Notch-mediated CBF-1/RBP-Jκ-dependent regulation of human vascular smooth muscle cell phenotype in vitro

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Morrow, David, Agnieszka Scheller, Yvonne A. Birney, Catherine Sweeney, Shaunta Guha, Philip M. Cummins, Ronan Murphy, Dermot Walls, Eileen M. Redmond, and Paul A. Cahill. Notch-mediated CBF-1/RBP-Jκ-dependent regulation of human vascular smooth muscle cell phenotype in vitro. Am J Physiol Cell Physiol 289: C1188–C1196, 2005. First published June 29, 2005; doi:10.1152/ajpcell.00198.2005.—Vascular smooth muscle cell (VSMC) phenotype modulations are key factors in vascular pathology. We have investigated the role of Notch receptor signaling in controlling human vascular smooth muscle cell (hVSMC) differentiation in vitro and established a role for cyclic strain-induced changes in Notch signaling in promoting this phenotypic response. The expression of α-actin, calponin, myosin, and smoothelin was examined by performing immunocytochemistry, Western blot analysis, and quantitative real-time PCR in hVSMCs cultured under static conditions after forced overexpression of constitutively active Notch 1 and 3 receptors, inhibition of endogenous Cbp-binding factor 1 (CBF-1)/recombination signal sequence-binding protein-Jκ (RBP-Jκ) signaling, and exposure to cyclic strain using a Flexercell Tension Plus unit. Overexpression of constitutively active Notch intracellular (IC) receptors (Notch 1 IC and Notch 3 IC) resulted in a significant downregulation of α-actin, calponin, myosin, and smoothelin expression, an effect that was significantly augmented after inhibition of Notch-mediated, CBF-1/RBP-Jκ-dependent signaling by coexpression of RPM-1 (Epstein-Barr virus-encoded gene product) and selective knockdown of basic helix-loop-helix factors [hairy enhancer of split (HES) gene and Hes-related transcription (Hrt) factors Hrt-1, Hrt-2, and Hrt-3] using targeted small interfering RNA. Cells cultured under conditions of defined equi-biastropic cyclic strain (10% strain, 60 cycles/min, 24 h) exhibited a significant reduction in Notch 1 IC and Notch 3 IC expression concomitant with a significant increase in VSMC differentiation marker expression. Moreover, this cyclic strain-induced increase was further enhanced after inhibition of CBF-1/RBP-Jκ-dependent signaling with RPM-1. These findings suggest that Notch promotes changes in hVSMC phenotype via activation of CBF-1/RBP-Jκ-dependent pathways in vitro and contributes to the phenotypic response of VSMCs to cyclic strain-induced changes in VSMC differentiation.

basic helix-loop-helix; cyclic strain; myosin; smoothelin

MODIFICATIONS IN THE STRUCTURE, integrity, and function of arterial blood vessels are central to the pathogenesis of many vascular diseases (20). Numerous studies have demonstrated a distinct heterogeneity of vascular smooth muscle cell (VSMC) phenotype in the vessel walls of both human and animal models and in cell culture studies (6). Because adult VSMCs are not terminally differentiated, they are capable of changing phenotype in response to changes in local environmental cues, including growth factors and/or inhibitors, mechanical influences, cell-cell and cell-matrix interactions, and various inflammatory mediators (17, 18). The overall control of VSMC differentiation and the regulation of its responses to changing environmental cues is extremely complex and involves the cooperative interaction of many factors and signaling pathways that are only beginning to be understood (17, 18).

Studies in cultured VSMCs have implicated a large number of factors in regulating VSMC differentiation, including mechanical forces (5, 17), contractile agonists, extracellular matrix components, neuronal factors, reactive oxygen species, and endothelial cell-VSMC interactions, all of which promote expression of VSMC marker genes in vitro (18). Surprisingly, despite the facts that cultured VSMC lines are highly modulated and that phenotypic modulation is a critical process in atherogenesis (29) and vascular injury repair (3), few factors and/or pathways have been identified that selectively and directly promote phenotypic modulation of the VSMC, with the exception of platelet-derived growth factor (PDGF)-BB. Researchers in a recent study (19) suggested that activation of Notch signaling mediated through members of the hairy enhancer of split (HES) gene and Hes-related family of transcription (Hrt) factors of basic helix-loop-helix (bHLH) represses smooth muscle cell (SMC) differentiation and the expression of genes encoding SMC contractile markers in a reporter cell line and in embryonic SMCs.

