miR-22 Is a Novel Mediator of Vascular Smooth Muscle Cell Phenotypic Modulation and Neointima Formation

BACKGROUND: MicroRNA-22 (miR-22) has recently been reported to play a regulatory role during vascular smooth muscle cell (VSMC) differentiation from stem cells, but little is known about its target genes and related pathways in mature VSMC phenotypic modulation or its clinical implication in neointima formation following vascular injury.

METHODS: We applied a wire-injury mouse model, and local delivery of AgomiR-22 or miR-22 inhibitor, as well, to explore the therapeutic potential of miR-22 in vascular diseases. Furthermore, normal and diseased human femoral arteries were harvested, and various in vivo, ex vivo, and in vitro models of VSMC phenotype switching were conducted to examine miR-22 expression during VSMC phenotype switching.

RESULTS: Expression of miR-22 was closely regulated during VSMC phenotypic modulation. miR-22 overexpression significantly increased expression of VSMC marker genes and inhibited VSMC proliferation and migration, whereas the opposite effect was observed when endogenous miR-22 was knocked down. As expected, 2 previously reported miR-22 target genes, MECP2 (methyl-CpG binding protein 2) and histone deacetylase 4, exhibited a regulatory role in VSMC phenotypic modulation. A transcriptional regulator and oncoprotein, EVI1 (ecotropic virus integration site 1 protein homolog), has been identified as a novel miR-22 target gene in VSMC phenotypic modulation. It is noteworthy that overexpression of miR-22 in the injured vessels significantly reduced the expression of its target genes, decreased VSMC proliferation, and inhibited neointima formation in wire-injured femoral arteries, whereas the opposite effect was observed with local application of a miR-22 inhibitor to injured arteries. We next examined the clinical relevance of miR-22 expression and its target genes in human femoral arteries. We found that miR-22 expression was significantly reduced, whereas MECP2 and EVI1 expression levels were dramatically increased, in diseased in comparison with healthy femoral human arteries. This inverse relationship between miR-22 and MECP2 and EVI1 was evident in both healthy and diseased human femoral arteries.

CONCLUSIONS: Our data demonstrate that miR-22 and EVI1 are novel regulators of VSMC function, specifically during neointima hyperplasia, offering a novel therapeutic opportunity for treating vascular diseases.
Clinical Perspective

What Is New?

- We show that microRNA-22 (miR-22) is a novel mediator of vascular smooth muscle cell phenotypic modulation and neointima formation.
- We demonstrate that miR-22 controls vascular smooth muscle cell phenotype and injury-induced arterial remodeling by modulating multiple target genes (MECP2, HDAC4, and EVI1).
- We observe that miR-22 expression is suppressed in the human femoral arteries with atherosclerotic plaques and have uncovered an inverse relationship between miR-22 and its target genes in healthy and diseased arteries.

What Are the Clinical Implications?

- Our findings on the miR-22–EVI1 and miR-22–MECP2 signaling axes in vascular smooth muscle cell phenotypic modulation present miR-22 and its target genes (EVI1 and MECP2) as novel biomarkers for peripheral arterial diseases.
- Local delivery of miR-22 in the injured arteries prevents adverse arterial remodeling, suggesting that the site-specific delivery of miR-22 mimics as a potential therapy for in-stent restenosis.

Vascular smooth muscle cell (VSMC) phenotype switching, or the phenotypic modulation of VSMCs from a differentiated, contractile state to a dedifferentiated, synthetic phenotype, has been shown to play a vital role in intima remodeling and in many cardiovascular diseases. Various environmental stimuli, such as growth factors, reactive oxidative species, and even mechanical injury, have been identified to lead to dramatic changes in VSMC phenotype and behavior. Both transcriptional and epigenetic mechanisms have been extensively implicated in VSMC phenotype switching and regulation of smooth muscle cell (SMC)–selective marker genes such as smooth muscle α-actin (SMαA), smooth muscle 22α (SM22α), smooth muscle calponin (CNN1), smooth muscle myosin heavy chain (SM-myh11), and smoothelin-B (SMTN-B). Of great interest to us is the growing evidence that supports a role for a novel class of gene regulators, microRNAs (miRs), in regulating VSMC differentiation from stem/progenitor cells and VSMC phenotype switching in response to vascular injury. Despite the growing number of identified miRs that have been implicated in VSMC differentiation and phenotypic modulation in response to injury, such as miR-1, miR-15b/16, miR-21, miR-34a, miR-133, miR-143/145 cluster, miR-182-3p, miR-214, miR-221/222, and miR-638, the epigenetic regulation of VSMC phenotype switching has yet to be fully understood.

