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　衛生管理教育訓練個人記録書
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自己点検手順書
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教育訓練手順書
　教育訓練計画書・記録書
　教育訓練実施状況結果報告書
　教育訓練個人記録書
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　ＧＭＰ教育年間基本計画
　ＧＭＰ教育訓練実施結果報告書
　ＧＭＰ教育訓練実施記録
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バリデーションマスタープラン
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製造管理基準書

目的
適用範囲
用語の定義
製造管理部門に関する業務
原材料の受入・保管
製品の保管
製造番号・ロット番号・管理番号付与
製造計画日程作成
製造指図書・記録書の発行
製造作業手順・製造記録
製造記録書等　記録類の点検・管理保管

製品の出荷
製造工程で発生する廃棄物の処理・処置

事故発生時の処置
工程管理項目逸脱時の措置
設備機器の管理
記録類の保存
作業員の教育
製造管理基準書の改廃

手順書
製造番号・ロット番号・管理番号付与手順書
原材料の受入管理手順書
製品の保管手順書
製造計画日程作成手順書
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製品ラベル発行手順書
製品出荷手順書
廃棄物処理手順書
異常発生報告書
逸脱管理手順書
教育訓練手順書

品質管理基準書

目的
適用範囲
用語の定義
品質管理部門に関する業務
受入れ原料の入庫判定
原材料、中間体、製品の品質試験検査実施
試験検査結果の判定及び報告
製品の出荷判定
試験不適合時の処置
試験検査の信頼性の確保
試験検査に使用する設備機器の管理
標準品、試薬の管理
委託試験の管理
試験検査記録の保管
作業員の教育訓練
品質管理基準書の改廃

手順書
原料入庫管理手順書
検体採取手順書
試験番号付与手順書
試験検査実施手順書
試験検査報告手順書
製品出荷判定手順書
逸脱管理手順書
バリデーション実施手順書
校正実施手順書
標準品、試薬管理手順書
委託試験手順書
教育訓練手順書

製品標準書

品名
成分及び分量
原料、容器、中間体、製品の規格及び試験方法
製造方法　製造手順書
標準的仕込量
製品の保管条件及び使用期限
標準書の改廃

D. 考察と結論

CPCでの製造に関するGMP文書体系を作成することにより、他施設で行われるCPCを用いた細胞培養技術の確立にも寄与できたと考えている。今後は、技術員のGMP教育とそれを担保する教育システムの確立が課題となる。

E. 研究発表

1. 論文発表

なし

2. 学会発表

日本細胞組織工学会 2007 年 11 月 28 日

“CPC における細胞製造”の講演

F. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（医療技術実用化総合研究事業：基礎研究成果の臨床応用推進研究）

分担研究報告書

虚血性疾患細胞治療臨床試験計画・データマネージメント

分担研究者　福島　雅典　臨床研究情報センター研究部　事業統括

研究要旨

虚血性疾患を対象とした増幅血管内皮前駆細胞移植治療の臨床適用を目指して、Cell Processing Center 関連手順書および臨床試験プロトコルの作成に対する適切な助言を行った。特に、プロトコル作成のために必要な前臨床研究成果の評価と対象患者選定のための根拠の確立を行った。

A. 研究目的

増幅血管内皮前駆細胞（Endothelial progenitor cell: EPC）移植治療の臨床適用を目指して、同治療の Cell Processing Center（CPC）関連手順書および臨床試験プロトコルを完成させる。

B. 研究方法

増幅 EPC を臨床適用するためには、同治療の安全性を担保し、有効性を支持しうる基礎研究・前臨床研究・先行臨床研究成果が必須である。先端医療センターの研究グループと共同で、これまでの研究成果を基盤として、CPC 関連手順書および臨床試験プロトコルの作成に着手した。

（倫理面への配慮）

臨床試験プロトコルは、完成後に先端医療センターの再生医療審査委員会、さらに厚生労働省における審査を請求する予定である。

C. 研究結果

CPC 関連手順書については、主として加工細胞の規格の設定、安全性の担保の方法について助言し、同書類の完成に貢献した。

新たに実施された基礎・前臨床研究（動物実験）から、有効性・安全性の両面から移植細胞の至適用量が明らかになったので、臨床試験プロトコルにおける細胞用量の設定根拠を確立しえた。