Notch receptor-ligand interactions are a highly conserved mechanism that were originally described in developmental studies using Drosophila. These interactions regulate intercellular communication and direct individual vascular cell fate decisions (10, 28). The intracellular (IC) Notch receptor (Notch IC) is translocated to the nucleus, where it interacts with the CSL {Cbp-binding factor 1 (CBF-1)/recombination signal sequence-binding protein-Jκ (RBP-Jκ), Suppressor of hairless [Su(h)], and Lag-1} family of transcription factors to become a transcriptional activator that can then modulate the expression of Notch target genes that regulate cell fate decisions (10, 28). These include the HES gene and Hrt factors that are critically involved in mammalian cell differentiation (28). Notch receptor-ligand interactions are known to regulate intercellular communication and direct individual cell fate decisions.
during the development of the embryonic vasculature (10, 28). We and others have recently shown that Notch receptors and downstream target genes (HES and HRT) are crucial in controlling the modulation of SMC growth, migration, and apoptosis in vitro and in vivo (23, 26, 27). Moreover, we have established that equibiaxial cyclic strain inhibits SMC proliferation while enhancing SMC apoptosis through inhibition of Notch receptor and downstream target gene expression (16). In the present study, we have addressed the role of Notch receptors in controlling human VSMC (hVSMC) phenotype and describe for the first time that Notch 1 and Notch 3 receptors repress SMC differentiation in a CBF-1/RBP-Jk-dependent manner in vitro. In addition, equibiaxial cyclic strain down-regulates SMC Notch receptor expression while promoting SMC differentiation.

MATERIALS AND METHODS

Materials. All materials were of molecular biology grade and were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Antibodies. Polyclonal rabbit anti-Notch 1 IC and Notch 3 IC antibodies were obtained from Upstate Cell Signaling Solutions (Milton Keynes, UK). Polyclonal anti-smoothelin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-myosin (smooth) clone hSM-V, anti-α-smooth muscle actin clone 1A4, and anti-calponin clone hCP antibodies were obtained from Sigma-Aldrich. Alexa Fluor 488-conjugated antibodies of rabbit anti-α-actin and goat anti-mouse IgG were purchased from Molecular Probes (Leiden, The Netherlands). Peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG, as well as anti-mouse and anti-goat fluorescein isothiocyanate (FITC) conjugates, were obtained from Amersham Biosciences (Little Chalfont, UK) and Sigma-Aldrich, respectively.

Expression vectors. Vectors expressing hemagglutinin (HA)-tagged Notch 1 IC (pCMV-ED1-HA) were generously donated by B. Kemppes (8). The Notch 3 IC expression construct (pCMX-Notch 3 IC-HA) was a kind gift from U. Lendahl (12). RPMS-1 (pcDNA3-SM-CIR) was a gift from P. Farrell (22). RPMS-1 is an Epstein-Barr virus (EBV)-encoded viral gene product that specifically inhibits the CBF-1/RBP-Jk interactions (17). RPMS-1 is therefore a specific negative regulation of HES signaling in VSMCs. We previously demonstrated the potency of expression of RPMS-1 (23). The product of the EBV RPMS-1 open-reading frame has been shown to regulate the activity of Notch IC negatively by specifically binding to CBF-1/RBP-Jk and the CBF-1/RBP-Jk-associated corepressors Sin3A and conserved inverted repeat (CIR) and to partially reverse Notch IC-mediated inhibition of differentiation in muscle cells by blocking relief of CBF-1-mediated repression and interfering with Ski-interacting protein (SKIP)-CIR interactions (17). RPMS-1 is therefore a specific negative regulator of Notch IC trans-activation of Notch target genes through interactions with proteins in the Notch corepressor complex. The plasmid pGK3-puro encoding puromycin resistance (25) was a kind gift from P. Ling.