miR-22, originally proposed to act as a tumor suppressor, has been implicated in a variety of cardiac diseases and recently reported to play a regulatory role during VSMC differentiation from stem cells. However, little is known about its downstream targets and whether there is functional involvement of miR-22 in mature VSMC phenotypic modulation and vascular injury–induced neointima formation. In this study, we have identified EVI1 (ecotropic virus integration site 1 protein homolog) as a novel target of miR-22 and have demonstrated that the miR-22/EVI1 signaling axis plays an important role in VSMC phenotype switching and arterial remodeling in both mouse and human femoral artery disease models. Our work offers a possible mechanistic basis for the beneficial effect of EVI1 inhibition using arsenic trioxide–eluting stent (AES) on in-stent restenosis.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

miR-22 Promoter, EVI1 3′–Untranslated Region Reporter, and Mutation of miR-22 Binding Site Within EVI1 3′–Untranslated Region Reporter

miR-22 promoter DNA was amplified from mouse genomic DNA by polymerase chain reaction using primers shown in Table I in the online-only Data Supplement. Amplified DNA fragments were cloned into the Kpn I and Mlu I sites of the pGL3-basic vector (Promega), designated as pGL3-miR-22. Reporter vectors harboring 3′–untranslated region (3′-UTR) sequences of the murine EVI1 were created using cDNA from VSMCs. The 3′-flanking 3′-UTR (11142 nucleotides of the 3′-UTR region) of murine EVI1 gene (NM_007963) was ampliﬁed by polymerase chain reaction with primers shown in Table I in the online-only Data Supplement and cloned into the Sac I and Mlu I sites of the pmiR-reporter-basic vector (Ambion, Applied Biosystems), designated as pmiR-Luc-EVI1-WT. miR-22 binding site mutation was introduced into pmiR-Luc-EVI1 by using the QuikChange site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions and designated as pmiR-Luc-EVI1-mutant. All plasmids were veriﬁed by DNA sequencing at GATC Biotech.

Mouse Femoral Artery Denudation Injury and Perivascular Delivery of miR-22 AgomiRs or LNA-miR-22

Mouse femoral arterial injury models were performed as described in our previous studies. To investigate the therapeutic effects of miR-22 on wire injury–induced vascular remodeling, 100 μL of 30% pluronic gel containing chemically modiﬁed and cholesterol-conjugated miR-22 (2.5 nmol) or scrambled AgomiRs were applied perivascularly to the femoral artery for local delivery of AgomiRs. miR-22 loss of function was conducted by local application of LNA-miR-22.
miR-22 in SMC Function and Neointima Formation

RESULTS
miR-22 Expression Is Modulated During VSMC Phenotype Switching In Vivo, Ex Vivo, and In Vitro

To explore the potential function of miR-22 in VSMC phenotype switching, we examined in vivo, ex vivo, and in vitro mouse models of VSMC phenotype switching. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) data showed that miR-22 was significantly decreased in the injured versus uninjured femoral arteries (Figure 1A, in vivo). RT-qPCR data also showed downregulation of miR-22, and the VSMC markers (Smα and SM-myh11), as well, in the explanted cultured thoracic aortic tissues (Figure 1B, ex vivo). As expected, we found that gene expression levels of VSMC markers were maintained in the VSMCs in early passages (up to passage 8, P8) but significantly downregulated later (P9 to P12) (Figure 1C, in vitro). Thus, VSMCs between P5 and P8 were used in the current study. It is interesting to note that a similar decreased expression pattern was observed for miR-22 (Figure 1C). Furthermore, miR-22 expression in cultured VSMCs was reduced in response to platelet-derived growth factor BB (PDGF-BB) and serum stimulation (Figure 1D), whereas the opposite effect was seen in the serum-starved VSMCs (Figure 1E). The induction of miR-22 in VSMCs was further enhanced by transforming growth factor β1 (TGFB1) treatment 24 hours and 48 hours after serum starvation (Figure 1E). Altogether, these data confirm that expression of miR-22 is altered during phenotype switching in vivo, ex vivo, and in vitro.

