

Stretch-induced alternative splicing of serum response factor promotes bronchial myogenesis and is defective in lung hypoplasia

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Smooth muscle (SM) develops only in organs and sites that sustain mechanical tensions. Therefore, we determined the role of stretch in mouse and human bronchial myogenesis. Sustained stretch induced expression of SM proteins in undifferentiated mesenchymal cells and accelerated the differentiation of cells undergoing myogenesis. Moreover, bronchial myogenesis was entirely controlled in lung organ cultures by the airway intraluminal pressure. Serum response factor (SRF) is a transcription factor critical for the induction of muscle-specific gene expression. Recently, a SRF-truncated isoform produced by alternative splicing of exon 5 has been identified (SRF Δ 5). Here we show that undifferentiated mesenchymal cells synthesize both SRF and SRF Δ 5 but that SRF Δ 5 synthesis is suppressed during bronchial myogenesis in favor of increased SRF production. Stretch induces the same change in SRF alternative splicing, and its myogenic effect is abrogated by overexpressing SRF Δ 5. Furthermore, human hypoplastic lungs related to conditions that hinder cell stretching continue to synthesize SRF Δ 5 and show a marked decrease in bronchial and interstitial SM cells and their ECM product, tropoelastin. Taken together, our findings indicate that stretch plays a critical role in SM myogenesis and suggest that its decrease precludes normal bronchial muscle development.

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Introduction

Visceral smooth muscle (SM) originates from local mesenchymal cells that in early-midgestation begin to synthesize SM proteins, including SM α -actin, desmin, SM myosin, SM22, and calponin in a specific periairway distribution (1–5). In the mouse developing respiratory system, cells expressing SM proteins are first detected in the trachea on day 11 of gestation (3, 4), and then SM differentiation proceeds in a cranial-to-caudal fashion to form the bronchial musculature (1–5). The other type of visceral SM cells found in the lung are interstitial SM cells, also known as interstitial contractile cells, or myofibroblasts. Interstitial SM cells are originally located at the sites of future alveolar septae, and, in the mature organ, they form part of the septae tips (6). Except for the aorta, the development of the vascular musculature lags behind that of visceral SM by several days (4, 7–9).

Unlike striated muscle differentiation, on which considerable information was gathered over the years, the mechanisms and genetic program that control SM myogenesis remain, for the most part, unknown. We and others have observed that lung mesenchymal cell precursors change their shape from round to elongated before undergoing bronchial SM differentiation (ref. 3; Y. Yang and L. Schuger, unpublished observations). Based on this observation we recently examined

whether changes in cell shape might play a role in airway myogenesis. Unexpectedly, our studies demonstrated that essentially all undifferentiated embryonic mesenchymal cells are potential SM precursors (10–12). These studies also confirmed the critical role of cell shape in myogenesis. Specifically, we found that cell rounding prevents myogenesis, regardless of the normal fate of the cell in vivo, whereas cell spreading/elongation induces SM differentiation, even in mesenchymal cells from nonmuscular organs (10–12).

Developing tubular tissues, such as those of the respiratory, gastrointestinal, and urinary systems, are filled with liquid. As a consequence, the periluminal mesenchymal cells are subjected to mechanical tension/stretch exerted by the liquid's hydrostatic pressure (13). These forces likely represent a significant factor in determining the periluminal mesenchymal cell shape. In the developing lung, cells are additionally subjected to repeated stretch caused by intrauterine breathing (13). The fact that mechanical stretch causes cell elongation and that cell elongation is likely to be sensed by the cell as a mechanical stimulus suggested to us that cell tension/stretch may play an important role in the process of visceral myogenesis.

Here we used a combination of lung cell and organ cultures from fetal mouse and human origin to determine the effect of mechanical stretch upon SM myogenesis.

We found that sustained stretch was by itself sufficient to induce expression of SM proteins in lung undifferentiated mesenchymal cells and to accelerate synthesis of SM proteins in mesenchymal cells already undergoing myogenic differentiation. Conversely, SM myogenesis did not take place in the absence of mechanical stimuli.

Many transcription factors have been linked to the control of SM-specific gene expression. Among them, one of the best studied is serum response factor (SRF) (14–16), a member of the MADS (MCM-1, agamous and deficiens, and SRF) box family of transcription factors. SRF binds to the CA_rG box or CA_rG box–like motif, an essential *cis*-element present in muscle-specific proteins such as SM α -actin, SM22, SM myosin, β -tropomyosin, and caldesmon, and stimulates their transcription.

Although SRF has been studied for many years, only recently has it been demonstrated that several truncated isoforms are produced by alternative splicing from the same SRF pre-mRNA (17, 18). The newly identified SRF isoforms are SRF Δ 5 (17), also referred to as SRF-M (18), lacking exon 5, SRF-S lacking exons 4 and 5, and SRF-I lacking exons 3, 4, and 5. All these SRF species lack regions of the COOH-terminal transactivation domain but have intact DNA-binding domains located in the NH₂ terminus. Here we show that stretch induces myogenic differentiation by suppressing SRF Δ 5 in SM cell precursors in favor of increased SRF production.

Finally, our studies demonstrate that human hypoplastic lungs related to conditions that diminish lung distention exhibit a severe decrease in visceral muscle and elastin and continue to synthesize SRF Δ 5. These new findings underscore the importance of mechanical stimuli in the control of visceral myogenesis.

Methods

Cell-stretching device. A cell-stretching device was manufactured according to our specifications by Michael Monford (Department of Bioengineering, University of California at San Diego, San Diego, California, USA). It consisted of a rectangular acrylic dish with a silicone membrane (Silastic; Specialty Manufacturing Inc., Saginaw, Michigan, USA) fitted to the bottom and attached to removable clamps (Figure 1a). The cells were plated on the membrane coated with 0.1% poly-L-lysine to maintain the cells in an undifferentiated state (round in shape) or 0.01% poly-L-lysine or collagen I (10 μ g/cm²) to allow spread-induced SM differentiation (10, 12). Static axial-stretching forces were applied after cell attachment was complete by placing the clamps into different slots (Figure 1a). The magnitude of stretching was represented as the percentage of membrane distention from its original length to its length after stretching.

