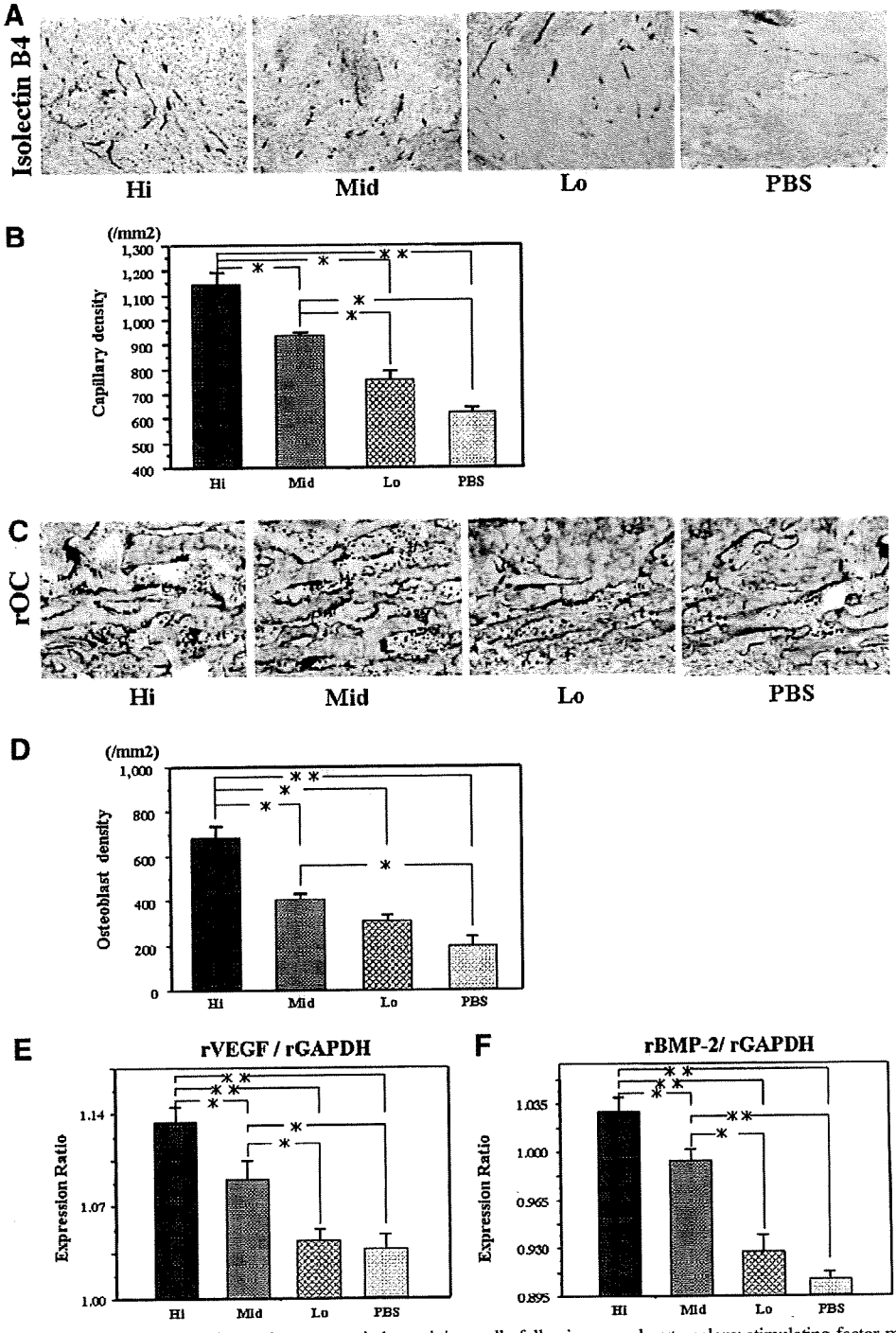


Figure 4. Enhancement of neovascularization and osteogenesis by recipient cells following granulocyte colony-stimulating factor-mobilized peripheral blood (GM-PB) CD34+ cell transplantation. **(A):** Representative vascular staining with isolectin B4 using tissue samples of perifracture sites collected at week 2 in the Hi, Mid, or Lo dose of GM-PB CD34+ cells and the PBS group (×100). **(B):** Neovascularization assessed by capillary density at week 2 was significantly greater in the Hi group than all other groups, as well as in the Mid group compared with the Lo and PBS groups. **, $p < .01$; *, $p < .05$ ($n = 4$ in each group). **(C):** Representative osteoblast staining with rOC at week 2 in animals treated with a Hi, Mid, or Lo dose of CD34+ cells or PBS alone (×200). **(D):** Osteogenesis assessed by osteoblast density at week 2 was significantly greater in the Hi group than other groups, as well as in the Mid group compared with the PBS group. **, $p < .01$; *, $p < .05$ ($n = 4$ in each group). **(E, F):** Gene expression of intrinsic cytokines for angiogenesis and osteogenesis at week 2. The mRNA expression ratio of rVEGF **(C)** and rBMP-2 **(D)** to rGAPDH at the fracture sites was significantly greater in the Hi group than other groups, as well as in the Mid group compared with the Lo and PBS groups. **, $p < .01$; *, $p < .05$ ($n = 4$ in each group). Abbreviations: Hi, 10^5; Lo, 10^3; Mid, 10^4; PBS, phosphate-buffered saline; rOC, rat osteocalcin; rBMP-2, rat bone morphogenic protein 2; rVEGF, rat vascular endothelial growth factor.

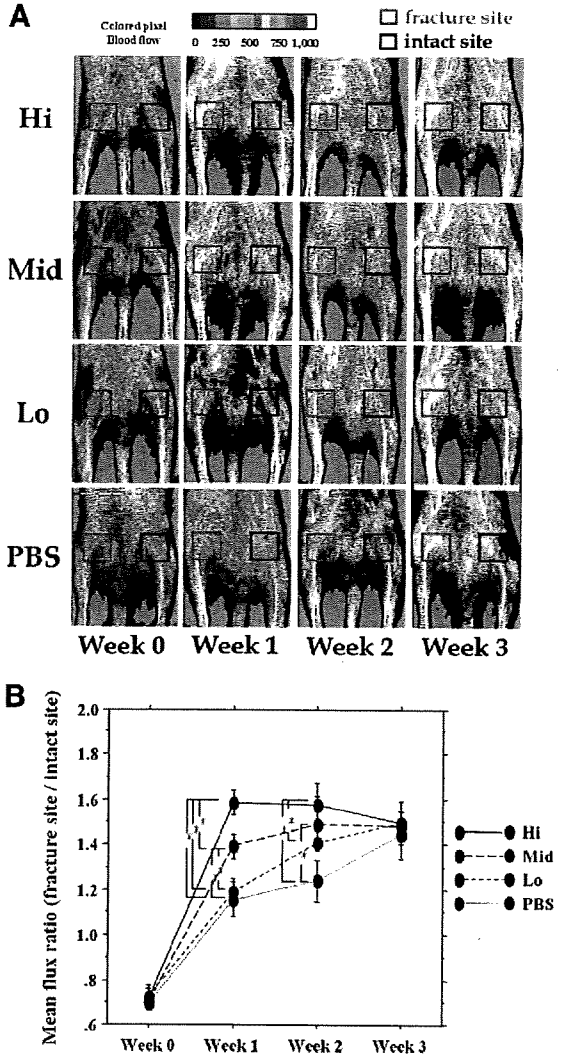


Figure 5. Improvement of blood flow at fracture sites following CD34+ cell transplantation. **(A):** Representative laser Doppler perfusion imaging (LDPI) at week 0 (1 hour after fracture), 1, 2 and 3 are shown. In these digital color-coded images, maximum perfusion values are indicated in white, medium values in green to yellow, and lowest values in dark blue. The skin blood flow within fracture site (red square) and intact contralateral site (black square) were evaluated as mean flux, and the ratio of the mean flux in the fractured site to that in the contralateral site (mean flux ratio) was calculated. **(B):** Severe reduction of the blood flow was observed 1 hour after nonhealing fracture was created (week 0) in all groups, whereas the mean flux ratio at week 1 was significantly higher in the Hi group compared with other groups, as well as in the Mid group compared with the Lo and PBS groups. At week 2, the ratio was significantly higher in the Hi group compared with the Lo and PBS groups, as well as in the Mid group compared with the PBS group. *, $p < .05$. Abbreviations: Hi, 10^5; Lo, 10^3; Mid, 10^4; PBS, phosphate-buffered saline.

Improvement of Blood Flow in Animals Receiving GM-PB CD34+ Cells After Fracture

To evaluate blood flow recovery at the fracture sites, LDPI was serially examined after fracture. LDPI analysis demonstrated severely low blood flow at the fracture site 1 hour after fracture creation (week 0) and subsequent recovery at weeks 1, 2, and 3 in all groups (Fig. 5A). In all groups, the ratio of fractured to intact (contralateral) blood flow significantly increased by week 1. There was no significant difference in the blood flow ratio of fractured to intact (contralateral) limbs 1 hour after fracture creation among any group, whereas the ratio at week 1 was significantly higher in animals receiving a high-dose of GM-PB CD34+ cells compared with the other groups, as well as in the middle-dose group compared with the low-dose and PBS groups (Hi, 1.587 ± 0.042; Mid, 1.397 ± 0.013; Lo, 1.193 ± 0.054; PBS, 1.190 ± 0.042, respectively; $p < .05$ for Hi vs. Mid, Lo, or PBS and for Mid vs. Lo or PBS group). At week 2, the blood flow ratio was still significantly higher in the Hi group compared with Lo and PBS groups, as well as in the Mid group compared with PBS group (Hi, 1.515 ± 0.035; Mid, 1.485 ± 0.015; Lo, 1.370 ± 0.040; PBS, 1.350 ± 0.020, respectively; $p < .05$ for Hi vs. Lo or PBS and for Mid vs. PBS group). At week 3, the flow ratio was similar in all groups (Hi, 1.445 ± 0.045; Mid, 1.485 ± 0.019; Lo, 1.495 ± 0.054; PBS, 1.498 ± 0.055, respectively; p = not significant) (Fig. 5B). These results indicate that local transplantation of human GM-PB CD34+ cells contributes to rapid improvement of tissue perfusion at the fracture site in a dose-dependent manner.

