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Experimental reduction of miR-92a mimics arterial aging

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Abstract

MicroRNAs (miRs) are small non-coding RNAs that are important regulators of aging and cardiovascular diseases. MiR-92a is important in developmental vascular growth and tumorigenesis and two of its putative targets, tumor necrosis factor alpha receptor (TNFR1) and collagen type1, play a role in age-related arterial dysfunction. We hypothesized that reduced miR-92a expression contributes to age-related arterial dysfunction characterized by endothelial dysfunction and increased large artery stiffness. MiR-92a is reduced 39% ($p < 0.05$, RT-PCR) in arteries of older adults compared to young adults. Similarly, there was a 40% reduction in miR-92a in aortas of old (29 mo, $n = 13$) compared to young (6 mo, $n = 11$) B6D2F1 mice, an established model of vascular aging. To determine if reduced miR-92a contributes to arterial dysfunction; miR-92a was inhibited *in vivo* in young mice using antagomirs (I.P., 4 weeks). Antagomir treatment was associated with a concomitant 48% increase in TNFR1 (Western blot, $p < 0.05$), 19% increase in type 1 collagen (immunohistochemistry, $p < 0.01$), and a reduction in endothelial dependent dilation (max dilation: 93 ± 1 v 73 ± 5 %, $p < 0.01$) in response to acetylcholine (ACh, 10^{-9} to 10^{-4} M). Treatment with the nitric oxide (NO) synthase inhibitor, L-NAME (10^{-4} M), revealed that impaired ACh dilation after antagomir treatment resulted from reduced NO bioavailability. Inhibition of miR-92a also increased arterial stiffness (309 ± 13 vs. 484 ± 52 cm/s, $p < 0.05$, pulse wave velocity). Together, these results suggest that experimental reductions in arterial miR-92a partially mimic the arterial aging phenotype and we speculate that modulating miR-92a may provide a therapeutic strategy to improve age-related arterial dysfunction.

Keywords

microRNA; age related arterial dysfunction; endothelial dependent dilation

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1. Introduction

Advancing age is the major risk factor for the development of cardiovascular disease (CVD), the leading cause of morbidity and mortality in the United States.¹ Arterial dysfunction is a major contributing factor to the development of CVD and aging confers its deleterious effects primarily via its effects on the arteries.¹ With advancing age, arterial dysfunction is primarily characterized by impaired endothelial function evidenced by reductions in endothelium-dependent dilation (EDD) and reduced nitric oxide (NO) bioavailability with concomitant alterations in extracellular matrix composition (i.e. collagen deposition) and increased stiffness (reduced compliance) of large elastic arteries leading to increased aortic pulse wave velocity (PWV).^{1, 2} Furthermore, impaired arterial EDD and increased PWV are independent predictors of future CVD.² Although the importance of endothelial dysfunction and arterial stiffening with age are well established, the molecular mechanisms involved and strategies to prevent these adverse effects have not been elucidated.

Aging is a complex process that is impacted by both environmental and genetic factors. Factor that has been recently established as an important regulator of aging are microRNAs (miRs). miRs are small, 21–25 nucleotide, highly conserved non-coding RNAs that regulate gene expression. miRs contribute to the regulation of cell function (e.g. proliferation and apoptosis), as well as the regulation of inflammation³ and other biological processes that are involved in vascular diseases⁴ including vascular calcification and cellular senescence. It has been suggested that close to 60% of genes are regulated by miRs, implying their critical role in controlling biological processes. In *C. elegans*, studies have shown that several miRs are differentially expressed in aging⁵ and recently this has been extended to mice, primates and humans.⁶

Studies have revealed that miR-92a, plays an important role in vascular growth during both normal fetal development and tumorigenesis.⁷ miR-92a is of particular interest to aging and vascular research owing to its negative association with important factors in arterial dysfunction. An age-related reduction in miR-92a was reported in human CD8⁺ T cells.⁸ miR-92a has also been found to be downregulated in senescent aortic endothelial cells and was associated with enhanced inflammation.⁹ miR-92a has also been found to be associated with several markers of large artery stiffness and inflammation, important macromechanistic processes involved in age-associated arterial dysfunction.¹⁰ Specifically, miR-92a is predicted to target the tumor necrosis factor alpha (TNF- α) receptor, the receptor for a key pro-inflammatory molecule; and collagen type I, a major constituent of the arterial wall that confers arterial stiffness.¹¹

