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Histone Deacetylase 3 Is Critical in Endothelial Survival and Atherosclerosis Development in Response to Disturbed Flow

Anna Zampetaki, PhD*; Lingfang Zeng, PhD*; Andriana Margariti, PhD; Qingzhong Xiao, PhD; Hongling Li, Msc; Zhongyi Zhang, Msc; Anna Elena Pepe, BSc; Gang Wang, BSc; Ouassila Habi, PhD; Elena deFalco, PhD; Gillian Cockerill, PhD; Justin C. Mason, PhD; Yanhua Hu, MD; Qingbo Xu, MD, PhD

**Background**—Histone deacetylase 3 (HDAC3) is known to play a crucial role in the differentiation of endothelial progenitors. The role of HDAC3 in mature endothelial cells, however, is not well understood. Here, we investigated the function of HDAC3 in preserving endothelial integrity in areas of disturbed blood flow, ie, bifurcation areas prone to atherosclerosis development.

**Methods and Results**—En face staining of aortas from apolipoprotein E–knockout mice revealed increased expression of HDAC3, specifically in these branching areas in vivo, whereas rapid upregulation of HDAC3 protein was observed in endothelial cells exposed to disturbed flow in vitro. Interestingly, phosphorylation of HDAC3 at serine/threonine was observed in these cells, suggesting that disturbed flow leads to posttranscriptional modification and stabilization of the HDAC3 protein. Coimmunoprecipitation experiments showed that HDAC3 and Akt form a complex. Using a series of constructs harboring deletions, we found residues 136 to 206 of HDAC3 to be crucial in this interaction. Enforced expression of HDAC3 resulted in increased phosphorylation of Akt and upregulation of its kinase activity. In line with these findings, knockdown of HDAC3 with lentiviral vectors (shHDAC3) led to a dramatic decrease in cell survival accompanied by apoptosis in endothelial cells. In aortic isografts of apolipoprotein E–knockout mice treated with shHDAC3, a robust atherosclerotic lesion was formed. Surprisingly, 3 of the 8 mice that received shHDAC3-infected grafts died within 2 days after the operation. Miller staining of the isografts revealed disruption of the basement membrane and rupture of the vessel.

**Conclusions**—Our findings demonstrated that HDAC3 serves as an essential prosurvival molecule with a critical role in maintaining the endothelial integrity via Akt activation and that severe atherosclerosis and vessel rupture in isografted vessels of apolipoprotein E–knockout mice occur when HDAC3 is knocked down. (Circulation. 2010;121:132-142.)

**Key Words:** proto-oncogene proteins c-akt ■ atherosclerosis ■ endothelial cells ■ histone deacetylase 3

Endothelial cells play a pivotal role in maintaining vessel integrity. Besides regulating the vascular tone by controlling the contractility of vascular smooth muscle cells, the endothelial monolayer also functions as a permeable barrier between the blood and the subendothelial matrix proteins that prevents inflammatory cell infiltration, thrombus formation, and vascular smooth muscle cell proliferation.1,2 Endothelial dysfunction acts as a trigger in the initiation of atherosclerosis,3 which is closely related to local hemodynamic forces. For instance, low or disturbed shear stress that occurs in vessel branch points, the outer wall of bifurcations, and the inner wall of curvatures is considered proatherogenic. Disturbed flow is associated with higher mitotic and apoptotic activity of endothelial cells, decreased endothelial nitric oxide (NO) synthase expression, impaired alignment, increased reactive oxygen species production, widening of the junctions between cells, and increased cytokine and adhesion molecule production. On the other hand, areas experiencing high laminar shear stress are considered atheroprotected. Endothelial cells in these regions tend to align to the direction of flow, exhibit a quiescent phenotype, and express significantly lower inflammatory mediators.4–7

**Clinical Perspective on p 142**

Histone deacetylases (HDACs) have previously been shown to be important in the biology of endothelial progen-
itor cells, but their role in atherosclerosis prone areas is unknown. HDAC3 belongs to the class I HDACs. It can catalyze the removal of acetyl groups from histone and nonhistone proteins. It is expressed ubiquitously, and its activity depends on its association with multisubunit repressor complexes containing the silencing mediator for retinoid and thyroid hormone receptors and with nuclear-receptor corepressors. HDAC3 is present in both the nucleus and cytoplasm of cells. Global loss of HDAC3 leads to embryonic lethality before embryonic day 9.5 as a result of proliferation defects. Our previous studies showed that HDAC3 is involved in the differentiation of embryonic stem cells into endothelial progenitors. Nevertheless, its part in maintaining endothelial integrity and developing atherosclerosis in vivo is unknown.

In the present study, we investigated the role of HDAC3 expression in mature endothelial cells in areas of disturbed flow. Our experiments revealed that HDAC3 is critical for endothelial cell survival and acts as a prosurvival molecule that could serve as a potential target for novel therapeutic interventions.

Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated from the human umbilical cord and cultured on collagen I–coated flasks in M199 medium supplemented with 3 ng/mL endothelial cell growth factor (Sigma, St Louis, Mo), 1 μg/mL endothelial growth supplement from bovine neural tissue (Sigma), 10 U/mL heparin, 1.25 μg/mL thymidine, 5% fetal bovine serum (PAA, A15–108), and 100 μg/mL penicillin and streptomycin in a humidified incubator supplemented with 5% CO2.

Flow Experiments
Laminar flow and disturbed flow were applied to HUVECs as described previously. When inhibitors were used, the reagents were applied 1 hour before the initiation of stress at the following concentrations: LY294002, 5 μmol/L; PD98059, 5 μmol/L; SU1498, 1 μmol/L; and sodium nitroprusside, 100 μmol/L (all purchased from Sigma). Cells were subsequently exposed to disturbed flow for 4 hours.

En Face Staining
All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Mice were anesthetized and perfused with 4% paraformaldehyde to fix endothelial cells, followed by dissection of the aortic tissue. The artery was mounted onto a rubber with the lumen opened and facing up. Staining was performed as described previously. Images were assessed with a Zeiss Axioplan 2 and processed by Adobe Photoshop software.

Mouse Aortic Isograft Model
The procedure for the mouse aortic isograft was similar to that described recently. Aortas were isolated from donor Tie2-LacZ/ApoE−/− mice. One end and main branches were ligated. Then, 300 μL viral solution (6×105 TU/mL) supplemented with 10 μg/mL polybrene (Sigma) was injected into the lumen, and the other end was ligated. The tissue was incubated with the viral solution for 2 hours at 37°C and subsequently cut into 2 pieces and isografted into the recipient Tie2-LacZ/ApoE−/− mice. Three weeks later, the grafts were harvested and fixed with formalin. The sections were stained with hematoxylin and eosin. Details on materials and methodology are available in the online-only Data Supplement.

