MicroRNA-15b/16 Attenuates Vascular Neointima Formation by Promoting the Contractile Phenotype of Vascular Smooth Muscle Through Targeting YAP

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Objective—To investigate the functional role of the microRNA (miR)-15b/16 in vascular smooth muscle (SM) phenotypic modulation.

Approach and Results—We found that miR-15b/16 is one of the most abundant mRs expressed in contractile vascular smooth muscle cells (VSMCs). However, when contractile VSMCs get converted to a synthetic phenotype, miR-15b/16 expression is significantly reduced. Knocking down endogenous miR-15b/16 in VSMCs attenuates SM-specific gene expression but promotes VSMC proliferation and migration. Conversely, overexpression of miR-15b/16 promotes SM contractile gene expression while attenuating VSMC migration and proliferation. Consistent with this, overexpression of miR-15b/16 in a rat carotid balloon injury model markedly attenuates injury-induced SM dedifferentiation and neointima formation. Mechanistically, we identified the potent oncoprotein yes-associated protein (YAP) as a downstream target of miR-15b/16 in VSMCs. Reporter assays validated that miR-15b/16 targets YAP’s 3′ untranslated region. Moreover, overexpression of miR-15b/16 significantly represses YAP expression, whereas conversely, depletion of endogenous miR-15b/16 results in upregulation of YAP expression.

Conclusions—These results indicate that miR-15b/16 plays a critical role in SM phenotypic modulation at least partly through targeting YAP. Restoring expression of miR-15b/16 would be a potential therapeutic approach for treatment of proliferative vascular diseases. (Arterioscler Thromb Vasc Biol. 2015;35:2145-2152. DOI: 10.1161/ATVBHA.115.305748.)

Key Words: microRNA ▪ neointima formation ▪ phenotypic modulation ▪ smooth muscle ▪ YAP
and heart regeneration. The Hippo signaling is an evolutionarily conserved pathway that controls organ size and tumorigenesis. Yes-associated protein (YAP) is a major downstream effector of this signaling pathway that is inactivated by phosphorylation by its upstream large tumor suppressor kinases. YAP is overexpressed in a spectrum of human primary tumors and is a potent oncoprotein because of its ability to induce expression of genes involved in cell cycle progression. Recently, several studies showed that the Hippo–YAP pathway plays a critical role in cardiovascular development and vascular disease in mouse. In particular, our recent study showed that YAP expression is induced during SM phenotypic modulation, and this increased expression of YAP inhibits SM-specific gene expression while promoting SM proliferation and migration in vitro and in vivo.13 In vivo, the mechanistic pathways through which YAP is upregulated during SM phenotypic switching remains elusive.

In this study, we demonstrate that miR-15b/16 promotes VSMC contractile phenotype, at least in part, by targeting YAP, and therefore, restoring expression of miR-15/16 would be a potential therapeutic approach for treatment of proliferative vascular diseases.

Materials and Methods

Rat aortic SMCs were prepared as previously reported. Rat carotid artery balloon injury was performed as described in our previous reports. Full Materials and Methods are available in the online-only Data Supplement.

Results

Expression of miR-15b/16 Is Downregulated During SM Phenotypic Switching From a Contractile to Synthetic State In Vitro and In Vivo

To explore the potential function of the miR-16 family miRs in VSMCs, we first examined their expression in vascular tissue and primary VSMCs. By quantitative reverse transcription polymerase chain reaction, we found miR-16 highly expressed in the rat thoracic aorta and found it to be ≈20% of miR-145, the most abundant miR that was previously identified in the vascular tissue. (Figure 1A in the online-only Data Supplement). Moreover, in both rat primary aortic SMCs and human coronary artery SMCs, miR-16 and its co-transcribed family member, miR-15b, are the most abundant miRs found among the miR-16 family (Figure 1B and IC in the online-only Data Supplement). Compared with endothelial cells and macrophages in mouse, primary VSMCs have highest expression level of miR-15b and miR-16 (Figure 1D in the online-only Data Supplement). We therefore first examined the expression of miR-15b/16 during SM phenotypic switching in vitro and in vivo. In culture, rat aortic tissue undergoes a dramatic switch toward a synthetic proliferative phenotype that was associated with significantly decreased expression of miR-15b/16 along with SM contractile phenotype marker Hic-5 (Figure 1A). In response to platelet-derived growth factor BB, a growth factor known to further inhibit SM contractile protein expression while promoting VSMC proliferation and migration, expression of miR-15b/16 was significantly downregulated, whereas expression of miR-221 was significantly induced as previously reported (Figure 1B). Consistent with the in vitro data, we found that miR-15b/16 was significantly reduced in the balloon-injured rat carotid arteries, a model resembling angioplasty in humans that promotes VSMC proliferation and migration while reducing expression of SM differentiation markers (Figure 1C). Taken together, these data demonstrate that expression of miR-15b/16 is attenuated in phenotypically modulated synthetic VSMCs in vitro and in vivo.

