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Epigenetic Regulation of Vascular Smooth Muscle Cell Proliferation and Neointima Formation by Histone Deacetylase Inhibition

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Abstract

Objective—Proliferation of smooth muscle cells (SMC) in response to vascular injury is central to neointimal vascular remodeling. There is accumulating evidence that histone acetylation constitutes a major epigenetic modification for the transcriptional control of proliferative gene expression; however, the physiological role of histone acetylation for proliferative vascular disease remains elusive.

Methods and Results—In the present study, we investigated the role of histone deacetylase (HDAC) inhibition in SMC proliferation and neointimal remodeling. We demonstrate that mitogens induce transcription of HDAC 1, 2 and 3 in SMC. siRNA-mediated knock-down of either HDAC 1, 2 or 3 and pharmacologic inhibition of HDAC prevented mitogen-induced SMC proliferation. The mechanisms underlying this reduction of SMC proliferation by HDAC inhibition involve a growth arrest in the G_1-phase of the cell cycle due to an inhibition of retinoblastoma protein phosphorylation. HDAC inhibition resulted in a transcriptional and posttranscriptional regulation of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip}$. Furthermore, HDAC inhibition repressed mitogen-induced cyclin D1 mRNA expression and cyclin D1 promoter activity. As a result of this differential cell cycle-regulatory gene expression by HDAC inhibition, the retinoblastoma protein retains a transcriptional repression of its downstream target genes required for S phase entry. Finally, we provide evidence that these observations are applicable *in vivo* by demonstrating that HDAC inhibition decreased neointima formation and expression of cyclin D1 in a murine model of vascular injury.

Conclusion—These findings identify HDAC as a critical component of a transcriptional cascade regulating SMC proliferation and suggest that HDAC might play a pivotal role in the development of proliferative vascular diseases, including atherosclerosis and in-stent restenosis.

Keywords

Vascular smooth muscle cells; HDAC; cell cycle; proliferation; cyclin D1

The majority of cardiovascular diseases are the result of vascular remodeling underlying the development of atherosclerosis and its occlusive complications.[1] In addition to endothelial dysfunction and macrophage-derived inflammation, proliferation of SMC constitutes an essential component for atherosclerosis formation and neointimal remodeling.[2] Vascular SMC resident in the normal arterial wall are quiescent but migrate, proliferate, and secrete

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extracellular matrix proteins in response to cues released at sites of vascular injury.[1] Once luminal obstruction in the course of atherosclerotic remodeling occurs, revascularization procedures represent primary treatment strategies.[3] Unfortunately, the clinical success of these procedures is limited by postangioplasty restenosis, transplant vasculopathy, and coronary artery bypass graft failure; and SMC proliferation has been attributed a critical role in the pathophysiology of these treatment failures.[1–2] Therefore, understanding the molecular mechanisms governing SMC proliferation may yield valuable insights into the pathogenesis of all major vascular diseases that display detrimental SMC phenotype behavior.

Expression profiling of SMC has revealed a multitude of genes that are transcriptionally regulated in response to mitogenic stimulation, which suggests that there is an upper level of regulation in SMC controlling entire gene expression programs rather than a single gene.[4] Transcriptional gene activation involves dynamic chromatin remodeling and histone modifications mandatory either for restricting or permitting binding of transcription factors and assembly of functional pre-initiation complexes.[5] This hierarchic upper-level of chromatin remodeling provides an additional layer of transcriptional control beyond those associated with variation in the sequence of RNA or DNA itself and is referred to as epigenetic mechanism of gene regulation.[6]

Modification of histones by acetylation, regulated by the balance of histone acetyltransferases (HAT) and histone deacetylases (HDAC), results in the loosening of histone-DNA contacts making DNA more accessible for transcription and gene activation.[7] Pharmacological HDAC inhibitors have been developed and shown to induce growth arrest and apoptosis in cancer cells, indicating that the net effect of histone acetylation is an inhibition of cell proliferation.[8] Although surprisingly little is known about the epigenetic regulation of vascular gene expression programs, recent studies have indicated that histone acetylation regulates SMC differentiation,[9] endothelial cell proliferation,[10] and inflammatory pathways,[11] processes that are critical for cardiovascular disease development. In the current study, we report that HDAC expression is increased in response to mitogenic stimulation of SMC and required for proliferation by regulating $G_1 \rightarrow S$ phase progression of the cell cycle. Furthermore, our *in vivo* experiments characterize histone acetylation as a previously unrecognized critical event for neointima formation in response to vascular injury.

Materials and Methods

Cell culture

Rat aortic SMC were isolated and cultured as described previously.[12] Cells were serum-deprived in 0.01% fetal bovine serum (FBS, Gibco) for 48 h, pretreated with 6 μM of the broad-spectrum HDAC-Inhibitor Scriptaid[13] (Enzo Life Sciences) or DMSO for 30 min followed by stimulation with 10% FBS or 50 ng/ml PDGF as indicated. For all data shown, cells were used between passages 3 and 7, and individual experiments were repeated at least 3 times with different cell preparations.

Cell proliferation assays

SMC proliferation was analyzed by cell counting using a hemocytometer. In addition, proliferation was analyzed by means of cell division using 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma). Cells were labeled with CFSE at 37° C for 10 min and washed. One plate was immediately fixed in 10% formalin and stored in 80 % ethanol for baseline analysis. The remaining cells were split at a density of 6×10^3 cells/cm^2 and allowed to adhere overnight. The following day cells were starved for 24 h, pretreated with

Scriptaid or DMSO for 30 min and stimulated with 10 % FBS. After an additional 48 h, cells were analyzed by FACS to determine fluorescence of the daughter cells.

Apoptosis-Assay

Serum-deprived SMC were pretreated with Scriptaid or DMSO and stimulated with 10 % FBS. Apoptosis was analyzed after 24h using the Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen).

Cell cycle analysis

Serum-deprived SMC were pretreated with Scriptaid or DMSO and stimulated with 10 % FBS or 50 ng/ml PDGF. At baseline and 24 h after stimulation cells were fixed in ice cold 70% ethanol. Cells were incubated in PBS containing 40 μg/ml RNase for 30 min at 37°C and resuspended in PBS containing 50 μg/ml propidium iodide. Cell cycle distribution was analyzed using a FACSCalibursorting system (Becton Dickinson).

RNA isolation and quantitative real-time RT-PCR

RNA was isolated and reverse transcribed as described.[12] Quantitative real-time PCR analysis of target gene expression was performed using an iCycler and SYBR Green I system (Bio-Rad) as described.[12] Each samplewas analyzed in triplicate and normalized to mRNA expression of the house-keeping gene rpl13a. Primer sequences are available on request.

Western blotting

Western Blotting for protein expression was performed as described previously.[12] SMC were harvested in cell lysis buffer, and whole cell proteins were subjected to immunoblotting using the following antibodies: HDAC 1 (5356, Cell Signaling), HDAC 2 (5113, Cell Signaling), HDAC 3 (3949, Cell Signaling), p21 (ab7960, Abcam), p27 (ab7961, Abcam), Cyclin D1 (2922, Cell Signaling), GAPDH (sc-25778, Santa Cruz), Phospho-Rb (Ser807/811) (9308S, Cell Signaling) and Total Rb (554136, BD Pharmingen). Quantification of the Western blots was performedby densitometry and normalization to GAPDH.

HDAC siRNA transfection

SMC were transfected twice during 24 h with 100 nM siRNA against HDAC 1, HDAC 2, HDAC 3 or HDAC 6 (Dharmacon) using Lipofectamine 2000 (Invitrogen). Following serum-deprivation for 24 h, synchronized cells were stimulated with 10 % FBS and used for analysis of cell proliferation or mRNA expression.

