SIRT1 Acts as a Modulator of Neointima Formation Following Vascular Injury in Mice

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Rationale: Vascular smooth muscle cell (VSMC) proliferation and migration are crucial events involved in the pathophysiology of vascular diseases. Sirtuin 1 (SIRT1), a class III histone deacetylase (HDAC), has been reported to have the function of antiatherosclerosis, but its role in neointima formation remains unknown.

Objective: The present study was designed to investigate the role of SIRT1 in the regulation of neointima formation and to elucidate the underlying mechanisms.

Methods and Results: A decrease in SIRT1 expression was observed following carotid artery ligation. Smooth muscle cell (SMC)–specific human SIRT1 transgenic (Tg) mice were generated. SIRT1 overexpression substantially inhibited neointima formation after carotid artery ligation or carotid artery wire injury. In the intima of injured carotid arteries, VSMC proliferation (proliferating cell nuclear antigen (PCNA)–positive cells) was significantly reduced. SIRT1 overexpression markedly inhibited VSMC proliferation and migration and induced cell cycle arrest at G1/S transition in vitro. Accordingly, SIRT1 overexpression decreased the induction of cyclin D1 and matrix metalloproteinase-9 (MMP-9) expression by treatment with serum and TNF-α, respectively, whereas RNAi knockdown of SIRT1 resulted in the opposite effect. Decreased cyclin D1 and MMP-9 expression/activity were also observed in injured carotid arteries from SMC-SIRT1 Tg mice. Furthermore, 2 targets of SIRT1, c-Fos and c-Jun, were involved in the downregulation of cyclin D1 and MMP-9 expression.

Conclusions: Our findings demonstrate the inhibitory effect of SIRT1 on the VSMC proliferation and migration that underlie neointima formation and implicate SIRT1 as a potential target for intervention in vascular diseases. (Circ Res. 2011;108:1180-1189.)

Key Words: SIRT1 • neointima formation • vascular smooth muscle cells • proliferation • migration

The process of neointima formation is common to various forms of vascular diseases such as atherosclerosis, instant restenosis, vein bypass graft failure, and transplant vasculopathy.1,2 In response to vascular injury, the activated medial smooth muscle cells (SMCs) proliferate and migrate into the intima, where they proliferate and subsequently produce an abundant extracellular matrix (ECM) to form the neointima.2,3 Although recent studies have shed light on some of the pathophysiologic mechanisms that are involved in vascular smooth muscle cell (VSMC) proliferation and migration, the molecular modulators that link these coordinated responses of VSMCs to injury remain to be elucidated.

Sirtuin 1 (SIRT1), the closest homology to the yeast Sir2 protein (silent information regulator 2) in human sirtuins,4 has been implicated in aging, metabolism, and tolerance to oxidative stress via its ability to deacetylate a variety of substrates, including histones, transcription factors, and coregulators.5 SIRT1 also serves as a key regulator in vascular endothelial homeostasis by controlling angiogenesis, vascular tone, and endothelial dysfunction as well as by decreasing atherosclerosis.6,7 Growing evidence has shown that SIRT1 is involved in multiple disease-relevant pathways such as cell-cycle regulation, cell apoptosis, and migration.8–10 A small-molecule activator of SIRT1, resveratrol, which is abundant in grape skin and red wine, has been shown to block neointima formation after arterial injury11 and to exert beneficial effects on vascular diseases such as atherosclerosis and hypertension.12,13 We therefore hypothesized that SIRT1 may play a critical role in the development of vascular diseases, which are characterized by neointima formation.
To determine the role of SIRT1 in the regulation of neointima formation, we performed both ligation and wire injury of the carotid arteries in SMC-SIRT1 transgenic (Tg) and wild-type (WT) mice. Here, we report direct evidence that SIRT1 in VSMC acts as a modulator in neointima formation, and suppression of both VSMC proliferation and migration by SIRT1 results in reduced neointima formation.

**Methods**

An expanded Materials and Methods section is available in the online Data Supplement available at http://circres.ahajournals.org.

**Generation of SMC-SIRT1 Transgenic Mice**

All of the animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC). The transgenic mouse lines were produced in a C57BL/6 background via the microinjection of human SIRT1 cDNA under the control of a minimal SM22α promoter.

**Carotid Artery Ligation and Carotid Artery Wire Injury Model**

Nine- to 12-week-old male SMC-SIRT1 Tg mice and their littermate WT controls underwent complete carotid artery ligation14 or carotid artery wire injury15 as described previously.

**Histological and Morphometric Analysis**

Carotid arteries were harvested at the indicated times after injury. The cross-sectional areas of the intima and media were measured in H&E-stained sections in a blinded manner by a single observer using Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). Neointima formation was determined as the ratio of the intimal area to medial area (I/M). The immunohistochemical analyses were processed according to standard procedures.

**Cell Culture, Adenovirus Generation, and Infection**

Rat aortic SMCs were isolated from the thoracic aorta of male Sprague–Dawley rats by enzymatic digestion and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The replication-defective adenoviral vectors expressing SIRT1 (Ad-SIRT1) or control green fluorescent protein (Ad-GFP) and adenovirus-mediated knockdown of SIRT1 (Ad-SIRT1 RNAi) or control vector (Ad-U6) were generated using the AdEasy Vector kit (Quantum Biotechnologies, Randburg, South Africa) as described previously.7,16 VSMCs were infected for 12 or 24 hours with the above adenovirus using a multiplicity of infection (MOI) of 100, washed and incubated in serum-free medium without virus for at least 24 hours.

**[^3]H[^]Thymidine Incorporation, Flow Cytometry Analysis and Migration Assay**

The VSMC proliferation capacity was analyzed by [3H]-thymidine incorporation and flow cytometry analysis, and the VSMC migratory capacity was measured by a scraping injury and transwell assay. Details are provided in the online Data Supplement.

**Southern Blotting, RT-PCR, Western Blotting, and Luciferase Reporter Assay**

Target gene expression levels were probed either by reverse transcription-polymerase chain reaction (RT-PCR) using the corresponding primers (online Table II) or by Western blot analysis. The indicated reporter constructs (0.2 μg) were cotransfected with the indicated expression vectors and the internal control thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK, 30 ng, Promega, Fitchburg, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega).

