Notch signaling is essential for vascular patterning and response of the vasculature to injury and growth factor stimulation. Despite these findings, the molecular basis of Notch signaling in the vasculature is poorly understood. Here we report that activation of Notch receptors by Jagged1 or forced expression of the constitutively active Notch1 intracellular domain in C3H10T1/2 fibroblasts inhibited myocardin-dependent transcription of SMC-restricted genes and activity of multiple SMC-restricted transcriptional regulatory elements. Consistent with these findings, forced expression of HRT2 inhibited myocardin-induced expression of SMC-restricted genes and activity of SMC-restricted transcriptional regulatory elements. Moreover, forced expression of HRT2 repressed transcription of multiple SMC-restricted transcriptional regulatory elements in A10 SMCs. The repressive function of HRT2 was not mediated via the capacity of HRT2 to bind SMC CArG elements or by disruption of myocardin-SRF protein complexes. Structure-function analyses of HRT2 indicated that repression required the basic DNA binding domain and additional C-terminal sequence. Taken together, these results demonstrate that Notch signaling represses myocardin-dependent SMC transcription. These data are consistent with a model wherein Notch signaling represses SMC differentiation and maintenance of the contractile SMC phenotype.

Vascular smooth muscle cells (VSMCs) express a unique set of genes encoding SMC-restricted contractile and cytoskeletal proteins required for the modulation of arterial tone and vascular homeostasis (reviewed in Ref. 1). In contrast to the skeletal and cardiac muscle cell lineages, where cellular differentiation is functionally coupled to irreversible exit from the cell cycle, SMCs retain the capacity to proliferate and modulate their phenotype during postnatal development (2). Although a reproducible transition from a “contractile” to a “synthetic” SMC phenotype is observed with passage of primary vascular SMCs in culture, in the intact vasculature SMCs are pleiotropic, expressing unique permutations and combinations of both contractile and synthetic genes that are continuously modulated in response to various stimuli and developmental cues (3). For example, during embryonic angiogenesis, SMCs express relatively high levels of SMC contractile proteins as well as proteins required for cell migration, adhesion, and proliferation (4, 5). This developmental program is also partially recapitulated in SMCs following arterial injury and in atherosclerotic plaques (6).

We and others have reported that VSMC differentiation is controlled by a serum response factor (SRF)- and myocardin-dependent transcriptional regulatory program (for a review, see Ref. 7). SRF belongs to the ancient MADS box family of transcription factors (8, 9). Functionally important SRF binding sites, or CArG boxes, have been identified in transcriptional regulatory elements controlling expression of most, but not all, SMC contractile genes (3). Mutations of these SMC CArG boxes totally abolished SMC-restricted transcription in transgenic mice (10–12). SRF activates SMC contractile genes by physically associating with the recently identified cardiac and SMC-restricted SAP domain transcription factor, myocardin (13, 14). Forced expression of myocardin in undifferentiated embryonic stem cells (which express SRF) activates a set of genes encoding SMC contractile proteins (15, 16). Moreover, there is no evidence of vascular SMC differentiation in myocardin-deficient mouse embryos, and these mice fail to survive past embryonic day 10.5, most likely due to vascular insufficiency (17).

By contrast, relatively little is understood about the molecular mechanisms regulating vascular patterning and the response of vascular SMCs to growth factor stimulation and vessel wall injury. Increasing evidence suggests that Notch receptors and downstream Notch effectors in the Hairy-related transcription (HRT) family play important roles during embryonic and postnatal vascular development (for a review, see Ref. 18). The evolutionarily conserved Notch signaling pathway regulates cell fate decisions and cellular differentiation mediated through cell-cell interactions (for a review, see Ref. 19). When bound by either Delta-like or Jagged ligands, Notch receptors on the cell surface are activated, resulting in proteolytic cleavage and translocation of the Notch intracellular domain (ICN) to the nucleus. In the nucleus, ICN associates with the transcription factor CSL (C/EBF1/Su(H)/Lag-1/RBP-J), providing DNA-binding specificity to the ICN-CSL complex. The ICN-
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CSL complex physically associates with transcriptional coactivators activating or derepressing transcription of target genes in the HES (Hairy-and-enhancer of split) and HRT (Hairy-related) transcription factor families (20). HRT1, HRT2, and HRT3 are direct targets of Notch signaling and are expressed at high levels in the embryonic vasculature (21–23). There is some controversy regarding the expression of Notch and HRT family members following vascular injury, most likely resulting from variations in the animal models employed and/or temporal assessment of Notch and HRT expression following injury (24–26).

Analyses of spontaneous and genetically engineered mutations in Notch receptors and HRTs has provided strong evidence that Notch signaling is critical for normal vascular development, patterning, and homeostasis. Notch1-deficient mice die at embryonic day 9.5 and exhibit disorganized vascular patterning in the embryo and yolk sac (27). Similarly, the combined absence of HRT1 and HRT2 in mice leads to defective angiogenesis and embryonic lethality at embryonic day 9.5–10, whereas loss of HRT2 alone results in perinatal lethality due to cardiac and outflow tract defects (28–30). However, some HRT2-deficient mice survive into adulthood and exhibit dysfunctional vascular smooth muscle responses to arterial injury and growth factor stimulation (31). In the zebrafish, mutation of the gridlock gene, an HRT2 ortholog, results in failure to establish arterial versus venous patterning (32). In mice, mutation of Jagged1 results in lethality at embryonic day 10.5 and is accompanied by abnormal vascular patterning and failure to remodel the primary vascular plexus in the yolk sac (33). In humans, Alagille syndrome, caused by a mutation in the Notch ligand Jagged1 gene, is associated with arteriopathy, most notably involving the pulmonary artery (34). Similarly, the human CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) syndrome is a heritable arteriopathy caused by a mutation in the human Notch3 gene (35).

