Vascular microRNA-204 is remotely governed by the microbiome and impairs endothelium-dependent vasorelaxation by downregulating Sirtuin1

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Gut microbiota promotes atherosclerosis, and vascular endothelial dysfunction, signalled by impaired endothelium-dependent vasorelaxation, is an early marker of atherosclerosis. Here we show that vascular microRNA-204 (miR-204) expression is remotely regulated by the microbiome, and impairs endothelial function by targeting the Sirtuin1 lysine deacetylase (Sirt1). MiR-204 is downregulated, while Sirt1 is upregulated, in aortas of germ-free mice. Suppression of gut microbiome with broad-spectrum antibiotics decreases miR-204, increases Sirt1 and bioavailable vascular nitric oxide, and improves endothelium-dependent vasorelaxation in mouse aortas. Antibiotics curtail aortic miR-204 upregulation, and rescue decline of aortic Sirt1 and endothelium-dependent vasorelaxation, triggered by high-fat diet feeding. Improvement of endothelium-dependent vasorelaxation by antibiotics is lost in mice lacking endothelial Sirt1. Systemic antagonism of miR-204 rescues impaired endothelium-dependent vasorelaxation and vascular Sirt1, and decreases vascular inflammation induced by high-fat diet. These findings reveal a gut microbe-vascular microRNA-Sirtuin1 nexus that leads to endothelial dysfunction.
The gut flora, consisting of trillions of microbes, plays an essential role in dietary energy harvest. The gut microbiome has been implicated in the pathogenesis of metabolic disorders such as diabetes and obesity. The microbiome can also contribute to the development of atherosclerotic vascular disease. Metabolites of dietary phospholipids and nutrients produced by gut flora and found in the systemic circulation contribute to vascular inflammation and atherosclerosis. On the other hand, the microbiome also participates in retarding atherosclerosis. Gut bacteria generate metabolites of a class of dietary flavonoids that decrease atherosclerotic burden by stimulating reverse cholesterol transport in macrophages.

Endothelial dysfunction, manifested as impaired endothelium-dependant vasorelaxation and vascular inflammation, is a precursor and strong predictor of atherosclerosis. Sirtuin1 (Sirt1) is a class III histone deacetylase expressed in cell types comprising the vascular wall that stimulates endothelial nitric oxide (NO) by deacetylating endothelial nitric oxide synthase (eNOS), promotes endothelium-dependant vasorelaxation, and protects against endothelial dysfunction and atherosclerosis. Sirt1 expression is governed by microRNAs. For example, miR-217 and miR-212, both of which directly target Sirt1, promote endothelial cell senescence and suppress in vitro angiogenesis. We hypothesized and proved that the expression of vascular Sirt1 is an important determinant of gut microbiome-dependant endothelial function, and vascular miR-204, one of the microRNAs whose expression is remotely controlled by the microbiome, targets endothelial Sirt1 leading to endothelial dysfunction.

**Results**

**Microbiome stimulates the expression of vascular miR-204.** Initially, we sought to identify the effect of the microbiota on vascular microRNA expression profiles. Unbiased microRNA nCounter expression arrays were performed and compared in aortas of microbiota-free germ-free mice (GFM) and control pathogen-free mice (PFM). Of the 578 microRNAs assayed, 15 were found to be significantly downregulated, while 5 were upregulated in aortas of GFM compared with PFM (Fig. 1a; Supplementary Fig. 1a). Global microRNA expression, and expression of the most abundant vascular microRNAs, did not differ between GFM and PFM (Supplementary Fig. 1b,c). Similarly, aortic expression of microRNA processing enzymes Dicer and Drosha were not altered in germ-free conditions (Supplementary Fig. 1d). Among the microRNAs downregulated by nCounter array in GFM aortas (Supplementary Fig. 2d), miR-204 and miR-148a were confirmed by quantitative PCR (qPCR), with a strong trend for miR-9 (Supplementary Fig. 2e). Since miR-204 was the most robustly downregulated microRNA, we henceforth focused our attention on it. Decrease of miR-204 in GFM aortas (Fig. 1b) was validated using different internal controls (Supplementary Fig. 2c) and expression of miR-204 precursors in the aorta followed a trend similar to that of the mature microRNA (Fig. 1b). In situ hybridization showed that miR-204 is expressed in both the endothelium and tunica media of mouse aortas (Supplementary Fig. 3).

