Splicing of Histone Deacetylase 7 Modulates Smooth Muscle Cell Proliferation and Neointima Formation Through Nuclear β-Catenin Translocation

Boda Zhou, Andriana Margariti, Lingfang Zeng, Ouassila Habib, Qingzhong Xiao, Daniel Martin, Gang Wang, Yanhua Hu, Xian Wang, Qingbo Xu

Objective—Vascular smooth muscle cell (SMC) proliferation has an indispensable role in the pathogenesis of vascular disease, but the mechanism is not fully elucidated. The epigenetic enzyme histone deacetylase 7 (HDAC7) is involved in endothelial homeostasis and SMC differentiation and could have a role in SMC proliferation. In this study, we sought to examine the effect of 2 HDAC7 isoforms on SMC proliferation and neointima formation.

Methods and Results—We demonstrated that overexpression of unspliced HDAC7 (HDAC7u) could suppress SMC proliferation through downregulation of cyclin D1 and cell cycle arrest, whereas spliced HDAC7 (HDAC7s) could not. Small interfering RNA (siRNA)–mediated knockdown of HDAC7 increased SMC proliferation and induced nuclear translocation of β-catenin. Additional experiments showed that only HDAC7u could bind to β-catenin and retain it in the cytoplasm. Reporter gene assay and reverse transcription polymerase chain reaction revealed a reduction of β-catenin activity in cells overexpressing HDAC7u but not HDAC7s. Deletion studies indicated that the C-terminal region of HDAC7u is responsible for the interaction with β-catenin. However, the addition of amino acids to the N terminus of HDAC7u disrupted the binding, further strengthening our hypothesis that HDAC7s does not interact with β-catenin. The growth factor platelet-derived growth factor-βB increased the splicing of HDAC7 while simultaneously decreasing the expression of HDAC7u. Importantly, in an animal model of femoral artery wire injury, we demonstrated that knockdown of HDAC7 by siRNA aggravates neointima formation in comparison with control siRNA.

Conclusion—Our findings demonstrate that splicing of HDAC7 modulates SMC proliferation and neointima formation through β-catenin nuclear translocation, which provides a potential therapeutic target in vascular disease. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: HDAC □ beta catenin □ mouse model □ neointima □ smooth muscle
Pre-mRNA alternative splicing is a central mode of genetic regulation in higher eukaryotes. Variability in splicing patterns is a major source of protein diversity from the genome. Recently, the importance of alternative splicing has drawn increasing attention in cardiovascular research. Conventional splicing involves the excision of introns during pre-mRNA processing, which leaves only exons in the mature mRNA. However, in certain cases, specific exons are spliced out or parts of an intron are conserved and expressed. These events lead to alternative primary amino acid sequences and can alter the conformation and binding partners of a protein.

β-Catenin is both an adhesion molecule and a signal transducer of the Wnt pathway, which plays a critical role in SMC proliferation and arteriosclerosis. β-Catenin promotes cell proliferation by inducing the transcription of β-catenin responsive genes through activation of the T-cell factor (TCF)/lymphoid enhancer factor family of transcription factors. β-Catenin predominantly localizes to the cell-cell junctions. Cytoplasmic and nuclear levels of β-catenin are kept low by glycogen synthase kinase-3–mediated phosphorylation and subsequent degradation. The stability and subcellular localization of β-catenin are pivotal in determining its function. However, the detailed mechanism controlling the nuclear-cytoplasmic shuttling event of β-catenin and any interactions with unspliced and spliced HDAC7 isoforms during SMC proliferation are not clear. We have recently found that HDAC7 interacts with β-catenin in endothelial cells. In this study, we demonstrated that splicing of HDAC7 occurs in SMCs. In addition, the unspliced isoform is crucial for SMC proliferation and neointima formation by modulating the nuclear translocation of β-catenin.

Methods
SMCs were isolated by enzymatic digestion of mouse aortas (wild-type mice) as described elsewhere. SMCs were cultured in conditions as previously described. Small interfering RNA (siRNA) transfection (Supplemental Tables I to III, available online at http://atvb.ahajournals.org) and transient transfection procedures were similar to those reported before. Cell proliferation assays were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Morphological observations were performed as previously described. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Femoral artery wire injury was performed as previously stated. An expanded Materials and Methods section is available in the Supplemental Methods.

Results
Alternative Splicing of HDAC7 in Mice and Humans
Alternative splicing gives rise to numerous protein isoforms with different functions and is a common phenomenon in cardiovascular diseases. We identified and studied 2 isoforms of HDAC7: unspliced HDAC7 (HDAC7u) and spliced HDAC7 (HDAC7s). The HDAC7s mRNA is 57 bp longer in the N-terminal region than HDAC7u mRNA (Supplemental Figure IA and Table IV and V). We examined the genomic sequence surrounding the 57-bp fragment and found that directly adjacent are consensus 5′ and 3′ intron splice sites (Supplemental Figure IA and IIA). Thus, we hypothesize that this fragment is a previously unreported cassette exon. To elucidate the function of both HDAC7 isoforms we subcloned HDAC7 cDNA to produce HDAC7u (deletion of the sequences before the second ATG site) and HDAC7s (deletion of the 57-bp sequence) plasmids. These were further subcloned into adenoviral DNA vectors. A comparison of the major HDAC7 isoforms in mice (Supplemental Figure IB) and humans (Supplemental Figure IC) revealed that a similar pattern of alternative splicing exists in the N terminus of human HDAC7 (Supplemental Figure ID). Human HDAC7u protein shares 85% similarity with mouse HDAC7u protein (Supplemental Figure IIB). It would be interesting to determine the reason for alternative splicing in the N terminus of HDAC7. We examined the expression of both HDAC7 isoforms in mouse aorta and cultured proliferating aortic vascular SMCs (Supplemental Figures IE and IIID). In animal aorta, the expression of HDAC7u and HDAC7s mRNA is almost equal, whereas in cultured SMCs HDAC7s is the dominant isoform detected. Furthermore, we found that HDAC7 mRNA (Supplemental Figure IIIA) and protein (Supplemental Figure IIIB and IIIC) levels were lower in proliferating SMCs in comparison with quiescent SMCs, indicating a correlation with SMC proliferation.

