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Circ Res. Author manuscript; available in PMC 2009 March 30.

Published in final edited form as:
Circ Res. 2008 March 28; 102(6): 661–668. doi:10.1161/CIRCRESAHA.107.165134.

Hairy-Related Transcription Factors Inhibit Notch-Induced Smooth Muscle α-Actin Expression by Interfering With Notch Intracellular Domain/CBF-1 Complex Interaction With the CBF-1–Binding Site

Yuefeng Tang, **Sumithra Urs**, and **Lucy Liaw**
From the Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough.

Abstract

Notch signaling regulates smooth muscle cell phenotype and is critical for vascular development. One Notch target is smooth muscle α-actin (SMA), a differentiated smooth muscle cell marker. The Notch intracellular domain (NotchICD) forms a complex with CBF-1 (C-promoter– binding factor-1) and directly induces SMA expression. Using primary human smooth muscle cells, we show that expression of the constitutive active ICD of human Notch1, Notch2, or Notch4 receptors increase SMA levels. NotchICD also induce expression of the transcriptional repressors HRT1 (Hairy-related transcription factor 1) and HRT2, in a CBF-1–dependent manner. However, unlike the activating effects of NotchICD, HRT1 or HRT2 represses basal SMA expression, and both are strong antagonists of NotchICD-induced SMA upregulation. This antagonism does not depend on histone deacetylase activity and occurs at the transcriptional level. Competitive coimmunoprecipitation experiments demonstrate that HRT does not disrupt the association of NotchICD and CBF-1, which form a complex in the presence or absence of HRTs. However, HRT suppresses NotchICD/CBF-1 binding to the SMA promoter, as measured by chromatin immunoprecipitation, and transactivation of an SMA promoter reporter spanning sequences −124/+32. SMA expression was regulated similarly following endogenous Notch activation in smooth muscle cells by coculture with endothelial cells, and this effect was also sensitive to HRT inhibition. Temporally defined HRT activity may constitute a negative feedback mechanism of Notch signaling. Our study presents a novel mechanism by which a balance between Notch signaling and HRT activity determines the expression of smooth muscle differentiation markers including SMA.

Keywords

vascular smooth muscle cells; smooth muscle α-actin; signaling pathways; endothelial cells

Notch signaling is important in vascular development and the pathogenesis of vascular diseases.[1,2] Following Notch activation by Jagged/Delta ligands, Notch intracellular domain (ICD) is proteolytically liberated, enters the nucleus, and participates in a multiprotein complex including CBF-1 (C-promoter– binding factor-1) that transcriptionally activates genes, including the Notch effector genes Hes, HRT1 (Hairy-related transcription factor 1) (also

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

Disclosures
None.

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known as Hey1, Herp2, Hesr1), and HRT2 (Hey2, CHF1, Herp1, Hesr2).[3–5] In smooth muscle cells (SMCs), Notch/HRT regulates cell proliferation, survival, and migration.[6–11]

Smooth muscle α-actin (SMA) is a marker of the differentiated SMCs with well-characterized transcriptional regulation.[12–14] NotchICD/CBF-1 activity induces SMA expression in multiple cell types.[15] This is consistent with studies showing that Jagged1 activation of Notch signaling supports SMC differentiation[10] and that Notch signaling is required in vivo for proper SMC differentiation from neural crest progenitors.[16] Interestingly, other studies indicate that Notch1 or Notch3 activation or HRT activity suppresses SMA expression and cell differentiation[6] and that Notch1ICD or HRT2 expression inhibits myocardin-activated SMC marker gene transcription.[17] We focused on SMA regulation in human primary aortic SMCs (HASMCs) and found that Notch1, Notch2, and Notch4 activation increases SMA expression in a CBF-1–dependent manner. We characterized a novel negative-feedback pathway by which expression of HRT1 or HRT2, alone or in combination with Notch activation, suppresses SMA transcript and protein levels. This is the first report showing that SMA regulation by Notch signaling depends on a balance between Notch activity and HRT activity, providing a mechanism for the activation and suppression of SMA expression. We propose a model by which HRT interacts with SMA promoter elements to consequently inhibit NotchICD/CBF-1 activation of SMA transcription.

Materials and Methods

Cell Culture

HASMCs, human aortic endothelial cells (HAECs), and human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Walkersville, Md). HASMCs were maintained in SmGM2 medium and used between passages 4 and 7. Endothelial cells were maintained in EGM2 medium and used between passages 3 and 7. For coculture, HAECs or HUVECs were cultured in 6-well plates until confluent and overlaid with HASMCs (control or expressing CBF-1 luciferase or HRTs). Endothelial cells were retrovirally transduced with green fluorescent protein (GFP) or soluble Jagged1.[18] γ-Secretase inhibitor IX (Calbiochem) was used at 10 μmol/L. Cell lysates were collected for luciferase assays or immunoblot.

Constructs and Gene Expression

Adenoviral constructs in pAdlox were generated as described.[19] The Notch1ICD, Notch2ICD, Notch4ICD, HRT1, and HRT2 constructs contain C-terminal epitope tags.[8] pCMX-CBF-1 for wild-type CBF-1 expression and pEF-BOSneo-R218H for dominant-negative (DN)-CBF-1 have been characterized by T. Honjo and colleagues,[20] and pEF-BOSneo-R218H was provided by I. Prudovsky (Maine Medical Center Research Institute, Scarborough). The mouse HRT2 mutant constructs, mHRT2 B(−) and HRT2 C(−) were provided by E. Olson (University of Texas Southwestern Medical Center, Dallas).[5] Cells were transduced 12 hours after plating for 24 hours with 100 $TCID_{50}$ (tissue culture 50% infective dose) virus particles per cell, optimized empirically by immunohistochemistry to achieve >90% transduction with minimal cytotoxicity. Endothelial cells were transduced with GFP or soluble Jagged1[18] using PINCO retroviruses (provided by Cathrin Brisken, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland).

Immunoblotting and Immunohistochemistry

Whole cell extracts were prepared as described.[8] Immunoblot analyses used anti-V5 (Invitrogen), anti-β-actin, anti-SMA, anti-hemagglutinin, and anti-FLAG (Sigma). For immunostaining, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100/PBS. Primary antibodies were diluted 1:400, followed by detection using tetramethylrhodamine B isothiocyanate (TRITC)-conjugated secondary antibody.

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RT-PCR and Quantitative RT-PCR

Total RNA was extracted, treated with RNase-free DNAase1 (Promega), and reverse-transcribed using 20 pmol/L oligo(dT) with AMV reverse transcriptase (Promega). Quantitative RT-PCR was performed using the iCycler (Bio-Rad) using SYBR green (Bio-Rad) with 20 ng of cDNA as template, in triplicate. Threshold cycle numbers were calculated at log phase of amplification and normalized to cyclophilin. Primer sequences are provided in Table I of the online data supplement, available at http://circres.ahajournals.org.