Cell culture and cyclic strain. Human aortic SMCs were purchased from Cell Applications (San Diego, CA). Cells were maintained in a 37°C humidified atmosphere of 5% CO2-95% air in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg of streptomycin. Cells were routinely subcultured (passages 2–18) after treatment for 5 min with 0.125% trypsin-EDTA at 37°C. For cyclic strain studies, hVSMCs were seeded into six-well Bioflex plates (Dunn Labortechnik, Asbach, Germany) at a density of ~5 × 10⁵ cells/well. Bioflex plates contain a pronectin-coated silicon membrane bottom that enables the precise deformation of cultured cells with the use of a microprocessor-controlled vacuum (2). Once 90% confluence was reached, cells were exposed to a physiological level of equibiaxial cyclic strain (10% strain, 60 cycles/min, 24 h, cardiac simulation waveform) using a Flexercell Tension Plus FX-4000T unit (Flexcell International, Hillsborough, NC).

Plasmid preparation and transient transfection. Plasmids were prepared for transfection according to the manufacturer’s instructions using a Qiagen plasmid midi kit (Qiagen, Crawley, UK). Cells were plated onto six-well plates 2 days before transfection at a density of 1 × 10⁵ cells/well and transfected at 70% confluence. Plasmid transfection was performed using Lipofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The cells were transfected with various expression constructs, with the addition of a total of 2.0 μg of DNA/well. Cells were harvested 16–24 h posttransfection using 1× reporter lysis buffer (Promega, Madison, WI). Transfection efficiency was verified by performing β-galactosidase assays, and Western blot analysis was used to confirm overexpression of effector proteins. For constitutively active Notch IC transient overexpression studies, cells transfected with vectors expressing Notch 1 IC, Notch 3 IC, and RPMS-1 were cotransfected with pGK3-puro and selected after treatment of cells with 0.8 mg/ml puromycin for 48 h (16, 23). At this concentration of puromycin, ~80% of cells that survived after 48 h were transfected with the plasmid of interest.

Preparation of cell lysates. Harvested cells were pelleted by performing low-speed centrifugation. The cell pellet was placed in ice-cold lysis buffer [20 mM Tris, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100 (vol/vol), 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin, pH 7.5] and subjected to ultrasonication with a sonic dismembrator (Vibracell; Sonics and Materials, Gland, Switzerland). Samples were divided into aliquots and stored at −80°C before use. Protein concentration was determined using the method of Bradford and BSA as a standard.

Western blot analysis. Proteins from cell lysates (10–20 μg) were analyzed by SDS-PAGE with the use of 7% or 12% resolving gels, followed by transfer to nitrocellulose membranes. Equal protein loading and transfer efficiency were determined by staining the nitrocellulose membranes with Ponceau S (Amersham Biosciences). Membranes were rinsed in wash buffer (PBS containing 0.05% Tween 20) and blocked for 60 min in wash buffer containing 5% nonfat dried milk at room temperature and gently agitated. After three 15-min washes in wash buffer, membranes were incubated overnight at 4°C with primary antibody (α-actin, 1:2,000 dilution; calponin or myosin, 1:500 dilution) in PBS/Tween 20/ml. The dilution factor for each antibody was determined empirically. After three 10-min washes in wash buffer, membranes were incubated with the appropriate secondary antibody (1:1,000 dilution for calponin and myosin; 1:2,000 dilution for α-actin) in PBS/Tween 20/ml for 3–4 h at room temperature on an orbital shaker. After three final 15-min washes, the ECL detection reagent (Amersham Biosciences) was placed on the membranes for 5 min before they were exposed to ECL HyperFilm. The signal intensity of the appropriate bands on the autoradiogram was calculated using the EDAS 120 system (Kodak, Rochester, NY).