Thus, we hypothesized that miR-92a is reduced with aging in human subjects as well as in a mouse model of aging. We also hypothesized that the aged arterial phenotype would be associated with increases in downstream targets of miR-92a, such as collagen type I and tumor necrosis factor alpha (TNF- α) receptor. We further hypothesized that in vivo inhibition of miR-92a in young mice using commercially available antagomirs would recapitulate the reduction in miR-92a seen in aging models and lead to arterial dysfunction similar to aging, i.e. reduced maximal endothelial dependent dilation (EDD) and increased stiffness of arteries.

2. Materials & Methods

2.1 Human subject selection and characterization

Arterial samples were collected from young (n=15) and older adults (n=12) during sentinel node biopsies. Skeletal muscle feed arteries were excised from the inguinal (e.g., hip adductors or quadriceps femoris) or axillary (e.g. serratus anterior or latissimus dorsi) regions and were free of melanoma cells.¹² Individuals having HIV, Hepatitis B or C, ongoing malignancy, current pregnancy or history of organ transplantations were excluded. Additionally, patients with a prior diagnosis of metastatic melanoma, prior chemotherapy treatment, and/or indication of melanoma metastasis (blood lactate dehydrogenase N618 U/L or positive sentinel lymph node biopsy) were excluded. Characteristics of the individuals are described in Table 1a. This study was conducted under Institutional Review Board of University of Utah (IRB) approval. Participants gave written informed consent and Declaration of Helsinki protocols were followed. Human samples were used only for data presented in Fig. 1A, all other data was collected using mouse tissues.

2.2 Animal assurances

All animal procedures conform to the *Guide to the Care and Use of Laboratory Animals* (version 8, revised 2011) and have been approved by the Institutional Animal Care and Use Committee at the University of Utah and the Veteran's Administration Medical Center-Salt Lake City.

2.3 Animals

Young (5–6 months, n=20) and old (29–31 months, n=10) male B6D2F1 mice were purchased from Charles River Inc. (Wilmington, MA) or the National Institute of Aging colony maintained at Charles River, respectively. Groups of young and old mice were left untreated (young: 11, old: 12) and aortas collected for the assessment of arterial miR-92a expression. Additional groups of young mice were treated with an *in vivo* processed negative control oligomer (8 mg/kg, 1/wk, 4 wks, ip) (Dharmacon, Lafayette, CO) or an *in vivo* processed miR-92a inhibitor (8 mg/kg, 1/wk, 4 wks, ip) (miRIDIAN microRNA mmu-miR-92a-1-3p hairpin, Dharmacon). The animal characteristics are listed in Table 1b.

2.4 Quantification of miRs expression by Real Time RT-PCR

RNA was extracted from excised aortas using RNeasy mini kit (Qiagen, Foster City, CA). miR analysis was done using TaqMan kits and primers (Applied Biosystems, Foster City, CA). FAM-labeled primer for miR-92a and RNU6B was used (ABI). Cycle threshold values (C_t) were determined using ABI 7500 Fast PCR system and miR expression values were calculated using RNU6B as an endogenous control following the 2^{-C_t} method.

2.5 Endothelium Dependent Dilation (EDD)

EDD to acetylcholine (ACh, 10^{-9} to 10^{-4} M) was assessed after pre-constriction to phenylephrine (2 μ M) in excised carotid arteries. To assess NO bioavailability (NO-mediated EDD), ACh dose responses were repeated in the presence of NO synthase inhibitor, NG-

nitro-L-arginine methyl ester (L-NAME: 10^{-4} M).¹³ Endothelium independent dilation to sodium nitroprusside (SNP, 10^{-10} to 10^{-4} M) was also assessed.

