Molecular Cardiology

Smooth Muscle Notch1 Mediates Neointimal Formation After Vascular Injury

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Background—Notch1 regulates binary cell fate determination and is critical for angiogenesis and cardiovascular development. However, the pathophysiological role of Notch1 in the postnatal period is not known. We hypothesize that Notch1 signaling in vascular smooth muscle cells (SMCs) may contribute to neointimal formation after vascular injury.

Methods and Results—We performed carotid artery ligation in wild-type, control (SMC-specific Cre recombinase transgenic [smCre-Tg]), general Notch1 heterozygous deficient (N1+/−), SMC-specific Notch1 heterozygous deficient (smN1+/−), and general Notch3 homozygous deficient (N3−/−) mice. Compared with wild-type or control mice, N1+/− and smN1+/− mice showed a 70% decrease in neointimal formation after carotid artery ligation. However, neointimal formation was similar between wild-type and N3−/− mice. Indeed, SMCs derived from explanted aortas of either N1+/− or smN1+/− mice showed decreased chemotaxis and proliferation and increased apoptosis compared with control or N3−/− mice. This correlated with decreased staining of proliferating cell nuclear antigen–positive cells and increased staining of cleaved caspase-3 in the intima of N1+/− or smN1+/− mice. In SMCs derived from CHF1/Hey2−/− mice, activation of Notch signaling did not lead to increased SMC proliferation or migration.

Conclusions—These findings indicate that Notch1, rather than Notch3, mediates SMC proliferation and neointimal formation after vascular injury through CHF1/Hey2 and suggest that therapies that target Notch1/CHF1/Hey2 in SMCs may be beneficial in preventing vascular proliferative diseases. (Circulation. 2009;119:2686-2692.)

Key Words: molecular biology ■ remodeling ■ signal transduction

The Notch receptors are highly conserved membrane-bound receptors that regulate binary cell fate determination and embryonic development.1 Binding of the Notch receptor ligands Jagged or Delta-like leads to enzymatic cleavage and nuclear translocation of the Notch intracellular domain (NICD). NICD binds to and derepresses the transcriptional repressors of the CSL family such as Cp-binding factor 1, recombination signal sequence-binding protein-Jc, suppressor of hairless, and Lag-1, leading to transcriptional activation and cell fate determination.2,3

Clinical Perspective on p 2692

Mutation of Notch3 is responsible for the autosomal dominant inherited disorder called CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) syndrome.4 Alternatively, mutation of the Notch ligand Jagged leads to Alagille syndrome.5 Interestingly, deletion of Notch3 in mice does not recapitulate CADASIL syndrome, suggesting that hypermorphism of Notch3 mutation rather than its deletion may contribute to the observed disorder. In contrast, Notch1-deficient mice exhibit widespread apoptosis of the central nervous system, impaired somitogenesis, and defective angiogenesis and are lethal at embryonic day 9.5 to 10.5.6 Interestingly, all of these abnormal phenotypes, including the cause of lethality of global Notch1-deficient mice, are recapitulated in mutant mice with endothelial-specific deletion of Notch1,7 suggesting the critical role of endothelial Notch1 in embryonic development. The precise role of Notch1 in SMCs, however, remains unknown.

Although recent in vitro studies have suggested that Notch1 may be important in regulating SMC proliferation8 and differentiation,9 the pathophysiological correlate of these findings in vivo, especially in the postnatal period, has not

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been demonstrated. This is due, in part, to the embryonic lethality of Notch1-deficient mice and the lack of availability of mice with tissue-specific deletion of Notch1 in SMCs. Accordingly, we have developed the SMC-specific Notch1 heterozygous deficient mice, which were created by mating conditional mice harboring the Notch1-loxP–targeted allele with the SMC-specific Cre recombinase transgenic (smCre-Tg) mice. These mice, along with Notch1 heterozygous deficient (N1+/−) and Notch3 homozygous deficient (N3−/−) mice, were subjected to vascular injury to determine the role of Notch1 and Notch3 in mediating neointimal formation and vascular remodeling. Here we show that Notch1, rather than Notch3, is critical for SMC proliferation, migration, and survival.

