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ORIGINAL ARTICLE

Nicotinamide mononucleotide (NMN) supplementation promotes anti-aging miRNA expression profile in the aorta of aged mice, predicting epigenetic rejuvenation and anti-atherogenic effects

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Abstract Understanding molecular mechanisms involved in vascular aging is essential to develop novel interventional strategies for treatment and prevention of age-related vascular pathologies. Recent studies provide critical evidence that vascular aging is characterized by NAD+ depletion. Importantly, in aged mice, restoration of

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cellular NAD+ levels by treatment with the NAD+ booster nicotinamide mononucleotide (NMN) exerts significant vasoprotective effects, improving endothelium-dependent vasodilation, attenuating oxidative stress, and rescuing age-related changes in gene expression. Strong experimental evidence shows that dysregulation of microRNAs (miRNAs) has a role in vascular aging. The present study was designed to test the hypothesis that age-related NAD+ depletion is causally linked to dysregulation of vascular miRNA expression. A corollary hypothesis is that functional vascular rejuvenation in NMN-treated aged mice is also associated with restoration of a youthful vascular miRNA expression profile. To test these hypotheses, aged (24-month-old) mice were treated with NMN for 2 weeks and miRNA signatures in the aortas were compared to those in aortas obtained from untreated young and aged control mice. We found that protective effects of NMN treatment on vascular function are associated with anti-aging changes in the miRNA expression profile in the aged mouse aorta. The predicted regulatory effects of NMN-induced differentially expressed miRNAs in aged vessels include anti-atherogenic effects and epigenetic rejuvenation. Future studies will uncover the mechanistic role of miRNA gene expression regulatory networks in the anti-aging effects of NAD+ booster treatments and determine the links between miRNAs regulated by NMN and sirtuin activators and miRNAs known to act in the conserved pathways of aging and major aging-related vascular diseases.

Keywords Senescence · Atherosclerosis · Vascular cognitive impairment · Epigenetics · Vascular aging · Endothelial dysfunction · Oxidative stress

Introduction

Age-related diseases of the cardiovascular system are a leading cause of morbidity and mortality in the elderly (Abdellatif et al. 2018; Minamino and Komuro 2007; Wang and Bennett 2012; Alfaras et al. 2016; Ungvari et al. 2018). Vascular aging is associated with stiffening of the large arteries, endothelial dysfunction, oxidative stress, and inflammation, promoting the development of atherosclerotic vascular diseases (ischemic heart diseases, stroke, peripheral artery disease) and aorta aneurysm (Wang and Bennett 2012; Ungvari et al. 2018). Microvascular aging is also a major contributing factor to the pathogenesis of vascular cognitive impairment (VCI), Alzheimer's disease, cerebral microhemorrhages, sarcopenia, heart failure, chronic kidney disease and (Ungvari et al. 2018; Mullins et al. 2014; Ungvari et al. 2017a; Toth et al. 2017; Tarantini et al. 2017a; Tarantini et al. 2016a; Sagare et al. 2013; Sweeney et al. 2018; Montagne et al. 2017; Kisler et al. 2017; Payne 2006; Hoenig et al. 2008; Long et al. 2012). Understanding molecular mechanisms involved in vascular aging is essential to develop novel interventional strategies for treatment and prevention of age-related vascular pathologies.

MicroRNAs (miRNA) are short, endogenous, non-coding transcripts that repress gene expression at the post-transcriptional level in both physiological and pathological conditions. Strong experimental evidence suggest that miRNAs have a role in regulation of lifespan in model organisms (Boehm and Slack 2005; Grillari and Grillari-Voglauer n.d.; Ibanez-Ventoso et al. 2006) and that alterations in cellular miRNA expression profile also play a role in mammalian aging (Bates et al. n.d.; Maes et al. 2008; Inukai et al. 2012; Inukai and Slack 2013; Ito et al. 2010; Mercken et al. 2013; Smith-Vikos and Slack 2012; Ungvari et al. 2013a; Zhang et al. 2012; Zovoilis et al. 2011; Smith-Vikos et al. 2016; ElSharawy et al. 2012). Importantly, miRNAs were also reported to regulate several important aspects of endothelial biology and vascular function (Bonauer et al. 2009; Doebele et al. n.d.; Kuehbacher et al. 2007; Chen et al. 2015a; Hergenreider et al. 2012; Kim et al. 2014; Leung et al. 2013; Lovren et al. 2012; O'Rourke and Olson 2011; Rotllan et al. 2013; Stellos and Dimmeler 2014; Weber et al. 2014; Zampetaki et al. 2014). Several studies have demonstrated that age-related miRNA dysregulation importantly contributes to the development of vascular aging phenotypes (Ito et al. 2010; Ungvari et al. 2013a,b; Menghini et al. 2014; Badi et al. 2018; Guo et al. 2017; Hazra et al. 2016; Regina et al. 2016; Boon et al. 2013; Csiszar et al. 2014) and promotes the pathogenesis of atherosclerotic diseases (Ono et al. 2011) encompassing every step from sterile vascular inflammation, plaque formation to plaque destabilization and rupture (Hartmann et al. 2016; Lu et al. 2018; Zhang et al. 2018). Dysregulation of miRNA expression has also been linked to microvascular aging phenotypes, including impaired angiogenesis (Ungvari et al. 2013b; Csiszar et al. 2014; Che et al. 2014; Jansen et al. 2015). Experimental interventions that both extend lifespan and prevent/delay age-related vascular dysfunction in rodents, including caloric restriction (Csiszar et al. 2014) and induction of early-life IGF-1 deficiency (Tarantini et al. 2016b), were shown to reverse aging-induced alterations in vascular miRNA

expression. Despite these advances, fundamental cellular and molecular processes of aging that are responsible for dysregulation of vascular miRNA expression have not been elucidated.

