Regulation of MicroRNAs by Brahma-related Gene 1 (Brg1) in Smooth Muscle Cells*1

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Background: It is unknown whether Brg1 regulates microRNA expression during smooth muscle differentiation.

Results: Brg1, SRF, and myocardin are required for transcription of miRs-143/145. Brg1 and SRF together with other factors regulate transcription of miR-133.

Conclusion: Brg1 interacts with distinct factors to regulate expression of microRNAs.

Significance: Brg1-containing chromatin-remodeling complexes regulate expression of both protein coding and noncoding genes to control smooth muscle differentiation.

MicroRNAs are involved in phenotypic switching of smooth muscle cells (SMCs). Brg1-containing SWI/SNF chromatin-remodeling complexes also play an important role in controlling the phenotype of SMCs. We thus determined whether Brg1 influences the transcription of microRNAs in SMCs. Microarray and quantitative RT-PCR analysis of smooth muscle from mice harboring smooth muscle-specific deletion of Brg1 revealed altered expression of several microRNAs, including miRs-143/145 and miR-133. Ablation of Brg1 in SMCs in vitro either by expression of dominant negative Brg1 or Brg1 knock-out attenuated miRs-143/145 expression. Knockdown of serum response factor (SRF) in SMCs significantly reduced the expression levels of miRs-143/145 and miR-133, whereas knockdown of myocardin only attenuated miRs-143/145 expression. Myocardin induced expression of miRs-143/145 and miR-133a and increased SRF binding to these genes in 10T1/2 cells. This myocardin-mediated induction was attenuated by dominant negative Brg1. In Brg1-null SW13 cells, miRs-143/145 were dramatically induced by myocardin only in the presence of Brg1, whereas miR-133 was not induced by myocardin in a Brg1-dependent manner. Chromatin immunoprecipitation assays demonstrated that in the presence of Brg1, myocardin increased SRF binding to both the miRs-143/145 and miR-133a loci. Together, these data suggest a mechanism in which Brg1-containing SWI/SNF complexes are required for myocardin to induce expression of miRs-143/145 in smooth muscle cells. In contrast, miR-133 expression appears to be regulated by Brg1-containing chromatin remodeling complexes in a partially SRF-dependent, although largely myocardin-independent manner. SWI/SNF-mediated chromatin remodeling thus regulates the phenotype of smooth muscle by affecting expression of protein-coding genes and microRNAs.

MicroRNAs (miRs)3 are endogenous small noncoding RNAs (~22 nucleotides) which have emerged as key regulators of gene expression through inhibiting translation and/or promoting degradation of their mRNA targets. During microRNA biogenesis, primary microRNAs are first transcribed by RNA polymerase II from genes that lie either between or within protein-coding genes. Primary microRNA transcripts are then sequentially processed by endonucleases Drosha and Dicer into mature microRNAs. MicroRNAs are widely expressed but exhibit tissue-specific and dynamic expression patterns during development and pathophysiological processes.

The importance of microRNAs in smooth muscle cell (SMC) differentiation and function has been demonstrated through analysis of mice lacking Dicer in these cells (1). Deletion of Dicer in mouse SMCs by an SM22α promoter-driven Cre transgene resulted in embryonic lethality at embryonic day 16–17 with underdeveloped vessels and extensive hemorrhage (1). Deletion of Dicer in more mature SMCs mediated by a smooth muscle myosin heavy chain (smMHC)-driven Cre transgene did not result in embryonic lethality, although the mice developed severe intestinal dysmotility with loss of the external smooth muscle layers (2). Postnatal deletion of Dicer in smooth muscle cells using a tamoxifen-regulated smMHC-Cre transgene resulted in a dramatic reduction of blood pressure and loss of contractile proteins within the vasculature (3). These studies demonstrate that microRNAs play a key role in the development and maintenance of smooth muscle cells in both vascular and gastrointestinal tissues.

