A Necessary Role of miR-221 and miR-222 in Vascular Smooth Muscle Cell Proliferation and Neointimal Hyperplasia

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Abstract — MicroRNAs (miRNAs) comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate gene expression. Functionally, an individual miRNA is as important as a transcription factor because it is able to regulate the expression of its multiple target genes. Recently, miR-221 and miR-222 have been found to play a critical role in cancer cell proliferation. However, their roles in vascular smooth muscle cell (VSMC) biology are currently unknown.

In the present study, the time course changes and cellular distribution of miR-221 and miR-222 expression were identified in rat carotid arteries after angioplasty, in which their expression was upregulated and localized in VSMCs in the injured vascular walls. In cultured VSMCs, miR-221 and miR-222 expression was increased by growth stimulators. Knockdown of miR-221 and miR-222 resulted in decreased VSMC proliferation in vitro. Using both gain-of-function and loss-of-function approaches, we found that p27(Kip1) and p57(Kip2) were 2 target genes that were involved in miR-221– and miR-222–mediated effect on VSMC growth. Finally, knockdown of miR-221 and miR-222 in rat carotid arteries suppressed VSMC proliferation in vivo and neointimal lesion formation after angioplasty. The results indicate that miR-221 and miR-222 are novel regulators for VSMC proliferation and neointimal hyperplasia. These findings may also represent promising therapeutic targets in proliferative vascular diseases. (Circ Res. 2009;104:476-487.)

Key Words: microRNAs ■ vascular smooth muscle cells ■ gene regulation ■ proliferation ■ vascular disease

Recently, the most significant breakthrough regarding gene expression regulation has been the discovery of microRNAs (miRNAs).1 miRNAs comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs.1-5 More importantly, one miRNA is able to regulate the expression of multiple genes because it can bind to its mRNA targets as either an imperfect or perfect complementarity. Thus, one miRNA is as functionally important as a transcription factor.6 More than 700 miRNAs have been identified and sequenced in humans,7 and the estimated number of miRNA genes is as high as 1000 in the human genome.8 As a group, miRNAs may directly regulate at least 30% of the genes in the human genome.9 It is not surprising that miRNAs are involved in the regulation of almost all major cellular functions, such as cell differentiation, proliferation/growth, mobility, and apoptosis.1 For that reason, miRNAs could be the pivotal regulators in normal development and physiology and disease development, including cancer and cardiovascular disease.10-13

The biological function of an individual miRNA is cell specific. One miRNA may have different cellular effects on different cells. For example, miR-21 has an antiapoptotic effect on glioblastoma cells but increases Hela cell apoptosis.14,15 Recent studies have revealed that miR-221 and miR-222 are upregulated in cancer cells.16-21 Both miR-221 and miR-222 have a proproliferative effect on cancer cells.16-21 It is well established that aberrant proliferation of vascular smooth muscle cells (VSMCs) is a key cellular event in the pathogenesis of a variety of proliferative vascular diseases.22 In our recent study, we have demonstrated via microarray analysis that miR-221 and miR-222 are upregulated in vascular walls with neointimal lesion formation.23 However, their roles in VSMC biology are currently unknown. The objective of the present study is to establish the roles miR-221 and miR-222 in VSMC growth and neointimal formation and their molecular mechanisms using both cultured VSMCs in vitro and balloon-injured rat carotid arteries in vivo.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

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Rat Carotid Artery Balloon Injury Model
Carotid artery balloon injury was induced in male Sprague–Dawley rats (250 to 300 g) as described.23

Local Oligo Delivery Into the Injured Vascular Walls
miR-221 and miR-222 were downregulated in the balloon-injured rat carotid artery via miR-222 and miR-221 inhibitor (2′OMe-miR-222) using an established local oligo delivery model with little modification.23

Morphometric Analysis for Neointimal Lesion Formation
Morphometric analysis was performed in carotid artery sections stained with Masson’s trichrome staining as described.23

Immunofluorescence of Proliferating-Cell Nuclear Antigen and Smooth Muscle α-Actin
Proliferating cells were evaluated in vessel sections by using the immunofluorescence of proliferating-cell nuclear antigen (PCAN). Smooth muscle (SM) α-actin was detected by immunofluorescence.

Cell Culture
VSMCs and endothelial cells were obtained from rat aortas.23

Oligo Transfection, miR-221 and miR-222 Knockdown, miR-221 and miR-222 Overexpression, and p27(Kip1) and p57(Kip2)
Gene Knockdown in Cultured VSMCs
Oligo transfection, miR-221 and miR-222 knockdown, miR-221 and miR-222 overexpression, and p27(Kip1) and p57(Kip2) gene knockdown in cultured VSMCs were performed as described in the expanded Materials and Methods section (see the online data supplement).

VSMC Proliferation In Vitro
VSMC proliferation in vitro was determined by cell counting and bromodeoxyuridine (BrdUrd) incorporation assay.23

Detection of miR-221 and miR-222 in Vessel Sections by Fluorescence In Situ Hybridization
In situ hybridizations (ISH) of miR-221 and miR-222 and Commmunofluorescence with the smooth muscle marker SM α-actin were performed in 10-μm vessel sections.24,25

Western Blot Analysis
Proteins were isolated from cultured VSMCs and carotid arteries and protein levels were determined by Western blot analysis.