Transient Transfection and Luciferase Assay

HASMCs were plated at 20 000 cells per well in a 24-well plate and transduced with adenovirus (100 $TCID_{50}$ virus particles per cell), 0.25 μg reporter plasmid, 0.75 μL of Gene Juice (Invitrogen), and 25 ng of *Renilla* luciferase plasmid per well. Two days after transfection, cells were collected for luciferase assay.[8] All experiments were repeated at least 3 times, and representative results shown. Human SMA-S promoter fragments were generated by PCR from genomic DNA, ligated into pGL3 basic vector, and sequenced. PCR primers amplify 157 bp (−124/+32) of the SMA-S promoter.[21]

Coimmunoprecipitation Assay

Cell lysates (300 μg) were incubated with 1.5 μg of antibody, followed by the addition of protein A/G agarose beads. Samples were washed to remove unbound proteins, boiled in sample buffer, centrifuged, and removed from beads. Immunoprecipitated proteins were subjected to SDS-PAGE, and immunoblotting was performed with anti-V5, anti-Flag, or anti–CBF-1 antibodies.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as described.[8] Cross-linked chromatin was immunoprecipitated with anti-Flag (M2, Sigma), anti-V5 (Invitrogen), normal mouse IgG (Santa Cruz Biotechnology), anti–CBF-1 (H-50, Santa Cruz Biotechnology), or normal rabbit IgG (Santa Cruz Biotechnology). Input DNA and immunoprecipitated chromatin were subjected to PCR using primers encompassing the CBF-1–binding site from the SMA promoter.

Statistical Analysis

Statistical analyses were performed using Student's *t* test, with a significant difference determined as $P<0.05$. Data are presented as means±SD.

Results

Expression of NotchICD and HRTs in HASMCs

Initial studies characterized expression conditions in human primary cells. All adenoviral constructs were titered over an 8-fold concentration to determine optimal protein (immunoblot analysis) with minimal cytotoxicity (Figure 1). We achieved high nuclear expression of each construct (Figure 1A and 1B), and NotchICD induced transactivation of a CBF-1 binding element (not shown). HRTs are transcriptional targets of Notch signaling in SMCs.[3,5,8] In HASMCs, HRT1 and HRT2 transcripts were significantly increased with NotchICD transduction compared with control (Figure 1C). In contrast, CBF-1 mRNA levels were not different in any of the transfectants (Figure 2A).

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Notch and HRT Differentially Regulate SMC Marker Gene Expression

Activation of Notch through Jagged1 induces SMA transcription in a CBF-1–dependent manner.[15] We found that activation of Notch1, Notch2, or Notch4 also increased SMA transcripts and also induced SM myosin heavy chain (SM-MHC), while having minimal effects on transcript levels of desmin, myocardin, GATA-6, and SM22α, other markers of SMC differentiation (Figure 2A). By contrast, HRT1 or HRT2 did not mimic the effects of NotchICD but suppressed transcripts for SMA, desmin, SM-MHC, and GATA-6 (Figure 2A). By immunoblot (Figure 2B), we confirmed that SMA was highly elevated following Notch activation and suppressed by HRT1 or HRT2. The Notch-induced increase of SMA in HASMCs was also evident by immunofluorescence staining (Figure 2C).

Because Notch regulates SMA transcriptionally, we analyzed the SMA gene. There are 2 potential transcripts for SMA, although they both encode for the same protein sequence with the translational start site in exon 2 (Figure 3A). The difference in these 2 transcripts is the size of intron 1, which is 3777 and 41 985 bp for the short (SMA-S) and long (SMA-L) forms, respectively. The SMA-S promoter contains a CBF-1 consensus binding site TGGGAA at position −64 relative to the transcriptional start site,[15] and a less conserved CBF-1 motif at −1309 (Figure 4A and supplemental Table II). The SMA-L promoter contains 2 consensus CBF-1 sites and 3 consensus HRT-binding sites. We designed primers, SMA-S, to detect the short intron 1 transcript, another pair of primers, SMA-L, to detect the long intron transcript, and a third pair of primers, SMA-W, to detect both. Although both transcripts were detectable in primary HASMCs, the SMA-S form was expressed at a 10-fold higher level (Figure 3B and Figure 4). Activation of Notch1, Notch2, or Notch4 significantly increased SMA-S transcript, (Figure 4B), whereas HRT1 or HRT2 decreased transcripts. In contrast, activation of Notch did not affect SMA-L, although HRT1 or HRT2 strongly suppressed expression from 15– to 20-fold compared with controls (Figure 4C). Notch-induced elevation of SMA protein is likely attributable to increased SMA-S transcript, thus we focused on SMA-S for subsequent experiments.

Notch Induction of SMA Is Dependent on CBF-1 and Antagonized by HRTs

Because Notch has CBF-1–independent functions, we tested whether CBF-1 was required for SMA regulation. We used the DN-CBF-1 (R218H)[20] and found a dose-dependent inhibition of Notch-induced SMA expression (Figure 5A) showing a requirement for CBF-1 activity. The same was true whether Notch1, Notch2, or Notch4 was activated.

Hes1 and HRT2 have negative autoregulation activity,[5,22] and we asked whether HRT had any effect on Notch-mediated SMA expression. We used cotransfection of Notch-ICD with HRT1 or HRT2 in HASMCs. As described, all of the NotchICDs strongly induced SMA expression (Figure 5B). When HRT1 or HRT2 were simultaneously expressed, however, induction of SMA by NotchICD was dramatically decreased. Importantly, neither HRT1 nor HRT2 affected CBF-1 expression (Figure 2A). These findings suggest that HRT1 and HRT2 inhibit Notch-induced SMA expression by functionally antagonizing NotchICD/CBF-1 activity, not by modulation of CBF1 expression. Quantitative PCR showed that HRTs repressed NotchICD-mediated SMA expression at the transcript level (Figure 5C). We also took advantage of HRT mutant constructs to query selected HRT structural domains. When cells were cotransfected with NotchICD and the HRT2 B(−) or HRT2 C(−) mutant, both HRT2 mutants decreased SMA protein levels compared with NotchICD control (Figure 5D). There are at least 2 possible mechanisms by which HRTs antagonize Notch regulation of SMA expression. First, HRT may interfere with complex formation between NotchICD and CBF-1, or, secondly, HRT may inhibit NotchICD/CBF-1 complex binding to its recognition site in the SMA promoter.

