a transcribed nucleic acid or translated protein.

In a preferred embodiment, the RBPMS2 expression level is assessed by analyzing the expression of mRNA transcript or mRNA precursors, such as nascent RNA, of RBPMS2 gene. Said analysis can be assessed by preparing mRNA/cDNA from cells in a biological sample from a subject, and hybridizing the mRNA/cDNA with a reference polynucleotide. The prepared mRNA/cDNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses, such as quantitative PCR (TaqMan), and probes arrays such as GeneChip(TM) DNA Arrays (AFFYMETRIX).

Advantageously, the analysis of the expression level of mRNA transcribed from the gene encoding for RBPMS2 involves the process of nucleic acid amplification, e. g., by RT-PCR (the experimental embodiment set forth in U. S. Patent No. 4,683, 202), ligase chain reaction (Barany, 1991), self sustained sequence replication (Guatelli et al., 1990),

transcriptional amplification system (Kwoh et al., 1989), Q-Beta Replicase (Lizardi et al., 1988), rolling circle replication (U. S. Patent No. 5,854, 033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

In another preferred embodiment, the RBPMS2 expression level is assessed by analyzing the expression of the protein translated from said gene. Said analysis can be assessed using an antibody (e.g., a radio-labeled, chromophore- labeled, fluorophore-labeled, or enzyme-labeled antibody), an antibody derivative (e.g., an antibody conjugate with a substrate or with the protein or ligand of a protein of a protein/ligand pair (e.g., biotin-streptavidin)), or an antibody fragment (e.g., a single-chain antibody, an isolated antibody hypervariable domain, etc.) which binds specifically to the protein translated from the gene encoding for RBPMS2.

Said analysis can be assessed by a variety of techniques well known from one of skill in the art including, but not limited to, enzyme immunoassay (EIA), radioimmunoassay (RIA), Western blot analysis and enzyme linked immunoabsorbant assay (RIA).

A reference value can be a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. Preferably, the person skilled in the art may compare the RBPMS2 expression levels (obtained according to the method of the invention) with a

defined threshold value. In one embodiment of the present invention, the threshold value is derived from the RBPMS2 expression level (or ratio, or score) determined in a biological sample derived from one or more subjects having a chronic intestinal pseudo-obstruction (CIPO). Furthermore, retrospective measurement of the RBPMS2 expression levels (or ratio, or scores) in properly banked historical subject samples may be used in establishing these threshold values.

In one embodiment of the invention, the reference value may consist in expression level measured in a biological sample associated with a healthy subject not afflicted with chronic intestinal pseudo-obstruction (CIPO) or in a biological sample associated with a subject afflicted with chronic intestinal pseudo-obstruction (CIPO).

According to the invention, high RBPMS2 expression level is indicative of subject having a chronic intestinal pseudo-obstruction (CIPO) and low RBPMS2 expression level is indicative of subject not having a chronic intestinal pseudo-obstruction (CIPO).

The present invention also relates to a method of treating chronic intestinal pseudo-obstruction (CIPO) in a subject in need thereof comprising the steps of:

i) providing a sample from said subject,

ii) determining the expression level of RBPMS2 in the biological sample obtained at step i),

iii) comparing said expression level measured in step ii) with a reference value, wherein high expression level of RBPMS2 is indicative of subject having a chronic intestinal pseudo-obstruction (CIPO), and

iv) treating said subject having a chronic intestinal pseudo-obstruction (CIPO) with a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors.

RBPMS2 sequence

SEQ ID NO:1 for RBPMS2 sequence

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MSNLKPDGEH GGSTGTGSGA GSGGALEEEV RTLFVSGLPV DIKPRELYLL FRPFKGYEGS      60
LIKLTARQPV GFVIFDSRAG AEAAKNALNG IRFDPENPQT LRLEFAKANT KMAKSKLMAT     120
PNPSNVHPAL GAHFIARDPY DLMGAALIPA SPEAWAPYPL YTTELTPAIS HAAFTYPTAT     180
AAAAALHAQV RWYPSSDTTQ QGWKYRQFC                                       209
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The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Sustained *RBPMS2* expression alters chick gastrointestinal development, SMC differentiation, and contractile function.

Measurement of contractile activity in isolated E15 gizzards. Typical recording showing stimulation with 10^{-6} to 10^{-4} mol/L carbachol in normal and *RBPMS2*-overexpressing organs (*left panels*). Maximum tension after 2 minutes of 10^{-4} mol/L carbachol stimulation in normal (n = 23) and *RBPMS2*-overexpressing (n = 21) gizzards (*right panel*). Values are the mean ± standard error of the mean.

Figure 2: *RBPMS2* overexpression hinders SMC differentiation and ability to contract.

(A) Gene expression analysis by QPCR in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 3 days. Normalized expression levels were converted to fold changes ± standard deviation (RCAS-*RBPMS2* vs RCAS-empty). ***P*< .01; ****P*< .001.

(B) Quantification of mitotic cells using anti-PH3 antibodies in primary cultured SMCs infected with RCAS-empty or RCAS-*RBPMS2* retroviruses for 7 days. Values are the mean ± standard error of the mean of 2 independent experiments. ***P*< .01.

Figure 3: *RBPMS2* overexpression hinders SMC ability to contract.

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* < .05. *CHRM3* mRNA level was decreased by 56% and *calponin* mRNA level by 67% in *RBPMS2*-overexpressing SMCs compared with control SMCs.

Figure 4: RBPMS2 inhibits BMP signaling through *Noggin* up-regulation.

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.

Figure 5: The impact of *RBPMS2* misexpression on SMC differentiation in the presence or absence of BMP4.

(A) Quantification of calponin-positive cells in primary cultured SMCs infected with RCAS-*RBPMS2* retroviruses in the presence or not of BMP4 for 4 days.

(B) QPCR analysis of gene expression in primary SMCs infected with RCAS-*RBPMS2* retroviruses for 7 days alone or in the presence of BMP4. Normalized expression levels then were converted to fold changes ± standard deviation (treatment vs RCAS-*RBPMS2*). $*P < .05$ and $***P < .001$ both compare with RCAS-*RBPMS2*.

EXAMPLE 1:

Material & 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 (16).

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 (17). 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 (11,16). 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 (10,16). 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 (18). Briefly, the tunica muscularis 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). 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. 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 muscarinergic agonist, as previously published (18). 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 (11). 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 $CaCl_2$ continuously bubbled with 95% O_2/5% CO_2 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^{-4} mol/L carbachol concentration.