Among Dicer-dependent microRNAs, the most abundant in smooth muscle cells are miRs-143/145. miR-143 and miR-145 are two mature microRNAs that are encoded by the same bicistronic primary microRNA transcript (4). Although miR-143

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3 The abbreviations used are: miR, microRNA; Brg1, Brahma-related gene 1; Brm, Brahma; Cre, Cre recombinase; DN, dominant negative; MRTF, myocardin-related transcription factor; qPCR, quantitative PCR; SMC, smooth muscle cell; smMHC, smooth muscle myosin heavy chain; SRF, serum response factor; SWI/SNF, switch/sucrose nonfermentable.
and miR-145 knock-out mice are viable, they have a significant reduction in blood pressure, thinner vascular smooth muscle layers, incomplete differentiation of SMCs, and altered neointima formation in response to vascular injury (4–6). miRs-143/145 have been shown to cooperatively modulate a network of targets via feedback, feed-forward, or double-negative feedback mechanisms (7). For example, miRs-143/145 can target Kruppel-like factor 4 (Klf4), myocardin, and Elk1 (8), as well as versican (9) to promote differentiation and repress proliferation of SMCs. Moreover, several targets have been identified that regulate actin dynamics and cytoskeletal organization, such as myocardin-related transcription factor B (MRTFB), Adducin-3 (ADD3), Slingshot 2 phosphatase (Ssh2), Slit-Robo GTPase-activating protein 1 (Srgap1) and Srgap2 (4). Using LacZ reporter mice, a 0.9-kb promoter region was shown to be sufficient to direct miRs-143/145 expression to cardiac and smooth muscle cells. Within this region, a highly conserved CARG element (SRF binding site) and Nkx2–5 binding region have been identified. Moreover, the mirs-143/145 gene is a direct transcriptional target of SRF, myocardin, and Nkx2–5, and it is up-regulated in more differentiated smooth muscle cells (8).

Recently, miR-133, which is conventionally considered as a cardiac- or skeletal muscle-specific microRNA, has also been shown to be highly expressed in smooth muscle cells and inhibit vascular SMC proliferation in vitro and after balloon injury in vivo, at least partially through regulating the expression of Sp1 and moesin (10). In cardiomyocytes, deletion of miR-133 causes aberrant cardiomyocyte proliferation and ectopic expression of smooth muscle genes in the heart, partially through the up-regulation of miR-133 target genes SRF and cyclin D2 (11, 12). Moreover, overexpression of miR-133 reduces cardiac hypertrophy, whereas inhibiting miR-133 resulted in hypertrophy (13). In skeletal muscle, miR-133 has been shown to repress myogenesis but promote proliferation, partially through targeting SRF (12). As SRF is a key regulator for skeletal, cardiac, and smooth muscle development, it is likely that miR-133 might be an important regulator of all three linesages. The miR-133 isoforms miR-133a and miR-133b are encoded by three genomic loci: miR-133a–1, miR-133a–2, and miR-133b. Each of these loci is transcribed as a bicistronic primary transcript containing one miR-133 isoform together with another microRNA (14). In skeletal muscle cells, expression of these microRNAs is regulated by the myogenic factors MyoD and myogenin, and in cardiac muscle miR-133a expression is regulated by MEF2 (14, 15). Although the miR-133 family are now recognized as intriguing regulators of vascular smooth muscle cells, little is known regarding the transcriptional regulation of these microRNAs in smooth muscle cells. There is also little information on the epigenetic regulation of microRNAs in smooth muscle cells.

In skeletal muscle cells, the Brg1 ATPase subunit of the SWI/SNF chromatin remodeling complex has been shown to be required for MyoD to activate transcription of both skeletal muscle contractile protein genes and miR-133a (16). Previously, it has also been shown that Brg1 is required for myocardin or MRTFs such as MRTF A to induce expression of smooth muscle-specific contractile proteins (17, 18). In the current study, we investigated the role of Brg1 in regulating microRNA expression in smooth muscle cells. Our results demonstrate that several microRNAs, including miRs-143/145 and miR-133, are regulated by Brg1 in smooth muscle. Brg1 is required for myocardin to induce binding of SRF to the regulatory region of miR-143/145, which is sufficient to activate its transcription. In contrast, the regulation of miR-133 expression by Brg1 requires other factors to cooperate with SRF to activate transcription.

**EXPERIMENTAL PROCEDURES**

**Animals**—All of the protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. To generate smooth muscle-specific Brg1 knock-out mice, female Brg1^flox/flox^ mice (obtained from C.-P. Chang at Stanford University) (19) were bred with male smMHC-Cre/eGFP mice (from Michael Kotlikoff at Cornell University) (20). Because of transient expression of Cre in the sperm of the male mice (21), the floxed allele transmitted from these mice is recombined, resulting in a global heterozygous null allele of Brg1 in all tissues. Genotyping was performed as described previously (22). Global heterozygous Brg1 mice (Brg1^flox/−^) were used as control mice, whereas smooth muscle-specific Brg1-null mice (smBrg1^−/−^) with global heterozygous Brg1 background were the experimental smBrg1 knock-out mice.