Chromatin immunoprecipitation assay (ChIP)

ChIP-assays were performed using the MAGnify ChIP-Kit (Invitrogen) according to the manufacturer's instructions. Briefly, serum-deprived SMC were pretreated with Scriptaid or DMSO and stimulated with 10 % FBS for two hours. Cells were harvested and soluble chromatin was prepared. Chromatin was immunoprecipitated using an antibody directed against histone H3 acetylated at lysine 9 (9649S, Cell signaling) or control IgG (Invitrogen). Final DNA extractions were PCR-amplified using the following primer pairs that cover the E-box or EGR1 binding sites in the rat cyclin D1 promoter: E-box forward: 5′-CTT CGC CTG TTA CAC AGT TCC TGA AT -3′ and reverse: 5′-ACA CCC TAT ACT TAA GCG GAG AGA ATG -3′; EGR1 forward: 5′-CGG CGA TTT GCA TAT CTA CGA AGG-3′ and reverse: 5′-AAG CCG GGC AGA GAA AAA GGA G-3′.

Endovascular femoral artery guide wire injury

Guide wire endothelial denudation injuries were performed in C57BL/6J mice (The Jackson Laboratory) at 8 weeks of age as described.[12] Mice were injected with Scriptaid (3.5 μg/g body weight i.p.) or DMSO the day before injury, on the day of the injury, and every other day thereafter for 28 days. The dose of Scriptaid used in this study was based on the doses used in previous murine studies.[14–15] Vessels for analysis of mRNA expression were collected in TRIzol 48 h after injury. The intimal and medialareas were measured after 28 days by computerized morphometry using the Image-Pro Plus 5.0 software (MediaCybernetics) as described.[12]

Histology

Paraffin sections were stained with an elastic Verhoeff-van Gieson staining kit (Sigma-Aldrich) to visualize the internal and external elastic lamina. Immunohistochemistry for PCNA was performed as described.[12] Paraffin sections were incubated with a primary antibody (ab2426, Abcam) followed by incubation with a biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories).

Statistics

One-way ANOVA or 2-way ANOVA was used to compare multiple groups as appropriate with Bonferroni's *t* test for post hoc analysis. Unpaired *t* test was performed for statistical analysis of two groups. Data were reported as means ± SEM. P values < 0.05 were considered statistically significant.

Results

Expression of HDAC in response to mitogenic stimulation of SMC

To investigate whether HDAC regulation might modulate SMC proliferation, we first assessed expression levels of HDAC members in primary rat aortic SMC. As depicted in Figure 1A-D, mitogenic stimulation of quiescent SMC resulted in increased transcript and protein levels of the class I HDAC 1, 2, and 3. Considering this mitogenic induction of HDAC 1–3 expression, we hypothesized that HDAC might contribute to the coordinate regulation of SMC proliferation. To further address this question, we performed cell counting and CFSE proliferation assays following siRNA-mediated knock down of class I HDAC members (Supplemental Figure I). As depicted in Figure 1E and Supplemental Figure II, knock-down of either HDAC 1, 2, or 3 reduced proliferation of serum-stimulated SMC. In contrast knock down of HDAC 6, a class II HDAC, did not alter SMC proliferation (Supplemental Figures I and III). These data demonstrate differential functions of HDAC in SMC and establish an essential role of class I HDAC in SMC proliferation.

Pharmacological HDAC inhibition prevents mitogen-induced SMC proliferation

To induce a broad inhibition of HDAC activity, we next employed a pharmacological approach using the HDAC inhibitor Scriptaid.[13] Scriptaid, structurally similar to the widely studied HDAC inhibitor trichostatin A (TSA), confers a higher efficiency in inhibiting endogenous histone deacetylation while exhibiting less cytotoxic activity compared to TSA.[13] As depicted in Figure 2A, pretreatment of quiescent SMC with Scriptaid completely prevented mitogen-induced cell proliferation. This inhibition of SMC proliferation by Scriptaid was dose-dependent and revealed a maximal inhibition of proliferation at 6 μM, as reflected by the increase in CFSE geo-mean fluorescence (Figure 2B). As illustrated in Supplemental Figure IV, this concentration did not induce apoptosis in SMC and corresponds to the reported maximal HDAC inhibition of more than 100-fold at a dose of 6 μM Scriptaid.[13]

HDAC activity is required for $G_1 \rightarrow S$ phase progression of the cell cycle

To further determine the mechanisms by which HDAC inhibition prevents SMC proliferation, we next determined cell cycle distribution using flow cytometry. As depicted in Figure 2C, treatment with Scriptaid inhibited mitogen-induced $G_1 \rightarrow S$ progression of SMC. A similar G_1-arrest was observed following mitogenic stimulation with platelet-derived growth factor (PDGF, Supplemental Figure V). Cell cycle progression through the $G_1 \rightarrow S$ phase checkpoint is governed by the retinoblastoma protein (Rb), which is phosphorylated in response to mitogenic stimulation by cyclin-dependent kinase (CDK)/cyclin complexes allowing cells to enter S phase.[16] Consistent with the G_0/G_1 arrest induced by HDAC inhibition, Scriptaid treatment completely prevented mitogen-induced phosphorylation of Rb (Figure 2D). Collectively, these findings demonstrate that Scriptaid inhibits SMC proliferation by preventing mitogen-induced Rb phosphorylation and subsequent cell cycle progression.

HDAC inhibition modifies the expression of cyclin-dependent kinase inhibitors

Considering the profound inhibition of Rb phosphorylation by Scriptaid in SMC, we next investigated whether HDAC inhibition modulates the expression of cyclin-dependent kinase inhibitors (CDKI) $p21^{Cip1}$ and $p27^{Kip1}$, which negatively regulate the activity of CDK/cyclin complexes and exhibit growth-inhibitory effects on SMC.[17] Analysis of gene expression profiles revealed that Scriptaid treatment increased mRNA expression levels of $p21^{Cip1}$ and $p27^{Kip}$ (Figure 3A and B). Albeit this transcriptional induction, HDAC inhibition attenuated the increase in $p21^{Cip1}$ protein levels observed in response to mitogenic stimulation (Figure 3C). Furthermore, mitogen-induced downregulation of $p27^{Kip1}$ protein levels was slightly altered in SMC treated with Scriptaid (Figure 3C). Collectively, these observations demonstrate increased transcript levels of the CDKI $p21^{Cip1}$ and $p27^{Kip}$ in response to Scriptaid treatment and point to a previously unrecognized translational block of $p21^{Cip1}$ transcription by HDAC inhibition.

HDAC inhibition represses cyclin D1 transcription

We next focused our analysis on the regulation of the CDK4/cyclin D1 complex by Scriptaid, the critical complex phosphorylating Rb. The induction of CDK4 mRNA expression in response to mitogenic stimulation was not modulated by HDAC inhibition (Figure 4A). In contrast, Scriptaid treatment profoundly repressed mitogen-induced cyclin D1 mRNA expression (Figure 4B and Supplemental Figure VI), despite a considerable increase in lysine 9 histone H3 acetylation at the cyclin D1 promoter (Supplemental Figure VII). This inhibition of cyclin D1 mRNA expression by Scriptaid translated into a complete prevention of mitogen-induced cyclin D1 protein expression by HDAC inhibition (Figure 4C). Transient transfection experiments further confirmed a solid inhibition of mitogen-induced cyclin D1 promoter activity in response to Scriptaid (Figure 4D). To investigate the specific contributions of class I HDAC to the observed inhibition of cyclin D1 transcription, SMC were transfected with siRNA against HDAC 1–3. As depicted in Figure 4E, knock down of HDAC 2 and 3 significantly reduced cyclin D1 mRNA levels. In concert, these data indicate that HDAC inhibition silences cyclin D1 expression through a transcriptional mechanism acting on the gene promoter, an effect that results in decreased Rb phosphorylation and cell cycle progression.