NAD^+ is a rate-limiting co-substrate for sirtuin enzymes, which are key regulators of pro-survival pathways in the vasculature (Das et al. 2018; Csiszar et al. 2009a; Csiszar et al. 2009b; Csiszar et al. 2008). Aging is associated with cellular NAD^+ depletion (Gomes et al. 2013; Massudi et al. 2012), which has been proposed to be a critical driving force of aging processes. In support of this theory, it was demonstrated that enhancing NAD^+ biosynthesis extends lifespan in lower organisms (Anderson et al. 2002) and improves health-span in mouse models of aging (Mitchell et al. 2018). Recent studies provide critical evidence that vascular aging is also characterized by NAD+ depletion (Tarantini et al. 2019; Csiszar et al. 2019; Kiss et al. 2019). Importantly, we 69 and other laboratories demonstrated (Das et al. 2018; de Picciotto et al. 2016) that in aged mice restoration of cellular NAD^+ levels by treatment with the NAD+ precursor nicotinamide mononucleotide (NMN) (Yoshino et al. 2018) confers potent anti-aging vascular effects, reversing endothelial dysfunction, improving mitochondrial function, and attenuating oxidative stress.

The present study was designed to test the hypothesis that age-related NAD+ depletion is causally linked to dysregulation of vascular miRNA expression. A corollary hypothesis is that functional vascular rejuvenation in NMN-treated aged mice is also associated with restoration of a youthful vascular miRNA expression profile. To test these hypotheses, aged mice were treated with NMN for 2 weeks and miRNA signatures in the aortas were compared to those in aortas obtained from untreated young and aged control mice.

Methods

Animals, NMN supplementation

Young (3-month-old) and aged (24-month-old) male C57BL/6 mice were purchased from the aging colony maintained by the National Institute on Aging at Charles River Laboratories (Wilmington, MA). The biological age of 24-month-old mice corresponds to that of ~ 60-year-old humans. Mice were housed under specific pathogen-free barrier conditions in the Rodent Barrier Facility at University of Oklahoma Health Sciences Center under a controlled photoperiod (12 h light; 12 h dark) with unlimited access to water and were fed a standard AIN-93G diet (ad libitum). Mice in the aged cohort were assigned to two groups. One group of the aged mice was injected daily with NMN (i.p. injections of 500 mg NMN/kg body weight per day) or the equivalent volume of PBS for 14 consecutive days at 6 PM and 8 AM on day 14 and were sacrificed 4 h after last injection. Similar dosages of NMN have been shown to exert potent anti-aging effects on mouse health span (de Picciotto et al. 2016). All procedures were approved by the Institutional Animal Use and Care Committees of the University of Oklahoma Health Sciences Center. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The effects of NMN treatment on cognitive function, cerebromicrovascular responses, and aorta endothelial function in the same cohort of mice have been recently reported (Tarantini et al. 2019).

Quantitative real-time RT-PCR and miRNA expression profiling

A quantitative real time RT-PCR technique was used to analyze miRNA expression profiles in the aorta of mice from each experimental group as reported (Ungvari et al. 2013b; Csiszar et al. 2014; Tarantini et al. 2016b). In brief, total RNA was isolated with a mirVana™ miRNA Isolation Kit (ThermoFisher Scientific) and was reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit as described previously (Ungvari et al. 2013b; Csiszar et al. 2014; Tarantini et al. 2016b). The expression profile of mouse miRNAs in aortas derived from young and aged control mice and aged NMN-treated mice was analyzed using the TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 (ThermoFisher Scientific). The qPCR data were quantified using the $\Delta\Delta$Ct method (Livak and Schmittgen 2001). Predicted and experimentally validated microRNA targets were obtained from the TargetScan database (Agarwal et al. 2015), and Gene Ontology enrichment analysis was performed on differentially expressed microRNA targets using Fisher's exact test between TargetScan targets and annotations from the Gene Ontology database (Harris et al. 2004). To identify relationships between miRNA targets and terms in the biomedical literature, we utilized

the IRIDESCENT system (Wren and Garner 2004). IRIDESCENT uses a statistical model to determine whether each target gene co-occurs with a term of interest more frequently than would be expected by chance, and quantifies this in terms of the mutual information measure.

Results

Changes in vascular miRNA expression profile in mice associated with aging and with NMN treatment

We assessed changes in miRNA expression in the mouse aorta associated with aging and with NMN treatment. Hierarchical clustering (Fig. 1a) and principal component analysis (Fig. 1b) of miRNA expression showed a clear separation between the young and aged groups. Aged control mice and aged NMN-treated mice were also separated in the principal component analysis and hierarchical clustering. In contrast, miRNA expression in young mice and NMN-treated aged mice was similar and these groups did not separate well in the principal component analysis and hierarchical clustering. The Venn diagram in Fig. 1c shows that expression of several miRNAs, which are differentially expressed in the aortas of young and aged mice, was restored to youthful levels in aortas of NMN-treated aged mice. These data suggest that NAD^+ depletion has a critical role in age-related dysregulation of vascular miRNA expression. Figure 2 shows changes in expressions of individual miRNAs in the mouse aorta associated with age and NMN treatment.

