Vascular Smooth Muscle Cell Sirtuin 1 Protects Against DNA Damage and Inhibits Atherosclerosis

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Background—Vascular smooth muscle cells (VSMCs) in human atherosclerosis manifest extensive DNA damage and activation of the DNA damage response, a pathway that coordinates cell cycle arrest and DNA repair, or can trigger apoptosis or cell senescence. Sirtuin 1 deacetylase (SIRT1) regulates cell ageing and energy metabolism and regulates the DNA damage response through multiple targets. However, the direct role of SIRT1 in atherosclerosis and how SIRT1 in VSMCs might regulate atherosclerosis are unknown.

Methods and Results—SIRT1 expression was reduced in human atherosclerotic plaques and VSMCs both derived from plaques and undergoing replicative senescence. SIRT1 inhibition reduced DNA repair and induced apoptosis, in part, through reduced activation of the repair protein Nijmegen Breakage Syndrome-1 but not p53. Fat feeding reduced SIRT1 and induced DNA damage in VSMCs. VSMCs from mice expressing inactive truncated SIRT1 (Δex4) showed increased oxidized low-density lipoprotein–induced DNA damage and senescence. ApoE−/− mice expressing SIRT1Δex4 only in smooth muscle cells demonstrated increased DNA damage response activation and apoptosis, increased atherosclerosis, reduced relative fibrous cap thickness, and medial degeneration.

Conclusions—SIRT1 is reduced in human atherosclerosis and is a critical regulator of the DNA damage response and survival in VSMCs. VSMC SIRT1 protects against DNA damage, medial degeneration, and atherosclerosis. (Circulation. 2013;127:386–396.)

Key Words: aneurysm • atherosclerosis • sirtuins • vascular disease

Advanced atherosclerosis is characterized by cell senescence, apoptosis, persistent DNA damage, and activation of the DNA damage response (DDR),1–3 a canonical pathway triggered by DNA double-strand breaks. The DDR can be activated by oxidant stress and promotes the recruitment of key nuclear proteins to damage sites, including the MRE11/RAD50/NBS1 complex and ataxia telangiectasia mutated (ATM) kinase.4 γ-phosphorylation of Ser139 of the histone variant H2AX (γ-H2AX) by ATM recruits repair factors to DNA breaks,5 whereas ATM phosphorylation activates downstream signaling pathways, ultimately activating multiple effector proteins including the tumor suppressor p53 and cell cycle regulators p16ink4a and p21cip1/waf1.6 The consequent growth arrest allows DNA repair to occur, although these same pathways can induce apoptosis and cell senescence when DNA damage is extensive. Normal arteries show no detectable apoptosis and do not express DNA damage markers; in contrast, human atherosclerotic plaques show increasing DNA damage with disease severity, cell senescence, and apoptosis, in particular, in vascular smooth muscle cells (VSMCs).7 Arterial DNA damage can be promoted by oxidant stress and hypercholesterolemia1–3; however, the pathways that protect VSMCs from DNA damage and its consequences are unclear.

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Sirtuin 1 (SIRT1) is a NAD+-dependent lysine deacetylase that has multiple roles in chromatin remodeling, cell ageing, organism longevity, energy metabolism, genomic stability, stress responses, and apoptosis.6 SIRT1 ameliorates many degenerative diseases associated with ageing, including some forms of neurodegeneration, cancer, and metabolic decline including glucose intolerance and insulin resistance.7 SIRT1 deacetylase targets include histone proteins and several transcription factors, including p53,8 E2F-1,9 and forkhead genes,10 to suppress DNA damage-induced apoptosis. SIRT1 also promotes double-strand break repair,11,12 in part, through activation of the repair protein Nijmegen Breakage Syndrome-1 (NBS1), increasing resistance to DNA damage.13 SIRT1 has received extensive attention recently for its potential role in extending longevity and ameliorating degenerative diseases, and therapeutics and lifestyle changes that increase SIRT1 have been developed. However, there is controversy over SIRT1’s role in longevity and degenerative disease.
and the specificity of some agents whose actions increase SIRT1. In particular, SIRT1’s longevity effects may be strain, species, and context-dependent in many organisms.

The role of SIRT1 in atherosclerosis is complex. SIRT1 increases endothelial nitric oxide synthase-derived nitric oxide and has anti-inflammatory functions in endothelial cells (ECs) and macrophages, downregulating the expression of various proinflammatory cytokines by interfering with the nuclear factor-κB signaling pathway. Indeed, EC overexpression of SIRT1 can retard atherosclerosis. RelA/p65-nuclear factor-κB deacetylation by SIRT1 in macrophages also suppresses expression of lectin-like oxidized low-density lipoprotein (oxLDL) receptor (Lox-1), a scavenger receptor for oxLDL, preventing macrophage foam cell formation and reducing atherosclerosis. SIRT1 also regulates the activity of liver X receptor A, promoting reverse cholesterol transport and reducing atherosclerosis. SIRT1’s longevity effects may be strain, species, and context-dependent in many organisms.

We therefore examined the role of SIRT1 on DNA repair in VSMCs and consequences of its inactivation on atherosclerosis. We show that endogenous SIRT1 is reduced in human atherosclerosis, particularly in VSMCs. SIRT1 deficiency reduces DNA repair and promotes the activation of DNA damage markers and senescence. ApoE−/− mice with reduced VSMC SIRT1 show elevated DNA damage markers and VSMC apoptosis, reduced relative fibrous cap thickness, medial degeneration, aneurysm formation, and aortic dissection. Altogether, our results demonstrate that SIRT1 is a critical protective gene in the vasculature and that decreased SIRT1 expression may promote atherosclerosis and medial degeneration due to DNA damage, apoptosis, and accelerated cell aging.

