

Histone Deacetylase Activity Selectively Regulates Notch-Mediated Smooth Muscle Differentiation in Human Vascular Cells

Yuefeng Tang, PhD*; Joshua M. Boucher, BS; Lucy Liaw, PhD

Background—Histone deacetylases (HDACs) modify smooth muscle cell (SMC) proliferation and affect neointimal lesion formation by regulating cell cycle progression. HDACs might also regulate SMC differentiation, although this is not as well characterized.

Methods and Results—Notch signaling activates SMC contractile markers and the differentiated phenotype in human aortic SMCs. Using this model, we found that HDAC inhibition antagonized the ability of Notch to increase levels of smooth muscle α -actin, calponin1, smooth muscle 22 α , and smooth muscle myosin heavy chain. However, inhibition of HDAC activity did not suppress Notch activation of the HRT target genes. In fact, HDAC inhibition increased activation of the canonical C-promoter binding factor-1 (CBF-1)-mediated Notch pathway, which activates HRT transcription. Although CBF-1-mediated Notch signaling was increased by HDAC inhibition in human SMCs and in a C3H10T1/2 model, SMC differentiation was inhibited in both cases. Further characterization of downstream Notch signaling pathways showed activation of the c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and PI3K/Akt pathways. The activation of these pathways was sensitive to HDAC inhibition and was positively correlated with the differentiated phenotype.

Conclusions—Our studies define novel signaling pathways downstream of Notch signaling in human SMCs. In addition to the canonical CBF-1 pathway, Notch stimulates c-Jun N-terminal kinase, mitogen-activated protein kinase, and PI3K cascades. Both canonical and noncanonical pathways downstream of Notch promote a differentiated, contractile phenotype in SMCs. Although CBF-1-mediated Notch signaling is not suppressed by HDAC inhibition, HDAC activity is required for Notch differentiation signals through mitogen-activated protein kinase and PI3K pathways in SMCs. (*J Am Heart Assoc.* 2012;1:e000901 doi: 10.1161/JAHA.112.000901)

Key Words: signal transduction • muscle, smooth • vasculature • cell differentiation

Smooth muscle cells (SMCs) have a unique ability to modify their contractile phenotype to a transitional state during the pathogenesis of vascular diseases. This transitional state can include alterations in morphology, gene expression, contraction, and proliferation. Typically, reduction in contractile proteins is concurrent with entry into the cell cycle and increased migratory ability. Several signaling pathways are well-characterized regulators of SMC phenotype, and many function in a cooperative manner.^{1–4} Our laboratory has focused on the Notch signaling pathway as a critical regulator of SMC phenotype. Notch signaling promotes SMC

differentiation via direct activation of contractile gene transcription as well as regulation of SMC microRNAs,^{5–9} and mutations in the Notch pathway are implicated in human vascular pathologies.^{10–12}

There is interest in epigenetic modifications that might potentially impact human cardiovascular diseases.¹³ In particular, histone acetylation and deacetylation are major determinants of chromatin structure and gene transcription. Enzymes of the histone deacetylase (HDAC) family generally inhibit transcription. A major mechanism of tumor suppressor gene silencing in cancers by HDACs has led to the use of HDAC inhibitors as anticancer therapeutics.^{14,15} Several lines of evidence implicate HDACs as a target for regulation of SMC phenotype. Inhibition of HDAC activity can alter SMC proliferation.^{16–19} Although there is some discrepancy, most *in vivo* studies show that HDAC inhibition suppresses neointimal lesion formation,^{18,20} which suggests a potential therapeutic target for cardiovascular diseases. Indeed, HDAC inhibitors prevent cardiac hypertrophy, heart failure, and hypertension in rodent models.^{21–24}

A few studies have addressed HDAC regulation of SMC differentiation. Suppression of SMC markers by platelet-derived growth factor-BB is mediated partially by recruitment of HDACs

From the Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, ME.

*Dr Tang is currently with the University of Massachusetts Medical School, Worcester, MA.

Correspondence to: Lucy Liaw, PhD, Maine Medical Center Research Institute, 81 Research Dr, Scarborough, ME 04074. E-mail liawl@mmc.org

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to contractile gene promoters.²⁵ In addition, suppression of SMC differentiation by oxidized phospholipids was mediated by Krüppel-like factor 4, E twenty-six-like transcription factor 1 (Elk 1), and HDAC activity on genes, including smooth muscle α -actin (SM actin).²⁶ Transforming growth factor β (TGF β)-mediated induction of smooth muscle 22 α (SM22 α) expression was concurrent with hyperacetylation of this locus, and HDAC inhibitors enhanced TGF β promotion of SM22 α transcription.²⁷ Recently, a link of HDACs to Notch signaling was discovered in studies that conditionally deleted HDAC3 in neural crest cells, which give rise to subpopulations of arterial SMCs.²⁸ Loss of HDAC3 impaired development of arterial SMCs in the aortic arch, and this defect was concomitant with reduced expression of Jagged1. These data suggest that epigenetic regulation of SMC precursors can function upstream of Notch signaling. HDAC activity is also associated with a regulatory function in Notch signaling in other cells,^{29–32} but effects on Notch-mediated SMC differentiation are unknown.

In the present study, we tested the idea that SMC differentiation mediated by the Notch pathway is regulated by HDAC activity. Our studies in human primary SMCs with Notch as a differentiation factor revealed distinct phenotypes compared to rat SMC differentiation induced by TGF β .²⁷ Inhibition of HDAC activity in human SMCs downregulated Fbw7 and increased Notch1 protein. However, HDAC inhibition suppressed Notch-mediated SMC differentiation, but this was not due to inhibition of canonical C-promoter binding factor-1 (CBF-1)-mediated signaling. Other pathways activated by Notch in human SMCs include PI3K/Akt, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) signaling, all of which were suppressed by HDAC inhibition. Our studies show that regulators of SMC differentiation that might use multiple signal mediators can be selectively sensitive to epigenetic modifiers of gene expression.

