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.

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

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.

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. *,

$P<0.05$ (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).

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

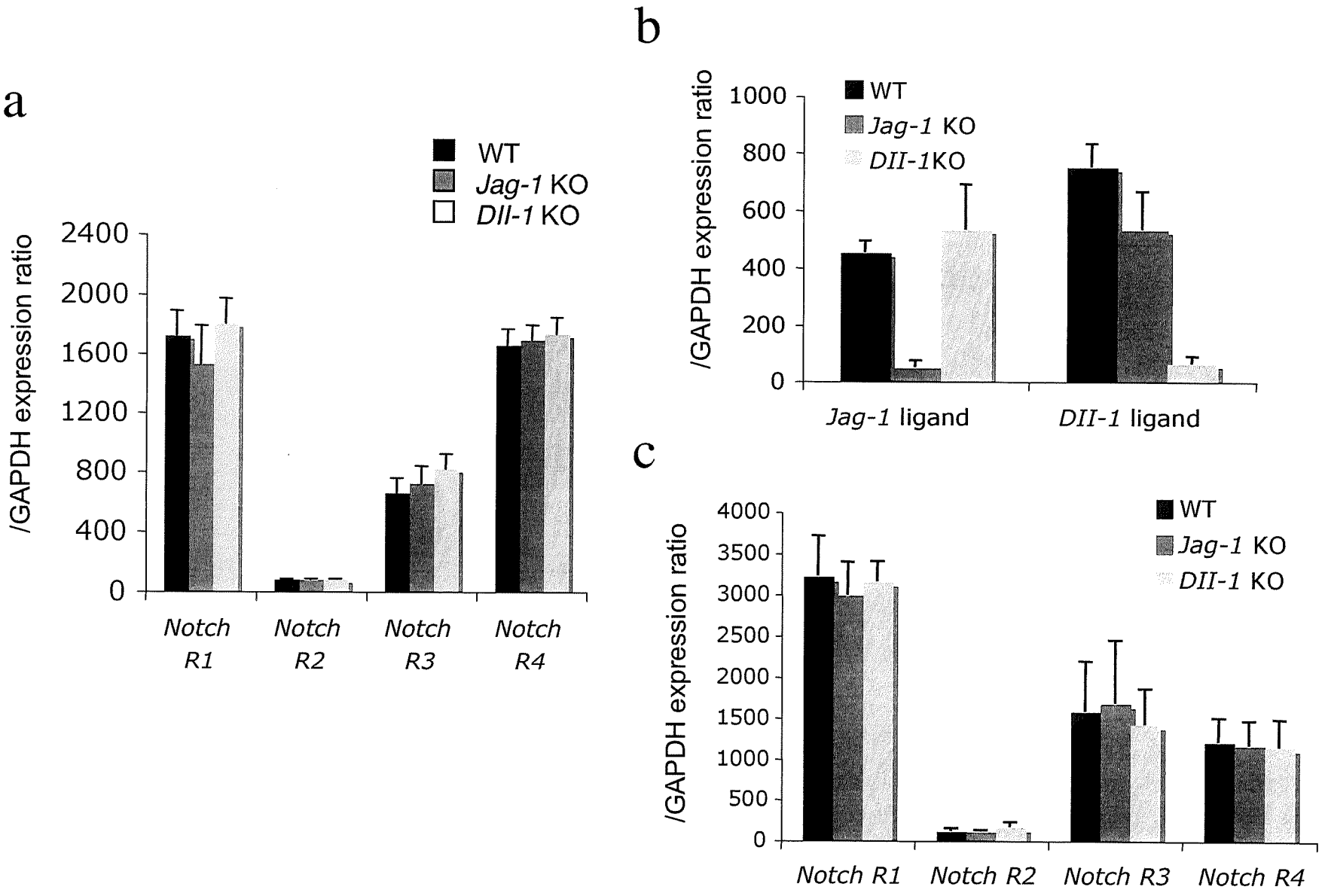
EPC colony forming assay was performed by incubating BM-KSLs from WT, *Jag-1* KO or *DII-1* KO mice 4 days after the insert culture with stromal cells overexpressing *Jag-1*, *Dll-1* or empty vector. *, $P<0.05$, **, $P<0.01$. ***, $P<0.001$ ($n = 3$ in each group).

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

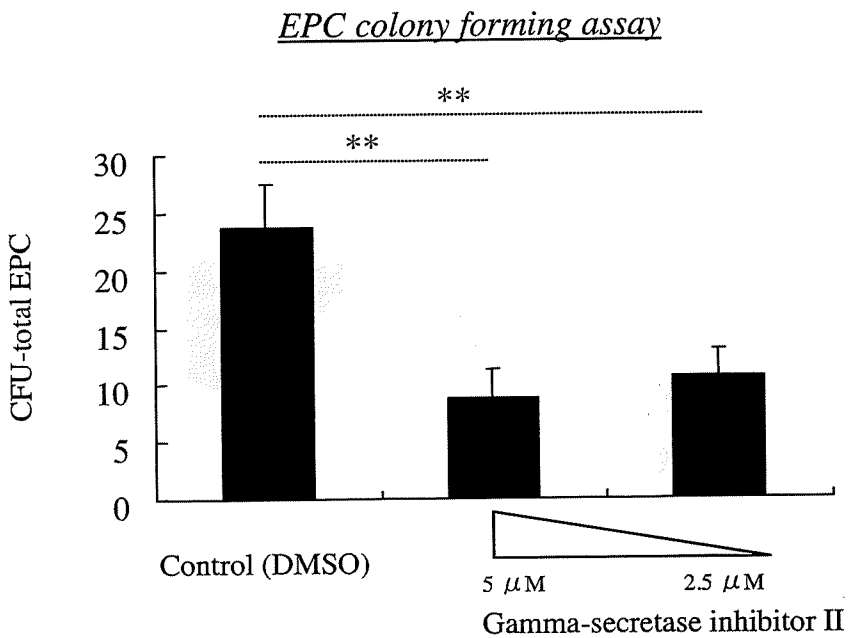
a: Representative TUNEL staining of the ischemic hindlimb tissue (3 days after ischemia) obtained from WT, *Jag-1* KO, or *DII-1* KO mice receiving BM Lin^- cells 4 days after insert culture with the stromal cells overexpressing *Jag-1*, *Dll-1* or empty vector.

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

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).