**Methods**

**Human Atherosclerotic Plaques and Normal Vessels**

Plaques and normal vessels (internal mammary artery, aorta, and saphenous vein) were obtained from patients undergoing carotid endarterectomy or coronary artery bypass grafting/valve replacement, respectively, with informed consent and approval from the local Research Ethical Committee.

**Cell Culture**

Human aortic medial VSMCs were isolated from recipients undergoing cardiac transplant or valve replacement and plaque VSMCs from carotid endarterectomies.

**Quantitative Real-Time Polymerase Chain Reaction**

RNA was isolated by using a NucleoSpin RNA II kit (Macherey-Nagel GmbH) and cDNA prepared with a Reverse Transcription System kit (Promega Corp). Quantitative real-time polymerase chain reaction was performed using predesigned Taqman Gene Expression Assays and AmpliTaq Gold DNA polymerase following the manufacturer’s instructions (Applied Biosystems Inc). Standard curves were generated and results expressed as ratios by using GAPDH as a reference gene. PCRs were performed on a Rotor-Gene 6000 real-time polymerase chain reaction system (Corbett Research, AU).

**Immunoprecipitation and Western Blotting**

Immunoprecipitation and Western blots were performed as described previously.

**Comet Assay and Immunofluorescence**

Comet assay and immunofluorescence were performed as described previously and in the online-only Data Supplement.

**Transfections and Virus Infections**

Transfections and retrovirus infections were performed as described previously and in the online-only Data Supplement.

**Mice and Atherosclerosis Protocols**

SM22-SIRT1−/−, SIRT1−/−, and SM22-SIRT1 mice were fed either normal chow or high-fat diet (HFD, 21% total fat, 0.2% cholesterol, 0% sodium cholate) from 6 to 22 weeks. In some experiments, ApoE−/− mice were fed HFD for 24 weeks, and tissues preserved in RNA Later (Sigma). Serum lipids were analyzed using a Dade-Behring Dimension autoanalyzer and low-density lipoprotein (LDL) calculated by using the Friedwald formula.

**Histological Analysis**

Histological analysis was performed as described previously and in the online-only Data Supplement.

**Statistical Analysis**

Statistical analysis was performed with the use of the Student t test where data were normally distributed. Data presented are means±standard errors of the mean with significance at P<0.05. Linear regression with the use of the least-squares approach was used to determine the best-fit line between plaque size and exon 4 recombination. Correlation and χ² coefficients were obtained with Excel (Microsoft). Because of the exploratory nature of the study, there is no adjustment for multiple comparisons.

**Results**

**SIRT1 is Downregulated in VSMCs in Human Atherosclerosis**

We first examined SIRT1 expression in human carotid endarterectomy atherosclerotic plaques and normal vessels obtained from aorta, internal mammary artery, or saphenous vein from patients undergoing coronary artery bypass grafting and/or valve replacement. There was no difference in patient demographics between groups including age, sex, the percentage of patients with hypertension or diabetes mellitus, or who smoked or had used statins within the last 3 months. SIRT1 mRNA and protein expression were markedly reduced in plaques versus control vessels using real-time polymerase chain reaction and Western blotting (Figure 1A and B), associated with an increase in the DNA damage marker γ-H2AX (Figure 1B), similar to previous findings using immunohistochemistry. Endarterectomies comprise both the vessel intima and part of the media with a heterogeneous mixture of cells, whereas the normal vessel media comprises mostly VSMCs; we therefore dissected the media from carotid...
plaques and reexamined SIRT-1 mRNA and other markers of VSMC senescence, including p21<sup>p16<sup>kip1</sup> and p16<sup>ink4</sup>. SIRT1 mRNA was significantly decreased in the media of plaques versus normal vessels (Figure 1C), associated with significantly increased p16<sup>ink4</sup>; the difference in p21<sup>p16<sup>kip1</sup></sup> mRNA did not reach statistical significance. To confirm that VSMCs were the major cell type affected in plaques, we cultured VSMCs from human carotid plaques or normal aorta (passage 2–3), and normal VSMCs to replicative senescence (≈passage 16). SIRT1 mRNA and protein expression were reduced in plaque VSMCs and senescent aortic VSMCs versus early-passage normal human VSMCs (Figure 1D and 1E).

Previous studies have shown that SIRT1 activity is required for double-strand break repair<sup>12,13</sup>, suggesting that SIRT1 downregulation or inactivation in VSMCs might promote DNA damage. We therefore examined DNA strand breaks by using the comet assay in plaque and normal VSMCs, including senescent normal VSMCs. We also treated normal VSMCs with the pro-oxidant tert-butyl hydrogen peroxide (BHP), which induces a concentration-dependent increase in double-strand breaks in VSMCs (online-only Data Supplement Figure I). Plaque and senescent VSMCs showed increased comet tails versus passage 2 normal VSMCs (Figure 1F).

Thus, SIRT1 expression is reduced in VSMCs from human atherosclerotic plaques, including medial VSMCs underlying the plaque, associated with markers of DNA damage and cell senescence. SIRT1 is also downregulated when VSMCs undergo replicative senescence or DNA damage.