Immunocytochemistry. hVSMCs were seeded onto six-well plates 2 days before staining at 2 × 10⁵ cells/well. Cells were stained for differentiation markers or Notch signaling pathway component protein expression at 80–90% confluence. In brief, confluent hVSMCs were permeabilized, fixed with ice-cold methanol for 10 min, and subsequently rehydrated with PBS containing 3% BSA for 10 min. Cells were then incubated in the appropriate primary antibody (1:200 dilution for α-actin; 1:50 dilution for calponin, myosin, and smoothelin in PBS/3% BSA) at 4°C overnight with gentle agitation. After five 10-min washes in PBS, cells were incubated in the appropriate secondary antibody (1:200 dilution in PBS/3% BSA using FITC or anti-mouse Alexa Fluor) for 2 h at 37°C in a humidified chamber. Cells were then washed three times in PBS before visualization using an Olympus DP-50 fluorescent microscope (with appropriate excitation and emission spectra at ×200 and ×400 magnification) coupled with Studio Life software.

Quantitative real-time RT-PCR. For quantitative measurement of mRNA, we used quantitative real-time RT-PCR (QRT-PCR) with

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GAPDH mRNA levels as an internal control. Total RNA from cell pellets was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s specifications before 1 μg of total RNA was reverse transcribed in a reaction containing 1/100 μl Moloney murine leukemia virus (MMLV) reverse transcriptase buffer (Promega, Madison, WI), 5 μM oligo(dT) 12-18 (Invitrogen), 1 mM 2-deoxynucleotide 5'-triphosphate, 2 μg of acetylated BSA, and 200 U of MMLV reverse transcriptase (Promega) at 37°C for 1 h before 2 U/μl RNase H (Promega) was added for 20 min at 37°C. QRT-PCR was performed using the Rotor Gene (RG-3000) and the SYBR Green PCR kit (Qiagen) as described by the manufacturer. PCR was performed using the following specific primers (MWG Biotech, Ebersberg, Germany): β-actin reverse primer, 5'-GTA CGT CCA GAG GCA TAG AG-3', forward primer, 5'-ATC TGG CAC CAC TCT TTC TA-3'; myosin forward primer, 5'-GGAGGATGAGATCCTGGTCA-3', reverse, 5'-TTAGCCGGAAGATCTGCTTCT-3'; and smoothelin forward primer, 5'-GGCAGTGTCACTCACGTCAC-3', reverse, 5'-CTGATTCCAGCATCTTGTCCA-3'. The conditions for amplification were 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. The specificity of PCR products was also validated by performing electrophoresis on 2% agarose gels and visualized using ethidium bromide staining.

siRNA transfection. For gene silencing studies, Lipofectamine 2000 reagent (Invitrogen, The Netherlands) was used for transient transfection of SMCs with gene-specific small interfering RNA (siRNA) duplexes as previously described (16). The siRNA duplexes for Hrt-1, Hrt-2, and Hrt-3 were as follows: scrambled, aa-auucua uccacaguacgac; Hrt-1, aa-gacggagaggacauacgc; Hrt-2, aa-cacccucucaguauugc; and Hrt-3 aa-gcggagaggacauacagag. All duplexes were acquired from MWG Biotech (Milton Keynes, UK).

Data analysis. Results are expressed as means ± SE. Experimental points were performed in triplicate, with a minimum of three independent experiments. An unpaired Student’s t-test and a Wilcoxon signed-rank test were used for comparison of two groups. *P < 0.05 was considered statistically significant.