Cells. Crl: CD-1 (ICR) BR mice (Charles River, Wilmington, Massachusetts, USA) were mated, and the day a vaginal plug was identified was designated as day 0 of embryonic development. Undifferentiated mesenchymal cells were isolated by differential plating (19) from

embryonic intestine and lung on day 11 and from kidney on day 12 of gestation. Absence of SM proteins in these organs at this stage was confirmed by previous studies (11). Human lung embryonic mesenchymal cells were also obtained from voluntary pregnancy terminations (see below) by differential plating. The cells were isolated directly on the silicone membranes and cultured in MEM with 10% FBS (Irvine Scientific, Santa Ana, California, USA) for up to 24–48 hours. To obtain fully differentiated SM cells (SM protein synthesis and electrical responses comparable to those of mature SM cells), the mesenchymal cells were cultured for 96 hours under conditions that allow cell spreading (11) before stretching and/or transfection. In some experiments, cell proliferation was prevented by reducing the FBS to 1% (10–11).

Lung organ culture. Lungs were microdissected from day 11 CD-1 mice and cultured embedded in a thin collagen gel (5 milligrams collagen I per milliliter of medium) layered on a transwell device (Figure 1b). Lung samples from ten voluntary pregnancy terminations (16–18 weeks) were obtained from Advanced Bioscience Resources Inc. (ABR, Alameda, California, USA). Fragments of peripheral lung parenchyma (including terminal sacs and surrounding mesenchyme) ranging in size from 1 to 4 mm³ were microdissected and cultured as described for the mouse lung. The airway intraluminal fluid was microaspirated from the organ cultures and replaced with a solution of MEM-10% FBS and various concentrations of dextran (Life Technologies Inc., Grand Island, New York, USA). Dextran is 68-kDa polysaccharide used clinically as an osmotic volume expander. Here we used dextran to increase the airway intraluminal pressure and thereby stretch the surrounding mesenchymal cells. Alternatively, dextran was dissolved in the culture medium surrounding the tissues to accomplish the opposite effect (Figure 1b). Explants were cultured submerged in serumless medium (BGjb; Life Technologies Inc.) for 24 hours. At the end of the culture period, the lungs were lysed or fixed as a whole mount for SM-specific protein detection. In some experiments, the main airways, including the peribronchial mesenchyme, were microdissected from fresh or cultured murine lungs and trypsinized. The peribronchial mesenchymal cells were then further selected by 45 minutes' differential plating (19) and immediately lysed for SRF isoform evaluation.

Measurement of stretch-induced cell elongation. Round-cell cultures were stretched by 0%, 1%, 5%, and 10% of the total silastic membrane length and fixed in 1% paraformaldehyde immediately and after 1, 2, and 6 hours of stretching. The airway intraluminal fluid of mouse lung organ cultures was replaced with 0%, 1%, 5%, 10%, or 15% dextran in BGjb or 1% dextran was added to the culture medium outside the lung. Preliminary studies showed that dextran concentrations higher than 2.5% in the culture medium cause osmotic damage. The tissues were frozen after 4 and 24 hours in culture. Preliminary studies indicated that the

increment in peribronchial mesenchymal cell diameter was fully elicited 4 hours after dextran microinjection and remained constant for up to 24 hours in culture. Five micrometer-thick sections were cut and stained with hematoxylin and eosin for light-microscopic observation. Stretch-induced cell elongation was determined on computer-scanned images. In cell cultures, the cell diameter for each stretching force was measured in 50 cells per treatment. In lung organ cultures (in which the cell boundaries are not defined), the distance between the centers of adjacent peribronchial mesenchymal cell nuclei was measured in the distal bronchial buds. Increments in this distance were considered a measure of cell stretch. At least 40 distances per treatment were recorded.

Human hypoplastic lungs. Formalin-fixed, paraffin-embedded tissue samples from nine hypoplastic lungs and nine gestational age-matched controls ranging in gestational age from 20 to 25 weeks were selected from autopsy files. Four hypoplastic lungs (two female, two male) were related to oligohydramnion due to renal agenesis and five (two female, three male) to left diaphragmatic hernia. The lungs had a 35–60% reduction in their expected weight, the most extreme reduction found in the lungs ipsilateral to a diaphragmatic hernia and the less severe in lungs contralateral to it. None of the neonates survived for more than a few minutes after delivery.

Immunoblot analysis. The following Ab's were used for detection of SM-specific proteins: a mouse mAb to SM α -actin (Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA) at a concentration of 0.25 μ g/ml, a mouse mAb to desmin (DAKO Corp., Carpinteria, California, USA) at a concentration of 1.125 μ g/ml, rabbit polyclonal Ab's to SM-myosin (Biomedical Technologies, Stoughton, Massachusetts, USA) at a concentration of 10 μ g/ml, and rabbit polyclonal Ab's to SM22 (gift from Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey, USA) at a concentration of 2 μ g/ml. A rabbit polyclonal Ab against mouse tropoelastin and another against human tropoelastin (Elastin Products, St. Louis, Missouri, USA) were used at a dilution of 1:1000. Rabbit polyclonal Ab against SRF/SRF Δ 5 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used at a 1:200 dilution. Cell cultures were lysed, and immunoblots were performed as described previously (10, 11).

Immunohistochemistry. Five micrometer-thick sections from formalin-fixed human lung explants, hypoplastic lungs, and matched controls were immunostained with Ab's against SM α -actin. Hypoplastic lungs and their controls were additionally stained with Ab's against tropoelastin, low-molecular-weight cytokeratins (rabbit polyclonal Ab from DAKO Corp.) and PECAM-1 (mouse monoclonal Ab from DAKO Corp.). Ab's to SM α -actin and PECAM-1 were used at a concentration of 1 μ g/ml. Ab's to cytokeratins and tropoelastin were used at a dilution of 1:200. Mouse embry-

onic lung explants were immunostained as whole mounts (without sectioning). Staining was completed using commercial peroxidase-anti-peroxidase kits (DAKO Corp.) or FITC-conjugated secondary Ab's as described previously (10, 20).

RNA isolation. RNA was isolated from cells and tissues with TRIzol reagent (Life Technologies Inc.) following the manufacturer's instructions. To isolate RNA from formalin-fixed, paraffin-embedded lungs, six 20- μ m-thick sections were cut, collected in Eppendorf tubes, and dewaxed using two changes of xylene for 10 minutes each at 60°C. The samples were dehydrated in decreasing concentrations of alcohol (100%, 70%, 50%) for 10 minutes each and dried in a Speed Vac for 3–4 minutes. Lysis buffer was added to the tubes (20 mM Tris, pH 7.5, 20 mM EDTA, 2.5 μ g/ml proteinase K, 1% SDS), and these were incubated overnight at 55°C. The samples were then spun down, the buffer was removed, and RNA was extracted with TRIzol.