### SIRT1 Inhibition Delays DNA Repair in VSMCs

To examine the function of SIRT1 in VSMCs, we stably expressed wild-type human SIRT1 or a deacetylation-deficient mutant SIRT1<sup>H364Y</sup> in rat VSMCs by using retrovirus-mediated gene transfer. Wild-type SIRT1 and the SIRT1<sup>H364Y</sup> mutant were expressed in nuclei at similar levels (Figure 2A). Basal levels of the DDR marker γ-H2AX were not significantly different between wild-type and mutant SIRT1-expressing cells (not shown). We therefore examined the kinetics of DNA repair by using BHP-induced DNA damage and both the appearance/disappearance of γ-H2AX DNA foci and comet assay. DNA damage indicated by γ-H2AX foci and comet tails was induced by 80 µmol/L BHP and was repaired in 4 to 5 h (Figure 2B and 2C). SIRT1<sup>H364Y</sup> VSMCs showed delayed appearance and resolution of γ-H2AX foci in comparison with cells containing a control empty vector (Figure 2B), whereas the kinetics of DNA repair was unchanged in wild-type and mutant SIRT1-expressing cells (not shown). However, SIRT1 overexpression promoted survival across a range of BHP concentrations (online-only Data Supplement Figure II). SIRT1<sup>H364Y</sup> did not affect initial DNA damage after BHP on comet assay, but DNA repair was significantly slower (Figure 2C and 2D). These results suggest that the deacetylase activity of SIRT1 is required for optimal DDR activation and DNA repair. Finally, with the use of time-lapse videomicroscopy, we found that SIRT1 decreases and SIRT1<sup>H364Y</sup> increases VSMC apoptosis after BHP treatment (Figure 2E).

### SIRT1 Binds and Deacetylates NBS1 in VSMCs

We have previously shown that VSMCs deficient in NBS1 show impaired DNA repair after oxidant stress.<sup>3</sup> Because
NBS1 is a known SIRT1 target that requires deacetylation for its activation, we examined whether NBS1 was recruited to DNA during oxidative DNA damage and whether it binds SIRT1 in VSMCs. Rat VSMCs expressing wild-type human SIRT1 were treated with increasing BHP concentrations and cells fractionated into non–chromatin-containing and chromatin-containing fractions. In untreated cells, SIRT1 and NBS1 were mostly present in the non–chromatin-containing fraction, indicating they were not bound to DNA. BHP increased SIRT1 and NBS1 in the chromatin fraction, associated with increased γ-H2AX (Figure 3A). Immunoprecipitation with anti-SIRT1 antibodies and blotting with antibodies to NBS1 demonstrated that SIRT1 binds to NBS1, without changing total NBS1 expression (Figure 3B). We next examined the ability of SIRT1 to deacetylate NBS1 in response to DNA damage in cells expressing SIRT1, SIRT1HY or the vector control using immunoprecipitation with anticetylated lysine antibodies and blotting with antibodies to NBS1. NBS1 Lys acetylation was slightly decreased by BHP but significantly decreased by overexpressing SIRT1 both with and without BHP (Figure 3B). SIRT1HY did not cause NBS1 deacetylation (online-only Data Supplement Figure IIB). To determine whether NBS-1 mediated the protective effects of SIRT-1 on both DNA damage and apoptosis, we inhibited NBS1 expression by using small interfering RNA (Figure 3C). NBS-1 small interfering RNA increased γ-H2AX DNA foci after BHP treatment (Figure 3D) and reduced both basal and SIRT1-induced survival (Figure 3E). Since deacetylation of NBS1 is required for its activation, our results suggest that SIRT1 activity is required for efficient DNA repair and protection against oxidative stress-induced apoptosis in VSMCs.

SIRT1 has multiple targets, including the tumor suppressor gene p53, which is also involved in the DDR and regulates cell cycle arrest and apoptosis. In contrast to NBS1, changes in p53 acetylation were not seen after SIRT1 inhibition alone in VSMCs; this was not due to a failure to detect changes in Ac-p53 expression, because the type I histone deacetylase inhibitor trichostatin A could induce Ac-p53 (Figure 3F). Similarly, there was no change in p53 acetylation in human VSMCs after nicotinamide, a noncompetitive inhibitor of SIRT1 activity, while trichostatin A did increase acetylation after BHP (Figure 3G). Thus, SIRT1 could deacetylate p53...
when p53 was preacetylated, but AcP53 was not found in control or BHP-treated unless trichostatin A was also present. This suggests that p53 is not a major target of SIRT1 in VSMCs, including its protective effects against oxidative stress.

**VSMC-Specific SIRT1 Deletion Induces DNA Damage and Promotes Atherosclerosis**

To examine the effect of SIRT1 inhibition on atherogenesis, we first examined the effect of a HFD on SIRT1 expression and markers of DNA damage in apolipoprotein E null (ApoE−/−) mice. Fat feeding for 24 weeks reduced SIRT1 mRNA expression in aortas of these mice (online-only Data Supplement Figure IIIA). VSMCs cultured from HFD mice showed elevated basal levels of γ-H2AX foci in comparison with control ApoE−/− mice on chow, indicating DNA damage. BHP increased DNA damage, but had no additional effect on VSMCs from HFD mice, suggesting that HFD mediates DNA damage through oxidant stress (online-only Data Supplement Figure IIIB). We next tested the effect of LDL cholesterol or oxidized LDL on DNA damage in human aortic VSMCs. Although both LDL and oxLDL were taken up by VSMCs to a similar extent, oxLDL induced γ-H2AX foci, which was greatly potentiated by nicotinamide (online-only Data Supplement Figure IV). Thus, fat feeding in vivo is associated with reduced SIRT-1 expression, and both fat feeding in vivo and oxLDL in vitro increases DNA damage.

