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MicroRNA-29 in Aortic Dilation: Implications for Aneurysm Formation

Short Communication

Reinier A. Boon, Timon Seeger, Susanne Heydt, Ariane Fischer, Eduard Hergenreider, Anton J.G. Horrevoets, Manlio Vinciguerra, Nadia Rosenthal, Sergio Sciacca, Michele Pilato, Paula van Heijningen, Jeroen Essers, Ralf P. Brandes, Andreas M. Zeiher, Stefanie Dimmeler

Rationale: **Aging represents a major risk factor for coronary artery disease and aortic aneurysm formation. MicroRNAs (miRs) have emerged as key regulators of biological processes, but their role in age-associated vascular pathologies is unknown.**

Objective: **We aim to identify miRs in the vasculature that are regulated by age and play a role in age-induced vascular pathologies.**

Methods and Results: **Expression profiling of aortic tissue of young versus old mice identified several age-associated miRs. Among the significantly regulated miRs, the increased expression of miR-29 family members was associated with a profound downregulation of numerous extracellular matrix (ECM) components in aortas of aged mice, suggesting that this miR family contributes to ECM loss, thereby sensitizing the aorta for aneurysm formation. Indeed, miR-29 expression was significantly induced in 2 experimental models for aortic dilation: angiotensin II-treated aged mice and genetically induced aneurysms in Fibulin-4$^{R/R}$ mice. More importantly, miR-29b levels were profoundly increased in biopsies of human thoracic aneurysms, obtained from patients with either bicuspid ($n=79$) or tricuspid aortic valves ($n=30$). Finally, LNA-modified antisense oligonucleotide-mediated silencing of miR-29 induced ECM expression and inhibited angiotensin II-induced dilation of the aorta in mice.**

Conclusion: **In conclusion, miR-29-mediated downregulation of ECM proteins may sensitize the aorta to the formation of aneurysms in advanced age. Inhibition of miR-29 in vivo abrogates aortic dilation in mice, suggesting that miR-29 may represent a novel molecular target to augment matrix synthesis and maintain vascular wall structural integrity. (*Circ Res*. 2011;109:1115-1119.)**

Key Words: microRNA ■ aging ■ aneurysm

Age is one of the major risk factors for cardiovascular diseases. With increasing life expectancy, the prevalence of aging-associated cardiovascular diseases will even increase in the near future.[1] One particular age-associated disease is abdominal aortic aneurysm formation, which affects approximately 9% of elderly men and has a high mortality rate.[2] On the other hand, aneurysms in the ascending part of the thoracic aorta are less age-associated and are often the result of genetic defects involving extracellular matrix (ECM) components.[3] On a mechanistic level, analysis of human pathological sections revealed that aneurysm formation and rupture are characterized by thinning of the vascular wall and blood vessel dilation.[4] Decreased formation and/or increased degradation of ECM are believed to be the key pathophysiological processes leading to vascular wall thinning.[5,6]

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Non-standard Abbreviations and Acronyms	
Ang-II	Angiotensin II
ECM	extracellular matrix
LNA	locked nucleic acid
miR	microRNA
RT-PCR	real time PCR

MicroRNAs (miRs) have recently emerged as key regulators of several (patho-) physiological processes. MiRs are short noncoding RNAs that regulate protein expression by inducing degradation of the targeted mRNA or by blocking protein translation. Whereas various studies showed that specific miRs control vessel growth and cardiac function,[7] the involvement of miRs in aortic wall pathologies are less well known.

Methods

Comprehensive methods are available as an Online Supplement at http://circres.ahajournals.org.

Results

miRs Are Affected by Aging in the Aorta

miR and mRNA microarray expression profiles comparing aortas of aged with young mice revealed 18 miRs that are regulated (fold increase/decrease >1.5 and $P<0.01$) (Figure 1A and Online Table I). To establish which of these miRs affect mRNA expression changes, we used 2 distinct unbiased bioinformatics tools that use mRNA expression data to identify putative regulation by miRs. Both these tools, Sylamer[8] and MirExTra,[9] identified the miR-29 family (miR-29a, b, and c) to be the only 1 of the 18 regulated miRs to functionally affect mRNA levels (Figure 1B and 1C). The upregulation of the miR-29 family by age in aortic tissue was confirmed by real-time PCR (Figure 1D). The miR-29 family is transcribed as 2 bicistronic primary miRs (Figure 1E). We found that only the pri-miR-29b1/a cluster is transcriptionally induced by aging (Figure 1F), suggesting that miR-29 family members are increased by both transcriptional and post-transcriptional mechanisms. Together, these data indicate that miR-29 is the only age-regulated miRNA, which significantly affects mRNA expression levels in the aorta.

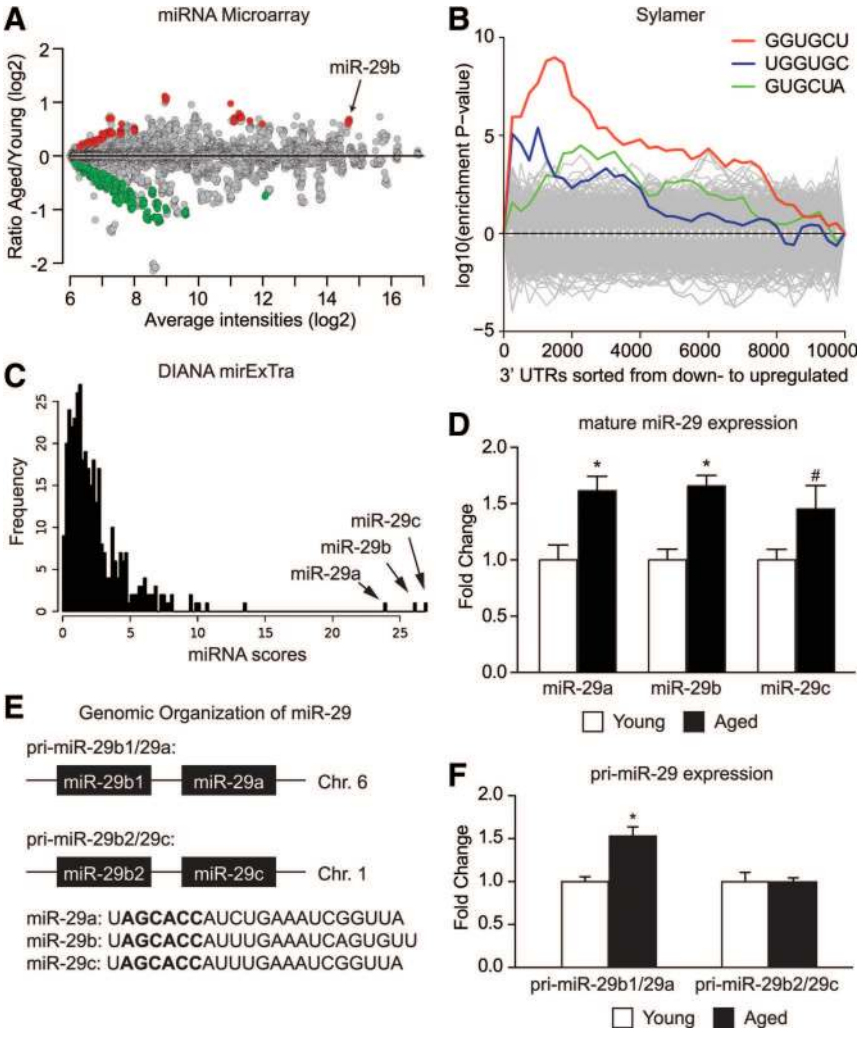

Figure 1. Aging regulates various miRs. MicroRNA and mRNA microarray profiles of aortas of aged (18 months old, n=4) and young mice (6 weeks old, n=4). **A**, M/A plot of the miRNA array data. Hybridization probes for upregulated miRs ($P<0.01$) are shown in red and for downregulated miRs ($P<0.01$) in green. mRNA profiling data were used to calculate enrichment of putative seed target sequences in the 3′UTR of genes that are regulated by aging, either with Sylamer (**B**) or mirExTra (**C**). RT-PCR of miR-29 levels in aortas (**D**) (n=8). **E**, miR-29 genomic organization. **F**, RT-PCR of primary miR-29 clusters in aortas of young and aged mice (n=5).*$P<0.05$, #$P=0.07$.