Results

Disturbed Flow Increases HDAC3 Expression in Endothelial Cells
Atherosclerosis develops at specific sites of the vasculature that experience disturbed blood flow. To determine whether HDAC3 expression in endothelial cells is affected by flow patterns, we performed en face staining of mouse aorta using an HDAC3 antibody. Immunostaining revealed that HDAC3 expression was upregulated in areas in close vicinity to branch openings where disturbed flow occurs (Figure 1A) compared with areas of undisturbed blood flow (Figure 1B). In the in vitro experiments, HUVECs expressed HDAC3 in static conditions but, when exposed to disturbed flow, displayed a transient stabilization of HDAC3 protein (Figure 1C). On the contrary, HDAC3 protein expression was not affected by laminar shear flow (data not shown). It is noteworthy that the expression of HDAC3 in vascular smooth muscle cells is not affected by flow patterns in vivo (Figure I of the online-only Data Supplement). Interestingly, no increase in deacetylase activity was observed in HUVECs exposed to disturbed flow (Figure 1D).

Effect of HDAC3 Knockdown on Endothelial Cell Viability
To investigate whether HDAC3 could play a role in endothelial cell survival, we knocked down HDAC3 expression in endothelial cells using shRNA lentiviral constructs. Two lentiviral vectors encoding an shRNA oligonucleotide targeting HDAC3 exon 14 and exon 15, respectively, gave the best results and therefore were used in the rest of this study.
Figure 2. Effect of HDAC3 knockdown on endothelial cell survival. A, Knockdown of HDAC3 with lentiviruses shHDAC31 and shHDAC32 (clones TRCN0000004826 and TRCN0000004828, respectively). A nontargeting lentivirus was used as a negative control. Bottom, Quantification data of HDAC3 knockdown in 3 independent experiments (n=3). B, Cell morphology in shHDAC3-infected HUVECs. HDAC3 knockdown led to a dramatic decrease in cell number (n=4; C) and MTT-detected survival of endothelial cells (n=4; D) even at baseline conditions (n=3). Enrichment of the cytosol with nucleosomes (n=3; E) and increased annexin V staining (F), both indications of apoptosis, were observed in shHDAC3-infected HUVECs. Data are mean±SD for 3 independent experiments (n=3). *Significant difference from the nontargeting control. Bar=100 μm.
HDAC3 protein expression was reduced to 25% to 30% (Figure 2A). HDAC3 knockdown had a dramatic effect on cell morphology, with the majority of cells rounded up and showing extensive membrane blebs (Figure 2B). Cell number was also reduced by almost 50% (Figure 2C). MTT assay confirmed that these cells have a severe defect in survival (Figure 2D). Cell-death assay demonstrated an enhanced presence of nucleosomes in the cytosol of HUVECs after HDAC3 knockdown (Figure 2E), an indication of apoptosis. Similar results were obtained after annexin V staining (Figure 2F). These findings suggest that HDAC3 plays a critical role in endothelial cell survival in vitro. Further experiments with aortic segments from Tie2-LacZ/ApoE/−/−/− mice showed that knockdown of HDAC3 led to loss of endothelial cells in these fragments (Figure 3), implying that HDAC3 knockdown affects endothelial cell survival both in vitro and ex vivo.

**HDAC3 and Akt Form a Complex**

Aiming to delineate the signaling pathways involved in HDAC3 stabilization in response to disturbed flow, we treated endothelial cells with various inhibitors before the initiation of flow. Cells were exposed to disturbed flow for 4 hours. As shown in Figure 4A, these experiments indicated that stabilization of the HDAC3 protein was abolished when inhibitors for vascular endothelial growth factor receptor (VEGFR2) and PI3 kinase were included, indicating an involvement of VEGFR2/PI3 kinase signaling pathways. Because the serine/threonine protein kinase Akt is an important mediator of PI3 kinase signaling, we decided to assess the Akt and HDAC3 expression levels at the membrane fraction. Our experiments revealed an increased presence of both proteins at the membrane in HUVECs exposed to disturbed flow (Figure 4B). Moreover, immunoprecipitation experiments showed that Akt and HDAC3 could form a complex. The interaction was evident in static conditions and became stronger under conditions of disturbed flow, when increased phosphorylation at serine and threonine residues was also observed on HDAC3 (Figure 4C). Deletion analysis with a series of constructs harboring deletions of HDAC3 protein showed that amino acids 136 to 206 of HDAC3 were crucial for Akt interaction (Figure 4D). Aiming to determine whether Akt can phosphorylate HDAC3, we performed an in vitro kinase assay using recombinant active Akt and immunoprecipitated HDAC3 as a substrate. No phosphorylation event was observed (data not shown), which suggests that HDAC3 is not a substrate for Akt.

Disturbed flow areas are associated with reduced NO levels. On the contrary, in areas exposed to high laminar flow, increased levels of NO are known to occur. To determine whether NO levels could affect the formation of the HDAC3–Akt complex, we pretreated HUVECs with an NO donor (sodium nitroprusside 100 μmol/L) and subsequently exposed the cells to disturbed flow in the presence of the NO donor. Static samples treated in a similar manner were used as control. As shown in Figure 4E, increased NO levels did not abolish the formation of the HDAC3–Akt complex but inhibited the enhanced stabilization of the HDAC3–Akt complex observed under disturbed flow, which suggests that in areas of disturbed flow patterns, reduced NO levels are crucial for the increased binding of HDAC3 to Akt. Interestingly, only negligible deacetylase activity was detected in the HDAC3–Akt complexes (Figure 4F).

