Multi-phenotypic Role of Serum Response Factor in the Gastrointestinal System

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Serum response factor (SRF) is a master transcription factor of the actin cytoskeleton that binds to highly conserved CArG boxes located within the majority of smooth muscle cell (SMC)-restricted promoters/enhancers. Although most studies of SRF focus on skeletal muscle, cardiac muscle, and vascular SMCs, SRF research has recently expanded into the gastrointestinal (GI) system. Genome scale analyses of GI SMC transcriptome and CArG boxes (CArGome) have identified new SRF target genes. In addition to circular and longitudinal smooth muscle layers, SRF is also expressed in GI mucosa and cancers. In the GI tract, SRF is the central regulator of genes involved in apoptosis, dedifferentiation, proliferation, and migration of cells. Since SRF is the cell phenotypic modulator, it may play an essential role in the development of myopathy, hypertrophy, ulcers, gastric and colon cancers within the GI tract. Given the multi-functional role displayed by SRF in the digestive system, SRF has received more attention emerging as a potential therapeutic target. This review summarizes the findings in SRF research pertaining to the GI tract and provides valuable insight into future directions.

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Key Words
Gastrointestinal diseases; Knockout; MicroRNAs; Myocytes, Smooth muscle; Serum response factor

Introduction
Serum response factor (SRF) was first identified by Treisman in 1986. He discovered that SRF transcriptionally activates the c-fos gene in susceptible cells after serum stimulation by binding a conserved DNA sequence having dyad symmetry (SRE, serum response element) located within the immediate promoter region. The SRE contains a core 10-nucleotide [CC (A/T)6 GG] sequence (now called a CArG box), which was subsequently identified in the promoter and intronic regions of most smooth muscle cell (SMC)-restricted genes. SRF binds to CArG boxes and transcriptionally activates target genes through direct association with more than 60 cofactors. The identification of functional CArG boxes in the genome (i.e., CArGome) has begun to be elucidated, though there are likely many more CArG-containing genes awaiting discovery. A smooth muscle genome and CArGome browser containing genome-wide CArG boxes alongside SMC transcriptome data has been built and available to search and identify new CArG-containing genes.

In addition to SRF-dependent protein-coding contractile mRNA genes, the SRF was recognized as an important regulator of many microRNA (miRNA) genes. Many SRF-induced miRNA genes have been identified and they appear to be abun-
dantly and predominantly expressed in SMCs. The mechanism of SRF regulation of miRNA genes is the same as mRNA genes: both use CArG boxes. Many highly expressed miRNA genes in SMCs are activated by SRF suggesting that SRF-dependent miRNAs largely drive the SMC phenotype.

SRF has a multi-functional role in regulating SMC growth, differentiation, and death in the gastrointestinal (GI) tract. The diverse roles of the protein were uncovered by use of transgenic Srf knockout studies using cell-specific Cre-lox systems. Loss of the functional Srf gene shows that SRF is required for cardiac and smooth muscle development both in embryos and maintenance in adults.

Abnormal expression of SRF is common in several GI diseases. Normal expression of the protein is essential for GI SMC differentiation. Loss or reduction of SRF may trigger myopathy or hypertrophy of SMCs or GI cancers, while overexpression of the protein may be linked to ulcers.

Smooth Muscle Cell Transcriptome

SMC transcriptomes were recently obtained from the jejunum and colon. SMCs express 16,000 genes, which are transcribed into 55,000 transcriptional variants. The most highly expressed genes are related to muscular contraction. SMC contractility is regulated by Ca\(^{2+}\) via ion channels and transporters. They express as many as 447 ion channel and transporter isoforms, indicating that SMC excitability is regulated by a complex coordinated effort of numerous ion channels and transporters. Within the ion channel family, calcium channels were the most abundantly expressed in SMCs, and the predominance of calcium channel expression is consistent with the current paradigm for excitation-contraction coupling, which is primarily regulated by Ca\(^{2+}\) via calcium channels. Many highly expressed and SMC-restricted ion channel isoforms and regulators such as Kcnmb1, Ryr3, Jph2, and Dmpk appear to be regulated by SRF.

CArGome and Serum Response Factor Binding Sites

Genome-wide SRF binding CArG boxes were mapped in mice and humans. A large number of CArG boxes (98,236) are conserved between the 2 species. In addition, over 1,000 genes are associated with SRF binding CArG boxes, most of which are found within the promoter region, exon 1 and/or intron 1. A hundred SRF-associated genes are highly expressed in SMCs, most of which appear to be specific to the cells.