**Tissue Harvest and RNA Extraction**—Two-month-old control and knock-out mice were sacrificed. Colons and bladders were dissected quickly and were put in cold PBS buffer on ice. The colon was cleaned, cut open longitudinally, and then the epithelial layer removed by scraping with a scalpel. The bladder was washed in ice-cold PBS. All the tissues were then frozen in liquid nitrogen immediately. To extract RNA from tissues, samples were first pulverized in liquid nitrogen and then homogenized in guanidinium isothiocyanate using a Polytron (Kinematica). Total RNA was extracted following standard protocols (23).

**MicroRNA Microarray**—Total RNA samples from colon smooth muscle tissues were checked for an RNA integrity number on an Agilent Bioanalyzer (Agilent Biotechnology). Four samples from each group with an RNA integrity number > 8 were then used for microarray analysis. Total RNA samples were labeled using the Genisphere FlashTag HSR kit. The labeled samples were hybridized to Affymetrix GeneChip miRNA arrays. They were stained and washed using the standard microRNA protocol. Affymetrix GeneChip Command Console Software was used to scan the arrays and generate CEL files. CEL files were imported into a custom microRNA QC Tool to generate expression levels and detection calls. Probe sets that were absent in more than two samples for both controls and knockouts were removed prior to importing the expression levels into Partek Genomics Suite for analysis (24). A t-test was performed using the log base 2 transformation of the expression levels. -Fold changes were calculated using the raw expression levels.

**Primary Smooth Muscle Cells**—Primary colon smooth muscle cells were isolated from 1-month-old wild type C57BL/6 mice. After colons were dissected, cleaned, and cut open, epithelial layers were removed by scraping. The smooth muscle
layers were then minced in Hanks’ buffered saline solution on ice and digested with 0.6 unit/ml Liberase™ (Roche Applied Science) and 0.25 mg/ml DNase I in Hanks’ buffered saline solution at 37 °C for 45 min with shaking. The digested cells were filtered through a 100-μm filter, washed in SMC growth medium (containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin), or SMC maintenance medium (containing DMEM supplemented with 1% FBS, 2% chick extract, 1% N2, 2% B27, 20 ng/ml mouse basic FGF, 100 μM retinoic acid, 50 μM β-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin). Washed cells were plated in 6-well plates (1 well/colon), and the medium was changed once per day until cells reached 100% confluence (usually about a week). Confluent primary cells were collected by trypsin digestion and were plated in 6- or 12-well plates for adeno viral transduction.

Expression Plasmids and Adenoviral Transduction—Human Brg1, Brm, and dominant negative-Brg1 (DN-Brg1) plasmids were obtained from AddGene (25). MRTFA cDNA was purchased from Invitrogen. Mouse myocardin pcDNA3.1-myc/His vector was kindly provided by Dr. Eric N. Olson (University of Texas Southwestern Medical Center, Dallas). These plasmids were used to generate adenoviral expression vectors, and adenoviral transductions were performed as described previously (17, 18). Adenovirus encoding nuclear localized yellow fluorescent protein (YFP) was used as negative control.

Quantitative RT-PCR—Total RNA was extracted using TRIzol reagent. MicroRNAs were quantitated using the small RNA quantitation system (SBI System Biosciences, Mountain View, CA) with some modifications. Poly(A) tails were added to the RNA by incubation with poly(A) polymerase at 37 °C for 30 min. An oligo(dT) adaptor conjugated with a sequence complementary to a universal reverse primer (CGA ATT CTA GAG CTC GAG GCA GG) was annealed to poly(A)-tailed RNAs at 65 °C for 10 min followed by chilling on ice for 10 min. Then, cDNA was synthesized and diluted as template for qPCR using microRNA-specific forward primers (miR-143 forward primer, TGA GAT GAA GCA CTG TAG CTC; miR-145 forward primer, GTC CAG TTT TCC CAG GAA TCC CT; miR-133a forward primer, TTT GGT CCC CTT CAA CCA GCT G; miR-133b forward primer, TTT GGT CCC CTT CAA CCA GCT A) and universal reverse primer. U6 snRNA was used as internal control (human U6 snRNA control forward primer sequence, CGC AAG GAT GAC ACG CAA ATT C; mouse U6 snRNA control forward primer sequence, TGG CCC CTG CGC AAG GAT G). mRNA expression levels were quantitated through reverse transcription-qPCR as described previously (22).