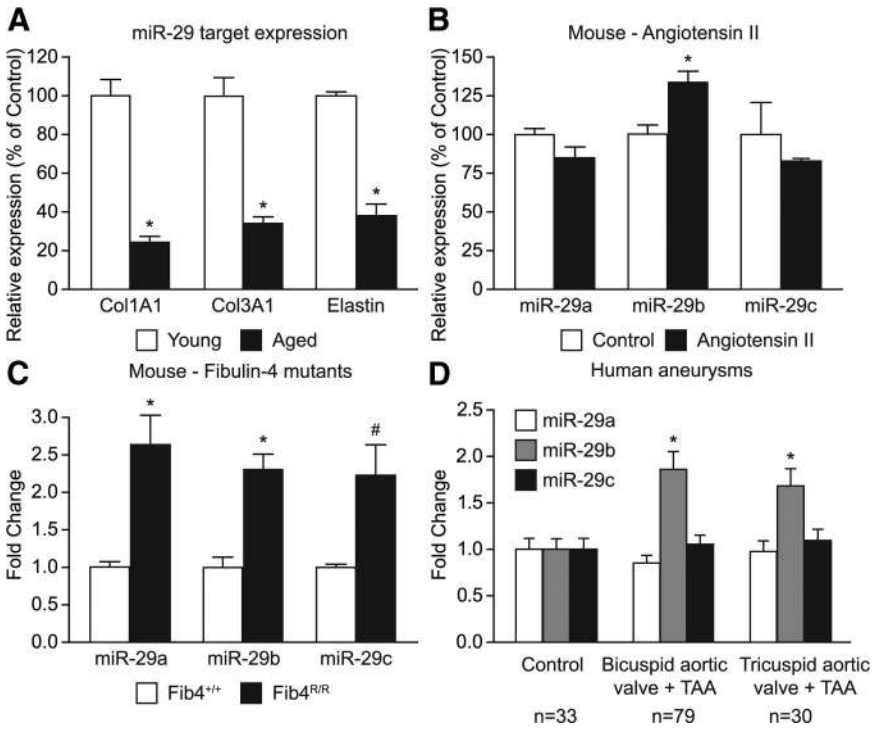

Figure 2. Increased expression levels of miR-29 in aneurysms. A, RT-PCR in entire aortas of young and aged mice (n=8) (**A**), in entire aortas of PBS-treated and Ang-II treated (1 mg/kg/d) mice (n=8) (**B**) and in aortic arches of Fibulin-4 mutant mice and wild-type littermates (n=3) (**C**). RT-PCR for miR-29 in human aorta biopsies (**D**).*$P<0.05$, #$P=0.1$.

MiR-29 Is Induced in Aortic Dilation and Aneurysms

In the heart, the miR-29 family has been shown to control tissue fibrosis after acute myocardial infarction by targeting mRNA coding for ECM proteins such as collagens, fibrillin and elastin.[10] All of these known targets of miR-29 were downregulated by age in the aorta (Figure 2A and Online Table II), including elastin, recently described to be crucially regulated by miR-29 in aortic development.[11] Because reduced ECM expression is a hallmark of aneurysm formation,[6,12] and age is the major risk factor for abdominal aortic aneurysm formation,[2] we hypothesized that the induction of miR-29 may link aging to aneurysm formation. Therefore, we next studied the role of miR-29 in 18-month-old mice that were infused with angiotensin II (Ang-II) for 1 week to induce aortic dilation in vivo. Ang-II infusion significantly increased the expression of miR-29b in the aorta, whereas miR-29a and miR-29c were not affected (Figure 2B).

Because defects in ECM components also contribute to the formation of inherited aneurysms, we next evaluated whether miR-29 family members are also regulated in the genetically induced thoracic aortic aneurysms that develop due to ECM defects in Fibulin-$4^{R/R}$ knockdown mice.[13] Fibulin-$4^{R/R}$ mice demonstrated a profound increase in expression of all 3 miR-29 family members in the aortic arch as compared to wild-type littermates (Figure 2C).

Finally, we analyzed the expression of miR-29a, b, and c in aortic biopsy samples obtained from a large series of patients with a thoracic aortic aneurysm undergoing aortic valve replacement surgery. The patients were divided into 2 groups, patients with a bicuspid aortic valve and patients with a tricuspid aortic valve, and were compared to control subjects without a thoracic aortic aneurysm, who underwent coronary bypass surgery (Online Table III). The expression of miR-29b is significantly increased in both aneurysm patient groups as compared to control subjects, whereas miR-29a and miR-29c are not affected (Figure 2D).

Inhibition of miR-29 In Vivo Prevents Ang II-Induced Dilation of the Aorta in Aged Mice

In order to assess whether miR-29 expression is causally linked to aortic dilation, we next designed LNA-modified antisense oligonucleotides[14] (LNA-29) to silence the expression of miR-29 in vivo. LNA-29 dose-dependently inhibited miR-29a, b, and c expression (Figure 3A), whereas it did not affect the expression of its closest homologues (Online Figure I). LNA-29 significantly increased expression of the miR-29 targets Col1A1, Col3A1, and elastin in aortic tissue (Figure 3B, Online Figure I). To determine whether inhibition of miR-29 prevents aortic dilation, we treated 18-month-old mice with Ang-II and measured the diameter of the aorta proximal to the origin of the renal arteries by ultrasonography before treatment and 7 days after. LNA-29 potently inhibited the expression of miR-29 in the aorta, even during continuous Ang-II infusion (Online Figure II), and induced a concomitant increase in protein levels of the miR-29 target elastin (Figure 3C, Online Figure III). LNA-29 abrogated the Ang-II-mediated increase in aortic diameter, whereas the aorta significantly dilated in the PBS- and control LNAotreated groups, as compared to untreated mice (Figure 3D). Likewise, aortic dilation in Ang-II–infused 6-month-old $ApoE^{-/-}$ mice was reduced by LNA-29 treatment at 1 week (Online Figure IV and V). Blood pressure was unaffected by miR-29 silencing, indicating that the lack of aortic diameter increase in Ang-II/LNA-29 cotreatment is not due to indirect effects on blood pressure (Online Figure VI). MiR-29 is known to regulate fibrosis in the heart, liver, and kidney.[10] However, LNA-29 did not affect cardiac function (data not shown) and