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 (11, 17). For immunodetection, anti-αSMA (Sigma-Aldrich), anti-Myc (Ozyme), antiavian 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 (11, 16). 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* (24). 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 (11). 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) (11). For QPCR, gene expression levels were measured using LightCycler technology (Roche Diagnostics). PCR primers 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 $P < .001$ (***).

Results

Dynamic Expression of RBPMS2 in Chick Gastrointestinal Mesenchyme

To determine the role of RBPMS2 in the digestive visceral smooth muscle, first the inventors analyzed its expression during chick gastrointestinal tract development. *RBPMS2* started to be expressed at embryonic day 4 (E4), an early stage of gastrointestinal tract development, in the regions of the future stomach, midgut, and colon. At E5, *RBPMS2* was expressed strongly in the developing stomach, small intestine, and colon, with the exception of the cecum. Additional expression was observed in the developing lungs. At E6, *RBPMS2* transcripts accumulated in the stomach, small intestine, and colon. *RBPMS2* expression was temporally and spatially comparable with that of α*SMA*, the earliest known SMC marker, which is expressed in the developing and differentiated visceral smooth musculature. By using paraffin sections the inventors showed that, at E6, *RBPMS2* and αSMA expression overlapped in the undifferentiated visceral mesenchyme. 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. 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, the inventors 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. Specifically, the proventriculus, which is the glandular part of the chick stomach, was hypertrophied, whereas the gizzard, the muscular part, was denser and malformed in comparison with controls that overexpressed GFP alone. 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. Similarly, the determination of visceral SMCs was not affected because positive αSMA cells still were observed in the smooth muscle layer; however, SMC differentiation was hindered as indicated by the reduction of calponin expression in *RBPMS2* misexpressing stomachs in comparison with GFP controls. HuC/D-positive neurons were organized into well-defined plexuses, suggesting that *RBPMS2* misexpression did not induce detectable changes in ENS migration and

differentiation. Finally, the SMC proliferation rate was 1.55-fold higher in *RBPMS2* misexpressing stomachs than in GFP controls, 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. 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, inventors 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 1). 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, the inventors 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 (18, 19). Control primary cultured SMCs were spindle-shaped and homogenously expressed αSMA and calponin, 2 SMC contractile markers, in highly organized filament bundles. 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, in SMCs infected with Myc-tagged *RBPMS2* their expression was lost. However, Mycpositive cells still expressed the mesenchymal marker Desmin, 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 2A). Analysis of the effect of *RBPMS2* overexpression on *serum response factor* (*SRF*) and its co-activator *Myocardin*, which control numerous steps of SMC differentiation (20), indicated that *Myocardin* mRNA was up-regulated (2-fold), whereas *SRF* expression was not significantly affected (Figure 2A). Similarly, *four and a half LIM domains 2* (*FHL2*) mRNA, which inhibits the induction of smooth muscle contractile genes regulated by SRF (21), was up-regulated (Figure 2A). To analyze the impact of ectopic *RBPMS2* expression on Myocardin protein level, the inventors 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. Because myocardin accumulates in response to proteasome inhibition by MG132 (22), the inventors transfected primary cultured SMCs with a plasmid encoding for myocardin with or without 5 μmol/L MG132. Ectopic myocardin expression increased calponin expression. Moreover, in accordance with published work (22), proteasome inhibition by MG132 induced accumulation of myocardin, but reduced calponin expression. 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. As before, *RBPMS2*-overexpressing cells were desmin-positive. Moreover, the number of PH3-positive cells was 6-fold higher in cells that overexpressed *RBPMS2* than in controls (Figure 2B), 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. 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. 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) (23). Similarly, the inventors found that *cholinergic receptor muscarinic 3* mRNA expression was decreased by 56% in *RBPMS2*-overexpressing SMCs compared with controls (Figure 3). 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. 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, the inventors analyzed the gene expression profiles of primary cultured SMCs infected with RCAS-*RBPMS2* or RCAS-empty retroviruses for 3 days by microarray analysis. *RBPMS2* overexpression in differentiated SMCs induced the down-regulation of *calponin* and also of markers of SMC differentiation, such as *caldesmon* and *SM-MHC*. Conversely, *Noggin*, the

BMP signaling pathway inhibitor, was 360-fold up-regulated, whereas BMP transcriptional targets, including *PITX2*, *ID2*, and *ID4*, were down-regulated, suggesting a major inhibition of BMP activity. *Noggin* up-regulation was confirmed by QPCR using primary SMCs harvested after 3 days of RCAS-*RBPMS2* infection (Figure 4).

The inventors performed in situ hybridization and showed that *Noggin* was expressed in gut mesenchymal derivatives as early as *RBPMS2*. Moreover, because misexpression of *Noggin* in stomach induces a hypertrophic phenotype (16) that is highly reminiscent of the defects observed upon *RBPMS2* misexpression, the inventors 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. Conversely, misexpression of *Noggin* had a moderate impact on the spatiotemporal expression of *RBPMS2*. 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, the inventors 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 total RNA from E6 gastrointestinal mesenchyme. QPCR showed that *Noggin* was strongly amplified (6% of total *Noggin* mRNA) after immunoprecipitation of Myc-tagged RBPMS2, indicating that *Noggin* mRNA and RBPMS2 are present in a common RNA-protein complex. Then, the inventors investigated the impact of the RBPMS2/*Noggin* interaction on the activity of the BMP signaling pathway with antibodies against the activated and phosphorylated intracellular BMP effectors SMAD1, 5, and 8 (namely PSMAD1) that the inventors previously characterized as tools to evaluate the activity of BMP pathway (10,25). In control primary SMCs, high expression of PSMAD1 was associated with calponin expression. In cells infected with RCAS-*RBPMS2* for 3 days, calponin expression decreased concomitantly with a strong reduction of PSMAD1 expression, showing an inhibitory effect of RBPMS2 on the BMP signaling pathway, but also indirectly a positive effect of RBPMS2 on *Noggin* mRNA transcription or stabilization.

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, the inventors focused on Noggin function during visceral SMC development and differentiation. The inventors 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 an alteration of visceral SMC differentiation without significant changes of the proliferative rate in vivo. 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. QPCR analysis of these cells showed that *calponin* and *SM22* were down-regulated, whereas *αSMA*, *myocardin*, and *FHL2* were induced. *Noggin* overexpression also increased myocardin protein level, 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). 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, the inventors 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 (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 5A for quantification). Indeed, *RBPMS2* overexpression in SMCs decreases calponin expression in infected (Myc-positive) and uninfected (Myc-negative) neighboring cells, 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 5B).

