

Cathepsin S Activity Controls Injury-Related Vascular Repair in Mice via the TLR2-Mediated p38MAPK and PI3K–Akt/p-HDAC6 Signaling Pathway

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Objective—Cathepsin S (CatS) participates in atherogenesis through several putative mechanisms. The ability of cathepsins to modify histone tail is likely to contribute to stem cell development. Histone deacetylase 6 (HDAC6) is required in modulating the proliferation and migration of various types of cancer cells. Here, we investigated the cross talk between CatS and HDAC6 in injury-related vascular repair in mice.

Approach and Results—Ligation injury to the carotid artery in mice increased the CatS expression, and CatS-deficient mice showed reduced neointimal formation in injured arteries. CatS deficiency decreased the phosphorylation levels of p38 mitogen-activated protein kinase, Akt, and HDAC6 and toll-like receptor 2 expression in ligated arteries. The genetic or pharmacological inhibition of CatS also alleviated the increased phosphorylation of p38 mitogen-activated protein kinase, Akt, and HDAC6 induced by platelet-derived growth factor BB in cultured vascular smooth muscle cells (VSMCs), and p38 mitogen-activated protein kinase inhibition and Akt inhibition decreased the phospho-HDAC6 levels. Moreover, CatS inhibition caused decrease in the levels of the HDAC6 activity in VSMCs in response to platelet-derived growth factor BB. The HDAC6 inhibitor tubastatin A downregulated platelet-derived growth factor–induced VSMC proliferation and migration, whereas HDAC6 overexpression exerted the opposite effect. Tubastatin A also decreased the intimal VSMC proliferation and neointimal hyperplasia in response to injury. Toll-like receptor 2 silencing decreased the phosphorylation levels of p38 mitogen-activated protein kinase, Akt, and HDAC6 and VSMC migration and proliferation.

Conclusions—This is the first report detailing cross-interaction between toll-like receptor 2–mediated CatS and HDAC6 during injury-related vascular repair. These data suggest that CatS/HDAC6 could be a potential therapeutic target for the control of vascular diseases that are involved in neointimal lesion formation. (*Arterioscler Thromb Vasc Biol.* 2016;36:1549-1557. DOI: 10.1161/ATVBAHA.115.307110.)

Key Words: cathepsin S ■ HDAC6 ■ neointimal formation ■ vascular remodeling

The process of neointima formation is common to various forms of vascular diseases, such as atherosclerosis, restenosis, and transplant vasculopathy.^{1,2} In response to vascular injury, the migration of vascular smooth muscle cells (VSMCs) from the media to the intima and the proliferation of intimal

VSMCs are key events in restenotic lesion development.³ One essential factor required for VSMC migration is degradation of the basement membrane and surrounding extracellular matrix.⁴ Cathepsins are a family of cysteine proteases localized in lysosomes.^{5–7} Over the past decade, emerging data revealed

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Nonstandard Abbreviations and Acronyms	
CatS	cathepsin S
Erk1/2	extracellular signal-regulated protein kinases 1/2
HDAC6	histone deacetylases 6
HIF-1α	hypoxia-inducible factor-1 α
PDGF-BB	platelet-derived growth factor BB
p-p38MAPK	phospho-p38 mitogen-activated protein kinase
TLR2	toll-like receptor 2
VSMC	vascular smooth muscle cell

unexpected roles of cathepsins in pathological conditions, such as metabolic disorder and atherosclerosis-based cardiovascular disease.^{8–10} Among cathepsin family, cathepsin S (CatS) was the first cathepsins found to be expressed in human atherosclerotic lesions.¹¹ Previous studies showed that vascular atherosclerotic lesions associated with the injury overexpress the elastolytic and collagenolytic CatS but show no change in cystatin C expression, their endogenous inhibitor,^{4,12} suggesting a shift in the balance between cathepsins and their inhibitor that favors the remodeling of cardiovascular wall.

Histone deacetylases (HDACs) are a family of enzymes that remove acetyl groups from lysine residues of histone proteins and nonhistone proteins, a modification that results in epigenetic modulation of gene expression.^{13,14} Previous study reported that HDACs play a crucial role in the development of proliferative vascular diseases, including

atherosclerosis and restenosis.¹³ Histone acetyltransferases and HDACs have been shown to regulate the expression of inflammatory and other genes involved in VSMC functions.¹⁵ Findeisen et al reported that short interfering RNA-mediated knockdown of HDAC 1, 2, or 3 prevented mitogen-induced SMC proliferation and that the pharmacological inhibition of HDAC decreased neointima formation.¹³ Yan et al found that HDACs modulate VSMC migration induced by cyclic mechanical strain.¹⁶ HDAC4 was recently reported to control neointimal hyperplasia via the stimulation of the proliferation and migration of VSMCs.¹⁷ HDAC6 has been found to play a pivotal role in modulating the migration and proliferation of cancer cells,¹⁸ and normal cells, including fibroblasts and epithelial cells.¹⁹ On the other hand, over the last couple of years' researches highlighted that the cathepsin family is closely related to many critical signaling pathways, such as the Notch signaling pathway (cathepsin K),⁹ the peroxisome proliferator-activated receptor γ pathway (CatS),²⁰ and histone H3 (cathepsin L).²¹ However, the relationship between CatS activity and the HDAC6 activation in the cellular events and injury-induced vascular repair is unclear.

In this study, we examined the hypothesis that CatS activity could modulate HDAC6 activation to stimulate VSMC migration and proliferation in vascular remodeling and neointimal hyperplasia in response to injury. We believed that this newly discovered CatS–HDAC6 interaction-mediated cellular mechanism has key roles in the hyperproliferative restenosis associated with endovascular treatments.

