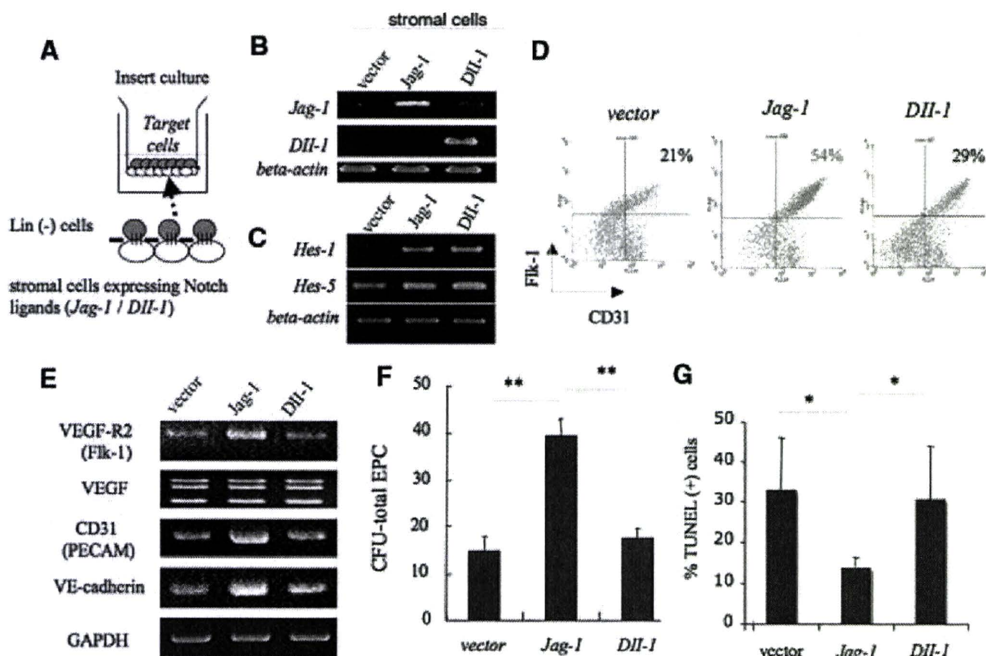

Figure 4. Effects of gain of function of Notch ligand–mediated signals on EPC differentiation. A, Scheme of the insert culture system with 0.4-μm pores, in which receptors on the surface of the target stem cells in the upper chamber are capable of directly interacting with ligands on the cells in the 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-

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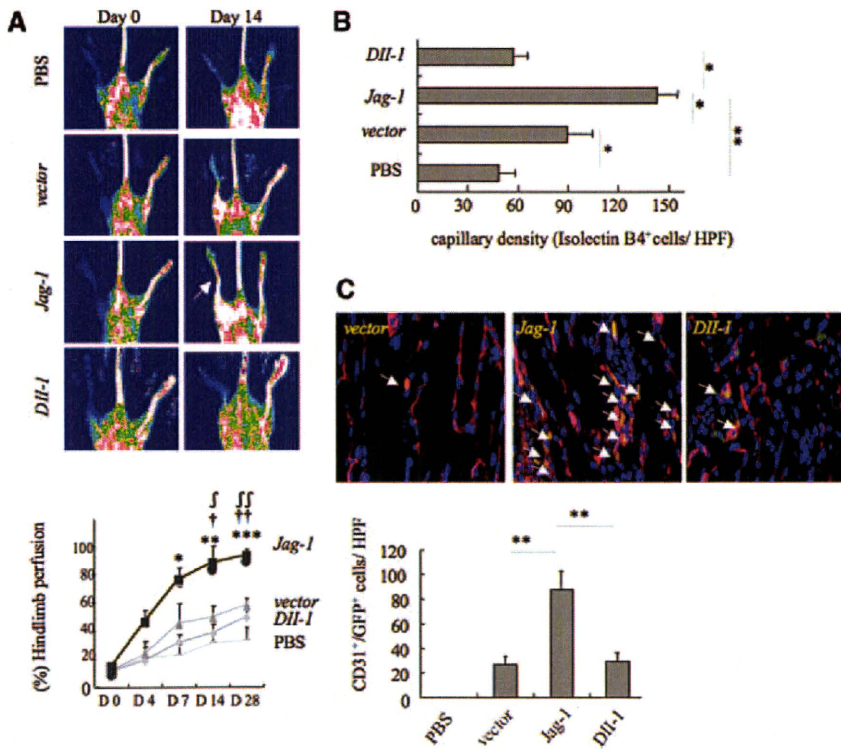