Methods

Cell Culture

Human aortic SMCs (Cambrex, Walkersville, MD) were maintained in SmGM2 medium and were used between passages 4 and 7. Murine C3H10T1/2 fibroblasts were cultured in minimum essential medium with Earle's salts containing 10% fetal bovine serum, L-glutamine (2 mmol/L), 1% nonessential amino acids, 100 IU/mL penicillin, and 100 μ g/mL streptomycin.

Reagents

Trichostatin A (TSA) and dimethyl sulphoxide were from Sigma Aldrich (St. Louis, MO), and the HDAC class I inhibitor MS-275 was from Selleck (Houston, TX). Kinase inhibitors U0126, SB203580, SP600125, and LY294002 and the protease inhibitor cocktail were obtained from EMD Biosciences (Madison, WI).

Quantitative Reverse-Transcription Polymerase Chain Reaction and Immunoblotting

Total RNA was extracted with Tri-reagent (Sigma), treated with RNase-free DNase I (Promega, Madison, WI), and reverse-transcribed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed in the iCycler (Bio-Rad, Hercules, CA) using SYBR Green (Bio-Rad) with 20 ng cDNA as template, in triplicate. Threshold cycle numbers were calculated at log phase of amplification and normalized to cyclophilin. Soluble cell extracts were prepared, and aliquots containing 20 μ g of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were probed with antibodies against corresponding proteins, as described.⁵

Gene Silencing With siRNA

Transfections were performed with the human aortic SMC-optimized Amaxa nucleofector system (Lonza, Walkersville, MD). Knockdown of CBF-1 was accomplished using 180 pmol siRNA or scrambled All Star Control (Qiagen, Valencia, CA) with 1×10^6 cells per reaction, and transfection was done with the V-025 program. Cells were plated for 24 hours before transduction adenoviral constructs. Knockdown of serum response factor (SRF) was accomplished using 125 pmol siRNA or scrambled All Star Control with 5×10^5 cells, and electroporation was done with the U-025 program. Cells were cultured for 48 hours before transduction with adenoviral constructs for subsequent analysis.

Transient Transfections and Luciferase Assay

Human aortic SMCs were plated at 40 000 cells per well in a 12-well plate and were transduced with adenovirus (100 TCID₅₀ virus particles per cell), 0.25 μ g reporter plasmid, 0.75 μ L Gene Juice (Invitrogen), and 25 ng of Renilla luciferase plasmid per well. Two days after transfection, cells were collected for luciferase assay, as described.⁶ All experiments were repeated ≥ 3 times, and representative results are shown. The SM actin promoter reporter constructs p125 and pA125³³ were generously provided by Gary K. Owens, University of Virginia.

Statistical Analysis

Statistical analyses to test differences between groups were performed with analysis of variance (ANOVA) in conjunction with the Tukey range test to determine significant differences at $P < 0.05$. Data are presented as mean \pm standard error of the mean (SEM). All experiments were performed independently ≥ 3 times. For quantitative RT-PCR analysis, each experimental

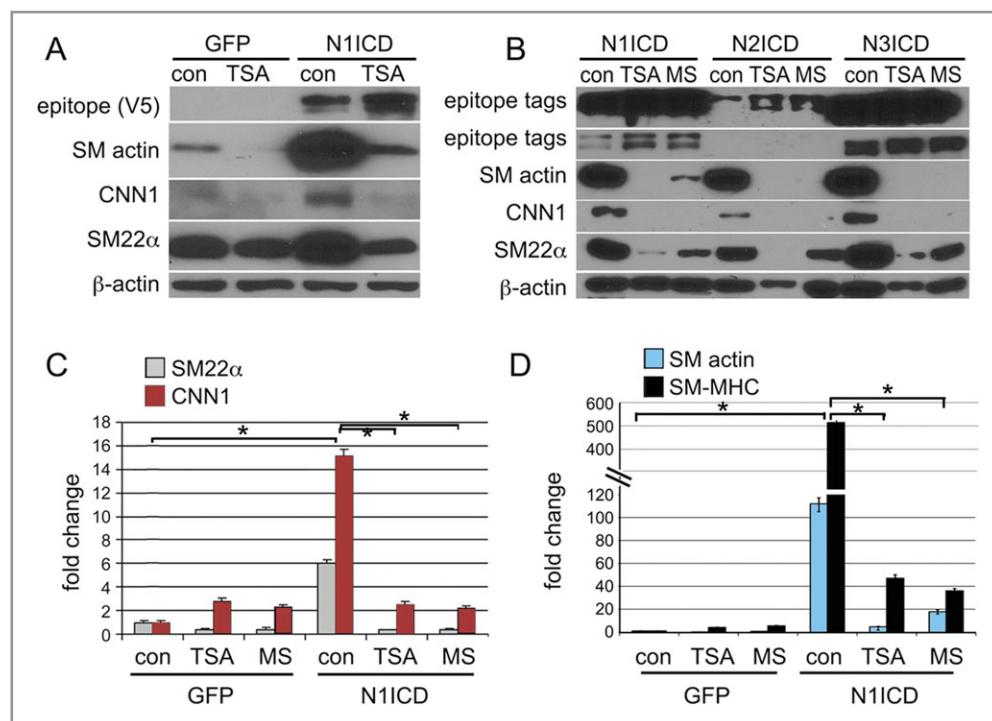


Figure 1. HDAC inhibition represses Notch-mediated SMC differentiation. **A**, Primary human aortic SMCs were transduced with Notch1ICD (N1ICD) or green fluorescent protein (GFP) and then were treated with the HDAC inhibitor TSA or control vehicle dimethyl sulphoxide (DMSO) (con) for 48 h before collection of cell lysates for immunoblot analysis. Expression of N1ICD was confirmed using the V5 epitope tag. Smooth muscle α -actin (SM actin), calponin1 (CNN1), and SM22 α were analyzed and compared to levels of β -actin. **B**, Notch1ICD, Notch2ICD, and Notch3ICD were transduced into human aortic SMCs, which were then treated with HDAC inhibitors TSA or MS-275 or with vehicle DMSO (con). The top 2 rows are different exposures of the same blot to detect the epitope tags on the NICD constructs. Longer (top row) and shorter (second row) exposures are shown because the level of N2ICD expression was lower than that of N1ICD and N3ICD. SMC markers were analyzed and were similarly induced by activation of each Notch receptor. Both TSA and MS-275 significantly suppressed the induction of SMC proteins by Notch activation. **C** and **D**, Total RNA was collected under the same conditions, and transcripts for SMC markers were quantitatively measured in comparison to control SMCs without HDAC inhibitor treatment. Data are presented as mean \pm SEM and were analyzed for statistical significance by ANOVA / Tukey test. The asterisks indicate $P < 0.05$. N1ICD significantly increased transcripts for all SMC markers tested, and TSA and MS significantly suppressed this induction.

group was tested in duplicate for each trial, and for luciferase assays, each group was tested in triplicate for each trial.