The experiments described herein explore the molecular basis of Notch function in cultured cells with particular emphasis on determining the effect of Notch signaling on SMC differentiation and maintenance of the SMC phenotype. We observed that Notch-induced HRT2 expression represses myocardin-induced SMC transcription and gene expression. Furthermore, HRT2 repressed multiple transcriptional regulatory elements controlling expression of SMC contractile genes in SMCs. Surprisingly, the repressive function of HRT2 is not mediated by disruption of myocardin-SRF protein complexes bound to DNA; nor does it reflect recruitment of HDAC activity by HRT2. Structure-function studies revealed that Notch-mediated repression is dependent upon the basic DNA binding domain and C terminus of HRT2. Together, these data suggest a model wherein Notch signaling converges upon and functionally antagonizes myocardin-dependent SMC differentiation and the contractile SMC phenotype.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid (pcDNA3-ICN1) encoding the mouse Notch1 intracellular domain (ICN1) was prepared by subcloning the PCR-amplified ICN1 cDNA into pcDNA3 (Invitrogen). Expression plasmids encoding HA epitope-tagged mouse SRF (pcDNA3 HA-SRF) and untagged mouse myocardin (pcDNA3-myo5) were prepared by subcloning the mouse SRF and myocardin cDNAs, respectively, into the expression vector pcDNA3. An amino-terminal FLAG-tagged myocardin expression plasmid (pFLAG-myocardin) was a gift of E. Olson (University of Texas Southwestern) and described previously (36). The expression plasmid pcDNA3-GATA4 was derived from subcloning the PCR-amplified HRT2, within which the basic domain was replaced by two amino acids, LE (HRT2-B), from pcDNA3.1 by TOPO directional cloning including a C-terminal V5 epitope tag (Invitrogen). All plasmids were sequenced to ensure accuracy of PCR amplification. A plasmid expressing N-terminal Myc-tagged HRT2, within which the basic domain was replaced by two amino acids, LE (HRT2-B), was described previously (37). Expression plasmids for mouse HRT1 (pHRT-V5) and HRT3 (pHRT-V5) were prepared by subcloning PCR-amplified HRT1 and HRT3 cDNA, respectively, into pcDNA3.1 with a C-terminal V5 epitope tag. The luciferase reporter plasmids pMS22α-luc, pMS22α-luc, pMS44-luc, pPI-Act-luc, and pPI-Myo-luc were described previously (15). The ARNT expression plasmid, pcDNA3-Arnt, was kindly provided by M. C. Simon (University of Pennsylvania).

Cell Culture and Transfections—C3H10T1/2 fibroblasts, 293T cells, and A10 rat aortic smooth muscle cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (HyClone) at 37°C. Plasmid transfections were performed using Fugene 6 (Roche Applied Science) with a Fugene 6/DNA ratio of 3:1. For 10T1/2 cells, maintenance medium was changed to Dulbecco’s modified Eagle’s medium with 2% fetal bovine serum 2 h prior to transfection, and the cells were harvested after 48 h. Where indicated, 24 h after plasmid transfection, 10T1/2 cells were placed in medium containing 300 nM trichostatin-A (TSA; Sigma) and harvested 24 h later (38).

Retrovirus Preparation and Cell Transduction—Control MIGR1 and MIGR1-expressing GFP-expressing virions were prepared as described (40). 2 × 10⁵ 10T1/2 cells were plated on a 60-mm tissue culture dish containing maintenance medium and incubated for 24 h at 37°C. Cells were infected with 1 ml of virus stock (titer >50%) in the presence of 4 µg/ml Polybrene. After an 8-h incubation at 37°C, 3 ml of fresh maintenance medium was added, and the cells were harvested after 40 h. The cell population was sorted for green fluorescent protein (GFP) expression by fluorescence-activated cell sorting. GFP-positive cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and exhibited stable fluorescence expression for at least 10 passages.

Notch Ligand Stimulation Assay—Notch signaling was induced in 10T1/2 cells plated on dishes displaying immobilized Jagged1 ligand as described (41) except that the Myc-tagged Jagged1 was substituted with an Fc-Jagged1 fusion protein (kindly provided by T. Radesch, University of Pennsylvania).

Real Time RT-PCR Analysis—For quantitative analysis of endogenous gene expression levels, total RNA was harvested from either ES cells or 10T1/2 cells using Trizol Reagent (Invitrogen), and 2 µg was subjected to oligo(dT)-primed reverse transcription (Invitrogen). cDNAs were used in each reaction as described in the representative experiments (5 µm) and SYBR Green 2× Master mix (Applied Biosystems) followed by thermal cycling between 95 and 60°C in a DNA Engine Option 2 System (MJ Research). mRNA quantification was calculated according to the 2⁻ΔΔCT method (42). Expression levels of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. All experiments were repeated at least twice, and results from a representative experiment are shown with S.D. values. Primer sequences for real time PCR of cDNAs for glyceraldehyde-3-phosphate dehydrogenase, SM22α-actin, SM-MyHC, SM22x, SM-calponin-h1, myocardin, HRT1, HRT2, and HRT3 were described previously (15, 25) with the exception of the reverse primer for HRT3, which was 5’-TAG-CTGACTGCTCAGGGAAGGCA-3’. Luciferase Reporter Assays—10T1/2 or A10 cells were seeded at 20,000 cells/well in a 24-well tissue culture plate and transfected with 25 ng of luciferase reporter construct, 25 ng of myocardin expression plasmid, and the indicated amounts of ICN1 or HRT expression plasmids with additional pcDNA3 vector added to normalize total DNA concentration in each transfection. The Renilla expression plasmid, pRL-TK (5 ng), was co-transfected for normalization of transfection efficiency. 48 h post-transfection, cells were harvested, and luciferase activities were measured by a dual luciferase assay (Promega). Each experiment was repeated at least two times with each reaction performed in triplicate. Results displayed with S.D. are from representative experiments.