Kwon, et al. Supplemental Fig. 1



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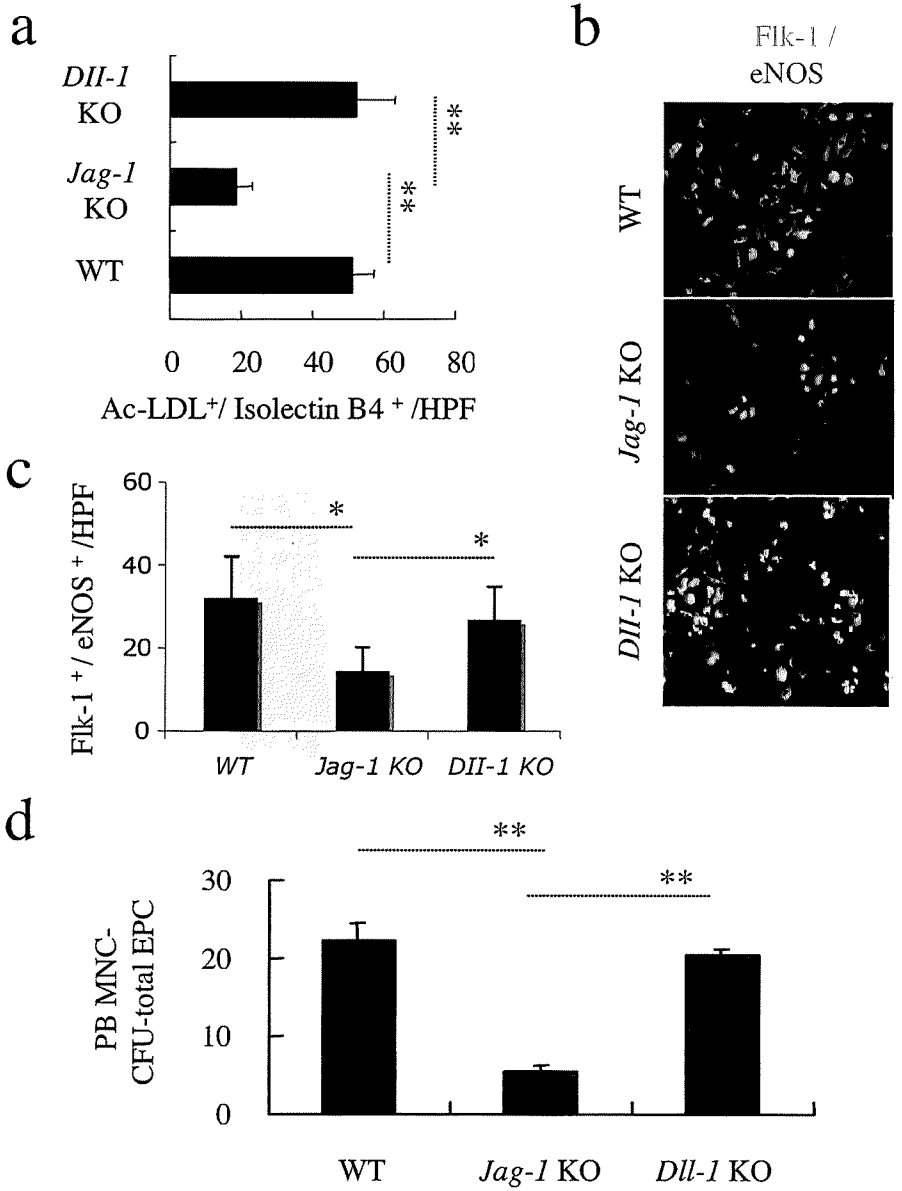

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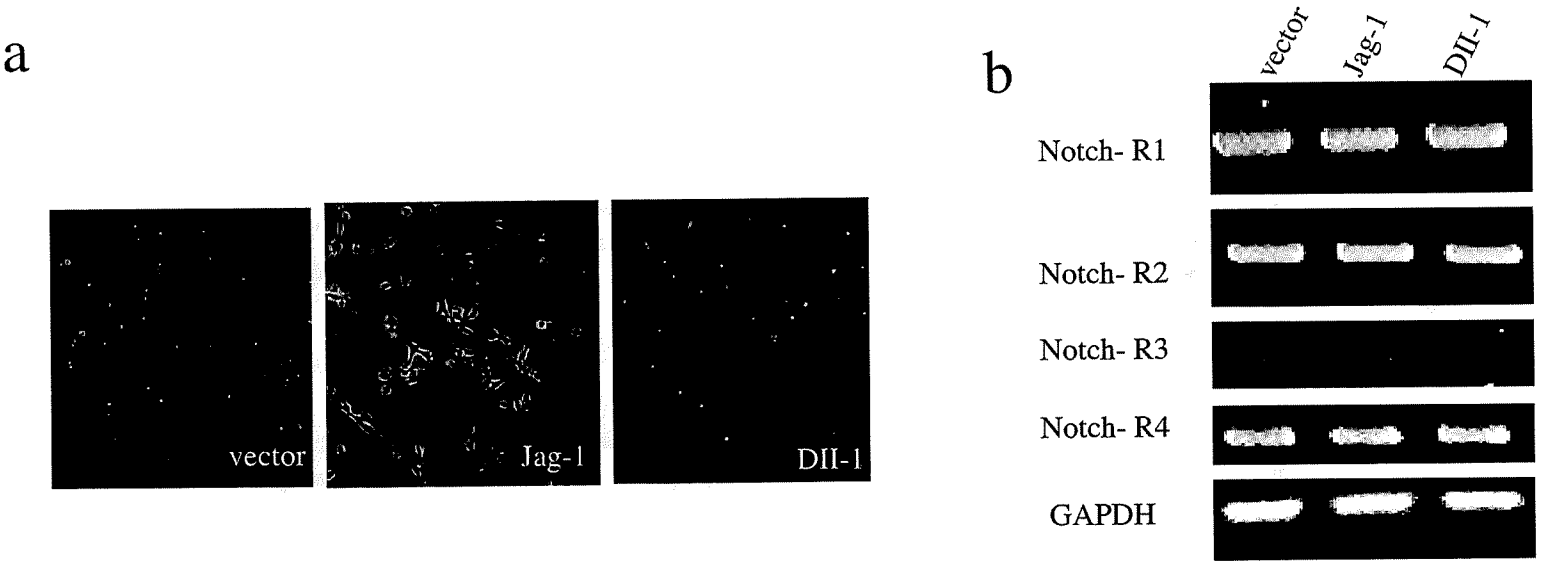