**Statistical Analysis**

Data analysis was performed by using SPSS version 13.0 (SPSS, Inc., Chicago, IL). Data are presented as the means±SEM. Paired data were compared by Student t tests. Differences among groups were determined with 1-way or 2-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni post hoc test. A probability value of <0.05 was considered significant.

**Results**

**SIRT1 Expression Is Downregulated by Vascular Injury**

To investigate the role of SIRT1 in VSMC function and neointima formation, we performed a mouse left common
carotid artery (LCCA) ligation and then examined the expression of SIRT1 after injury. The Western blot analyses showed that SIRT1 expression was significantly decreased during neointima formation at 7, 14, and 28 days after injury (Figure 1). This finding suggests that SIRT1 may be involved in the development of neointimal lesions.

**Generation of SMC-Specific SIRT1 Tg Mice**

To study the role of SIRT1 in the regulation of neointima formation, particularly in VSMCs, we generated SMC-SIRT1 Tg mice in a C57BL/6 background. The transgenic construct contained human SIRT1 cDNA under the control of a VSMC-specific mouse minimal SM22α promoter (Figure 2A). Two independent strains of SMC-SIRT1 Tg mice were identified by Southern blot analysis (Figure 2B), and the transgene inheritance followed a Mendelian segregation pattern. No significant differences in heart function (analyzed by echocardiography) or basal systolic blood pressures were observed between the WT and SMC-SIRT1 Tg mice (online Table I). Genomic Southern blot analyses indicated that 2 transgenic strains carried similar copy numbers of the transgene. Human SIRT1 mRNA and protein were detected in the arteries of SMC-SIRT1 Tg mice (Figure 2C and 2D). Moreover, as expected, increased immunostaining of SIRT1 was observed in the media of carotid arteries in SMC-SIRT1 Tg mice (Figure 2E).

**SIRT1 Represses Neointima Formation in Response to Carotid Ligation and Carotid Artery Wire Injury**

To determine whether SIRT1 modulates neointima formation, we examined the effect of both carotid ligation and carotid artery wire injury in transgenic and littermate WT mice. Four
weeks after carotid ligation, the luminal area of carotid arteries was significantly reduced. Most of the cells in the neointima were VSMCs (SMα-actin staining; data not shown). At day 14 after ligation, a decreased tendency of I/M ratio in SMC-SIRT1 Tg mice was observed. At 28 days after ligation, I/M ratio was significantly reduced in SMC-SIRT1 Tg mice than in WT mice (Figure 3A). Consistent with these findings, at 14 days after the carotid artery wire injury, I/M ratio was significantly suppressed in SMC-SIRT1 Tg mice than in WT mice (online Figure I, A).

Proliferating cell nuclear antigen (PCNA)–staining analyses were performed to determine the level of cellular proliferation in the neointima. Staining for PCNA and intimal PCNA-positive cells were significantly reduced in SMC-SIRT1 Tg mice than in WT mice in each of the 2 mouse models of vascular injury (Figure 3B, online Figure I, B). However, no change in apoptosis was observed between WT and SMC-SIRT1 Tg mice in carotid arteries after ligation (online Figure II).

**SIRT1 Inhibits VSMC Proliferation and Migration In Vitro**

Because VSMC proliferation and migration are 2 key events in neointima formation following vascular injury, we investigated whether SIRT1 inhibits VSMC proliferation in vitro. The overexpression of SIRT1 in VSMCs resulted in a significant reduction of cell proliferation, as determined by [3H] thymidine incorporation following serum induction (Figure 4A). Furthermore, FACS analyses of both VSMCs and A7r5 cells with SIRT1 overexpression revealed a significant accumulation of the cells in G0/G1 phase (Figure 4B, online Figure III). These findings suggest that SIRT1 inhibits VSMC proliferation by blocking cell-cycle entry into S phase. Next, we examined the potential role of SIRT1 in VSMC migration using TNF-α as a chemoattractant. Wound healing assays indicated that the overexpression of SIRT1 resulted in a significant inhibition of VSMC migration (Figure 4C). Boyden chamber migration assays further confirmed the inhibitory effect of SIRT1 overexpression on VSMC migration (Figure 4E). In contrast, an RNAi-mediated SIRT1 knockdown resulted in a significant increase in VSMC migration (Figure 4D and 4F).

**SIRT1 Inhibits the Expression of Cyclin D1 and MMP-9 in VSMCs**

Because cyclin D1 and matrix metalloproteinase-9 (MMP-9) are 2 key regulators in VSMC proliferation and migration,18,19 we investigated whether cyclin D1 and MMP-9 were involved in the inhibitory effect of SIRT1 on VSMC proliferation and migration. Western blot analyses of cyclin D1 and MMP-9 protein levels showed that the overexpression of SIRT1 significantly decreased cyclin D1 and MMP-9 expression in VSMCs (Figure 5A and 5C), and a similar finding of reduced cyclin D1 expression was observed in the SMC cell line A10 (online Figure IV). SIRT1 knockdown by RNAi produced an opposite effect (Figure 5B and 5D). In addition, we found that overexpression of SIRT1 reduced cyclin E and CDK2 expression (online Figure V, A), although SIRT1 knockdown by RNAi did not affect cyclin E and CDK2 expression (online Figure V, B).

Using gelatin zymography, we assayed the enzymatic activity of secreted MMP-9. As shown in Figure 5E, SIRT1 overexpression significantly inhibited the enzymatic activity of MMP-9. Consistent with these results, VSMC treatment with sirtinol (a chemical inhibitor of SIRT1) plus TNF-α increased MMP-9 protein expression (Figure 5F). The overexpression of SIRT1 in VSMCs did not have significant effects on other VSMC
migration-associated genes, including MMP-2, TIMP-1, TIMP-2, and MMP-3 (online Figure VI). Collectively, these results indicate that MMP-9 plays an important role in the inhibitory effects of SIRT1 on VSMC migration.

