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「重症心不全患者の自己心筋幹細胞を用いた

心筋・血管ハイブリッド組織シート移植治療の臨床研究開発」

# 平成20年度～22年度総合研究報告書

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# I 総括研究報告書

厚生労働科学研究費補助金（再生医療実用化研究事業）

# 総合研究報告書

## 重症心不全患者の自己心筋幹細胞を用いた心筋・血管ハイブリッド組織シート移植治療の臨床研究開発

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**研究概要**：平成２０年度から２２年度までの３年間のプロジェクトで、心筋幹細胞の採取および維持培養標準化のための基礎開発を進めた。技術開発により、培養心筋幹細胞は臨床治療効果を期待できる細胞品質を保つことが可能になった。細胞シートの移植方法は多岐にわたって検討されたが、心筋再生のための心筋幹細胞シートと、血管再生のための血管内皮前駆細胞移植の組み合わせで、慢性心不全モデルに心筋再生および血管再生効果をもたらし、機能回復を期待できることが判明した。この成果をもとに、先端医療センターおよび大阪大学の共同で、臨床研究を具体的に検討する段階まで進んだ。

### A．分担者名

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### 1．研究目的

以下の３つの研究目的が設定された。

(1) 心筋幹細胞の採取および培養の標準化

(2) 心筋幹細胞シートの開発

(3) 心筋幹細胞心筋・血管ハイブリッド治療の確立

(4) 臨床心筋幹細胞シート移植治療法の開発

### 2．3．研究結果および考察

(1) 心筋幹細胞の採取および培養の標準化

臨床応用のための hCSC 細胞採取・培養方法の標準化のための基礎開発研究を進めた。

(A)臨床の心筋採取部位とhCSCの分離効率の関係を追求したところ、右心房が最も心臓幹細胞の分離効率が高い傾向にあることが判明した。

(B) 各種心疾患と心臓幹細胞の分離効率の関係を検討したところ、全ての心疾患患者から心臓幹細胞は単離可能であること、が明らかになった。

(C) 臨床採取組織から分離する際のコラゲナーゼ反応時間と心臓幹細胞の分離効率の関係を追求したところ、１２時間以内のコラゲナーゼ反応時間が最も hCSC の分離効率が高いことが判明。

(D) 播種細胞密度と１継代当りの培養日数が C-Kit 陽性率に与える効果を検討したところ、340cells/cm2, 5 days/passage の条件が最も C-Kit 陽性率の低下を抑制し、細胞倍化時間の遅延化を抑制できることが判明。

(E) 心筋サンプルの維持培養条件を確認するために、9人の患者から９検体を採取しhCSC培養を試み、全例で CSC は採取され、細胞倍加時間 (doubling time) を測定したところ、患者の年齢と正の相関関係が確認され、高齢患者においては女性より男性由来 hCSC の boubling time が延長される傾向が確認

された。さらに多くの経験を必要とするが、基礎疾患とともに年齢・性別などを考慮した培養経過の観察が必要と考えられた。

以上、臨床のための hCSC 培養方法の開発に伴い、米国の本法開発者 Piero Anversa 教授との会合も続け、研究者を派遣して、臨床における CSC 培養の開発の同期化を計ってきた。

(2) 心筋幹細胞シートの開発

採取 hCSC 細胞シート作製の適正化研究を行った。

(A) hCSC シート作成方法:条件検討を加えたところ、シート作製のための播種細胞密度は $1.5x10^6$ 個または $3.0x10^6$ 個/3.5cm ディッシュが最適であることが判明した。細胞シート移植時には、シートが心臓組織に確実に接着するまでしばらく静置する必要があるため、あらかじめ積層化したものを移植することが適切であることが判明した。

(B) hCSC シートの移植生着検討:hCSC のシートが移植された場合の、炎症・低酸素組織の移植環境での有効性を推測するために、酸化ストレス下での hCSC シートの細胞活性について検討が加えられた。心筋分化誘導細胞シートの場合は、熱ショックや hypoxia を組み合わせても、in vitro における強い酸化ストレス(1mM H2O2)に対して細胞生存活性や細胞増殖活性は有意に向上することはなかった。心筋分化誘導細胞シートはこれらの処理を全く行わなくても酸化ストレスに対して細胞生存活性等の有意な亢進を示したことから、確立した心筋分化誘導条件で作製した心筋・血管細胞シートは抗酸化ストレス活性を十分に有しており、虚血下でも細胞増殖活性を示して、細胞保護作用を持つことが示唆された。

しかし、未分化の hCSC 細胞シートを作成した場合、熱ショックと hypoxia の両方で処理した時に心筋分化誘導細胞シートと同様の細胞活性を保てることが判明し、未分化 hCSC シートの場合は、熱ショックおよび低酸素などの耐性強化により、移植率を上げられる可能性が示唆された。