Methods

Animals

Notch1 general heterozygous knockout (Notch1+/−) and Notch3 general homozygous knockout (N3−/−) mice were generated as described previously.6,10–12 SMC-specific deletion of Notch1 was accomplished by crossing Notch1floxed mice with smCre-Tg mice.13 The background of all mice is C57Bl/6. Wild-type (WT) (Notch1+/−), smCre-Tg mice, and corresponding littermates served as controls for N1+/− and smN1+/− mice. The Standing Committee on Animal Care at Harvard Medical School approved all animal experimentation protocols.

Isolation and Mouse Endothelial Cells and SMCs

Endothelial cells (ECs) from hearts and SMCs from aortas were isolated from WT, smCre-Tg, Notch1floxed, N1+/−, and smN1+/− mice. ECs were isolated with the use of 2-sort antibody-coated magnetic beads as described previously.14 The SMCs were obtained with the use of collagenase and elastase digestion of aortas as described previously.15 The ECs were cultured with Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, heparin, endothelial cell growth factor, and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate). The SMCs were cultured with Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, nonessential amino acids (GIBCO), and antibiotics. All ECs and SMCs used were of passages between 2 and 5.

Western Blot Analysis

Cells were incubated in lysis buffer (20 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mg leupeptin per liter, and 1 mmol/L phenylmethylsulfonyl fluoride). The cell lysates were separated on SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with polyclonal antibody for Notch1 (Santa Cruz Biotechnology, Santa Cruz, Calif; sc-6014), Notch3 (Santa Cruz Biotechnology; sc-7424), and actin (Sigma, St Louis, Mo; A2066).

Carotid Artery Ligation

The mouse carotid artery was ligated with a 6-0 silk suture just proximal to the carotid bifurcation as described.16 At 1 and 4 weeks after the procedure, the carotid arteries were harvested and perfusion-fixed with 4% paraformaldehyde. The samples were then embedded in OCT compound and frozen. Five cryosections (each 6 μm thick) at 3 to 4 mm proximal to the ligation site were obtained in each animal. Areas of lumen, intima, and media were measured in sections stained with hematoxylin and eosin and analyzed with the NIH Image program (National Institutes of Health) as described previously.17 A mean value between 5 sections in each animal was used for analysis.

Immunofluorescence

Immunofluorescence for smooth muscle actin (Sigma; A2547), Notch1 (Santa Cruz Biotechnology; sc-6014), activated Notch1 (NICD; Abcam, ab8925), Notch2 (Rockland; 100-401-406), Notch3 (Santa Cruz Biotechnology; sc-7424), cleaved caspase-3 (Cell Signaling Technology; 9664), and proliferating cell nuclear antigen (PCNA) (Laboratory Vision; RB-9055) was performed by standard procedures.

SMC Migration and Chemotaxis Assay

Explants of SMC outgrowth from aortas were assessed as described previously.16 Briefly, aortic explants (1×1 mm) from WT, smCre-Tg, N1+/−, and smN1+/− mice were placed with the luminal side down and allowed to attach to the surface of a 24-well plate. The SMC culture medium was changed every 3 days. The number of SMCs migrated from the explants was assessed after 3 and 7 days. In some studies, SMCs from WT or CHF1 mutant mice were infected with Ad.GFP or Ad.NICD at a multiplicity of infection of 100 for 24 hours. Chemotactic migration was measured with the use of a modified Boyden chamber with gelatin-coated 8-μm-pore polycarbonate filter as described previously.19 Briefly, 5×103 SMCs from control and Notch mutant mice were suspended in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum in the upper chamber of a 48-well Boyden chamber apparatus with serum stimuli in the lower chamber for 4 hours. The cells that migrated to the lower sides of the filter membranes were fixed and stained with Diff-Quik stain set (Dade Behring). Experiments were performed at least 3 times in quadruplicate.