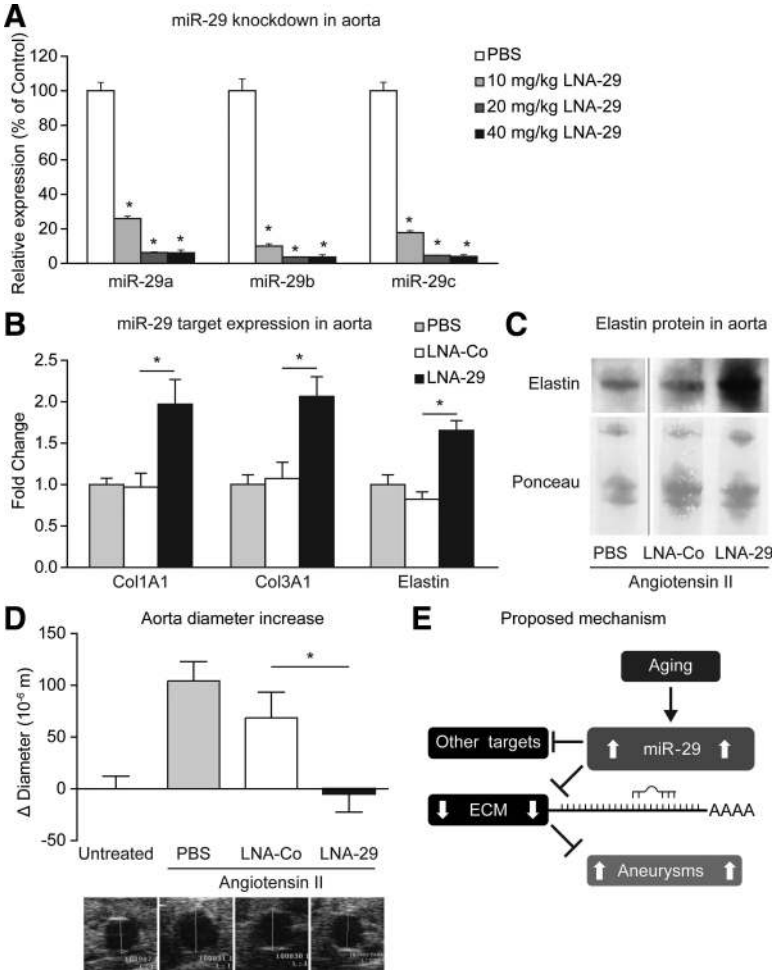

Figure 3. Inhibition of miR-29 induces ECM expression and prevents Ang-II-mediated aortic dilation. Mice (8 weeks old) were treated intravenously with LNA-29 at 10, 20, or 40 mg/kg or with PBS. After 7 days, miR-29 was measured by RT-PCR (n=4) (**A**). **B**, Mice (8 weeks old) received PBS, 20 mg/kg nontargeting control LNAs (LNA-Co) or 20 mg/kg LNA-29 intravenously. After 7 days, miR-29 was measured by RT-PCR (n=5–8). Eighteen-month-old mice were infused with Ang-II by subcutaneous mini-pumps (1 mg/kg/d) and received PBS, LNA-Co, or LNA-29 intravenously at day 0 (20 mg/kg). After 7 days, aortas were harvested and extracellular proteins were extracted. Elastin levels were analyzed by Western blot (**C**). Ponceau staining was used as loading control. The diameter of the aorta was measured in vivo by ultrasonography at day 0 and 7 and the difference in diameter between day 7 and 0 is shown in (**D**) (n=5–15). (**E**) Schematic representation of the proposed mechanism where aging triggers miR-29 expression, leading to a reduction in the expression of ECM components, which in turn causes aortic dilation. *$P<0.05$.

liver or kidney fibrosis, as investigated by histological analysis (Online Figure VI).

Discussion

Here, we demonstrate that vascular aging and age-associated pathologies induce a significant upregulation of the miR-29 family in the aorta of mice and humans. Whereas aging and genetically induced aneurysms were associated with increased expression of all family members, miR-29b was preferentially upregulated in Ang-II–induced aortic dilation in mice and in human thoracic aortic aneurysm biopsies. The upregulation of the miR-29 family in aging is consistent with a recent report, showing an increased expression of all miR-29 family members in an accelerated aging model in mice.[15]

MiR-29 targets several ECM proteins, which are known to play a key role in maintaining the integrity of the vascular wall. MiR-29 was additionally shown to induce apoptosis in cancer cells by targeting Mcl-1, an antiapoptotic Bcl-2 family member,[16] and by augmenting p53 levels.[17] Smooth muscle cell apoptosis is considered to favor aneurysm formation[18] and, because miR-29 is highly expressed in smooth muscle cells (Online Figure V and VII), this mechanism may contribute to miR-29–mediated destabilization of the vascular wall. Interestingly, inhibition of miR-29 decreased the expression of matrix metalloproteinase MMP9 in the aorta (Online Figure VIII), which may additionally prevent further degradation of matrix proteins. However, whereas miR-29 inhibition also blocked the early dilation in the Ang-II-induced aneurysm model in ApoE$^{-/-}$ mice (Online Figure IV), it did not prevent aneurysm formation in this rapidly accelerated inflammatory model at 4 weeks suggesting that inhibition of miR-29 predominantly maintains the structural integrity of the vessel wall.

In conclusion, aging and miR-29 repress the expression of target genes that mainly encode ECM proteins and may, thus, sensitize the aorta for aneurysm formation (Figure 3E). Local inhibition of miR-29, eg, by drug-eluting balloons or stents, may provide a promising novel therapeutic approach to interfere with vascular aging by augmenting matrix synthesis.

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Disclosures

Reinier A. Boon, Andreas M. Zeiher, and Stefanie Dimmeler applied for a patent regarding this work.

References

1. Lakatta EG. Age-associated cardiovascular changes in health: Impact on cardiovascular disease in older persons. *Heart Fail Rev*. 2002;7:29–49.
2. Singh K, Bonaa KH, Jacobsen BK, Bjork L, Solberg S. Prevalence of and risk factors for abdominal aortic aneurysms in a population-based study: The tromso study. *American Journal of Epidemiology*. 2001;154:236–244.
3. Ince H, Nienaber CA. Etiology, pathogenesis and management of thoracic aortic aneurysm. *Nat Clin Pract Cardiovasc Med*. 2007;4:418–427.
4. Tang PCY, Coady MA, Lovoulos C, Dardik A, Aslan M, Elefteriades JA, Tellides G. Hyperplastic cellular remodeling of the media in ascending thoracic aortic aneurysms. *Circulation*. 2005;112:1098–1105.
5. Guo DC, Papke CL, He R, Milewicz DM. Pathogenesis of thoracic and abdominal aortic aneurysms. *Ann N Y Acad Sci*. 2006;1085:339–352.
6. Mangrum WI, Farassati F, Kadirvel R, Kolbert CP, Raghavakaimal S, Dai D, Ding YH, Grill D, Khurana VG, Kallmes DF. Mrna expression in rabbit experimental aneurysms: A study using gene chip microarrays. *American Journal of Neuroradiology*. 2007;28:864–869.
7. Bonauer A, Boon RA, Dimmeler S. Vascular micrornas. *Curr Drug Targets*. 2010;11:943–949.
8. van Dongen S, breu-Goodger C, Enright AJ. Detecting microrna binding and sirna off-target effects from expression data. *Nat Meth*. 2008;5:1023–1025.
9. Alexiou P, Maragkakis M, Papadopoulos GL, Simmosis VA, Zhang L, Hatzigeorgiou AG. The diana-mirextra web server: From gene expression data to microrna function. *PLoS One*. 2010;5:e9171.
10. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microrna after myocardial infarction reveals a role of mir-29 in cardiac fibrosis. *Proceedings of the National Academy of Sciences*. 2008;105:13027–13032.
11. Ott CE, Grunhagen J, Jager M, Horbelt D, Schwill S, Kallenbach K, Guo G, Manke T, Knaus P, Mundlos S, Robinson PN. Micrornas differentially expressed in postnatal aortic development downregulate elastin via 3′ utr and coding-sequence binding sites. *PLoS One*. 2011;6:e16250.
12. Mueller BT, Modlich O, Prisack HB, Bojar H, Schipke JD, Goecke T, Feindt P, Petzold T, Gams E, Muller W, Hort W, Sandmann W. Gene expression profiles in the acutely dissected human aorta. *European Journal of Vascular and Endovascular Surgery*. 2002;24:356–364.
13. Hanada K, Vermeij M, Garinis GA, de Waard MC, Kunen MGS, Myers L, Maas A, Duncker DJ, Meijers C, Dietz HC, Kanaar R, Essers J. Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. *Circ Res*. 2007;100:738–746.
14. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S, Orum H. Therapeutic silencing of microrna-122 in primates with chronic hepatitis c virus infection. *Science*. 2010;327:198–201.
15. Ugalde AP, Ramsay AJ, de la Rosa J, Varela I, Marino G, Cadinanos J, Lu J, Freije JMP, Lopez-Otin C. Aging and chronic DNA damage response activate a regulatory pathway involving mir-29 and p53. *EMBO J*. 2011;30:2219–2232.
16. Mott JL, Kobayashi S, Bronk SF, Gores GJ. Mir-29 regulates mcl-1 protein expression and apoptosis. *Oncogene*. 2007;26:6133–6140.
17. Park SY, Lee JH, Ha M, Nam JW, Kim VN. Mir-29 mirnas activate p53 by targeting p85α and cdc42. *Nat Struct Mol Biol*. 2009;16:23–29.
18. Jones JA, Beck C, Barbour JR, Zavadzkas JA, Mukherjee R, Spinale FG, Ikonomidis JS. Alterations in aortic cellular constituents during thoracic aortic aneurysm development: Myofibroblast-mediated vascular remodeling. *Am J Pathol*. 2009;175:1746–1756.

Novelty and Significance

What Is Known?

- Advanced age is a major risk factor for developing aneurysms.
- MicroRNA-29 inhibits expression of several extracellular matrix proteins.

What New Information Does This Article Contribute?

- Advanced age induces microRNA-29 expression in the aorta and represses extracellular matrix expression.
- MicroRNA-29 levels are increased in the aorta in animal models of aneurysm formation and patients with thoracic aneurysms.
- Inhibition of microRNA-29 in vivo prevents aortic dilatation in mice.

