Cyclic Strain Inhibits Notch Receptor Signaling in Vascular Smooth Muscle Cells In Vitro

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Abstract—Notch signaling has been shown recently to regulate vascular cell fate in adult cells. By applying a uniform equibiaxial cyclic strain to vascular smooth muscle cells (SMCs), we investigated the role of strain in modulating Notch-mediated growth of SMCs in vitro. Rat SMCs cultured under conditions of defined equibiaxial cyclic strain (0% to 15% stretch; 60 cycles/min; 0 to 24 hours) exhibited a significant temporal and force-dependent reduction in Notch 3 receptor expression, concomitant with a significant reduction in Epstein Barr virus latency C promoter-binding factor-1/recombination signal-binding protein of the Jk immunoglobulin gene–dependent Notch target gene promoter activity and mRNA levels when compared with unstrained controls. The decrease in Notch signaling was Gi-protein– and mitogen-activated protein kinase–dependent. In parallel cultures, cyclic strain inhibited SMC proliferation (cell number and proliferating cell nuclear antigen expression) while significantly promoting SMC apoptosis (annexin V binding, caspase-3 activity and bax/bcl-xL ratio). Notch 3 receptor overexpression significantly reversed the strain-induced changes in SMC proliferation and apoptosis to levels comparable to unstrained control cells, whereas Notch inhibition further potentiated the changes in SMC apoptosis and proliferation. These findings suggest that cyclic strain inhibits SMC growth while enhancing SMC apoptosis, in part, through regulation of Notch receptor and downstream target gene expression. (Circ Res. 2005;96:567-575.)

Key Words: notch ■ cyclic strain ■ apoptosis ■ proliferation ■ vascular ■ G-proteins

Hemodynamic forces associated with the flow of blood play an important role in the physiological control of vascular tone, remodeling, and associated vascular pathologies. These forces include cyclic circumferential strain, which is caused by a transmural force acting perpendicular to the vessel wall.1–4 Mechanotransduction is known to play a central role in the highly coordinated cellular response of the vasculature to changes in hemodynamic stimulation. Transduction of biomechanical stimuli leads to activation of cellular signaling mechanisms that ultimately lead to adaptive, and sometimes maladaptive, changes in cell and tissue fate.5,6 The ultimate arbiter of vascular cell fate (growth, migration, differentiation, and apoptosis) in response to hemodynamic stimulation is unclear but considered fundamental to the pathogenesis of vascular disease. Strain-induced changes in smooth muscle cell (SMC) growth, defined as the balance between SMC proliferation and apoptosis, participates in the local vascular reaction to hypertension,3,7 late lumen loss, and restenosis after vascular interventions, as well as plaque vulnerability during atherosclerosis.1,8

Because changes in vascular cell fate are also apparent during vascular morphogenesis and modeling of the embryonic vasculature,9,10 the control of these cell fate decisions in adult cells may share similar signaling patterns. Notch receptor–ligand interactions are a highly conserved mechanism, originally described in developmental studies using Drosophila, that regulate intercellular communication and direct individual vascular cell fate decisions.9,10 Notch receptors and ligands are transmembrane proteins that have been identified in mammalian cells (Notch receptors 1 through 4; Notch ligands Delta, Serrate, and Jagged). Studies using constitutively activated Notch receptors missing their extracellular domains (Notch intracellular [IC]) have shown that Notch signaling determines proliferation, differentiation, and, more recently, apoptosis in several mammalian cell types.11–13 Notch IC is translocated to the nucleus, where it interacts with the CSL family of transcription factors (Epstein Barr virus latency C promoter-binding factor-1/recombination signal-binding protein of the Jk immunoglobulin gene [CBF-1/RBP-Jk], suppressor of hairless, and longevity assurance gene-1) to become a transcriptional activator that can then modulate the expression of Notch target genes that regulate cell fate decisions. These include the “Hairy Enhancer of Split” (hes)
gene and HES-related transcription factors (Hrts) that are critically involved in mammalian cell differentiation. Recent studies demonstrate that Notch receptors and hrt genes are coordinately upregulated in neointimal cells but downregulated in medial cells after vascular injury, an effect that is mimicked by addition of serum mitogens (platelet-derived growth factor) to cultured cells in vitro. Furthermore, Notch signaling may be a critical determinant of SMC survival and vascular structure by modulating the expression of downstream mediators of apoptosis.

Biomechanical signals induce a highly restricted transcriptional response in vascular SMCs that include genes that can modify vascular structure. Because components of the Notch-signaling pathway are regulated coordinately in vascular tissue after injury, the present study examined the specific role of uniform equibiaxial cyclic strain on endogenous Notch-signaling components in SMCs and their contributory role in controlling the growth response of these cells after strain.

Materials and Methods

Materials
All items were of the highest purity commercially available and purchased from Sigma Aldrich unless otherwise stated.

