Role of Jagged1 in Arterial Lesions After Vascular Injury

Xiaojing Wu, Yunzeng Zou, Qi Zhou, Lan Huang, Hui Gong, Aijun Sun, Kaoru Tateno, Ken-ichi Katsube, Freddy Radtke, Junbo Ge, Tohru Minamino, Issei Komuro

Objective—Impaired regeneration of endothelial cells (EC) and overactivity of vascular smooth muscle cells (VSMC) are hallmarks of the arterial lesions associated with aging. The occurrence of 2 opposing cellular processes in the same arterial milieu makes pharmaceutical treatment difficult to develop. We previously reported that endothelial expression of a Notch ligand (Jagged1) was reduced in aged animals and that growth of the neointima was enhanced in these animals.

Methods and Results—Similar to aged animals, Tie2-cre<sup>+</sup> Jagged1<sup>lox/lox</sup> mice (with heterologous knockout of Jagged1 in EC) showed exaggerated intimal and medial thickening after carotid artery ligation. Unexpectedly, these mice showed little increase of Jagged1 expression not only in EC but also in VSMC, in contrast to a significant upregulation of Jagged1 in wild-type arteries after ligation. Coculture of VSMC with Jagged1-null EC resulted in the transition of VSMC from the contractile to the synthetic phenotype, along with decreased Jagged1 expression by VSMC. Conversely, overexpression of Jagged1 by EC or VSMC was shown to prevent the unfavorable phenotypic transition of VSMC, under both monoculture and coculture conditions.

Conclusion—These findings suggest a unidirectional effect of Jagged1 on both EC and VSMC that contributes to inhibition of arterial lesions after vascular injury. Our data also indicate that Jagged1 may be a novel therapeutic target for aging-related vascular diseases. (Arterioscler Thromb Vasc Biol. 2011;31:2000-2006.)

Key Words: aging ♦ endothelium ♦ smooth muscle cell ♦ vascular injury

Aging is an independent risk factor for the development of cardiovascular disease.<sup>1</sup> Age-associated remodeling of the vascular wall leads to intimal and medial thickening as well as increased vascular stiffness.<sup>2,3</sup> Although both impaired regeneration of endothelial cells (EC) and enhanced proliferation of vascular smooth muscle cells (VSMC) contribute to the progression of age-associated vascular remodeling, how aging regulates these opposing processes in the same arterial milieu remains largely unknown. Moreover, the contrary behavior of these 2 types of cells makes pharmaceutical treatment of aging-associated vascular disease difficult to achieve. For instance, although drug-eluting stents for coronary intervention can lessen restenosis by inhibiting VSMC proliferation, their inhibition is nonselective and can also disturb endothelial repair, forcing patients to prolong the use of dual antiplatelet therapy to prevent late thrombosis.<sup>4</sup>

An agent that promotes both EC and VSMC protection could be ideal to overcome such issues.

Among factors that influence the functions of both EC and VSMC and are also related to the ageing process, we focused on Notch. This is an evolutionarily conserved intercellular signaling pathway, which has been shown to contribute to decisions about cellular fate during embryogenesis. Recent investigations have revealed a potential link between Notch signaling and the repair of aged skeletal muscle.<sup>5,6</sup> In the cardiovascular system, Notch signaling is known to be involved in various physiological and pathological processes, including regulation of blood vessel sprouting and branching during angiogenesis and the regulation of arterial and venous differentiation during embryonic vessel formation.<sup>7</sup>

There have also been several reports that Notch signaling, in company with its ligand Jagged1, has a role in responses of EC and VSMC during development or postnatal remodeling after vascular injury.<sup>8-10</sup> Thus, we hypothesized that Notch signaling might play a pivotal role in the development and progression of age-related vascular diseases.