RT-PCR. The following primers were used for PCR: SM α -actin: 5' forward primer, 5'-TCCCTGGAGAAGAGC-TACGA-3', and 3' reverse primer, 5'-GGGCTTTAATCTC-CTTCGG-3'. Desmin: 5' forward primer, 5'-GTGAAGATGGCCTTGATGT-3', and 3' reverse primer, 5'-GTAGCCTCGCTGACAACCTC-3'. SM22: 5' forward primer, 5'-TGTGACCAAAAACGATGGAA-3', and 3' reverse primer, 5'-ATAGGCATTTGTGAGGCAGG-3'. SM-myosin: 5' forward primer, 5'-GACAACCTCTCTCGCTTTGG-3', and 3' reverse primer, 5'-GCTCTCCAAAAGCAGGTCCAC-3'. Tropoelastin: 5' forward primer, 5'-AGATGGCTCCTCA-CACTGGT-3', and 3' reverse primer, 5'-AGCACCACCAC-CTGGATAAA-3'. SRF isoforms: 5' forward primer, 5'-ATCACCAACTACCTGGCACC-3', and 3' reverse primer, 5'-CACCTGTAGCTCGGTGAGGT-3'. The same set of primers was used to amplify both SRF isoforms in mice

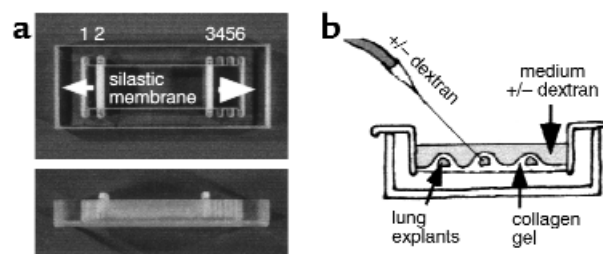
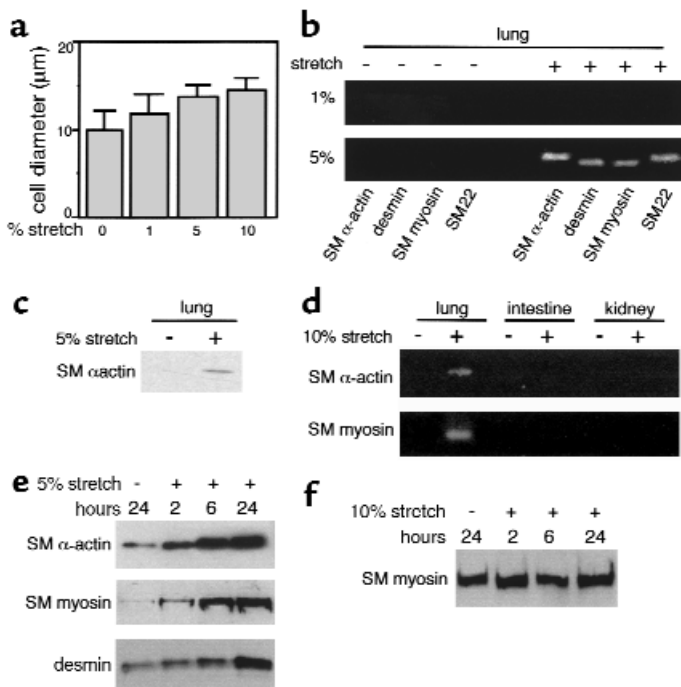


Figure 1

(a) The cell-stretching device consists of a rectangular acrylic dish with a stretchable silastic membrane fitted to the bottom and attached to removable clamps. The embryonic mesenchymal cells are cultured on the membrane coated with 0.1% poly-L-lysine to maintain the cells' round shape and thereby prevent spread-induced SM differentiation or on 0.01% poly-L-lysine or collagen I to allow spread-induced SM differentiation. The membrane is longitudinally stretched up to several lengths by placing the clamps into different slots (labeled 1 to 6). (b) Diagram of the organ culture system used in this study is shown. Mouse and human embryonic lung explants are cultured embedded in a thin collagen gel layered on a transwell device. Dextran, a volume expander polysaccharide, is microinjected into the airways to increase the intraluminal pressure by attracting liquid. Alternatively, dextran is dissolved in the culture medium to obtain the opposite effect.

Figure 2

(a) Measurement of mesenchymal cell elongation caused by different uniaxial stretching forces. Undifferentiated (round) cells were measured in this study. Each column represents the mean maximal diameter of 50 cells \pm SD. (b) RT-PCR shows induction of SM-specific mRNA in undifferentiated embryonic lung mesenchymal cells after 2 hours of 5% uniaxial stretch. (c) Immunoblot shows SM α -actin translation in embryonic lung mesenchymal cells after 18 hours of uniaxial stretch. (d) Lack of myogenic response to stretch in undifferentiated mesenchymal cells from the intestine and kidney after 2 hours of uniaxial stretch are shown. This study was done under conditions that inhibit cell proliferation (1% FBS). (e) Immunoblots show stretch-induced upregulation of SM-specific protein synthesis in embryonic mesenchymal cells undergoing spread-induced myogenic differentiation. Notice that desmin requires more time to be stimulated. The cells are plated under conditions that allow cell spreading and then stretched for 2, 6, or 24 hours (lanes 2 and 3). The nonstretched controls (lane 1) were cultured for 24 hours. (f) Upon reaching full SM differentiation (96 hours in culture, SM-specific protein levels and electrical properties of mature SM cells; ref. 11), mesenchymal cells stop responding to stretch under the conditions tested here. The studies presented in b-f were repeated more than three times with similar results.



and humans. RT-PCR was performed with the GeneAmp RNA PCR kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA), as described previously (11). Twenty cycles were run for all amplifications besides tropoelastin, which was run for 28 cycles, and SRF, which was run for 20 to 35 cycles. Message for all the SM-specific proteins, except SM myosin, was detected in the round cells if the number of cycles was increased above 30. SM myosin mRNA was not detected in round cells even after 50 cycles.