Figure 5. Promotion of in vivo neovascularization by transplantation of putative EPCs stimulated by Jag-1–mediated signals. A, Representative laser Doppler perfusion imaging findings in nude mice receiving PBS (no cells) or BM-Lin⁻ cells cocultured with empty-vector– or specific Notch ligand (Jag-1, 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; ʃ$P<0.05$ vs Dll-1; ʃʃ$P<0.01$ 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

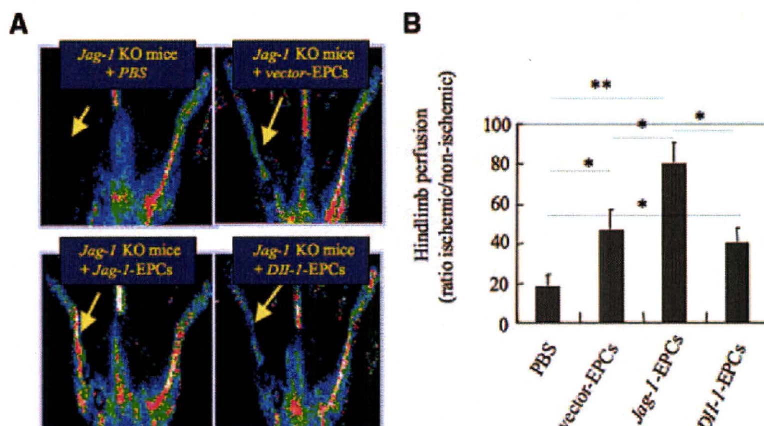

Figure 6. Rescue of impaired neovascularization in *Jag-1* knockout (KO) mice by transplantation of putative EPCs stimulated by specific Notch ligand–mediated signals. A, Representative laser Doppler perfusion imaging findings in *Jag-1* KO mice with hindlimb ischemia receiving PBS or BM-Lin⁻ cells cocultured 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).

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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.

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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.

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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

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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

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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)

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followed by quantitative analysis with FACSCalibur (Becton Dickinson) and Cell Quest software. To evaluate the frequency of $CD31^+$/ Flk-1^+ cells or $CD31^+$/ Flt-1^+ cells in Sca-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

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vehicle was placed in the lower compartment of the chamber. A total of 1×10^5 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-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, 1×10^4 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.

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Thereafter, 10 μl of the cell proliferation assay reagent WST-1 (Roche Applied Science, Indianapolis, IN), was added to each well and incubated for 3 hours. Absorbance at 450 nm was measured for each well using an enzyme-linked immunosorbent assay reader.

TUNEL assay

To evaluate the apoptotic potential of Notch ligand-deficient BM cells, we performed the TUNEL assay with a commercial *in situ* cell death detection kit (Roche, Penzberg, Germany) according to the manufacturer's protocol. In brief, the EPC-enriched cells (BM-Sca-1^{+} /Lin^{-} cells) preconditioned by the activated Notch ligand signal, followed by serum starvation for 12 hours *in vitro*, and the tissue samples obtained from ischemic hindlimb *in vivo* were fixed with 4% PFA for 1 hour at room temperature. After brief incubation in permeabilazation solution containing 0.1% Triton X100 in 0.1% sodium citrate for 2 minutes, the samples were washed twice with PBS and incubated with 50 μl of TUNEL reaction solution for 1 hour in a humidified chamber at 37°C in the dark. After washing with PBS, apoptotic cells were quantified by flow cytometric analysis or fluorescence microscopy.

Rescue of the impaired vasculogenic potential by supplying the distinct Notch ligand signals for EPCs

To evaluate the rescue potential of the distinct Notch ligand signal for BM KSLs, we carried out an *in vitro* EPC colony forming assay as described above using KSLs from *Jag-1* KO or *Dll-1* KO or wild type mice 4 days after co-culturing with 3T3 stromal cells expressing *Jag-1, Dll-1* or empty vector. We quantified the number of EPC colonies 8 days after starting the assay.