Construction of the Adenoviruses
The adenoviruses expressing miR-221 (Ad-miR-221) or miR-222 (Ad-miR-222) and control viruses expressing green fluorescent protein (GFP) (Ad-GFP), miR-31 (Ad-miR-31), or miR-145 (Ad-miR-145) were generated using the Adeno-X Expression Systems 2 kit according to the protocols of the manufacturer (Clontech, Mountain View, Calif).

Luciferase Assay
A construct in which a fragment of the 3′ untranslated region (3′-UTR) of p27(kip1) or p57(kip2) mRNA containing the putative or mutated miR-221 and miR-222 binding sequence was used.

Results
The Expression of miR-221 and miR-222 Are Increased in Vascular Walls With Neointimal Lesion Formation After Angioplasty
In the present study, the upregulation of miR-221 and miR-222 was verified by qRT-PCR, and the time course expression changes of both miRNAs in carotid arteries after angioplasty were displayed in Figure 1A. Undoubtedly, both miR-221 and miR-222 were upregulated in the balloon-injured arteries. To further confirm the upregulation of miR-221 and miR-222 was unique for injured arteries, we also measured the expression of several miRNAs that did not change based on the microarray data. We found via qRT-PCR that the expression some miRNAs such as miR-152, miR-181C, and miR-142 did not change after balloon injury (supplemental Figure I).

Both miR-221 and miR-222 Are Localized in VSMCs of the Vascular Wall
To determine the distribution of miR-221 and miR-222 expression in the vascular wall, in situ hybridization was performed on the rat carotid arteries. Vessel structure was demonstrated via Masson’s trichrome staining in frozen sections of balloon-injured rat carotid arteries as shown in Figure 1B. In situ hybridization of miR-221 (Figure 1F) and miR-222 (Figure 1G) showed that it was expressed in the vessel media and neointima where VSMCs were localized. To confirm that VSMC contained miR-221 and miR-222, communofluorescence with the smooth muscle marker SM α-actin was performed. As expected, SM α-actin was observed in VSMCs that were located in the media and neointima (Figure 1E through 1G). In addition, both miR-221 (Figure 1F) and miR-222 (Figure 1G) were clearly colocalized with VSMCs. In contrast, there was no miR-221 and miR-222 expression in 3 control sections: negative control (had SM α-actin antibody but had no SM α-actin antibody and scrambled miRNA probe) (Figure 1E), scrambled probe control 1 (no SM α-actin antibody but had scrambled miRNA probe) (Figure 1D), and scrambled probe control 2 (had SM α-actin antibody and scrambled miRNA probe) (Figure 1E).

It should be noted that we performed in situ staining in balloon-injured vessel sections, in which endothelial cells were removed by balloon catheter during the injury. To further confirm the cellular distribution, we had also determined the levels of miR-221 and miR-222 in isolated rat aortic VSMCs and endothelial cells using qRT-PCT. As shown in supplemental Figure II, miR-221 and miR-222 were also expressed in endothelial cells, even though the expression levels were a little lower than those in VSMCs.
The Expression of miR-221 and miR-222 Are Increased in Proliferative VSMCs Stimulated by Either Platelet-Derived Growth Factor or Serum

To investigate the potential link between VSMC proliferation and miR-221 and miR-222 expression, the expression of miR-221 and miR-222 was determined in nonproliferative VSMCs and in proliferative VSMCs stimulated by either platelet-derived growth factor (PDGF)-BB (20 ng/mL) or 10% serum. As shown in Figure 2A and 2B, the expression of miR-221 and miR-222 in both PDGF-BB– and serum-treated VSMCs was significantly higher than that in vehicle-treated VSMCs, which was time-dependent. The dose-dependent response in miR-221 and miR-222 expression to PDGF was measured in cultured VSMCs after 48 hours of treatment with different concentrations of PDGF, and the result is shown in Figure 2C. These results demonstrated that the expression of miR-221 and miR-222 was upregulated in proliferating VSMCs.

Knockdown of miR-221 and miR-222 Inhibits Proliferation of Cultured VSMCs

To further determine the potential roles of miR-221 and miR-222 in VSMC proliferation, we applied antisense oligonucleotide-mediated miRNA depletion to knock down miR-221 and miR-222. After 48 hours of treatment, the miR-221 inhibitor 2’OMe-miR-221 inhibited miR-221 expression but had no effect on miR-222 expression (Figure 3A). However, the miR-222 inhibitor 2’OMe-miR-222 inhibited the expression of both miR-221 and miR-222 (Figure 3A). Addition of both miR-221 and miR-222 inhibitors could not give additional inhibition on the expression of miR-221 and miR-222 compared with 2’OMe-miR-222 only, although the reason was unclear (Figure 3A). We therefore used 2’OMe-miR-222 as the inhibitor for both miR-221 and miR-222.