SRF and SRF Δ 5 transfections. SRF and SRF Δ 5 cDNAs cloned into pGEM-T easy (Promega Corp., Madison, Wisconsin, USA) (18) were released from the vector by *Eco*RI and ligated into *Eco*RI-digested pCDNA3 expression vector (Invitrogen Corp., Carlsbad, California, USA). The orientation of the clones was determined by restriction digestion with *Pst*I, and the sequence was confirmed. Primary cultures of mouse lung embryonic mesenchymal cells were transfected 1 hour after attachment was completed using Lipofectamine plus reagent (Life Technologies Inc.), following the manufacturer's instructions. The plasmids and empty vector were mixed with the Lipofectamine reagent in a 1:3.5 wt/vol proportion, and the cells were transfected for 3 hours in the presence of 10% FBS. In the experiments in which the cells were stretched, transfection and stretching were initiated concomitantly. In this case the cells were allowed to spread since, in our experience, round cells are difficult to transfect. The cells were lysed 18 hours after transfection. This experimental approach does not allow for determination of transfection efficiency. However, studies using hemagglutinin-tagged plasmid constructs unrelated to SRF indicated that approximately 30–35% of the cells in our primary cultures are transfected (S. Beqaj and L. Schuger, unpublished observation).

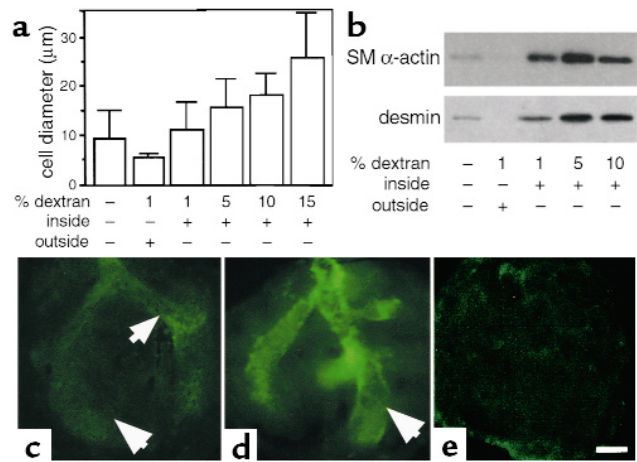
To determine whether transfections affected total protein synthesis, control and transfected cells were metabolically labeled with 100 μ Ci/well of [35 S]-methionine (NEN-Dupont, Boston, Massachusetts, USA). [35 S]-methionine was added to the cultures concomitantly with the plasmid constructs. At the end of the culture period, the cells were lysed, and the protein was precipitated with 10% trichloroacetic acid. The [35 S]-methionine incorporated into 1 μ g of precipitated protein was determined as cpm in a β -scintillator counter.

Results

Stretch induced mouse-lung undifferentiated mesenchymal cells to express SM proteins and stimulated differentiation of SM myoblasts. In the first set of studies mesenchymal cells were prevented from spreading (and undergoing spread-induced SM differentiation) by plating on 0.1% poly-L-lysine. Upon stretching, the cells increased their maximal diameter from $16 \pm 2.5\%$ with 1% stretching to up to $38 \pm 1.6\%$ with 10% membrane stretching (Figure 2a). Lung undifferentiated mesenchymal cells responded to 2 hours of 5% and 10% uniaxial stretching by turning on the expression of SM myosin and drastically increasing mRNA levels for SM α -actin, desmin, and SM22 (Figure 2, b and d). In these cells SM-specific protein translation was initiated after 18 hours of stretch (Figure 2c). Immunohistochemical studies indicated that all of the cells in culture synthesized equal levels of SM protein (not shown). Control (nonstretched) cells and cells exposed to 1% stretch remained undifferentiated for the whole period studied (Figure 2, b and c) and synthesized high levels of α -fetoprotein, an embryonic marker (not shown). Stretching forces of 15% or higher did not induce SM myosin expression and decreased SM α -actin, desmin, and SM22 to levels undetectable by

Figure 3

(a) Determination of periluminal mesenchymal cell elongation caused by different concentrations of dextran within the airways. Since mesenchymal cell boundaries are indistinguishable *in vivo*, we measured the distance between the centers of adjacent nuclei (the more the stretch, the more the distance). Each column represents the mean measurement of 40 distances \pm SD. (b) Immunoblots show modulation of SM protein synthesis in embryonic lung organ cultures by dextran-induced peribronchial cell stretch. A dose-response increase in SM protein synthesis is seen in day-11 lung explants containing dextran inside the airways after 24 hours in culture (lanes 3–5). No SM protein synthesis is observed when dextran is present in the culture medium outside the lung, preventing development of intraluminal pressure (lane 2). Minimal synthesis of SM proteins is seen in the control lung explants in which some hydrostatic pressure is likely to be produced by the fluid secreted by epithelial cells (lane 1). (c–e) Immunofluorescence shows modulation of bronchial myogenesis in embryonic lung organ cultures by dextran-induced peribronchial cell stretching. Day-11 lung explants were cultured for 24 hours and then immunostained for SM α -actin as whole-mount preparations. (c) SM α -actin in lung explants cultured without dextran. Notice the occurrence of some bronchial SM development (arrows). (d) SM α -actin in lung explants cultured with 5% dextran inside the airways, where significant bronchial myogenesis took place (arrows). (e) In lung explants cultured in the presence of 1% dextran in the culture medium, notice the absence of bronchial SM development. Results shown in b–e are representative of three experiments, each performed on duplicate lung explants per treatment. Bar, 100 μ m.