RESULTS

SMC differentiation marker and Notch 1 and Notch 3 receptor expression in hVSMCs in vitro. The presence of SMC differentiation markers in hVSMCs was confirmed using immunocytochemical and Western blot analyses. While β-actin and calponin were abundantly expressed in hVSMCs, the...
expression of myosin and smoothelin was significantly less robust (data not shown). The presence of Notch 1 and Notch 3 receptors in hVSMCs was also confirmed using Western blot analysis and immunocytochemistry. To maximize the percentage of cells expressing constitutively active Notch IC receptors after transfection, cells were pooled after puromycin selection. Using a green fluorescent protein (GFP) reporter plasmid, we confirmed that >80% of cells coexpressed GFP after a 48-h pretreatment with puromycin (0.8 mg/ml) compared with mock-transfected cells (Fig. 1A). The expression of Notch 1 IC and Notch 3 IC proteins in puromycin-selected cells was significantly enhanced after transfection compared with mock-transfected controls (Fig. 1B). The specificity of the Notch IC antibodies was confirmed using competitive blocking peptides, which successfully blocked the detection of the Notch IC domains with the anti-Notch antibodies as previously described (23). Immunocytochemical analysis revealed significant cytoplasmic and nuclear localization for endogenous Notch 1 IC and Notch 3 IC receptors in hVSMCs (Fig. 1C). In cells that overexpressed constitutively active Notch IC, there was also significant cytoplasmic localization in addition to nuclear staining, which were confirmed using specific anti-HA antibodies targeted against plasmid-encoded, HA-tagged Notch 1 IC and Notch 3 IC (Fig. 1C). We finally confirmed the functional activity of Notch IC in these cells after constitutive overexpression of Notch 3 IC by demonstrating a significant increase in Notch target gene (hrt-1, hrt-2, and hrt-3) mRNA expression, an effect that was blocked after coexpression of RPMS-1, an inhibitor of Notch IC-mediated CBF-1/RBP-Jκ-dependent gene expression (Fig. 1D).

**Fig. 2. Notch signaling inhibits hVSMC differentiation.** The effect of Notch signaling on hVSMC differentiation in static cultures was determined after forced overexpression of constitutively active Notch IC and subsequent examination of the expression of α-actin, calponin, and myosin in these cells. There was a significant decrease in the expression of SMC-specific α-actin after overexpression of constitutively active Notch 1 IC and Notch 3 IC as determined using immunocytochemical and Western blot analyses (Fig. 2, A and B). This was further confirmed using QRT-PCR, in which α-actin mRNA expres-

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**A**

**α-actin**

non-immune control

Mock

CMV Notch 1 IC

CMX- Notch 3 IC

**B**

Mock

CMV- Notch 1 IC

CMX- Notch 3 IC

**C**

Mock

RPMS-1

CMX- Notch 3 IC

CMX-Notch 3 IC + RPMS-1
sion was substantially reduced after overexpression of constitutively active Notch 1 IC and Notch 3 IC compared with mock-transfected controls (Fig. 2B).

It has previously been shown that RPMS-1 is a specific negative regulator of Notch IC trans-activation of Notch target genes through interactions with proteins in the Notch corepressor complex (22). Coexpressing constitutively active Notch 3 IC with the CBF-1/RBP-Jk inhibitor RPMS-1 determined the effect of inhibition of Notch signaling on hVSMC differentiation. The marked inhibition of α-actin protein expression by Notch IC was attenuated after coexpression with RPMS-1 (Fig. 2C). Similarly, myosin and calponin protein expression in hVSMCs was decreased after overexpression of constitutively active Notch 1 IC and Notch 3 IC, compared with mock-transfected controls (Fig. 3A), an effect that was reversed after

![A](image1.png)

![B](image2.png)

![C](image3.png)
coexpression of the CBF-1/RBP-Jκ inhibitor RPMS-1 (Fig. 3A and B). This finding was further confirmed using QRT-PCR in that α-actin, smoothelin, and myosin mRNA levels were substantially reduced after overexpression of constitutively active Notch 3 IC compared with mock-transfected controls (Fig. 3C). Moreover, the inhibitory effect of Notch IC on SMC differentiation marker mRNA expression was significantly reversed by coexpression with RPMS-1 (Fig. 3C).

Notch signaling inhibits hVSMC differentiation through bHLH factors. To assess the role of specific bHLH factors in mediating the expression of SMC differentiation markers in vitro, we examined the expression of myosin and smoothelin after selective knockdown of Hrt-1, Hrt-2, and Hrt-3 siRNA within these cells. The ability of each siRNA to decrease HRT expression was confirmed by performing real-time PCR (Fig. 4A, inset). Selective knockdown of Hrt-1 and Hrt-2 increased myosin expression compared with the scrambled control. Hrt-2 knockdown appeared to have the most potent effect (Fig. 4A). In a similar manner, selective knockdown of Hrt-1, Hrt-2, and Hrt-3 significantly increased smoothelin mRNA levels in these cells (Fig. 4B).