We previously reported that endothelial expression of a Notch ligand (Jagged1) was reduced in aged animals, in association with increased VSMC proliferation, and that the formation of neointima was enhanced in these animals.<sup>11</sup>

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vitro study suggested that EC and VSMC may interact with each other via Notch and that reduced endothelial expression of Jagged1 may be crucial for age-associated vascular remodeling. However, the detailed mechanisms underlying such cell-to-cell interactions remain unclear. More importantly, it is still unknown whether such interactions have any significance in vivo. In the present study, we demonstrate a causative role of endothelial Jagged1 in the occurrence of intimal and medial thickening after vascular injury in vivo. We also provide novel data indicating that endothelial Jagged1 induces VSMC to express Jagged1, presumably through a paracrine mechanism, and that this influences the phenotypic transition of VSMC. These findings suggest a unidirectional influence of Jagged1 on both EC and VSMC that inhibits arterial lesion formation after vascular injury. Our findings support the possibility that Jagged1 may be a novel therapeutic target for aging-related vascular diseases.

Materials and Methods

Animals and Carotid Artery Ligation
C57BL/6J mice were purchased from SLC Japan, and the Tie2-cre mice were obtained from Jackson Laboratories. The floxed Jagged1 mice have been described elsewhere. Deletion of Jagged1 in EC was accomplished by crossing male Tie2-cre+ mice with female Jagged1lox/lox mice (Tie2-cre+ Jag1lox+/+), and the corresponding littermate without Cre transgene (Tie2-cre- Jag1lox/lox) served as controls. These mice also served as a donor for the bone marrow transplantation models. The mouse carotid artery was ligated with a 6-0 silk suture just proximal to the carotid bifurcation as previously described. All experimental procedures were done according to the guidelines established by Chiba University for animal experiments and all protocols were approved by our institutional review board.

Morphology Analysis
The carotid arteries were harvested at 4 weeks after the ligation surgery and were then perfusion-fixed with 4% paraformaldehyde and embedded in paraffin. Sections (each 5 μm thick) at 3 to 4 mm proximal to the ligation site were obtained in each animal. Areas of the lumen, intima, and media were measured in sections stained with hematoxylin and eosin and analyzed with the National Institutes of Health Image Program, as previously described.

Immunofluorescence
The carotid arteries were harvested at 2 weeks after the ligation surgery and were then embedded in OCT compound, frozen, and sectioned. Immunofluorescence for Jagged1 (Santa Cruz Biotechnology: sc-6011), α-smooth muscle actin (SMA, Sigma:A2547), and CD31 (BD Pharmingen:558736) was performed by standard procedures.

Cell Culture and Assay
Primary cultures of human aortic EC (HAEC) and VSMC were from Bio Whittaker (Walkersville, MD). The cells of passage 5 to 7 were used for the experiments. The proliferation assay by MTT16 and cell count were performed as previously described. Peripheral blood cells of mice were harvested as previously described.

Retroviral Infection
Retroviral gene transfer of Jagged1 in pLNCX2 vectors (Clontech) was done as previously described. Briefly, retroviral stocks were generated by transient transfection of the HEK-293 packaging cell line and stored at −80°C until use. HAEC or VSMC were plated at 4×10^5 cells per 100-mm diameter dish 24 hours before infections. For infections, the culture medium was replaced by retroviral stocks supplemented with 8 μg/mL polybrene (Sigma). At 48 hours after infection, the infected cell populations were selected by culture in 500 μg/mL G418 for 6 days. After selection, the cells were cultured in fresh complete medium and subjected to experiments. Information about the retroviral vector encoding the Jagged1 gene is available on request.

Transfection of siRNA
RNA interference was used to knock down the expression of Jagged1 in the EC or VSMC. A small interfering RNA (siRNA) of Jagged1 (sequence: UAAUGUGAUGUUGCCAGUGUAUC) was designed according to the human Jagged1 sequence (NM_000214), and the oligo was synthesized by Invitrogen. The control siRNA (The BLOCK-iT Alexa FluorR Red Fluorescent Oligo, Cat. No. 14750–100) and the transfection reagents (Lipofectamine RNAi Max) were obtained from Invitrogen. After preparation with the siRNA transfection reagent, the siRNA for Jagged1 or the control siRNA was transfected into the cells (at 30% to 50% confluence) according to the manufacturer’s instructions. To confirm the effect of siRNA on the expression of Jagged1, some of the cells were subjected to Western blot analysis, using an anti-Jagged1 antibody.