2.6 Arterial Stiffness

The day before euthanasia, mice were anesthetized with 2% isoflurane and aortic pulse wave velocity (PWV) was measured as previously described.¹⁴

2.7 Quantification of type I and type III collagen

10uM sections of aortic segment were stained with picrosirius red to identify total collagen as described.¹⁴ Green channel images from a RGB stack were used for collagen assessment. Images were captured using an Olympus IX81 inverted microscope with epifluorescence and DIC (Nomarski) illumination lamps under polarized light to identify the type I and type III collagen fibers. Olympus DP Controller imaging software was used for image acquisition. All images were analyzed by Image J. Collagen content was calculated as percentage of the selected area (arterial wall) with positive color.

2.8 Western blotting

Aortic lysates were separated by SDS-PAGE and subsequently electro blotted to PVDF membranes. Blots were incubated overnight with beta actin (ab8227, Abcam) and TNFR1 (ab19139, Abcam) antibody. Target bands were normalized to the amount of protein loaded, as determined by densitometric analysis of the corresponding beta actin lane.

2.9 Statistical Analysis

Data were analyzed with GraphPad Prism using student's t-tests when comparing two conditions or with analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons. The EDD data were analyzed using repeated measures of ANOVA with SPSS. Probability values less than 0.05 were considered significant. Outliers were detected by ESD test. Data are presented as mean $\pm$ SEM.

3. Results

3.1 miR-92a is down regulated in arteries of older humans and mice

Arterial samples from older adults demonstrated a 39% reduction ($p<0.05$) in miR-92a expression compared to younger adults (Fig. 1A). Likewise, aortas isolated from old mice demonstrated a 40% reduction ($p<0.05$) in miR-92a expression compared to young mice (Fig. 1B).

3.2 Arterial expression of the miR-92a targets increases after anti-miR-92a treatment

Similar to arteries from older humans and mice, anti-miR-92a treatment resulted in a 33% reduction ($p<0.01$) in arterial miR-92a expression in young compared to mice treated with scrambled (scr) miR (Fig. 1C). Body and tissue masses as well as fed blood glucose were similar between treatment groups (Table 1b).

The putative miR-92a target gene, type 1 collagen, was higher ($p<0.05$) in the medial layer of the aortas of anti-miR-92 treated mice compared to scr-miR treated mice (Fig. 1D).

However, the total collagen and type III collagen remained unchanged (data not shown). Likewise, anti-miR-92a increased arterial TNFR1 expression (Fig. 1E) compared to scr-miR treated mice.

3.3 Inhibition of miR-92a impairs endothelial function in young mice

There was a significant dose by group interaction for ACh between carotid arteries from scr-miR and anti-miR-92a treated mice (Fig. 2A, $P < 0.001$) and maximal carotid artery vasodilation to ACh was also reduced ($p = 0.01$) in anti-miR-92a compared to scr-miR treated mice (Fig. 2C). Sensitivity (IC_{50}) to ACh did not differ between anti-miR-92a (-8.17 ± 0.09 log M) and scr-miR (-7.92 ± 0.11 log M) treated mice ($p = 0.14$).

After treatment with L-NAME, a competitive inhibitor of eNOS, phenylephrine-induced pre-constriction tended to increase in anti-miR-92a ($28.8 \pm 2.4\%$, $p = 0.06$) but not in scr-miR treated mice ($27.5 \pm 3.1\%$). L-NAME reduced vasodilation to incremental doses of ACh in carotid arteries (Fig. 2A) from scr-miR ($p = 0.05$) but not anti-miR-92a ($p = 0.36$) treated mice. Likewise, maximal dilation to ACh was reduced after L-NAME in scr-miR ($p < 0.001$) but not in anti-miR-92a ($p = 0.22$) treated mice, a finding that is suggestive of reduced NO bioavailability after anti-miR-92a treatment (Fig. 2C) or a shift in the contribution of NO relative to other vasodilators such as prostanoids or endothelial derived hyperpolarizing factors. Sensitivity to ACh did not differ after L-NAME in either the scr-miR (-7.3 ± 0.1 log M) or anti-miR-92a (-7.2 ± 0.2 log M) treated mice.

Pre-constriction to phenylephrine did not differ between scr-miR and anti-miR-92a (24.7 ± 1.3 vs. $24.0 \pm 2.9\%$) treatment groups ($p > 0.05$). Endothelium independent dilation in response to SNP was unaltered between treatments groups (Fig. 2B).

