cells/HPF; 障害部 8.3±2.1 cells/HPF)、脂肪間葉系細胞では、健常部、障害いずれにも生着は確認されなかった。Western blot にて、移植後の細胞の生着率を定量化すると、CPC シート移植群では、移植時の約 2 割の細胞が生着していたのに対して、脂肪間葉系細胞では、わずか 0.8%の細胞が生着しているに過ぎなかった。また、生着した CPC の約 30%の細胞が、明瞭なサルコメア構造を呈し、かつαアクチニンを発現しており、一方、他の 30%の細胞は、管腔構造を呈しており、血管への分化も確認された。

D. 考察

今回の検討により、細胞シートを用いて心筋前駆細胞を心筋梗塞心に移植することにより、心臓のリモデリングの抑制及び心収縮力の改善が確認された。心筋前駆細胞は、対照として用いた脂肪間葉系細胞に比し、移植後の高い生着率を認め、結果心機能改善効果を示した。また結果には示していないものの、同数の CPC を心筋内に直接移植した際には、移植 1 週目の段階で、すでに約 1 割程度しか生着が確認されなかったことから、心筋前駆細胞の有用性のみならずシート移植の有用性も確認された。定量的評価より、今回の CPC シート移植によって創生された心筋細胞は、マウス心臓全体の約 5%に相当すると考えられ、心収縮力改善の一因と考えられた。

E. 結論

以上より、心筋前駆細胞は、他の組織幹細胞とは異なる特性を有し、また細胞シートを用いた細胞移植法により、心筋細胞創生、血管新生促進効果により、心筋梗塞後心機能障害を改善することが明らかとなった。

F. 健康危惧情報

なし

G. 研究発表

1. 論文発表

   なし

2. 学会発表

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H. 知的財産権の出願・登録状況

1. 特許出願　　なし
2. 実用新案登録　　なし
3. その他

研究協力者

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分担研究報告書

心筋・血管内皮ハイブリッド細胞シート作成を目的とした心筋幹細胞及び血液幹細胞由来血管内皮前駆細胞の分化増幅法の確立

分担研究者　増田治史　東海大学医学部基盤診療学系再生医療科学　准教授

研究要旨：ヒト心筋細胞・血管内皮細胞ハイブリッド細胞シートによる心・血管再生療法の確立を目的として、血管内皮前駆細胞シート作成技術を確立する。本年は、その細胞源獲得の基盤技術として、1）ヒト心筋幹細胞自身及び2）血液系幹細胞由来血管内皮前駆細胞の分化増幅法の至適化を行った。1）採取ヒト心筋幹細胞(心筋組織c-kit+細胞)を単離・継代培養を行い、特定培養条件における血管内皮前駆細胞への分化促進が可能であった。また、2)採取ヒト血液幹細胞(CD133+細胞)の特定成長因子組み合わせによる血管内皮前駆細胞の分化促進が可能であった。次年度は、各血管内皮前駆細胞由来内皮細胞シート培養を確立する。

A. 研究目的

採取ヒト心筋幹細胞(cardiac stem cell)及び血液幹細胞(hematopoietic stem cell)由来血管内皮前駆細胞(endothelial progenitor cell=EPC)シート作成を目的とした各EPCの分化増幅法の確立を目的とした。

B. 研究方法

1）CSC由来EPCについて；

① 採取ヒト心組織を初代培養し、c-kit+細胞をFACS sortingにてCSCとして採取。

② CSCをbFGF, erythropoietin 添加Ham's F12培地を用いて、HYCLONE社、VITROMEX社製FBSにて血管内皮系細胞分化能をFACS及びqPCR法にて検討した。

2）HSC由来EPCについて；

①臍帯血由来CD133+細胞を採取し、8GF(SCF, TPO, SDF, angiopoietin-1, IGF, EGF, VEGF, IL6)添加EPC分化増幅培養を行い、EPC colonyassayにてEPC分化能を検討した。

（倫理面への配慮）

東海大学における「医の倫理委員会」の承認を得た。

C. 研究結果

1）CSC由来EPCについて；

HYCLONE社製FBSに比較してVITROMEX社製FBSでは、CD31(内皮系マーカー)の発現がFACSにて約4倍上昇した。またqPCRではKDR及びVE-cadherinの発現がいずれも約２倍に上昇した。VITROMEX社製FBS添加培地の内皮系分化能が高かった。

2）HSC由来EPCについて；

8GFから各１成長因子削除培地によるEPC増幅培養によるEPC分化能を検討したところ、8GFからangiopoietin-1削除培養条件において、最も高い分化能を示した（他の1.7ー6倍）。

D. 考察

1）CSCから心筋細胞と共に内皮細胞への効率的な分化誘導が可能であることが示された。

2）HSC由来EPCの効率的誘導が可能であることが示された。

以上の結果より、シート作成に必要な内皮細胞の取得において、心筋幹細胞由来血管内皮細胞のみならず、血液幹細胞由来EPCから分化する内皮細胞の増幅分化培養が可能であることが示唆された。

現在、これら至適化培養条件下における各EPCの温度感受性培養皿を用いた細胞シートの作成の播種細胞数、培養期間等の観点から効率のよい細胞シート作成技術の確立を検討している。

E. 結論

本年度は、第一段階として、心筋細胞とのハイブリッド細胞シートに供する血管内皮細胞の細胞源の取得を目的とした培養法の至適条件を検討した。その増幅培養により本来、希少な幹細胞からの細胞供給が可能であることが示唆され、次年度は第二段階として細胞シート作成における内皮細胞培養条件の確立を実施し、そのin vivoでの効果をする。

F. 健康危険情報

特記なし。

G. 研究発表

1. 論文発表

① Kwon S, Masuda H, Asahara T et al. Specific jagged-1 Signal from Bone Marrow microenvironment is required for Endthelial Progenitor Cell development for neovascularization. *Circulation*.2008;118:157-165.

② Eguchi M, Masuda H, Kwon S, Asahara T etal.Lesion-targeted Thrombopoietin Potentiates Vasculogenesis by Enhancing Motility and Enlivenment of Transplanted Endothelial Progenitor Cells via Activation of Akt/mTOR/p70S6kinase Signaling Pathway. *JMCC*. 2008:45:661-669.