Sirt1 is targeted by miR-204 and is upregulated in GFM. Computational microRNA–mRNA algorithms predicted Sirt1 to be in the top 100 genes (0.5 percentile) targeted by the conserved microRNAs downregulated by nCounter array in aortas of GFM (Fig. 1c). Among these microRNAs, miR-204, miR-9, miR-142-3p and miR-199a-5p have been experimentally shown to down-regulate Sirt1 (refs 14–16). Focusing on miR-204, we confirmed that it targets Sirt1 in vascular endothelial cells using miR-204 modulators (mimic, inhibitor) and Sirt1 3'-untranslated region (UTR) reporter assay (Fig. 1d,e; Supplementary Fig. 4). Furthermore, Sirt1 was upregulated in several tissues, including the aortas (Fig. 1f), skeletal muscle and lungs, but not in the heart and liver of GFM (Supplementary Fig. 2f). Because miR-204 is in intron 6 of the Trpm3 gene located on chromosome 9q21.12, and is co-expressed with Trpm3 (ref. 17), we also measured Trpm3 expression. Paralleling miR-204 expression, Trpm3 was downregulated in GFM aortas (Fig. 1g).

**Antibiotics decrease miR-204 and increase vascular Sirt1.** We next asked whether suppressing gut flora biomass with broad-spectrum oral antibiotics results in changes in vascular miR-204 and Sirt1 expression similar to that observed in GFM. Wild-type C57Bl/6 mice isolated from the colony, housed in separate cages and treated with antibiotics in drinking water for 6 weeks were slightly leaner than their untreated counterparts but had similar plasma lipids (Supplementary Fig. 5a,b). Mice on antibiotics had markedly lower faecal microbial load (Supplementary Fig. 6). Administration of antibiotics decreased aortic miR-204 and Trpm3 (Fig. 2a–c), while increasing aortic Sirt1 (Fig. 2d–g). Discontinuation of antibiotics and co-housing with mice not on antibiotics reversed changes in aortic expression of miR-204, Trpm3 and Sirt1 (Fig. 2a–g). Immunohistochemistry showed that total aortic Sirt1 as well as endothelial Sirt1 was upregulated in mice receiving antibiotics (Fig. 2d–g).

**Antibiotics promote endothelial function via Sirt1.** Because of the important role of Sirt1 in eNOS signalling, we questioned whether the gut microbiome stimulates vascular NO and endothelium-dependant vasorelaxation. Antibiotic administration to mice upregulated eNOS (Fig. 2d,e), and improved endothelium-dependant vasorelaxation (Fig. 2h), in mouse aortas. Repopulation of the microbiome by discontinuation of antibiotics and co-habitation with mice not on antibiotics reversed changes in aortic expression of eNOS and decreased endothelium-dependant vasorelaxation (Fig. 2d,e,h). To determine the role of endothelial Sirt1 in the microbiota-vascular axis, we examined the effect of antibiotics on endothelium-dependant vasorelaxation in mice with conditional deletion of endothelial Sirt1 (cSirt1<sup>−/−</sup>). In contrast to control wild-type, Sirt1<sup>fl/fl</sup> and VE-Cad-Cre mice, antibiotics paradoxically impaired endothelium-dependant vasorelaxation in eSirt1<sup>−/−</sup> mice (Fig. 2h–k), showing an obligatory role of endothelial Sirt1 in improved vasorelaxation achieved by suppressing the microbiome.