Proliferation Assay

Proliferation assay was performed as described previously.20 Briefly, SMCs (100 μL, 2500 cells) were cultured on gelatin-coated 96-well plates. In some studies, SMCs from WT or CHF1 mutant mice were infected with Ad.GFP or Ad.NICD at a multiplicity of infection of 100 for 24 hours. After 72 hours of incubation, cells were fixed and stained with 0.1% crystal violet solution. The number of cells was quantified by measurement of absorbance at 590 nm with a microplate reader (SPECTRA Fluor, TECAM). Experiments were performed at least 3 times in triplicate.

Cellular DNA synthesis was assessed by [3H]thymidine uptake as shown previously.21 SMCs were seeded on 1% gelatin-coated 24-well plates at 104 cells per well and incubated in culture medium for 24 hours, followed by serum starvation. Cells were incubated in SMC culture medium for 16 hours. Then 0.5 μCi of [3H] thymidine and 0.5 μg of thymidine (Sigma) were added per well. After 6 hours, cells were washed and fixed (10 minutes on ice with 5% trichloroacetic acid), the DNA was released by alkaline lysis (0.5 N NaOH), and supernatants were quantified in a beta counter (Beckmann LS6000SC; Beckman Coulter, Somerset, NJ). Experiments were performed at least 3 times in triplicate.

Assessment of Apoptosis

Annexin V staining was used for assessment of apoptotic cells after 24-hour exposure to 50 μmol/L H2O2 followed by serum starvation.22 After staining treated SMCs (passages 2 to 4) with enhanced green fluorescent protein–annexin V solution (1:40; Clontech) and 4′, 6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma), enhanced green fluorescent protein–annexin V–positive cells with nuclear cleavage were evaluated. Random fields were chosen, and up to 2000 cells were counted for the analysis.23

Statistical Analysis

Results were expressed as mean±SD. The data among groups were compared with either 1-way or 2-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. A P value of <0.05 was taken to be statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Vascular Notch1 Expression in N1+/− and smN1+/− Mice

Primary cultures of ECs and SMCs from WT, smCre-Tg, Notch1flox+/−, N1+/−, and smN1+/− mice were obtained. In ECs, the Notch1 expression was decreased by half in N1+/− but not smN1+/− mice compared with that of WT or control mice (Figure 1A). However, in SMCs, Notch1 expression was decreased by more than half in both N1+/− and smN1+/− mice (Figure 1B). These findings indicate specificity of Cre-mediated excision of Notch1 in SMCs, but not in ECs, in smN1+/− mice.

Expression of Notch1, Notch2, and Notch3 After Carotid Artery Ligation

Immunofluorescence staining of smooth muscle actin and Notch1, Notch2, or Notch3 was performed in intact and ligated carotid artery (14 days after surgery) from WT mice (Figure 1C). In intact carotid artery, Notch1 was expressed mostly in ECs, Notch2 was expressed in both ECs and SMCs, and Notch3 was expressed mostly in medial SMCs. After ligation injury, Notch1 was highly expressed in neointimal SMCs, whereas Notch2 and Notch3 were constitutively expressed in medial and neointimal SMCs. These different patterns of expression suggest that Notch1, Notch2 and Notch3 may have distinct roles in SMC function. Indeed, compared with carotid artery from WT mice, activated Notch signaling, as determined by staining for NICD, was substantially reduced in SMCs of N1+/− and smN1+/− mice (Figure 1D). Thus, Notch1 and its downstream signaling pathway may be more temporally responsive and critical to SMC proliferation after vascular injury.

