Myocardin Regulates Vascular Response to Injury Through miR-24/-29a and Platelet-Derived Growth Factor Receptor β

Amarnath Talasila, Haixiang Yu, Matt Ackers-Johnson, Martine Bot, Theo van Berkel, Martin Bennett, Ilze Bot, Sanjay Sinha

Objective—Myocardin, a potent transcriptional coactivator of serum response factor, is involved in vascular development and promotes a contractile smooth muscle phenotype. Myocardin levels are reduced during vascular injury, in association with phenotypic switching of smooth muscle cells (SMCs). However, the direct role of myocardin in vascular disease is unclear.

Approach and Results—We show that re-expression of myocardin prevents the vascular injury response in murine carotid arteries, with reduced neointima formation due to decreased SMC migration and proliferation. Myocardin reduced SMC migration by downregulating platelet-derived growth factor receptor β expression. Expression of platelet-derived growth factor receptor β was regulated by myocardin-induced miR-24 and miR-29a expression, and antagonizing these microRNAs restored SMC migration. Furthermore, using miR-24 and miR-29a mimics, we demonstrated that miR-29a directly regulates Pdgfrb expression at the 3′ untranslated region while miR-24 has an indirect effect on Pdgfrb levels. Myocardin heterozygous-null mice showed an augmented neointima formation with increased SMC migration and proliferation, demonstrating that endogenous levels of myocardin are a critical regulator of vessel injury responses.

Conclusions—Our results extend the function of myocardin from a developmental role to a pivotal regulator of SMC phenotype in response to injury, and this transcriptional coactivator may be an attractive target for novel therapeutic strategies in vascular disease. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: carotid arteries ▪ microRNAs ▪ muscle, smooth ▪ signal transduction ▪ transcriptional coactivator

One of the principal roles of a mature vascular smooth muscle cell (SMC) is contraction. SMCs in adult arteries have extremely low rates of proliferation and express a characteristic set of contractile proteins.1 However, unlike cardiac or skeletal muscle cells, adult SMCs retain the plasticity to undergo a reversible phenotypic switch to an immature form, characterized by downregulation of the contractile apparatus, and higher rates of migration, proliferation, and deposition of extracellular matrix.2 Phenotypic switching of SMCs is associated with vascular injury or disease responses, for example in restenosis after angioplasty, atherogenesis, and accelerated graft disease. Although SMC plasticity is important for vessel repair after injury, it may also predispose to the development and progression of vascular disease.2 Thus, understanding the molecular mechanisms that control SMC differentiation in disease may lead to novel approaches to treatment.

 Mature SMCs are characterized by expression of a range of SM-specific contractile proteins, including SM α-actin, SM myosin light chain kinase, SM myosin heavy chain, SM-22α, smoothelin, and h1-calponin. The transcription of most SMC marker genes is regulated by serum response factor (SRF) binding to a cis-acting DNA sequence known as a CARG box (CC[A/T][GG]). Myocardin is a potent coactivator of SRF found only in smooth and cardiac muscle and regulates gene expression by forming a higher order complex with SRF rather than binding directly to DNA.3,4 Mice with homozygous null mutations for myocardin die during early aortic development and embryos fail to express SMC markers, whereas cardiac development seems normal.5 However, myocardin is not absolutely required for SMC development, as homozygous myocardin-null embryonic stem cells can differentiate into SMCs in vitro, and in vivo these myocardin-null cells contribute to vascular smooth muscle in tissues, albeit to a lesser extent than wild-type cells.6,7 Loss of myocardin might, in part, be compensated for by the myocardin-related transcriptional factors A (MKL1) and B (MKL2).8,9 Apart from inducing SMC differentiation, myocardin has been reported to inhibit cell proliferation,4 possibly by its interaction with nuclear factor (NF)-xB and suppression of NF-xB transcriptional activity.10 Myocardin expression is associated with a contractile SMC phenotype, with reduced expression or function during phenotypic switching in vitro. For example, platelet-derived growth factor (PDGF)-BB represses myocardin-induced SMC marker expression through a variety of mechanisms including...
Kruppel-like factor 4–mediated inhibition of myocardin expression and function. Despite extensive studies in culture, our understanding of the role of myocardin in SMC phenotypic switching and the injury response in vivo is significantly limited because only correlative data have been reported from animal models. For example, we have previously shown that myocardin decreases soon after carotid artery injury, in parallel with reduced SMC marker gene expression, while others have recently documented similar reductions after coronary angioplasty and femoral artery wire injury. Critically, there is no direct evidence for the role of myocardin in SMC phenotypic switching in the injury response in vivo. Here, we present for the first time, data from myocardin gain and loss of function in mouse models of arterial injury and remodeling. We demonstrate that myocardin decreases neointima formation by reducing both SMC migration and proliferation. We identify a previously unknown role for myocardin as a regulator of SMC migration by inducing specific microRNAs (miR-24 and miR-29a) that downregulate PDGF receptor β (PDGFRB) expression. Conversely, reduced myocardin augments neointima formation in myocardin heterozygous-null mice. These studies demonstrate conclusively that modulating myocardin levels in vivo has a direct effect on the vascular injury response, and this may represent a new approach for the treatment of vascular diseases.

Materials and Methods

Results

Loss of Myocardin Expression in Arterial Injury or Atherosclerosis

Previous studies have shown that SMC markers such as Acta2, Myh11, and myocardin are reduced after balloon injury to rat carotid arteries. To validate that myocardin is also reduced in a murine model, we examined RNA from wire-injured murine carotids. Myocardin mRNA was significantly decreased within 1 week of wire injury (P<0.01; Figure 1A). The decrease in myocardin levels also correlated with significant downregulation of Acta2 and Myh11 (Figure 1B). Together with previous studies on vascular injury, these data suggest that loss of myocardin expression is an early and consistent feature of vascular pathology.

Expression of Myocardin Inhibits Neointima Formation After Vascular Injury

To investigate the role of myocardin after vascular injury, we re-expressed myocardin using adenovirus (Ad.)-mediated gene transfer. Mice underwent carotid wire injury followed by intraluminal instillation with adenoviruses. To ensure optimal transduction, we first examined efficiency in vivo using an Ad.LacZ virus. Efficient transduction was confirmed throughout the media using virus at 1.5×10^10 pfu/mL incubated intraluminally for 20 minutes (Figure 1A in the online-only Data Supplement). We then confirmed expression of the myocardin transgene and elevated levels of total myocardin, 3 days after Ad.Myocardin infection in vivo (Figure 1B and IC in the online-only Data Supplement). Ad.Myocardin treatment also enhanced the expression of smooth muscle marker genes such as Tagln, Myh11 and Acta2 (Figure 1D–IF in the online-only Data Supplement). More extensive studies were then performed to compare the injury response at 28 days in the Ad.LacZ versus Ad.Myocardin groups (Figure 1C; Figure II in the online-only Data Supplement). Ad.Myocardin reduced neointimal area by 45% (P<0.01) and total neointimal cell number compared with the Ad.LacZ treated control mice (Figure 1D and 1E). Ad.Myocardin also decreased medial area by 52% (P<0.001) but did not alter medial cell number suggesting that myocardin affected medial remodeling either through an effect on cell size or extracellular matrix deposition (Figure 1F and 1G).