**Effect of HDAC3 on Akt Activity**

Akt is fully active when T308 and S473 are phosphorylated.17 To evaluate the effect of HDAC3 on Akt activity, we used lentiviral vectors to knock down HDAC3 expression. Interestingly, knockdown of HDAC3 with either vector led to decreased phosphorylation of both T308 and S473 on Akt (Figure 5A). In a similar manner, overexpression of HDAC3 induced a robust upregulation of Akt phosphorylation at both regulatory sites (Figure 5B). In vitro kinase assay was
performed to determine the effect of HDAC3 overexpression on Akt activity. Whole-cell lysate (200 μg) was used to immunoprecipitate Akt from HUVECs overexpressing HDAC3, and a GSK3 fusion protein served as a substrate. Because of the small amount of protein used, the interaction of HDAC3 and Akt was not evident. However, significantly higher Akt kinase activity, as displayed by increased phosphorylation of the substrate, in HDAC3-overexpressing cells (Figure 5C) was observed. In line with these findings, increased levels of phospho-Akt were detected in close proximity to branching points where HDAC3 expression is elevated (Figure 5D). In a similar manner, when aortic segments were infected ex vivo with shHDAC3 lentiviral particles, reduced phospho-Akt levels were observed (Figure 5E). Interestingly, inhibition of HDAC activity with trichostatin A did not abrogate this response, as indicated by experiments using various concentrations of the reagent (Figure 5F) and a time-course experiment (Figure 5G), suggesting that this is a novel function of HDAC3 that is independent of its deacetylase activity. Aiming to delineate the mechanisms involved, we wondered whether overexpression of HDAC3 would facilitate the transport of Akt to the cell membrane and therefore act as a carrier. Isolation of the membrane fraction in these cells revealed an increased presence of HDAC3 but not Akt, suggesting that this is not the case (Figure 6A). Next, we examined the role of phosphatase PP2A, which was previously shown to dephosphorylate Akt. Decreased phosphatase activity could lead to an increased half-life of phosphorylated Akt. Knockdown of HDAC3 and inhibition of PP2A activity with okadaic acid (1 nmol/L) failed to restore the phosphorylation levels of Akt, indicating that PP2A activity is not involved in this regulation (Figure 6B). However, treatment of cells with a PI3 kinase inhibitor (5 μmol/L LY294002) reduced the Akt phosphory-
lation levels observed after HDAC3 overexpression (Figure 6C). In a similar manner, PI3 kinase activity was increased after enforced expression of HDAC3 (Figure 6D).

Overexpression of Active Akt Can Partially Rescue the Apoptotic Response Observed After HDAC3 Knockdown

To determine whether the apoptotic response induced by HDAC3 knockdown was due to reduced Akt activation,20,21 overexpression of a myristoylated Akt protein was performed. Reconstitution of active Akt in cells infected with shHDAC3 viruses led to an increased number of cells (Figure 7B) and improved cell morphology (Figure 7A). Interestingly, most HUVECs infected with shHDAC3 died after 7 days in culture, whereas the growth of these cells when overexpressing active Akt could be partially rescued (Figure 7B). Reduced apoptosis (Figure 7C) and increased cell survival (Figure 7D) and proliferation

Figure 5. Effect of HDAC3 on Akt activation. A, Effect of HDAC3 knockdown on Akt phosphorylation. Bottom, Average band density in 3 independent experiments (n=3). B, Effect of HDAC3 overexpression on Akt phosphorylation. Bottom, Average band density in 3 independent experiments (n=3). C, Akt kinase activity was increased in HUVECs after HDAC3 overexpression. Bottom, Average band density in 2 independent experiments (n=2). D, En face staining of mouse aorta with a phospho-AktS473 antibody. Nuclei were counterstained with hematoxylin. Bar=100 μm. E, En face staining for phospho-AktS473 in aortic segments after ex vivo infection with lentiviral particles. CD31 was used to identify endothelial cells. Bar=10 μm. F, Effect of inhibition of HDAC activity using different concentrations of trichostatin A (TSA) for 24 hours on Akt phosphorylation. Bottom, Average band density in HDAC3-overexpressing samples in 3 independent experiments (n=3). G, A time-course experiment highlighting the effect of TSA (100 nmol/L) on Akt phosphorylation. Bottom, Average band density in HDAC3-overexpressing samples in 3 independent experiments. *Significant difference from control.
HDAC3 interaction with Akt regulates its activity and plays a key role in endothelial cell viability.

Robust Neointima Formation in Aortic Isografts After HDAC3 Knockdown in Endothelial Cells

To investigate whether HDAC3 is critical for endothelial cell survival in vivo, we used the mouse aortic isograft model. Aortas from apolipoprotein E–knockout (ApoE−/−) mice were harvested, infected with shHDAC3 or the nontargeting control for 2 hours, and then isografted into the carotid artery of recipient ApoE−/− mice. To confirm that HDAC3 downregulation occurs specifically in endothelial cells in the isograft, the mouse aortas were infected with shHDAC3 or the nontargeting control and harvested 48 hours after grafting. Immunofluorescence staining showed that only the endothelial monolayer was infected. HDAC3 expression was significantly reduced in endothelial cells in the shHDAC3 grafts compared with the nontargeting control, whereas HDAC3 expression in the media was not affected. CD31 was used as a marker for endothelial cells (Figure II of the online-only Data Supplement). To evaluate the role of HDAC3 in neointima formation, the isografts were harvested 3 weeks later. The isografts infected with the nontargeting lentivirus showed small neointimal lesions (Figure 8A). On the contrary, isografts infected with the shHDAC3 virus exhibited a robust atherosclerotic lesion (Figure 8B and 8C). Interest-
ingly, 3 of 8 mice that received an shHDAC3-infected isograft died within 2 days after the operation. Close examination of the isografts showed a rupture of the vessel. Miller staining of the isografts revealed a disruption of the basement membrane and elastic fibers (Figure 8D). Of note, immunostaining of the isografts demonstrated that the HDAC3 levels in the vascular smooth muscle cells were not affected (Figure 8E), suggesting that HDAC3 expression in endothelial cells is crucial for maintaining the vascular integrity.
HDAC3. Bar 50/H11005 determined by immunocytochemistry with an antibody against /H11005 100 Bar elastic fibers in shHDAC3-infected isografts as demonstrated. The 2 groups (n 5). D, Miller staining indicated disruption of the whole area of intima plus media. *Significant difference between is presented as a percentage of the area of neointima to the

ent role of HDAC3 in maintaining the integrity of the vessel using the mouse aortic isograft model confirmed the prominent role of HDAC3 in stress-induced cell apoptosis and cell viability.29,30 Animal models targeting global HDAC3 protein expression yielded very early embryonic lethality in that no null embryo was detected on embryonic day 9.5.11 Deletion of HDAC3 in the postnatal mouse liver, however, did not lead to lethality. Instead, these mice developed hepatomegaly, which was the result of hepatocyte hypertrophy. These morphological changes coincided with significant imbalances between carbohydrate and lipid metabolism.31 In a similar manner, cardiac-specific deletion of HDAC3 led to massive cardiac hypertrophy and metabolic abnormalities.32 In the present study, we assessed the role of HDAC3 in adult endothelial cells and found that HDAC3 expression was increased in close proximity to branch openings compared with areas of undisturbed flow, which was also confirmed by exposure of endothelial cells to disturbed flow in vitro because a transient stabilization of the HDAC3 protein was detected. These findings established the impact of HDAC3 on endothelial function in response to disturbed flow.