Electrophoretic Mobility Shift Assays—EMSAs—EMSAs were performed with in vitro transcribed and translated HA-SRF, FLAG-myocardin, and HRT2-V5 and the radiolabeled SMC4 CArG-box containing
oligonucleotide probe as described previously (43). 1 μg each of pcDNA3 HA-SRF, pFLAG-myocardin, and pHRT2-V5 were added to a T7 transcription-coupled rabbit reticulocyte lysate per the manufacturer’s instructions (Promega), and reaction products were verified by SDS-PAGE and Western blotting. Equal amounts of lysate reactions were incubated with 32P-labeled SME-4 probe in the presence of 1 μg of poly(dI:dC) (Amersham Biosciences) and binding buffer and incubated for 15 min on ice as described previously (43). Reaction products were separated on a 4% polyacrylamide gel containing 0.25 M urea. Reaction products were verified by SDS-PAGE and Western blotting. Equal amounts of lysate reactions were incubated with 32P-labeled SME-4 probe in the presence of 1 μg of poly(dI:dC) (Amersham Biosciences) and binding buffer and incubated for 15 min on ice as described previously (43). Reaction products were separated on a 4% polyacrylamide gel containing 0.25 M urea.

**RESULTS**

**Activated Notch Signaling Represses Myocardin-dependent SMC Gene Expression and Transcription**—To examine whether Notch signaling modulates SMC differentiation, we utilized 10T1/2 fibroblasts, which, in response to myocardin, differentiate and express a set of genes encoding SMC contractile markers, including SMα-actin, SM-MHC, calponin-h1, and SM22α (45). First, we tested whether exposure of 10T1/2 cells to the Notch ligand Jagged1 alters myocardin-induced SMC differentiation. Jagged1 can activate Notch signaling whether it is on a neighboring cell or immobilized on a tissue culture plate (46). 10T1/2 cells plated on either Jagged1-coated or control tissue culture dishes were transfected with the pcDNA3-myocardin expression plasmid or the control plasmid pcDNA3. 48 h post-transfection, the cells were harvested, and relative level of myocardin gene expression assessed by real time RT-PCR. Consistent with our previous report (15), cells transfected with pcDNA3-myocardin exhibited a dramatic increase in SMα-actin, SM-MHC, and SM22α mRNA were quantified by real time RT-PCR. Consistent with our previous report (15), cells transfected with pcDNA3-myocardin exhibited a dramatic increase in SMα-actin, SM-MHC, and SM22α gene expression (Fig. 1A). However, when 10T1/2 cells grown on Jagged1-coated plates were transfected with pcDNA3-myocardin, a 50–80% inhibition of myocardin-induced SMα-actin, SM-MHC, and SM22α gene expression was observed (Fig. 1A). Of note, the relative level of myocardin gene expression assessed by real time RT-PCR was equivalent whether cells were plated on Jagged1 or control plates (data not shown). As expected, the
repression of myocardin-induced SMC gene expression is regulated, at least in part, at the level of transcription.

**Notch Signaling Modulates SMC Gene Expression in ES Cells**—Relative levels of SMC contractile gene expression were measured by real-time RT-PCR in Notch1+/− and Notch1−/− ES cells. In multiple independent Notch1−/− ES cells, expression of genes encoding early SMC differentiation markers including SMα-actin, SM22α, and calponin-h1 was induced relative to the level of gene expression observed in control Notch1+/− ES cells (Table I). Interestingly, expression of the late SMC differentiation gene, SM-MHC, was not induced in Notch1-deficient ES cells, further substantiating the postulate that the transcriptional programs regulating early and late SMC differentiation differ fundamentally (1).

**HRT Transcription Factors Repress Myocardin-induced SMC Gene Expression**—The Notch target genes HRT1, HRT2, and HRT3 are expressed in vascular SMCs and are important in mediating Notch signaling activity in the vasculature (18). To determine whether one or more of these proteins acts downstream of the Notch receptor to repress myocardin-inducible SMC gene expression, 10T1/2 cells were transiently co-transfected with an expression plasmid encoding HRT2 as well as the myocardin expression plasmid, and the expression level of SMC genes encoding early SMC differentiation markers including SMα-actin, SM22α, and calponin-h1 was measured relative to the level of gene expression observed in control Notch1−/− ES cells (Fig. 3A). A similar inhibitory response was observed when HRT3 was substituted for HRT2 in these experiments (data not shown).

**TABLE I**

| Gene                  | Relative mRNA levels | (Notch1+/− vs. Notch1−/− ES cells) |
|-----------------------|----------------------|------------------------------------|
| GAPDH                 | NC                   |                                    |
| SMα-actin             | 79 ± 7/− 79          |                                    |
| SM-calponin-h1        | 12 +/− 1             |                                    |
| SM-MHC                | NC                   |                                    |
| SM22α                 | 150 +/− 19           |                                    |

* Gene-specific primer sequences and reaction conditions are described under “Experimental Procedures.”