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HRT Suppression of SMA Is Independent of Histone Deacetylase Activity

HRT interacts with the corepressors mSin3 and SIRT1, which have histone deacetylase (HDAC) activity.[23,24] To determine whether HRT activity in the context of SMA expression requires recruitment of HDACs, we treated HASMCs with trichostatin A, a potent HDAC inhibitor. Even high concentrations of 500 ng/mL trichostatin A were ineffective in altering HRT activity in suppressing SMA expression (Figure 6A). Likewise, the expression of SMA following Notch activation with HRT coexpression was not significantly altered by the presence of trichostatin A (Figure 6B). These data suggest that HRT does not need to recruit HDACs to inhibit SMA expression and antagonize Notch.

HRT Does Not Disrupt NotchICD/CBF-1 Complex Formation

HRT and HES can complex with CBF-1 in Cos1 cells[25]; thus, we tested whether HRTs bind CBF-1 in HASMCs. This is a potential mechanism by which HRT can disrupt NotchICD/CBF-1 activity at the SMA promoter. HASMCs were transduced with constructs separately or in combination, and immunoprecipitation of total cell lysates were performed, followed by immunoblot to detect coimmunoprecipitating proteins (Figure 7A). When NotchICD was expressed alone (lanes 1 to 2) and immunoprecipitated with anti-V5, endogenous CBF-1 was undetectable by immunoblot. However, coexpression of NotchICD and CBF-1 led to coimmunoprecipitation of the complex (lanes 3 to 4). When NotchICD and CBF-1 were coexpressed with either HRT 1 (lanes 5 and 7) or HRT2 (lanes 6 and 8), there was no difference in the ability to immunoprecipitate NotchICD and detect CBF-1. Under no conditions were HRT1 or HRT2 (Flag) detected following immunoprecipitation of NotchICD. Likewise, we found no evidence of HRT binding to CBF-1 (not shown). Our findings show that HRTs do not disrupt the NotchICD/CBF-1 complex and do not bind to the complex. Similar results were obtained using Notch4ICD (not shown).

HRTs Interfere With NotchICD/CBF-1 Binding to the SMA Promoter

To test whether HRT1 and HRT2 dissociate CBF-1 from its binding site in SMA-S (−64 in relation to the transcriptional start), we performed chromatin immunoprecipitation (Figure 7B). HASMCs were transduced with GFP, Notch1ICD, or Notch1ICD in combination with either HRT1 or HRT2. Protein/DNA complexes were cross-linked, and lysates were tested for the amplification of SMA-S (input). Immunoprecipitation was performed using either normal IgG or anti-V5 to detect NotchICD. Following specific immunoprecipitation, we amplified SMA-S in Notch1ICD-expressing cells but not in the GFP group. However, coexpression of HRT1 or HRT2 eliminated immunoprecipitation of SMA-S with NotchICD, suggesting that HRTs were inhibiting NotchICD/CBF-1 complex binding to the SMA promoter. The functional consequence of this inhibition was tested using an SMA reporter construct encompassing the sequence −124/+32. Activation of Notch, particularly Notch2, was effective in increasing reporter transactivation compared with GFP-expressing cells (Figure 7C). The activity of HRTs varied in this assay but, in general, was effective in inhibiting NotchICD-induced SMA promoter transactivation.

Activation of Endogenous Notch Signaling in SMCs via Coculture With Endothelial Cells Induces HRT-Sensitive Expression of SMA

Notch activation facilitated molecular studies of the SMA promoter and analysis of protein complex formation. Our next question was whether placing HASMCs under conditions to mimic physiological interactions would regulate gene expression similarly. We previously demonstrated that endothelial denudation in vivo induced reciprocal expression of Jagged1 and Notch receptors in endothelium and SMC, respectively.[26] The regenerating endothelial wound edge expressed localized, high levels of Notch ligands, and adjacent SMCs had elevated Notch expression, supporting the hypothesis that heterotypic Notch stimulation from

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endothelial cells affects SMC behavior. We established a coculture system of primary human endothelial cells in contact with HASMCs. Using RT-PCR and immunoblot, we verified that HAECs and HUVECs express both Jagged1 and Delta-like1, and Notch1, Notch2, and Notch3 are expressed endogenously in HASMCs (not shown). We established monolayers of endothelium expressing GFP (Figure 8A) and then overlaid the monolayer with HASMCs transfected with the CBF-1 luciferase reporter construct to test for Notch activation in heterotypic cell culture. HASMCs alone have low levels of endogenous CBF-1 activation that increased 7-fold when HASMCs were in contact with endothelial cells (Figure 8B). This induction was significantly inhibited when endothelial cells were expressing dominant negative sJagged1.[18] Using this system, we tested HAECs and HUVECs in coculture with HASMCs to determine effects on SMA. Although SMA is expressed in HASMCs, protein levels were enhanced in coculture with HAECs (Figure 8C) or HUVECs (Figure 8D). Similar to its effects on CBF-1 reporter activation, sJagged1 expression in HAECs blocked the increase in SMA induced by coculture (Figure 8C), showing specificity of this effect to Notch signaling. Similarly, γ-secretase inhibitor decreased SMA levels in cocultures of endothelial/SMCs (Figure 8E). These results show that coculture of endothelial cells with HASMCs induces Notch signaling in SMCs, leading to increased SMA production.

To determine whether HRT can impact SMA expression in our coculture system, HRT1 or HRT2 were expressed in HASMCs before coculture with endothelial cells (Figure 8F). In the absence or presence of endothelial cells, HRT expression was a strong suppressor of SMA accumulation. Taken together, our data show that HRT is a major inhibitor of SMA expression in SMCs, whether it is basal expression induced by constitutive Notch signaling or induced via endogenous Notch signaling following coculture with endothelial cells.

Discussion

Phenotypic modulation of SMCs occurs during embryonic development, vascular injury, and cardiovascular disease. Transcriptional pathways regulating SMC genes include serum response factor and myocardin,[27] and accumulating evidence suggests that Notch is an additional pathway critical for SMC differentiation.[6,10,15,28] Functional roles of Notch/HRT signaling in the cardiovasculature in vivo were uncovered by extensive mouse genetic studies.[29] Notch signaling components are temporally and spatially expressed following injury in vivo,[8,30] and HRT2 is present in human atherosclerotic lesions.[28] Mutations of the Notch pathway in Alagille syndrome and CADASIL emphasize the clinical importance of these pathways in human diseases.[31]