Morphological Fracture Healing in Animals Receiving GM-PB CD34+ Cell Transplantation

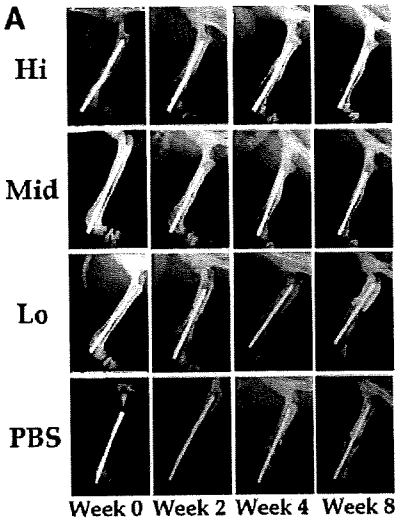
Morphological fracture healing was evaluated by radiographic and histological examinations. Thirty-three percent of animals at week 4 and all animals at week 8 that received a high-dose of CD34+ cells, as well as 11% of animals at week 4 and 50% of animals at week 8 that received a middle dose CD34+ cells, demonstrated fractures that radiographically appeared healed with bridging callus formation. Fracture sites in all animals receiving a low dose of CD34+ cells or PBS showed no bridging callus formation and subsequently displayed nonunions after 8 weeks, which is consistent with the previous reports of the natural course of this animal model [23, 24] (Fig. 6A, 6B).

Fracture healing was also histologically evaluated with toluidine blue staining. The degree of fracture healing at week 8 was assessed by the classification of Allen et al. [29] and was significantly higher in the Hi group compared with the other groups, as well as the Mid group compared with the Lo and PBS groups (Hi, 3.8 ± 0.13; Mid, 2.1 ± 0.16; Lo, 0.4 ± 0.48; PBS, 0.0 ± 0.00, respectively; $p < .01$ for Hi vs. Lo or PBS group and Mid vs. PBS group; $p < .05$ for Hi vs. Mid group and Mid vs. Lo group) (Fig. 6C, 6D). These results indicate that a nonhealing femoral fracture in a rat created by periosteal cauterization may be healed in a dose-dependent manner by local administration of human GM-PB CD34+ cells.

DISCUSSION

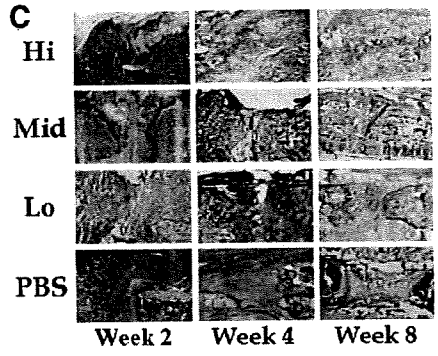
Although most fractures typically heal with callus formation that bridges the fracture gap, a significant proportion (5%–10%) of fractures fail to heal and result in delayed union or persistent nonunion, caused mainly by inappropriate neoangiogenesis [30–33]. The importance of angiogenesis in bone formation/fracture healing has been noted since as early as 1763 [30], and adequate blood supply has been considered to be a key contributor to the osteogenic process [34].

In various fields of regenerative medicine, therapeutic neovascularization induced by EPC transplantation has been preclinically and even clinically tested in ischemic diseases, and promising outcomes have been reported [11–17]. Apart from potential for vasculogenic induction, adult human CD34+ cells, an EPC/hematopoietic stem cell (HSC)-enriched population, are also capable of differentiation into cardiomyocytes in vitro [35] and into cardiomyocytes and smooth muscle cells in vivo [11].



B

Period after fracture	GM-PB CD34+ cells			PBS
	High	Middle	Low	
Week 2	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)
Week 4	3/9 (33%)	1/9 (11%)	0/9 (0%)	0/9 (0%)
Week 8	6/6* (100%)	3/6* (50%)	0/6 (0%)	0/6 (0%)



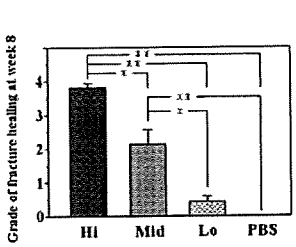


Figure 6. Radiographical and histological evidence of fracture healing following CD34+ cell transplantation. **(A):** Representative radiographs of fractured sites at weeks 0, 2, 4, and 8 in each group. **(B):** The fracture healing ratio in all groups. At week 8, in 100% of animals receiving a Hi dose of CD34+ cells and in 50% of animals receiving a Mid dose of CD34+ cells, the fracture radiographically healed with bridging callus formation. Fracture sites in all animals receiving a Lo dose of CD34+ cells or PBS showed no bridging callus formation and fell into nonunions, an outcome consistent with the previous report showing natural course of this animal model. *, $p < .05$. **(C, D):** Histological evaluation with toluidine blue staining demonstrated enhanced endochondral ossification consisting of numerous chondrocytes and newly formed trabecular bone at week 2, bridging callus formation at week 4, and complete union at week 8 in animals receiving Hi and Mid doses of CD34+ cells. In contrast, although a thick callus formation was observed at week 2, the healing process stopped by week 4, and finally the callus was absorbed at week 8 in animals receiving a Lo dose of CD34+ cells or PBS. The degree of fracture healing assessed by the classification of Allen et al. [29] was significantly higher in the Hi group than in the other groups at week 8, as well as the Mid group compared with the Lo and PBS groups. **, $p < .01$; *, $p < .05$. Abbreviations: GM-PB, granulocyte colony-stimulating factor-mobilized peripheral blood; Hi, 10^5; Lo, 10^3; Mid, 10^4; PBS, phosphate-buffered saline.

Human circulating CD133+ cells and BM CD34+ cells, both of which are EPC/HSC-enriched fractions, have been reported to differentiate into OBs in vitro [20–21]. These findings suggest that human CD34+ cells obtained from BM or PB may have the potential to differentiate into not only hematopoietic and endothelial lineages but also mesenchymal lineages, including osteogenic cells. On the basis of these studies, we previously investigated and reported the efficacy of i.v. transplantation of human circulating CD34+ cells for morphological and physiological recovery from unhealing fracture [22].

In the present study, we successfully confirmed the in vitro differentiation of human GM-PB CD34+ cells into OBs, which show matrix mineralization and calcium deposition, as well as the expression of hOC and hCol1A1 mRNA. Following our previous study, the efficacy of local transplantation of adult human GM-PB CD34+ cells was also examined in an unhealing fracture model of nude rats. To compare the incorporation efficiency between local and systemic transplantation, the human cells located in fracture sites 1 week after cell administration were detected by immunostaining for HLA-ABC. Quantification of the histological staining revealed 2.7-fold and 2.6-fold more incorporation of GM-PB CD34+ cells in the granulation area and the newly formed bone area following local transplantation with atelocollagen compared with i.v. infusion. Although homing efficiency of CD34+ cells after systemic infusion in this study was similarly impressive to that in previous reports of hind limb ischemia [14], myocardial infarction [13], and fracture [22], the cell recruitment was more efficient following local transplantation compared with systemic infusion. The result suggests that local transplantation may overcome the critical issue of CD34+ cell scarcity in clinical situations. Therefore, the effect of local transplantation of not only Hi, which was the same dose as in the previous study for i.v. infusion [22] but also Mid and Lo GM-PB CD34+ cells was examined in this study. Immunohistochemistry and RT-PCR analysis for human-specific markers revealed that direct vasculogenesis and osteogenesis by transplanted GM-PB CD34+ cells were detected in the Hi and Mid groups but not in the Lo and PBS groups. In regards to the paracrine effects of the transplanted cells on the recipient cells, immunostaining for rat-specific markers indicated significant enhancement of intrinsic angiogenesis and osteogenesis in the Hi and Mid groups but not in the other groups. We hypothesized that various cytokines and growth factors secreted from GM-PB CD34+ cells may exert a paracrine effect for intrinsic angio-osteogenesis. Endogenous vascular endothelial growth factor (VEGF) is one of the most important molecules for endochondral bone formation [36–38], and VEGF is expressed in the same temporal and spatial pattern in the fracture callus as occurs during bone development [39, 40]. VEGF activity is essential for normal angiogenesis and appropriate callus architecture and mineralization in response to bone injury [41–43]. Another growth factor, bone morphogenic protein (BMP), is also a key molecule for the process of fracture healing [44–46]. BMPs are newly synthesized by callus-forming cells near the fracture site, and BMP-2 stimulates angiogenesis via upregulation of VEGF at the fracture sites [47]. In this study, real-time RT-PCR analysis revealed overexpression of rVEGF and rBMP-2 at the perifracture sites of the Hi and Mid groups, which may be one of the mechanisms underlying the intrinsic angio-osteogenesis. Physiological assessment by LDPI showed significant recovery of blood flow at the fracture sites in the Hi and Mid groups but not in the other

groups. Finally, radiological healing of the fractures was observed only in the Hi and Mid groups. The frequency of the healing was 100% in the Hi group, 50% in the Mid group, and 0% in the Lo and PBS groups at week 8. This radiological outcome was consistent with histological evaluation of the fracture healing by the classification of Allen et al. [29]. These findings strongly suggest that local transplantation of GM-PB CD34+ cells may have the potential to repair fractures by autocrine and paracrine mechanisms of neovascularization and osteogenesis. Most importantly, the significant efficacy of the local transplantation of GM-PB CD34+ cells is found at doses equal to or more than middle dose, which is lower than the effective dose of i.v. infusion found in the previous study [22]. In contrast, transplantation of the low dose of human CD34+ cells did not significantly contribute to vasculo-osteogenesis for fracture repair. The results regarding dose effects in this preclinical study might provide helpful information for establishing a clinical strategy for this novel modality.