Decreased Cyclin D1 and MMP-9 Expression/Activity in SMC-SIRT1 Tg Mice
To validate that cyclin D1 and MMP-9 function as downstream effectors of SIRT1 in the modulation of neointima formation in vivo, we analyzed cyclin D1 and MMP-9 expression in carotid arteries following ligation. Cyclin D1 expression was significantly decreased in the ligated arteries of SMC-SIRT1 Tg mice (Figure 6A). Furthermore, the increased MMP-9 expression observed after 1 day of ligation was significantly attenuated in SMC-SIRT1 Tg mice (Figure 6B). Zymography assays were performed using protein extracts from ligated and sham-operated carotid arteries of WT and SMC-SIRT1 Tg mice. The MMP-9 enzymatic activity in the ligated carotid arteries of SMC-SIRT1 Tg mice was significantly reduced in comparison with that detected in WT mice (Figure 6C). An immunostaining assay also revealed that the expression levels of cyclin D1 and MMP-9 were reduced in the ligated arteries of SMC-SIRT1 Tg mice in comparison with those in their WT littermates at the indicated time points (Figure 6D).

AP-1 Is Implicated in the Transcriptional Regulation of Cyclin D1 and MMP-9 by SIRT1
We next sought to determine whether SIRT1 regulated cyclin D1 and MMP-9 transcription. We found that SIRT1 overexpression significantly decreased cyclin D1 and MMP-9 mRNA levels that were induced by serum and TNF-α, respectively (Figure 7A). Because transcription factor activa-
tor protein-1 (AP-1) is important in the regulation of cyclin D1 and MMP-9 transcription,20–22 we performed a transient transfection analysis to determine whether AP-1 played a role in the transcriptional regulation of cyclin D1 and MMP-9 by SIRT1. Using 2 luciferase reporter vectors under the controls of the cyclin D1 (H11002/903 to H11001/202) and MMP-9 (H11002/711 to H11001/19) promoters, we found that c-Fos/c-Jun increased the promoter activity of both cyclin D1 and MMP-9, and the induction of the cyclin D1 and MMP-9 promoters by c-Fos/c-Jun was blocked by pcDNA3.1-SIRT1 (Figure 7B). Furthermore, point mutations or deletions of the AP-1 DNA-binding site significantly decreased the ability of SIRT1 to repress the cyclin D1 and MMP-9 promoters (Figure 7C).

To investigate whether endogenous SIRT1 regulates cyclin D1 and MMP-9 transcription by associating with the chromatin region that contains the AP-1 DNA binding site, we performed chromatin immunoprecipitation (ChIP) assays. As shown in Figure 8A and 8B, chromatin fragments containing the AP-1 DNA binding site from the cyclin D1 and MMP-9 promoter region were observed specifically in the immunoprecipitates obtained using the antimSirt1, antic-Fos, or c-Jun antibodies. Furthermore, point mutations or deletions of the AP-1 DNA-binding site significantly decreased the ability of SIRT1 to repress the cyclin D1 and MMP-9 promoters (Figure 7C).

We further determined whether SIRT1 affected c-Fos and c-Jun activity. The immunofluorescent staining results showed that SIRT1 and c-Fos/c-Jun colocalized in the cell nucleus (online Figure VII), and the coimmunoprecipitation analyses revealed that SIRT1 and c-Fos/c-Jun interacted physically (online Figure VIII). Because SIRT1 usually functions via the deacetylation of target proteins, transient transfection and immunoprecipitation analyses were performed to determine whether SIRT1 regulated the acetylation status of AP-1 in A10 cells. SIRT1 overexpression markedly reduced the acetylation level of both c-Fos and c-Jun under TNF-α-inducing conditions (online Figure IX).

**Discussion**

In the present study, we demonstrated for the first time that SIRT1 expression was decreased in the process of neointima formation and that overexpression of SIRT1 in VSMCs inhibited neointima formation following vascular injury in mice. Given that increased VSMC proliferation is one of the central features associated with the development of neointimal lesions, several molecules have been shown to regulate the formation of neointima by inhibiting VSMC proliferation.1 The mouse carotid artery ligation model is a very suitable model for the study of SMC proliferation, and in some cases, it mimics the vascular lesions that develop in humans at sites of altered hemodynamics that are associated with low shear stress.14,23 Using this mouse model, we found that SIRT1 expression was substantially decreased with the development of the neointima, which indicated that SIRT1 was involved in this process and might act as...
a growth suppressor. This presumption was confirmed in both carotid artery ligation and carotid artery wire injury models by a reduction of the neointimal thickening in the injured artery of SMC-SIRT1 Tg mice. It was further strengthened by a remarkable reduction of in situ cell proliferation, as determined by the number of PCNA-positive cells in the intima. Still, the mechanism by which SIRT1 expression is downregulated during neointima formation remains to be elucidated.

The mechanism responsible for the growth suppression induced by SIRT1 was found to be a cell-cycle arrest at the G1 phase and a reduction in DNA synthesis; these results were consistent with recent findings showing that the increased expression of SIRT1 causes a delay in S-phase entry and greatly reduces the growth of some specific cells.8,9,24,25

In contrast, the inhibition of SIRT1 by shRNAs in telomerase-immortalized human cells has been shown to enhance cell growth under normal and nutrient-limiting conditions,26 and SIRT1-deficient mouse embryonic fibroblasts (MEFs) were also demonstrated an enhanced proliferative capacity under conditions of chronic, sublethal oxidative stress.27 Nevertheless, a growth arrest effect, but not a growth-inducing effect, has been previously observed in human umbilical-vein endothelial cells (HUVECs) in which SIRT1 is inhibited by sirtinol or siRNA.28 Similarly, we also observed a reduction of DNA synthesis in serum-induced VSMCs during the knockdown of endogenous SIRT1 by RNAi (online Figure X), which suggests that endogenous SIRT1 is required for both growth and proliferation in some certain types of cells including VSMCs.