In subsequent experiments, we determined the effect of 2’OMe-miR-222 on VSMC proliferation by using 2 different methods: cell counting and BrdUrd incorporation assay as described. Consistent with the levels of miR-221 and miR-222 in Figure 3A, 2’OMe-miR-222 significantly decreased cell numbers and BrdUrd incorporation at 48 hours after culture with DMEM containing 10% FBS (Figure 3B and 3C). Representative BrdUrd-stained cell photomicrographs (top), their corresponding total cell photomicrographs stained by 4’,6-diamidino-2-phenylindole (DAPI) (middle), and merged photomicrographs (bottom) are shown in Figure 3D. Because we found that miR-221 inhibitor, 2’OMe-miR-
221 could only inhibit miR-221, we determined the effect of miR-221 inhibition on VSMC proliferation. We found that miR-221 inhibition also inhibited VSMC proliferation. However, the inhibitory effect on VSMC proliferation is smaller than that in both miR-221– and miR-222–inhibited cells via 2′OMe-miR-222 (supplemental Figure III). The results indicated that both miR-221 and miR-222 had a proproliferative effect on cultured VSMCs. In contrast, control oligo had no effect on VSMC proliferation.

**p27(Kip1) and p57(Kip2) Are Target Genes of miR-221 and miR-222 in VSMCs**

Computational analysis from previous studies have suggested that p27(Kip1) and p57(Kip2) are 2 potential target genes of miR-221 and miR-222. Supplemental Figure IV shows that both p27(Kip1) and p57(Kip2) have miR-221 and miR-222 binding sites in their 3′-UTR.

If p27(Kip1) and p57(Kip2) are the target genes of miR-221 and miR-222, their expression should be downregulated in proliferative VSMCs because both miR-221 and miR-222 are upregulated in these cells. Indeed, as shown in Figure 4A (top), both p27(Kip1) and p57(Kip2) were downregulated in proliferative VSMCs stimulated by PDGF-BB (20 ng/mL). Quantification of p27(Kip1) and p57(Kip2) protein levels is shown in Figure 4A (bottom).

To verify that p27(Kip1) and p57(Kip2) are the target genes of miR-221 and miR-222, both gain-of-function and loss-of-function approaches were applied. As shown in Figure 4, 2′OMe-miR-222 decreased (Figure 4B), whereas Ad-miR-221 and Ad-miR-222 increased (Figure 4C) the expression of miR-221 and miR-222 in cultured VSMCs. In contrast, unrelated adenovirus control, Ad-miR-31, had no effect on the expression of miR-221 and miR-222 (Figure 4C). As expected, p27(Kip1) and p57(Kip2) were upregulated by 2′OMe-miR-222 (Figure 4D) and were downregulated by Ad-miR-221 or Ad-miR-222 but not by unrelated adenovirus Ad-miR-31 (Figure 4E) at protein levels. Furthermore, overexpression of miR-221 or miR-222 by Ad-miR-221 or Ad-miR-222 decreased p27(Kip1) mRNA level but had no effect on p57(Kip2) mRNA level (Figure 4F).

To further confirm that miR-221 and miR-222 are able to directly bind to p27(Kip1) and p57(Kip2) and inhibit their expression, a construct in which a fragment of the 3′-UTR of either p27(Kip1) or p57(Kip2) mRNA containing the putative miR-221 and miR-222 binding sequences was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle, an empty plasmid (pDNR-CMV), a plasmid expressing miR-221 (pmiR-221), miR-222 (pmiR-222), or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145), following the transfection procedures provided by Invitrogen. The constructs with mutated fragment of the 3′-UTR of either p27(Kip1) or p57(Kip2) mRNA without the putative miR-221 and miR-222 binding sequences were used as mutated controls. As expected, pmiR-221 and miR-222, but not pmiR-145 or pDNR-CMV, increased miR-221 or miR-222 expression in HEK 293 cells (Figure 5A). Consequently, pmiR-221 and miR-222, but not pDNR-CMV or pmiR-145, inhibited luciferase activity (Figure 5B). In the mutated control groups, the inhibitory effect of pmiR-221 and miR-222 disappeared (Figure 5B). The results suggested that miR-221 and miR-222 were able to bind to p27(Kip1) and p57(Kip2) directly and inhibit their expression.

**p27(Kip1) and p57(Kip2) Are Involved in miR-221– and miR-222–Mediated Effects on VSMC Proliferation**

As shown in Figure 6A and 6B, both p27(Kip1) and p57(Kip2) were depleted by their small interfering (si)RNAs. We next...
Figure 3. The effect of miR-221 and miR-222 inhibitor on VSMC proliferation in vitro. A, Knockdown of miR-221 and miR-222 expression by their inhibitors, 2′OMe-miR-221 (100 nmol/L), 2′OMe-miR-222 (100 nmol/L), and 2′OMe-miR-221 plus 2′OMe-miR-222 (100 nmol/L). B and C, 2′OMe-miR-222 (100 nmol/L) decreased cell numbers (B) and BrdUrd incorporation at 48 hours after culture with DMEM containing 10% FBS (C). D, Representative BrdUrd-stained cell photomicrographs (top), their corresponding total cell photomicrographs stained by DAPI (middle), and merged photomicrographs (bottom). n=8. *P<0.05 compared with vehicle control.
determined the involvement of p27(Kip1) and p57(Kip2) in 2′OMe-miR-222–induced inhibitory effect on proliferation. In this experiment, we defined the relative inhibition of cells without 2′OMe-miR-222 as 0\% and the relative inhibition of 2′OMe-miR-222 plus vehicle–treated cells as 100\%. As shown in Figure 6C, in the target gene–depleted VSMCs, 2′OMe-miR-222–induced inhibitory effect on proliferation was decreased compared with that in VSMCs without target gene depletion. The results suggested that p27(Kip1) and p57(Kip2) were functional target genes of miR-221 and miR-222 that were involved in miR-221– and miR-222–mediated effect on VSMC proliferation.
p27(Kip1) and p57(Kip2) Are Target Genes of miR-221 and miR-222 in Vascular Walls After Angioplasty  
As shown in Figure 7A, both p27(Kip1) and p57(Kip2) were down regulated in balloon-injured rat carotid arteries. The negative relationship with the expression of miR-221 and miR-222 indicated that p27(Kip1) and p57(Kip2) could be miR-221 and miR-222 target genes in vivo. To further verify this, the expression of miR-221 and miR-222 in balloon-injured rat carotid arteries was modulated by their inhibitor, 2′OMe-miR-222. As shown in Figure 7B, miR-221 and miR-222 were downregulated by 2′OMe-miR-222. Interestingly, in 2′OMe-miR-222–treated vessels, expression of p27(Kip1) and p57(Kip2) was upregulated (Figure 7C). The results demonstrated that p27(Kip1) and p57(Kip2) were target genes of miR-221 and miR-222 in vivo.