(3) 心筋幹細胞心筋・血管ハイブリッド治療の確立

心筋再生および血管再生の共同再生メカニズムを探り、ハイブリッドhCSC 移植治療の最適化を計った。

(A) 心筋分化誘導シート作製研究:心筋分化誘導 hCSC シート化には「細胞増殖効率」と「温度応答性培養皿への接着効率」の 2 つが重要な因子となる。様々な培養・播種条件を検討したところ、細胞播種密度 $3.0 \times 10^5$ cells / 3.5cm ディッシュが細胞シート形成には必須であることが明らかになった。hCSC の心筋細胞への分化誘導に影響を与える因子として「分化培地に含まれる血清」と「分化誘導剤のタイミング」が考えられ検討した。人工血清 KSR は 2.5%の濃度において、細胞シートを構築される 10〜14 日目にかけて心筋初期遺伝子の持続的な発現と心筋構造タンパク質の有意な増加が確認できた。分化誘導剤のタイミングは、dexamethasone を連続投与すると寧ろ、心筋・血管内皮・平滑筋遺伝子の発現を抑制する結果になり、初期投与で十分であることが判明した。

(B) hCSC シートの血管再生作用の検討:hCSC、ヒト骨格筋芽細胞、ヒト骨髄間葉系細胞の非コンフルエント、コンフルエント、細胞シート培養の比較、分泌蛋白比較、血管形成効果の検討を行った。hCSC の培養上清中の VEGF、bFGF 濃度は、ヒト骨格筋芽細胞、ヒト間葉系細胞培養上清に比して有意に高かった。またこの差は、コンフルエント、細胞シート状態でより顕著であった。また培養上清を matrigel 上で培養した HUVEC に添加したところ、増殖因子の濃度の同様に、hCSC 培養上清を添加した群で管腔形成能が亢進し、さらにコンフルエント培養、細胞シートから回収した培養上清ではより管腔形成能が亢進した。以上より hCSC は、ヒト骨格筋芽細胞、ヒト骨髄間葉系細胞より、多くの血管

新生増殖因子を産生し、またこの産生された増殖因子は血管新生作用として機能的であると考えられた。この hCSC シートにおける顕著な血管新生促進効果の機序として、Notch シグナルの活性化に伴う VEGF の産生亢進が、シグナル検索で推定された。

(C) 移植環境下での hCSC 分化誘導研究: 移植環境下での hCSC 分化条件を探るため、流れズリ応力および低酸素での hCSC 分化機序を研究した。静的条件下および低酸素条件下では細胞の配列は一定の方向性を示さなかったが、流れずり応力条件下では流れの方向に細胞が伸展・配勾した。この反応は血管内皮細胞に特異的であり、hCSC が流れずり応力により血管内皮細胞系に分化誘導されたことを示している。これは、FACS による血管内皮のマーカーである KDR と Tie2 の流れずり応力負荷および低酸素負荷で発現率が増大したことからも推定できる。また、増殖作用・抗アポプトーシス作用も、流れずり応力負荷および低酸素負荷で確認され、hCSC シートの移植環境下での分化誘導機序と、再生機序の有効性も推察できた。

(4) 臨床心筋幹細胞シート移植治療法の開発

(A) hCSC シートのラット移植予備検討

心筋梗塞モデルラットに hCSC シートを移植して細胞シートの治療効果を検証した。

シートの細胞密度とシート枚数について、移植検討したが、1 枚 Low シート移植では治療効果が認められなかったが、3 枚 Low および High シート移植群では、対照群と比較して心機能と毛細血管密度の有意な改善が認められた。よって hCSC シートは 1 枚ではなく複数枚積層化して移植する必要があることが示唆された。移植した hCSC がより効率よく心筋細胞に分化するような移植方法等の検討は進める余地があると考えられた。

(B) hCSC シートと血管内皮前駆細胞(EPC)の同時移植効果検討

hCSC シート単独移植群では、心機能が改善する傾向が見られた。hCSC シートと EPC を同時移植した群では、移植 2 週目と 4 週目において、細胞を移植しなかった群に比べて、さらに、移植後 2 週目において、hCSC シート単独移植群と比べて、有意な心機能改善効果（LVFS、左室内径短縮率）が検出された。組織学的にも、心筋分化と血管形成の促進が hCSC シート・EPC 同時移植群で明確に確認され、心筋血管両方の分化機序が機能改善に伴われたことを示唆した。