3.4 Inhibition of miR-92a increases large elastic artery stiffness without impacting medial wall thickness or blood pressure

Aortic stiffness, assessed by PWV, was not different between groups prior to the initiation of treatment. PWV increased 37% in anti-miR-92a ($P < 0.05$) but was unchanged in scr-miR treated mice (Fig. 2D). Anti-miR-92a treatment did not impact carotid artery medial area (scr-miR: 53903.7 ± 7940.08 mm², anti-mir-92a: 96768.08 ± 40760.36 mm²) or total collagen content (scr-miR: $70.61 \pm 6.45\%$, anti-mir-92a: $78.17 \pm 4.60\%$). Neither systolic blood pressure (131 ± 7 vs. 136 ± 7 mm Hg) nor heart rate (699 ± 10 vs. 714 ± 18 bpm) differed between treatment groups.

3.5 Treatment with scrambled oligomers appears to be without effect on young mice

Compared to our previously published studies, body mass was similar in untreated young mice and young mice treated with scrambled miR (young: 32.7 ± 1.34^{15} vs. scr-miR: 30 ± 0.3 , g). In addition, mean arterial pressure (young: 130 ± 1^{16} vs. scr-miR: 131 ± 7 , mm Hg) and pulse wave velocity (young: 324 ± 14^{15} vs. scr-miR: 309 ± 12 , cm/s) were also similar between young untreated and scr-miR treated mice. Carotid artery maximal dilation to ACh was 95% in untreated mice¹⁶ and 92% in scr-miR treated mice. The EDD response to SNP was also similar with maximal vasodilation in young mice 98%¹⁴ and scr-miR treated mice

97%. Together, these data suggest that there was no effect of treatment with a scrambled oligomer.

4. Discussion

In our study, we demonstrate for the first time that miR-92a is reduced with aging in the arteries of older adults. We also demonstrate that there is a similar reduction of miR-92a in aortas from aged mice. In addition, we also demonstrate that the level of reduction of miR-92a in experimental mice after antagomir treatment was similar to what we found in older humans and aged mice without any treatments. Furthermore, we find that this reduction in miR-92a is associated with increased arterial expression of a major structural protein, type 1 collagen, and the pro-inflammatory receptor, TNFR1. Furthermore, similar to advancing age, anti-miR-92a resulted into arterial dysfunction that is characterized by impaired carotid artery EDD and reduced NO bioavailability as well as increased aortic stiffness, evidenced by increased pulse wave velocity. These findings suggest that interventions aimed at increasing arterial miR-92a should be explored in the setting of age-related arterial dysfunction.

In this study, we demonstrated that aging leads to reduced arterial expression of miR-92a in older adults (Fig. 1A). We also demonstrated that there was a similar reduction in miR-92a in aorta isolated from aged mice (Fig. 1B) as observed in human subjects. Downregulation of miR-92a has been reported in human diploid fibroblasts and in trabecular meshwork cells in stress induced premature senescence¹⁷ and also in human CD8⁺ T cells with aging.⁸ Previously, we have demonstrated that miR-92a is down-regulated in senescent human aortic endothelial cells,⁹ taken together these studies suggest that miR-92a may play a role in aging in a variety of tissues. Our novel results are in agreement with others demonstrating that miR-92a is downregulated in various tissues with aging, and extend these findings to demonstrate an effect of aging on arterial miR-92a expression.

To test whether downregulation of miR-92a recapitulates arterial aging phenotype, we have modulated miR-92a using commercially available antagomirs in young mice, which resulted in a similar reduction in miR-92a as seen in older human arteries and aged mouse aorta (Fig. 1C). Target prediction algorithms suggest that a key marker of inflammation, TNFR1, and collagen type1, a major constituent of the arterial wall conferring stiffness, may both be direct targets of miR-92a. Thus, we measured their expression in arterial tissues after antagomir treatment. We found that type 1 collagen and TNFR1 were increased in anti-miR-92a treated mice (Fig. 1D and E). Consistent with the results of the present study, an increase in arterial TNFR1 expression has been previously associated with aging in the carotid arteries of C57BL/6 mice¹⁸ and in the setting of age related atherosclerosis.¹⁸ In addition, our laboratory have previously demonstrated that aging is associated with increased type I collagen in the carotid artery.¹⁰