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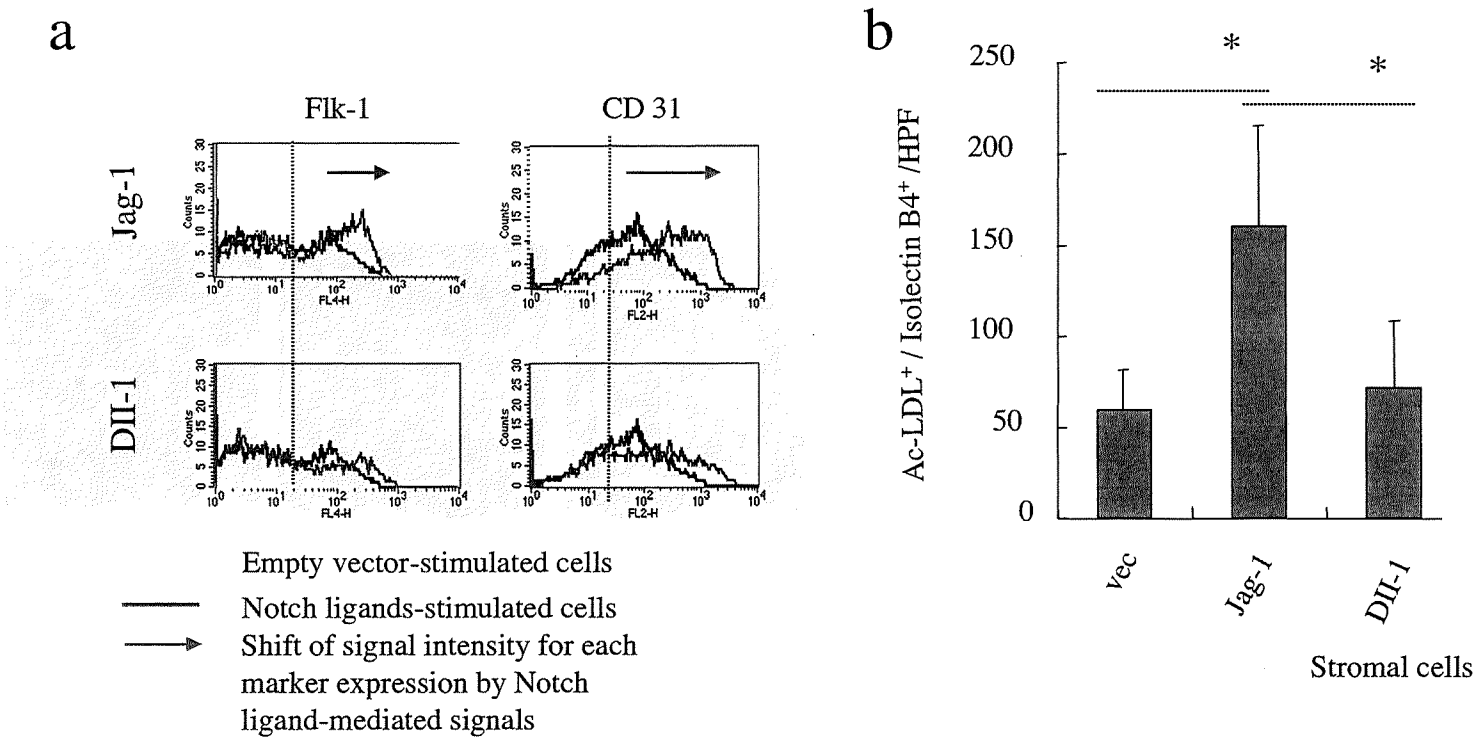

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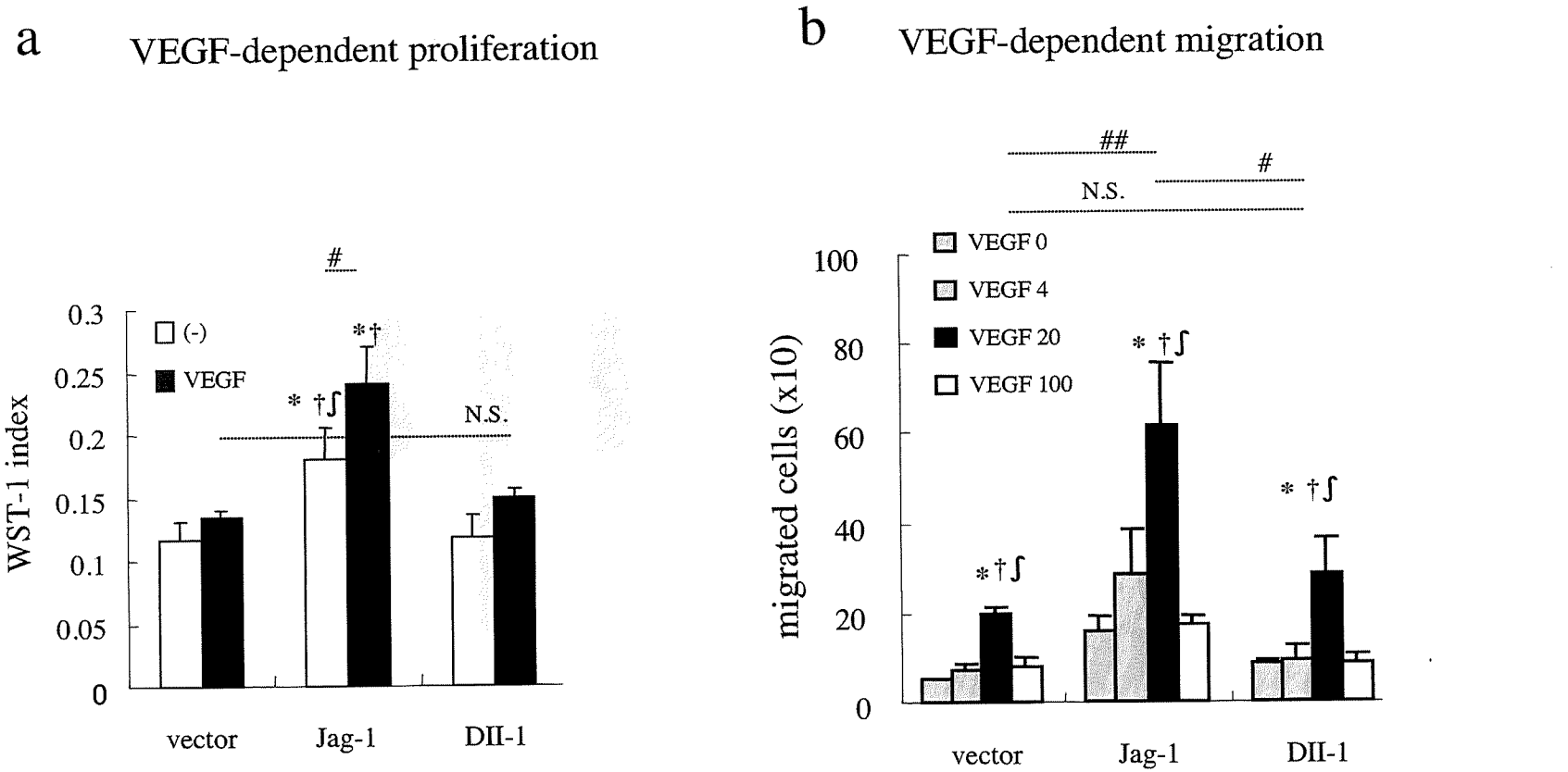