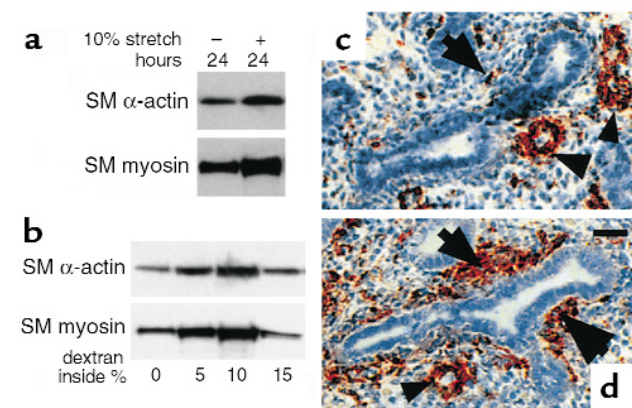
RT-PCR (not shown). Mesenchymal cells from kidney and intestine subjected to 1–15% stretching forces remained undifferentiated (Figure 2d). These cells, however, differentiated into SM cells upon spreading (11). The response to stretching was observed irrespective of whether the cells were cultured under conditions that promoted or inhibited cell proliferation, as indicated by the expression of SM markers in mesenchymal cells cultured in 1% FBS (Figure 2d). Lung mesenchymal cells undergoing spread-induced SM differentiation (plated on 0.01% poly-L-lysine or collagen I) responded to mechanical stretch by further upregulating the production of SM proteins (Figure 2e). Differentiated SM cells (after 96 hours in culture) showed no response to uniaxial stretching (Figure 2f).

Stretch induced SM myogenesis in mouse embryonic lung organ cultures. In mouse embryonic lung organ cultures, maximal diameter of distal peribronchial mesenchymal cells increased from $10 \pm 6 \mu$ m (with no dextran) to $26 \pm 8 \mu$ m with 15% intraluminal dextran (Figure 3a)

(*in vivo* normal SM cell diameter is $20 \pm 10 \mu$ m). Although the magnitude of stretch and the dextran concentration are graded similarly (1–15%), notice that these are not equivalent. For instance, 5% dextran caused more cell elongation than 10% stretch. Also, unstretched cells have a slightly smaller diameter in explants than in cell culture. In the absence of intraluminal dextran, the lung explants developed some bronchial muscle after 24 hours in culture (Figure 3, b and c). This may reflect the secretion of fluid by the epithelial cells (21), which is likely to cause some intraluminal hydrostatic pressure. By comparison, a prominent bronchial SM layer was developed in response to the stretch generated by 1–5% dextran (approximately 20–60% cell elongation) (Figure 3, b and d). Dextran concentrations higher than 15% resulted in less SM protein synthesis (not shown). When intraluminal pressure was maintained at a minimum by increasing the osmotic pressure outside the lung, no bronchial SM development was observed (Figure 3, b and e).

Figure 4

(a) Immunoblot shows stretch-induced upregulation of SM-specific protein synthesis in human lung mesenchymal cells undergoing myogenic differentiation. (b) Immunoblots show stimulation of SM protein synthesis in human fetal lung organ cultures by dextran-induced tissue stretching. In a and b the lungs were obtained from 18-week fetuses, after the onset of visceral and vascular SM differentiation. Therefore, the initial levels of SM protein are higher than in murine cells. (c and d) Immunohistochemistry shows SM α -actin in histological sections from human fetal lungs (18 weeks) cultured for 48 hours with 1% dextran in the culture medium outside the explant (c) and 1% dextran inside the airways (d). Notice the significant increment in bronchial SM cells in c compared with d (arrows). The vascular SM shows no differences (arrowheads). Results are representative of three experiments, each performed on quadruplicate lung explants per treatment. Bar, 20 μ m.



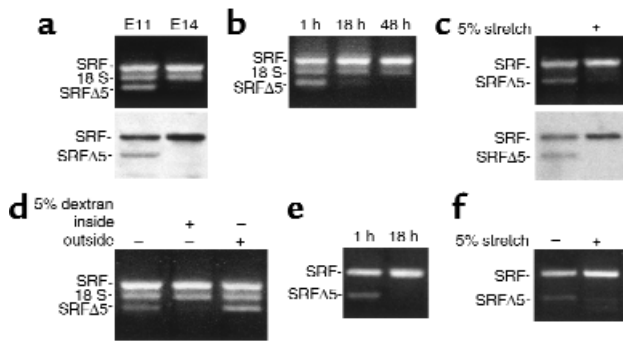


Figure 5
 (a) RT-PCR and immunoblot demonstrate the presence of SRFΔ5 in fresh (uncultured) mouse undifferentiated peribronchial mesenchymal cells on day 11 (E11) and absence of SRFΔ5 with concomitant increment in SRF on day 14 (E14), after the peribronchial cells become SM cells. S18 represents an internal control. The increment in SRF isoforms was best seen at the protein rather than at the message level. (b) RT-PCR shows SRF and SRFΔ5 mRNA changes along with cell spread-induced SM differentiation in culture. Notice the increment in SRF mRNA and the decrease and disappearance of SRFΔ5 mRNA. (c) RT-PCR demonstrates rapid disappearance of SRFΔ5 mRNA and increments in SRF mRNA upon 4 hours of sustained stretch. The same change is seen at the protein level after 12 hours in the immunoblot in the lower panel. (d) Effect of peribronchial mesenchymal cell stretch on SRF and SRFΔ5 isoforms in lung organ cultures. One percent dextran inside the airways led to suppression of SRFΔ5 and increase in SRF (lane 2), whereas dextran in the medium outside the lung explants maintained the SRF isoform profile characteristic of undifferentiated mesenchymal cells (lane 3). (e and f) Effect of cell spreading (e) and stretching (f) on SRFΔ5 in human fetal mesenchymal cells. Notice that the very low levels of SRFΔ5 mRNA found in the human cells are likely a reflection of their more advanced stage of SM differentiation. Results shown are representative of three experiments, each done on duplicate samples per treatment.

Stretch leading to approximately 60% cell elongation stimulated myogenesis in lung explants, but inhibited SM differentiation in cell cultures. These differences are likely to be related to the more artificial conditions created by cell culture compared with organ culture.

Stretch stimulated the expression of SM proteins in human lung embryonic mesenchymal cells and organ cultures. Human differentiating SM cells in primary cultures increased their expression of SM proteins upon stretching in a manner similar to that of mouse SM-differentiating cells (Figure 4a). Similarly, human lung explants responded to the stretch generated by 1–10% dextran (approximately 20–60% cell elongation) by increasing the synthesis of SM proteins (Figures 4, b–d). Concentrations greater than 15% resulted in less SM protein synthesis. The observation that SM markers are higher in unstretched human cells, compared with the murine counterparts, likely reflects the more advanced developmental stage of the human lungs and the presence of some differentiated SM cells before organ culture.