miR-22 Is Transcriptionally Regulated During VSMC Phenotypic Modulation

Transcriptional modulation and regulation of microRNA biogenesis are 2 main mechanisms through which miR activity can be regulated. Here, we sought to determine whether either of these mechanisms was responsible for regulating miR-22 expression dur-
Figure 1. miR-22 expression is closely modulated during VSMC phenotype switching. 
A, miR-22 was significantly downregulated after injury in the in vivo mouse model. Total RNA was collected from the uninjured (day [D] 0) and injured femoral arteries (D3, D7, D14, and D28 postinjury). RT-qPCR analyses were conducted to obtain relative expression levels. 
B, miR-22 was decreased in the ex vivo cultured thoracic aortas. RT-qPCR analysis was used to examine the mRNA (SMαA and SM-myh11) and miR (miR-22) levels in the freshly isolated aortas and the aortas cultured (Continued)
ing VSMC phenotype switching in cultured VSMCs. We measured expression of miR-22, its primary (Pri-
miR-22) transcript, and its precursor (Pre-miR-22) transcript. We found that all were significantly down-
regulated by PDGF-BB and serum (Figure 2A) but upregulated by TGF-β1 (Figure 2B), suggesting that
miR-22 was transcriptionally regulated during VSMC phenotypic modulation. Such a notion was further
supported by data from our luciferase activity assays using miR-22 gene promoter reporter (Figure 2C). A
recent study showed that miR-22 expression is regu-
lated by a P53-dependent mechanism during cardiac
aging, and such a mechanism may be responsible for
miR-22 regulation during VSMC phenotypic modulation.
Indeed, we found that the P53-specific inhibitor,
Pifithrin-α (15 µmol/L), actively reduced miR-22 ex-
pression in TGF-β1–treated VSMCs (Figure 2D). These
data demonstrate that TGF-β1 can regulate miR-22
transcription in VSMCs, likely through a P53-depen-
dent mechanism.

**Functional Role of miR-22 in VSMC Phenotype Switching**

To test our hypothesis that miR-22 plays a role in
VSMC phenotype switching, we transfected murine
and human VSMCs with miR-22 mimics or miR-22
inhibitor and then subjected the transfected cells to
various analyses. RT-qPCR data showed that miR-22
expression in murine VSMCs was significantly upreg-
ulated by miR-22 mimics (Figure IA in the online-only
Data Supplement) and downregulated by the miR-22
inhibitor (Figure IB in the online-only Data Supple-
ment). The expression of 4 VSMC genes (SmxA, SM22α, SM-myh11, and SMTN-B) was also sig-
ificantly increased in VSMCs transfected with miR-22
mimics (Figure IA in the online-only Data Supple-
ment) and significantly decreased in VSMCs treated
with the miR-22 inhibitor (Figure IB in the online-only
Data Supplement). It is important to note that we
found that, whereas PDGF-BB treatment reduced
expression of SM-myh11 and SMTN-B, the addition
of miR-22 mimics significantly increased expression
of both genes even with PDGF-BB treatment (Figure
IC and ID in the online-only Data Supplement), sug-
uggesting that miR-22–induced contractile gene ex-
pression is PDGF-BB–independent. RT-qPCR analysis
verified that miR-22 was successfully overexpressed
and knocked down in VSMCs by miR-22 mimics and
inhibitor, respectively, in control, serum, and PDGF-
BB treatments (Figure 3A and 3B). It is important to
note that VSMC proliferation, growth, and migration
were significantly inhibited by miR-22 overexpression
as demonstrated in bromodeoxyuridine incorpora-
tion analysis (Figure 3C), cell counting (Figure IIA in
the online-only Data Supplement), transwell migra-
tion (Figure 3D), and wound-healing assays (Figure
IIB in the online-only Data Supplement). Conversely,
an increased capacity to proliferate, grow, and mi-
grate was observed in VSMCs transfected with the
miR-22 inhibitor (Figure 3E and 3F, Figure IIC and IID
in the online-only Data Supplement). Similar findings
from human aortic SMCs also supported that miR-22
plays an important role during human VSMC pheno-
typic modulation (Figure III in the online-only Data
Supplement).

**VSMC Apoptosis Is Not Regulated by
miR-22**

Apart from VSMC migration and proliferation, VSMC
apoptosis has been suggested to be another major
contributing factor to atherosclerotic lesion formation
and plaque phenotype. To explore any potential
roles of miR-22 in VSMC apoptosis, serum-starved
VSMCs were subjected to extended serum starva-
tion (96 hours) (Figure IVA and IVB in the online-only
Data Supplement) or incubation with 10 µmol/L H2O2
(Figure IVC and IVD in the online-only Data Supple-
ment) to induce apoptosis. Data from flow cytometry

