Jagged1-selective Notch Signaling Induces Smooth Muscle Differentiation via a RBP-Jk-dependent Pathway*

The Notch signaling pathway plays a crucial role in specifying cellular fates by interaction between cellular neighbors; however, the molecular mechanism underlying smooth muscle cell (SMC) differentiation by Notch signaling has not been well characterized. Here we demonstrate that Jagged1-Notch signaling promotes SMC differentiation from mesenchymal cells. Overexpression of the Notch intracellular domain, an activated form of Notch, up-regulates the expression of multiple SMC marker genes including SM-myosin heavy chain (SM-MHC) in mesenchymal 10T1/2 cells, but not in non-mesenchymal cells. Physiological Notch stimulation by its ligand Jagged1, but not Dll4, directly induces Sm-mhc expression in 10T1/2 cells without de novo protein synthesis, indicative of a ligand-selective effect. Jagged1-induced expression of SM-MHC was blocked by γ-secretase inhibitor, N-(3,5-difluorophenyl)-t-alanyl-L-phenylglycine t-butyl ester, which impedes Notch signaling. Using Rbp-jk-deficient cells and site-specific mutagenesis of the SM-MHC gene, we show that such an induction is independent of the myocardin-serum response factor-CArG complex, but absolutely dependent on RBP-Jk, a major mediator of Notch signaling, and its cognate binding sequence. Of importance, Notch signaling and myocardin synergistically activate SM-MHC gene expression. Taken together, these data suggest that the Jagged1-Notch pathway constitutes an instructive signal for SMC differentiation through an RBP-Jk-dependent mechanism and augments gene expression mediated by the myocardin-SRF-CArG complex. Given that Notch pathway components are expressed in vascular SMC during normal development and disease, Notch signaling is likely to play a pivotal role in such situations to modulate the vascular smooth muscle cell phenotype.

Vascular smooth muscle cells (VSMC) maintain considerable phenotypic plasticity throughout life and this plasticity is essential for vascular development and the pathogenesis of vascular disease such as atherosclerosis and restenosis following angioplasty (1). Differentiated VSMC exhibit a contractile phenotype that is characterized by a low rate of proliferation, low synthetic activity, and expression of a unique repertoire of proteins such as smooth muscle α-actin (SM α-actin), SM22α, h-caldesmon (h-Cad), smoothelin-B, and smooth muscle-myosin heavy chain (SM-MHC). Once VSMC are exposed to injurious stimuli, they dedifferentiate into a so-called synthetic phenotype that exhibits a high rate of proliferation, high synthetic activity, and down-regulated expression of SMC marker genes accompanied by the induction of some marker genes for immature SMC.

Among the many transcription factors involved in SMC differentiation, the myocardin-SRF complex is a key regulator as its expression is essential for SMC differentiation (2–5). Promoter/enhancer regions of most SMC marker genes such as SM-MHC and SM22α include several SRF binding elements, referred to as CArG or SRE. SRF recruits a potent coactivator, myocardin, resulting in the induction of SMC marker genes. Most importantly, mouse embryos deficient in myocardin show no evidence of vascular SMC development (6), suggesting an indispensable role of myocardin in SMC differentiation; however, several lines of evidence indicate that the myocardin-SRF complex does not govern the entire program of VSMC differentiation. First, forced expression of myocardin did not induce a subset of SMC marker genes such as smoothelin-B and aortic carboxypeptidase-like protein (7). Second, the Sm α-actin and Sm22α genes are expressed within the developing aorta as early as E9.5 (8), whereas myocardin is undetectable until E12.5 (3). To date, transcription factors that function prior to the expression of myocardin remains to be determined.

The evolutionarily conserved Notch signaling pathway controls various cell fates in metazoans through local cell-cell interactions (9). Interaction of Notch receptors with their ligands (Delta-like or Jagged) leads to proteolytic cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus. In the nucleus, NICD associates with transcription factor RBP-Jk (also known as CSL for CBF1/Su(H)/Lag-1) and activates transcription from its cognate DNA binding sequence, GTGGGAA.

Vascular smooth muscle cells

* This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan, a grant from the Japan Cardiovascular Foundation (to M. K.), and a Japan Heart Foundation Grant for Research on Atherosclerosis Update (to T. I.). The costs of publication of this article were defrayed in part by the payment of costs. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Gunma University Graduate School of Medicine 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. Tel.: 81-27-220-8140; Fax: 81-27-220-8150; E-mail: mkuraba@med.gunma-u.ac.jp.

2 The abbreviations used are: VSMC, vascular smooth muscle cell; SM, smooth muscle; MHC, myosin heavy chain; HES, hairy and enhancer of split; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

SEPTEMBER 29, 2006•VOLUME 281•NUMBER 39
© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Notch Signaling Induces Smooth Muscle Differentiation

In mammals, the NICD-RBP-Jκ complex up-regulates the expression of primary target genes of Notch, such as hairy and enhancer of split (HES) and HES-related repressor protein (HERP) families that act as transcriptional repressors as well as Notch effectors (10).

Notch activation has been shown to inhibit cell fate determination through its effector and maintain cells in an undifferentiated state (9). Recent studies have also demonstrated that Notch signaling promotes cell fate in various lineages during organogenesis, such as gliogenesis, hematopoiesis, adipogenesis, and osteogenesis, although there are some controversial reports (11–14). Importantly, Notch signaling often induces target genes other than HES/HERP without mediating putative Notch effectors, HES/HERP. For instance, Notch signaling directly induces brain lipid-binding protein and interleukin-4, markers for glia and the Th2 lineage of T cells, respectively, in an RBP-Jκ-dependent manner (11, 15). Thus, the regulation of cell-fate specification by Notch is highly pleiotropic and indeed context dependent.