HDAC7u Suppresses SMC Proliferation
To identify the HDAC7 isoform involved in cell proliferation HDAC7u and HDAC7s were overexpressed in SMCs by adenoviral gene transfer at a multiplicity of infection of 5. Adenovirus (Ad)–tetacycline-controlled transactivator–treated and nontreated cells were used as controls. Overexpression of HDAC7u significantly suppressed SMC proliferation, as revealed by 5-bromo-2′-deoxyuridine incorporation assay (Figure 1A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide–based mitochondrial enzyme assay (Figure 1B), and Beckman Coulter–mediated cell number counts (Figure 1C). Morphological observations (Figure 1E) showed that there were significantly fewer cells present after overexpression of HDAC7u with no change in cell size (Supplemental Figure IVA and IVB). Western blot analysis (Supplemental Figure VA and VB) demonstrated successful overexpression of the HDAC7 isoforms. Furthermore, SMCs with enhanced levels of HDAC7u showed G1 phase elongation (Figure 1F) and lowered cyclin D1 protein expression (Figure 1D). These results suggest that overexpression of the different HDAC7 isoforms has different impacts on SMC proliferation. Only HDAC7u is able to suppress SMC proliferation by downregulation of cyclin D1.

Knockdown of HDAC7 Increases SMC Proliferation
Knockdown of HDAC7 significantly increased SMC proliferation compared with nontreated cells, as revealed by 5-bromo-2′-deoxyuridine incorporation (Figure 2A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide activity assay (Figure 2B), and Beckman-Coulter–mediated cell number count (Figure 2C). Control siRNA (ctl siRNA) from 2 different sources did not influence the above parameters. Treatment of SMCs with siRNA targeted against HDAC7 (siHDAC7) resulted in an 80% reduction at the protein level (Supplemental Figure VC and VD). Images of cells 72 hours
after transfection demonstrated that siHDAC7 treatment resulted in increased cell number with no distinguishable differences in cell size (Figure 2E; Supplemental Figure IVC and IVD). Importantly, both HDAC7u and HDAC7s could be significantly inhibited by siHDAC7 (Figure 2F and Supplemental Figure III). As expected, the protein level of cyclin D1 was increased in siHDAC7-treated cells (Figure 2D).

To elucidate the underlying mechanism, we performed double immunofluorescence staining of HDAC7 and β-catenin. Interestingly, in control siRNA-treated (Figure 2G) and untreated cells (Figure 3D, bottom), HDAC7 localized to both the nucleus and the cytoplasm. In these cells, β-catenin localized to the pericellular junctions in most cells and to both the pericellular junctions and the nucleus in a small fraction of cells (Figure 2G, top). In contrast, siRNA-mediated knockdown of HDAC7 increased the fraction of cells containing β-catenin within the nucleus (Figure 2G, bottom, and Supplemental VIE). A cell fraction experiment revealed more β-catenin in the nuclear fraction of siHDAC7-treated SMCs (Supplemental Figure VID and VIF). Real-time polymerase chain reaction (PCR) confirmed this finding as the β-catenin responsive genes, such as cyclin D1, Axin2, Id2, Tcf3, and Tcf4, were upregulated on HDAC7 knockdown (Figure 2H). Cyclin D1 protein level was elevated, and there was no obvious change in β-catenin or active β-catenin level (Figure 2D). These results indicate that knockdown of HDAC7 increases SMC proliferation through increased nuclear translocation of β-catenin and upregulation of β-catenin responsive genes.

**HDAC7u Binds to β-Catenin and Prevents Its Nuclear Localization**

Having determined that knockdown of HDAC7 led to nuclear localization of β-catenin, we hypothesized that HDAC7 may directly interact with β-catenin in SMCs. To test this, we performed endogenous coimmunoprecipitation in SMCs. SMCs in the growth phase were lysed and precipitated with antibodies against HDAC7 and β-catenin. We found that HDAC7 directly binds to β-catenin (Figure 3A and 3B), but not HDAC4 (Supplemental Figure VIIA). We then asked whether both isoforms of HDAC7 can bind to β-catenin with the same affinity, because only HDAC7u is able to suppress SMC proliferation. Interestingly, β-catenin was only detectable in the HA immunoprecipitate after overexpression of HDAC7u (Figure 3C), whereas 14-3-3ζ was detectable in both HDAC7u and HDAC7s precipitate (Supplemental Figure VB). This indicates that only HDAC7u could bind to β-catenin in SMCs.

We subsequently performed double immunofluorescence staining of HA and β-catenin to determine the cellular location of both HDAC7 isoforms and β-catenin. Endoge-
nous HDAC7 localized to both the cytoplasm and the nucleus, whereas β-catenin was found predominantly in the pericellular area (Figure 3D). HA staining demonstrated that HDAC7s was present in both the nucleus and the cytoplasm, whereas HDAC7u was located exclusively in the cytoplasm. We also demonstrated colocalization of HDAC7u and β-catenin. In Ad-HDAC7u-infected cells, β-catenin was barely detectable in the nucleus (Figure 3D and Supplemental Figure VIB), whereas there was no obvious change in β-catenin localization in Ad-HDAC7s-infected SMCs. Cell fraction assay confirmed the localization of HDAC7 isoforms and showed less β-catenin in the nuclear fraction in HDAC7u overexpressing SMCs (Supplemental Figure VIA and VIC). Active β-catenin increased, with no significant change in total β-catenin level (Figure 1D). Furthermore, using a TOPflash/FOPflash reporter assay, we revealed that reduced luciferase activity was induced by the TCF/lymphoid enhancer factor binding site in SMCs cotransfected with HDAC7u (Figure 3E). This indicates reduced nuclear localization of β-catenin. As expected, real-time PCR confirmed that overexpression of HDAC7u downregulated the expression of the β-catenin-responsive genes cyclin D1, Axin2, Tcf3, and Tcf4 (Figure 3F). In summary, these results suggest that HDAC7u and HDAC7s function differently because of their different binding affinities with β-catenin. Specifically, only HDAC7u can bind to β-catenin and retain it in the cytoplasm.

**Identification of the HDAC7 Binding Site With β-Catenin**

After confirming the interaction between HDAC7u and β-catenin, we wanted to identify the region of HDAC7u that is responsible. The sole difference between HDAC7u and HDAC7s is the additional 22 N-terminal amino acids of HDAC7s. We therefore hypothesized that the binding site responsible for the interaction with β-catenin is present in the N-terminal region of HDAC7u. We produced several deletion mutants of HDAC7u which involved deleting different lengths in the N terminus (D1 to D4) and deletion of the C terminus (D5) (Figure 4A). Cotransfection of D1 to D5 alongside Flag-tagged mouse β-catenin in HEK-293 cells indicated that D1 to D4 retained the ability to bind β-catenin, whereas D5 lost this ability. Surprisingly, this identifies the β-catenin binding site to the C-terminal region. If the β-catenin binding site is present in the C terminus, how is it possible that alteration of the N-terminal region affects this interaction?

