Inhibition of MicroRNA-29 Enhances Elastin Levels in Cells Haploinsufficient for Elastin and in Bioengineered Vessels

Pei Zhang, Angela Huang, Jacopo Ferruzzi, Robert P. Mecham, Barry C. Starcher, George Tellides, Jay D. Humphrey, Frank J. Giordano, Laura E. Niklason, William C. Sessa

Objective—The goal of this study was to determine whether antagonizing microRNA (miR)-29 enhances elastin (ELN) levels in cells and tissues lacking ELN.

Methods and Results—miR-29 mimics reduced ELN levels in fibroblasts and smooth muscle cells, whereas miR-29 inhibition increased ELN levels. Antagonism of miR-29 also increased ELN levels in cells from patients haploinsufficient for ELN and in bioengineered human vessels.

Conclusion—miR-29 antagonism may promote increased ELN levels during conditions of ELN deficiencies. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: aneurysms • extracellular matrix • microRNA

Elastin (ELN) is a major component of the extracellular matrix in arteries that is essential for normal structural integrity and function. In humans, a single ELN gene is found on chromosome 7, and mutations in 1 allele can lead to several elastinopathies, including supravalvular aortic stenosis (SVAS) and a congenital narrowing of the ascending aorta or other vessels.1 SVAS is also a prominent component of Williams-Beuren syndrome (WBS), a frequent heterozygous deletion of a ≈1.5-Mb segment at chromosome 7q11.23 that includes ELN.2 Despite having 1 intact allele, the expression of ELN is less than 50% of normal in WBS, thus suggesting posttranscriptional modulation of the mRNA.3

ELN is secreted into the extracellular space as soluble tropoelastin, which is cross-linked by lysyl oxidase and lysyl oxidase-like 1 to form insoluble, mature ELN fibrils.4 Rodent studies have shown that elastogenesis begins during midgetstage, peaks in the perinatal period, and drops sharply thereafter to low levels that persist into adulthood.1 Interestingly, tropoelastin premRNA levels remain elevated in adult rat lungs despite considerably reduced steady-state mRNA levels, thus suggesting that mRNA posttranscriptional regulation may be a predominant mechanism to regulate ELN mRNA and protein levels in adults.5

In the past decade, microRNAs (miRNAs) have emerged as important regulators of gene expression robustness.6 miRNAs predominantly target the 3′-untranslated region of mRNAs, either destabilizing the mRNA transcript or interfering with its translation into protein. Previous work has shown that microRNA (miR)-29 mimics downregulate the expression of

ELN, COL1A1, and COL3A1.7 Here, we show that inhibition of miR-29a can dramatically increase ELN expression in human cells and miR-29 inhibition upregulates ELN levels in cells from patients with ELN haploinsufficiencies and in bioengineered human blood vessels. Thus, antagonizing the actions of miR-29 may promote increased ELN levels during conditions of enhanced elastinolysis or deficiencies.

Materials and Methods

Cell Culture and miRNA Treatment

Human dermal fibroblasts, isolated from human foreskin or adult skin, were purchased from Coriell. WBS and age-matched normal human dermal fibroblasts were purchased from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD). Human vascular smooth muscle (VSM) cells and SVAS pulmonary VSM cell were explanted by outgrowth as described.8 Cells were treated with miRIDIAN miR-29 mimic, hairpin inhibitors or corresponding controls (Dharmacon, Chicago, IL) at 60 nmol/L for 15 hours unless specified otherwise and then were grown in complete media for 48 hours before harvest for RNA or protein studies.

Western Blot

To measure tropoelastin expression in the media, cells were grown in 0.5% FBS Dulbecco’s modified Eagle’s medium for the final 24 hours and media proteins were precipitated by 10% trichloroacetic acid and resolved on 10% SDS-PAGE gels. To measure ELN protein associated with cell membrane, equal number of cells were lysed in 6 mol/L urea-containing sample loading buffer and loaded onto 10% SDS-PAGE. Western membranes were probed with a polyclonal antibody recognizing human ELN. Ponceau red staining (for media

Received on: September 2, 2011; final version accepted on: November 2, 2011.

From the Vascular Biology and Therapeutics Program (P.Z., A.H., G.T., J.D.H., F.J.G., L.E.N., W.C.S.) and the Departments of Pharmacology (W.C.S.), Anesthesiology (L.E.N.), Biomedical Engineering (A.H., J.F., J.D.H.), Medicine (P.Z., F.J.G.), and Surgery (G.T.), Yale University School of Medicine, New Haven, CT; Department of Cell Biology, Washington University School of Medicine, St. Louis, MO (R.P.M.); University of Texas Health Science Center at Tyler, Tyler, Texas (B.C.S.).