We studied human primary SMCs and their coculture with endothelial cells. One SMC marker, SMA, is exquisitely sensitive to Notch and HRT signaling. Although distinct Notch receptors may provide variable signals,[8,32–34] all Notch pathways we tested (N1ICD, N2ICD, N4ICD) had conserved activity in the induction of SMA. HRT1 and HRT2 have similar functions in repressing basal SMA-S and SMA-L transcript levels and antagonizing Notch induction of SMA-S. The positive (Notch) and negative (HRT) balance in the regulation of SMA is a novel finding that provides a mechanism by which SMC differentiation genes can be sensitively fine tuned. Indeed, vascular injury corresponds to induction of Notch ligands and receptors,[8,30] and we previously noted a reciprocal relationship between endothelial cell expression of ligand and SMC expression of Notch receptors in vivo[26] following injury. We propose that HRT is a candidate suppressor of mature SMC markers, including SMA, which are downregulated following vascular injury in vivo.[35] The physiological relevance of our work is highlighted by the conserved regulation of SMA in endothelial/SMC coculture, a condition that activates Notch signaling in SMCs. Our work thus adds to our understanding of the positive and negative regulators of Notch signaling that are important in modulating Notch responsiveness in vascular cells.[36]

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There are still discrepancies about whether Notch induces or inhibits differentiation, which may be partly attributable to different experimental systems and species, as well as phenotypic readouts. For example, there is an established crossregulation of myocardin target genes by Notch/HRT2,[17] but other SMC gene regulatory pathways may be differentially impacted by Notch signaling. In vitro studies showed that Notch signaling induces SMC differentiation and identified SMA and SM-MHC as NotchICD/CBF-1 targets.[10,15] However, Proweller et al[17] and Morrow et al[37] reported that HRT2-mediated Notch signaling inhibits SMC differentiation, and Doi et al[28] reported that HRT repressed myocardin-induced SMC differentiation. These apparent discrepancies should be considered in light of our findings that although NotchICD directly activates markers such as SMA and SM-MHC, concomitant expression of HRT1 or HRT2 is sufficient to turn off these signals. We envision a regulatory loop that in vivo would allow for temporally restricted Notch signaling and induction of target gene expression until HRT production rises above a threshold needed to antagonize the signal.

Members of the Hes and HRT families of bHLH (basic helix–loop–helix) proteins have been characterized as Notch effectors that mediate Notch activity.[3,5] In some cases, expression of Hes or HRT alone recapitulate the phenotype of activated Notch signaling,[8,38] and some Notch-induced vascular phenotypes are inhibited by blocking Hes/HRT function.[6] However, Hes/HRT can participate in specific negative-feedback loops that may limit the extent of Notch/Hes/HRT signaling. Hes-1 inhibits its own transcription by binding to N boxes (CACNAG) in its promoter,[22] providing negative feedback. Although HRT2 also shows characteristics of negative autoregulation of its expression,[5] this appears to be a novel mechanism distinct from HRT2 binding to its own promoter. We also observed that expression of HRT2 led to accumulation of HRT2 protein but not HRT2 mRNA, consistent with a negative autoregulatory activity. Another related bHLH transcription factor, Stra13/DEC1/SHARP2, also negatively autoregulates its expression in an HDAC-dependent manner.[39] Our study extends this concept to show that other genes such as SMA are also susceptible to negative-feedback regulation by HRT through a mechanism involving disruption of Notch/CBF-1 interaction with the SMA promoter in an HDAC-independent manner. This disruption is not attributable to disassembly of the NotchICD/CBF-1 complex, and extensive coimmunoprecipitation experiments did not show evidence for ternary complex formation with HRTs. The matrix group from MatInspector[40] did not identify strong consensus HRT binding sites in the SMA-S promoter, but there are several E box motifs at −258, −330, −1222, and −1742 and HRT consensus motifs in SMA-L. Although the SMA-S luciferase promoter (−124/+32; Figure 7C) showed responsiveness to both Notch activation and accompanying HRT expression with induction and suppression, respectively, the extent of the response was not as dramatic as expected based on the quantitative changes in SMA-S transcript levels. This leaves the likely possibility that important Notch and HRT regulatory sites are upstream of −124 in the SMA-S promoter, and these upstream sequences are currently under investigation. We also need to consider the possibility that HRT is interacting with DNA via another DNA-binding protein or through previously uncharacterized DNA motifs. Experiments to test the direct binding of HRTs to the SMA promoter have been as yet inconclusive, because of the lack of information regarding HRT binding sites in the SMA gene.

In summary, our studies provide the novel findings that HRT1 and HRT2 are repressors of SMA expression and, further, are able to antagonize Notch-induced SMA expression. HRT does not inhibit NotchICD/CBF-1 complex formation, although it decreases NotchICD/CBF-1 binding and activation of the SMA promoter. SMC responsiveness to Notch signaling, therefore, may be limited temporally and spatially by the production of HRT factors, which feed back to inhibit specific Notch target gene activation.

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Supplementary Material

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Acknowledgments

We are grateful to our colleagues who generously shared reagents. We appreciate the work of Nancy Chandler-Conrey and Jeong Yoon, PhD, of our Viral Vector core for production of adenoviral constructs. We also thank Drs Doug Spicer, Jeong Yoon, and Howard Crawford for critical discussions and expertise.

Sources of Funding

This work was supported by NIH grants R01 HL070865 (to L.L.) and P20RR1555 (to R.E. Friesel and L.L.).

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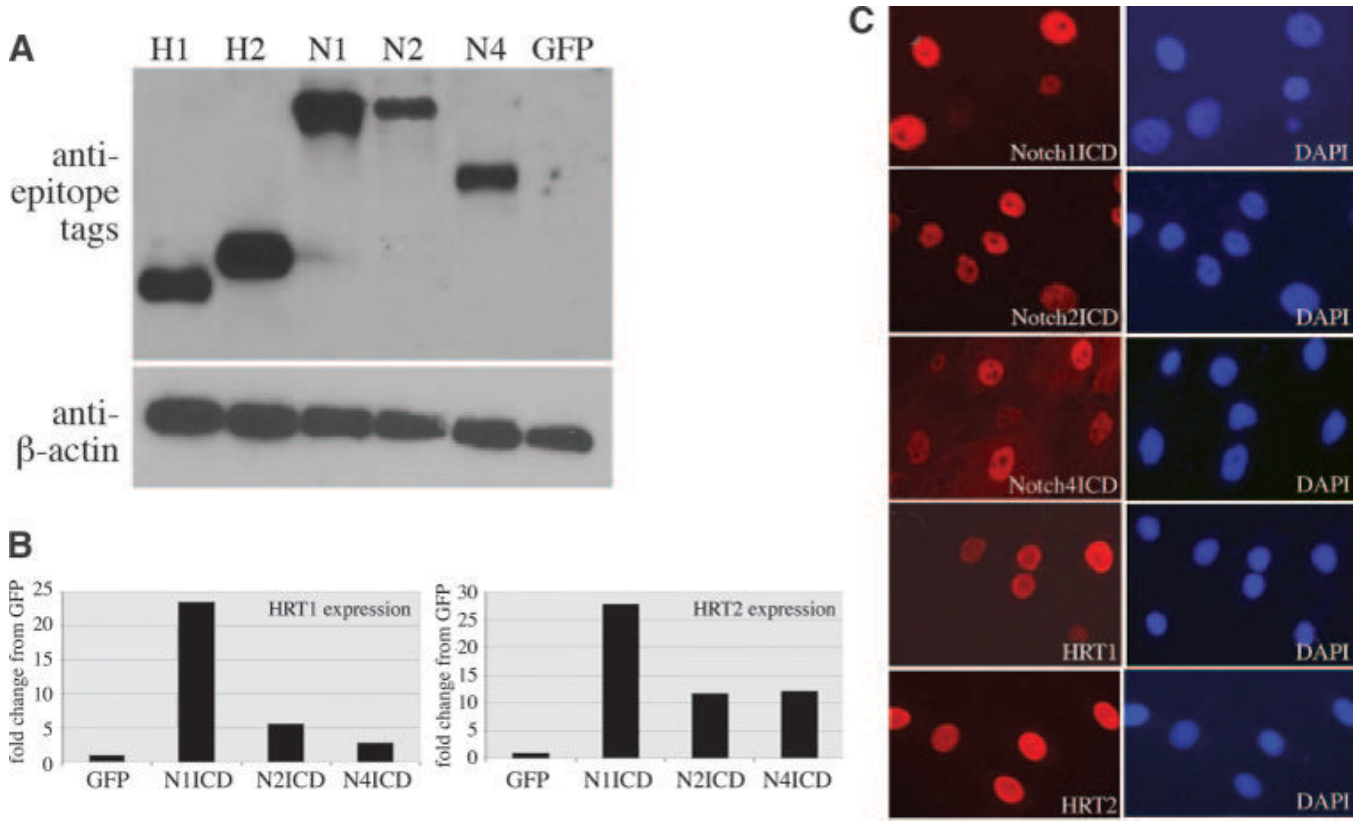