Activation of VEGFR2/PI3 kinase leads to phosphorylation of membrane phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], which generates PI(3,4,5)P3, some of which is converted to phosphatidylinositol 3,4-trisphosphate [PI(3,4)P3] by a phospholipid phosphatase. PI(3,4)P2 and PI(3,4,5)P3 recruit Akt, a major downstream effector of PI3 kinase, to the cell membrane.17 Here, we present the first evidence of functional interaction between Akt and HDAC3 and identify a key role for HDAC3 in maintaining baseline Akt activation levels. Deletion analysis identified amino acids 136 to 206 of HDAC3 as critical for this interaction. Intriguingly, the levels of NO affect the stabilization of the complex and the enhanced binding observed in disturbed flow, where reduced NO levels are known to occur. Previous studies have revealed that NO donors markedly reduced the enzymatic activity of HDAC2, minimally influenced that of HDAC1, and had no effect on HDAC3.33 Our data suggest a novel role of NO as a bioactive molecule in the regulation of HDAC3 function through modulation of HDAC3–Akt binding. To further elaborate on the effect of this binding, we used lentiviral vectors encoding shRNA targeting HDAC3. Because we also identified multiple isoforms of HDAC3 in endothelial cells (data not shown), we found that by using oligonucleotides targeting exons 14 and 15, we could achieve the most efficient downregulation and provided further evidence that HDAC3 functions as a prosurvival factor in the vasculature.

HDAC3 protein is expressed ubiquitously and conserved across many species.23 HDAC3 is a repressor for many transcription factors (c-jun, E2F/Rb, nuclear factor-κB), but it also regulates transcription through binding to specific promoters, where it represses transcription through deacetylation of the histone tails.24–27 Interestingly, its function seems to differ in primary and immortalized cells. Previous studies have reported the localization of HDAC3 to the mitotic spindle and have shown that HDAC3 knockdown leads to chromosome misalignment, impaired kinetochore–microtubule attachment, and mitotic spindle collapse in immortalized cells,28 whereas in primary cells, HDAC3 gene deletion does not lead to mitosis defects.11 Other studies highlighted the role of HDAC3 in stress-induced cell apoptosis and cell viability.29,30 Animal models targeting global HDAC3 protein expression yielded very early embryonic lethality in that no null embryo was detected on embryonic day 9.5.11 Deletion of HDAC3 in the postnatal mouse liver, however, did not lead to lethality. Instead, these mice developed hepatomegaly, which was the result of hepatocyte hypertrophy. These morphological changes coincided with significant imbalances between carbohydrate and lipid metabolism.31 In a similar manner, cardiac-specific deletion of HDAC3 led to massive cardiac hypertrophy and metabolic abnormalities.32 In the present study, we assessed the role of HDAC3 in adult endothelial cells and found that HDAC3 expression was increased in close proximity to branch openings compared with areas of undisturbed flow, which was also confirmed by exposure of endothelial cells to disturbed flow in vitro because a transient stabilization of the HDAC3 protein was detected. These findings established the impact of HDAC3 on endothelial function in response to disturbed flow.

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Discussion

Atherosclerotic lesions occur preferentially in regions of low or disturbed shear stress. These mechanical forces, which modulate the structure and function of the endothelium, determine the outcome of lesion formation.7,22 In the present study, we demonstrate for the first time that disturbed flow induces transient stabilization of the HDAC3 protein in endothelial cells through activation of the VEGFR2 and PI3 kinase signaling pathways. In addition, we show that HDAC3 is critical for endothelial cell survival, at least partially by controlling the baseline activation of Akt. We identified translocation of Akt and HDAC3 to the cell membrane under disturbed flow and formation of a complex that affects Akt activity in endothelial cells. Moreover, in vivo experiments using the mouse aortic isograft model confirmed the prominent role of HDAC3 in maintaining the integrity of the vessel

Figure 8. Robust neointima formation in aortic isografts after HDAC3 knockdown in endothelial cells. Thoracic aortas were isolated from donor ApoE−/− (C57BL/C) mice and infected with nontargeting (A) or shHDAC3 (B) virus in vitro and isografted into recipient ApoE−/− (C57BL/C) mice of the same background. Neointima formation on the grafts was checked 3 weeks later. Bar=100 μm. C, Mean±SD of neointima area from each group is presented as a percentage of the area of neointima to the whole area of intima plus media. *Significant difference between the 2 groups (n=5). D, Miller staining indicated disruption of the elastic fibers in shHDAC3-infected isografts as demonstrated. Bar=100 μm. E, HDAC3 expression in the aortic isografts as determined by immunocytochemistry with an antibody against HDAC3. Bar=50 μm.
of HDAC3 protein. Infections with either vector led to a sharp downregulation of Akt phosphorylation. In a similar manner, transient overexpression of HDAC3 resulted in increased Akt phosphorylation and enhanced kinase activity.

Akt phosphorylation at steady state is tightly regulated, representing a balance between kinase-activating and phosphatase-inactivating events. Aiming to delineate the molecular mechanisms involved, we examined the Akt localization to the cell membrane, the PI3 kinase, and the PP2A phosphatase activity. Our experiments showed that overexpression of HDAC3 did not induce translocation of Akt to the cell membrane, which would lead to constitutive activation of Akt; instead, Akt phosphorylation was enhanced through increased activity of PI3 kinase.

Cell proliferation and survival depend significantly on Akt activation. Could the diminished Akt activation observed in shHDAC3-infected HUVECs have an effect on cell viability? Our experiments showed a dramatic effect on cell survival and proliferation. Reduced cell numbers, extensive membrane blebbing, and apoptosis were observed in these cells. This response could be partially rescued by the overexpression of constitutively active Akt, implying that HDAC3 expression was critical for cell survival through regulation of Akt activity at baseline. Furthermore, ex vivo experiments using aortic segments from Tie2-LacZ/ApoE−/− mice showed a significant loss of endothelial cells after HDAC3 knockdown. Additional evidence supporting the importance of HDAC3 in the survival of primary endothelial cells was provided by an in vivo model of atherosclerosis. Infecting the endothelial monolayer with shHDAC3 in aortic isografts led to a robust atherosclerotic lesion formation and, in some cases, even vessel rupture. Interestingly, it seems that HDAC3 is essential for endothelial monolayer survival and integrity.