* Mean ± S.E. of fold-target mRNA level change between three independent Notch1+/− and Notch1−/− ES cell lines.

* Glyceraldehyde-3-phosphate dehydrogenase; housekeeping gene used for quantitation of relative target gene expression levels.

* NC, no change.

To determine whether forced expression of either HRT1, HRT2, or HRT3 repressed myocardin-induced transcriptional activation of the SMC transcriptional regulatory elements, 10T1/2 cells were co-transfected with expression plasmids encoding myocardin and either HRT1, HRT2, or HRT3 and a luciferase reporter plasmid under the transcriptional control of the SM22α promoter. Luciferase activity was measured by 42%, and the SM-MHC promoter/enhancer by 42%, and the SM-MHC promoter/enhancer by 42% compared with cells co-transfected with the control pcDNA3 expression plasmid (Fig. 2).

These results extend our findings above showing Notch-mediated repression of endogenous SMC gene expression and suggest that repressing Notch signaling represses myocardin-dependent transcriptional activity.
plasmid (the total amount of DNA was held constant in this series of experiments). As shown in Fig. 3D, myocardin-induced activation of the SM22α promoter was progressively repressed by increasing concentrations of HRT2. Taken together, these results strongly implicate the HRT factors in Notch-mediated repression of myocardin-dependent SMC gene expression.

HRT2 Represses Activity of Multiple SMC-restricted Transcriptional Regulatory Elements in SMCs—To address whether HRT2 modulates maintenance of the SMC phenotype, A10 SMCs, which express high levels of genes encoding contractile SMC markers (49), were transiently co-transfected with luciferase reporter plasmids under the control of the SM22α, SM-MyHC, or SMα-actin promoter/enhancer elements, respectively, and expression plasmids encoding either HRT2 or the HRT2 basic domain mutant HRT2(B−) (see Fig. 7). Forced expression of HRT2, but not the HRT2(B−) mutant, repressed activity of the SM22α promoter, the SM-MyHC promoter/enhancer, and the SMα-actin promoter/enhancer (Fig. 4). Furthermore, when myocardin was overexpressed in SMCs by co-transfection with the pcDNA3-myocardin expression plasmid, forced expression of HRT2, but not HRT2(B−), repressed myocardin-induced superactivation of the −441 SM22α-LUC, pPI-SMα-actin-LUC, and pPI-SM-MyHC-LUC reporter plasmids (Fig. 4). These results indicate that under basal conditions in A10 SMCs and in response to overexpression of myocardin, HRT2 represses transcription of multiple SMC-restricted contractile transcriptional regulatory elements. Hence, Notch/HRT2 signaling can modulate the transcriptional programs regulating both SMC differentiation and maintenance of SMC phenotype.

**HRT2-mediated Repression of the SM22α Promoter Is Transduced via CArG Boxes—**HRT2 repression of basic helix-loophelix proteins in the Hairy-related family repress transcription by directly binding to nuclear protein binding sites containing N- or E-box DNA elements in concert with transcriptional co-repressors and/or via their capacity to physically associate with other transcription factors (for a review, see Ref. 20). We previously reported that a multimerized copy of either the SME-1 or SME-4 CArG box-containing elements (also referred to as CarG-far and CarG-near (11)) in the SMC-restricted SM22α promoter is necessary and sufficient to restrict transgene expression to arterial SMCs in transgenic mice (10). To determine whether HRT2-mediated repression of the SM22α promoter is mediated through the CArG box-containing regulatory element and not other nuclear protein binding sites in the SM22α promoter (43), a series of transient co-transfection experiments were performed by transfecting 10T1/2 cells with either the CarGx4-luc reporter plasmid, containing four copies of the SME4 consensus CArG box subcloned immediately 5’ of the minimal SM22α promoter (bp −90 to +1) or the SME4x4-luc reporter plasmid, containing four copies of the SME-4 nuclear protein binding site subcloned immediately 5’ of the min-
HRT2 inhibits expression of smooth muscle contractile gene promoter elements in SMCs. A10 rat aortic smooth muscle cells (2 × 10^4/24-well) were grown to 70% confluence and co-transfected with 200 ng of pHRT2-V5 or pHRT2(B-), with or without 25 ng of pcDNA3-myocardin, and 25 ng of luciferase reporter construct under the control of a −441 SM22α promoter (SM22α-LUC) or promoter/intragenic enhancers for SMα-actin (pPI-Act-luc or SMα-ACTIN-LUC) and SM-MyHC (pPI-Myo-luc or SM-MyHC-LUC). 48 h following transfection, cells underwent passive lysis, and extracts were assayed for luciferase activity as described under “Experimental Procedures.” Data are displayed as relative luciferase activity with reference to pcDNA3-only sample.

![Graph](http://www.jbc.org/)

**Fig. 4.** HRT2 inhibits expression of smooth muscle contractile gene promoter elements in SMCs. A10 rat aortic smooth muscle cells were transiently transfected with pHRT2-V5 or pHRT2(B-), with or without 25 ng of pcDNA3-myocardin. The graphs show the relative luciferase activity compared to the control (pcDNA3-only sample).