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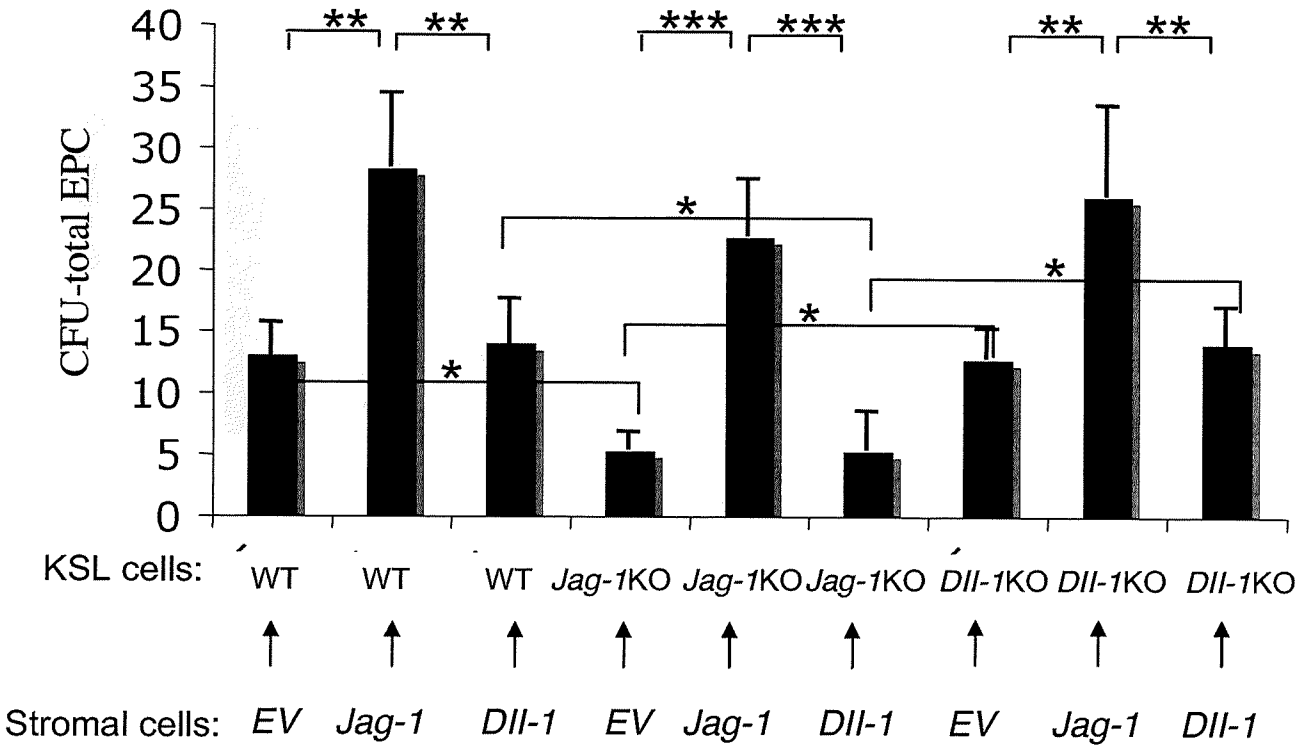

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Kwon, et al. Supplemental Fig. 8

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Original article

Lesion-targeted thrombopoietin potentiates vasculogenesis by enhancing motility and enlivenment of transplanted endothelial progenitor cells via activation of Akt/mTOR/p70S6kinase signaling pathway

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ABSTRACT

Thrombopoietin (TPO), a physiological regulator of megakaryocyte and platelet development, is a multifunctional positive regulator in early hematopoiesis by hematopoietic stem cells. In this study, we investigated the effect of TPO on endothelial progenitor cells (EPCs) for therapeutic vasculogenesis *in vitro* and *in vivo*, and the intracellular signaling mechanism exerting the activity of EPCs. 7-day culture-expanded EPCs derived from human peripheral blood mononuclear cells were applied to each assay. Flow cytometry demonstrated the expression of c-Mpl, the receptor of TPO, in cultured EPCs. *In vitro* experiments revealed enhanced migration and survival of cultured EPCs by TPO. *In vivo*, TPO was intramuscularly administered into the foci of ischemic hindlimbs in athymic nude mice, immediately followed by intravenous injection of cultured EPCs, to assess the booster effect of TPO on vascular regeneration. At day 4 post-transplantation, transplanted EPCs were 1.7-fold higher in TPO-treated animals compared to control. At day 28, blood perfusion was recovered in the TPO-treated group, accompanied by an increase in microvascular density. The signaling transduction pathway underlying TPO-mediated activities of cultured EPCs was assessed by Western blotting. TPO induced sequential phosphorylations of Akt to p70S6kinase through mTOR. Inhibition of the PI3-kinase/Akt/mTOR/p70S6kinase signaling pathway negated the biological functions of cultured EPCs, either migration (by LY294002 for PI3-kinase and Rapamycin for mTOR) or survival and tubulogenesis (by Rapamycin). These findings provide evidence that TPO possesses booster potential for therapeutic vasculogenesis, by activating the PI3-kinase/Akt/mTOR/p70S6kinase pathway crucial to the biological activities of EPCs.

1. Introduction

Postnatal vasculogenesis, contributed by bone marrow (BM) derived endothelial progenitor cells (EPCs), has been recognized as one of the key events in physiological or pathological neovessel formation, concurrent with angiogenesis by resident endothelial cells (ECs) [1]. Based on a new dogma of blood vessel formation in postnatal life, "therapeutic vasculogenesis" targeting ischemic diseases has been recently developed, consisting of transplantation of total mononuclear cells from bone marrow, cultured EPCs, or autologous EPC fractioned in G-CSF-mobilized CD34+ cells [2,3]. However, the scarcity of original EPCs and the impairment of biological functions in patients suffering from risk factors of hyperlipidemia, hypertension, cigarette smoking, diabetes, aging, etc. [4] limits the efficacy of EPC transplantation on vasculogenesis in ischemic diseases. Therefore, methods of improving the quality and quantity of EPCs are desired for therapeutic purposes. Recently, several hematological regulators such as erythropoietin [5], stromal cell derived factor-1 (SDF-1) [6], granulocyte-colony stimulating factor (G-CSF), and estrogen have been described as versatile agents affecting the biological activities of endothelial lineage cells, including EPCs.