Previous studies suggested that HDACs play a role in the vasculature. Investigation of HDAC1 and HDAC2 expression in situ in atherosclerotic lesions of human coronary arteries indicated reduced expression of HDAC2, whereas HDAC5 phosphorylation and nuclear export were reported to mediate angiostatin II–induced mouse embryonic fibroblast-2 activation and vascular smooth muscle cell hypertrophy. Additionally, trichostatin A, a potent inhibitor of HDACs, was shown to have a proatherogenic effect in Ldlr−/− mice fed a Western-type diet. Our experiments indicate that the interaction of HDAC3 and Akt is critical for endothelial cell survival. Interestingly, this novel function of HDAC3 is independent of its deacetylase activity. Because the association of HDAC3 with multiunit repressor complexes (silencing mediator for retinoid and thyroid hormone receptors and nuclear-receptor corepressors) is essential for its enzymatic activity, the data presented here provide direct evidence that the unbound HDAC3 protein also has specific functions in endothelial cells.

Conclusions

We have identified HDAC3 as a key regulator of endothelial cell viability that functions in part through controlling the activation of Akt. We uncovered a novel interaction between HDAC3 and Akt, determined the residues involved, and elaborated on the molecular mechanisms in place. The specific upregulation of HDAC3 expression in atherosclerosis-prone areas through VEGFR2 activation and the robust atherosclerotic lesion formation in shHDAC3-infected isografts further underlined the importance of HDAC3 in endothelial cell function. The coexistence of proinflammatory and antioxidative gene expression in endothelial cells in areas of disturbed flow that facilitate the survival of endothelial cells has been highlighted by previous studies. Here, we propose that HDAC3 acts as a prosurvival molecule for endothelial cells in these areas, implying that HDAC3 might serve as a target for novel therapeutic approaches in cardiovascular diseases.

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Disclosures

Dr Mason has received honoraria (lecture fee) from Pfizer. The other authors report no disclosures.

References


CLINICAL PERSPECTIVE

Atherosclerotic lesions form at distinct sites in the arterial tree, suggesting that hemodynamic forces influence the initiation of atherosclerosis. In the present study, we show that disturbed flow stabilizes histone deacetylase 3 (HDAC3) protein in endothelial cells and demonstrate that HDAC3 levels correlate with Akt activity. We uncover a novel direct interaction between HDAC3 and Akt and elaborate on the molecular mechanisms involved. Our experiments indicate that the HDAC3–Akt complex formation is critical for the survival of endothelial cells. Clinically, endothelial dysfunction is identified as a major trigger of atherosclerosis. Conventional treatments for vascular diseases involve lowering blood cholesterol levels, which may have a role in endothelial protection of the vessel wall. Our experiments identify HDAC3 as a crucial molecule for endothelial viability. Moreover, in vivo experiments in which the mouse aortic isotrophy model was used confirmed the prominent role of HDAC3 in maintaining the integrity of the vessel and provided further evidence that HDAC3 functions as a prosurvival factor in the vasculature. Therefore, HDAC3 may serve as a putative target for novel therapeutic approaches in cardiovascular diseases. Detailed elucidation of the mechanism involved would enable us to design new drugs that could interfere with HDAC3 function in endothelial cells and successfully retard disease progression.
SUPPLEMENTAL MATERIAL

HDAC3 is Critical in Endothelial Survival and Atherosclerosis Development in Response to Disturbed Flow

Anna Zampetaki,1* PhD; Lingfang Zeng;1* PhD; Andriana Margariti,1 PhD; Qingzhong Xiao,1 PhD; Hongling Li,1 Msc; Zhongyi Zhang,1 Msc; Anna Elena Pepe,1 Bsc; Gang Wang1, BSc; Ouassila Habi1, PhD; Elena deFalco,1 PhD; Gillian Cockerill,2 PhD; Justin C Mason,3 PhD; Yanhua Hu,1 MD; and Qingbo Xu1 MD, PhD.
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1Cardiovascular Division, King’s College London BHF Centre, London SE5 9NU, United Kingdom
2Department of Cardiovascular Medicine, St. George’s University of London, London SW17 0RE, United Kingdom
3Bywaters Center for Vascular Inflammation, Imperial College, Hammersmith Hospital, London, W12 ONN, United Kingdom

* A Zampetaki and L Zeng contributed equally to this study.

Correspondence should be addressed to Professor Qingbo Xu, Division of Cardiology, King’s College London, James Black Centre, 125 Coldharbour Lane London SE5 9NU, UK. Tel: +44-2078485322, Fax: +44-2078485296, qingbo.xu@kcl.ac.uk

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METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from the human umbilical cord and cultured on collagen I-coated flasks in M199 medium supplemented with 1ng/ml -endothelial cell growth factor (Sigma), 3 µg/ml endothelial growth supplement from bovine neural tissue (Sigma), 10U/ml heparin, 1.25µg/ml thymidine, 5% foetal bovine serum (PAA, A15-108), 100µg/ml penicillin and streptomycin in a humidified incubator supplemented with 5% CO₂. The cells were subcultured every three days at a ratio of 1:4. HEK 293 and 293T cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. Cell culture images were taken using a Nikon Eclipse TS100 microscope with Ph1 ADL 10x/0.25 objective lenses and Nikon DS-Fil camera.

Flow experiments

Laminar flow was applied to HUVECs as described previously. Disturbed flow was created by placing the cells on a platform shaker (Labnet, model Rocker 25) with parameters of 2.0 mm culture medium depth, 10 cm length of flask, ±7 ° rotating angle and frequency of 0.5Hz (i.e. 2 sec per cycle) respectively. The magnitude of the shear stress experienced by cells is approximately 4.5dyn/cm². In addition, cells are subjected to spatial and temporal varying shear stress, with the spatial gradient of the shear stress ranging between 0 and ~20 dyn/cm² in a rotating cycle, and the average temporal gradient of the shear stress being 4.5 (dyn/cm²)/sec. These values are estimated based on a numerical simulation of the oscillatory shear flow system using computational fluid dynamics (CFD) software. In experiments where inhibitors were used, the reagents were
applied one hour prior to the initiation of the stress at the following concentrations: LY294002 (5 μM), PD98059 (5 μM), SU1498 (1 μM), SNP (100μM) (all purchased from Sigma). Cells were subsequently exposed to disturbed flow for 4h.

**Deacetylase activity Assay**

Deacetylase activity was assessed using a colorimetric HDAC activity assay kit (Biovision, cat. K331-100). In brief, cells were exposed to disturbed flow or kept under static conditions for 4h. Subsequently, 200μg of cell lysates were incubated at 37°C for 1hr in the presence of an HDAC colorimetric substrate, consisting of an acetylated lysine side chain. Deacetylation of the substrate sensitizes it so that in a second step treatment with a Lysine developer produces a chromophore. HDAC activity was quantified using the absorbance measurements at 405nm, with that of static samples set as 1. To assess the deacetylase activity in the HDAC3-Akt complex, 1mg of cell lysates was used to immunoprecipitate total Akt and deacetylase activity was determined as described above. Static samples were used as a control and data were normalized for protein content.