Results

HDAC Inhibition Abrogates Notch-Induced SMC Differentiation

It was shown previously that HDAC inhibition suppresses TGF β -induced fibroblast–myofibroblast differentiation^{34,35} and regulates SMC proliferation and migration.^{13,18} We have characterized Notch signaling as a strong inducer of the contractile phenotype in human SMCs.^{5,6} No previous work has addressed the interaction of Notch signaling and HDAC activity in human SMCs. Therefore, our study was designed to test the effect of HDAC inhibition on Notch-induced SMC differentiation

using transient expression of the constitutively active Notch intracellular domain (ICD) in the presence or absence of the HDAC inhibitor TSA for 2 days. Consistent with our previous report,⁶ human SMCs with activated Notch1 signaling dramatically increased levels of SM actin, calponin1 (CNN1), and SM22 α (Figure 1A). Inhibition of HDAC activity reduced basal and Notch-induced activation of all differentiation markers (Figure 1A). We previously showed that human SMCs also express Notch2 and Notch3, which also promote a contractile phenotype when activated.⁵ To determine if HDAC inhibition also affected signaling downstream of different Notch receptor activation, cells were transduced with Notch1ICD, Notch2ICD, or Notch3ICD and then treated with TSA or MS-275 (entinostat), an HDAC class I-specific inhibitor. Inhibition of HDAC activity also repressed Notch2ICD- and Notch3ICD-mediated induction of SM actin, SM22 α , and calponin1 (Figure 1B). Quantitative analysis of transcript levels determined that the effects of HDAC inhibition also were seen at the mRNA level. Induction of SM22 α

and calponin1 transcript was significantly suppressed in the presence of either HDAC inhibitor (Figure 1C), as were transcripts for SM actin and smooth muscle myosin heavy chain (Figure 1D). These data show that multiple SMC differentiation markers are inhibited by HDAC inhibition, with similar effects downstream of Notch1, Notch2, and Notch3 signaling.

TSA Does Not Suppress the Notch/CBF-1 Pathway

To explore the mechanism of the inhibitory effect of TSA on Notch-mediated SMC differentiation, we examined whether TSA exerts its effects by antagonizing the Notch/CBF-1-dependent signaling pathway, which is central in promoting the SMC contractile phenotype.^{5,6,36} To assess this, CBF-1