To determine the effect of reduced VSMC SIRT1, and thus the role of endogenous SIRT1 in VSMCs in atherosclerosis, we generated mice expressing a truncated inactive SIRT1 as heterozygotes (SIRT1+/△ex4) or homozygotes (SIRT1△ex4/△ex4) from the smooth muscle cell–specific SM22α (transgelin, Tagln) promoter. SM22-cre mice were crossed with mice expressing SIRT1△ex4 flanked by LoxP sites. Unlike whole-body SIRT1 knockout mice, 25 SM22-SIRT1+/△ex4 and SM22-SIRT1△ex4/△ex4 mice were viable with no apparent phenotype at birth. Mice were backcrossed with ApoE−/− null mice. SM22-SIRT1+/△ex4/ApoE−/− and SM22-SIRT1△ex4/△ex4/ApoE−/− mice but not SM22-SIRT1+/+/ApoE−/− mice expressed the truncated
SIRT1 (Figure 4A). SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) mice also showed reduced expression of full-length SIRT1. To investigate whether VSMCs expressing SIRT1\(^{\Delta ex4}\) showed DNA damage we cultured aortic VSMCs from 6 separate 24-week-old SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice fed normal chow and examined DNA damage using \(\gamma\)-H2AX and micronuclei, a feature of VSMCs expressing chronic DNA damage. 26 Although there was some heterogeneity between isolates from different mice, most likely based on different recombination efficiencies (see below), early passage (passage 2) VSMCs from SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice showed significantly increased \(\gamma\)-H2AX fluorescence (6.9±1.0 versus 3.8±0.5 arbitrary units, \(P<0.05\), \(n=6\)) and percentage of cells showing micronuclei (23.7±3.6\% versus 14.5±2.0\% \(P<0.05\), \(n=6\)) compared with cells expressing wild-type SIRT1 (Figure 4B and 4C). Although VSMCs expressing wild-type SIRT1 proliferated to at least passage 6, all SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) VSMCs underwent premature senescence by passage 4, as characterized by no cell proliferation and the typical large flattened phenotype of senescent VSMCs (Figure 4D). SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) VSMCs showed increased DNA damage at baseline and delayed kinetics of DNA repair after BHP treatment on comet assay (Figure 4E).

We next fat-fed 6-week-old littermate SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\), SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\), and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice for 16 weeks, a time point when extensive atherosclerosis is present. Although SIRT1 is a key regulator of energy metabolism, and defects in genes regulating SIRT1 can result in obesity in whole-body knockout mice,27 body weights and serum lipids of SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice were similar to SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) littermate controls (online-only Data Supplement Figure V). In contrast, SIRT1 inhibition in VSMCs had marked effects on atherogenesis and plaque composition, with additional striking effects on the vessel media. SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice had significantly increased plaque areas in comparison with control SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) mice (Figure 5A and 5B). Because there was variability in exon 4 excision rates in mutant mice, we determined whether plaque area correlated with full-length SIRT1 mRNA expression for each mouse. Excision rates were assayed by measuring SIRT1 mRNA in each mouse, with exon 4/exon 8 ratios used to indicate recombination efficiency (Figure 5C). Average excision rates were 35\% and 54\% deletion (range, 20\%–81\%) for heterozygote and homozygote \(\Delta ex4\) mice, respectively. Plaque area showed a significant negative correlation with full-length SIRT1 mRNA levels (Figure 5C).

Figure 4. Inhibition of SIRT1 induces DNA damage and early senescence in VSMCs. A, Western blot for SIRT1 in aortas of SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\), SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\), and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice showing expression of wild-type (wt) and truncated SIRT1 (\(\Delta ex4\)). B, \(\gamma\)-H2AX foci and micronuclei in 6 different VSMC isolates from SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) (1–6) or SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice (7–12). Data are means, error bars represent SEMs. C, \(\gamma\)-H2AX foci or DAPI nuclear staining in VSMCs from SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) mice. Arrows and inset show micronuclei. D, Phase contrast micrographs (10\times) of passage 4 SM22-SIRT1\(^{+/+}\)/ApoE\(^{-/-}\) and SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\)/ApoE\(^{-/-}\) VSMCs. E, Comet assay for VSMCs from SM22-SIRT1\(^{+/+}\) (Control) or SM22-SIRT1\(^{\Delta ex4/\Delta ex4}\) mice (Un, untreated; BHP, BHP treatment, followed by 1 to 5 hours of recovery. *\(P<0.05\). VSMCs indicates vascular smooth muscle cells; SIRT1, sirtuin 1; DAPI, 4\ʹ,6-diamidino-2-phenylindole; and SEM, standard error of the mean.

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We next examined plaques for morphological features of plaque vulnerability, including necrotic core size and fibrous cap thickness. Although necrotic core and cap thickness individually were not significantly different in SM22-SIRT1$^{∆ex4/∆ex4}$ and SM22-SIRT1$^{∆ex4/∆ex4}$ mice versus controls, there was a significant decrease in cap/intima area ratio and in medial thickness in SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice (Figure 5D). In contrast to studies where we induced massive VSMC apoptosis,28 we did not see foci of inflammation. We next examined plaques for evidence of DDR activation and VSMC apoptosis. Double-labeling by using α-smooth muscle actin and ATM/Ataxia telangiectasia and Rad3 related-phosphorylated substrate-specific antibodies showed that VSMCs in SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice showed DDR activation as evidenced by increased detection of ATM/ATR substrates, most marked in the media under the plaque (Figure 6A). We identified apoptotic VSMCs by labeling adjacent sections with α-smooth muscle actin and terminal deoxynucleotidyl transferase dUTP nick end labeling, and quantified them by using double labeling (online-only Data Supplement Figure VI). VSMCs in SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice showed increased apoptosis; this was significantly increased overall and in the media, with a trend with genotype in the intima and fibrous cap (Figure 6B and 6C).