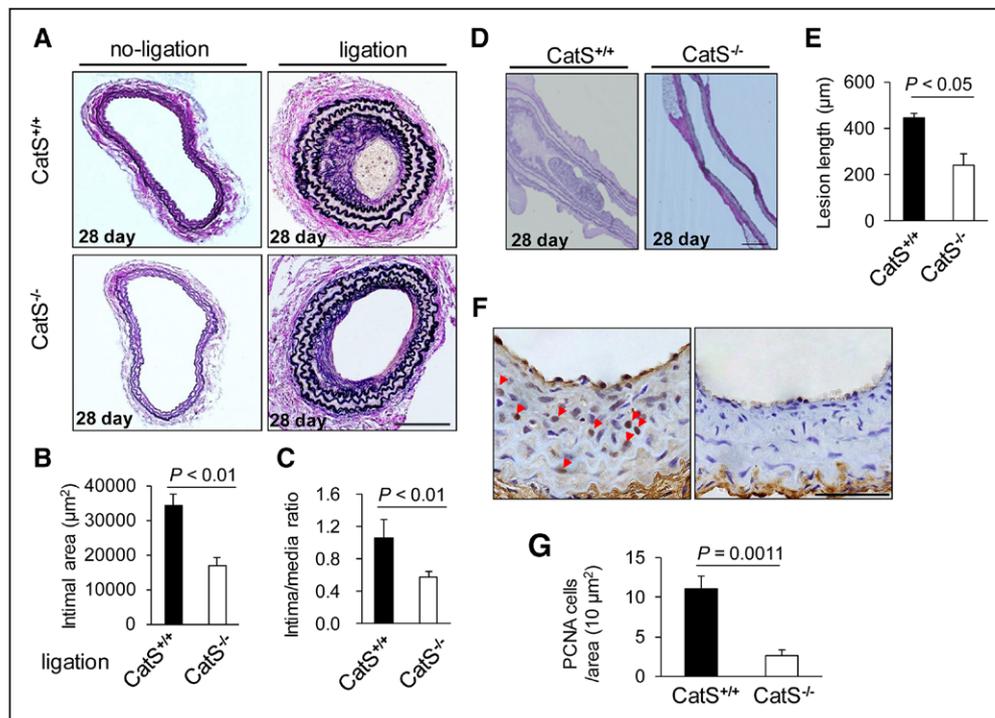


Figure 1. CatS^{-/-} reduces neointimal formation in ligation-injured carotid arteries of mice. **A**, Representative images (van Geison staining) and quantitative data of hematoxylin and eosin (H&E) staining on cross-sections from injured carotid arteries of CatS^{+/+} and CatS^{-/-} mice at day 28. Scale bar: 100 μ m. **B** and **C**, The intimal area and intima/media ratio of injured arteries on day 28 were lower in CatS^{-/-} mice. Data are mean \pm SEM (n=8), Student unpaired *t* test. **D** and **E**, Representative images (van Geison staining) and quantitative data of H&E on longitudinal sections from injured carotid arteries of CatS^{+/+} and CatS^{-/-} mice. Scale bar: 200 μ m. Data are mean \pm SEM (n=12 and 10, respectively), Student unpaired *t* test. **F** and **G**, Representative images and quantitative data of proliferating cell nuclear antigen (PCNA) staining on cross-sections from injured carotid arteries of CatS^{+/+} and CatS^{-/-} mice. Scale bar: 50 μ m. Data are mean \pm SEM (n=6), Student unpaired *t* test. Triangles indicate PCNA-stained cells. CatS indicates cathepsin S.

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results

Reduced Neointimal Formation in the CatS^{-/-} Mice

The ligation injury induced CatS expression in carotid arteries. The relative mRNA levels of CatS on day 1, 2, 4, 14, and 28 after ligation were increased by 25-fold over those of the uninjured control vessels of CatS^{+/+} mice (Figure IA in the [online-only Data Supplement](#)). CatS protein expression was also increased in the injured arteries by 4-fold (Figure IB in the [online-only Data Supplement](#)). As shown in Figure 1A and 1D, marked intimal lesion formation and long lesion lengths were observed in the samples from CatS^{+/+} mice at day 28 after the ligation injury. Much less intimal lesion formation was observed in the samples from the CatS^{-/-} mice. The quantitative measurements revealed significantly lower levels of intimal areas from CatS^{-/-} mice compared with those from the CatS^{+/+} mice (Figure 1B and 1E), but no significant difference in media thickness was observed between the CatS^{+/+} and CatS^{-/-} mice (32.3 ± 1.7 versus $29.1 \pm 1.4 \times 10^3 / \mu\text{m}^2$; $P=0.182$). Therefore, the ratio of intimal to medial area was higher in the CatS^{+/+} mice (Figure 1C). The proliferating cell nuclear antigen staining showed a low level of proliferative activity in both the intima and media of injured carotid arteries from CatS^{-/-} mice compared with those of CatS^{+/+} mice (Figure 1F and 1G). As shown in Figure 1C and 1D in the [online-only Data Supplement](#), we could not detect the expression of CatS gene and protein in the tail tissues and in the cultured VSMCs of CatS^{-/-} mice.

The macrophage activation-related release of inflammatory chemokines is an important hallmark of human and animal vascular repair and is mediated by a toll-like receptor

(TLR) signaling pathway in cardiovascular disease.^{10,22} Here, we evaluated TLRs and inflammatory chemokine expressions. The quantitative polymerase chain reaction revealed that compared with the CatS^{+/+} mice, the lesions in CatS^{-/-} mice that received a ligation injury had lower mRNA levels of TLR2, as well as monocyte chemoattractant protein-1, whereas TLR4 exhibited no significant difference (Figure IIA–IIC in the [online-only Data Supplement](#)). As shown in Figure IIF in the [online-only Data Supplement](#), the TLR2-positive cells were higher in the neointima of the injured vessels from CatS^{+/+} mice on days 4 than in that of CatS^{-/-} mice. There was also no significant difference in the cathepsin K or cystatin C mRNA expressions between the CatS^{+/+} and CatS^{-/-} mice (Figure IID and IIE in the [online-only Data Supplement](#)).