**Sirt1 rescues miR-204-induced endothelial dysfunction.** To establish a causal role for miR-204-induced downregulation of Sirt1 in endothelial dysfunction, miR-204 mimic was transfected into mouse aortas<sub>ex vivo</sub> resulting in robust upregulation of aortic miR-204 (Supplementary Fig. 7a). MiR-204 mimic suppressed endothelial Sirt1 (Supplementary Fig. 7b) and decreased endothelium-dependant vasorelaxation (Fig. 2l). This decrease in endothelium-dependant vasorelaxation was rescued by adenoviral-mediated reconstitution of endothelial Sirt1 (lacking the 3'-UTR) in mouse aortas (Fig. 2l; Supplementary Fig. 7b), indicating that miR-204 mimic impairs endothelial function, at least in part, by suppressing Sirt1 expression.

**Diet-microbiome interaction governs miR-204 and Sirt1.** We then questioned whether diet-induced endothelial dysfunction is also dependent on the microbiome and triggered by miR-204-mediated downregulation of vascular Sirt1. To address this, we first measured aortic expression of Sirt1 and miR-204 in a high-fat...
Antibiotics reverse HFD-induced endothelial dysfunction via Sirt1. HFD feeding impaired endothelium-dependent vasorelaxation (Fig. 3h), and reduced bioavailable vascular NO, in mouse aortas (Fig. 3i). Broad-spectrum antibiotics rescued this decline in endothelium-dependent vasorelaxation (Fig. 3h) and bioavailable NO (Fig. 3i). Cessation of antibiotics and re-housing the mice with those not on antibiotics negated improvement in endothelium-dependent vasorelaxation and bioavailable NO (Fig. 3h,i). Importantly, rescue of endothelium-dependent vasorelaxation by antibiotics was lost in cSirt1/−/− mice (Fig. 3j), underscoring the role of endothelial Sirt1 in mediating the beneficial effect of gut microbiota suppression on vascular endothelial function.

Antibiotics promote endothelial Stat3 signalling. We next sought to understand the molecular mechanism responsible for the regulation of vascular miR-204 expression by the gut microbiome. The transcription factor Stat3 represses miR-204 expression in vascular smooth muscle cells and pancreatic beta cells. We therefore explored the role of Stat3 signalling in regulation of vascular miR-204 by the gut microbiome. Inhibition of Stat3 signalling in endothelial cells with a dominant-negative Stat3 construct stimulated miR-204 expression (Supplementary Fig. 8a). Moreover, activated (phosphorylated) Stat3 was diminished in aortas of mice on HFD (Fig. 3f; Supplementary Fig. 8b,c). Suppression of the microbiome with antibiotics rescued aortic Stat3 signalling, while reconstitution of the gut flora diminished phospho-Stat3 (Fig. 3f; Supplementary Fig. 8b,c). Importantly, phosphorylation of Stat3, and changes in its phosphorylation with HFD and antibiotics, was seen principally in the endothelium (Supplementary Fig. 8b,c), indicating that endothelial Stat3 signalling is an important target of the gut microbiome.

MiR-204 mediates HFD-induced endothelial dysfunction. We then asked whether vascular miR-204 plays a causative role in endothelial dysfunction and vascular inflammation secondary to HFD feeding. Locked nucleic acid anti-miR-204 was systemically delivered with osmotic mini-pumps for the final 6 weeks of the 8-week HFD feeding period, resulting in robust suppression of miR-204 in mouse aortas (Fig. 4a; Supplementary Fig. 9a), but no change in weight gain, fat mass or blood glucose (Supplementary Fig. 9b–d). In vivo delivery of anti-miR-204 prevented HFD-triggered downregulation of aortic Sirt1 (Fig. 4b) and HFD-induced decline of endothelium-dependent vasorelaxation (Fig. 4c) and bioavailable NO (Fig. 4d). In addition, HFD-induced vascular inflammation, as assessed by aortic macrophage content, was reduced in mice receiving anti-miR-204 (Fig. 4e,f). Thus, miR-204 mediates impaired endothelial function and vascular inflammation secondary to a HFD.

Systemic delivery of miR-204 inhibitor may affect expression of non-vascular miR-204 targets. We therefore examined the effect of miR-204 inhibition on isolated aortas. Ex vivo inhibition of miR-204 with anti-miR-204 in aortas of mice subjected to HFD feeding improved endothelium-dependent vasorelaxation (Fig. 4j), indicating that the extravascular effects of miR-204...
inhibition do not play a major role in improving vascular endothelial function.