Coculture of HAEC With VSMC
VSMC and HAEC were cocultured, respectively, in cell culture inserts and corresponding companion 6- or 12-well plates (BD Biosciences). HAEC overexpressing Jagged1, or transfected with Jagged1 siRNA, were cultured in complete medium (EGM-2, Lonza) until 90% confluence. VSMC were inoculated onto the insert at a density of 10^5 cells/mL. Twenty-four hours later, the inserts with VSMC were inserted into the culture dishes of the EC. After 24 hours of incubation with or without 10% serum, VSMC in the upper chamber were harvested for further experiments.

Western Blotting
Total cell protein was extracted with RIPA buffer (Santa Cruz Biotechnology), and nuclear protein was extracted with NE-PER Nuclear Protein Extraction Kit (Thermo Scientific), and probed with monoclonal antibody for Jagged1, cleaved Notch1 (Notch intracellular domain, NICD, Cell Signaling, No. 2421,) and actin (Santa Cruz Biotechnology, sc-8432) by standard procedures.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
RNA was isolated from carotid artery or cultured cells using the RNeasy mini kit (Qiagen). Reverse transcription and real-time polymerase chain reaction (PCR) conditions are described in the online Data Supplement available at http://atvb.ahajournals.org. Relative mRNA expression was calculated with the comparative C_{T} method and normalized to the expression of endogenous 18s or GAPDH.

Statistical Analysis
Data are expressed as mean±SEM. Differences were determined either by unpaired Student t test or by 1-way ANOVA followed by a post hoc test to compare the differences between 2 groups. Values of P<0.05 were considered significant.

Results

Tie2-cre+ Jag1lox/+ Mice Show Increased Intimal Thickening After Carotid Artery Ligation
Real-time PCR analyses demonstrated that the most prominent Notch receptor in EC was Notch1, and the ligand Jagged1, suggesting a major role for these molecules (Supplemental Figure IA, IB). We previously reported that endothelial expression of Jagged1 decreased with advancing age and the neointimal proliferation was enhanced in aged rats. Likewise, endothelial Jagged1 expression was reduced in...
aged mice (Supplemental Figure IIA, IIB). To investigate whether endothelial expression of Jagged1 had an influence on arterial lesion formation in vivo, we developed endothelial-specific Jagged1 heterozygous deficient (Tie2-cre/Jag1lox/lox) mice. Expression of Jagged1 mRNA in the intact carotid artery was significantly decreased in Tie2-cre/Jag1lox/lox mice compared with that in the carotid artery of control (Tie2-cre–Jag1lox/lox) mice, falling to 30.29 ± 5.26% of the level in control mice (n = 5, P < 0.01) (Supplemental Figure IIC). In contrast, Jagged1 expression was similar in the carotid arteries of Tie2-cre/Jag1lox/lox mice and control mice after the carotid endothelium was removed (n = 5, P NS) (Supplemental Figure IID). After carotid artery ligation, the area of the neointima was significantly larger in Tie2-cre/Jag1lox/lox mice than in control mice at 28 days after carotid ligation (intimal area: 7.59 ± 0.46 × 10^4 μm^2 versus 4.93 ± 0.57 × 10^4 μm^2, n = 5, P < 0.01; N/M 1.49 ± 0.11 versus 1.10 ± 0.09, n = 5, P < 0.01) (Figure 1A through 1C).