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 (4, 5). Recently, specific smooth muscle defects were shown to be involved in the pathogenesis of pediatric digestive motility disorders (7). Because our findings suggests that RBPMS2 might be involved in visceral SMC development and differentiation, the inventors 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 (4). 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. 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).

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

Discussion

In this invention, the inventors 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. α*SMA* 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.

The inventors 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.

The inventors 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.

The inventors demonstrated 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 own function and that proteosomal degradation of myocardin is required for its full transcriptional activity. Similarly, the inventors show that myocardin accumulation results in a reduction of calponin expression (a marker of differentiated SMCs). In addition, *RBPMS2* and *Noggin* misexpression led to up-regulation of the SRF target gene *FHL2*, which interacts with SRF and inhibits induction of smooth muscle contractile genes by SRF (21). Altogether, these results support the notion that activation of the RBPMS2/Noggin pathway inhibits SMC contractile genes through functional alteration of the myocardin/SRF pathway.

In conclusion, the inventors show that, in chick embryos, *RBPMS2* 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 *RBPMS2* 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. The inventors show that regulated expression of *RBPMS2* is important for the correct development and differentiation of visceral SMCs. The inventors then found that *RBPMS2* 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) (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 (6, 7). In summary, the inventors identified *RBPMS2* as a new marker of visceral SMC remodeling that could be useful for the characterization of smooth muscle alteration in visceral myopathies.

EXAMPLE 2:

Material & Methods

Plasmids

The human *RBPMS2* cDNA sequence coding to the aminoacid 27 to 117 was subcloned into pET22 (pET22-human-RBPMS2-Nter). Substitution of Leucine by Glutamic Acid in position 49 of the human RBPMS2 sequence (L49E) was introduced by QuikChange site-directed mutagenesis method (Stratagene) in order to create pET22-human-RBPMS2-Nter L49E plasmid. The full-length human RBPMS2 cDNA was subcloned in the pCS2 vector with an in frame N-terminal HA tag and the CMV promoter (pCS2-HA-human-RBPMS2). The full-length human RBPMS2 and RBPMS2 L49E were subcloned in the pHRTK vector with an in frame N-terminal Myc tag and the CMV promoter (respectively pHRTK-Myc-human-RBPMS2 and pHRTK-Myc-human-RBPMS2 L49E). HA-tagged human TC10 was previously described (Coisy-Quivy et al., 2009). Myc-tagged chick full-length RBPMS2 with corresponding Leu40Glu substitution was cloned into the RCAS vector to produce replication-competent retroviruses that express Myc-RBPMS2 Leu40Glu. Myc-tagged chick full-length RBPMS2 (RCAS-Myc-gallus-RBPMS2), GFP (RCAS-GFP) and Myc-NICD were previously described (Notarnicola et al., 2012; Moniot et al., 2004; Shih and Holland, 2006). All plasmids were checked by DNA sequencing and protein expression.

DuoLink analysis

For DuoLink in situ Proximity Ligation Assay (PLA) (adapted from Soderberg et al.), DF1 cells transfected with different plasmid combination were labeled with mouse anti-HA (Santa Cruz Biotechnologies) and rabbit anti-Myc (Ozyme) primary antibodies and incubated with a pair of nucleotide-labeled secondary antibodies (rabbit PLA probe MINUS and mouse PLA probe PLUS; OLINK Biosciences, Uppsala Sweden) in hybridization solution. In addition, secondary mouse and rabbit anti-IgG respectively coupled to Alexa 488 and 555 were incubated to detect protein expression. Interactions between the PLA probes, possible when within a distance less than 40 nm, were revealed by adding a ligase and by amplification of a rolling-circle product using far red labeled oligonucleotides and a polymerase, according to the manufacturer's instructions. Cells were counterstained using Duolink II Mounting Medium with 4',6-Di-Amidino-2-Phenyl-Indole. Signals indicative of interactions were detected by confocal microscopy as fluorescent dots.

Recombinant Proteins

pET22-human-RBPMS2-Nter and pET22-human-RBPMS2-Nter L49E plasmids were transformed into Escherichia coli strain BL21λDE3 for protein overexpression using T7 RNA polymerase. Proteins were purified as previously described (Yang et al., 2009).

NMR Spectroscopy

All NMR experiments were carried out at 27°C on a Bruker Avance III 700 spectrometer equipped with 5 mm z-gradient TCI cryoprobe, using the standard pulse sequences (Sattler et al., 1999). NMR samples consist on approximately 0.5 mM ^{15}N - or ^{15}N,^{13}C-labeled protein dissolved in 10 mM acetate buffer, 50 mM NaCl, pH 4.6 with 5% D2O for the lock. ^{1}H chemical shifts were directly referenced to the methyl resonance of DSS, while ^{13}C and ^{15}N chemical shifts were referenced indirectly to the absolute ^{15}N /^{1}H or ^{13}C/^{1}H frequency ratios. All NMR spectra were processed and analysed with GIFA (Pons et al., 1996). Structures were validated using PROCHECK (Laskowski et al., 1993).

Fluorescence Anisotropy

Human RBPMS2-Nter and RBPMS2-Nter L49E were labelled with the NHS ester of ATTO647N in 20 mM Na-phosphate buffer pH 7.5 with 50 mM KCl during 3 hours at room temperature. Labelled proteins were separated from the free dye using a 2 ml Zeba spin desalting column (Thermo Scientific) equilibrated in binding buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl). Anisotropy measurements were carried out at 25°C in dilution mode. RBPMS2-Nter–ATTO3457N and RBPMS2-Nter L49E-ATTO647N (2nM final) were then mixed with different RNAs (2μM final) in binding buffer. The mixture was serially diluted with the same buffer containing only 2nM RBPMS2-Nter-ATTO647N or RBPMS2-Nter L49E-ATTO647N. Measurements were made at each dilution in Corning black 384 wells assay plate with a TECAN Safire2 in polarization mode.

Using the pCMV6-XL5 plasmid that contains the human *NOGGIN* cDNA including the 5' and 3' untranslated regions and the open reading frame (OriGene), different DNA matrix were constructed by PCR with forward primers that carried T7 promoter sequence. These amplified DNA were used as template to synthesize in vitro human *NOGGIN* RNA using T7 high yield RNA synthesis Kit (New England Biolabs). Following sequences were used : 214-1447, 214-838, 214-538, 518-838, 818-1447.