As impaired endothelial function resulting from reduced NO bioavailability, is one of the main features of arterial aging¹⁹ we assessed EDD and NO bioavailability after anti-miR-92a treatment. We have previously demonstrated that carotid artery dilation in response to acetylcholine (ACh) was ~26% lower in older mice compared with young mice¹⁶, here we

find that anti-miR-92 treatment lowered dilation ~20% in young mice compared to young mice treated with scr-miR. Treatment with the scr-miR appears to be without any effect on EDD as maximal dilation to ACh was 95% in untreated¹⁶ and 92% in scr-miR treated young mice. Similar to aging, we found that inhibition of miR-92a promotes arterial dysfunction, as indicated by reduced maximal vasodilation to ACh that may be mediated by a reduction in NO bioavailability or a shift away from a reliance on NO as a vasodilator (Fig. 2C).

Stiffening of large conduit arteries, assessed by aortic PWV in mice and humans, is another hallmark of vascular aging¹⁴ and is associated with increased CVD risk in older adults. Similar to what we have observed with aging in this mouse model,¹⁴ Our group has previously shown that PWV of old mice is 22% higher compared to young mice,¹⁵ and here, we demonstrate a 37% increase in PWV in mice treated with anti-miR-92a compared to scr-miR treated mice. Scr-miR treatment also appears to be without effect on PWV in young mice (untreated: $324 \pm 14 \text{ cm/s}$ ¹⁵ vs. scr-miR: $309 \pm 12 \text{ cm/s}$). Although IMT has been demonstrated to increase with advancing age, IMT was unaltered by anti-miR-92a treatment (date not shown). The lack of effect on IMT could result from the short duration of treatment with antagomirs, which was insufficient to alter blood pressure or pulse pressure, important stimuli for increase in IMT. Despite a lack of increase in IMT, changes in primary structural matrix proteins of the aortic wall, such as collagen and elastin as well as the accumulation of advanced glycation end products, play a major role in stiffening of large elastic arteries. As collagen synthesis is directly inhibited by the miR-17-92 cluster,²⁰ collagen content was measured in the mouse aorta after treatments. In the anti-miR-92a treated mice, total collagen content tended to increase and aortic type I collagen content was upregulated (Fig. 1D), a finding similar to what is observed in advancing age.¹⁰ This increase in type I collagen occurred in the absence of changes of another arterial collagen isoform, collagen type 3, an isoform not predicted to be a target of miR-92a. Thus, from the observations mentioned above, it can be concluded that experimental reduction of miR-92a mimic some of the phenotypes commonly seen in arterial aging, such as increased arterial stiffness, reduced endothelial dependent dilation, increased arterial collagen and TNFR1 content.¹⁶

The present study includes limitations that should be noted. First, we aimed to determine if inhibition of miR-92a could mimic arterial aging, and therefore the studies were carried out in young mice. Future studies should be conducted using miR-92a mimics to determine if increases in miR-92a can improve vascular function in aged mice. Second, the duration of the treatment may also play a role in not observing all the age related changes in the young mice. Thus, future studies could be planned to assess the long-term effect of miR-92a inhibition and the development of aging phenotype. In addition, the effect of inhibition of miR-92a on longevity of the mice should be investigated.

5. Conclusions

Our findings demonstrated that miR-92a is downregulated in aging and experimental reductions of miR-92a in young mice led to an aging-like phenotype as evident by reduced EDD and large artery stiffening. Moreover, we also provide insights into potential mechanism of action of miR-92a inhibition and development of aging phenotype. These

findings suggest the potential for the development of new therapies targeting miR-92a to correct age related arterial dysfunctions.