Recent studies have documented that Notch signaling is involved in multiple aspects of vascular development including differentiation and homeostasis of VSMC (16). In vertebrates, several receptors, ligands, and other components of Notch signaling are expressed in VSMC. The human disease CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is caused by highly stereotyped mutations of the receptor Notch3 (17). They cause a defect in vessel homeostasis due to VSMC degeneration, resulting in the early onset of ischemic stroke (17). Notch3-deficient mice also revealed that Notch3 is required for arterial differentiation and maturation of VSMC (18). Recently, we and others have shown that HERP1, a Notch effectors, HES/HERP. For instance, Notch signaling directly induces brain lipid-binding protein and interleukin-4, markers for glia and the Th2 lineage of T cells, respectively, in an RBP-Jκ-dependent manner (11, 15). Thus, the regulation of cell-fate specification by Notch is highly pleiotropic and indeed context dependent.

Recent studies have documented that Notch signaling is involved in multiple aspects of vascular development including differentiation and homeostasis of VSMC (16). In vertebrates, several receptors, ligands, and other components of Notch signaling are expressed in VSMC. The human disease CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is caused by highly stereotyped mutations of the receptor Notch3 (17). They cause a defect in vessel homeostasis due to VSMC degeneration, resulting in the early onset of ischemic stroke (17). Notch3-deficient mice also revealed that Notch3 is required for arterial differentiation and maturation of VSMC (18). Recently, we and others have shown that HERP1, a target gene of Notch, inhibits myocardin-mediated SMC differentiation (19, 20). Morrow et al. (21) also reported that Notch signaling inhibits SMC differentiation through an RBP-Jκ/Hrt-dependent mechanism. On the other hand, several reports have suggested that Notch positively regulates VSMC differentiation. In Notch3-deficient mice, the expression of several VSMC marker genes such as Sm22α and smoothelin was markedly down-regulated (18). In addition, SM α-actin was strongly induced by NICD in human umbilical vein endothelial cells during the process of epithelial-mesenchymal transition (22). Very recently, Noseda et al. (23) reported that SM α-actin was a direct target of Notch signaling via RBP-Jκ-dependent pathways. Taken together, these findings suggest that the function of Notch signaling in VSMC differentiation remains to be determined.

Here we report that Jagged1-selective activation of Notch signaling positively and directly regulates SMC differentiation. Our data also implicate functional interaction between NICD-RBP-Jκ and myocardin-SRF complexes on SMC differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—Culture conditions for C3H10T1/2 cells (murine embryonic fibroblasts), L cells stably expressing GFP alone, Jagged1 and DI4 (L-GFP, L-Jagged1, and L-DI4, respectively), Rbp-jk−/− mouse embryonic fibroblast (OT11), and the wild-type cell line (OT13) were previously described (24, 25). Rat aorta smooth muscle cells were cultured in M199 medium supplemented with 10% fetal bovine serum. HASMC (human aortic smooth muscle cells, KS4001, Kurabo Industries, Ltd., Osaka, Japan) were cultured in Humedia-SG2 (KS-2150S, Kurabo Industries, Ltd.). The γ-secretase inhibitor IX, DAPT (N-(N-(3,5-difluorophenyl)-1-allyl)-S-phenylglycine t-butyler ester), was purchased from Calbiochem. Cyclohexamide was purchased from Sigma. The co-culture experiment was performed as described elsewhere (24, 25).

Expression Plasmids—Expression plasmids for N1-ICD (Notch1 intracellular domain), N3-ICD (Notch3 intracellular domain), myocardin, and RBP-Jκ were kindly provided by Drs. Weinmaster (26), Lendahl (27), Izumo (28), and Honjo (29), respectively. N1-ICD and myocardin with the FLAG tag at the amino terminus was subcloned into pCDNA3.1(−) (Invitrogen) by PCR (FLAG-N1-ICD and FLAG-myocardin, respectively). The myocardin carboxyl-terminal truncation mutant (myoCA585) was constructed as described previously (4). RBP-Jκ with the hemagglutinin tag at the amino terminus was subcloned into pCDNA3.1(−) by PCR (HA-RBP-Jκ).

siRNA—The target sequences of the HERP1 siRNA duplex and control (non-silencing) siRNA (Dharmacon Research) were 5′-GCAGUGAUGACAUCUCCA-3′ and 5′-UAUA CAUUGCGCGCGUGUUU-3′, respectively. Two hundred nm control siRNA or HERP1 siRNA concentrations were used.

Luciferase Reporter Gene Constructs, Transfection, and Luciferase Assay—The following promoter regions were cloned into the pGL3 basic vector (Promega). The MHC-Luc construct (nucleotides −1226 to +47 of the mouse Sm-mhc gene) was described previously (19). MHC-860Luc was prepared by PCR with the following primers: 5′-cagcgctgctgctgccacctcgaggggattgg-3′ and 5′-cagcgctgctgctgccacctcgaggggattgg-3′. To generate the mutated reporter construct (MHC-RmLuc), the RBP-Jκ binding site (ctttcccccaac, nucleotides −624 to −618 in the Sm-mhc promoters was changed by PCR to ctgtatatccac. The RBP-Jκ binding sequence is underlined and mutated residues are written in bold. 10T1/2 cells were transfected with plasmid DNA by the modified calcium phosphate precipitation method as described (19). Forty-eight hours later, luciferase assays were performed according to the manufacturer’s protocol (Promega). These assays were done in triplicate and repeated several times.