Stretch regulated the synthesis of SRF and SRFΔ5 in lung embryonic mesenchymal cell and organ cultures of mouse and human origin. Immunoblot analysis of peribronchial

mesenchymal tissue microdissected from mouse lungs before the onset of myogenesis (day 11) and lysed after brief plating (to separate epithelial cells) demonstrated that these cells synthesize both SRF and SRFΔ5 isoforms in vivo (Figure 5a). However, bronchial SM cells isolated in a similar manner from day 14 lungs (after the onset of bronchial muscle) synthesize only SRF (Figure 5a). Furthermore, levels of SRF message and protein were higher in SM cells compared with their undifferentiated counterparts, consistent with a change in SRF pre-mRNA alternative splicing. Since undifferentiated embryonic mesenchymal cells undergo myogenic differentiation upon spreading/elongation in culture (10, 11), these were evaluated for concomitant changes in SRF alternative splicing. RT-PCR confirmed that in the first 24–48 hours of myogenic differentiation SRFΔ5 isoform synthesis is gradually suppressed, whereas SRF isoform production increases (Figure 5b). We next determined the effect of mechanical stretch upon SRF isoform production in mouse lung cell and organ culture. Mechanical stretch elicited the same change in SRF isoform production observed during in vivo and in vitro SM myogenesis. Four hours of sustained uniaxial stretch caused suppression of SRFΔ5 mRNA synthesis with a concomitant increase in SRF mRNA levels (Figure 5c). Consistent with these changes, after 12 hours in culture nonstretched cells synthesized SRF and SRFΔ5 while stretched cells synthesized only SRF (Figure 5c). A similar pattern of SRF isoform production in response to dextran-induced peribronchial mesenchymal stretch was seen in mouse lung organ cultures (Figure 5d). Mesenchymal cells isolated from human lungs at week 16 of gestation also synthesized SRF and SRFΔ5 but, upon spread- and stretch-induced SM differentiation,

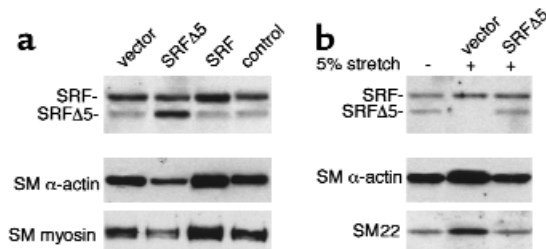


Figure 6
 (a) Transfection of SM-differentiating cells with SRF and SRFΔ5 expression vectors, empty vector, or nontransfected controls. Primary cultures of mouse lung embryonic mesenchymal cells were plated under conditions that allow cell spreading. The cells were transfected 1 hour after attachment was completed. In the experiments in which the cells were stretched, transfection and stretching were initiated concomitantly. The cells were lysed 18 hours after transfection. SRF overexpression stimulates and SRFΔ5 overexpression inhibits SM differentiation. The vector alone does not affect differentiation (compare with nontransfected control cells). (b) SRFΔ5 overexpression inhibits stretch-induced SM differentiation. Notice that, as expected, untransfected stretched cells synthesize higher levels of SRF than untransfected nonstretched ones (lane 1). These experiments were repeated four times with similar results.

SRFΔ5 isoform synthesis was suppressed whereas SRF isoform production increased (Figure 5, e and f). The very low levels of SRFΔ5 found in these cells are likely a reflection of a more advanced stage of myogenesis compared with the murine counterparts.

SRF stimulated and SRFΔ5 inhibited spontaneous and stretch-induced myogenic differentiation. We first determined the effect of overexpressing SRF and SRFΔ5 isoforms in mouse embryonic mesenchymal cells undergoing spread-induced SM differentiation in culture. The process of SM differentiation was either inhibited or facilitated by transfecting these cells with SRFΔ5 or SRF (Figure 6a). We then determined the effect of transfecting SRFΔ5 into stretched cells. In these studies the cells were transfected and stretched immediately upon attachment and were also allowed to spread to facilitate transfection. These studies showed that stretch-induced myogenesis was blocked by expression of SRFΔ5 (Figure 6b). Metabolic radiolabeling demonstrated no difference in protein synthesis between transfected and untransfected cells. At the end of the culture period nontransfected cells incorporated [³⁵S]-methionine at a rate of 21,540 ± 410 cpm/μg protein, cells transfected with

empty vector incorporated 20,440 ± 520 cpm/μg protein, and cells transfected with SRFΔ5 plasmid incorporated 21,030 ± 560 cpm/μg protein. This indicated that the inhibitory effect of transfected SRFΔ5 was not due to a nonspecific decrease in protein synthesis.

Human hypoplastic lungs related to conditions that impair pulmonary stretch/distention showed poor visceral myogenesis with a consequent decrease in elastin deposition. Immunostaining with anti-SM α-actin demonstrated a severe decrease in the amount of visceral SM, including bronchial and interstitial SM cells, in all of the human hypoplastic lungs studied (Figure 7, arrows). The maximal inhibition in visceral myogenesis was seen in areas of collapsed lung parenchyma resulting from diaphragmatic hernia where there were almost no visceral SM cells (Figure 7c). Epithelial, endothelial, and vascular SM cells in the hypoplastic lungs did not show significant differences in amount and distribution compared with controls (Figure 7, d–g, and arrowheads for vascular SM). Immunohistochemical studies demonstrated elastin deposition in the bronchi and bronchioli of normal fetal lungs (Figure 7h, arrows), whereas hypoplastic fetal lungs of the same gestational age