Vascular SMC (VSMC) proliferation and migration across the internal elastic lamina are key processes in neointima formation after vascular injury. To quantify proliferation and migration, mice received BrdU continuously throughout the postinjury period. Any BrdU-positive cell must have undergone proliferation after wire injury. Migration is harder to measure directly. However, as previously described, we reasoned that because mice do not normally have intimal SMCs, then any BrdU-negative SMCs in the neointima must have migrated there from the media or transluminally and were classified as migratory cells. Clearly a cell could migrate into the neointima and then proliferate, in which case we would be underestimating the number of migratory cells. We used high-resolution confocal microscopy and ACTA2 expression to identify SMCs and to colocalize with BrdU (Figure 2A). Ad.Myocardin reduced neointimal ACTA2 positive cells by...
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47% \( (P<0.01) \), with no change in the number of medial SMCs (Figure 2B and 2C). As predicted by previous in vitro studies,\textsuperscript{4,11,18} Ad.Myocardin reduced cell proliferation in both neointima and media \( (P<0.05; \) Figure 2D and 2E). Importantly, Ad.Myocardin significantly reduced the number of BrdU-negative SMCs in the neointima \( (P<0.01) \) with no change in the media, indicating a novel role for myocardin in inhibiting SMC migration into the neointima (Figure 2F and 2G).

**Myocardin Attenuates PDGF-BB–Induced SMC Migration by Downregulating PDGFRB Expression**

To determine the mechanisms responsible for myocardin-mediated reduction in SMC migration, which has not previously been described, we performed in vitro experiments on cultured vascular SMCs. As SMCs undergo phenotypic modulation when placed in culture, and therefore express low levels of SM-contractile genes and myocardin, we performed gain of function studies using Ad.Myocardin, which led to detectable levels of myocardin in cultured SMCs (Figure IIIA in the online-only Data Supplement). To assess cell migration, we used a chemotaxis assay using transwell inserts coated with matrigel to simulate the in vivo basement membrane in the vessel media. Ad.Myocardin reduced PDGF-BB–induced migration by 36% compared with Ad.LacZ treated cells \( (P<0.01; \) Figure 3A). Ad.Myocardin also attenuated PDGF-BB–induced migration by 75% in a scratch assay model \( (P<0.001; \) Figure IIIB and IIC in the online-only Data Supplement). PDGF-BB is known to activate PDGFRB and regulate SMC migration, proliferation, and cell survival during vascular injury.\textsuperscript{19,20} We, therefore, examined whether myocardin regulated PDGFRB levels. Ad.Myocardin treatment significantly decreased expression of PDGFRB at both mRNA and protein (Figure 3B and 3C). In keeping with these findings in rat aortic SMCs, similar results were also obtained using human embryonic stem cell–derived SMCs\textsuperscript{21} (Figure IVA and IVB in the online-only Data Supplement). Taken together, these results suggest that myocardin attenuates PDGF-BB–induced SMC migration by downregulating PDGFRB.

**Myocardin Regulates PDGFRB Expression Through miR-24 and miR-29a**

We next examined the mechanism of reduction in PDGFRB expression by myocardin, focusing on microRNAs (miRNAs) targeting PDGFRB. miRNAs are an emerging class of non-coding RNA that regulate gene expression at the post-transcriptional level by degrading the target mRNAs or inhibiting their translation.\textsuperscript{22} Indeed, previous studies have shown that miR-26a, miR-143, miR-145, and miR-221 control SMC phenotypic switching\textsuperscript{23,24} and miR-21, miR-143,
and miR-145 also regulate SMC migration. We, therefore, investigated the expression pattern of these miRNA in myocardin-treated cells. Myocardin significantly upregulated miR-143 and miR-145 and downregulated miR-26a and miR-221; miR-21 levels did not change (Figure VA in the online-only Data Supplement). Moreover, blocking miR-143 and miR-145 by antagomirs in myocardin-treated cells rescued SMC migration only in part ($P<0.01$; Figure VB in the online-only Data Supplement). Moreover, antagonizing miR-143 and miR-145 did not alter the inhibition of PDGFRB by myocardin (Figure VC in the online-only Data Supplement), suggesting that modulation of PDGFRB by myocardin proceeded through an alternative pathway. The transfection efficiency of antagomirs was confirmed by visual fluorescent microscopy (>50% of all cells; data not shown).

To identify potential miRNAs that regulate PDGFRB expression, we performed an in silico search using the online prediction algorithm from www.targetscan.org. The software identified Pdgfrb as a possible target for miR-9, mir-24, miR-29a, and miR30. Indeed, miR-24 and mir-29a were significantly upregulated and miR-30c was downregulated in SMCs treated with Ad.Myocardin compared with control cells (Figure 3D and Figure VA in the online-only Data Supplement), with no change in miR-9 expression (Figure VA in the online-only Data Supplement). Similarly, increases in miR-24, miR-29a, miR-143, and miR-145 expression were also observed in Ad.Myocardin-treated hESC-derived SMCs compared with rat VSMCs (Figure IVC in the online-only Data Supplement). Although consensus CArG and CArG-like boxes were previously detected at both miR-24 and miR-29a promoter regions, through which myocardin could possibly function, there was poor conservation between mouse and rat. We, therefore, targeted promoter regions where there was at least partial interspecies conservation (Figure VIA and Table III in the online-only Data Supplement). Using chromatin immunoprecipitation with antibodies against myocardin and SRF, we were not able to amplify miR-24/-29a products from myocardin-transduced SMC, despite effective enrichment of the Acta2 and Myh11 promoter regions (Figure VIB–VID in the online-only Data Supplement). Interestingly, Park et al previously identified that expression of miRs-24 and -29a was SRF-dependent. However, functionality of the putative CArG or CArG-like boxes in their promoters was not tested, and indeed the poor
cross-species conservation of the CArG(-like) elements is consistent with our observation of a lack of cis-element function. These results indicate that myocardin is inducing miR-24 and miR-29a expression either indirectly or via a CArG-independent mechanism (Figure VI in the online-only Data Supplement). A previous study also identified an inhibitory interaction between myocardin and NF-κB.1 However, inhibition of NF-κB activity using APQ, a cell permeable inhibitor, did not replicate the effect of myocardin overexpression in the Ad.LacZ control group or enhance the blockade of SMC migration in the Ad.Myocd treatment group (Figure VII in the online-only Data Supplement). Therefore, we concluded that myocardin did not modulate SMC migration by inhibition of the NF-κB pathway.