The mechanism of multilineage differentiation potential in human CD34+ cells into ECs and OBs is still being investigated. However, single-cell PCR assessment indicated the expression of OC in 4 of 20 CD34+ cells in our previous study [22], suggesting that direct differentiation of human CD34+ cells into OBs may contribute to the multipotent plasticity even at a low rate. Microenvironmental interaction between vascular and osteoblastic lineage cells through paracrine regulatory factors and direct cellular communications may also be involved in developing CD34+ cells and may be conducive to fracture healing. We speculate that an enhanced vasculogenesis signal may cause the cellular commitment and development of CD34+ cells into osteoblastic cells as a cooperative organogenesis mechanism.

Several research groups have demonstrated the usefulness of local transplantation of total BM cells for fracture healing [48–51]. Hernigou et al. reported that in 88% of patients with noninfected nonunions of the tibia, bone union was achieved by percutaneous grafting of autologous total BM cells accompanied by external fixation or cast immobilization [51]. Compared with transplantation of purified CD34+ cells, total BM cell transplantation does not require a magnetic cell sorting process, indicating that time and cost of the cell preparation can be diminished. However, our group recently reported that intramyocardial transplantation of human GM-PB total MNCs represents a possible risk of severe hemorrhagic myocardial infarction in nude rats through the excessive inflammation induced by abundant infiltration of hematopoietic cells [52]. Thus, additional preclinical/clinical studies would be warranted to compare the feasibility, safety, and efficacy of both strategies for bone repair.

Concerning future clinical application, the biological risk of G-CSF may also become a problem. G-CSF has been used in thousands of clinical cases; however, severe complications, such as spleen rupture, interstitial pneumonitis, and acute coronary syndrome, are rarely reported [53]. This rare but potential risk of G-CSF, as well as the high cost of CD34+ cell isolation, would need to be overcome in the future.

CONCLUSION

In conclusion, the present findings suggest that local transplantation of GM-PB CD34+ cells could be a promising clinical strategy for enhancing bone repair in patients suffering from unhealing fracture.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Specific Jagged-1 Signal From Bone Marrow Microenvironment Is Required for Endothelial Progenitor Cell Development for Neovascularization

Sang-Mo Kwon, Masamichi Eguchi, Mika Wada, Yo Iwami, Katsuhito Hozumi, Hideki Iwaguro, Haruchika Masuda, Atsuhiko Kawamoto and Takayuki Asahara

Circulation 2008;118;157-165; originally published online Jun 30, 2008;
DOI: 10.1161/CIRCULATIONAHA.107.754978
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Specific Jagged-1 Signal From Bone Marrow Microenvironment Is Required for Endothelial Progenitor Cell Development for Neovascularization

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Background—Despite accumulating evidence that proves the pivotal role of endothelial progenitor cells (EPCs) in ischemic neovascularization, the key signaling cascade that regulates functional EPC kinetics remains unclear.

Methods and Results—In this report, we show that inactivation of specific Jagged-1 (Jag-1)–mediated Notch signals leads to inhibition of postnatal vasculogenesis in hindlimb ischemia via impairment of proliferation, survival, differentiation, and mobilization of bone marrow–derived EPCs. Bone marrow–derived EPCs obtained from *Jag-1*$^{-/-}$ mice, but not Delta-like (*Dll*)-*1*$^{-/-}$ mice, demonstrated less therapeutic potential for ischemic neovascularization than EPCs from the wild type. In contrast, a gain-of-function study using 3T3 stromal cells overexpressing Notch ligand revealed that Jag-1–mediated Notch signals promoted EPC commitment, which resulted in enhanced neovascularization. The impaired neovascularization in *Jag-1*$^{-/-}$ mice was profoundly rescued by transplantation of Jag-1–stimulated EPCs.

Conclusions—These data indicate that specific Jag-1–derived Notch signals from the bone marrow microenvironment are critical for EPC–mediated vasculogenesis, thus providing an important clue for modulation of strategies for therapeutic neovascularization. (***Circulation.*** **2008;118:157-165.)**

Key Words: angiogenesis ■ progenitor cells ■ ischemia ■ signal transduction

Growing evidence indicates that the perturbation of Notch signaling leads to dysfunctional behavior of the vascular system.[1] A human degenerative vascular disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), is related to mutations in the Notch3 receptor. Alagille syndrome, caused by mutation of the *Jagged-1* (*Jag-1*) gene, is a pleiotropic developmental disease that is accompanied by features of congenital heart defects with cardiovascular anomalies.[2] Murine genetic studies that generate loss or gain of function of Notch receptors or ligands have exhibited abnormalities in blood vessel formation, such as impaired proliferation and migration of endothelial cells (ECs)[3] and arterial-venous identification.[4–7] These findings indicate the involvement of Notch1,[7] Notch3,[8] and Notch4[7] receptors, as well as Delta-like ligand (Dll)-4[4,5] and Jag-1[9] ligands, in vascular formation. Recently, Notch ligand, especially Dll-4, has been focused on as an essential regulator for tumor angiogenesis and vascular development in terms of ligand signal control from tissue environment for EC bioactivity through Notch receptors.[10,11]

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Although pioneered in the field of vascular biology, especially in terms of EC morphogenesis for blood vessel development and EC determination of arterial-venous specification, the role of Notch signal in stem cell–related postnatal vasculogenesis has not been investigated. Endothelial progenitor cells (EPCs) derived from bone marrow (BM) play an important role in the promotion of vascular and tissue repair in physiological and pathological conditions, such as coronary or peripheral vascular diseases.[12–14] BM-derived EPCs are committed and differentiated from hemangioblastic stem cells,[15–17] a common stem cell for EPCs and hematopoietic stem cells (HSCs), into endothelial lineage and mobilized from BM into circulating blood, then recruited into sites of ischemia and interaction with tissue-specific cells to regenerate blood vessels in organs. Because vasculogenesis is essential for adult neovascularization,[12–14] and given the angiogenesis mechanism, the Notch ligand/receptor systems could play a key role in the functional kinetics of BM-derived EPCs.

Received November 25, 2007; accepted April 18, 2008.

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The online-only Data Supplement, consisting of Methods and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.754978/DC1.

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Circulation **is available at http://circ.ahajournals.org**

DOI: 10.1161/CIRCULATIONAHA.107.754978

In BM, Notch ligands, especially Jag-1 and Dll-1, are expressed mainly by osteoblasts, stromal cells, ECs, and hematopoietic stem/progenitor cells.[3,18–20] These cells consist of microenvironmental niches for HSC self-renewal and commitment for hematopoietic maintenance, which has been of great interest recently.[21–24] The interaction between osteoblasts that express Notch ligands and HSCs that express Notch receptors is considered to be one of the key molecular mechanisms underlying the regulation of HSC function in the BM niche.

Considering the common origin and localization of HSCs and EPCs,[15–17] we were interested in controlling EPC maintenance and kinetics by modulating Notch signals in BM niches. EPC proliferation, commitment from hemangioblast, differentiation as an endothelial phenotype, and mobilization into circulation for vascular maintenance could be regulated by certain pathways triggered by specific Notch ligand–mediated signals in BM environments. The purpose of the present study was to investigate the role of specific Notch ligands, Jag-1 and Dll-1, on EPC biology in BM through a loss-of-function study using conditional knockout mice and a gain-of-function study using a coculture system with a gene-modified stromal cell line.

Methods

Animals and Stromal Cell Line

Conditional *Jag-1*$^{-/-}$ mice (loxP/loxP, mxCre) or conditional *Dll-1*$^{-/-}$ mice (loxP/loxP, mxCre) were generated as reported previously.[20,25] For gene targeting, polyinosinic:polycytidylic acid (poly I:C; 200 μg/200 μL) was administered intravenously 4 times over a period of 12 days (once every 3 days). For the gain-of-function study, 3T3 stromal cells in which *Jag-1*, *Dll-1*, or empty vector was transduced retrovirally were cultured in DMEM with 10% fetal bovine serum.

Evaluation of EPC Bioactivity: EPC Colony Assay, Migration Assay, Proliferation Assay, Apoptosis, and Gene or Protein Assay

After BM c-kit$^+$/Sca-1$^+$/lineage (Lin)$^-$ cells (KSLs) and peripheral blood (PB)–mononuclear cells were isolated, we performed an EPC colony-forming assay, recently established in our laboratory. To investigate the different functions of EPCs under various conditions, a migration assay, proliferation assay, apoptosis assay, and expression analysis of both gene and protein were performed.

Evaluation of EPC Kinetics in the Hindlimb Ischemia Model

A hindlimb ischemia model was generated to evaluate in vivo EPC functions, such as capacity for blood vessel regeneration, mobilization from BM, incorporation into sites of neovascularization, and survival of endogenous cells. A more detailed and expanded description of the materials and methods used is provided in the online-only Data Supplement.

Statistical Analysis

All data are presented as mean±SEM. The results were analyzed statistically with the use of the software package Statview 5.0 (Abacus Concepts Inc, Berkeley, Calif). A paired *t* test was performed to compare the bromodeoxyuridine (BrdU) incorporation rate of EPCs before and after hindlimb ischemia. Scheffé's test was performed for multiple comparisons after ANOVA between each group. A P value <0.05 was considered to denote statistical significance.