To shed some light on this conundrum, we produced a deletion mutant lacking the first 15 N-terminal amino acids of HDAC7s, which has the same effect as adding an additional 7 amino acids to the N terminus (D6) (Figure 4A). Cotransfection of D1 to D5 alongside Flag-tagged mouse β-catenin in HEK-293 cells indicated that D1 to D4 retained the ability to bind β-catenin, whereas D5 lost this ability. Surprisingly, this identifies the β-catenin binding site to the C-terminal region. If the β-catenin binding site is present in the C terminus, how is it possible that alteration of the N-terminal region affects this interaction?

To shed some light on this conundrum, we produced a deletion mutant lacking the first 15 N-terminal amino acids of HDAC7s, which has the same effect as adding an additional 7 amino acids to the N terminus (D6). A second deletion mutant, lacking amino acids 23 to 178 of HDAC7s, was also produced (D7). This mutation added the 22 N-terminal amino acids of HDAC7s to the D1 mutant. As revealed by HA-antibody–mediated immunoprecipitation, the D6 mutation lost the ability to bind β-catenin, whereas the D7
because the D6 mutation does not bind to amino acids in the N terminus can disrupt this interaction. Exogenous HDAC7 was indicated by HA staining (red). Scale bars indicate 10 μm. SMCs were infected with adenovirus for tetracycline-controlled transactivator (Ad-tTA), HA-tagged unspliced HDAC7 (Ad-D7u), and HA-tagged spliced HDAC7 (Ad-D7s) at a multiplicity of infection of 5. Seventy-two hours later, the cells were harvested for IP. β-Catenin was detected only in the HA-IP of the Ad-D7u-infected group. D. Unspliced HDAC7 (HDAC7u) overexpression retains β-catenin in the cytoplasm. In nontreated (NT) cells and Ad-D7s infected cells, β-catenin was localized to the pericellular area, cytoplasm, and nucleus. In Ad-D7u-infected cells, β-catenin was localized to the cytoplasm, and very little nuclear localization of β-catenin was detected. Exogenous HDAC7 was indicated by HA staining (red). Scale bars indicate 10 μm. E and F, TOPflash/FOPflash reporter assay (E) and real-time polymerase chain reaction analysis (F) of β-catenin responsive genes, normalized to 18s. *P<0.05, **P<0.01 as compared with empty vector transfection (E) and Ad-tTA (F). Results are derived from 3 independent experiments.

PDGF-BB Increases HDAC7 Splicing
PDGF-BB is a potent stimulator of SMC migration, as well as proliferation, but the mechanism through which PDGF-BB enhances SMC proliferation is not fully understood.36 In contrast to HDAC7s, HDAC7u can suppress SMC proliferation. We hypothesized that PDGF-BB could modulate the ratio of HDAC7u to HDAC7s. There is no antibody currently available that can distinguish between the 2 protein isoforms of HDAC7; therefore, we used reverse transcription PCR to detect the mRNA level of the endogenous HDAC7 isoforms. We have previously found that PDGF-BB increases the promoter activity of HDAC7 and promotes its transcription.11 In this study, we used actinomycin D to inhibit transcription. Treatment of SMCs with PDGF-BB (10 ng/mL) for 30 minutes significantly increased HDAC7s expression (Figure 5A). Using primers specific for HDAC7u, we showed that HDAC7u expression decreased after PDGF treatment, whereas the total level of HDAC7 mRNA was unaffected (Figure 5B). We then performed Western blot analysis to examine the effect of PDGF-BB on HDAC7 splicing at the protein level. The construct of full-length HDAC7 adenovirus was described previously11 (Supplemental Figure IIC). As expected, PDGF-BB significantly increased the expression of HDAC7s at the protein level (Figure 5C). After 6 hours of PDGF-BB (10 ng/mL) treatment, there was a significant upregulation in the Flag:HA ratio (Figure 5D), with no obvious change in the total HA or HDAC7 protein levels. Furthermore, PDGF could abolish genomic HDAC7’s effect on SMC proliferation (Supplemental Figure IX). These results provide solid evidence that PDGF-BB increases HDAC7 splicing in SMCs and offers a novel pathway responsible for PDGF stimulated SMC proliferation.

HDAC7 siRNA Aggravates Neointima Formation in Injured Arteries
To examine whether the role of HDAC7 in SMC proliferation has relevance to disease models, we performed in vivo experiments to examine the role of HDAC7 during neointima formation. HDAC7 siRNA and control siRNA were mixed with pluronic gel-127 and delivered to the adventitial side of injured vessels to assess the effect of HDAC7 knockdown on neointimal formation after endothelial denudation. Previously, a high efficiency of transfection was achieved with pluronic gel containing siRNA that was perivascularly applied to wire-injured artery.37 Two weeks after the operation, the arteries were harvested, fixed, sectioned, and stained with hematoxylin and eosin. Real-time reverse transcription PCR demonstrated that the HDAC7 mRNA and protein levels in HDAC7 siRNA-treated femoral artery were significantly decreased compared with the control group (Figure 5E).
lower than those of the control siRNA-treated arteries (Supplemental Figure X), indicating successful in vivo knockdown. HDAC7 siRNA aggravated neointima formation (Figure 6A). Quantitative analysis and statistics also confirmed this observation (Figure 6B and 6C). Furthermore, we performed proliferation marker Ki67 staining in the arteries 14 days after injury (Supplemental Figure XI) and found significantly increased proliferating cells in intima of siHDAC7-treated arteries. Quantification of reendothelialization revealed no difference in both groups (Supplemental Figure XII). Seven days after injury, we performed HDAC7 and β-catenin double immunofluorescent staining. As indicated in Supplemental Figure XA, there were more cells with nuclear β-catenin localization in siHDAC7-treated arteries (Supplemental Figure XB). Cyclin D1 protein and mRNA levels were also elevated in siHDAC7-treated arteries (Supplemental Figure XC and XD). These results highlight the crucial role of HDAC7 during the pathogenesis of restenosis, which may result from its impact on SMC proliferation.