Immunoprecipitation

The avian DF-1 chicken fibroblast cell line (ATCC-LGC) was grown in DMEM supplemented with 10% FBS and transfected using Lipofectamine 2000 (Invitrogen, France) with above described constructs. Cells were analyzed after 24h. Cells were lysed using Lysis Buffer (20 mM Tris pH8, 50 mM NaCl, 1% NP40, cOmplete, EDTA-free Protease Inhibitor

Cocktail (Roche)). 50 mg of total DF1 protein lysates were incubated directly with the rabbit anti-Myc antibodies (Ozyme) pre-adsorbed to protein A-Sepharose CL-4B (GE Healthcare) for 1 h at 4°C in Immunoprecipitation Buffer (50 mM Tris pH8, 150 mM NaCl, 0.4% NP40, cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche)). After extensive washing, bound proteins were eluted by boiling in SDS-PAGE sample buffer, analyzed by 12% SDS-PAGE, and transferred to nitrocellulose. The membrane was blocked with 10% nonfat milk in TBS + 0.1% Tween and probed with mouse anti-HA or rabbit anti-Myc polyclonal antibodies overnight. After several washes, membranes were incubated with the relevant horseradish peroxidase-conjugated secondary antibodies (Perkin Elmer). Detection was performed by chemiluminescence (Santa Cruz Biotechnologies) on Kodak films.

Avian retroviral misexpression system

Fertilized White Leghorn eggs from Haas Farm (France) were incubated at 38°C in humidified incubators. Gastrointestinal tissues from chick embryos were dissected as described (Moniot et al., 2004). 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 (Moniot et al., 2004; Notarnicola et al., 2012). Eggs were then placed at 38°C until harvested.

Primary cultured SMCs and analysis

Primary cultures from E15 gizzard muscle were prepared as described (Notarnicola et al., 2012). Briefly, the tunica muscularis was carefully separated from the serosa and tunica mucosa before collagenase dissociation. Isolated cells were cultured in Dulbecco's modified Eagle medium (DMEM) in presence of 0.2% BSA and 5 μg/ml insulin in Matrigel-coated plates to maintain the cell differentiation status (more than 95% of isolated cells were Desmin- and αSMA-positive, data not shown). Differentiated SMCs were then infected with RCAS-empty, or RCAS-Myc-gallus-RBPMS2, or RCAS-Myc-gallus-RBPMS2 L40E retroviruses and maintained in culture for 3 days. In our conditions, avian retroviruses have a high tropism to infect SMCs. For immunodetection, anti-Myc (Ozyme), anti-avian Calponin (Sigma-Aldrich), and anti-Phospho-Histone H3-Ser10 (Millipore) antibodies were used. Nuclei were stained with Hoechst (Molecular Probes).

Immunofluorescence and in situ hybridization on chick stomach

Immunofluorescence experiments with chick paraffin-embedded sections and in situ hybridization experiments using dissected gut were carried out as described (Moniot et al., 2004; Notarnicola et al., 2012). For immunodetection, anti-Myc (Ozyme), and anti-avian Calponin (Sigma-Aldrich) antibodies were used. Nuclei were stained with Hoechst

(Molecular Probes). The chick *Noggin* template was used (Notarnicola et al., 2012). Anti-sense *Noggin* riboprobes was generated by reverse transcription using chick *Noggin* template with incorporation of digoxigenin-UTP (Roche). Anti-digoxigenin antibodies coupled to alkaline phosphatase (Roche) were used to detect *Noggin* sens/antisens complexes with BM Purple solution (Roche). Images were acquired using and a Carl-Zeiss AxioImager microscope (for immunofluorescence) and a Nikon-AZ100 stereomicroscope (for whole-mount in situ hybridization).

Results

In order to determinate the structure of the human RBPMS2 protein, the inventors looked at the structural organization of RBPMS2 by molecular modeling using the server @TOME-2 (Pons and Labesse, 2009). The inventors found that the N-terminus part of the human RBPMS2 protein (residues 27-117) was predicted to be structured as a RRM domain. Based on this result, the inventors have produced this domain in bacteria (RBPMS2-Nter) and purified it. Using NMR experiments, the inventors have confirmed the RRM fold for the N-terminus part of RBPMS2 protein, and found that this domain is exclusively present in the homodimeric form in solution.

To examine RBPMS2 homodimerization in vitro, the inventors conduct coImmunoprecipitation assays (coIP) using DF1 cell lysates expressing Myc- or HA-tagged RBPMS2 proteins and anti-Myc antibodies. The inventors observe that HA-tagged RBPMS2 coprecipitates with Myc-tagged RBPMS2. To test whether the interaction is specific or mediated by bridging RNA, the inventors performed coIP from RNase-treated assays and observe a specific homodimerization between RBPMS2 proteins. To confirm our results, the inventors include the small GTPase TC10 protein fused to HA tag as an additional negative control and observe no interaction with RBPMS2.

The inventors also investigate RBPMS2 homodimerization in cell culture using DuoLink technology, an in situ proximity ligation assay (PLA) that detects two proteins only when they are in close proximity. The inventors find that HA-RBPMS2 interacts with Myc-RBPMS2 in DF1 cells expressing both Myc- and HA-RBPMS2 proteins. For negative control, the inventors also test the interaction of RBPMS2 with unrelated Myc- or HA-tagged proteins, but we do not observe interaction. These data support that RBPMS2 is present as a homodimer in vivo.

Using NMR experiments done on human RBPMS2-Nter protein, the inventors found that homodimerization of RBPMS2 protein could involve the interaction of both Leucine 40

from each RBPMS2 protein. This Leucine 49 is conserved into all RBPMS2 homologs (the presence of the similar Leucine at position 40 in gallus). In order to test the hypothesis that Leucine 49 is involved in the dimerization complex of RBPMS2, the inventors substitute Leucine 49 by Glutamic Acid (L49E) that is predicted to avoid the dimerization process without alteration of global structure. The inventors analyze by NMR experiment their structure and observe that RBPMS2-Nter L49E is essentially present as a monomeric form in solution. The inventors previously showed that RBPMS2 is a RNA-Binding Protein that can bind RNAs via its RRM domain and using immunoprecipitation of tagged avian RBPMS2 protein we found that *Noggin* mRNA and RBPMS2 are present in a common RNA-protein complex (Notarnicola et al., 2012). In order to analyze the impact of L49E substitution of RBPMS2, the inventors evaluate the capacity of human purified RBPMS2-Nter protein that contains the RRM domain to bind to the human *NOGGIN* mRNA synthesised in vitro by fluorescence anisotropy-based binding assays. The inventors find that RBPMS2-Nter binds to human *NOGGIN* mRNA and identify that sequence between 518 to 838 is involved in this binding. The inventors also find that human RBPMS-Nter L49E binds to similar *NOGGIN* sequence without affinity difference, suggesting that L49E substitution did neither alter its capacity to bind RNA nor its structure. These data support that L49E substitution in RBPMS2 protein does not alter the structure of RBPMS2 protein nor its ability to bind *NOGGIN* RNA, but conducts to the presence of monomeric RBPMS2 protein in solution.