SMC Notch1 and Neointimal Formation

To determine whether Notch1 in SMCs is involved in vascular lesion formation, we performed carotid artery ligation in WT, smCre-Tg, N1+/−, and smN1+/− mice (n=10 in each group) (Figure 2A). No differences were found in hemodynamic parameters such as blood pressure and heart rate between the groups of mice, either basally or after carotid arterial ligation. Carotid arteries were collected 28 days after the procedure. Hematoxylin and eosin staining was performed on sections from WT, smCre-Tg, N1+/−, N3+/−, and smN1+/− mice. Arrows indicate internal elastic lamina. A, Representative sections are shown. Bar=100 μm. B, Quantitative morphometric analysis of intimal area in WT, smCre-Tg, N1+/−, N3+/−, and smN1+/− mice. n=10 in each group; †P<0.01 vs WT; ‡P<0.01 vs smCre-Tg. C, Attenuated intima/media (I/M) area ratio in Notch1 mutant but not N3+/− mice compared with control mice. n=10 in each group; †P<0.01 vs WT; ‡P<0.01 vs smCre-Tg.
artery ligation (data not shown). After carotid artery ligation, WT and smCre-Tg mice exhibited substantial increase in the neointima as measured by the area between the lumen and the internal elastic lamina (2.86±0.14 and 2.77±0.13 × 10^4 μm^2, respectively) (Figure 2B). However, the ligated carotid arteries from N1^-/- and smN1^-/- mice revealed less neointimal area compared with those in WT and smCre-Tg mice (0.99±0.10 and 0.98±0.07 × 10^4 μm^2, respectively; *P<0.01 for both compared with WT or smCre-Tg). In contrast, neointimal area of N3^-/- mice (2.66±0.18 × 10^4 μm^2) was not different from that of WT (*P=NS). The medial areas of the ligated carotid arteries were not different between the groups. Therefore, the intima-to-media ratio was substantially reduced in ligated carotid arteries from N1^-/- and smN1^-/- mice (0.44±0.05 and 0.45±0.05, respectively) compared with that of WT or smCre-Tg mice (1.36±0.20 and 1.25±0.12, respectively; *P<0.01) (Figure 2C). Furthermore, no difference was found in the neointimal areas and intima-to-media ratio between ligated carotid arteries of N1^-/- and smN1^-/- mice, indicating that Notch1 in intimal SMCs, and not in other cell types, is primarily responsible for neointima formation.

**Notch1 Mediates SMC Migration**

To determine whether the decrease in neointima formation in N1^-/- and smN1^-/- mice was due to decreased SMC migration and/or proliferation, we first investigated whether Notch1 regulates SMC migration. Using explants of aortas, we found that outgrowth of SMCs from aortas of N1^-/- and smN1^-/- mice were substantially less compared with that of WT or N3^-/- mice (WT versus N1^-/-, smN1^-/-, and N3^-/- mice: 7.9±1.0 versus 6.2±0.8, 6.5±1.1, and 7.3±1.1 × 10^3 cells at 3 days, *P<0.05; 3.1±0.4 versus 2.4±0.5, 2.4±0.4, and 3.3±0.33 × 10^3 cells at 7 days, *P<0.05; n=8 in each group) (Figure 3A and 3B). The SMC outgrowth was similar between aortas of WT and smCre-Tg mice. To determine whether Notch1 could also mediate SMC chemotaxis, we isolated SMCs from WT, N1^-/-, smN1^-/-, and N3^-/- mice and determined their chemotactic response to serum in a modified Boyden chamber. Compared with WT or N3^-/- mice, the migration of SMCs in response to serum was decreased in SMCs derived from N1^-/- and smN1^-/- mice (WT versus N1^-/-, smN1^-/-, and N3^-/- mice: 100±10% versus 83±9%, 81±9%, 96±12%; *P<0.05; n=12 in each group) (Figure 3C). These findings indicate that Notch1 is critically important for SMC migration in response to serum.

**Notch1 Mediates SMC Proliferation**

To determine whether Notch1 mediates SMC proliferation, we assessed whether indices of SMC proliferation are altered in SMCs from N1^-/- and smN1^-/- mice. In response to serum, the cell counts for SMCs from N1^-/- and smN1^-/- mice were decreased compared with that of WT or smCre-Tg mice (WT versus N1^-/- mice: 100±6% versus 85±5%, *P<0.05; smCre-Tg versus smN1^-/- mice: 104±6% versus 86±4%, *P<0.05, n=10 in each group) (Figure 4A). The cell counts were similar between WT and N3^-/- mice (WT versus N3^-/- mice: 100±6% versus 93±5%; *P=NS). This correlated with decreased [3H]thymidine incorporation in SMCs of N1^-/- and smN1^-/- mice (WT versus N1^-/- mice: 100±10% versus 81±13%, *P<0.05; smCre-Tg versus smN1^-/- mice: 98±10% versus 80±9%, *P<0.05; n=6 in each group) (Figure 4B). The [3H]thymidine incorporation in SMCs was similar between WT and N3^-/- mice (WT versus N3^-/- mice: 100±10% versus 92±15%; *P=NS). After carotid artery ligation, increased PCNA staining was observed in the neointima of WT mice (18±4% PCNA-positive cells; n=6) (Figure 4C). In contrast, PCNA staining was substantially reduced in the neointima of N1^-/- mice (7±3% PCNA-positive cell; n=6). The PCNA staining in the media was comparable between WT and N1^-/- mice. These findings suggest that SMC Notch1 mediates neointima formation after vascular injury.