Western Blotting Analysis—Proteins were extracted with radioimmuno precipitation assay lysis buffer on ice. Protein concentrations were determined using a BCA Protein Assay kit (Pierce). 20 μg of proteins was fractionated on 7.5 or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were then probed with a series of primary antibodies. Antibodies used for Western blotting were: β-actin antibody (Sigma, 1:10,000), FLAG tag antibody (Sigma, M2, 1:5000) for exogenous Brg1 and Brm; HA tag antibody (Covance, 1:3000) for exogenous MRTFA; and Omni antibody (Santa Cruz, M-21, sc-499, 1:3000) for myocardin. Primary antibodies were then detected using horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescence. Chemiluminescent signals were collected and analyzed on a G Box imager (Syngene).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays using cells were performed as described (17). Briefly, cells were fixed in 1% formaldehyde for 10 min at room temperature and harvested using cold PBS with protease inhibitors. After collecting cells by centrifugation, cell pellets were lysed using 1% SDS lysis buffer with protease inhibitors (200 μl of lysis buffer/10⁶ cells). For each group, 1 ml of lysate was sonicated at setting High with 30-s burst and 30-s silence for 9 cycles in a bioruptor (Diagenode) at 4 °C. A similar protocol was used for chromatin immunoprecipitation assays from smooth muscle tissue except that tissues were finely minced before fixation, and the sonication condition for tissues was 45 cycles of 30 s on and 30 s off for each cycle. 200-μl aliquots of chromatin were immunoprecipitated using 6 μg of specific antibodies or rabbit IgG as negative control. The precipitated genomic DNA was purified, and the presence of specific promoters was detected by real-time qPCR, using gene promoter-specific primers (supplemental Table 1). Primers for the telokin promoter were used as positive control.

RESULTS

Altered MicroRNA Expression in Smooth Muscle Tissues from smBrg1 Knock-out Mice—To determine which microRNAs are regulated by Brg1, we analyzed microRNA expression in smooth muscle tissues of control and smBrg1 knock-out mice using Affymetrix GeneChip microRNA arrays. This analysis identified several microRNAs with decreased expression levels in knock-out tissues, including miR-133a, 133b, and miR-206 (supplemental Table 2). Several microRNAs including miR-423–5p, 423–3p, 34a, 28, 212, and 674–5p exhibited increased expression in Brg1 knock-out smooth muscle (>2-fold at a false discovery rate of <0.2). We did not observe a large change in expression of miR-143/145 in the array analysis, although miR-143 was decreased approximately 1.4-fold. Quantitative RT-PCR analysis of mature microRNA expression levels was used to confirm the microarray results. This approach revealed that in colonic smooth muscle tissues of smBrg1 knock-out mice, there was a significantly lower level of expression of miR-143, 145, 133a, 133b, and 30a-3p compared with tissues from control mice, whereas miR-34a, 28, and 674–5p were significantly up-regulated (Fig. 1A). Similar decreases in miR-143, 145, 133a, and 133b were seen in bladder smooth muscle, whereas only miR-28 was increased in bladders of smBrg1 knock-out mice (Fig. 1A). Because miR-143, 145, 133a, and 133b were decreased in both colon and bladder, and these were the most abundant microRNAs analyzed in these tissues (Fig. 1B), we focused on determining how Brg1 regulates the expression of these four microRNAs.

Brg1 Regulates the Expression of miRs-143/145 in Cultured Smooth Muscle Cells—Altered microRNA expression observed in the tissues of smBrg1 KO mice could be a consequence of the pathological changes that occur in these mice, as opposed to direct regulation of microRNA expression by Brg1. To distin-
To distinguish between these possibilities, we introduced a dominant negative form of Brg1 (K798R) (DN-Brg1) directly into cultured primary smooth muscle cells by adenoviral transduction. Consistent with a previous report (17), the DN-Brg1 attenuated expression of smooth muscle markers such as smMHC, telokin, and calponin (Fig. 2A). The DN-Brg1 also attenuated expression of miRs-143/145, suggesting that miRs-143/145 may be directly regulated by Brg1 (Fig. 2B). Surprisingly, expression of miRs-133a and 133b were not significantly affected by DN-Brg1 (Fig. 2A). To further confirm the results, we cultured primary colon smooth muscle cells from Brg1flox/flox mice and transduced them with Cre-expressing adenovirus to delete Brg1 in vitro. Using this approach, knock-out of Brg1 significantly decreased expression of miRs-143/145 without affecting the expression of miRs-133a/b (Fig. 2B). These findings suggest that the attenuated expression of miRs-143/145 observed in vivo in smBrg1 knock-out mice is likely a direct consequence of loss of Brg1, whereas the decreased expression of miR-133 observed in vivo may be a secondary result of pathological changes rather than a direct affect of Brg1 on miR-133 expression. However, we noted that there is a much larger decrease in miR-133 relative to miRs-143/145 expression levels in the primary colon smooth muscle cells compared with intact tissue (Fig. 2C). The residual miR-133 expression observed in cells may thus be Brg1-independent with the Brg1-dependent regulatory mechanism being lost during the partial dedifferentiation of smooth muscle cells that occurs in primary culture.