Figure 1 Continued. in DMEM containing 20% serum for 3 days. Data and error bars in A and B represent the mean±SEM (n=4), where up to 5 femoral arteries were pooled as 1 experiment. *P<0.05 (versus D0 [A], or fresh aorta [B]). C, miR-22 expres-
sion was downregulated in the extended culture of murine VSMCs. Total RNA, including miR (miR-22) and miRNA (SmxA, SM-
myh11, and CNN1), was harvested from freshly cultured VSMCs (cultured until day 7 and then split, designated P0), and VSMCs
with the indicated passage number (P0, P3, P8, P9, or P12) were subjected to RT-qPCR analysis to obtain relative expression levels.
D, Serum (left) and PDGF-BB (right) downregulated miR-22 in cultured VSMCs. VSMCs were subjected to serum starvation for 48
hours, followed by incubation with 20% serum or PDGF-BB (10 ng/mL), respectively. Total RNA was harvested at each indicat-
ed time point. E, Serum starvation and TGF-β1 upregulated miR-22 in cultured VSMCs. VSMCs in normal culture were used as
control (TGF-β1−, Serum+) or subjected to serum starvation for 48 hours and then harvested after the indicated stimulations and
in time points: no stimulation (TGF-β1−, Serum−) at 0 hour, TGF-β1 stimulation at 24 hours (TGF-β1−24hrs, Serum−), and TGF-β1
stimulation at 48 hours (TGF-β1−48hrs, Serum−). Total RNA was extracted and subjected to RT-qPCR analysis with a specific miR-
22 forward primer and a universal miR reverse primer. Data and error bars in C through E represent mean±SEM (n=5). *P<0.05,
**P<0.01, ***P<0.001 (versus P0 [C], 0 hours [D], or normal culture [E]); #P<0.05 (versus 0 hours). DMEM indicates Dulbecco’s
modified Eagle’s medium; miR-22, microRNA-22; PDGF-BB, platelet-derived growth factor BB; RT-qPCR, reverse transcription
quantitative polymerase chain reaction; TGF-β1, transforming growth factor β1; and VSMC, vascular smooth muscle cell.
analyses showed that the percentages of live, early apoptotic, late apoptotic, and necrotic VSMCs were not significantly changed by either miR-22 overexpression (Figure IVA and IVB in the online-only Data Supplement) or inhibition (Figure IVC and IVD in the online-only Data Supplement), indicating that miR-22 was not involved in either extended serum starvation or H2O2-induced VSMC apoptosis.

### Potential Downstream Targets of miR-22 During VSMC Phenotype Switching

Conserved miR-22 binding site(s) were found within 270 genes by using Targetscan. Among them, 23 validated/predicted targets of miR-22 were selected for further study, because they are known to play key regulatory roles in both VSMC functions (differentiation, proliferation, migration, apoptosis, and cell cycle)\(^{11,42-45}\) and vascular biology\(^{34}\) (Figure V in the online-only Data Supplement). Our data showed that the expression levels of TP53INP1, TGFβ1, P21, MAP2K4, and SP1 were significantly increased in both serum-starved and TGFβ1–treated VSMCs, whereas the expression levels of MECP2, ARPC5, EVI1, MYST4, and histone deacetylase 4 (HDAC4) were significantly inhibited by either serum starvation or TGFβ1 stimulation (Figure V in the online-only Data Supplement). It is important to note that MECP2 and EVI1 expression in serum-starved VSMCs was further decreased by TGFβ1 treatment (Figure V in the online-only Data Supplement). Because TGFβ1 treatment increased miR-22 expression (Figure 1E) and decreased expression of MECP2, EVI1, and HDAC4, these observations indicated these genes could be potential targets of miR-22.
MECP2 and HDAC4 Are 2 Functional Target Genes of miR-22s During VSMC Phenotype Switching

Our previous study showed that miR-22 targets MECP2 during VSMC differentiation from stem cells, leading to our hypothesis that MECP2 may also be a downstream target of miR-22 during VSMC phenotype switching. Data from RT-qPCR and Western blot analyses showed that both MECP2 mRNA and protein expression levels are decreased in VSMCs by miR-22 overexpression (Figure VIA and VIB in the online-only Data Supplement). The MECP2 3′-UTR reporter activity was also significantly inhibited by miR-22 mimics (Figure VIC in the online-only Data Supplement). Moreover, all 5 VSMC genes (SMαA, SM22α, CNN1, SM-myh11, SMTN-B), but not miR-22, were significantly upregulated by MECP2 inhibition (Figure VID in the online-only Data Supplement), supporting that MECP2 is the downstream target of miR-22. It is important to note that VSMC proliferation (Figure VIE in the online-only Data Supplement) and migration (Figure VIF in the online-only Data Supplement) were significantly decreased by MECP2 knockdown, demonstrating that MECP2 suppression can recapitulate the effects of miR-22 overexpression in VSMC phenotypic modulation. In addition to MECP2, HDAC4 is another reported target of miR-22. miR-22 overexpression decreased the expression level of HDAC4 (Figure VIIA in the online-only Data Supplement). Inhibition of HDAC4 mimics miR-22 overexpression during VSMC phenotype switching (Figure VIIB through VIIID in the online-only Data Supplement). These data suggested that miR-22 also targets HDAC4 in mature VSMCs.
EVI1 Is a Novel Target Gene and Responsible for miR-22–Mediated VSMC Phenotype Switching

The transcriptional regulator and oncoprotein EVI1 was predicted as one of the target genes of miR-22 by Targetscan (Figure 4A). Opposite from miR-22 (Figure 1C), EVI1 gene expression was dramatically increased in the extended passages (P8, P9, and P12) of cultured murine VSMCs (Figure 4B). These observations prompted us to investigate whether or not EVI1 is a novel target gene of miR-22 during VSMC phenotype switching. As expected, expression levels of both the EVI1 mRNA (Figure 4C) and protein (Figure 4D) were significantly downregulated by miR-22 mimics but upregulated by miR-22 inhibitor in VSMCs. The luciferase activity of EVI1 3′-UTR reporter was significantly repressed by miR-22 mimics but enhanced by miR-22 inhibition (Figure 4E). Once the miR-22 binding site within EVI1 3′-UTR was mutated, the miR-22 mimic–induced inhibition of EVI1 expression was abrogated (Figure 4F), suggesting that miR-22 directly downregulates EVI1 via its 3′-UTR binding site.