Thrombopoietin (TPO) is a hepatocyte-derived growth factor consisting of a 332 amino acid residue polypeptide which serves as a ligand for the TPO receptor (TPO-R; c-Mpl; CD110), which stimulates the development of human megakaryocyte (HuMK) progenitors and increases circulating platelets. Furthermore, TPO has recently been shown to play a pivotal role in hematopoietic stem/progenitor cells as well. Murine and human hematopoietic stem/progenitor cells have been shown to highly express c-Mpl [7], in which TPO stimulates VEGF expression in an autocrine manner to promote self-

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renewability [8]. Also, various studies have already revealed the ability of TPO to enhance the survival of hematopoietic stem/progenitor cells [9]. On the other hand, patients with unstable angina show higher serum levels of TPO as compared to healthy subjects or patients with stable angina [10], along with other heamatopoietic and angiogenic regulators elevated in patients with acute myocardial infarction, such as VEGF, angiopoietins and erythropoietin [11], or G-CSF [12]. Given such accumulated findings, TPO is intriguingly hypothesized to possess a preferential effect on EPC bioactivity, leading to a booster effect when co-administered in EPC transplantation.

Numerous studies in diverse cell types have demonstrated that TPO gives rise to biological functions of motility, survival, proliferation etc., through the orchestration of intracellular signaling pathways p44MAPK, JAK-STAT, or PI3K/Akt [13]. The Akt pathway has been revealed to be a key mediator for vasculogenic functions, such as migration, survival, differentiation, and proliferation in EPCs as well as ECs, concomitantly with phosphorylation of eNOS [14]. The mTOR (mammalian target of rapamycin)/p70S6kinase pathway, as the downstream effector of Akt activation, has also been indicated to regulate various biological functions of ECs for angiogenesis [15]. TPO is speculated to augment vasculogenic functions of EPC through the activation of the Akt/mTOR/p70S6kinase pathway. TPO has also been indicated to have angiogenic potential, not only by activating migration and tubulogenesis of human umbilical vein ECs (HUVECs) expressing c-Mpl *in vitro*, via the JAK-STAT pathway, but also by enhancing neovessel formation *in vivo* [16]. However, the association of PI3K/Akt and/or mTOR/p70S6kinase with TPO in an endothelial lineage for activating angio/vasculogenesis has not been evidenced as yet. Furthermore, the importance of sequential activation of the PI3K/Akt/mTOR/p70S6kinase pathway for EPCs remains to be elucidated, despite evidence that the mTOR/p70S6kinase pathway essential for producing EPCs from circulating ancestral CD133+ stem cells [17].

In the present study, we not only investigated the capability of TPO as a preferential mediator to ameliorate the efficacy of EPC transplantation for therapeutic vasculogenesis, but also assessed the intracellular mechanisms underlying the activities of EPCs.

2. Materials and methods

2.1. Materials

The following materials and antibodies were used: recombinant human TPO (KIRIN, JAPAN), recombinant human SDF-1α (PEPROTECH, France), LY294002 (SIGMA, USA), wortmannin (SIGMA, USA), rapamycin (Calbiochem, Germany), for FACS analysis; monoclonal anti-VEGF receptor-2 (KDR) (SIGMA, USA), purified mouse anti-human CD110 (c-Mpl), fluorescein isothiocyanate (FITC)-conjugated anti-human CD31, FITC-conjugate anti-human CD45, and phycoerythrin (PE)-conjugated CD31, CD133 (BD Pharmingen, USA), for immunohistochemistry; rabbit anti-human c-Mpl antibody (H-300) (SANTA CRUZE USA), Isolectin GSIB4 Alexa Fluor-488, Isolectin GSIB4 Alexa Fluor 594 (Molecular Probes, USA), for Western blotting analysis; anti-Actin (SIGMA, USA), phospho-mTOR (Ser2448), phospho-p70 S6 kinase (Thr421/Ser424), phospho-Akt (Ser473), Akt antibody (Cell Signaling, USA).

2.2. Ex vivo preparation of human cultured EPCs

Human cultured EPCs were cultured as described previously [1,2]. Briefly, total human peripheral blood mononuclear cells (PBMNC) isolated from healthy human volunteers by density-gradient centrifugation were cultured in human fibronectin coated Primaria tissue culture dishes (BD Falcon, USA), using 5% FBS-EBM-2 medium with EGMV-2 growth factor supplement (Clonetics, USA). Following the removal of suspended cells with PBS at day 4, adherent cells were cultured for 3 more days.

2.3. Cellular staining

After 7 days, cultured EPCs were detected by fluorescent staining of double positive cells with FITC-labeled *Ulex europaeus* agglutinin (UEA)-1 (Vector Lab, USA) and 1,1′-dioctadecyl-3,3,3,3′,3′-tetramethylindocarbocyyanine (DiI-I) labeled acetylated low density lipoprotein (DiI-acLDL Biomedical Technologies, USA). Cells were first incubated with DiI-acLDL at 37 °C for 4 h and later fixed with 1% paraformaldehyde for 10 min. After washing twice, the cells were reacted with FITC-conjugated UEA-1 (10 μg/ml) for 1 h. After staining, cells were observed with a fluorescence microscope (Olympus IX70, Japan). Cultured EPCs were also stained by mouse anti-human CD110 (c-Mpl) antibody with anti-mouse IgG1 Alex 594 as second antibody.

2.4. FACS analysis of cultured EPCs

Cultured EPCs from six healthy volunteer underwent FACS analysis not only to confirm their endothelial lineage but also assess c-Mpl expression, using PBS containing 2 mM EDTA, 1% BSA, and 0.1% $NaNO_3$. Cultured EPCs were stained with propidium iodide (PI) in order to exclude dead cells. The monoclonal antibodies for FACS staining were as follows: KDR (VEGFR-2), CD110 (c-Mpl), FITC-conjugated CD31, CD45 and PE-conjugated CD31, CD133. The cells were analyzed by FACS Calibur (BD Biosciences USA).

2.5. Migratory activity assay of cultured EPCs

To investigate EPC migratory activity, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber with an 8 μm pore sized polycarbonate membrane (Corning Inc, USA), as described elsewhere [18]. The protocol was described in SM-I.

2.6. Proliferative activity assay of cultured EPCs

The assay for EPC proliferative activity effected by TPO was performed, according to the detail description in SM-II.

2.7. Survival assay of cultured EPCs

The assay for EPC survival effected by TPO was performed, according to detail description in SM-III.

2.8. RT-PCR for endothelial gene expression in cultured EPCs

The protocol for RT-PCR assay was described in SM-IV.

2.9. Lesion-targeted administration of TPO along with EPC transplantation in vivo

The impact of local administration of TPO after EPC transplantation on therapeutic neovasculogenesis was assessed in a hindlimb ischemic model, using athymic nude mice (Clea Japan Inc.) [2] for a TPO group or control (PBS). Soon after operative ligation of one femoral artery, 1 μg recombinant human TPO (rhTPO) in PBS (total 12 μl) per mouse was intramuscularly injected into ischemic thigh and lower muscles (total 3points), followed immediately by intravenous transplantation of (2.0×10^5 cells/mouse) cultured EPCs. To survey the transplanted EPCs incorporated into neovasculatures in ischemic muscles, four mice per group were injected with EPCs labeled with Cell Tracker CM-DiI (Molecular probes, USA), according to the manufacturers' protocol.