Antagonists for miR-24 or miR-29a partially rescued PDGFRB inhibition by myocardin, with a complete recovery of PDGFRB at both mRNA and protein levels observed with a combination of anti–miR-24 and anti–miR-29a (Figure 3E and 3F). To determine whether Pdgfrb is a direct target of myocardin-induced miRs-24 and -29a, we cotransfected the SMCs with a firefly luciferase reporter constructs containing the full length rat Pdgfrb 3′ untranslated region (UTR) and antagonists for miR-24 and miR-29a (Figure VIII in the online-only Data Supplement). Ad.Myocardin expression in VSMCs repressed the 3′ UTR Pdgfrb luciferase activity by 50%, and blocking miR-24 and miR-29a rescued the myocardin-induced inhibition of luciferase activity, suggesting that myocardin-mediated miRs-24 and -29a may bind directly to target RNA and inhibit PDGFRB expression (Figure 3G). Furthermore, inhibition of both miR-24 and miR-29a markedly enhanced the migration of Ad.Myocardin-treated VSMCs (Figure 3H).

To determine whether miR-24-29a acted directly on the 3′Pdgfrb-UTR, we performed additional experiments with miR-24-29a mimics investigating the expression of Pdgfrb mRNA, activity at 3′Pdgfrb-UTR and SMC cell migration (Figure VIII in the online-only Data Supplement). These experiments demonstrated that miR-29a was able to inhibit Pdgfrb mRNA expression, 3′Pdgfrb-UTR luciferase activity, and SMC migration, whereas miR-24 alone downregulated Pdgfrb gene expression and inhibited SMC migration without a significant effect on 3′Pdgfrb-UTR activity (Figure VIIIIB–VIIIID in the online-only Data Supplement). Further scrutiny revealed that although the miR-29a seed sequence was conserved among human, mouse, and rat, the 2 binding sites for miR-24 were different between human and mouse/rat (Figure VIIIIE in the online-only Data Supplement). Given
the lack of effect of miR-24 on the 3′ Pdgfrb-UTR and the lack of conservation of its seed sequence, we proceeded to mutate just the miR-29a binding site on rat Pdgfrb 3′ UTR that abolished the action of myocardin (Figure VIII in the online-only Data Supplement; Figure 3I). It is noteworthy that combination of miR-24/-29a mimics further potentiated the inhibition of 3′ Pdgfrb-UTR activity and SMC migration compared with the mimics alone (Figure VIIC and VIID in the online-only Data Supplement). We, therefore, conclude that miR-29a acts directly on the Pdgfrb 3′ UTR while miR-24 likely has an indirect effect on Pdgfrb mRNA. However, we speculate that cooperativity of miRNA binding at the Pdgfrb 3′ UTR is seen in the presence of miR-29a, leading to a direct effect by miR-24 and synergistic effects on the luciferase assay.

To corroborate that myocardin also worked through miRs-24 and -29a in vivo, we measured the expression of miRs-24 and -29a in a murine vascular injury model. Mice underwent carotid wire injury followed by intraluminal administration of either Ad.LacZ or Ad.Myocardin. Due to the minimal amounts of RNA that can be extracted from the injured segment of a single mouse carotid artery, it was necessary to pool 5 vessels per group before RNA extraction. Results, therefore, represent the mean of 5 vessels, but error bars and statistics cannot be applied because the samples were pooled. Vascular injury by itself (Ad.LacZ control groups) caused a profound reduction of miR-24, miR-29a, miR-143, and miR-145 in the injured vessel compared with its uninjured counterpart (Figure 3J and 3K and Figure IXB in the online-only Data Supplement). Taken together, these experiments suggest that myocardin downregulates PDGFRB levels by promoting miR-24 and miR-29a expression indirectly or in a non-CARG dependent manner.

Myocardin Deficiency Potentiates Neointima Formation

The studies above have used a gain of function approach to show that re-expressing myocardin can inhibit both the SMC migration and proliferation associated with the vascular injury response. However, this does not prove that endogenous levels of myocardin directly inhibit these processes in vivo. We, therefore, examined myocardin−/− mice, which we established have lower myocardin expression in the aorta but similar levels of Mkl1 and Mkl2 compared with their wild-type littermates (Figure X in the online-only Data Supplement). To more closely study the effects of low levels of myocardin on SMCs, these experiments were performed after carotid artery ligation, a model with reduced complexity compared with atherosclerosis models. We also chose a time point of 14 days, when the neointima is not fully formed, to be able to determine any increase in neointima formation.

Neointimal area (3-fold increase; P<0.01) and total neointimal cell number were significantly increased in myocardin−/− mice 14 days after ligation compared with wild-type littermates (Figure 4A–4C and Figure XI in the online-only Data Supplement). In myocardin−/− mice, the medial area was also increased by 30% (P<0.01) but medial cell numbers were unchanged (Figure 4D and 4E). Neointimal lesions from both myocardin−/− and wild-type mice contained mainly SMCs, staining positive for ACTA2 (Figure 5A), and SMC number was significantly higher in myocardin−/− mouse neointima with no changes in medial SMC number (Figure 5B and 5C). Myocardin−/− mice had significantly increased SMC proliferation in both neointima and media (Figure 5D and 5E) and increased migrating (BrdU-negative) neointimal SMCs (P<0.01) with no alteration in medial nonproliferative BrdU-negative SMCs (Figure 5F and 5G). Consistent with previous in vitro studies in which myocardin suppressed SMC growth and cyclin D1 expression, loss of myocardin in vivo augmented the mRNA expression of cyclin D1 and E1 (Figure XIIA and XIIB in the online-only Data Supplement).

**Figure 4.** Loss of myocardin promotes neointima formation in a carotid artery ligation model. A, Hematoxylin and eosin staining of representative sections of carotid arteries from wild-type (WT) and myocardin heterozygous-null (Myocardin+/−) mice, 14 days after ligation. Scale bar, 100 mm. B, Quantification of cross-sectional neointimal area (B), total number of neointimal cells (C), medial area (D), and total number of medial cells (E) in the ligated carotid arteries from WT and Myocardin−/− animals. Data are presented as mean±SEM. *P<0.05, **P<0.01. ns indicates no statistical significance. WT n=7, Myocardin−/− n=8.
Furthermore, Ad. Myocardin reduced cyclin D1 and E1 RNA levels in hESC-derived SMCs (Figure XIIC in the online-only Data Supplement).