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Hindlimb ischemia model and cell transplantation

Operative resection of the femoral artery was performed in C57BL6/J, Balb/C-nude mice or *Jag-1*$^{-/-}$ mice to generate hindlimb ischemia model as described previously[29]. The 2.5 x 10^5 putative EPCs (Sca-1^+/Lin^- cells) or BM Lin^- cells cocultured with the stromal cells expressing Notch ligands were intravenously administered immediately after induction of ischemia. To evaluate the perfusion recovery from hindlimb ischemia, laser Doppler perfusion imaging (LDPI) was conducted to measure blood flow recovery ratio (ischemic/non-ischemic limb) as previously described[14 29].

***In vivo* incorporation assay and immunohistochemistry**

To determine the effect of Notch signaling on the capacity of putative EPCs to incorporate into sites of ischemic tissues, BM-derived Lin^- cells from GFP-transgenic mice (kindly provided by Dr. M. Okabe, Osaka University, Osaka, Japan) were cultured onto stromal cells transferred with each Notch ligand for 4 days in EBM-2 medium. The 2.5 x 10^5 Notch ligand-stimulated EPCs were transplanted into nude mice with hindlimb ischemia. Ischemic muscle samples were embedded in OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and cut into 6 μm-thick sections on day 7. Frozen sections of the ischemic hindlimb muscles were stained with rat anti-mouse CD31 antibody (Becton Dickinson) overnight at 4°C, followed by staining with Alexa fluor 564-conjugated anti-rat antibody to identify capillaries in the ischemic tissue. The capacity of the transplanted cells to be incorporated was elucidated by counting the number of double-positive cells for CD31 and GFP/ low power field (20 x magnification). The frozen samples were also stained with FITC-labeled isolectin B4 (Molecular Probes) to evaluate capillary density by counting capillaries at 25 different randomly selected low power fields per

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sample (n=4).

Statistical Analysis

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

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Supplementary Figure Legends

Supplementary Fig. 1: Realtime RT-PCR to quantify the expression levels of Notch receptor mRNA or Notch ligand mRNA in both BM stromal cells (a) and KSLs (b, c) obtained from WT, *Jag-1* KO and *Dll-1* KO mice

Targeted deletion of the Notch ligands did not affect the expression of Notch receptor 1, 2, 3 and 4 in BM stromal cells and KSLs. Notch R1, Notch receptor 1; Notch R2, Notch receptor 2; Notch R3, Notch receptor 3; Notch R4, Notch receptor 4.

Supplementary Fig. 2: Impairment of EPC vasculogenic capacity by inactivation of Notch signals

The effect of gamma secretase inhibitor on vasculogenic capacity of BM KSLs was evaluated by EPC colony forming assay. **, P<0.01 (n = 3 per group).

Supplementary Fig. 3: Impairment of endothelial lineage commitment in PB-MNCs in conditional *Jag-1* KO, but not *DII-1* KO mice.

a: Number of EPCs showing capability of Ac-LDL uptake and positivity for isolectin B4, a typical endothelial marker, was significantly lower in *Jag-1* KO mice, but not *DII-1* KO mice, compared with WT mice. **, P<0.01 (n = 4 in each group).

b: Representative fluorescent images of the *in vitro* EPC culture assay using PB-MNCs obtained from WT, *Jag-1* KO and *DII-1* KO mice. Red fluorescence indicates positivity for eNOS (nitric oxide synthase, endothelial), which is strongly expressed in EPCs, while the green signal demonstrates positivity for Flk-1 (VEGFR2), a typical endothelial marker.

c: Number of EPCs showing positivity for both eNOS and Flk-1 was significantly lower in *Jag-1* KO mice, but not *DII-1* KO mice, compared with WT mice. *,

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P<0.05 (n = 4 in each group).

d: Vasculogenic capacity of circulating MNCs obtained from WT, *Jag-1* KO and *Dll-1* KO mice was analyzed by EPC colony forming assay. The number of EPC colonies was significantly lower in *Jag-1* KO, but not *Dll-1* KO mice, compared to WT mice.**, P<0.01 (n = 3 in each group).