Cell Culture
Rat vascular SMCs (R354-05) were purchased from Cell Applications Inc. For cyclic strain studies, cells were seeded into 6-well Bioflex plates (Dunn Labortechnik) at a density of \(~6 \times 10^5\) cells/well. When cells had reached \(\approx 80\%\) confluence, a Flexercell Tension Plus FX-4000T system (Flexcell International Corp.) was subsequently used to apply a physiological level of cyclic strain to each plate (0% to 15% strain; 60 cycles/min; 0 to 24 hours using the Heartbeat simulation).

Notch-Expressing Vectors/Luciferase Reporter Plasmids
Notch 3 IC expression vectors (cytomegalovirus X-Notch 3 IC) were a kind gift from Prof Urban Lendahl, Karolinska Institute, Stockholm, Sweden. Notch 1 IC delta RAM domain (mNotch 1 IC) was a kind gift of Prof Bettina Kemkens, GSF Institute of Clinical Molecular Biology, Neuherberg, Germany. R218H was a kind gift of Prof Paul J. Farrell, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London, UK.

Plasmid Preparation, Transient Transfection, Luciferase, and \(\beta\)-Galactosidase Assays
Plasmids were prepared for transfection according to manufacturer instructions using a Qiagen plasmid Midi kit (Qiagen) as described previously.

Western Blot Analysis
Proteins from cell lysates (12 to 15 \(\mu\)g) were resolved on SDS-PAGE (12% resolving; 5% stacking) before transfer onto nitrocellulose membrane (Amersham Biosciences). Membranes were stained in Ponceau S to ensure equal protein loading and rinsed in wash buffer (PBS containing 0.05% Tween 20) before being probed, as described previously.

Caspase-3 Assay
Activation of the caspase cascade was determined using a colorimetric assay based on the cleavage of acetyl-Asp-Glu-Val-Asp-pNitroaniline (Ac-DEVD-pNA) as described previously.

Acridine Orange/Ethidium Bromide and Annexin V-Fluor/Propidium Iodide Cell Staining
Apoptotic cells were stained and visualized using the annexin V–fluor/propidium iodide (AV/EtBr) stain (Roche Diagnostics) and acridine orange/ethidium bromide (AO/EtBr) stain as described previously.

Quantitative Real-Time RT-PCR
Quantitative real-time RT-PCR was performed using the Rotor Gene (RG-3000; Corvett Research) and the SYBR green PCR kit (Qiagen) as described previously.

Small Interfering RNA Transfection
For gene silencing studies, Lipofectamine 2000 reagent (Invitrogen) was used for transient transfection of SMCs with gene-specific small interfering RNA (siRNA) duplexes as described previously. The siRNA duplexes for Hrt-2 corresponded to position 123 to 143 in the rat Hrt-2 sequence (Accession number: XM.344806). All duplexes were acquired from MWG Biotech.

Data Analysis
Results are expressed as mean±SEM. Experimental points were performed in triplicate, with a minimum of 3 independent experiments. An unpaired Student’s t test and a Wilcoxon signed rank test were used for comparison of two groups. A value of \(P<0.05\) was considered significant. More information is available online at http://circres.ahajournals.org.

Results
Notch 3 Receptor Promotes SMC Proliferation and Inhibits Apoptosis Through Activation of Notch Target Genes in a CBF-1/RBP-Jk–Dependent Manner
Expression of constitutively active Notch 3 IC in serum-deprived cells resulted in a significant increase in the number of apoptotic nuclei when compared with mock controls (Figure 1a). Overexpression of constitutively active Notch 3 IC in serum-deprived cells resulted in a significant decrease in the percentage of apoptotic nuclei (67.75±4.5% in mock compared with 38.53±2.0% in cells overexpressing Notch 3 IC; \(n=3; P<0.05\), an effect that was reversed after coexpression with RPMs-1 (data not shown). In addition, inhibition of endogenous CBF-1/RBP-Jk–dependent Notch signaling after expression of RPMs-1, R218H, and mNotch 1 IC in cycling SMCs significantly increased caspase-3 activity when compared with mock controls (Figure 1b). Overexpression of constitutively active Notch 3 IC also resulted in a significant reduction in the \(bax/bcl-xL\) ratio such that steady-state \(bax\) mRNA levels decreased, whereas \(bcl-xL\) mRNA levels increased when compared with mock controls (Figure 1c and 1d). Moreover, inhibition of endogenous CBF-1/RBP-Jk–dependent signaling after expression of RPMs-1 resulted in an increase in the proapoptotic \(bax\) mRNA levels while concomitantly inhibiting antiapoptotic \(bcl-xL\) mRNA levels (Figure 1c and 1d).