In addition to increased atherosclerosis, reduced cap/intima ratio, and VSMC apoptosis, there was marked medial degeneration in SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice. Furthermore, the suprarenal abdominal aortas in 2/12 SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice with the highest SIRT1 recombination rates (80% and 81%) showed extensive aneurysm formation with dissection and bleeding into the vessel wall (Figure 7). These features were seen particularly in areas with significant atherosclerosis (Figure 7), but were not seen in SM22-SIRT1$^{∆ex4/∆ex4}$ or SM22-SIRT1$^{∆ex4/∆ex4}$/ApoE$^{-/-}$ mice without fat feeding. The media underlying these aneurysms showed signs of cystic medial degeneration, including loss of VSMCs and collagen with consequent appearance of cysts and extensive elastin fragmentation.

**Discussion**

SIRT1 has been identified as a longevity gene, with multiple actions predicted to slow a variety of degenerative diseases, via regulation of energy balance and protection against DNA damage, cell senescence, and apoptosis. SIRT1 expression in cells comprising the plaque has been shown to protect against atherosclerosis, through effects on ECs, macrophages, and thrombosis,29 although recent studies indicate that transgenic expression of SIRT1 is proatherogenic via effects on serum lipids.20 The advanced atherosclerotic plaque is characterized by low cell proliferation rates, cell senescence, and increased apoptosis especially in VSMCs. In vitro, plaque VSMCs...
show reduced cell proliferation, premature senescence, multiple DNA damage markers, and increased apoptosis.3,31 DDR activation with impaired DNA repair induces cell cycle arrest, senescence, and apoptosis. As SIRT1 targets regulate all of these processes, and atherosclerosis is associated with both organismal ageing and cellular senescence, it is important to define the role of SIRT1 in atherosclerosis, particularly before the widespread use of agents to augment SIRT-1 activity. Furthermore, the nonspecific nature of pharmacological agents that both activate and inhibit SIRT1 means that genetic manipulation is an appropriate mode of investigation.

We demonstrate that SIRT1 expression is reduced in human atherosclerosis. While normal vessels from a variety of sites show similar SIRT1 expression, endogenous levels of both SIRT1 mRNA and protein are reduced in plaques versus normal vessels, and in VSMCs cultured from human plaques versus VSMCs from normal vessels, associated with increased DNA damage and cell senescence markers. We also have not excluded the possibility that normal vessels from patients with coronary artery disease have lower SIRT1 expression than those from patients without atherosclerosis. Although the precise mechanisms underlying reduced SIRT1 activity in
atherosclerosis are not known, plaque VSMCs show increased intracellular oxidant stress that can induce SIRT1 degradation. For example, the RNA-binding protein HuR associates with and stabilizes SIRT1 mRNA; oxidative stress triggers HuR-SIRT1 mRNA complex dissociation, promoting SIRT1 mRNA decay, reducing SIRT1 abundance. SIRT1 activity is also regulated posttranslationally, via changes in cellular NAD⁺ levels and protein:protein interactions, and SIRT1 can be reduced by a HFD and increased by caloric restriction. Indeed, fat feeding reduced aortic SIRT1 expression in vivo, associated with DNA damage in VSMCs derived from these vessels, and human VSMCs exposed to oxLDL showed increased DNA damage that was exacerbated by nicotinamide. This suggests that the combination of LDL and oxidant stress in the vessel wall downregulates SIRT1 expression in VSMCs in atherosclerosis. SIRT1 was also reduced in VSMCs undergoing senescence in culture, raising the possibility that specific pathways promote both cell senescence and downregulate SIRT1. For example, chronic oxidant stress can downregulate SIRT1 and promote stress-induced premature senescence simultaneously.

Although SIRT1 has multiple functions, we show that reduced SIRT1 activity in VSMCs is associated with defective DNA repair, persistent DNA damage, DDR activation, reduced cell proliferation, premature senescence, and apoptosis, dependent partly on the SIRT1 substrate NBS1. After DNA damage, NBS1 and SIRT1 were recruited to the chromatin fraction, and associate with each other. SIRT1 binding to NBS1 causes deacetylation of NBS1, a prerequisite for NBS1 activation. NBS1 downregulation in VSMCs also increased the DNA damage marker γ-H2AX. NBS1 deficiency has been shown to increase apoptosis in neurons in knockout animals, and in VSMCs NBS-1 is upregulated within 30 minutes after oxidative stress and is a critical regulator of DNA repair. Importantly, we also find that p53 was not a major SIRT1 deacetylation substrate in VSMCs. Although the targets of SIRT1 are both cell type and context-specific, our results also suggest that the effects of agents such as nicotinamide (a nonspecific SIRT1 inhibitor) on p53 are indirect and not through SIRT1. Although our results support NBS1 as an important SIRT1 substrate of SIRT1, we cannot preclude the involvement of other specific SIRT1 inhibitors (eg, shown to increase apoptosis in neurons in knockout animals, and in VSMCs NBS-1 is upregulated within 30 minutes after oxidative stress and is a critical regulator of DNA repair. Importantly, we also find that p53 was not a major SIRT1 deacetylation substrate in VSMCs. Although the targets of SIRT1 are both cell type and context-specific, our results also suggest that the effects of agents such as nicotinamide (a non-specific SIRT1 inhibitor) on p53 are indirect and not through SIRT1. Although our results support NBS1 as an important substrate of SIRT1, we cannot preclude the involvement of other SIRT1 targets in DNA repair or apoptosis. For example, SIRT1 recruitment to sites of DNA damage after oxidative stress is accompanied by derepression of previously silenced genes; this epigenetic silencing may be due to specific histone methyltransferase enzymes being directly regulated by SIRT1.