To investigate the effect of reduced myocardin on PDGFRB, miRs-24 and -29a expression, carotids from myocardin+/− mice and wild-type littermate controls were harvested 2 days postligation. miRNAs in injured vessels were normalized to expression levels in contralateral uninjured vessels. After carotid ligation, there was enhanced downregulation of myocardin mRNA levels in myocardin+/− mice compared with their wild-type littermates (Figure 6A). Additionally, greater reduction of miRs-24 and -29a (by 50%, P<0.05; Figure 6B and 6C) and miRs-143 and -145 (by 70% P<0.01; Figure XIII in the online-only Data Supplement) were observed in myocardin+/− mice compared with their wild-type littermates. Finally, we demonstrated that myocardin+/− mice have higher expression of PDGFRB in the neointimal SMCs with no changes in medial PDGFRB expression compared with the wild-type littermate controls (Figure 6D–6F). These loss of function in vivo studies are consistent with the virally mediated gain of function experiments and strongly suggest that endogenous levels of myocardin regulate neointima formation by decreasing both the migration and proliferation of VSMCs.

Discussion

Vascular SMCs respond to injury or disease by undergoing phenotypic switching and display a range of characteristic features including enhanced migration, proliferation, and deposition of ECM. Extensive in vitro studies and developmental models suggest that myocardin has a central role in regulating SMC phenotype and SMC-specific gene expression through its interaction with SRF. However, there are very limited data on the role of myocardin in vascular injury or disease. Previous studies have reported a reduction of myocardin in association with the loss of SMC contractile gene expression. However, these data are correlative only and there is no direct evidence that the loss of myocardin is causal in the vascular injury response in vivo. Moreover, the mechanisms by which myocardin might regulate this response are unclear.

We present here the first in vivo studies on the effects of gain and loss of myocardin on the neoointimal response in injured and remodeling vessels. We show that myocardin regulates neointima formation after vascular injury through effects on SMC proliferation and a previously undescribed effect on SMC migration. The following novel observations support this conclusion: (1) ectopic expression of myocardin in a murine carotid artery injury model decreased neointimal formation by reducing both SMC migration and proliferation;
(2) myocardin<sup>−/−</sup> mice had reduced levels of myocardin expression and displayed augmented neointima formation due to increased SMC migration and proliferation; (3) myocardin reduced PDGFRB expression in cultured VSMCs whereas myocardin<sup>−/−</sup> mice exhibited increased PDGFRB expression in the neointima; and (4) the inhibitory effects of myocardin on SMC migration and PDGFRB expression were mediated, in part, through miR-24 and miR-29a. Based on these findings, we propose that endogenous levels of myocardin have a key role in regulating SMC phenotype in vivo, and expression or activity of this transcription factor are key determinants of the vascular injury response. Moreover, we outline a novel molecular mechanism involving miR-24 and miR-29a that underlies, in part, the action of myocardin in vascular disease.

**Myocardin-Mediated Modulation of Proliferation and Migration In Vivo**

We identify that myocardin repression is a key requirement for the development of vascular disease and that modulation of myocardin levels and activity regulate the injury or disease response. After arterial injury, medial SMCs undergo proliferation or migration or both and synthesize extracellular matrix, resulting in neointima formation. Although it has been suggested that bone marrow–derived SMC-like cells may also colonize the neointima after vascular injury, such cells represent a very small fraction of the total neointimal cells, and high-resolution confocal studies have shown that most neointimal SMCs are derived from the vessel wall. Myocardin has previously been shown to regulate negatively SMC proliferation in vitro, possibly through an interaction with NF-xB. We extend these findings using 2 distinct animal models to show that myocardin similarly regulates SMC proliferation and the neointimal response in vivo. Moreover, we demonstrate, to our knowledge for the first time, that myocardin also regulates SMC migration, both in vitro and in vivo, in both a simple flow cessation model and a more complex wire injury/atherosclerosis model. To summarize, we have identified myocardin as a key regulator of the vascular injury response that modulates multiple aspects of SMC phenotypic
switching including proliferation and migration, leading to the development of a neointimal lesion.

**Modulation of PDGF-BB Pathway by Myocardin and miRNAs**

In this study, myocardin inhibited PDGF-BB–induced smooth muscle migration by downregulating the expression of PDGFRB. A previous study has demonstrated that FOXO4 binding represses the activity of myocardin and prevents SMC differentiation. On stimulation of the phosphatidylinositol 3'-kinase/Akt pathway, which may be activated by PDGF signaling, FOXO4 was exported from the nucleus resulting in activation of myocardin. However, in other studies, myocardin was inhibited by PDGF-BB signaling via Kruppel-like factor 4 and Elk-1. Combining these observations with our results, we propose an autoregulatory model whereby myocardin is either activated or inhibited by PDGF-BB signaling in a context-dependent manner and in turn regulate PDGFR-B activity through miR-24 and miR-29a. Such context dependency may explain why PDGF-BB has repressive effects on SMC gene expression in mature SMCs while stimulating SMC gene expression and differentiation in SMC progenitors. Interestingly, Chan et al recently showed that miR-24 was upregulated by PDGF-BB and was required for the inhibition of the bone morphogenetic protein–induced contractile phenotype. This apparent discrepancy with our results in which myocardin inhibits PDGFRB and SMC migration in part through miR-24 and miR-29a. Such context dependency may explain why PDGF-BB has repressive effects on SMC gene expression in mature SMCs while stimulating SMC gene expression and differentiation in SMC progenitors. Additionally, Chan et al showed that miR-24 and miR-29a to overcome the increased development of neointima in the Myocd−/− mice could be performed to further test our proposed mechanism in vivo.