Furthermore, the changes in the \(bax/bcl-xL\) ratio after overexpression of constitutively active Notch 3 IC were reversed after coexpression with RPMs-1 (Figure 1c). In
addition, selective knockdown of Notch target gene *Hrt-2* with siRNA resulted in a significant increase in *bax* mRNA levels while significantly decreasing *bcl-xL* mRNA when compared with the scrambled siRNA control (Figure 1c).

**Cyclic Strain Induces Changes in SMC Growth In Vitro**

Cyclic strain (10%) significantly decreased expression of proliferating cell nuclear antigen (pCNA), a marker for cell proliferation, after 24 hours while concomitantly decreasing the proliferation rate of SMC for up to 10 days after strain (Figure 2a). The lack of an effect of cyclic strain on SMC viability was confirmed by measuring LDH levels in media from strained cells (data not shown).

In parallel cultures, cyclic strain promoted SMC apoptosis because annexin V positive binding was significantly increased after strain when compared with unstrained controls (Figure 2b). This was confirmed using an AO/EtBr stain of...
apoptotic nuclei (8.25±1.25% in the unstrained controls to 30.75±8.98%; n=3; P<0.05 in the strained cells). Serum deprivation of SMCs for 48 hours, a known ubiquitous proapoptotic stimulus for SMCs, caused a marked increase in SMC bax protein expression while concurrently inhibiting bcl-xL expression (Figure 2c). Cyclic strain also increased the bax/bcl-xL ratio by increasing the expression of bax and decreasing the expression of bcl-xL, respectively, when compared with unstrained cells (Figure 2c). The change in the bax/bcl-xL ratio was further confirmed by examining bax and bcl-xL mRNA levels after strain. Strain significantly increased the levels of proapoptotic bax mRNA and decreased the levels of bcl-xL mRNA when compared with unstrained cells (Figure 2d). The levels of caspase-3 activity were also significantly increased after exposure to cyclic strain when compared with unstrained controls (Figure 2d).

**Cyclic Strain Inhibits Notch Signaling in SMC In Vitro**

Cyclic strain (10%; 24 hours) caused a significant decrease in Notch 3 protein expression (Notch IC and the full-length receptor [Notch extracellular]) concomitant with a significant decrease in the expression of Notch ligand jagged-1 and the Notch target gene products Hrt-1 and Hrt-3 (Figure 3a).
Cyclic strain also significantly decreased Notch 1 and Notch 3 receptor and Jagged-1 steady-state mRNA levels concomitant with a significant decrease in hrt-1, hrt-2, and hrt-3, and hes-1 and hes-5 mRNA levels when compared with unstrained controls (Figure 3a).

The effects of cyclic strain on the Notch-signaling pathway were force and time dependent. Notch 3 mRNA levels were temporally reduced after exposure to strain for up to 24 hours when compared with the unstrained controls (Figure 3b). Similarly, hes-5 mRNA levels were significantly decreased in a force- and time-dependent manner in SMCs subjected to 0, 5, 10, and 15% cyclic strain for up to 24 hours when compared with the unstrained controls (Figure 3b). For subsequent studies, regulation of Notch signaling was examined after exposure to 10% cyclic strain for 24 hours. There was a significant temporal decrease in baseline transactivation CBF-1/RBP-Jκ–dependent promoter activity in cells exposed to strain when compared with unstrained controls at all times examined (6, 10, and 24 hours) with strain maximally decreasing CBF-1/RBP-Jκ transactivation after 6 hours (Figure 3e). Furthermore, the strain-induced reduction in transactivation of CBF-1/RBP-Jκ–dependent promoter activity was maintained after overexpression of Notch 3 IC when compared with unstrained controls (Figure 3c). Cyclic strain also significantly decreased Notch target gene promoter activity (hrt-1 and hes-5) at all times examined after strain, with maximal inhibition occurring after 8 hours (Figure 3c).

**Cyclic Strain Inhibits Notch Signaling in a Gi–Mitogen-Activated Protein Kinase–Dependent Manner**

Because cyclic strain regulates SMC fate through various mechanosensitive pathways,5,6,20,23,24 the role of inhibitory Gi-proteins and mitogen-activated protein kinase (MAPK) in mediating the strain-induced response was examined. Pretreatment of cells with the Gi-protein inhibitor pertussis toxin (PTX; 100 ng/mL for 24 hours) significantly inhibited baseline levels of Notch3 IC protein expression and Hrt-2 mRNA in unstrained cells (Figure 4a). Moreover, the strain-induced decrease in Notch 3 IC expression and Hrt-2 mRNA levels was blocked after PTX treatment (Figure 4a). Furthermore, pretreatment of cells with the MAPK inhibitor PD98059 significantly inhibited baseline levels of Notch 3 IC protein expression while concurrently inhibiting the strain-induced decrease in Notch IC expression (Figure 4b).

In parallel studies, pretreatment of cells with PTX significantly inhibited the strain-induced decrease in pcNA expression (Figure 4c) while concomitantly inhibiting the strain-induced increase in caspase-3 activity in these cells (Figure 4d).