In ApoE−/− mice, reduced SIRT1 in VSMCs promoted DDR activation, atherosogenesis, and some features of vulnerable plaques (reduced cap/intima, VSMC apoptosis), indicating that SIRT1 is a critical protective protein in vascular disease. Indeed, there was a significant negative correlation between SIRT1 mRNA expression and plaque area, indicating that even small reductions in SIRT1, eg, seen after a HFD, could promote atherosogenesis. Reduced SIRT1 was associated with increased apoptosis, and we have previously shown that VSMC apoptosis can increase atherosclerosis, associated with increased necrotic core, reduced fibrous cap thickness, and foci of inflammation within the cap.

SIRT1 has been shown previously to regulate resistance to cellular stress and thus the threshold for apoptosis, in part, through interactions with Forkhead box class O (FOXO) transcription factors, which regulate both energy status and stress resistance. In particular, FOXO3a is bound and deacetylated by SIRT1, increasing resistance to cellular stress. In addition, poly(ADP-ribose) polymerase 2 (PARP-2), another NAD⁺-consuming enzyme, directly downregulates SIRT1, whereas deletion of poly(ADP-ribose) polymerase 1 (PARP-1) increases NAD⁺ content and SIRT1 activity. Human plaque VSMCs show increased apoptosis in culture and in vivo, in part, because of reduced expression of the insulin-like growth factor 1 receptor, leading to reduced signaling through the serine threonine kinase Akt. FOXO3a is inhibited by Akt, and this inhibition mediates much of the protective effect of both IGF-1 and Akt in VSMCs. Thus, reduced SIRT1 activity would augment the potent proapoptotic effect of FOXO3a in VSMCs.

Although some of the effects of reduced SIRT-1 in vivo may be due to increased VSMC apoptosis, other consequences of reduced SIRT-1 may be important. Thus, primary VSMCs cultured from aortas of SM22-SIRT1Δex4/ApoE−/− mice showed increased DNA damage and early senescence in comparison with control cells, both of which may promote atherosclerosis independent of apoptosis. In addition, SIRT1 can regulate lipid metabolism, in part, through effects on the nuclear receptors LXR and FXR, which regulate reverse cholesterol transport, although other studies show that mice constitutively expressing SIRT1 have an atherogenic profile of serum lipids. In the current study, although VSMCs deficient in SIRT1 showed increased DNA damage after oxLDL, there was no difference in serum lipids in SM22-SIRT1Δex4/ApoE−/− mice versus controls. Finally, although our mouse model selectively inhibits SIRT1 only in smooth muscle cells, SIRT1 can deacetylate and regulate EC nitric oxide activity and inhibit nuclear factor-kB signaling, protecting vessels from inflammation and procoagulant stress. SIRT-1 overexpression in ECs can also prevent senescence induced by oxidative stress and reduce atherosclerosis, whereas downregulation of SIRT1 promotes EC senescence.

Importantly, we show that SIRT1 deficiency promotes medial degeneration, a common finding in ageing, inherited vascular disease such as Marfan syndrome, aortic dissection, and aneurysm formation. Again, it is unclear which of the effects of reduced SIRT1 are primarily responsible for medial degeneration, although chronic VSMC apoptosis can induce features of cystic medial necrosis in ApoE−/− mice, including fragmentation of elastic laminae, loss of VSMCs with consequent cysts, abnormal deposition of extracellular matrix components, and spotty calcification. However, VSMC apoptosis alone did not induce aneurysm formation or aortic dissection in this previous study. It is therefore possible that other sequelae of the DDR, including cell cycle arrest and cell senescence, or of downregulation of SIRT1 may contribute to an inability of the media to replace lost VSMCs, with more extensive medial degeneration and dissection.

Our data are consistent with the following model. Oxidant stress and LDL reduce SIRT-1 expression in VSMCs in atherosclerosis, both in the intima and the underlying media. Reduced SIRT-1 inhibits DNA repair, in part, through...
defective deacetylation and activation of NBS1, contributing to the persistence of DNA damage in these cells. DNA damage and reduced resistance to cellular stress promotes VSMC growth arrest, senescence, and apoptosis in intimal and medial VSMCs, which result in increased atherosclerosis, reduced relative fibrous cap thickness, and cystic medial necrosis. The latter promotes aneuysm formation and dissection. These proatherosclerotic effects of reduced SIRT1 in VSMCs may be augmented by reduced SIRT1 in ECs, with resultant EC dys-

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Disclosures
None.