In addition, the inventors conduct coIP using DF1 cell lysates expressing HA-tagged RBPMS2 or Myc-RBPMS2 or Myc-RBPMS2 L40E and anti-Myc antibodies. The inventors observe that HA-tagged RBPMS2 coprecipitates with Myc-tagged RBPMS2 but faintly with Myc-tagged RBPMS2 L40E. The Leucine to Glutamic Acid substitution abrogates 83,5% of the dimerization. The inventors also test with DuoLink technique the impact of Leucine to Glutamic Acid substitution in cell culture. The inventors find that HA-RBPMS2 does not interact with Myc-RBPMS2 L40E in DF1 cells expressing both Myc- and HA-RBPMS2 proteins. These data demonstrate that substitution of Leucine 49 to Glutamic acid of RBPMS2 protein avoid RBPMS2 dimerization in vitro and in vivo.

To test the function or the requirement of the RBPMS2 dimerization in cellulo, the inventors analyze the impact of RBPMS2 L40E in primary SMC cell culture and compare it to the action of RBPMS2. The inventors establish primary cultures on Matrigel of visceral differentiated SMCs from E15 gizzard muscles in serum-free medium supplemented with insulin (Notarnicola et al., 2012). Control primary cultured SMCs in the presence of replication-competent retroviruses without transgene (RCAS-empty) were spindle-shaped and

homogenously expressed aSMA and calponin, 2 SMC contractile markers, in highly organized filament bundles. SMCs then were infected with replication-competent retroviruses (RCAS-RBPMS2 or RCAS-RBPMS2 L40E construct or RCAS-empty [control]) for 3 days. Although in control cells the expression of calponin remained unchanged, in SMCs infected with Myc-tagged RBPMS2 calponin expression is lost. The inventors also observe that SMCs infected with Myc-tagged RBPMS2 L40E calponin expression remain unchanged. The inventors also investigate the impact of RBPMS2 L40E on primary cultured SMCs with the analysis of the expression of phosphorylated Histone 3-Ser10 (PH3), a standard marker of G2/M transition. After 6 days of culture, the number of PH3-positive cells was 4.5-fold higher in cells that over-expressed *RBPMS2* than in controls (Figure 3). In addition, the inventors observe no change in the number of PH3-positive cells in cells that over-expressed *RBPMS2* L40E compare to the controls, demontrating that RBPMS2 dimerization is essential to induce the dedifferentiation of the SMCs.

To test the function or the requirement of the RBPMS2 dimerization in vivo, the inventors analyze the impact of RBPMS2 L40E and RBPMS2 during the development of the avian gastrointestinal tract and compare it to the action of RBPMS2. The inventors use the avian replication-competent retroviral misexpression system that allows in vivo targeting of specific genes in the stomach mesenchyme and the sustained expression of transgene throughout visceral muscle development and differentiation. As previously demonstrated (Notarnicola et al., 2012), sustained RBPMS2 expression results in a dramatic alteration of the stomach morphology. Specifically, the proventriculus, which is the glandular part of the chick stomach was hypertrophied, whereas the gizzard was denser and malformed in comparison with controls that overexpressed GFP alone. In contrast, to the action of RBPMS2, the sustained expression of *RBPMS2* L40E does not induce any morphological change of the infected stomachs (n=26), stomachs presenting expression of transgenes.

The inventors previously showed that sustained *RBPMS2* expression in the GI tract induces the upregulation of *Noggin* mRNAs in vivo (Notarnicola et al., 2012). Using the avian retroviral misexpression technique, the inventors analyze the impact of *RBPMS2* L49E, *RBPMS2* and GFP as control on *Noggin* mRNA expression by in situ hybridization. The inventors observe that RBPMS2 misexpression in the gastrointestinal mesenchyme is always associated to the upregulation of *Noggin* mRNA in infected stomach in comparison to controls. In contrast, the inventors find that *RBPMS2* L49E misexpression does not induce any up-regulation of *Noggin* mRNA (n=17, infected stomachs analyzed by in situ

hybridization), demontrating that RBPMS2 dimerization is essential to induce upregulation of *Noggin* mRNA.

The inventors show that the conserved RBPMS2 protein, homodimerizes via its RRM domain and that this interaction is essential for its function. The inventors also demonstrate that the newly identified RRM-homodimerization motif (residues 47-50 of the SEQ ID NO:1) is crucial for the function of RBPMS2 at the cell and tissue levels.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of treating chronic intestinal pseudo-obstruction in a subject in need thereof, comprising the step of administering to said subject a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors.

2. A method of screening a candidate compound for use as a drug for the treatment of chronic intestinal pseudo-obstruction in a subject in need thereof, wherein the method comprises the steps of:

(i) providing a RBPMS2, providing a cell, tissue sample or organism expressing the RBPMS2,

(ii) providing a candidate compound such as small organic molecule, peptide, polypeptide, aptamer or intra-antibodies,

(iii) measuring the activity of the RBPMS2,

(iv) and selecting positively candidate compounds that blocks RBPMS2 dimerization, inhibits RBPMS2 activity by blocking the binding of RBPMS2 to RNAs via its RNA recognition motif domain or blocks RBPMS2 binding to Noggin inducing Noggin up-regulation and then inhibiting BMP signalling.

3. A method of identifying a subject having a chronic intestinal pseudo-obstruction which comprises the step of analyzing a biological sample from said subject for:

(i) determining the RBPMS2 expression level,

(ii) comparing the RBPMS2 expression level in the sample with a reference value,

(iii) detecting differential in the RBPMS2 expression level between the sample and the reference value is indicative of a subject having a chronic intestinal pseudo-obstruction.