**MiR-204 acts primarily via endothelial Sirt1.** Because microbiome-dependent changes in vascular miR-204 and Sirt1 expression were not limited to the endothelium (Figs 2a–g and 3a–l), we more carefully gauged the role of endothelial Sirt1 in improving endothelial function rendered by miR-204 inhibition. Lack of endothelial Sirt1 (eSirt1+/− mice) did not completely negate the effect of miR-204 inhibition on HFD-induced endothelial dysfunction, despite a marked decrease in aortic miR-204 (Fig. 4g,h). However, comparison of overall impairment of endothelial-dependent vasorelaxation in wild-type and eSirt1+/− mice indicated that the absence of endothelial Sirt1 significantly diminishes the beneficial impact of miR-204 inhibition on vascular endothelial function (Fig. 4i). Thus, endothelial Sirt1 is a key but not the only target of miR-204 in HFD-induced endothelial dysfunction. Given the finding that neither antibiotics nor miR-204 inhibition impacted endothelium-independent vasorelaxation (Supplementary Fig. 12), it is likely that targets of miR-204, other than Sirt1, that are partly responsible for miR-204-mediated endothelial dysfunction with HFD feeding are also expressed in the endothelium.

**Microbiome-dependent serum factor(s) regulate miR-204.** Remote regulation of endothelium-dependent vasorelaxation by the microbiome suggests the presence of second messenger(s) in the systemic circulation that govern expression of vascular miR-204 and Sirt1. Supporting this line of thought, endothelial cells incubated with serum from mice in which the gut microbiome was suppressed with antibiotics showed decreased miR-204 and Trpm3, and increased Sirt1, expression (Supplementary Fig. 10a–c). In contrast, serum from mice in which the microbiome was repopulated by discontinuation of antibiotics did not downregulate miR-204 in endothelial cells (Supplementary Fig. 10d). In addition, serum from antibiotic-treated mice led to the increase in endothelial Stat3 signalling (Supplementary Fig. 10a).
The effect of the microbiome on atherosclerosis, glucose homeostasis and adiposity has been attributed, in part, to circulating metabolites such as trimethylamine-oxide and short-chain fatty acids (SCFAs) produced by gut bacteria. The receptors for SCFAs, including Gpr41 and 43, and Olfr78, are expressed in the vasculature such as the aorta, renal arteries and myometrial arteries. We tested whether SCFAs serve as second messengers responsible for the effect of the microbiome on aortic miR-204 and Sirt1 expression, and endothelial function. Supplementation of drinking water with acetate and butyrate, two SCFAs produced by gut bacteria, did not negate the effect of antibiotics on aortic miR-204 expression and endothelium-dependent vasorelaxation (Supplementary Fig. 11a,b). In addition, a mixture of SCFAs did not decrease Sirt1 in endothelial or smooth muscle cells in vitro (Supplementary Fig. 11c,d). The lack of effect of SCFAs suggests that they are not responsible for the communication between the gut microbiome and vascular miR-204.

In summary, these findings identify a gut–vascular axis in which microbes remotely upregulate miR-204 expression in the vessel wall. Among the genes targeted by miR-204 in the vascular wall is Sirt1, downregulation of which promotes endothelial dysfunction (Fig. 5). In addition, this work demonstrates that nutritional stress in the form of a western diet negatively impacts the endothelium via this same gut–vascular axis. Although the data do not identify the mediator(s) responsible for this remote communication between the gut and the vasculature, they do suggest that these messengers are in the systemic circulation, and regulate endothelial miR-204 expression through the Stat3 transcriptional program. It is important to acknowledge that while orally administered antibiotics led to a striking diminution of the gut flora, because a fraction of these antibiotics may be systemically absorbed, their effect on vascular miR-204/Sirt1 expression and endothelial function could also be due to their impact on the composition of the microbiome in niches other than the gut. Finally, given that microbiome-dependent expression of Sirt1 was not limited to the vasculature, miR-204 was not the only microRNA differentially regulated in the vasculature in germ-free conditions, these findings may provide a

