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Original Article

Serum Response Factor Is Essential for Prenatal Gastrointestinal Smooth Muscle Development and Maintenance of Differentiated Phenotype

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Background/Aims
Smooth muscle cells (SMCs) characteristically express serum response factor (SRF), which regulates their development. The role of SRF in SMC plasticity in the pathophysiological conditions of gastrointestinal (GI) tract is less characterized.

Methods
We generated SMC-specific *Srf* knockout mice and characterized the prenatally lethal phenotype using ultrasound biomicroscopy and histological analysis. We used small bowel partial obstruction surgeries and primary cell culture using cell-specific enhanced green fluorescent protein (EGFP) mouse lines to study phenotypic and molecular changes of SMCs by immunofluorescence, Western blotting, and quantitative polymerase chain reaction. Finally we examined SRF change in human rectal prolapse tissue by immunofluorescence.

Results
Congenital SMC-specific *Srf* knockout mice died before birth and displayed severe GI and cardiac defects. Partial obstruction resulted in an overall increase in SRF protein expression. However, individual SMCs appeared to gradually lose SRF in the hypertrophic muscle. Cells expressing low levels of SRF also expressed low levels of platelet-derived growth factor receptor alpha (PDGFRα^{low}) and Ki67. SMCs grown in culture recaptured the phenotypic switch from differentiated SMCs to proliferative PDGFRα^{low} cells. The immediate and dramatic reduction of *Srf* and *Myh11* mRNA expression confirmed the phenotypic change. Human rectal prolapse tissue also demonstrated significant loss of SRF expression.

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Conclusions
SRF expression in SMCs is essential for prenatal development of the GI tract and heart. Following partial obstruction, SMCs down-regulate SRF to transition into proliferative PDGFRα^{low} cells that may represent a phenotype responsible for their plasticity. These findings demonstrate that SRF also plays a critical role in the remodeling process following GI injury.
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Key Words
Gastrointestinal tract; Myocyte; Platelet-derived growth factor receptor alpha; Rectal prolapse; Serum response factor; Smooth muscle cell

Introduction

Serum response factor (SRF) is a transcription factor that plays a critical role in embryonic development as well as the growth and maintenance of all differentiated muscle types.[1-3] SRF belongs to the MADS (MCM1, Agamous, Deficiens, and SRF) superfamily of transcription factors, which recognize 10-nucleotide (CC [A/T]$_6$ GG) sequences called CArG boxes through a DNA binding domain.[4] In smooth muscle cells (SMCs), SRF drives expression of contractile and cytoskeletal proteins through recognition and binding of these CArG boxes, which are primarily located in the promoter and intronic regions of most SMC-specific genes.[5,6] The role of SRF as a "master switch" for contractility is further demonstrated by the loss of contractile phenotype with loss of SRF expression in muscle cells and the differentiation of fibroblasts to myofibroblasts with SRF overexpression in cell culture.[7]

The importance of SRF in embryonic development has been previously demonstrated in a SM22α-Cre knockout (KO) system in mice that resulted in *Srf* deletion in the majority of cardiomyocytes and vascular smooth muscle cells.[8] Interestingly, the SM22α-Cre *Srf* KO mice died at embryonic day (E) 11.5 and had severe structural defects in cardiomyocytes and SMCs.

In a similar transgenic mouse model that restricts deletion of *Srf* to primarily SMCs through the expression cassette Myh11-Cre-EGFP, we confirm the prenatal lethality of congenital *Srf* deletion in GI and cardiac muscle cells. We also provide new evidence that expression of SRF in SMCs is lost in a surgically induced hypertrophy model, in human rectal prolapse tissue, and in cell culture. Importantly, we present a new finding that the loss of SRF protein expression during dedifferentiation of SMCs may be accompanied by a gain of platelet-derived growth factor receptor alpha (PDGFRα) expression during proliferative expansion. Collectively, our data further supports the critical role of SRF in contractile tissues and its loss in pathologic states.

Materials and Methods

Generation of Congenital *Srf* Knockout Mice

The SMC-specific congenital *Srf* KO mouse line, *Tg(Myh11-Cre-EGFP);Srf*$^{lox/lox}$, was generated by cross-breeding a *Srf*$^{lox/lox}$ female homozygote mouse (The Jackson Laboratory, Bar Harbor, ME, USA) with a *Tg(Myh11-Cre-EGFP)* male mouse[9] according to procedures approved by the Institutional Animal Care and Use Committee at the University of Nevada, Reno.

Ultrasound Biomicroscopy

Pregnant dams were sedated with isoflurane and scanned with a high frequency ultrasound system containing a 32-MHz linear array transducer (VisualSonics Vevo 2100, Toronto, ON, Canada) for diagnosis of congenital heart defects. Ultrasound scanning was performed according to the manufacturer's instructions and recorded in real-time.

Partial Obstruction Surgery

One month old transgenic mice with the genotypes *Tg(Myh11-Cre-EGFP)*, *Tg(Pdgfra-EGFP)*, or *Tg(Myh11-Cre-EGFP)*; *Tg(Pdgfra-EGFP)* were used for intestinal partial obstruction surgeries as previously described.[10] Briefly, a silicon ring was surgically placed on the distal ileum just oral to the cecum. As a modification to the previously described protocol, a 5-0 polyglycolic acid suture was placed through the ends of the silicon ring opening to secure its closing. After ~2 weeks, the obstructed mice were sacrificed along with the sham operated control mice for histological and molecular analyses. Of note, the

Tg(Myh11-Cre-EGFP) mice expressed cytoplasmic EGFP (cEGFP) in SMCs, whereas *Tg(Pdgfra-EGFP)* expressed nuclear EGFP (nEGFP) in PDGFRα^+ cells. The surgical procedure was approved by the Institutional Animal Care and Use Committee at the University of Nevada, Reno, USA.

Histological Analysis

Srf KO and WT fetuses from gestational days 18 to 19 were fixed in Bouin's fixative for at least 24 hours followed by washing in 70% ethanol for several days for picric acid removal. Fixed embryos were dehydrated through an alcohol gradient, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Three sections from at least 2 different embryos were analyzed. Small intestine tissues from partially obstructed and sham control animals were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 1 hour 20 minutes at 4°C prior to dehydration through an alcohol gradient and H&E staining. Paraffin embedded sections of human rectal prolapse and control colon tissues were obtained from Stanford University Medical Center, Palo Alto, California, USA, where Institutional Review Board approval was obtained. Sections were examined using the iScan Coreo scanner (Ventana Medical Systems, Tucson, AZ, USA).

Immunohistochemical Analysis

Immunofluorescence microscopy of the jejunum was performed as previously described with minor modifications.[11] For immunohistochemistry, tissues were fixed in 4% paraformaldehyde/PBS for 1 hour 20 minutes at 4°C prior to dehydration overnight in 20% sucrose/PBS at 4°C. The tissues were cut and placed into Tissue-Tek Crymold (Sakura Finetek, Torrence, CA, USA) containing one part Tissue-Tek OCT Compound (Sakura Finetek) and one part 20% sucrose/PBS prior to flash freezing with liquid nitrogen. Embedded molds were sectioned with a cryostat at 8 μm thickness onto glass slides and stained with primary antibodies for SRF (1:100, Cat No: sc-13029; Santa Cruz Biotechnology, Dallas, TX, USA), Ki67 (1:100, Cat No: RM-9106-S0; Thermo Scientific, Fremont, CA, USA) overnight at 4°C followed by staining with the secondary antibody Alexa Fluor 594-Conjugated AffiniPure Donkey Anti-Rabbit IgG (1:500, Cat No: 711-585-152; Jackson Immuno Research, West Grove, PA, USA) for 1 hour at room temperature prior to mounting onto slides with 4,6-diamidino-2-phenylindole (DAPI)-Prolong Gold (Cat No: P36931; Invitrogen, Carlsbad, CA, USA). Images were collected using the Fluoview FV10-ASW 3.1 Viewer software (Olympus, Tokyo, Japan) with an Olympus FV1000 confocal laser scanning microscope.