Figure 1. Expression and localization of NotchICD and HRT in human primary SMCs

Cells were adenovirally transduced with Notch1ICD (N1), Notch2ICD (N2), Notch4ICD (N4), HRT1 (H1), or HRT2 (H2). A, Cell lysates were collected after 3 days and immunoblotted using antibodies against the epitope tags: flag for HRT1 and HRT2, V5 for N1 and N2, and HA for N4. The control was GFP, and total protein levels were evaluated with anti–β-actin. B, Immunofluorescence was used to detect the epitope tags at 72 hours after adenoviral transduction (left). DAPI staining (right) allowed for quantification of transduction efficiency. C, Quantitative PCR was performed using HRT1 and HRT2 primers to detect transcript levels 72 hours after NotchICD transduction. Graphed are fold changes compared with GFP-transduced cultures.

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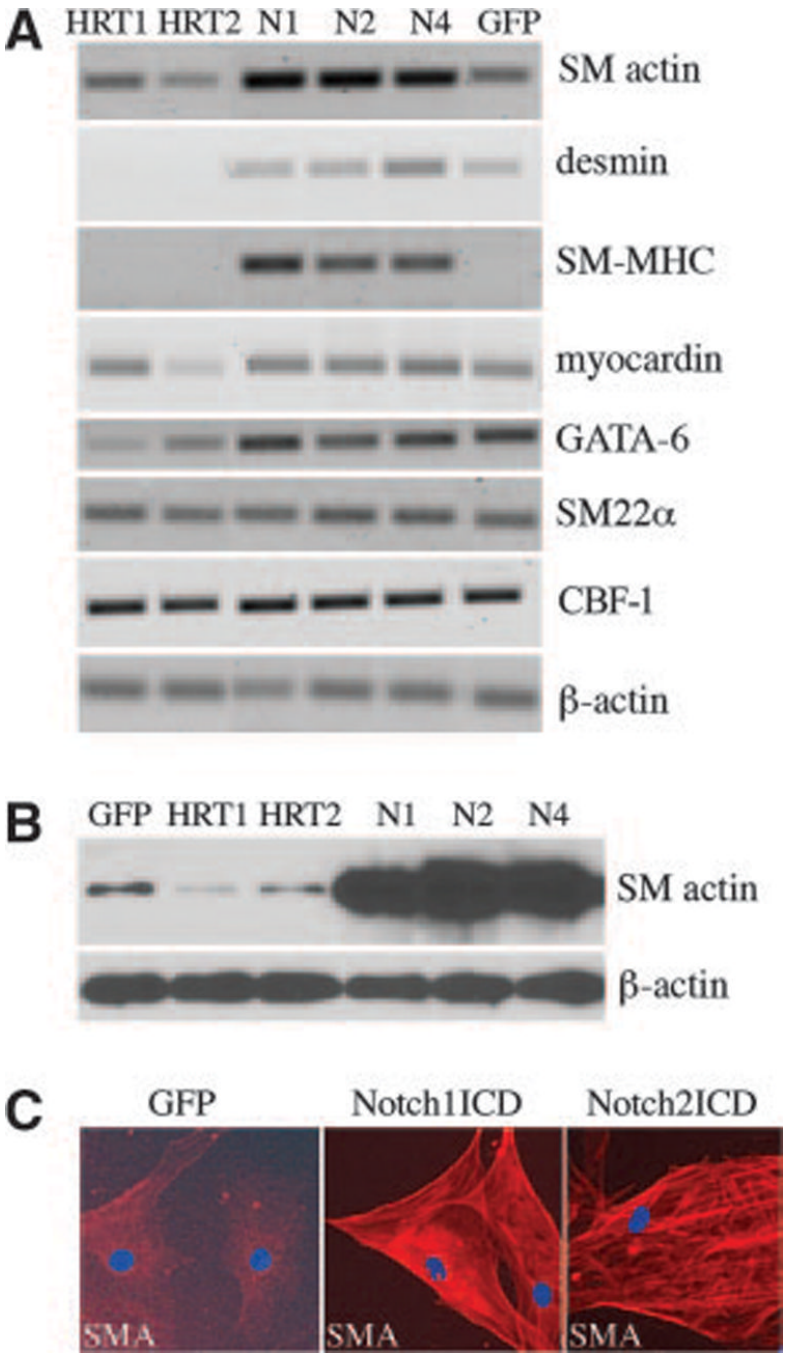

Figure 2. Notch and HRT differentially regulate SMC markers such as SMA

HASMCs were transduced with GFP, NotchICD, or HRTs. A, Total RNA was collected after 72 hours and reverse-transcribed. PCR amplification was performed for indicated transcripts. B, Cell lysates were collected and immunoblotted for SMA and β-actin. C, Immunofluorescence staining was performed to detect SMA localization within GFP or Notch-activated HASMCs (red fluorescence). Cells were also stained with DAPI to detect nuclei (blue fluorescence).