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**HRT2 Does Not Physically Associate with SRF or Myocardin or Disrupt SRF-Myocardin Complexes Bound to DNA**—The finding that HRT2-mediated repression of the SM22α promoter is mediated via a CArG box-containing nuclear protein binding site rather than another nuclear protein binding site within the −441-bp SM22α promoter, suggests the possibility that HRT2 does not physically associate with any of these proteins. To test this hypothesis, a series of EMSAs were performed to examine the physical association between HRT2 and SRF or myocardin or disrupt myocardin-SRF protein complexes under experimental conditions where HRT2 represses transcription.

EMSAs were performed using a radiolabeled oligonucleotide corresponding to the SM-4 CArG box–containing nuclear protein binding site in the mouse SM22α promoter, which contains a consensus CArG box (43). The addition of in vitro translated SRF to the labeled probe resulted in one specific nuclear protein complex (labeled S in Fig. 6A, lane 2). This complex was supershifted when anti-SRF antibody was included in the incubation mixture (Fig. 6A, lane 4) but not with preimmune serum (Fig. 6A, lane 3), demonstrating that it contains SRF or an antigenically related protein. Each nuclear protein complex was abolished when cold competitor oligonucleotide was added to the incubation mixture (Fig. 6A, lanes 5 and 6). The addition of in vitro translated HA epitope-tagged SRF and FLAG epitope-tagged myocardin revealed a second low mobility complex (labeled S-M in Fig. 6A, lane 7). This low mobility complex was abolished when anti-FLAG antibody was added to the incubation mixture (Fig. 6A, lane 9), demonstrating that it contains myocardin and SRF. However, when in vitro translated HRT2 was added to the reaction, the myocardin-SRF nuclear protein complex (S-M) was neither supershifted nor abolished (compare Fig. 6A, lanes 7 and 10). An identical result was obtained despite a severalfold increase in the amount of HRT2 added to a fixed amount of myocardin/SRF in vitro translation products (data not shown). These data demonstrate that under the reaction conditions employed in this assay, HRT2 does not disrupt myocardin-SRF protein complexes bound to DNA.

In addition, EMSAs were performed with nuclear extracts prepared from 293T cells expressing SRF, SRF and FLAG-tagged myocardin, or SRF, FLAG-tagged myocardin, and V5-tagged HRT2, respectively. As expected, incubation mixtures containing SRF-only nuclear extracts exhibited a specific nuclear protein complex that co-migrated with in vitro translated SRF (compare Fig. 6B, lanes 2 and 4). This nuclear protein complex was supershifted by the addition of an anti-SRF antibody (Fig. 6B, lane 5). A second, low mobility nuclear protein complex (labeled S-M) was observed when the radiolabeled SME-4 oligonucleotide was incubated with myocardin/SRF-containing nuclear extracts (Fig. 6B, lane 6). The lower complex (labeled S) was supershifted when anti-SRF antibody was included in the incubation mixture, and the upper complex was abolished when anti-FLAG antibody was included in the binding reaction (Fig. 6B, lanes 7 and 8). These data demonstrate that the higher mobility complex (S) contains SRF (or an antigenically related protein) and the lower mobility complex (S-M) contains myocardin (and SRF). Once again, nuclear extracts prepared from 293T cells expressing SRF, myocardin, and HRT2 did not alter the nuclear protein complexes observed in binding reactions (Fig. 6B, compare lanes 6 and 9). These results strongly suggest that HRT2 does not bind to either SRF or myocardin or disrupt myocardin-SRF protein complexes under the experimental conditions employed in this series of EMSAs.

To more rigorously test for physical interactions between HRT2 and SRF or myocardin, co-immunoprecipitation experiments were performed. Whole cell lysates from 293T cells co-transfected with expression plasmids encoding V5-tagged HRT2 and either SRF (Fig. 6C, lanes 3 and 4) or FLAG-tagged myocardin (Fig. 6C, lanes 5 and 6) were incubated with anti-V5 antibody. As a positive control, whole cell extracts were prepared from 293T cells expressing SRF, myocardin, and HRT2 did not alter the nuclear protein complexes observed in binding reactions (Fig. 6B, compare lanes 6 and 9). These results strongly suggest that HRT2 does not bind to either SRF or myocardin or disrupt myocardin-SRF protein complexes under the experimental conditions employed in this series of EMSAs.

Finally, to address the possibility that HRT2 disrupts the myocardin-SRF protein complex when bound to DNA only in specific cellular contexts, chromatin immunoprecipitation assays were performed. 10T1/2 cells were transiently co-transfected with myocardin or myocardin and HRT2 expression plasmids under experimental conditions where HRT2 represses...
myocardin-induced SM-MyHC gene expression. SRF-DNA complexes were immunoprecipitated with anti-SRF antibody, and the cross-linked DNA was subjected to PCR amplification of the SM-MyHC 5’ promoter CArG box (44). Surprisingly, SRF bound to the SM-MyHC promoter was not observed in the absence of myocardin (Fig. 6D, lane 1). In contrast, in cells expressing myocardin and chromatin immunoprecipitated with anti-SRF antibody, a distinct PCR product corresponding to the expected size of the amplified SM-MyHC promoter sequence was observed, strongly suggesting that myocardin stabilizes SRF bound to the 5’ CArG element in the SM-MyHC promoter (Fig. 6D, lane 3). Since inclusion of HRT2 repressed myocardin-dependent SM-MyHC gene activation by more than 90% (see Fig. 3A), we anticipated a similar reduction in the amount of PCR-amplified cross-linked DNA corresponding to the SM-MyHC 5’ promoter CArG if HRT2 disrupted the SRF bound to DNA. However, co-expression of HRT2 with myocardin followed by chromatin immunoprecipitation with anti-SRF antibody did not reveal a significant difference in the amount of PCR-amplified SM-MyHC 5’ CArG box DNA (Fig. 6D, lane 5). In control chromatin immunoprecipitation reactions performed with polyclonal rabbit antiserum, amplified DNA was not observed (Fig. 6D, lanes 2, 4, and 6). Together, these data demonstrate that HRT2 does not disrupt SRF binding to SM CArG elements in vivo.