To explore the functional significance of EVI1 in VSMC phenotype switching, an EVI1 small hairpin RNA lentivirus was produced and used to generate a stable knockdown of EVI1 in VSMCs as validated by RT-qPCR (Figure 5A) and Western blot (Figure 5B). We found that expression of 5 VSMC genes and 2 SMC transcription factor genes (SRF and Myocd) was significantly increased in EVI1 knockdown VSMCs (Figure 5C). Similarly, VSMC proliferation (Figure 5D) and migration (Figure 5E) in response to both serum and PDGF-BB stimulation were significantly decreased. As expected, expression levels of both the EVI1 mRNA and protein (Figure 5F) were significantly increased in EVI1 knockdown VSMCs. The luciferase activity data showed that EVI1 knockdown induced both SMαA and SM22α gene promoter activity, but this was completely lost once the SRF binding element within the gene promoter was mutated (Figure 6A and 6B), confirming that EVI1 regulates VSMC marker expression through a transcriptional mechanism requiring SRF binding. Moreover, we found that both SRF and Myocd gene promoter reporter activity was significantly increased in EVI1 knockdown VSMCs (Figure 6C and 6D), indicating that EVI1 also represses SRF and Myocd gene regulation.

To understand the underlying molecular mechanisms of EVI1 regulation, chromatin immunoprecipitation assays were conducted in control and EVI1 knockdown VSMCs using an EVI1-specific antibody. We found significant enrichment of EVI1 within the promoter regions of SMαA, SM22α, SRF, and Myocd genes in control VSMCs (Figure 6E). Such enrichment disappeared in EVI1 knockdown VSMCs (Figure 6E), confirming that EVI1 directly binds to these 4 gene promoters. In our previous publication, H3K9me3 was shown to bind to promoter regions of VSMC marker genes and suppress their expression. This led to the hypothesis that H3K9me3-induced suppression is dependent on EVI1. A chromatin immunoprecipitation assay with a H3K9me3-specific antibody showed a significantly lower H3K9me3 enrichment within the promoter regions of VSMC marker genes in EVI1 knockdown cells (Figure 6F), suggesting that the binding of H3K9me3 to the promoter regions of VSMC genes is EVI1-dependent.