Aging is a major risk factor for aneurysm formation. Because microRNAs play pivotal roles in various physiological processes, we assessed changes in the microRNA expression profile in the aorta in aged mice. MicroRNA-29, which is known to attenuate extracellular matrix expression, is induced in the aorta of old mice. Moreover, microRNA-29 is induced in mouse models of aortic aneurysm and human aneurysm biopsies. Inhibition of microRNA-29 prevents aortic dilation in aged mice. Thus, inhibition of microRNA-29 may constitute a promising treatment against aortic aneurysms.

Supplement Material

Supplemental Methods

Laboratory animals, human material and LNA-antimirs

C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany) and Janvier (Le Genest Saint-Isle, France). Fibulin-4 mutant mice were described before [1]. The animal experiments were approved by the Regional Board of the State of Hessen, Germany. Patient material was obtained under informed consent and according to the declaration of Helsinki. Atherosclerotic risk factors were determined as described before [2]. Echography was performed using a Vevo-770 (Visualsonics, Toronto, Canada) and aorta diameters were measured proximal of the renal arteries by a researcher blinded to the experimental procedure. Blood pressure measurements were performed with a Visitech 4 channel blood pressure analysis system (Visitech, Apex, NC). LNA-antimirs were synthesized by Exiqon (Vedbaek, Denmark). The following sequences were used in this study: LNA-Co: ACGTCTATACGCCCA, Tm 75°C; LNA-29: GATTTCAAATGGTGCT, Tm 73°C. Angiotensin II was purchased from Bachem (Bubendorf, Switzerland), osmotic pumps from Durect (Cupertino, CA).

RNA isolation, Real-time PCR, micro-arrays and bioinformatics

Total RNA was isolated with miRNeasy and miRNeasy FFPE kits from Qiagen and real-time PCR was performed with Applied Biosystems (Carlsbad, CA) microRNA assays run on an Applied Biosystems StepOnePlus machine. SNO202 and RNU6 were used for normalization. Transcriptional profiling was performed by DNAvision (Charleroi, Belgium) on the Agilent-v1 platform (570 miRs) or on the Affymetrix 430 v2.0 platform (mRNA, full genome). Microarray expression data are available at the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/project/geo). Data was background-corrected and normalized by quantile normalization. Statistical comparisons were done using a Bayesian one-way

ANOVA and probability-values were corrected for multiple testing with the Benjamini/Hochberg false discovery rate method. Sylamer analysis was performed with software from http://www.ebi.ac.uk/enright/sylamer/ using a genelist of microarray expression data sorted by regulation factor (fold change/p-value$^{-0.5}$) and publicly available mouse 3' UTR data (from Biomart). For DIANA mirExTra analysis (http://diana.cslab.ece.ntua.gr/hexamers), regulated gene lists (fold change<-1.5 and p-value<0.05) and unchanged gene lists (fold change= -1.1 to 1.1) were used. Sylamer and mirExTra identify statistical enrichment in a ranked list, more specifically these tools identify combinations of nucleotides that are present in the 3'UTR of genes that are regulated. MirExTra uses target prediction software rather than random nucleotide combinations.

Protein extraction and Western blots

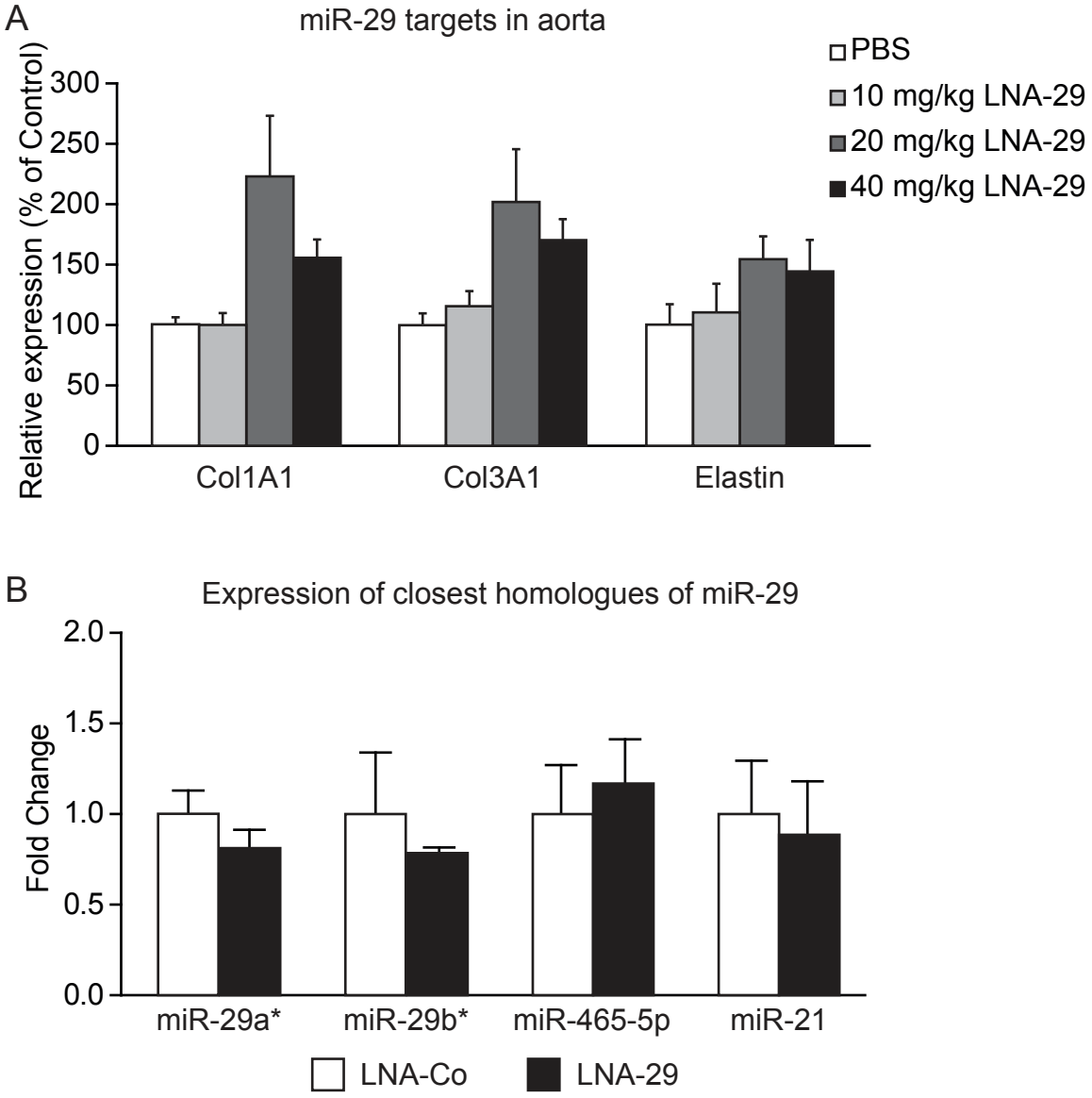
Extracellular proteins were extracted as previously described [3]. In short, adventitial tissue was removed from mouse aortas and washed 5 times in PBS with 25mM EDTA. Then the aortas were vortexed at room temperature for 72h in 4M guanidine HCl, 50mM sodium acetate, 25 mM EDTA, pH5.8. Proteins were then precipitated in 80% ethanol for 16h at -20°C and centrifugation. Pellets were washed with 90% ethanol, dried and resuspended in deglycosylation buffer (150mM NaCl, 50mM sodium acetate, 10mM EDTA, proteinase and phosphatase inhibitors, containing 0.05 units each of chondriotinase ABC, keratinase and heparitinase II (Sigma Aldrich, St. Louis, MO)). After 16h incubation at 37°C, each sample was boiled in denaturing sample buffer and loaded entirely on a single 8-16% polyacrylamide gradient gel (Thermo-Fischer). After separation, gels were blotted to PVDF membranes and total proteins were stained using PonceauS as loading control. The antibody used for Elastin was from Abcam (Cambridge, MA).