4. A method of treating chronic intestinal pseudo-obstruction in a subject in need thereof comprising the steps of:

(i) identifying a subject having a chronic intestinal pseudo-obstruction by performing the method according to claim 3, and

(ii) treating said subject having a chronic intestinal pseudo-obstruction with a compound which is selected from the group consisting of RBPMS2 antagonists or RBPMS2 expression inhibitors.

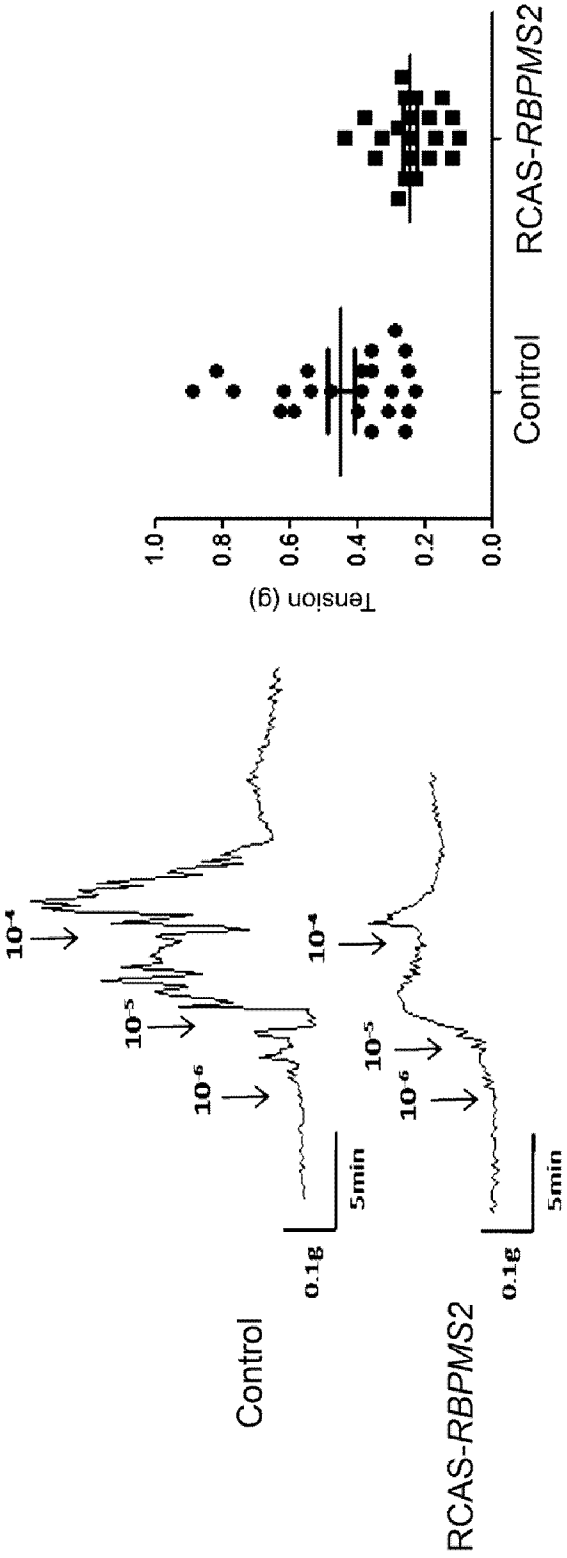
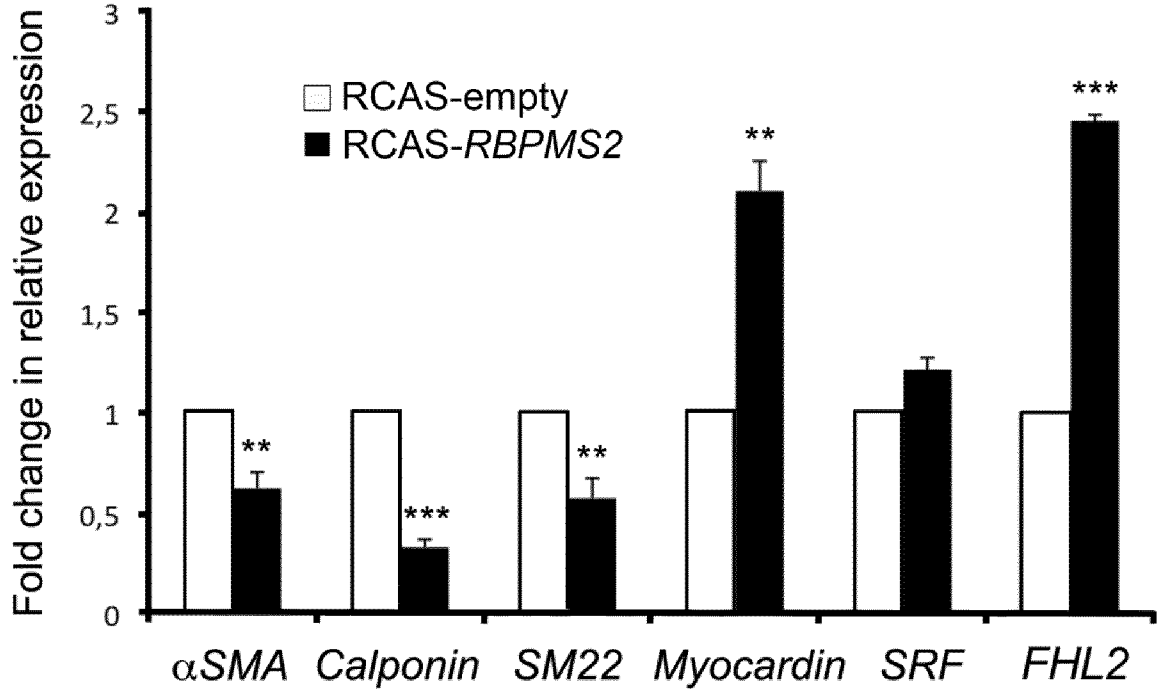

Tension (g)
1.0
0.8
0.6
0.4
0.2
0.0
Control
RCAS-RBPMS2
Control
RCAS-RBPMS2
10⁻⁴
10⁻⁵
10⁻⁶
0.1g
5min

Figure 1

A


Fold change in relative expression
3
2,5
2
1,5
1
0,5
0
RCAS-empty
RCAS-RBPMS2
**
***
**
**
***
αSMA
Calponin
SM22
Myocardin
SRF
FHL2