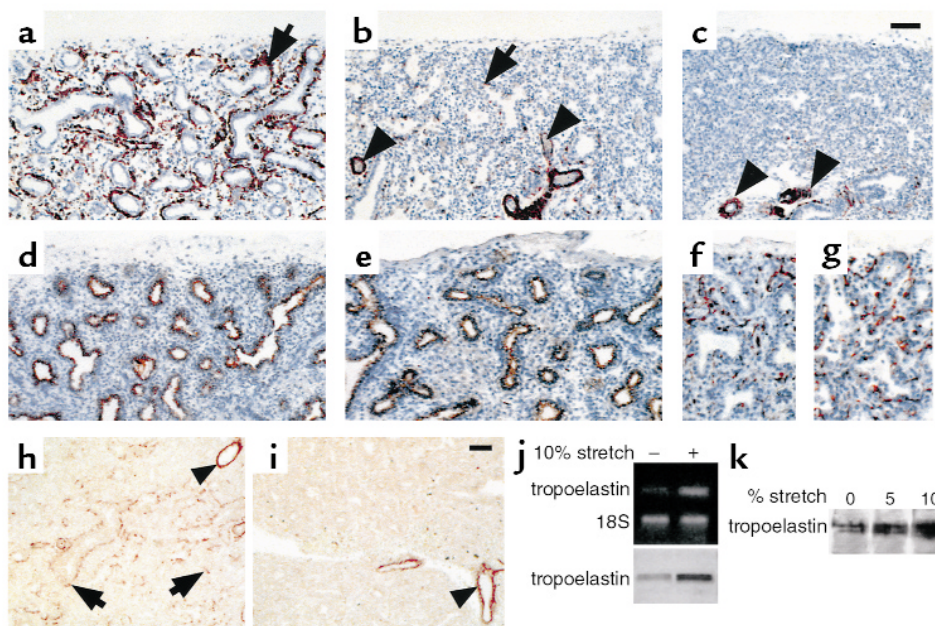


Figure 7

Immunohistochemistry showing paucity of visceral SM cells in human fetal hypoplastic lungs. Shown are histological sections from normal lung (a), hypoplastic lung caused by oligohydramnion (b), and hypoplastic lung caused by diaphragmatic hernia (c), all at 22 weeks of gestation, immunostained for SM α-actin. There is a significant decrease in bronchial and interstitial SM cells (arrows) in the hypoplastic lungs (b and c), particularly in those compressed by intrathoracic herniation of abdominal viscerae due to diaphragmatic hernia (c). The vascular musculature seems unaffected (arrowheads). In the same hypoplastic lung shown in b, the epithelial cells, immunostained for low-molecular-weight cytokeratins (e), and the endothelial cells, immunostained for PECAM-1 (g), show no changes compared with controls (d and f). Photos (h and i) demonstrate immunohistochemistry showing decrease in tropoelastin deposition in human hypoplastic lungs. (h) Histological sections from the normal lung at 20 weeks of gestation demonstrate tropoelastin deposition around bronchi and bronchioli and at scattered interstitial sites (arrows). (i) Histological sections from same age hypoplastic lung reveals essentially no tropoelastin deposition, with the exception of vascular SM that shows no changes in tropoelastin when compared with controls (arrowheads). Bar, 60 μm in a–e and h and i. Bar, 100 μm in f and g. (j) RT-PCR and immunoblot show stretch-induced upregulation of tropoelastin expression in mouse lung embryonic mesenchymal cells undergoing myogenic differentiation. (k) Immunoblot shows stretch-induced upregulation of tropoelastin synthesis in human lung embryonic mesenchymal cells undergoing myogenic differentiation. Results shown in j and k are representative of three experiments conducted in duplicate sample per treatment.

showed no elastin deposition except for the blood vessels (Figure 7i, arrowheads). Mouse and human SM-differentiating cells subjected to stretch increased their synthesis of tropoelastin (Figure 7, j and k). These studies suggested that the decrease in elastin seen in hypoplastic lungs may be the combined effect of fewer cells producing less tropoelastin per cell.

SRFΔ5 was present in hypoplastic lungs but not in normal age-matched controls. Intact RNA was isolated from five out of the nine hypoplastic lungs, ages 20 to 25 weeks (two samples of diaphragmatic hernia, both isolateral to the hernia, and three cases of oligohydramnion). In addition, RNA was isolated from five age-matched controls. RT-PCR amplified both SRF and SRFΔ5 in the five hypoplastic lungs, but only SRF isoform in the controls (Figure 8). Furthermore, the levels of SRF appeared higher in the controls than in the hypoplastic lungs.

Discussion

Stretch plays a critical role in initiating and maintaining lung visceral myogenesis. Although the morphological aspects of visceral myogenesis are well documented, the stimuli that initiate and control the process have remained elusive. Based on the hypothesis that mechanical stimuli are an important factor in SM development, we devised cell and organ culture systems to determine the role of stretch in bronchial myogenesis. These studies demonstrated that mechanical stretch alone is sufficient to induce lung undifferentiated mesenchymal cells to follow a myogenic pathway. Similarly, SM-differentiating cells responded to stretch by upregulating the expression of SM proteins. The critical contribution of cell stretching to visceral myogenesis was best exemplified in mouse lung organ cultures, where bronchial SM differentiation was either induced or prevented by modulating the airway intraluminal pressure.

Fully differentiated SM cells and embryonic mesenchymal cells from gut or kidney did not respond to stretch under the conditions studied. These observations suggest a lower susceptibility to mechanical stimuli. Interestingly, in a previous study we found that embryonic mesenchymal cells from lung, gut, and kidney equally differentiated into SM upon spreading (11). The reasons for this unexpected difference in response to stretch are unclear. However, they may be related to the unique type of mechanical stimuli sustained by the lung. Besides the stretch generated by intraluminal hydrostatic pressure common to all three organs, the lung also sustains the rhythmic stretch produced by intrauterine breathing. Therefore, it could be possible that lung mesenchymal cells are highly dependent on stretch as the main stimulus for muscle differentiation, while additional factors are required in the intestine and urinary tract.

The most extensively reported effect of cell stretching is the stimulation of cell proliferation (22, 23). However, we found that induction of myogenesis by cell stretching was independent of cell growth, as it took place despite serum deprivation. In this regard, our pre-

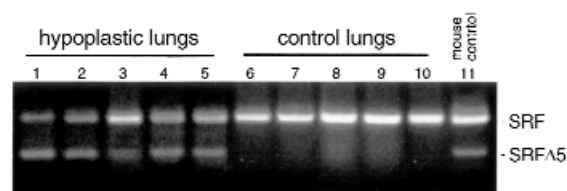


Figure 8

RT-PCR shows SRFΔ5 and SRF isoforms in five human hypoplastic lungs: lane 1, lung isolateral to diaphragmatic hernia, week 20; lane 2, lung isolateral to diaphragmatic hernia, week 22–23; lane 3, lung from oligohydramnion, week 25; lane 4, lung from oligohydramnion, week 21; lane 5, lung from oligohydramnion, week 24. Lanes 6–11 show lungs from five matching controls with the presence of higher SRF levels and absence of SRFΔ5. Lane 11 represents SRF isoforms in day-11 mouse lung, and it has been used as an additional control.

vious studies demonstrated that SM myogenesis induced by cell spreading/elongation is also independent of cell growth (10, 11).