**Validation of Mechanisms In Vivo**

These and other studies confirm that myocardin expression is reduced in cultured SMCs or after vascular injury. In vitro, we present considerable evidence that re-expression of myocardin inhibits SMC migration at least, in part, through miR-24–mediated and miR-29a–mediated inhibition of PDGFRB. In vivo we were also able to confirm myocardin transgene expression in the Ad.Myocardin group by the presence of the Flag tag on Western blotting. Interestingly, myocardin mRNA and expression of SMC markers were only increased moderately in the Ad.Myocardin-treated vessels compared with Ad.LacZ controls (Figure 1 in the online-only Data Supplement), whereas Pdgfrb showed only a small reduction (Figure IXB in the online-only Data Supplement). These modest changes, although consistent with the mechanism identified in vitro, were in contrast to the large changes detected in level of miR-24 and miR-29a with Ad.Myocardin treatment. It is possible that there may be a relatively low threshold above which myocardin is able to inhibit the injury response which even a modest increase in myocardin expression in the Ad.Myocardin group is able to reach. The adventitia may also have contributed to the background reducing our ability to discriminate differences in expression between the Ad.LacZ and Ad.Myocardin groups at 3 days postinjury. In addition, there may be further increases in myocardin expression level in the Ad.Myocardin group at later time points to mitigate the profound reduction seen at 1 week in nontransduced injured vessels (Figure 1A). Finally, it is possible that the differences in PDGFRB expression between in vitro and in vivo studies reflect differences in mode of action of the miRNAs. Recent studies suggest that miRNA-mediated gene silencing operates through multiple mechanisms including a combination of mRNA decay and translational repression, and different elements may predominate in different settings. Further studies involving the delivery of miR-24 and miR-29a to overcome the increased development of neointima in the Myocd−/− mice could be performed to further test our proposed mechanism in vivo.
In conclusion, using a series of in vivo gain and loss of function studies and in vitro mechanistic studies, we have identified that myocardin is a key regulator of the neointimal response. This includes a previously unidentified role in regulating VSMC migration, via a novel mechanism involving induction of miR-24 and miR-29a and inhibition of the PDGFRB pathway (Figure 6G). We propose that, in addition to its well-documented developmental role, myocardin is a vital regulator of smooth muscle phenotype in response to pathological stimuli. The profound effects of myocardin on the vascular response to injury suggest that this transcriptional coactivator may be an attractive target for novel therapeutic strategies in vascular disease.

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Disclosures
None.

References
Myocardin is a smooth muscle cell and cardiomyocyte restricted transcriptional coactivator, principally known for its role in smooth muscle cell development and gene expression. Although myocardin expression decreases in phenotypically modulated smooth muscle cells, there are no studies demonstrating a direct causal role for myocardin in the vascular injury response. Here, we have performed both gain and loss of myocardin function experiments in vivo, and we report for the first time that myocardin has a direct role in the vascular injury response. We identify a previously unknown role for myocardin in the regulation of smooth muscle cell migration and delineate a novel molecular mechanism involving miR-24 and miR-29a by which myocardin regulates platelet-derived growth factor receptor β and cell migration. The clinical significance of our findings is that the myocardin level seems to be a pivotal regulator of the vascular injury response, and myocardin or associated microRNAs may be attractive targets for novel therapeutic strategies in vascular disease.
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Supplemental Methods

Mouse model of carotid artery wire injury

Transluminal arterial injury was induced as previously described (1) in 8-week old apoE<sup>−/−</sup> mice (C57BL/6 background) fed with a western diet for 1 week before and up to 4 weeks post-injury. Briefly, mice were anesthetized using subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health, The Netherlands) and hypnorm (5%; VetaPharma, UK). Wire injury was induced with a 0.36-mm flexible angioplasty guide-wire by transverse arteriotomy of the external carotid artery and denuding the endothelial layer by 3 rotational passes. Immediately after injury, the left common carotid artery (LCCA) was cannulated and the biclamped segment incubated with 10 µl of adenovirus at 1.5 x 10<sup>10</sup> pfu/ml encoding myocardin (n=14) or LacZ (n=14) for 20 minutes. 0.8 mg/mL bromodeoxyuridine (BrdU; Sigma Aldrich, UK) and 1% sucrose were added to drinking water immediately after surgery and maintained throughout to identify proliferating and migrating VSMCs. BrdU-negative neointimal SMCs were classified as migratory cells as previously described (2, 3). After 28 days, animals were sacrificed and pressure-perfused with 10% formalin and vessels embedded in paraffin for morphometric and histological analysis. Animals were excluded from analysis if they died prematurely, if there was no injury response or if the injured vessel was totally occluded at time of harvest. For miRNA and mRNA studies, carotid vessels were harvested after 3 days post wire injury. These animal experiments were performed in compliance with the University of Leiden and Dutch government guidelines.

Mouse model of carotid artery ligation

Myocardin heterozygous-null 129/C57BL/6 mixed background mice were kindly provided by Prof. Eric Olson, Southwestern Medical Center, University of Texas (4). Littermates were
inter-bred to expand the colony. Age-matched wild type male littermates (n=12) and myocardin heterozygous-null mice (n=12) were anesthetized by intraperitoneal ketamine hydrocholride (80 mg/kg) and xylazine (5 mg/kg). LCCA ligation was performed near the bifurcation, and 0.8 mg/mL BrdU and 1% sucrose added to drinking water immediately after surgery and maintained throughout. Mice were euthanized 14 days post-surgery and pressure-perfused with 10% formalin. Animals were excluded from analysis if they died prematurely. These animal experiments were performed under UK Home Office licensing.

**Histology and Immunohistochemistry**

Carotid arteries were cut into 3 pieces of identical length and multiple 5 µm sections at 225 µm intervals stained with hematoxylin and eosin. Double immuno-fluorescence labelling used antibodies to smooth muscle α-actin and BrdU using an Alexa Fluor-568 conjugated secondary antibody (Catalogue No: A11077, Life Technologies). Details of the primary antibodies are listed in Supplementary Table I. Coverslips were mounted using Vectashield with DAPI. Cells were imaged using a Zeiss LSM 700 Laser Scanning Microscope. Morphometric analysis and cell counting were performed by an investigator blinded to treatment groups. Right common carotid artery sections served as internal controls for each animal. Medial and neointimal thickness and cell numbers were calculated from eight serial sections from each carotid portion and analysed by ImageJ software.

For PDGFRB quantification, the carotid sections were dual-labelled with antibodies against smooth muscle α-actin and PDGFRB using an Alexa Fluor-568 conjugated secondary antibody. Cells were imaged using a Zeiss LSM 700 Laser Scanning Microscope. Smooth muscle cells were identified by smooth muscle α-actin staining (green colour) and PDGFRB by red colour. PDGFRB level in medial and neointimal regions was calculated by the red pixel intensity per smooth muscle cell. Analysis was carried out by ImageJ software.
Cell culture

Aortic vascular SMCs isolated from Wistar rats were maintained in 1:1 Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin, 5 μg/ml L-glutamine and 10% fetal bovine serum. Cells at 70-80% confluence were serum starved for 24 hours before in vitro experiments.