**Notch1 Mediates SMC Survival**

To determine whether haploinsufficiency of Notch1 leads to increase SMC apoptosis, we induced SMC apoptosis with 24-hour exposure to H2O2 (50 μmol/L) followed by immunofluorescence staining with DAPI and annexin V. Haploinsufficiency of Notch1 in SMCs led to a substantial increase in the percentage of apoptotic nuclei (WT versus N1^-/- mice: 4±1% versus 14±2%; *P<0.01; n=10; smCre-Tg versus smN1^-/- mice: 4±1% versus 14±3%; *P<0.01; n=10) (Figure 5A). The percentage of apoptotic nuclei was comparable...
between WT and N3−/− mice (WT versus N3−/− mice: 4±1% versus 5±1%; P=NS). This correlated with increased caspase-3 cleavage in the neointima of N1−/− mice compared with that of WT mice (WT versus N1−/− mice: 6±1% versus 9±2%; P<0.05; n=6) (Figure 5B). These findings indicate that the decrease neointimal formation after vascular injury in N1−/− and smN1−/− mice is attributed to both decreased SMC proliferation and survival.

**CHF1/Hey2 Mediates Notch Signaling in SMCs**

Previous studies show that CHF1/Hey2 regulates neointimal formation and vascular smooth muscle proliferation. Because CHF1/Hey2 is the mammalian homologue of the zebrafish gridlock and gridlock lies downstream of Notch, we investigated whether CHF1/Hey2 mediates Notch-induced SMC proliferation and migration. Quiescent SMCs from WT mice proliferated in response to infection with adenovirus carrying NICD (Figure 6A and 6B). However, SMCs from CHF1−/− mice did not proliferate in response to Ad.NICD. Furthermore, increased migration was observed with Ad.NICD in SMCs from WT but not CHF1−/− mice (Figure 6C). These findings suggest that CHF1 may be the downstream mediator of Notch-induced SMC proliferation and migration.

**Discussion**

We have shown that Notch1, but not Notch3, in SMCs mediates neointimal formation after vascular injury. The mechanism is due, in part, to mediation of SMC migration, proliferation, and survival. These findings indicate that Notch1 is an important regulator of SMC function in the postnatal period and suggests that therapy that modulates Notch1 signaling in SMCs may be beneficial in treating vascular proliferative diseases. However, the role of Notch1 and Notch3 in SMCs could also be dependent on the model of vascular injury. Thus, it remains to be determined whether Notch1 is the sole Notch receptor in SMCs mediating the response to vascular injury.

The downstream mediators of Notch signaling in the vasculature are incompletely understood. Previous studies have identified CHF1/Hey2 as a downstream target of Notch.
and as a regulator of neointimal formation after vascular injury. Loss of CHF1/Hey2 attenuates the response of SMCs to growth factors and leads to decreased SMC proliferation in vivo and in vitro, through decreased activation of the small GTPase Rac1. To determine whether CHF1/Hey2 functions downstream of Notch in SMCs, we attempted to rescue the proliferation and migration defect in CHF1/Hey2-null SMCs by transfection of the Notch1 intracellular domain. Both proliferation and migration were blocked, indicating that CHF1/Hey2 is an important downstream mediator of Notch1 in SMCs. Identification of additional downstream components remains under investigation.