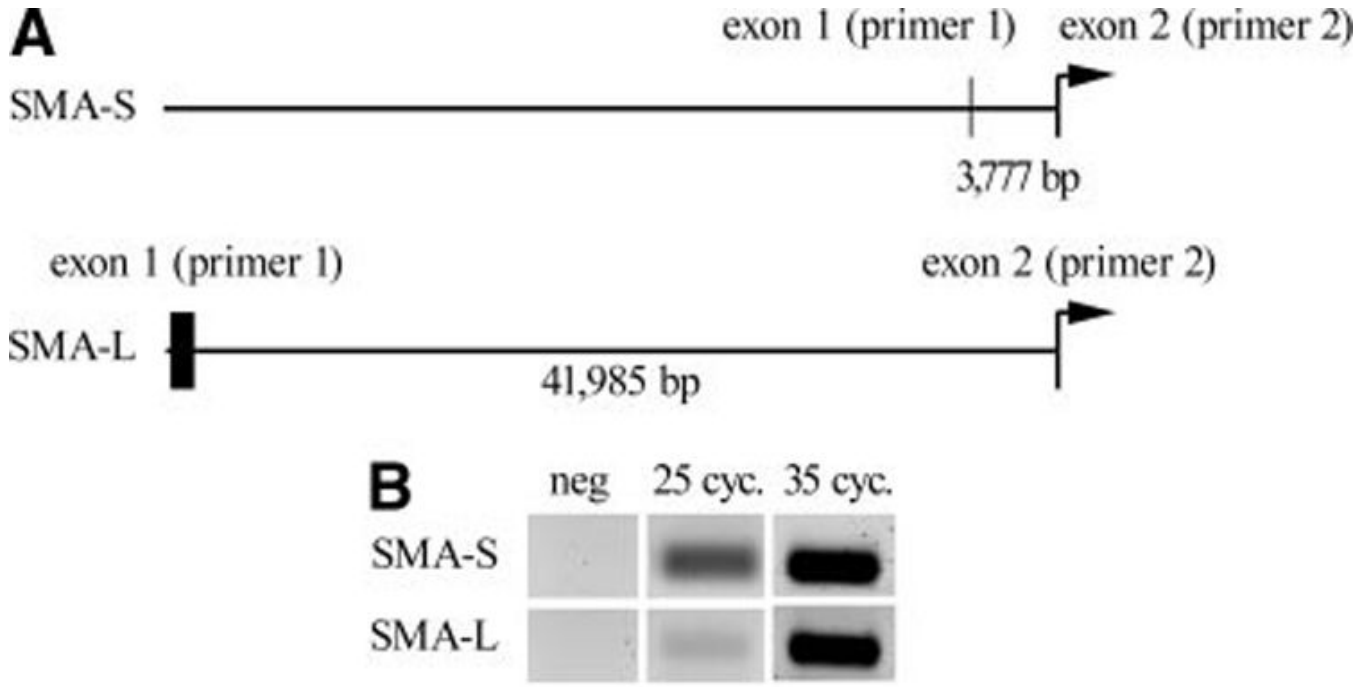

Figure 3. Two SMA transcripts are expressed in HASMCs

A, Schematic representation of the SMA gene. The SMA-S form is transcribed from an exon 1 that is ≈3.8 kb upstream of the translational start site in exon 2. Exon 1 of the SMA-L form is located ≈42 kb upstream of exon 2. Primers to amplify the alternate forms were generated within exons 1 and 2. B, HASMCs were used for RT-PCR for amplification of the SMA-S or SMA-L forms. The SMA-S primers amplify a ≈100-bp product, and the SMA-L primers amplify a ≈200-bp product. Although both transcripts are expressed in HASMCs, SMA-S was more abundant. Samples were PCR-amplified for 25 or 35 cycles.

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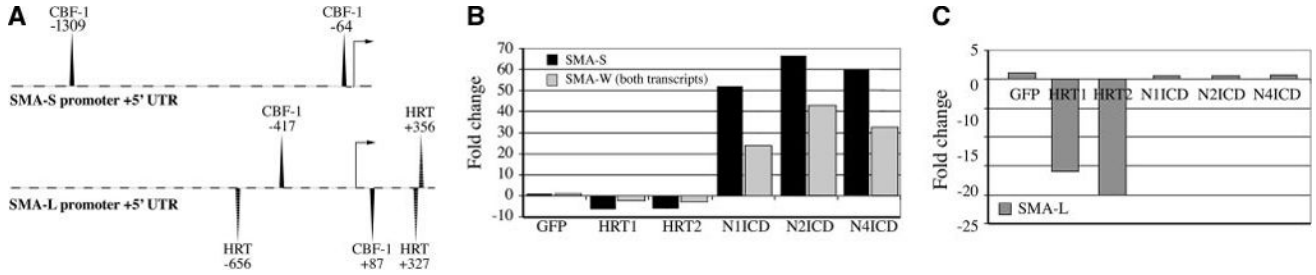

Figure 4. The SMA transcripts are regulated differently by Notch

A, Diagrammed are the short (SMA-S) and the long (SMA-L) promoter sequences with relation to the transcriptional start site (arrow). The 2000-bp stretch of the upstream promoter sequence was analyzed for transcription factor motifs using Genomatix weight matrices for transcription factor binding sites (MatInspector40), and significant consensus sites for CBF-1 binding and HRT binding are indicated, relative to the transcriptional start site. Quantitative PCR was performed using primers for SMA-S and SMA-W (recognizing all SMA mRNA forms) (B) or SMA-L primers (C) under different HASMC transduction conditions. Data were compared with expression with control GFP transduction. SMA-S is expressed at an ≈10-fold higher quantity than SMA-L, although both are detectable in HASMCs. Notch activation selectively increased SMA-S transcript, and HRTs suppress levels of both SMA-S and SMA-L. UTR indicates untranslated region.