Inhibition of HDAC activity alters E2F target gene expression

Phosphorylation of Rb releases its repressive function on the transcription factor E2F and allows transactivation of gene promoters that drive cells into S phase.[16] To validate the notion that histone deacetylase activity operates to promote this transcriptional cascade at the $G_1 \rightarrow S$ transition, we next analyzed downstream Rb/E2F target gene expression,

including cyclin A and MCM6. As predicted, the inhibition of Rb phosphorylation in response to HDAC inhibition resulted in a solid silencing of the downstream *bona fide* E2F target genes MCM6 and cyclin A (Supplemental Figure VIII and IX). These data indicate that the inhibition of Rb phosphorylation by Scriptaid translates into a downstream stable repression of E2F target genes.

HDAC inhibition reduces neointima formation following vascular injury

Finally, we analyzed HDAC expression in mice following vascular injury and tested whether the observed attenuation of SMC proliferation by HDAC inhibition is applicable *in vivo*. Consistent with our *in vitro* studies, expression of class I HDAC increased in femoral arteries following vascular injury (Figure 5A). Next, we treated mice with Scriptaid and performed an endothelial denudation injury. The treatment dose of Scriptaid in these experiments was based on prior studies in mice and results in a solid inhibition of HDAC *in vivo* without toxicity.[14–15] As depicted in Figure 5B, endovascular injury of the mouse femoral artery resulted in a concentric neointima formation. In contrast, treatment with Scriptaid considerably attenuated neointima formation, and morphometric quantification revealed an approximately 60% reduction in the intima-to-media ratio following HDAC inhibition (Figure 5B and Supplemental Table I). To recapitulate our *in vitro* findings, we analyzed cyclin D1 expression in femoral arteries of DMSO and Scriptaid-treated mice following vascular injury. As demonstrated in Figure 5C, HDAC inhibition significantly decreased injury-induced cyclin D1 mRNA expression. Finally, to assess SMC proliferation *in vivo* we performed immunohistochemical staining of PCNA. As depicted in representative stainings in Figure 5D, Scriptaid treatment reduced neointimal PCNA expression following vascular injury. These findings establish that HDAC inhibition in mice prevents proliferative remodeling in response to endovascular injury.

Discussion

Epigenetic control of gene expression constitutes a conserved regulatory layer of transcriptional control that orchestrates important cellular processes, including proliferation, differentiation, and inflammation.[9–11] However, whether epigenetic modifications of chromatin accessibility operate to modulate SMC proliferation remains elusive. In the present study, we report that HDAC expression is increased in response to mitogenic stimulation of SMC and required for proliferation by regulating $G_1 \rightarrow S$ phase progression of the cell cycle. Furthermore, our *in vivo* experiments characterize histone acetylation as a previously unrecognized critical event for neointima formation in response to vascular injury.

During the course of atherosclerotic lesion formation, pathways activated by growth factors and cytokines ultimately converge to the transcriptional modulation of cell cycle-regulatory genes promoting proliferation of SMC.[1] In our study, we illustrate that class I HDAC represent a group of mitogen-responsive genes in SMC that are upregulated at a transcriptional level. Functional experiments established that siRNA-mediated knock-down of class I HDAC 1–3 reduces SMC proliferation, illustrating a novel mitogenic function for HDAC activity in SMC proliferation. These observations are consistent with previous reports documenting an induction of HDAC 1 in different murine cell types in response to growth factors,[18] and reduced proliferation of tumor cells and embryonic stem cells following knock-down or genetic knockout of class I HDAC.[19–21]

Inhibitors of HDAC stabilize the acetylation of histone proteins and have been primarily studied in cancer cells leading to their investigation in clinical trials.[22] One of the best described events following HDAC inhibitor treatment is the induction of growth arrest in G_1- and/or G_2/M-phase of the cell cycle.[8, 23] In our study, treatment of quiescent SMC with

the HDAC inhibitor Scriptaid induced G_1 arrest following mitogenic stimulation. The molecular mechanisms by which histone acetylation serves its mitogenic function are likely multifactorial and involve transcriptional events orchestrating the activation of a mitogenic gene expression program. In SMC, phosphorylation of Rb by CDK/cyclin complexes was completely abolished by HDAC inhibition resulting in decreased expression of downstream E2F target genes. Considering the complete prevention of mitogen-induced Rb phosphorylation by HDAC inhibition, we focused our initial mechanistic studies on the upstream CDKI and CDK/cyclin complexes, which mediate the phosphorylation of Rb during cell cycle progression. Transcript levels of $p21^{CIP1}$, the CDKI upregulated by HDAC inhibitors in most cell types,[8, 24] were induced by Scriptaid treatment in SMC. However, this transcriptional induction of $p21^{CIP1}$ mRNA did not translate to increased protein levels, pointing to previously unrecognized translational or posttranslational regulation of $p21^{CIP1}$ protein by HDAC inhibition in SMC. Considering that induced $p21^{CIP1}$ limits SMC proliferation,[25] this finding indicates that the inhibition of SMC proliferation by histone acetylation likely operates independent of $p21^{CIP1}$. In several prior studies the growth arrest induced by HDAC inhibition has been attributed to the induction of $p21^{CIP1}$.[8, 24] Similarly, Okamoto et al. have noted an induction of $p21^{CIP1}$ protein in rat SMC by TSA treatment and suggested a $p21^{CIP1}$-dependent growth arrest.[26] However, our data is more consistent with a recent study by Wilting et al. using a genetic model of HDAC 1 and HDAC 2 deficiency.[27] In this compelling study, it was confirmed that the growth arrest induced by histone acetylation occurs independently of $p21^{CIP1}$.[27] A potential explanation for this discrepancy might be differences in experimental design and, more likely, the less potent inhibition of HDAC by the frequently used TSA in these studies.[13] Finally, the CDKI $p27^{Kip}$ was mildly upregulated at a transcriptional level and its decrement following mitogenic stimulation was modestly prevented in response to HDAC inhibition in SMC. Since $p27^{Kip}$ has previously been described to be upregulated by HDAC inhibition and to inhibit SMC proliferation, a contribution of $p27^{Kip}$ to the growth arrest induced by HDAC inhibition in SMC may be conceivable.[17, 28] However, collectively, neither the moderate induction of $p27^{Kip}$ by Scriptaid nor the block of mitogen-induced $p21^{CIP}$ protein expression can explain the profound inhibition of SMC proliferation observed by HDAC inhibition in our experiments.