Reduced Levels of Phospho-HDAC6 in CatS^{-/-} Mice

Representative immunoblots showed that the level of phospho-HDAC6 (p-HDAC6) was increased in the injured arteries of CatS^{+/+} mice, and this increased expression was ablated in the CatS^{-/-} mice on day 1 after ligation injury (Figure 2A and 2B). However, there are no significant differences in the total HDAC6 protein or HDAC6 mRNA levels between the injured and uninjured arteries of CatS^{+/+} mice (Figure 2A; Figure IIIA and IIIB in the [online-only Data Supplement](#)). With the exception of HDAC4, HDAC5, HDAC8, and HDAC9, we also observed that there were no between-group differences in other HDAC family members (including HDAC1, HDAC2, HDAC3, HDAC6, and HDAC7). As shown in Figure 2A, 2C, and 2D, we observed lower levels of phospho-p38 mitogen-activated protein kinase (p-p38MAPK) and p-Akt proteins in the injured arteries of CatS^{-/-} mice. On operative day 1, compared with

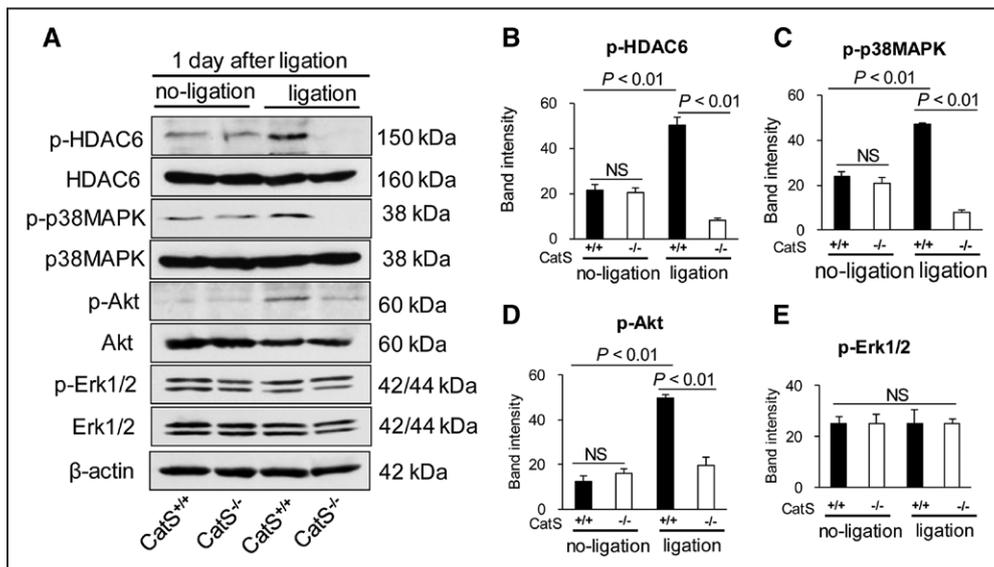


Figure 2. The levels of targeted protein in the ligation-injured arteries of the 2 experimental groups. On day 1 after injury, equal amounts of total protein extraction were immunoblotted using p-HDAC6, t-HDAC6, p-p38MAPK, t-p38MAPK, p-Akt, t-Akt, p-Erk1/2, t-Erk1/2, and β -actin antibodies. Representative immunoblots (A) and quantitative data (B–E) show reduced levels of p-HDAC6, p-p38MAPK, and p-Akt proteins in the injured arteries of CatS^{-/-} mice, but show no changes in the levels of p-Erk1/2 between the CatS^{+/+} mice and CatS^{-/-} mice (A and E). Data are mean \pm SEM of 4 independent experiments, Student unpaired *t* test or ANOVA and Bonferroni post hoc tests. ANOVA indicates analysis of variance; CatS, cathepsin S; ERK1/2, extracellular signal-regulated protein kinases 1/2; HDAC6, histone deacetylase 6; p-HDAC6, phospho-histone deacetylase 6; and p-p38MAPK, phospho-p38 mitogen-activated protein kinase.

uninjured arteries, we observed an increase in the levels of hypoxia-inducible factor-1 α (HIF-1 α) gene in the injured arteries of CatS^{+/+} mice, indicating that ligation injury contributes to vascular local hypoxia (Figure IIG in the [online-only Data Supplement](#)). CatS deficiency caused decrease in HIF-1 α gene change (Figure IIIH in the [online-only Data Supplement](#)). The levels of plasma platelet-derived growth factor-BB (PDGF-BB) were increased on day 4 after ligation in the CatS^{+/+} mice and regressed on day 28 (Table I in the [online-only Data Supplement](#)). Interestingly, we observed that this increased expression of plasma PDGF was blunted in the CatS^{-/-} mice on day 4, but there was no difference on day 28 between the 2 genotype groups.

HDAC6 Inhibition With Tubastatin A Decreased the Mitogen-Induced VSMC Proliferation and Migration and Induced Cell-Cycle Arrest

As shown in Figure 3A, the HDAC6-specific inhibitor tubastatin A reduced the PDGF-BB–induced proliferation of VSMCs at 48 hours after incubation. We also observed that tubastatin A reduced the PDGF-BB–induced migration of VSMCs at 36 hours after incubation (Figure 3B and 3C). To further explore the mechanisms by which HDAC inhibition prevents VSMC proliferation, we determined the cell-cycle distribution using flow cytometry. As shown in Figure 3D–3G, tubastatin A reduced the proportion of cells at S and G2/M stage. As anticipated, tubastatin A mitigated PDGF-induced HDAC6 phosphorylation in cultured VSMCs (Figure IV in the [online-only Data Supplement](#)). Moreover, HDAC6 silencing

mitigated VSMC proliferation and migration in response to PDGF-BB (Figure V in the [online-only Data Supplement](#)). Thus, these findings indicate that HDAC6 activity may play a pivotal role in VSMC proliferation and migration, which are main processes of vascular remodeling. However, tubastatin A exhibited no effect on proliferation (0.11 ± 0.02 versus 0.13 ± 0.02 ; $P=0.46$) and migration (7.3 ± 0.9 versus 9.1 ± 1.0 ; $P=0.81$; $n=7$ for each) in cultured VSMCs without PDGF-BB stimulation.

