Published in final edited form as:

Gastroenterology. 2011 July ; 141(1): 164–175. doi:10.1053/j.gastro.2011.03.058.

SRF-Dependent microRNAs Regulate Gastrointestinal Smooth Muscle Cell Phenotypes

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Abstract

Background & Aims—Smooth muscle cells (SMCs) change phenotypes under various pathophysiological conditions. Such change is controlled by the serum response factor (SRF), a transcription factor that binds to CArG boxes in SM contractile genes. MicroRNAs (miRNA) regulate transitions among SMC phenotypes. The SMC miRNA transcriptome (SMC miRNAome) and the regulation of the SMC miRNAome by SRF have not been determined.

Methods—We performed massively parallel sequencing to identify gastrointestinal (GI) SMC miRNAomes in mice and humans. SMC miRNAomes were mapped to identify all CArG boxes (miCArGome), which were confirmed by SRF knockdown and microarrays. Quantitative PCR was used to identify SMC-phenotypic miRNAs in differentiated and proliferating SMCs. Bioinformatics and target validation analysis showed regulation of SMC phenotype by SRF-dependent, SMC-phenotypic miRNAs.

Results—We cloned and identified GI miRNAomes using genome-wide analyses of mouse and human cells. The SM miRNAome consisted of hundreds of unique miRNAs that were highly conserved among both species. We mapped miCArGomes and found many had a SRF-dependent signature in the SM miRNAome. The SM miCArGome had several distinct features. We also identified ~100 SMC-phenotypic miRNAs that were induced in differentiated or proliferative SMC phenotypes. We showed that SRF-dependent, SMC-phenotypic miRNAs bind and regulate *Srf* and its cofactors, *Myocd* and *Elk1*.

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Author Contributions: Conceived and designed the experiments: CP, KMS, SR. Performed the experiments: CP, JHC, WJH, SR. Analyzed the data: CP, GW, JHC, DR, JKP, SMW, JMM, WY, SR. Contributed reagents/materials/analysis tools: GW, KMS, JMM, YW, SR. Wrote the paper; JMM, KMS, SR.

Supplementary Data Supplementary data associated with this article can be found in the online version at doi: and www.gastrojournal.org.

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Conclusions—The GI SMC phenotype is controlled by SRF-dependent, SMC-phenotypic miRNAs that regulate expression of SRF, MYOCD, and ELK1.

Keywords

development; genomics; gene expression profile; GI contraction

Introduction

SMCs undergo changes in phenotype and function in GI motility disorders. Phenotypic and functional changes result from altered gene expression driven largely by SRF.¹ SRF is a master switch for expression of contractile and cytoskeletal genes in virtually all cells across diverse species.² Actions of SRF are regulated via transcriptional co-activators MYOCD and ELK1.³ During SMC differentiation, SRF regulation occurs by the 10-nucleotide [CC (A/T)₆ GG] sequence (CArG box) found primarily in promoter and intronic regions of many SM-restricted genes.^{1,4} SRF binds to CArG boxes, which activates SM gene transcription. Several functional CArG boxes have been determined in the genome (i.e., CArGome),⁵⁻⁷ however the nature of many CArG-containing genes is unknown.

Recent studies show that SMC proliferation and differentiation are regulated by miRNAs, including miR-1, miR-21, miR-133a, miR-143, and miR-145, which are key regulators of SMC growth and differentiation.⁸⁻¹³ Some of these SM miRNAs are regulated by SRF through a conserved CArG box.^{9,11,13,14} One-third of 500 or more miRNA genes in mammalian genomes contain at least one CArG box in their promoter regions.¹⁵ Despite these advances, it is important to characterize the entire SM miRNAome and its regulation in differentiated or proliferating SMCs to understand the phenotypic regulation of SMCs by miRNAs in various pathophysiological conditions. Here we report the GI SM miRNAomes and miCArGomes from mice and humans. We identified genome-wide SRF-dependent, SMC phenotype-specific miRNAs and confirmed that SMC phenotype is regulated by a subset of these miRNAs.

Materials and Methods

Animal and Tissue Preparation

Tissues were dissected from C57 or transgenic *smMHC/Cre/eGFP* (*smMHC-eGFP*) mice¹⁶ at ~3 weeks of age. Mucosa and submucosa were removed from the muscularis. Animal protocols were approved by Institutional Animal Care and Use Committee at the University of Nevada.

Isolation of Total and Small RNAs from Tissues and Cells

Total RNAs and/or small RNAs were isolated from multiple tissues, sorted SMCs from *smMHC-GFP* mice, and from the rat pulmonary arterial smooth muscle cell line (PAC1)¹⁷ as previously described.¹⁸ Proliferating PAC1 cells were harvested at subconfluency (70%) for small RNA isolation. Human small intestine (SI) total RNA, pooled from 5 male and female Caucasians ages 20-61, was purchased (BD Biosciences). Small RNA was isolated from total RNA. Small and total RNAs were used for gene expression analyses using RT-PCR and/or qPCR (for all primers used in this study see Supplementary Table 1 online at www.gastrojournal.org).