The CDK4/cyclin D1 complex initiates the phosphorylation-dependent inactivation of the retinoblastoma protein allowing S phase entry and cell proliferation.[29] As expected, HDAC inhibition resulted in an increase of lysine 9 histone H3 acetylation at the cyclin D1 promoter. This histone modification has been shown to correlate with activation of gene expression.[7] However, in SMC HDAC inhibition completely abolished the transcriptional induction of cyclin D1 in the early G_1 phase. Transient transfection experiments suggest that HDAC inhibition silences cyclin D1 expression through a transcriptional mechanism. Consistent with this observation in SMC, an inhibition of cyclin D1 mRNA and protein expression by HDAC inhibitor treatment has been observed in cancer cells and certain somatic cell types.[30–32] The transcriptional repression of cyclin D1 transcription in cancer cell types appears to be stimulus-dependent and involves an inhibition of either a NF-κB or AP-1-dependent activation of the cyclin D1 promoter.[30–31] HDAC inhibition impairs the binding of NF-κB through post-translational modifications including the acetylation of the NF-κB subunit p52.[31] In contrast AP-1-dependent activation of the cyclin D1 promoter is inhibited through impaired recruitment of the preinitiation complex to the c-jun promoter resulting in reduced AP-1 protein levels.[30] Following siRNA-mediated knock down of class I HDAC, we observed the largest decrease in cyclin D1 expression using siRNA against HDAC 3. Interestingly, the repression of c-jun transcription by HDAC inhibition was also conferred through HDAC 3.[30] Both the NF-κB and AP-1 transcriptional signaling pathways are activated during the mitogenic response of SMC, and CDK4/cyclin D1 activity is induced during neointima formation.[33–35] Considering further that overexpression of cyclin D1 induces SMC proliferation,[36] we would infer that the silencing of cyclin D1 expression

by histone acetylation contributes at least in part to the inhibition of SMC proliferation by the HDAC inhibitor Scriptaid.

Using a murine model of endovascular endothelial denudation,[37] HDAC inhibition reduced cyclin D1 and PCNA expression and neointima formation in response to vascular injury. This observation confirms that the inhibition of SMC proliferation by histone acetylation noted in SMC culture systems is applicable to proliferative vascular remodeling *in vivo*. Furthermore, this anti-proliferative activity of HDAC inhibition in the vascular wall is consistent with the well-established induction of growth arrest and associated reduction in tumor growth in various species treated with HDAC inhibitors.[38–39] Interestingly, in further support of the reduction of neointima formation by HDAC inhibition in the present study, the HDAC inhibitor tributyrin was previously found to inhibit SMC migration,[40] a critical early event for pathological vascular remodeling.[1] Therefore, in addition to an inhibition of mitogen-induced proliferation, the HDAC inhibitor Scriptaid may prevent neointima formation by limiting SMC migratory responses.

In summary, we demonstrate in the present study that class I HDAC serve a mitogenic role in SMC. HDAC inhibition induces cell cycle arrest by preventing mitogen-induced Rb phosphorylation. This inhibition of Rb phosphorylation occurs through a differential regulation of cell cycle regulatory genes orchestrating the G1→S phase transition checkpoint, including a transcriptional repression of cyclin D1. Finally, we establish that HDAC inhibition limits the proliferative response underlying neointima formation. These findings identify HDAC as a critical component of a transcriptional cascade regulating SMC proliferation and suggest that HDAC might play a pivotal role in the development of proliferative vascular diseases, including atherosclerosis and in-stent restenosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1A

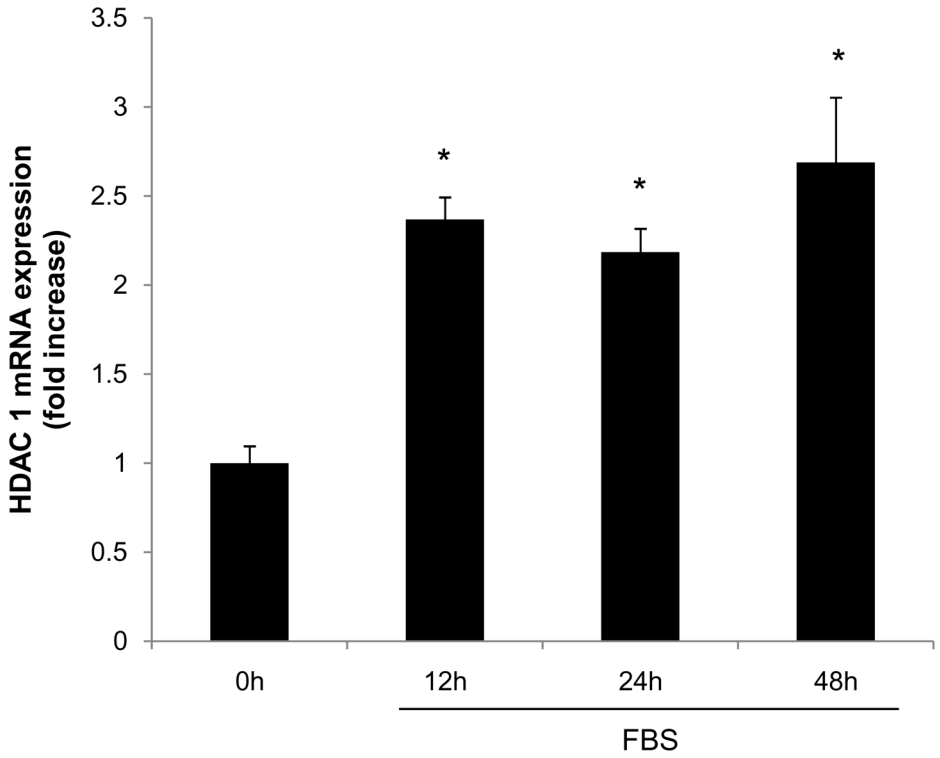


Figure 1B

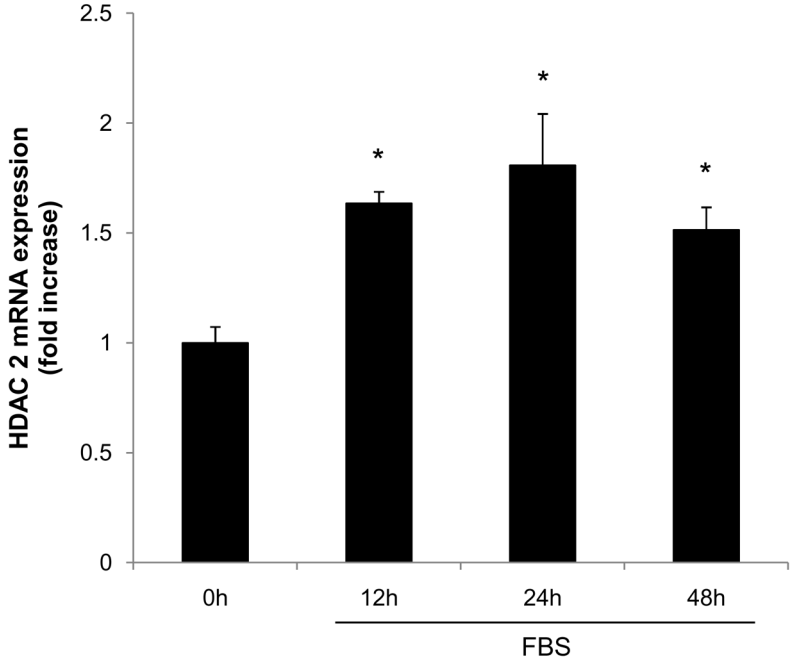


Figure 1C

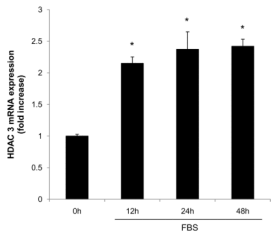
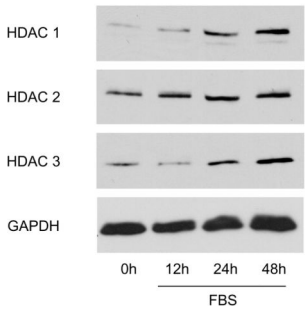


Figure 1D



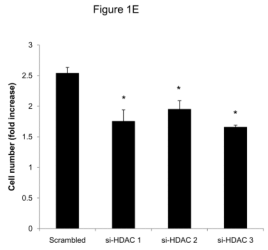
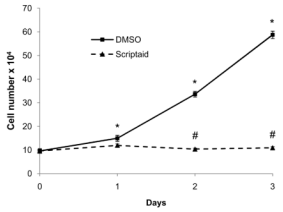


Figure 1.