MiR-15b/16 Attenuates VSMC Proliferation and Migration

Proliferation and migration of VSMCs are key events for the development of neointima thickening. Because miR-15b/16 was reduced in phenotypically modulated VSMCs and these cells are known to be proliferative and migratory, we directly examined the effects of miR-15b/16 on VSMC proliferation and migration. Using WST-1 assays, we found that overexpression of miR-15b/16 (≈15-fold; Figure 1A in the online-only Data Supplement) significantly impaired VSMC proliferation in a variety of cell culture media (Figure 2A). Cell cycle analysis further demonstrated that miR-15b/16 overexpression resulted in an accumulation of cells in the G0/G1 phase and
Assay was performed to examine cell migration. ** were transfected into rat aortic SMCs, and the Boyden chamber that were transduced by either GFP or miR-15b/16 adenovirus. BB indicates platelet-derived growth factor BB.

Counts were performed into rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. **P<0.05. Counting was performed into rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. **P<0.05. **P<0.05. After overexpression of miR-15b/16, rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. **P<0.05. Human coronary artery SMCs (HCASMCs) were transduced by control GFP or miR-15b/16 virus, and cell numbers were counted as described in C. **P<0.05. C, After overexpression of miR-15b/16, rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. **P<0.05. Data from these gain/loss-of-function assays suggest that YAP is a potential target of miR-15b/16. Consistent with this idea, overexpression of miR-15b/16 was found to attenuate YAP protein expression without affecting expression of the YAP-associated protein, TEA domain family member 1 (Figure 3A and 3B). The inhibition of YAP by miR-15b/16 is through blocking of YAP translation because YAP mRNA levels were unaltered (Figure 3C). In contrast, a known miR-16 target, cyclin D1, appears to be regulated through altered mRNA stability (Figure 3A and 3C). Furthermore, silencing endogenous miR-15b/16–induced YAP expression inhibited expression of SM-specific differentiation genes and upregulated cyclin D1 expression (Figure 3D). Data from these gain/loss-of-function assays suggest that YAP is a potential target of miR-15b/16. Targetscan.org and microRNA.org prediction algorithms indicated that the 3′-UTR of the human YAP gene harbors a putative consensus site for miR-15b/16, and this binding site is evolutionarily conserved among vertebrates (Figure 3E). To experimentally validate YAP as a miR-15b/16 target gene, we first generated luciferase reporters containing the YAP 3′-UTR region harboring the few cell numbers in S phase (Figure 2B), indicating a cell cycle arrest in G0/G1. This cell cycle retardation resulted in a significant decrease in cell growth (Figure 2C) of rat VSMCs and human coronary artery SMCs (Figure 2D). To test the functional role of endogenous miR-15b/16 on VSMC proliferation, we used antisense oligonucleotides to knock down endogenous miR-15b/16. Quantitative reverse transcription polymerase chain reaction revealed that the endogenous miR-15b/16 was almost completely depleted after transfection with antisense oligonucleotide (Figure 1B in the online-only Data Supplement). Silencing miR-15b/16 resulted in enhanced rates of VSMC proliferation and growth (Figure 2E and 2F). Moreover, overexpression of miR-15b/16 in rat aortic primary SMCs also significantly attenuated VSMC migration as assessed by Boyden chamber assays (Figure 2G). In contrast, silencing endogenous miR-15b/16 promoted VSMC migration (Figure 2H). Together, these data demonstrate that miR-15b/16 is a potent inhibitor for VSMC proliferation and migration.

**Figure 2.** MicroRNA (miR)-15b/16 inhibits vascular smooth muscle cell (VSMC) proliferation and migration. **A**, Adenovirus encoding control green fluorescent protein (GFP) or miR-15b/16 was transduced into rat primary aortic smooth muscle cells (SMCs), and then proliferation was measured using a cell proliferation WST-1 kit (Roche) in the culture media as indicated. **P<0.05. **B**, After transduction with miR-15b/16 or control GFP adenovirus, rat primary aortic SMCs were harvested for propidium iodide staining to analyze cell cycle by a flow cytometry. **P<0.05. **C**, After overexpression of miR-15b/16, rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. N=3. **P<0.05. **D**, Human coronary artery SMCs (HCASMCs) were transduced by control GFP or miR-15b/16 virus, and cell numbers were counted as described in C. **E**, WST-1 assay was performed in rat primary aortic SMCs cultured in the different culture media as indicated after transfection either with control or with antisense oligonucleotides against miR-15b/16. **P<0.05. **F**, After silencing miR-15b/16, rat primary aortic SMCs were seeded at equal density at day (D) 0 and counted at each time point as indicated. N=3. **P<0.05. **G**, Quantification of Boyden chamber assay to assess cell migration after 4 hours plating of rat aortic SMCs cells that were transduced by either GFP or miR-15b/16 adenovirus. **P<0.05. **H**, Control or miR-15b/16 antisense oligonucleotides were transected into rat aortic SMCs, and the Boyden chamber assay was performed to examine cell migration. **P<0.05. PDGF-BB indicates platelet-derived growth factor BB.