2. 学会発表

The American Heart Association（AHA）, November 9, Poster presentation (Notch and vascular biology, first author), New Orleans, USA. Endothelial Specific Jagged-1/Notch Signal in Bone Marrow Niche Regulates Endothelial Progenitor Cell Development.

（発表誌名巻号・頁・発行年等も記入）

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　特記なし。
2. 実用新案登録　特記なし。
3. その他　特記なし。

Ⅲ　研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kwon SM, Eguchi M, Wada M, Iwami Y, Hozumi K, Iwaguro H, Masuda H, Kawamoto A, Asahara T.	Specific Jagged-1 signal from bone marrow microenvironment is required for endothelial progenitor cell development for neovascularization.	Circulation	118(2)	157-65	2008
Mifune Y, Matsumoto T, Kawamoto A, Kuroda R, Shoji T, Iwasaki H, Kwon SM, Miwa M, Kurosaka M, Asahara T.	Local Delivery of Granulocyte Colony Stimulating Factor-Mobilized CD34-Positive Progenitor Cells Using Bioscaffold for Modality of Unhealing Bone Fracture.	Stem Cells	26(6)	1395-405	2008
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Eguchi M, Masuda H, Kwon S, Shirakura K, Shizuno T, Ito R, Kobori M, Asahara T	Thrombopoietin Potentiates Vasculogenesis by Enhancing Motility and Enlivenment of Transplanted Endothelial Progenitor Cells via Activation of Akt/mTOR/p70S6kinase Signaling Pathway.	JMCC	45	661-669	2008

Ⅳ　研究成果の刊行物・別刷

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

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

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]

Clinical Perspective p 165

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).

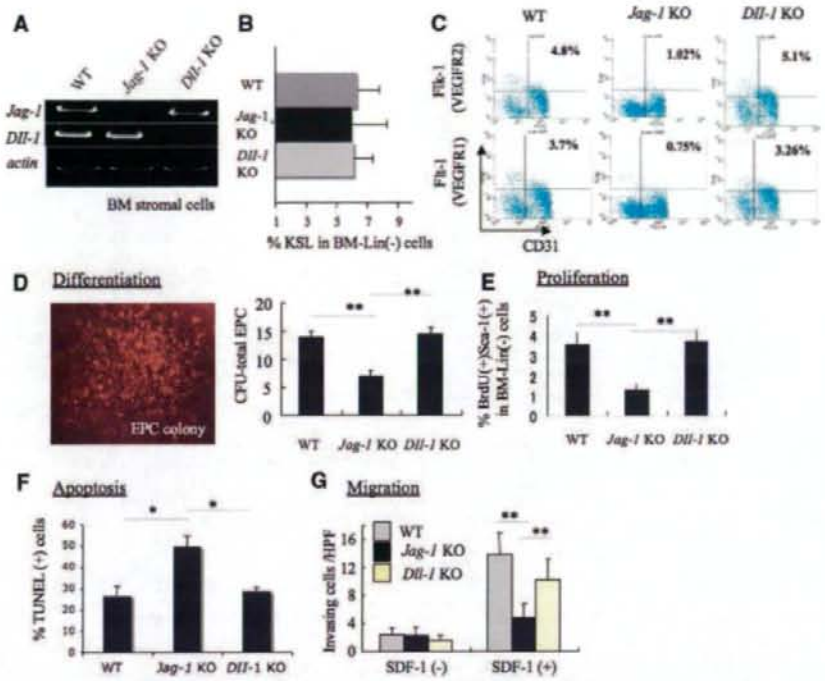

Figure 1. Impairment of endothelial commitment, proliferation, and invasiveness of EPCs in BM of conditional *Jag-1*$^{-/-}$ mice. A, Generation of conditional knockout (KO) mice lacking *Jag-1* or *Dll-1* gene. Reverse-transcription polymerase chain reaction confirmed specific deletion of *Jag-1* or *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

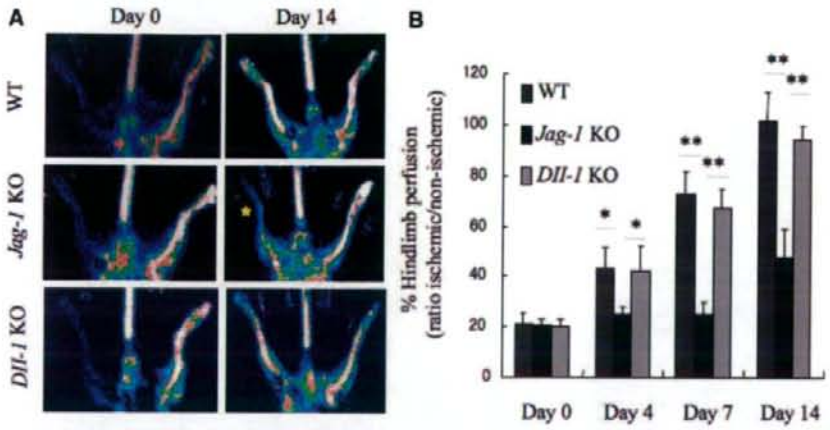

Figure 2. Delayed and impaired postnatal revascularization in *Jag-1* knockout (KO) mice. A, Representative findings of laser Doppler perfusion imaging in wild-type (WT), *Jag-1* KO, and *Dll-1* KO mice immediately after (Day 0) and 14 days after (Day 14) hindlimb ischemia. Recovery of blood flow at 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