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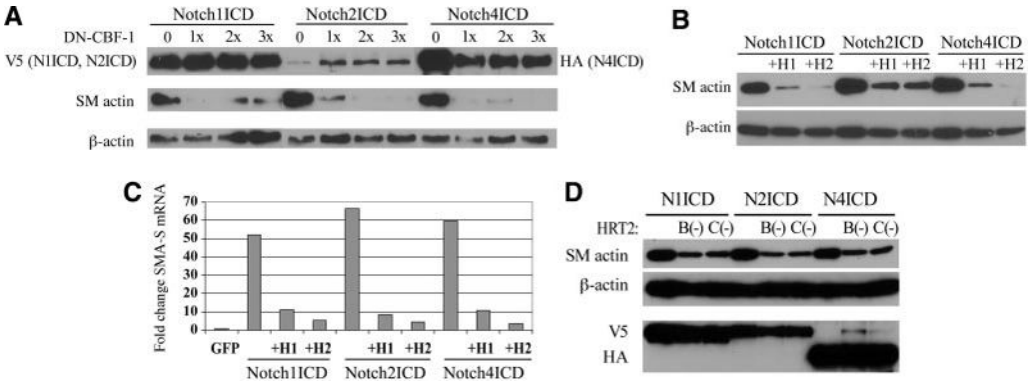

Figure 5. HRTs antagonize Notch induction of SMA expression

HASMCs were transfected as indicated, and total cell lysates collected 3 days after transduction. A, Cells were cotransfected with NotchICD and increasing amounts (3-fold range) of DN-CBF-1. Cell lysates were immunoblotted using antibodies against NotchICD, SMA, and β-actin. B, Cells were cotransfected with NotchICD and either HRT1 (H1) or HRT2 (H2), and cell lysates were immunoblotted for SMA and β-actin. C, Quantitative PCR was performed using SMA-S primers. Data are expressed as fold changes in relation to GFP-transduced cells. All NotchICD significantly increased SMA-S transcripts, which was antagonized by either HRT1 or HRT2 coexpression. D, This experiment was repeated using the HRT2 mutants. Both HRT2 B(−) and HRT2 C(−) suppressed Notch-induced SMA protein levels.

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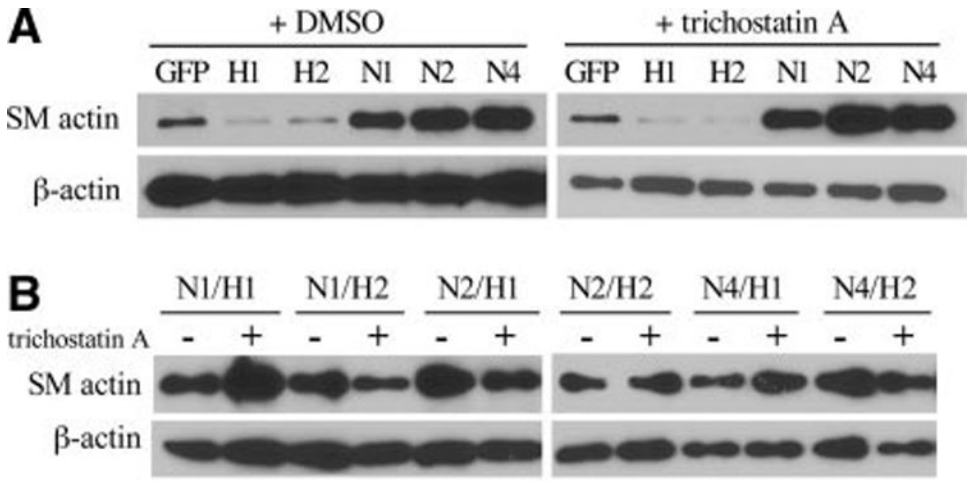

Figure 6. HRT inhibition of SMA expression is not dependent on histone deacetylase activity

HASMCs were transduced as indicated, and after 48 hours, either DMSO (control,−) or trichostatin A were added for 24 hours before total cell lysate collection (3 days after transduction). A, SMA levels, in comparison with GFP controls, was decreased in HRT1 (H1)- or HRT2 (H2)- expressing cells and increased in NotchICD-expressing cells. B, Cells expressing ICD forms of Notch1 (N1), Notch2 (N2), or Notch4 (N4) in combination with either HRT1 (H1) or HRT2 (H2) were analyzed for SMA levels. There were no major differences in the presence or absence of trichostatin A.

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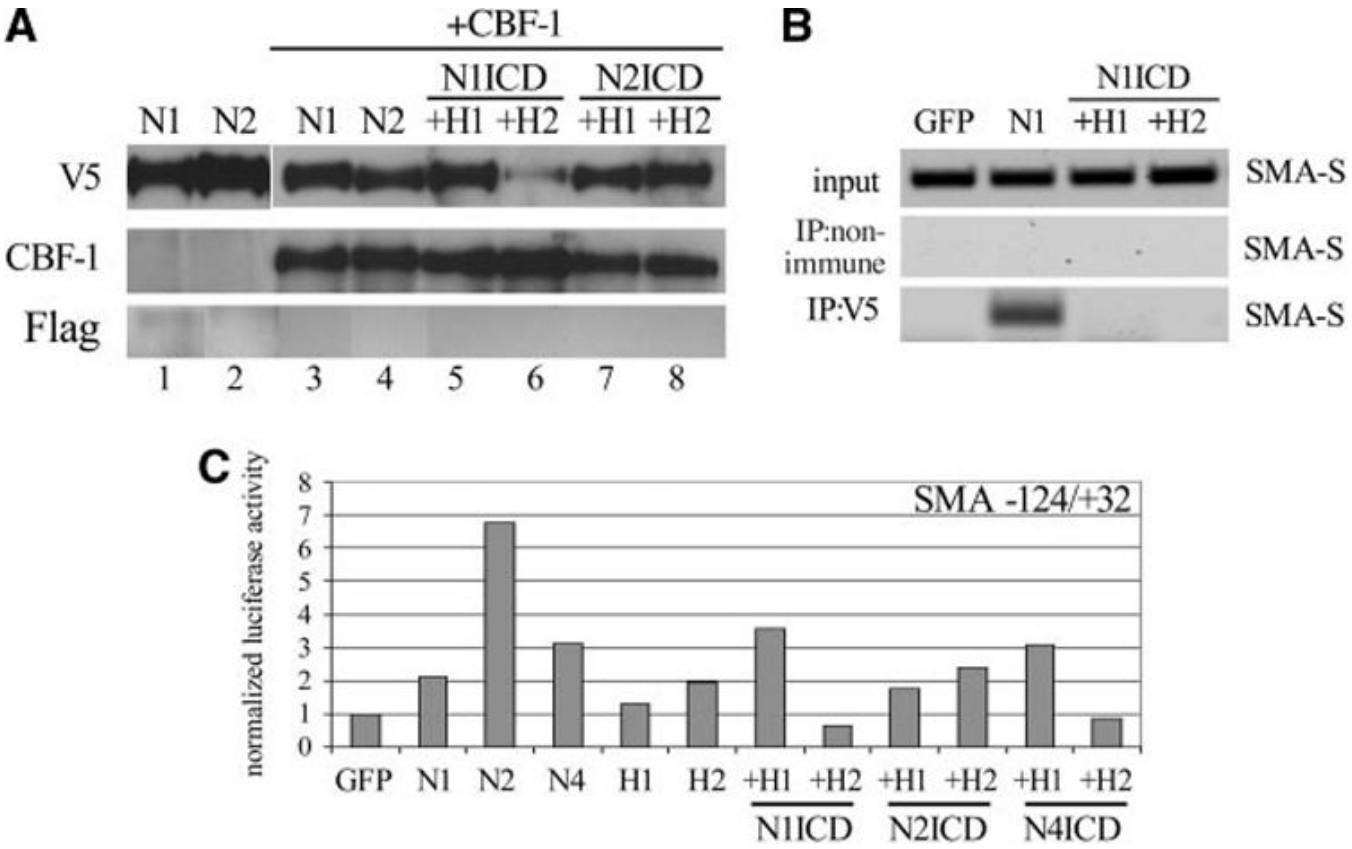