Supplementary Fig. 4: Characterization of BM Lin⁻ cells pre- and post- gain-of-function of Notch signals

a: Representative images of BM Lin⁻ cells 4 days after an insert coculture with 3T3 cells overexpressing Notch ligands. Spindle-like cells forming tubular structures, whose morphology indicates active vasculogenesis *in vitro*, were frequently observed in the *Jag-1* group, but not in the empty vector and *Dll-1* groups.

b: RT-PCR for Notch receptors in BM Lin⁻ cells 4 days after coculture with the Notch ligand-overexpressing stromal cells. Expression of the Notch receptors was similar in all groups.

Supplementary Fig. 5: Characterization of BM Lin⁻ cells by gain-of-function of Notch signals in terms of endothelial commitment

a: The signal intensity by FACS analysis indicating the degree of positivity for Flk-1 and CD31 was augmented in the *Jag-1* group but not in the Dll-1 group, when compared with the empty vector group.

b: *In vitro* EPC culture assay using PB-MNCs was obtained from each group. The number of EPCs showing capability of Ac-LDL uptake and positivity for isolectin B4 was significantly greater in the *Jag-1* group than in the *Dll-1* and empty vector groups.*, P<0.05 (n = 3 in each group).

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Supplementary Fig. 6: Effect of Notch activation on EPC proliferation and migration

a: The proliferation capacity of BM Lin$^-$ cells cocultured with Notch ligand-expressing 3T3 cells was evaluated by WST-1 assay. WST-1 index indicated that the *Jag-1* group showed higher proliferation activity compared with the *Dll-1* and empty vector groups. †, P<0.05 vs vector; ∫, P<0.05 vs DII-1. #, P<0.05 vs VEGF(-).

b: VEGF-dependent migration assay. In the presence of 20 ng/ ml of VEGF, the migrated cell number was significantly greater in the *Jag-1* group than the *Dll-1* and empty vector groups. Intragroup difference: *, P<0.05 vs VEGF 0; †, P<0.05 vs VEGF 4; ∫, P<0.05 vs VEGF 100. Intergroup difference: #, P<0.05; ##, P<0.01.

Supplementary Fig. 7: Rescue of downregulated EPC colony forming potential in BM KSLs from *Jag-1* null mice by gain of Notch signals

EPC colony forming assay was performed by incubating BM-KSLs from WT, *Jag-1* KO or *DII-1* KO mice 4 days after the insert culture with stromal cells overexpressing *Jag-1*, *Dll-1* or empty vector. *, P<0.05, **, P<0.01. ***, P<0.001 (n = 3 in each group).

Supplementary Fig. 8: Rescue of ischemia-induced apoptosis in *Jag-1* null mice by transplantation of EPCs stimulated by *Jag-1* signals

a: Representative TUNEL staining of the ischemic hindlimb tissue (3 days after ischemia) obtained from WT, *Jag-1* KO, or *DII-1* KO mice receiving BM Lin$^-$ cells 4 days after insert culture with the stromal cells overexpressing *Jag-1*, *Dll-1* or empty vector.

b: Frequency of TUNEL-positive cells in the hindlimb tissue was significantly

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lower in the *Dll-1* and empty vector groups than in the PBS group. Further, the frequency of the apoptotic cells was lower in the *Jag-1* group compared with the *Dll-1* and empty vector groups. *, P<0.05, **, P<0.01 (n = 4 in each group).

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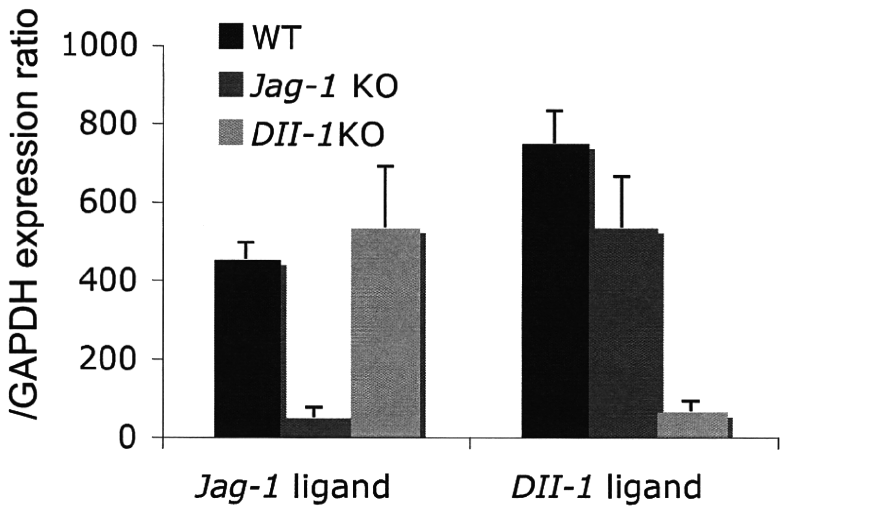