Knockdown of Myocardin or SRF in Primary Smooth Muscle Cells Attenuates miRs-143/145 Expression—Because SRF and myocardin have been previously reported to regulate miR-143/5 expression (4) and there are also several conserved SRF binding sites or CArG boxes within the miR-133 loci (Fig. 3A), we examined the role of these proteins in regulating expression of endogenous miRs-143/145 and 133 in colonic smooth muscle cells. Knockdown of myocardin or SRF in primary colonic smooth muscle cells using adenovirus-encoded shRNA attenuated expression of smooth muscle-specific contractile proteins and miRs-143 and 145 (Fig. 3, B and C). In contrast, myocardin knockdown had no significant effect on miR-133a or miR-133b expression (Fig. 3C), whereas SRF knockdown decreased expression of miRs-133a and 133b (Fig. 3B).

DN-Brg1 Attenuates Myocardin-mediated Induction of miRs-143/145 and miR-133a—To circumvent problems resulting from lowered miR-133 expression in primary SMC cultures, we next utilized a cell system in which smooth muscle differentiation was induced. Overexpression of myocardin in 10T1/2 cells has been previously shown to increase expression of most smooth muscle-specific genes, and this increase can be blocked by DN-Brg1 (18, 26). Myocardin has also been shown to induce expression of miR-143 in 10T1/2 cells (9). We therefore used this system to examine the effects of DN-Brg1 on microRNA expression during smooth muscle differentiation. Similar to the smooth muscle-specific contractile proteins, expression of miRs-143, 145, and 133a was increased by myocardin, and this induction was attenuated by DN-Brg1 (Fig. 4A). In con-
Myocardin did not induce miR-133b expression (Fig. 4A).

Myocardin Increases SRF Binding to the miR-143/145 and miR-133a Genes—Brg1 has been shown to enhance myocardin or MRTFA-mediated activation of smooth muscle-specific genes via facilitating SRF binding to their promoter regions (17, 18). Because myocardin induced expression of miRs-143/145 and miR-133a in 10T1/2 cells (Fig. 4A), we examined the ability of myocardin to affect SRF binding to these microRNA genes during the myocardin-induced differentiation of 10T1/2 cells (Fig. 4B). Consistent with previous reports (8) we observed a myocardin-mediated increase in SRF binding to the CArG box within the miR-143/5 promoter (Fig. 4B). The increased SRF binding was also associated with increased binding of Brg1 (Fig. 4B). Myocardin also increased SRF binding to both CArG elements within the miR-133a-1 locus and the CArG1 site, but not the CArG2 site, within the miR-133a-2 locus (Fig. 4B). The increased SRF binding was associated with significantly increased Brg1 binding to the 133a-2 CArG1 site, and it was also associated with increased Brg1 binding to the 133a-1 CArG sites although these did not reach statistical significance.

Brg1 Together with Myocardin/MRTFA Synergistically Induces Expression of miRs-143/145, but Not miRs-133a/b—To further explore the role of Brg1 in regulating induction of microRNAs, we utilized SW13 cells, which lack endogenous Brg1 or Brm. In these cells, we found that the ability of myocardin or MRTFA to induce expression of miRs-143/145 is dependent on exogenously added Brg1 (Fig. 5). SW13 cells were transduced with adenoviruses encoding Brg1, myocardin, MRTFA, or YFP control. Western blotting confirmed the successful expression of exogenous Brg1, myocardin, or MRTFA in SW13 cells (data not shown). Brg1, myocardin, or MRTFA alone did not dramatically influence the expression of any of the four microRNAs analyzed, whereas Brg1 together with myocardin or MRTFA robustly induced the expression of miR-143 and miR-145 about 20-fold and 10-fold, respectively (Fig. 5). Although myocardin alone resulted in a small 2-fold induction of miR-133a, this was not further enhanced by Brg1 (Fig. 5).
Consistent with the data obtained from 10T1/2 cells (Fig. 4A), miR-133b expression was not significantly affected by myocardin either in the presence or absence of Brg1. Together, these data suggest that expression of miRs-143/145 is induced by myocardin or MRTFA in a Brg1-dependent manner, whereas miR-133a is only weakly activated by myocardin, and miR-133b is refractory to myocardin and MRTFA activation. Similar results were obtained when Brm was used in place of Brg1 (data not shown).