Therapeutic Potential of miR-22 for Postinjury Arterial Remodeling

To determine the therapeutic potential of miR-22 in postinjury arterial remodeling, 2.5 nmol of miR-22 or Cel-miR-67 AgomiRs (negative control) was perivascularly applied to femoral arteries immediately after wire-induced injury as described in our previous studies. In comparison with uninjured arteries, injured arteries treated with control Cel-miR-67 AgomiRs displayed significantly decreased expression of miR-22 and
VSMC genes (SMαA and SM-myh11) and significantly increased expression of cell proliferation marker gene, PCNA, and the identified miR-22 target genes (MECP2, HDAC4, and EVI1), whereas the opposite effects were observed in injured arteries treated with miR-22 AgomiR (Figure 7A). It is important to note that increased miR-22 expression via perivascular transfection of miR-22 AgomiR was specific to injured arteries, yet absent in other organs/tissues (eg, heart, skeletal muscle, spleen, liver, kidney, and lung) (data not shown). As expected, injury-induced MECP2, EVI1, HDAC4, and PCNA gene expression was blunted, whereas the expressions of
VSMC genes (SMαA and SM-myh11) were enhanced by local ectopic expression of miR-22 (Figure 7A). These findings are consistent with the notion that miR-22 promoted VSMC phenotype switching from its proliferative, synthetic state to a contractile phenotype after vascular injury. Consequently, although the application of control AgomiRs (Cel-miR-67 AgomiR) in the injured artery exhibited pronounced neointima hyperplasia after 28 days, miR-22 overexpression significantly inhibited neointima formation, as evidenced by decreased
intima area and intima/media ratio in the miR-22 AgomiR–treated injured artery, although the media area experienced no significant change (Figure 7B and 7C). To better understand the functional role of miR-22 in postinjury arterial remodeling, we also conducted miR-22 loss-of-function experiments using LNA-miR-22 and found that miR-22 inhibition produces the opposite effects of miR-22 overexpression on VSMC marker
Figure 7. Modulation of miR-22 expression in the injured arteries influences neointima formation.
A through C, Local enforced expression of miR-22 reduces neointima formation in the injured femoral arteries. After wire-induced injury, 100 µL of 30% pluronic gel containing 2.5 nmol control AgomiR (Cel-miR-67 AgomiR) or miR-22 AgomiR per artery per mouse was immediately applied and packed around the injured femoral arteries. Three days (A) or 4 weeks (B and C) later, injured segments of femoral arteries were harvested for analyses. A, Perivascular delivery of miR-22 AgomiRs reversed the gene expression profiles in wire-induced femoral artery injury. Total RNA was harvested from uninjured and AgomiR-applied injured femoral arteries before undergoing RT-qPCR analyses. Data and error bars represent mean±SEM (n=3) (5 femoral arteries were pooled for each experiment). *P<0.05 (versus uninjured arteries). #P<0.05 (miR-22 AgomiRs versus Cel-miR-67 AgomiRs in the injured arteries).
B and C, Locally enforced expression of miR-22 inhibited neointima formation in wire-injured femoral arteries. Paraffin sections from both groups (n=15 mice for Cel-miR-67 and n=13 mice for miR-22 AgomiRs) were prepared and subjected to H&E staining. Representative images (B) and morphological characteristics (C), including media area (left), intima area (middle), and intima/media (I/M) ratio (right) at 4 weeks after injury were presented. #P<0.05 (versus Cel-miR-67 AgomiRs). D through F, miR-22 inhibition promotes neointima formation in the injured arteries. After wire injury, 100 µL of 30% pluronic gel containing vehicle (mock transfection, Mock), 2.5 nmol control LNA (scrambled LNA, Scrbl-LNA), or LNA-miR-22 per artery per mouse was immediately applied and packed around injured femoral arteries. Seven days (Continued)
expression and injury-induced neointima hyperplasia (Figure 7D through 7F). These findings suggest therapeutic potential for harnessing local ectopic expression of miR-22 to suppress neointima hyperplasia after arterial injury.

**miR-22 Is Significantly Downregulated, Whereas Its Target Genes (MECP2 and EVI1) Are Dramatically Upregulated in the Diseased Human Arteries**

To translate our findings from murine into human, we collected 30 diseased and 30 healthy femoral arterial specimens from patients who underwent leg amputation at the First Affiliated Hospital of Zhejiang University, China. Diseased femoral artery specimens featured SMC-rich atherosclerotic lesions with severe neointima hyperplasia, whereas healthy femoral arteries displayed no atherosclerotic lesions (Figure 8A). In comparison with healthy arteries, diseased femoral arteries showed a significantly decreased gene expression level of miR-22 and dramatically increased expression levels of its target genes (MECP2 and EVI1) (Figure 8B). It is more important that significant inverse relationships between miR-22 and its downstream targets, MECP2 and EVI1, were observed in both healthy and diseased femoral arteries (Figure 8C and Figure IX in the online-only Data Supplement). It is interesting to note that an abnormally high expression level of EVI1 was observed in 3 healthy and 3 diseased femoral arteries harvested from the patients with hypertension and hyperlipidemia, indicating that the coexistence of these 2 comorbidities could significantly affect EVI1 expression. Thus, these data provide critical information about the functional relevance of miR-22 and its target genes in human atherosclerotic lesions.

**DISCUSSION**

By using various in vivo, ex vivo, and in vitro models of VSMC phenotypic modulation, we identified a novel role of miR-22 as a mediator of VSMC phenotype switching and neointima formation. We specifically show that miR-22 is transcriptionally regulated by serum, PDGF-BB, and TGF-β1. Moreover, VSMC contractile gene expression, proliferation, and migration, but not apoptosis, were modulated by miR-22. Mechanically, we have confirmed that MECP2, HDAC4, and EV11 are the authentic downstream targets of miR-22 during VSMC phenotype switching. We have also identified EV11 as a novel transcriptional repressor of VSMC contractile genes. It is important to note that we observed that miR-22 expression is suppressed in the human femoral arteries with atherosclerotic plaques and have uncovered an inverse relationship between miR-22 and its target genes, MECP2 and EVI1, in healthy and diseased arteries.