Human embryonic stem cells (hESC) are differentiated into SMCs using defined growth factor combinations as previously described (5). Briefly, human ESCs were induced to a mesodermal fate using FGF-2, BMP-4 and LY294002 for 36 hours then FGF-2 and BMP-4 for a further 3.5 days. Cells were then treated with PDGF-BB and TGF-β1 for 12 days to generate SMCs. The hESC-derived SMCs were maintained in 1:1 Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin, 5 μg/ml L-glutamine and 10% fetal bovine serum. Cells at 70-80% confluence were serum starved for 24 hours before in vitro experiments.

Adenovirus Infection

Adenovirus vectors for LacZ and FLAG-tagged myocardin were purchased from the Gene Transfer Vector Core, University of Iowa. For infection, virus was added in a minimal volume of serum-free media supplemented with insulin, transferrin and selenium covering SMCs and incubated for 8 hours. Virus-containing media was then removed and replaced with serum-free media for 18 hours before experiments.

Transwell migration Assay

Chemotaxis assays were performed using a transwell insert with a polycarbonate membrane (8μm pores) coated with matrigel (BD Biosciences). VSMCs were added to the upper
chamber and serum-free DMEM/F-12 media supplemented with PDGF-BB (10 ng/ml) added to the lower chamber. After 8 hours migrated cells on the lower surface were quantified by crystal violet staining.

**Scratch Assay**

VSMCs were grown to confluence, scratched using a 200 µl pipette tip over the monolayer, and stimulated with platelet-derived growth factor-BB (PDGF-BB; 10ng/ml, PeproTech). At indicated time-points, cells were washed with PBS, fixed with 4% paraformaldehyde and stained with crystal violet. The cell-free wound area was determined using ImageJ software.

**miRNA quantification**

VSMCs were lysed with QiazolTM reagent and total RNA isolated using the miRNeasy kit (Qiagen) according to the manufacturer’s instructions. For carotids, the vessels were homogenised with lysing matrix beads D (MP Biomedicals) and RNA extracted using miRvana miRNA isolation kit (Life Technologies). RNA was reverse transcribed using a Taqman microRNA reverse transcription kit (Life Technologies) following the manufacturer’s protocol. Taqman kits (Life Technologies) were used to measure rno-miR-21, rno-miR-24, rno-miR26a, rno-miR-29a, rno-miR-143, rno-miR-145, rno-miR221, and U6 snRNA (internal control). Amplification took place on a LightCycler (Life Technologies).

**mRNA quantification**

mRNA was isolated using a RNAeasy mini kit (Qiagen) following the manufacturer’s protocol. cDNA was synthesised using a RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific) and qPCR performed using a Rotor Gene 6000 system (Corbett Life Science) using gene-specific primers and SYBR GreenER (Life Technologies). 18S, Actb and
*Gapdh* were used as housekeeping genes. Supplementary Table II summarises the primer sequences.

**Western blot analysis**

Proteins were extracted using lysis buffer {10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Sigma Aldrich, UK)}, and concentrations determined using a BCA Protein Assay Kit (Thermo Scientific). Samples were separated by SDS-PAGE and proteins transferred on to polyvinylidene difluoride membranes, blocked in 5% milk in Tris-buffered saline and 0.05% Tween 20, incubated with primary antibodies against PDGFRB, (Cell Signalling Technologies), ACTB and myocardin (Sigma Aldrich, UK), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Dako). Details of the primary antibodies are listed in the Supplementary Table I. Signals were detected using ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences), with densitometric analysis using ImageJ software.

**Transfection of cells**

VSMCs were transfected with anti-hsa-miR-24, anti-hsa-miR29a and anti-hsa-miR-let-7c using siPORT™ NeoFX™ reagent (Life Technologies) 8 hours after transfection, cells were washed and maintained in serum-free DMEM/F12 media for 18 hours before *in vitro* studies.

**Luciferase Assay and Site-directed mutagenesis**

VSMCs were co-transfected with the 1,743 bp full-length rat *Pdgfrb* 3’-UTR (RmiT051132, Genecopoeia; NCBI ref NM_031525.1) inserted downstream of the firefly luciferase sequence in a pEZX-MT01 vector system (Genecopoeia) using FuGene 6 (Promega) and antimiRs - 24 and -29a or antimiR-let-7c (control) followed by Ad.LacZ or Ad.Myocd
treatment. Mutagenesis of the miR-29a-binding sites on Pdgfrb 3’-UTR was performed according to the manufactures protocol (QuikChange II Site-Directed Mutagenesis Kit, Aligent Technologies). Mutagenesis primers were generated using QuikChange Primer Design software (Aligent Technologies) Forward 5’- GGGG AAGA TTTT AATA TTAA ACTT GACG CTTC TCAC TGAA TAGC CAGT C-3’ and Reverse 5’- ACTG GCTA TTCA GTGA GAAG CGTC AAGT TTAA TATT AAAA TCTT CCCC-3’. The miR-29a binding site was mutated from UGUGCU to UGACGCU. Cell extracts were prepared 48 h after transfection and luciferase activity was measured using the Luc-Pair™ miR Luciferase Assay Kit (Genecopoeia). Luciferase readings were obtained on a Glomax 96 microplate luminometer (Promega).

**Chromatin Immunoprecipitation Assays (ChIP)**

Rat VSMCs (1 x 10^6) were transduced with Ad.LacZ or Ad.Myocardin for 8 h. The virus was removed and cells were allowed to recover for further 24 h. Cells were fixed in formaldehyde for 15 min, lysed, and sonicated. The cell lysates were immunoprecipitated by incubation with either IgG (Sigma) or Myocardin (Sigma) or SRF (Santa Cruz Biotechnology) antibodies overnight. Formaldehyde crosslinking was reversed by incubation with 200 mM NaCl, overnight at 65°C and protein was degraded by further incubation with proteinase K (Thermo Scientific) overnight at 45°C. DNA was then isolated by phenol-chloroform extraction and ethanol precipitation, and quantified by quantitative PCR using specific promoter-targeted primers (Supplementary Table III). Primers were designed in accordance to previously identified regions in murine miR-24 and miR-29a promoters which contain CArG boxes (6).
References:


**Supplemental Table I**: List of antibodies used in Western Blotting and Immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Application</th>
<th>Concentration</th>
<th>Source</th>
<th>Manufacturer (Catalogue number)</th>
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<tr>
<td>ACTB</td>
<td>Western Blotting</td>
<td>1:10,000</td>
<td>Mouse</td>
<td>Sigma-Aldrich (A1978)</td>
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<td>BrdU</td>
<td>Immunofluorescence</td>
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<td>Rat</td>
<td>Abcam (Ab6326)</td>
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<td>Myocardin</td>
<td>Western Blotting</td>
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<td>Mouse</td>
<td>Sigma-Aldrich (M8948)</td>
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<tr>
<td>MKL1</td>
<td>Western Blotting</td>
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<td>Rabbit</td>
<td>Abcam (Ab49311)</td>
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<tr>
<td>MKL2</td>
<td>Western Blotting</td>
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<td>Rabbit</td>
<td>Santa Cruz (sc98989)</td>
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<tr>
<td>PDGFRB</td>
<td>Western Blotting</td>
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<td>Rabbit</td>
<td>Cell Signalling Technology (3169)</td>
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<td></td>
<td>Immunofluorescence</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle-α Actin conjugated to FITC</td>
<td>Immunofluorescence</td>
<td>1:200</td>
<td>Mouse</td>
<td>Sigma-Aldrich (F3777)</td>
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Supplemental Table II: List of Primers used in real time PCR

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>18S (Mouse, Rat, Human)</td>
<td>CGGCTACCA[CATCCAAGGAA AGCTGGAAATTACCGCGGC]</td>
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<td>36B4 (Mouse)</td>
<td>GGACCCGAGAAGACCTCCTTT GCACATCACTCAGAATTTCAATGG</td>
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<td>Actb (Mouse, Rat)</td>
<td>GCTCGGT[TTTACACCCCTTTC GTTTGCTCCAACCAACTGC]</td>
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<td>Cyclin D1 (Mouse)</td>
<td>GAGCTGCTGCAAAATGGAACGTGAA GACATCACTCAGAATTTCAATGG</td>
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<td>Cyclin D1 (Human)</td>
<td>AGCTGCTGCAAAATGGAACGTGAA GACATCACTCAGAATTTCAATGG</td>
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<td>Cyclin E1 (Mouse)</td>
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<td>Cyclin E1 (Human)</td>
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<td>GAPDH (Mouse, Rat, Human)</td>
<td>GCTCGGTTTTACACCCCTTTC GTTTGCTCCAACCAACTGC</td>
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<td>Myocd (Mouse)</td>
<td>GCAGTTTCAGATATCAACCCAGCCCCCTTGGCATGTCGGCATTT</td>
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<td>Pdgfrb (Rat)</td>
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<td>RPS13 (Mouse)</td>
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<td>Tagln (Mouse)</td>
<td>AAGCAGCTGTGCTGGCTTCAAGGTGACTGATGACATTC</td>
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<td>Myh11 (Mouse)</td>
<td>GCTAATCCACCCGGAGGATGCAAGGGAGGAAA</td>
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**Supplemental Table III:** List of primers used in ChIP analysis