Downregulation of miR-221 and miR-222 Decreases Cell Proliferation and Neointima Formation in Rat Carotid Artery After Angioplasty  
To determine the effects of miR-221 and miR-222 on VSMC proliferation and neointimal growth in vivo, the carotid arteries were isolated 14 days after balloon injury. Representative immunofluorescence of PCAN and its negative control is shown in Figure 8A. Compared with vehicle-treated vessel, fewer cells were proliferating in the injured vascular walls treated with 2′OMe-miR-222 (Figure 8B). Furthermore, downregulation of the miR-221 and miR-222 resulted in a nearly 40% decrease in neointima formation after angioplasty (Figure 8C). Representative Masson’s trichrome–stained photomicrographs of rat carotid arteries from different groups are shown in Figure 8D.

Discussion  
mir-221 and miR-222 are encoded by a gene cluster on the X chromosome. They share the same seed and appear to have identical target genes and similar functions. Recent studies have revealed that miR-221 and miR-222 are overexpressed in several types of cancers, in which they are related to cancer cell proliferation.16–21 VSMC proliferation–mediated expansion of the neointima is a unifying facet of a variety of proliferative vascular diseases such as atherosclerosis, restenosis after angioplasty or bypass, diabetic vascular complication, and transplantation arteriopathy. In our recent report,23 we demonstrate that miR-221 and miR-222 are abundant miRNAs in the vascular wall; miR-221 is ranked 39th and miR-222 is ranked 50th among the 140 detected mature miRNAs. Interestingly, miR-221 and miR-222 are significantly upregulated in the vascular walls with neointimal lesion formation, as determined by microarray analysis.23 However, the roles of miR-221 and miR-222 in VSMC proliferation and proliferative vascular disease are currently unknown.
In the present study, the upregulation of miR-221 and miR-222 is further verified by qRT-PCR. We found that the increase in their expression is larger than that determined by microarray analysis. We think the different method of miRNA quantification could be the major reason for it. This opinion is also supported by a recent report from Miller et al.\textsuperscript{26} demonstrating a 2-fold increase in miR-221 and miR-222 expression in tamoxifen-resistant MCF cells compared with that in tamoxifen-sensitive MCF cells, as determined by microarray analysis. However, by qRT-PCR analysis, the authors demonstrate a >10-fold increase in miR-221 and miR-222 expression in tamoxifen-resistant cells. qRT-PCR might be better than microarray when we quantify a specific miRNA. Another important reason is that the samples for microarray analysis and for qRT-PCR are from a different group of rats. In addition, the rats used for microarray data are older (≈320 g) than those used for qRT-PCR (≈250 g). The age might also be a potential reason for the difference. The effect of age on miRNA expression is also supported by recent reports. For example, miR-21 expression is significantly increased in neonatal rat cardiac myocytes compared with that in adult rat cardiac myocytes.\textsuperscript{27} Another finding regarding miR-221 and miR-222 expression is that the extent of miR-221 and miR-222 upregulation in vivo in balloon-injured rat arteries is larger than that in vitro in cultured VSMCs stimulated with either PDGF or serum. The result indicates that there are multiple stimuli in vivo that could synergistically determine the upregulation of miRNAs.

It is well established that tissue- and cell-specific expression is one important characteristic of miRNA expression. One miRNA may be highly expressed in one type of cell but has no or low expression in another type of cell. To investigate the role of miR-221 and miR-222 in VSMC...
biology, we initially determined their cellular distribution in the vascular walls to see whether they were located in VSMCs. The results from in situ hybridization clearly showed that both miR-221 and miR-222 were localized in VSMCs of the injured vascular wall.

Cellular functions of a miRNA are cell type–specific. For example, miR-21 has an antiapoptotic function in glioblastoma cell and VSMCs14,15,23 but has no such a function in HeLa cells.15 In cultured cells, we found for the first time that miR-221 and miR-222 have a proproliferative effect on VSMCs. The cellular effect of miR-221 and miR-222 on VSMCs is consistent with that on cancer cells.