Figure 7. Transcriptional regulation of cyclin D1 and MMP-9 by SIRT1 in VSMCs. A, Representative semiquantitative RT-PCR results for Ad-GFP- or Ad-SIRT1-infected VSMCs (bottom panel). Quantitative analyses of PCR (normalized to that of β-actin, top panel). Values represent the means ± SEM (n = 3). **P < 0.01 versus Ad-GFP–infected VSMCs treated with 10% FBS. ***P < 0.001 versus each Ad-GFP group. B, Regulation of the promoter activities of cyclin D1 and MMP-9 by SIRT1 in A10 (cyclin D1-luc) or A7r5 (MMP-9-luc) cells, respectively. Data represent the means ± SEM (n = 3). *P < 0.05, **P < 0.001 versus each pcDNA3.1 group. C, Modulation of the repressive effect of SIRT1 on the promoter activities of cyclin D1 and MMP-9 by the indicated cyclin D1 and MMP-9 promoter deletions or mutations, respectively. The relative cyclin D1 or MMP-9 luciferase activities are presented as the ratio of the indicated pcDNA3.1-SIRT1–modified to the pcDNA3.1-modified cyclin D1 or MMP-9 promoter activities. Data represent the means ± SEM (n = 3). *P < 0.01, **P < 0.001 versus the fold change in cyclin D1-Luc and MMP-9-Luc, respectively.
During formation of the neointima, the processes of VSMC proliferation and migration are interrelated. After stimulation, most of the migrating VSMCs are in late G1 phase of the cell cycle. A reduction in VSMC proliferation by SIRT1 overexpression may result in reduced VSMC migration. As we proposed, the results of our cell migration experiments demonstrated that SIRT1 overexpression resulted in a reduction of the VSMC migratory capacity. Thus, the reduced numbers of migratory VSMCs might be due to the effects of SIRT1 overexpression or caused indirectly by the decreased VSMC proliferation. Hence, the inhibitory effects of SIRT1 on VSMC proliferation and migration contributed to the reduced neointimal lesion observed in the injured vessels of SMC-SIRT1 Tg mice.

Cyclin D1 acts as a mitogenic signal sensor and promotes progression through the G1-S phase of the cell cycle. A reduction in VSMC proliferation by SIRT1 overexpression may result in reduced VSMC migration. As we proposed, the results of our cell migration experiments demonstrated that SIRT1 overexpression resulted in a reduction of the VSMC migratory capacity. Thus, the reduced numbers of migratory VSMCs might be due to the effects of SIRT1 overexpression or caused indirectly by the decreased VSMC proliferation. Therefore, the inhibitory effects of SIRT1 on VSMC proliferation and migration contributed to the reduced neointimal lesion observed in the injured vessels of SMC-SIRT1 Tg mice.

Cyclin D1 acts as a mitogenic signal sensor and promotes progression through the G1-S phase of the cell cycle. Increased cyclin D1 protein expression and medial VSMC growth have also been observed in rat balloon-injured carotid arteries. In addition, DNA synthesis defects are apparent in MEFs and 3T3 cells derived from cyclin D1−/− mice. We found that SIRT1 overexpression inhibited VSMC proliferation and reduced cyclin D1 expression at both the mRNA and protein levels. The inhibitory effect of SIRT1 on cyclin D1 protein expression was also observed in injured arteries from SMC-SIRT1 Tg mice. MMP-9 has been reported to be a major regulator in VSMC proliferation and migration and in neointima formation. Nonspecific MMP inhibition has also been proposed to oppose remodeling, as evidenced by an inhibition of outward arterial remodeling. SIRT1 overexpression in VSMCs resulted in decreased MMP-9 mRNA and protein expression, enzymatic activity and VSMC migration following stimulation with TNF-α. Moreover, ligated vessels from SMC-SIRT1 Tg mice displayed reduced MMP-9 protein levels, enzymatic activity, and immunoactivity. In addition, cyclin D1 has been reported to modulate the invasive capacity of cells by increasing MMP-9 activity and cell motility, whereas MMP-9−/− VSMCs display reduced proliferation and cyclin D1 protein levels. Therefore, the inhibitory effects of SIRT1 on VSMC proliferation and migration may be caused by a downregulation of these two effectors and their interactions. Although the effects of cyclin D1 and MMP-9 on VSMC proliferation and migration have been well documented, additional studies are needed to confirm that the effects of SIRT1 are mediated directly by SIRT1 overexpression inhibited VSMC proliferation and reduced cyclin D1 expression at both the mRNA and protein levels. The inhibitory effect of SIRT1 on cyclin D1 protein expression was also observed in injured arteries from SMC-SIRT1 Tg mice. MMP-9 has been reported to be a major regulator in VSMC proliferation and migration and in neointima formation. Nonspecific MMP inhibition has also been proposed to oppose remodeling, as evidenced by an inhibition of outward arterial remodeling. SIRT1 overexpression in VSMCs resulted in decreased MMP-9 mRNA and protein expression, enzymatic activity and VSMC migration following stimulation with TNF-α. Moreover, ligated vessels from SMC-SIRT1 Tg mice displayed reduced MMP-9 protein levels, enzymatic activity, and immunoactivity. In addition, cyclin D1 has been reported to modulate the invasive capacity of cells by increasing MMP-9 activity and cell motility, whereas MMP-9−/− VSMCs display reduced proliferation and cyclin D1 protein levels. Therefore, the inhibitory effects of SIRT1 on VSMC proliferation and migration may be caused by a downregulation of these two effectors and their interactions. Although the effects of cyclin D1 and MMP-9 on VSMC proliferation and migration have been well documented, additional studies are needed to confirm that the effects of SIRT1 are mediated directly

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**Figure 8.** The repression of AP-1 activity by SIRT1 leads to decreased cyclin D1 and MMP-9 expression by SIRT1. A through D, ChIP of cyclin D1 (A and C) and MMP-9 (B and D) promoter complexes. Primary VSMCs (A and B) and primary VSMCs with Ad-U6 or Ad-SIRT1 RNAi infection (C and D) were used for immunoprecipitation with antiSirt1 (antibody from Millipore), anti–c-Fos, anti–c-Jun antibody, or control IgG. Immunoprecipitated chromatin fragments were applied to routine PCR (bottom panel of A and B) and quantified by realtime-PCR (top panel of A and B, C and D) using specific primers sets. The images and data are representative of 3 independent experiments.
through cyclin D1 and MMP-9 in vivo. Considering that there are many other SIRT1 target proteins (such as survivin, FOX1, and angiotensin II type I receptor (AT1R)) that play important roles in VSMC proliferation, neointimal hyperplasia, and hypertension, we also detected the expression of these proteins in ligated vessels from WT and SM-CeSIRT1 Tg mice (online Figure XI). It will be interesting to further elucidate the involvement of these proteins in the regulation of neointima formation by SIRT1.