Smooth Muscle Genome and CArGome Browser

Although many SRF-regulated genes were identified and validated, there are still many protein-coding and non-coding genes in the genome that need to be discovered. To facilitate the analysis of genes in relation to SRF binding sites and CArG boxes, we built an interactive SMC genome and CArGome browser for mice: http://medicine.nevada.edu/physio/transcriptome (requires Google Chrome and takes 1-2 minutes to upload the large files). This genome browser offers a new perspective into the alternative expression of genes in the context of SRF binding sites in SMCs, thus providing a valuable reference for future functional studies. For example, Figure 1 shows 6 transcriptional variants expressed in jejunal and colonic SMCs, 2 CArG boxes, and 1 SRF binding site.
site in an oncogene Fhl2 on the browser. The SRF binding site in the proximal promoter region contains a conserved CArG box “CCATATAAGG” that overlaps with CpG island (DNA methylation sites), histone methylation H3K4me3 marks (active or poised gene), and histone acetylation H3K27ac marks (active gene).

**Serum Response Factor Knockout Phenotype**

Several animal studies have shown that SRF is a key regulator in the development and maintenance of both embryonic and adult muscle cells. All SRF deficient phenotypes generally have defects in the development and/or maintenance of the heart and GI tract. However, the phenotypes depend on when the gene is knocked out and which promoter drives the expression of Cre recombinase (Table 1). For example, in congenital knockout systems, Srf gene knockouts are embryonic lethal and exhibit the cardiac or GI smooth muscle defects. However, embryonic survival varies between E10.5 and E18. This suggests that each promoter used is activated to knockout the gene at different time points. Myh11-driven knockout of Srf leads to extended survival of embryos (E18) as compared to the survival observed when Srf is inactivated with other promoters, including SM22α. Consistent with the congenital studies, inducible knockout of the gene in adult SMCs resulted in severe GI dilation with thinning of the smooth muscle layers. In addition, Myh11-driven knockout mice survived longer than SM22α-driven knockouts. This phenotypic variation in 2 different promoters likely relates to the activation time and the strength of each promoter. Indeed, SM22α-Cre is activated earlier at E9.5 than Myh11-Cre at E13.5. SM22α (Tagln) is expressed higher than Myh11 in jejunal and colon SMCs. Furthermore, Myh11 and SM22α are differentially expressed in SMCs. Myh11 is expressed exclusively in differentiated SMCs, whereas SM22α is expressed in a less restrictive manner in proliferating as well as differentiated SMCs. Nevertheless, the 2 phenotypes are similar in congenital and inducible knockout animals, suggesting that SRF is necessary for cardiac and smooth muscle development in embryos and maintenance in adults.

### Serum Response Factor-induced MicroRNAs

miRNAs are required for the development and maintenance of GI SMCs. There are 2 important RNAse III enzymes in miRNA biogenesis, Dicer and Drosha that cleave primary transcripts to generate precursor miRNAs and mature miRNA duplexes respectively. We demonstrated that GI SMCs could not survive without miRNAs in the RNAse III enzyme Dicer deficiency model. The SMC-specific Dicer null mice developed severe dilation of the intestinal tract associated with the thinning and degeneration of the smooth muscle layers. A similar phenotype in SMC-specific Drosha null mice was observed although Drosha null mice showed a more severe pathological phenotype than Dicer null mice (unpublished data). This SMC degeneration also resembles that of SMC-specific Srf null mice, suggesting that SRF regulates expression of a large number of miRNAs in SMCs. We previously found that GI smooth muscle of mice expressed 312 miRNAs, of which 36 were SRF-dependent as evidenced by in vitro Srf knock-down. Furthermore, using an advanced miRNA-seq technology, we identified 891 miRNAs from Srf wild type and deficient smooth muscle, of which 124 were induced by SRF. SRF-induced miRNAs are highly expressed in GI SMCs as evidenced by the fact that over 95% of miRNAs were decreased in Srf deficient smooth muscle. The most highly expressed miRNAs in GI SMCs are miR-145 and miR-143 (account for 78% of all miRNAs) in which expression is SRF-dependent in in vitro knock-down and in vivo knockout systems. In fact, miR-143 and miR-145 are generated from the same primary transcript, and binding of SRF to a conserved