One surprising result from the study is that the inhibitor against miR-222 also suppresses miR-221 expression. To further confirm that the 2′OMe-miR-222–mediated inhibition is miR-221– and miR-222–specific, we determined the effect of the miRNA inhibitor on other unrelated miRNAs such as miR-24 and miR-146. No inhibitory effect was found on these unrelated miRNAs (supplemental Figure V). Although the reason that 2′OMe-miR-222 can inhibit both miR-221 and miR-222 is not clear, we think the similarity of the sequence might explain the finding.

miRNAs have multiple mRNA targets that are responsible for miRNA-mediated biological functions. First, based on the proproliferative effect of miR-221 and miR-222 on VSMCs, we hypothesize that their target gene(s) might have negative effect on cell growth. p27(Kip1) and p57(Kip2) are tumor suppressors and cell cycle inhibitors21 and might be the target genes of miR-221 and miR-222 based on recent cancer cell studies.16–21 Computational analysis for miRNA target prediction demonstrates that p27(Kip1) and p57(Kip2) have binding sites for both miR-221 and miR-222. The direct binding and inhibitory functions of miR-221 and miR-222 on p27(Kip1) and p57(Kip2) are validated in HEK 293 cells using luciferase constructs, in which the 3′-UTR fragments of these target gene mRNAs are included. To further confirm that p27(Kip1) and p57(Kip2) are target genes of miR-221 and miR-222 in VSMCs, both gain-of-function and loss-of-function approaches are applied. We have confirmed that both genes are targets of miR-221 and miR-222 in our experimental cells. Furthermore, both p27(Kip1) and p57(Kip2) are involved in miR-221– and miR-222–mediated effect on VSMC proliferation as the decreased inhibitory effect on VSMC proliferation is found in these gene deficient VSMCs. However, miR-221– and miR-222–mediated effect on cell proliferation is only partially inhibited in both p27(Kip1)- and p57(Kip2)-depleted cells, suggesting that other unidentified gene targets should be determined to completely understand their molecular mechanisms.

miRNA binds to complementary sites in the mRNA target to negatively regulate gene expression via degradation or translational inhibition of their target mRNAs. To further understand the detailed mechanisms of miR-221– and miR-222–mediated regulatory effect on expression of p27(Kip1) and p57(Kip2), we investigated their effects on expression of p27(Kip1) and p57(Kip2) in balloon-injured rat carotid arteries using antibodies to p27(Kip1) and p57(Kip2) and Western blotting.

Figure 7. p27(Kip1) and p57(Kip2) are target genes of miR-221 and miR-222 in the vascular walls after angioplasty. A, Both p27(Kip1) and p57(Kip2) proteins were downregulated in balloon-injured rat carotid arteries. B, miR-221 and miR-222 were downregulated by 2′OMe-miR-222 in balloon-injured vascular walls. C, Representative Western blot of p27(Kip1) and p57(Kip2) protein in the injured vascular walls treated with vehicle, control oligo, or 2′OMe-miR-222. D, In 2′OMe-miR-222–treated vessels, expression of p27(Kip1) and p57(Kip2) was upregulated. n=6. *P<0.05 compared with vehicle control.
Figure 8. Downregulation of miR-221 and miR-222 decreases cell proliferation and neointima formation in rat carotid artery after angioplasty. A, Representative immunofluorescence of PCNA in rat carotid arteries at 14 days after balloon injury. Green is the immunofluorescence of PCNA that represents proliferating cells. Blue is cell nuclear staining by DAPI that reflects total cells. Red is the autofluorescence in elastic laminae. B, Quantification of the proliferative cells showed that, compared with vehicle-treated and control oligo-treated vessels, fewer cells were proliferating in the injured vascular walls treated with 2’OMe-miR-222. C, Downregulation of the miR-221 and miR-222×2’OMe-miR-222 decreased neointimal formation. D, Representative Masson’s trichrome–stained photomicrographs of rat carotid arteries from different groups. n=8. *P<0.05 compared with vehicle control.
and p57(Kip2), the expression of the 2 target genes at mRNA level is determined. We have found that overexpression of miR-221 or miR-222 decreases p27(Kip1) mRNA level but has no effect on p57(Kip2) mRNA level. Recent studies have revealed that degradation of the target gene mRNA is binding site–dependent. Target gene mRNA with multiple binding sites, even with partial complementarity, is easily degraded.28 Our results match the results from computational analysis in which p27(Kip1) has 4 binding sites with partial complementarity, whereas p57(Kip2) has only 1 binding site with partial complementarity, for both miR-221 and miR-222 at their 3′-UTRs.

In addition to the effect of miR-221 and miR-222 on p27(Kip1) and p57(Kip2) expression, other miRNAs may be also involved in the regulation of p27(Kip1) and p57(Kip2) expression in injured arteries via indirect mechanisms. For example, in our previous study we have found that balloon injury of injury is able to cause an increase in miR-21 expression and that PTEN is among the targets of miR-21.23 PTEN is reported to be able to reduce the ubiquitination of p27,29 so it could be possible that the reduced PTEN levels could cause a reduction of p27 levels.

VSMC proliferation is the critical cellular event in vascular neointimal lesion formation and p27(Kip1) and p57(Kip2) are negative regulators for VSMC proliferation in vivo.30,31 In the present study, we demonstrated that p27(Kip1) and p57(Kip2) are 2 gene targets of miR-221 and miR-222 in rat carotid artery in vivo. Finally, downregulation of the overexpressed miR-221 and miR-222 significantly decreases VSMC proliferation and neointimal lesion formation in rat carotid arteries after angioplasty.

