Induction of MicroRNA-1 by Myocardin in Smooth Muscle Cells Inhibits Cell Proliferation

Jie Chen, Hao Yin, Yulan Jiang, Sarvan Kumar Radhakrishnan, Zhan-Peng Huang, Jingjing Li, Zhan Shi, Elisabeth Petronella Catharina, Yu Gui, Da-Zhi Wang, Xi-Long Zheng

Objective—Myocardin is a cardiac- and smooth muscle–specific transcription factor that potently activates the expression of downstream target genes. Previously, we demonstrated that overexpression of myocardin inhibited the proliferation of smooth muscle cells (SMCs). Recently, myocardin was reported to induce the expression of microRNA-1 (miR-1) in cardiomyocytes. In this study, we investigated whether myocardin induces miR-1 expression to mediate its inhibitory effects on SMC proliferation.

Methods and Results—Using T-REx inducible system expressing myocardin in human vascular SMCs, we found that overexpression of myocardin resulted in significant induction of miR-1 expression and inhibition of SMC proliferation, which was reversed by miR-1 inhibitors. Consistently, introduction of miR-1 into SMCs dramatically inhibited their proliferation. We isolated spindle-shaped and epithelioid human SMCs and demonstrated that spindle-shaped SMCs were more differentiated and less proliferative. Correspondingly, spindle-shaped SMCs had significantly higher expression levels of both myocardin and miR-1 than epithelioid SMCs. We identified Pim-1, a serine/threonine kinase, as a target gene for miR-1 in SMCs. Western blot and luciferase reporter assays further confirmed that miR-1 targets Pim-1 directly. Furthermore, neointimal lesions of mouse carotid arteries display downregulation of myocardin and miR-1 with upregulation of Pim-1.

Conclusion—Our data demonstrate that miR-1 participates in myocardin-dependent SMC proliferation inhibition. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: Pim-1 • microRNA-1 • myocardin • proliferation • vascular smooth muscle cells

During the early embryonic stages of vasculogenesis, smooth muscle cells (SMCs) are highly proliferative and migratory. However, in adult blood vessels, SMCs become quiescent and express a repertoire of contractile proteins necessary for the contractile function of fully differentiated and migratory SMCs. Interestingly, SMCs, unlike cardiac and skeletal myocytes, are not terminally differentiated, and they are capable of regaining their highly proliferative and migratory characteristics under certain conditions, such as vascular injury. Expression of a majority of SMC marker genes is known to be dependent on CArG boxes in their promoters/enhancers. Serum response factor (SRF) is essential for the regulation of muscle specific genes through its interaction with the muscle-enriched SRF cofactor myocardin. Intriguingly, many SRF cofactors are antagonistic in action and are therefore involved in regulating phenotypic switching of SMCs between proliferation and differentiation. In addition, heterogeneity of vascular SMCs has been well established in many species, with potential pathophysiological significance in the vascular system. Spindle-shaped human vascular SMCs are more differentiated and less proliferative than epithelioid SMCs. We have recently reported that myocardin is able to repress SMC proliferation by functionally antagonizing the nuclear factor (NF)-κB signaling pathway. SMC proliferation is a complicated process regulated by numerous signaling mediators. For instance, Pim-1, an oncogenic serine/threonine kinase, is reported to promote proliferation of cultured SMCs and neointimal hyperplasia. However, it remains unknown whether Pim-1 or other signaling molecules could be targeted in myocardin inhibition of SMC proliferation.

Recently, the involvement of microRNAs (miRNAs) in phenotypic modulation of SMCs has been reported. microRNA-21 (miR-21), miR-24, miR-25, and miR-221/222 are reported to modulate SMC proliferation. Importantly, myocardin has been reported to induce the expression of the smooth muscle–enriched miRNA gene miR-143/145. miR-143/145 promotes the contractile phenotype of vascular SMCs through regulating myocardin-dependent expression of SMC differentiation markers and cytoskeletal dynamics. However, whether myocardin induces miR-
NAs to mediate its antiproliferative effects in SMC remains largely unknown.