SRF Binding to the miRs-143/145 and miR-133a Loci Is Brg1-dependent—To examine the Brg1 dependence of myocardin-mediated changes in SRF binding we examined SRF binding to the miR genes in SW13 cells with and without Brg1 and myocardin (Fig. 6). As reported previously myocardin increased SRF binding to the telokin promoter in Brg1-dependent manner (data not shown). Myocardin similarly increased SRF binding to the miR-143/145 promoter in a Brg1-dependent manner (Fig. 6, left). However, Brg1 did not affect myocardin binding to the miR-143/145 promoter region, and myocardin did not affect Brg1 binding (Fig. 6, middle and right).

Although myocardin and Brg1 together were not sufficient to induce miR-133 expression in SW13 cells (Fig. 5), ChIP assays demonstrated that the combination of Brg1 and myocardin induced SRF binding to both CArG sites within the miR-133a-1 gene and to the CArG1 site within the miR-133a-2 gene, without affecting SRF binding to the miR-133a-2 CArG2 site (Fig. 6). These data together with those shown in Figs. 3 and 5 suggest that SRF binding to the miR-133a-1 and miR-133a-2 genes...
is necessary but not sufficient to activate miR-133a transcription. There are numerous other highly conserved potential transcription factor binding sites in the miR-133a loci, including E box elements and MEF2 binding sites (Fig. 3A). However, expression of either E12, E47, MEF2A, MEF2B, MEF2C, or MEF2D was also not sufficient to significantly induce expression of miR-133 either with or without Brg1 or myocardin in SW13 cells (data not shown).

To confirm the importance of Brg1 in regulating SRF binding to the miR-143/145 and miR-133 loci, we performed ChIP assays on colonic smooth muscle tissue isolated from smBrg1 knock-out mice. Consistent with the in vitro data, we observed significantly decreased SRF binding to the miR-143/145 promoter in smBrg1 knock-out mice compared with controls (Fig. 7). SRF binding to the CArG2 site in the miR-133a-1 gene and CArG1 site in the miR-133a-2 gene also decreased although these did not quite reach significance. In contrast, there was no detectable SRF binding to either the miR-133a-1 CArG1 site or the miR-133a-2 CArG2 site in colonic tissues (Fig. 7).

DISCUSSION

Our current results demonstrate that the SWI/SNF chromatin remodeling complex acts together with transcription factors and cofactors to regulate the expression of microRNAs in smooth muscle cells. The SWI/SNF complex is required for myocardin or MRTFA to induce expression of miRs-143/145 (Fig. 8). Although Brg1 is also required for miR-133 expression in vivo and SRF is involved in the regulation of miR-133 expres-
mation, myocardin is a weak activator of miR-133a and does not significantly activate miR-133b. These data suggest that Brg1 regulates miR-133 expression through SRF acting together with other, as yet unidentified, transcription factors (Fig. 8).

The Brg1- and myocardin/MRTFA-dependent regulation of miR-143/145 is very analogous to the previously reported mechanism by which these proteins regulate expression of many smooth muscle-specific contractile proteins (17, 18). These findings are also consistent with previous studies that have shown that expression of miRs-143/145 can be regulated by myocardin and SRF. Myocardin has been shown to be able to increase expression of miRs-143/145 in cardiomyocytes (4) and 10T1/2 cells (9), and a CArG box within the miR-143/145 promoter has been shown to be required for reporter gene expression in smooth muscle cells in vivo in mice (4, 8). The current studies extend these results to demonstrate that both myocardin and SRF are required for expression of miRs-143/145 in smooth muscle cells and that the SWI/SNF complex is required for myocardin-mediated induction of these microRNAs. ChIP data in 10T1/2 cells suggest that the myocardin-mediated increase in SRF binding to the miR-143/145 promoter is associated with increased binding of Brg1 (Fig. 4B). This would be consistent with previous studies that showed direct binding of Brg1 and myocardin (18). This suggests a mechanism by which myocardin recruits Brg1 to the miR-143/145 promoter to facilitate chromatin remodeling, increased SRF binding, and transcriptional activation (Fig. 8). However, in SW13 cells we observed no effect of myocardin on Brg1 binding to the miR-143/145 promoter and no effect of Brg1 on myocardin binding. The reason for these cell-specific differences is not clear; however, in either cell type Brg1 binding to the promoter is low (only approximately 2-fold greater than background), suggesting that this interaction is either very weak or transient and thus not readily detectable by ChIP assay. Overall these findings are consistent with a model in which the gene encoding miRs-143/145 is subjected to similar transcriptional and epigenetic regulation to genes encoding contractile proteins in smooth muscle cells.