Construction of Small RNA cDNA Libraries and Massively Parallel Sequencing

Two small RNA cDNA (srcDNA) sequencing libraries were made from mouse and human SI small RNAs as described.¹⁹ After quality confirmation, the two libraries were combined

and sequenced using the high-throughput Genome Sequencer 20 DNA Sequencing System (Roche Applied Science).

Bioinformatics Analysis of the Sequence Reads

We developed software to extract small RNA sequences, sort, and compare them to known pre-miRNAs and miRNA libraries (see Extended Materials and Methods). Mouse and human SM miRNAs were annotated with this software.

Flow Cytometry and Fluorescence-Activated Cell Sorting

Cells were dispersed from SI and large intestine (LI) muscularis of 3-week old *smMHC-GFP* mice, as described.²⁰ Cells labeled with Hoechst 33258 were analyzed and sorted with a FACSaria II (Becton Dickinson) and used to isolate SMC-specific total and small RNAs.

Bioinformatics Analysis of CArG and CArG-like Boxes

Genomic location, cluster, CArG and CArG-like boxes of SM miRNAs cloned from mice and humans were analyzed using the miRNA database miRBase²¹, the UCSC mouse database^{22,23}, and the human genome browser^{22,24}.

Knockdown of SRF or Overexpression of MYOCD

SI smooth muscle tissue from ~3-week old female C57 mice was cultured in SMC culture media at 37°C overnight, and then transduced with an adenovirus expressing a short hairpin RNA against *Srf*²⁵, an adenovirus expressing MYOCD (Ad-MYOCD)²⁵, a control adenovirus expressing eGFP (Ad-eGFP)²⁶, and no adenovirus (No Ad). Tissue strips were cultured 3 days and transduction efficiency in longitudinal and circular SM layers examined with eGFP expression. Tissues were collected for qPCR and microarray analyses. Subconfluent (70%) PAC1 cells were transduced with Ad-MYOCD or Ad-eGFP, and harvested 2 days post-transduction for qPCR analysis.

microRNA Microarrays

Mouse microRNA microarrays were performed using μ Paraflo® technology and proprietary probe design from the Sanger miRBase database (Release 14.0) (LC Sciences). Four small RNA samples (2 Ad-ShSRF and 2 Ad-eGFP transduced tissues) were used for the arrays. Twenty one randomly selected miRNAs were confirmed with the srcDNAs through qPCR.

miRNA-Target Validation Luciferase Assay

Each of the precursor miRNAs amplified by PCR using each primer set, along with each miRNA-target site and seed sequence mutant chemically synthesized for *Srf*, *Myocd*, or *Elk1* 3' UTRs (Supplementary Table 2 online at www.gastrojournal.org), were subcloned into miRNA-target validation vector pGL-miTar²⁷. The effects of miRNAs on target genes in HEK-293 were examined using a luciferase assay system as described.²⁷

miRNA Functional Analysis in PAC1

The miRIDIAN™ microRNA Hairpin Inhibitors designed against mouse miR-143, miR-145, miR-199a-3p, miR-214, or the scrambled negative control #1 were transfected into PAC1 cells at subconfluency or postconfluency according to the manufacturer's instructions (Dharmacon). SRF and ELK1 expression in the transfected cells were analyzed by Western blot, and smooth muscle (SM) α -actin expression was analyzed by confocal microscopy.

Results

SM miRNA Transcriptomes of Mice and Humans

SMC miRNAs from mouse and human SI were cloned and identified using massively parallel sequencing. Mouse and human sequencing templates were prepared from small RNAs amplified using sequence tags. The two templates were combined into a single sample and sequenced. We obtained 83,825 reads, which were sorted into 44,552 (mice) and 37,297 reads (humans) using each tag (Supplementary Table 3 at www.gastrojournal.org). Bioinformatics analysis of sequence reads identified 4,210 mouse and 1,754 human miRNAs, which were grouped into 312 and 181 unique miRNAs respectively (Supplementary Table 3). All cloned miRNAs are shown in Supplementary Table 4 (details in Supplementary Tables 5 and 6 at www.gastrojournal.org). Among the cloned miRNAs, 25 are novel miRNAs (21 from mice and 4 from humans), which include 19 passenger (miRNA*) strands (17 from mice and 2 from humans) and 6 guide (miRNA) strands (4 from mice and 2 from humans).