B

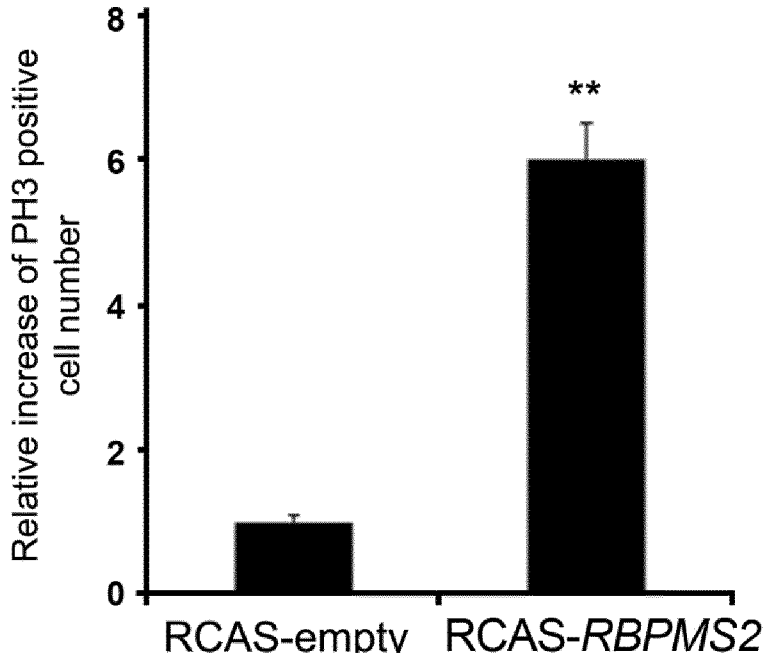

Relative increase of PH3 positive cell number
8
6
4
2
0
**
RCAS-empty
RCAS-RBPMS2

Figure 2

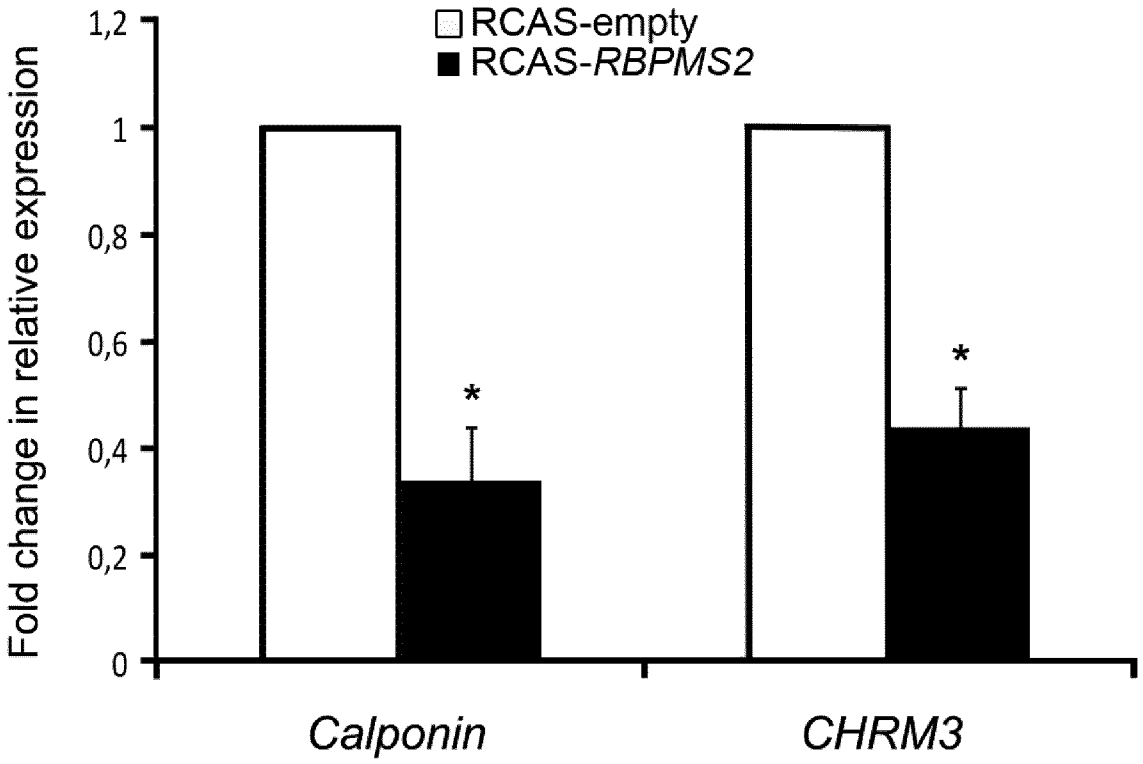

RCAS-empty
RCAS-RBPMS2
Fold change in relative expression
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Calponin
CHRM3