(C) ブタ慢性心筋梗塞モデルを用いた hCSC シート移植および EPC 心筋内投与併用療法の前臨床試験

将来的な臨床試験を目指し、ブタ大動物モデルを用いて、hCSC シートと hEPC 心筋内投与併用療法の安全性および治療効果を検討した。移植 8 週後の心臓超音波検査上、hCSC シート群では 7.9%の EF の改善を認め、hCSC シート＋hEPC 併用群では 11.3%と治療効果の増大が確認された。全例が耐術し、経過観察期間中、致死性不整脈を含めた主要な有害事象を認めず、これらの細胞移植の安全性は確認できた。さらにこれまでの小動物実験同様、慢性心筋梗塞モデルに対する hCSC シートと hEPC 併用による左室収縮能の改善効果が確認された。今後は、包括的評価の後、臨床応用の際のプロトコールを作成する予定である。

## 4. 結論

平成20年度から22年度までの 3 年間のプロジェクトで、hCSC 採取および維持培養の標準化のための基礎開発は完成した。培養されたhCSC は臨床治療効果を期待できる細胞品質を保つことが可能になった。細胞シ

ートの移植方法は多岐にわたって検討されたが、hCSCシートとEPC移植の組み合わせで、慢性心不全モデルに心筋再生および血管再生効果をもたらし、機能回復を期待できることが判明した。この成果をもとに、先端医療センターおよび大阪大学の共同で、臨床研究を計画している。

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# II　研究成果の刊行に関する一覧表

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# Ⅲ　研究成果の刊行物・別刷

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

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# 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.

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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).

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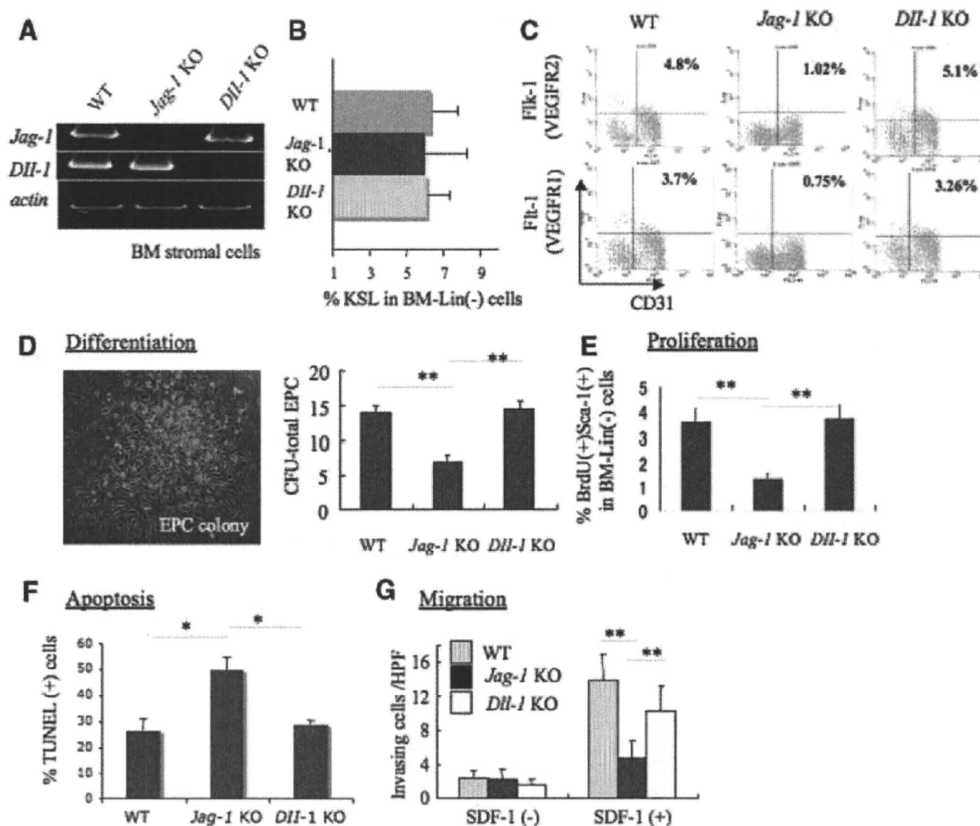


**Figure 1.** Impairment of endothelial commitment, proliferation, and invasiveness of EPCs in BM of conditional *Jag-1*$^{-/-}$ mice. A, Generation of conditional knockout (KO) mice lacking *Jag-1* or *Dll-1* gene. Reverse-transcription polymerase chain reaction confirmed specific deletion of *Jag-1*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