Increase of Jagged1 in VSMC After Carotid Ligation Is Blunted in Tie2-cre/Jag1lox/lox Mice

In an attempt to confirm the expression of Jagged1 in mice, we performed double immunofluorescence staining of sections obtained from the animals at 14 days after carotid artery ligation. Using anti-Jagged1 and anti-CD31 antibodies, we found that the expression of Jagged1 in EC was significantly increased in the control mice, whereas (as expected) this increase was minimal in Tie2-cre/Jag1lox/lox mice (Figure 2A). Unexpectedly, this was accompanied by marked elevation of Jagged1 expression in the intima and media of the control mice. Double immunofluorescence staining with Jagged1 and SMA revealed that the majority of the Jagged1-expressing cells in the intima and media were VSMC (Figure 2B). More surprisingly, the increase of Jagged1 in VSMC was clearly impaired in the Tie2-cre/Jag1lox/lox mice (Figure 2B). Because Jagged1 expression in VSMC should not be genetically affected in Tie2-cre/Jag1lox/lox mice, the results
suggested that expression of Jagged1 by VSMC might be regulated through Jagged1 expression by EC, at least in the context of arterial ligation injury.

**Coculture With Jagged1 Knockout EC Results in Jagged1 Attenuation and VSMC Transition**

To confirm that endothelial Jagged1 affects the expression of Jagged1 in VSMC and to find out whether this influences the phenotype of VSMC (directing these cells toward neointimal formation or medial hyperplasia), we developed a cell coculture model (Figure 2C). We found that expression of Jagged1 mRNA by VSMC was significantly decreased after coculture with Jagged1 knockout HAEC (Figure 2D). Although proliferation of VSMC was not affected under the basal conditions without serum stimulation (data not shown), VSMC proliferation determined by the MTT assay was increased after coculture with Jagged1 knockdown HAEC in the presence of 10% serum (scRNA versus siRNA 0.53 ± 0.02 versus 0.84 ± 0.04, n = 4, P < 0.01) (Figure 2E). We then examined the effects of endothelial Jagged1 on expression of phenotypic genes, including calponin or SMC22α (contractile VSMC markers) and vimentin (a synthetic VSMC marker). We found that coculture with Jagged1 knockdown EC resulted in decreased expression of both calponin and SMC22α by VSMC, as well as increased expression of vimentin (Figure 2F), indicating phenotypic transition of these cells from the contractile to the synthetic type. Thus, endothelial Jagged1 had a significant influence on Jagged1 expression and phenotypic transition of VSMC.

**Loss of Jagged1 in VSMC Induces Synthetic Transition**

To examine the relationship between expression of Jagged1 by VSMC and their phenotypic transition, we studied VSMC monocultures. VSMC were transfected with a small interfering RNA (siRNA) for Jagged1 and with control RNA (scRNA). Western blotting of nuclear protein revealed that the activated form of the Notch1 receptor (NICD; Notch intracellular domain) simultaneously decreased after transfection with Jagged1 siRNA (Figure 3A). Expression of Jagged1 by VSMC did not influence the growth of these cells under basal conditions without stimulation (data not shown), but blocking of Jagged1 expression increased the proliferation of VSMC (as determined by cell counting and the MTT assay) after stimulation with 10% serum for 24 hours (MTT: 0.32 ± 0.04 versus 0.54 ± 0.06, n = 6, P < 0.01; cell counting: 3.57 ± 0.33 versus 5.01 ± 0.20 × 10^4 cells, n = 4, P < 0.01) (Figure 3B and 3C). Blocking of Jagged1 expression by VSMC also resulted in decreased expression of calponin and SMC22α, whereas there was increased expression of vimentin (Figure 3D). These findings indicate that loss of Jagged1 expression was sufficient to induce the synthetic transition of VSMC.