2.10. Blood flow assessment of ischemic hindlimb in transplanted mice

Laser Doppler perfusion imaging (LDPI) (Moor Instrument, UK) was used to record serial blood flow measurements for 4 weeks after EPC transplantation, as previously described [2].

2.11. Histological assessment of ischemic hindlimb in transplanted mice

Cross-sectional tissue samples at 6–8 μm thickness were sliced from tissue blocks of the muscles of ischemic and contralateral limbs embedded into O.C.T compound (Tissue-Tek, USA) and frozen in liquid nitrogen at day 4 and day 28, then subjected to each assessment described below.

At day 4, the muscles from the thigh and lower leg muscles (tibialis anterior (TA), extensor digitorum lounges, soleus, plantaris, gastrocnemius (ESPG), and quadriceps femoris (QF)) of ischemic hindlimbs injected with DiI-labeled EPCs were harvested and stained with Isolectin GSIB4 Alexa Fluor 488 (Molecular Probes, USA) to assess the incorporation of transplanted EPCs into vasculatures of ischemic muscles concurrently delivered TPO. DiI-labeled EPCs in ischemic hindlimbs were counted all inside a section in 3 cross sections (proximal, middle, distal) from each mouse (N=4) under a fluorescence microscopy (Olympus IX70, Japan, ×200 magnification). Also the sections were stained with rabbit anti-human c-Mpl antibody to assess the c-Mpl expression on DiI-labeled EPCs.

On the other hand, as an independent experiment, microvascular density (MVD) was evaluated at day 28 by counting the capillaries stained with Isolectin GSIB4 Alexa Fluor 594 (Molecular Probes, USA) under fluorescence microscopy. A total 24 different fields were randomly selected (3 cross sections from each animal, each groups were consist with 8 animals N=8), and capillary were counted (×100 magnification).

2.12. Note on experiments in animal subjects

All animal experiments were performed in accordance with the institutional Animal Care and Use Committee of the Isehara Campus, Tokai University School of Medicine. The experimental animal protocols for making ischemic models or LDPI were performed under adequate anesthetization by intraperitoneal pentobarbital injection (50 mg/kg).

2.13. Western blotting to assess phosphorylation of intracellular signaling pathway of cultured EPCs

The protocol for Western blotting assay was described in SM-V.

2.14. In vitro angiogenesis assay of HUVECs cocultured with cultured EPCs using Matrigel

Cultured EPCs were incubated in EBM-2 medium containing 0.1% BSA for 4 h to render the cells quiescent, harvested, and then incubated with or without TPO at serial concentrations for 60 min, following preincubation of Rapa (50 nM) for 10 min. After washing with ice-cold PBS twice, cultured EPCs were suspended (1.0×10^3/50 μl) in EBM-2 with 2% FBS. HUVECs cultured in 10 cm culture dishes were harvested at subconfluent conditions with trypsin/EDTA, and suspended (1.5×10^4/50 μl) in the same medium. Fifty μl from each cell suspension was cocultured at 37 °C for 12 h in a 96 well tissue culture plate provided with an equal volume of Matrigel (BD Bioscience, USA) in advance. Tube formation in each well was observed at ×40 magnification by a light microscope.

2.15. Statistical analysis

All results are expressed as mean±SEM. Statistical significance was evaluated using the unpaired Student t-test for comparisons between 2 means. Multiple comparisons between >3 groups were done by ANOVA. Probability value of $p< 0.05$ denoted statistical significance.

3. Results

3.1. c-Mpl expression in ex vivo cultured EPCs

Only EPCs cultured for 7 days were characterized as endothelial lineage cells by DiI-acLDL uptake and UEA-1 lectin-FITC binding (Fig. 1a) [2], but also by double positive of endothelial specific antigen such as CD31 and VEGF receptor-2 (KDR) (13.46±1.41) by FACS analysis. The expression of c-Mpl on cultured EPCs were 30.1±3.36% and 15.3±3.63% of them were double positive for c-Mpl and CD31 (Fig. 1b). The expression of pan-leukocyte maker CD45 was 96.6± 1.08%. These data suggest that EPCs cultured for 7 days are similar to early EPC reported by Hur et al. [19]. The c-Mpl expression on cultured EPC was also confirmed by fluorescence immunohistochemistry, as described in supplemental Figure (SF)-1. About 62.4±8.4% of cultured EPCs were stained with c-Mpl.

3.2. TPO-stimulated bioactivity of cultured EPCs

3.2.1. Migration

TPO enhanced the migratory potential of cultured EPCs in a dose-dependent manner (Migrated cells/100×HPF=9.4±1.3 at 1 ng/ml; 12.9±1.9 at 10 ng/ml, $p< 0.005$; 15.5±1.9 at 100 ng/ml, $p< 0.0001$ vs. 6.7±0.8 for control) (Fig. 2a).

3.2.2. Proliferation

The proliferation activity of cultured EPCs was not affected by low or middle doses of TPO and decreased with a high dose (O.D. 460 nm=0.541±0.014 at 1 ng/ml; 0.528±0.008 at 10 ng/ml; 0.505± 0.015 at 100 ng/ml, $p< 0.01$ vs. 0.554±0.009 for control) (Fig. 2b).

3.2.3. Apoptosis

The percentage of pyknotic nuclei in cultured EPCs was significantly decreased by TPO (% pyknotic nuclei=19.0±2.4 at 10 ng/ml, $p< 0.05$; 19.3±3.78 at 100 ng/ml, $p< 0.05$ vs. 28.5±1.7 for control) (Fig. 2c). Furthermore in the cell death detection assay, value at O.D. 405 nm in the TPO-treated group was significant smaller vs control (O.D. 405 nm=0.118±0.01 at 100 ng/ml TPO, $p< 0.05$ vs. 0.143±0.006 for control) (Fig. 6c). These findings revealed that TPO potentiated the migration and survival of cultured EPCs *in vitro*, but not proliferation.

3.2.4. Gene expression

In day 7 cultured EPCs, endothelial gene expressions, such as von Willbrand Factor (vWF) and CD31 were detected. The expression of VE-cadherin on cultured EPCs was increased with 60 min stimulation of TPO (SF-II). This finding indicates that TPO stimulated EPCs differentiation.