Figure 3 | Antibiotics rescue endothelial Stat3 and Sirt1 in high-fat diet-induced vascular dysfunction. (a–c) Suppression of gut microbiota with broad-spectrum antibiotics inhibits HFD-induced aortic miR-204. (a) qPCR for mature and precursor miR-204 in mouse aortas. n = 4 for NPD and HFD-AB-R, n = 6 for HFD and HFD-AB. (b) In situ hybridization for miR-204 in mouse aortas (blue, magnification ×100, Scale bar; 20 μm). (c) Quantification of miR-204 in b. n = 5 images from different regions of aortic sections. (d–f) Suppression of gut microbiota with antibiotics prevents HFD-induced downregulation of aortic Sirt1 and Stat3 signalling. (d) Immunofluorescence for Sirt1 in mouse aortic sections (magnification ×63, Scale bar; 20 μm). (e) Quantification of total aortic (n = 4 sections) and endothelial (n = 15 endothelial cells) Sirt1 immunostaining in d. (f) Western blot for Sirt1 and phospho-Stat3 in mouse aortas. Immunoblots were done with pooled aortic samples; n = 4 for NPD and HFD-AB-R and n = 6 for HFD and HFD-AB. (g) Suppression of gut microbiota rescues HFD-induced decrease in total aortic NOx [nitrate + nitrite]. NPD: n = 9, HFD: n = 6, HFD-AB: n = 6. (h,i) Suppression of gut microbiota rescues HFD-induced reduction of endothelium-dependent vasorelaxation and nitric oxide bioavailability. NPD: n = 10, HFD: n = 12, HFD-AB: n = 10, HFD-AB-R: n = 10. (j) Suppression of gut microbiota does not rescue HFD-induced impairment of endothelium-dependent vasorelaxation in eSirt1+/− mice. eSirt1−/− HFD: n = 6, eSirt1−/− HFD-AB: n = 12. *P < 0.05 versus NPD. #P < 0.05 versus HFD. A, acetylcholine; AB, antibiotics; eSirt1−/−, mice conditionally lacking endothelial Sirt1; HFD, high-fat diet; NPD, normal pellet diet; PE, phenylephrine; R, stoppage of antibiotics and recolonization. n represents the number of aortic rings in h–j. IF, immunofluorescence. Independent sample t-test was used. Data shown as mean and error bar represents s.e.m.
miR-204 inhibitor infusion. RT–PCR was done with pooled aortic samples. (a) Systemic delivery of miR-204 inhibitor in HFD-fed mice (a) prevents upregulation of aortic miR-204, (b) downregulation of aortic Sirt1 and (c,d) rescues endothelium-dependent vasorelaxation and bioavailable vascular NO. n = 4 for NPD-SC, n = 6 for HFD-SC and HFD-204-I in a; n = 4 for NPD-SC, n = 6 for HFD-SC and HFD-204-I in b. NPD-SC: n = 15, HFD-SC: n = 16, HFD-204-I: n = 18 in c,d. n represents number of aortic rings. (e,f) Systemic in vivo delivery of miR-204 inhibitor suppresses HFD-induced vascular inflammation. Expression of macrophage marker F4/80 was assessed by immunoblot in aorta (e) and by immunohistochemistry (f) in aortic roots of mice (magnification × 4 or × 100; scale bars, 500 μm (× 4), 20 μm (× 100)). Adjacent sections of aortic root sections were stained with haematoxylin and eosin (magnification × 4 and × 100). n = 4 for NPD-SC, n = 6 for HFD-SC and HFD-204-I. *P < 0.05 versus NPD-SC. HFD, high-fat diet; NPD, normal pellet diet; SC, scrambled control microRNA; 204-I, miR-204 inhibitor. (g-i) Inhibition of miR-204 with HFD feeding improves endothelium-dependent vasorelaxation to a lesser degree in eSirt1+/− mice compared with wild-type mice. (g) Aortic expression of miR-204 in eSirt1+/− mice with HFD and with miR-204 inhibitor infusion. RT–PCR was done with pooled aortic samples. (h) Systemic delivery of miR-204 inhibitor partially improves endothelium-dependent vasorelaxation induced in eSirt1+/− mice on HFD. n represents the number of aortic rings. NPD-SC: n = 13, HFD-SC: n = 12, HFD-204-I: n = 9. (i) Per cent impairment in endothelium-dependent vascular relaxation in c,h. (j) Ex vivo inhibition of miR-204 rescues HFD-induced impairment of endothelium-dependent vasorelaxation. n represents the number of aortic rings. NPD-SC: n = 5, HFD-SC: n = 10, HFD-204-I: n = 10, *P < 0.05 versus NPD-SC. P < 0.05 versus HFD-SC. A, acetylcholine; eSirt1+/−, mice conditionally lacking endothelial Sirt1; HFD, high-fat diet; L, lumen; NPD, normal pellet diet; PE, phenylephrine. Independent sample t-test was used. Data shown as mean and error bar represents s.e.m.
Infusion of locked nucleic acid microRNA inhibitor, ALZET 2006 osmotic pups per day for a period of 6 weeks.