We also observed that pharmacological intervention targeted toward CatS mitigated VSMC proliferation and migration in response to PDGF-BB or 2% FBS (Figure VIA, VIC, and VID in the [online-only Data Supplement](#)). Likewise, CatS deficiency impaired VSMC proliferation in response to PDGF-BB (Figure VIB in the [online-only Data Supplement](#)). Compared with the CatS^{+/+} explants, the sprouting VSMC numbers and areas were markedly decreased in the aorta explants of the CatS^{-/-} mice during the follow-up periods (Figure VII in the [online-only Data Supplement](#)). These data showed that both CatS and HDAC6 play an important role in VSMC proliferation and migration. CatS inhibitor had no effect on migration (4.0 ± 1.2 versus 5.7 ± 0.9 ; $P=0.38$) and proliferation (0.09 ± 0.01 versus 0.10 ± 0.00 ; $P=0.91$; $n=5$ for each) in cultured VSMCs without PDGF-BB stimulation. In vivo, CatS inhibitor also had no effects on the neointima areas (14971 ± 2371 versus 15747 ± 2101 μm^2 ; $P=0.23$) and the ratio of intima to media (0.48 ± 0.07 versus 0.51 ± 0.08 ; $P=0.66$; $n=6$ for each) in CatS deficiency mice.

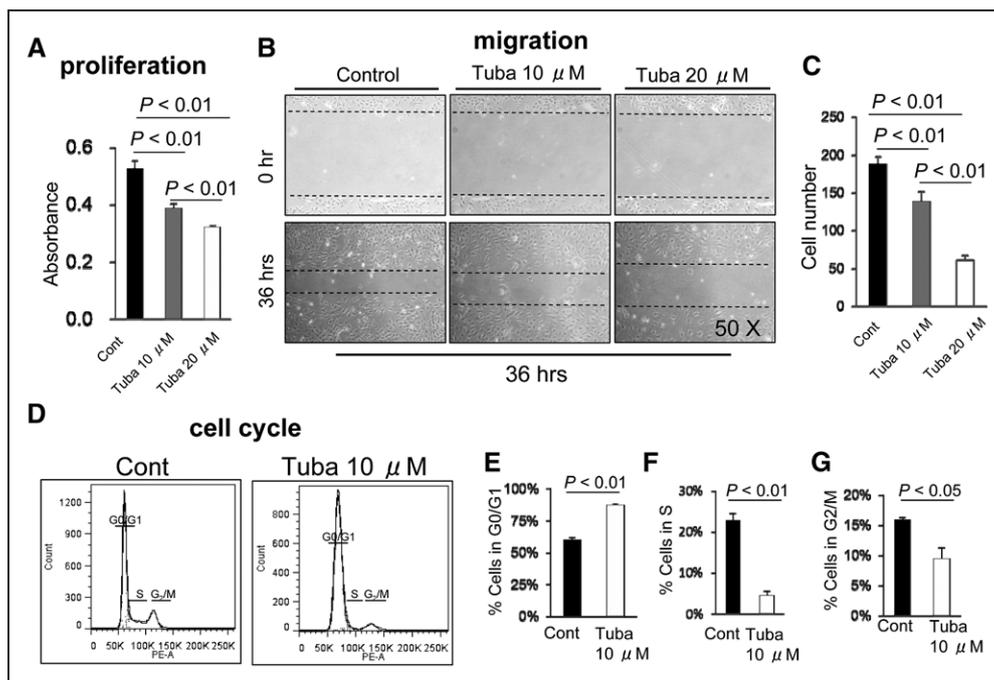


Figure 3. Histone deacetylase 6 (HDAC6) inhibition suppressed the growth factor–stimulated vascular smooth muscle cell (VSMC) proliferation and migration and blocked cell-cycle progression in vitro. **A**, VSMCs were grown in a 96-well plate in the absence or presence of tubastatin A (Tuba, 10 μM or 20 μM) with platelet-derived growth factor BB (PDGF-BB; 20 ng/mL) for 48 h. Data are mean \pm SEM ($n=7$). Representative images (**B**) and quantitative data (**C**) of effect of tubastatin A on the PDGF-BB–induced migration of VSMCs. Mouse VSMCs were scratched with a 1-mL pipet tip and cultured for 36 h in DMEM containing PDGF-BB (20 ng/mL) with or without tubastatin A (Tuba, 10 μM and 20 μM ; $n=7$). The dotted lines define the areas lacking cells; ANOVA and Bonferroni post hoc tests. **D–G**, Representative images (**D**) and the quantitative data (**E**, **F**, and **G**) show the distribution of cells in G1 (**E**), S (**F**), and M (**G**) stage expressed as percentage of total cells. Data are mean \pm SEM ($n=4$), Student unpaired t test.

HDAC6 Inhibition Reduced the Neointima Formation After Vascular Injury

Compared with the mice treated with vehicle, HDAC6 inhibition with tubastatin A reduced the neointimal formation in the ligation-injured arteries of CatS^{+/+} mice as determined by hematoxylin and eosin staining (Figure 4A and 4C). On day 14 after the surgery, we detected double-positive staining of bromodeoxyuridine and hematoxylin for proliferative VSMCs in the neointima of injured arteries from the CatS^{+/+} mice. The immunostaining of these cells was greatly decreased in the tubastatin A–treated mice (Figure 4B and 4D), indicating that HDAC6 activity plays a role in the process of proliferation of VSMCs. We also observed a decreased level of plasma HDAC6 activity in tubastatin A–treated group (Figure 4E). However, tubastatin exhibited no beneficial effects on neointima areas (15 392±1736 versus 16 079±2383 μm²; *P*=0.87) and the ratio of intima to media (0.52±0.10 versus 0.49±0.11; *P*=0.71; *n*= 6 for each) in CatS deficiency mice.