**MiR-15b/16 Promotes the Smooth Muscle Contractions Phenotype by Targeting YAP**

Because miR-15b/16 was downregulated in tandem with the reduction of SM-specific markers during SM phenotypic switching (Figure 1), we next determined the role of miR-15b/16 on SM differentiation. Overexpression of miR-15b/16 in rat primary aortic SMCs promoted the expression of SM contractile genes, including SM22α, Hic-5, SM α-actin, and calponin, whereas expression of proliferative markers, such as PCNA (proliferating cell nuclear antigen) and cyclin D1, was decreased (Figure 3A). Similar results can be obtained in human coronary artery SMCs (Figure 3B). Recently, we showed that the expression of YAP is induced in all of in vitro and in vivo SM phenotypic modulation models as described in Figure 1. Furthermore, we found that the induced expression of YAP plays an integrated role in inhibiting SM-specific gene expression while promoting VSMC migration and proliferation. Because the expression of miR-15b/16 and YAP is negatively correlated and their functions exert opposite effects in VSMCs, we postulated that YAP may be a potential target of miR-15b/16. Role of miR-15b/16 in Smooth Muscle Cells
wild-type or mutated putative miR-15b/16–binding site, and then transient transfection experiments were performed. Data from these experiments revealed that luciferase activity of the wild-type YAP 3′-UTR luciferase reporter was significantly decreased in a dose-dependent manner in the presence of miR-15b/16, whereas luciferase activity of constructs in which the miR-15b/16–binding site was mutated was unaffected by miR-15b/16 overexpression (Figure 3F). To further determine the role of YAP in the miR-15b/16–mediated inhibition of VSMC growth, adenovirus encoding miR-15b/16 was co-transduced into rat primary aortic SMCs with or without YAP adenovirus, which lacks its 3′-UTR, and then the SMC growth was evaluated by WST-1 assays and cell number counting. Data from these experiments revealed that in the presence of YAP lacking its 3′-UTR, the miR-15b/16–mediated VSMC growth inhibition was significantly attenuated (Figure 3G and 3H),
suggesting that YAP as a downstream target of miR-15b/16 was involved in miR-15b/16–mediated effects on VSMC proliferation. Taken together, these data indicate that miR-15b/16 promotes SM contractile phenotype through directly targeting YAP.

Restoring the Expression of miR-15b/16 Attenuates Arterial Injury–Induced Neointima Formation in the Rat Carotid Artery Balloon Injury Model

Given that miR-15b/16 strongly inhibits VSMC proliferation and migration while promoting SMC contractile gene expression through targeting YAP in vitro (Figure 2 and 3), we next tested miR-15b/16 function in vivo in the rat carotid artery balloon injury model. After the protocol we recently established for the local viral infusion in the balloon-injured carotid artery,13 we first confirmed that local delivery of miR-15b/16 adenovirus increased the expression of miR-15b/16 2- to 4- folds compared with the control right carotid artery (Figure 4A). Moreover, overexpression of miR-15b/16 in vivo significantly inhibited neointima formation as measured by decreased neointima/media ratio and reduced neointima area as compared with control green fluorescent protein–transduced carotid arteries (Figure 4B–4D). These data demonstrate that restoring the expression of miR-15b/16 attenuates arterial injury–induced neointima formation in the rat carotid artery balloon injury model.

Overexpression of miR-15b/16 Promotes VSMC Contractile Phenotype In Vivo

Previously we have shown that the expression of YAP was significantly induced in rat balloon injury model, and blocking the induction of YAP significantly upregulated SM marker expression and decreased SMC proliferation, thereby attenuating neotima formation after arterial injury.13 Because overexpression of miR-15b/16 in vivo attenuated neointima formation after arterial injury (Figure 4) and YAP was identified as a direct target of miR-15b/16 (Figure 3), we sought to determine whether the beneficial effects of miR-15b/16 were associated with decreased YAP expression in vivo. Western blotting of proteins isolated from either green fluorescent protein control or miR-15b/16 virus–treated vessels 14 days after balloon injury revealed that exogenous overexpression of miR-15/16 impeded the arterial injury–induced YAP expression and significantly alleviated injury–induced downregulation of SM–specific differentiation genes (Figure 5A and 5B). Furthermore, overexpression of miR-15b/16 significantly decreased neointima VSMC proliferation by 40% as indicated by Ki-67 staining (Figure 5C and 5D). Taken together, these data demonstrate that miR-15b/16 promotes the VSMC contractile phenotype by targeting YAP, thereby attenuating arterial injury–induced neointima formation (Figure 5E).