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Human atherosclerotic plaques show extensive DNA damage, cell death (through apoptosis), and cell senescence. Although these processes are thought to promote an unstable plaque phenotype predisposing to acute coronary syndromes, the mechanisms underlying their regulation in vivo is not known. The sirtuin 1 (SIRT1) deacetylase enzyme has been implicated in regulating multiple degenerative diseases, through effects on energy metabolism, longevity, cell proliferation, and cell death (among others). We show that SIRT1 is reduced in human atherosclerosis, in particular, in vascular smooth muscle cells in both the plaque and underlying vessel wall. SIRT1 controls DNA repair, apoptosis, and cell senescence in the vessel. SIRT1 is reduced by a high-fat diet, and both fat feeding in vivo and oxidized low-density lipoprotein in vitro increase DNA damage. Smooth muscle cell–specific loss of SIRT1 in mice increases DNA damage and apoptosis, accelerates atherosclerosis, and causes extensive medial degeneration. Our data show that SIRT1 is a powerful protective protein in the vessel wall, regulating oxidative DNA damage and keeping cells alive. Loss of SIRT1 promotes atherosclerosis and other vascular pathologies, indicating that elective augmentation of SIRT1 becomes an attractive therapeutic target. The results of clinical studies with agents that increase SIRT1 activity in atherosclerosis are awaited.
Vascular Smooth Muscle Cell Sirtuin 1 Protects Against DNA Damage and Inhibits Atherosclerosis
Isabelle Gorenne, Sheetal Kumar, Kelly Gray, Nichola Figg, Haixiang Yu, John Mercer and Martin Bennett

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**SUPPLEMENTAL MATERIAL**

**Cell culture**
Human VSMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% FCS. Senescent human VSMCs were obtained by culturing aortic cells until no division was observed for 3 weeks. In some experiments cells were treated with LDL or oxidized LDL (Autogen Bioclear UK Ltd.) in serum-free conditions as indicated. Rat VSMCs were isolated from aortas of Wistar rats. Where indicated, cells were treated with tert-butyl hydrogen peroxide (BHP, Sigma), nicotinamide (NA, Sigma), or trichostatin A (TSA, Sigma) as indicated. Cell survival in rat cells was measured by MTT assay as previously described. Mouse aortic smooth muscle cells were prepared by dissecting out surrounding tissues and enzymatic dispersion using Type I collagenase (1 mg/ml, Sigma) and elastase (0.5 mg/ml, Worthington Biochemical Corp.) in serum-free medium for 1h at 37°C. Culture medium was DMEM containing 20% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2.5 µg/ml Amphotericin B.

**Comet assay**
DNA strand breaks were analyzed by single cell electrophoresis (comet assay). Cells were trypsinized and 30 000 cells per condition were centrifuged, resuspended in 80 µl of 1% low melting point agar, transferred onto slides coated with 1% normal melting point agar, covered, and incubated for 10 min at 4°C. After removing the coverslip, cells were then lysed in alkaline buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10), electrophoresed for 30 min in 0.3 M NaOH and 1 mM EDTA at 24 V and 0.3 Amps constant current, neutralized in 0.4 M Tris-HCl buffer, pH 7.5, and stained with 6 µg/ml ethidium bromide. For each slide, 6 random pictures were taken using an Olympus BX51 microscope at 10x magnification, and the comet length of at least 50 cells measured using NIH Image analysis software.

**Transfections and virus infections**
Retrovirus infection was used to produce stable expression of wild type SIRT1 or the deacetylase-deficient mutant SIRT1<sup>H365Y</sup> in rat aortic VSMCs. pBabe-puro vectors encoding human SIRT1 or SIRT1<sup>H365Y</sup> (from Anthony Kouzarides, Cambridge, UK) were used to transfect Phoenix™ packaging cells (Orbigen, San Diego, CA) using SuperFect (Qiagen). Virus-producing cells were selected with hygromycin B (300 µg/ml; Calbiochem). VSMCs were infected with the virus suspension in the presence of 8 µg/ml Polybrene (hexadimethrine bromide, Sigma), and selected with 5 µg/ml of puromycin (Sigma). Expression plasmids pCMV-p53 (from Dr R.S. Foo, Cambridge, UK) were transfected into rat VSMCs using Lipofectamine™ transfection reagent (Invitrogen Corp). For gene silencing experiments, rat aortic VSMCs were transfected with pre-designed small interference RNAs (siRNAs) for NBS1 (Applied Biosystems Inc) or non-related scrambled nucleotides using an Amaza Nucleofector II system and Amaza Basic Nucleofector kit solutions for primary VSMCs (Lonza-Amaza, Basel, SW). EGFP fluorescence and Western blotting 48 hours after transfection were used to control transfection and silencing efficiency, respectively.

**Immunofluorescence**
For immunofluorescence experiments, cells were cultured in chamber slides (Fisher Scientific). After treatment, cells were rinsed with PBS, fixed with 4% paraformaldehyde for 10 min, rinsed again then permeabilized with 0.3% Triton x100 for 5 min. After blocking for 1 h with BSA 1%, cells were incubated for 2h with primary antibodies (1:500 dilution). After rinsing, Alexa Fluor 568-conjugated secondary antibodies (1:500; Invitrogen, Eugen, OR) were added for 1h. After rinsing, slides were mounted with DAPI-containing medium.
(Vectashield, Vector Labs Inc., Burlingame, CA) and visualized with a BX51 fluorescent microscope (Olympus). Image processing was performed with Cell-D software (Soft Imaging Processing). For lipids staining, human VSMCs were cultured in chamber slides. Paraformaldehyde fixed cells were stained with Nile red for 15 min in PBS and rinsed before mounting.

**Immunoprecipitation and Western blotting**

Immunoprecipitation and Western blots were performed as described previously. Total extracts and immunoprecipitates were resolved on SDS-polyacrylamide gels, transferred onto PVDF membranes (Millipore) and incubated with primary antibodies as indicated. Chromatin fractions were isolated as previously reported. Antibodies against acetyl lysine, SIRT1, phosphoSer139 H2AX, acetylLys382 p53, NBS1, were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against NBS1 (IP), H2AX were from Abcam plc (Cambridge, UK), anti-human p53 (DO1) was from Merck (Darmstadt, GE). Anti-b actin was from Sigma-Aldrich Co. (St Louis MO, USA).