Discussion
This study sheds light on the functional importance of N-terminal alternative splicing of HDAC7 in modulating SMC proliferation and neointima formation. First, we demonstrated that N-terminal alternative splicing of HDAC7 is a common phenomenon in both mice and humans. Second, we found that HDAC7s and HDAC7u have different impacts on SMC proliferation in mice, which is due to distinct binding affinities with β-catenin. Furthermore, we identified that HDAC7 interacts with β-catenin through a C-terminal binding site, although N-terminal amino acids play a crucial role in regulation of this interaction. The careful balance between HDAC7s and HDAC7u can be influenced by the growth factor PDGF-BB (Supplemental Figure XIII). Finally, siRNA-mediated knockdown of HDAC7 promoted SMC proliferation in vitro and aggravated neointima formation in vivo. Our findings provide a novel mechanistic insight into SMC proliferation and identify a signaling pathway that could be a potential target for disrupting cell growth and a key event during restenosis.
Alternative splicing determines the inclusion of a portion of coding sequence in mRNA and can give rise to protein isoforms that differ in their peptide sequence and biological activity. It is estimated that the minimum number of human gene products that undergo alternative splicing is 60%. Cassette exons, a common form of alternative splicing, are not always present in mature mRNA, as they can either be included or spliced out. Our previous studies have revealed that in mouse embryonic stem cells, there is an additional 57 bp exon located in introns 1 to 2 of HDAC7. This 57-bp sequence contains 3 stop codons that halt transcription from the first start codon (ATG) (Supplemental Figure IA). A shorter protein product, HDAC7u, is produced as transcription subsequently initiates from a second ATG site. However, as stem cells differentiate into SMCs, the expression of the spliced isoform of HDAC7 increases. This isoform lacks the 57-bp exon, allowing transcription to initiate from the first ATG site, giving rise to a protein containing 22 additional amino acids at the N terminus (Supplemental Figure IIB). The function of this domain is not clear. Our results clearly show that the N terminus of HDAC7 is not responsible for the interaction with β-catenin (Figure 4). However, when 7 amino acids were added to the N terminus of HDAC7u (HDAC7-D6), the binding between HDAC7 and β-catenin was abolished. HDAC7-D7, involving the addition of 22 amino acids to the N terminus of HDAC7-D1, allowed us to assess whether additional N-terminal amino acids are sufficient to disrupt the interaction with β-catenin. As indicated in Figure 4B, HDAC7-D7 was still able to bind to β-catenin, implying that the exact sequence of the additional amino acids is not specific. Although the 3-dimensional structure of the HDAC7 protein is not yet available, it is possible that the N-terminal region of HDAC7 is spatially adjacent to the...
C-terminal site, which mediates the binding of HDAC7 to β-catenin. Additional amino acids in the N-terminal region might exclude β-catenin from the binding site, thus inducing a loss of function.

PDGF-BB activates various signal pathways and promotes SMC proliferation,4 but the mechanism is not entirely clear. Several groups discovered that PDGF-BB-induced proliferation of SMCs and fibroblasts requires HDAC activity.39,40 In this study, we found that PDGF-BB induces HDAC7 splicing, leading to an increase in HDAC7s levels while decreasing HDAC7u levels. As HDAC7u suppresses SMC proliferation, the role of PDGF-BB in promoting HDAC7 splicing might be a novel mechanism through which PDGF-BB exerts its function during SMC proliferation. Another interesting finding is that the ratio of HDAC7s:HDAC7u is different between aorta and cultured cells. HDAC7s is the dominant isoform in proliferating cells (Supplemental Figure IE). However, in normal mouse aorta, HDAC7u is almost as abundant as HDAC7s. Proliferating SMCs have lower expression of HDAC7u compared with quiescent SMCs, as well as cells from mouse aorta, indicating a physiological relevance between HDAC7u and SMC proliferation.

As described above, SMCs are mostly in a quiescent phase in normal vessels and only became proliferative in response to endothelial injury, which is a key event in the development of restenosis.41 Here, we used an in vivo mouse injury model and found that siRNA-mediated knockdown of HDAC7 aggravates neointimal formation. Additional experiments reasoned that the increased proliferating cells but not the difference in reendothelialization was responsible for the increased neointima formation. Immunofluorescent staining revealed increased nuclear translocation of β-catenin in siHDAC7-treated arteries, with elevated cyclin D1 mRNA and protein levels. These findings indicate that HDAC7 plays a crucial role in mediating SMC proliferation in vivo.

In summary, the growth factor PDGF can induce SMC proliferation in which HDAC7 exerts its role in signal pathways leading to gene expression. HDAC7 is normally maintained as an unspliced isoform in cytoplasm, where it binds to β-catenin and keeps SMCs in a quiescent state. After PDGF stimulation HDAC7 is spliced and no longer binds to β-catenin. The released β-catenin translocates to the nucleus and binds to TCF to activate gene expression related to cell proliferation (Supplemental Figure XIII). Thus, these findings suggest that HDAC7 might be a promising therapeutic target in atherosclerosis, in particular prompting investigation of whether certain molecules (eg, nitric oxide) could modulate HDAC7 splicing process would be beneficial.

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Disclosures
None.

References


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Supplemental Material

Splicing of HDAC7 Modulates Smooth Muscle Cell Proliferation and Neointima Formation through beta-Catenin Translocation

Boda Zhou, Andriana Margariti, Lingfang Zeng, Ouassila Habi, Qingzhong Xiao, Daniel Martin, Gang Wang, Yanhua Hu, Xian Wang and Qingbo Xu

Department of Physiology (B.Z., X.W.), Peking University, Beijing, China
Cardiovascular Division (B.Z., A.M., L.Z., O.H., D.M., G.W., Y.H., Q.X.), King’s College London BHF Centre, London, UK
Clinical Pharmacology (Q. Xiao), William Harvey Research Institute, Queen Mary University of London, London, UK

Correspondence to: Professor Qingbo Xu, Cardiovascular Division, King’s College London BHF Centre, 125 Coldharbour Lane, London SE5 9NU, Telephone: +44 20 7848 5295 Fax: +44 20 7848 5296, Email: qingbo.xu@kcl.ac.uk

Materials and Methods

Materials
Antibodies [goat anti-HDAC7 (C-18, sc-11491), rabbit anti-HDAC7 (sc-1142), rabbit anti-β-catenin (H102, sc-7199), goat anti-β-catenin(C-18, sc-1496), mouse anti-Cyclin D1 (A-12, sc-8396), rabbit anti-14-3-3 ζ (C-16, sc-1019)] were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA); Antibodies [mouse anti-α-tubulin (Clone B-5-1-2, T5168), mouse anti-HA (Clone HA-7, H9658), mouse anti-Flag (clone M2, F3165), mouse anti β-actin (clone AC-15, A1978), monoclonal anti-HA agarose conjugated antibody (A2095) and monoclonal anti-Flag agarose conjugated antibody (F2426)] were purchased from Sigma (St Louis, MO, USA). Antibodies [rabbit anti-Ki67 (ab15580), rabbit anti-HDAC7 (ab1441), rabbit anti-HDAC4 (ab11968)] were from Abcam (Cambridge, UK). Anti active β-catenin antibody (05-665) was from Millipore (Billerica, MA). All secondary antibodies for western blot were from Dako (Glostrup, Denmark). Secondary antibodies or immunofluorescence were from Invitrogen and Dako. Recombinant PDGF-BB (P4056) was purchased from Sigma. The Topflash and Fopflash reporter plasmids were from Millipore (21-170, 21-169).