Figure 3

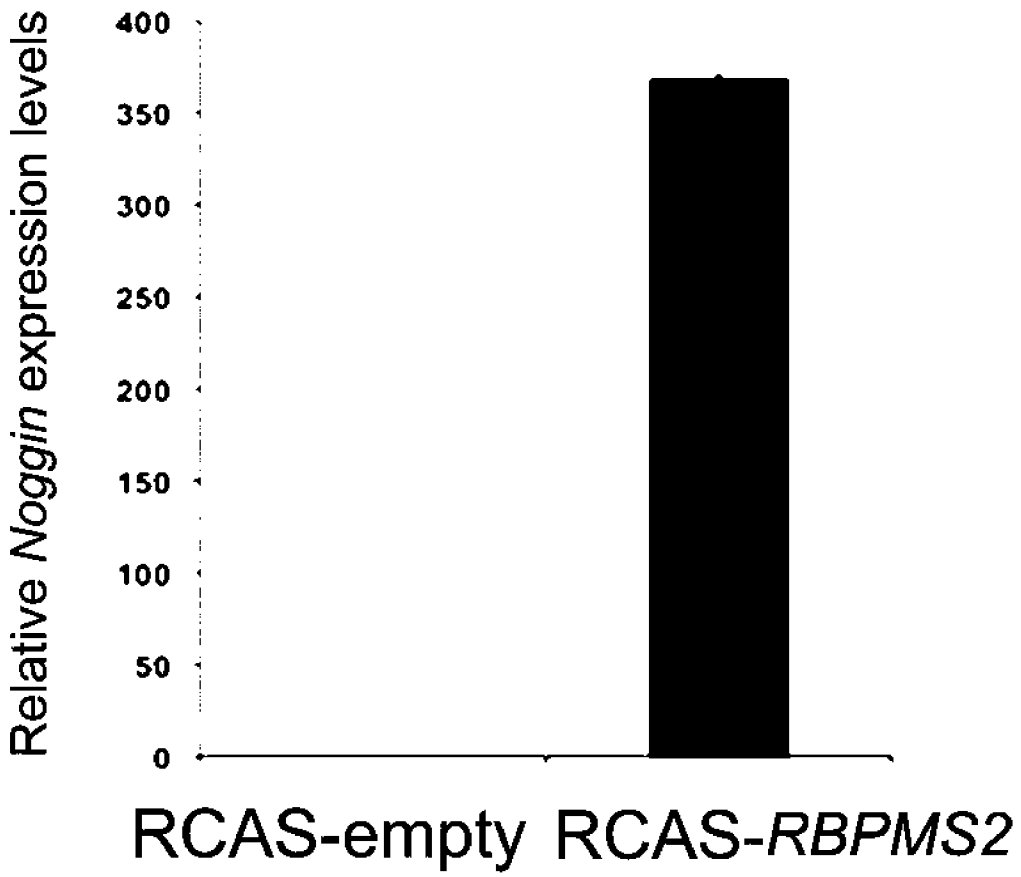

Relative Noggin expression levels
400
350
300
250
200
150
100
50
0
RCAS-empty
RCAS-RBPMS2

Figure 4

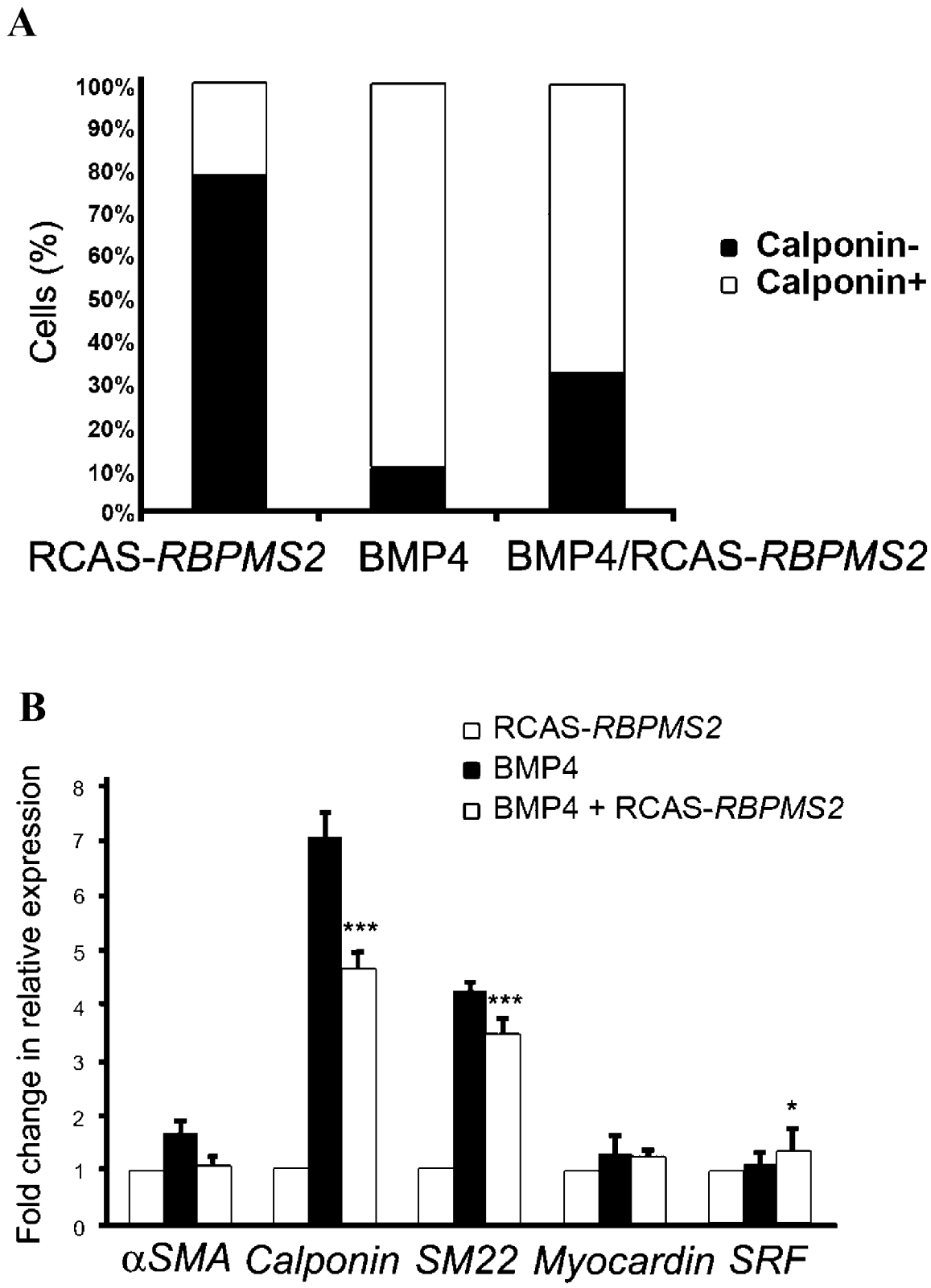

A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cells (%)
Calponin-
Calponin+
RCAS-RBPMS2
BMP4
BMP4/RCAS-RBPMS2
B
RCAS-RBPMS2
BMP4
BMP4 + RCAS-RBPMS2
Fold change in relative expression
0
1
2
3
4
5
6
7
8
***
***
*
αSMA
Calponin
SM22
Myocardin
SRF

Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/052296

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/68 G01N33/50 C12N15/113 A61P1/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CÉCILE NOTARNICOLA ET AL: "The RNA-Binding Protein RBPMS2 Regulates Development of Gastrointestinal Smooth Muscle", GASTROENTEROLOGY, vol. 143, no. 3, September 2012 (2012-09), pages 687-697.e9, XP055054509, ISSN: 0016-5085, DOI: 10.1053/j.gastro.2012.05.047 abstract final paragraph on page 693 onwards	1-4

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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 2014	21/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilling, Stephen

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NOTARNICOLA C ET AL: "The RNA-binding protein RBPMS2 controls the development and the phenotypic plasticity of the visceral smooth muscle", NEUROGASTROENTEROLOGY AND MOTILITY, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 22, no. Suppl.1, August 2010 (2010-08), page 90, XP009167974, ISSN: 1350-1925 [retrieved on 2010-08-31] abstract -----	1-4