Neointimal formation does not only rely on VSMCs but also is related to reendothelialization and inflammation. Our results demonstrated miR-221 and miR-222 are expressed in both VSMCs and endothelial cells, which is consistent with other recent studies showing that miR-221 and miR-222 are also expressed in endothelial cells and hematopoietic cells.32,33 miR-221 and miR-222 are found to be able to inhibit endothelial cell migration.33 Thus, the biological effects of miR-221 and miR-222 on endothelial cells may be also involved in miR-221– and miR-222–mediated effect on vascular neointimal growth. Currently, the role of hematopoietic cells in miR-221– and miR-222–mediated vascular effect is unclear and needs to be investigated in future studies.

In summary, the present study reveals that the noncoding small miRNAs miR-221 and miR-222 are novel regulators of VSMC proliferation and vascular neointimal lesion formation via their target genes p27(Kip1) and p57(Kip2). These novel findings may have extensive implications for the treatment of a variety of proliferative vascular diseases.

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Disclosures
None.

References


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Supplement Material

Expanded Materials and Methods

**Rat carotid artery balloon injury model.** Carotid artery balloon injury was induced in male Sprague-Dawley rats (230 to 300 g) as described in our previous studies (1-3). Briefly, rats were anesthetized with ketamine (80 mg/kg)/xylazine(5 mg/kg). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Baxter Edwards) was introduced via an arteriotomy in the external carotid artery, and then the catheter was advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it three times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After injury, the external carotid artery was permanently ligated with a 6-0 silk suture, and blood flow in the common carotid artery was restored. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey and were consistent with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

**Local oligo delivery into the injured vascular walls.** miR-221 and miR-222 were downregulated in the balloon-injured rat carotid artery via their inhibitors. miR-222 and miR-221 inhibitor (2'OMe-miR-222) was synthesized by Integrated DNA Technologies and had the following sequence and structure: 5'-mAmCmCmCmAmGmUmAmGmCmCmGmAmUmGmUmAmGmCmU-3'. To
deliver the miRNA inhibitor or control oligonucleotide (Control oligo) (Ambion, Inc.) into the injured vascular tissue and to avoid any potential systemic side effects, we applied an established local oligo delivery model via F-127 pluronic gel as described in our recent report with little modification (1). Briefly, immediately after balloon injury of the right common carotid artery, transfection solutions (100 µl 0.2% transfection reagent in DMEM, Qiagen, Valencia, CA) were mixed with 100 µl 2'OMe-miR-222 (10 µg), vehicle (DMEM), or control oligonucleotides (10 µg) and infused into the ligated segment of the common carotid artery for 30 min. Then, 90 µg of these oligonucleotides, preloaded into 200 µl 30 % F-127 pluronic gel (Sigma) and 1% transfection reagent (Qiagen, Valencia, CA) at 4 °C, was applied locally to the adventitia around injured artery segments.

**Morphometric analysis for neointimal lesion formation.** Morphometric analysis via computerized image analysis system (Scion Image CMS-800) was performed on sections stained with Masson's trichrome staining as described (1-3). Six sections (5 µm thick) sectioned at equally spaced intervals of injured carotid arteries were used. The medial area was calculated by subtracting the area defined by the internal elastic lamina (IEL) from the area defined by the external elastic lamina (EEL), and the intimal area was determined by subtracting the lumen area from the area defined by the IEL. Finally, the intimal to medial area ratio (I/M) of each section was calculated. The average I/M of the six sections was used as the I/M of this animal.
Immunofluorescence of proliferating cell nuclear antigen (PCAN) and smooth muscle (SM) α-actin. Proliferating cells were evaluated in vessel sections by using the immunofluorescence of PCAN. The freeze (5 µm) sections were incubated with anti-PCAN antibodies (Santa Cruz Biotechnology) followed by fluorescein conjugated secondary antibodies (1:200 dilution, Vector Laboratories). Cell nuclei were stained with DAPI. For SM α-actin staining, the sections were then incubated with anti-SM α-actin (1:400 dilution, Dako) followed by fluorescein conjugated secondary antibodies (1:200 dilution, Vector Laboratories). Cell nuclear was stained by DAPI (blue color). The fluorescence was observed by Nikon Eclipse 80i immunofluorescence microscope.

Cell culture. VSMCs and endothelial cells (Cell Applications, Inc.) were obtained from the aortic media of male Sprague-Dawley rats (5 weeks old) by using an enzymatic dissociation method as described (1). The basic culture medium was DMEM VSMCs and M199 for endothelial cells. Cells between passage 3 and 6 were used.

Oligo transfection, miR-221 and miR-222 knockdown, miR-221 and miR-222 overexpression, and p27(Kip1) and p57(Kip2) gene knockdown in cultured VSMCs. Oligo transfection was performed according to a pre-established protocol (1, 4). Briefly, cells were transfected using a transfection reagent (Qiagen, Chatsworth, CA) 24 h after seeding into the wells. Transfection complexes were prepared according to the manufacturer's instructions. For miR-221 and miR-222 knockdown, 2’OMe-miR-222 (Integrated DNA Technologies) was added to the complexes at final oligonucleotide concentration of 100 nmol/L.
p27(Kip1) and p57(Kip2) gene knockdown was performed using p27(Kip1) and p57(Kip2) siRNA (25 nM). The transfection medium was replaced 4 h posttransfection by the regular culture medium. Vehicle and oligo controls (Ambion, Inc.) were applied. For miR-221 or miR-222 overexpression, adenovirus expressing miR-221 (Ad-miR-221) or miR-222 (Ad-222) was added the culture medium at 30 MOI. Adenovirus expressing GFP (Ad-GFP) was used as an adenovirus control.