Cell Culture
SMCs were isolated by enzymatic digestion of mouse aortas (wild-type mice) as described elsewhere 1,2 and were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2. The medium was changed every 2 days, and cells were passaged by treatment with 0.2% trypsin, 0.02% EDTA solution. Cells up to passage 10 were used for experiments. HEK-293 cells were purchased from ATCC (CRL-1573), and maintained in DMEM supplemented with 10% FBS and penicillin / streptomycin. Living cell images were assessed by Nikon Eclipse TS100 microscope with Ph1
ADL 10X/0.25 objective lenses and Nikon DS-Fil camera at room temperature and processed by Adobe Photoshop software.

Quiescent SMCs were SMCs in confluent condition and kept culturing for 3 days with medium changed everyday.

**Plasmid and Adenovirus Construction**

The expression vectors pShuttle2-HDAC7u (HDAC7u) and pShuttle2-HDAC7s (HDAC7s) and corresponding Ad-HDAC7 viral DNA (Ad-HDAC7u and Ad-HDAC7s) were constructed as described previously. Mouse β-catenin (NM_007614.2) was cloned into Flag-tagged pCDNA3 vector (invitrogen). The deletions of HDAC7 (D1-D7) were subcloned from the HDAC7u and HDAC7s plasmids. The primers used for plasmid construction are listed in supplemental Table S1. All plasmids were verified by DNA sequencing.

**RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Real Time PCR**

RT-PCR and Real time PCR were performed as described previously. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 1 µg RNA were reverse transcribed into cDNA using random primers by MMLV reverse transcriptase (RT) (Promega). 20-50ng cDNA (relative to RNA amount) was amplified by standard PCR with Taq DNA polymerase (Invitrogen) or real time PCR SYBR master mix (Applied Biosystems). Primers are listed in supplemental Table S2 and S3. PCR primers for real time PCR were designed using Primer Express software (Applied Biosystems).

**Immunoblotting**

Cells were harvested and washed with cold PBS, resuspended in lysis buffer (25mM Tris-Cl pH 7.5, 120mM NaCl, 1 mM EDTA pH 8.0, 0.5% Triton X100) supplemented with protease inhibitors (Roche) and lysed by ultrasonication (twice, 6 seconds each) (Bradson Sonifier150) for whole cell lysate.

For cell fraction assay, Cells were harvested and lysed in hypotonic buffer (10 mM HEPES-KOH pH 7.2, 1.5 mM MgCl₂, 10 mM KCl) and high-salt buffer (20 mM HEPES-KOH pH 7.2, 25% glycerol, 1.5 mM MgCl₂, 420 mM KCl, 0.2 mM EDTA) supplemented with protease inhibitors and 0.5% NP-40 for nuclear and cytoplasmic fractions.

For western blot analysis of tissue from mice, the tissue was lysed in lysis buffer (25mM Tris-Cl pH 7.5, 120mM NaCl, 1 mM EDTA pH 8.0, 0.5% Triton X100, PI) with the assistance of Fastprep FP 120(Thermo) meachine and Lysing Matrix D (MP Biomedicals).

The protein concentration was determined using the Biorad Protein Assay Reagent. 50µg of whole lysate was applied to SDS-PAGE and transferred to Hybond PVDF membrane (GE Health), followed by standard western blot procedure. The bound primary antibodies were detected by the use of horseradish peroxidase (HRP)-conjugated secondary antibody and the ECL detection system (GE Health). The band density was semi-quantified by Adobe Photoshop software as previously described.

**Indirect Immunofluorescence Assay**

Immunostaining of SMCs overexpressing HDAC7 or control was performed as previously described. Briefly, the cells were fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS for 10 min and blocked in 10% serum in PBS for 30 min at 37°C. Incubation with the primary antibodies (goat HDAC7& rabbit β-catenin; mouse HA& rabbit β-catenin) was performed overnight at 4°C. The bound primary antibodies were revealed by...
incubation with fluorescence conjugated secondary antibody (donkey anti goat ALEXA 546 & swine anti rabbit FITC; goat anti mouse ALEXA 594 & goat anti rabbit ALEXA 488) at 37°C for 30 min. Cells were counterstained with 4', 6- diamidino-2-phenylindole (DAPI; Sigma) and mounted in Floromount-G (DAKOCytomation, Glostrup, Denmark) and examined with SP5 confocal microscope (Leica, Germany). Magnification is indicated as scale bar in Figures.

For the staining of animal tissue, the tissue was fixed with cold acetone for 15 min in 4°C, permeabilised with 0.1% Triton X-100 in PBS for 15 min, following by standard indirect immunofluorescent protocol. Primary antibodies were incubated 1 hour at 37°C (rabbit HDAC7 (abcam) & goat β-catenin). Secondary antibodies were incubated 30 min at 37°C (swine anti rabbit TRITC & donkey anti goat ALEXA 488).

**Immunohistochemical staining.**
For immunohistochemical staining, femoral arteries were harvested and frozen in liquid nitrogen directly. Sections were fixed in cold acetone for 15 min, permeabilised with 0.1% Triton X-100 in PBS for 15 min. Endogenous peroxidase activity was quenched by incubation with 3% H2O2 for 20 min. After washing with PBS, the sections were blocked with 10% normal goat serum in PBS for 1 hour and incubated with the primary antibody rabbit Ki67 1 hour at 37°C. Subsequently, the bound primary antibodies were detected by incubation with goat anti-rabbit IgG–HRP conjugated for 30 min. Peroxidase activity was assessed using DAB reagent, all sections were counterstained with Mayer’s hematoxylin for 3 min and mounted on the slides.

**Adenoviral Gene Transfer**
Ad-HDAC7u and Ad-HDAC7s were prepared as described before. For adenoviral gene transfer SMCs were seeded on gelatin-coated flasks 24h prior to infection. Infection was performed for 12 h and fresh medium was added to the cells. Ad-tTA virus (Tetracycline-controlled transactivator tTA adenovirus, commercially available) was included as control and to compensate for the MOI. Medium was changed 24h after infection, and cells were harvested at the time indicated after infection.

**siRNA Transfection**
The HDAC7 siRNA (sc-35547) and control siRNA (sc-37007) were purchased from Santa Cruz Biotech, another control siRNA (Cat. No: 4611) was purchased from Ambion Ltd (Huntingdon, UK). SMCs were seeded in 6-well plate at 5 X 10^5 cells/well 24 h before transfection. For siRNA transfection, 10 µl/well of 10 µM HDAC7 siRNA or control siRNA was transfected with siIMPORTER reagents (Millipore) according to protocol provided. Medium was changed 24h after transfection, and cells were harvested for analysis 72h after transfection.