**Effect of Notch Signaling on Cyclic Strain–Induced Changes in SMC Proliferation and Apoptosis In Vitro**

Overexpression of constitutively active Notch 3 IC was confirmed using an anti-hemagglutinin (HA) antibody specific for cells expressing the HA-tagged plasmid encoding Notch 3 IC (Figure 5a, inset). The recovery from strain-induced decreases in Notch signaling was confirmed by demonstrating that overexpression of Notch 3 IC recovered the strain-induced decrease in Notch 3 and hrt-2 mRNA to levels that were comparable to unstrained cells (Figure 5a and 5b). In parallel cultures, cyclic strain decreased pcNA expression in mock controls, an effect that was significantly attenuated after overexpression of Notch 3 IC (Figure 5c). Moreover, the cyclic strain–induced increase in the percentage of apoptotic nuclei in mock controls was significantly attenuated after overexpression of Notch 3 IC (Figure 5d).

The effect of Notch 3 IC overexpression on cyclic strain–induced changes in caspase-3 and Bcl-2 family mRNA levels was also investigated. The cyclic strain–induced increase in caspase-3 activity was attenuated after overexpression of constitutively active Notch 3 IC (Figure 6a). Cyclic strain...
significantly decreased bcl-xL mRNA levels when compared with mock controls, an effect that was significantly attenuated after overexpression of Notch 3 IC (Figure 6b). In contrast, the cyclic strain–induced increase in bax mRNA levels in mock controls was further enhanced after overexpression of constitutively active Notch 3 IC (Figure 6d). In parallel studies, inhibition of Notch IC by coexpression with RPMS-1 further enhanced the strain-induced increase in bax mRNA while concomitantly potentiating the strain-induced decrease in bcl-xL mRNA levels when compared with mock controls (Figure 6b). The inhibition of Notch IC also further enhanced the strain-induced decrease in pCNA expression compared with mock controls (Figure 6b).

Discussion

Biomechanical signals can induce a highly restricted transcriptional response in vascular SMCs that include genes that can modify vascular structure.5–8 The current study examined the effect of equibiaxial cyclic strain on Notch signaling in adult SMCs and determined the contributory role of Notch
receptors in regulating strain-induced changes in SMC proliferation and apoptosis. We established that cyclic strain induces a significant decrease in the proliferative and antiapoptotic effects of Notch by decreasing the expression and activity of components of the Notch 3–signaling pathway in a force- and time-dependent manner. This decrease in Notch signaling was Gi-protein– and MAPK-dependent. Moreover, overexpression of constitutively active Notch 3 IC reversed the strain-induced inhibition of SMC proliferation to levels comparable to that of unstrained cells while attenuating strain-induced SMC apoptosis in vitro. Furthermore, Notch inhibition potentiated the strain-induced decrease in Notch signaling. Collectively, these studies suggest for the first time that biomechanical stimulation of SMC inhibits endogenous Notch receptor signaling, resulting in fundamental changes in vascular SMC proliferation and apoptosis in vitro. Understanding these responses may provide new insights into the pathogenesis and treatment of vascular diseases such as atherosclerosis and intimal hyperplasia.

Previous studies have presented conflicting reports on the effects of cyclic strain on SMC growth in vitro. Depending on the species of SMC, the phenotype studied, the extracellular matrix environment, the cell cycle status (whether quiesced or cycling), and the type of cyclical strain regime applied, SMCs can either increase or decrease their proliferative capacity. In the current study, cyclic strain decreased SMC proliferation while concomitantly increasing SMC apoptosis. This concurs with several other studies that report increased SMC apoptosis in vitro and in vivo in response to strain or pressure. The cyclic strain–induced SMC apoptosis is, at least in part, associated with an increase in caspase-3 activity and a change in the bax/bcl-xL ratio in favor of apoptosis. It is clear that strain-induced regulation of Bcl-2 proteins is consistent with strain-induced changes in caspase-3 activity in these cells, and mirrors changes during vascular remodeling in vivo. The stimulus for the observed increase in bax expression after strain is likely attributable to increased p53 activity because bax is a direct transcriptional target for p53, and two recent studies have shown increases in p53 activity and bax expression attributable to cyclic strain. The strain-induced decrease in bcl-xL is consistent with previous studies in vitro and validates previous in vivo studies that demonstrate a correlation between downregulation of bcl-xL and increased SMC apoptosis. Together, this study has clearly established that cyclic strain decreases proliferation and increases SMC apoptosis through a mechanism involving modulation of the Bcl-2 family and activation of caspase-3.