The decreased expression of miRs-133a/b in vivo in smBrg1 knock-out mice is consistent with previous studies that showed that Brg1 plays a critical role in the regulation of miR-133 expression in skeletal muscle. In skeletal muscle cells, it has been proposed that MyoD recruits Brg1 to E box sequences within the miR-133a promoters (16). Because MyoD is restricted to skeletal muscle cells the regulation of miR-133 must be tissue-specific, and other transcription factors must recruit Brg1 to the miR-133 loci in smooth muscle cells. Analysis of the regulatory regions of all three miR-133 genomic loci revealed the presence of several highly conserved cis-acting regulatory elements, including E box elements and potential binding sites for SRF and MEF2 (Figs. 3A and 8). Knockdown of SRF in primary cultures of smooth muscle cells resulted in a

![Figure 5](https://example.com/figure5.png)
small decrease in expression of both miR-133a and miR-133b, suggesting that SRF may play a role in regulating expression of these genes in smooth muscle cells (Fig. 3B). Consistent with this observation we observed decreased binding of SRF to CArG elements within the miR-133a-1 gene in smBrg1 knock-out mouse colon smooth muscle (Fig. 7).
that Brg1-mediated regulation of miR-133a occurs, at least in part, through SRF (Fig. 8). Although myocardin induced SRF binding to several CArG elements within the miR-133a loci, unlike miRs-143/145, expression of miR-133 was not significantly affected by myocardin knockdown in colonic smooth muscle cells (Fig. 3C). In addition, myocardin was a very poor activator of miR-133a in SW13 cells and 10T1/2 cells, and miR-133b expression was completely refractory to myocardin stimulation in either cell type (Figs. 4A and 5). These data would suggest that miR-133 expression is SRF-dependent but myocardin-independent. However, the dramatic decrease in miR-133 expression seen in cultured smooth muscle cells compared with smooth muscle tissue (Fig. 2C) suggests that results obtained from these cells should perhaps be interpreted with caution. The importance of myocardin in regulating the expression of miR-133 thus remains to be determined in vivo. Nevertheless, the ability of myocardin to increase SRF binding to the miR-133a loci in a Brg1-dependent manner in SW13 cells, without a concomitant increase in endogenous miR-133a expression, suggests that SRF binding to the miR-133a loci is not sufficient to activate transcription. This observation together with the observed decrease in miR-133a/b expression seen following SRF knockdown suggests that SRF is necessary but not sufficient to regulate miR-133 transcription in smooth muscle cells (Fig. 8). E box-binding proteins and MEF2 have been reported to play important roles in regulating expression of smooth muscle differentiation genes (27–29); however, the role of these proteins in regulating microRNA expression in smooth muscle has not been determined. Because MEF2 has also been shown to bind to myocardin to autoregulate its own expression in cardiomyocytes (30) we evaluated the possibility that myocardin may cooperate with MEF2 proteins to induce miR-133 expression. However, in the SW13 cell system MEF2 proteins with or without myocardin were not able to induce miR-133 expression even in the presence of Brg1 (data not shown). Similarly, E12/47 proteins together with Brg1 were not able to induce miR-133 expression either. However, in these cells we were also not able to demonstrate a MyoD/Brg1-mediated induction of miR-133 even though MyoD has been shown to regulate miR-133 expression in skeletal muscle cells (data not shown and Ref. 16). These findings may suggest that SW13 cells are missing another critical factor that is required for miR-133 expression or that this locus is epigenetically silenced in these cells. Additional studies will be required to resolve these
microRNA observed to drive expression of miR-133 in smooth muscle cells. Although miR-133 was the most significantly decreased microRNA observed in vivo in smBrg1 knock-out mice, in vitro experiments more clearly demonstrate the direct Brg1-dependence of expression of miRs-143/145. Knockdown of Brg1 or expression of DN-Brg1 attenuated expression of miRs-143/145 without affecting miR-133 expression in cultured colonic smooth muscle cells (Fig. 2). In 10T1/2 cells the myocardin-mediated induction of miRs-143/145 was dependent on Brg1 whereas the small myocardin-mediated induction of miR-133a was not. Yet in these same cells there appears to be a significant Brg1-dependent, myocardin-mediated increase in SRF binding to at least some of the CArG elements in the miR-133a loci (Fig. 6). Together, these data clearly show that miRs-143/145 are regulated by a myocardin-, Brg1-, and SRF-dependent mechanism (Fig. 8). The Brg1-dependent regulation of miR-133 remains more ambiguous, such that we cannot rule out the possibility that in smooth muscle cells Brg1 either indirectly regulates miR-133 expression or that Brg1 is acting through an as yet unidentified transcription factor.