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

Loss of Jag-1–Mediated Notch Signal Impairs EPC Commitment and Mobilization From BM

To prove the significance of Notch ligand for EPC biology, we analyzed EPC kinetics in conditional *Jag-1* or *Dll-1* null mice, which were generated by cre/loxP systems and induced in a timely manner by administration of poly I:C in postnatal stages. In this system, reverse-transcription polymerase chain reaction revealed that expression of *Jag-1* or *Dll-1* was decreased drastically in BM stromal cells (Figure 1A) and KSLs (online-only Data Supplement Figure Ib) in *Jag-1* null or *Dll-1* null mice, respectively, compared with wild-type mice. In contrast, no significant differences between *Jag-1* null, *Dll-1* null, and wild-type mice were found with regard to expression levels of Notch receptors in BM stromal cells (online-only Data Supplement Figure Ia) or KSLs (online-only Data Supplement Figure Ic). The frequency of KSLs in BM-Lin$^-$ cells was similar in *Jag-1* null, *Dll-1* null, and WT mice (Figure 1B). Flow cytometry analysis of BM mononuclear cell samples demonstrated that the frequency of Flk-1 (VEGFR2 [vascular endothelial growth factor receptor-2])$^+$/CD31$^+$ or Flt-1 (VEGFR1)$^+$/CD31$^+$ cells in Sca-1$^+$/Lin$^-$ cells, the EPC-enriched cells, decreased significantly in *Jag-1*$^{-/-}$ mice compared with *Dll-1*$^{-/-}$ mice and wild-type mice (Figure 1C). To determine whether the impaired EPC commitment was accompanied by a defect in vasculogenic capacity in *Jag-1*$^{-/-}$ mice, BM KSLs from *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, or wild-type mice were allowed to form a cluster of EPC colonies with spindle-like morphology. KSLs from *Jag-1*$^{-/-}$ mice indeed formed fewer EPC colonies than wild-type KSLs, although the absence of *Dll-1* in KSLs did not lead to significant defects in vasculogenic capacity (Figure 1D). The effect of Notch signaling on the vasculogenic capacity of EPCs was also evaluated by experimental inhibition of Notch signals in BM-KSLs with γ-secretase II, which blocks the cleavage steps of the intracellular domain of Notch receptors, revealing that the inhibition of Notch signals resulted in a significant decrease in EPC colony-formation activity (online-only Data Supplement Figure II).

To evaluate the kinetics of EPCs mobilized from BM, PB mononuclear cells were isolated and analyzed by both EPC culture assay and EPC colony-forming assay. Importantly, the EPC culture assay indicated that the number of attached EPCs that represented uptake of acetylated LDL and expression of the endothelial markers isolectin B4, Flk-1 (VEGFR2), and/or endothelial nitric oxide synthase was significantly less in *Jag-1*$^{-/-}$ mice than in either *Dll-1*$^{-/-}$ or wild-type mice (online-only Data Supplement Figures IIIa, IIIb, and IIIc). The EPC colony-forming assay also demonstrated the significantly impaired vasculogenic capacity of PB mononuclear cells in *Jag-1*$^{-/-}$ mice compared with *Dll-1*$^{-/-}$ or wild-type mice (online-only Data Supplement Figure IIId).

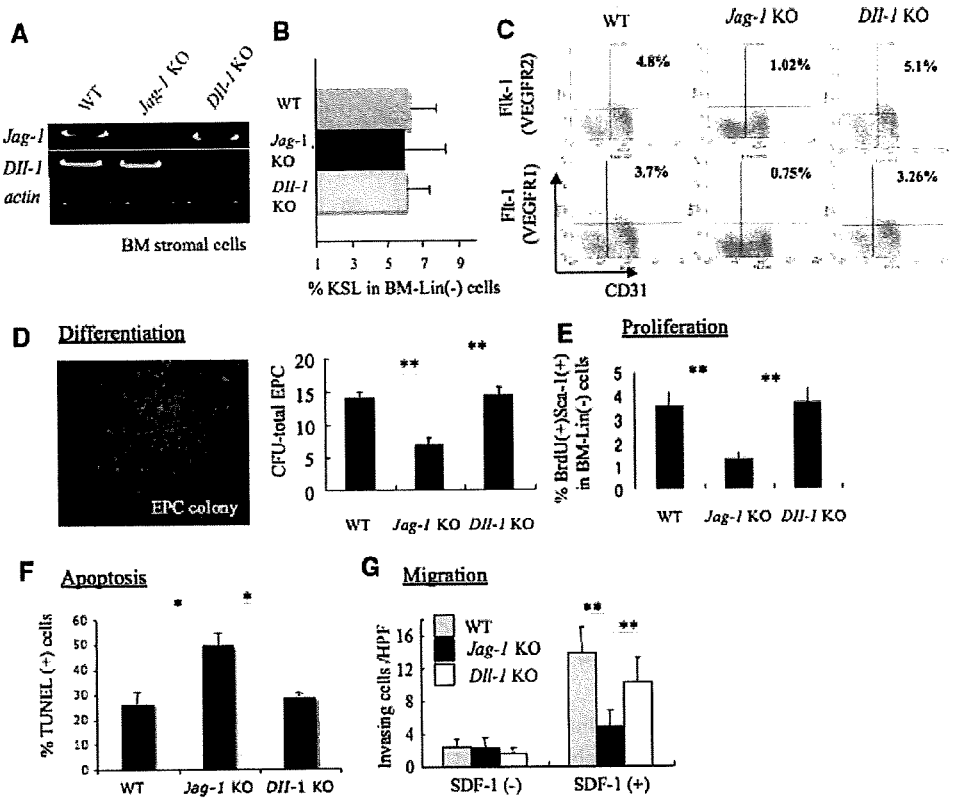

Figure 1. Impairment of endothelial commitment, proliferation, and invasiveness of EPCs in BM of conditional *Jag-1*$^{-/-}$ mice. A, Generation of conditional knockout (KO) mice lacking *Jag-1* or *Dll-1* gene. Reverse-transcription polymerase chain reaction confirmed specific deletion of *Jag-1* or *Dll-1* mRNA expression in BM stromal cells obtained from each of the KO mice. WT indicates wild type. B, Frequency of KSLs in BM was similar in WT, *Jag-1* KO, and *Dll-1* KO mice (n=3 in each group). C, Fluorescence-activated cell sorting analysis for CD31 and Flt-1 (VEGFR1) or Flk-1 (VEGFR2) using BM Sca-1$^+$/Lin$^-$ cells obtained from WT, *Jag-1* KO, or *Dll-1* KO mice. Frequency of Flt-1$^+$/CD31$^+$ or Flk-1$^+$/CD31$^+$ cells, which are EPC-enriched populations, in Sca-1$^+$/Lin$^-$ cells was drastically lower in *Jag-1* KO mice but not in *Dll-1* KO mice compared with WT mice (n=3 in each group). D, EPC colony-forming assay was performed by incubation of BM-KSLs from WT, *Jag-1* KO, or *Dll-1* KO mice in methyl cellulose–containing medium, supplemented by several cytokines as the driving force for endothelial differentiation. Left, Representative EPC colony clusters with a spindlelike morphology; Right, significant inhibition of EPC colony-forming capacity in *Jag-1* KO mice but not *Dll-1* KO mice compared with WT mice. CFU indicates colony-forming unit. **P<0.01 (n=3 in each group). E, BrdU proliferation assay was performed with BM Sca-1$^+$/Lin$^-$ cells of WT, *Jag-1* KO, or *Dll-1* KO mice 14 days after induction of hindlimb ischemia. Frequency of BrdU$^+$/Sca-1$^+$ cells in BM-Lin$^-$ cells was significantly lower in *Jag-1* KO mice but not *Dll-1* KO mice compared with WT mice. **P<0.01 (n=5 per group). F, Frequency of TUNEL-positive cells in BM Sca-1$^+$/Lin$^-$ cells 3 days after hindlimb ischemia was significantly greater in *Jag-1* KO mice than in *Dll-1* KO and WT mice. *P<0.05 (n=3 in each group). G, In vitro invasiveness assay with BM Sca-1$^+$/Lin$^-$ cells from WT, *Jag-1* KO, or *Dll-1* KO mice 3 days after hindlimb ischemia. Number of cells invading into methyl cellulose was significantly lower in *Jag-1* KO mice but not *Dll-1* KO mice compared with WT in the presence of stromal cell–derived factor-1 (SDF-1), although invasiveness activity was similar in all groups in the absence of stromal cell–derived factor-1. HPF indicates high-power field. **P<0.01 (n=4 per group).

These findings suggest that Notch signaling, especially the Jag-1–mediated signal, is crucial for EPC commitment and mobilization in BM.

Loss of Jag-1–Mediated Notch Signal in BM Impairs EPC Bioactivities In Vitro

To test the effect of switching off Notch signals on ischemia-induced EPC proliferation, we examined the frequency of BrdU$^+$ cells in Sca-1$^+$/Lin$^-$ cells obtained from *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, or wild-type mice before and 4 days after hindlimb ischemia. Ischemia partially induced BrdU incorporation in wild-type EPCs (preischemia 1.71±0.45%, postischemia 3.4±0.55%, P<0.01) and *Dll-1* null EPCs (preischemia 1.65±0.52%, postischemia 3.51±0.43%, P<0.01); however, such an ischemia-induced effect was not observed in *Jag-1* null EPCs (preischemia 1.52±0.32%, postischemia 1.72±0.65%, P=NS; Figure 1E). These data support the hypothesis that Jag-1–mediated signals are critical for the proliferation of EPC-enriched cells in response to ischemia.

To clarify the effect of Notch signals on the survival potential of EPCs, we performed an in vitro terminal dUTP nick end-labeling (TUNEL) assay using BM Sca-1$^+$/Lin$^-$ cells obtained from *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, or wild-type mice 4 days after hindlimb ischemia. As shown in Figure 1F, the frequency of apoptotic cells in EPC-enriched cells was significantly greater in *Jag-1*$^{-/-}$ mice than in *Dll-1*$^{-/-}$ or wild-type mice.