| Promoter | Specificity   | Knockout     | Phenotype              | Survival    |
|----------|--------------|--------------|------------------------|-------------|
| Srf¹⁵    | Global       | Congenital   | Gastrulation defect    | E9.5        |
| Myh6¹⁷   | Cardiac muscle| Congenital   | Cardiac defect         | E12.5       |
| Myh7¹⁹   | Cardiac muscle| Congenital   | Cardiac defect         | E10.5-13.5  |
| SM22α¹⁶   | Smooth muscle| Congenital   | Cardiac and GI defects | E11.5       |
| Myh11¹⁸   | Smooth muscle| Congenital   | Cardiac and GI defects | E18         |
| SM22α¹²,²⁶ | Smooth muscle| Inducible    | GI and bladder dilation| PT8-22      |
| Myh11¹²   | Smooth muscle| Inducible    | GI dilation            | PT21-28     |

E, embryonic day; GI, gastrointestinal; PT, post tamoxifen injection day.
CARG box located in the distal promoter region modulates their expression. SRF-induced miR-143 and miR-145 expression promotes GI SMC differentiation and suppression of proliferation. In addition, deficiency of Dicer in SMCs induces expression of genes involved in cell killing and death, suggesting that the miRNAs may also suppress apoptotic genes. Taken together, miRNA studies indicate that SRF-induced miRNAs suppress proliferation and apoptosis of SMCs, and thereby promote differentiation of the cells in the GI tract. These miRNA regulatory pathways add to the complexity of SRF influence on epigenetic regulation of the GI SMC phenotype.

Apoptosis

Recent studies showed that SRF regulates apoptosis. We found that SMCs undergo massive apoptosis in the absence of SRF expression in a transgenic knockout mouse model. In SMC-restricted Srf inducible knockout mice, SMC degeneration occurs by abnormally overexpressed apoptotic proteins in SRF-dependent and anti-apoptotic miRNA deficiency. This new role of SRF as an anti-apoptotic regulator is supported by recent findings (Table 2). Parlakian et al first observed that cardiac-restricted Srf depletion induces caspase 3 and apoptosis in the embryonic heart. Wiese et al recently reported that restoration of SRF antagonized Myc repression of SRF target genes, attenuated Myc-induced apoptosis, and reverted a Myc-dependent decrease in Akt phosphorylation and activity. Sisson et al also recently confirmed that a SRF/MRTF pathway inhibitor CCG-203971 promotes myofibroblast apoptosis, decreases alveolar plasminogen activator inhibitor-1, and leads to significantly reduced lung collagen content, thereby decreasing lung fibrosis. Furthermore, Bae et al showed that antisense inhibition of SRF expression in SH-J1 cells significantly enhanced the apoptotic effects of sorafenib, an oral multi-kinase inhibitor, while reducing expression of mesenchymal markers and restoring expression of E-cadherin. Chen et al also recently reported that miR-320a contributed to atherogenesis by down-regulating SRF, inhibiting human-derived endothelial cell proliferation and inducing apoptosis. Lastly, Huang et al reported that apoptotic activities (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling [TUNEL], caspase 3, caspase 9, p53, nuclear chromatin aggregation, nuclear fragmentation, and cytoplasmic apoptotic body formation) are increased in the aortic smooth muscle with conditional knockout of Myocd, which are strikingly similar to those of our DNA fragmentation, ultrastructural and TUNEL assay findings in the GI smooth muscle of Srf knockout mice. The remarkable similarities in the gross, microscopic, and molecular findings of Myocd knockout and Srf knockout mouse models are not surprising given that myocardin is an important transcriptional coactivator that binds directly to SRF to activate the transcription of a subset of SRF-regulated genes encoding cytoskeletal and contractile proteins. All of the evidence above clearly indicates that SRF suppresses apoptotic activities in SRF-restricted cells including GI SMCs.