Generation of Recombinant Adenovirus—Empty adenovirus (Ad-empty) and adenoviruses expressing N1-ICD and RBP-Jκ (Ad-N1-ICD and Ad-RBP-Jκ, respectively) have been created as described elsewhere (24, 25). Adenoviruses expressing N3-ICD and myocardin (Ad-N3-ICD and Ad-myocardin, respectively) have been constructed as described previously (23). Protein expression was confirmed by Western blot analysis (data not shown).

RNA Isolation, Northern Blot Analysis, and Reverse Transcriptase (RT)-PCR—Total RNA was isolated from various cells using the IsoGen reagent (Nippon Gene, Tokyo). Northern blot analysis was carried out as described (19). Probes for mouse Sm22α, mouse Herp2, rat Herp1, and rat Herp2 were described elsewhere (24, 30, 31). The probe for mouse Sm-mhc corresponded to nucleotides 5726–6088 of mouse Sm-mhc. Semi-quantitative RT-PCR was performed with an RT-PCR kit (TAKARA, Japan) according to the manufacturer’s protocol.
**Notch Signaling Induces Smooth Muscle Differentiation**

Gene-specific primers for cDNA were as follows: mouse h-CaD, 5'-aaagctgacacagtgaagatgagatgagagtaaaaagatgag-3' and 5'-agatcagagatgagagagatgagagtaaaaagatgag-3'; mouse smoothelin-B, 5'-cagagtccttgagagaggtcagctg3'-5'gagaattgtggtcagctgatt-3'; mouse Herp2, 5'-catgaagaagagctgacttc-3' and 5'-aatgtgtccgaggccac-3'; rat Rbp-j, 5'-gcctgctggttacctgctg3'-5'aggcaacatgagctg-3'; human SM-MHC, 5'-ggagatgagatgagacatg-3' and 5'-aagctgccacctgctg-3'; human HERPI, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'; and human SM22α, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'. The gene-specific primers for mouse Sm-mhc and Herp1 were the same sequences as semi-quantitative RT-PCR, respectively. The gene-specific primers for GAPDH were 5'-aacacccctctgctg-3' and 5'-tccagcatactcag-3'. All experiments were repeated at least three times, and results from a representative experiment are shown with S.D. values.

**Western Blot Analysis**—Western blot analyses were carried out as previously described (19). Antibodies against FLAG tag (M2, Sigma), α-tubulin (Sigma), SM1 (a kind gift from KYOWA HAKKO KOGYO Co., Ltd, Tokyo), and h-CaD (a kind gift from Dr. M Walsh, University of Calgary) were used.

**Electrophoretic Mobility Shift Assay**—In vitro translated proteins of RBP-Jκ were prepared with a TnT T7-coupled reticulocyte lysate system (Promega). The nucleotide sequence of the SM-MHC probe containing the RBP-Jκ binding site (underlined) was 5'-ctcttctttctcaccacc-3'. The mutated sequence (in bold) for competition assay was 5'-ctcttctttctcaccacc-3'. Electrophoretic mobility shift assay was performed as previously described (19). For the supershift assay, anti-RBP-Jκ antibody (H-50, Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology) was used.

**Chromatin Immunoprecipitation Assay**—The chromatin immunoprecipitation assay was performed as described (19). Cross-linked chromatin was immunoprecipitated with 10 μl of anti-SRF antibody (G20, Santa Cruz Biotechnology), normal rabbit IgG (Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology).

**RESULTS**

**Overexpression of the Constitutively Activated Form of Notch (NICD) Increases the Expression of Smooth Muscle Marker Genes**

Gene-specific primers for cDNA were as follows: mouse h-CaD, 5'-aaagctgacacagtgaagatgagatgagagtaaaaagatgag-3' and 5'-agatcagagatgagagagatgagagtaaaaagatgag-3'; mouse smoothelin-B, 5'-cagagtccttgagagaggtcagctg3'-5'gagaattgtggtcagctgatt-3'; mouse Herp2, 5'-catgaagaagagctgacttc-3' and 5'-aatgtgtccgaggccac-3'; rat Rbp-j, 5'-gcctgctggttacctgctg3'-5'aggcaacatgagctg-3'; human SM-MHC, 5'-ggagatgagatgagacatg-3' and 5'-aagctgccacctgctg-3'; human HERPI, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'; and human SM22α, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'. The gene-specific primers for mouse Sm-mhc and Herp1 were the same sequences as semi-quantitative RT-PCR, respectively. The gene-specific primers for GAPDH were 5'-aacacccctctgctg-3' and 5'-tccagcatactcag-3'. All experiments were repeated at least three times, and results from a representative experiment are shown with S.D. values.

**Western Blot Analysis**—Western blot analyses were carried out as previously described (19). Antibodies against FLAG tag (M2, Sigma), α-tubulin (Sigma), SM1 (a kind gift from KYOWA HAKKO KOGYO Co., Ltd, Tokyo), and h-CaD (a kind gift from Dr. M Walsh, University of Calgary) were used.