MicroRNA-1 (miR-1) is a critical mediator of cell proliferation and differentiation in cardiacc19,20 and skeletal21 muscles. In particular, it was reported that miR-1 was highly enriched in cardiac and skeletal muscle cells as compared to nonstriated muscle tissues during animal development and in adults.19–21 miR-1 promotes cardiac and skeletal muscle gene expression and muscle differentiation, in part, by repressing the key transcriptional regulators HDAC421 or hand2.22 Conversely, it has been shown that tissue-specific expression of miR-1 is dependent on SRF in the heart22 and MyoD family of transcription factors in skeletal muscle.21,23 Given that SRF was necessary for miR-1 expression in myocardium22 and that myocardin is also highly expressed in SMCs,3 we hypothesize that myocardin induces the expression of miR-1 in vascular SMCs, thereby contributing to myocardin regulation of SMC proliferation and differentiation. In the current study, we report that myocardin induces miR-1 expression in SMCs, and induced miR-1 expression consequently inhibits SMC proliferation. We identify Pim-1 as one of the target genes of miR-1 in SMCs and show decreases in myocardin and miR-1 and an increase in Pim-1 in neointimal lesions of mouse carotid arteries. Our studies therefore provide a novel molecular mechanism by which myocardin and miRNAs are involved in the control of SMC proliferation and differentiation.

Materials and Methods

Reagents
RPMI 1640 medium, bromodeoxyuridine (BrdU), propidium iodide, and antibodies for Flag tag and β-actin were obtained from Sigma-Aldrich Canada Ltd (Oakville, Canada). Fetal bovine serum, 1% penicillin and streptomycin, Alamar Blue reagent, and PureLink miRNA Isolation Kit were from Invitrogen Canada Inc (Burlington, Canada). The RNasey Plus Mini Kit, primer sets of myocardin, and QuantiTect SYBR Green PCR Kit were purchased from Qiagen Canada. The RNeasy Plus Mini Kit, primer sets of myocardin, and QuantiTec SYBR Green PCR Kit were purchased from Qiagen (Mississauga, Canada). The TaqMan MicroRNA Reverse Transcription Kit, primers of miR-1 and U6B, and Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, Calif). miR-1 mimic, miRNA negative control, miR-1 inhibitor, and miRNA hairpin inhibitor negative control were purchased from ThermoFisher (Lafayette, Colo). Antibodies for myocardin (M-16) and blocking peptide, Pim-1 (12H8), HDAC4 (A-4), Ras homolog enriched in brain (Rheb) (C-19) and RIPA buffer were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). GAPDH antibody was from Cell Signaling Technology, Inc (Danvers, Mass).

Cell Culture, Cloning of Spindle-Shaped and Epitheliod Phenotypes, and Transient Transfection
Human aortic SMCs (CRL-1999) were purchased from American Type Culture Collection. Human aortic SMCs harboring the tetracycline-regulated T-REx system (Invitrogen) to express myocardin were established and cultured as previously described.24 Two SMK phenotypes of spindle-shaped and epitheloid SMCs were cloned using a limiting dilution cloning method as previously described.24,25 To transfect miRNAs into human aortic SMCs, cells were plated in 96-well plates (4000 cells per well) overnight in the presence of 10% serum, followed by transfection with miRNAs using Lipofectamine 2000 (Invitrogen) for 5 hours as instructed by the manufacturer. The miRNA transfection efficiency in all cells was above 95%, as monitored by paralleled transfections with Cy3-conjugated negative control short interfering RNA (siRNA) (Invitrogen). Then, cells were cultured in fresh medium for further assays.

Western Blot Analysis
Western blot analysis was performed as described previously.26 Briefly, protein was extracted from cells with RIPA buffer, and equal amounts of protein from each sample (20 µg) were separated by 9% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies. The chemiluminescence signals were detected with the ECL Detection Kit (GE Healthcare, Piscataway, NJ). The densitometric analysis was conducted with ImageMaster (Pharmacia Biotech, Uppsala, Sweden). To detect endogenous myocardin in cultured SMCs, the membranes were preblocked with PBS-T (PBS with 0.15% [v/v] Tween-20) containing 5% skim milk overnight at 4°C, followed by incubation at room temperature for 1 hour with myocardin (M-16, sc-21561) antibody (1:200 diluted in PBS-T). After being washed, the membranes were subsequently incubated at room temperature for 1 hour with horseradish peroxidase–coupled donkey anti-goat secondary antibody (sc-2020, Santa Cruz Biotechnology, 1:4000 diluted in PBS-T containing 0.5% skim milk). The chemiluminescence signals were detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Northern Blot Analysis
Northern blot analysis was performed as described previously.21 Briefly, total RNA was extracted from cells with TRIzol (Invitrogen). Forty micrograms of each RNA sample (5 µg in skeletal muscle control sample) was processed with polyethylene glycol to remove large RNAs and to facilitate the detection of miRNAs. The miR-1 probe sequence was 5′-TACATACCTTTCAATTCCCA-3′. Supplemental Figure II (available online at http://atvb.ahajournals.org) shows the results of overnight exposure, and Figure 1C shows the results of exposure for 5 days.