Numerous studies have demonstrated that AP-1, a transcription factor that consists of a homodimer and heterodimers of JUN family members and heterodimers of the Fos and Jun families, is crucial for the mediation of VSMC proliferation and neointima formation in response to vascular injury. The activity of AP-1 is regulated by posttranslational modifications, including extensively studied phosphorylation and less-studied acetylation modifications. An increase in c-Fos and c-Jun gene expression and enhanced AP-1 DNA-binding activity activated by MAP kinases have been observed during neointima development following arterial injury. Recently, our group and others have shown that SIRT1 can interact with AP-1, and then inhibit its transcriptional activation. However, little information is available concerning the effect of AP-1 acetylation on the function of VSMCs. In the present study, we found that endogenous SIRT1, c-Fos, and c-Jun bound to the AP-1 binding site of the cyclin D1 and MMP-9 promoters, and that SIRT1 inhibition resulted in an enrichment of c-Fos and c-Jun at the promoters of cyclin D1 and MMP-9, respectively. Moreover, SIRT1 interacted and colocalized with both c-Fos and c-Jun. SIRT1 overexpression markedly reduced the acetylation level of both c-Fos and c-Jun induced by TNF-α, which suggested that the repressive effect of SIRT1 on AP-1 activity and downstream events was due to its deacetylase activity in VSMCs, although the in vivo effects of SIRT1 on AP-1 acetylation remain to be verified. Taken together, our findings suggest that the decreased VSMC proliferation and migration and the reduced neointima formation are, at least in part, due to a decrease in the transcriptional activity of AP-1 and a downregulation of cyclin D1 and MMP-9 expression.

In summary, our findings demonstrate that SIRT1 is a novel modulator that is involved in the process of neointima formation and further elucidate that the repression of AP-1 activity by SIRT1 contributes to the decreased expression of cyclin D1 and MMP-9. These effects, in turn, lead to a decrease in VSMC proliferation and migration and reduced neointima formation. The present findings indicate that SIRT1 may be an attractive therapeutic target for the prevention of vascular diseases.

Acknowledgments

We thank Dr Li-Min Li from PUMC for critical reading of the manuscript; Xi-Ping Chen from PUMC for assistance with the histological assay; Dr Zhu-Qin Zhang, Dr Rui-Feng Yang, Dr Wei Zheng, Dr Yun-Biao Lu, and Ying He from PUMC for their technical assistance; and Dr Lan Sun from PUMC for assistance with the statistical analyses.

Sources of Funding

This work was supported by grants from the National Basic Research Program of China (Nos. 2006CB503801 and 2011CB503902), the Special Fund of the National Laboratory of China (No. 2060204), and the National Natural Science Foundation of China (No. 31021091).

Disclosures

None.

References

Novelty and Significance

What Is Known?

- Vascular smooth muscle cell (VSMC) proliferation and migration are crucial events in the development of vascular diseases.
- SIRT1 has been reported to exert functions that antagonize atherosclerosis, but its role in neointimal formation is unknown.

What New Information Does This Article Contribute?

- SIRT1 expression is downregulated by vascular injury.
- SIRT1 represses neointima formation in response to carotid ligation and carotid artery wire injury.
- SIRT1 inhibits VSMC proliferation and migration.
- SIRT1 represses AP-dependent transcriational regulation of cyclin D1 and MMP-9.

The process of neointima formation is common to various forms of vascular diseases such as atherosclerosis, in-stent restenosis, vein bypass graft failure, and transplant vasculopathy. In this study, we demonstrate that SIRT1, a longevity gene, inhibits VSMC proliferation and migration that underlie neointima formation. Our work provides evidence that SIRT1 acts as a novel modulator linking the coordinated responses of VSMCs to injury. This finding expands our knowledge about the function of SIRT1 in addition to its roles in regulating aging and metabolism, and advances our understanding of molecular mechanisms of neointima formation. SIRT1 represents a potential new target for intervention in vascular diseases.
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_Circ Res._ 2011;108:1180-1189; originally published online April 7, 2011; doi: 10.1161/CIRCRESAHA.110.237875

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIAL

SIRT1 acts as a modulator of neointima formation following vascular injury in mice

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Detailed Methods

Generation of SMC-SIRT1 transgenic mice

All of the animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS&PUMC). Transgenic (Tg) mouse lines were produced in a C57BL/6 background via the microinjection of human SIRT1 cDNA under the control of a minimal SM22α promoter (a region of the murine SM22α promoter that contains 445 base pairs of 5'-flanking sequence: a kind gift from Dr. J. M. Miano, University of Rochester School of Medicine & Dentistry, New York, USA)1. The successful insertion of the SIRT1 transgene was confirmed by Southern blot analysis, and SIRT1 overexpression was assessed by PCR and western blot analyses.

Blood pressure evaluation and echocardiography

The heart rate and systolic blood pressure of the animals were measured using tail-cuff plethysmography (BP-2000 System, Visitech Systems, Apex, NC). Echocardiography was performed using a Vevo770 High-Resolution Imaging System (Visual Sonics Inc., Toronto, ON, Canada) with a 30-MHz lineararray ultrasound transducer.

Carotid artery ligation and carotid artery wire injury models

Nine- to 12-week-old male SMC-SIRT1 Tg mice and their littermate wild-type (WT) controls underwent a complete carotid artery ligation2 or wire injury3. Briefly, the mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). For the carotid artery ligation model, the LCCA was completely ligated just near its bifurcation using a 6-0 silk ligature. For the carotid artery wire injury model, the left internal carotid artery was exposed by a blunt-end dissection, tied off distally, and looped proximally on the external branch using a 5.0 silk black suture. A transverse section was generated in the
proximal portion of the internal carotid artery, and a straight guide wire (C-SF-15-15, Cook Belgium NV) was passed toward the aortic arch and withdrawn 3 times with a rotating motion. After removal of the guide wire, the proximal portion of the internal carotid artery was tied off, and the skin incision was closed. The animals were then processed for morphological and biochemical studies at specific time points after the initial surgery.

**Histological and morphometric analysis**

The carotid arteries were harvested at 0, 1, 7, 14 or 28 days after injury. The animals were euthanized by an intraperitoneal injection of ketamine/xylazine. The left ventricle was cannulated and perfused with PBS containing heparin, and then perfused and fixed with 4% paraformaldehyde in phosphate-buffered saline under physiological pressure. Then the left carotid artery was removed, further fixed for 16 hours, and paraffin-embedded without further dissection. Serial sections (5-μm thick) were created at 500μm proximal to the ligation site (carotid artery ligation model) or throughout the whole approximate 350-μm from the junction of the external and internal branches of the left carotid artery (carotid artery wire injury model). The cross-sectional areas of the intima and media were measured in H&E-stained sections in a blinded manner by a single observer using Image Pro Plus 6.0 software (Media Cybernetics). A mean value was determined from at least 4 sections in each animal. Neointima formation was determined as the ratio of the intimal area to the medial area (I/M).