Linear regression showed a strong correlation for the number of clones for each unique miRNA present in both mice and humans ($R^2 = 0.74$; Figure 1A). The distribution frequency of the difference between the two groups of miRNAs showed that 87% of the miRNAs have a difference of less than ± 0.3 (Figure 1B). Among the total number of miRNAs, 87% (mice vs. humans) and 94% (humans vs. mice) were in both species (SM common miRNAs) while 13% and 6% were found in only human or mouse (SM unique miRNAs) (Figure 1C). When individual miRNAs were compared between species, percentages (46-79%) of common miRNAs were lower while percentages (21-54%) of unique miRNAs were higher (Figure 1D), suggesting that common miRNAs were abundantly expressed while unique miRNAs were rarely expressed.

Expression of 10 miRNAs in multiple tissues was analyzed by qPCR (Figure 1E). miR-143 and miR-145 appeared most prevalent in GI SM tissues. miR-26a, let-7b, and let-7c were also highly expressed. Other miRNAs were enriched in different tissues: miR-26a, miR-24, and miR-23b were highly abundant in the lung, miR-181a and miR-125b in the brain, and miR-30c in the kidney.

Identification of miRNAs Induced in Differentiated SMCs and Proliferative PAC1

To obtain differentiated SMCs, we used a *smMHC-eGFP* mouse line that expresses eGFP under the control of the myosin heavy chain gene, *Myh11* (SM-MHC) promoter, in a SMC-specific manner.¹⁶ SM-MHC is a marker for SM lineages, and its expression is restricted to differentiated SMCs throughout development.²⁸ Fluorescence images from whole mounts and cryostat sections displayed distinct longitudinal and circular SM layers in *smMHC-eGFP* mice (Figure 2A). Expression of eGFP in isolated SMC was also confirmed (Figure 2A). SMCs were purified from dispersed SI muscularis using FACS. eGFP⁺ cells were detected from non-dead cells (Figure 2B). Sorted eGFP⁺ cells were confirmed as SMCs by microscopy and RT-PCR. SI and LI tissue contain several cell types including SMCs, neuronal cells, interstitial cells of Cajal (ICC), and cells of hematopoietic origin. The eGFP⁺ cells purified from the SI and LI expressed the SMC marker, *Myh11*,²⁸ but not *Ncam1* (neuronal cells), *Kit* (ICC), *Ptpnc* (*Cd45*, hematopoietic cells including macrophages), and *Cma1* (mast cells) (Figure 2C). Cells positive for *Myh11* also expressed SMC genes, *Cald1*, *Cnn1*, *Des*, *Myh11*, *Tagln* (*SM22a*), and *Acta2*. SI SMCs expressed all of the genes with a high abundance of *Cnn1* and *Myh11*; LI SMCs expressed a higher abundance of *Cnn1* and *Des* (Figure 2D). Both SMCs expressed all predominant miRNAs and a high abundance of miR-143 and miR-145, which have been reported to enhance SMC differentiation⁹ (Figure

2E). These data confirmed the purity and phenotype-specific gene expression of sorted SMCs.

To observe the quantity of miRNAs expressed, we made extensive expression profiles of 310 cloned SM miRNAs in whole SI tissue and isolated differentiated SMCs using qPCR. All qPCR amplicons were confirmed on 2% agarose gels and Ct values were corrected accordingly. qPCR detected 301 and 275 miRNAs in SI tissue and differentiated SMCs, respectively (see Supplementary Table 7 online at www.gastrojournal.org). Among 275 differentiated SMC miRNAs, 82 were more abundantly expressed in the SMCs than in the tissue, while 37 were enriched in the SMCs >2 fold (Supplementary Table 7). Highly enriched SMC miRNAs included miR-1937b, miR-143, miR-29c, miR-21, and miR-125b-5p. Interestingly, miR-1937b was most abundantly expressed in both SI tissue and differentiated SMCs (Supplementary Table 7). An expression profile of the tissue and SMC miRNAs is shown in Figure 3A. The SI tissue expressed more miRNAs in number and amount than differentiated SMCs except for 82 SMC enriched miRNAs (Figure 3A; details in Supplementary Table 7). The correlation coefficient between tissue and SMC miRNAs was $R^2 = 0.60$ (Figure 3B). When 82 SMC enriched miRNAs were compared to the tissue miRNAs, the correlation coefficient ($R^2 = 0.94$) increased significantly, suggesting that the expression pattern of SMC enriched miRNAs is closely related to that of tissue miRNAs.

In order to compare the miRNA expression profiles of differentiated SMCs and proliferating SMCs, we attempted to culture differentiated SI SMCs. However, these SMCs failed to grow in SM cell media, which contained several growth factors, even though the mixed, dispersed cells of SI grew well in this medium. As an alternative, we used the PAC1 SMCs for identification of miRNAs induced in proliferating SMCs. PAC1 cells rapidly proliferate up to 79% of subconfluence and then switch phenotype into differentiated SMCs during confluence and postconfluence conditions.¹⁷ Proliferating PAC1 were used for qPCR analysis with each miRNA-specific primer for the 310 SM miRNAs. qPCR detected 285 miRNAs in PAC1 and expression levels of miRNAs were then compared to 278 miRNAs found in the SI SMCs (Supplementary Table 8 online at www.gastrojournal.org). Most miRNAs (273 miRNAs: 96-98%) were found in both cells while 12 miRNAs were found only in PAC1, and only 2 in SI SMC. In general, proliferating PAC1 more abundantly expressed miRNAs in number and amount than differentiated SMCs (Figure 3C).