Discussion

This study provides the first evidence demonstrating a novel role for miR-15b/16 in targeting YAP to regulate the phenotype of VSMCs. We found that miR-15b/16 was highly expressed in contractile VSMCs and was downregulated in phenotypically modified VSMCs concomitant with increased YAP expression (Figure I in the online-only Data Supplement and Figure 1).13 We identified YAP as a direct downstream target of miR-15b/16 (Figure 3E and 3F). Because miR-15b and miR-16 share the identical seed sequence to bind to YAP 3′UTR, it is conceivable that individual miR-15b and miR-16 have similar effects on YAP 3′UTR reporter activity. Furthermore, we found that overexpression of miR-15/16 significantly represses YAP expression in vitro and in vivo (Figure 3A and 3B and Figure 5A). Conversely, depletion of endogenous miR-15b/16 resulted in an upregulation of YAP expression and decreased expression of contractile proteins (Figure 3D). Therefore, our study suggests that the downregulation of miR-15b/16 in response to vascular injury alleviates its repression of YAP in vitro and in vivo, permitting YAP to drive VSMC from a contractile phenotype toward a synthetic proliferative phenotype. In further support of the negative relationship between miR-15b/16 and YAP expression,
previous studies have shown that miR-16 family members are drastically upregulated, whereas the expression of YAP is significantly downregulated during the arrest of cardiomyocyte proliferation in the postnatal mouse heart. Similarly, the expression of a subset of miR-16 family miRs is strikingly upregulated during the maturation of mouse aorta when the VSMCs transit from a proliferative to a quiescent phenotype.

In addition to directly targeting YAP, it is likely that miR-15b/16 modulates VSMC proliferation by also targeting YAP-regulated genes that control cell cycle. The miR-16 family has been proposed to be a set of master inhibitors for cell proliferation by silencing many overlapping target genes involved in the cell cycle progression, especially G1-S transition, including cyclin D1, cyclin E1, and cdc25A. Furthermore, several other cell cycle genes were revealed to be regulated by miR-16 family, including cyclin D2, cyclin D3, cyclin-dependent kinase 6, check point kinase 1, cdc2, Birc5, and cdc42. YAP activates a spectrum of genes involving in cell cycle that significantly overlap with the miR-16 family targets, including cyclin D1, cyclin E1, check point kinase 1, cdc2, and Birc5. Among these, cyclin D1 was initially identified as a bona fide target gene of YAP and can be targeted by miR-16 to both promote mRNA degradation and inhibit protein translation. Previous study has shown that increased cyclin D1 expression was sufficient to mediate the injury-induced neointima formation by promoting VSMC proliferation. Moreover, we have shown that YAP can induce cultured VSMC proliferation in vitro through induction of cyclin D1.

Together, these findings suggest that the inhibitory effects of overexpressed miR-15b/16 on neotima formation in vivo likely result from a combination of its ability to target both YAP and cyclin D1. Therefore overexpression of miR-15b/16 would have more potent inhibitory effects on SM cell proliferation than silencing YAP alone because miR-15b/16 may target additional cell cycle regulators other than the shared target cyclin D1 with YAP. Although miR-195, another member of the miR-16 family, has been reported to inhibit VSMC proliferation, we found that expression of miR-195 is low compared with the expression of miR-15b/16 in VSMCs (Figure I in the online-only Data Supplement).

In this study, we provide a novel mechanistic insight into the post-transcriptional regulation of YAP expression in VSMCs by miR-15b/16. Because YAP protein is overexpressed in an array of tumors in humans and in injured vessels, studying the regulation of endogenous YAP expression is of great clinical importance. Initial studies have concentrated on investigating YAP phosphorylation, degradation, and nuclear localization. However, emerging evidences suggest that the expression of YAP can be regulated at both transcriptional and post-transcriptional levels. For example, c-Jun, β-catenin, CAM response element-binding protein, and the Ets family member GA-binding protein can regulate YAP gene transcription. Recently, the activating transcription factor, ATF-4, has also been shown to promote YAP...
transcriptional induction in the cells experiencing endoplasmic reticulum stress, thereby promoting cell survival. YAP expression is known to be regulated post-transcriptionally by several miRs. During preparation of the article, we noticed that miR-15a/16 targeted YAP to inhibit cell proliferation and migration in gastric adenocarcinoma cells. In addition to miR-16 family members, YAP can be regulated by miR-375 in liver, lung, pancreatic cancer cell, and thyroid cancer cell. Additionally, miR-141, -200a, and -506 have been implicated in regulating YAP expression in esophageal squamous cell carcinoma, breast cancer cell, and hepatoma cancer cell, respectively. A recent report also demonstrated that YAP can be regulated at the epigenetic level. Together, these studies suggest that regulation of YAP gene expression is complex and is development stage-, cell- and tissue context–dependent. Interestingly, in this study, we found over-expression of miR-15b/16 specifically downregulates YAP protein expression without affecting YAP mRNA expression level (Figure 3A–3C), suggesting that the inhibitory effects of miR-15b/16 is through suppressing the translation of YAP. However, we previously reported that YAP protein and mRNA levels are both increased after vascular injury, suggesting that miR-15b/16–mediated decrease in YAP protein expression are only a part of mechanism accounting for increased YAP expression after vascular injury. Given that ATF-4 promotes YAP expression and ATF-4 is induced in VSMCs after arterial injury to mediate neointima thickening, it is likely that ATF-4 plays a role in the transcriptional upregulation of YAP in VSMCs after injury. Additional studies are needed to confirm this possibility.