Mitogenic stimulation of SMC induces class I HDAC expression. A–D: Serum-deprived rat aortic SMC were stimulated with 10% FBS. HDAC mRNA and protein expression was analyzed at the indicated time points. The autoradiograms shown are representative of three independently performed experiments using different cell preparations. E: Cells were transfected twice with siRNA against HDAC 1, 2, 3 and serum-deprived for 24 h. Following this starvation period, synchronized cells were stimulated with 10 % FBS for 48 h and counted using a hemocytometer. Data is presented as mean ± SEM (* $p < 0.05$ vs. baseline or scrambled siRNA).

Figure 2A



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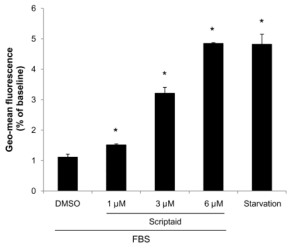

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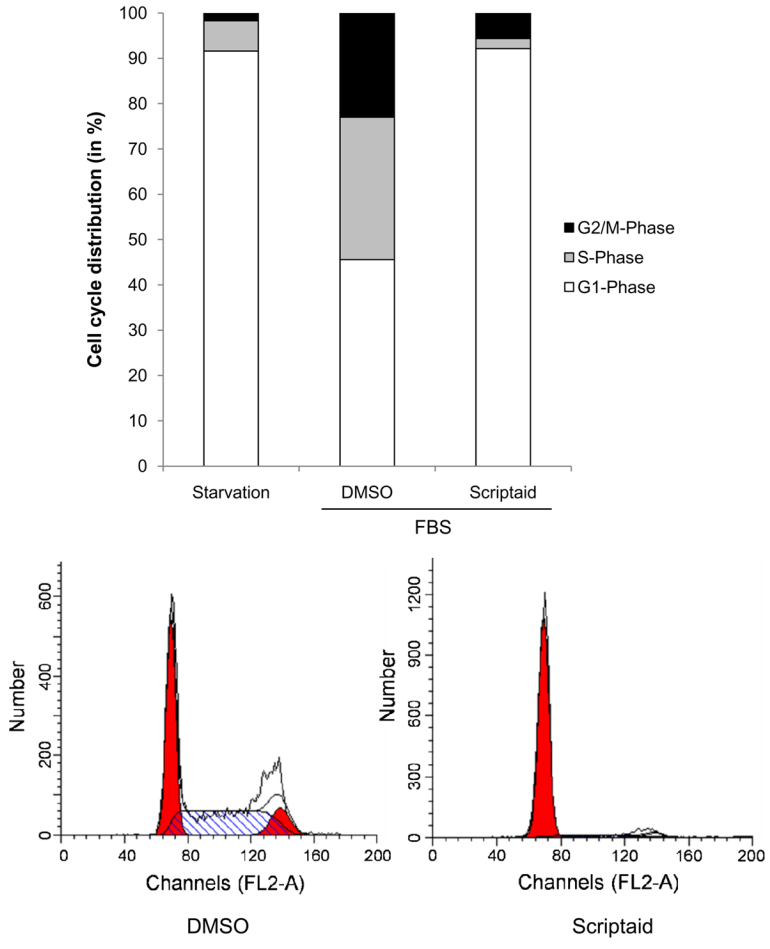

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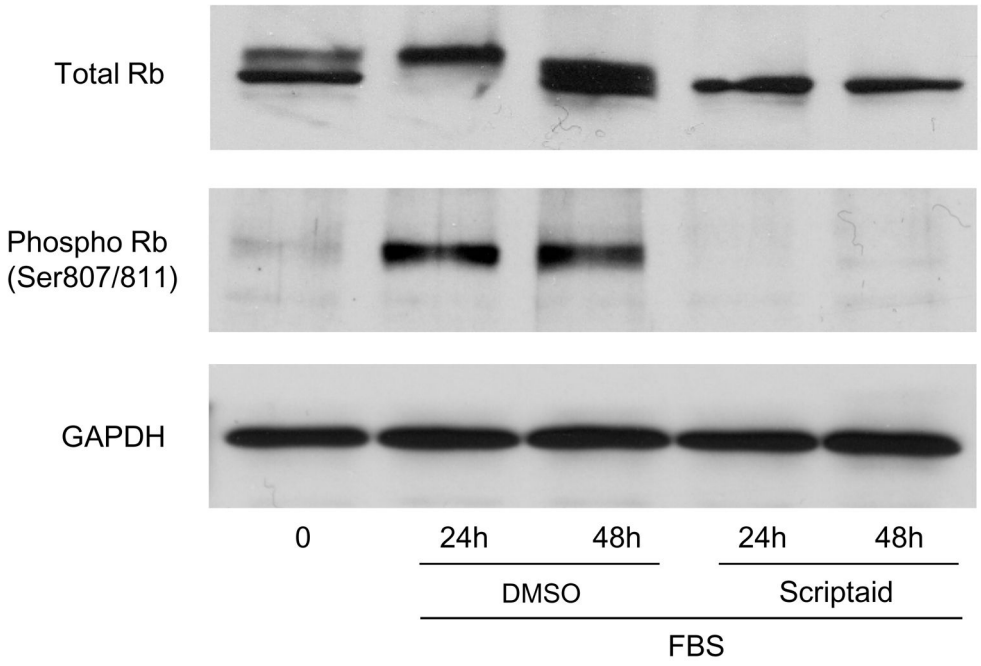


Figure 2.

The HDAC inhibitor Scriptaid prevents mitogen-induced SMC proliferation. A: Serum-deprived SMC were pretreated with DMSO or 6 μM Scriptaid, and stimulated with 10% FBS. Cells were counted at indicated time points using a hemocytometer. B: CFSE labeled SMC were starved for 24 h, treated with DMSO or different doses of Scriptaid, and stimulated with 10% FBS. After 48 h cells were analyzed by FACS. C: SMC were treated as described in A. Cell cycle distribution was assessed at baseline and 24 h after FBS stimulation using FACS analysis. D: Cells were stimulated as described in A. Whole cell lysate was collected at the indicated time points and analyzed for protein expression of total Rb, phosphorylated Rb and GAPDH. The autoradiograms shown are representative of three independently performed experiments using different cell preparations. Data is presented as mean ± SEM (* $p < 0.05$ vs. DMSO).