Western Blot Analysis

SRF and MYH11 protein expression in the jejunum tissues from the hypertrophy and sham mice were analyzed by Western blot. Hypotonic lysis buffer was directly applied to treated tissue, which were then grinded. Supernatants were boiled for 5 minutes, and the protein samples (30 μg) were then subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (0.45 μM, Cat No. 162-0115; Bio-Rad Laboratories) for overnight in cold-room. After blocking, blots were incubated overnight in a cold-room with rabbit anti-SRF (1:1000, Cat No: sc-13029; Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-MYH11 (1:1500, Cat No: sc-79079; Santa Cruz Biotechnology), and monoclonal mouse anti-human smooth muscle actin (SM22α, Cat No. M0851; Dako, Glostrup, Denmark) diluted in 5% blocking buffer. The blots were then washed 3 times (10 minutes each) in TBS-T (Tris buffered saline containing 0.1% Tween 20) followed by incubation with peroxidase-conjugated AffiniPure bovine anti-goat IgG (1:5000, Cat No: 805-035-180; Jackson Immuno Research), peroxidase-conjugated AffiniPure donkey anti-mouse IgG (1:5000, Cat No: 715-035-150; Jackson Immuno Research) and peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (1:5000, Cat No: 711-035-152; Jackson Immuno Research) for 1 hour. After washing, the blot was finally detected by exposure to ECL (Cat No: RPN 2135; Amersham Biosciences, Piscataway, NJ, USA) for between 10 seconds and 2 minutes.

Cell Culture

The jejunum was dissected from 3-week-old transgenic *Myh11-Cre-EGFP* mice, which expressed EGFP in SMCs, and the muscularis (n = 3) was gently peeled off from the mucosa and incubated for 30 minutes at 37°C in a collagenase-based dissociation solution containing 1.3 mg/mL collagenase (type II; Worthington Biochemical, Freehold, NJ, USA), 2 mg/mL bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), 2 mg/mL trypsin inhibitor (Sigma), and 0.27 mg/mL ATP (Sigma), 10 mL of calcium-free Hank's balanced salt solution (pH 7.0). The tissue was then bluntly triturated with a glass pipette until all of the tissue was broken down in the media. Single cells were obtained by using a 30 μm Pre-Separation Filter (Miltenyi Biotec, Bergisch Gladbach, Germany) and seeded in a 4-well glass slide Lab-Tek II CC2 glass chamber slide (Thermo

Scientific) at a concentration of 1.9 $\times 10^6$ cells/mL per well in Medium199 (Invitrogen) with 10% heat inactivated fetal bovine serum (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), and incubated at 37°C with 5% CO_2 for 1, 2, 4, and 8 days with daily change of fresh media. Cultured slides were used for epifluorescence imaging or harvested for quantitative polymerase chain reaction (QPCR) analysis.

Quantitative Polymerase Chain Reaction

Total RNAs were extracted from the cultured cells, cDNAs were reverse transcribed from the total RNAs, and QPCR was performed on the cDNAs as previously described.[12] All primers used for RT-PCR are shown in Table.

Statistical Methods

Western blot and QPCR data were analyzed for statistical significance by using Student's test. Measured variables were expressed as mean ± SEM. The differences in mean values between 2 animal groups (sham and hypertrophy) were evaluated and considered significantly different when $P < 0.05$ or $P < 0.01$.

Results

Serum Response Factor Is Required for Prenatal Gastrointestinal Tract and Cardiac Development

All congenital homozygous *Srf* KO (KO hereafter) mice died before birth while heterozygous *Srf* KO and wild type mice were viable. Multiple breeding combinations consisting of homozygous floxed ($Srf^{lox/lox}$) or heterozygous floxed ($Srf^{lox/+}$) mice with Cre males and females resulted in birth of ~100 pups that were either wild type or *Srf* KO heterozygotes. We, therefore, examined the embryos of pregnant females. Figure 1A shows a representative mother that had 7 embryos at E15. Genotyping of the embryos identified 4 heterozygous KO ($Srf^{+/-}$), 2 wild type (WT, $Srf^{+/+}$), and 1 homozygous KO ($Srf^{-/-}$) in agreement with Mendelian genetics. Grossly, there was no significant difference in size between homozygous KO, heterozygous KO, and WT embryos (Fig. 1B). However, sagittal cross sections of whole fetuses at E18 showed that internally, the KO fetus was grossly underdeveloped compared to the WT fetus, and the severe underdevelopment of the GI tract in the KO fetus was particularly striking (Fig. 1C).

Interestingly, real-time doppler ultrasonography of the fetal heart (echocardiogram) also revealed that cardiac contractility, ejection fraction, and blood flow were significantly weaker in the KO fetus than WT fetus at E18 (Fig. 1D and Supplementary Video). The fetal defects of the congenital *Srf* KO mouse suggested that SRF is essential for development of the GI tract and heart as well as overall survival of the fetus in utero. In a parallel set of experiments, we also generated an inducible SMC-specific *Srf* KO mouse system, which showed that SRF is required for maintenance of a differentiated phenotype and prevention of degeneration and apoptosis in adult GI SMCs (unpublished data). Collectively, these animal studies also indicated that SRF is essential for development and maintenance of mature GI SMCs within the GI tract.

Serum Response Factor and PDGFRα Are Linked to Smooth Muscle Cell Hypertrophy

Since SMCs dedifferentiate into a proliferating phenotype and hypertrophy under pathological obstruction,[13] we hypothesized that SRF expression in GI SMCs decreases during the re-

Table. Oligonucleotides Used for Quantitative Polymerase Chain Reaction in This Study

Name	Sequence (5' to 3')	Tm	Gene	Size (c/gDNA, bp)
Srf-2	CCACTGGCTTTGAAGAGCCAGATC	59°C	Srf	132/1997
Srf-2r	CTGTCTGGATTGTGGAGGTGGTAC	59°C	Srf	
Myh-1	GATGACCTGGTCGTGGACTTGG	59°C	Myh11	121/1537
Myh-1r	CATCCGCATACTTGGAGGAGATG	57°C	Myh11	
Myo-5	GTTCAGCAGTTTCAGATATCACCCAG	58°C	Myocd	155/4440
Myo-5r	CTGAAGACATGAATGATCTTCCCTGG	58°C	Myocd	
Elk-1	GTTCAAGTTGGTGGATGCAGAGG	57°C	Elk1	146/4136
Elk-1r	CAAACTTCTGGCCGCTCACCTTG	59°C	Elk1	
Gapdh-1	AATGGTGAAGGTCGGTGTGAACG	57°C	Gapdh	155/1989
Gapdh-1r	CGTGAGTGGAGTCATACTGGAAC	57°C	Gapdh	

bp, base pair.