**Electrophoretic Mobility Shift Assay**—In vitro translated proteins of RBP-Jκ were prepared with a TnT T7-coupled reticulocyte lysate system (Promega). The nucleotide sequence of the SM-MHC probe containing the RBP-Jκ binding site (underlined) was 5'-ctcttctttctcaccacc-3'. The mutated sequence (in bold) for competition assay was 5'-ctcttctttctcaccacc-3'. Electrophoretic mobility shift assay was performed as previously described (19). For the supershift assay, anti-RBP-Jκ antibody (H-50, Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology) was used.

**Chromatin Immunoprecipitation Assay**—The chromatin immunoprecipitation assay was performed as described (19). Cross-linked chromatin was immunoprecipitated with 10 μl of anti-SRF antibody (G20, Santa Cruz Biotechnology), normal rabbit IgG (Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology).

Gene-specific primers for cDNA were as follows: mouse h-CaD, 5'-aaagctgacacagtgaagatgagatgagagtaaaaagatgag-3' and 5'-agatcagagatgagagagatgagagtaaaaagatgag-3'; mouse smoothelin-B, 5'-cagagtccttgagagaggtcagctg3'-5'gagaattgtggtcagctgatt-3'; mouse Herp2, 5'-catgaagaagagctgacttc-3' and 5'-aatgtgtccgaggccac-3'; rat Rbp-j, 5'-gcctgctggttacctgctg3'-5'aggcaacatgagctg-3'; human SM-MHC, 5'-ggagatgagatgagacatg-3' and 5'-aagctgccacctgctg-3'; human HERPI, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'; and human SM22α, 5'-gcaacatgagacatg-3' and 5'-tcctctgtgctc-3'. The gene-specific primers for mouse Sm-mhc and Herp1 were the same sequences as semi-quantitative RT-PCR, respectively. The gene-specific primers for GAPDH were 5'-aacacccctctgctg-3' and 5'-tccagcatactcag-3'. All experiments were repeated at least three times, and results from a representative experiment are shown with S.D. values.

**Western Blot Analysis**—Western blot analyses were carried out as previously described (19). Antibodies against FLAG tag (M2, Sigma), α-tubulin (Sigma), SM1 (a kind gift from KYOWA HAKKO KOGYO Co., Ltd, Tokyo), and h-CaD (a kind gift from Dr. M Walsh, University of Calgary) were used.

**Electrophoretic Mobility Shift Assay**—In vitro translated proteins of RBP-Jκ were prepared with a TnT T7-coupled reticulocyte lysate system (Promega). The nucleotide sequence of the SM-MHC probe containing the RBP-Jκ binding site (underlined) was 5'-ctcttctttctcaccacc-3'. The mutated sequence (in bold) for competition assay was 5'-ctcttctttctcaccacc-3'. Electrophoretic mobility shift assay was performed as previously described (19). For the supershift assay, anti-RBP-Jκ antibody (H-50, Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology) was used.

**Chromatin Immunoprecipitation Assay**—The chromatin immunoprecipitation assay was performed as described (19). Cross-linked chromatin was immunoprecipitated with 10 μl of anti-SRF antibody (G20, Santa Cruz Biotechnology), normal rabbit IgG (Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or normal mouse IgG (Santa Cruz Biotechnology).
Notch Signaling Induces Smooth Muscle Differentiation

A

**NMuMg**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

**10T1/2**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

B

**HUVEC**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

**HASMC**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

C

**NHEK**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

**HASMC**

| No infection | + | Ad-empty | + | Ad-N1-ICD | + | SM-MHC | + + + |
|-------------|---|---------|---|-----------|---|--------|-------|
| Herb1       |   |         |   |           |   | GAPDH  |       |

**FIGURE 2. Effect of NICD on the induction of SM-MHC mRNA in various cells.** Normal murine mammary gland epithelial (NMuMg) (A), human umbilical vein endothelial cells (HUVEC) (B), or normal human epidermal keratinocytes (NHEK) (C) were infected with Ad-empty or Ad-N1-ICD at a multiplicity of infection of 20. Two days after infection, total RNA was isolated and gene expression was assayed by RT-PCR. GAPDH was used as an internal control. RNAs from 10T1/2 cells infected with Ad-N1-ICD or HASMC (human aorta smooth muscle cells) were used as a positive control for SM-MHC gene expression.

**in Human Aortic Smooth Muscle Cells and 10T1/2 Cells—**

As our previous study (19) revealed that HERP1 inhibits SMC differentiation by disrupting myocardin-SRF complex formation, we reasoned that Notch signaling represses SMC differentiation. To test this hypothesis, we first studied the effect of N1-ICD on SMC marker gene expression in human aortic SMC, HASMC (Fig. 1A). Contrary to our expectations, forced expression of N1-ICD increased the steady state level of mRNA for SM-MHC and SM22α despite the strong induction of HERP1 expression. These observations offer the intriguing idea that Notch signaling rather promotes SMC differentiation. Thus, we next chose a cell line, C3H10T1/2, a mesenchymal precursor cell line of murine embryonic origin, in which Notch signaling should recapitulate, at least partially, its effects in embryogenesis because many studies have revealed its pluripotency, that is, its ability to differentiate toward different mesenchymal lineages including SMC, skeletal muscle, adipocytes, chondrocytes, and osteoblasts (32–35). N1-ICD introduced by adenovirus increased the mRNA level of SM22α in a dose-dependent manner (Fig. 1B). The mRNA expression for a series of SMC marker genes such as Sm-mhc, h-Cad, and smoothelin-B were also induced by N1-ICD overexpression despite the induction of Herp1 (Fig. 1C). Importantly, the expression level of SM-MHC, the most reliable marker for mature SMC (1), was up-regulated by N1-ICD, strongly suggesting that NICD induces SMC differentiation, not merely several SMC marker genes. We further confirmed that N1-ICD induced the expression of SM-MHC (SM1) and h-CaD at the protein level as myocardin did (Fig. 1D). In contrast, we observed no induction of SM-MHC by N1-ICD in other non-mesenchymal cell lines tested (Fig. 2, A–C), which suggests that SMC differentiation by Notch signaling is cell-type specific.