Cyclic strain and hVSMC differentiation. The effect of 10% equibiaxial cyclic strain on Notch signaling was initially determined in hVSMCs after 24 h by measuring the expression of Notch IC in these cells under static and strain conditions. Cyclic strain significantly reduced Notch 1 IC and Notch 3 IC expression compared with unstrained cells (Fig. 5A). This correlated with a decrease in Notch 1 and Notch 3 receptor mRNA levels after exposure to strain (data not shown). The effect of 10% equibiaxial cyclic strain on adult hVSMC differentiation in vitro was also determined in parallel cultures by measuring the expression of α-actin, calponin, myosin, and smoothelin in strained and unstrained cells by performing immunocytochemistry and immunoblot analyses (Fig. 5B). Cyclic strain (10%, 24 h) significantly increased the expression of α-actin, myosin, calponin, and smoothelin in hVSMCs as determined using immunocytochemistry, an effect that was quantitatively confirmed by performing Western blot analysis (Fig. 5B). In addition, cyclic strain (10%, 24 h) significantly increased myosin and smoothelin mRNA levels compared with unstrained controls (Fig. 6A).

Because overexpression of constitutively active Notch 1 and 3 IC in unstrained cells inhibits SMC differentiation marker expression and because cyclic strain promotes the expression of hVSMC differentiation markers in these cells while concomitantly inhibiting Notch IC activation, the effect of inhibition of CBF-1/RBP-Jκ Notch signaling after expression of RPMS-1 on cyclic strain-induced SMC differentiation marker expression was subsequently determined. Expression of RPMS-1 in unstrained cells increased the protein expression of α-actin and calponin compared with mock controls (Fig. 6B). Moreover, in parallel cultures, expression of RPMS-1 in un-
phenotype in response to a wide variety of environmental stimuli, including growth factors and/or inhibitors, mechanical influences, cell-cell and cell-matrix interactions, and various inflammatory mediators (17, 18). However, little is known regarding upstream factors that regulate SMC differentiation in vivo. Several SMC-selective or SMC-specific genes and gene products have been identified that serve as useful markers of the relative state of differentiation and maturation of VSMCs. These include the smooth muscle isoforms of contractile apparatus proteins: α-actin, myosin, calponin, SM22, aortic carboxypeptidase-like protein, desmin, and smoothelin (18). In the present study, we used these SMC-selective markers of differentiation to describe for the first time the regulation of the hVSMC phenotype in vitro by the Notch receptor signaling pathway.

Recent studies have significantly advanced the understanding of the effect of Notch signaling on vascular development (1, 9, 7, 13). During the early stages of vasculogenesis, SMCs are highly migratory, undergo rapid cell proliferation, and exhibit high rates of synthesis of ECM components (17, 18). It is known that the loss of Notch signaling in zebra fish embryos leads to molecular defects in arteriovenous differentiation, including loss of artery-specific markers within the dorsal aorta (14). Moreover, Notch mutations are linked to some late-onset hereditary vascular pathological conditions such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), which further suggests the important contribution of this signaling pathway to vascular homeostasis (11). In contrast, in adult blood vessels, SMCs show a low rate of proliferation and/or turnover, are largely nonmigratory, show a low rate of synthesis of ECM components, and are focused primarily on contractile function (21). The model that has emerged for SMCs within adult mammals is a highly plastic cell population that is capable of rather profound alterations in phenotype in response to changes in local environmental cues that are important for differentiation (17, 18).