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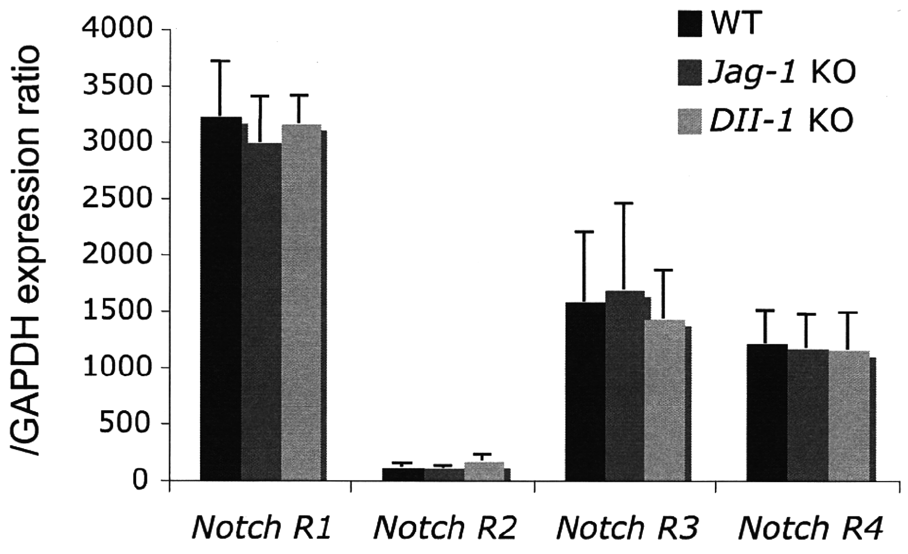

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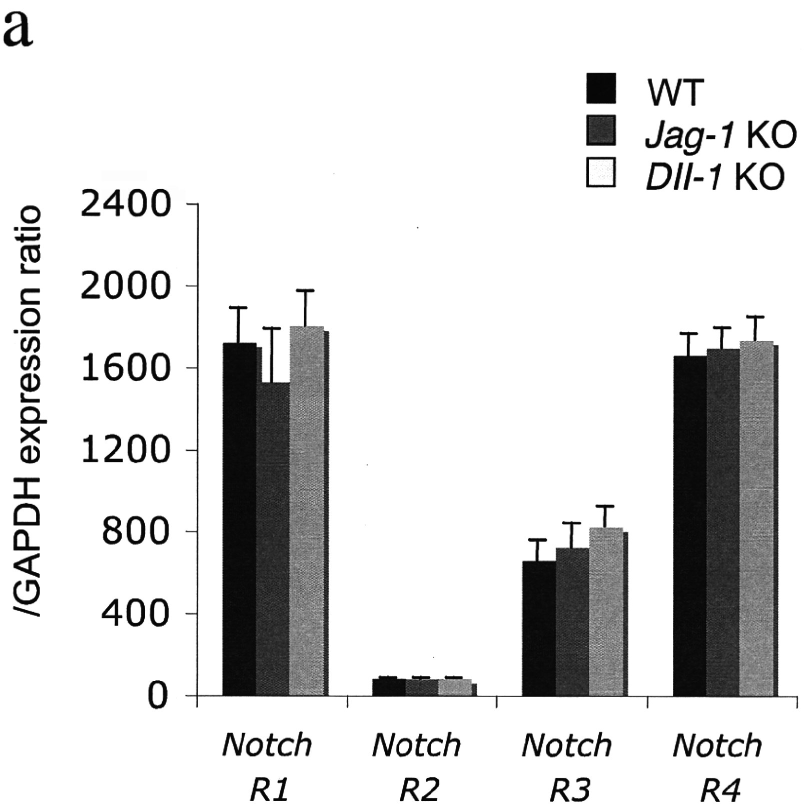