**Overexpression of Jagged1 Changes VSMC to the Contractile Phenotype**

Thus far, our results indicated that loss of Jagged1 expression by EC or VSMC caused these cells to contribute to neointimal formation or medial hyperplasia. Thus, we considered that enhancement of Jagged1 expression might be a novel therapeutic method for such pathological conditions. To further address this issue, we examined whether overexpression of Jagged1 in either VSMC or EC could induce VSMC to cause proliferation and/or synthesis. First, we infected VSMC with a retrovirus encoding Jagged1 (Figure 3E). Overexpression of Jagged1 induced the activation of Notch signaling (Figure 3E) and decreased the proliferation of VSMC (as determined by cell counting and the MTT assay) after stimulation with 10% serum for 24 hours (MTT: 0.45 ± 0.04 versus 0.23 ± 0.02, n = 6, P < 0.01; cell counting: 3.99 ± 0.25 × 10^5 versus 2.42 ± 0.47 × 10^4 cells, n = 4, P < 0.01) (Figure 3F, G). This also resulted in increased expression of calponin and SMC22α but decreased expression of vimentin (Figure 3H), in contrast to the results of siRNA experiments.

**Coculture With Jagged1-Overexpressing EC Elevates Jagged1 Expression by VSMC and Promotes the Contractile Transition**

Finally, we infected HAEC with the Jagged1 retrovirus (Figure 4A). VSMC cocultured with these Jagged1-overexpressing HAEC showed increased expression of Jagged1 mRNA (Figure 4B). Proliferation of VSMC (as determined by the MTT assay) was reduced after coculture with Jagged1-overexpressing HAEC (Figure 4C), whereas expression of calponin and SMC22α was increased significantly, and vimentin expression was markedly attenuated (Figure 4D). Thus, overexpression of Jagged1 in either EC or VSMC inhibited proliferation and synthesis by VSMC, strongly suggesting that enhancement of Jagged1 expression in the vascular wall might prevent neointimal formation or medial hyperplasia after vascular injury (Figure 5).

**Heterozygous Jagged1 Deficiency in Bone Marrow–Derived Cells Has Little Influence on VSMC Phenotype and the Arterial Lesion Formation**

The Cre transgene was driven by the Tie-2 promoter, which was predominantly activated in endothelial cells but also in hematopoietic stem cells to some extent. To exclude the possibility that reduced expression of Jagged1 in hematopoietic cells affects neointimal formation in Tie2-cre^-/- Jag1lox/+ mice, we harvested peripheral blood cells from Tie2-cre^-/- Jag1lox/+ mice or control littermates and coinoculated them with VSMC in the Boyden chamber. Although we found a modest decrease in platelet counts of the mutant mice, the number and fraction of white blood cells, or the number of red blood cells, did not differ between Tie2-cre^-/- Jag1lox/+ mice and control littermates (Supplemental Figure IIIA). Coculture with blood cells of Tie2-cre^-/- Jag1lox/+ mice did not affect expression of phenotypic genes and Jagged1 by VSMC compared with that with wild-type blood cells (Supplemental Figure IIIB, IIIC). To further confirm these results, we transplanted the bone marrow cells from either wild-type or Tie2-cre^-/- Jag1lox/+ mice into lethally irradiated wild-type mice, followed by carotid artery ligation. We found that heterozygous Jagged1-deficiency in bone marrow–derived cells had little influence on the arterial lesion formation after injury (Supplemental Figure IIID, IIIE).
Discussion

Many studies have demonstrated a link between aging and cell proliferation. In general, the decline of various cellular functions with aging is correlated with a decrease in the proliferative capacity of cells. In the vascular bed, however, aging is associated with reduced EC regeneration but paradoxically with increased VSMC proliferation in vivo. It is still unclear how aging exerts a different effect on these 2 major types of vascular cells in the same arterial milieu.

Among various factors that influence the functions of both EC and VSMC and are also related to the ageing process, Notch signaling is an important candidate. Because the most prominent Notch ligand and receptor are found to be Notch1 and Jagged1, we focused on these 2 molecules. We previously demonstrated that neointimal formation after vascular injury was exaggerated in aged rats and that the endothelial expression of Jagged1 after vascular injury decreased with aging. However, the actual role of endothelial Jagged1 in...
vascular remodeling in vivo was unproven. In the present study, we established Tie2-cre\(^+\) Jag1\(^{lox/+}\) mice with EC showing heterozygous Jagged1 deficiency. Using these animals, we successfully demonstrated a causative role of endothelial Jagged1 in neointimal formation and medial thickening after vascular injury.