Restoring expression of miR-15b/16 after balloon injury significantly attenuated neointima formation (Figure 4 and 5). This data together with strong effects of miR-15b/16 on promoting VSMC contractile phenotype suggest that overexpression of miR-15b/16 family may be an attractive therapeutic strategy for treating proliferative VSMC-related diseases, such as atherosclerosis and restenosis. However, the potent ability of miR-16 family to inhibit cell proliferation may be a double-edged sword because it also affects endothelial cell proliferation and migration that is involved in angiogenesis and re-endothelialization. Therefore, any therapies aimed at targeting miR-16 family in the cardiovascular system are required to be cell type–specifically targeted.

In summary, this study identifies miR-15b/16 as a critical modulator in promoting VSMC contractile phenotype by targeting, at least in part, YAP. Therefore, restoring miR-15b/16 expression in the VSMCs would be an attractive therapeutic approach for treatment of VSMC-related occlusive vascular diseases.

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Disclosures

None.

References

occlusive vascular diseases. Our study not only provides a novel mechanistic insight into the regulation of YAP expression at the post-transcriptional level, but also of silencing YAP and promotes the smooth muscle contractile phenotype, thereby attenuating neointima formation after arterial injury. Overexpression of miR-15b/16 mimics the effects of YAP and promotes the smooth muscle contractile phenotype, thereby attenuating neointima formation after arterial injury in vivo. Our study not only provides a novel mechanistic insight into the regulation of YAP expression at the post-transcriptional level, but also suggests that restoring miR-15b/16 expression in vascular smooth muscle cells would be a potential therapeutic strategy for treatment of occlusive vascular diseases.

Significance
Phenotypically switched vascular smooth muscle cells are the major contributor to the neointima formation after arterial injury. Previously, we found that yes-associated protein (YAP) is induced and plays a crucial role in the phenotypic switching of vascular smooth muscle cells in response to arterial injury. However, the mechanism by which YAP was upregulated during phenotypic switching of vascular smooth muscle cell is unknown. In this study, we demonstrate that YAP is a direct target of microRNA (miR)-15b/16. We find that expression of miR-15b/16 is significantly reduced in concomitant with the upregulation of YAP after arterial injury. Overexpression of miR-15b/16 mimics the effects of YAP signaling and promotes the smooth muscle contractile phenotype, thereby attenuating neointima formation after arterial injury. Our study not only provides a novel mechanistic insight into the regulation of YAP expression at the post-transcriptional level, but also suggests that restoring miR-15b/16 expression in vascular smooth muscle cells would be a potential therapeutic strategy for treatment of occlusive vascular diseases.
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Supplemental Methods

Primary VSMC culture and PDGF-BB treatment. Rat arterial tissues were harvested and rat primary aortic SMCs were prepared and cultured as described in our previous reports. Briefly, male Sprague-Dawley rats (200-250 grams; Taconic Farms, Germantown, NY) were euthanized by CO₂ and the thoracic aorta was dissected to remove adhering periadventitial tissue and the endothelium was denuded with a catheter. The aorta either was directly harvested for total RNA exaction or was digested with a Blend enzyme III solution (Roche, 0.5U/ml) for 10 min at 37°C followed by dissection to remove the adventitial layer, then the remaining medial layer was minced into small pieces for a second digestion with Blend enzyme III for 2 hours at 37°C. Following removal of digestion solution and re-suspending in 10% FBS DMEM medium, cells were gently liberated with a pipette and transferred into culture dishes. Every batch of smooth muscle cells was tested by smooth muscle marker SM α-actin staining to ensure the purity of primary VSMCs above 95%. Human coronary artery SMCs were purchased from Invitrogen (catalog number: C-017-5C) and cultured essentially following the manufacturer’s protocol. All SMC cultures used in this study were less than 6 passages. For PDGF-BB treatment experiments, rat primary aortic VSMCs were grown to 80-90% confluence and serum-starved for 24 hours and then treated with recombinant rat 50 ng/ml PDGF-BB (platelet-derived growth factor BB, Calbiochem) for 48 hours as described in our previous reports. Cells treated with vehicle served as control. Following PDGF-BB or vehicle administration cells were harvested for total RNA to detect microRNA expression by qRT-PCR.