CatS Regulates HDAC6 Phosphorylation via the p38MAPK/Akt Signaling Pathway in Cultured VSMCs

We have observed a reduced expression of p-HDAC6 in the injured vessels of CatS^{-/-} mice compared with those of CatS^{+/+} mice (Figure 2A and 2B). Here, we investigated how CatS regulates p-HDAC6 in cultured VSMCs. First, we observed that PDGF-BB increased the phosphorylation levels of HDAC6, p38MAPK, Akt, and extracellular signal-regulated protein kinases 1/2 (Erk1/2), with the peak at 10 to 30 minutes (Figure

VIIIA and VIIIB in the [online-only Data Supplement](#)) in CatS^{+/+} VSMCs. Interestingly, the increased levels of p-HDAC6, p-p38MAPK, and p-Akt were blunted in CatS^{-/-} VSMCs, with the exception of p-Erk (Figure 5A and 5B). And an increased HDAC6 activity in cell lysates of CatS^{+/+} VSMCs was also ablated by CatS^{-/-} (Figure 5C). These results indicate that CatS regulates HDAC6 phosphorylation and activity in VSMCs. As shown in Figure 5D and 5E, CatS inhibitor reduced the PDGF-induced phosphorylation of HDAC6, p38MAPK, and Akt, but had no effect on that of p-Erk1/2. Likewise, the HDAC6 activity in cell lysates of CatS^{+/+} VSMCs was increased by PDGF-BB, and these changes were ablated by CatS inhibition (Figure 5F). We also found that p38MAPK and Akt inhibitor blocked the PDGF-induced phosphorylations of HDAC6 or eEF-2 (eukaryotic elongation factor-2; Figure VIIIC–VIIE in the [online-only Data Supplement](#)), whereas Erk1/2 inhibitor alone had no effect (data not shown). These results indicate that CatS may regulate HDAC6 phosphorylation and activity through the p38MAPK/Akt signaling pathway in VSMCs. In addition, SMCs were transfected with a HDAC6 plasmid to dramatically increase HDAC6 expression, which resulted in enhanced levels of p-HDAC6 protein (Figure IX in the [online-only Data Supplement](#)).

HDAC6 Plasmid Transfection Increased the In Vitro VSMC Proliferation, Migration, and Cell-Cycle Progression

To further determine the effect of HDAC6 on VSMC proliferation and migration, we enhanced the HDAC6 expression by transfecting HDAC6 plasmid into VSMCs in vitro. As

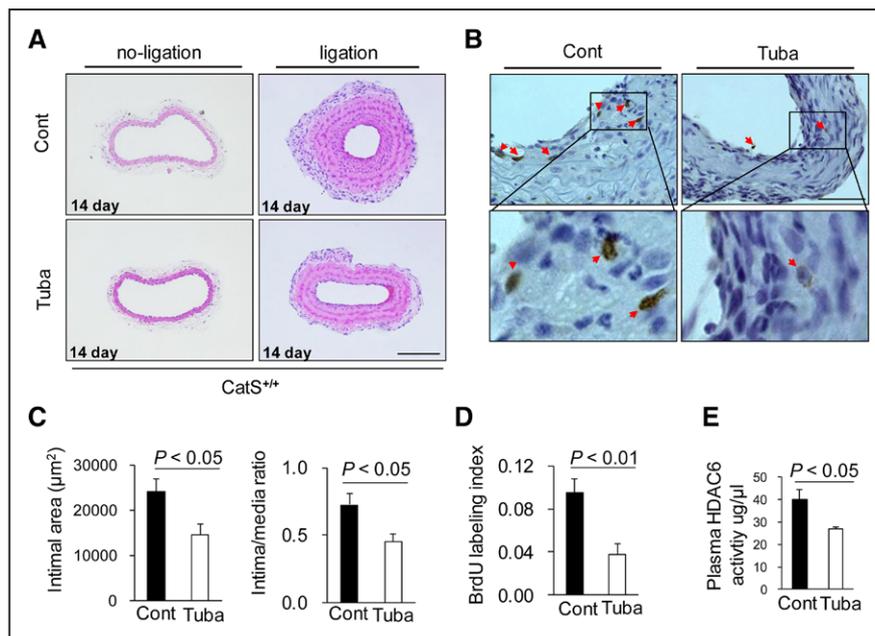


Figure 4. Histone deacetylase 6 (HDAC6) inhibition reduced the neointima formation after vascular injury. After tubastatin A (Tuba) was intraperitoneally administered to 10-week-old mice at a dose of 10 mg/kg every day for 2 weeks (*n*=6), the carotid artery was harvested and set in 5-μm paraffin sections. Representative images (A) and quantitative data (C) of hematoxylin and eosin (H&E) staining on cross-sections from injured carotid arteries of CatS^{+/+} mice treated with or without tubastatin A (Tuba). Scale bar: 100 μm. The intimal area and intima/media ratio of injured arteries were much lower in the tubastatin A–treated mice. Data are mean±SEM, Student unpaired *t* test. Representative images (B) of the bromodeoxyuridine (BrdU) staining on cross-sections from injured carotid arteries and quantitative data (D) of the BrdU labeling index. The BrdU labeling index was calculated by dividing the number of BrdU-labeled cells by the number of total cells in the neointima (*n*=5, each group). Triangles indicate BrdU-labeled cells. Tubastatin A reduced the plasma HDAC6 activity (E) as compared with control group (*n*=5, each group). Data are mean±SEM, Student unpaired *t* test. CatS indicates cathepsin S.