**En face staining**

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Mice were anesthetized and perfused with 4% paraformaldehyde to fix endothelial cells, followed by dissection of the aortic tissue. The artery was mounted onto a rubber with the lumen opened and facing up. Staining was performed as described previously using an anti-HDAC3 (H3034 from SIGMA, 1:50 dilution) or anti-phosphoAktS473 (sc-7985R from Santa Cruz 1:50) antibody. Images were assessed by Zeiss Axioplan 2 Imaging microscope with Plan-NEOPLUAR 10x/0.3, 20x/0.5 and 40x/0.75 objective lenses, AxioCam camera.
and Axiovision software at room temperature, and were processed by Adobe Photoshop software.

**Membrane Protein isolation**

HUVECs were subjected to disturbed flow for the time indicated or kept as a static control. Cell membrane proteins were labelled with biotin, 80% of the labelled cells were subjected to membrane protein purification using a commercially available kit (Cell Surface Protein Isolation Kit, Pierce) according to the protocol provided and the remaining 20% were used for whole cell lysate protein purification. In experiments where cells overexpressing HDAC3 were used, cells were incubated with AdHDAC3 or the Adnull virus (MOI 5) for 24 hrs. The viral solution was subsequently removed and membrane protein isolation was performed 48hrs later.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation assays, 1 mg of cell lysate was incubated with 2µg of target antibody or normal IgG and 20µl of Protein G-Sepharose beads, followed by Western blotting. For immunoblotting (Western blot), 20µg of cell lysate was applied to SDS-PAGE, as described previously. Antibodies against Akt, pAktS473, pAktT308 and GAPDH were from Santa Cruz Biotechnology. HDAC3 and α-tubulin antibodies were purchased from Sigma. PhosphoGSK3 antibody was from Cell Signalling. Specific antibody-antigen complexes were detected by using the ECL Western Blot Detection Kit (Amersham Pharmacia Biotech UK). In experiments where inhibitors were applied, cells were treated with the respective reagents (Okadaic acid 1nM, LY294002 5µM, TSA...
100nM) for 24hrs. Okadaic acid was purchased from Calbiochem, LY294002 and TSA were from Sigma.

**Co-Immunoprecipitation assay**

HEK293 cells were seeded at 1x10^6 cells/T75 flask 24 hr prior to transfection. 2µg of pShuttle-Flag-HDAC3 (or truncated variants) and 2µg of pShuttle-HA-Akt1 plasmids were co-tranfected into the 293 cells with Fugene 6 according to the protocol provided. Forty-eight hours post transfection, the cells were lysed in 600µl/flask IP-A buffer (25mM Tris-HCl, pH7.5, 150mM NaCl, 1mM EDTA, pH8.0, 1% Triton X-100 plus protease inhibitors) on ice for 45 min. The protein concentration was measured by Bradford method. 1mg of cell lysate was mixed with 2 volume of IP-B buffer (25mM Tris-HCl, pH7.5, 150mM NaCl, 1mM EDTA, pH8.0 plus protease inhibitors) and precleared with 2 µg of normal rabbit IgG and 5 µl of Protein G-agarose beads. The precleared lysate was then incubated with 2 µg of rabbit anti-HA antibody for 2hrs, followed by addition of 5 µl of Protein G-agarose beads and further incubation for 4hr or overnight. The precipitated HA-Akt1 and its associated proteins were subjected to standard western blot probed with mouse anti-Flag to detect associated HDAC3 or its variants.

**Akt kinase activity**

To assess the Akt kinase activity, we used the non radioactive Akt kinase activity Assay from Cell Signalling. In brief, cells were lysed in Cell Lysis Buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na₃VO₄, 1µg/ml Leupeptin) on ice for
10 min. After a brief sonication, cell debris was removed by centrifugation for 10 min at 4°C. The supernatant was transferred to a new tube and stored at –80°C. To assess Akt kinase activity, total Akt was precipitated from 200µg of cell lysate, using the immobilized Akt mouse antibody (cat. 9279 from Cell Signalling) in Cell Lysis Buffer overnight. The following day, IP pellets were washed (twice) with Cell Lysis Buffer and twice with Kinase Buffer (25mM Tris-HCl pH 7.5, 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na3VO4, 10mM MgCl2). Kinase activity was determined using a GSK3 fusion protein (cat. 9237) as a substrate. The pellet was suspended in 50µl of Kinase Buffer supplemented with 1µl of 10mM ATP and 1µg of GSK-3 fusion protein and incubated at 30°C for 30 min. The reaction was terminated by adding 25µl of 3xSDS Sample Buffer (187.5mM Tris-HCl (pH 6.8), 6%w/v SDS, 30% glycerol, 150mM DTT, 0.03%w/v bromophenol blue. Phosphorylation of GSK-3 was measured by Western blotting, using phospho-GSK-3alpha/beta (Ser21/9) antibody.

**PI3 Kinase Assay**

The PI3 kinase activity was assessed using the PI3 kinase ELISA kit by Echelon according to the manufacturer’s recommendations. In brief, cells were lysed in Buffer A (20mM Tris-HCl pH 7.4, 137mM NaCl, 1mM CaCl2, 1mM MgCl2, 1%NP-40, 1mM PMSF and 0.1mM sodium orthovanadate) on ice for 20 min and cell debris was removed by centrifugation. The enzyme was immunoprecipitated from 200µg of cell lysate using the anti-PI3 Kinase antibody (cat. 06195 from Upstate Biotechnology). In brief cell lysates were incubated with the above antibody for 2h at 4°C. Normal rabbit IgG was used as a control. Protein G agarose beads in PBS (60µl) were added to each tube and the samples were incubated for one hour at 4°C. The immunoprecipitated enzyme was
washed twice with Buffer A, three times with Buffer B (0.1M Tris-HCl pH 7.4, 5mM LiCl and 0.1mM sodium orthovanadate) and twice with TNE buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA) supplemented with 0.1mM sodium orthovanadate. PI3 Kinase reaction was performed using 240pmole of PI(4,5)P2 as a substrate. For each reaction 6µl of 10x PI3 Kinase Reaction Buffer (200mM Tris pH 7.4, 100mM NaCl, 40mM MgCl2, 250µM ATP) and 2.4µl of 100µM PI(4,5)P2 substrate (240pmol) were added to the immunoprecipitated PI3 kinase enzyme and the total volume was adjusted to 60µl with dH2O. Additional samples containing known concentrations of PI(3,4,5)P3 were used to generate the standard curve. Reactions were stopped by incubation with PI(3,4,5)P3 detector protein/EDTA for 1h at room temperature. After the PI3K reactions were completed, the reacted mixtures were added to a PI(3,4,5)P3-coated microplate for competitive binding and incubated further for 1hour at room temperature. The wells were subsequently washed with TBST and a peroxidase-linked secondary detector was added to the plate and incubated for 30 min. The wells were then washed with TBST and a colorimetric reaction was allowed to develop for 10 min in the dark through the addition of TMB. Reactions were stopped by adding 50µl of 1N H2SO4 stop solution. This colorimetric signal was used to assess PI(3,4,5)P3 detector binding to the plate and it is inversely proportional to the amount of PI(3,4,5)P3 produced by PI3K. PI3 Kinase activity was estimated by comparing the absorbance values to the standard curve. Data shown is average of three independent experiments.
Plasmid Construct