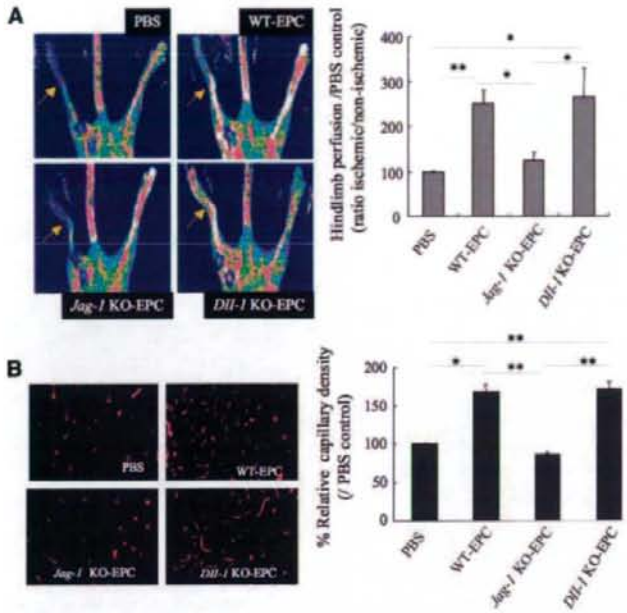

Figure 3. Effect of conditional deletion of distinct Notch signals on the therapeutic potential of EPCs for ischemic neovascularization. A, Representative laser Doppler perfusion imaging findings in nude mice 14 days after hindlimb ischemia and infusion of PBS or BM Sca-1$^+$/Lin$^-$ cells (EPC-enriched 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.

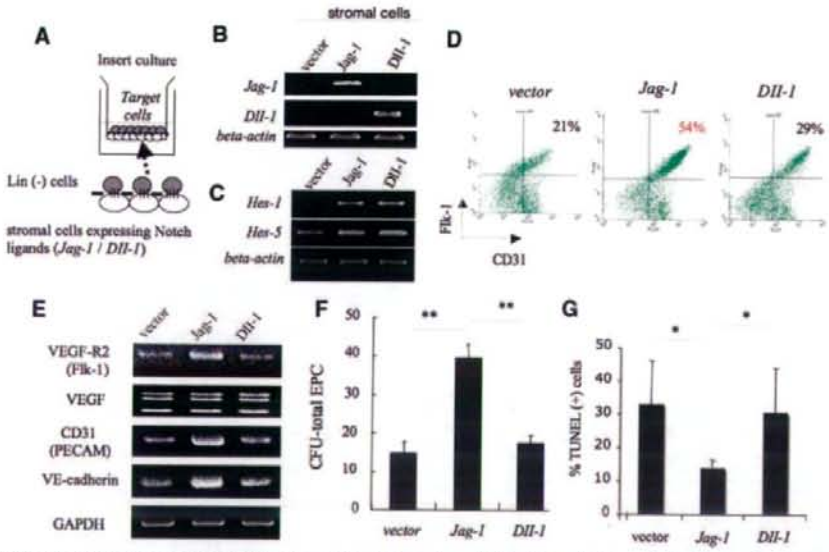

Figure 4. Effects of gain of function of Notch ligand–mediated signals on EPC differentiation. A, Scheme of the insert culture system with 0.4-μm pores, in which receptors on the surface of the target stem cells in the upper chamber are capable of directly interacting with ligands on the cells in the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

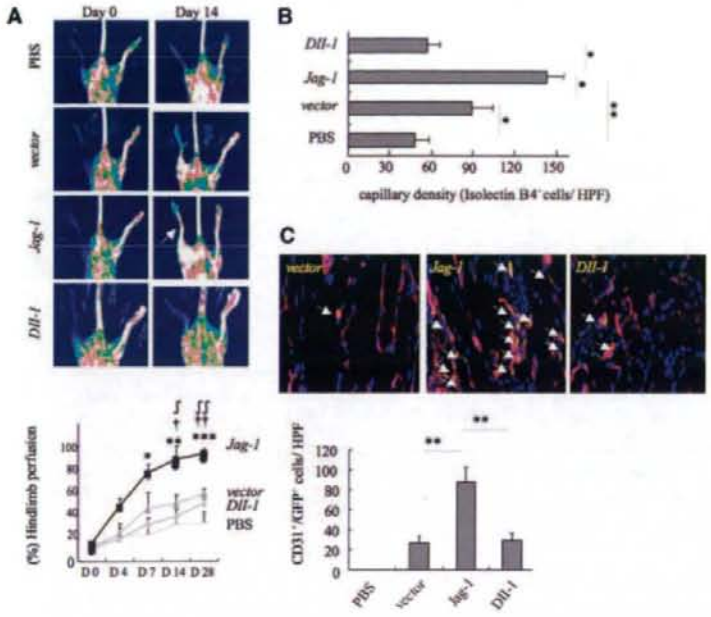

Figure 5. Promotion of in vivo neovascularization by transplantation of putative EPCs stimulated by Jag-1–mediated signals. A, Representative laser Doppler perfusion imaging findings in nude mice receiving PBS (no cells) or BM-Lin$^-$ cells cocultured with empty-vector– or specific Notch ligand (Jag-1,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

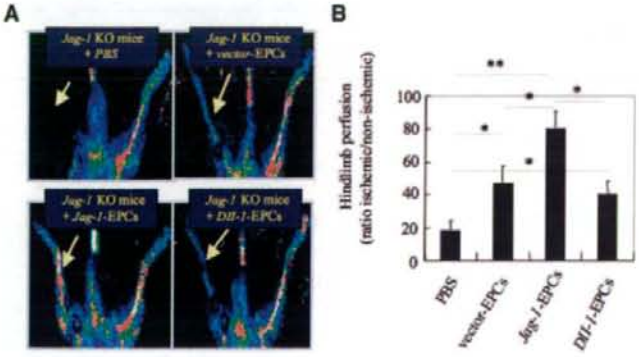

Figure 6. Rescue of impaired neovascularization in *Jag-1* knockout (KO) mice by transplantation of putative EPCs stimulated by specific Notch ligand–mediated signals. A, Representative laser Doppler perfusion imaging findings in *Jag-1* KO mice with hindlimb ischemia receiving PBS or BM-Lin$^-$ cells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

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.