For the immunohistochemical analysis, the sections were preincubated with 5% normal goat serum and then incubated with anti-hSIRT1 (1:100, cat. no. sc-15404, Santa Cruz Biotechnology Inc. ), anti-PCNA (1:100, cat. no. M0879, DAKO), anti-MMP-9 (1:100, cat. no. sc-6840, Santa Cruz Biotechnology Inc.) or anti-cyclin D1 (1:100, cat. no. sc-8396, Santa Cruz Biotechnology Inc.). Next, the sections were incubated with the biotinylated secondary antibody streptavidin–horseradish peroxidase (Dako) followed by diaminobenzidine (DAB kit, Vector Laboratories), and then they were counterstained with Gill’s hematoxylin (Fisher Scientific) and Scott’s solution. For the negative controls, the primary antibody was replaced with non-immune rabbit or mouse serum. The mean value obtained from at least 3 sections in each animal was used for the quantitative analysis of the number of PCNA–positive cells that were present in the injured vessel walls.

**Cell culture, adenovirus generation and infection**

The A10 and A7r5 rat embryonic aortic vascular smooth muscle cell (VSMC) lines were obtained from the American Type Culture Collection (ATCC). Rat aortic VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats by enzymatic digestion. All of the cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The VSMCs were maintained at 37 °C in a humidified atmosphere containing 5% CO₂, and passages 3 to 5 of these cells at 80% confluence were used in the experiments. The medium was changed before the experiments. The replication-defective adenoviral vectors expressing SIRT1 (Ad-SIRT1) or the control green fluorescent protein (Ad-GFP) and the adenovirus-mediated knockdown of SIRT1 (Ad-SIRT1 RNAi) or the control vector (Ad-U6) were generated using the AdEasy Vector kit (Quantum Biotechnologies) as described previously. The VSMCs were infected for 12 or 24 hours with the above adenovirus at a MOI of 100, washed and incubated in serum-free medium without virus for at
least 24 hours.

[^3]H thymidine incorporation

The VSMCs were plated in 12-well plates at a density of 50,000 cells/well, infected with the adenovirus overnight, and then serum-starved for 48 hours prior to a 24-hour stimulation with 10% FBS. The cells were then incubated with [^3]H thymidine (2 μCi per well, Amersham) for the last 5 hours. At the end of the experiment, the cells were washed with cold PBS, scraped off the well, and treated with 10% trichloroacetic acid at 4°C for 60 minutes. The precipitates were then dissolved in NaOH (0.4N) and counted with a liquid scintillation counter.

Flow cytometry analysis

Adenovirus-infected VSMCs were maintained in serum-free DMEM for 24 hours and then stimulated with 10% FBS for different periods. The cells were trypsinized, fixed in 70% ethanol at 4 °C overnight, washed twice with ice-cold PBS and incubated with RNase and propidium iodide. The cell-cycle phase was analyzed by flow cytometry using a Becton Dickinson FACStar flow cytometer and the Becton Dickinson CellFIT software.

Migration assay

The two- and three- dimensional cell migratory ability of the VSMCs was assessed. The two-dimensional cell migration was analyzed by the scratch wound assay. In brief, VSMCs were scratched at the beginning of the 24-hour infection with adenovirus. The cells were then cultured in the presence or absence of TNF-α (30 ng/mL, PeproTech) for an additional 24 hours, fixed and imaged using light microscopy. The furthest distance of cell migration from the wound edge was measured in an average of five independent microscopic fields.

The three-dimensional cell migration was examined in modified Boyden transwell cell culture chambers using a gelatin-coated polycarbonate membrane (Corning Life Science, Costar Inc.). Briefly, serum-starved infected cells at a density of 2 × 10^5/ml (100 μL) were added to the upper chamber, while 600 μL of DMEM containing the appropriate concentration of TNF-α or vehicle was placed in the lower chamber. After incubation at 37°C for 8 hours, the cells on the upper side were removed with cotton swabs. The membranes were then fixed with methanol at 4°C for 10 minutes and stained with crystal violet. The number of cells that had migrated was counted under a light microscope. Three randomly selected fields were counted per membrane.

Plasmid construction and luciferase reporter assay

The plasmid containing the coding sequence for human SIRT1 was a kind gift from Professor F. Ishikawa (Kyoto University, Kyoto, Japan). The full-length c-Fos and c-Jun expression plasmids (pcDNA3.1-c-Fos and pcDNA3.1-c-Jun, respectively) were constructed by inserting human c-Fos and c-Jun cDNA into pcDNA3.1. A 1,105-bp of rat cyclin D1 promoter fragment was cloned into the MluI/XhoI site of the reporter vector, pGL3-basic (cyclin D1-Luc). A series of deletion constructs was generated by PCR amplification. Constructs containing a fragment from -813 to +202 of the promoter and from -40 to +202 were denoted cyclin D1-900-Luc and cyclin D1-200-Luc, respectively. The mutant cyclin D1 promoter plasmid (cyclin D1-APm-Luc) was modified using a site-directed mutagenesis kit (Promega).
The AP-1 binding element (5'−GTAACGTCA−3' from -44 to -41) in cyclin D1-Luc was modified to 5'-GTAACGTTCG−3'. Underlined sequences denote the mutated sites. A 730-bp human MMP-9 promoter fragment was cloned into the MluI/BglII site of pGL3-basic (MMP-9-Luc). The AP-1 binding element in the mutant MMP-9 promoter plasmid MMP-9-Apm-Luc was modified from (5'-CTGAGTCA−3') to (5'-CTGAGTTG−3'). Underlined sequences denote the mutated sites. All constructs were confirmed by DNA sequencing. The purified luciferase plasmids were transiently transfected into A10 or A7r5 cells using the Lipofectamine 2000 reagent at a ratio of 1 μL Lipofectamine 2000/1 μg of DNA, according to the manufacturer’s instructions (Invitrogen). The indicated reporter constructs (0.2 μg) were cotransfected with the indicated expression vectors and the internal control pRL-TK reporter (30 ng, Promega). Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega).