Figure 3A

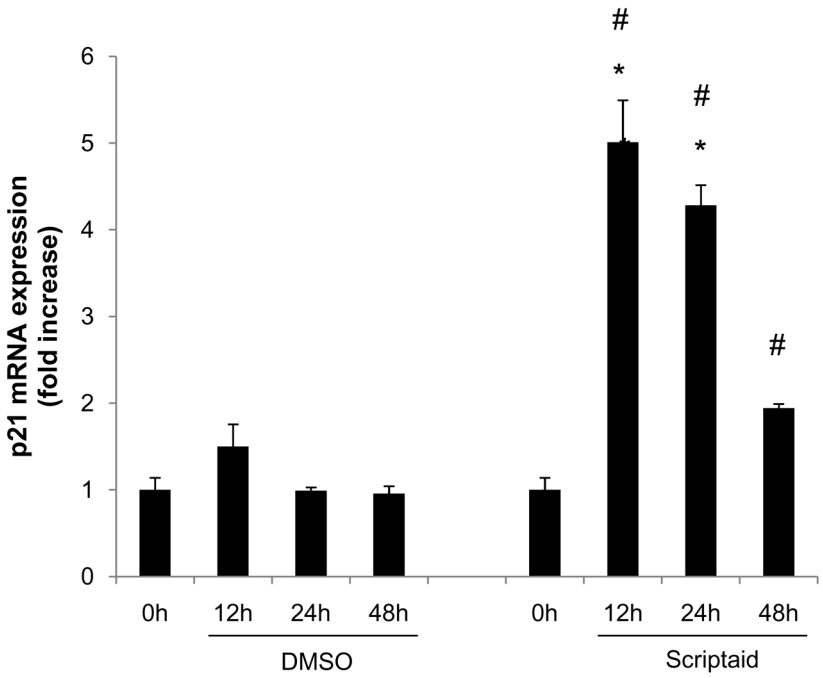


Figure 3B

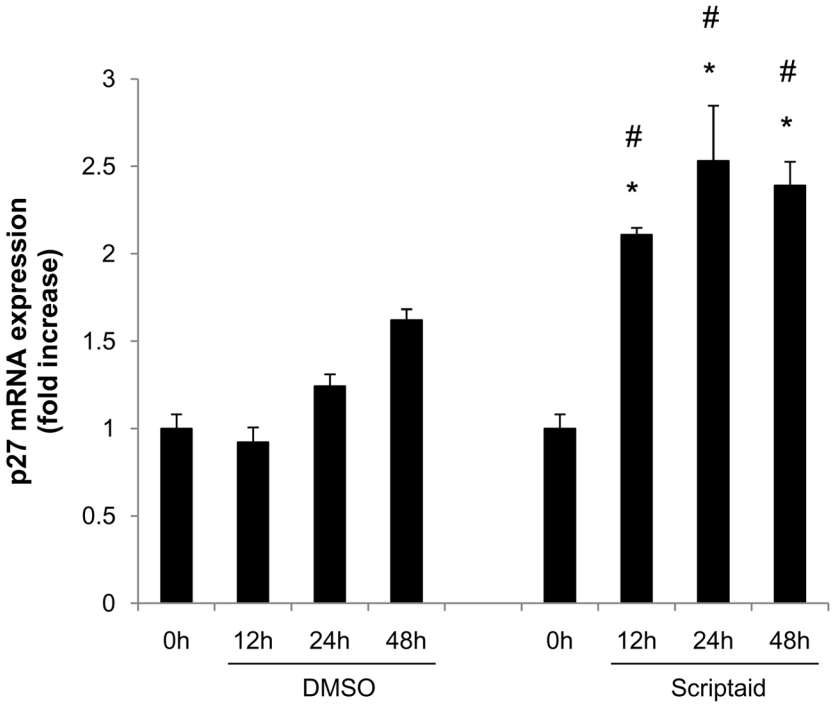
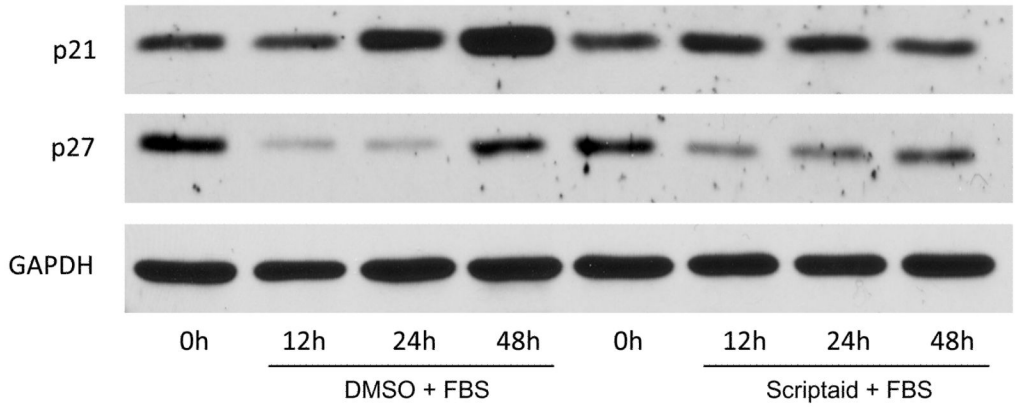


Figure 3C



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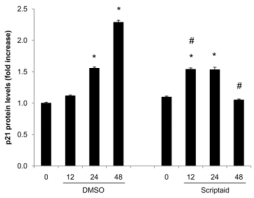


Figure 3E

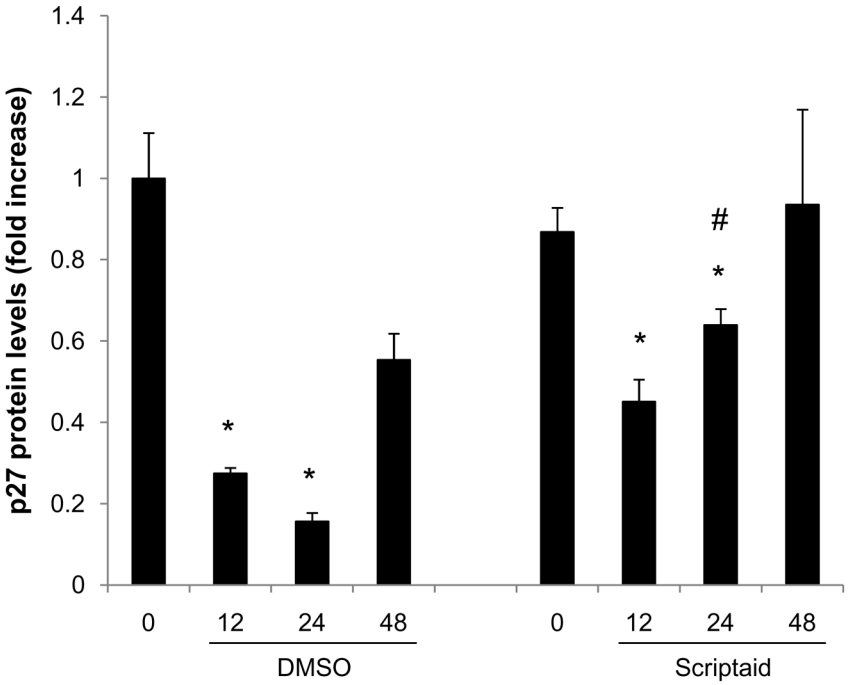


Figure 3.

HDAC inhibition modifies the expression of cyclin-dependent kinase inhibitors. A–C: Serum-deprived SMC were treated with DMSO or 6 μM Scriptaid, and stimulated with 10% FBS. Cells were harvested at the indicated time points and analyzed for mRNA and protein expression of $p21^{Cip1}$ and $p27^{Kip1}$ by real-time RT-PCR (A–B) and Western blotting (C). Quantification of the Western blotting experiments was performed by densitometry and normalization to GAPDH from three independently performed experiments. Data is presented as mean ± SEM (* $p < 0.05$ vs. baseline, # $p < 0.05$ vs. DMSO).