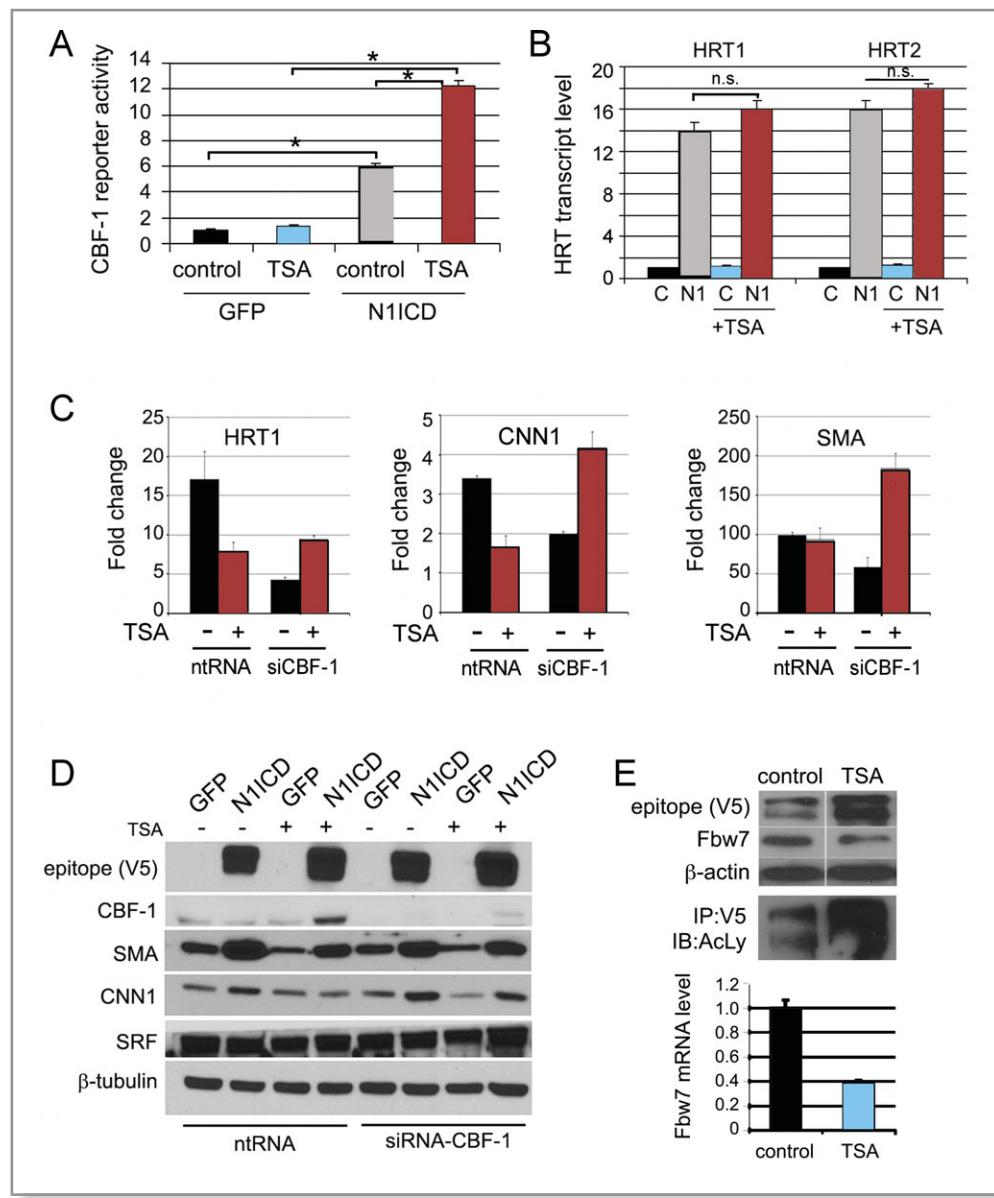


Figure 2. HDAC inhibition enhances Notch signaling and downregulates Fbw7. **A**, Primary human SMCs were transduced with green fluorescent protein (GFP) or Notch1ICD (N1ICD) and a CBF-1 luciferase reporter construct and were treated with TSA or control dimethyl sulphoxide (DMSO) for 48 h before analysis. Shown is normalized luciferase (mean \pm SEM). ANOVA / Tukey test was used for statistical analysis, and asterisks indicate $P<0.05$. Expression of N1ICD significantly increased reporter activity, even in the presence of TSA, and TSA further enhanced CBF-1 reporter activity in the presence of activated Notch signaling. **B**, GFP- or N1ICD (N1)-transduced SMCs were treated with TSA or control vehicle DMSO (C) for 48 h, and total RNA was collected for quantitative RT-PCR to measure HRT transcripts. Data were statistically analyzed by ANOVA / Tukey test, and HRT1 and HRT2 transcripts were significantly elevated with N1ICD or N1ICD+TSA compared to respective controls. There was no significant difference (n.s.) in N1ICD induction of HRT when TSA was included. **C**, CBF-1 was suppressed using specific siCBF-1 compared to nontargeting control (ntRNA), and quantitative RT-PCR was used to measure HRT1, calponin1 (CNN1), and SM actin (SMA) mRNA in the absence or presence of TSA. **D**, Protein lysates were collected from cells under the same conditions as in C and were used for immunoblot as indicated. **E**, N1ICD-transduced SMCs were treated with TSA or control vehicle DMSO for 48 h before analysis by immunoblot or quantitative RT-PCR to detect Fbw7 protein and transcript, respectively. Under the same conditions, 10% of total cell lysates were immunoprecipitated with anti-V5 and immunoblotted with an antibody recognizing acetylated lysine (AcLy).

luciferase reporter assays were performed. Although TSA treatment did not affect basal CBF-1 reporter activity, TSA enhanced Notch1ICD activation of CBF-1 activity (Figure 2A), which is consistent with studies in other cells.^{29–32} In addition, we quantified mRNA for HRT1 and HRT2, which are activated by Notch1ICD/CBF-1 complexes. TSA had no effect on the ability of Notch to activate these targets (Figure 2B). Thus, suppression of HDAC activity affects Notch targets in a gene-specific manner and does not appear to suppress Notch signaling via the canonical CBF-1 pathway. To further examine this pathway, CBF-1 was silenced in SMCs with siRNA, followed by activation of Notch1 signaling (Figure 2C). As expected, silencing CBF-1 protein decreased the ability of Notch1ICD to activate HRT1. Loss of CBF-1 also suppressed Notch induction of calponin and SM actin transcripts, but at only <50% reduction, which suggests that Notch is also activating alternative pathways leading to induction of SMC markers. Loss of CBF-1 with Notch1ICD expression led to a significant increase in calponin1 and SM actin transcripts with TSA treatment, which suggests that TSA activity is altered in the absence of CBF-1. Immunoblot analysis confirmed that suppression of CBF-1 protein did not completely block Notch1ICD-induced SM actin or calponin1 induction (Figure 2D). In addition, because SRF activity plays a dominant role in SMC differentiation, we analyzed SRF protein levels with Notch activation, TSA, and silenced CBF-1. The level