STEM CELLS
TRANSLATIONAL AND CLINICAL RESEARCH

Local Delivery of Granulocyte Colony Stimulating Factor-Mobilized CD34-Positive Progenitor Cells Using Bioscaffold for Modality of Unhealing Bone Fracture

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Key Words. CD34 stem cells • CD34 progenitors • CD34 cell dose • Cellular therapy • Cell transplantation • Osteoblast • Endothelial cell • Stem cell transplantation

ABSTRACT

We recently reported that i.v. transplantation of adult human circulating CD34+ cells, an endothelial/hematopoietic progenitor-enriched cell population, contributes to fracture healing through the enhancement of vasculogenesis and osteogenesis. However, the scarcity of CD34+ cells in the adult human is a critical issue for the future clinical application of this method. To overcome this issue, we assessed in vitro and in vivo capacity of granulocyte colony-stimulating factor-mobilized peripheral blood (GM-PB) human CD34+ cells for vasculogenesis and osteogenesis. First, we confirmed the differentiation capability of GM-PB CD34+ cells into osteoblasts in vitro. Second, local transplantation of GM-PB CD34+ cells on atelocollagen scaffold was performed in nude rats in a model of unhealing fractures. Immunostaining for human leukocyte antigen-ABC of tissue samples 1 week after fracture and cell therapy showed the superior incorporation after local transplantation compared with systemic infusion. Third, the effects of local transplantation of 10^5 (Hi), 10^4 (Mid), or 10^3 (Lo) doses of GM-PB CD34+ cells or phosphate-buffered saline (PBS) on fracture healing were compared. Extrinsic vasculogenic and osteogenic differentiation of GM-PB CD34+ cells, enhancement of the intrinsic angio-osteogenesis by recipient cells, augmentation of blood flow recovery at the fracture sites, and radiological and histological confirmation of fracture healing were observed only in the Hi and Mid groups but not in the Lo and PBS groups. These results strongly suggest that local transplantation of GM-PB CD34+ cells with atelocollagen scaffold is a feasible strategy for therapeutic vasculogenesis and osteogenesis needed for fracture healing. STEM CELLS *2008;26:1395–1405*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Adult stem/progenitor cells play important roles in tissue homeostasis and have important implications for regenerative medicine. A number of reports have suggested that stem cells derived from a variety of adult tissues are capable of maintaining, regenerating, and repairing other tissues derived from all three germ layers [1–3].

Endothelial progenitor cells (EPCs), which are involved in vascular development in the embryonic stage [4, 5], were first identified as CD34+ cells in adult peripheral blood (PB) [6]. Adult EPCs, originating mainly from bone marrow (BM), are mobilized into PB and recruited into neovascularization sites. Recruited EPCs contribute to postnatal neovascularization by proliferating, differentiating and migrating [6–8]. The therapeutic application of EPCs has been attempted for neovascularization in animal models of hind limb, myocardial, and cerebral ischemia/infarction [9–17] and wound healing [18].

Recent progress in stem cell biology has brought human EPCs/CD34+ cells to light for other fields of regenerative medicine. Eghbali-Fatourechi et al. [19] first identified circulating osteocalcin (OC)-positive cells in adult human, demonstrating osteogenic gene expression and mineralized nodule formation in vitro and bone regeneration in vivo. Chen et al. [20] identified osteoblast (OB) precursor cells in human BM CD34+ cells. Tondreau et al. [21] reported that granulocyte

Author contributions: Y.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; T.M. and A.K.: conception and design, administrative support, manuscript writing; R.K., T.S., H.I., S.-M.K., and M.M.: conception and design, administrative support; M.K. and T.A.: conception and design, financial support, final approval of manuscript.

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colony-stimulating factor-mobilized peripheral blood (GM-PB) CD133+ cells, another EPC-enriched population, can act as mesenchymal stem cells and contribute to osteogenesis in vitro. These findings strongly suggest the therapeutic potential of BM-derived CD34+ cells for osteogenesis, as well as vasculogenesis. We previously reported that human circulating CD34+ cells, systemically transplanted into immunodeficient rats with nonhealing fracture, were recruited into fracture sites, contributed to a favorable environment for fracture healing by enhancing vasculogenesis and osteogenesis, and finally led to functional recovery from fracture [22]. Although our previous animal study demonstrating the efficacy of systemic transplantation of CD34+ cells may encourage the application of cell-based therapy in patients with unhealing fracture, local transplantation, but not systemic infusion, of CD34+ cells may need to be considered for future clinical trials for the following reasons: (a) intravenous infusion is generally known as a less safe method than local administration because of adverse systemic effects, and (b) local transplantation may allow reduction of effective cell dose compared with systemic administration, and this may overcome the critical issue in the clinical application: scarcity of circulating CD34+ cells in adults. Use of granulocyte colony-stimulating factor (G-CSF) is also a favorable method to harvest abundant CD34+ cells from adult PB in clinical situation.

In the current preclinical study, we proved the hypothesis that local transplantation of GM-PB CD34+ cells may contribute to fracture healing through vasculogenesis and osteogenesis at the lower dose compared with the systemic infusion used in our previous study [22]. We also demonstrated the in vitro differentiation potential of human GM-PB CD34+ cells into OBs as a supportive mechanistic study.

Materials and Methods

Preparation of Human Cells

We purchased human GM-PB CD34+ cells obtained from a healthy female (43 years old, African-American) and human BM total mononuclear cells (MNCs) obtained from a healthy female (25 years old, African-American) from Lonza (Valais, Switzerland, http://www.lonza.com/group/en.html).

Flow Cytometry Studies

Regular flow cytometric profiles were analyzed with a FACSCalibur analyzer (BD Biosciences, San Diego, http://www.bdbiosciences.com) and CELLQuest software (Becton, Dickinson and Company, Mountain View, CA, http://www.bd.com) as described previously [22].

Induction of Osteogenic Differentiation of GM-PB CD34+ Cells In Vitro

We seeded 5×10^3 BM-MNCs in six-well plates with α-minimum essential medium (α-MEM) (Gibco-BRL, Tokyo, http://www.gibcobrl.com) supplemented with 10% fetal bovine serum (FBS) (Vitromex, Vilshofen, Germany) and 2 mM L-glutamine (Gibco-BRL). After 2 weeks of culture, we collected the medium with floating cells and centrifuged it at 7,500*g* for 15 minutes at 4°C; we then collected the supernatant as conditioned medium (CM) and stored at −80°C for further use.