Figure 7. HRT antagonizes NotchICD/CBF-1 at the level of SMA promoter binding and activation

HASMCs were transduced as indicated. A, Cell lysates were immunoprecipitated using anti-V5 (NotchICD) and immunoblotted with antibodies against V5, CBF-1, or Flag (HRTs). A NotchICD/CBF-1 complex was clearly detectable (lanes 3 and 4) and was not inhibited by the addition of HRT1 (lanes 5 and 7) or HRT2 (lanes 6 and 8). Endogenous levels of CBF-1 were not sufficient to be detected by immunoblot after immunoprecipitation (lanes 1 to 2). HRTs (Flag epitope) were never detectable in the NotchICD-immunoprecipitated lysates. B, Protein/DNA complexes in transduced cells were cross-linked, and the DNA was sheared. Lysates were used for PCR amplification of SMA-S using specific primers (input). Lysates were immunoprecipitated with anti-V5 to pull down protein/DNA complexes containing NotchICD. HRT1 (H1) or HRT2 (H2) expression inhibited immunoprecipitation of a NotchICD/DNA complex containing SMA-S amplifiable sequence. C, Luciferase promoter transactivation assays were performed with a construct containing the −124/+32 SMA sequence.

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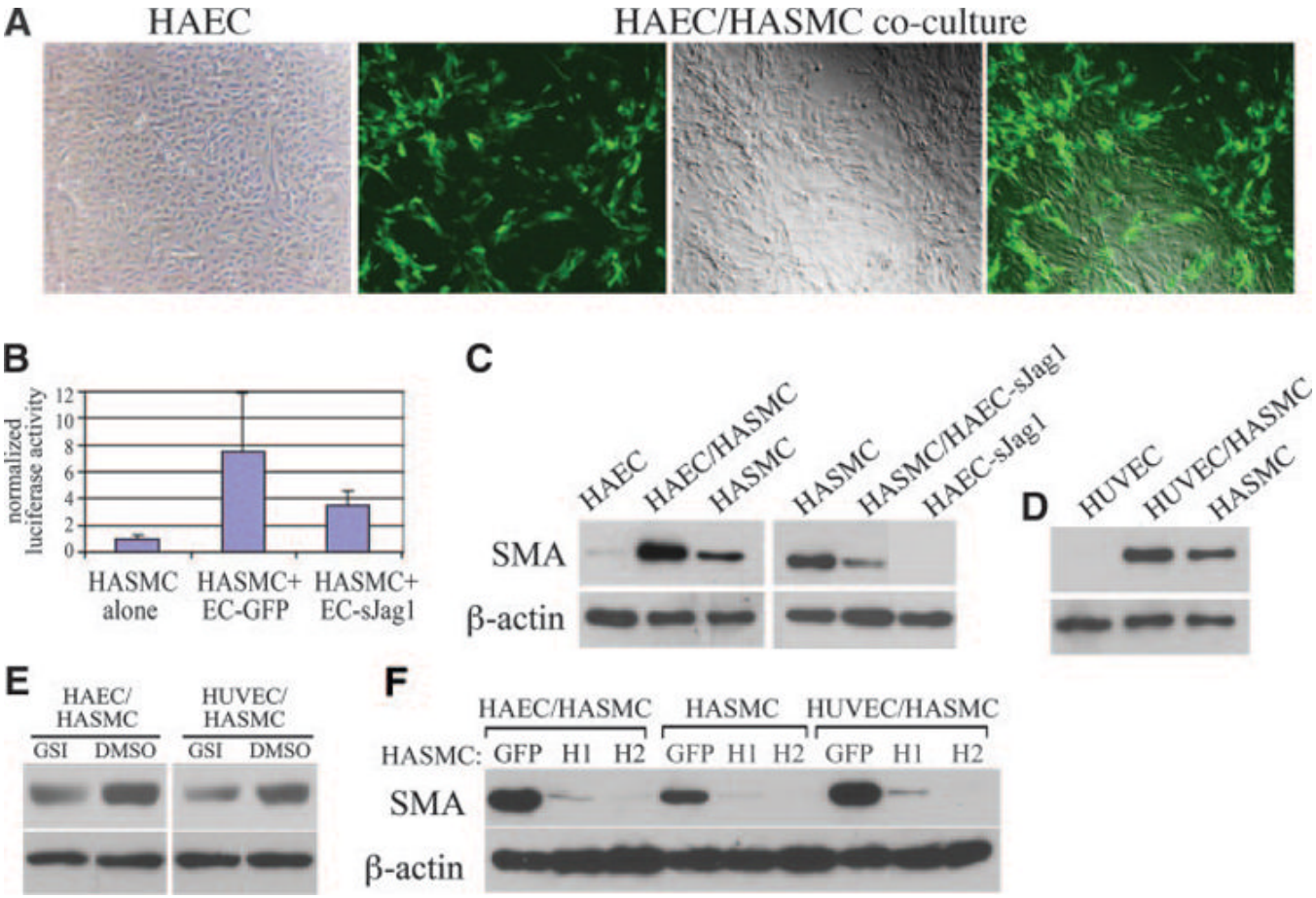

Figure 8. Activation of endogenous Notch signaling by endothelial cell and SMC coculture activates SMA, which is sensitive to HRT

A, We established a coculture system of fluorescently labeled (GFP) monolayer endothelial cells overlaid with HASMCs. Shown are HAECs alone (left) and HAECs/HASMC coculture (next 3 images) with HAECs retrovirally transduced with GFP. The images show cells under fluorescence, phase, or combined microscopy. B, Endothelial cells were transduced with GFP or sJagged1 and cocultured with HASMCs expressing a CBF-1 luciferase reporter construct and assayed after 48 hours. C through F, Endothelial cell/SMC cocultures were performed with control or transduced cells, and protein lysates were collected after 3 days for immunoblot analysis for SMA and β-actin. C, HAEC/HASMC coculture induced SMA expression, which was inhibited in coculture with sJagged1-expressing endothelial cells. D, HUVEC/HASMC coculture also increased SMA levels. E, Coculture was performed in the presence of γ-secretase inhibitor or DMSO (vehicle). F, HASMCs were transduced with GFP, HRT1 (H1), or HRT2 (H2) and plated alone or in coculture with HAECs or HUVECs as indicated.