Kwon, et al. Supplemental Fig. 1

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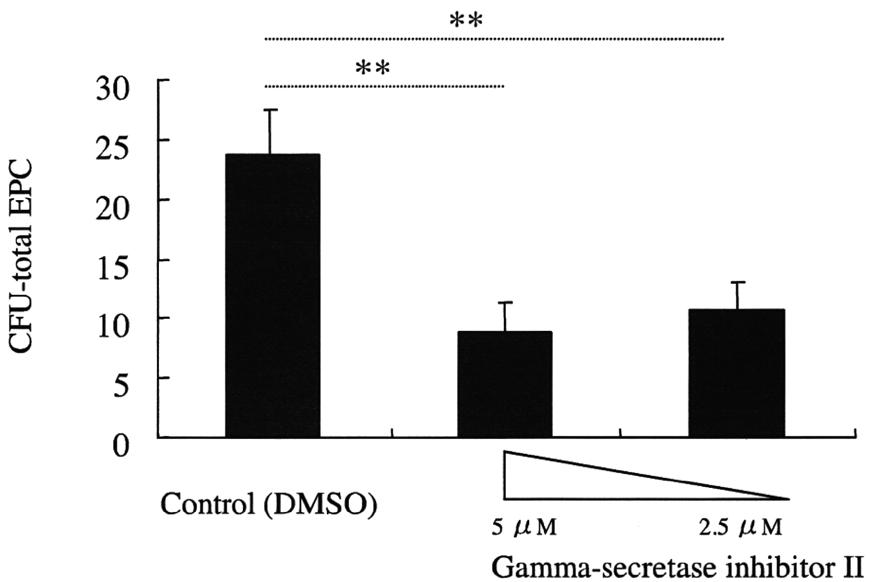

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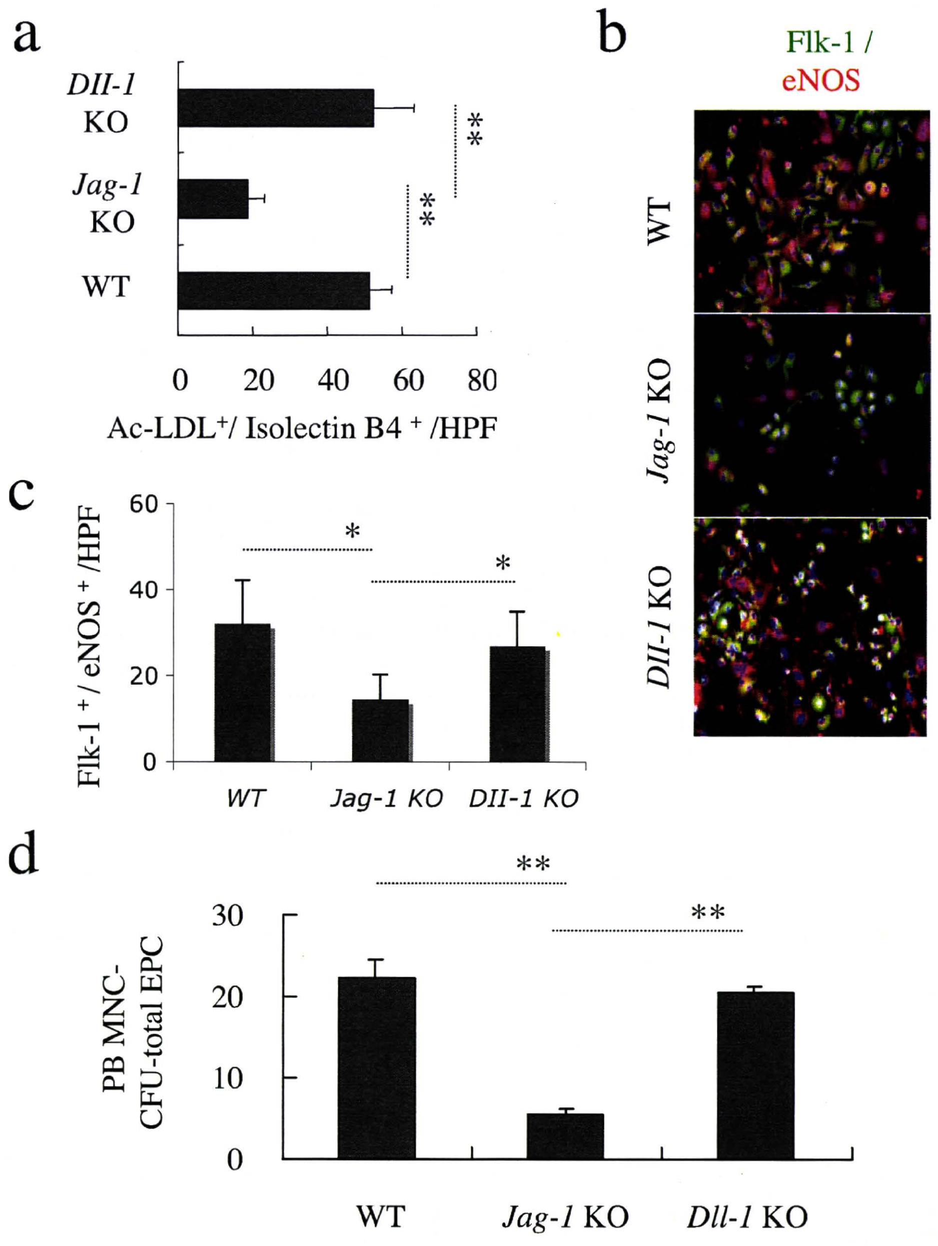