Cell Proliferation Assay
Cells were plated in 96-well plates (4000 cells per well) overnight and then transfected with miRNAs as described above. After 48 hours, Alamar Blue (10% v/v) was added for an additional 3 hours before fluorescence was measured in triplicate for each sample with a fluorescence plate reader, with excitation and emission at 560 and 590 nm, respectively.

Analysis of miRNA Expression
miRNAs from human vascular SMCs or mouse arteries were isolated with the PureLink miRNA Isolation Kit (Invitrogen). The expression levels of mature miR-1, miR-143, miR-145, and U6B were measured using the Applied Biosystems TaqMan MicroRNA Assay system. Real-time polymerase chain reaction (PCR) was performed in triplicate from each sample using universal PCR Master Mix in the iCycle iQ real-time PCR detection system (Bio-Rad, Mississauga, Ontario, Canada). iQ version 3.1 software (Bio-Rad) was used for data analyses. U6B expression was used to normalize the expression of miR-1, miR-143, and miR-145. The 2A-L method was used to analyze real-time PCR data.

Analysis of mRNA Expression
Total RNA was isolated from human vascular SMCs or mouse arteries using the RNeasy Plus Mini kit (Qiagen). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The mRNA levels of myocardin, Pim-1, SM22, and GAPDH were evaluated by real-time PCR as previously described (Chen et al, 2008). Briefly, real-time PCR was performed in triplicate for each sample using Quantitect SYBR Green PCR Kit (Qiagen) in the iCycle iQ real-time PCR detection system (Bio-Rad). The GAPDH primer set was obtained from PrimerDesign Ltd (Southampton, UK), and myocardin, Pim-1, and SM22 primer sets were obtained from Qiagen. The cycling conditions were as follows: 15-minute initial activation step at 95°C, then 94°C for 15 seconds, annealing at 55°C for 30 seconds and 72°C for 30 seconds, repeated for 40 cycles. Finally, the melting curve analysis was performed. Each assay included a no-template negative control and a no-reverse-transcription negative control.
Luciferase Assay

The luciferase reporter plasmid containing human Pim-1 3’ untranslated region (UTR) sequence was a generous gift of Dr Kalpana Ghoshal (Ohio State University, Columbus, Ohio).27 The mutation of the putative miR-1 seed sequence in the Pim-1 3’ UTR was conducted using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The reporter plasmids, together with miR-1 or miRNA control, were transfected into cells using Lipofectamine 2000 (Invitrogen), and luciferase activity was measured with the Dual-Luciferase Reporter Assay kit (Promega).

BrdU Incorporation Assay

The BrdU incorporation assay was performed and analyzed as described previously.24 Briefly, cells grown on glass coverslips were labeled with 10 μmol/L BrdU for 60 minutes. After being fixed in 80% and 100% ethanol, cells were permeabilized in 0.25% Triton X-100 in PBS (pH 7.4) for 20 minutes, incubated with 4 N hydrochloric acid for 20 minutes, neutralized with sodium borate (pH 8.5) for 2 to 3 minutes, preblocked with 1% bovine serum albumin and 0.1% Tween-20 in PBS for 30 minutes, and then incubated with anti-BrdU antibody (BD Biosciences) and Alexa Fluor 488-conjugated secondary antibody (Invitrogen). The nuclei were counterstained with propidium iodide in the presence of RNase A. Cells were analyzed for their BrdU uptake with a laser scanning cytometer, and the BrdU incorporation rate was expressed as the percentage of BrdU-positive cells in total scanned cells.