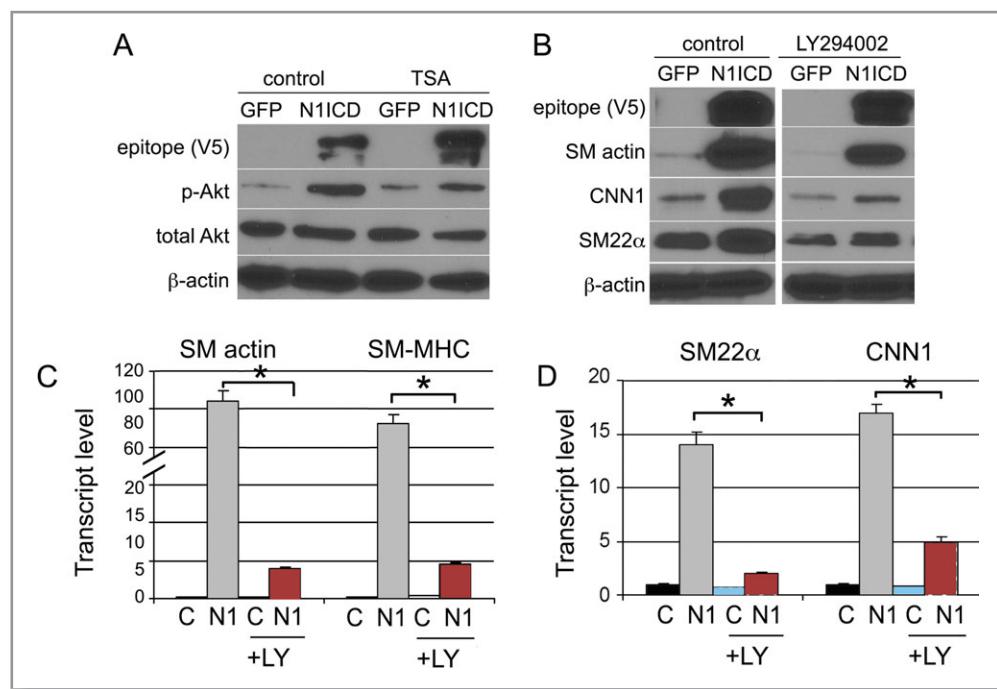


Figure 3. Notch activity requires PI3K/Akt signaling, which is sensitive to HDAC inhibition. A, Primary human SMCs were transduced with Notch1ICD (N1ICD) or green fluorescent protein (GFP) and then were treated with TSA or control vehicle dimethyl sulphoxide (DMSO) for 48 h before analysis. Cell lysates were collected for immunoblot to examine the activity of PI3K/Akt measured by p-Akt. B, N1ICD- or GFP-transduced SMCs were treated with the PI3K/Akt inhibitor LY294002 or DMSO control for 48 h before analysis. C and D, GFP- or N1ICD (N1)-transduced SMCs were treated with LY294002 (LY) or control vehicle (C) for 48 h, and total RNA was collected for expression analysis by quantitative RT-PCR for SMA and smooth muscle myosin heavy chain (SM-MHC) (C) and for SM22 α and CNN1 (D). Grafted are fold changes compared with control SMCs without LY294002 treatment. Data are presented as mean \pm SEM and were statistically analyzed by ANOVA / Tukey test. Asterisks indicate $P<0.05$; LY294002 treatment significantly suppressed induction of all SMC marker genes by Notch activation.

of SRF did not change significantly under any condition (Figure 2D).

We also observed an apparent increase in transfected Notch1ICD protein with TSA treatment and tested whether HDAC inhibition affected Notch protein levels. The levels of Notch1 transcript were unchanged (data not shown), but there was a consistent increase in Notch1ICD protein after HDAC inhibition (Figure 2E), which suggests regulation of protein degradation. Fbw7 is an F-box protein that facilitates the ubiquitination and degradation of Notch receptors.^{37–40} Thus, we assessed whether TSA regulates Fbw7 expression. The results indicated that TSA represses Fbw7 expression at both the protein and the mRNA levels (Figure 2E). These data suggest that TSA enhances Notch signaling activity, at least in part by stabilizing Notch1ICD via the downregulation of Fbw7 in SMCs. The stabilization of Notch1ICD was consistent with increased acetylation. Cell lysates from control or TSA-treated cells were immunoprecipitated with anti-V5 (Notch1ICD epitope tag) and were immunoblotted with an antibody to acetylated lysines (Figure 2E, bottom blot). Increased acetylated Notch1ICD was

associated with TSA treatment and is consistent with prior reports of acetylation-induced Notch1ICD stabilization.⁴¹

The PI3K/Akt Pathway Is Required for Notch-Mediated SMC Differentiation

Although HDAC inhibitors abrogate Notch-stimulated SMC differentiation, they also enhanced the Notch1ICD/CBF-1 pathway. This prompted us to assess whether HDAC inhibitors impede other pathways activated downstream of Notch signaling. It is known that the PI3K/Akt pathway is important for SMC differentiation,^{42–44} but Notch activation of this pathway is not well studied in SMCs. We first examined the extent to which Notch activation enhances PI3K/Akt activation. After expression of Notch1ICD, there was a significant increase in phosphorylated Akt (pAkt; Figure 3A), which is partially inhibited by HDAC inhibition.

Using the PI3K inhibitor LY294002, we found that PI3K activity is indeed required for Notch stimulation of SMC markers, particularly protein accumulation of calponin1 and SM22 α (Figure 3B). Notch induction of SM actin, smooth muscle myosin heavy chain, SM22 α , and CNN1 transcripts was blocked by inhibition of PI3K activity (Figure 3C through 3D). These data show that multiple pathways, including PI3K/Akt signaling, are required for the full extent of Notch-mediated SMC differentiation.

JNK and p38 MAPK Contribute to Notch1ICD-Mediated SMC Differentiation

Multiple signaling pathways are activated during SMC differentiation by other inducers, such as TGF β 1.^{45–47} We previously reported that activation of Notch signaling enhances TGF β 1 responsiveness in vascular SMCs,⁵ so we tested whether TGF β 1 pathways also were affected by Notch signaling. A pathway of interest is the MAPK pathway, including MEK/Erk, JNK, and p38. After Notch activation in SMCs, we observed significantly increased levels of the phosphorylated forms of JNK (p-JNK) and p38 (p-p38) but not Erk1/2 (Figure 4A and not shown).