Since the discovery of miRNA regulation of genes, several studies have been focused on predicting the biologically relevant target genes for miRNAs. We have used TargetScan database to predict putative biological targets of miRNAs differentially expressed with age whose expression is restored to youthful levels in aortas of aged mice by NMN supplementation (Table 1). GO terms enriched among miRNAs differentially expressed with age whose expression is restored to youthful levels in aortas of aged mice by NMN supplementation are shown in Table 2. Analysis of the differentially expressed miRNAs indicated that a statistically significant number of them had target sites within genes associated with pathways regulating the intracellular signaling, protein homeostasis, and inflammation (Table 2). The results are consistent with the predicted anti-aging effects of NMN treatment.

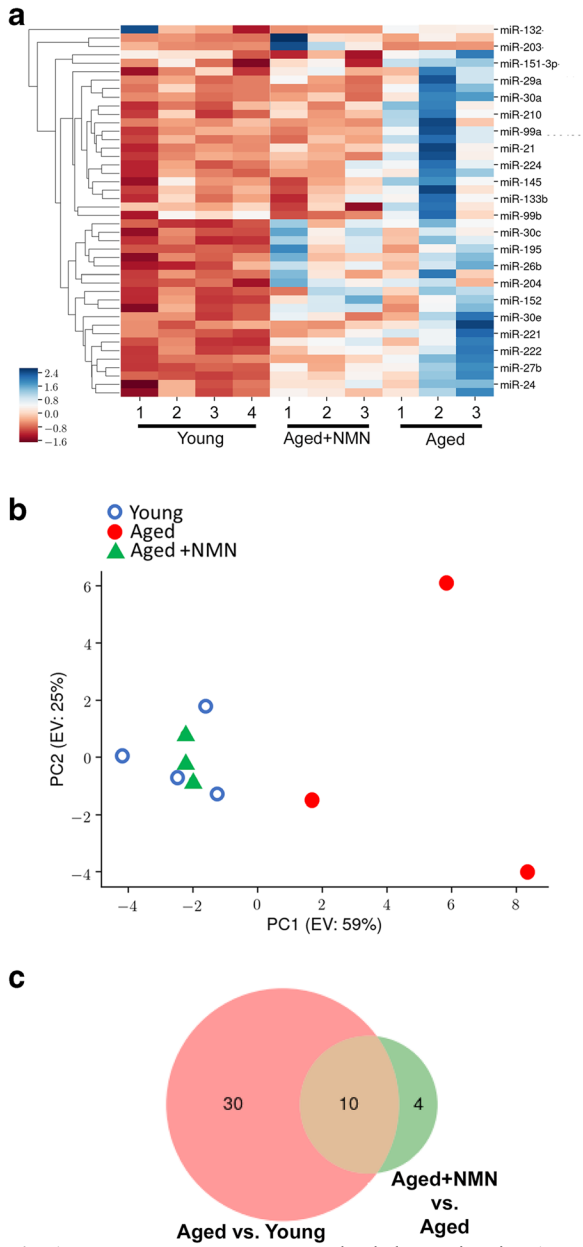

Fig. 1 NMN treatment reverses age-related changes in miRNA expression profile in the mouse aorta. **a** The heat map is a graphic representation of normalized miRNA expression values in aortas derived from young (3-month-old), aged (24-month-old), and NMN-treated aged mice. Hierarchical clustering analysis revealed the similarities on miRNA expression profiles of aortas from young and NMN-treated aged mice. **b** Principal component analysis (PCA) plot of miRNA expression profiles from aortas derived from young, aged control, and NMN-treated aged mice. The profiles from aged mice (red dots) cluster separately to clusters representative of young mice (blue circles) and NMN-treated aged mice (green triangles). PC1 and PC2: Principal components 1 and 2, respectively. **c** Venn diagrams showing the differentially expressed miRNAs in each group, which are significantly up- or down-regulated in aortas from aged mice compared to those from young mice or aged NMN-treated mice