We and others have established a functional coupling between Notch signaling, SMC proliferation, and apoptosis in SMCs in vitro and in vivo. The current study demonstrated for the first time that constitutively active Notch 3 IC increased bcl-xL expression while concomitantly inhibiting bax expression, an effect that was fully reversed after inhibition of CBF-1/RBP-Jκ with expression of RPMS-1. These data suggest that bax and bcl-xL are downstream targets of CBF-1/RBP-Jκ–dependent Notch signaling in these cells. This is further reinforced by studies that demonstrate constitutively active Notch 1 modulates expression of bcl-xL.

Moreover, we have shown using specific siRNA targeted against an individual Notch target gene (Hrt-2) selective hrt-2 knockdown resulted in a significant increase in bax and a decrease in bcl-xL expression in SMCs, further confirming the importance of Notch IC regulation of Bcl-2 family of proteins in these cells. At present, it is unclear why bax mRNA levels are enhanced in strained cells after constitutive Notch activation despite being significantly inhibited in unstrained cells. Additional factors affected by cyclic strain may act to regulate and potentiate bax expression in strained cells. This possibility is further reinforced because bax expression is significantly enhanced in strained cells irrespective of whether Notch IC is overexpressed or inhibited (after RPMS-1 expression).

Because cyclic strain can induce a highly restricted transcriptional response in vascular SMCs that regulates vascular structure, we hypothesized that Notch could represent a novel pathway for contributing to strain-induced changes in vascular fate. The current study demonstrates a functional role for cyclic strain–induced decreases in Notch-mediated CBF-1/RBP-Jκ–dependent signaling and subsequent changes in vascular cell fate. The level of inhibition of Notch signaling after exposure to cyclic strain suggests that Notch is a major target for biomechanical regulation of SMCs in vitro. Indeed, previous in vivo studies concur by demonstrating an acute decrease in Notch receptor and Notch target gene expression after balloon injury and subsequent cellular disarray, with the most dramatic decrease evident for the Notch 3 receptor and the hrt-1 target gene. This response to strain appears to be temporal. Platelet-derived growth factor and angiotsensin II also induced a coordinate downregulation of Notch signaling in SMCs through an extracellular signal-regulated kinase–dependent pathway. In the current study, the decrease in the expression of Notch components is consistent with the observed changes in SMC fate after strain. Moreover, selective inhibition of Gi-proteins and MAPK, respectively, which are known signaling pathways involved in mechanotransduction in vascular cells, despite decreasing baseline Notch signaling, blocked the strain-induced changes in Notch and corresponding changes in SMC fate. To our knowledge, this is the first demonstration that the expression of Notch receptors and downstream target genes are directly modulated by strain in mammalian cells in vitro. This finding further suggests a possible nexus by which the activation of a biomechanical signaling pathway is coupled to the Notch cellular fate program. Additional studies will be required to delineate the mechanism(s) by which cyclic strain regulates the expression of Notch in vitro.

The regulation of Notch signaling is known to occur at multiple levels, including patterns of ligand and receptor expression, Notch-ligand interactions, trafficking of the receptor and ligands, and covalent modifications, including glycosylation, phosphorylation, and ubiquitination. A possible mechanistic explanation for the effect of cyclic strain on Notch 3 IC activity may be post-translational modifications, such as phosphorylation, that may influence its transactivation capacity. Indeed, glycogen synthase kinase-3β (GSK3β) is known to modulate Notch signaling through phosphorylation of Notch IC, such that inhibition of...
GSK3β shortened the half life of Notch1 IC, whereas, conversely, activated GSK3β reduced the quantity of Notch IC that was degraded by the proteasome.\textsuperscript{46} Moreover, the regulatory phosphorylation of GSK3β and hence its activity is under the control of MAPK-dependent signaling pathways.\textsuperscript{46} Therefore, it is tempting to speculate that cyclic strain modulates this process, thereby influencing the translocation of Notch IC into the nucleus. However, because capacity of Notch 3 IC to transactivate various CBF-1/RBP-Jc-dependent promoters is inhibited by cyclic strain and because strain does not affect constitutively active Notch 3 IC expression, these data collectively suggest that other strain-induced events are critical to the ability of Notch IC (whether endogenous or vector encoded) to transactivate CBF-1/ RBP-Jc target promoters. Indeed, it is known that Notch IC interacts with coactivating proteins, including histone acetyltransferase proteins, which play a role in chromatin opening and initiation of transcription.\textsuperscript{50} Moreover, strain may inhibit endogenous inhibitors of Notch IC, such as Numb, that result in a loss of Notch IC functionality after strain. However, because Notch 3 mRNA levels and the expression of full-length Notch 3 receptors are also decreased after strain, it is clear that cyclic strain inhibits the expression of the Notch 3 receptor in addition to any post-translational modifications that may occur to shorten the half life of Notch IC.