Hypertrophy

SMCs change their behavior in response to intestinal injuries. Under the hypertrophic condition of small bowel partial obstruc-

### Table 2. Evidence of Serum Response Factor in Apoptosis

| Model                                                                 | Target gene                  | Genetic change                | Phenotype                      |
|-----------------------------------------------------------------------|------------------------------|--------------------------------|--------------------------------|
| Congenital knockout of Srf in heart                                   | Srf                          | Caspase 3 ↑                    | Apoptosis in embryonic heart ↑ |
| Conditional knockout of Srf in SMCs                                   | SRF-dependent miRNAs ↓       | Apoptotic proteins ↑           | SMC apoptosis ↑                |
| Myc/Miz1 mediated SRF repression in epithelial cells                  | SRF/MRTF target genes ↓      | Akt phosphorylation and activity ↓ | Myc-induced apoptosis ↑       |
| SRF/MRTF pathway inhibitor in lung fibrosis                          | SRF/MRTF target genes ↓      | Alveolar plasminogen activator inhibitor-1 ↓ collagen ↓ | Myofibroblast apoptosis ↑ fibrosis ↓ | Apoptotic effects of sorafenib ↑ |
| Antisense inhibition of SRF expression in SH-FJ1 cells                | Srf                          | Caspase 3, caspase 9 and p53 ↑ | Apoptosis ↑                   |
| miR-320a in atherogenesis                                             | SRF/MYOCID target genes ↓    | Caspase 3, caspase 9 and p53 ↑ | Apoptosis ↑                   |

SRF, serum response factor; SMCs, smooth muscle cells.
tion, we recently found that SMCs are dedifferentiated into myofibroblast-like cells (platelet-derived growth factor receptor α [PDGFαR]low cells) with low-level expression of SRF. Consistently, Srf expression is dramatically reduced in dedifferentiated and growing PDGFαRlow cells in a cell culture condition. The PDGFαRlow cells are highly proliferative and responsible for the thickness of the hypertrophied muscle. The partial obstruction bowel model results in a transient hyperplasia at the beginning, followed by a prolonged hypertrophic response of intestinal SMCs.

We also found that cell proliferation is transiently increased in smooth muscle layers at the beginning of SMC-restricted, inducible Srf knockout in adult mice. We also found that cell proliferation is transiently increased in smooth muscle layers at the beginning of SMC-restricted, inducible Srf knockout in adult mice. SMC proliferation is regulated by the myogenic repressor ELK1 bound to SRF. Since ELK1 competes with another cofactor MYOC for the same binding region of SRF, SMC phenotype depends on amount of these two antagonistic cofactors. Chen et al. showed that the expression of Elk1 is immediately and transiently induced within 6 hours in the partial obstruction model, but the expression level of the cofactor comes back to normal, and is not significantly changed during the development of hyperplasia and/or hypertrophy. If ELK1 is required for GI SMC hyperplasia, expression of the protein should be gradually increased to bind dominantly to SRF. We observed that expression of Elk1 is decreased as SMCs become proliferative PDGFαRlow cells. Chevigny et al. recently reported that expression of nuclear ELK1 is not changed in hyperplastic and hypertrophic airway smooth muscle in asthma. Further studies are obviously required to demonstrate if ELK1 regulates GI SMC proliferation. Nevertheless, the evidence above indicates that hyperplasia and hypertrophy develop in the GI smooth muscle when expression of SRF is reduced in SMCs.

**Contractility**

The role of SRF in SMC growth, differentiation, and phenotypic maintenance has been well established. SRF regulates expression of most SMC-specific contractile and contractile-associated proteins, including smooth muscle myosin heavy chain, SMα-actin, SM22, and calponin, by binding to highly conserved CArG boxes that are located within the majority of SMC-restricted promoters/enhancers. Lack or decrease of SRF is directly linked to a phenotypic change of SMCs, leading to hypomotility of smooth muscle in the GI tract. Deficiency of SRF in SMCs of Srf knockout mice results in impaired contractility in the GI smooth muscle. We identified 34 SRF-regulated proteins in the Srf knockout smooth muscle, many of which appear to be contractile and contractile-associated proteins. Furthermore, we found that expression of voltage-activated L-type calcium channel CACNA1C is also regulated by SRF-induced myotonic dystrophy protein kinases (manuscript in revision). In smooth muscle, the excitation-contraction coupling of smooth muscle is mainly regulated by the L-type Ca2+ channels. Reduction of CACNA1C in Srf knockout SMCs not only decreased intracellular Ca2+ spikes, but also disrupted their coupling between cells resulting in decreased contractility.