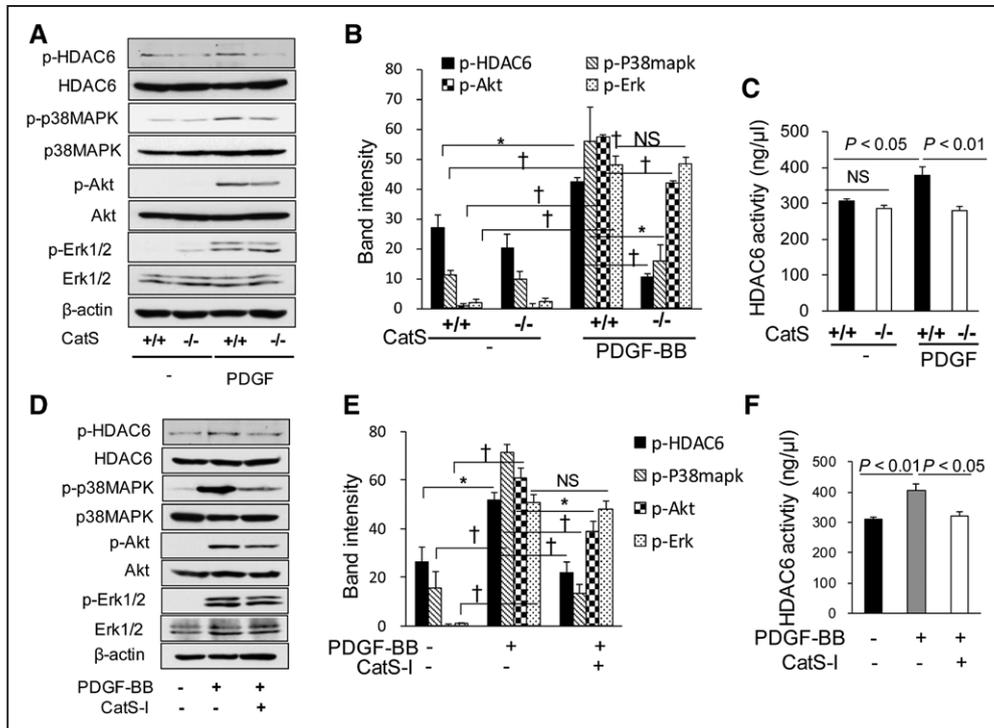


Figure 5. Cathepsin S (CatS) regulates histone deacetylase 6 (HDAC6) activity in vascular smooth muscle cells (VSMCs). Mouse aortic VSMCs were cultured in 10% FBS/DMEM medium and then subjected to serum-free medium for 12 h before the following treatment. Protein samples were isolated and used for a Western blotting analysis. Representative immunoblots (A) and quantitative data (B) show that the increased levels of p-HDAC6, p-p38MAPK, and p-Akt were blunted in CatS^{-/-} VSMCs, with the exception of p-Erk. CatS^{-/-} decreased HDAC6 activity in VSMCs in response to platelet-derived growth factor BB (PDGF-BB; C). Representative immunoblots (D) and quantitative data (E) show that the CatS inhibitor (CatS-I, 10 μ M) reduced the PDGF-induced phosphorylation of HDAC6, p38MAPK, and Akt, but had no effect on that of p-Erk1/2. PDGF-BB-induced HDAC6 activity of CatS^{+/+} VSMCs was reduced by CatS inhibition (F). PDGF-BB, 20 ng/mL, for 10 minutes. Data are mean \pm SEM of 4 independent experiments, ANOVA and Bonferroni post hoc tests. ERK1/2 indicates extracellular signal-regulated protein kinases 1/2; p-HDAC6, phospho-histone deacetylase 6; and p-p38MAPK, phospho-p38 mitogen-activated protein kinase.

anticipated, we observed increased proliferation and migration ability in VSMCs transfected with HDAC6 plasmid compared with those transfected with vector plasmids (Figure XA–XC in the [online-only Data Supplement](#)). HDAC6 transfection also enhanced the cell-cycle progression by increasing the proportion of cells in the S/G2/M stage (Figure XD and XE in the [online-only Data Supplement](#)).

Hypoxia Increased the HDAC6 Signaling and VSMC Proliferation and Migration In Vitro

Because we observed an increased expression of HIF-1 α gene in injured arteries, which is a marker of hypoxia, we further investigated whether hypoxia plays a role in the CatS/p38MAPK/Akt/HDAC6 signaling pathway. As shown in Figure 6A and 6B, hypoxia increased the levels of PDGF-BB-induced p-HDAC6, p-p38MAPK, and p-Akt, indicating that hypoxic stress could enhance p38MAPK/Akt/HDAC6 signaling cascade activation in VSMCs. However, hypoxia had no effect on the levels of p-Erk1/2 protein (Figure 6C). Moreover, we found that hypoxia increased the proliferation and migration of VSMCs in response to PDGF-BB (Figure 6D–6F). In addition, CatS ablation diminished hypoxia-induced VSMC proliferation (0.48 ± 0.6 versus 0.62 ± 0.08 ; $P<0.01$) and migration (23.0 ± 3.1 versus 47.2 , cells; $P<0.01$).

TLR2 Was Required in HDAC6 Signaling and VSMC Events

To further investigate the interaction between TLR2 and HDAC6 signaling pathways, siTLR (short interfering RNA against toll-like receptor-2) was applied to use in the cellular experiments. Results indicated that TLR2 silencing mitigated HDAC6 as well as p38MAPK and Akt activations in cultured VSMCs in response to PDGF-BB (Figure XIA and XIB in the [online-only Data Supplement](#)). As anticipated, siTLR ameliorated cell proliferation and migration (Figure XIC–XIE in the [online-only Data Supplement](#)).