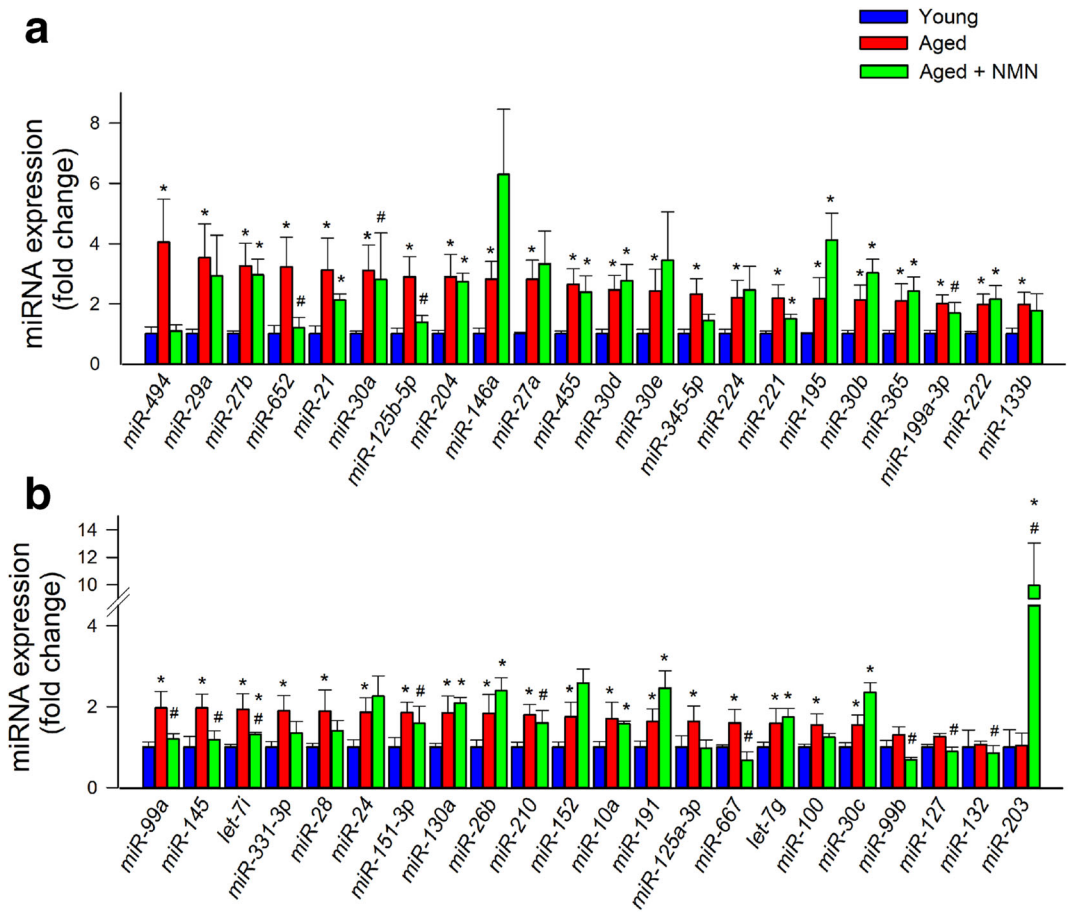

Fig. 2 Effects of aging and NMN treatment on miRNA expression in the mouse aorta. **a**, **b** qPCR data showing miRNA expression in aortas isolated from young (3-month-old), aged (24-month-old), and NMN-treated aged mice. Data are mean ± S.E.M. ($n = 3$–4 for each data point). *$P < 0.05$ vs. young; #$P < 0.05$ vs. aged

We also attempted to predict the biological effects of the differentially expressed miRNAs by identifying relationships between miRNA targets and terms in the biomedical literature utilizing the IRIDESCENT system (Wren and Garner 2004). The results of this analysis suggest that NMN supplementation likely promotes epigenetic rejuvenation and confers anti-atherogenic effects (Table 3).

Discussion

Our study demonstrates that protective effects of NMN treatment on vascular function is associated with anti-aging changes in the miRNA expression profile in the aorta in a mouse model of aging that recapitulates vascular alterations and deficits present in elderly humans at risk for cardiovascular and cerebrovascular diseases.

Age-related changes in vascular miRNA expression likely play important pathogenic roles targeting critical signaling pathways, inflammatory processes, and cellular mechanisms involved in protein homeostasis and thereby impairing the structural and functional integrity of the vasculature (Fig. 3). Among others, miR-29a (Huang et al. 2016), miR-27b (Signorelli et al. 2016), miR-652 (Pilbrow et al. 2014), miR-221 (Wei et al. 2013), miR-28 (Wang et al. 2017), miR-21 (Urbich et al. 2008), miR-125b-5p (Ohukainen et al. 2015) , miR-494 (Wezel et al. 2015), and miR-145 (Faccini et al. 2017), which are up-regulated in aging, have been implicated in vascular inflammation and atherogenesis.

Table 1 Selected genes, whose expression changes with age and are predicted to be targeted by NMN-dependent differentially expressed miRNAs. Shown are (1) the number of miRNAs targeting the gene, whose expression is significantly changed my NMN treatment; (2) relative age-dependent changes in gene expression, predicted by the AgeAtlas software; and (3) cellular function of the protein encoded by the gene and its putative role in vascular pathologies

Gene symbol	Gene full name	NMN-induced significant miRNAs	AgeAtlas change	Cellular function	Role in vascular pathology
Sec62	SEC62 homolog	4	− 0.0089	Component of the protein translocation apparatus	Single nucleotide polymorphism is associated with vulnerable plaque (de Boer et al. 2018)
Nbeal1	neurobeachin like 1	3	− 0.81	Plays a role in vesicle trafficking, membrane dynamics, receptor signaling, pre-mRNA processing, signal transduction and cyto-skeleton assembly	Single nucleotide polymorphism is associated with early atherogenesis (Hixson et al. 2017) and development of ischemic white matter hyperintensities in stroke patients (Traylor et al. 2016)
Fyn	Fyn proto-oncogene	2	− 0.31	Kinase	Genome-wide analysis of DNA methylation showed association with aortic atherosclerosis (Yamada et al. 2014); in vitro overexpressed in activated smooth muscle cells (Singh et al. 2017);
Mef2a	myocyte enhancer factor 2A	2	− 0.094	Transcription factor	In vitro increased expression in senescence endothelial cell; increased plasma level in coronary artery disease patients (Liu et al. 2019)
Tet2	tet methylcytosine dioxygenase 2	2	− 0.45	Epigenetic regulator	Contributes to the development of atherosclerosis by epigenetic modification (Aavik et al. 2019; Peng et al. 2016; Liu et al. 2013)
Ptch1	patched 1	2	− 0.0072	Hedgehog signaling pathway	Overexpressed in atherosclerotic plaque in mouse carotid artery (Ali et al. 2013)
Adra2b	adrenergic receptor, alpha 2b	2	− 0.061	Seven-pass transmembrane protein	role in hypertension (Kintsurashvili et al. 2009)
Abcg4	ATP-binding cassette, sub-family G (WHITE), member 4	2	− 0.0038	ABC-transporter	Cholesterol transporter, strongly linked to atherosclerosis and other cardiovascular disease (Schumacher and Benndorf 2017; Westerterp et al. 2014)
Epha6	Eph receptor A6	2	− 0.59	Ephrin receptor	GenSalt and MESA studies: SNP variant associated with hypertension (Li et al. 2016; Kim et al. 2017); in vitro activated in cells relevant for atherogenesis (Sakamoto et al. 2011)