**MTT Assay**
SMCs were infected with Ad-HDAC7u, Ad-HDAC7s and Ad-tTA, or transfected with HDAC7 siRNA and control siRNA. A sample of non-treated cells was used as a control in both groups. 24 h after adenovirus infection or siRNA transfection, the cells were sub-cultured into 96-well plate at 2000 cells/well with medium changed next day. Cell proliferation assay was performed 72h after infection or transfection with CellTiter 96 Queous One Solution Cell Proliferation Assay kit (Promega, Cat No. G3580) according to manufacturer’s protocol. The absorbance at A490nm was measured and analysed as an indication of MTT activity.

**BrdU Incorporation**
SMCs were infected with Ad-HDAC7u, Ad-HDAC7s and Ad-tTA, or transfected with HDAC7 siRNA and control siRNA. A sample of non-treated cells was used as a control. Forty-eight hours after infection or transfection, the cells were dispensed with trypsin and counted, followed by re-plating into 12-well plates at 1 X 10^4 cells/well, 3 wells in each plate for each group. 20 hours
later, cells in one 12-well plate were labeled with BrdU for 4h. Then, the BrdU labeled cells were subjected to BrdU incorporation assay (5-Bromo-2’-deoxy-Uridine Labeling and Detection Kit III, Ref 11444611001, Roche) according to protocol provided. All assays were performed at 72 hours after infection. The fold of induction for BrdU incorporation was defined as the ratio of A405nm for BrdU incorporation assay to A490nm for proliferation assay with that of control group set as 1.0.

**Cell Size Calculation and Cell Count**

For calculation of the cell size and cell count, SMCs were infected with Ad-HDAC7u, Ad-HDAC7s and Ad-tTA, or transfected with HDAC7 siRNA and control siRNA. Non-treated SMCs are included as a control in both groups. 72 hours after infection of SMCs, the cells were trypsinized and subjected to cell size calculation using a multisizer 3 counter (Beckman Coulter), according to the manufacturer’s instructions. For the live cell area analysis, live cells were taken picture with Nikon Eclipse TS100 microscope and Nikon DS-Fil camera. The image was analyzed later with Axiovision 4 software, the mean value of 100 cells from each treatment was statistically analyzed.

**FACS Analysis**

Forty-eight hours post infection of SMCs with Ad-HDAC7u, Ad-HDAC7s or Ad-tTA, the cells were harvested by incubating cell cultures with dissociation buffer (GIBCO). The harvested cells were fixed in 70% ethanol for at least 30 minutes and washed twice in PBS. Fixed cells were incubated with PBS containing propidium iodide (PI) and RNase at 37°C for 45 minutes, and analysed with a FACS scan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Forward and 90° side scatter were used to identify cell population and single cell fractions were gated out to perform further analysis of PI staining intensity. Data analysis was carried out using CellQuest software (Becton Dickinson).

**Co-immunoprecipitation (co-IP)**

SMCs were infected with Ad-HDAC7u, Ad-HDAC7s and Ad-tTA for 12h, followed by further incubation with growth medium for 36h. The cells were lysed by 1h rotation at 4°C in lysis buffer (25mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X100) supplemented with protease inhibitors (Roche). One milligram of whole lysate was subjected to standard co-immunoprecipitation procedure. Briefly, lysates were pre-cleared with normal IgG and then incubated with HA conjugated beads (Sigma) overnight at 4°C. Precipitated proteins were washed 3 times with IP-wash buffer (25mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0, 0.5 % Triton X100), resolved by SDS gel electrophoresis. Western blot was performed to analyse the precipitate. For endogenous co-IP, SMCs were harvested when 80% confluent, lysed, and the protein concentration measured. 1 mg of whole lysate were pre-cleared with normal IgG and then incubated with HDAC7 or β-catenin antibody for 12h at 4°C, then 2h with protein G beads (E3403 Sigma). Precipitated proteins were washed and resolved by SDS gel electrophoresis and subsequently western blot. Since the transfection efficiency of SMCs is low, the co-IP for deletions of HDAC7 and β-catenin was done in HEK-293 cells. HEK-293 cells were co-transfected with 2 µg HDAC7 or deletions (HDAC7 D1-D7) and 2 µg β-catenin using Fugene 6 (Roche 11814443001). Medium was refreshed 24h after transfection and cells were lysed and precipitated 48h after transfection with HA conjugated beads followed by western blot analysis.

**PDGF Treatment**

For PDGF-BB treatment, SMCs were seeded on gelatin coated plates/flasks for 24h cultured with complete medium (10% FBS in DMEM). Cells were cultured in the absence of serum for 12 hours prior to PDGF treatment. Then PDGF-BB (10ng/ml) was added into the medium. During PDGF treatment no serum was included in the media. For overexpression of HDAC7, SMCs were
infected with Ad-HDAC7 with complete medium and left for 36h. Then medium was changed to 0% FBS medium for 12h, before adding PDGF-BB (10ng/ml). Cells were harvested and subjected to RT-PCR or western blot after the time of PDGF treatment as indicated in the figures.

**Luciferase Activity Assay**
For transient transfection, SMCs were seeded on gelatin-coated 6 well plate at 5 X 10^5 cells/well 24h prior to co-transfection with TOPFLASH or FOPFLASH reporter (2μg/well) (Millipore) and HDAC7 or pshuttle 2 (2μg/well) using Fugene-6-Reagent (Roche Molecular Biochemicals), according to the manufacturer’s instructions. Renilla-Luc (0.5μg/well) was included in all transfection assays as an internal control. Forty-eight hours later, firefly and renilla luciferase activity was assessed with respective assay kit (Promega). The relative luciferase activity (RLU) was defined as the ratio of reading for firefly luciferase to that for renilla luciferase with that of control group set as 1.0.

**Animal Artery Injury**
All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animal. C57BL/6J mice were purchased from Charles River (Margate, Kent, UK), wild type mice were anesthetized and the surgical procedure was similar to that described previously 6. Removal of the endothelium of the femoral arteries was achieved by 3 passages of a 0.25 mm angioplasty guide wire (Brivant Ltd, Ireland). 5 μg (1μg/μl) of the HDAC7 siRNA (santa cruz sc-35547) and a random control siRNA (santa cruz sc-37007) dissolved in 30% pluronic gel-127 solution were perivascularly delivered to the left or right femoral artery immediately after injury, respectively. On contact with the tissue, the solutions gelled immediately, forming a translucent layer that enveloped the injured femoral artery. The wound was closed after gel layer formation. 14 days later, animals were euthanised, and injured arteries harvested, fixed, sectioned, and stained with H&E.