Neointimal Lesion Formation Through Ligation of Mouse Carotid Arteries

C57BL/6 male mice (6 to 8 weeks, 19 to 21 g) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China. Seven and 5 mice were used for extraction of miRNA and mRNA, respectively. The left common carotid artery was dissected and ligated near the carotid bifurcation as previously described.28 Fifteen days after ligation, both left and right carotid arteries (to serve as negative controls) were sampled and then examined for vascular wall morphology using hematoxylin-eosin staining or stored in 0.5 mL of RNAlater, followed by extraction of miRNA (7 mice) or mRNA (5 mice) as described above. All animal studies were approved by and performed according to the Guidelines for the Care and Use of Laboratory Animals of Nankai University (A5521-01), which strictly conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Data Analysis

Data are presented as the mean±SEM. Statistically significant differences were evaluated by the Student t test when 2 groups were compared and by ANOVA when 3 or more groups were compared. The Mann-Whitney U nonparametric analysis was used for data that did not show normality and homogeneity of variance. *P<0.05 was considered significant.

Results

Myocardin Induces miR-1 Expression in Human Vascular SMCs

Myocardin is a potent transcriptional coactivator for smooth muscle–specific gene expression,3,5 and it also strongly represses the proliferation of SMCs when overexpressed.7,29 We have previously generated a stable cell line through T-REX tetracycline-regulated system to overexpress Flag-tagged myocardin in human aortic SMCs.7 Addition of 1 μg/mL doxycycline (Dox) into culture medium induced the expression of myocardin as detected by Western blot analysis using antibodies against either the Flag tag or myocardin (Figure 1A), consistent with our prior report.7 The specificity of myocardin antibody (M-16, Santa Cruz Biotechnology) was confirmed using blocking peptide and myocardin siRNA. Preincubation with the blocking peptide made the band at the same position as detected by Flag tag antibody disappear (Supplemental Figure IA). Furthermore, we used myocardin siRNA to ensure the specificity of myocardin (M-16) antibody to detect endogenous myocardin protein. With protein lysates from cultured human aortic SMCs, myocardin (M-16) antibody specifically detected a band at the identical position as the overexpressed Flag-tagged myocardin protein (Supplemental Figure IB). Of note, the blot signal was reduced on transfection with myocardin siRNA as compared with transfect-
tion with a control siRNA, confirming the specificity of the myocardin (M-16) antibody for endogenous myocardin in SMCs (Supplemental Figure IB). The specificity of the M-16 antibody was further demonstrated by myocardin peptides, in which addition of the myocardin peptide used to generate myocardin M-16 antibody completely blocked the signal of overexpressed myocardin in Western blot (Supplemental Figure IA).

We then examined the myocardin induction of miR-1 using reverse-transcription real-time PCR. Our results showed that the mature miR-1 level increased $\approx 30$- and $\approx 250$-fold in response to Dox treatment for 1 and 3 days, respectively, in myocardin-inducible SMCs but not in control cells with empty vectors (Figure 1B and data not shown). Of note, after 3 days of Dox induction, the miR-1 level in cultured SMCs became comparable to that in intact carotid artery (Figure 1B). Subsequently, we used Northern blot assays to confirm the expression of mature miR-1 in response to myocardin induction. As anticipated, the mature miR-1 was detected in myocardin-inducible SMCs after 3 days of treatment with Dox, but not in the control cells with empty vector (Figure 1C and Supplemental Figure II).

Intriguingly, the induction of miR-1 on myocardin overexpression was even greater than that of many established myocardin target genes. As shown in Figure 1D to 1F, myocardin overexpression resulted in a $\approx 5$-fold increase in mRNA expression of SM22 (Figure 1D), $\approx 3$-fold increase in miR-143 and miR-145 levels (Figure 1F). A decrease in the mRNA level of KLF4, a negative regulator of SMC contractile phenotype, was also observed (Figure 1E). Therefore, our data demonstrate that myocardin is capable of inducing miR-1 expression in human vascular SMCs.

**Expression of Myocardin and miR-1 in 2 Phenotypes of Human SMCs**

Myocardin is an essential inducer of contractile phenotype of SMCs. To investigate the physiological significance of miR-1 in myocardin-dependent contractile phenotype, we sought to examine the potential difference of myocardin and miR-1 expression in 2 phenotypes of human SMCs. Using the limiting dilution method as previously described, we cloned 2 sub-populations from primary cultured human aortic SMCs: spindle-shaped (Figure 2A) and epithelioid (Figure 2B) SMCs. To characterize the phenotypic difference of SMCs, we showed that epithelioid SMCs are more proliferative than spindle-shaped SMCs, as indicated by BrdU incorporation rates in the presence of 10% serum (Figure 2C), and that spindle-shaped SMCs are more differentiated than epithelioid SMCs, as indicated by higher expression levels of a smooth muscle contractile marker gene, such as SM22 (Figure 2D). In addition, epithelioid SMCs exhibited a nonsignificant higher mRNA level of KLF4 than spindle-shaped SMCs (Figure 2E). Therefore, the characteristics of 2 phenotypes of human aortic SMCs are consistent with previous demonstrations of these 2 typical phenotypes of human SMCs.