Quantitative real time RT-PCR (qRT-PCR) analysis for mature microRNAs. Total RNA from rat thoracic aorta, rat primary vascular SMCs or human coronary artery VSMCs was isolated with TRIzol reagent. To measure mature microRNAs in tissue or cells, the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA for TaqMan MicroRNA Assays (Applied Biosystems) following the manufacturer’s protocol. The following probes were used: miR-145, 002278; miR-15a, 000389; miR-15b, 000390; miR-16, 000391; miR-195, 000494; miR-424, 001076; miR-497, 001346; U6 snRNA, 001973 for endogenous control.
qRT-PCR analysis for gene expression. Total RNA from rat primary VSMCs was isolated with TRIzol reagent and qRT-PCR was performed with respective gene-specific primers as we previously reported. All samples were amplified in duplicate and every experiment was repeated independently 2 times. Relative gene expression was converted using the \(2^{-\Delta\Delta CT}\) method against the internal control acidic ribosomal phosphoprotein P0 (RPLP0) house-keeping gene.

Generation of adenovirus for expression of rat miR-15b/16. A 600-bp fragment spanning the genomic region of rat miR-15b and miR-16-2 cluster was amplified by PCR using rat brain genomic DNA as a template (see the primer sequences in the Online Table 1) and cloned into a pAdTrack vector under the control of CMV promoter to express mature microRNAs. This vector contains another independent CMV-driven transcription cassette for GFP (green fluorescent protein), therefore the efficiency of transduction can be directly monitored by visualization of GFP expression. The linearized miR-15b/16-pAdTrack vector then was electroporated into BJ5183 competent AdEasy cells (Stratagene), resulting in a recombination of the expression cassettes into AdEasy adenoviral backbone. The recombinant adenoviral DNA was transfected into HEK293 cells for viral packaging and the adenovirus was harvested for cell infection as described previously. GFP control virus was generated by same method except no insert in pAdTrack vector.

Delivery of antisense oligonucleotides into VSMCs. Rat VSMCs were transfected either with control or miR-16 family (miR-15b and miR-16) LNA (locked nucleic acids) microRNA antisense oligonucleotides (Exiqon) at final concentration 25nM each using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s protocol. 72 hours later, cells were harvested for qRT-PCR or Western blot as described above.

VSMC proliferation assay. Proliferation of rat aortic VSMCs was measured by cell proliferation WST-1 kit (Roche) in 96-well format. Following silencing or over-expression of miR-15b/16, rat primary aortic VSMCs were maintained in 0.2% FBS medium for additional 24 hours to allow growth arrest. Cells were then treated with 1% FBS, 50 ng/ml PDGF-BB or 10% FBS medium as indicated in figures. After 24 hours the rates of proliferation were determined with incubation of 10 μl/well WST-1 for 4 hours, and then measured the absorbance at 450 nm with a plate reader. For VSMC counting, rat aortic VSMCs after miR-15b/16 over-expressing or knock-down
were seeded in 60mm dish at equal density and then cell numbers were manually counted at the days as indicated in figures by a hemocytometer.

**Cell cycle analysis by flow cytometry.** Flow cytometry was performed to analyze VSMC cell cycle as described in our recent report. Briefly, rat aortic VSMCs were transduced with control GFP adenovirus or adenovirus co-expressing miR-15b/16 and GFP for 48 hours. Cells were then harvested and washed with phosphate-buffered saline and fixed overnight with 70% ice-cold ethanol at -20 °C. The fixed cells were stained with a solution containing propidium iodide (25 μg/ml) and then analyzed with a Becton Dickinson FACSAria II SORP cell sorter flow cytometer at the core facility. Only GFP positive cells in both control and over-expressing miR-15b/16 groups were gated for further cell cycle analysis. G0/G1, S and G2/M phases were assessed by fitting the cell distribution using Cell Quest and Modfit software (Becton Dickinson).

**Assessment of VSMC migration by Boyden chamber assay.** Boyden chamber assays were carried out as previously described. Briefly, rat aortic primary VSMCs were grown in medium containing 10% FBS for 48 hours post infection with miR-15b/16 or control GFP adenovirus or post transfection 48 hours with miRCURY LNA miR-16 family inhibitors. Subsequently, the treated cells were trypsinized and seeded into Boyden chambers (PET track-etched, 8-μm pores, 24-well format; Becton Dickinson) in serum-free DMEM medium. Chambers were then immersed in 10% FBS medium for 5 hours. The top-side of the membranes was swabbed to remove cells, and then cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde, stained with DAPI to visualize nuclei, and counted under fluorescence microscopy. Five identically located fields per membrane were averaged for quantification of migrated cell numbers.