To further investigate the modulation of BM EPC biology by Notch signals, we performed an in vitro invasiveness assay, a modified Boyden chamber invasiveness analysis. The assay exhibited marked impairment of the stromal

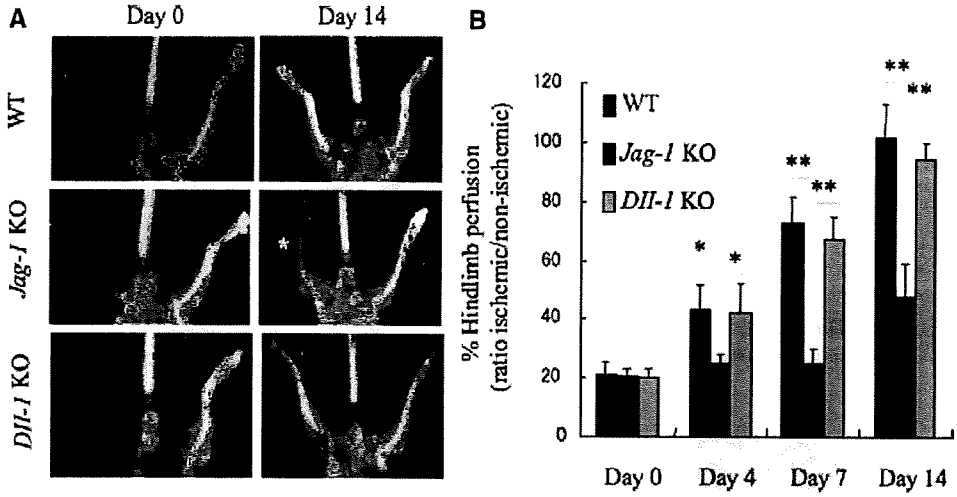

Figure 2. Delayed and impaired postnatal revascularization in *Jag-1* knockout (KO) mice. A, Representative findings of laser Doppler perfusion imaging in wild-type (WT), *Jag-1* KO, and *Dll-1* KO mice immediately after (Day 0) and 14 days after (Day 14) hindlimb ischemia. Recovery of blood flow at day 14 was impaired in a *Jag-1* KO mouse. B, Assessment of % hindlimb perfusion (percent ratio of perfusion in ischemic hindlimb to that in contralateral nonischemic hindlimb) by laser Doppler perfusion imaging revealed significant impairment of perfusion recovery at days 4, 7, and 14 after induction of ischemia in *Jag-1* KO mice but not *Dll-1* KO mice compared with WT. $^{*}P<0.05$, $^{**}P<0.01$ (n=5 per group).

cell–derived factor-1–induced invasiveness in *Jag-1* null EPCs but not *Dll-1* null EPCs (Figure 1G), which implies that Jag-1–mediated Notch signals play an important role in the motility of EPCs in the BM microenvironment. Thus, Jag-1–mediated Notch signals are crucial for various EPC functions such as proliferation, antiapoptosis, and invasiveness.

Loss of Jag-1–Mediated Notch Signals in BM Attenuates EPC Contribution for Vasculogenesis In Vivo

To test the involvement of Notch signals in vascular regeneration, we induced hindlimb ischemia by ligating the femoral arterial structure in conditional *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, or wild-type mice. The recovery of hindlimb perfusion was delayed significantly by inactivation of Jag-1–mediated but not Dll-1–mediated Notch signals (Figures 2A and 2B), which suggests a specific role for Jag-1–mediated Notch signals in ischemic neovascularization.

To evaluate the possible role and contribution of Notch signals for EPC function in ischemic recovery in vivo, we next transplanted the EPC-enriched cells (Sca-1^{+}/Lin^{-} cells) obtained from BM of *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, or wild-type mice into nude mice with hindlimb ischemia. Transplantation of the BM-EPCs from wild-type or *Dll-1*$^{-/-}$ mice significantly improved hindlimb perfusion compared with PBS injection. In contrast, BM-EPCs from *Jag-1*$^{-/-}$ mice failed to augment hindlimb perfusion (Figure 3A). Histological assessment of capillary density also revealed enhanced neovascularization after transplantation of EPCs from wild-type or *Dll-1*$^{-/-}$ mice but not *Jag-1*$^{-/-}$ mice (Figure 3B). These findings strongly indicate the essential role of Jag-1–mediated Notch signaling in BM-EPCs with regard to their vasculogenic potential in ischemic diseases.

Gain of Jag-1–Mediated Notch Signal From Stromal Cells Stimulates BM-EPC Commitment and Differentiation

The present data from loss-of-function studies indicate that Notch systems could regulate the kinetics of BM-EPCs for vasculogenesis. To further confirm the critical role of Notch signals from BM microenvironments for EPC bioactivity, we established an insert culture system by coculturing BM Lin^{-} cells together with 3T3 stromal cells overexpressing Notch ligand, Jag-1, or Dll-1 (Figure 4A), in which activation of each Notch signal was confirmed by reverse-transcription polymerase chain reaction analysis (Figure 4B and 4C) and luciferase assay (data not shown). This analysis revealed that expression of Notch receptor 1, 2, 3, and 4 was similar after coculture with either of the Notch ligand–expressing stromal cells (online-only Data Supplement Figure IVb).

To assess the effect of Notch ligand signaling on EPC differentiation, the percentage of BM-Lin^{-} cells positive for CD31 and Flk-1 (VEGFR 2), which are typical EPC markers, was determined by flow cytometric analysis. Importantly, the population of CD31^{+}/Flk-1^{+} cells was remarkably increased in BM Lin^{-} cells stimulated by Jag-1–mediated signals but not Dll-1–mediated signals (Figure 4D). The signal intensity of CD31 and Flk-1 in BM Lin^{-} cells also increased after stimulation with Jag-1–mediated signals but not Dll-1–mediated signals (online-only Data Supplement Figure Va). Moreover, the cellular mRNA level of EPC markers such as CD31, Flk-1, or vascular endothelial cadherin was elevated in the Jag-1 group compared with the Dll-1 and empty-vector groups. In contrast, the cellular mRNA level of vascular endothelial growth factor was similar in all groups (Figure 4E). To obtain more concrete evidence for enhancement of EPC differentiation by Notch signals, we performed in vitro EPC culture assay using BM-Lin^{-} cells cocultured with stromal cells expressing various Notch ligands. Fluorescent

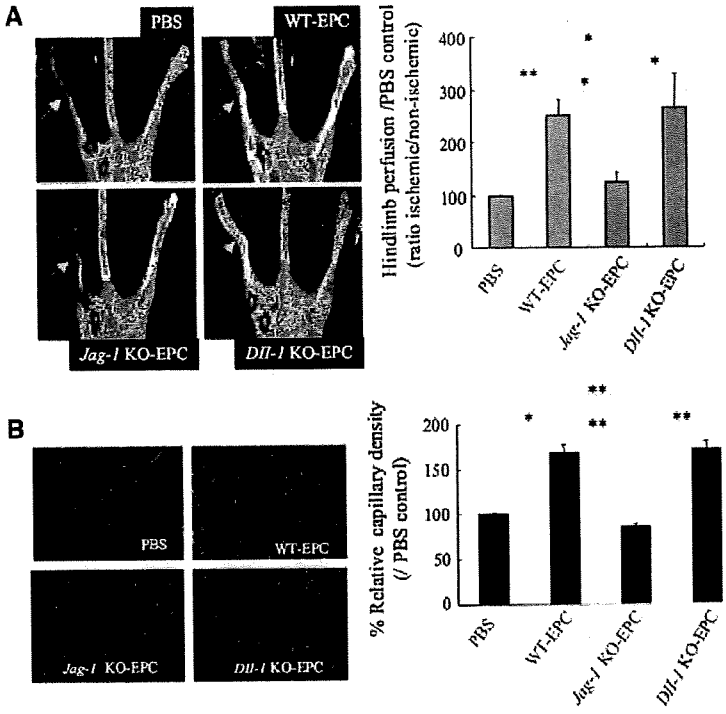

Figure 3. Effect of conditional deletion of distinct Notch signals on the therapeutic potential of EPCs for ischemic neovascularization. A, Representative laser Doppler perfusion imaging findings in nude mice 14 days after hindlimb ischemia and infusion of PBS or BM Sca-1$^+$/Lin$^-$ cells (EPC-enriched population) obtained from wild-type (WT), *Jag-1* knockout (KO), or *Dll-1* KO mice (Left). Arrows show ischemic hindlimbs in each group. Recovery of ischemic hindlimb perfusion on day 14 was significantly greater in mice receiving WT EPCs or *Dll-1* KO EPCs than in mice receiving PBS. In contrast, infusion of *Jag-1* KO EPCs did not contribute significantly to perfusion recovery (Right). *$P<0.05$; **$P<0.01$ (n=8 per group). B, Representative isolectin B4 chemical staining in the ischemic hindlimb tissue of nude mice 28 days after infusion of PBS or EPCs from WT, *Jag-1* KO, or *Dll-1* KO mice (Left). Capillary density was significantly greater in mice receiving WT-EPCs or *Dll-1* KO-EPCs than in mice receiving PBS, whereas transplantation of *Jag-1* KO EPCs did not significantly increase capillary density (Right). *$P<0.05$; **$P<0.01$ (n=6 per group).

microscopic examination revealed that the number of cells demonstrating both acetylated LDL uptake and isolectin B4 binding was significantly greater in the Jag-1 group than in the empty-vector group, whereas in the Dll-1 group, the number was comparable to that in the control group (online-only Data Supplement Figure Vb). EPC colony-forming assay also clearly disclosed that specific induction of Jag-1–mediated signals but not Dll-1–mediated signals contributed significantly to enhancement of the vasculogenic activity of BM-KSLs, which are considered to be the putative origin of EPCs in mice (Figure 4F). TUNEL staining further indicated that Jag-1–mediated, not Dll-1–mediated, signals significantly inhibited the apoptosis of the cultured EPCs (Figure 4G). Importantly, Jag-1–derived signals enabled the BM-Lin$^-$ cells to form a tubelike structure just 4 days after coculture. In contrast, Dll-1–derived signals, as well as empty-vector–derived signals, did not affect the morphological features of the EPC-enriched cells (online-only Data Supplement Figure IVa). These data indicate that Jag-1–mediated Notch signal augments the commitment and differentiation of BM stem/progenitor cells toward endothelial lineage.