Statistics

Data were analyzed with Graphpad Prism 5 using unpaired student's t-tests when comparing two conditions, or a one-way ANOVA with Bonferroni or Newman-Keuls correction for multiple comparisons. Probability-values of less than 0.05 were considered significant and tests were performed two-sided. Correlations were analyzed using the Pearson method. Patient characteristics were assessed by Chi^2-test. Data are presented as mean and error bars depict the standard error of the mean (SEM).

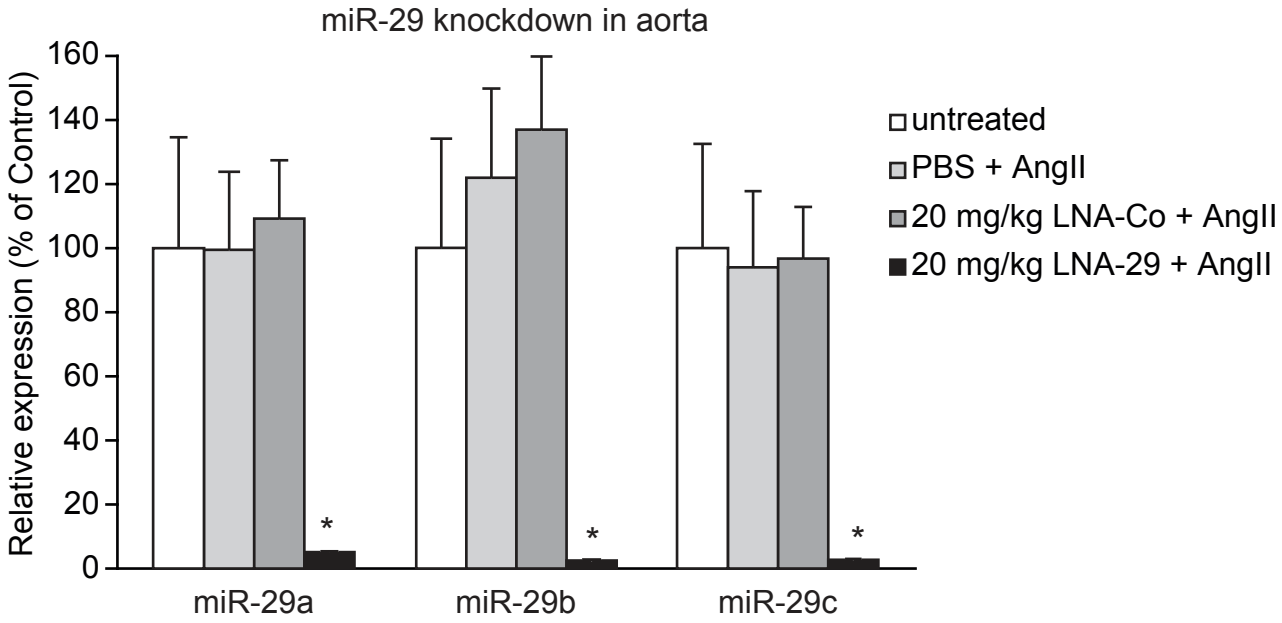
1. Hanada K, Vermeij M, Garinis GA, de Waard MC, Kunen MGS, Myers L, Maas A, Duncker DJ, Meijers C, Dietz HC, Kanaar R, Essers J. Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. *Circulation Research* 2007;100:738-746
2. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circulation Research* 2001;89:e1-e7
3. Didangelos A, Yin X, Mandal K, Baumert M, Jahangiri M, Mayr M. Proteomics characterization of extracellular space components in the human aorta. *Mol Cell Proteomics* 2010;9:2048-2062

Online figure I



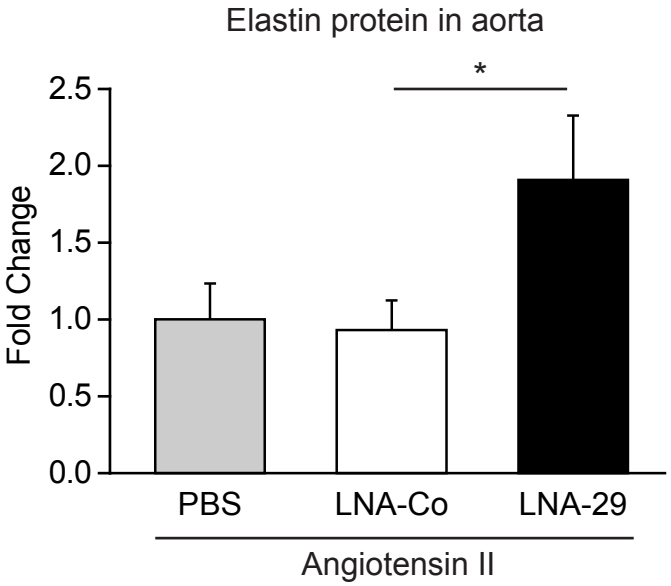
Online figure I. **MiR-29 inhibition dose-dependently induces miR-29 targets, but does not affect the expression levels of close homologues of miR-29a, b and c.** Mice (8 weeks old) were treated intravenously with LNA-29 at 10, 20 or 40 mg/kg or with PBS. After 7 days, collagen 1A1, 3A1 and elastin were measured by RT-PCR (n=4) (A). (B) RT-PCR was performed with RNA isolated from mouse aortas, one week after a single intravenous injection of 20 mg/kg LNA-29 (designed to target miR-29) or scrambled control LNA (LNA-Co). MiR-29 homologues were identified by BlastN at mirbase.org. MiR-21 is a fibrosis-related miRNA. (n=4)

Online figure II



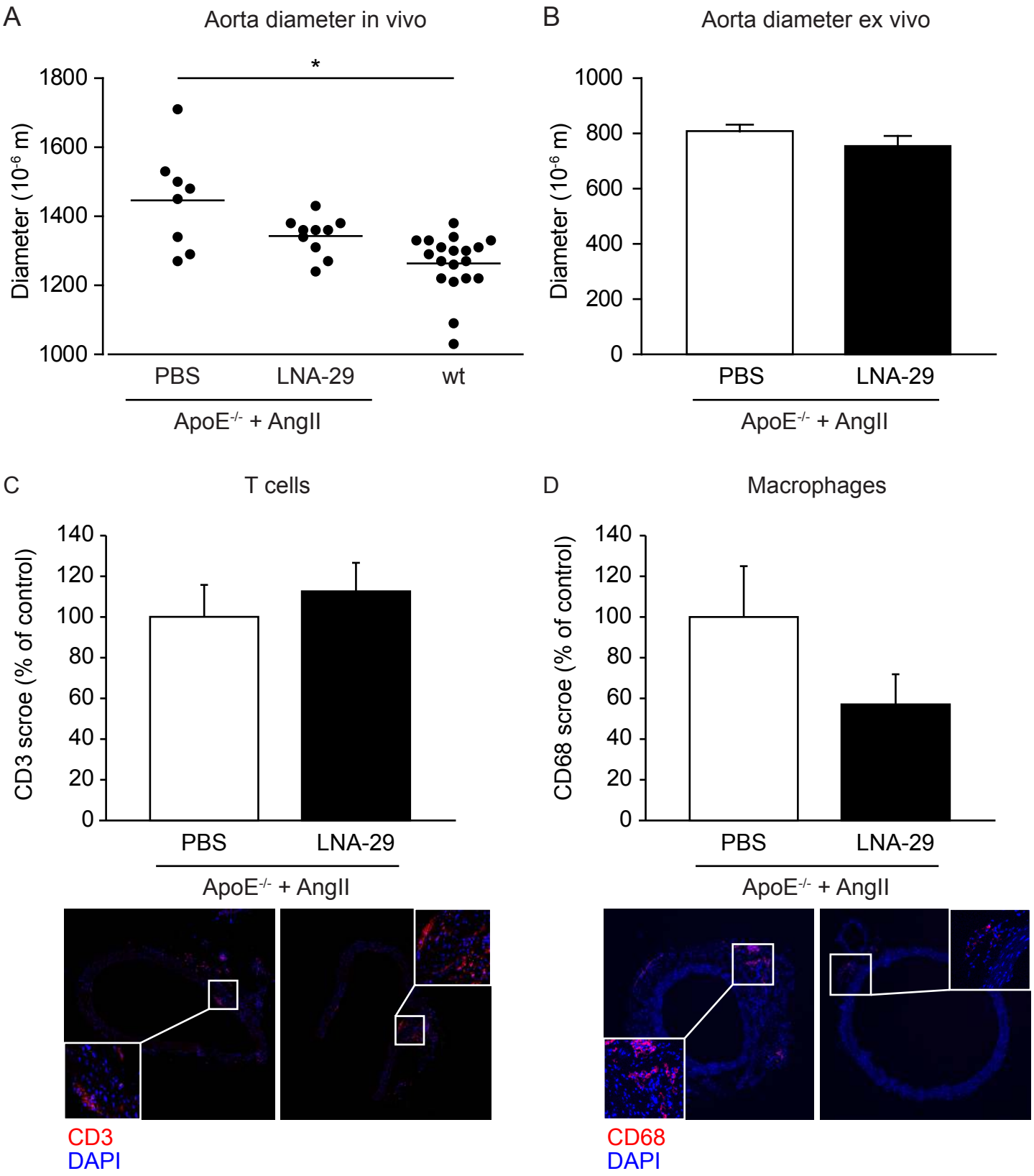
Online figure II. **LNA-29 inhibits the expression of miR-29 in the aorta in Ang-II treated mice.**
Real-time PCR was performed with RNA isolated from mouse aortas, four weeks after implantation of subcutaneous AngiotensinII-releasing minipump and weekly intravenous injection of PBS, 20 mg/kg LNA-29 (designed to target miR-29) or scrambled control LNA (LNA-Co). Untreated mice did not receive any intravenous injection nor AngII (n=4-10).