Expression levels between the two miRNA groups did not show extensive correlation (Figure 3D). In the differentiated SMCs, 65 miRNAs were induced >2 fold while 155 miRNAs were induced in proliferating PAC1 (Supplementary Table 8). A total of 94 miRNAs (25 and 69 miRNAs induced in the SMCs and PAC1, respectively) with a cutoff of >10 fold are shown in Figure 3E. The miRNAs induced in the differentiated SMCs include miR-145, miR-143, and miR-1, which are at the top of the list and are known to regulate SMC differentiation.^{9,12} There were almost three times more miRNAs induced in proliferating PAC1 than in differentiated SMCs. The miRNAs induced in PAC1 include let-7c, miR-21, let-7d, miR-484, and miR-214, which are at the top of the list (Supplementary Table 8) and have been reported, except for miR-484, to regulate cell proliferation.²⁹⁻³² Interestingly, miR-214 was completely absent in the differentiated SMCs, but was abundantly expressed in PAC1 (Supplementary Table 8).

CAR_G and CAR_G-like Boxes Identified in the Flanking Regions of SM pre-miRNAs

To identify miRNA CAR_G and CAR_G-like boxes, we analyzed the flanking genomic regions (−4 kb to +4 kb) as previously applied to the mammalian CAR_Gome⁶ using the UCSC mouse^{22,23} and human genome browser.^{22,24} All sequences and locations of CAR_G and CAR_G-like boxes are shown in Supplementary Tables 5 and 6. We found 251 and 104 CAR_G boxes, and 3,878 and 1,690 CAR_G-like boxes in mice and humans, respectively (Table 1). In mice, 56% (152 out of 272 total) unique miRNAs had one or more associated CAR_G boxes.

In humans, 46% (81 out of 178 total) had one or more associated CArG boxes (Supplementary Table 9 at www.gastrojournal.org). The average number of CArG boxes per miRNA was 1.7 for mice and 1.3 for humans (Table 1). CArG-like boxes were found for every miRNA and contained 14.3 CArG-like boxes per miRNA gene for mice, while all human miRNAs, except hsa-mir-126 and hsa-mir-199b, contained an average number of 9.5 CArG-like boxes per miRNA gene (Supplementary Table 9). In mice, more CArG boxes (185) were found in the downstream than upstream regions (66) while human CArG boxes were found in almost equal amounts in the upstream (53) and downstream regions (51) (Table 1). The distribution of CArG-like boxes up- or downstream is similar to those of CArG boxes found in mice and humans. The frequency of CArG and CArG-like boxes in the flanking regions (−4 kb to +4 kb) are shown in Figure 4A. Most CArG boxes of mice and humans were found from −3 kb to +1 kb while CArG-like boxes were spread more widely from −4 kb to +4 kb. The nucleotide sequence content of consensus CArG boxes was slightly different between the two species. Mouse CArG nucleotides 4-8 show preference for ‘T’ while human CArG nucleotides 4-8 show preference for ‘A’ except at nucleotide 5 where it prefers ‘T’ (Figure 4B). Human and mouse CArG-like nucleotides 3-8 are similar except at nucleotide 7 (Figure 4B).

Identification of SRF-Dependent miRNAs

To identify miRNAs regulated by SRF through CArG or CArG-like boxes, we knocked-down SRF in SM organ cultures using an adenovirus expressing SRF shRNA (Ad-ShSRF).²⁵ SI muscularis was transduced with either Ad-ShSRF or Ad-eGFP and cultured for 3 days. To confirm transduction efficiency, Ad-eGFP transduced tissue was analyzed by confocal microscopy. Images revealed robust eGFP expression (Figure 4C), and optical sectioning confirmed transduction in longitudinal and circular layers (LM and CM; Figure 4C). In CM, the morphology of single cells resembled SMCs identified by microscopy (CM high; Figure 4C). Next we looked at expression of *Srf* and the SMC marker *Myh11* in knocked-down tissues. *Srf* was significantly reduced in Ad-ShSRF tissue compared to Ad-eGFP or no-virus control tissues (Figure 4D). Expression of *Myh11* in vascular SMCs is regulated by SRF through two CArG elements in the promoter region³³. *Myh11* was notably reduced in the Ad-ShSRF compared to the controls (Figure 4D), suggesting that SRF regulated expression of *Myh11* in GI SMCs.