**PCR Analysis, Southern Blotting, Reverse transcription and real-time PCR**

For routine identification of SMC-SIRT1 Tg mice, PCR analysis was performed using the Primers: sense 5'−CTTCAGGTCAAGGGATGGTAT−3'; anti-sense 5'-GCGTGTCTATGTTCTGGGTAT−3', yielding a 233-bp product. Identification was further confirmed by standard Southern blot analysis of mouse tail genomic DNA. A 201bp DNA fragment amplified from the DNA for microinjection was used as the probe for Southern blot analysis. Primers sequences: sense 5'-CCCCAGAGCGTGAGGTGC-3'; anti-sense 5'-CTGACAAGTTTGTCGAGGCAGC-3'. Gene copy numbers in the founder mice were estimated by densitometric scanning of autoradiogram using a Phosphor Imager (Molecular Dynamics). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Two micrograms of total RNA was used for the first-strand synthesis with cDNA M-MuLV Reverse Transcriptase (New England Biolabs) using random primers. RT–PCR was used to analyze the cellular mRNA levels of SIRT1, cyclin D1, MMP-9, β-actin, MMP-2, TIMP-1, TIMP-2 and MMP-3. The primers that were used for the PCR are listed in Online Table II. The Quantititect SYBR Green RT-PCR Kit (QIAGEN) was employed for the amplifications reactions using the 1-step protocol described by the manufacturer with a BioRad iCycler iQ5 Real-Time PCR Detection System. The fluorescence curves were analyzed using iCycler iQ5 Optical System software (Version 2.0).

**Western blotting**

Cellular and mouse tissue proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). After a complete homogenization on ice, the samples were centrifuged, and the obtained supernatants were fractionated by 10% SDS–PAGE and electro-transferred onto a PVDF membrane. After blocking with Tris-buffered saline (TBS) containing 5% non-fat milk, the membranes were probed with primary antibodies against SIRT1 (anti-mSirt1, 1:2000, cat. no. 07-131, Millipore; anti-hSIRT1, 1:1000, cat. no. sc-15404, Santa Cruz Biotechnology Inc.), MMP-9 (1:1000, cat. no. sc-6840, Santa Cruz Biotechnology Inc.), cyclin D1 (1:1000, cat. no. sc-8396, Santa Cruz Biotechnology Inc.), MMP-2 (1:1000, cat. no. sc-13595, Santa Cruz Biotechnology Inc.), cyclin E (1:1000, cat. no. sc-481, Santa Cruz Biotechnology Inc.), CDK2 (1:1000, cat. no. sc-163, Santa Cruz Biotechnology Inc.),
CDK4 (1:1000, cat. no. sc-260, Santa Cruz Biotechnology Inc.), p27 (1:1000, cat. no. sc-1641, Santa Cruz Biotechnology Inc.), survivin (1:1000, cat. no. sc-10811, Santa Cruz Biotechnology Inc.) and AT1R (1:1000, cat. no. sc-1173, Santa Cruz Biotechnology Inc.) at 4°C overnight. A horseradish peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection. Immunoreactive peroxidase-conjugated secondary antibody was used for the ECL detection.

**Coimmunoprecipitation assay**

A10 whole-cell lysates were preincubated with 1.0 µg of non-immune rabbit IgG and 20 µL of protein A-Sepharose beads (Upstate) at 4°C for 3 hours and centrifuged. The cleared lysates (1 mg) were immunoprecipitated with the anti-hSIRT1 (cat. no. sc-15404, Santa Cruz Biotechnology Inc.), anti-c-Jun (cat. no. sc-1694, Santa Cruz Biotechnology, Inc.) or anti-HA (H6908, Sigma-Aldrich) antibody at 4°C for 1 hour and then incubated with protein A-Sepharose beads at 4°C overnight. The immunoprecipitated proteins were washed 6 times with lysis buffer and resuspended in electrophoresis sample buffer. Samples of the immunoprecipitated or total proteins (20–30 µg) were analyzed by western blotting using the following primary antibodies: anti-hSIRT1 (cat. no. sc-15404, Santa Cruz Biotechnology Inc.), anti-c-Jun (cat. no. SC-1694, Santa Cruz Biotechnology Inc.) anti-HA (cat. no. H6908, Sigma-Aldrich) and anti-acetyl-lysine(anti-Ac-Lys, cat. no. 05-515, Upstate).

**Immunofluorescence staining**

A10 cells were cotransfected with the expression plasmids pcDNA3.1-SIRT1-Myc, pcDNA3.1-c-Fos or pcDNA3.1-c-Jun for 30 hours and then fixed, blocked, and incubated with a mouse monoclonal anti-c-Myc antibody (Santa Cruz Biotechnology, Inc.) and a rabbit polyclonal anti-c-Fos (cat. no. SC-52, Santa Cruz Biotechnology Inc.)/anti-c-Jun antibody (1:100 dilution) at 4°C overnight. Fluorescein-conjugated secondary antibodies (anti-mouse TRITC, red; anti-rabbit FITC, green; 1:100 dilution) were then added to the samples, followed by a 1-hour incubation. The nuclei were visualized by staining with Hoechst 33258 in the dark for 5 minutes. The slides were washed and covered with mounting medium. Confocal microscopic images were obtained by simultaneous recording in the 488 λ, 560 λ, and /or 615 λ channels as appropriate.

**Gelatin zymography assay**

The gelatinolytic activity of MMP-9 in the 10-fold concentrated culture medium of VSMCs or in the extracts of individual carotid arteries exposed to different treatments was analyzed by zymography on gelatin-containing polyacrylamide. Equal amounts of sample were loaded in each lane. After washing in 2.5% Triton X-100, the gels were incubated overnight in buffer (10 mM CaCl₂, 0.01% NaN₃, and 50 mM Tris–HCl, pH 7.5) and subsequently stained with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad) for 2 hours and destained with 10% acetic acid and 40% methanol. Proteins with gelatinolytic activity were visualized as clear zones in an otherwise blue gel.