Gain of Jag-1–Mediated Notch Signal Promotes Vasculogenic Property of BM-EPCs

To explore the effects of gain of function from Notch signals on the therapeutic potential of EPCs, we serially examined perfusion recovery after hindlimb ischemia and transplantation of BM-Lin$^-$ cells in which Notch signals were stimulated by coculturing with 3T3 stromal cells. Laser Doppler perfusion imaging revealed that recovery of blood flow in the ischemic hindlimb was significantly enhanced by transplantation of EPC-enriched cells stimulated by Jag-1–mediated but not Dll-1–mediated signals compared with infusion of PBS or empty-vector–transduced EPCs (Figure 5A). The favorable effect of stimulating Jag-1–mediated signal was also confirmed by histological assessment of capillary density (Figure 5B). Thus, augmentation of Jag-1–mediated signal may specifically enhance the therapeutic potential of the BM EPC-enriched fraction for ischemic neovascularization.

Homing of EPCs to sites of ischemia is an essential step for neovascularization. Therefore, we examined the effect of specific Notch ligand stimulation on the incorporation of putative EPCs into blood vessels of ischemic tissues. BM-Lin$^-$ cells obtained from GFP (green fluorescent protein) transgenic mice, cocultured with stromal cells overexpressing the distinct Notch ligand, were infused intravenously into nude mice with hindlimb ischemia. Histochemical staining for CD31, a typical marker of endothelial cells, revealed significantly abundant incorporation of GFP$^+$/CD31$^+$ cells into ischemic tissue in the Jag-1 group but not the Dll-1 group compared with the empty-vector and PBS groups (Figure 5C).

Finally, we examined the therapeutic potency of Jag-1– or Dll-1–stimulated EPCs in *Jag-1*$^{-/-}$ mice with hindlimb ischemia, which is a model of Alagille syndrome and represents severe impairment of ischemic neovascularization. Recovery of hindlimb perfusion was augmented significantly in both the Dll-1– and empty-vector–stimulated EPC groups compared with the PBS group. Notably, perfusion recovery in *Jag-1*$^{-/-}$ mice was further enhanced after transplantation of Jag-1–stimulated EPCs compared with infusion of Dll-1– or empty-vector–stimulated EPCs (Figure 6A and 6B). These data provide critical evidence that augmentation of specific Jag-1–mediated signaling, not Dll-1–mediated signaling, from stromal cells enhances the vasculogenic potential of BM-EPCs for ischemic recovery.

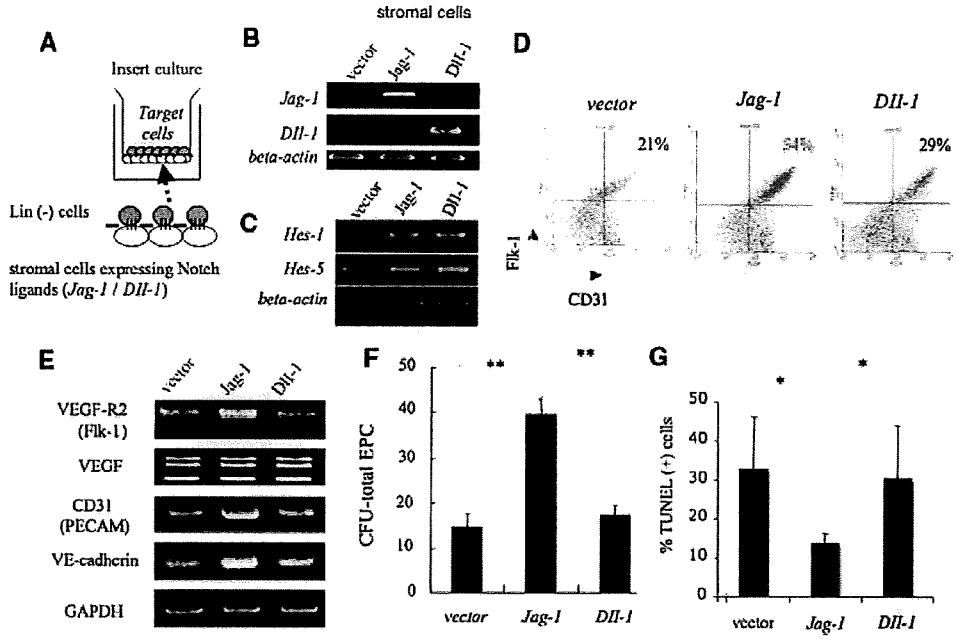

Figure 4. Effects of gain of function of Notch ligand–mediated signals on EPC differentiation. A, Scheme of the insert culture system with 0.4-μm pores, in which receptors on the surface of the target stem cells in the upper chamber are capable of directly interacting with ligands on the cells in the bottom chamber. In the present study, BM-Lin$^-$ cells were seeded in the upper chamber, whereas 3T3 stromal cells overexpressing a specific Notch ligand, *Jag-1* or *Dll-1*, were placed in the bottom chamber. B, Reverse-transcription polymerase chain reaction revealed enhanced expression of the specific Notch ligand in the stromal cells stimulated by Jag-1 or Dll-1 signal compared with those transduced with empty vector. C, Reverse-transcription polymerase chain reaction revealed expression of Hes-1 and Hes-5, target effector genes of active Notch, in BM-Lin$^-$ cells, which were cocultured with 3T3 stromal cells specifically expressing the target Notch ligand gene. D, Fluorescence-activated cell sorting analysis revealed more frequent expression of Flk-1 and CD31 in BM-Lin$^-$ cells stimulated by Jag-1–mediated signals, but not Dll-1–mediated signals, than in those stimulated by empty vector. E, Reverse-transcription polymerase chain reaction to detect expression of typical EPC surface markers and vascular endothelial growth factor in BM-Lin$^-$ cells stimulated by specific Notch ligand or empty vector. The cellular mRNA level of CD31, Flk-1, and vascular endothelial cadherin (VE-cadherin) was elevated in the Jag-1 group compared with the Dll-1 and empty-vector groups. In contrast, the cellular mRNA level of vascular endothelial growth factor and Flt-1 was similar in all groups. PECAM indicates platelet and endothelial cell adhesion molecule. F, EPC colony-forming assay using BM-KSLs stimulated by specific Notch ligand–mediated signals revealed significant augmentation of vasculogenic capacity in the Jag-1 group, but not the Dll-1 group, compared with the empty-vector group. CFU indicates colony-forming units. $^{**}P<0.01$ (n=3 in each group). G, Frequency of apoptotic cells (TUNEL-positive cells) in BM Sca-1$^+$/Lin$^-$ cells in vitro was significantly lower in EPC-enriched cells stimulated by Jag-1 signal than in those stimulated by Dll-1 signal or empty vector. $^*P<0.05$ (n=3 in each group).

Discussion

The novel finding in the present study is that the specific Jag-1–mediated Notch signal promotes adult neovascularization by regulating functional kinetics of stem/progenitor cells in the BM microenvironment. We demonstrated that the Jag-1–induced signal evokes EPC commitment and differentiation in an in vitro gain-of-function study, as well as in an in vivo loss-of-function study, which eventually resulted in improvement of ischemia-induced neovascularization. In contrast, the Dll-1–induced Notch signal appeared to be dispensable for both commitment of EPCs in BM and recovery of blood flow from organ ischemia, at least in the postnatal stage, although Limbourg et al[26] recently showed that inadequate Dll-1–induced Notch signal from the embryonic to adult stages appeared to affect arteriogenesis.

In the loss of Jag-1 ligand function, but not Dll-1, we observed (1) fewer BM cells expressing endothelium-specific genes; (2) lower EPC colony-forming ability in BM; (3) less proliferative activity, invasive capacity, and survival bioactivity of the EPC-enriched fraction in BM; (4) impaired neovascularization in ischemic tissue; and (5) impaired potential of therapeutic vasculogenesis after EPC transplantation. A surprising finding in Jag-1 knockout mice was the drastic decrease in functional EPCs (ie, an 80% decrease of Flk-1$^+$/CD31$^+$/Sca-1$^+$/Lin$^-$ cells in BM and >50% reduction in total EPC colony-forming capacity compared with wild-type or Dll-1 knockout mice). The fact that the loss of Jag-1 function resulted in a lower number of EPCs and impaired EPC biological function for vasculogenesis indicates the essential regulatory role of Jag-1 for EPC commitment from stem cells and EPC differentiation to acquire vasculogenic properties in BM.