Using microRNA microarrays on the Ad-ShSRF knocked-down tissues, we identified 55 miRNAs that were dysregulated by SRF (Figure 4E). Among these, 35 miRNAs including miR-214, miR-199a-3p, miR-143, and miR-145 were down-regulated, and 20 miRNAs were up-regulated (Supplementary Table 10; www.gastrojournal.org). Most down-regulated miRNAs were those identified in the cloning pool of mice and abundantly expressed in GI tissues. Conversely, all up-regulated miRNAs, except for miR-320, were not identified in the cloning pool. All down-regulated miRNAs, except 5 that have not yet been analyzed, contained at least one CArG or CArG-like box (Supplementary Table 10), suggesting these miRNAs are regulated by SRF through CArG or CArG-like boxes. Twenty three randomly selected SRF-dependent miRNAs (15 down-regulated and 8 up-regulated miRNAs) were validated by qPCR (n=2). All miRNAs, except for two (miR-1224 and miR-1892), matched changes observed in array data (Supplementary Table 10).

Regulation of SMC Phenotype by miR-143, miR-145, miR-199a, and miR-214

SMC phenotype is regulated by SRF, MYOCD, and ELK1.³ All miRNAs potentially targeting *Srf*, *Myocd*, and *Elk1* were retrieved from MicroCosm,³⁴ TargetScan,³⁵ and PicTar.³⁶ Supplementary Table 11 found online at www.gastrojournal.org summarizes the targeting miRNAs along with a cloning profile of mouse SM miRNAs, expression profiles of miRNAs in differentiated SI SMCs and proliferating PAC1, CArG and CArG-like boxes

per miRNA gene, and an expression profile of SRF-dependent miRNAs. All miRNAs targeting *Srf*, *Myocd*, and *Elk1* include the differentiated-SMC-induced miRNAs (miRNAs targeting *Srf*, 10; *Myocd*, 5; and *Elk1*, 6) and the proliferative-SMC-induced miRNAs (15, 9, and 15, respectively) which showed fold changes >10 (Supplementary Table 11). Among those miRNAs, four miRNAs: miR-143, miR-145, miR-199a-3p, and miR-214 were selected because 1) they were identified in the deep sequencing of the mouse SM (Supplementary Table 4), 2) miR-143 and miR-145 were highly expressed in the differentiated SI SMCs, but their expression was reduced in proliferating PAC1. Conversely, miR-214 and miR-199a-3p were highly expressed in the proliferating PAC1, but their expression decreased in differentiated SI SMC (Supplementary Table 8). 3) they contain 1-2 CArG and 5-20 CArG-like boxes (Supplementary Table 9), and 4) they are regulated by SRF (down-regulated by Ad-shSRF) (Supplementary Table 10). Interestingly, the four miRNAs are located on two miRNA clusters: miR-143 and miR-145 on chromosome 18(qE1), and miR-199a and miR-214 on chromosome 1(qH2.1), suggesting two miRNAs are generated from each clustered transcript. Multiple target sites were identified in the 3' UTRs of *Srf* (miR-199a and miR-214), *Myocd* (miR-145), and *Elk1* (two miR-143, miR-214, and miR-145) (Figure 5A). The targeting effect at each site was examined using the luciferase miRNA-target validation system²⁷. Expression of the luciferase reporter protein was reduced 17-63% in *Srf*-199a-3p, *Elk1*a-143, *Elk1*b-143, and *Elk1*d-145 compared to each seed sequence (binding site) mutant, while *Srf*b-214, *Myocd*-145, and *Elk1*c-214 induced 37-54% expression (Figure 5B). All targeting effect data is summarized on the maps (Figure 5A). This result of reduction in *Elk1*a/b-143 and induction in *Myocd*-145 was consistent with the report by Cordes et al⁹. In addition, we found the reduction of *Elk1*d-145 in this targeting assay, suggesting both differentiated SMC-specific miR-143 and miR-145 can suppress ELK1.

Functional roles of the SRF-dependent, phenotypic miRNAs were further investigated using their antisense inhibitors in PAC1. ELK1 was abundantly expressed in the subconfluent (proliferating) PAC1 as well as in the smooth muscle from mouse small intestine and colon (Figure 5C). As expected, ELK1 was down-regulated when PAC1 (postconfluent) was differentiated. miR-214 inh down-regulated ELK1 by 60% in the proliferating cells while miR-145 inh and miR-143 inh up-regulated ELK1 by 10% and 20%, respectively (Figure 5C). This expression profile of ELK1 regulated by the miRNA inhibitors matched the luciferase assay (Figure 5B). However, expression of SRF was suppressed in proliferating PAC1 and returned in differentiated PAC1 (Figure 5C). SRF was abundantly expressed in SM tissues. miR-199a-3p inh up-regulated SRF by 60%. miR-214 inh also slightly up-regulated SRF when contrasted to the luciferase assay (Figure 5B). Interestingly, miR-143 inh and miR-145 inh further decreased SRF by 60% and 80% respectively. Since the roles of differentiated SMC-specific miR-143 and miR-145 have already been reported in vascular SMCs⁹, we focused on proliferating SMC-specific miR-214 and miR-199a-3p and investigated if they could switch SMC phenotypes. When proliferating PAC1 was transfected with either miR-214 inh or miR-199a-3p inh, SM- α -actin was dramatically induced compared to scrambled Neg control (Figure 5D), suggesting miR-214 and miR-199a-3p are required for SMC proliferation.