**VSMC Proliferation in vitro.** VSMC proliferation *in vitro* was determined by cell counting and bromodeoxyuridine (BrdU) incorporation assay (1). For cell counting, the cells were detached by trypsinization and resuspended in PBS. The cells were then counted under a microscope. For BrdU incorporation assay, 10 mM BrdU was added to the culture medium for incorporation into the DNA of replicating cells. After 1 h of incubation, cells were fixed, and anti-BrdU antibody (*In Situ* Cell Proliferation Kit) was added to each well for 45 min. Finally, the proliferative cells were detected under a fluorescence microscope.

**RNA levels were determined by qRT-PCR.** Briefly, RNAs from VSMCs and rat carotid arteries were isolated with a RNA Isolation Kit (Ambion, Inc.). qRT-PCR for miRNAs was performed on cDNA generated from 50 ng of total RNA using the protocol of the mirVana qRT-PCR miRNA Detection Kit (Ambion, Inc). qRT-PCR for p27(Kip1) and p57(Kip2) was performed on cDNA generated from 200 ng of total RNA using the protocol of a qRT-PCR mRNA Detection Kit (Roche). Amplification and detection of specific products were performed with a Roche Lightcycler 480 Detection System. As an internal control, U6 was used for
miRNA template normalization and GADPH was used for other template normalizations. The sequences of the primers used are shown in supplementary Table 1. Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the U6 or GADPH Ct value, which provided the ∆Ct value. The relative expression level between treatments was then calculated using the following equation: relative gene expression = $2^{(\Delta Ct_{\text{sample}}-\Delta Ct_{\text{control}})}$ (1, 4).

**Detection of miR-221 and miR-222 in vessel sections by fluorescence in situ hybridization.** In situ hybridizations (ISH) were performed in 10-µm vessel sections (5, 6). Tissue sections were cut using a cryostat and transferred to SuperFrost/plus slides (Fisher). Slides were stored at −80°C until ISH. Vessel sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine, followed by washes in PBS. Sections were then pre-hybridized in hybridization solution (50% formamide, 5x SSC, 0.5 mg/mL yeast tRNA, 1x Denhardt’s solution) at 25°C below the predicted $T_m$ value of the LNA probe for 30 min. Probes (3 pmol) (LNA miRCURY probe; Exiqon) were DIG-labeled (DIG Oligonucleotide 3’ Tailing Kit; Roche Applied Sciences) and hybridized to the sections for 1 h at the same temperature as pre-hybridization. After post-hybridization washes in 0.1x SSC at 55°C, the ISH signals were detected using the tyramide signal amplification system (PerkinElmer) according to the manufacturer’s instructions. Slides were mounted in Prolong Gold...
containing DAPI (Invitrogen) and analyzed with a Nikon microscope equipped with a CCD camera and image software.

**Western blot analysis.** Proteins were isolated from cultured VSMCs and carotid arteries and protein levels were determined by western blot analysis. Briefly, equal amounts of protein were subjected to SDS-PAGE. Standard western blot analysis was conducted using p27 (Kip1) and p57 (Kip2) antibodies (Santa Cruz Biotechnology). GADPH antibody (1:5000 dilution; Cell Signaling) was used as a loading control.

**Construction of the adenoviruses.** The adenoviruses expressing miR-221 (Ad-miR-221) or miR-222 (Ad-miR-222), and control viruses expressing GFP (Ad-GFP), miR-31 (Ad-miR-31) or miR-145 (Ad-miR-145) were generated using the Adeno-X™ Expression Systems 2 kit (Clontech, CA) according to the manufacturer’s protocols. Briefly, fragments for the precursor miR-221, miR-222, miR-31 and miR-145 were amplified respectively with their primer pairs from rat genomic DNA and were inserted into pDNR-CMV donor vectors (Clontech, CA) at EcoR I and Xho I sites. These vectors were named pmiR-221, pmiR-222 and pmiR-31. The constructs were sequenced to confirm the DNA sequences. The miR-221, miR-222, miR-31 and miR-145 fragments were then excised from the pmiR-221, pmiR-222, pmiR-31 and pmiR-145 were inserted into the pLP-Adeno-X-CMV vectors using cre recombinase, which were termed pLP-Adeno-X-CMV-mir-221, pLP-Adeno-X-CMV-mir-222, pLP-Adeno-X-CMV-mir-31 and pLP-Adeno-X-CMV-mir-145. The pLP-Adeno-X-CMV-mir-221, pLP-Adeno-X-CMV-mir-31 and pLP-Adeno-X-CMV-mir-145 plasmids
digested by Pac I were used to transfect low-passage HEK 293 cells to produce recombinant adenoviruses with LipofectAMINE 2000 according to the manufacturer’s protocols (Invitrogen, CA). Adenovirus expressing GFP was generated as described (7). Briefly, the GFP DNA fragment was excised from pGFP-N3 (Clontech) by digestion of the plasmid with Sall and NotI and subcloned into an entry vector, pENTR3C (Invitrogen, CA), producing pENTR3C-GFP. pENTR3C-GFP was transformed into E. coli DH5α, and the plasmids were amplified. These plasmids were recombined with pAd/CMV/V5-DEST as described by the manufacturer (Invitrogen, CA), producing pAd-GFP plasmids, which were verified by DNA sequencing. The pAd-EGFP was linearized with Pael and transfected into HEK293A cells. The resulting adenoviruses (Ad-miR-221, Ad-miR-222, Ad-miR-31, Ad-miR-145 and Ad-GFP) were further amplified by infection of HEK293A cells and purified by cesium chloride gradient ultracentrifugation. The Ad-miR-221, Ad-miR-222, Ad-miR-31, Ad-miR-145 and Ad-GFP were titrated using a standard plaque assay.