Methods

Animals. All animal experiments were approved by Institutional Animal Care and Use Committee and were carried out according to National Institutes of Health (NIH) guidelines. Male GFM and PFM (Swiss-Webster, age 10 weeks) were procured from Taconic, New York, USA. All other studies were performed in C57BL/6, Sirt1fl/fl mice (age 10–12 weeks).

RNA isolation and RT-qPCR. Total aortic RNA was used as input for the NanoCounter reactions and was performed according to the manufacturer’s instructions. In brief, a unique oligonucleotide was tagged to the microRNAs for the specific and sensitive detection by nCounter analysis system. The system is based on target-specific probe pairs that are hybridized to sample in solution. A reporter probe carries the fluorescent signal, while capture probe allows the complex to be immobilized for data collection. The protocol does not involve any amplification steps. The panel was designed to screen 578 mouse microRNAs, 33 viral microRNAs, 3 non-mammalian miRNAs, 8 negative controls and a dilution control microRNA inhibitor, ALZET 2006 osmotic pumps were normalized for lane-to-lane variation by six dilution series spike positive controls. Global microRNA expression was used to normalize individual microRNA expression. Heat map for expression of microRNAs was prepared using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). In the absence of universally accepted internal control for normalization of aortic microRNA expression analysis by qPCR, we identified a microRNA that best resembles global microRNA expression as previously described. Differential regulated microRNAs in GEM were examined for their gene targets using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). The normalisation of microRNAs by miR-29b was found to best resemble the expression profile as achieved with normalization using global microRNA expression, followed by miR-29c, β-actin and Gapdh (Supplementary Fig. 2a), and thus miR-29b was used as an internal control for qPCR experiments in aortic samples. To ascertain change in the expression and to validate selection of internal control for qPCR, a single microRNA (miR-204) was normalized by another common housekeeping gene beta-actin, and the expression of multiple microRNAs was normalized with miR-29b, to see if the pattern observed with nCounter assay remains the same (Supplementary Fig. 2b–e).

Target gene prediction. MicroRNA-SVR is an algorithm that ranks microRNA-mRNA target sites interaction (SVR score). Lower SVR score indicates stronger interaction between a microRNA and mRNA target site. Differentially regulated microRNAs in GEM were examined for their gene targets using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). The normalization of microRNAs in GEM were examined for their gene targets using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). The normalization of microRNAs in GEM were examined for their gene targets using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). In the absence of universally accepted internal control for normalization of aortic microRNA expression analysis by qPCR, we identified a microRNA that best resembles global microRNA expression as previously described with some modifications. In brief, to select the microRNA most suitable as a normalization control, the pattern of microRNA expression in PFM and GFM mice normalized with (i) candidate microRNAs and (ii) commonly used normalization mRNAs (Actb (β-actin), R2m (β2-microglobulin) and Gapdh) was determined and compared with expression pattern as achieved with normalization with global microRNA expression. The data were clustered by pairwise complete linkage based on Pearson correlation using GenePattern (http://genepattern.broadinstitute.org/gp/pages/index.jsp). The normalization of microRNAs by miR-29b was found to best resemble the expression profile as achieved with normalization using global microRNA expression, followed by miR-29c, β-actin and Gapdh (Supplementary Fig. 2a), and thus miR-29b was used as an internal control for qPCR experiments in aortic samples. To ascertain change in the expression and to validate selection of internal control for qPCR, a single microRNA (miR-204) was normalized by another common housekeeping gene beta-actin, and the expression of multiple microRNAs was normalized with miR-29b, to see if the pattern observed with nCounter assay remains the same (Supplementary Fig. 2b–e).