Next we investigated if the SRF-dependent miRNAs were transcriptionally regulated by SRF-cofactor MYOCD. miR-143 and miR-145 were abundantly expressed in differentiated SMCs while miR-199a-3p and miR-214 had very low expression (Supplementary Table 8). When MYOCD was overexpressed in the smooth muscle tissue using an adenovirus expressing MYOCD (Ad-MYOCD)²⁵, the miRNAs had the opposite effect when compared to the negative control Ad-eGFP: expression of miR-143 and miR-145 increased, but miR-199a-3p and miR-214 were further decreased (Supplementary Figure 1A). Conversely, in proliferating PAC1, expression of miR-143 and miR-145 were dramatically reduced

while expression of miR-199a-3p and miR-214 significantly increased (Supplementary Table 8). When MYOCD was overexpressed in proliferating PAC1, the same opposite effect was observed: induction in miR-143 and miR-145, and suppression in miR-199a-3p and miR-214 (Supplementary Figure 1B). This data suggests MYOCD can act as both an activator of miR-143 and miR-145 and a repressor of miR-199a-3p and miR-214.

Collectively, our studies from the targeting analyses lead us to propose a model that SMC phenotype is controlled by four SRF-induced miRNAs: miR-143, miR-145, miR-199a, and miR-214 (Fig 6). miR-143 and miR-145 promote SMC differentiation by activation of MYOCD and through the inhibition of ELK1. Conversely, miR-214 and miR-199a suppresses SMC differentiation, but promotes its proliferation by activation of ELK1 and inhibition of SRF. It is interesting that SRF induces the miRNAs of the two clusters, each of which modulates SMC differentiation and proliferation respectively, depending on the cofactors MYOCD and ELK1.

Discussion

We found that SM miRNAomes are highly conserved in mice and humans, and that a hundred miRNAs are SRF- and SMC phenotype-dependent. We also demonstrated that SMC phenotype is modulated by the SRF-dependent, SMC-phenotypic miRNAs.

SM transcriptomes (SM miRNAomes) of mice and humans are genetically conserved (Supplementary Table 4), and SM miRNA expression in SI SM is similar to vascular SM. Most miRNAs (273 miRNAs: 96-98%) were found in SI and vascular PAC1 SMCs (Supplementary Table 8). Several miRNAs including miR-21,^{10,29} miR-221,^{37,38} miR-133a,¹¹ miR-143, and miR-145^{8,9,13,39} regulate SMC phenotype in vascular diseases. All of the vascular SMC regulatory miRNAs are expressed in mouse and human SI SM (Supplementary Table 4), particularly the SMC-specific miRNAs miR-143 and miR-145 (Figure 2E), suggesting these miRNAs play an important role in the GI tract, as they do in blood vessels.

We identified SM miRNA CARGomes (Table 1; details in Supplementary Tables 5, 6, and 9) and confirmed miRNAs are regulated by SRF (Figure 4E; details in Supplementary Table 10). The CARG or CARG-like boxes of the SM miRNAs are somewhat different than those of messenger RNA (mRNA) transcripts.⁶ 1) there are more SRF targets (CARG) found within the flanking regions of miRNAs (56% in mice and 46% in humans) than mRNAs (1%). SRF targets in mRNAs include 243 validated CARG boxes,⁶ (and unpublished data), encompassing approximately 1% of the protein-coding genes.²³ It was expected that some miRNAs might be targeted by SRF, but surprisingly, hundreds (152 CARG boxes) and thousands (3,878 CARG-like boxes) of miRNAs are potential SRF targets. 2) SRF target sites were more widely distributed in miRNAs (Figure 4A). 3) the miRNA CARG sequence motif (Figure 4B) is somewhat different than that of mRNA.⁶ Further studies on potential SRF target miRNAs will reveal whether the mechanism of SRF regulation is the same as mRNAs. SRF knockdown microarrays identified 36 miRNAs that were repressed in the knockdown (Figure 4E). We confirmed SRF regulation of miR-143 and miR-145 in intestinal SM, which is consistent with previous studies.^{9,13} We also found that miR-214, miR-199a-3p, and miR-21 are regulated by SRF. These miRNAs contain CARG and CARG-like boxes (Supplementary Table 5). Future work should further validate the necessity of CARG and CARG-like sequences for SRF-dependent miRNA expression and the role of SRF cofactors in activation of SM miRNAs.