**Luciferase assay.** A construct in which a fragment of the 3’-UTR of p27(kip1) or p57(kip2) mRNA containing the putative miR-221 and miR-222 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with an empty plasmid (pDNR-CMV) (0.2 µg/ml), a plasmid expressing miR-221 (pmiR-221) (0.2 µg/ml), a plasmid expressing miR-222 (pmiR-222) (0.2 µg/ml), or a control plasmid expressing an unrelated miRNA, miR-145 (pmiR-145) (0.2 µg/ml), following the transfection procedures provided by Invitrogen. The constructs with mutated fragment of the 3’-UTR of either p27(Kip1) or p57(Kip2)
mRNA without the putative miR-221 and miR-222 binding sequences were used as mutated controls. Relative luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system.

**Statistics.** All data are presented as mean ± standard error. For relative gene expression, the mean value of vehicle control group is defined as 1 or 100%. Two-tailed unpaired Student’s *t* tests and ANOVA were used for statistical evaluation of the data. Sigma stat statistical analysis program was used for data analysis. A *p* value < 0.05 was considered significant.

**References**


## Online Table I: PCR Primer Sequences

<table>
<thead>
<tr>
<th>Primer Names</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat p27kip1 real-time forward primer</td>
<td>GACGACCCCAAGCCTTCCG</td>
</tr>
<tr>
<td>rat p27kip1 real-time reverse primer</td>
<td>TCTCCACCTCTGCCACTCG</td>
</tr>
<tr>
<td>rat p57kip2 real-time forward primer</td>
<td>CCCCCATACACATCTTCACTTTACG</td>
</tr>
<tr>
<td>rat p57kip2 real-time reverse primer</td>
<td>CGACAGTCTGCTACCAAGTACG</td>
</tr>
<tr>
<td>rat GAPDH real-time forward primer</td>
<td>AAGCTCAGCTGGCATGAGC</td>
</tr>
<tr>
<td>rat GAPDH real-time reverse primer</td>
<td>CCGCATGTCAGATCCACAAC</td>
</tr>
<tr>
<td>rat p27kip1 3’ UTR forward primer</td>
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<td>rat p27kip1 3’ UTR reverse primer</td>
<td>ttgctgccgcTCCAAGGACTTAGGATTATGTTACGT</td>
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<tr>
<td>rat p57kip2 3’ UTR forward primer</td>
<td>ttctcgagTCTGCAGTAGGTTAGTTAGAGCC</td>
</tr>
<tr>
<td>rat p57kip2 3’ UTR reverse primer</td>
<td>ttgctgccgcTGTGGAATCTGAGC</td>
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Online Figure I. Vascular injury has no effect on the expression of miR-352, miR-181C and miR-342 in rat carotid arteries. Rat carotid arteries were isolated at 7 days after balloon-injury. miRNAs were measured by qRT-PCR. No difference was found in the expression of miR-352, miR-181C and miR-342 between injured vessels and uninjured controls. Note: n=6.
Online Figure II. miR-221 and miR-222 are expressed in both vascular smooth muscle cells and endothelial cells isolated from rat aortas. miR-221 and miR-222 expression was measured by qRT-PCR. Note: n=6; *P<0.05 compared with that in VSMCs.
Online Figure III. miR-221 inhibitor, 2'OMe-miR-221 inhibits VSMC proliferation. But the effect is smaller than both miR-221 and miR-22 inhibition via 2'OMe-miR-222. 2'OMe-miR-221 (100 nM) decreased cell numbers by about 20% at 48 h after culture with DMEM containing 10% FBS. Note: N=6; *P<0.05 compared with control.
Online Figure IV. miR-221 and miR-222 binding sites 3'-untranslated region (3'-UTR) of p27(Kip1) and p57(Kip2). A. One binding site for miR-221 and miR-222 in 3'-UTR of p57(Kip2). B. Four binding sites for miR-221 and miR-222 in 3'-UTR of p27(Kip1).
Online Figure V. miR-221 and miR-222 inhibitor, 2'OMe-miR-222 has no effect on other unrelated miRNAs such as miR-24 and miR-146 in cultured VSMCs. The cultured VSMCs were transfected with vehicle, control oligo or 2'OMe-miR-222 (100 nM). Twenty hours later, the RNAs were isolated and miRNAs were determined by qRT-PCR. miR-221 and miR-222 inhibition had no effect on miR-24 and miR-146 expression. Note: n=3.