**Quantification of Reendothelialization**
The procedure was similar to that described previously 7,8. TIE2-LacZ transgenic mice expressing β-gal under the control of the endothelial-specific protein TIE2 promoter (The Jackson Laboratory) were crossed to C57BL/6J mice 6 generations in our laboratory before performing the experiments. Endothelium-specific enhancer in the first intron of the mouse TIE2 gene was introduced. The combination of the TIE2 promoter with an intron fragment containing this enhancer allows it to target reporter gene expression specifically and uniformly to virtually all vascular ECs throughout embryogenesis and adulthood. Briefly, vessel segments were incubated at 37°C for 18 hours in PBS supplemented with 1 mg/mL X-Gal (Sigma). Vessel segments were rinsed with 3% DMSO in PBS and mounted with the endothelium facing up on a glass slide. Images were assessed by Zeiss Axioplan 2 Imaging microscope with 20X objective lenses, AxioCam camera and Axiovision software at room temperature, and were processed using Adobe Photoshop software. Beta-gal+ cells on the denudated surface of the artery were accounted under the microscope, and compared between two groups.

**Statistical Analysis**
Data expressed as the means±SEM were analysed 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.

**References**
muscle cell migration. *FASEB J.* 2003;17:2106-2108


Supplemental Figure I. Alternative splicing of HDAC7 in mice and humans. A. Mouse genomic HDAC7 is located on chromosome 15 and contains 24 exons (Ex1-Ex24) and 23 introns (intron1-2, intron2-3, etc). The 57bp sequence is located on chromosome 15: 97,657,222-97,657,166 and contains 3 stop codons which halts the transcription from the first ATG. The protein product of unspliced HDAC7 (HDAC7u) lacks the first 22 N-terminal amino acids as compared with spliced HDAC7 (HDAC7s). B. Major reported HDAC7 isoforms in mice; HDAC7s and HDAC7u. C. Major reported HDAC7 isoforms in humans. D. Comparison of mouse HDAC7s and HDAC7u with human HDAC7. The N-terminal alternative splicing event in humans is similar to that in mice. E. Ratio of HDAC7u and HDAC7s mRNA in mouse aorta and mouse aortic SMCs in culture. The total level of HDAC7 is lower in aorta and the ratio of HDAC7s:HDAC7u is lower in aorta than cultured cells. Real time PCR results are listed in Fig. S9D. *P<0.05. n=3 in E.
Supplemental Figure II. A novel HDAC7 exon. A. The mouse HDAC7 gene contains a 19.7kb long intron between Exon1 (Ex1) and Exon2 (Ex2). Cloning of HDAC7 in mouse embryonic stem cells revealed an additional 57bp exon between Ex1 and Ex2 (refer to FigS1A). The new exon is located at 97,657,166-97,657,222 of the 19.7kb intron. Further analysis found that adjacent sequences to the 57bp exon have the characteristics of intron start (GU) and intron end (AG) sites. Thus the 57bp exon might be an un-reported cassette exon.

B. Protein blast result of human unspliced HDAC7 (NP_001091886.1 or Q8WUI4-7) with mouse unspliced HDAC7 (Q8C2B3-5).

C. Model to investigate HDAC7 splicing in the protein level. A Flag-tag was fused to the N-terminus of full-length HDAC7, whilst an HA-tag was added to the C-terminus. In spliced HDAC7 the intron could be removed and the transcription could start from the first ATG, thus express both the Flag and HA tag. In unspliced HDAC7, transcription starts from the second ATG, thus only expresses the HA tag.
Supplemental Figure III. HDAC7 expression in quiescent, proliferating SMC and aortic cells.

A. Quiescent and proliferating SMCs were harvested for real-time reverse transcription PCR. B. Quiescent and proliferating SMCs were harvested for Western blot analysis. C. Densitometry quantification of Western blot in B, *p<0.05, comparing with quiescent SMC. D. Real-time PCR analysis of total HDAC7 and unspliced HDAC7 levels in mouse aorta and proliferating aortic vascular SMCs, normalized to 18s. **P<0.01 comparing with proliferating cell. Results are data of 3 independent experiments (A,B,C and D).
Supplemental Figure IV. SMC cell size after adenovirus infection and siRNA transfection.