Our study is the first to demonstrate in adult human arterial SMCs that Notch decreases SMC differentiation marker expression in a CBF-1/RBP-Jk-dependent manner. Our findings in hVSMCs are that inhibition of endogenous Notch IC with RPMS-1 or Hrt knockdown enhances SMC differentiation marker expression, while Notch activation directly decreases SMC contractile phenotype and concomitantly increases cell proliferation (23, 26, 27). This notion supports a potentially critical role for Notch signaling in mediating the response of VSMCs to arterial injury by regulating the expression of genes encoding contractile proteins and promoting cell proliferation. Indeed, investigators at our laboratory (16, 23) and others (26, 27) have previously reported that Notch regulates the growth, migration, and death of SMCs in vitro through a CBF-1/RBP-Jk-dependent pathway, a response that is mirrored in animal models of vascular injury in which Notch receptors are differentially regulated postinjury as changes in vascular cell fate occur unabated (15, 26). These findings strongly suggest a possible nexus in which the activation of Notch signaling is coupled to the control of SMC differentiation marker expression and phenotype in vitro and is mirrored in vivo.

The mechanisms of how Notch controls SMC phenotype are currently unknown, but recent studies have highlighted the role of Notch signaling in myocardin-dependent transcription of...
SMC-restricted genes in a reporter cell line (C3H10T1/2 fibroblasts) and in embryonic rat SMCs (19). Consistent with our findings in hVSMCs, forced expression of Hrt-2 in embryonic A10 cells inhibited myocardin-induced expression of SMC-restricted genes and the activity of SMC-restricted transcriptional regulatory elements (19). In addition, the repressive function of Hrt-2 was not mediated via the capacity of the bHLH to bind SMC CArG [CC(ATA) 6 GG] elements or by disruption of myocardin-serum response factor protein complexes. Using selective knockdown of HRT, we have demonstrated, in agreement with these findings, that Hrt-2 is an important bHLH factor in mediating Notch IC inhibition of SMC myosin expression. We have further demonstrated that Hrt-1 and Hrt-2 regulated the expression of myosin and smoothelin to varying degrees, suggesting a further complex interaction between individual bHLH factors and their respective downstream targets. Furthermore, a preliminary report by Berrou et al. (4) suggested that Notch 3 modulation of the α-actin cytoskeleton in SMCs, despite RhoA involvement in SMC phenotypic modulation, was RhoA independent. Collectively, these data are all consistent with a model whereby Notch receptor activation through CBF-1/RBP-Jk-dependent mechanisms represses SMC differentiation and maintenance of the contractile SMC phenotype.

In the present study, equibiaxial cyclic strain reduced both Notch 1 and Notch 3 activation concomitant with a significant increase in SMC α-actin, calponin, myosin, and smoothelin expression. The strain-induced decrease in Notch activation in human cells mirrors that observed in animal cells (16) and in vivo (15). Moreover, the cyclic strain-induced increase in SMC differentiation marker expression was further enhanced after inhibition of CBF-1/RBP-Jk-dependent signaling using RPMS-1. These data are in accord with those from previous studies that documented a dual effect of strain on SMC phenotype characteristics by potentiating SMC proliferation, an attribute of a dedifferentiated phenotype, while concomitantly increasing the expression of the high molecular weight form of caldesmon, considered a marker of a differentiated SMC state (5). Consistent with the current findings, cyclic strain increased α-actin protein expression and promoter activity through a mechanism involving MAPK family (Erk, JNK, and p38) (24). Because the strain-induced decrease in Notch IC expression and activity in SMC is Erk dependent (16), it is clear that cyclic strain may modulate SMC phenotype through similar mechanisms that promote changes in SMC growth (i.e., proliferation and apoptosis). Collectively, these studies indicate that in adult SMCs, mechanical strain leads to increased expression of smooth muscle differentiation markers, resulting in a more contractile phenotype. Moreover, biomechanical activation of SMCs enhances SMC differentiation in part through inhibition of Notch activation in these cells. Moreover, these data suggest that Notch, in addition to its role in vascular development, may also play a significant role in determining adult cell fate in vascular cells subjected to various environmental cues.

In conclusion, we have shown for the first time that Notch promotes changes in hVSMC phenotype via activation of CBF-1/RBP-Jk-dependent pathways in vitro and contributes to the phenotypic response of SMCs to cyclic strain. Future studies will address the regulation of SMC differentiation downstream of the activation of Notch target genes in an effort to provide new insights into the molecular mechanisms underlying changes in vascular cell fate that underlie vascular proliferative disease.

REFERENCES