The Basic Domain and C-terminal Region of HRT2 Are Required for Repression of Myocardin-induced SMC Gene Transcription—The findings that (i) HRT2-mediated repression of SMC promoters is transduced through SMC CArG elements, (ii) HRT2 does not bind directly to CArG elements, and (iii) HRT2 does not bind directly to SRF or myocardin or disrupt SRF-myocardin protein complexes bound to DNA led to the question of what domains in HRT2 mediate the observed transcriptional repression. To address this question, structure-function analyses were performed to define the domains in HRT2 that mediate transcriptional repression. A series of HRT2 truncation and deletion mutants were generated and assayed for their capacity to repress transcriptional activity of the SMC-restricted SM22a promoter in 10T1/2 cells. The domain structure of HRT2 is illustrated in Fig. 7 (center). The basic domain mediates binding to the consensus N/E-box elements of various promoters and may also be required for protein-protein interactions that result in transcriptional repression (20, 37, 51). Constructs expressing only the basic domain (HRT2:B); basic domain and helix-loop-helix domain (HRT2: BH); and basic domain, helix-loop-helix domain, and Orange domain (HRT2:BHO) did not repress myocardin-induced activation of the SM22a promoter in 10T1/2 cells (Fig. 7, right). However, the HRT2:BHO+ deletion mutant containing C-terminal sequences beyond the Orange domain, but not including the YRPW C-terminal amino acid domain, repressed myocardin-induced activation of the SM22a promoter. Moreover, HRT2-mediated repression was rescued by replacement of the basic domain with two amino acids (HRT2(B−)), a mutation that would be predicted to abolish the capacity of HRT2 to bind DNA (Fig. 7, right). Taken together, these results demonstrated that the basic domain is necessary, but not sufficient, to confer HRT2-mediated repression of smooth muscle gene transcription. In addition, HRT2-mediated transcriptional repression requires protein sequences C-terminal to the Orange domain. By contrast, the C-terminal YRPW domain is not required for HRT2-mediated repression of the SM22a promoter (Fig. 7, right).

HRT2-mediated Repression Is Not Dependent upon HDAC Activity—The basic domain of HRT family members has been shown to inhibit transcription via its capacity to heterodimerize with histone deacetylases (HDACs) (51). Similarly, histone acetylation has been shown to regulate SRF-dependent activation of the SM22a promoter (52). To examine the molecular basis of HRT2-mediated SMC gene expression with particular focus on whether HRT2-mediated transcriptional repression is mediated via the capacity of its basic domain to recruit HDAC activity, a series of transient co-transfection experiments were performed. 10T1/2 cells were co-transfected with pcDNA3-myocardin and the −441 SM22a luciferase reporter constructs in the presence or absence of the HDAC inhibitor TSA (53). As shown in Fig. 8, exposure of cells to either TSA or Me2SO (the TSA solvent) failed to rescue HRT2-mediated repression on myocardin-induced smooth muscle gene transcription. These data suggest that the mechanism of HRT2-mediated repression does not involve the recruitment of HDAC activity by HRT2.

DISCUSSION

The molecular mechanisms regulating SMC differentiation and maintenance of the contractile SMC phenotype are relevant to understanding the pathogenesis of common vascular proliferative syndromes including atherosclerosis. Our group and others have shown that differentiation of SMCs from mesodermal precursors is regulated by a SRF- and myocardin-dependent transcriptional program (for a review, see Ref. 1). By contrast, relatively little is currently understood about the molecular mechanisms underlying down-regulation of contractile SMC genes in response to vessel wall injury. We have shown that Notch signaling, mediated by HRT family members, represses myocardin-dependent smooth muscle differentiation of SMCs from mesodermal precursors and
FIG. 6. HRT2 does not directly affect myocardin-SRF complexes. A, HA-tagged SRF (SRF), FLAG-tagged myocardin (Myoc), and V5-tagged HRT2 (HRT2) proteins were expressed by in vitro transcription and translation and incubated with radiolabeled SME-4 probe as described under “Experimental Procedures.” Protein-DNA complexes were separated by nondenaturing polyacrylamide gel electrophoresis. Gels were vacuum-dried and exposed to film (BioMax MR; Eastman Kodak Co.). Comp, 75 ng of cold competitor oligonucleotide. 2x Comp, 150 ng of cold competitor oligonucleotide. Sera, rabbit sera. S/M, SRF-myocardin complex. *, nonspecific complexes.

B, nuclear extracts from 293T cells transfected with pcDNA3 (293T), pcDNA3-HA-SRF (SRF), pcDNA3-HA-SRF plus pFLAG-myocardin (SRF/Myoc), or pcDNA3-HA-SRF plus pFLAG-myocardin and pHRT2-V5 (SRF/Myoc/HRT2) were incubated with radiolabeled SME-4 probe, and complexes were separated by gel electrophoresis as in A. Each binding reaction contained 10 μg of extract. SRF, antibody to SRF. α-FLAG, antibody to FLAG epitope. α-V5, antibody to V5 epitope. S, SRF-only complex. S/M, SRF-myocardin complex. *, nonspecific complexes.