In vitro and ex vivo transfection of oligonucleotides. Oligonucleotides were incubated with Lipofectamine2000 (Invitrogen) at room temperature for 20 min before adding to cells in OPTI-MEM (Invitrogen). Four hours later, the medium was replaced by fresh medium and cells were cultured for an additional specified period of time (24–72 h). Ex vivo transfection of oligonucleotides into mouse thoracic aorta was performed using Lipofectamine (Invitrogen). Olignucleotides were incubated with Lipofectamine at room temperature for 20 min before adding to aortas in OPTI-MEM (Invitrogen). The aorta was kept in transfection mixture overnight. The sequence of oligonucleotides used in study is provided in Supplementary Table 1.

Luciferase reporter assay. The pRL-CMV and Luciferase-Sirt1-3’-UTR plasmids were transfected in the human umbilical vein endothelial cells (HUVECs) with scrambled microRNA or miR-204 mimic (20 pM). Lipofectamine 2000 was used as a transfection agent. The luciferase-to-renilla ratio was calculated to normalize for variations in transfection efficiencies.

Ex vivo adenoviral infections. Gene transfer was achieved ex vivo by incubating aortas from mice with 5 × 10^9 p.f.u. of the appropriate adenoviral stock at 37 °C for 24 h before recording vascular reactivity. For in vitro infections of HUVECs, 100 multidrug-resistant infection was used to infect cells with Ad-eSirt1 for Exochiria coli LacZ, (ii) AdSirt1 coding for Sirt1 and (iii) Ad-DNStat3 coding for non-phosphorylatable dominant-negative Stat3.

Vascular reactivity. Thoracic aortas of mice were used. The animals were rapidly killed by CO2 inhalation. The aorta was carefully dissected, rapidly removed and placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution. The vessel was carefully cleared of loose connective tissue and cut into 5–10 1.5 mm rings. A single ring from each mouse was suspended between two wire stirrups (150 mg) in aKrebs-Ringer (95% O2–5% CO2, pH 7.4, 37 °C). One stirrup was connected to a three-dimensional micromanipulator, and the other to a force transducer. The mechanical force signal was amplified, digitalized and recorded (PowerLab 8/30). All concentration–response curves were performed on arterial rings beginning at their optimum resting tension. This was determined by stretching arterial rings at 10 min intervals in increments of 100 mg to reach optimal tone (∼500 mg). One dose of...
KCl (60 mM) was administered to verify vascular smooth muscle viability. Vascular NO bioavailability was determined as the difference in cumulative dose–response curve for phenylephrine (10⁻⁹–10⁻⁵ M) in the presence and absence of the NO synthase inhibitor L-NAME. Data are presented as per cent change to the maximum contraction induced by PE (10⁻⁸ M). Endothelium-dependent and -independent vasorelaxation was determined by generating dose–response curves to acetylcholine (10⁻⁹–10⁻⁵ M) and sodium nitroprusside (10⁻⁸–10⁻⁷ M), respectively, on PE (10⁻⁶ M)-induced pre-contracted vessels. Vasorelaxation evoked by acetylcholine and sodium nitroprusside was expressed as per cent relaxation, determined by calculating the percentage of inhibition to the pre-constricted tension. Aortic rings not responding to initial dose of KCl or showing auto-relaxation were excluded. Per cent impairment in acetylcholine-induced vascular relaxation was determined by measuring the difference between the mean area under the curve for the NPD-scrambled control group and that of the test groups.