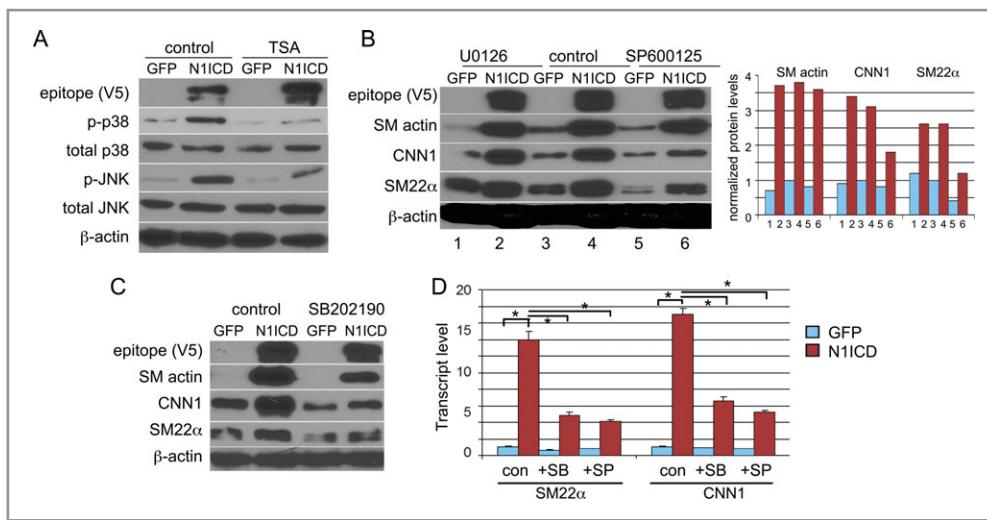


Figure 4. The MAPK pathway is involved in Notch-mediated SMC differentiation. A, Primary human SMCs were transduced with green fluorescent protein (GFP) or Notch1ICD (N1ICD) and were treated with TSA or control vehicle dimethyl sulphoxide (DMSO) for 48 h before analysis. Phosphorylated and total forms of p38 and JNK were measured. B, GFP- or N1ICD-transduced SMCs were treated with the MEK/Erk inhibitor U0126, JNK inhibitor SP600125, or control DMSO for 48 h before analysis by immunoblot for SMC markers. Protein levels are quantified in the graph on right. C, GFP- or Notch1ICD-transduced SMCs were treated with the p38 inhibitor SB202190 or control vehicle for 48 h before immunoblot analysis. D, GFP- or N1ICD-transduced SMCs were treated with the JNK inhibitor SP600125 (SP), the p38 inhibitor SB202190 (SB), or control vehicle DMSO (con) for 48 h, and total RNA was collected for expression analysis by quantitative RT-PCR for SM22 α and CNN1. Data are presented as fold change compared to SMCs with control treatment. Data are presented as mean \pm SEM and were statistically analyzed by ANOVA / Tukey test. Asterisks indicate $P<0.05$; both JNK and p38 inhibitors significantly suppressed N1ICD induction of these genes.

The inhibition of HDACs by TSA repressed Notch-induced JNK and p38 activation (Figure 4A). To further define the requirement of MAPKs in Notch activity in SMCs, we utilized inhibitors targeting MEK/Erk (U0126),⁴⁸ JNK (SP600125),⁴⁹ and p38 (SB201090).⁵⁰ The inhibition of p38 and JNK pathways repressed Notch-mediated calponin1 and SM22 α induction at both the mRNA and protein levels (Figure 4B through 4D). Also, inhibition of p38, but not JNK, moderately decreased Notch-mediated SM actin induction (Figure 4C). Erk signals are not required for Notch induction of SMC differentiation (Figure 4B) because Erk1/2 are not activated by Notch signaling, and U0126 did not change the response to Notch activation (Figure 4B). These data support the idea that HDAC inhibition represses Notch1ICD-mediated SMC differentiation via suppression of the p38 and JNK pathways.

SRF Is Required for Notch1ICD-Induced SMC Differentiation

One possible mechanism of action of HDAC inhibition is to alter the expression levels either of components of Notch signaling or of other factors that control smooth muscle differentiation. Although we found that TSA seemed to stabilize transduced Notch1ICD protein (Figure 2E), levels of the Notch ligands Jagged-1 and Dll-4 were not altered by TSA treatment

(Figure 5A). SRF and its coactivators, myocardin and members of the myocardin-related transcription factor (MRTF) family, are important regulators of SMC phenotype.^{51,52} In addition, Notch signaling was reported to repress myocardin-regulated SMC differentiation via the Notch target gene HRT2.^{53,54} Thus, we addressed the interaction of SRF with Notch-mediated SMC differentiation. First, we assayed SRF protein levels under a variety of conditions, with TSA (Figure 2E and Figure 5A), with silenced CBF-1 (Figure 2E), and with activated Notch1 signaling⁹ (Figure 2E), and we found no evidence of changes in SRF protein levels. Because no suitable antibodies are available to detect myocardin and MRTFA by immunoblot, we performed quantitative RT-PCR to detect mRNA levels (Figure 5B).

We observed a slight increase in the steady-state transcript levels for SRF, myocardin, and MRTFA. Although the changes in SRF mRNA did not translate into changes in protein level, we cannot exclude the possibility of increased levels of SRF cofactors. Therefore, we used siRNA to suppress SRF activity. SRF knockdown was confirmed by quantitative RT-PCR and immunoblotting (Figure 5C and 5D). SRF transcript was reduced by $\approx 80\%$, leading to significantly decreased SRF protein. In cells with reduced SRF, there was a dramatic decrease in basal SMC marker expression and the induction after Notch activation (Figure 5E). Thus, endogenous SRF contributes significantly to the differentiated phenotype of SMCs, but SRF is not the target of HDAC inhibition in these cells.

The CArG Box Is Required for Notch1ICD-Mediated SM Actin Promoter Activity

The SM actin gene has a well-characterized promoter, and its transcription is sensitive to both SRF/myocardin and Notch signaling. To analyze whether TSA affects SM actin promoter activity, we used a luciferase reporter (p125, SMA-125).³³ As expected, activation of Notch1 significantly enhanced SM actin promoter activity, and this was blocked by HDAC inhibition (Figure 5F). To investigate the role of the SRF-responsive CArG box in Notch transcriptional activity, a