Several reports have proposed that Notch signaling is actively involved in HSC maintenance/growth in osteoblastic niches in various experimental animal models. A study[23] using transgenic mice constitutively expressing active parathyroid hormone receptor under the control of collagen type IV promoter reported an increase in trabecular bone mass associated with overexpression of a Notch ligand, Jag-1, in osteoblasts. The authors of that report argued that the increase in BM HSCs is a direct consequence of the increased osteoblastic niche area and over-

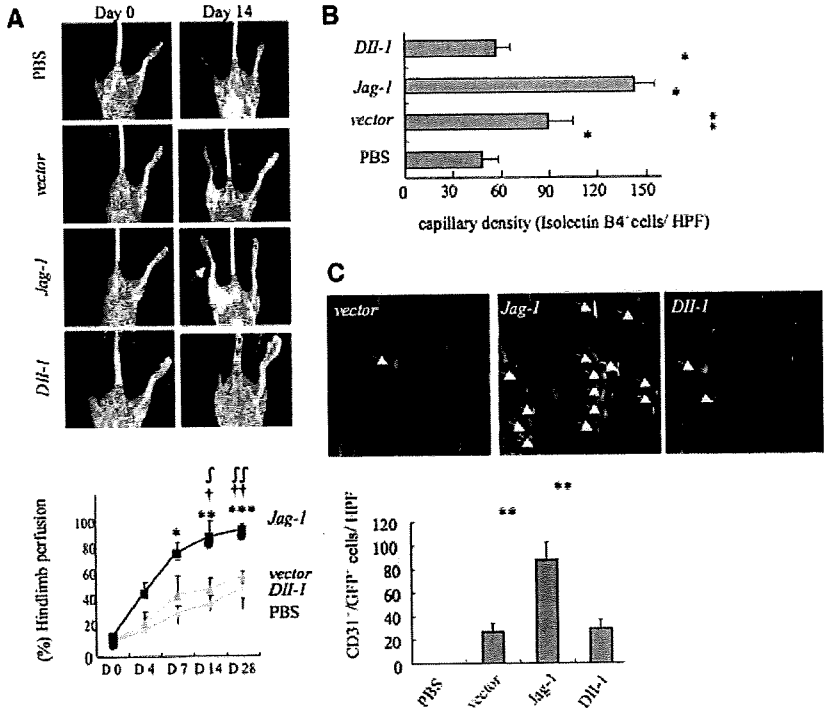

Figure 5. Promotion of in vivo neovascularization by transplantation of putative EPCs stimulated by Jag-1–mediated signals. A, Representative laser Doppler perfusion imaging findings in nude mice receiving PBS (no cells) or BM-Lin$^-$ cells cocultured with empty-vector– or specific Notch ligand (Jag-1, Dll-1)–transfected 3T3 stromal cells at days 0 and 14 (upper panel). Hindlimb perfusion recovery was significantly enhanced in the Jag-1 group compared with the Dll-1, empty-vector, and PBS groups (n=6 per group, lower panel). *P<0.05 vs PBS; **P<0.01 vs PBS; ***P<0.001 vs PBS; †P<0.05 vs vector; ††P<0.01 vs vector; ʃP<0.05 vs Dll-1; ʃʃP<0.01 vs Dll-1. B, Histological capillary density by isolectin B4 staining revealed augmented neovascularization in the Jag-1 group but not the Dll-1 group compared with the PBS group. *P<0.05; **P<0.01 (n=4 per group). HPF indicates high-power field. C, Histological density of putative EPCs (BM-Lin$^-$ cells obtained from GFP transgenic mice) incorporating into vasculature of ischemic tissue. The density of the incorporating EPCs identified as CD31$^+$/GFP$^+$ cells was significantly greater in the Jag-1 group than in the Dll-1, empty-vector, and PBS groups. Green fluorescence indicates GFP; red signal, CD31. *P<0.05; **P<0.01 (n=4 per group).

expression of Jag-1 in the niche cells.[23] Taken together with the present data in the loss-of-function studies, signal transmission between stromal cells expressing a Notch ligand, Jag-1, and EPCs expressing Notch receptors is considered to be the most essential molecular mechanism underlying the differential regulation of EPCs in the stromal niche in BM.

In the present analysis of *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$, and WT mice, we did not observe a significant difference in the number of KSLs (Figure 1B) or their hematopoietic colony-forming capacity (data not shown) among the 3 groups as reported previously.[19] However, the development of EPCs from the stem cell pool evaluated by the in vitro EPC colony-forming assay was significantly impaired only in *Jag-1*$^{-/-}$ mice. These facts suggest that the Jag-1–mediated Notch signal may exist in the marrow structure for specific regulation of EPC kinetics in response to demands of neovascularization, such as ischemic conditions.

To confirm the mechanism of regulating EPC commitment and differentiation in the BM stroma, we used a study of gain of Notch ligand function. An insert coculture system of BM-Lin$^-$ cells together with 3T3 stromal cells overexpressing Notch ligand (Jag-1 or Dll-1) demonstrated that precisely controlled gain of Jag-1 function in vitro promoted (1) endothelium-specific gene expression, (2) activity of EPC colony formation, (3) antiapoptosis bioactivity, (4) activity of both vascular endothelial growth factor–dependent proliferation and migration (online-only Data Supplement Figure Va and Vb), and (5) the potential of therapeutic vasculogenesis of hindlimb ischemia in BM-EPCs. These findings suggest that Jag-1 strongly drives the immature BM population to commit and differentiate into endothelial lineage, whereas

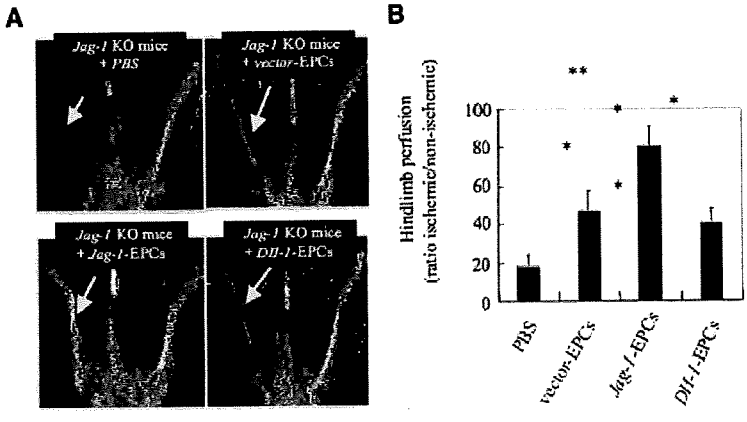

Figure 6. Rescue of impaired neovascularization in *Jag-1* knockout (KO) mice by transplantation of putative EPCs stimulated by specific Notch ligand–mediated signals. A, Representative laser Doppler perfusion imaging findings in *Jag-1* KO mice with hindlimb ischemia receiving PBS or BM-Lin$^-$ cells cocultured with 3T3 cells overexpressing specific Notch ligands at day 14. B, Recovery of hindlimb perfusion was significantly augmented in both the Dll-1– and empty-vector–stimulated EPC groups compared with the PBS group. Notably, perfusion recovery in *Jag-1* KO mice was further enhanced after transplantation of Jag-1–stimulated EPCs compared with Dll-1– or empty-vector–stimulated EPCs. *P<0.05; **P<0.01 (n=6 in each group).

Dll-1 is not involved at all, although downstream signals, such as Hes-1 and Hes-5, are equally stimulated.

The finding that EPCs preconditioned by specific Jag-1–dependent signaling were able to rescue the impaired vasculogenic potential in both athymic nude and *Jag-1* null mice may open a novel gate for enhancing the potential of therapeutic neovascularization. Key mechanisms underlying this favorable phenomenon may be upregulation of EPC functions, including proliferation, differentiation, and migration, by exogenous Jag-1 signal, because the impaired EPC bioactivity in the *Jag-1*–deficient KSLs could be rescued by Jag-1–mediated signals (Figure 4; online-only Data Supplement Figures VI and VII). Another possible mechanism may relate to the rescue signals for prevention of programmed cell death, because the present study indicates the antiapoptotic effect of preconditioning by Jag-1 signal on EPCs (Figure 4G). The antiapoptotic effect of Jag-1 signals was also confirmed in ischemic tissue after transplantation of the distinct Notch ligand–stimulated EPCs (online-only Data Supplement Figure VIII). These combined effects of Jag-1 signaling on EPCs may contribute to augmentation of the vasculogenic potential.

The predominant view of Notch signaling is that any Notch ligand is capable of inducing consequential structural changes of Notch receptors for their cleavage and initiating the proteolytic cascade that ultimately leads to generation of a Notch intracellular domain. Very recently, several reports have propounded the concept that each Notch ligand might independently communicate with the receptor for a separate signaling cascade even in the same cell for hematopoiesis or ear regeneration.[25,27] Ligand-specific signaling for vascular development in postnatal stages, however, has never been demonstrated. We demonstrated for the first time the specific role of Jag-1 in stimulating postnatal vasculogenesis, which was not observed in Dll-1–dependent signaling. As indicated by the recently discovered concept, elucidation of distinct Notch ligand/receptor communication would be fundamental to illustrating the governed and elaborated mechanisms of stem cell biology in BM environments, as well as the vascular biology involved in postnatal neovascularization for vascular repair.

Acknowledgments

We would like to thank Rie Ito and Michiru Kobori for providing technical assistance and Sachie Ota and Yumiko Masukawa for secretarial work. We appreciate assistance from the Research Center for Regenerative Medicine and from members of the animal facility in Tokai University School of Medicine, as well as technical advice and support from the Teaching and Research Support Center. We also thank all our colleagues for their helpful advice and encouragement.

Source of Funding

This work was supported by the Academic Frontier Promotion Program of the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

Disclosures

None.

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CLINICAL PERSPECTIVE

Although accumulating evidence has indicated that therapy with endothelial progenitor cells (EPCs) could be a promising modality for vascular regeneration, the problems of quantity and quality control need to be resolved to achieve translational application in humans. Pathological conditions such as aging, diabetes mellitus, and hypercholesterolemia lead to a decrease in circulating EPCs and impairment of their proliferative and migratory function. These limitations may be solved by the integration of both in vitro expansion and quality control of EPCs by genetic modification, such as transducing vascular endothelial growth factor, glycogen synthase kinase-1β, human telomerase reverse transcriptase expression, or adjunctive cytokines that promote EPC mobilization. The promise of our therapeutic strategy is that governed Notch signaling in culture can produce the preferred quality and quantity of EPCs needed to enhance vasculogenic potential. The manipulation of Jag-1 ligand–mediated signals in culture before transplantation would allow EPCs to increase in number and augment their vasculogenic potential in patients with ischemic diseases.