Online figure III



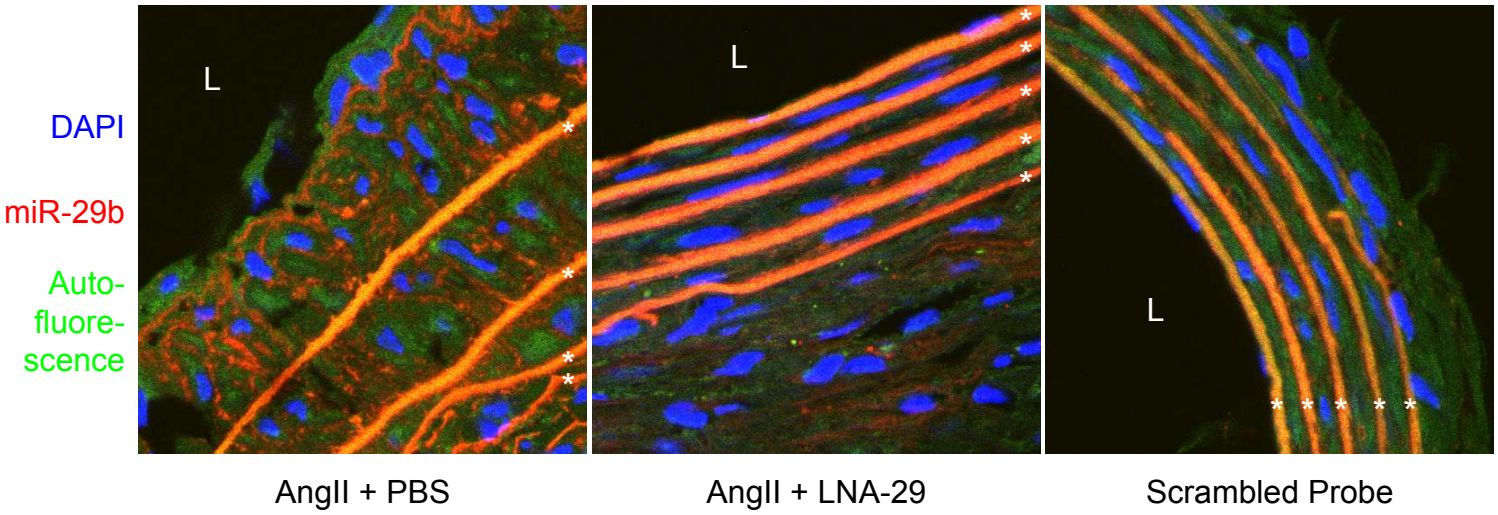
Online figure III. **Elastin protein expression in the aorta is induced by LNA-29 treatment.** 18-month old mice were infused with Ang-II by subcutaneous mini-pumps (1 mg/kg/day) and received PBS, LNA-Co or LNA-29 intravenously at day 0 (20 mg/kg). After 7 days, aortas were harvested and extracellular proteins were extracted. Elastin levels were analyzed by Western blot and quantified by densitometry (using ImageJ). n=4 *p=0.046 (one-way ANOVA with LSD multiple comparison post-hoc test)

Online figure IV



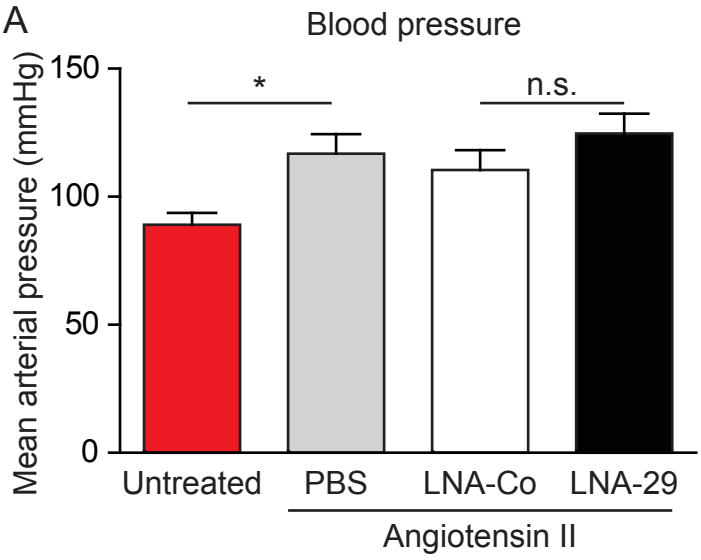
Online figure IV. **MiR-29 inhibition reduces AngII-mediated aorta dilation in ApoE$^{-/-}$ mice, without affecting inflammation.**
The diameter of the aorta was measured in vivo by ultrasonography after 7 days of AngII infusion in ApoE$^{-/-}$ mice and untreated wild-type mice (A) or after 4 weeks of AngII infusion from aortic histology sections ex vivo (non-pressurized) (B) (n=8-19). Vessel wall T lymphocyte and macrophage burden were quantified in histology sections, with CD3 and CD68 stainings (C and D). *P<0.05