In conclusion, we have shown for the first time that equibiaxial cyclic strain induces apoptosis and inhibits proliferation of rat SMC in vitro. Cyclic strain–induced changes in fate are attributable, at least in part, to a cyclic strain–induced decrease in expression and activity of Notch signaling pathway components. These studies provide further evidence that Notch receptors acting through CBF-1/RBP- Jc–dependent signaling pathways are an important determinant of SMC fate in vitro.

Acknowledgments

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References


Supplemental Material on-line

Materials:

All items were of the highest purity commercially available and purchased from Sigma Aldrich Ltd (Poole, Dorset, UK) unless otherwise stated. Polyclonal rabbit anti-Notch 1 and Notch 3 IC antibodies were obtained from Upstate Cell Signaling Solutions (Milton Keynes, UK) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Santa Cruz Notch 1 and 3 antibodies are affinity-purified goat polyclonal raised against a peptide mapping near the carboxy terminus of Notch 1 and 3 receptors and have been used widely to examine and detect Notch receptor expression in various cell lines and tissues [1-5]. The Upsate Cell Signaling anti-Notch 1 cytoplasmic domain antibody was raised using a GST fusion protein corresponding to residues 2272-2488 of rat Notch 1. The anti-Notch 3 was a His-tag fusion protein containing the carboxy-terminus (residues 5935-6454) of murine Notch 3. The specificity of the Notch IC antibodies was verified using competitive blocking peptides, which successfully blocked the detection of the Notch IC domains by each anti-Notch antibodies and by studies that demonstrated over-expressed constitutively active Notch 1 and 3 IC co-migrated with a similar molecular weight as endogenous Notch IC when detected using the same anti-Notch antibodies and using anti-HA antibodies targeted against the constitutively active Notch IC. Polyclonal goat anti-Hrt-1, Hrt-3 and Jagged-1 antibodies were obtained from Santa Cruz Biotechnology and validated using specific blocking peptides. The AlexaFluor secondary antibodies were purchased from Molecular Probes (Leiden, The Netherlands). Lipofectamine™ was purchased from Invitrogen (Carlsbad, CA, USA). Specific primers for Notch receptors and target genes were purchased from MWG Biotech (Ebersberg, Germany).

Cell Culture:

Rat vascular smooth muscle cells (SMC, R354-05) were purchased from Cell Applications Inc. (CA, USA). 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 streptomycin. Cells were routinely subcultured following treatment for 5 min
with 0.125% trypsin-EDTA at 37°C. Cells between passages 3 and 18 were used in experiments. Cells displayed typical ‘hill and valley’ morphology of smooth muscle cells in culture and stained positive for smooth muscle cell specific α-actin but stained poorly for myosin, calponin and smoothelin as previously described [6]. For cyclic strain studies, cells were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately 6 x10^5 cells/well. Bioflex® plates contain a pronectin-coated silicon membrane bottom which enables precise deformation of cultured cells by microprocessor-controlled vacuum [7]. When cells had reached approximately 80% confluency, a Flexercell® Tension Plus™ FX-4000T™ system (Flexcell International Corp. - Hillsborough, NC USA) was subsequently employed to apply a physiological level of cyclic strain to each plate (0-15% strain, 60 cycles/min, 0-24 h using the Heartbeat™ simulation).

**Preparation of Cell Lysates:** Harvested cells were pelleted by 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 (v/v); 2.5 mM sodium pyrophosphate; 1 mM γ-glycerophosphate; 1 mM sodium orthovanadate; 1 μg/ml leupeptin, pH 7.5) and subjected to ultrasonication with a sonic dismembrator (Vibra Cell, Sonics and Materials Inc. Gland, Switzerland). Samples were divided into aliquots and stored at -80°C before use. Protein concentration was measured by the method of Bradford with BSA used as a standard [8].

**Notch Expressing Vectors/Luciferase Reporter Plasmids:**
Notch 3 IC expression vector (CMX-Notch 3 IC) was a kind gift from U. Lendahl (19). Vectors expressing Notch 1 IC (CMV-Notch 1 IC) and Notch 1 IC delta RAM domain (mNotch 1 IC) and the luciferase reporter plasmids pGa98-1-6 and pGa50-7 were a gift from B. Kempkes [9]. The vector pGa981-6 contains the hexamerized 50-bp Epstein-Barr virus nuclear antigen (EBNA-2) response element of the TP-1 promoter (ERE-TP1) in front of the minimal β-globin promoter driving the luciferase gene. pGa50-7 is the corresponding vector without ERE-TP1. The luciferase reporter plasmids hrt-1 and hrt-3 were a gift from E. Olson [10]. The hes-1 and hes-5 luciferase reporter plasmids were
also a gift from U. Lendhl (11.). A dominant-negative CBF-1 construct, R218H was a kind gift from T. Honjo (12), and the pcDNA3-RPMS-1/FLAG (RPMS-1) was a gift from P. Farrell (13). The plasmid pGK3-puro that encodes puromycin resistance (14) was a kind gift from P. Ling. The product of the EBV RPMS-1 open reading frame has been shown to negatively regulate the activity of Notch IC by specifically binding to CBF-1/RBP-Jk and the CBF-1/RBP-Jk associated co-repressors Sin3A and 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 SKIP-CIR interactions (13). RPMS-1 is, therefore, a specific negative regulator of Notch IC trans-activation of Notch target genes through interactions with proteins in the Notch co-repressor complex. pEF-BOSneo-RPB-R218H (R218H) is a mutant of RBP-Jk that cannot bind to DNA (15). The R218H mutant competes with endogenous CBF-1/RBP-Jk for binding to Notch IC or unknown cofactors. Dominant-negative suppression by R218H of a given promoter suggests that the trans-activation activity of Notch IC is mediated by direct or indirect interaction with CBF-1/RBP-Jk (15).