A, B. SMCs were untreated (NT) or infected/with Ad-HDAC7u (Ad-7u), Ad-HDAC7s (Ad-7s), and Ad-tTA at 5 MOI. C, D. SMCs were untreated (NT) or transfected with control siRNA and HDAC7 siRNA. A, C. The cell size was measured with Beckman Coulter 72h after infection/transfection. Results were analysed by Beckman Multisizer 3 software following the manufacturers protocol. The mean particle diameter was statistically analyzed. Particles within 95% range of the mean particle diameter were analyzed in Figures 1C and 2C. B, D. Photographs were taken from live cells and the area of each cell was analyzed by Axiovision 4 software. 100 cells from each treatment were statistically analyzed. Results are derived from 3 independent experiments (A,B,C and D).
Supplemental Figure V. Western blot of overexpression and knockdown of HDAC7. A. Effect of adenovirus mediated overexpression of HDAC7. SMCs were untreated (NT) or infected with adenoviruses Ad-HDAC7u (Ad-7u), Ad-HDAC7s (Ad-7s), and Ad-tTA at 5 MOI. 72h after infection, cells were harvested for Western blot analysis. B. Densitometry statistical analysis of data from panel A (n=4). *p<0.05 comparing with NT. C. Effect of siRNA. SMCs were untreated (NT) or transfected with control (ctl siRNA) or siRNA targeted against HDAC7 (siHDAC7). 72h after infection, cells were harvested for Western blot analysis. D. Densitometry statistical analysis of data from panel C (n=4). **p<0.01 comparing with NT.
Supplemental Figure VI. HDAC7 and β-catenin nuclear translocation. A, D. Cellular localization of HDAC7 and β-catenin was detected by Western blot in the cytosolic and nuclear fractions. α-tubulin and histone H4 were included as loading controls for cytoplasmic and nuclear fractions. C, F. Densitometry and statistical analysis of nuclear/cytosolic β-catenin in panels A and D, B, E. Quantification of cells in Figure 3D and 2F. 300 cells were counted, the number of cells with nuclear localization of β-catenin was statistical analyzed. *p<0.05, n=3 in A, C, D and F.
Supplemental Figure VII. Co-Immunoprecipitation experiments. A. Co-immunoprecipitation (co-IP) assays between endogenous HDAC4 and β-catenin in SMCs. B. IP with HA. SMCs were infected with adenovirus for tTA (Ad-tTA), HA-tagged HDAC7u (Ad-D7u), and HA-tagged HDAC7s (Ad-D7s) at 5 MOI. 72h later the cells were harvested for IP. 14-3-3 ζ was only detected in the HA-IP of both the Ad-HDAC7u and Ad-HDAC7s infected group. C. protein expression of HDAC7 in human umbilical vein endothelial cells (HUVEC) and human vascular SMCs. Results were derived from 3 independent experiments in A, B and C.
Supplemental Figure VIII. Input for HDAC7 deletions. A. HEK-293 cells were co-transfected with the different HA-tagged HDAC7 deletion mutants alongside Flag-tagged mouse β-catenin. The whole cell lysate was subjected to HA-IP 48h after transfection. All deletions and Flag–tagged β-catenin were expressed in HEK-293 cells as demonstrated by the detection of HA and Flag in the input. Images are representative of 3 independent experiments. B. HEK-293 cells were co-transfected with HA-tagged HDAC7 deletions alongside Flag-tagged mouse β-catenin. Whole cell lysates were subjected to HA-IP 48h after transfection. Each deletion mutant was expressed in HEK-293 cells as demonstrated by the detection of HA in the precipitates. All the deletion mutants except D5, D6 and HDAC7s showed specific binding with Flag-tagged β-catenin.
Supplemental Figure IX. expression of HDAC7 isoforms. A. 1mg of whole lysate protein from growing phase SMCs was subject to co-immunoprecipitation (co-IP) with goat IgG or goat HDAC7, along with 25ug of input, were then probed with rabbit HDAC7. α-tubulin severed as a control for contamination. B. Same amount of SMCs were infected with adenovirus for tTA (Ad-tTA), HDAC7u (Ad-HDAC7u), HDAC7s (Ad-HDAC7s) and genomic HDAC7 (Ad-HDAC7o) at 5 MOI. 12h later the medium was changed into 1% serum medium, one group of Ad-HDAC7o infected cells had additional 10 ng/ml PDGF-BB in the medium. The medium was changed every 12 hours and BrdU synthesis was measured 48 hours later. Results were derived from 3 independent experiments in A, B. **P<0.01, *P<0.05 as compared with Ad-tTA.
Supplemental Figure X. Knockdown HDAC7 in femoral arteries induces β-catenin nuclear translocation. A. Femoral arteries were harvested 1 week after injury and frozen sections were prepared. HDAC7 and β-catenin double immunofluorescent staining was performed and images were taken with confocal microscope. Scale bars indicate 25 µm. B. The percentage of cells with nuclear localisation of β-catenin was statistically analyzed. *p<0.05 as compared with ctl siRNA. C. Femoral arteries were harvested 1 week after injury for Western blot analysis. D. Femoral arteries from both ctl siRNA and siHDAC7-treated mice were subjected to RNA extraction and subsequent reverse transcription real time PCR. Expression of HDAC7 was normalized to 18s. *P<0.05 as compared with ctl siRNA. n=3 in each group(A,B,C and D).
Supplemental Figure XI. Ki67 staining in siRNA-treated femoral arteries. A. Femoral arteries were harvested 14 days after injury and frozen sections were prepared. Ki67 immunohistochemical staining was performed. Scale bars indicate 25 µm. B. The number of Ki67 positive nuclei was divided by the number of total nuclei in media or in intima. The ratio was statistically analyzed. C. The number of all cells in media or intima was shown, p<0.05, n=3.
Supplemental Figure XII. Re-endothelialization in siRNA treated femoral arteries. Femoral arteries of TIE2-LacZ mice were harvested 7 or 14 days after injury and prepared for en face staining with beta-gal. A. Representative images 1 week after injury (lower panel). Scale bars indicate 50 µm. B. The number of beta-gal positive cells were accounted and statistically analyzed, Data are means±SEM (n=4).
Supplemental Figure XIII. Model for the role of HDAC7 splicing in modulating SMC proliferation. There are two HDAC7 isoforms in SMCs, spliced (HDAC7s) and unspliced (HDAC7u). HDAC7s is located in the cytoplasm and the nucleus whilst HDAC7u is only present in the cytoplasm. HDAC7u directly binds to β–catenin and sequesters it in the cytoplasm. This prevents its nuclear translocation and subsequent activation of TCF responsive genes e.g. cyclin D1. HDAC7s could not bind to β–catenin. Thus HDAC7u and not HDAC7s can suppress SMC proliferation. The ratio between the two isoforms is influenced by PDGF stimulation. PDGF-BB increases the expression ratio between HDAC7s to HDAC7u. This results in increased SMC proliferation.
Supplemental Figure XIV. HDAC7u suppresses human SMC proliferation.

**A.** Human SMCs were infected with Ad-HDAC7u (Ad-7u), Ad-HDAC7s (Ad-7s), and Ad-tTA at MOI of 5, BrdU incorporation was detected 72h after infection.

**B.** Representative images of the cells. C. Cells were harvested 72h after infection for Western blot analysis. D. Cells were harvested 72h after infection for co-immunoprecipitation analysis, *p<0.05 comparing with Ad-tTA. Results are representative of 3 independent experiments (A, B, C and D).
## Supplemental Table I. Primers used to clone HDAC7 and β-catenin.

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### Supplemental Table II. Primers used to investigate HDAC7 splicing

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### Supplemental Table III. Real time PCR primers of HDAC7 and β-catenin responsive genes.

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Supplemental Table IV. cDNA sequence of spliced HDAC7.

**HDAC7 cDNA sequence (spliced HDAC7) cloned from differentiated mouse embryonic stem cells (day 9 towards smooth muscle cells)**

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accctgcaac acccccaacg cctgcaccgc catctcttcc tggcaggctt acaccagcaa
cagcgctcag ccgagcccat gaggctctcc atggacccac caatgccgga gctgcagggg
ggacagcagg agcaagcagtc tgcgctgctg ccacgctgctg ccacgctgctg
```
Supplemental Table V. cDNA sequence of unspliced HDAC7.

HDAC7 cDNA sequence (unspliced HDAC7) cloned from undifferentiated mouse embryonic stem cells

```
atgcacagccccggcgcggactagaagtgagtccccacagcctgctaagacattctcggaagctccctgga
cccaggctgccc
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aagttgcgct acaaacccaa gaaatccctg gagagacgca agaatcccct gctcaggaag
```

**Note:** The sequence provided is a segment of the cDNA sequence of HDAC7 cloned from undifferentiated mouse embryonic stem cells. It includes the unspliced region of the gene, which is crucial for understanding the full-length transcript. Further analysis would be required to understand the complete gene structure and its expression in various cellular contexts.