C, whole cell extracts from 293T cells transiently co-transfected with 5 μg each of pHRT2-V5 and pcDNA3-GATA4, pcDNA3-HA-SRF, or pFLAG-myocardin were incubated with anti-V5 epitope antibody coupled to protein A/G-agarose beads as described under “Experimental Procedures.” Input protein and co-immunoprecipitants were detected by Western blot using antibodies against GATA4 (lanes 1 and 2), SRF (lanes 3 and 4), and FLAG epitope (lanes 5 and 6). Blots were reprobed with anti-V5 epitope antibody to verify the presence of HRT2-V5 in all lanes (data not shown). I, input whole cell lysate (lanes 1, 3, and 5); αV5, HRT2-V5 immunoprecipitation (lanes 2, 4, and 6). Note (in lane 6) trivial detection of anti-V5 antibody IgG heavy chain (arrow) by the anti-mouse secondary antibody employed in the FLAG-myocardin immune blot. D, HRT2 does not disrupt SRF binding to the endogenous SM-MyHC 5′ CArG promoter element. 10T1/2 cells were transfected with pcDNA3-myocardin (Myoc) with or without pHRT2-V5 (HRT2) expression plasmid. After 48 h, cells were formaldehyde cross-linked, and sonicated chromatin was immunoprecipitated with an anti-SRF antibody or rabbit serum. Recovered DNA was subjected to PCR using primers encompassing the 5′ CArG-box element within the SM-MyHC promoter (SM-MyHCp) as described (44). PCR amplification of the same promoter region from chromatin fragments prior to immunoprecipitation (Input DNA) served as positive control. Input, immunoprecipitated DNA.
antagonizes transcription of endogenous contractile genes in SMCs. As such, this study expands our understanding of the transcriptional programs that control SMC differentiation and begins to define, at a molecular level, the basis for SMC phenotypic modulation.

The data presented herein suggest a model wherein CSL-dependent Notch signaling results in repression of myocardin-dependent gene transcription (Fig. 9). Evidence supporting this model is the observed decrease in SMC contractile gene expression when 10T1/2 cells were retrovirally transduced with a virus encoding the constitutively active Notch1/H9004E or after exposure to Jagged1 ligand. These results suggested that activated Notch could itself be responsible for inhibiting myocardin activity or that a downstream target may be induced that mediates the observed inhibition of SMC gene transcription. The finding that myocardin-induced SMC gene expression is also decreased in cells expressing the Notch targets HRT1, HRT2, or HRT3 (which was observed in Notch1ΔE- and
Jagged1-stimulated cells). One or more of these Hairy-related basic helix-loop-helix transcription factors in turn directly or indirectly represses transcriptional activity of the myocardin-SRF-dependent SMC-restricted transcriptional regulatory element. In this regard, it is noteworthy that forced expression of HRT2 repressed transcription of a reporter gene controlled by a multimerized copy of a CArG-box-containing element, strongly suggesting that HRT- and Notch-mediated SMC transcription is mediated through CArG-box-containing regulatory elements rather than other nuclear protein binding sites. This model is consistent with the inhibitory role that Notch signals and HRT family members play in the differentiation of other cell lineages. In skeletal muscle, Notch signaling blocks the differentiation of skeletal myoblasts into skeletal myotubes. In differentiating C2C12 skeletal myoblasts, activation of Notch inhibited myotube formation and blocked induction of MyoD, myogenin, and myosin light chain gene expression (54). Forced expression of HRT1 recapitulated the inhibition of myotube formation by a mechanism potentially involving HRT1-MyoD heterodimer formation with resultant loss of MyoD function and down-regulation of the skeletal muscle program. Similarly, when 3T3-L1 preadipocytes are exposed to Jagged1 ligand or the Notch target gene Hes-1 is expressed in 3T3-L1 cells, the capacity of these cells to differentiate into adipocytes is significantly reduced (41). The target genes regulated by Hes-1 in preadipocytes remain largely undefined.

Compelling evidence has shown that Notch signaling plays a critical role in vascular patterning, remodeling, and arterial-venous cell fate decisions. Combinatorial deletions of murine Notch1/Notch4 or HRT1/HRT2 genes or the deletion of a single Delta-like 4 (Dll4) allele in mice results in early (embryonic day 9.5–10.5) embryonic lethality due to defective angiogenesis (27, 28, 55–57). However, it remains unclear whether the observed mutant phenotypes resulted primarily from Notch signaling mediated via endothelial cells and/or SMCs. The finding that Notch signaling represses myocardin-induced SMC gene expression from fibroblasts, coupled with the observation that Notch1-defective ES cells up-regulate expression of genes encoding early SMC markers, strongly suggests that Notch signaling transduced via SMCs are at least partially responsible for the observed defects in vascular patterning and arterial-venous cell fate decisions. Interestingly, a recent report suggested that Notch3, although not required for proper embryonic vascular development, has an essential function in promoting postnatal maturation of vascular SMCs at least in a subset of distal (nonelastic) arteries (58). However, Notch3-deficient vascular SMCs did not display altered levels of HRT gene expression, suggesting that a complete understanding of the molecular mechanisms of Notch signaling will be complex. Ultimately, cell lineage-restricted conditional loss or gain-of-function studies will be required to clarify the precise role of Notch signaling in the developing vasculature.