Table 1 (continued)

Gene symbol	Gene full name	NMN-induced significant miRNAs	AgeAtlas change	Cellular function	Role in vascular pathology
Atf2	activating transcription factor 2	2	− 0.34	Transcription factor	In mouse models participates in foam cells activation signaling (Raghavan et al. 2018); vascular smooth cell activation (Wu et al. 2014)
Homer2	homer scaffolding protein 2	2	− 0.31	Glutamate signaling pathway	Biomarker of atherosclerosis (Zhu et al. 2016)
Kcnb1	potassium voltage gated channel, Shab-related subfamily, member 1	3	− 0.21	Potassium channel subunit	Changed expression in arteries in rat model of hypertension (Cox et al. 2008)
Rap1a	RAS-related protein-1a	2	− 0.52	Ras signaling pathway	Potential role in carotid atherosclerosis (Mao et al. 2018)
Fryl	FRY like transcription coactivator	2	− 0.71	Transcription factor; Notch signaling	Downregulated in hypertensive mouse aorta (Rippe et al. 2017)

To our knowledge, this is the first study to demonstrate that NMN treatment in aged mice reverses, at least in part, age-related, pro-inflammatory, and pro-atherogenic alterations in miRNA expression profile in the aorta. These findings raise the possibility that changes in post-transcriptional control of expression of genes that encode critical targets for vascular health contribute to the beneficial effects of treatment with NAD+ boosters on health span. Demonstration of NMN-induced changes in miRNA biology in the vasculature is particularly important as alterations in miRNA expression profile have been causally linked to the development of cardiovascular aging phenotypes (Ungvari et al. 2013a; Boon et al. 2013; Csiszar et al. 2014) and the pathogenesis of cardiovascular diseases (Ono et al. 2011). A single miRNA can target up to several hundred mRNAs, thus capable of significantly altering gene expression regulatory networks. Systematic prediction of target pathways supports the concept that chronic NMN treatment may exert significant anti-atherogenic effects via epigenetic rejuvenation of the vasculature. These miRNA-mediated vasoprotective effects of NMN treatment appear to be synergistic with its endothelial protective, anti-aging, and pro-angiogenic effects demonstrated by recent studies (Tarantini et al. 2019; Csiszar et al. 2019; Kiss et al. 2019).

The molecular mechanisms contributing to aging-induced decline in NAD^+ in the vasculature are likely multifaceted and may include down-regulation of nicotinamide phosphoribosyltransferase (NAMPT, also known as NMN synthase; which catalyzes the rate limiting step in the biosynthesis of NAD^+) (Tarantini et al. 2019) and increased utilization of NAD^+ by activated Poly [ADP-ribose] polymerase 1 (PARP-1) (Csiszar et al. 2019; Pacher et al. 2002). Additional studies are warranted to determine the efficacy of combination treatments that simultaneously increase NAD+ production and inhibit its degradation (e.g., NMN plus a PARP-1 inhibitor) for the prevention of age-related vascular pathologies.

Previous studies demonstrate that restoration of NAD^+ levels by NMN treatment exert protective effects on endothelial vasodilation in aged rodents by reducing ROS generation and restoring mitochondrial function in a sirtuin-dependent manner (Tarantini et al. 2019). The mechanisms by which

Table 2 Predicted regulatory effects of miRNAs whose expression is restored to youthful levels in aortas of aged mice by NMN supplementation. Shown are GO terms enriched among miRNAs differentially expressed with age in the aorta whose expression is significantly affected by NMN treatment. *N* = genes in each GO category, targeted by miRNAs that are differentially regulated in the aged mouse aorta. Significance was determined by Fisher's exact test; odds ratio: (observed to expected ratio); SLPV: signed log10 *P* value

GO term ID	Name of biological process/molecular function	N	Odds Ratio	SLPV
6886	Intracellular protein transport	20	3.17	3.26
7218	Neuropeptide signaling pathway	7	7.32	2.54
5198	Structural molecule activity	6	9.40	2.45
51082	Unfolded protein binding	7	5.49	2.20
45778	Positive regulation of ossification	6	6.27	2.07
50839	Cell adhesion molecule binding	10	3.49	1.92
15137	Citrate transmembrane transporter activity	3	inf	1.84
48227	Plasma membrane to endosome transport	3	inf	1.84
8188	Neuropeptide receptor activity	3	inf	1.84
7217	Tachykinin receptor signaling pathway	3	inf	1.84
42594	Response to starvation	3	inf	1.84
70536	Protein K63-linked deubiquitination	6	4.70	1.77
71108	Protein K48-linked deubiquitination	6	4.70	1.77
5102	Receptor binding	27	1.82	1.72
90630	Activation of GTPase activity	10	2.85	1.71
31338	Regulation of vesicle fusion	7	3.66	1.68
1664	G-protein coupled receptor binding	6	3.76	1.52
6631	Fatty acid metabolic process	6	3.76	1.52
45777	Positive regulation of blood pressure	4	6.25	1.47
32924	Activin receptor signaling pathway	4	6.25	1.47
70530	K63-linked polyubiquitin binding	4	6.25	1.47
10863	Positive regulation of phospholipase C activity	4	6.25	1.47
16579	Protein deubiquitination	8	3.13	1.47
18107	Peptidyl-threonine phosphorylation	9	2.57	1.42
48015	Phosphatidylinositol-mediated signaling	5	3.91	1.36
7200	Phospholipase C-activating G-protein coupled receptor signaling pathway	5	3.91	1.36
71837	HMG box domain binding	5	3.91	1.36
61578	Lys63-specific deubiquitinase activity	3	9.37	1.33
33674	Positive regulation of kinase activity	3	9.37	1.33
43122	Regulation of I-kappaB kinase/NF-kappaB signaling	3	9.37	1.33
50995	Negative regulation of lipid catabolic process	3	9.37	1.33