**Protein extraction and Western blotting.** Rat carotid arteries were harvested by cutting open longitudinally and removing off the adventitia as described in our recent reports. Tissues were cut into small pieces and ground with a glass homogenizer in RIPA buffer (Fisher) with 1% proteinase inhibitor cocktail (Pierce) and 1% PMSF. After sonication and centrifugation of the cell lysate, proteins were quantified by BCA assay and then loaded in a 6-12.5% SDS-PAGE gel at 5-20 μg per lane. Protein was extracted from rat primary aortic SMCs as previously described. Antibodies used in this study were: β-actin (Sigma, A5316, 1:10000), calponin (Sigma, C2687, 1:5000), cyclin D1 (Cell Signaling, #2926, 1:1000), GAPDH (Santa Cruz, sc-20357,
1:2000), Hic-5 (BD, 611164, 1:5000), MLCK (Sigma, M7905, 1:5000), PCNA (Santa Cruz, sc-56, 1:500), SM22α (abcam, ab10135, 1:5000), SM α-actin (Sigma, A2547, 1:10000), TEAD1 (BD, 610922, 1:1000), vinculin (Sigma, V4505, 1:5000), YAP (Sigma, WH0010413M1, 1:1000).

Images were taken by ImageQuan LAS4000 Imaging Station (GE) and band densities were quantified using the ImageQuant TL software (GE).

**Dual luciferase reporter assay.** For generating human YAP 3'-UTR reporter, a 1.8-k bp fragment of YAP 3'-UTR containing a putative miR-16 binding site was amplified by PCR with primers harboring SacI and Pmel restriction enzyme sites (primer sequences were listed in the Online Table I) from human genomic DNA and then inserted to 3' of the firefly luciferase gene in pMir-report vector (Ambion). Mutation of miR-16 seeding site in YAP 3'-UTR was carried out with QuickChange Site-Directed Mutagenesis kit (Stratagene). All plasmids were sequenced to verify the integrity of the insert. Transfection in HEK293 cells was carried out with X-tremeGENE 9 transfection reagent (Roche) as previously described. The level of luciferase activity was evaluated by measurement of the firefly luciferase activity relative to the internal control TK-renilla luciferase activity using the Dual Luciferase Assay System essentially as described by the manufacturer (Promega). A minimum of six independent transfections were performed and all assays were replicated at least twice. Results are reported as the mean ± SE.

**Adenoviral infusion in rat carotid balloon angioplasty.** Adenovirus expressing miR-15b/16 or GFP was generated as described above and purified using Fast-Trap adenovirus purification and concentration kit by EMD Millipore. Rat balloon angioplasty was carried out as previously described. Briefly, male Sprague-Dawley rats (350 g; Taconic Farms, Germantown, NY) were anesthetized with xylazine 4.6 mg/kg and ketamine 70 mg/kg via intraperitoneal injection. Following a midline cervical incision and muscular tissues separation, the left common carotid artery was exposed and blunt dissection was performed alongside the artery by dull forceps to expose the carotid artery bifurcation into the internal/external branches. Blood flow cessation was achieved by arterial clamps and a small arteriotomy was made in the external carotid artery. A 2F Fogarty balloon embolectomy (Edwards) was inserted through the small cut and passed into the common carotid artery. After balloon inflation at 1.6-2.0 atm of pressure, the catheter was partially withdrawn and reinserted 3 times. Concentrated adenoviral solutions encoding GFP or miR-15b/16 (100µl each) were infused into the injured segment of the common carotid artery and incubated for 30 minutes. After the viral treatment, residual viral solutions in lumen
were aspirated to avoid viral solutions into the systemic circulation. A permanent ligation was then placed in the external carotid artery, and the blood flow in the common carotid artery and its internal branch was restored by releasing arterial clamps. The right intact carotid artery served as a contralateral control. The use of experimental rats for arterial injury procedures, including BSL-2 viral work was approved by the IACUC and Biosafety committees at Georgia Regents University.

**Sections, hematoxylin & eosin (H&E) staining, immunofluorescence (IF).** Rats were euthanized by asphyxiation with CO₂ and fragments of carotid arteries were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin for histological assays as described in our previous reports. Sections were cut at 7-µm thickness, deparaffinized and antigen retrieval was done by using microwave to heat at 98°C for 5 minutes in citric acid buffer (10mM, pH6.0). After goat serum (10%, Invitrogen) blocking for 30 minutes, sections were then incubated with anti Ki-67 (Thermo Scientific, rabbit, 1:30) and anti SM α-actin (Sigma, mouse, 1:600) antibodies. The sections were then stained with fluorescence-labeled secondary antibody (488nm anti-rabbit secondary antibody or 647nm anti-mouse secondary antibody, 1:250 dilution, Invitrogen) diluted in blocking buffer for 1 hour at room temperature. Sections were immersed with mounting medium (ProLong Gold antifade reagent with DAPI, Invitrogen) to visualize nuclei. Sections after stained with fluorescence-labeled secondary antibody were imaged using confocal microscopy (LSM 780 upright, Zeiss) at 40x magnification. H&E staining was performed following standard protocol and images were acquired using an Olympus BX41 upright microscope. The areas of intima and media were measured by ImageJ software (NIH).