To induce mesenchymal stem cells from GM-PB CD34+ cells similarly to the previous method using BM-MNCs [21], we seeded 10^5 GM-PB CD34+ cells in six-well plates with α-MEM supplemented with 10% FBS, 2 mM L-glutamine, and 10% CM during the first 7 days of culture. Then, we cultured the cells in α-MEM supplemented with 10% FBS and 2 mM L-glutamine for the next 2 weeks.

Finally, to induce osteogenic differentiation, 10^5 cells were plated in six-well plates for 3 weeks under specific osteogenic conditions using α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 60 μM ascorbic acid (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 10 mM β-glycerophosphate (Sigma-Aldrich), and 0.1 μM dexamethasone (Sigma-Aldrich). Cells (10^5 cells) were also cultured with α-MEM supplemented with 10% FBS and 2 mM L-glutamine as a negative control.

Unhealing Fracture Model and Local/Systemic Transplantation of GM-PB CD34+ Cells

Female athymic nude rats (F344/N Jcl rnu/rnu; CLEA Japan, Tokyo, http://www.clea-japan.com) ages 8–12 weeks and weighing 150–170 g were used in this study. Nonhealing fracture was induced in femur with cauterizing periosteum according to the methods of Einhorn [23] and Kokubu et al. [24]. Immediately after the creation of nonhealing fracture, rats received local transplantation of 10^5 (Hi), 10^4 (Mid), or 10^3 (Lo) GM-PB CD34+ cells suspended in 100 μl of phosphate-buffered saline (PBS) using atelocollagen (Koken, Tokyo, http://www.kokenmpc.co.jp/english), which is a bovine-derived bioscaffold, or the same volume of PBS without cells using the same scaffold ($n = 15$ in the Hi group and $n = 12$ in all other groups). Atelocollagen was used as a bio-absorbable scaffold retaining the cells in the transplanted site [25, 26]. The left, unfractured femur of each animal served as a control. In addition, three rats received i.v. (systemic) transplantation of 10^5 GM-PB CD34+ cells suspended in 100 μl of PBS through the tail vein immediately after the creation of the fracture model.

Tissue Harvesting

Three rats receiving local or i.v. transplantation of 10^5 GM-PB CD34+ cells were sacrificed at week 1 for histological examination. Three rats in each group that received local transplantation were euthanized at weeks 2 and 4. Remaining rats receiving local transplantation were sacrificed at week 8. Bilateral femurs were harvested and quickly embedded as described previously [22].

Morphometric Evaluation of Capillary Density and OB Density

Histochemical staining with isolectin B4 (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) as a rat endothelial cell (EC) marker or immunostaining for OC (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) as a rat and human OB marker was performed. Capillary or OB density was morphometrically evaluated as the average value in five randomly selected fields of soft tissue in the perifracture site. To address the location of chondrocytes in fractured sections, toluidine blue was used for counterstaining. All morphometric studies were performed by two blinded examiners.

Reverse Transcriptase Polymerase Chain Reaction Analysis of RNA Isolated from GM-PB CD34+ Cells and Tissue Samples at the Perifracture Site

Total RNA was obtained from GM-PB CD34+ cells and the rat tissues in perifracture sites at week 2 using Trizol (Life Technologies, Gaithersburg, MD, http://www.lifetech.com) according to the manufacturer's instructions. The synthesization of first-strand cDNA and polymerase chain reaction (PCR) were performed as described previously [22]. Subsequently, PCR products were visualized in 1.5% ethidium bromide-stained agarose gels. Human umbilical vein endothelial cells and human OBs (NHOst cells; Cambrex) were used for positive controls for human-specific endothelial and bone-related genes.

To avoid interspecies cross-reactivity of the primer pairs between human and rat genes, we designed human-specific primers using Oligo software (Takara Bio, Shiga, Japan, http://www.takara-bio.com). Each primer sequence is shown in supplemental online Information 1. No primers showed cross-reactivity to rat genes (data not shown).

Real-Time PCR Analysis to Detect Expression of Cytokines in Recipients Following Human GM-PB CD34+ Cell Transplantation

Total RNA was obtained from the rat tissues at perifracture sites at week 2 as described for reverse transcription (RT)-PCR analysis. After the first-strand cDNA was synthesized, the converted cDNA samples (2 μl) were amplified in triplicate by real-time PCR (ABI Prism 7700, Applied Biosystems, Foster City, CA, http://www3.appliedbiosystems.com/index.htm) in a final volume of 20 μl using SYBR Green Master Mix reagent (Applied Biosystems). Melting curve analysis was performed to ensure that only a single product was amplified using Dissociation Curves software (Applied Biosystems). Specificity of the reactions was confirmed by 2.0% agarose gel electrophoresis. Results were obtained using sequence detection software (ABI Prism 7700) and evaluated using Excel (Microsoft, Redmond, WA, http://www.microsoft.com). Each primer sequence is shown in supplemental online Information 2.

Physiological Assessment of Tissue Perfusion by Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging (LDPI) (Moor Instruments, Wilmington, DE, http://www.moor.co.uk) [27, 28] was used to measure blood flow in both legs at 0, 1, 2, and 3 weeks postfracture. The ratio of fractured to intact (contralateral) blood flow was calculated to evaluate serial blood flow recovery after fracture.

Immunohistochemical Staining

To detect the transplanted human cells in the rat tissue, immunohistochemistry was performed with following human-specific antibodies: human leukocyte antigen (HLA)-ABC (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) to detect various lineage of human cells, *Ulex europaeus* lectin type 1 (UEA-1) (Vector Laboratories) for human ECs, human-specific osteocalcin (hOC) (Biomedical Technologies, Inc., Stoughton, MA, http://www.btiinc.com/index.html) for human OBs, and smooth muscle actin (SMA) for both human and rat smooth muscle cells. The secondary antibodies for each immunostaining were as follows: Alexa Fluor 594-conjugated goat anti-mouse IgG_1 (Molecular Probes, Tokyo, http://probes.invitrogen.com) for HLA-ABC staining, Cy3-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) for UEA-1 staining, Alexa Fluor 488-conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for SMA, and Cy3-conjugated AffiniPure goat anti-rabbit IgG(H+L) (Jackson Immunoresearch Laboratories) for hOC. 4,6-Diamidino-2-phenylindole solution was applied for 5 minutes for nuclear staining.