**ChIP assay**
VSMCs were cultured in complete medium until they were 80% confluent and infected with Ad-U6 or Ad-SIRT1 RNAi for 24 hours. The cells were then washed, and the medium was replaced with medium lacking serum for an additional 48 hours. At two and a half hours prior to harvesting, the VSMCs were washed with serum-free DMEM medium. The cells were sonicated and immunoprecipitated using the indicated antibodies. Extracted DNA was then PCR-amplified using specific primers for rat cyclin D1 promoter with an AP-1 binding site (from -44 to -41), rat MMP-9 promoter with an AP-1 binding site (from -80 to -73), 3'UTR of cyclin D1 and MMP-9 and the β-actin coding region. An equal volume of non-precipitated (input) genomic DNA at a 1:45 dilution was amplified as a positive control.

**TdT-mediated dUTP nick end labeling assessment of apoptosis**

The TdT-mediated dUTP nick end-labeling (TUNEL) assay was performed using a DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer’s instructions. Briefly, 5-μm-thick tissue sections were obtained from the samples. The slides were washed with 1 mL of the wash buffer provided in the kit and labeled with the DNA labeling solution at 37°C for 60 minutes. After the slides were rinsed and incubated with equilibration buffer, nucleotide mix and rTdT enzyme at 37°C for 1 hour in the dark, they were counterstained with Hoechst and then examined using confocal fluorescence microscopy. TUNEL-positive cells were identified by a green fluorescence (apoptotic cells) and analyzed using the Image Pro Plus 6.0 program.

**Statistical analysis**

Data analysis was performed by using SPSS version 13.0 (SPSS, Inc., Chicago, IL). Data are presented as the means ± SEM. Paired data were compared by Student's t tests. Differences among groups were determined with one-way or two-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni’s post hoc test. A probability value of <0.05 was considered significant.

**Supplemental References**


Supplemental Tables:

**Online Table I.**

**Echocardiographic Characteristics in WT and SMC-SIRT1 Tg Mice**

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<td>FS, %</td>
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SBP indicates systolic blood pressure; HR, heart rate; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; IVSd, left ventricular septum, diastolic; LVPWd, left ventricular posterior wall, diastolic; FS, fractional shortening. All of the values are means ± SEM, *P* = NS.
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H: Human, m: mouse, r: rat, s: Sense, a: Antisense
Supplemental Figures:

**A**

**Online Figure I.** SIRT1 overexpression inhibits neointima formation following carotid artery wire injury. **A,** Representative cross-sections of hematoxylin/eosin-stained carotid arteries without injured (control) or injured for 14 days. Scale bar: 50 μm. **B,** Quantitative analyses of the I/M ratio using histological sections from WT or SMC-SIRT1 Tg mouse arteries. ***P < 0.001 for SMC-SIRT1 Tg versus WT mice (n = 6 mice per group). **C,** Left, Representative sections of mouse carotid arteries at 14 days following wire injury that were immunostained for markers of proliferation (PCNA to identify cells in S phase, brown). Scale bar: 25 μm. Right, Quantification of PCNA-positive cells in the neointima of carotid arteries. *P < 0.05 for SMC-SIRT1 Tg versus WT mice (n = 4 mice per group). The internal elastic lamina is indicated by black arrows.
**Online Figure II.** Number of TUNEL-positive cells in carotid arteries of WT and SMC-SIRT1-Tg mice 28 days after ligation. Values are means ± SEM (n = 5 mice per group).
Online Figure III. FACS analysis to measure the DNA content in Ad-GFP- or Ad-SIRT1-infected A7r5 cells following serum starvation for 24 hours and then serum stimulation at the indicated time points. Data are expressed as the percentage of the total cells and represent the means ± SEM (n=4). *P < 0.05 versus Ad-GFP at the indicated time points.
Online Figure IV. Bottom, Representative western blots of pcDNA3.1- or pcDNA3.1-SIRT1 transfection in A10 cells following 24 hours serum starvation and then serum induction for 12 hours. Top, Quantification of densitometric analysis. Values are means ± SEM (n = 3). *P < 0.05 , ***P < 0.001 versus each pcDNA3.1 group treated with 10%FBS for 12 hours.
Online Figure V. Bottom, Representative Western blots of Ad-SIRT1- (A) or Ad-SIRT1 RNAi-infection (B) in VSMCs. Top, Quantification of densitometric analysis. Values are means ± SEM (n = 3). *P < 0.05 , **P < 0.01 versus each Ad-GFP group by treatment with 10% FBS for 12 hours.
Online Figure VI. A and B, Bottom, Semi-quantitative RT-PCR (A) and Western blot (B) to detect MMP-2 expression in VSMCs incubated with TNF-α (30 ng/mL) for indicated time points. β-actin served as loading control. Top, Quantification of densitometric analysis. Values are means ± SEM (n =3). C, Semi-quantitative RT-PCR showed mRNA levels of TIMP-1, TIMP-2 and MMP-3 in VSMCs following TNF-α (30 ng/mL) induction for indicated time points. β-actin served as loading control. Quantitative analyses (top panel). Values are means ± SEM (n =3).
**Online Figure VII.** Immunofluorescence staining to detect the localization of SIRT1 and c-Fos or c-Jun in A10 cells. Expression of SIRT1 was confirmed by detection of the Myc-tag (red) (upper left panel). TRITC-labeled secondary antibody (red) for Myc-SIRT1 (A and B, upper left panel) and FITC-labeled secondary antibody (green) for c-Fos (A, lower left panel) and c-Jun (B, lower left panel). Nuclei were stained with Hoechst 33258.
Online Figure VIII. Coimmunoprecipitation assays demonstrated that SIRT1 interacted with c-Fos or c-Jun. A10 cells were cotransfected with the plasmids encoding HA-tagged c-Fos, c-Jun and hSIRT1 for 36 h and were lysed for immunoprecipitation (IP) and western blotting (WB).
Online Figure IX. Immunoprecipitation assays showed that SIRT1 deactylated c-Fos and c-Jun in A10 cells following TNF-α (30 ng/mL) induction for 36 hours.
**Online Figure X.** DNA synthesis in Ad-U6- or Ad-SIRT1 RNAi-infected (MOI=100) VSMCs following serum starvation for 48 hours and then serum induction for 24 hours. Data represent the means ± SEM (n=4). ***P < 0.001 versus Ad-U6 treated with 10%FBS.
Online Figure XI. Representative western blots for p27 - an important target protein of Foxo1 (A), survivin (B) and AT1R (C) showing pooled samples of carotid artery from WT and SMC-SIRT1 Tg mice 28 days after ligation or the sham-operation, respectively (n=6 mice in each pool).