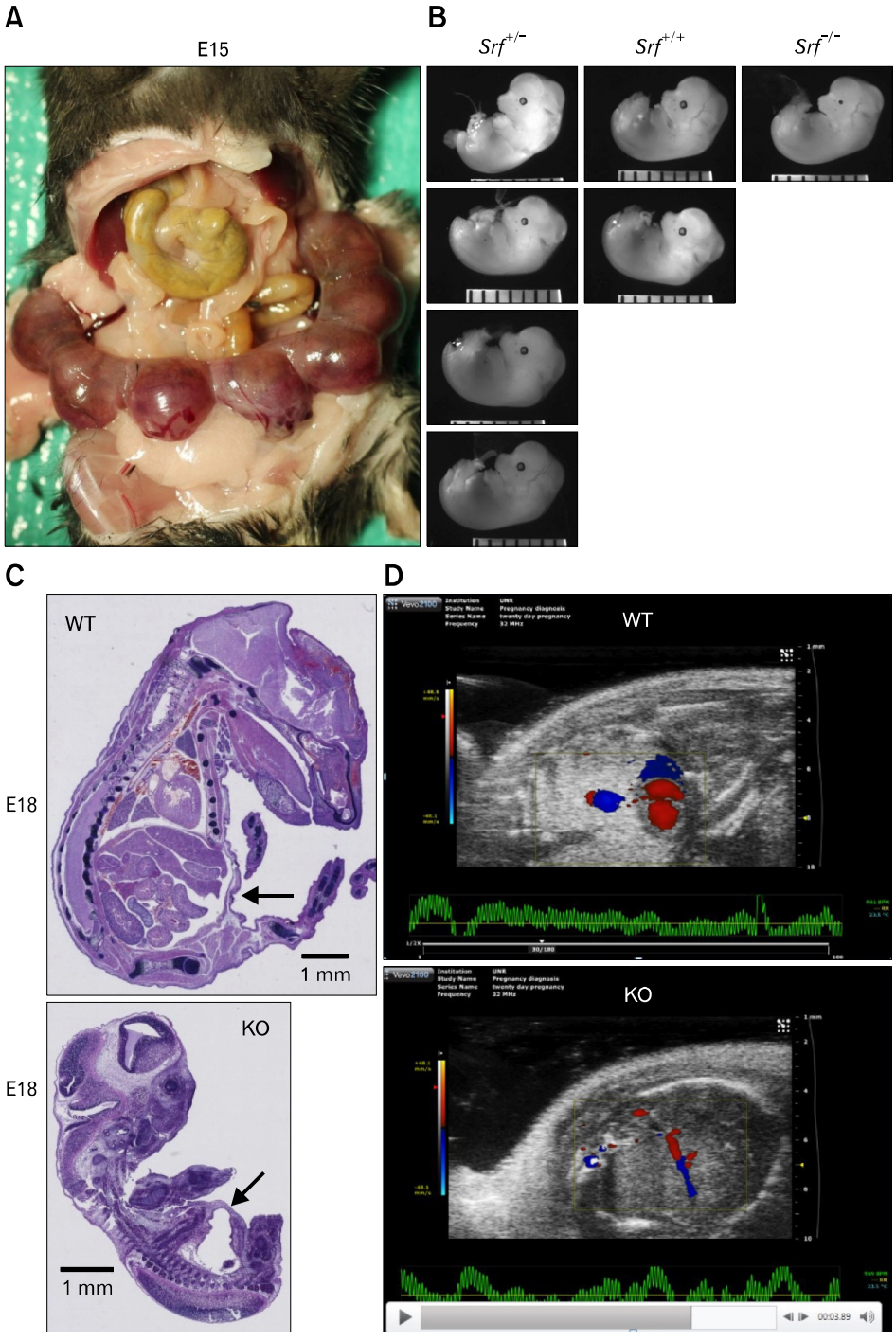

Figure 1. Fetal defects of congenital smooth muscle cell (SMC)-specific *Srf* knockout (KO). (A) A pregnant *Srf* KO mouse with the uterus containing 7 developing embryos at E15 days. (B) Genotypes and photomicrographs of fetuses showing wild type (WT), heterozygous ($Srf^{+/-}$), and homozygous Srf KO ($Srf^{-/-}$). (C) Sagittal cross sectional images of WT and KO fetuses with H&E staining at E18. Note significant global underdevelopment of the KO fetus with paucity of GI track development. (D) Doppler ultrasonography of viable WT and KO fetuses, respectively, showing red and blue areas indicating significantly lowered cardiovascular activity in KO fetus (see real-time images in Supplementary Video).

sulting compensatory process. To test our hypothesis, we looked for changes in SRF expression in hypertrophic GI tissue using a surgically induced partial obstruction mouse model.[10] Two weeks after placement of a small silicon ring on the distal ileum, the jejunum and ileum were severely distended compared to the large ring sham control (Fig. 2A). H&E staining confirmed the presence of significant hypertrophy in the longitudinal and circular muscle layers of partially obstructed jejunum (Fig. 2B), and western blot analysis showed that expression levels of SRF protein in the jejunum smooth muscle increased with partial obstruction, whereas expression levels of MYH11 and SM22α did not change (Fig. 2C and 2D). To examine the localization of