Radiographic Assessment of the Fracture Healing

Radiographs of the fractured hind limbs were taken on weeks 0, 2, 4, and 8 following creation of the fracture. Fracture union was identified by the presence of a bridging callus on two cortices. Radiographs of each animal were examined by three blinded observers.

Histological Assessment of the Fracture Healing

Toluidine blue staining was performed to histologically evaluate the process of endochondral ossification at weeks 2, 4, and 8. The degree of fracture healing was evaluated using a five-point scale proposed by Allen et al. [29].

Statistical Analysis

The results were statistically analyzed using a software package (Statview 5.0; Abacus Concepts Inc, Berkeley, CA, http://www.abacus.com/abacus/home). All values were expressed as mean ± SE. Paired *t* tests were performed for comparison of data before and after treatment, and unpaired *t* tests were performed for comparison of local and i.v. transplantation. The multiple comparisons among groups were made using a one-way analysis of variance. Post hoc analysis was performed by Fisher's Protected Least Significant Difference test. The comparison of radiological results was performed with a χ^2 test. A probability value of $<.05$ was considered to denote statistical significance.

RESULTS

Phenotypic Characterization of GM-PB CD34+ Cells

GM-PB CD34+ cells from the healthy females used in this study were analyzed by flow cytometry. GM-PB CD34+ cell fraction was mainly positive for CD133, CD31, c-Kit, and CD45 but negative for kinase insert domain protein receptor and CD14 (supplemental online Fig. 1A). RT-PCR analysis of the GM-PB CD34+ cells revealed weak expression of the human-specific gene of CD31 (hCD31) and OC (hOC) but no expression of VE-cadherin (hVE-cad) and collagen1A1 (hCol1A1) (supplemental online Fig. 1B). RT-PCR analysis of the bovine-derived atelocollagen that we used as a scaffold showed no expression of human-specific genes, suggesting no cross-reaction of human-specific primers with bovine mRNAs.

In Vitro Differentiation of Human GM-PB CD34+ Cells into OBs

During the primary culture for mesenchymal stem cell induction, part of the GM-PB CD34+ cells exhibited a fibroblast-like spindle shape (Fig. 1A), proliferating quickly to form colonies (Fig. 1B). Treatment for 3 weeks with specific conditions for osteogenic induction resulted in a morphological transformation of the cells from long and spindle-like into a cuboidal shape (Fig. 1C). In contrast, no transformation was observed in the negative control group (Fig. 1D). As shown in Figure 1E, following osteogenic induction, matrix mineralization (calcium deposition) was clearly demonstrated by alizarin red staining. In contrast, no mineralization was observed in negative control wells (Fig. 1F). As shown in Figure 1G, mRNA of hOC and hCol1A1 was highly expressed in the CD34+ cells after osteogenic induction compared with those not induced. These results indicate that human GM-PB CD34+ cells are capable of differentiating into OBs under specific culture conditions.

More Efficient Incorporation of Human GM-PB CD34+ Cells at Fracture Site Following Local Transplantation Compared with Systemic Infusion

To test the potential therapeutic superiority of local transplantation over systemic infusion, rats received 10^5 GM-PB CD34+ cells locally or intravenously and were sacrificed 1 week later to obtain tissue samples at the fracture sites. Immunostaining for HLA-ABC was performed to quantify the number of recruited human cells in the rat granulation area (Fig. 2A, 2B) and newly bone formed area (Fig. 2D, 2E). The double immunostaining for HLA-ABC and SMA demonstrated more abundant recruitment of human cells in the granulation area compared with the inner layer of SMA-positive smooth muscle cells, which were morphologically compatible with endothelial cells in arterioles, in both groups (Fig. 2A, 2B). The number of human cells in the granulation area was significantly higher in the local transplantation group compared with the i.v. infusion group (local, 105.6 ± 14.8; i.v., 38.0 ± 16.6 cells per mm^2; $p < .05$) (Fig. 2C). Similarly, the number of human cells in the newly formed bone area was significantly higher in the local transplantation group than in the i.v. infusion group (local, 96.0 ± 10.5; i.v., 37.9 ± 15.4 cells per mm^2; $p < .05$) (Fig. 2F). These findings strongly indicate that local transplantation is superior to i.v. infusion in terms of efficiency of human GM-PB CD34+ cell incorporation into fracture sites.