GI motility is largely regulated by the activities of three electrically coupled cell types, SMCs, interstitial cells of Cajal, and PDGFαRα cells (called SIP), which form a multicellular functional syncytium via gap junctions. Disruption of coordination of these coupled cells alters GI motility patterns. Defective SMCs with loss or reduced expression of SRF may impair the SIP activity and GI motility.

**Cancer**

Although several studies demonstrated that SRF is linked to tumorigenesis, SRF seems to both negatively and positively contribute to GI cancers depending on pathways. The promoter and exon 1 of the SRF gene became hypermethylated in gastric carcinoma, which downregulated the mRNA expression. Overall patient survival from gastric carcinoma metastasis in China, Japan, and Korea has been linked to the differential methylation of SRF, GFRA1, and ZNF382. In addition, a truncated SRF isoform, SRFΔ5 appears to be abnormally overexpressed in colon cancer. Over-expression of this isoform increased cell survival, suggesting that this truncated protein may contribute to colon tumorigenesis. However, in the truncated SRF study, it was not made clear whether the truncated protein alone induced cell growth or whether this truncated protein simply attenuated the effect of SRF. The downregulated SRF that induced gastric cancer suggested the latter may be the case in colon cancer. Another pathway contributing to GI cancers is by the oncogene FHL2 whose expression is induced by SRF. FHL2 is a cell cycle and growth modulator that is highly expressed in GI cancers such as colon cancer. FHL2 is required for cancer cell invasion, migration, and adhesion to the extracellular matrix. Our SMC genome and CArGome browser identified the SRF binding site and multiple transcriptional variants of Fhl2 gene (Fig. 1). It is of interest to investigate how this gene is activated by SRF, DNA methylation, or histone modifications in cancer, and which transcriptional variant is responsible for tumorigenesis. Taken together, the positive and negative regulation of genes by SRF in...
GI cancers suggests a multifunctional role of SRF in cell phenotype and tumorigenesis.

Ulcer

Other than circular and longitudinal SMCs, SRF is also expressed in the SMCs of the muscularis mucosa, proliferative cells of mucosal epithelium, and endothelial cells of microvessels. SRF is required for the wound healing process in gastric and esophageal ulcers. SRF appears to induce VEGF-induced angiogenesis in endothelial cells. Overexpression of SRF in gastric epithelial cells and SMCs promotes proliferation and migration of cells, which lead to re-epithelialization and restoration of smooth muscle structures damaged by ulcers. In addition, SRF is also critical for TGFβ-induced myofibroblast differentiation during esophageal ulcer healing.

Conclusions

SRF is a multifunctional phenotypic modulator that is linked to several GI diseases (Fig. 2). SMCs require a normal expression of SRF for their differentiation. Complete loss of SRF in SMCs induces apoptosis of the cells, which results in degeneration of the muscle. Reduction of SRF in SMCs triggers the cells to dedifferentiate and become proliferative, leading to hypertrophic muscle, gastric and colon cancer. Conversely, overexpression of SRF induces proliferation of cells (endothelial cells, epithelial cells, and SMCs) in ulcer healing that results in re-epithelialization and restoration of smooth muscle.

Future Directions

Given that SRF plays a critical role in controlling SMC behavior in different pathological conditions, the protein is a good therapeutic target gene to aid in recovery from GI injury. For example, normalization of SRF expression may thwart or delay the progression of SMC death in myopathy, SMC dedifferentiation and proliferation in hypertrophy, and cancer cell proliferation and migration in gastric and colon cancers. For ulcers, overexpression of SRF may also accelerate the healing process of re-epithelialization and restoration of smooth muscle. However, we still do not know the identity of SRF susceptible cells that abnormally change their phenotype in ulcers and cancers. Further studies should be performed to identify SRF susceptible cells and target genes in the pathological condi-
tions. In addition, tools for in vivo overexpression or restoration of SRF should be developed. These tools can be viral gene delivery systems, nanoparticles, or chemicals that induce or restore SRF expression. SRF itself induces its own expression. Thus subtle increases in SRF expression may be enough to trigger a positive feedback reaction that would restore SRF in the protein deficient diseases. Demethylation of the hypermethylated SRF gene may provide a new anti-cancer therapy to stop or kill proliferating cancer cells. Although we are still far away from treating SRF deficient diseases, this multi-phenotypic protein could offer potential clinical applications in medicine that can reverse some of the unwanted pathological changes occurring in these GI diseases.

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