3.3. Enhanced neovessel formation in vivo by EPC transplantation with lesion-targeted TPO co-administration

Histological examination disclosed that TPO significantly increased the number of DiI-labeled EPCs colocalized into vasculatures stained with Isolectin B4-Alex 488 in ischemic hindlimbs as compared with control (DiI-labeled EPCs No./section=QF 49.8±10.2 in TPO, $p< 0.05$ vs 21.8±2.1 in control; TA 87.7±8.8 in TPO, $p< 0.05$ vs 60.2±3.5 in control; ESPF 125.2±7.0 in TPO, $p< 0.01$ vs 73.2±11.8 in control;) (Figs. 3a,b). Some of the DiI stained transplanted EPCs incorporated into ischemic muscle, also expressing c-Mpl by fluorescence immunohistochemistry (Fig. 3c). This finding indicates that the recruitment potential of transplanted EPCs into the vasculature of ischemic hindlimbs was promoted by TPO co-administration. MVD was assessed by histological examination of tissue sections in ischemic hindlimbs

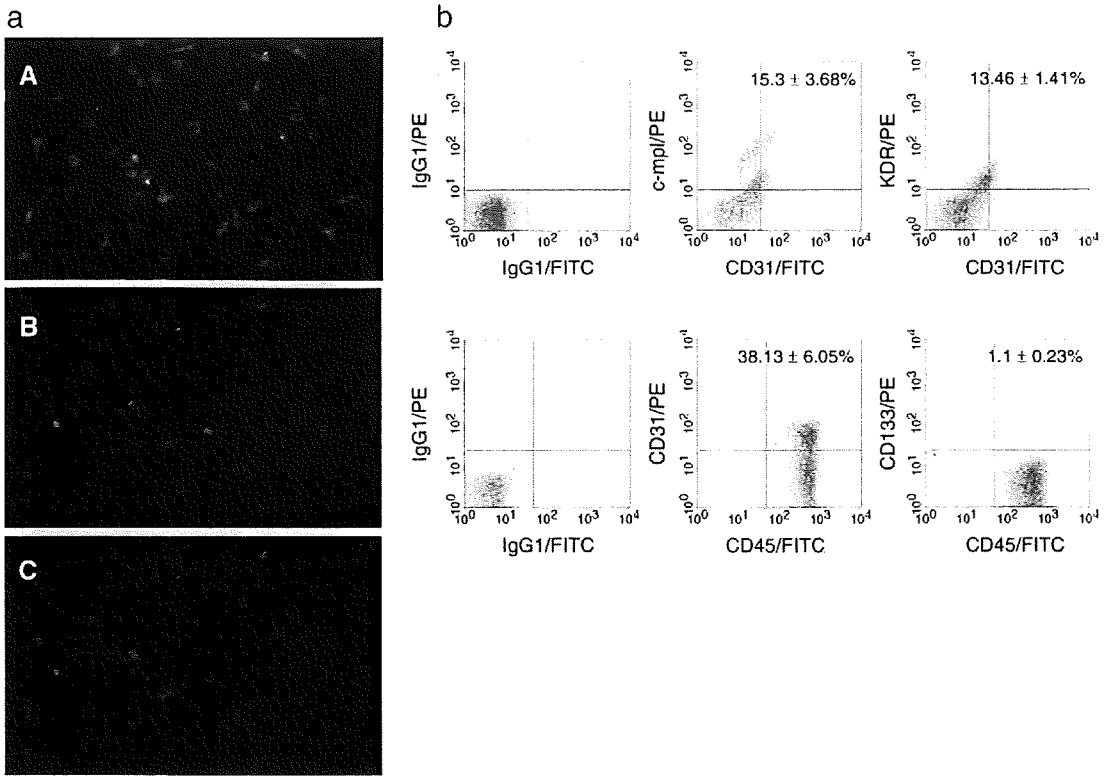

Fig. 1. Cultured EPCs expresses c-Mpl as well as endothelial specific antigens. (a) Cultured EPCs were shown to simultaneously endocytose DiI-acLDL (A) and bind FITC-UEA-1 (B) Mirror image (C). (b) Analysis of c-Mpl expression on cultured EPCs by flow cytometry. Cultured EPCs was analyzed for double positive of c-Mpl and CD31, KDR and CD31, CD31 and CD45, and CD133 and CD45.

stained with an endothelial marker, Isolectin B4-Alexa594, at day 28 (Fig. 3d). MVD was significantly augmented in the TPO-treated group as compared to the control (MVD/×100 HPF=156.32±50.824 in TPO, $p< 0.0001$ vs. 107.89±55.95 in control) (Fig. 3e). Thus, neovessel formation by cultured EPC transplantation was augmented by lesion-targeted TPO co-administration.

3.4. Induced blood flow recovery of ischemic limb by TPO co-administration

At day 14 and day 28, the blood flow of ischemic hindlimbs in the TPO treatment group significantly improved, as compared with control (ischemic/contralateral perfusion ratio=at day 4: 0.15±0.02 in TPO vs. 0.20±0.05 in control; at day 7: 0.46±0.06 in TPO vs. 0.31±0.06 in control; at day 14: 0.86±0.14 in TPO, $p< 0.05$ vs. 0.50±0.09 in control; at day 28: 0.81±0.07 in TPO, $p< 0.05$ vs. 0.55±0.10 in control) (Figs. 4a,b). These results indicated that lesion-targeted TPO co-administration enhanced neovessel formation by EPC transplantation, leading to the functional improvement of blood flow in ischemic hindlimbs.

3.5. TPO activation of the Akt/mTOR/p70S6K signal pathway in cultured EPCs

We next examined the effect of TPO on signaling transduction pathways in cultured EPCs. Akt phosphorylation was induced by TPO in 5 min. and reached a peak at 30 min. (Figs. 5a,b). Furthermore, phosphorylation of mTOR and p70S6K in the mTOR/p70S6K pathway downstream of Akt was also induced by TPO (Figs. 5c,d) (in Fig. 5b: phospho Akt intensity ratio/0 min=1.429±0.092 at 5 min, $p< 0.05$;

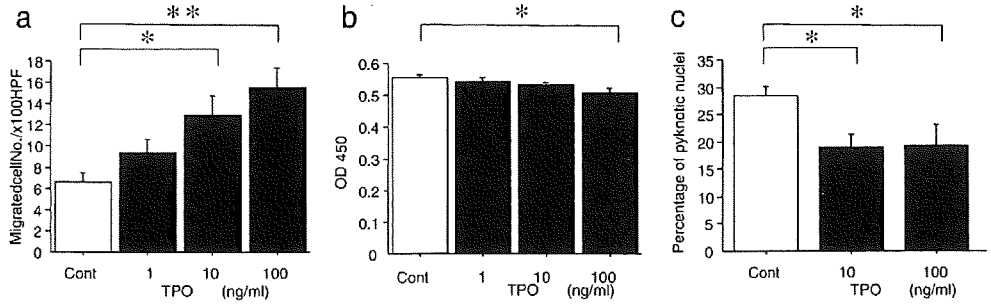

Fig. 2. TPO enhanced bioactivity of cultured EPCs. (a) TPO induced EPC migration. Migratory response of EPCs toward different dosages of TPO was measured by modified Boyden chamber migration assay. Cultured EPCs demonstrated a potent dose-dependent activity toward TPO (*$p< 0.005$, **$p< 0.0001$, $N=10$). (b) Increment of proliferative activity of cultured EPCs in response to TPO (*$p< 0.01$, $N=8$). (c) TPO attenuated cultured EPCs apoptosis (*$p< 0.05$, $N=5$).