We examined the expression of myocardin and miR-1 in spindle-shaped and epithelioid SMCs using Western blot analysis and real-time PCR, respectively. As shown in Figure 2G, myocardin is expressed at significantly lower level in epithelioid SMCs than in spindle-shaped cells. This observation is consistent with the role of myocardin in promoting...
Importantly, our results revealed that expression levels of mature miR-1 were significantly lower in epithelioid SMCs than spindle-shaped cells (Figure 2H). Similarly, we found that the expression of miR-143 and miR-145 (Figure 2F) was lower in epithelioid SMCs. Taken together, these data suggest that endogenous myocardin also regulates the expression of miR-1. Our results also suggest that myocardin-dependent miR-1 expression may contribute to lower activity of proliferation and the phenotypic heterogeneity of human vascular SMCs.

miR-1 Mediates Myocardin-Dependent Inhibition of SMC Proliferation

Given that miR-1 could enhance cardiac and skeletal muscle differentiation and inhibit muscle cell proliferation, as well as its induction by myocardin in differentiated SMCs,14,20 we hypothesized that miR-1 may contribute to myocardin-mediated inhibitory effects of SMC proliferation. To test this hypothesis, we first examined the effects of miR-1 inhibitor on myocardin-dependent inhibition of SMC proliferation. Human vascular SMCs harboring the T-REx inducible system for overexpression of myocardin were transfected with miR-1 inhibitor (1 nmol/L) or miRNA hairpin inhibitor negative control for 5 hours, followed by induction with Dox for 48 hours. Cell proliferation was measured by the Alamar Blue assay. The y-axis shows the normalized fluorescein value. *Significant difference compared with the control cells with transfection of inhibitor negative control and without Dox induction (P<0.01, n=5). **Significant difference compared with cells with transfection of inhibitor negative control and with Dox induction (P<0.01, n=5).

Next, we investigated whether miR-1 is sufficient to inhibit SMC proliferation. It is well known that phenotypically different SMCs have different proliferative responses to various stimuli, in which the epithelioid SMCs are more proliferative than spindle-shaped SMCs.2,6 We examined the effects of miR-1 on the proliferation of these 2 phenotypes of human SMCs. The cells were transfected with either miR-1 mimic or negative control miRNA (50 nmol/L) for 48 hours, followed by determination of cell proliferation with Alamar Blue assays and BrdU incorporation assays. Indeed, transfection of miR-1 led to a modest but statistically significant reduction in cell proliferation of both spindle-shaped (Figure 4A) and epithelioid (Figure 4B) SMCs. In addition, a greater inhibitory effect of miR-1 mimic was observed for proliferation of epithelioid SMCs (≈15% reduction) than that of spindle-shaped cells (≈5% reduction). Furthermore, BrdU incorporation analyses confirmed the decrease in cell proliferation of both phenotypes on miR-1 transfection (Figure 4C and 4D). Together, our data demonstrate that miR-1 is sufficient to inhibit cell proliferation in SMCs.

Pim-1 Is a Target Gene of miR-1 in Human Vascular SMCs

To further reveal the molecular mechanism underlying miR-1-mediated inhibition of SMC proliferation, we attempted to identify miR-1 target genes in human SMCs. Several miR-1
target genes have been reported to mediate miR-1-dependent effects on cell proliferation or differentiation,\textsuperscript{21,22} which include Pim-1, an oncogenic serine/threonine kinase\textsuperscript{27}; HDAC4, a histone deacetylase mediating cell proliferation-differentiation in skeletal muscle\textsuperscript{24}; hand2, a member of bHLH transcription factor involving cardiomyocyte development\textsuperscript{22}; and Rheb, a GTPase essential for cell growth regulation.\textsuperscript{32} We evaluated the effects of miR-1 on the expression levels of Pim-1, HDAC4, hand2, and Rheb in human SMCs. Western blot analysis showed that transfection of miR-1 significantly decreased the expression level of endogenous Pim-1 protein (Figure 5A and 5B). In addition, there was no significant decrease in the mRNA level of Pim-1 on miR-1 transfection, although a tendency for the decreased expression of Pim-1 was observed in spindle-shaped SMCs (Figure 5C). In contrast, the protein expression levels of HDAC4, hand2, and Rheb were not significantly changed by miR-1 in either spindle-shaped or epithelioid SMCs (Figure 5D and data not shown).