NAD+ boosters regulate miRNA expression are likely multifaceted and may include both transcriptional and post-transcriptional regulatory mechanisms (Fig. 3). NMN-induced transcriptional regulation may involve changes in the expression of miRNA genes due to altered transcription factor activity, changes in genome accessibility (e.g., histone modifications), and altered methylation status of the promoter of the miRNA genes. Post-transcriptional mechanisms affected by NMN treatment may include rescue of miRNA processing pathways (Ungvari et al. 2013b) and miRNA stability. Activation of sirtuins by NAD+ boosters, which has been linked to attenuation of age-related vascular oxidative stress (Tarantini et al. 2019; Kiss et al. 2019), may potentially contribute to both transcriptional and post-transcriptional regulation of miRNA expression in the vasculature. In particular, future

Table 3 Literature commonalities of the genes targeted by miRNAs whose expression is restored to youthful levels in aortas of aged mice by NMN supplementation. The IRIDESCENT literature-mining software was used to identify commonalities (e.g., genes, diseases, phenotypes, biological processes) of the genes predicted to be targeted by the miRNAs. A network of related objects was established by their co-occurrence within MEDLINE records, shared relationships were identified, and their statistical relevance was scored by comparing observed frequencies with what would be expected in a random network model. Number of shared relationships is the number of genes (out of the top 100 most significant) co-mentioned with the terms in the left-hand column. The observed to expected (obs/exp) ratio is the enrichment for the term. References and notes on how each one relates to vascular pathophysiology of aging are shown in the rightmost column

Literature associations	Remark/full name	# shared relationships	Obs/exp	Score	Biological process/function
CTNNB1	Catenin Beta 1	63	2.25	139.9	Adherent junctions; Wnt/beta-catenin signaling in VSMCs contribute to Intimal thickening (Tsaousi et al. 2011)
Wnt		56	2.02	110.8	Wnt signaling regulates atherogenesis (Zhao et al. 2018; Bhatt et al. 2012)
PTEN	Phosphatase and tensin homolog	43	2.2	93	Regulates VSMC phenotype (Moulton et al. 2018)
epithelial-mesenchymal transition		41	2.07	83.3	Endothelial to menesnchymal transition contributes to atherogenesis (Bostrom et al. 2016; Evrard et al. 2016; Moonen et al. 2015)
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	22	3.73	81	Chromatin remodeling; genome wide association study showed its potential role in atherosclerosis (Mao et al. 2017)
FGFR1	Fibroblast growth factor receptor 1	28	2.63	72.3	Receptor; FGF receptor signaling regulates atherogenesis (Che et al. 2011)
EP300	E1A binding protein p300	32	2.27	71.3	Transcriptional coactivator; VEGFA triggers changes in transcriptional activity of endothelial cells via epigenetic regulation with the help of EP300 (Zhang et al. 2013)
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit	25	2.78	68.6	Histone methyltransferase; epigenetic suppression of gene expression
sumoylation		24	2.56	60.6	Sumolylation reactions play a role in atherogenesis (Heo et al. 2013; Heo et al. 2015; Stein et al. 2014)
RNA polymerase II		29	2.08	58.9	mRNA transcription
SOX2	Sex determining region Y-box 2	27	2.15	57.7	Transcription factor, stem cell function; upregulated in aortic endothelial cells

Table 3 (continued)

Literature associations	Remark/full name	# shared relationships	Obs/exp	Score	Biological process/function
					in atherosclerotic mice (Bostrom et al. 2016). Limiting Sox2 decreases calcification in aortas of ApoE(-/-) mice (Bostrom et al. 2016).
chromatin remodeling		29	2.03	57.3	(Khyzha et al. 2017)
CDH2	N-cadherin	27	2.11	55.5	Adherent junctions; neointima formation (Jones et al. 2002)
CDKN1A	Cyclin-dependent kinase inhibitor 1; p21	27	2.02	53.3	Senescence; regulates atherogenesis and neointima fromation (Yang et al. 1996)
KMT2D	Histone-lysine N-methyltransferase 2D	15	3.6	53	Histone methyltransferase; epigenetic regulation of gene expression
SMAD4	Mothers against decapentaplegic homolog 4	23	2.26	51	Transcription factor, mediates TGFβ signaling, regulates pathways involved in atherogenesis (Kintscher et al. 2002)
KDM1A	Lysine-specific histone demethylase 1A	16	3.02	47.2	Histone methyltransferase; epigenetic regulation of gene expression (Pojoga et al. 2011)
FGFR2	Fibroblast growth factor receptor 2	23	2.08	47	FGF receptor signaling regulates atherogenesis (Che et al. 2011)
BCOR	BCL-6 corepressor	12	3.96	46.4	transcription repressor
PAX6	Paired box 6	20	2.34	46.1	Transcription factor;
DNMT1	DNA methyltransferase (cytosine-5) 1	22	2.05	44.5	Chromatin remodeling; mediate macrophage activation and participate inflammation in atherosclerotic lesions (Yu et al. 2016)
polycomb		19	2.31	42.7	Chromatin remodeling, regulate ABCA1 expression in the macrophages and consequently has an important role in the development of the inflammation in the atherosclerotic lesion (Lv et al. 2016)
MECP2	Methyl CpG binding protein 2	18	2.25	39.5	Key epigenetic factor regulating global gene transcription by gathering the histone deacetylase complex to the