Notch signaling has been implicated in cell-to-cell communication and the regulation of cell fate in a variety of cell types. However, the role of Notch signaling in vascular cells, especially in the postnatal period, is not entirely known. Previous studies suggest that the expressions of several Notch components, including Notch1, Notch2, and Notch3, were altered after vascular injury. Our results showed different expression patterns of Notch1, Notch2, and Notch3 after vascular injury. Neointimal SMCs transiently expressed Notch1 after carotid artery ligation, whereas Notch2 and Notch3 expressions were constitutive in both medial and neointimal SMCs. These findings suggest that Notch1, Notch2, and Notch3 may have distinct roles in SMC function, with Notch1 being more responsive to vascular injury and critical to SMC proliferation.

Because bone marrow–derived vascular progenitor cells could also contribute to neointimal formation in the carotid artery ligation model, we compared neointimal formation in N1−/− and smN1−/− mice in response to vascular injury. Notch1 expression in bone marrow cells in smN1−/− mice was similar to that of WT mice (data not shown). Furthermore, little or no difference was found in the amount of neointimal formation between global and SMC-specific Notch1 haploinsufficient mice, suggesting that Notch1 in SMCs, and not in other cell types including bone marrow–derived progenitor cells, mediates neointimal formation. As a control for potential nonspecific effects of Cre recombinase expression in SMCs, we also found no difference in neointimal formation between WT and smCre-Tg mice.

Transfection of Epstein-Barr virus–encoded gene product RPMS-1 in SMCs leads to attenuation of Notch signaling and decreased proliferation and survival of SMCs. In contrast, activation of Notch signaling by overexpression of NICD increases SMC proliferation and survival. These results are consistent with our findings that SMCs from Notch1 mutant mice exhibit impairment of cellular migration, proliferation, and survival. Indeed, a recent study suggests that Notch1 and Notch3 signaling are mediated by Cp-binding factor 1/recombination signal sequence-binding protein-Jx. These effects of Notch1 may be the underlying mechanism contributing to the observed decrease in neointimal formation in our Notch1 mutant mice. However, the role of Notch signaling in SMC differentiation is somewhat controversial. In vitro studies suggest that augmentation of Notch signaling represses myocardin-induced SMC differentiation. In contrast, Notch3-deficient mice were reported to exhibit impairment of maturation and differentiation of SMCs in small arteries. Thus, the role of Notch signaling in SMC differentiation requires further investigation.

In summary, we have shown that Notch1 in SMCs is critical for neointimal formation after vascular injury. Notch1 mediates SMC migration, proliferation, and survival and may be an important therapeutic target in vascular diseases involving excessive SMC proliferation such as restenosis, atherosclerosis, and transplant-associated arteriopathy. The nature of some of the downstream targets of Notch1 and the manner in which they regulate Notch1-mediated SMC function remain to be determined.

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Disclosures
None.

References
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CLINICAL PERSPECTIVE

The signaling mechanisms that govern the response of smooth muscle after vascular injury and during neointimal formation are not known. The Notch receptors are highly conserved membrane-bound receptors that regulate binary cell fate determination and embryonic development. However, their role in postnatal vascular remodeling is not well understood. In this study, we showed that after carotid artery ligation, Notch1 was highly expressed in neointimal smooth muscle cells (SMCs), whereas Notch2 and Notch3 were constitutively expressed in medial and neointimal SMCs. These different patterns of expression suggest that Notch1, Notch2, and Notch3 may have distinct roles in SMC function. Mutant mice with targeted deletion of Notch1, but not Notch3, in SMCs exhibited decreased neointimal formation after carotid artery ligation. Indeed, compared with wild-type mice, activated Notch signaling was substantially reduced after carotid artery ligation in global and SMC-specific Notch1 hemizygous knockout mice. This was associated with decreased SMC chemotaxis and proliferation and increased apoptosis. In SMCs derived from CHF1/Hey2 knockout mice, activation of Notch signaling did not lead to increased SMC proliferation or migration. These findings indicate that Notch1 mediates SMC proliferation and neointimal formation after vascular injury through CHF1/Hey2 and suggest that therapies that target Notch1/CHF1/Hey2 in SMCs may be beneficial in preventing vascular proliferative diseases.
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