In addition to the described decreased expression of miRs-143/145 and miR-133 in smBrg1 knock-out mice, we also observed significantly increased levels of several microRNAs. This observation suggests that either these microRNAs are transcriptionally repressed by Brg1 or they are increased as a result of pathological changes that occur in the knock-out mice. Because DN-Brg1 did not increase expression of these microRNAs in smooth muscle cells in vitro (data not shown), it is probable that the changes observed in vivo are not a direct result of Brg1-mediated repression.

SWI/SNF complexes contain one of two alternative ATPases, Brg1 or Brm. Both of these proteins are ubiquitously expressed in almost all tissues and have both redundant and distinct functions in regulating gene expression. In vitro, both Brg1 and Brm can facilitate myocardin- or MRTFA-mediated induction of smooth muscle-specific genes (17, 18) and miRs-143/145 (Fig. 5 and data not shown). However, the knock-out of Brg1 alone in smooth muscle cells, in vivo, resulted in decreased expression of smooth muscle-specific contractile proteins and microRNAs in the gastrointestinal tract (22) (Fig. 1). Thus, in vivo, Brg1 must have specific functions in smooth muscle cells that cannot be performed by Brm. Why there are specific requirements for Brg1 in vivo but not in vitro remains a mystery but may reflect expression levels of the proteins in vivo. In vitro, in overexpression experiments it is possible that Brm may be able to access complexes that Brm cannot access at normal expression levels in vivo. Specific functions of Brg1 have also been revealed in other cell types. For example, Brg1 is specifically required for T cell development (31). Moreover, global Brg1-null mice die early in embryonic development due to growth arrest of the inner cell mass and trophoblast (32), whereas global Brm-null mice are viable and slightly larger than normal (33). Knock-out of Brg1 in smooth muscle cells in mice results in a myriad of defects including altered vascular remodeling leading to persistent ductus arteriosus and altered gastro-intestinal contractility leading to intestinal blockade (22). Results of the current study suggest that these pathological changes likely result from altered microRNA expression in addition to altered expression of protein-coding genes. In support of this proposal, knock-out of miRs-143/145 results in numerous defects in smooth muscle cells including reduction in blood pressure, presumably due to decreased contractility of vascular smooth muscle cells, thinner smooth muscle layers, incomplete differentiation of SMCs, and altered neointima formation in response to vascular injury (4–6). The decreased expression of miRs-143/145 could thus be contributing to the decreased expression of contractile proteins and decreased smooth muscle contractility observed in the smBrg1 knock-out mice (22). Similarly miR-133 has been shown to inhibit vascular SMC proliferation and promote differentiation in vitro and after balloon injury in vivo, at least partially through regulating the expression of Sp1 and moesin (10). Decreased miR-133 expression in smBrg1 knock-out mice could thus also be contributing to the decreased expression of contractile proteins seen in these mice.

In conclusion, we demonstrated for the first time that the SWI/SNF chromatin-remodeling complex plays an important role in regulating microRNA expression in smooth muscle cells, in vitro and in vivo. Expression of miRs-143/145 is regulated by SRF/myocardin complexes, in a SWI/SNF-dependent manner. In contrast, expression of miR-133 genes appears to be SRF-dependent, although largely myocardin-independent. In smooth muscle cells, microRNAs and protein-coding genes are thus regulated by similar transcriptional and epigenetic mechanisms to coordinate the phenotype of the cells.

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