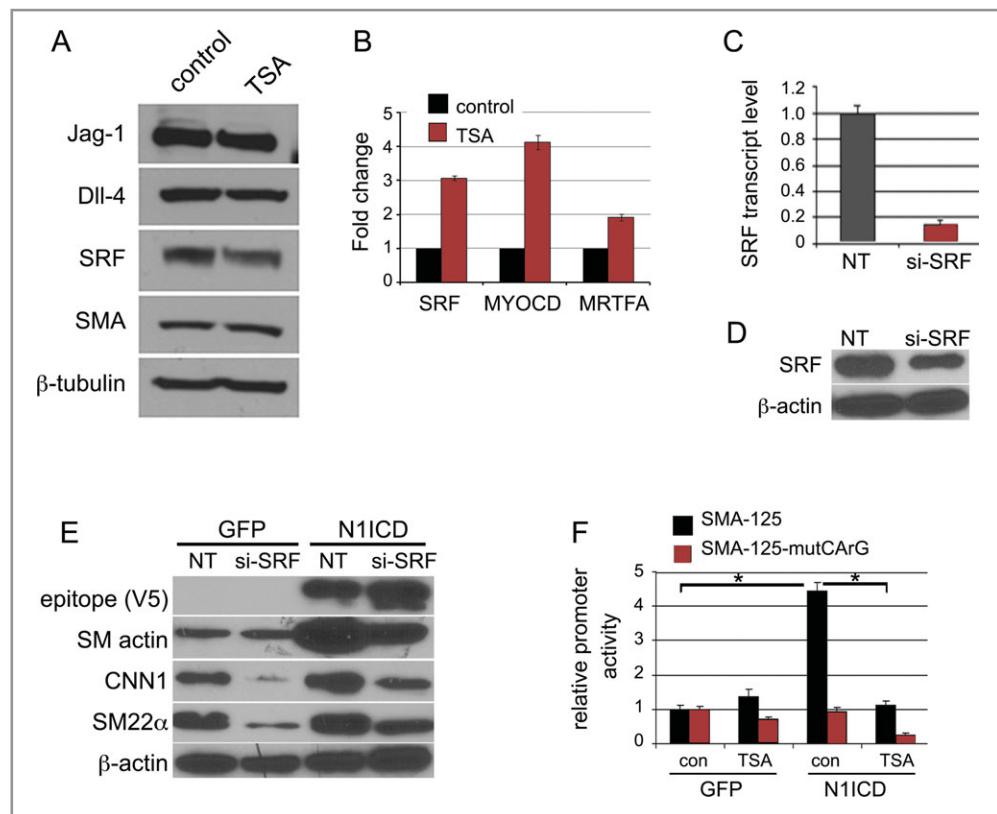


Figure 5. SRF is required for Notch-mediated SMC differentiation. A and B, Primary human SMCs were treated with TSA or vehicle control dimethyl sulphoxide (DMSO) for 48 h before Western blotting for Jag-1, DII-4, SRF, and SMA protein levels (A) or quantitative RT-PCR analysis for SRF, myocardin (MYOCD), and MRTFA transcript levels (B). Data are presented as fold change as compared to SMCs treated with DMSO control. Human SMCs were transduced with a nontargeting siRNA (NT) or with siRNA targeted against SRF (si-SRF) for 4 days and were collected to examine the efficiency of knockdown by quantitative RT-PCR (C) and immunoblot (D). E, NT- or si-SRF-transduced SMCs were infected with green fluorescent protein (GFP) or Notch1ICD (N1ICD) for 3 days and were collected for analysis of SMC markers. F, SMCs were transduced with GFP or N1ICD with the SM actin promoter reporter construct (SMA-125) or the construct with the mutant CArG box (SMA-125-mutCArG). Cells were treated with TSA or control vehicle DMSO (con) before analysis. Data are presented as fold change compared to SMCs with control DMSO treatment. Data are presented as mean \pm SD, and asterisks indicate $P<0.05$. SMA-125 activity was significantly increased with N1ICD compared to GFP, and this activity was significantly reduced with TSA.

CArG mutant (-62) p125 reporter plasmid (pA 125, SMA-125-mutCArG) was used.³³ SMCs were transduced with green fluorescent protein, Notch1ICD, and SMA-125-mutCArG and then were treated with TSA or vehicle for 48 hours before luciferase assay. Activation of Notch was not able to promote transcription of the CArG mutant SM actin promoter (Figure 5F), consistent with the requirement of SRF activity for Notch-mediated differentiation.

HDAC Inhibition Repressed Notch1ICD-Mediated SMC Differentiation From 10T1/2 Cells

We showed that Notch-mediated activation of several SMC markers is sensitive to HDAC inhibition in human primary SMCs. To test whether this mechanism is conserved in the

differentiation of progenitor cells into SMCs, we used the C3H10T1/2 model, a murine embryonic mesenchymal precursor line that previously has been characterized to respond to Notch signaling by differentiation into the SMC lineage.³⁶ To test the effects of TSA on this differentiation, we transduced cells with Notch1ICD in the absence or presence of TSA (Figure 6A). Similar to human SMCs, HDAC inhibition in C3H10T1/2 cells dramatically repressed Notch activity to induce these markers. This trend also was seen at the transcript level, because Notch activation increased levels of transcript for SMC markers, and this was suppressed by HDAC inhibition (Figure 6B). Finally, TSA enhanced Notch1ICD/CBF-1 pathway activation (Figure 6C), which is similar to enhanced CBF-1 pathway in human SMCs. These data show that the induction of the SMC contractile phenotype by Notch signaling in human primary SMCs and a murine progenitor cell require HDAC activity but through a mechanism that is independent of canonical CBF-1 signaling.

Discussion

SMCs maintain considerable phenotypic plasticity at the molecular and cellular levels, which is essential for vascular development and is a hallmark of the pathogenesis of vascular diseases. The molecular mechanisms regulating SMC differentiation and maintenance of the contractile SMC phenotype are not completely understood, although multiple pathways regulate SMC phenotypic transitions. Our study is the first to examine the effects of HDAC inhibition on Notch induction of the SMC contractile phenotype. Because inhibition of HDAC activity reduces the level of neointimal lesion formation in vivo,^{18,20} this is an area of interest in considering novel therapies for vascular diseases. Therefore, it is important to understand the relationship of HDAC activity and multiple pathways that regulate SMC phenotype. The HDAC inhibitor

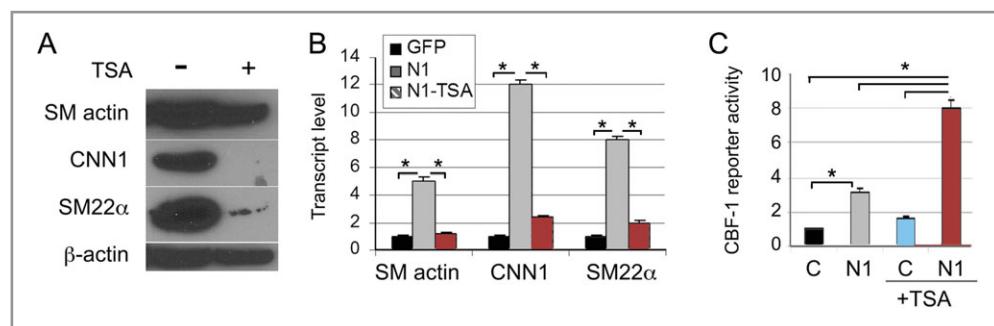


Figure 6. HDAC inhibition blocks Notch-mediated SMC differentiation in C3H10T1/2 progenitor cells. A and B, C3H10T1/2 cells were transduced with green fluorescent protein (GFP) or Notch1ICD (N1ICD) and were treated with TSA (+) or control (−) for 4 days before collection for immunoblot (A) or quantitative RT-PCR. C, C3H10T1/2 cells were transduced with the CBF-1 luciferase reporter construct and GFP control (C) or Notch1ICD (N1) and then were treated with TSA for 2 days. Graphed are fold changes compared to SMCs with control dimethyl sulphoxide (DMSO) treatment. Data are presented as mean±SEM, with asterisks indicating $P<0.05$.