Supplementary Methods

Animals and stromal cell lines

All animal experiments were conducted in accordance with the institutional guidelines of Tokai University School of Medicine (Isehara, Japan).

Mice. Conditional *Jag-1*$^{-/-}$ mice (loxP/loxP, mxCre) or conditional *Dll-1*$^{-/-}$ mice (loxP/loxP, mxCre) were generated as reported previously.[20] [25] For gene targeting, poly I/C (200 μg/ 200 μl) was administered intravenously 4 times over a period of 12 days (once every 3 days). Male C57BL6/j and Balb-C nude mice were purchased from Clea Japan Inc. (Tokyo, Japan).

Stromal cells. For the purpose of transduction of various Notch ligands (*Jag-1, Dll-1*), 3T3 stromal cells were cultured in Dubecco Modified Eagles Medium (D-MEM) with 10% fetal bovine serum (FBS). Retroviral transduction of the Notch ligands was performed as described previously[26]. Gene transduction was confirmed by RT-PCR analysis. To prepare for the primary stromal cells, murine BM MNCs were cultured as described previously.

EPC colony forming assay

After collecting the total BM cells aseptically by flushing tibias and femurs of C57BL6/J mice, and further isolating BM MNCs by density-gradient centrifugation with Histopaque 1083 (Sigma, St. Louis, MO), Lin-committed cells were depleted from the BM-MNCs using MACS™ system after incubating with a cocktail of biotin-conjugated antibodies against specific lineage markers (B220, CD3, Gr-1, Mac-1, TER-119) and streptavidin-coupled micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany). KSLs were then isolated from the BM Lin$^-$ cells using FACS Vantage sorting equipment (Becton Dickinson, Franklin Lakes, NJ) with a purity of more than

98%. MNCs were also isolated from PB by density-gradient centrifugation as previously described[1 12 13]. The number of EPC colonies was assessed after culturing 500 BM-KSLs or PB-MNCs for 10-12 days in methyl cellulose-containing medium M3236 (StemCell Technologies, Vancouver, Canada) with 20 ng/ml stem cell factor (SCF, Kirin, Tokyo, Japan), 50 ng/ml vascular endothelial growth factor (VEGF, R&D Systems, Minneapolis, Minn), 20 ng/ml interleukin-3 (Kirin, Tokyo, Japan), 50 ng/ml basic fibroblast growth factor (bFGF, Wako, Osaka, Japan), 50 ng/ml epidermal growth factor receptor (EGF, Wako, Osaka, Japan), 50 ng/ml insulin-like growth factor-1 (IGF-1, Wako, Osaka, Japan) and 2 U/ml heparin (Ajinomoto, Tokyo, Japan). Endothelial phenotype of the EPC colonies was confirmed by high uptake of acetyl LDL (Ac-LDL), cytochemical positivity for isolectin B4 (Molecular Probes, Carlsbad, CA), Flk-1 (VEGFR2), VE-cadherin or eNOS (**data not shown**).

Insert culture system for EPC differentiation

To enable direct interaction between Notch ligands on the surface of the stromal cells and Notch receptors on the target cells, a novel culture system with 0.4 μm pores (1×10^8, high density pores) was developed by modifying the cell culture insert system as reported previously[26]. In brief, 2.5×10^5 stromal cells (3T3 cells), in which Notch ligand gene was transduced by a retroviral system using GFP encoding vectors, were cultured onto the lower side of the track-etched membrane of the insert in D-MEM supplemented with 10% FBS for 3 hours. Luciferase assay confirmed activation of Notch receptor 2 and reporter gene in CHO cells following insert culture with the 3T3 cells (**data not shown**). BM-derived Lin^- cells (1×10^6), were cultured on the upper side in endothelial cell basal medium-2 (EBM-2) (Clontech, Mountain View, CA) supplemented with 5% FBS, human VEGF, human bFGF, human EGF, human IGF-1, ascorbic acid, and penicillin/streptomycin antibiotics. After 4 days in

culture, the target cells were harvested with 2 mM EDTA-PBS in preparation for further analysis.

Semi-quantitative and realtime RT-PCR analysis

The ability of putative EPCs, which were co-cultured with Notch ligand-expressing 3T3 cells, to express endothelial surface markers was determined by semi-quantitative RT-PCR analysis. Cellular mRNA was extracted from each sample using RNeasy Micro Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using 1 μg of mRNA. To investigate the expression of EPC-specific marker genes, PCR was performed for 25 cycles using the TITANIUM ™ Taq RT-PCR kit system (BD Biosciences, San Jose, CA). Sequences of the specific primers used in RT-PCR and PCR conditions can be requested. Realtime PCR profiles in the expression of Notch receptors and ligands were analyzed using PrimeScrift ™ RT reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol.

Flow cytometric analysis

To detect cell surface markers and endothelial lineage antigens on putative EPCs, fluorescence-activated cell sorting (FACS) analysis was conducted 4 days after coculturing with 3T3 stromal cells expressing Notch ligands as described previously. Surface expression of mouse CD31 was determined by directly phycoerythrin (PE)-conjugated antibody (Becton Dickinson) against mouse CD31. Biotin-conjugated anti-mouse Flk-1 (VEGFR 2) antibody (e-Bio, San Diego, CA) and anti-streptavidin APC-conjugated secondary antibody (e-Bio, San Diego, CA) were used to determine the surface expression of Flk-1. Each staining procedure was performed for 20 minutes at 4°C, and the stained cells were fixed with 2% paraformaldehyde (PFA)

followed by quantitative analysis with FACSCalibur (Becton Dickinson) and Cell Quest software. To evaluate the frequency of $CD31^+$/ $Flk\text{-}1^+$ cells or $CD31^+$/ $Flt\text{-}1^+$ cells in $Sca\text{-}1^+$/ Lin^- cells obtained from *Jag-1* null, *Dll-1* null or WT mice, we used Sca-1-FITC (Becton Dickinson), CD31-PE (Becton Dickinson), Flk-1-APC (Becton Dickinson) and Flt-1-APC (Becton Dickinson) antibodies after isolating the Lin- depleted cells with a purity of more than 97% by the MACS system.

Acetyl LDL uptake analysis and *in vitro* EPC differentiation analysis

To investigate *in vitro* differentiation of the target cells into endothelial lineage following the insert culture, BM Lin^- cells 4 days after coculture with Notch ligand-overexpressing stromal cells were seeded onto fibronectin-coated 4 chamber slides (BD Falcon). The attached spindle-shaped cells were assayed by costaining with DiI-conjugated Ac-LDL (DiI-Ac-LDL) (Biomedical Technologies Inc., Stoughton, MA) and FITC-conjugated isolectin B4 (Sigma Chemical Co., Milwaukee, WI), a typical marker of endothelial lineage. EPCs identified as the double positive cells were randomly counted using fluorescence microscopy (25 random images per sample, 3 times in independent experiments).

In vitro EPC differentiation was similarly assessed for PB MNCs from *Jag-1*$^{-/-}$ or *Dll-1*$^{-/-}$ mice. To determine the number of EPCs, the double positive cells for Flk-1 (e-Bio, San Diego, CA) and nitric oxide synthase (eNOS, Sigma) were randomly counted using fluorescence microscopy (25 random images per sample).

Migration and invasiveness analysis

To explore EPC migratory capacity, an *in vitro* Boyden chamber assay (Costar, Cambridge, MA) was performed as described previously[2]. 600 μl of EBM-2 media with 0.5% lipid-free FBS (Sigma Chemical Co.) and 4, 20 or 100 ng/ ml of VEGF or

vehicle was placed in the lower compartment of the chamber. A total of 1 x 10^5 BM Lin^- cells, cultured for 4 days in the insert culture system with Notch ligand-overexpressing 3T3 cells in 100 μl of EBM-2 supplemented with 0.5% bovine serum albumin (BSA), were seeded in the upper compartment of the chamber. Cell migration activity was quantified by counting cells migrating from the upper to lower chamber in four randomly selected high-power fields (40 x magnification). All groups were studied in triplicate.

To determine the invasive capacity of BM $Lin^-/Sca\text{-}1^+$ cells, an EPC-enriched population, the cells were seeded in the upper chamber coated with 0.2% methyl cellulose (StemCell Technologies), while 100 ng/ ml of SDF-1 or no growth factor was added in the lower compartment as reported previously[28]. In similar fashion to the migration assay, the number of invasing cells was counted under each condition.

BrdU proliferation and WST-1 assay

For BrdU proliferation assay, BM Lin^- cells from *Jag-1*$^{-/-}$, *Dll-1*$^{-/-}$ or wild type mice were collected as described above, and pulsed with 10 μM BrdU (BrdU flow kit, BD pharmingen) for 45 min before immunostaining with PE-conjugated rat anti-mouse Sca-1 antibody. After fixation and permeabilization with Cytofix/ Cytoperm buffer (BD pharmingen) according to the manufacturer's instructions, the cells were stained with APC-conjugated antibody against BrdU, and analyzed with FACSCalibur using CellQuest software (BD pharmingen).

In a gain of function study for Notch ligands, the effect of EPC proliferation was assessed by WST-1 assay. In brief, 1 x 10^4 Notch ligand-stimulated cells were seeded to each well of fibronectin-coated 96-well plates and incubated for 48 h. For the evaluation of cell proliferation in response to VEGF (4 ng/ml), the cultured cells were starved in EBM-2 culture medium with 1% FBS without any growth factor.