The expression vectors pShuttle(HD3FL), pShuttle(HD3N1), pShuttle(HD3N2), pShuttle(HD3C1), pShuttle(HD3C2), and pShuttle(Akt1) encoding various mutants of mouse HDAC3 and human Akt1 protein respectively, were generated by PCR using mouse or human cDNA as a template. Mouse and human HDAC3 proteins share more than 99% homology. The PCR product was then digested, gel purified and cloned into the modified pShuttle2 vector, in which flag tag is fused to the N-terminal of HDAC3 and variants, while HA tag is fused to the N-terminal of Akt1 protein. All primers and cloning sites are shown in Table 1. All vectors were verified by sequencing.

Adenoviral DNA transfer

AdHDAC3 viral DNA was constructed from the pShuttle2-HD3FL plasmid using BD Adeno-X Viral DNA system (Clontech). The resulting viral particles were generated and amplified in HEK293 cells according to standard procedures. AdMyrAkt was described previously. For Adenoviral DNA transfer experiments, HUVECs were seeded on collagen I coated plates and left to adhere overnight. The next day cells were incubated with AdHDAC3 or the Adnull at MOI 5 for 24hrs. AdMyrAkt virus was used at MOI 10. Subsequently, fresh medium was added to the cells and the plates were returned to the incubator and harvested 48hrs later.

Lentiviral Particle transduction

Lentiviral particles were produced using MISSION shHDAC3 plasmid DNA (Sigma) according to the company’s recommendations. The shRNA non targeting vector was used as a negative control. In brief, 293T cells were transfected with the lentiviral vector and the packaging plasmids, pCMV-dR8.2 and pCMV-VSV-G (both obtained
from Addgene) using Fugene 6. The supernatant containing the lentivirus was harvested 48hrs later filtered, aliquoted and stored at −80°C. p24 antigen ELISA (Zeptometrix) was used to determine the viral titer. The transducing unit (TU) was calculated using the conversion factor recommended by the manufacturer (10⁴ physical particles per pg of p24 and 1 TU per 10³ physical particle for a VSV-G pseudotyped lentiviral vector), with 1 pg of p24 antigen converted to 10 TU. For lentiviral infection, HUVECs were seeded on collagen I coated plates and left to adhere overnight. The next day cells were incubated with shHDAC3 or non targeting control (1x10⁷ TU/ml) in complete medium supplemented with 10µg/ml of polybrene for 24hrs. Subsequently, fresh medium was added to the cells and the plates were returned to the incubator and harvested 48hrs later.

**Transient Transfection**

Transient transfection experiments were performed using Fugene-6-Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. After 24 hrs, fresh medium was added to the transfected cells and the plates were returned to the incubator and harvested 48hrs later. Data was confirmed by three independent experiments.

**Proliferation Assay**

Cell proliferation assay was performed with CellTiter 96 AQuesous One Solution Cell Proliferation Assay kit (Promega) according to the protocol provided. HUVECs were infected with shHDAC3 or non targeting lentiviral particles as described above. In experiments where Akt overexpression was performed, cells were incubated with the lentiviral particles and AdMyrAkt or Adnull (MOI 10) for 24hrs. The cells were then subcultured into 24-well plates at 3x10⁴ cells/well. The next day fresh medium was added
and the cells were either treated with 50µM H₂O₂ or left untreated. Survival was assessed 24hrs later by measuring the optical density at 490nm. Data shown are representative of four independent experiments.

**Cell Death Detection ELISA.**

Enrichment of the cytosol with nucleosomes was assessed as an indicator of cell apoptosis. The cytoplasmic fraction was analysed for the presence of histone proteins on the released nucleosomes, by incubation with anti-histone biotin-conjugated antibody and anti-DNA peroxidase-conjugated antibody in a streptavidin-conjugated microplate. The Cell Death Detection ELISA Plus (Roche) was used according to the manufacturer’s instructions. Briefly, cells were infected as described above. Cells were lysed by incubation in Lysis buffer for 30 min at room temperature. After cell lysis, intact nuclei were pelleted by centrifugation at 200g for 10 min. The supernatant representing the cytoplasmic fraction was harvested and 20µl (1x10⁴ cells) were transferred to a streptavidin coated microplate and 80µl of Immunoreagent, a mixture of anti-histone biotin antibody and anti-DNA peroxidase conjugate antibody were added to the plate. During the incubation period the anti-histone antibody binded to the histone component of the nucleosomes and simultaneously facilitated the binding of the immunocomplex to the streptavidin coated microplate via its biotinylation. Additionally the anti-DNA-peroxidase conjugate antibody reacted with the DNA-component of the nucleosomes. After 2 hours, unbound components were removed by washing with incubation buffer and quantitative assessment of the amount of nucleosomes in the samples was performed by a colorimetric reaction using a peroxidase substrate and absorbance measurement at 405nm. Three independent experiments were performed for each condition.
Annexin V staining

Annexin V staining was performed using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen), according to manufacturer’s recommendations. In brief, cells were trypsinized, washed with PBS and incubated with Annexin V for 15 min at room temperature. Flow cytometric analysis was performed with a FACS Calibur Flow cytometer (Becton Dickinson). Data shown is average of three independent experiments.

BrdU incorporation

HUVECs were seeded in a 6-well plate at 1.5x10^5 cells/well and infected with lentiviral particles and Adnull or AdMyrAkt as described above. Thirty six hours after infection, the cells were trypsinized and plated in a 24-well plate at 1x10^4 cells/well and left to adhere overnight. In the morning, the cells were labelled with BrdU for 6h using the 5-Bromo-2’-deoxy-Uridine Labeling and Detection Kit III (Roche, Ref 11444611001) according to the company’s recommendations. Proliferation was measured as BrdU incorporation during DNA synthesis using an anti-BrdU-peroxidase conjugate antibody. Quantitative assessment was performed by a colorimetric reaction using a peroxidase substrate and absorbance measurement at 405nm with that of the control group set as 100. Three independent experiments were performed in quadruplicate for each condition.