NO₂ (nitrate/nitrite) measurement. Aortic rings were carefully isolated and immediately frozen. Aorta (5–7 mm) was used for the nitrate/nitrite assay and was performed as per the manufacturer’s instructions (Cayman-780001, Ann Arbor, MI, USA).

**Immunoblotting.** Protein samples were resolved on 10–12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Antigen–primary antibody complexes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by western blotting luminol reagent (Thermo, USA). Images were captured and quantified using the Image Lab (BioRad, USA) software, and intensity values were normalized to Gapdh. Uncropped blots are shown in Supplementary Fig. 13.

**Histological processing and immunostaining.** Sections (5 μm) of formalin-fixed paraffin-embedded tissues were heated (95°C, 20 min) in citrate buffer (10 mM) for antigen retrieval, followed by incubation with primary antibodies. The working concentration for the primary antibodies were as follows; Sirt1; 1: 200, p-Stat3; 1: 200. Sections were dehydrated with alcohols and xylene, and mounted in DPX. Aortic root cryosections (20 μm) of formalin-fixed paraffin-embedded tissues were heated (95°C, 20 min) in citrate buffer (10 mM) for antigen retrieval, followed by incubation with primary antibodies. The working concentration for the primary antibodies were as follows; Sirt1; 1: 200, p-Stat3; 1: 200. Sections were dehydrated with alcohols and xylene, and mounted in DPX. Alternatively, antigen–primary antibody complexes were probed with fluorescence-tagged secondary antibodies. Aortic root cryosections (20 μm) were examined for anti-F4/80 staining using goat anti-rabbit secondary antibody and streptavidin–horseradish peroxidase system to amplify the signals, followed by detection with dianibenzamide as a chromogen. Slides were counterstained with haematoxylin, dehydrated with alcohols and xylene, and mounted in DPX. Immunofluorescence images were captured using the ImageXpress (Molecular Devices, USA) software. 10 μm sections were captured and quantified using the ImageXpress (Molecular Devices, USA) software.

**In situ hybridization.** Deparaffinized and rehydrated aortic sections (6 μm) were treated with proteinase K (10 μg/ml, 5 min, 37°C). Sections were dehydrated using ethyl alcohol and incubated with pre-hybridization buffer, followed by hybridization mixture containing either double digoxigenin(DIG)-tagged miR-204-5p probe or DIG-tagged scrambled probe (36h, 57°C). Sections were washed with sodium saline citrate and probed with either Dylight 594 labelled anti-DIG goat antibody (working concentration; 1:500, Vector Laboratories, Burlingame, CA, USA) or alkaline phosphatase-conjugated anti-DIG FAB, BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium) was used as a chromogen. Slides were counterstained with either Nuclear Fast Red or DAPI. Microscopic images were captured using the ImageXpress (Molecular Devices, USA) software. Sequence of miR-204-5p double DIG-tagged probe is provided in Supplementary Table 1.

**Cell culture.** HUVECs (Cat# CC-2519) were purchased from Clonetics (San Diego, CA, USA) and cultured in growth factor- and fetal bovine serum-supplemented EGM-2 (Lonza, Walkersville, MD, USA). To examine the effects of mouse serum, 2% fetal bovine serum in EGM-2 was replaced with pooled serum from the specific group of mice.

**Statistical analysis.** To determine sample sizes, power analysis was carried out using preliminary data sets. Considering the independent sample t-test, a sample size of 5 achieved >90% power for studies involving gene and protein expression. However, for vascular reactivity experiments, a sample size of 10 achieved >95% power to reject null hypothesis at the significance level of 0.05. All the data meet assumptions of the statistical test and different groups had similar variance.

**Data availability.** Microarray data were deposited in the Gene Expression Omnibus database (Accession code GSE46360). The authors declare that the data supporting the findings of this study are available within the article and from the authors on request.

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A.V. and K.I. conceived the project; A.V., Y.-R.K., S.K., Q.L., M.K. and J.S.J. performed the experiments; K.I. obtained funding; A.V. and K.I. analysed the data and wrote the manuscript.

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