Discussion

Endovascular treatment-related maladaptive vascular remodeling represents the leading cause of restenosis and cardiovascular events.¹⁰ Identifying novel targets to suppress vascular negative remodeling will contribute to therapeutic strategies to preempt restenosis.¹⁰ The significant finding of our present study is that mice lacking the CatS gene are resistant to acute injury-induced intimal hyperplasia via the HDAC6-induced proliferation and migration of VSMCs. At the molecular level, CatS deletion retards injury-induced TLR2 gene and its downstream inflammatory genes and p38MAPK, Akt, and HDAC6 signaling activations. The pharmacological inhibition of HDAC6 activity also results in vascular protective actions via the reduction of

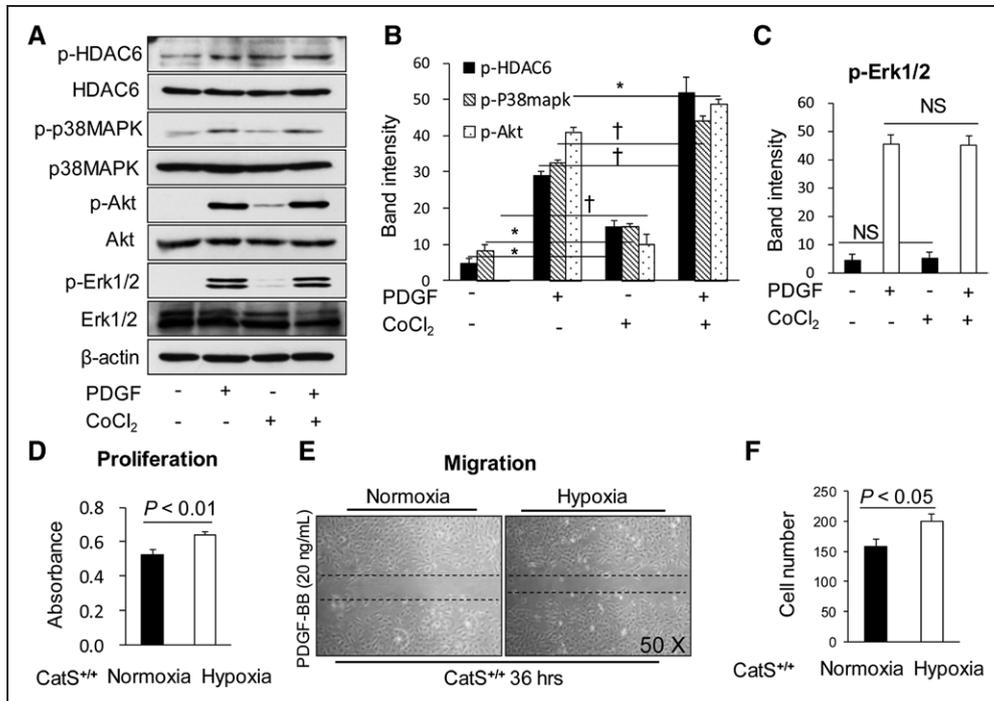


Figure 6. Hypoxia increased the histone deacetylase 6 (HDAC6) signaling and vascular smooth muscle cell (VSMC) proliferation and migration in vitro. Mouse VSMCs were cultured in 10% FBS/DMEM medium and then subjected to serum-free medium for 12 h. After starvation, cells were incubated with or without CoCl₂ (600 μM) for 20 minutes and then stimulated with or without platelet-derived growth factor BB (PDGF-BB; 20 ng/mL) for 10 minutes. Protein samples were isolated and used for a Western blotting analysis as indicated. The representative image (A) and quantitative data (B) show that hypoxia increased the PDGF-BB-induced p-HDAC6, p-p38MAPK, and p-Akt levels, but had no effect on p-ERK1/2 signaling (C, n=3). Hypoxia increased VSMC proliferation (D, n=7). Representative images (E) and quantitative data (F) that hypoxia increased PDGF-BB-induced VSMC migration. Data are mean±SEM, Student unpaired t test, or ANOVA and Bonferroni post hoc tests. ERK1/2 indicates extracellular signal-regulated protein kinases 1/2; p-HDAC6, phospho-histone deacetylase 6; and p-p38MAPK, phospho-p38 mitogen-activated protein kinase.

VSMC proliferation. In vitro, the specific inhibition of CatS or HDAC6 attenuated VSMC migratory and proliferative abilities. We also observed that CatS regulates HDAC6 phosphorylation through the p38MAPK/Akt signaling pathway in cultured VSMCs. Moreover, TLR2 silencing mitigated the phosphorylations of HDAC6, p38MAPK, and Akt and VSMC functions. To the best of our knowledge, this is the first study to report that CatS controls injury-related vascular repair in mice via the TLR2-mediated p38MAPK/Akt-HDAC6 signaling pathway (Figure XII in the online-only Data Supplement).

Cathepsin cysteine proteases can degrade the basement membrane and surrounding extracellular matrix of artery walls.²³ Cathepsin family members, such as CatS and CatK, have been demonstrated to play an important role in atherosclerosis-based proliferative diseases.^{10,24} Our research and previous studies by other groups have demonstrated increased expression and activity of CatS in balloon-injured arteries of rat¹² or rabbit.⁴ In the present study, we also observed an increased expression of CatS in ligation-injured arteries of mice (Figure IA in the online-only Data Supplement). Interestingly, we observed that neointimal formation was reduced markedly in CatS^{-/-} mice compared with wild-type mice. Intimal hyperplasia is believed to be the consequence of VSMC proliferation and migration from the media into the intima. In our present work, we observed that cell proliferative activity in the intima and media of injured arteries was decreased in the absence of CatS in vivo (Figure 1), and the pharmacological

inhibition of CatS prevented the mitogen-induced VSMC proliferation and migration in vitro (Figure VI in the online-only Data Supplement). Moreover, the impaired VSMC migration from the arterial explants of CatS^{-/-} reflects that the intrinsic ability of cells to migrate and degrade the local extracellular matrix proteins is controlled by CatS activity (Figure VII in the online-only Data Supplement). Collectively, these observations demonstrated that CatS controls injury-related vascular repair in mice by affecting VSMC migration and proliferation.