Correspondence to William C. Sessa, PhD, Yale University School of Medicine, Amistad Research Bldg, 10 Amistad St, New Haven, CT 06520. E-mail william.sessa@yale.edu

© 2011 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.111.238113
Culture and Analyses of Tissue Engineered Vessels
Engineered arteries 1 mm in diameter and 3 cm in length were grown as previously described. Biaxial tests were performed using a custom device as previously described. Desmosine and Hydroxyproline Analysis
Desmosine and hydroxyproline, demonstrating cross-linked ELN and collagen respectively, were measured as described.

Results and Discussion
Human dermal fibroblasts were transfected with miRNA mimics for miR-29a, -29b, and -29c or inhibitors (60 nmol/L of each) for 48 hours, and the levels of ELN mRNA were quantified using quantitative reverse transcription–polymerase chain reaction. Figure 1A shows that miR-29a, -29b, and -29c mimics significantly reduced ELN mRNA levels (to 2%–3% of control), whereas transfection with miR-29 inhibitors increased ELN mRNA levels (4–6-fold). Because of homology within the seed sequence of miR-29 family members, miR-29 inhibitors also reduced the levels of other miR-29 miRNAs (Supplemental Figure I, available online at http://atvb.ahajournals.org). Computational analyses have predicted 3 seed sequences (38–44, 297–303, 310–316) for miR-29 binding in the 3′-untranslated region of the human ELN mRNA and these sequences are highly conserved among mammals. As seen in Figure 1B, treatment of human
dermal fibroblasts with mimics reduced tropoelastin protein levels, whereas miR-29a, -29b, and -29c inhibitors increased levels compared with controls. To further test whether the miR-29 inhibitor increases major ELN isoforms, we used exon-specific primers to detect the existence of major ELN exons. As seen in Figure 1C, ELN mRNAs containing the known exons 3, 10, 13, 23, and 25 were upregulated to a similar degree with the miR-29a inhibitor.

Because all miR-29 members are abundantly present in the cell types in this study and can potentially regulate ELN levels, we performed additional experiments using only miR-29a. To test the specificity of the miR-29 inhibitor, it was titrated (0–100 nmol/L) in the presence or absence of 60 nmol/L of the miR-29a mimic. As seen in Figure 1D, more miR-29a inhibitor was needed to achieve the same fold increase of ELN mRNA, but this difference disappeared between 30 and 100 nmol/L of the inhibitor. These data together with data in Figure 1A suggested that (1) the miR-29a inhibitor specifically antagonizes endogenous miR-29, as well as the miR-29a mimic, in a dose-dependent manner, and (2) the miR-29a inhibitor specifically protects ELN mRNA from endogenous miR-29a dependent decay.

Computer analyses also predict several other genes with miR-29 seed sequences, including COL1A1, COL3A1, and VEGF. Overexpression of miR-29 can decrease the activity of luciferase reporter plasmids containing 3’-untranslated regions of COL1A1, COL3A1, and ELN1, and inhibiting miR-29 in vivo can modestly upregulate COL1A1 and COL3A1 mRNA levels in the heart.7 Indeed, miR-29a mimic (60 nmol/L) reduced ELN, COL1A1, COL3A1, and VEGF-A (not shown) mRNA levels in human VSM (Figure 1E). In contrast, the miR-29a inhibitor enhanced ELN expression but did not significantly change the levels of COL1A or COL3A.

Additional genes involved in matrix assembly, fibrillin-1 to
-3, emilin1, lysyl oxidase 1, and lysyl oxidase-like 1/2, were also tested in VSM and fibroblasts, and miR-29a-I did not increase their expression levels (Supplemental Figure II). The miR-29a inhibitor increased soluble and cell-associated ELN levels in VSM (Figure 1F, left and right). This suggests that miR mimics may achieve concentrations that destabilize multiple transcripts having seed sequences, whereas miR inhibition may selectively upregulate physiological mRNA partners regulated by the endogenous miRNA levels.