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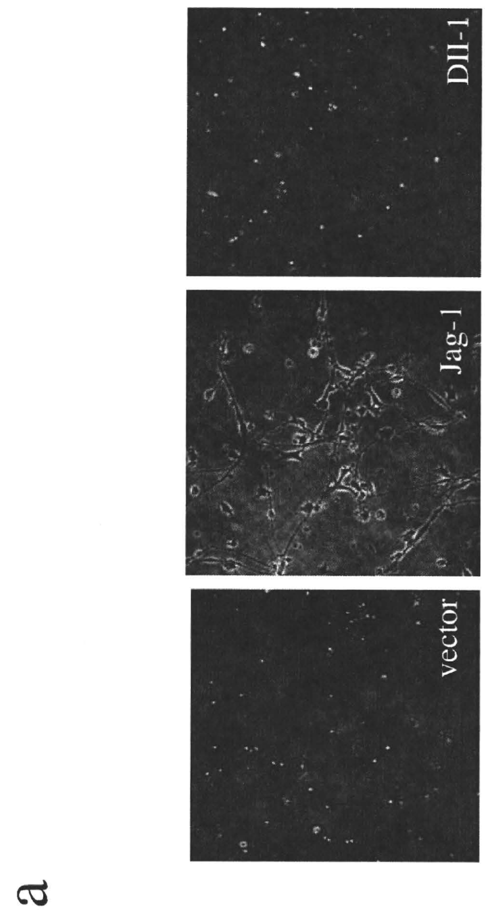
a



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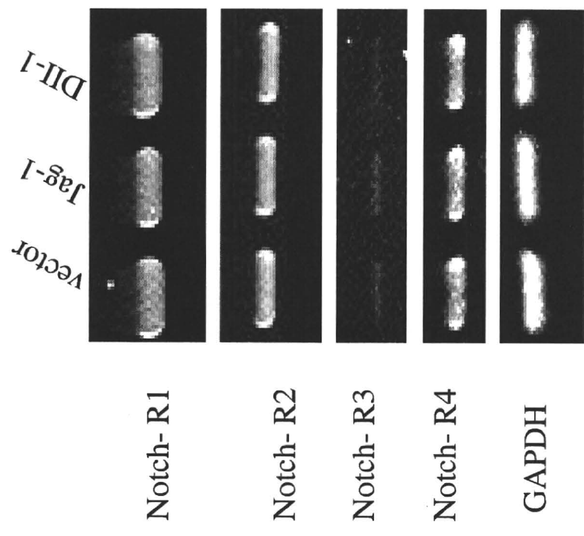

Kwon, et al. Supplemental Fig. 4

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