Table 3 (continued)

Literature associations	Remark/full name	# shared relationships	Obs/exp	Score	Biological process/function
					promoter regions of the genes; with polycomb, it regulates ABCA1 expression in the macrophages (Lv et al. 2016)
TSC1	TSC complex subunit 1	16	2.48	38.9	Tumor suppressor gene; regulates mammalian target of rapamycin complex 1 (mTORC1) signaling, thought the mTHOR pathway it plays a critical role in the development of atherosclerotic lesions (Kurdi et al. 2016)
CREBBP	CREB binding protein	18	2.18	38.4	Involved in the transcriptional coactivation of many different transcription factors
ID2	Inhibitor of DNA binding 2	16	2.42	37.9	Transcriptional regulator, inhibit the functions of basic helix-loop-helix transcription factors; vascular smooth muscle phenotypic change in atherosclerosis (Zhu et al. 2015)
BAP1	BRCA1 associated protein 1	12	3.02	35.9	Ubiquitin C-terminal hydrolase, removes ubiquitin from proteins
TCF4 (alias of TCF7L2)	transcription factor 7 like 2	17	2.12	35.5	Transcription factor; epidemiological data shows SNP polymorphism associated with atherosclerosis (Estrada-Velasco et al. 2013; Muendlein et al. 2011)
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	16	2.12	33.5	Kinase activity; somatic mutation causes venous (Limaye et al. 2015) and lymphatic malformation (Blesinger et al. 2018)
ARHGEF2	Rho/Rac guanine nucleotide exchange factor 2	16	2.05	32.2	Participates in the rho-dependent signaling pathway
PAX8	Paired box 8	13	2.56	32.2	Transcription factor
FGF8	Fibroblast growth factor 8	14	2.35	31.5	Mitogenic and cell survival activities; regulates the cardiovascular development (Brown et al. 2004)
BTG2	BTG anti-proliferation factor 2	11	2.87	30.8	Involved in the regulation of the G1/S transition of the cell cycle

Table 3 (continued)

Literature associations	Remark/full name	# shared relationships	Obs/exp	Score	Biological process/function
FBXW7	F-box and WD repeat domain containing 7	12	2.48	29.6	Phosphorylation-dependent protein ubiquitination; regulates angiogenesis (Izumi et al. 2012) and the barrier function of endothelial cells (Pronk et al. 2019)
FMR1	Fragile X mental retardation 1	14	2.09	28.9	Controls the proliferation and angiogenesis of endothelial cells via the miR-181a-mediated calmodulin (CaM)/CaMKII pathway (Zhao et al. 2018)
MSTN	Myostatin	14	2.11	28.8	Participates in TGF-beta signaling; increased expression in atherosclerotic lesions, especially in vascular smooth muscle cells (Verzola et al. 2017)
HDAC3	Histone deacetylase 3	14	2.07	28.5	Transcriptional regulator by epigenetic modification of DNA; regulats the atherosclerotic phenotype of macrophages (Hoeksema et al. 2014)
CCNE1	Cyclin E1	12	2.35	27.9	Regulator of cell cycle
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	11	2.54	27.5	Chromatin remodeling
AXIN2	Axin 2	12	2.31	27.3	Participates in G-protein signaling; participate in Wnt signaling which known to regulates atherosclerosis (Zhao et al. 2018; Bhatt et al. 2012; Tian et al. 2017)
RASSF1	Ras association domain family member 1	12	2.29	26.9	regulator of DNA methylation and DNA repair
FOXP1	Forkhead box P1	11	2.48	26.7	Transcription factor; in atherosclerosis downregulation of miR-206 causes the upregulation of FOXP1 contributing the development of the plaque (Xing et al. 2017)
TOP2A	DNA topoisomerase II alpha	12	2.21	26.1	Controls and alters the topologic states of DNA during transcription; Topo II inhibitor teniposide reduce

Table 3 (continued)

Literature associations	Remark/full name	# shared relationships	Obs/exp	Score	Biological process/function
					calcification of atherosclerotic lesions (Liu et al. 2018)
ASCL1	Achaete-scute family bHLH transcription factor 1	11	2.42	26	Transcription factor; presence of ASCL1 polymorphism correlate with the development of subclinical atherosclerosis (Lopez-Mejias et al. 2016)
FOXA1	Forkhead box A1	11	2.21	23.9	Transcription factor
AXIN1	Axin 1	11	2.15	23.2	Participate in G-protein signaling
SOX10	Sex determining region Y-box 10	11	2.08	22.4	Transcription factor
MAP2K4	Mitogen-activated protein kinase kinase 4	11	2	21.6	Protein kinase; participates in VEGF signaling (Sullivan et al. 2019)