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<th>Gene / miR Targeted</th>
<th>Region relative to TSS in rat</th>
<th>Homologous region in mouse</th>
<th>CArG Conservation between mouse and rat</th>
<th>Primer Sequences</th>
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<tr>
<td><em>Myh11</em></td>
<td>-3920</td>
<td>-1080</td>
<td>Yes</td>
<td>CTGC GCGGGACCATATTTAGTCAGGGGAG CTGGGC GGAGAC ACAACCCAAAAGGCCAGG</td>
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<td><em>Acta2</em></td>
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<td>-2843</td>
<td>Yes</td>
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<td>-495</td>
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<tr>
<td>miR-24 Site-2</td>
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<td>-3135</td>
<td>One mismatch</td>
<td>CTCTAATTGCAAACACAGC ACAT GCC TGAATTTGAGGA GACTTC</td>
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<tr>
<td>miR-24 Site-3</td>
<td>1014, 1140</td>
<td>1765, 1957</td>
<td>Yes</td>
<td>TCTTAATAAGTACC GGATTTGCTG GCC AACTAAATCTCC CTCTTACTTCC</td>
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<tr>
<td>miR-29a Site-1</td>
<td>-90</td>
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<td>TGCC TCTCATCT GTGAGCTG CAT GCTCAGC AGG GGT TAAA</td>
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<td>miR-29a Site-2</td>
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<td>-2786</td>
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<td>ACCT CGGTTGTG GTGATAGC C GGG CCTTCTG TCTGTGTTGA</td>
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<td>miR-29a Site-3</td>
<td>-2326</td>
<td>-3723</td>
<td>No</td>
<td>GGTACCTTCAGCTT TAGGTTAGAC GTCTGTGAGAA CAAT CCTCTCTGCT</td>
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Supplemental Figure I: Adenoviral transfection efficiency in injured carotid artery vessels. (A) β-galactosidase expression in medial layers of the injured carotid artery. Carotid artery was stained for β-galactosidase expression (blue) 5 days following wire injury and transluminal infection of Ad.LacZ. Scale bar: 100 μm. (B) Quantification of myocardin expression in mouse carotid arteries, 3 days following wire injury and transluminal infection of Ad.Myocd. Tissue lysates underwent Western blotting using antibodies against FLAG and ACTB. (C-F) mRNA expression of myocardin and smooth muscle markers – Tagln, Myh11 and Acta2 quantified by real-time RT-PCR. Note, carotid lysates and mRNA were isolated from pooled carotid arteries (n=5), so no statistical analysis was carried out. Data are presented as a mean fold change to the uninjured right carotid artery.
Supplemental Figure II: Ectopic expression of myocardin inhibits neointima formation in carotid arteries after vascular injury. Hematoxylin and eosin staining of injured carotid arteries from mice transduced with adenoviral vector (Ad) expressing LacZ or myocardin (Ad.Myocd), 28 days post-injury. Scale bar: 100 µm.
Supplemental Figure III: Myocardin inhibits vascular SMC migration in a wound healing model. SMCs were transfected with adenoviral vectors (Ad) expressing either LacZ (control) or myocardin (Ad.Myocd). (A) Expression of myocardin in cultured SMCs. Cell lysates were analysed by Western blotting using antibodies against myocardin and ACTB. (B) Wound healing assays were performed on SMCs in the presence of PDGF-BB (10 ng/ml). (C) Quantification of the scratch area from the wound healing experiments. Data are presented as mean ± S.E.M. (n=2-5). Asterisk indicates statistical significance; ***P<0.001.
Supplemental Figure IV: In human embryonic stem cell (hESC)-derived smooth muscle cells myocardin inhibits cell migration and regulates PDGFRB mRNA expression and miRNA expression similar to rat VMSCs. SMCs were derived from hESCs using defined growth factor combinations as previously described (Cheung et al. 2012). SMCs were transduced with adenoviral LacZ (Ad.LacZ) or myocardin (Ad.Myocd) vectors. (A) Wound healing assays were performed on SMCs in the presence of PDGF-BB (10 ng/ml) for 24 hours. Quantification of PDGFRB mRNA (B) and miRNA expression (C) was carried by RT-PCR analysis. Data are presented as mean ± S.EM fold change to the Ad.LacZ group (n=3). Asterisk indicates statistical significance. **P<0.01 and ***P<0.001
Supplemental Figure V: miR-143 and miR-145 regulate SMC migration but not through PDGFRB expression. (A) Quantification of miRNA expression by real time PCR in SMCs following adenoviral transduction with LacZ (Ad.LacZ) or myocardin (Ad.Myocd) vectors. (B – C) SMCs were transfected with anti-miRs -143 and -145 or control anti-miR-let-7c prior to Ad.LacZ or Ad.Myocd transduction. Although inhibition of miRs -143 and -145 by respective antagonirs rescued the effect of myocardin on SMC migration in part (B), there was no change in the expression of PDGFRB (C). Data are presented as mean ± S.E.M. (n=3). Asterisk indicates statistical significance; *P<0.05, **P<0.01, ***P<0.001, # indicates statistical significance from Ad.myocd; #P<0.05 and ns– not significant.
Supplemental Figure VI: Myocardin induces miR-24 and miR-29a expression either indirectly or via a CArG-independent mechanism. (A) A schematic diagram representing the relative positions of the Sites (1-3) in the rat miR-24 and -29a promoters where the ChIP was carried out, the putative CArG boxes and their alignment to mouse genome. The cell lysates were immunoprecipitated by incubation with either IgG (control) or myocardin (MYOCD) or SRF antibodies. ChIP analysis was carried out at miR-24 (B), miR-29a (C) and Myh11 and Acta2 (D; positive controls promoter regions. Data are presented as mean ± S.E.M relative to the total DNA input signal (n=2-3).
Supplemental Figure VII: Myocardin-induced inhibition of SMC migration does not involve NF-κB pathway. Following Ad.LacZ or Ad.Myocd infection SMCs were pre-incubated for 30min with a cell-permeable NF-κB transcriptional activation inhibitor, APQ (1nM). Wound healing assays were performed on SMCs in the presence of PDGF-BB (10ng/ml). Data are presented as mean ± S.EM percentage change (n=3). Asterisk indicates statistical significance compared to the respective 0 hour. ***P<0.001
Supplemental Figure VII: miR-29a mimic directly inhibits Pdgfrb expression and SMC migration, whereas miR-24 may have an indirect effect on the PDGF-BB-induced SMC migration. Combination of miRs-24/-29a mimics robustly targets Pdgfrb and antagonists SMC migration. (A) Schematic representation of the luciferase reporter construct used to measure miRNA repressive activity at the full length Pdgfrb 3’UTR. A mutated plasmid with the alterations at miR-29a binding site was also generated. WT – wild type (B) Quantification of Pdgfrb mRNA expression by RT-PCR analysis. (C) Pdgfrb 3’UTR luciferase activity in VSMCs co-transfected with miRs -24 and -29a or miR-let-7c mimics (control). Data are presented as mean ± S.EM fold change to the miR-let-7c control group (n=4-5). (D) Wound healing assays were performed on SMCs transfected with either miR-24 or miR-29a in the presence of PDGF-BB (10 ng/ml; n=5). (E) Conservation of the miRs-24/-29a seed sequences between the human, mouse, and rat Pdgfrb 3’UTR. Asterisk indicates statistical significance; *P<0.05, **P<0.01, #P<0.05, ###P<0.001.
Supplemental Figure IX: Re-expression of myocardin regulates miRs -24, -29a, -143 and -145 and Pdgfrb expression after vascular injury. (A) Quantification of miRs -24, -29a, -143 and -145 and Pdgfrb (B) expression in mouse carotid arteries, 3 days following wire injury and transluminal infection of Ad.LacZ or Ad.Myocd. The uninjured vessel is the right carotid artery and the injured is the contralateral left carotid artery treated with either Ad.LacZ or Ad.Myocd. Note, miRNA was isolated from pooled carotid arteries (n=5), so no statistical analysis was carried out. Data represent fold change to the uninjured right carotid artery from Ad.LacZ treated group.
Supplemental Figure X: Expression of myocardin family members in myocardin heterozygous-null mice. (A) Total mRNA was isolated from wild type (WT) and myocardin heterozygous-null (Myocd+/−) mice aortas and analysed by real time PCR. (B) Aortic tissue lysates were analysed by Western blotting using antibodies against myocardin (MYOCD), myocardin-related transcription factor A (MKL1), myocardin-related transcription factor B (MKL2), and β-actin (ACTB). White arrowhead points to MKL1 band. Data are presented as mean ± S.EM. (n=3). mRNA expression is shown in arbitrary units following normalisation with three housekeeping genes. Asterisk represents statistical significance; **P<0.01, ns – not significant.
Supplemental Figure XI: Myocardin haploinsufficiency promotes neointima formation in a carotid artery ligation model. Hematoxylin and eosin staining of carotid artery sections from various wild type (WT) and myocardin heterozygous-null (Myocd+/−) mice, 14 days after ligation. Scale bar: 100 μm.
Supplementary Figure XII: Myocardin inhibits cell cycle genes. (A-B) Quantification of cyclin D1 (CcnD1) and cyclin E1 (CcnE1) mRNA expression in carotid arteries from wild type (WT) and myocardin heterozygous-null (Myocd+/−) mice, 2 days after ligation. Data are presented as a mean ± S.E.M fold change to the respective unligated right carotid artery (n=3). *P<0.05. (C) Analysis of Cyclin D1 and E1 genes in human embryonic stem cell (hESC)-derived smooth muscle cells transduced with adenoviral LacZ (Ad.LacZ) or myocardin (Ad.Myocd) vectors. Data are presented as mean ± S.E.M fold change to the Ad.LacZ group (n=3). Asterisk indicates statistical significance. *P<0.05 and **P<0.01.
Supplementary Figure XIII: Expression profile of miRs in wild type and Myocardin haploinsufficiency carotids under normal and vascular injury conditions. (A) Quantification of miRs 24, -29a, -143 and -145 expression in normal healthy mouse carotid arteries. (B-E) Expression profile of miRs 24, -29a, -143 and -145 levels in ligated and contralateral unligated mouse carotids arteries, 2 days post-ligation. miRNA expression is shown in arbitrary units following normalisation to U6 levels. Data are presented as a mean ± S.EM. (n=4-5).