We compared the effect of the N1-ICD and N3-ICD because both Notch receptors are highly expressed in vascular SMC (18, 36, 37). Fig. 1E showed that N3-ICD induced mRNA expression for a series of SMC markers including Sm-mhc, Sm22α, h-Cad, and Smoothelin-B as N1-ICD did. We confirmed that the protein expression level of those Notch proteins was comparable as assessed by Western blot (data not shown). Altogether, our data strongly suggest that activated Notch signaling, represented by NICD, is a potent inducer, not a repressor, of SMC differentiation.

**Jagged1-selective Notch Stimulation Induces SMC Differentiation without de Novo Protein Synthesis—**

As the expression of natural NICD is maintained at an extremely low level under physiological conditions (38), forced expression of NICD might elicit physiologically irrelevant results. On the other hand, the differential effect of Notch ligands in the vascular system has never been addressed although several Notch ligands such as Jagged1 and Dll4 are expressed (16). To avoid the concerns and to study the ligand-selective effect, we performed co-culture experiments using L cells stably expressing either Jagged1 or Dll4. We confirmed that both ligands are biologically active because they equally induced the expression of HERP2, a known target gene of Notch, in C2C12 myoblasts (25). Of particular interest, Jagged1 stimulation, but not Dll4, obviously induced Sm-mhc expression in 10T1/2 cells (Fig. 3A), suggesting that Jagged1-selective activation of the Notch pathway brings about SMC differentiation in 10T1/2 cells. We next examined whether Jagged1-induced Sm-mhc expression is dependent on activation of the Notch pathway, or proteolytic cleavage of signal-transducing NICD by γ-secretase. As shown in Fig. 3B, the γ-secretase inhibitor DAPT (39) abolished Jagged1-induced Sm-mhc expression. To further study whether Sm-mhc is a direct target gene of Notch, we carried out a co-culture experiment in the presence of cycloheximide, a protein synthesis inhibitor. Even in the presence of cycloheximide, Sm-mhc mRNA was induced by co-culture with L-Jag1 (Fig. 3C), which suggests that SMC marker genes are directly up-regulated by Notch without producing second activating proteins. We next asked whether the basal expression of endogenous SMC marker genes in rat aorta smooth muscle cells also depends on Notch signaling. In the presence of DAPT, sm-mhc expression as well as herp1, was obviously down-regulated.
Notch Signaling Induces Smooth Muscle Differentiation

although the expression of other genes such as rbp-jk and gapdh was not affected (Fig. 3D), suggesting that γ-secretase-dependent proteolytic cleavage of Notch receptor(s) is, at least in part, required for endogenous Sm-mhc expression in rat aorta smooth muscle cells. Collectively, we established that Jagged1-selective, but not Dll4-selective, stimulation of Notch receptors induces SMC marker gene expression in 10T1/2 cells without de novo protein synthesis and that endogenous SMC marker genes in vascular SMC are partly transactivated by Notch signaling.

RBP-Jk and Its Binding Sequence Are Essential for the Induction of SMC Marker Genes by NICD—In the nucleus, NICD binds to transcription factor RBP-Jk, forming a complex that activates the expression of target genes from the RBP-Jk binding site (9). To address the role of RBP-Jk in Notch-mediated vitro translated RBP-Jk protein bound to the RBP-Jk binding site within the promoter (Fig. 5B). We further performed chromatin immunoprecipitation assays to study whether NICD associates with the RBP-Jk binding sequence via RBP-Jk protein in vivo. Fig. 5C showed that NICD overexpression in 10T1/2 markedly induced in NICD binding to the RBP-Jk binding site of the SM-MHC promoter, presumably via the RBP-Jk protein, within intact chromatin. These findings provide compelling evidence that RBP-Jk and its binding sequence are indispensable for NICD-mediated induction of the Sm-mhc gene.