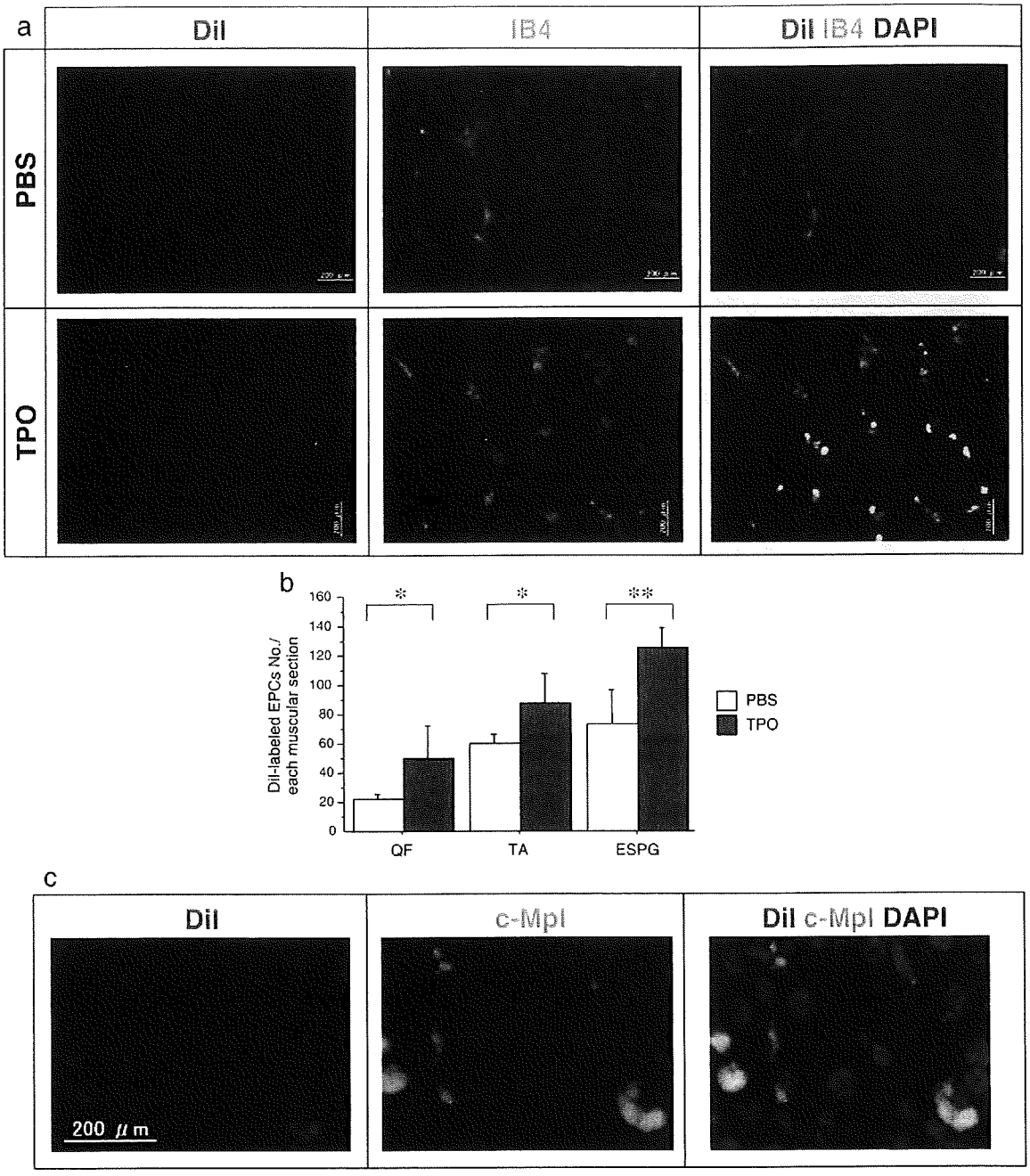

Fig. 3. Recruitment potential of transplanted EPC into vasculature of ischemic hindlimb was ameliorated by TPO co-administration at early time point. (a) Representative microscopic photographs of double fluorescence in ischemic muscles (tibialis anterior (TA), extensor digitorum lounges, soleus, plantaris, gastrocnemius (ESPG), and quadriceps femoris (QF) at day 4. Transplanted EPCs were labeled with a DiI fluorescence marker (red) in histological sections retrieved from ischemic muscles. Host mouse vasculatures were identified by isolectin B4 (green) in same tissue sections. Double positive cells were recognized as transplanted EPCs incorporate into mouse vasculature. The upper row: Ipsilateral hindlimb injected with PBS, The lower row: Ipsilateral hindlimb injected with TPO. (b) Quantitative analysis of incorporated EPCs in the muscle by counting number of DiI-labeled EPCs (red fluorescence) in PBS group at day 4 (*$p< 0.05$ **$p< 0.01$, $N=4$). (c) The c-Mpl expression (green) on DiI positive transplanted EPC (red). (d) Representative histology of microvasculature stained by Isolectin B4-Alexa594 in ischemic tissue at day 28. left: PBS group, right: TPO-treated group. (e) Quantitative analysis of MVD at 28 days (**$p< 0.0001$, $N=8$).

1.804±0.160 at 10 min, $p< 0.005$; 2.263±0.152 at 30 min, $p< 0.0001$ vs. 1.000±0.00 at 0 min; in Fig. 5d: phospho-mTOR intensity ratio/0 min=1.448±0.049 at 5 min; 2.032±0.072 at 10 min, $p<0.0001$; 1.978±0.140 at 30 min, $p<0.0001$ vs. 1.000±0.00 at 0 min; in Fig. 5d: phosph p70S6k intensity ratio/0 min=1.219±0.084 at 5 min; 2.957±0.267 at 10 min, $p< 0.005$; 3.559±0.310 at 30 min, $p< 0.0001$ vs. 1.000±0.00 at 0 min).

3.6. Essential role of the TPO-induced PI3K/Akt/mTOR/p70S6 kinase signaling pathway for migration and survival of cultured EPCs in vitro

To address the mechanism of the augmented effect of TPO on the *in vitro* bioactivity of cultured EPCs, we examined the effect of a phosphatidylinositol-3 kinesis (PI3K) inhibitor, Ly, and an mTOR inhibitor, Rapa, on augmented migration and survival capabilities