Stretch induces a change in SRF RNA alternative splicing that promotes SM-specific gene expression. Using these culture systems, we began to explore the molecular mechanisms underlying stretch-induced myogenesis. We focused initially on SRF because this transcription factor stimulates expression of a broad range of SM-specific proteins. The biological activity of SRF was therefore compatible with the effects of stretch. It has been shown recently that SRF has three alternative spliced variants in addition to the full SRF molecule (17, 18). We found that one of them, SRFΔ5 (lacking exon 5), is synthesized by lung undifferentiated mesenchymal cells, which also express relatively high levels of SRF. Our studies demonstrated that during the first stages of myogenesis, SRFΔ5 synthesis is suppressed in favor of increased SRF production. This change in SRF isoform profile seems to play an important functional role in stimulating SM differentiation, as indicated by the transfection studies. In this regard, it should be stressed that since these were done on primary cultures, without further selection for transfected cells, the results reflect a cell pool that also included nontransfected cells. Therefore the differences in SM protein expression are likely to be greater than those detected.

Our findings correlated well with those of Belaguli et al. (17). Using promoter-reporter constructs, this group showed that SRFΔ5 behaves as a powerful dominant-negative isoform inhibiting SM α -actin and SM22 expression elicited by SRF. However, SRFΔ5 can also stimulate muscle-specific gene expression, depending upon the cell type as well as other factors (18).

SRFΔ5 binds to SRF and to DNA-SRF elements (17). Therefore, it has been proposed that the repression of SM protein expression by SRFΔ5 is due to the formation of SRF-SRFΔ5 heterodimers as well as the interaction of SRFΔ5 with SRF promoter-binding sites (17). These possibilities seem to be refuted by the fact that SRF is present in lung undifferentiated mesenchymal cells in significantly higher levels than SRFΔ5. However, SRFΔ5

may function only as a selective antagonist of SRF myogenic activity without participation in other SRF-mediated processes. In such a case the small amount of SRF Δ 5 could be recruited to few specific DNA sites by means of their comparatively high binding affinity to SRF Δ 5 or through binding to their associated proteins.

SRF Δ 5 mRNA has been identified in adult aorta and striated muscle (17, 18). This may suggest that other SRF isoforms could substitute for SRF Δ 5 antimyogenic function, depending on the type of muscle (smooth or striated), or even more, that myogenesis in different sites proceeds through different pathways. Alternatively, SRF Δ 5 may be re-expressed in some muscles after their differentiation is completed, perhaps to serve other functions. Supporting the last possibility, SRF Δ 5 mRNA has been identified in lysates of adult organs bearing SM, including stomach (17) and uterus (18), but was not detected in adult intestinal SM (Y. Yang and L. Schuger, unpublished observation).

Presence of SRF Δ 5 and paucity of bronchial and interstitial SM cells in human hypoplastic lungs suggest that visceral myogenesis may be impaired by decreased stretching. Lung hypoplasia is a severe and often fatal neonatal condition characterized by a low lung-to-body weight ratio (small lungs) (21). The two main causes of lung hypoplasia are diaphragmatic hernia and oligohydramnion (21). Although the pathogenesis of lung hypoplasia associated with these two conditions is not well understood, low mechanical tension is thought to play an essential role. The developing lung is filled with liquid, mainly amniotic fluid but also fluid secreted by the lung itself (13, 21). In oligohydramnion the amniotic fluid volume is diminished, producing a decrease in intrapulmonary hydrostatic pressure (24, 25). In diaphragmatic hernia the abdominal organs are displaced into the thoracic cavity, thereby precluding normal lung expansion and indirectly decreasing the airway hydrostatic pressure (26, 27). Diminished intrapulmonary pressure and impaired distention result in less mechanical stretch (24–30), inasmuch as correcting the latter in utero by artificially increasing the airway hydrostatic pressure ameliorates these two types of lung hypoplasia (26, 29, 30).

Although no bronchial or interstitial SM abnormalities have been reported in hypoplastic lungs, our experimental data suggested that they may be defective. Indeed, immunohistochemistry demonstrated a severe decrease in bronchial as well as interstitial SM cells in human hypoplastic lungs compared with age-matched controls and to the other main cell types in the lung. The normal development of vascular SM in hypoplastic lungs was consistent with our in vitro observations. If mechanical stretching also induces vascular myogenesis, then the stretching forces would have to come from the hydrostatic pressure within blood vessels, which is not decreased in hypoplastic lungs.

Elastin represents a critical structural and functional component of the lung. Since tropoelastin, the monomeric form of elastin, is synthesized mainly by

SM cells (6, 31), we determined the deposition of this extracellular matrix constituent in hypoplastic lungs. Consistent with the lack of SM cells, immunohistochemical studies indicated that hypoplastic lungs contain significantly less peribronchial and interstitial tropoelastin than controls. Absence of elastin was also reported in an ultrastructural study of human oligohydramnion-related hypoplastic lungs (32). In addition, our studies suggested that stretch induces elastin deposition not only by promoting SM differentiation, but also by stimulating tropoelastin synthesis by differentiating SM myoblasts. Therefore, the decreased elastin levels found in hypoplastic lungs may be the combined effect of fewer SM cells and less tropoelastin production by each of them.

Our studies demonstrated the presence of SRF Δ 5 and relatively lower levels of SRF in hypoplastic lungs compared with age-matched controls. These findings indicated an abnormal pattern of SRF splicing in the hypoplastic lungs and lent additional support to the role of mechanical stimuli in visceral myogenesis. In addition, the higher level of SRF Δ 5 in the hypoplastic lungs compared with the embryonic mouse suggested that this isoform might be more prevalent in the early human lung than in its murine counterpart. The signaling pathways connecting cell stretch to SRF alternative splicing are currently unknown. Likewise there is no information on other factors that may regulate SRF isoform profile in addition to mechanical stimuli. Therefore, it is presently unclear how specifically SRF-splicing regulation is associated with the latter. In fact, the possibility exists that abnormalities in SRF RNA alternative splicing represent a common denominator for diseases characterized by abnormal SM or SM-related cells in the lung, such as chronic lung fibrosis, asthma, or lymphangiomyomatosis. Further studies are required to address this possibility.

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