Figure 4A

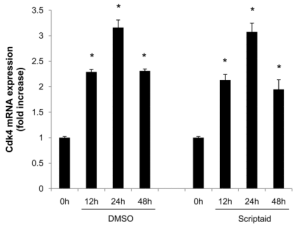
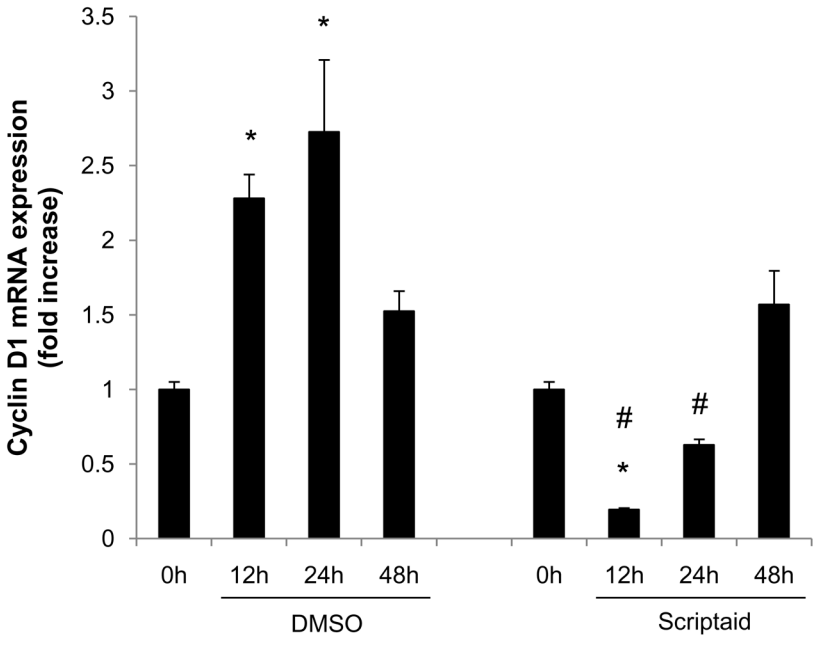


Figure 4B



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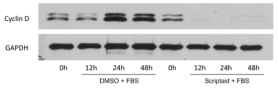
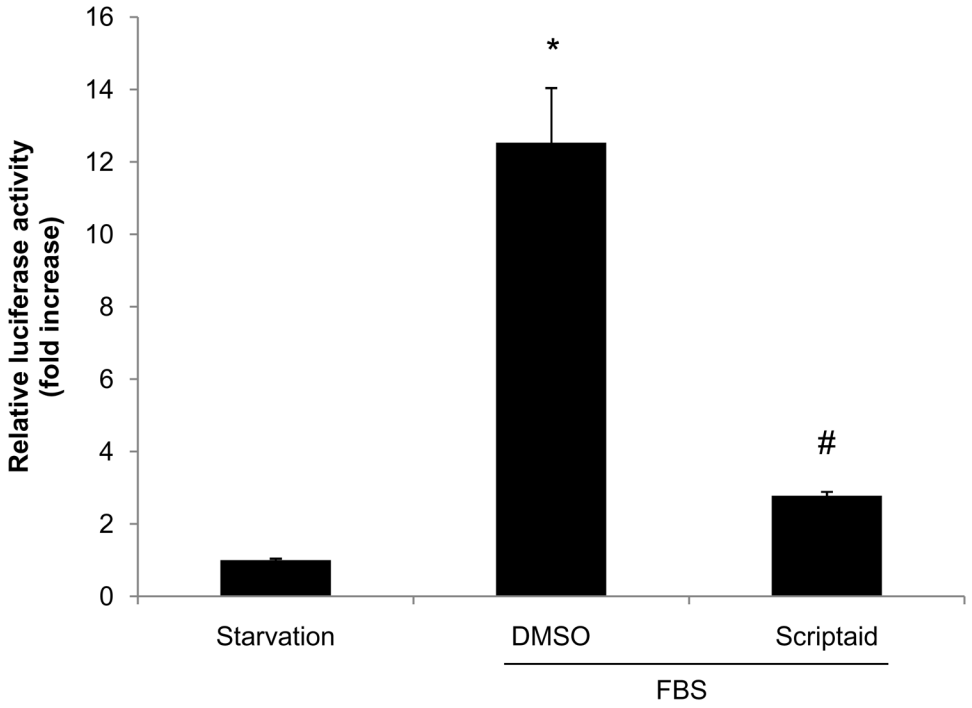


Figure 4D



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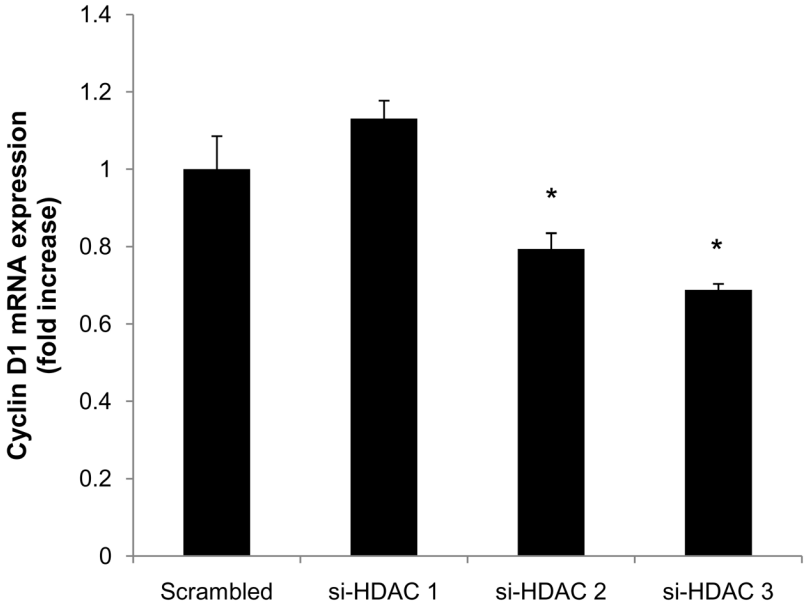

Figure 4.

HDAC inhibition represses cyclin D1 transcription. A and B: Serum-deprived SMC were treated with DMSO or 6 μM Scriptaid, and stimulated with 10% FBS. Cells were harvested at indicated time points and analyzed for mRNA expression of Cdk4 (A) and cyclin D1 (B). C: Cells were treated as described in A. Whole cell lysate was collected and analyzed for protein expression of cyclin D1. D: SMC were transfected with a cyclin D1 promoter luciferase reporter plasmid. Following transfection, cells were treated with DMSO or 6 μM Scriptaid in 10% FBS. Luciferase activity was assayed after 24 h. Transfection efficiency was adjusted by normalizing firefly luciferase activities to renilla luciferase activities generated by cotransfection with pRL-CMV. E: Cells were transfected twice with siRNA against HDAC 1, 2, 3 and serum-deprived for 24 h. Following this starvation period, synchronized cells were stimulated with 50 ng/ml PDGF and mRNA expression of cyclin D1 was analyzed after 6h. Data is presented as mean ± SEM (* $p < 0.05$ vs. baseline or scrambled siRNA, # $p < 0.05$ vs. DMSO).