**Statistical analysis.** Data are expressed as means ± SE, and statistical analysis using unpaired t test (two group comparison) or two-way ANOVA (multiple group comparison) was done with Prism software (Graphpad). Differences with p values < 0.05 were considered significant.
References


Online Figure Legends

Online Figure I. Expression abundance of miR-16 family in rat aortic tissue and VSMCs.
A. qRT-PCR was performed to assess miR-145 and miR-16 family microRNA expression in the rat thoracic aortae. The miR-145 expression was set to 1 (red line). N=4. B. Rat primary aortic SMCs or human coronary artery SMCs (C) were harvested for qRT-PCR to measure miR-145 and miR-16 family microRNA expression as described in panel “A”. N=3. D. qRT-PCR was performed to measure miR-15b and miR-16 expression in mouse aortic VSMCs, endothelial cells and peritoneal macrophages.

Online Figure II. Validation of adenovirus-mediated over-expression of miR-15b/16 and knock-down efficacy after transfection of antisense oligonucleotides against miR-16 family in rat aortic primary SMCs
A. After transduction of miR-15b/16 adenovirus for 48 hours, rat primary aortic SMCs were harvested for qRT-PCR to evaluate microRNA expression as indicated. N=3. B. Total RNA was extracted from the rat primary aortic SMCs that were transfected either with control or antisense oligonucleotides against miR-15b and miR-16, and then qRT-PCR was carried out to measure the microRNA expression as indicated. MiR-145 served as a negative control to demonstrate the specificity of the antisense oligonucleotides.
Online Figure II

A

B

GFP
miR-15b/16

miR-15a
miR-15b
miR-16

miR-15b
miR-16
miR-15

Anti-control
Anti-miR-15b/16

Relative Expression Level

Relative Expression Level

*
Online Table I. List of primer sequences used in the study.

A. Taqman probes used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>miR Name</th>
<th>Species</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>miR-15a</td>
<td>Mouse/rat/human</td>
<td>UAGCAGCACAUAAUGGUUUGUG</td>
</tr>
<tr>
<td>miR-15b</td>
<td>Mouse/rat/human</td>
<td>UAGCAGCACAUCAUGGUUUACA</td>
</tr>
<tr>
<td>miR-16</td>
<td>Mouse/rat/human</td>
<td>UAGCAGCACGUAUAUUGGCG</td>
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<tr>
<td>miR-145</td>
<td>Mouse/rat/human</td>
<td>GUCCAGUUUUCCCAGAAUCCC</td>
</tr>
<tr>
<td>miR-195</td>
<td>Mouse/rat/human</td>
<td>UAGCAGCACAGAAUAUUGGC</td>
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<tr>
<td>miR-424</td>
<td>Mouse/rat/human</td>
<td>CAGCAGCAUUCAUGUUUGGA</td>
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<tr>
<td>miR-497</td>
<td>Mouse/rat/human</td>
<td>CAGCAGCACACUGGUUUGUA</td>
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B. Primers used for cloning of human YAP 3'-UTR luciferase reporter and mutagenesis

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<td>YAP 3'-UTR luci F (Sacl):</td>
<td>5'-AGAGCTCAGCCCTCAGGCAGACTGAATTCTAAAT-3'</td>
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<tr>
<td>R (Pme1):</td>
<td>5'-AGTTAAACACATTGAAAGACCCCTTTAAACACAGAGG-3'</td>
</tr>
<tr>
<td>YAP 3'-UTR mut luci F:</td>
<td>5'-TTTTGCTCTTCTTTGCTCAGTACGTAAATGTATTGCTGACCTC-3'</td>
</tr>
<tr>
<td>R:</td>
<td>5'-GAGTGTCAGCAATACATTAACGTACCTGATGGCAAGGAAGAGCAAAA-3'</td>
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C. Primers used for cloning of rat miR-15b/16-2 cluster

<table>
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<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>miR-15b/16-2 adenovirus F (NotI):</td>
<td>5'-TGCGGCGCTAAGTGGAAGAGACATTAAAGA-3'</td>
</tr>
<tr>
<td>R (EcoRV):</td>
<td>5'-AAGCTAGCTAGCCATTGTGACAAATTTAAAACCCAC-3'</td>
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