Recent evidence has shown that HDACs play a crucial role in the development of proliferative vascular diseases, including atherosclerosis and in-stent restenosis.¹³ Our present findings revealed that the level of phospho-HDAC6 protein was increased in injured arteries of CatS^{+/+} mice compared with noninjured arteries (Figure 2). The pharmacological inhibition of HDAC6 with tubastatin A markedly reduced the neointimal formation and neointimal VSMC proliferation in the ligation-injured arteries of CatS^{+/+} mice (Figure 4). Our in vitro study showed that tubastatin A reduced the mitogen-induced proliferation and migration of VSMCs and that the overexpression of HDAC6 exerted the opposite effect (Figure 3; Figure X in the online-only Data Supplement). These observations indicate that HDAC6 activity plays an important role in the process of intimal hyperplasia after vascular injury via the control of VSMC proliferation and migration. We further observed that the increased expression of p-HDAC6 protein in injured arteries was ablated in CatS^{-/-} mice, and both the genetic

ablation and pharmaceutical inhibition of CatS significantly suppressed the PDGF-induced HDAC6 phosphorylation and activities in cultured VSMCs (Figures 2 and 5). Thus, the present data suggest that CatS may control injury-related vascular repair via HDAC6-mediated VSMC migration and proliferation. It should be noted that the levels of p-HDAC6 but not the total HDAC6 expression changed in the injured lesions of CatS^{+/+} mice because neither HDAC6 mRNA nor protein changed from day 1 to day 4 after ligation in wild-type mice (Figure IIIB in the [online-only Data Supplement](#)), suggesting that HDAC6 activity plays the key role in vascular remodeling.

The engagement of TLRs on the cell surface by specific ligands leads to an increase in the expressions of pro-inflammatory cytokines and chemokines, such as MCP-1 (monocyte chemoattractant protein-1).^{10,22} Several lines of investigation demonstrated that TLR2 and TLR4 are required in the vascular SMC proliferation via MAPK activation.^{10,25} Previous studies reported that p38MAPK, Akt, and Erk1/2 signaling have critical roles in SMC proliferation and migration and in the process of neointimal formation.²⁶ Our present observations show that CatS deletion reduced the levels of TLR2, p-p38MAPK, and p-Akt in the arterial lesions (Figure 2A, 2C, and 2D; Figure IIA and IIF in the [online-only Data Supplement](#)). Furthermore, both the genetic ablation and pharmaceutical inhibition of CatS significantly decreased the PDGF-BB-stimulated phosphorylation of p38MAPK and Akt but not that of Erk1/2 in cultured VSMCs (Figure 5A, 5B, 5D, and 5E), indicating that CatS controls vascular repair and that neointimal formation may act through p38MAPK and Akt signaling pathways. In addition, our present findings demonstrated that the inhibition of p38MAPK and Akt blocked the PDGF-induced phosphorylation of HDAC6 (Figure VIII C–VIII E in the [online-only Data Supplement](#)). HDAC6 inhibition has been shown to disrupt STAT3-related anti-inflammatory response in the antigen-presenting cells.²⁷ A recent single study reported that PDGF regulated vascular SMC proliferation via STAT3 signaling pathway activation.²⁸ Here, we have observed that TLR2 silencing mitigated VSMC proliferation and migration accompanied with the reduction of p-HDAC6, p-p38MAPK, and p-Akt levels (Figure XI in the [online-only Data Supplement](#)). Taken together, these results indicate that CatS controls injury-related vascular repair in mice via the TLR2-mediated p38MAPK/Akt-HDAC6 signaling pathway. PDGF-BB has been known to be a key player in vascular remodeling and restenosis in response to mechanical stress. It should be noted that here the plasma PDGF-BB concentrations were lower in the CatS^{-/-} mice than in the CatS^{+/+} mice at an early stage (day 4) after vascular injury, indicating that CatS deficiency-mediated vascular benefits may also be attributable, at least in part, to an attenuation of circulating PDGF-BB levels in a mouse carotid artery-injury model.

Recent studies have supported the existence of hypoxia in human and murine intima plaques, which augments plaque progression by stimulating plaque angiogenesis, altering glucose metabolism, promoting plaque proteolysis, inciting plaque inflammation, and increasing lipid accumulation in macrophage foam cells.^{29,30} In the present study, we observed that HIF-1 α mRNA increased in the injured

arteries, suggesting that ligation injury contributes to vascular local hypoxia (Figure IIG in the [online-only Data Supplement](#)). CatS deficiency had decreased levels of HIF-1 α mRNA in injured arteries (Figure IIH in the [online-only Data Supplement](#)). The results of our in vitro study showed that hypoxia increased the levels of p-HDAC6, p-p38MAPK, and p-Akt in the PDGF-BB group (Figure 6A and 6B), indicating that hypoxia enhances the CatS/p38MAPK/Akt/HDAC6 signaling pathway in VSMCs. We also observed that hypoxia increased the proliferation and migration of VSMCs induced by PDGF-BB in vitro (Figure 6D–6F). These observations indicate that hypoxia may be involved in and may enhance the role of CatS in vascular repair. On the other hand, CatS ablation mitigates the levels of p-p38MAPK and p-HDAC6 (Figure 2C) as compared with the corresponding control, whereas it had increased levels of p-p38MAPK and p-HDAC6 proteins in cultured VSMCs in response to PDGF (Figure 5A and 5B). As known, it is too hard to completely reproduce in vivo conditions in one experiment. Although we have no direct evidence, this discrepancy might be because of the differences in the experimental conditions (in vivo and in vitro) and protein extractions (whole vessels or SMCs lysates).

In conclusion, our study demonstrated that CatS controls injury-related vascular repair via TLR2-mediated p38MAPK/Akt signaling activation and HDAC6-mediated VSMC migration and proliferation. Moreover, our present findings suggest that targeting CatS and HDAC6 represent an attractive therapeutic approach to treat vasculopathies that are involved in neointima formation and intima-media thickening.

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Disclosures

None.

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Highlights

- Cathepsin S gene and protein levels increased in mouse carotid vasculature to injuries.
- Cathepsin S deficiency attenuated injury-related inflammatory actions and medial smooth muscle cellular events (including proliferation and migration) and mitigated vascular remodeling.
- There is cross-interaction between toll-like receptor 2–mediated cathepsin S and histone deacetylase 6 signaling pathways during injury-related vascular repair.
- Targeting the cross talk between cathepsin S and histone deacetylase 6 could provide a potential therapeutic route for the clinical treatment of proliferative vascular diseases that are involved in vascular remodeling and neointima hyperplasia.