**Plasmid Preparation, Transient Transfection, Luciferase and β-Galactosidase Assays:**

Plasmids were prepared for transfection according to the manufacturers instructions using a Qiagen plasmid midi kit (Qiagen, Crawley, UK) as described previously [4]. Briefly cells were plated onto 6-well plates 2 days prior to transfection, at a density of 1 x 10⁵ cells/well and transfected at 70% confluency. Plasmid transfection was performed using Lipofectamine™ Reagent (Invitrogen). For constitutively active Notch IC over expression studies, cells transfected with vectors expressing Notch 1 and 3 IC (CMV-Notch 1 IC and CMX-Notch 3 IC, both containing HA tags), delta RAM deleted Notch IC (mNotch 1 IC), RPMS-1 (pcDNA3-RPMS-1/FLAG) and R218H (pEF-BOSneo-(RPB)-R218H) were co-transfected with a pGK3-puro and selected following treatment of cells with 0.8 mg/ml puromycin for 48 h [14]. At this concentration of puromycin, we have demonstrated that, after 48 h treatment, the majority of pGK3-puro remaining cells (~ 80%) survived as compared to 0% cell survival for mock controls. In order to maximise the transfection efficiency for SMC, puromycin resistant cells were pooled and
used in the subsequent expression studies. The transfection efficiencies for both Notch 1 and 3 IC were confirmed by immunocytochemistry using anti-Notch 1 and 3 IC antibodies and by anti-HA antibodies, respectively (data not shown).

**Western Blot Analysis:**

Proteins from cell lysates (12-15 µg) were resolved on SDS-PAGE (12% resolving, 5% stacking) prior to transfer onto nitrocellulose membrane (Amersham Biosciences). Membranes were stained in Ponceau S to ensure equal protein loading and rinsed in wash buffer (PBS containing 0.05% Tween-20). Membranes were blocked for 2 h in wash buffer containing 5% non-fat dried milk at room temperature with gentle agitation. Following three 15 min washes in wash buffer, membranes were incubated in primary antibody (1:1000 dilution in PBS containing 0.05% Tween 20 and 5% non-fat dried milk) at 4°C overnight with gentle agitation. Membranes were incubated, following three 15 min washes, in 1:1000 dilution of horseradish peroxidase conjugated anti-mouse or rabbit IgG (in PBS containing 0.05% Tween 20 and 5% non-fat dried milk) for 2 h. Following three final 15 min washes, the ECL™ detection reagent (Amersham Biosciences) was placed on the membranes for 5 min before they were exposed to Hyperfilm ECL. The signal intensity of the appropriate bands on the autoradiogram was calculated using the EDAS 120 system from Kodak (Kodak, Rochester NY, USA).

**Caspase-3 Assay:**

Activation of the caspase cascade was determined using a colorimetric assay based on the cleavage of acetyl 1-Asp-Glu-Val-Aps–p Nitroanaline (Ac-DEVD-pNA). Caspase-3 activity was measured by adding 10 µl caspase-3 substrate (2 mM Ac-DEVD-pNA containing 10% DMSO in 20 mM HEPES, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) to 5 µl cell lysate, 5 µl caspase-3 inhibitor (200 µM Ac-DEVD-CHO containing 10% DMSO in 20 mM HEPES, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) and diluted in assay buffer (20 mM HEPES, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) to a final volume of 100 µl. Samples were incubated for 90 min and the absorbance measured at 405 nm using a Tecan Spectra™ plate reader. Appropriate negative controls and blanks were included. A pNA standard curve (0-20 µM) coupled to protein quantification by the
BCA Assay (Pierce Chemical Co, Rockford IL, USA) allowed for the specific activity of caspase-3 (pmol pNA released/min/mg) to be calculated for each sample.