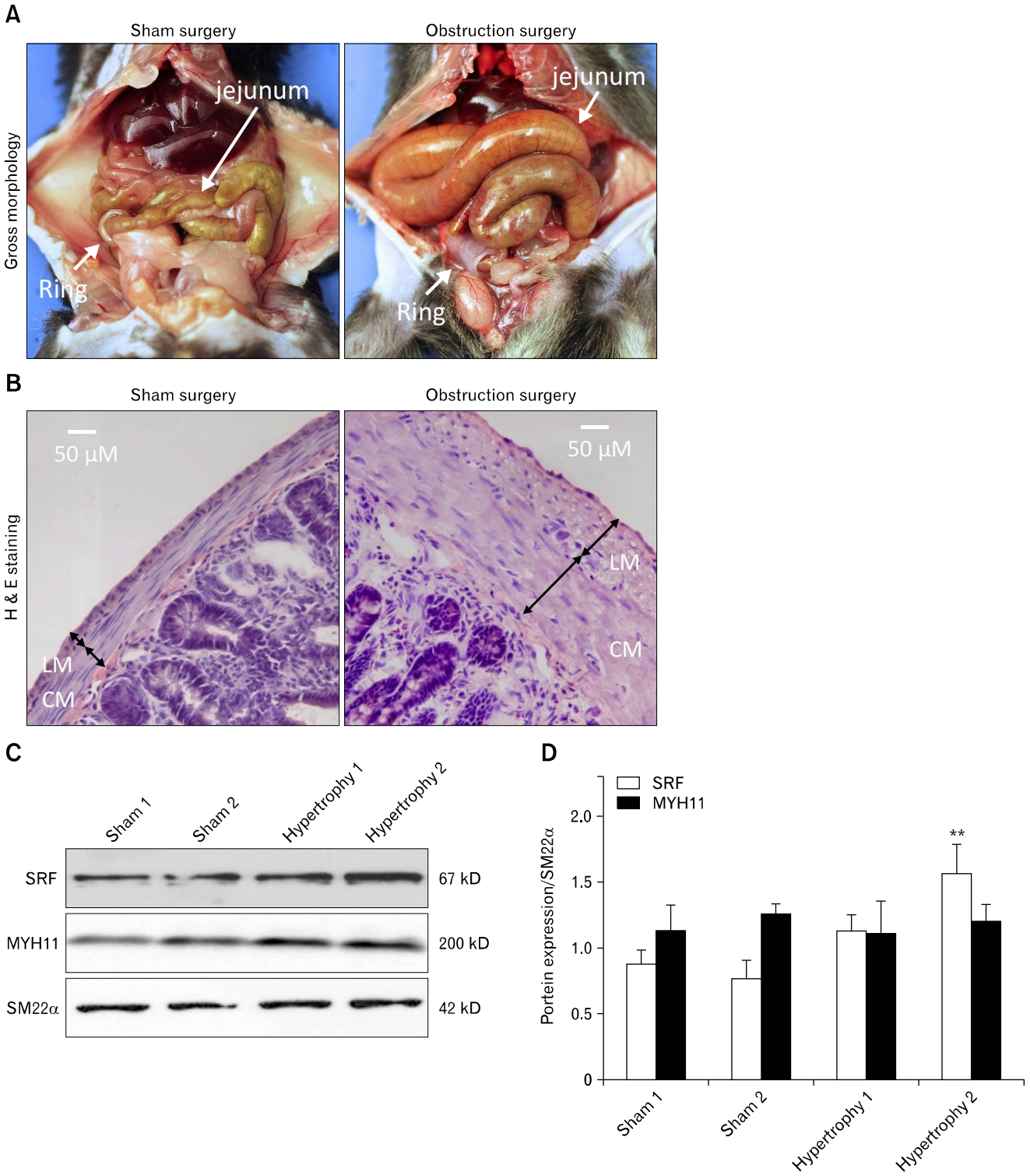

Figure 2. Gastrointestinal (GI) smooth muscle hypertrophy in mouse partial obstruction model. Partial obstruction related hypertrophy was surgically induced for ~2 weeks by placing a small silicon ring on the distal ileum of mice. (A) Gross images of GI tract in sham (large ring) and obstruction (small ring) surgeries. (B) Representative H&E staining of jejunal cross sections from sham control and partially obstructed mice. Hypertrophied jejunum contained significantly thicker circular (CM) and longitudinal muscle (LM) layers compared to a sham control. (C) Western blot analysis of SRF, MYH11, and SM22α in hypertrophied and sham control jejunum. (D) Summary of Western blot analysis. Samples were run in triplicates for each animal (n = 2), and bars represent standard error of the mean. $^{**}P < 0.01$.

SRF in the GI tract, we generated *Myh11*$^{cytoplasmic\ EGFP}$; *Pdgfra*$^{nuclear\ EGFP}$ mice and looked at SRF expression in the smooth muscle via immunofluorescence labeling. We discovered that there were at least 2 distinct populations of cells expressing SRF in the muscularis (Fig. 3A and 3B). The sham control jejunum had populations of SMCs expressing robust levels of SRF (SRF^{high} cells; Fig. 3A, white arrows) within the circular and longitudinal muscle layers. However, a magnified view of the hypertrophied jejunum revealed the presence of cells expressing low levels of SRF (SRF^{low} cells; Fig. 3B, yellow arrowheads) adjacent to a population of SRF^{high} cells (Fig. 3B, white arrows) located in the longitudinal muscle layer just outside the myenteric border. The layer of SRF^{high} cells in hypertrophied jejunum was notably thinner than that of the sham control, and most of these SRF^{high} cells expressed cytoplasmic EGFP (cEGFP) indicative of MYH11 expression and hence, SMC phenotype (Fig. 3B). However, these SMCs (cEGFP/SRF^{high}) also expressed low levels of nuclear EGFP (nEGFP), indicating a PDGFRα^{low} SMC/myofibroblast-like phenotype (Fig. 3B, white arrows). In contrast to the cEGFP/SRF^{high} SMCs of the longitudinal muscle, most cells in the subserosal hypertrophic region had little or no cEGFP expression along with low levels of SRF and nEGFP

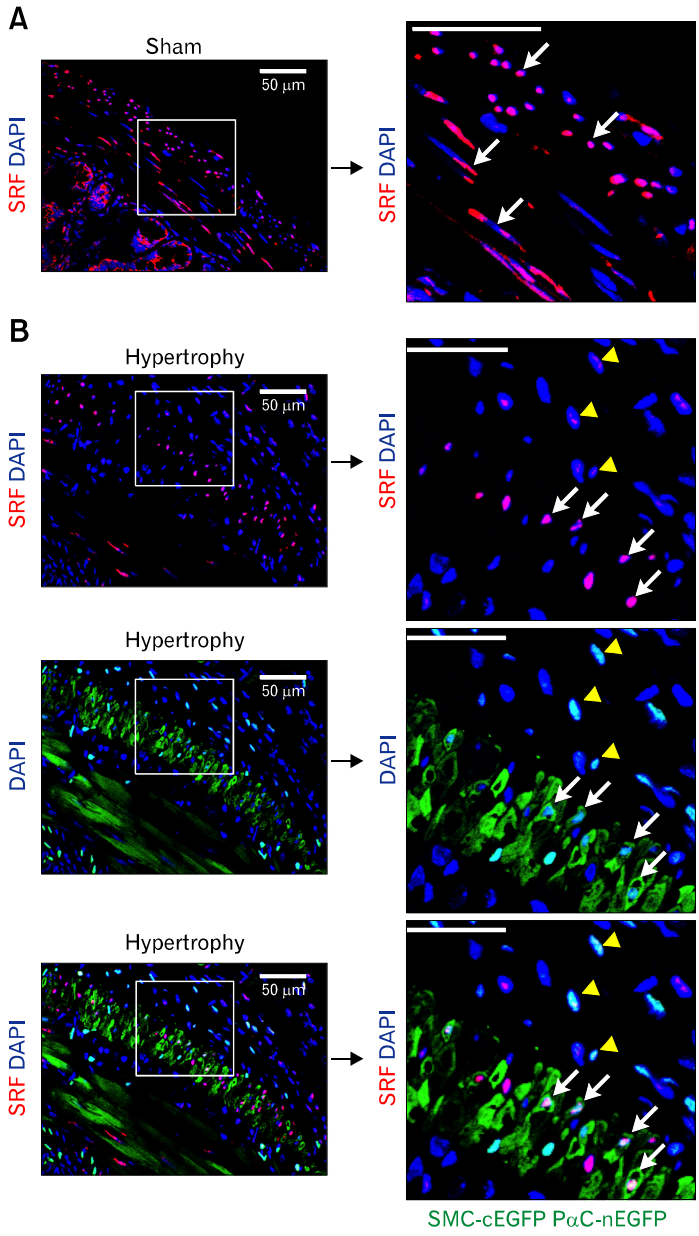

Figure 3. Differentiated serum response factor (SRF)^{high} smooth muscle cells (SMCs) become SRF^{low} platelet-derived growth factor receptor alpha positive (PDGFRα^{+}) cells during hypertrophy. Representative confocal laser scanning images of jejunal cross sections from sham control (A) and partial obstruction (B) mice following immunohistochemical staining with anti-SRF antibody (red) and counterstaining with DAPI (blue). Note robust levels of SRF expression in SMC nuclei of sham control (A; SRF^{high} cells, white arrows) compared to significantly decreased SRF expression in hypertrophied muscle layers (B; SRF^{low} cells, yellow arrowheads). Panels on right are high-resolution images magnified from the square boxes. Cytoplasmic enhanced green fluorescent protein (cEGFP; MYH11) is expressed in differentiated SMCs while nuclear EGFP (nEGFP; PDGFRα) is expressed in PDGFRα^{+} cells (PαC) from double transgenic *Myh11-Cre-EGFP; Pdgfra-EGFP* mice. Note that longitudinal SMCs expressing cEGFP with high levels of SRF also express low levels of nEGFP (SRF^{high}/cEGFP [MYH11]^{high}/nEGFP [PDGFRα]^{low}, white arrows]. These cells are located in the vicinity of SRF^{low}/cEGFP (MYH11)$^{-}$/nEGFP (PDGFRα)^{low} cells in the subserosal area of hypertrophied smooth muscle (yellow arrowheads). The latter may represent a transitional phenotype between mature SMCs and PDGFRα^{+} cells. All scale bars are 50 μm.