Next, we tested the ability of miR-29a inhibitor to upregulate ELN expression in pulmonary VSM cells isolated from a patient with SVAS. A GTAT insertion in exon 9 of the ELN gene caused a frame shift and a premature stop codon in exon 10. Quantitative polymerase chain reaction experiments using primers recognizing ELN exon 18 showed that SVAS cells had low levels of ELN mRNA (~10% of normal VSM) but equal amounts of endogenous miR-29a compared with normal VSM (Supplemental Figure IIIA, left). Transfection of SVAS cells with miR-29a inhibitor upregulated ELN expression 3.5-fold (Figure 2A). The expression of COL1A1 and COL3A1 mRNAs were moderately higher in SVAS compared with normal cells (0.68±0.24 versus 0.23±0.09 for COL1A1; 17.5±2.2 versus 9.6±4.6 for COL3A1), and miR-29a inhibitor (60 nmol/L) increased COL1A1 (1.7-fold) but not COL3A1 mRNA levels. miR-29a inhibition increased tropoelastin levels in SVAS cells dose dependently, and in contrast, miR-29 mimic decreased tropoelastin (Figure 2B).

Next, we examined whether miR-29 regulates ELN in dermal fibroblasts isolated from 2 donors with WBS. Comparative genomic hybridization verified that these cells had a typical ~1.5-Mb microdeletion at 7q11.23 encoding 25 genes, including ELN (Supplemental Figure IV).5 These ELN-haplodeficient cells expressed only 26% to 36% of ELN mRNA levels but comparable levels of miR-29a (Supplemental Figure IIIA, right, and IIIB, left) compared with age-matched normal fibroblasts from the same bank. Treatment of WBS cells with miR-29 inhibitor upregulated ELN mRNA levels close to those seen in normal cells (Figure 2C and Supplemental Figure IIIB, middle). In contrast, the levels of COL1A1 were comparable in WBS cells, and miR-29 inhibition increased COL1A1 levels. Western blot analysis showed ELN protein was increased by 40% in WBS cells 48 hours after miR-29 inhibitor treatment (Figure 2D).

Bioengineered human blood vessels are collagen replete but ELN deficient,12 and this paucity of ELN may compromise their mechanical properties. To examine whether miR-29 inhibition can enhance ELN levels and therefore improve vascular mechanics, polyglycolic acid scaffolds were seeded with early passage human VSM as described,12 and vessels were grown in a bioreactor for 6 weeks in the absence or presence of miR-29a inhibitor (60 nmol/L) increased COL1A1 (1.7-fold) but not COL3A1 mRNA levels. miR-29a inhibition increased tropoelastin levels in SVAS cells dose dependently, and in contrast, miR-29 mimic decreased tropoelastin (Figure 2B).

Next, we examined whether miR-29 regulates ELN in dermal fibroblasts isolated from 2 donors with WBS. Comparative genomic hybridization verified that these cells had a typical ~1.5-Mb microdeletion at 7q11.23 encoding 25 genes, including ELN (Supplemental Figure IV).5 These ELN-haplodeficient cells expressed only 26% to 36% of ELN mRNA levels but comparable levels of miR-29a (Supplemental Figure IIIA, right, and IIIB, left) compared with age-matched normal fibroblasts from the same bank. Treatment of WBS cells with miR-29 inhibitor upregulated ELN mRNA levels close to those seen in normal cells (Figure 2C and Supplemental Figure IIIB, middle). In contrast, the levels of COL1A1 were comparable in WBS cells, and miR-29 inhibition increased COL1A1 levels. Western blot analysis showed ELN protein was increased by 40% in WBS cells 48 hours after miR-29 inhibitor treatment (Figure 2D).

Bioengineered human blood vessels are collagen replete but ELN deficient,12 and this paucity of ELN may compromise their mechanical properties. To examine whether miR-29 inhibition can enhance ELN levels and therefore improve vascular mechanics, polyglycolic acid scaffolds were seeded with early passage human VSM as described,12 and vessels were grown in a bioreactor for 6 weeks in the absence or presence of miR-29a inhibitor. As seen in Figure 2E (left), there was little ELN (via transmission electron microscopy) in the control vessels, whereas miR-29a inhibition increased the appearance of ELN “islands” (Figure 2E, right) and its detection by Western blotting (Figure 2F). This ELN was cross-linked as quantified by desmosine content (Figure 2G), and levels of hydroxyproline were comparable between the vessels (Figure 2H). The number of VSM in the vessels were not different between groups (1434±437 versus 1797±453 4’,6-diamidino-2-phenylindole–positive/mm² for treated versus control vessels, respectively).

Finally, biaxial mechanical tests revealed that miR-29a inhibition increased the distensibility of the bioengineered vessels at low pressures (Figure 2I); this response is similar to that of native vessels and suggests that the ELN conferred some structural benefit to the vessel.