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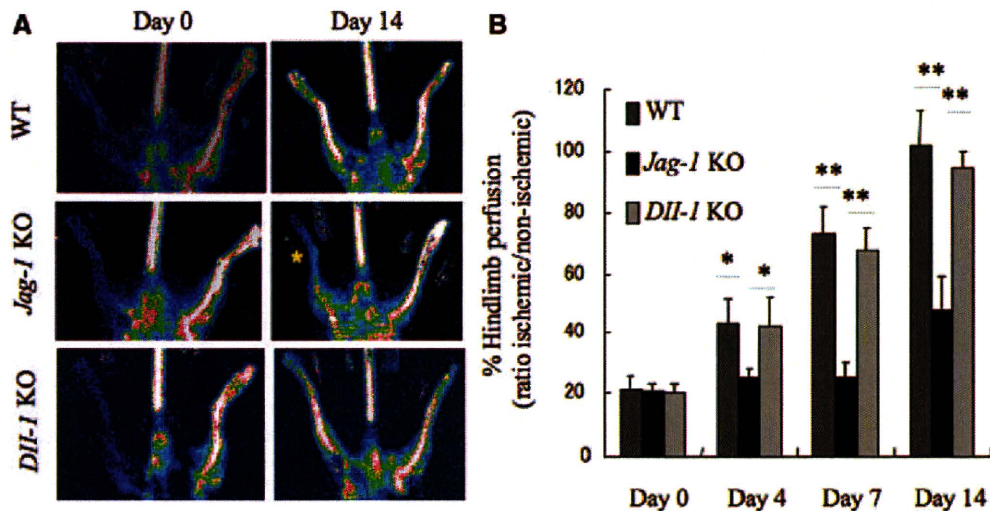


**Figure 2.** Delayed and impaired postnatal revascularization in *Jag-1* knockout (KO) mice. A, Representative findings of laser Doppler perfusion imaging in wild-type (WT), *Jag-1* KO, and *Dll-1* KO mice immediately after (Day 0) and 14 days after (Day 14) hindlimb ischemia. Recovery of blood flow 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 $Jag\text{-}1^{-/-}$, $Dll\text{-}1^{-/-}$, 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 $Jag\text{-}1^{-/-}$, $Dll\text{-}1^{-/-}$, or wild-type mice into nude mice with hindlimb ischemia. Transplantation of the BM-EPCs from wild-type or $Dll\text{-}1^{-/-}$ mice significantly improved hindlimb perfusion compared with PBS injection. In contrast, BM-EPCs from $Jag\text{-}1^{-/-}$ 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 $Dll\text{-}1^{-/-}$ mice but not $Jag\text{-}1^{-/-}$ 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

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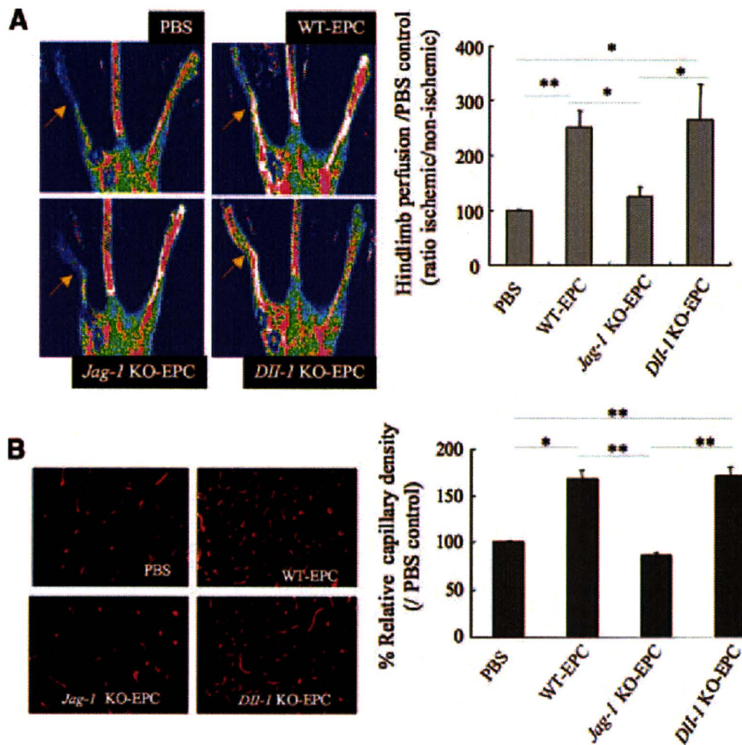


**Figure 3.** Effect of conditional deletion of distinct Notch signals on the therapeutic potential of EPCs for ischemic neovascularization. A, Representative laser Doppler perfusion imaging findings in nude mice 14 days after hindlimb ischemia and infusion of PBS or BM Sca-1$^+$/Lin$^-$ cells (EPC-enriched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

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