(PDGFRα) expression, indicating a non-SMC PDGFRα^{low} phenotype (Fig. 3B, yellow arrowheads). This transitional evidence suggests that SMCs dedifferentiate into PDGFRα^{low} cells during the hypertrophic process and is dependent on SRF expression levels.

Smooth Muscle Cells May Dedifferentiate and Gain PDGFRα Expression to Proliferate During Intestinal Smooth Muscle Hypertrophy and Hyperplasia

In both surgically obstructed and sham control mice, there was little proliferative activity, as detected by anti-Ki67 staining, in differentiated SMCs expressing cEGFP (Fig. 4). However, cells expressing low levels of nEGFP (PDGFRα) in hypertrophied jejunum, which were located just outside of the longitudinal muscle layer (subserosal area), displayed higher levels of cell cycling although the Ki67 signal was relatively weaker in this population of cells (Fig. 4, bottom panels). Other areas of the cross section that showed high levels of Ki67 staining included the mucosal epithelia and submucosa in both obstructed and sham control jejunum (Fig. 4).

To recapture the possible phenotypic switch from SMCs to PDGFRα^{+} cells in vitro, we cultured primary jejunal SMCs from *Myh11-Cre-EGFP* or *Pdgfra-EGFP* mice for up to 8 days (Fig. 5). cEGFP expression in differentiated SMCs from *Myh11-Cre-EGFP* mice was detectable by epifluorescence microscopy at day 0 and day 1 of cell culture but was abruptly lost by day 2 (Fig. 5A). In contrast, dispersed SMCs from *Pdgfra-EGFP* mice began to express nEGFP at day 2 indicating a phenotypic transition to PDGFRα^{+} cells (Fig. 5B). QPCR analysis of the dispersed cells in culture at differing time points revealed that transcription of *Srf* and its target gene *Myh11* essentially shut down within 24 hours of incubation (Fig. 5C). *Srf* expression began to resume at low levels on day 8, whereas *Myh11* expression remained silent. The data indicated that both *Srf* and *Myh11* genes turned off quickly in cell culture conditions as SMCs began to proliferate and that the residual *Myh11* promoter driven cEGFP remained in SMCs up to 1 day following silencing of the genes. The cell culture and QPCR data also showed that SMCs grown in culture may lose contractile proteins, such as MYH11, with the gain of PDGFRα expression as early as day 2 of culture and that the SRF^{low} cells expressing PDGFRα seen in the partial obstruction model (Figs. 3 and 4) may represent a population of cells that may have dedifferentiated from SMCs to proliferate as a hypertrophic and hyperplastic response.

The vascular SMC phenotype is regulated by SRF and its cofactors myocardin (MYOCD) and ELK1, an ETS domain-containing protein.[14] Therefore, we also examined the *Myocd* and *Elk1* mRNA transcript levels of cultured SMCs and hypertrophied jejunum smooth muscle. *Myocd* expression declined gradually during cell culture and was also significantly decreased in hypertrophic tissue (Fig. 5D). However, expression levels of *Elk1* mRNA did not change significantly in cell culture although there was a decrease in hypertrophic tissue. Collectively, the QPCR data was consistent with the dedifferentiated proliferative phenotype of SRF^{low}/nEGFP(PDGFRα)^{low} cells in Figures 5A, 5B, and 3B and indicated that SRF by itself may directly modulate the SMC phenotype.

Loss of Serum Response Factor in Smooth Muscle Cells Is Implicated in the Pathophysiology of Rectal Prolapse in Humans

To assess the relevance of our studies to human GI pathologies, we examined expression of SRF in human colorectal prolapse tissue. The histological morphology of healthy colon was well organized with distinct circular and longitudinal muscle layers present in between the submucosa and subserosa (Fig. 6A). In contrast, prolapse tissue contained disorganized and dysplastic muscle layers that were in abnormal contact with the mucosa juxtaposed to a highly disorganized and dysplastic serosa layer. Robust levels of SRF expression were detected by immunohistochemistry in the nuclei of SMCs of control colon but barely detectable in rectal prolapse tissue (Fig. 6B). These findings mirrored those of the mouse partial obstruction model (Fig. 3B). Taken together, our human rectal prolapse tissue studies support the hypothesis that SRF is required for SMC differentiation and maintenance and that pathological loss of SRF in SMCs may trigger SMC dedifferentiation and growth leading to hypertrophy and hyperplasia in the GI smooth muscle.

Discussion

To our knowledge, this is the first animal study that has demonstrated a developmental defect of the GI smooth muscle caused by a congenital *Srf* deficiency. SRF is an essential regulator for muscle cell proliferation and differentiation in skeletal,[15] cardiac,[16] and smooth muscle,[5] and a global *Srf* gene mutagenesis results in a severe gastrulation defect that is embryonic lethal.[1] Cardiac-specific (*Myh6* promoter driven) and vascular-specific (*SM22α*