In addition, we observed that forced expression of HRT2 repressed transcription of multiple genes encoding SMC contractile markers in SMCs, strongly suggesting that HRT2 (and Notch signaling) regulates maintenance of the SMC phenotype. In these experiments, repression of SMC-restricted transcriptional regulatory elements was not complete, thus recapitulating the observed partial repression of SMC contractile genes observed in SMCs following vessel wall injury (59). This suggests that repression of SMC genes is mediated via multiple signals. Consistent with this postulate, Owens and colleagues reported recently that the SM22α promoter contains a G/C-rich nuclear protein binding site that mediates transcriptional repression via platelet-derived growth factor-BB (60). Moreover, these data are consistent with and may partially explain the recent observation that, following wire injury, HRT2 null mice exhibit reduced neointimal proliferation (31).

These data may also be relevant to the outstanding question of whether phenotypic modulation of SMCs and SMC proliferation in response to vessel wall injury are functionally coupled. Notch signals have been shown to promote cell proliferation in various developmental and cellular contexts. When A7r5 SMCs are forced to express HRT1 or the Notch-3 intracellular domain, they displayed postconfluence growth coincident with diminished p21^{WAF1/CIP1} (26, 61). Consistent with this finding, cyclin D1, which promotes progression through the G phase of the cell cycle, has been identified as a target of ICN1 (62). Cultured VSMCs isolated from HRT2 knock-out mice demonstrated a decreased proliferative capacity and diminished migratory response to platelet-derived growth factor (31). Furthermore, response to wire injury of the femoral artery in HRT2 knock-out mice revealed reduced neointimal hyperplasia consistent with repressed medial VSMC proliferation and migration (31). Taken together, our demonstration in SMCs that enhanced HRT2 expression represses contractile gene regulatory elements and the demonstration that Notch signaling directly represses the SMC contractile phenotype and promotes SMC proliferation support a critical role for Notch signaling in mediating the response of vascular SMCs to arterial injury by repressing expression of genes encoding contractile proteins and promoting cell proliferation.

Whereas these data serve to identify Notch- and HRT-mediated signals as direct antagonists of myocardin-dependent SMC differentiation and function, the mechanism of inhibition remains elusive. The findings that superactivation of the SM22α promoter is repressed by forced expression of HRT2 in SMCs coupled with the finding that Notch1-deficient ES cells express early SMC-restricted genes suggests that myocardin and Notch signaling are in dynamic balance modulating SMC phenotype. Potential mechanisms include direct interactions between HRT and factors associated with transcriptosomes governing myocardin-dependent contractile smooth muscle genes. Such factors include SRF, myocardin, and potentially other proteins such as ternary complex factors, which may play a role in regulating myocardin-dependent promoters. However, under the experimental conditions employed, we failed to demonstrate physical association between HRT and factors associated with transcriptosomes governing myocardin-dependent contractile smooth muscle genes. Such factors include SRF, myocardin, and potentially other proteins such as ternary complex factors, which may play a role in regulating myocardin-dependent promoters. However, under the experimental conditions employed, we failed to demonstrate physical association between HRT2 and SRF or myocardin. Using either in vitro translated proteins or nuclear extracts in EMSAs and co-immunoprecipitation experiments, inclusion of HRT2 did not alter the SRF-myocardin protein complex. However, these experiments do not exclude the possibility that 293T cells are missing a HRT2-regulated factor that directly associates with either SRF or myocardin. Consistent with the EMSA results, chromatin immunoprecipitation experiments revealed that the relative amount of SRF associated with the SM-MyHC promoter in vivo was not noticeably different in the absence or presence of HRT2. Interestingly, myocardin was required for recruitment of SRF to the promoter, a finding not previously published (compare Fig. 6D, lanes 1 and 3). Finally, it does not appear that association between HRT2 and HDACs is a critical determinant of HRT-mediated repression of SMC-restricted transcriptional regulatory elements. Taken together, these data demonstrate that HRT2-mediated repression of myocardin-dependent SMC gene expression does not occur by competitive binding with myocardin to SRF, by altering the recruitment of SRF to CArG boxes by myocardin and/or disruption of the SRF-DNA complex at the SM-MyHC promoter in vivo.

Whereas the data presented clearly show that Notch signaling mediated via HRTs represses myocardin-dependent SMC
transcription, mechanistic studies failed to demonstrate direct interactions between HRT2 and SRF or myocardin at least under the experimental conditions employed. Moreover, we did not find evidence that HRTs repress SMC transcription by binding directly to CaRg elements or other sites in SMC-restricted transcriptional regulatory elements. Rather, these findings raise the important and intriguing hypothesis that HRT2, through its basic DNA-binding domain, represses the gene expression of a required co-factor for myocardin-dependent gene transcription in the context of SMC differentiation (Fig. 9). The observation that HRT2 C-terminal sequence also contributes to its repressive activity clearly suggests that the molecular basis of HRT-mediated repression of SMC genes is complex. This is not surprising, since whatever mechanism(s) regulate SMC phenotype must allow the spectrum of SMC molecular basis of HRT-mediated repression of SMC genes is complex. This is not surprising, since whatever mechanism(s) contribute to its repressive activity clearly suggests that the molecular basis of HRT-mediated repression of SMC genes is complex. This is not surprising, since whatever mechanism(s) regulate SMC phenotype must allow the spectrum of SMC gene transcription in the context of SMC differentiation.

Acknowledgments—We gratefully acknowledge the support and advice of Tom Kadesch. We thank Hong Sai for assistance with virus preparation and Shara Kabak for advice and critical reading of the manuscript.

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J. Biol. Chem. 2005, 280:8994-9004.
doi: 10.1074/jbc.M413316200 originally published online January 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413316200

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