Acridine Orange/Ethidium Bromide and Annexin V-Fluos/Propidium Iodide Cell Staining:
Apoptotic cells were stained and visualized using the Annexin V-Fluos/Propidium Iodide (AV/PI) Stain (Roche Diagnostics Ltd, Sussex UK) and acridine orange/ethidium bromide stain (AO/EtBr) as described previously [4]. The cells were analysed using an Olympus DP-50 fluorescent microscope (excitation 450-500 nm, emission 515-565 nm). Acridine orange (AO) is a vital dye that stains both viable and dead cells. Ethidium bromide (EB) stains cells that have lost membrane permeability and therefore stains late apoptotic cells undergoing secondary necrosis. Acridine orange stained cells appear green under a narrow band fluorescein (FITC). Annexin-V was used as it serves as a fluorescent probe for apoptotic cells. It does not bind normal, intact cells. However, since necrotic cells are leaky enough to give Annexin-V access to inner membrane PS, apoptotic cells have to be differentiated from necrotic cells. This is achieved by examining the number of necrotic cells using Propidium iodide (PI). The number of apoptotic cells vs. necrotic cells was then corrected. Thus, the assay involves simultaneous staining with both Annexin-V- (green) and the DNA stain propidium iodide (red).

Quantitative Real-Time RT-PCR (QRTPCR):
For quantitative measurement of mRNA, we used RealTime PCR with GAPDH mRNA levels as an internal control. Total RNA from cell pellets was extracted using TRIzol reagent (Invitrogen) before 1 µg of total RNA was reverse transcribed in a reaction containing 1 x Molony murine leukemia virus reverse (MMLV) transcriptase buffer (Promega, Madison, WI, USA), 5 µM oligo (dT) 12–18 (Invitrogen), 1 mM dNTPs, 2 µg acetylated BSA and 200 U 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. Quantitative real-time RT-PCR was carried out using the Rotor Gene (RG-3000, Corvett Research, Australia) and the SYBR green PCR kit (Qiagen) as described by the manufacturer. The gene-specific
oligonucleotide sequences were:  **Notch 1:** forward -5’ GAGTCACCCCATGGCTAC 3’, reverse -5’ GTGGCTGCACCTGCTGGG 3’, **Notch 3:** forward -5’ GACCGTGTGGCCTCTTTC TATTGT 3’, reverse -5’ GACACTGATAACGGTGGGCT 3’,  **hrt-1:** forward -5’GAAGCGGCGACGAGACCGAATCAA 3’, reverse -5’ CAGGGCG TGCGCGTCAA AATAACC 3’, **hrt-2:** forward -5’ CATCAGAGTCAACGCCATGT 3’, reverse -5’ GACACTGATAACGGTGGGCT 3’,  **hrt-3:** forward -5’ GACCGTGTGGCCTCTTTC TATTGT 3’, reverse -5’ GCTGAGATA GGTTTTGTTGCCATTCTGG, **bax:** forward -5’ TTGCCCTCTTCTACTTTGCT 3’, reverse -5’ CAAAGATGGTCACTGTCTGC 3’,  **hes-1:** forward -5’ TCAACACGACACCGGACAAAC 3’, reverse -5’ GTGGCACA GGTTCTTT GAT 3’, **hes-5:** forward -5’ GCTCAGTCCCAAGGAGAAAA 3’, reverse -5’ GCAGCTGAAGC CATTGACTCTATCCT 3’, **Jagged 1:** forward -5’ AACAGAACACA GGGATTGCC 3’, reverse -5’ GTGGCACA GGTTCTTT GAT 3’, **bcl-xL:** forward -5’ AATGACCACCTAGAGCCTTG 3’, reverse -5’ AGTGAGCCCA GCAGA ACTAC 3’.  

The conditions for amplification were 5 minutes at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 minutes at 72°C. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The specificity of PCR products was confirmed by Southern blot hybridisation. Images were captured digitally using the Kodak 1D image analysis software.

**Cell Counts:**
Changes in SMC cell number following serum stimulation were determined by cell counting following transient over expression of Notch 1 and Notch 3 IC. Cells were serum starved for 48 h and then seeded at 1 x 10⁴ cells/well onto 6-well plates. At two-day intervals the average of 3 wells was quantified using a haemacytometer. At two-day intervals the cells were also fixed, stained with Harris hematoxylin solution (Sigma) and digitally photographed.
siRNA Transfection

For gene silencing studies, Lipofectamine™ 2000 Reagent (Invitrogen, The Netherlands) was used for transient transfection of SMC with gene-specific siRNA duplexes as previously described [16]. The siRNA duplexes for Hrt-2 corresponded to position 123-143 in the rat Hrt-2 sequence (Accession Number: XM.344806). All duplexes were acquired from MWG Biotech (Milton Keynes, UK).

Data Analysis:

Results are expressed as mean ± SEM. Experimental points were performed in triplicate, with a minimum of 3 independent experiments. An unpaired Student’s t test and a Wilcoxon signed rank test were used for comparison of two groups. A value of p<0.05 was considered significant.
References