Ex vivo experiments

Arteries were isolated from Tie2-LacZ/ApoE-/- mice and cut into 2 mm² segments. The tissue was subsequently incubated with 1 ml M199 medium that contained either control non targeting or shHDAC3 lentiviral particles (1x10^7 TU/ml) supplemented
with 5% FBS and 10µg/ml of polybrene, in a 12-well plate for 16 hrs. Three segments were included in each group. The viral solutions were then removed, fresh medium was added to each well and the plates were returned to the incubator for an additional 48hrs. For apoptosis experiments, the tissue segments were treated with 50µM of H2O2 during the last 24hrs. Subsequently, the segments were fixed with 4% formaldehyde and 1% gluteraldehyde in PBS for 5 minutes, followed by X-gal staining overnight. The segments were mounted on a slide with vessel lumen facing up. Images were assessed and processed as described above. LacZ expression, the fold induction of endothelial cell loss, was defined as the ratio of cell number/mm² of shHDAC3 infected group to that of non targeting infected group. Three independent experiments were performed.

For en face staining preparations using double immunofluorescence, the thoracic artery was harvested from mice and cut into 2 mm² segments. The tissue was subsequently infected as described above, incubated for 48h and fixed with 4% paraformaldehyde. The aortic segments were then pinned on a silicon base, permeabilised with 0.1% Triton X-100 in PBS for 40 min and blocked in 10% swine serum in PBS for 1h at 37°C. Incubation with the primary antibodies, HDAC3 (H3034 from SIGMA 1:50), phospho AktS473 (sc-7985R from Santa Cruz 1:50) and CD31 (sc-1506 from Santa Cruz 1:200) was performed overnight at 4°C. The bound primary antibodies were detected by incubation with an ALEXA 546 conjugated donkey anti-goat IgG and a FITC-conjugated swine anti-rabbit IgG at 37°C for 2h. The tissue was counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) and mounted on microscope slides in Floromount-G.
(DAKOCytomation, Glostrup, Denmark) and examined with SP5 confocal microscope (Leica, Germany).

**Mouse aortic isograft model**

The procedure for the mouse aortic isograft was similar to that described recently². Aortas were isolated from donor *Tie2-LacZ/ApoE-/-* mice³. One end and main branches were ligated. 300µl of viral solution (6x10⁶ TU/ml) supplemented with 10µg/ml of polybrene (Sigma) was injected into the lumen and then the other end was ligated as well. The tissue was incubated with the viral solution for 2 hrs at 37ºC with the whole aorta immersed in M199 medium plus 5% FBS. Each aorta was then cut into two pieces and isografted into the recipient *Tie2-LacZ/ApoE-/-* mice. To confirm that shHDAC3 viral vectors target specifically the endothelial cells, grafts were harvested 48h after grafting and HDAC3 expression was assessed by immunofluorescence staining using cross sections. To evaluate the role of HDAC3 in neointima formation, the recipient mice were sacrificed after three weeks and the grafts were harvested and fixed with formalin. The sections were stained with H.E. and neointimal lesions were quantified using the software Aniox.

Neointimal lesions were defined as the region between the lumen and the media. Lesion quantification for each sample was performed by subtracting the area of the lumen from the area enclosed by the line inside of the media and dividing to the area of the whole aorta.
**Immunohistochemistry**

Paraffin sections from the aortic isografts were used to confirm that HDAC3 knockdown occurred specifically in endothelial cells. In brief, the slides were deparaffinized and antigen unmasking was performed by heating the sections with 10mM sodium citrate for 10 min. The slides were air dried and washed in dH₂O and endogenous peroxidase activity was quenched by incubation with 3% H₂O₂ for 20 min. After a short wash with PBS, the sections were blocked with 5% normal swine serum in PBS for 20 min and incubated with the primary antibody for HDAC3 (H3034 from SIGMA) for 1h. Subsequently, the bound primary antibodies were detected by incubation with swine anti-rabbit IgG–HRP conjugate for 30 min. Peroxidase activity was assessed using DAB reagent and the sections were counterstained with haematoxylin, dehydrated and mounted on microscope slides. Staining was visualized as described above.

To assess the effect of flow on HDAC3 expression in vascular smooth muscle cells *in vivo*, cryosections were prepared from mouse thoracic artery and the aortic arch and used for double immunofluorescence staining. The slides were fixed with ice-cold acetone for 10 min and air-dried. The tissue was permeabilized with 0.1% Triton X-100 in PBS. After a brief wash with PBS, the sections were blocked with 5% normal swine serum in PBS for 30 min and incubated with the primary antibodies for HDAC3 and SMA (H3034 (1:150) and A5228 (1:500) from SIGMA, respectively) for 1h. Subsequently, the bound primary antibodies were detected by incubation with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG for 30 min. The slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) in
Floromount-G (DAKOCytomation, Glostrup, Denmark) and examined with SP5 confocal microscope (Leica, Germany).

**Miller’s elastin staining**

To assess the elastic lamina in the isograft sections, we used the Miller’s elastin staining. In brief, paraffin sections were deparaffinised and rehydrated. They were then immersed in potassium permanganate, then oxalic acid and finally rinsed in 70% ethanol. The tissue was incubated in Miller’s elastin stain (BDH Laboratory Supplies, Poole UK) for 3hrs followed by counterstaining with van Gieson solution (50% saturated picric acid solution, 0.09% acid fuchsin) for 5 min. Sections were subsequently dried, dehydrated and mounted on microscope slides. Staining was visualized as described above.

**Statistical analysis.**

Data expressed as the mean±SD were analyzed with a two-tailed student’s *t*-test for two-groups or pair-wise comparisons. A value of *P*<0.05 was considered to be significant.
Table 1. Primers used to construct HDAC3 truncated variants

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Figure S2

CD31

HDAC3

DAPI

MERGE

Non Targeting       shHDAC3
Supplemental Figures

Figure S1. (A) Flow patterns do not affect the expression levels of HDAC3 in vascular smooth muscle cells as indicated by double immunofluorescence staining of sections from the mouse thoracic artery and the aortic arch. Bar: 50µm (B) Quantification of HDAC3 expression. Graphics show statistical data of mean±SD from three independent areas with that of the thoracic artery set as 100.

Figure S2. HDAC3 shRNA viral particles target specifically the endothelial monolayer of the aortic graft as indicated by double immunofluorescence staining of cross sections. CD31 was used as a marker for endothelial cells. Bar: 50µm.