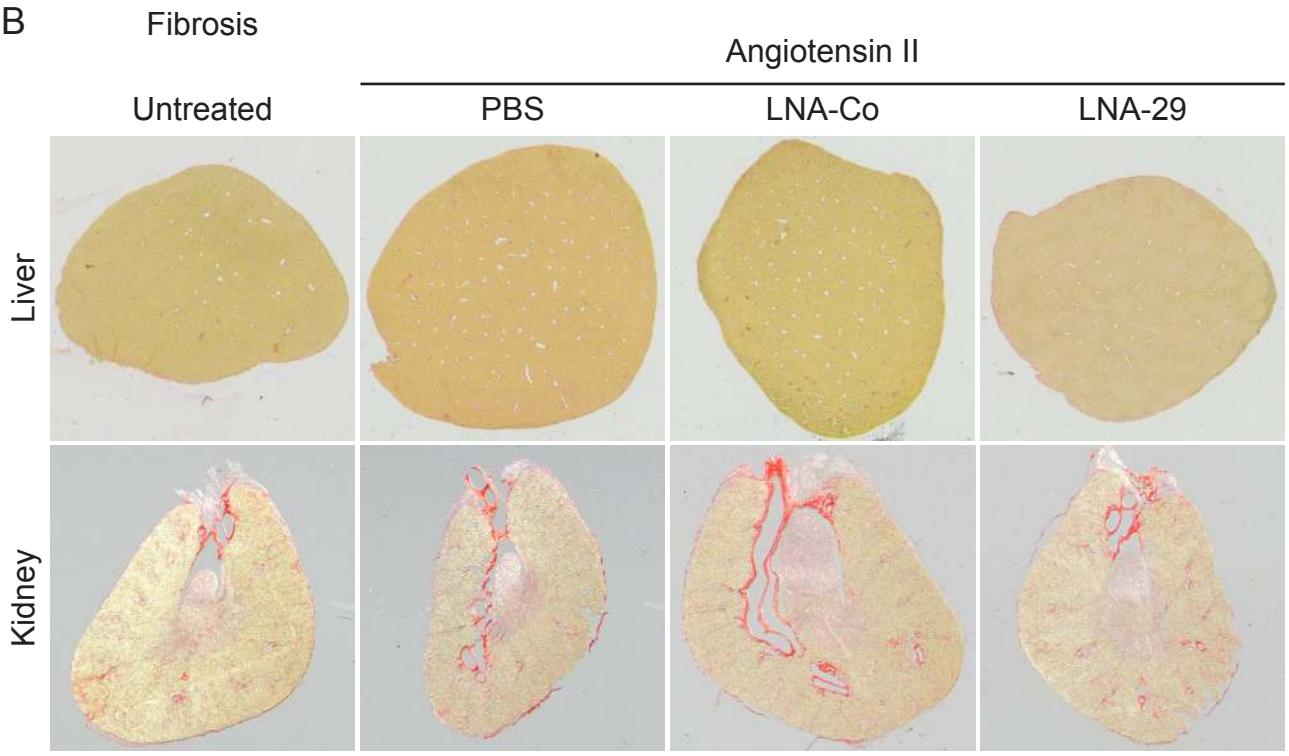
Online figure V



Online figure V. **MiR-29b is expressed in the vessel wall of Angiotensin II treated ApoE$^{-/-}$ mice.** In situ hybridization for miR-29b with double-DIG-labeled LNA detection probes on aorta tissue from 6 month-old ApoE$^{-/-}$ mice that were treated with Angiotensin II and either PBS or LNA-29 for 4 weeks. Autofluorescence is depicted in green. The miR-29b or scrambled (non-specific) detection probe is visualized in red. Yellow color indicates autofluorescent signal in both the green and red channels. (L) depicts the vessel lumen and (*) indicate autofluorescent internal elastic laminae.

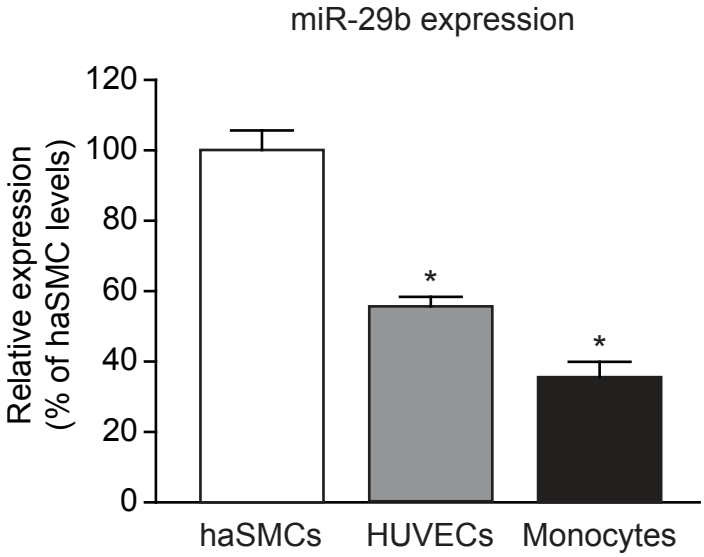
Online figure VI





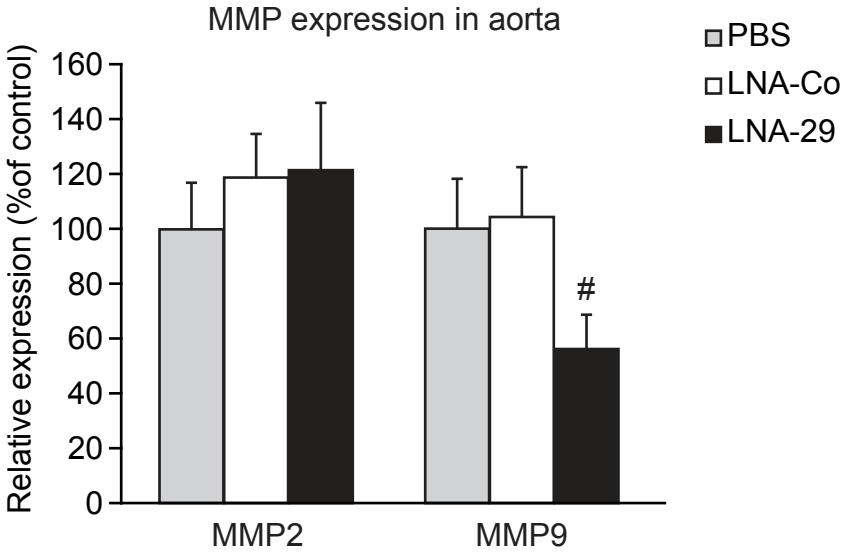
Online figure VI. **LNA-29 treatment does not affect blood pressure, nor fibrosis, in combination with Angiotensin II infusion.**
(A) Blood pressure was measured using tail-cuffs, 5 days after implantation of subcutaneous Angiotensin II-releasing minipump and a single intravenous injection of PBS, 20 mg/kg LNA-29 (designed to target miR-29) or scrambled control LNA (LNA-Co). Untreated mice did not receive any intravenous injection nor AngII. (n=6-8) (B) Liver and kidney sections were obtained from mice that received continuous Angiotensin II infusion and weekly treatment with intravenous PBS, LNA-Co (20 mg/kg) or LNA-29 (20 mg/kg) at 4 weeks after start of treatment. Collagen was stained using Sirius Red.

Online figure VII



Online figure VII. **miR-29b expression levels in vascular cell types.** Real-time PCR was performed for miR-29b with RNA isolated from human aorta smooth muscle cells (white bar), human umbilical vein endothelial cells (grey bar) and human CD14+ cells isolated from peripheral blood mononuclear cells (black bar). *$p<0.05$

Online figure VIII



Online figure VIII. **LNA-29 reduces the expression of MMP9 but not MMP2 in the aorta.**
Real-time PCR was performed for MMP2 and MMP9 with RNA isolated from mouse aortas, one week after a single intravenous injection of PBS, 20 mg/kg LNA-29 (designed to target miR-29) or scrambled control LNA (LNA-Co) (n=5-8). #p=0.068

Online table I. MicroRNAs that are regulated by age in the mouse aorta (absolute fold change>1.5 and p<0.01)

Upregulated miRs	Fold	FDR p	Role
mmu-miR-146a	2.02	0.00671	Inflammation
mmu-miR-142-3p	1.63	0.00599	Hematopoiesis
mmu-miR-29b	1.61	0.00381	Fibrosis/apoptosis
mmu-miR-223	1.58	0.00649	Hematopoiesis

Downregulated miRs	Fold	FDR p	Role
mmu-miR-299*	-2.21	0.00032	Unknown
mmu-miR-181c	-2.14	0.00028	Hematopoiesis
mmu-miR-127	-2.10	0.00013	Lung development
mmu-miR-154	-1.95	0.00145	Unknown
mmu-miR-337-5p	-1.86	0.00163	Unknown
mmu-miR-379	-1.76	0.00150	Unknown
mmu-miR-136	-1.71	0.00244	Unknown
mmu-miR-329	-1.70	0.00017	Unknown
mmu-miR-31	-1.67	0.00246	Oncogenesis
mmu-miR-322	-1.67	0.00675	Angiogenesis/differentiation
mmu-miR-377	-1.63	0.00359	Fibrosis
mmu-miR-434-3p	-1.61	0.00325	Unknown
mmu-miR-411	-1.54	0.00052	Unknown
mmu-miR-181d	-1.51	0.00237	Hematopoiesis