Myocardin-SRF-CArG Complex Is Dispensable for NICD-induced Expression of the SM-MHC Gene—We examined whether SM-MHC induction by Jagged1 is mediated by the induction of myocardin and SRF. Co-culture of 10T1/2 cells with L-Jag1 cells showed no measurable induction of mRNA expression for Sm-mhc mRNA expression in OT11 (Fig. 4B). In contrast, when both NICD and RBP-Jk were co-expressed, Sm-mhc mRNA expression was remarkably induced (lanes 3 and 4), indicating that RBP-Jk is necessary and sufficient for the expression of Sm-mhc induced by NICD. Indeed, we found putative RBP-Jk binding elements within promoter regions of various SMC marker genes including SM-MHC (Table 1). To test the requirement of the RBP-Jk binding sequence, we next performed reporter gene assays using the mouse Sm-mhc promoter (MHC-Luc) and its derivative carrying a mutation of the RBP-Jk binding site, Jagged1-selective activation of Notch signaling directly induces the expression of Sm-mhc mRNA without de novo protein synthesis. A, 10T1/2 cells were co-cultured with L-GFP, L-Jag1, or L-Dll4. Two days after co-culture, total RNA was isolated for RT-PCR. Gapdh was used as an internal control. B, 10T1/2 cells were co-cultured with either L-GFP or L-Jag1 with or without daily treatment of 1 μM DAPT. Two days after co-culture, total RNA was isolated for RT-PCR. C, 10T1/2 cells were co-cultured with L-GFP or L-Jag1 in the presence of 10 μM cyclohexamide (CHX). Cells were harvested at the indicated time points and total RNA was extracted for RT-PCR. D, rat aorta smooth muscle cells were treated daily with 1 μM CHX, rat aorta smooth muscle cells were treated daily with 1 μM DAPT. Two days after co-culture, total RNA was isolated for RT-PCR. E, 10T1/2 cells were co-cultured with L-GFP or L-Jag1 in the presence of 10 μM cyclohexamide (CHX). Cells were harvested at the indicated time points and total RNA was extracted for RT-PCR. F, 10T1/2 cells were co-cultured with L-GFP or L-Jag1 in the presence of 10 μM cyclohexamide (CHX). Cells were harvested at the indicated time points and total RNA was extracted for RT-PCR.
Notch Signaling Induces Smooth Muscle Differentiation

TABLE 1
Putative RBP-Jκ consensus binding sites in the promoter regions of various SMC marker genes

| Promoter Accession no. Position | Consensus, TTTCCCAAC Similarity |
|--------------------------------|---------------------------------|
| Mouse SM-MHC U53469 -624       | CTTTTCCCAACACA 7/7              |
| Rat SM-MHC U55179 -567         | AGTTTCCCAACAGC 6/7             |
| Human SM22 AF013710 -1709      | CTTTTCCCAAGGCC 7/7             |
| Mouse SM22a U36589 -369        | TATTTCCCAACAAAA 7/7            |
| Chicken caldesmon D17553 -629  | CTTTTCCCAACTGC 7/7             |
| Mouse smoothelin AF327749 -1350| GCCCTTTCCCAACTCA 7/7           |

| Promoter Accession no. Position | Consensus, GTGGGAAA Similarity |
|--------------------------------|--------------------------------|
| Rabbit SM-MHC U15514 -928      | GACGTTGGGAAAAACGC 7/7          |
| Rat SM22a Z48607 -98           | GGCAGTTGGGAAAAACGC 7/7        |
| Human SM α-actin J05193 -50    | CAAAAGTTGGGAAAAACGC 6/7       |
| Rat SM α-actin S76001 -53      | CAGACTGGGAAAAACGC 6/7         |
| Human calpain D85611 -237      | ACAGCTGGGAAAAACGC 6/7         |

A MHC-Luc

FIGURE 5. RBP-Jκ binding element is required for Notch signal-mediated induction of the Sm-mhc gene.

A, 10T1/2 cells were transfected with the indicated reporter genes in the presence or absence of either myocardin or N1-ICD expression vector. Three days later, cells were lysed for luciferase assay. Data are shown as the fold induction of luciferase activity relative to basal activity of each promoter with no expression vector. MHC-Luc, promoter of the wild-type Sm-mhc gene in pGL3 vector; MHC-RmLuc, MHC-Luc with mutation of the RBP-Jκ binding site. *p < 0.05 relative to MHC-Luc. Values represent the mean ± S.D. B, EMSA was performed with incubated RBP-Jκ and a radiolabeled probe containing the RBP-Jκ binding element of the mouse Sm-mhc promoter. C, 10T1/2 cells were transfected with the indicated expression vectors. Three days later, cells were cross-linked with formaldehyde, and sonicated chromatin was immunoprecipitated with anti-FLAG antibody or normal IgG. Recovered DNA was subjected to PCR using primers encompassing the RBP-Jκ binding element within the mouse Sm-mhc promoter.

both Myocardin and Srf (data not shown). To further study whether myocardin function is required for the Notch-mediated induction of SMC marker gene expression, we utilized a dominant-negative form of myocardin, myoCΔ585 (4). As reported, myoCΔ585 abrogated the induction of Sm-mhc gene expression by wild-type myocardin (Fig. 6A, lanes 3 and 4); however, overexpression of myoCΔ585 did not alter the Sm-mhc gene expression induced by NICD (lanes 5 and 6). We also performed luciferase reporter gene assays using Sm-mhc promoter, which showed essentially the same results (Fig. 6B). We next performed reporter gene assays to study the requirement of CArG elements in the NICD-mediated induction of SMC marker genes. Although myocardin-activated transcription of the MHC-Luc construct was markedly reduced in the MHC-860Luc construct lacking CArG boxes, the transactivation of two reporter constructs by NICD was barely affected (Fig. 6C). Moreover, the chromatin immunoprecipitation assay revealed that Notch signaling did not alter intensity of interaction between SRF and CArG box (Fig. 6D). These findings suggest that myocardin/SRF and CArG elements are not required for NICD-mediated induction of the Sm-mhc gene.