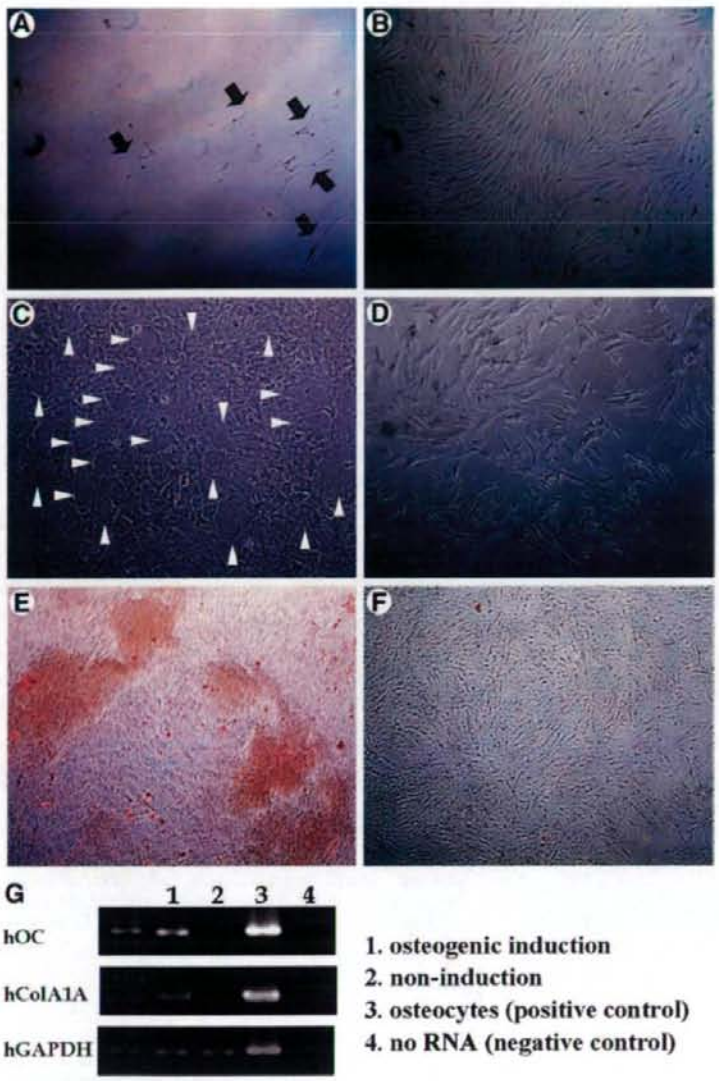

Figure 1. Osteogenic differentiation of granulocyte colony-stimulating factor-mobilized peripheral blood (GM-PB) CD34+ cells in vitro. **(A, B):** Morphology of the GM-PB CD34+ cells, which were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10% conditioned medium (CM) during the first 7 days and in the same medium without CM for the next 1 week, changed into fibroblast-like spindle shape (×200) **(A)**. Then, these spindle-shaped cells proliferated quickly to form colonies (×40) **(B)**. **(C, D):** After osteogenic induction with α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 60 μM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone, the cell morphology changed from spindle-shaped to a cuboidal shape (arrowheads) (×40) **(C)**. In contrast, no transformation was observed in cultured CD34+ cells with α-MEM supplemented with 10% FBS and 2 mM L-glutamine only (no osteogenic induction) (×40) **(D)**. **(E, F):** In wells with osteogenesis-inducing conditions, the matrix mineralization was clearly demonstrated by alizarin red staining, indicating existence of calcium **(E)**. In contrast, no mineralization was observed in noninducing conditions (×40) **(F)**. **(G):** The mRNA of hOC and hCol1A1 was markedly expressed in osteogenesis-induced cells but not in noninduced cells. Abbreviations: hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; hOC, human-specific osteocalcin.

Vasculogenesis and Osteogenesis Induced by Human GM-PB CD34+ Cells

Next, we performed experiments to characterize the transplanted human CD34+ cells incorporating into fracture sites. To histologically validate the phenomenon of human cell-derived vasculogenesis, histochemical staining for UEA-1, a human-specific EC marker, was performed using the tissue samples obtained 2 weeks after local cell transplantation. Differentiated human ECs in the vasculature of the perifracture area were detected as UEA-1-positive cells in the Hi and Mid GM-PB CD34+ cell groups but not in the Lo and PBS groups (Fig. 3A–3F). These findings suggest that GM-PB CD34+ cells have the potential to differentiate into ECs; however, transplantation of more than 10^4 CD34+ cells may be necessary for significant vasculogenesis. To further verify this phenomenon, RT-PCR analysis of tissue RNA isolated from the perifracture site for human-specific EC markers (hVE-cad and hCD31) was performed (Fig. 3G). The expression ratio of hVE-cad to rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) was significantly greater in the Hi group than in all others; the ratio was also higher in the Mid group than in the Lo and PBS groups (Hi, 0.579 ± 0.043; Mid, 0.399 ± 0.023; Lo, 0.197 ± 0.011; PBS, 0.191 ± 0.018; $p < .01$ for Hi vs. Lo or PBS group; $p < .05$ for Hi vs. Mid and for Mid vs. Lo or PBS group). The expression ratio of hCD31 to rGAPDH was also greater in the Hi group than in the other groups, and the ratio was higher in the Mid group than in the Lo and PBS groups. The ratio was also significantly higher in the Lo group than in the PBS group (Hi, 0.974 ± 0.064; Mid, 0.563 ± 0.023; Lo, 0.429 ± 0.025; PBS group, 0.159 ± 0.011; $p < .01$ for Hi vs. PBS group; $p < .05$ for Hi vs. Mid or Lo, for Mid vs. Lo or PBS, and for Lo vs. PBS group) (Fig. 3H).

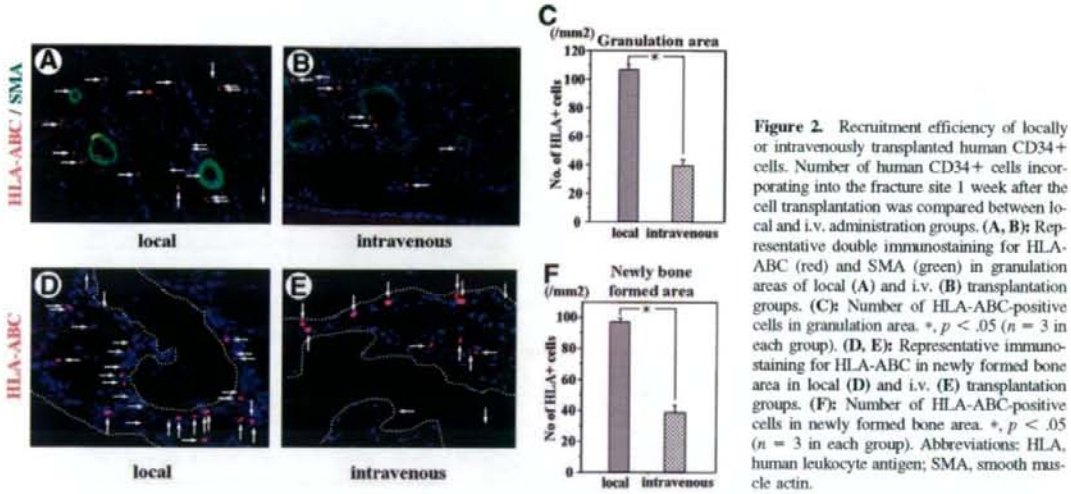

Figure 2. Recruitment efficiency of locally or intravenously transplanted human CD34+ cells. Number of human CD34+ cells incorporating into the fracture site 1 week after the cell transplantation was compared between local and i.v. administration groups. **(A, B):** Representative double immunostaining for HLA-ABC (red) and SMA (green) in granulation areas of local **(A)** and i.v. **(B)** transplantation groups. **(C):** Number of HLA-ABC-positive cells in granulation area. *, $p < .05$ ($n = 3$ in each group). **(D, E):** Representative immunostaining for HLA-ABC in newly formed bone area in local **(D)** and i.v. **(E)** transplantation groups. **(F):** Number of HLA-ABC-positive cells in newly formed bone area. *, $p < .05$ ($n = 3$ in each group). Abbreviations: HLA, human leukocyte antigen; SMA, smooth muscle actin.