To further confirm that Pim-1 was a direct target gene of miR-1 in SMCs, we used the luciferase reporter assay using the Pim-1 3' UTR luciferase reporter gene. Our results showed that overexpression of miR-1 significantly inhibited the activity of the Pim-1 3' UTR luciferase reporter gene (Figure 5E). More specifically, mutations introduced into the miR-1 seed sequences resulted in loss of inhibition by miR-1 (Figure 5E). Together, our results demonstrated that Pim-1 is a direct regulatory target of miR-1 in SMCs. Given that Pim-1 is known to stimulate the proliferation of SMCs,\textsuperscript{8} our results provide an explanation for miR-1-mediated SMC proliferation repression.

**Downregulation of miR-1 Expression in Neointima Formation of Mouse Carotid Arteries**

To further study the expression of myocardin and miR-1 in SMCs in vivo, we performed carotid artery ligation to induce neointimal lesions in mice, a well-established experimental procedure to study in vivo SMC proliferation.\textsuperscript{28} Two weeks after the ligation, carotid arteries were harvested for tissue cross-sectioning and extraction of mRNA and miRNA. Consistent with previous reports, significant neointima formation was detected in ligated carotid arteries through hematoxylin-eosin staining (Figure 6A, uninjured, and 6B, injured). Real-time PCR results showed that miR-1 expression was significantly downregulated in neointimal lesions compared with uninjured arteries (Figure 6C), accompanied by significant downregulation of myocardin (Figure 6D). We also observed a modest but statistically significant increase of Pim-1 mRNA in neointima lesion (Figure 6E). Taken together, our studies suggested that the regulatory cascade of myocardin-miR-1–Pim-1 is likely involved in the regulation of vascular SMC proliferation, both in vitro and in vivo.

**Discussion**

In current study, we have revealed that the expression of miR-1 is induced by myocardin in human vascular SMCs and that miR-1 mediates the inhibitory effects of myocardin on SMC proliferation. This study has provided a novel mechanism underlying myocardin-induced inhibition of SMC proliferation. Our results are consistent with the role of myocardin and miR-1 in growth and proliferation of cardiomyocytes and skeletal myoblasts as previously reported.\textsuperscript{19–22}

The expression and functions of miR-1 were not previously reported in vascular SMCs. Earlier studies using Northern blot revealed the enriched expression of miR-1 in cardiac and skeletal muscle.\textsuperscript{21,22} Moreover, recent reports with the more
sensitive real-time PCR assay have documented the presence of miR-1 in nonmuscle tissues, including mouse cerebral cortex and lung. Most importantly, we revealed for the first time that expression of miR-1 in nonmuscle tissues, including mouse cerebral cortex and lung.33

One of the major functions of myocardin in SMCs is to induce differentiation and the contractile phenotype,3,5,31 associated with an increase in the expression of contractile marker genes. Conversely, myocardin was also shown to inhibit SMC proliferation.7 It is notable that miR-1 was induced by myocardin to an even greater (≈30-fold) degree than well-established myocardin targets, including SM22 (≈10-fold) and miR-143/145 (≈3-fold), suggesting that the expression of miR-1 may contribute to the cellular effects of myocardin in SMCs. The role of miR-1 in myocardin-induced SMC contraction is currently investigated in our laboratory (Y. Jiang and X.-L. Zheng, unpublished observations). In the present study, we have revealed that miR-1 mediates myocardin-dependent inhibition of SMC proliferation. Our conclusion is based on 3 lines of evidences: (1) miR-1 inhibitor partially reverses the inhibitory effects of myocardin on SMC proliferation, (2) exogenous miR-1 inhibits SMC proliferation, and (3) miR-1 directly downregulates the expression of target gene Pim-1, which promotes SMC proliferation.