**Role of miR-22 in Heart Disease and Vascular Remodeling**

miR-22 has been primarily identified as a tumor suppressor, but later studies have identified miR-22 as a prohypertrophic miR.32,33,46,47 A phenotypic screen with primary rat cardiomyocytes has suggested that miR-22 has prohypertrophic potential,46 which was further confirmed by using transgenic mice: specifically, global or cardiac-specific deletion of miR-22 blunted stress-induced cardiac hypertrophy and remodeling,32,33 whereas cardiac-specific overexpression of miR-22 induced a prohypertrophic gene expression profile and elicited cardiac dilatation and heart failure.47 In a more clinically relevant study, pharmacological inhibition of miR-22 promoted cardiac functional recovery after myocardial infarction by eliciting cardiac autophagy.30 These important studies provide clear evidence to suggest that a fine balance of miR-22 expression and regulation is critical for maintaining adequate cardiac functions. Although the majority of miR-22 studies were conducted in cancer cells or cardiac cells, we recently reported an important role for miR-22 in VSMC differentiation from stem cells both in vitro and in vivo,34 inferring a regulatory role for miR-22 in VSMC biology. However, the role for miR-22 in VSMC phenotypic modulation had not been explored until our present study. By performing miR gain- and loss-of-function studies, we demonstrated that miR-22 promotes VSMC contractile gene expression and inhibits VSMC proliferation and migration. We show that local transfer of miR-22 onto the injured arteries can restore synthetic VSMCs to a contractile phenotype, providing a basis for using site-specific delivery of miR-22 mimics via miR-22–coated stents to prevent or inhibit in-stent restenosis. Our study was...
also the first to identify an inverse relationship between the expression levels of miR-22 and its target genes, MECP2 and EVI1, in both healthy and diseased human femoral arteries, suggesting that miR-22 could be a potential therapeutic agent in coronary atherosclerosis. Therefore, extending our studies to other human arteries (eg, coronary/carotid arteries) would be a promising next step for exploring the therapeutic application of miR-22 in various cardiovascular diseases.

Transcriptional Regulation of miR-22

The regulatory mechanisms of miR-22 expression in VSMCs are only partially known. Our previous publication suggested that miR-22 is transcriptionally regulated by PDGF-BB and TGF-β1 during VSMC differentiation from stem cells, but no direct evidence has been obtained. PDGF-BB is a member of the platelet-derived growth factor family primarily expressed by vascular endothelial cells and platelets at the vascular injury sites, and it is a key stimulant of VSMC proliferation and migration. In addition to upregulating miR-22 during VSMC differentiation from stem cells, PDGF-BB signaling has also been reported to transcriptionally induce miR-15b expression to mediate VSMC dedifferentiation and inhibit miR-221 to promote proliferation in pancreatic cancer cells.

TGF-β1, initially identified as a tumor suppressor, is known to transcriptionally regulate genes involved in proliferation, growth, and differentiation by binding their response elements within target promoters. Similar to TGF-β1 signaling, the P53 signaling pathway can also

Figure 8 Continued. MECP2 (relative to 18S, %) and EVI1 (relative to 18S, %), are shown for each HFA and DFA specimen (dots). Lines represent the mean in each patient group. *P<0.05 (DFA versus HFA, Mann-Whitney U test). The expression level of miR-22 was significantly decreased, whereas the expression levels of MECP2 and EVI1 were dramatically increased in DFA. C, Spearman rank correlation analyses were performed to characterize the relationships between the gene expression levels of MECP2/EVI1 and miR-22 in HFA and DFA specimens. The y axis represents the expression level of miR-22 (relative to U6, %); the x axis represents the expression level of its target genes (MECP2 and EVI1) (relative to 18S, %). The solid line indicates the fitted linear regression line; the dotted line indicates 95% confidence interval level. r is the Spearman rank correlation coefficient between the expression levels of MECP2/EVI1 and miR-22. A value closer to −1 indicates a stronger negative correlation, and a value closer to 1 indicates a stronger positive correlation. P is the P value indicating the significant level of correlation. EVI1 indicates ecotropic virus integration site 1 protein homolog; H&E, hematoxylin and eosin; MECP2, methyl-CpG binding protein 2; miR-22, microRNA-22; and RT-qPCR, reverse transcription quantitative polymerase chain reaction.
modulate miR expression and maturation, and thereby cell proliferation and differentiation. A recent study showed that miR-22 expression is regulated by a P53-dependent mechanism during cardiac aging. In this study, we have provided definitive evidence that miR-22 gene expression in VSMCs is regulated by PDGF-BB and TGF-β1 through modulation of gene promoter activity (Figure 2C). Furthermore, our data also show that TGF-β1 transcriptionally regulates miR-22 in VSMCs via a P53-dependent mechanism. Our observation first establishes the TGF-β1/P53/miR-22 signaling axis and uncovers the regulatory role of PDGF-BB, TGF-β1, and P53 signaling pathways in VSMC phenotypic modulation. Because these major signaling pathways may represent novel therapeutic targets, the development of agents that target these signaling pathways is likely to have a significant therapeutic impact on vascular disease.