Hundreds of SMC phenotypic miRNAs were induced or repressed in differentiated or proliferating SMCs (Figure 3E; details in Supplementary Table 8). This finding is significant

for SMC miRNA research because: 1) this is the first genome-wide identification of SMC phenotypic miRNAs; 2) expression profiling was made from a pure population of differentiated SMCs compared to other SMC miRNA studies that have been performed on SM tissues containing mixed cell-types. This genome-wide expression profile of SMC phenotypic miRNAs (Figure 3E; details in Supplementary Table 8) will provide a foundation for future research into the phenotypic regulation of SMCs by miRNAs in pathophysiological conditions.

We propose that two SRF-induced miRNA clusters, miR-143/miR-145 and miR-199a/miR-214, regulate SMC differentiation and proliferation, respectively (Figure 6). We confirmed the role of miR-143/miR-145 in SMC differentiation, reported previously by Cordes et al.⁹ In addition, we identified a novel role for miR-199a/miR-214 in SMC proliferation. The contrasting roles of these clustered miRNA, are achieved by different targeting (positive or negative) effects on SRF and its cofactors MYOCD and ELK1 (Figure 5). Induction of miR-199a/miR-214 in proliferating SMCs is particularly interesting since SMC hyperplasia and hypertrophy occur in some GI motility disorders such as Hirshsprung's disease. Induction of miR-199a/miR-214 occurs in hypertrophic SMCs (data not shown). Inhibition of miR-199a/miR-214 in proliferating SMCs switched the SMC phenotype to be differentiated (Figure 5D). Further studies are needed to understand the role of these miRNAs in GI motility disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M.I. Kotlikoff for the *smMHC-eGFP* mice; S. Chung for the summer undergraduate research work; H. Shin and K. Lee for experimental support; N. Horowitz for technical support regarding tissue and cell culturing; G. Hultin for flow cytometric SMC sorting; L. Peri for supplying buffers and cell lines; T. Gardner for technical support on computational analysis; Nevada Genomics Center for GeneChip array and sequencing services; Nevada INBRE for the undergraduate research opportunities fund. This work was supported by the NIH grant P20 RR018751 to K.M. Sanders, NIH grants HD048855, HD60858, and HD050281 to W. Yan, and HL-62572 to J.M. Miano.

Funding sources: NIH grant P20 RR018751 to K.M. Sanders and HL-62572 to J.M. Miano

Abbreviation used in this paper

SMCs	smooth muscle cells
miRNA	microRNAs
miRNAome	microRNA transcriptome
CArG box	CC (A/T) ₆ GG
CArG-like box	a 1-bp deviation from the CArG box
miCarGome	microRNAs CArG boxes
SRF	serum response factor
SI	small intestine
LI	large intestine
LM	longitudinal smooth muscle
CM	circular smooth muscle

smMHC-eGFP	smMHC/Cre/eGFP
srcDNA	small RNA cDNA
Ad-ShSRF	adenovirus expressing a short hairpin RNA against <i>Srf</i>
Ad-eGFP	adenovirus expressing eGFP
PAC1	rat pulmonary arterial smooth muscle cell line
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
FACS	fluorescence activated cell sorting

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Figure 1.

Expression profiles of SM miRNAs cloned from mouse and human SI using massively parallel sequencing. (A) Linear regression of SM miRNAs cloned from mouse and human ($P < 0.0001$). (B) Distribution frequency of the difference between the normalized percentage of mouse and human miRNAs. (C) Percentage of total mouse and human miRNAs that were found in both species (common) and those that were found in only one (unique). (D) Percentage of common and unique miRNA species found in mouse and human. (E) Relative expression profile of the most prevalent miRNAs found in mouse tissue analyzed by qPCR. The expression level of each miRNA was normalized by an average Ct of the five control snoRNA genes, and then the normalized values were converted to a number between 1 (lowest) and 10 (highest) on the heat map.

Figure 2.

Isolation of a pure population of differentiated SMCs from the SI muscularis of *smMHC/Cre/eGFP* mice, and the expression profiles in the isolated SMCs. (A) Fluorescent images of the whole mount and cryostat section of the SI muscularis showing longitudinal SM (LM) and circular SM (CM), and of an isolated SMC. (B) Flow cytometric identification of eGFP⁺ SMCs sorted from the mice. SI muscle was dissociated and cells were stained with Hoechst 33258 double stranded DNA labeling in cells. Cells were analyzed and sorted using a Becton Dickinson FACSaria II flow cytometry system. Non-dead cells were gated out and eGFP⁺ cells were detected and sorted from the non-dead cells. (C) The identity of eGFP⁺ SMCs sorted from SI and LI tissue was confirmed by RT-PCR using gene specific primers. (D) mRNA enrichment of SM genes in sorted SI and LI SMCs were tested using qPCR. The expression level of each gene was normalized to that of *Gapdh*. Normalized expression levels were then converted to fold changes (sorted vs. tissue). (E) Expression profiles of SM miRNAs in sorted SMCs analyzed by qPCR. The expression level of each miRNA was normalized using the 6 control snoRNAs.