In models of vascular injury, the expression of Notch1, Notch3, and Jagged1 has been reported to show to be modulated by a putative paracrine factor that influences VSMC behavior. Thus, a pharmacotherapy that enhances Jagged1 expression in EC, which in turn might inhibit neointimal and medial thickening. The paracrine factor from EC or VSMC could prevent proliferation or synthesis by VSMC. We also found that Jagged1 expression by EC had a paracrine effect, upregulates Jagged1 in vascular smooth muscle cells (VSMC). The increase of Jagged1 in VSMC induces its contractile transition, presumably through Notch signaling, which in turn might inhibit neointimal and medial thickening. Thus, a pharmacotherapy that enhances Jagged1 expression in vascular wall might be a novel strategy to prevent vascular remodeling associated with aging. The paracrine factor from EC that is responsible for the regulation of VSMC Jagged1 may also be used for this purpose.

ligands and receptors play some roles in our model. Nevertheless, we believe that Jagged1 and Notch1 play a prominent role because they are expressed predominantly in EC. It should also be noted that the Cre transgene of these mice was driven by the Tie-2 promoter, which is predominantly activated in endothelial cells but also in hematopoietic stem cells to some extent.\(^7,21\) We found that peripheral blood cells from mutant mice did not differ from wild-type blood cells in the effect on VSMC phenotype transition in vitro. Nor could we find any influence of mutant bone marrow on arterial lesion formation in vivo. Thus, it is unlikely that heterozygous Jagged1 deficiency in blood cells contribute to enhanced formation of neointima in Tie2-cre\(^+\) Jag1\(^{lox/+}\) mice.

The mechanism by which endothelial Jagged1 influences the phenotype of VSMC is another issue. High et al\(^8\) reported that the primary role of endothelial Jagged1 is to potentiate the development of neighboring VSMC during embryonic vessel formation. Liu et al\(^22\) found that EC Jagged1 induces mural cell differentiation through activation of Notch3 by using their in vitro system. Although Jagged1 is a major ligand of Notch, we suppose a paracrine mechanism rather than a direct cell-to-cell contact, as shown in these reports. In conduit arteries including the carotid arteries, EC and VSMC are separated by a thick basement membrane that is likely to inhibit direct contact of these cells. Our coculture model was also designed to prevent such contact, favoring a paracrine mechanism that influences VSMC behavior. Thus, in blood vessels, there may be 2 distinct mechanistic cascades governed by Jagged/Notch signaling, according to its anatomic or situational context.

Unexpectedly, we found in Tie2-cre\(^+\) Jag1\(^{lox/+}\) mice that the lack of Jagged1 expression by EC resulted in lack of Jagged1 expression by VSMC as well. This was also reproduced in the coculture model, suggesting that the effect results from EC/VSMC interaction. Moreover, in the monoculture experiment, deletion or introduction of Jagged1 was sufficient to influence the phenotypic transition of VSMC along with attenuation or enhancement of activated Notch1. Notch signaling has been reported to govern the differentiation and proliferation of VSMC.\(^23\) Doi et al\(^24\) reported that Jagged1/Notch signaling promotes VSMC differentiation. Also, Noseda et al\(^25\) showed that the VSMC gene \(\alpha\)-SMA is a direct downstream target of Notch/C/CSL. Similar to these reports, our study confirmed negative regulation of the proliferation of VSMC by Jagged1. Taken together, it is likely that a paracrine factor or factors released from EC (driven by a Jagged1-dependent mechanism) influences the expression of Jagged1 by VSMC, which then regulates Notch signaling to finally drive the changes of VSMC phenotypic proteins.