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Highlights

- Expression of miR-92a is reduced in arteries from aged human subjects and mice
- Experimental reductions in miR-92a impair large artery function
- Reductions in miR-92 are associated with increases in type 1 collagen and TNFR1

Figure 1. miR-92a expression is reduced in arteries from older human subjects, aortas of aged mice and in arteries of young mice after anti-miR-92a treatment and this is associated with an upregulation of the putative targets of miR-92a (type 1 collagen and tumor necrosis factor receptor-1) in anti-miR-92a treated mice

(A) miR-92a expression in arteries from young and older adults (n=12–15 per group), *p<0.05 vs. young adults and in (B) young and old mouse aortas (n=11–12 per group), *p<0.05 vs. young. (C) miR-92a expression in aortas of young mice treated *in vivo* with scrambled miR (scr-miR) or anti-miR-92a (n=6–10 per group), **p<0.01 vs. scr-miR. (D) Type I collagen content in the thoracic aorta was measured by picosirius red staining in histological sections of aorta from scr-miR and anti-miR-92a treated mice, followed by image acquisition under polarized light, *p<0.05 vs. scr-miR. (E) Tumor necrosis factor receptor-1 expression in aorta from scr-miR and anti-miR-92a treated mice measured by Western blotting, *p<0.05 vs. scr-miR, N=4–6 per group, Values are means ± sem

Figure 2. Reduced endothelial function and increased arterial stiffness in anti-miR-92a treated mice

(A) Endothelium-dependent dilatation (EDD) to ACh in the absence or presence of L-NAME measured by pressure myography in carotid arteries of scr-miR and anti-miR-92a treated mice. * $p < 0.05$ scr-miR vs. anti-miR-92a, † $p < 0.05$ ACh vs. ACh with L-NAME, (B) endothelium independent dilation to sodium nitroprusside, $n = 6$ per group. (C) maximal dilation to ACh in the absence or presence of L-NAME in scr-miR and anti-miR-92a treated mice. * $p < 0.05$ anti-miR-92a ACh vs. scr-miR ACh, † $p < 0.001$ ACh vs. ACh in the presence of L-NAME (D) Aortic stiffness measured by *in vivo* pulse wave velocity (PWV) in scr-miR and anti-miR-92a treated mice pre and post treatment. * $p < 0.05$ denotes difference from pre. Values are means $\pm$ sem. ($n = 8-10$ per group)

Table 1a

Characteristics of young and old individuals used for measuring miR-92a expression

	Young	Old	p value
Number	15	12	
Gender M/F	6/9	7/8	
Age (years)	30 ± 2	67 ± 2	p<0.001
Height (cm)	177 ± 2	168 ± 2	p<0.010
Weight (kg)	74 ± 4	84 ± 3	p=0.060
BMI	24 ± 1	30 ± 1	p<0.001
Systolic Blood Pressure (mm Hg)	125 ± 3	142 ± 6	p<0.050
Diastolic Blood Pressure (mm Hg)	73 ± 3	74 ± 4	p=0.870
Medical History			
Hypertension	0(0%)	7 (58%)	
Coronary Artery Disease	0(0%)	1 (8%)	
Peripheral Vascular Disease	0(0%)	0 (0%)	
Diabetes Mellitus	1(6%)	3 (25%)	
Chronic Kidney Disease	0(0%)	0 (0%)	
Hypothyroid	1(6%)	1(8%)	
Asthma	1(0%)	1(8%)	
Medication			
Calcium Channel Blocker	0(0%)	4(33%)	
Beta Blocker	0(0%)	1(8%)	
ACE inhibitor	0(0%)	2(16%)	
Angiotensin receptor blocker	0(0%)	1(8%)	
Diuretics	0(0%)	1(8%)	
Statin	0(0%)	6(50%)	

Table 1b

Characteristics of mice treated with scrambled miR and anti-miR-92a

	scr-miR	anti-miR-92	p value
Body mass, g	30.0 ± 0.30	30.7 ± 0.70	p=0.380
Heart, g	0.15 ± 0.00	0.15 ± 0.01	p=0.670
Heart: BW g/g x100	0.49 ± 0.01	0.49 ± 0.03	p=0.990
Soleus, g	0.011 ± 0.001	0.011 ± 0.001	p=0.520
Soleus: BW g/gx100	0.038 ± 0.003	0.034 ± 0.001	p=0.410
WAT, g	0.67 ± 0.15	0.46 ± 0.07	p=0.770
WAT: BW g/gx100	2.2 ± 0.50	1.5 ± 0.20	p=0.340
Blood Glucose (mg/dl)	125 ± 7	136 ± 13	p=0.450