studies should determine how NMN treatment and sirtuin activation affect activity/expression of the Dicer/TRBP complex (Ungvari et al. 2013b). Further, the anti-aging vascular effects of caloric restriction also have been causally linked to sirtuin activation (Csiszar et al. 2009a). Importantly, caloric restriction also promotes significant anti-inflammatory and anti-atherogenic changes in vascular miRNA expression (Csiszar et al. 2014). Various humoral factors (e.g., hormones, cytokines) can also affect vascular miRNA expression. Additional studies are needed to determine the indirect effects of NMN-induced changes in humoral factors (e.g., adipokines) on vascular miRNA expression profile. The available evidence also supports the concept that a bi-directional link exists between NAD+ levels and miRNA expression (Choi et al. 2013). Recent studies identify the miR-34a/NAMPT (nicotinamide phosphoribosyltransferase) regulatory axis, which regulates SIRT1 activity through altering NAD+ levels (Choi et al. 2013). Interestingly, miR-34a tends to be increased in the aged mouse aorta (~2.9-fold), which associates with a down-regulation of NAMPT (Tarantini et al. 2019).

Conclusions

In conclusion, rescue of vascular function and attenuation of oxidative stress in the vasculature of NMN-treated aged mice is accompanied by anti-aging changes in miRNA expression profile in the aorta. The predicted regulatory effects of NMN-induced differentially expressed miRNAs in aged vessels include anti-atherogenic affects and epigenetic rejuvenation (Fig. 3) and are consistent with the anti-aging functional effects of treatment with both NMN (Das et al. 2018; Tarantini et al. 2019; Kiss et al. 2019; de Picciotto et al. 2016) and sirtuin activators (Pearson et al. 2008; Csiszar et al. 2012; Mattison et al. 2014; Toth et al. 2015; Toth et al. 2014; Zhang et al. 2009; Oomen et al. 2009; Minor et al. 2011; Chen et al. 2015b; Gano et al. 2014) observed both *in vivo* and *ex vivo*. We hope that our findings will facilitate future endeavor of uncovering the mechanistic role of miRNA gene expression regulatory networks in the anti-aging effects of NAD+ booster treatments. Future studies should also investigate the links between miRNAs

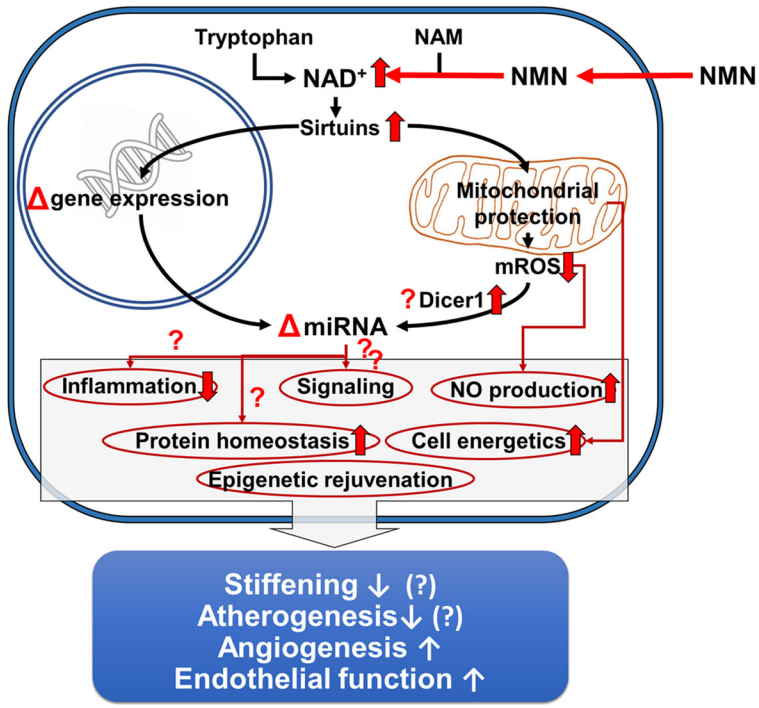

Fig. 3 Proposed scheme for the mechanisms by which restoration of NAD+ levels in the aged vasculature by NMN supplementation promotes anti-aging miRNA expression profile, rescues endothelial function, and prevents atherogenesis. The model, based on our present and previous findings and earlier data from the literature (Tarantini et al. 2019; Csiszar et al. 2019), predicts that increased NAD+ activates sirtuin-mediated pathways, restores cellular energetics and attenuates mitochondrial ROS (mtROS) production, which lead to epigenetic changes promoting youthful gene/miRNA expression, restore Dicer1-mediated miRNA processing, increase NO bioavailability, decrease inflammation, and improve protein homeostasis. All of these effects are predicted to act to decrease large artery stiffness, inhibit atherogenesis, improve vasodilation, and promote angiogenesis at the level of the microcirculation

regulated by NMN and sirtuin activators and miRNAs known to act in the conserved pathways of aging (Ungvari et al. 2018; Menghini et al. 2014; Tarantini et al. 2016b; Kennedy et al. 2014; An et al. 2017; Ashpole et al. 2017; Bennis et al. 2017; Deepa et al. 2017; Fang et al. 2017; Fulop et al. 2018; Lee et al. 2018; Reglodi et al. 2018; Menghini et al. 2009; Fan et al. 2018) and major aging-related diseases (Csiszar et al. 2017; Meschiari et al. 2017; Tarantini et al. 2017b; Tucsek et al. 2017; Ungvari et al. 2017b; Carlson et al. 2018; Csipo et al. 2018; Tana et al. 2017; Feinberg and Moore 2016). Potentially, miRNA-regulated anti-aging mechanisms of NAD+ booster treatments and sirtuin activators could be harnessed for development of new pharmacological approaches for the prevention and treatment of age-related vascular diseases.

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