Notch and Myocardin Synergistically Induce SMC Differentiation—We next addressed the possibility that Notch signaling synergistically induces SMC gene expression in collaboration with the SRF-myocardin complex. Of interest, both semi-quantitative and real-time RT-PCR analyses showed that co-expression of NICD and myocardin synergistically induced the expression of Sm-mhc (Fig. 7A). Reporter gene assay also revealed synergistic transactivation of the Sm-mhc promoter by simultaneous overexpression of NICD and myocardin (Fig. 7B). We further studied the requirement of DNA binding sequences for SRF and RBP-Jκ. Fig. 7C showed no synergistic transactivation by co-expression of NICD and myocardin when using the MHC-RmLuc construct (with mutation of the RBP-Jκ binding sequence)
Notch Signaling Induces Smooth Muscle Differentiation

DISCUSSION

Notch Signaling Is a Potent Inducer of SMC Differentiation—We demonstrated, for the first time, that Notch signaling instructively regulates SMC differentiation in mesenchymal cell lines. Constitutively active NICD induced multiple SMC marker genes including SM-MHC, the most definite differentiation marker. This finding is remarkable because, to date, there are no reports that a single transcription factor (complex) induces multiple SMC marker genes at both mRNA and protein levels except the myocardin-SRF complex (41). We also demonstrated the requirement of RBP-Jk and its cognate binding sequence in the induction of the Sm-mhc gene by various methods such as loss- and gain-of-function studies using Rbp-jk deficient cells and site-directed mutagenesis of the reporter gene assay. We found RBP-Jk binding sequences in promoter regions of many other SMC marker genes in various species. In fact, we observed the induction of SMC marker genes by NICD overexpression in cell lines from several species including humans and mice (i.e. HASMC, 10T1/2, and OT11/13 cells). These findings of the presence of RBP-Jk binding sites and induction of SMC marker genes in multiple species strongly suggest that Notch signaling is an evolutionarily conserved and therefore crucial mechanism controlling SMC differentiation. Of importance, we showed that Notch-mediated SMC differentiation is independent of myocardin-SRF complex, the central regulator of SMC differentiation. Furthermore, we found that Jagged1 selectively and directly induces SMC differentiation via the activation of Notch signaling. Of importance, we showed that Jagged1-triggered Notch signaling is a potent inducer of SMC differentiation in

and MHC-860Luc construct (lacking two CArG boxes), suggesting that both DNA binding sequences for SRF and RBP-Jk are required for synergistic transactivation. We failed to detect protein-protein interaction between NICD and SRF, myocardin and RBP-Jk, and NICD and myocardin by co-immunoprecipitation (data not shown). Collectively, these findings suggest that both complexes individually bind to their own cognate binding sequences without direct protein-protein interaction, leading to synergistic transactivation.

Notch Signaling Promotes SMC Differentiation Independent of HERP1—Our previous study demonstrated that HERP1 represses myocardin-induced SMC gene expression (19). Because HERP1 is the direct target of Notch signaling, we determined the role of HERP1 in Notch-induced SMC gene expression. We performed loss-of-function study by employing siRNA (Fig. 8). Quantitative RT-PCR analysis showed that HERP1-specific siRNA significantly reduced the expression level of Herp1 mRNA with or without overexpression of N1-ICD. A decreased level of Herp1 mRNA did not result in a significant change of the gene expression for Sm-mhc (Fig. 8, lane 4). Thus, it is most likely that Notch signaling promotes SMC differentiation independent of HERP1 and that Notch-induced HERP1 barely affects SMC differentiation.
mesenchymal cell lines through an RBP-Jk-dependent, but myocardin/SRF-independent mechanism.

Notch Signaling Complements and Augments the Function of Myocardin-SRF Complex during SMC Differentiation—Although the discovery of myocardin had a significant impact on the clarification of molecular mechanisms underlying SMC differentiation (2–5), several studies have revealed that myocardin does not govern the entire program of SMC differentiation. For instance, myocardin is not capable of inducing all SMC-related genes such as smoothelin-B and aortic carboxypeptidase-like protein (7). Of note, we found that NICD strongly induced smoothelin-B expression. A previous study also showed that the expression of smoothelin was markedly down-regulated in Notch3-deficient mice (18). These findings suggest that Notch signaling up-regulates some myocardin-independent genes, resulting in more differentiated SMC in collaboration with myocardin. Alternatively, Notch may play an important role in vascular SMC differentiation in early developing aortas. Myocardin expression is detectable within the aorta as early as E12.5 (3), 3 days after SMC marker genes such as SM α-actin and SM22α are detected (8). Of importance, Notch components are expressed earlier than myocardin (42–44), suggesting that Notch may play a major role in early vascular SMC differentiation before myocardin is expressed.

Functional cooperation between Notch and myocardin is also noteworthy. Our quantitative RT-PCR and reporter gene assays showed that NICD synergistically enhanced myocardin-induced transactivation of Sm-mhc. As described above, Notch components are expressed earlier than myocardin in developing aortas. These findings suggest that the early expression of Notch might make ready the subsequent amplification by myocardin to efficiently establish more differentiated SMC in developing aortas.

We currently have no definitive explanation for the mechanism of synergistic activation by NICD-RBP-Jk and myocardin-SRF complexes. Although we found that cis elements for both NICD-RBP-Jk and myocardin-SRF proteins are required for the cooperative effect, we failed to show that the NICD-RBP-Jk complex physically interacted with the myocardin-SRF complex at the protein level (data not shown). Given that multiple transcription factors plus promoter/enhancer regions of DNA often constitute higher order three-dimensional complexes, termed enhanceosomes (45), we assume that a common transcriptional coactivator for NICD and myocardin such as p300 (46, 47) may facilitate higher complex formation, which augments transcription.