Finally, we found that overexpression of Jagged1 by either EC or VSMC could prevent proliferation or synthesis by VSMC. We also found that Jagged1 expression by EC had a protective effect on EC themselves (X. Wu, K. Tateno, and T. Minamino, unpublished data). These findings strongly suggest that Jagged1 might act on both EC and VSMC to inhibit arterial lesion formation after vascular injury in vivo. Thus, it would be a plausible strategy to introduce a factor that enhances Jagged1 expression in the vascular wall to prevent...
intimal/medial thickening in persons with ageing-related vascular diseases. Sethi et al\textsuperscript{26} reported that in certain tumors, Jagged1 promotes its growth and metastasis. Thus, it should be noted with caution that enhancement of Jagged1 in vivo may potentially be hazardous in such situations. Another therapeutic approach would be to identify a Jagged1-driven paracrine factor that is secreted by EC and inhibits the overactivity of neighboring VSMC. Accordingly, it could be worthwhile to perform microarray analyses of samples derived from Jagged1 knockout EC.

In conclusion, Jagged1 had a unidirectional effect on both EC and VSMC that inhibited arterial lesion formation after vascular injury. Our data support the possibility that Jagged1 may be a novel therapeutic target for aging-related vascular diseases.

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Disclosures
None.

References
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Supplemental Materials

Supplemental methods

Quantitative Real-time Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from carotid artery or cultured cells using the RNeasy mini kit (Qiagen), and then the extract RNA was reverse transcribed by QuantiTect Reverse Transcription kit (Qiagen). Amplification was performed with a LightCycler® 480 (Roche Diagnostic Ltd) according to the manufacturer’s instructions. Sequences of the primers and number of the probe (Universal probelibrary set, Roche, Indianapolis, IN, USA) are indicated (supplement Tab). Relative mRNA expression was calculated with the comparative $C_T$ method and normalized to the expression of the endogenous 18s and GAPDH.
### Supplemental Table

#### Primers and conditions for real-time PCR

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Supplemental Figure I. Expression of Notch receptors and ligands. Relative copy numbers of Notch receptors (A) and ligands (B) quantified by real-time PCR.
Supplemental Figure II. Expression of Jagged1 in the carotid artery. A and B, Jagged1 expression of carotid artery wall of young and aged mice quantified by real-time PCR. Endothelial monolayer was left intact (A), or denuded with a scraper (B). C and D, Jagged1 expression of carotid artery wall of Tie2-cre Jag1^lox/+ mice. Endothelial monolayer was left intact (C), or denuded with a scraper (D).
Supplemental Figure III. Significance of bone marrow derived cells from the Tie2-cre Jag1\textsuperscript{lox/+} mice. A, Blood cell count of adult Jagged1 hetero-deficient mice. Data indicate mean ± SE. B and C, Co-culture of VSMC with peripheral blood cells from Jagged1 hetero-deficient mice. Phenotypic gene expression in VSMC after co-culture, quantified by real-time PCR (B). Note that PCR value is in logarithm. Expression of Jagged1 in VSMC after co-culture (C). D and E, Neointimal area (D) and I/M ratio (E) following carotid artery ligation of wild type mice, in which bone marrow was replaced with the one from Jagged1 hetero-deficient mice.

A.

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<th>Hb g/dL</th>
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<th>Plt x10E4</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5057±756</td>
<td>14.2±1.8</td>
<td>1.6±0.2</td>
<td>84.0±1.9</td>
<td>13.6±0.3</td>
<td>54.0±1.0</td>
<td>91.2±2.4</td>
</tr>
<tr>
<td>KO</td>
<td>6325±1232</td>
<td>10.5±2.5</td>
<td>1.5±0.5</td>
<td>87.5±2.5</td>
<td>14.0±0.8</td>
<td>55.3±0.9</td>
<td>68.5±8.5</td>
</tr>
</tbody>
</table>

B

C

Expression of Jagged1 in VSMC after co-culture (C).