TSA caused significant inhibition of the Notch-mediated SMC contractile phenotype, both in human primary aortic SMCs and in a mouse mesenchymal progenitor model. Because TSA is a relatively wide-spectrum HDAC inhibitor, we also used a class I HDAC inhibitor with specificity to HDAC1 and HDAC3 (MS-275). This inhibitor yielded the same activity in regulating SMC markers as TSA. In addition, we utilized a class II inhibitor, which was unable to block Notch activity in inducing SMC markers (data not shown). Therefore, antagonism of Notch signaling is a selective feature of inhibition of class I HDAC. Class I HDAC (HDAC1, HDAC2, and HDAC3) also were the molecules shown to mediate cytokine-stimulated proliferation in rat SMCs.¹⁸

The effects of HDAC inhibition in human SMCs were gene specific, as the Notch targets of the HRT family were not affected, unlike SMC contractile proteins. This specificity for selective Notch target genes can be explained by our observation that HDAC inhibition does not inhibit canonical CBF-1 activity induced by Notch signaling. In addition, knockdown of CBF-1 activity was not sufficient to completely block the induction of SMC differentiation by Notch. These observations led us to discover alternative signaling pathways activated by Notch signaling. We found that some of the effects of Notch signaling in SMCs can be accounted for by activation of the PI3K/Akt, JNK, and p38 pathways. These pathways are sensitive to HDAC inhibition and are particularly important for increasing calponin1 and SM22α levels. Overall, HDAC inhibition plays a dual role of enhancing CBF-1-mediated Notch signals in SMCs but repressing the Notch-mediated SMC differentiation phenotype. We suggest this dual function is enabled by the activation of multiple pathways downstream of Notch. The canonical CBF-1 and HRT target gene activation is independent of HDAC activity, whereas signaling via PI3K/Akt, JNK, and p38 pathways requires HDAC activity. The requirement

of SRF for Notch-mediated SMC differentiation suggests interplay between Notch, HDACs, and SRF in SMC differentiation.

There might be multiple mechanisms of HDAC activity that regulate SMC differentiation. In other studies that examined cytokines that inhibit SMC differentiation (eg, platelet-derived growth factor-BB in rat SMCs²⁵), it was shown that suppression of some SMC markers is partially due to HDAC2, HDAC4, and HDAC5 activity in a traditional role of deacetylating histone H4 to silence gene transcription.

Likewise, oxidized phospholipid suppression of SM actin in rat SMCs was also associated with recruitment of HDAC2 and HDAC5 and hypoacetylation of histone H4 at the SM actin promoter.²⁶ TGFβ, an inducer of SMC differentiation, promotes SM22α transcription, and HDAC inhibition enhances this effect by acetylation of the SM22α promoter.²⁷ Although we found that Notch-induced SM22α induction was suppressed by HDAC inhibition, we did observe that the canonical Notch-mediated CBF-1 transcriptional activity was increased, similar to the reported increase in Smad activity. In combination, these data show that the upstream signal activator (eg, platelet-derived growth factor, TGFβ, Notch) and the mechanism of gene target regulation (direct transcriptional activation versus secondary mechanism) will modify the outcome of blocking HDAC activity in SMCs. Class I HDACs also have multiple nonhistone substrates, including transcription factors. This brings up the possibility that both histone and nonhistone targets of HDACs are important to consider in the potential therapeutic use of HDAC inhibitors to regulate SMC differentiation, proliferation, or neointimal lesion formation.

Although substantial evidence shows that Notch signaling regulates SMC differentiation and phenotypic modulation, there are some functional discrepancies, which could be due partly to different experimental systems, species, and phenotypic read-outs. This is further complicated by the fact that Notch interacts with and is regulated by other signaling pathways. For example, we found that the Notch direct target genes of the HRT family have a negative feedback role in Notch-induced SMC differentiation⁶ and also negatively regulate myocardin and TGFβ-mediated SMC differentiation.^{5,53,54} Notch signaling transcriptionally activates SM actin and smooth muscle myosin heavy chain expression through the CBF-1-binding sites in their promoters,^{8,36} and HRTs can repress Notch1ICD induction of SM actin expression by inhibiting

Notch1ICD/CBF-1 binding to the SM actin promoter. We also recently found that miR143/145 is a direct transcriptional target of Jagged1/Notch signaling via CBF-1-binding sites in the promoter.⁹ However, the mechanism by which Notch regulates other SMC contractile proteins, such as calponin and SM22 α , is still unclear. Although there are CBF-1 consensus-binding sites in the calponin and SM22 α promoters, we have not been successful in demonstrating NICD/CBF-1 binding to these promoters with chromatin immunoprecipitation assays in human SMCs (not shown). Therefore, it is likely that although some SMC marker genes are direct transcriptional targets, Notch signaling can induce other contractile genes in an indirect manner, possibility via other signaling pathways or transcriptional mechanisms, including SRF or Smad transcriptional activity.⁵ We found that PI3K/Akt, JNK, and p38 MAPKs are required for Notch-mediated induction of calponin1 and SM22 α . However, known direct transcriptional targets such as SM actin were less affected by inhibition of the MAPKs or PI3K pathways. These findings are consistent with our model that different signaling mechanisms act downstream of Notch activation to regulate SMC contractile gene expression.

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Disclosures

None.

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