Our data that miR-1 inhibits SMC proliferation are in line with its role in cardiomyocytes, skeletal myoblasts, and lung cancer cells.21,22,27 More importantly, the roles of miR-1 in myocardin-dependent cellular effects have important physiological relevance to vascular smooth muscle functions, given that myocardin is a master regulator of the contractile phenotype of SMCs.5,31 The phenotypic plasticity is an integral feature of mature SMCs, and distinctive phenotypes of SMCs exist in the normal blood vessel wall.1,2,6 Our data suggest that differential expression of myocardin and miR-1 is suppressed during neointima formation, deregulation of myocardin-miR-1 signaling could facilitate SMC proliferation in some pathophysiological contexts, such as restenosis after angioplasty.

We previously reported that myocardin physically interacts with the NF-κB p65 subunit so as to inhibit NF-κB-dependent gene transcription and cell proliferation.7 Our current study, in which we show that myocardin also reduces proliferation through myocardin-induced miRNA, has provided a complementary mechanism for myocardin inhibition of SMC proliferation. The inhibitory effect of miR-1 on SMC proliferation is produced, at least in part, through the down-regulation of the expression of Pim-1 gene encoding an onco-genic serine/threonine kinase, which is required for injury-induced neointima formation and SMC proliferation.8 Intriguingly, the interdependence between Pim-1 expression and NF-κB signaling has been demonstrated in the context of chemoresistance of prostate cancer34 and CD40-dependent proliferation of B cells.35 It will be interesting to investigate the interplay between the miR-1–Pim-1 cascade and NF-κB signaling in SMC proliferation under the control of myocardin.
In summary, we have demonstrated myocardin induction of miR-1 inhibits SMC proliferation, probably through down-regulation of Pim-1. It will be important to determine whether the myocardin–miR-1–Pim-1 pathway could serve as a therapeutic target in proliferative vascular diseases.

Sources of Funding
This work was supported by an operating grant from the Canadian Institute of Health Research (R7734076 to X.-L.Z.) and by National Institutes of Health Grant R01-HL085635 (to D.-Z.W.). Funding to pay the Open Access publication charges for this article was provided by the Canadian Institute of Health Research. Dr Zheng is the recipient of Senior Scholarship Award from Alberta Heritage Foundation for Medical Research. Dr Huang is a Postdoctoral Fellow and Dr Wang is an Established Investigator of the American Heart Association.

Disclosures
None.

References
Induction of MicroRNA-1 by Myocardin in Smooth Muscle Cells Inhibits Cell Proliferation

Jie Chen, Hao Yin, Yulan Jiang, Sarvan Kumar Radhakrishnan, Zhan-Peng Huang, Jingjing Li, Zhan Shi, Elisabeth Petronella Catharina, Yu Gui, Da-Zhi Wang and Xi-Long Zheng

Arterioscler Thromb Vasc Biol. published online November 4, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2010/11/04/ATVBAHA.110.218149.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/11/04/ATVBAHA.110.218149.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**Supplemental Fig I** Myocardin antibody (M-16, Santa Cruz) recognizes the overexpressed and endogenous myocardin proteins in SMCs

*A*: Protein lysates (50 µg) from adult rat skeletal muscle (SkM, serving as negative control) and Dox-treated myocardin T-REx SMCs were subjected to western blotting analysis with myocardin M-16 antibody with or without pre-incubation of blocking peptide (sc-21561 p, Santa Cruz). Note that the presence of blocking peptide completely blocked the blot signals of M-16 antibody. *B*: Human aortic SMCs were transfected with 50 nM ON-TARGETplus SMARTpool myocardin siRNA (siMyocd, lane 3) or non-targeting siRNA (siControl, lane 2) (Dharmacon, Lafayette, CO) with TransIT-TKO (Mirus Bio, Madison, WI). 48 h after transfection, proteins were extracted. 50 µg proteins were subjected to western blotting analysis for endogenous myocardin (lane 2 & 3) with myocardin (M-16) antibody. To ensure the position of endogenous myocardin, 8 µg protein lysate from Dox-treated myocardin T-REx SMCs was run in parallel (lane 1, overexpressed). β-actin was detected as a loading control.

**Supplemental Fig II** Northern blots for miR-1 induction upon myocardin overexpression in SMCs

Total RNAs from adult rat skeletal muscle (SkM, serving as positive control, 5 µg) and empty vector (EV) or myocardin T-REx SMCs treated with or without Dox for 3 d SMCs (40 µg) were subjected northern blotting analysis. Mature and precursor of miR-1 was indicated. U6 serves as a loading control.