Figure 5A

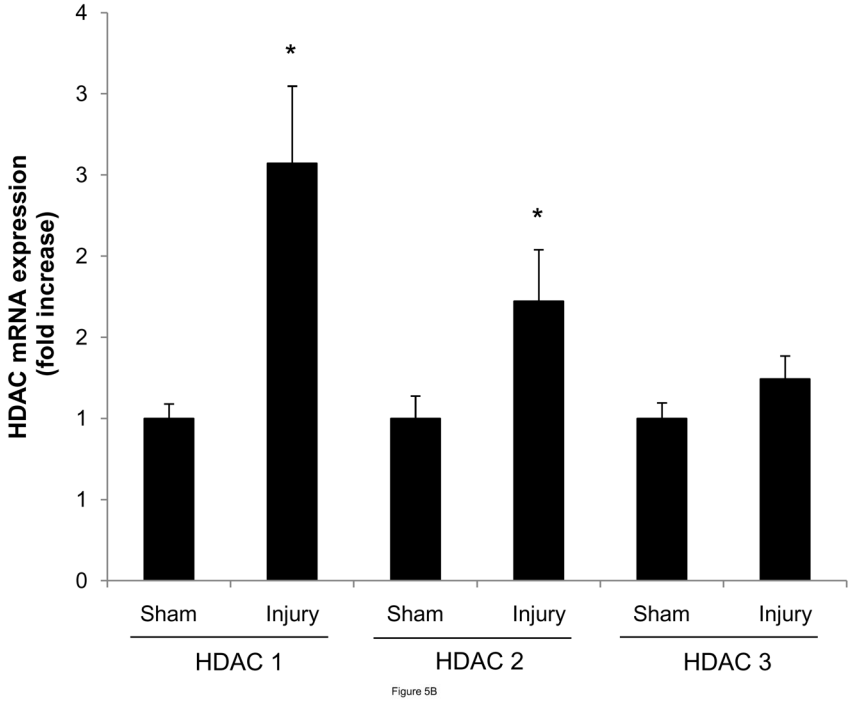

Figure 5B

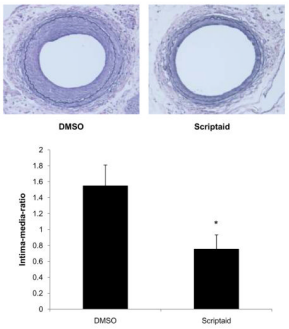

Figure 5C

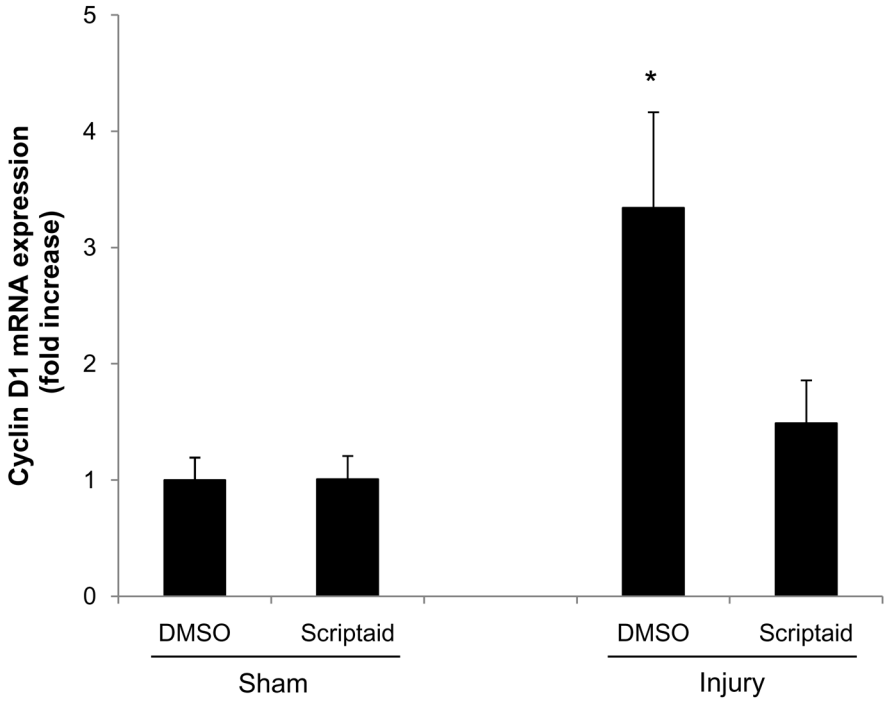

Figure 5D

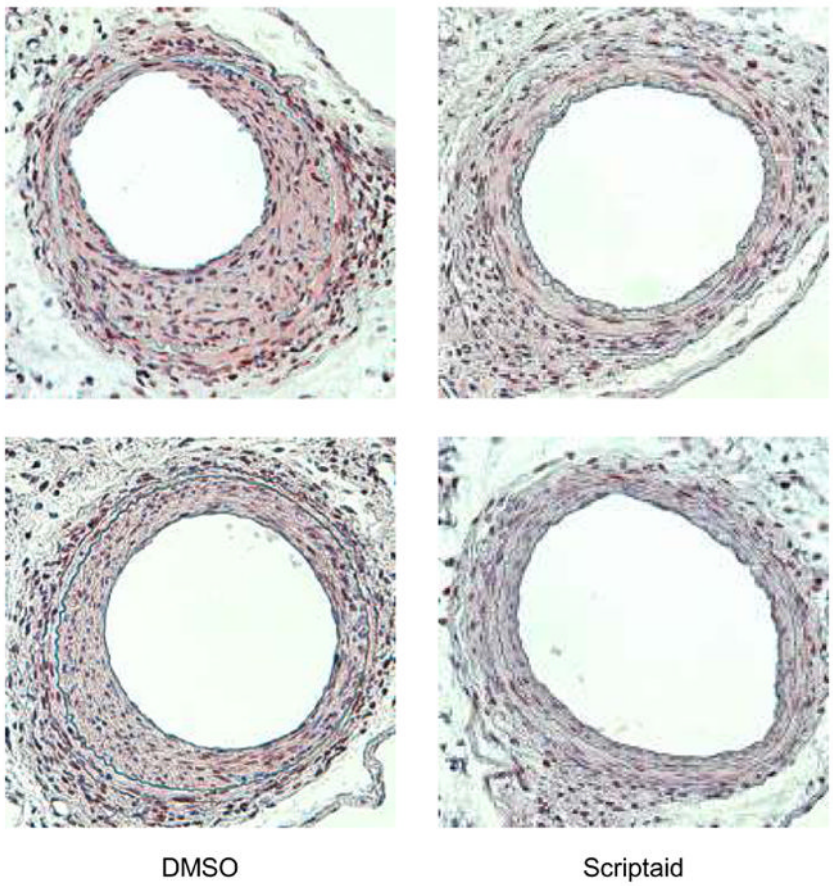

Figure 5.

HDAC inhibition reduces neointima formation following vascular injury. A: Guide wire-induced endothelial denudation injuries were performed on the left femoral artery of C57BL/6J mice (n = 4). mRNA expression of HDAC 1–3 was analyzed after 48 h. B: Following endothelial denudation injuries, mice were treated with Scriptaid or DMSO for 28 days (n = 9 each group). Tissues were harvested and elastic Verhoeff-van Gieson stain was performed to visualize the internal and external elastic lamina. The ratio of intima to media was calculated as the intimal area divided by medial area. C: Cyclin D1 mRNA expression was analyzed 48 after endothelial denudation injuries (each group n = 4) or sham surgery (each group n = 3) and treatment with Scriptaid or DMSO. D: Representative immunohistochemical stainings of PCNA expression. Tissues were harvested after 28 days of Scriptaid or DMSO treatment. Data is presented as mean ± SEM (* $p < 0.05$ vs. baseline)

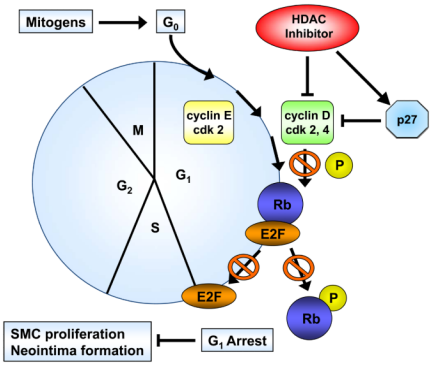

Figure 6.

HDAC inhibition targets cell cycle progression. Cell cycle progression is dependent on the orchestrated expression, activation and holoenzyme formation of cyclins and cyclin-dependent kinases (CDKs). HDAC inhibition blocks cell cycle progression through repression of cyclin D1. Impaired activation of cyclin–CDK complexes inhibits mitogen-induced Rb phosphorylation and downstream activation of E2F-regulated genes, resulting in G1 arrest and reduced SMC proliferation.