Collectively, these data suggest that antagonizing miR-29 may increase functional ELN levels in conditions of ELN haploinsufficiency or during enhanced elastinolysis secondary to aneurysms, emphysema, or aging.

While this work was in review, a new study documented that anti-miR-29a reduces the extent of aneurysms.13

Statistical Analysis
Comparisons between 2 groups were made by unpaired Student t test. Statistical analyses were performed using Prism 4 software (GraphPad). Probability values were 2-tailed, and values <0.05 were considered statistically significant.

Acknowledgments
We thank Dr Barbara Pober for all her help and advice with this project.

Sources of Funding
This work was supported by grants from the Kiev Foundation (to W.C.S., F.J.G., and F.T.) and National Institutes of Health Grants R01-HL64793, R01-HL61371, R01-HL081190, and HL096670 (to W.C.S.).

Disclosures
None.

References
Inhibition of MicroRNA-29 Enhances Elastin Levels in Cells Haploinsufficient for Elastin and in Bioengineered Vessels

Pei Zhang, Angela Huang, Jacopo Ferruzzi, Robert P. Mechem, Barry C. Starcher, George Tellides, Jay D. Humphrey, Frank J. Giordano, Laura E. Niklason and William C. Sessa
Supplementary figure legends

Figure I: HDF cells were treated with miR-29a-b or-c inhibitor (90nM) and RNAs were isolated 48 h post-treatment. qPCR values of miR-29abc were normalized to 5S and then compared to those in a control group, which were set as 1. Data are mean ± SEM.

Figure II: VSM or human dermal fibroblast (HDF) were treated with control mimic/inhibitor, miR-29a mimic (29a-M) or miR-29a inhibitor (29a-I) and the levels of several genes implicated in elastic matrix biogenesis assessed by qPCR. Data are mean ± SEM, n= 3.

Figure III: In A, the levels of miR-29a were measured in VSM, SVAS (left panel), or WBS (donor 1, right panel). In B, the levels of miR-29a (left panel), ELN (center panel) and COL1A1 (right panel) were measured in normal fibroblasts (NF) and an additional WBS donor.

Figure IV. Genotyping of two WBS donors.
Supplemental Figure I

miR-29a inhibitor

miR-29b inhibitor

miR-29c inhibitor
Supplemental Figure II

VSM

HDF

Normalized mRNA

Fibrillin1  Fibrillin2  Fibrillin4  Emilin1  LOX  LOXL1  LOXL2  ELN

Normalized mRNA

Fibrillin1  Fibrillin2  Fibrillin4  Emilin1  LOX  LOXL1  LOXL2  ELN

Legend:
- Ctr-M
- Ctr-I
- 29a-M
- 29a-I
Supplemental Figure III

**A**

Graphs showing normalized miR-29a levels:
- VSM vs. VSM + miR-29a-1: N.S.
- SVAS vs. SVAS + miR-29a-1: N.S.

**B**

Graphs showing:
- Normalized miR-29a levels:
  - NF, Ctr-I, 29a-I: WBS donor 2
- Normalized elastin mRNA:
  - NF, Ctr-I, 29a-I: WBS donor 2
- Normalized COL1A1 mRNA:
  - NF, Ctr-I, 29a-I: WBS donor 2

All graphs indicate no significant difference (N.S.).
Supplemental figure IV

Method and outcome of genetic sequence of WBS specimens:

A Chromosome Microarray Comparative Genomic Hybridization (CM-CGH) test was performed on a slide with an array of 180,000 60-mer oligonucleotide probes (Agilent 180K) in Yale cytogenetics laboratory.

WBS donor 1
The analysis of signal patterns revealed an XY male with a 822 Kb microduplication at 5q12.3 (chr5:65,489,514-66,312,145, involving genes SFRS12 and MAST4) and 1.413 Mb microdeletion at 7q11.23 (chr7:72,364,314-73,777,467.NCBI36/hg18, including genes from TRIM50 to GTF2I). The 7q11.23 microdeletion is pathognomonic for Williams-Beuren syndrome (OMIM #194050).

WBS donor 2
The analysis of signal patterns revealed an XY male with a 1.413 Mb microdeletion at 7q11.23 (chr7:72,364,314-73,777,467.NCBI36/hg18, including genes from TRIM50 to GTF2I). The 7q11.23 microdeletion is pathognomonic for Williams-Beuren syndrome (OMIM #194050).