How Does a Specific Notch Ligand Regulate SMC Differentiation?—In this study, we showed that Jagged1 but not Dll4 induces SMC marker gene expression, highlighting the differential effects of two Notch ligands on SMC differentiation. Likewise, we observed that only Dll4 stimulation, but not Jagged1, induced an arterial marker ephrinB2 expression in endothelial cells, suggestive of the Dll4-selective effect (25). Several previous reports have demonstrated ligand-type specific effects, rather than a common end point of Notch signaling by different ligands. Amsen et al. (11) showed that Dll1 promotes Th1 responses, and Jagged1 instructs naive CD4 T cells to differen-
tiate into an alternate Th2 lineage. Rutz et al. (48) also reported that Dll1 and Jagged1 induce the dose-dependent inhibition of early activation of T cells, whereas triggering of Notch by Dll4 enhances T cell activation and proliferation; however, the precise molecular mechanism underlying ligand-selective effects remains elusive. One possibility is that different ligands preferentially activate different Notch receptors that may have distinct functions. The available data, however, show no special relationship between Notch ligands (Dll1, Jagged1, and Jagged2) and receptors (Notch1–3) (49). An alternative possibility is the glycosylation of Notch receptors by Fringe proteins. It is known that Fringe glycosylates O-fucosylated residues within epidermal growth factor-like motifs present in Notch receptors (50, 51) and that the glycosylation potentiates Notch signaling induced by Delta while inhibiting signaling by Serrate/Jagged (52, 53). This mechanism accounts for Delta-selective effects, but not Jagged-selective effects. Although there are three Fringe proteins in higher vertebrates, none potentiates Jagged1-selective effects (54, 55), suggesting that Fringe does not provide a simple explanation for the phenomena observed. Clarifying the mechanism underlying ligand-selective effects is a critical issue to be addressed to understand the diverse biological functions of Notch signaling, including vascular biology.

Notch Signaling Regulates SMC Differentiation in a Context-dependent Manner—In this study, we showed that Notch signaling directly induces SMC differentiation independent of HERP1 and that Notch-induced HERP1 barely affects SMC differentiation. Very recently, Noseda et al. (23) reported that SM α-actin was a direct target of Notch signaling via RBP-Jκ-dependent pathways, which is consistent with the results of this study that Notch signaling directly promotes SMC differentiation. However, Proweller et al. (20) and Morrow et al. (21) have reported that HERP1-mediated Notch signaling inhibits SMC differentiation and we have also reported previously that forced expression of HERP1 suppressed myocardin-induced SMC differentiation (19). These apparent discrepancies can be reconciled by our proposed model in which NICD released from the plasma membrane by ligand binding directly activates the transcription of SMC marker genes, but simultaneous induction of HERP is insufficient to inhibit the NICD- and myocardin-mediated expression of SM marker genes. However, when other exogenous stimuli as well as specifically expressed endogenous proteins in certain cells cooperatively enhance NICD-induced HERP expression within the cells, the abundant expression of HERP may counteract the function of NICD and myocardin. Indeed, HERP expression has been reported to be up-regulated by several factors such as the transforming growth factor-β superfamily (56, 57), c-jun (58), and heparin-binding epidermal growth factor.3 Those factors or others influencing HERP induction may modulate Notch-mediated SMC differentiation. Opposing effects of Notch signaling have been often observed in a context-dependent manner during differentiation even in other systems such as adipogenesis (12, 59) and osteogenesis (14, 60, 61), although their precise molecular mechanisms have not been clarified. Future studies will be necessary to elucidate the molecular mechanisms underlying context-dependent effects of Notch in various situations including SMC differentiation.

Acknowledgments—We are grateful to Drs. G. Weinmaster, K. Stark, T. Ueyama, S. Izumo, T. Honjo, U. Lendahl, M. Walsh, Y. Sagabe, and O. Ishikawa for critical reagents. We thank Yoshiko Nonaka for excellent technical assistance.

REFERENCES

1. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Physiol. Rev. 84, 767–801
2. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) J. Mol. Cell Cardiol. 34, 1345–1356
3. Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K., and Parmacek, M. S. (2003) Mol. Cell. Biol. 23, 2425–2437
4. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Cell 105, 851–862
5. Yoshida, T., Sinha, S., Dandre, F., Wamhoff, B. R., Hoofnagle, M. H., Kremer, B. E., Wang, D. Z., Olson, E. N., and Owens, G. K. (2003) Circ. Res. 92, 856–864
6. Li, S., Wang, D. Z., Wang, Z., Richardson, J. A., and Olson, E. N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9366–9370
7. Yoshida, T., Kawai-Kowase, K., and Owens, G. K. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 1596–1601
8. Li, L., Miano, J. M., Cserjesi, P., and Olson, E. N. (1996) Circ. Res. 78, 188–195
9. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Science 284, 770–776
10. Iso, T., Kedes, L., and Hamamori, Y. (2003) J. Cell. Physiol. 194, 237–255
11. Amsen, D., Blander, J. M., Lee, G. R., Tanigaki, K., Honjo, T., and Flavell, H. D., ISO, and M. Kurabayashi, unpublished observation.

FIGURE 8. HERP1 represses NICD-mediated SMC differentiation. 10T1/2 cells were transfected with the indicated expression vectors or siRNA. Three days after transfection, total RNA was isolated for quantitative RT-PCR.