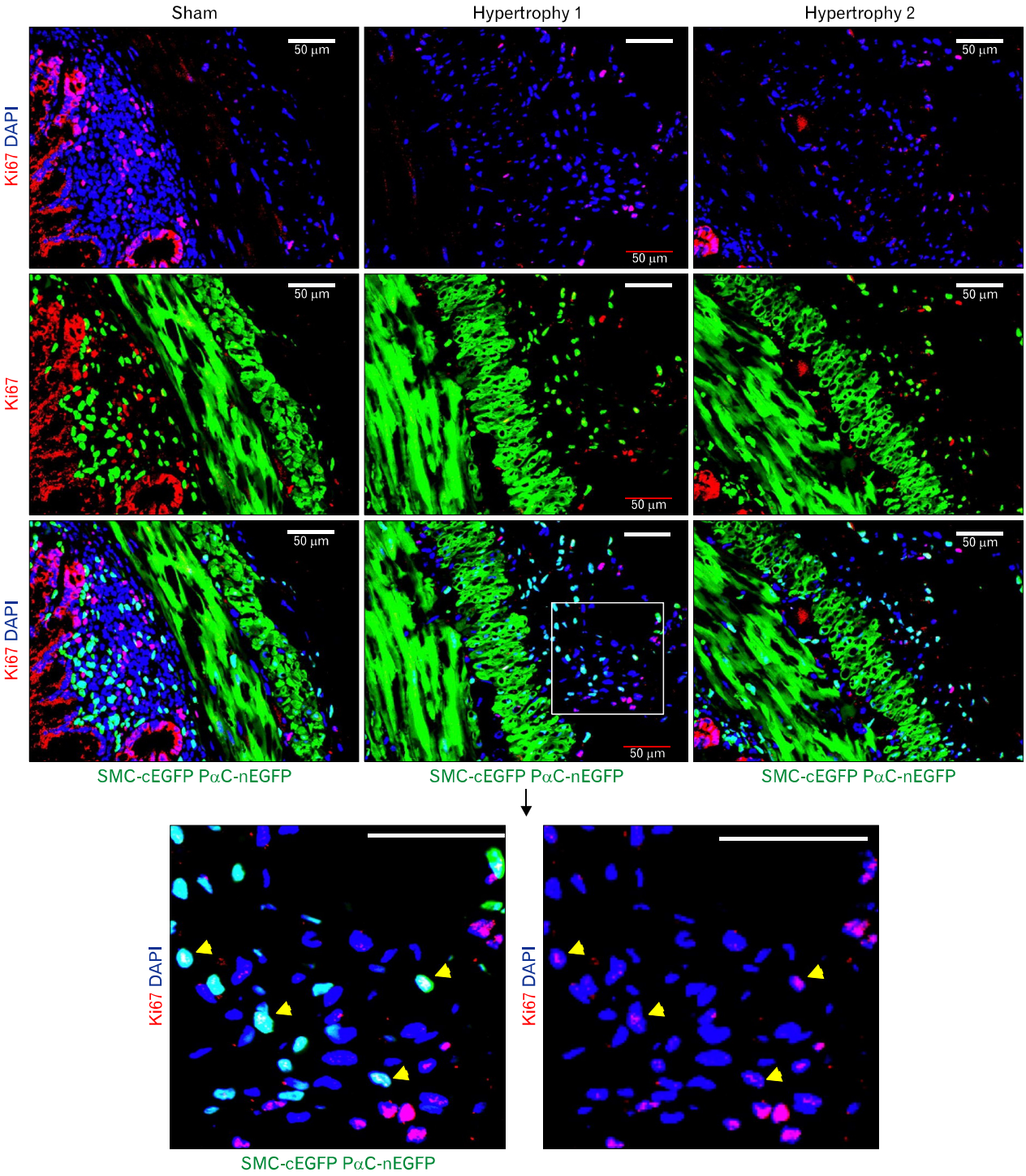

Figure 4. Dedifferentiated platelet-derived growth factor receptor alpha (PDGFRα^{+}) cells proliferate during gastrointestinal smooth muscle hypertrophy and hyperplasia. Proliferative activity as detected by anti-Ki67 antibody staining in a sham control and partially obstructed jejunal tissues from double transgenic *Myh11-Cre-EGFP; Pdgfra-EGFP* mice. In sham controls, cycling cells are abundant in the mucosal epithelia and submucosal regions. However, smooth muscle layers show little proliferative activity. Two hypertrophic jejunal tissues samples show high proliferative activity in the hypertrophic region just outside of the longitudinal muscle layers. Bottom panels are high-resolution images magnified from the square boxes that show nuclear colocalization of Ki67, nEGFP, and DAPI signals. Note Ki67 positive cells also expressing low levels of PDGFRα (nEGFP; yellow arrow heads). All scale bars are 50 μm.

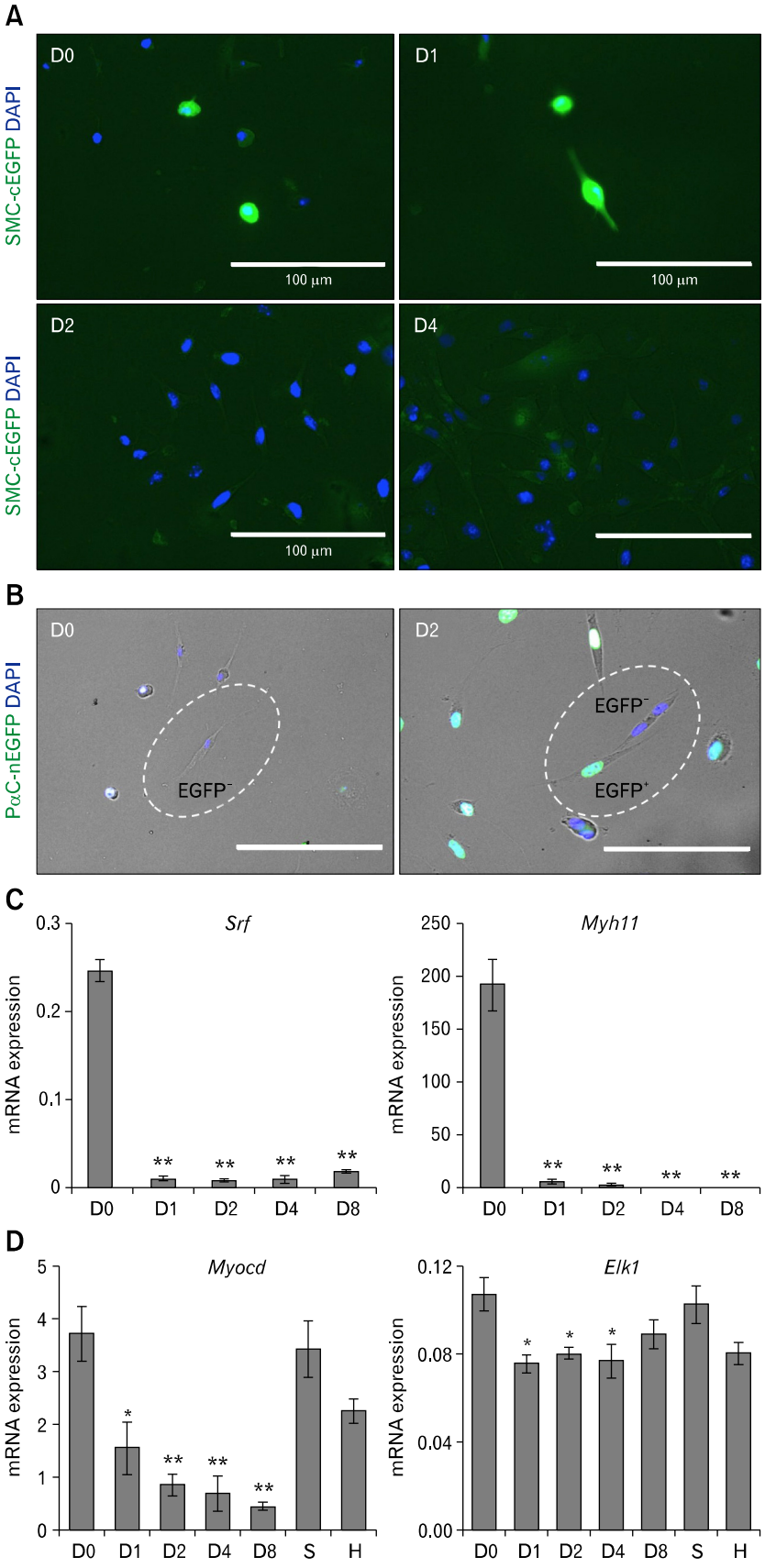

Figure 5. Loss of Srf, Myocd, and Myh11 with gain of platelet-derived growth factor receptor alpha (PDGFRα) expression in dedifferentiated smooth muscle cells (SMCs) during cell culture. Jejunal smooth muscle was dissected from 2-week-old *Myh11-Cre-EGFP* or *Pdgfra-EGFP* mice, and the cells were dispersed and cultured for 8 days. At designated time points, cultured cells were examined by epifluorescence imaging and/or harvested for quantitative polymerase chain reaction (QPCR) analysis. (A) Epifluorescence imaging of SMCs from a *Myh11-Cre-EGFP* mouse showing loss of cEGFP (green) after day 2 in culture. DAPI (blue) marks nuclei in cells. (B) Epifluorescence imaging showing gain of nEGFP (green) expression in SMCs from a *Pdgfra-EGFP* mouse after 2 days in culture indicating activation of PDGFRα expression in SMCs. (C) QPCR analysis showing immediate and dramatic loss of *Srf* and *Myh11* mRNA transcripts in primary SMCs after day 1 of culture. (D) QPCR analysis showing gradual loss of *Myocd* mRNA transcripts and sustained expression of *Elk1* in primary SMCs after day 1 of growth in culture, in hypertrophic jejunum (H), and in sham controls (S). Expression of each gene was normalized by housekeeping gene, *Gapdh* (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$.