To identify osteogenesis from human CD34+ cells, immunohistochemical staining for human-specific OC, an OB marker, was performed using tissue samples obtained 2 weeks after cell transplantation. Differentiated human OBs derived from high and middle doses of GM-PB CD34+ cells were detected as hOC-positive cells in the perifracture area (Fig. 3I, 3J), whereas hOC-positive cells were not identified in the low-dose group of GM-PB CD34+ cells or the PBS group (Fig. 3K, 3L). These findings suggest that transplanted GM-PB CD34+ cells may have the potential to differentiate into OBs; however, a greater than middle dose of CD34+ cells may be necessary for significant osteogenesis, as suggested in the vasculogenesis assessment. RT-PCR analysis also demonstrated dose-dependent expression of human-specific bone-related markers (hOC and hCol1A1) following human GM-PB CD34+ cell transplantation (Fig. 3M). The expression ratio of hOC to rGAPDH was significantly greater in the Hi group than in the other groups (Hi, 0.591 ± 0.032; Mid, 0.279 ± 0.021; Lo, 0.287 ± 0.014; PBS, 0.288 ± 0.011; $p < .05$ for Hi vs. Mid, Lo, or PBS group). The expression ratio of hCol1A1 to rGAPDH was also significantly greater in the Hi group than in the other groups, as well as in the Mid group compared with the Lo and PBS groups (Hi, 0.988 ± 0.077; Mid, 0.366 ± 0.030; Lo, 0.227 ± 0.021; PBS, 0.031 ± 0.004, respectively; $p < .01$ for Hi vs. PBS group; $p < .05$ for Hi vs. Mid or Lo, for Mid vs. Lo or PBS, and for Lo vs. PBS groups) (Fig. 3N).

These results indicate that human GM-PB CD34+ cells dose-dependently differentiate into both EC and OB lineages in the fracture-induced environment. For efficient vasculogenesis and osteogenesis, local transplantation of at least 10^4 CD34+ cells may be essential in this animal model.

Enhancement of Intrinsic Angiogenesis and Osteogenesis in Animals Receiving GM-PB CD34+ Cells

Enhanced angiogenesis and osteogenesis by the paracrine effect of the transplanted cells on recipients' cells were confirmed by immunostaining for rat-specific markers. Vascular staining with isolectin B4, a rat-specific marker for EC, using tissue samples collected 2 weeks postfracture, demonstrated enhancement of intrinsic neovascularization around the endochondral ossification area in animals treated with high and middle doses of GM-PB CD34+ cells (Fig. 4A). Capillary density was significantly greater in the Hi group compared with the other groups, as well as in the Mid group compared with the Lo and PBS groups (Hi, 1,136.8 ± 95.5; Mid, 928.7 ± 61.6; Lo, 752.5 ± 49.3; PBS, 616.5 ± 57.4 cells per mm^2, respectively; $p < .01$ for Hi vs. PBS group; $p < .05$ for Hi vs. Mid or Lo group and for Mid vs. Lo or PBS group) (Fig. 4B).

OB staining with anti-rat OC using tissue samples collected 2 weeks postfracture revealed augmentation of intrinsic osteogenesis in the area of new bone formation in animals treated with high and middle doses of GM-PB CD34+ cells (Fig. 4C). OB density was significantly greater in the Hi group than in the other groups, and density in the Mid group was significantly higher than that in the PBS group (Hi, 641.3 ± 54.3; Mid, 399.6 ± 21.3; Lo, 301.7 ± 12.4; PBS group, 213.6 ± 19.2 cells per mm^2, respectively; $p < .01$ for Hi vs. PBS group; $p < .05$ for Hi vs. Mid or Lo group and for Mid vs. PBS group) (Fig. 4D).

A possible explanation for the enhancement of intrinsic angiogenesis and osteogenesis following CD34+ cell therapy is the upregulation of angiogenesis- and osteogenesis-related cytokines at the perifracture site. Accordingly, we performed real-time RT-PCR to quantify the expression of rat vascular endothelial growth factor (rVEGF) and rat bone morphogenetic protein 2 (rBMP-2) around the fracture sites. The expression ratio of rVEGF to rGAPDH at week 2 was greater in animals receiving a high dose of GM-PB CD34+ cells compared with other groups, and the ratio in the middle-dose group was higher than that in the low-dose and PBS groups (Hi, 1.131 ± 0.284; Mid, 1.085 ± 0.269; Lo, 1.037 ± 0.215; PBS, 1.035 ± 0.231, respectively; $p < .01$ for Hi vs. Lo or PBS group; $p < .05$ for Hi vs. Mid group and for Mid vs. Lo or PBS group) (Fig. 4E). The expression ratio of rBMP-2 to rGAPDH at week 2 was also significantly greater in animals receiving a high dose of GM-PB CD34+ cells compared with other groups, and the ratio in the middle-dose group was higher than that in the low-dose and PBS groups (Hi, 1.028 ± 0.276; Mid, 0.991 ± 0.271; Lo, 0.923 ± 0.216; PBS, 0.907 ± 0.244, respectively; $p < .01$ for Hi vs. Lo or PBS and for Mid vs. PBS group; $p < .05$ for Hi vs. Mid group and for Mid vs. Lo group) (Fig. 4F). These results indicate that high and middle doses of human GM-PB CD34+ cells enhance both intrinsic angiogenesis and osteogenesis, at least in part by upregulating rVEGF and rBMP-2 at the fracture sites.