**EVI1 as Novel Gene Target of miR-22**

Previous studies have shown that EVI1 functions as a transcriptional regulator that binds DNA sequences in the promoter region of target genes and regulates a number of biological processes, such as hematopoesis, apoptosis, development, and cell differentiation and proliferation. However, little is known about its potential involvement in VSMC function and cardiovascular disease. In this study, we provide compelling evidence that EVI1 is the target gene of miR-22-mediated VSMC phenotypic modulation and is a transcriptional repressor for multiple VSMC contractile genes. We specifically demonstrate that EVI1 transcriptionally inhibits VSMC-specific gene expression by providing the following evidence: (1) suppression of EVI1 expression and its reporter activity by miR-22 mimics; (2) increase in gene expression of all 5 VSMC-specific contractile markers and 2 transcription factors by EVI1 knockdown; (3) increase in gene promoter activity of SmαA, SM22α, SRF, and Myocd by EVI1 inhibition; (4) requirement of SRF binding sites(s) for EVI1-mediated SmαA and SM22α gene repression; and (5) direct binding and enrichment of EVI1 at the promoter regions of SmαA, SM22α, SRF, and Myocd confirmed by chromatin immunoprecipitation assay.

Emerging evidence has suggested that EVI1 regulates transcription, in part, through epigenetic modification. For example, the aberrant DNA hypomethylation signature in EVI1-directed acute myeloid leukemia is likely through interaction with DNA methyltransferases 3A and 3B. In this study, we observed a significant H3K9me3 enrichment within the promoter regions of VSMC-specific genes (SmαA, SM22α, SRF, and Myocd), and this enrichment was significantly inhibited by EVI1 knockdown (Figure 6F). Combined with the previous finding that another miR-22 target, MECP2, also modulates H3K9me3 enrichment within promoter regions of VSMC-specific genes, these results imply that epigenetic modification within VSMC gene promoters may be one of the underlying pathways through which miR-22 regulates VSMC phenotype switching. Whether other epigenetic mechanisms are involved in EVI1-mediated VSMC phenotype switching remains to be seen. Further investigations are warranted to identify genome-wide targets for EVI1 in VSMCs by conducting an unbiased EVI1 chromatin immunoprecipitation sequencing to better understand the global regulatory role of EVI1 in VSMC phenotype modulation and potentially in cardiovascular diseases.

It is noteworthy that our identification of the miR-22/EVI1 signaling axis in human atherosclerotic plaques presents potential clinical application toward treating cardiovascular disease. Because the expression of miR-22 was significantly decreased, whereas expression of its target genes, MECP2 and EVI1, was dramatically increased in femoral atherosclerotic lesions in comparison with healthy femoral arteries, inhibiting EVI1 may offer a therapeutic opportunity to decrease neointima formation and restenosis. Furthermore, it has been reported that EVI1 protein can be specifically degraded by an anticancer drug, arsenic trioxide. Several preclinical and clinical studies suggest AES is a promising alternative to widely used sirolimus derivative–eluting stents. In a rabbit iliac artery injury model, AES significantly suppressed in-stent restenosis by reducing proliferation and inducing apoptosis of VSMCs. The beneficial effects of AES on in-stent restenosis in a porcine coronary model were also attributed to an early anti-inflammatory effect of arsenic trioxide in the stented vessels. A 2-year follow-up clinical study also demonstrated that AES has a comparable efficacy and safety to durable polymer sirolimus–eluting stent for the treatment of de novo coronary artery lesions. Our finding that the miR-22/EVI1 signaling axis plays an important role in VSMC phenotypic modulation and arterial remodeling may offer a possible mechanistic basis for the beneficial effect of AES on in-stent restenosis, and suggests that correcting the dysregulation of miR-22 and EVI1 in atherosclerotic arteries through a site-specific delivery of miR-22 mimics to the stented vessels by using a miR-22–coated balloon or stent, or ultrasound-triggered nanodelivery technology, could be a potential treatment to prevent or inhibit in-stent restenosis.

We recognize a few limitations of our study. First, we chose the mouse wire-injury model to study the therapeutic potential of miR-22 for treating postangioplasty restenosis, because it partially mimics balloon angioplasty and intraluminal stent placement, but further
investigation using a stent model would increase the translational feasibility of our current findings. Another limitation is that this model fails to accurately emulate neointima formation attributable to native atherosclerosis, a major underlying cause of cardiovascular disease. Hence, extending our studies to a hyperlipidemia-induced atherogenic animal model is required to validate the therapeutic potential of miR-22 in other cardiovascular diseases.

Taken together, we present in this study compelling evidence that miR-22 is a novel regulator of VSMC phenotype switching and vascular neointima lesion formation, which acts via its target genes, MECP2, HDAC4, and EVI1. Our data have shown for the first time that ectopic expression of miR-22 in the injured arteries can reverse the process of VSMC phenotype switching and prevent postangioplasty restenosis, supporting a potential role for miR-22 and its target genes in a variety of proliferative vascular diseases. These findings may have extensive implications for the treatment of human atherosclerosis.

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Disclosures

None.

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