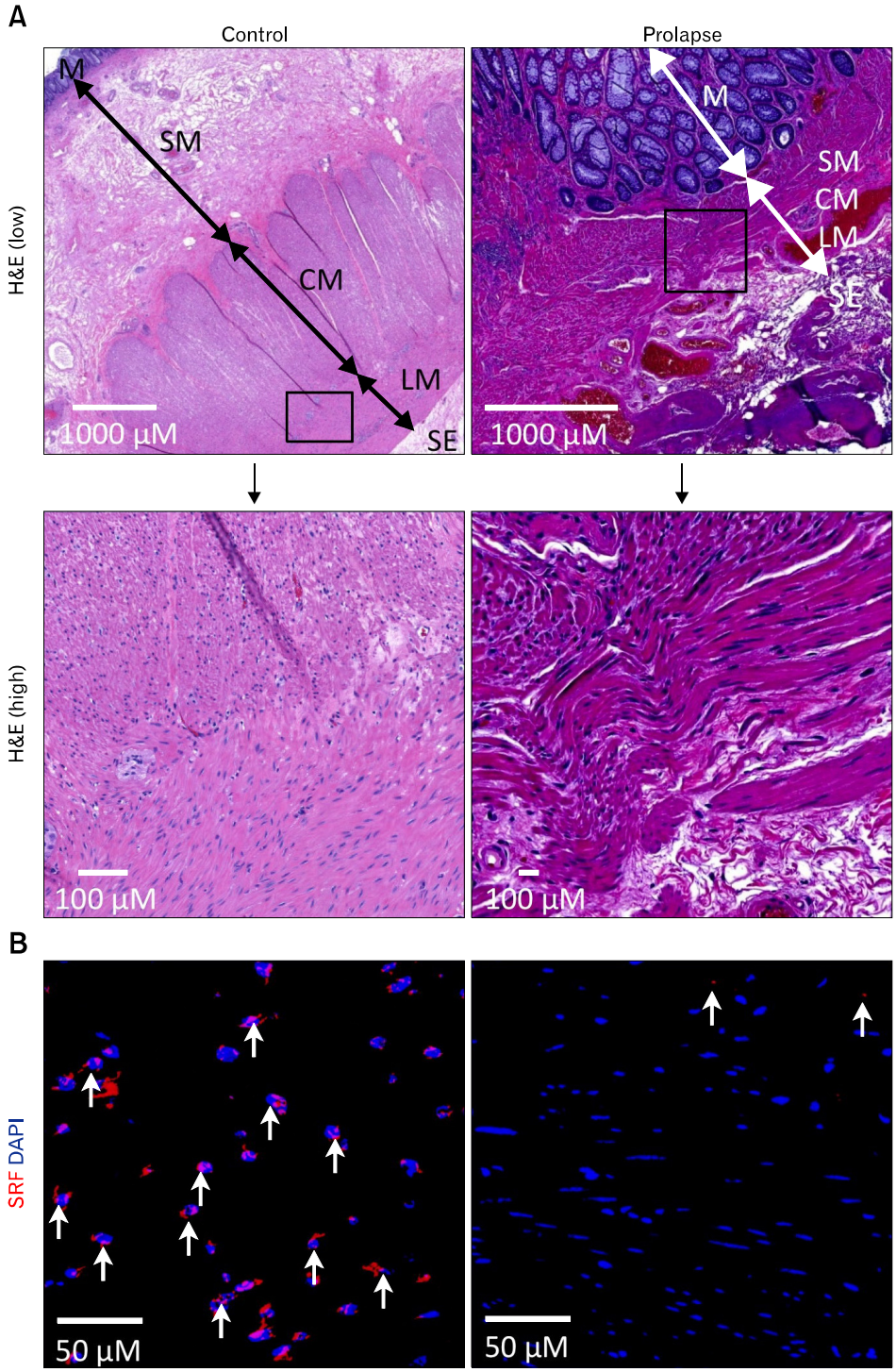

Figure 6. Loss of serum response factor (SRF) protein expression in dysplastic smooth muscle cells (SMCs) of human rectal prolapse tissue. (A) H&E staining of control colon and rectal prolapse tissue. Control colon sections show a well-organized muscularis with distinctive circular (CM) and longitudinal muscle (LM) layers, whereas prolapse tissue contains a disorganized and disrupted muscularis. (B) Immunohistochemical staining of SRF in control colon and rectal prolapse tissues. SMCs in control colon express abundant SRF (red) in nuclei counterstained with DAPI (blue). Rectal prolapse SMCs display minimal SRF expression (n = 3). M, mucosa; SM, submucosa; SE, serosa.

promoter driven) knockouts of *Srf* are also embryonic lethal due to a severe cardiac defect.[8,17] The expression of *SM22α* (*Tagln*) is relatively restricted to vascular SMCs in adults[18] in addition to cardiac muscle cells in embryos.[19] However, *SM22α* is also expressed in proliferating and differentiated SMCs[20] as well as PDGFRα$^+$ cells in GI smooth muscle (transcriptome analysis, unpublished data). To target SMCs specifically, we generated a *Myh11* promoter driven *Srf* KO mouse model. MYH11, which is expressed in SMCs of the GI tract by E13.5,[9] is widely considered the most selective marker for differentiated SMCs.[21] In this

KO system, the *Srf* gene is deleted by Cre under the control of the *Myh11* promoter at E13.5. However, SRF protein synthesized prior to E13.5 may continue to reside within cells for several days. In our congenital studies, the cardiac and GI defects from *Myh11* promoter driven *Srf* KO manifested at E18, which is later than the time of manifestation for *Myh6* (E12.5) and *SM22α* (E11.5) promoter driven KOs.[8,17] This phenotypic discrepancy may be partially explained by the differential timing of promoter activation in different cell populations during development. Since MYH11 is the most distinctive marker for differentiated SMCs, the prenatal GI and cardiac defects revealed in this study most accurately represent the phenotypes that result from congenital deletion of *Srf* in SMCs. The manifestation of cardiac dysfunction was not anticipated since MYH11 is not expressed in cardiomyocytes. However, it is conceivable that the *Srf* KO may have disrupted the normal vascularization of the developing heart in utero since MYH11 is expressed in vascular SMCs. Our congenital *Srf* KO studies along with the 2 previously described phenotypes emphasize the critical nature of SRF in the development of prenatal GI smooth muscle and cardiac muscle.

The phenomena of SMC hypertrophy and hyperplasia, which occurs during partial bowel obstruction, has been previously well described.[13,22,23] Inflammation may also cause SMC hypertrophy within the GI tract.[24,25] However, the molecular mechanism remains less understood. Our in vivo and in vitro data indicated that under the physiologic stress of partial obstruction, intestinal SMCs may lose expression of SRF and characteristic contractile proteins in favor of a dedifferentiated phenotype capable of proliferation. In vascular SMCs, SRF regulates expression of contractile proteins mainly through its cofactors MYOCD (differentiation) and ELK1 (dedifferentiation).[14] In contrast, however, our data in Figures 3 and 5 suggest that the SMC phenotype in intestinal obstruction may be regulated directly by SRF rather than via ELK1. *Elk1* expression was minimal at the beginning and did not increase in cell culture and hypertrophied jejunal smooth muscle (Fig. 5) although previous studies have reported ELK1 induction in vascular injury.[26] In this vascular injury model, phosphorylated ELK1 repressed the *SM22α* gene. However, in our partial intestinal obstruction model, SM22α protein levels were not affected by hypertrophy of the jejunal smooth muscle (Fig. 2C). In addition, our transcriptome data showed that little or no Elk1 was expressed in both jejunal SMCs and colonic SMCs, whereas *Srf* was abundantly expressed in a SMC-specific manner.[27] These observations support a model for direct SRF regulation of the GI SMC phenotype during intestinal obstruction, which differs from that of vascular SMCs.