**Mice and atherosclerosis protocols**

All animal experimental procedures were undertaken under United Kingdom Home Office licensing. Re-derived Sv129 /SIRT1ex4/ex4 mice (floxed exon 4; Gift of Prof. F. Alt, Harvard Medical School, Boston, USA) were back-crossed for 4 generations with C57BL6/J mice then for 4 generations with C57BL6/J/ApoE−/− mice (Jackson Laboratory, Bar Harbor, Me) to obtain SIRT1ex4/ex4/ApoE−/− mice. These mice were then crossed with Tg(Tagln-cre)1Her/J mice on the C57BL/6J background, which express the cre recombinase under the control of the mouse transgelin (SM22) promoter (Jackson Laboratory) to achieve deletion of the SIRT1 exon 4 (Dex4) coding for the catalytic domain. Quantification of the exon 4 deletion was performed by measuring mRNA by real time RT-PCR using Taqman assays with 2 sets of primers and probes within exon 4-5 or exon 7-8 (ABI) and the ratio calculated. Genotyping of Tg(Tagln-cre)1Her/J and ApoE−/− mice was performed by PCR using company protocols (Jackson Laboratory). The genotyping of SIRT1 mice was performed by PCR using specific primers (F: 5′-GCCCATTAAGCAGTATGTG-3′; R: 5′-CATGTAATCTCAACCTTGAG-3′) and the following cycling parameters: 1 cycle of 90°C, 15 min and 40 cycles of 94°C, 30 s; 60°C, 1 min; 72°C, 45 s.

**Histological Analysis**

Tissues were formalin-fixed overnight and embedded in paraffin blocks. 5 µm sections of aortic roots or aortas were cut, deparaffinated and stained with hematoxylin and eosin (H&E). For immunohistochemistry, antigen retrieval was achieved by boiling in citrate buffer (pH 6.0). Primary antibodies were specific for the following: a-smooth muscle actin (SMA, 1A4, Dako) and phosphorylated ATM/ATR substrate (Cell Signaling). Sections were stained with biotinylated-alkaline phosphatase-coupled secondary antibodies detected with ABC reagents (Vector Laboratories) and/or horseradish peroxidase-coupled secondary antibodies visualized with diaminobenzidine (DAB, Vector Laboratories). Masson’s Trichrome staining was achieved with an Accustain Kit (HT-15). Collagen was visualized with Sirius red. TUNEL assay for apoptosis was performed using dUTP-digoxigenin incorporation (Roche), detection with an alkaline phosphatase-conjugated antibody to digoxigenin (Roche) and development with 5-bromo-4-chloro-3-indoyl-phosphate/p-nitroblue tetrazolium (Vector) and counterstained with 1% eosin. Images were captured using a BX51 microscope (Olympus), air-cooled CCD camera (CoolSnap) and imaging and analysis software (Soft Imaging Systems). Plaque morphometry and histological analysis for plaque composition were
performed as described previously\(^2\). Total number of apoptotic cells were counted per whole aortic root section and expressed per \(\mu m^2\) of SMA-positive pixels.

**Supplemental References**

Supplemental Figure 1

(A-B) Comet tail lengths of human normal VSMCs exposed to increasing concentrations of BHP.

Supplemental Figure 2

(A) Cell survival after BHP treatment in rat VSMCs expressing the empty vector (pB-EV) or pB-SIRT1. Data are means, error bars represent SEMs. *p<0.05, (n=3). (B) Immunoprecipitation (IP) using anti-acetyl-lysine (AcLYS) antibodies or non-specific IgG. Total cell lysates (input) from pB-EV and pB-SIRT1^{HY} VSMCs were blotted as indicated.
**Supplemental Figure 3**

(A) SIRT1 mRNA expression in aortas of ApoE−/− mice fed normal chow or high fat diet for 24 weeks. (B) γ-H2AX foci in passage 4 aortic smooth muscle cells of mice from (A), additionally treated or not with BHP.

**Supplemental Figure 4**

(A) Nile red fluorescence in human VSMCs that were serum starved for 24h then incubated with LDL (200 µM) or oxidized LDL (oxLDL, 100µM) for the indicated time. (B) Quantitation of γ-H2AX fluorescence in human VSMCs that were serum starved for 24h then incubated with LDL or oxidized LDL (oxLDL) at the indicated concentration for 24h. (C) γH2AX fluorescence in human VSMCs serum starved for 24h, then treated with NA (10 mM) 2h prior incubation with LDL (100 µM) or oxidized LDL (oxLDL, 50 µM) for 24h. Data are means, error bars represent SEMs. *p<0.05, (n=3).
Supplemental Figure 5

(A-B) Body weights (A) and serum concentrations of HDL, LDL, total cholesterol (TC) and triglycerides (TRI) (B) of SM22-SIRT1^{+/+}/ApoE^{-/-} (+/+), SM22-SIRT1^{+/Δex4}/ApoE^{-/-} (+/Δex4) and SM22-SIRT1^{Δex4/Δex4}/ApoE^{-/-} (Δex4/ Δex4) mice after 16 w of high fat feeding. Data are means, error bars represent SEMs. *p<0.05.

Supplemental Figure 6

(A-B) Double labeling for SMA (A) and TUNEL (B) on the same section for quantification of apoptotic VSMCs.