Figure 3.

Identification of SMC differentiation- or proliferation-dependent miRNAs. (A) Comparison of miRNA expression levels between SI tissue and differentiated SMCs. Expression of 310 cloned miRNAs was examined in the SI tissue and SMCs using qPCR. Expression of 301 miRNAs was detected in the SI tissue while 278 miRNAs were detected in the SI SMCs. Expression levels of each miRNA were normalized by an average expression level of the 5 snoRNA genes. Normalized expression levels were converted to a log scale and then plotted on the graph. (B) Correlation of expression profiles of differentiated SI SMC miRNAs vs. SI tissue miRNAs. (C) Comparison of miRNA expression levels between differentiated SI SMCs and proliferative PAC1. qPCR detected 285 miRNAs in proliferative PAC1, which were compared to 278 miRNAs found in the SI SMCs. (D) Correlation of expression profiles of differentiated SI SMC miRNAs vs. proliferative PAC1 miRNAs. (E) Differentially expressed 94 miRNAs between differentiated SI SMCs and proliferative PAC1 (cutoff: >10 fold change). Expression levels were converted to a log scale and then plotted on a heat map: red (high) to blue (low or not detected).

Figure 4.

Identification of SRF binding sites of SM miRNAs and miRNAs regulated by SRF knockdown. (A) Frequency of CArG and CArG-like boxes found in the flanking regions of mouse and human pre-miRNAs. (B) Conserved CArG and CArG-like motifs of mouse and human SM pre-miRNAs. (C) SI SM tissue transduced with Ad-ShSRF, Ad-eGFP, or no adenovirus (No Ad). A digital composite of a confocal Z-stack showing expression of eGFP in the muscularis. Digital composites of subsets of Z-slices taken from the longitudinal SM (LM) and the circular SM (CM) region from the entire confocal stack showing the expression of eGFP within these regions. A higher magnification of CM (CM high) showing an individual circular SMC expressing eGFP. (D) Reduction of *Srf* and *Myh11* expression in Ad-ShSRF, Ad-eGFP, and No Ad SM tissue confirmed by qPCR. (E) SRF-regulated miRNAs detected by miRNA arrays (n=2).

Figure 5.

SRF-induced miRNAs targeting SRF, MYOCD, and ELK1 regulate SMC phenotypes. (A) Maps of predicted targeting sites of SRF-induced miRNAs (miR-199a-3p, miR-214, miR-143, and miR-145) in the 3' UTRs of *Srf*, *Myocd*, and *Elk1*. (B) Luciferase studies of the SRF-induced miRNAs. Targeting effect in B is summarized in A with arrows: upregulation (↑) and downregulation (↓). (C) Western blot of SRF and ELK1 in subconfluent (70%) and postconfluent (100%) PAC1, respectively, transfected with each of the miRNA inhibitors (miR-143 inh, miR-145 inh, miR-199a-3p inh, or miR-214 inh). GAPDH was used for endogenous control to normalize SRF and ELK1; normalized values are shown. (D) Immunocytochemistry of subconfluent PAC1 using smooth muscle (Sm) α-actin antibodies (green) transfected with miRNA inhibitors indicated; nuclear stain, DAPI (purple).

Figure 6.

A model of SMC fate and plasticity regulated by SRF-induced miRNAs. The SRF-induced miRNAs, miR-143/miR-145 and miR-199a/miR-214, regulate the differentiated and proliferative phenotype of SMCs by fine tuning SRF, MYOCD, and ELK1, respectively.

Summary of miCArGome in the mouse and human genome

Table 1

Species	Motif	Frequency			Distribution	
		pre-miRNAs	Hit ¹	Average ²	Upstream	Downstream
Mice	CArG	272	152(56%)	1.7(251)	66	185
Humans	CArG	178	81(46%)	1.3(104)	53	51
Mice	CArG-like	272	272(100%)	14.3(3,878)	1173	2,705
Humans	CArG-like	178	177(99%)	9.5(1,690)	827	863

¹ Number of unique pre-miRNA sequences that contained CArG or CArG-like boxes found in the 4 kb-upstream/downstream regions from the pre-miRNA sequence.

² Average numbers of CArG or CArG-like boxes per pre-miRNA that had one or more CArG/CArG-like matches. Total numbers of CArG/CArG-like matches shown are in the brackets.