Whether expression of SRF and contractile proteins (myosin heavy chains) increases or decreases with intestinal smooth muscle hypertrophy has been the subject of controversy.[13,28] The discrepancy between contradictory findings by different investigators may be explained by the dynamic stages of the hypertrophic response at which the tissues were examined. For instance, Chen et al[13] found that SRF and contractile protein in whole muscle tissue were down-regulated at the beginning of hypertrophy but later restored to the control levels. Our Western blot data indicated that SRF levels increased after 2 weeks of partial obstruction but that MYH11 levels remained unchanged. The immunohistochemistry actually showed a thinner band of SMCs expressing high levels of SRF in hypertrophied tissue relative to sham controls. Furthermore, we identified 2 distinctive SMC populations that appeared to be responsible for these changes: 1) mature hypertrophic SMCs that are SRF^{high}/$cEGFP(MYH11)^{high}$ located near the myenteric border and 2) less differentiated SMCs that are $SRF^{low\ or\ -}$/$cEGFP(MYH11)^{low\ or\ -}$ juxtaposed to the longitudinal smooth muscle layer. Collectively, our data indicated that after 2 weeks of intestinal partial obstruction, there were overall more cells (SMCs and hyperplastic $PDGFR\alpha^{low}$ cells) expressing SRF, albeit at lower levels. The increased number of cells expressing SRF resulted in a higher total amount of SRF expression compared to non-obstructed intestinal tissue.

A hybrid phenotype between fibroblasts (similar to $PDGFR\alpha^{+}$ cells) and SMCs (myofibroblast) has been reported in fibrosis of Crohn's disease,[29] and vascular SMCs have been shown to display plasticity from a quiescent contractile phenotype to a myofibroblast-like proliferative synthetic state during vascular repair.[6] However, it has been technically difficult to validate and study dedifferentiated SMCs since they lose their distinctive cell markers. We partially overcame this limitation with a double transgenic mouse line that labeled SMCs with cEGFP and $PDGFR\alpha^{+}$ cells with nEGFP. Using this murine intestinal injury model, we report the first *in vivo* evidence of mature SMCs converting into proliferative $PDGFR\alpha^{+}$ cells. This finding offers a new opportunity to isolate and study dedifferentiated SMCs after loss of their characteristic markers.

Interestingly, we discovered that the dedifferentiated $SRF^{low\ or\ -}$/$cEGFP(MYH11)^{low\ or\ -}$ cells in hypertrophic jejunum robustly expressed the proliferative marker Ki67 and low levels of $PDGFR\alpha$ ($nEGFP^{low}$) implicating their role in smooth muscle hyperplasia. Although it is possible that these $SRF^{low\ or\ -}$/

cEGFP(MYH11)$^{low\ or\ -}$/nEGFP(PDGFRα)^{low} cells could represent fibroblast-like cells *in situ* that may be differentiating into mature SMCs to compensate for the increased physiologic stress of partial obstruction, our cell culture and immunofluorescence data (thinner band of SRF^{+} SMCs) indicate that this cell population likely represents SMCs that have dedifferentiated to accommodate for cellular proliferation and creation of more mature SMCs. The discovery of nEGFP(PDGFRα)^{low} cells was particularly intriguing because these cells were a separate and distinct population from that of nEGFP(PDGFRα)^{high} cells and had higher levels of Ki67 expression. The latter finding suggested that nEGFP(PDGFRα)^{low} cells may be more primitive than nEGFP (PDGFRα)^{high} cells. Moreover, previous studies have implicated PDGF-AA, PDGF-BB, and their receptors in the proliferation of intestinal subepithelial myofibroblasts (ISEMFs) although a similar interplay in the subserosa has been less characterized.[29,30]

Although we have demonstrated that nEGFP(PDGFRα)^{low} cells may derive from dedifferentiated SMCs, other potential sources for these cells include subserosal PDGFRα^{+} cells,[31,32] myofibroblasts,[29,33] or bone marrow-derived mesenchymal stem cells.[34,35] In the GI tract, PDGFRα^{+} cells have a similar anatomical distribution as interstitial cells of Cajal (ICC), both of which have close contact with SMCs.[36] Within the small intestine, PDGFRα^{+} cells are grouped into the subtypes PDGFRα^{+}-IM (muscle bundles), PDGFRα^{+}-MY (myenteric plexus), PDGFRα^{+}-DMP (deep muscular plexus), and PDGFRα^{+}-SS (subserosal surface).[31,37] We found that each subtype was differentially remodeled during partial intestinal obstruction. However, further studies are needed to determine whether any of these subtypes can display plasticity in the remodeling response.

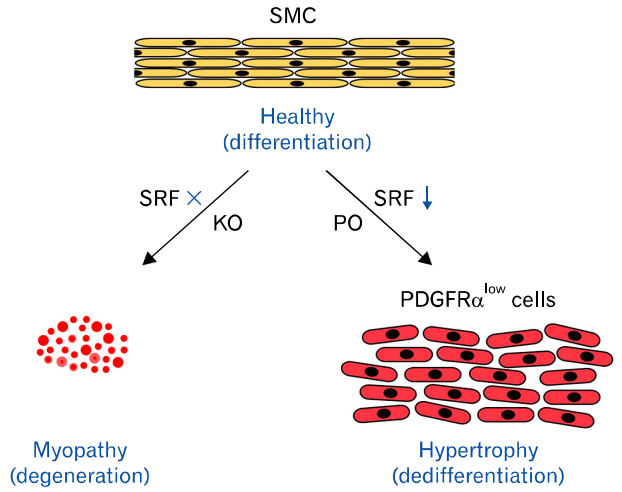

Figure 7. Model of smooth muscle cell (SMC) phenotype regulated by SRF. In absence (knockout [KO]) of serum response factor (SRF), SMCs cannot grow and differentiate, but they degenerate and undergo apoptosis. With down-regulation of SRF during intestinal injury (partial obstruction [PO]), SMCs dedifferentiate into proliferative SRF^{low} PDGFRα^{low} cells, which result in hypertrophy of smooth muscle. PDGFRα, platelet-derived growth factor receptor alpha.

Finally, we have also demonstrated the pathologic loss of SRF expression in human rectal prolapse tissue. The question of whether loss of SRF in human SMCs is associated with a gain of PDGFRα expression and proliferative activity as is in mice should be addressed by further studies. In summary, we have shown that SRF is required for the development of prenatal SMCs and the maintenance of its differentiated phenotype in adult SMCs. The loss or down-regulation of SRF is directly linked to the pathological transitions of SMCs, which includes degeneration during myopathy or proliferation of PDGFR^{low} cells during obstruction induced hypertrophy (Fig. 7). This study offers new insight into the direct role of SRF in the plasticity of pathologic SMCs.

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Supplementary Materials

Note: To access the supplementary Video mentioned in this article, visit the online version of *Journal of Neurogastroenterology and Motility* at http://www.jnmjournal.org/, and at http://dx.doi.org/10.5056/jnm15063.

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