Online table II Extracellular matrix expression is repressed by age in the aorta

Symbol	fold change	Bayes.p	FDR	GenBank	UniGene	Description
Fbn2	-6.0323876	0.00037421	0.013589	NM_010181	Mm.20271	fibrillin 2
Col1a1	-4.7324502	1.00E-06	0.000192	U08020	Mm.277735	procollagen, type I, alpha 1
Mmp16	-3.7790897	0.00024836	0.010372	BB378819	Mm.204820	matrix metallopeptidase 16
Col3a1	-3.4727743	3.52E-09	2.41E-06	AW550625	Mm.249555	procollagen, type III, alpha 1
Col1a2	-3.376066	1.32E-08	6.85E-06	BF227507	Mm.277792	procollagen, type I, alpha 2
Fbn2	-3.2689534	0.00084569	0.023661	AV010392	Mm.20271	fibrillin 2
Eln	-3.0622485	7.60E-07	0.000154	BB229377	Mm.275320,	elastin
Col1a1	-2.9436819	1.60E-07	4.83E-05	BI794771	Mm.277735	procollagen, type I, alpha 1
Col3a1	-2.6254093	6.91E-07	0.000144	BG075843	Mm.249555	procollagen, type III, alpha 1
Col11a1	-2.5613174	0.00011755	0.006237	NM_007729	Mm.209715	procollagen, type XI, alpha 1
Col5a2	-2.3009434	8.75E-09	4.93E-06	AV229424	Mm.10299	procollagen, type V, alpha 2
Eln	-2.2686736	1.23E-08	6.43E-06	BB229377	Mm.275320,	elastin
Col5a2	-2.2310021	6.31E-07	0.000134	AV229424	Mm.10299	procollagen, type V, alpha 2
Fbn1	-2.2188853	6.14E-09	3.74E-06	NM_007993	Mm.271644	fibrillin 1
Col4a5	-2.1432228	3.78E-07	9.03E-05	BM250666	Mm.286892	procollagen, type IV, alpha 5
Col11a1	-2.1242418	0.00045111	0.015402	NM_007729	Mm.209715	procollagen, type XI, alpha 1
Col5a3	-2.0269518	1.83E-06	0.000307	NM_016919	Mm.334994	procollagen, type V, alpha 3
Col4a3	-2.0197026	8.73E-05	0.005068	NM_007734	Mm.389135	procollagen, type IV, alpha 3
Fbn1	-1.9968533	3.12E-08	1.37E-05	AF007248	Mm.271644	fibrillin 1
Col4a3	-1.9927252	0.00066508	0.020131	AV366831	Mm.389135	procollagen, type IV, alpha 3
Fbln1	-1.9910311	0.0003049	0.011968	BC007140	Mm.297992	fibulin 1
Col1a2	-1.9752956	3.92E-05	0.002916	BB150460	Mm.277792	procollagen, type I, alpha 2
Col4a5	-1.9659698	6.34E-07	0.000134	BM250666	Mm.286892	procollagen, type IV, alpha 5
Col3a1	-1.9630329	9.64E-10	8.53E-07	AW550625	Mm.249555	procollagen, type III, alpha 1
Sparc	-1.9372812	1.88E-08	9.21E-06	NM_009242	Mm.291442	secreted acidic cysteine rich glycoprotein
Sparc	-1.9282242	3.2E-08	1.39E-05	NM_009242	Mm.291442	secreted acidic cysteine rich glycoprotein
Col5a1	-1.9075833	4.73E-05	0.003337	AW744319	Mm.7281	procollagen, type V, alpha 1
Col6a1	-1.8665417	8.34E-08	2.89E-05	NM_009933	Mm.2509	procollagen, type VI, alpha 1
Col9a1	-1.8332169	0.00064798	0.019854	AK004383	Mm.154662	procollagen, type IX, alpha 1
Col6a2	-1.8325502	9.13E-08	3.05E-05	BI455189	Mm.1949	procollagen, type VI, alpha 2
Col14a1	-1.8100671	1.68E-05	0.001563	BB521934	Mm.297859	procollagen, type XIV, alpha 1
Col1a2	-1.7775253	5.26E-09	3.30E-06	BF227507	Mm.277792	procollagen, type I, alpha 2
Col15a1	-1.7486951	1.15E-05	0.001213	AF011450	Mm.233547	procollagen, type XV
Col10a1	-1.7486624	0.00842435	0.106487	NM_009925	Mm.443177	procollagen, type X, alpha 1
Mmp2	-1.7453837	1.77E-06	0.000302	NM_008610	Mm.29564	matrix metallopeptidase 2
Col4a1	-1.741257	2.16E-07	6.17E-05	BF158638	Mm.738	procollagen, type IV, alpha 1
Fbln1	-1.7110513	0.00090112	0.024646	NM_010180	Mm.297992	fibulin 1
Col2a1	-1.6905208	0.00819705	0.104878	NM_031163	Mm.2423	procollagen, type II, alpha 1
Col4a6	-1.6892918	0.00054146	0.017569	BB794645	Mm.155586	procollagen, type IV, alpha 6
Mmp2	-1.6854596	9.07E-06	0.001	BF147716	Mm.29564	matrix metallopeptidase 2
Col6a2	-1.6838415	2.10E-06	0.000336	BI455189	Mm.1949	procollagen, type VI, alpha 2
Col5a1	-1.6635513	6.34E-06	0.00079	AW744319	Mm.7281	procollagen, type V, alpha 1
Col6a3	-1.6634733	1.32E-06	0.000239	AF064749	Mm.7562	procollagen, type VI, alpha 3
Mmp16	-1.6234235	0.00702838	0.096144	AF282844	Mm.204820	matrix metallopeptidase 16
Mmp14	-1.5735841	0.00022699	0.009806	NM_008608	Mm.280175	matrix metallopeptidase 14 (membrane-inserted)
Col4a2	-1.5601997	4.79E-05	0.003336	BC013560	Mm.181021	procollagen, type IV, alpha 2
Col4a1	-1.5557434	8.08E-05	0.004815	BF158638	Mm.738	procollagen, type IV, alpha 1
Col14a1	-1.5535355	4.16E-05	0.00301	AJ131395	Mm.297859	procollagen, type XIV, alpha 1
Col4a6	-1.4977489	0.00340145	0.060043	BB794645	Mm.155586	procollagen, type IV, alpha 6
Lamc1	-1.483957	0.0000757	0.004612	BG066605	Mm.1249	laminin, gamma 1
Col4a4	-1.4692681	0.0029215	0.054855	BB530633	Mm.40253, M	procollagen, type IV, alpha 4
Timp4	-1.4546069	0.00454159	0.072609	BI788452	Mm.255607	tissue inhibitor of metalloproteinase 4
Mmp14	-1.4037513	0.00214608	0.044604	NM_008608	Mm.280175	matrix metallopeptidase 14 (membrane-inserted)
Fbln5	-1.3734461	0.00075926	0.021909	NM_011812	Mm.288381	fibulin 5
Col19a1	1.96123182	3.39E-05	0.002587	BB459641	Mm.329196	procollagen, type XIX, alpha 1
Mmp9	1.99019903	0.00015219	0.007413	NM_013599	Mm.4406, Mr	matrix metallopeptidase 9
Mmp13	2.01744927	0.00843435	0.106494	NM_008607	Mm.5022	matrix metallopeptidase 13
Mmp9	2.04005973	5.36E-05	0.00361	NM_013599	Mm.4406, Mr	matrix metallopeptidase 9
Col19a1	2.25942133	4.84E-05	0.003349	NM_007733	Mm.329196	procollagen, type XIX, alpha 1
Mmp3	2.29322653	1.32E-08	6.78E-06	NM_010809	Mm.4993	matrix metallopeptidase 3
Mmp12	5.26819181	8.64E-08	2.95E-05	BC019135	Mm.2055	matrix metallopeptidase 12

Confirmed target of Mir29 (van Rooij et al PNAS (2008) 105, p13027-32)

Confirmed target of Mir29 (Li et al JBC (2009) 284, p15676-84)

Confirmed target of Mir29 (Luna Mol Vis (2009) 15, p2488-97)

Confirmed target of Mir29 (Liu Hypertension (2010) 55, p974-82)

Up in AAA (Rush et al BMC Gen (2009) 10, p298-314 and Pyo et al JCI (2000) 105, p1641-9)

Online table III. Patient characteristics for the obtained aortic biopsies.

	Control	Bicuspid aortic valve + TAA	Tricuspid aortic valve + TAA	p-value
n=	33	79	30	
Age	67.5(8.9)	58.3(12.3)	69(10.2)	<0.0001
Hypertension	70%	65%	80%	0.1905
Angiotensin medication	58%	56%	14%	0.0120
Hypercholesterolemia	42%	34%	27%	0.2442
Diabetes	33%	6%	3%	<0.0001
Atrial fibrillation	3%	10%	20%	0.0776
Smoker	30%	30%	17%	0.3715
Females	18%	30%	52%	0.0067