さらに先行第 I/II 相下肢血管再生治療臨床試験が終了したので、全症例における（非培養の）EPC の採取・分離・移植時から移植後 1 年までの安全性、有効性データを解析した。これにより、高齢者・透析患者・動脈硬化患者などでは非培養 EPC の採取・分離効率が低いことが明らかになり、これらの患者群は培養増幅 EPC 治療のより良い適応になると考えられた。

D. 考察

臨床試験計画の骨子はほぼ固まってきたので、今後は細部を検討し、倫理的でかつ再現性が高く、科学的評価に耐える臨床試験計画の完成に貢献していきたい。

E. 結論

CPC 関連手順書の完成に貢献した。臨床試験プロトコルは未完成であるが、その骨子を確定させた。

F. 研究発表

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G. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得

特記事項なし

2.実用新案登録

特記事項なし

3.その他

特記事項なし

Ⅲ　研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Murasawa S., Asahara T.	細胞・組織利用製品、遺伝子治療用医薬品、その他	早川　堯夫	バイオ医薬品の品質・安全性管理	エルアールシー	日本	2007	485-497

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
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Iwasaki H, Fukushima K, Kawamoto A, Umetani K, Oyamada A, Hayashi S, Matsumoto T, Ishikawa M, Shibata T, Nishimura H, Hirai H, Mifune Y, Horii M, Sugimura K, Suehiro S, Asahara T.	Synchrotron radiation coronary microangiography for morphometric and physiological evaluation of myocardial neovascularization induced by endothelial progenitor cell transplantation	Arterioscler Thromb Vasc Biol	27(6)	1326-1333	2007
Losordo DW, Schatz RA, White CJ, Udelson JE, Veereshwarayya V, Durgin M, Poh KK, Weinstein R, Kearney M, Chaudhry M, Burg A, Eaton L, Heyd L, Thorne T, Shturman L, Hoffmeister P, Story K, Zak V, Dowling D, Traverse JH, Olson RE, Flanagan J, Sodano D, Murayama T, Kawamoto A, Kusano KF, Wollins J, Welt F, Shah P, Soukas P, Asahara T, Henry TD.	Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina. A phase I/IIa double-blind, randomized controlled trial	Circulation	115(25)	3165-3172	2007

Kawamoto A, Asahara T.	Role of progenitor endothelial cells in cardiovascular disease and upcoming therapies	Catheter Cardiovasc Interv	70(4)	477-484	2007
Kawamoto A, Losordo DW.	Endothelial progenitor cells for cardiovascular regeneration	Trends Cardiovasc Med	18(1)	33-37	2008
Matsumoto T, Mifune Y, Kawamoto A, Kuroda R, Shoji T, Iwasaki H, Suzuki T, Oyamada A, Horii M, Yokoyama A, Nishimura H, Lee SY, Miwa M, Doita M, Kurosaka M, Asahara T.	Fracture induced mobilization and incorporation of bone marrow-derived endothelial progenitor cells for bone healing	J Cell Physiol	215(1)	234-242	2008

Ⅳ　研究成果の刊行物・別刷

Estrogen-Mediated Endothelial Progenitor Cell Biology and Kinetics For Physiological Postnatal Vasculogenesis

Haruchika Masuda, Christoph Kalka, Tomono Takahashi, Miyoko Yoshida, Mika Wada, Michiru Kobori, Rie Itoh, Hideki Iwaguro, Masamichi Eguchi, Yo Iwami, Rica Tanaka, Yoshihiro Nakagawa, Atsuhiko Sugimoto, Sayaka Ninomiya, Shinichiro Hayashi, Shunichi Kato, Takayuki Asahara

Abstract—Estrogen has been demonstrated to promote therapeutic reendothelialization after vascular injury by bone marrow (BM)–derived endothelial progenitor cell (EPC) mobilization and phenotypic modulation. We investigated the primary hypothesis that estrogen regulates physiological postnatal vasculogenesis by modulating bioactivity of BM-derived EPCs through the estrogen receptor (ER), in cyclic hormonally regulated endometrial neovascularization. Cultured human EPCs from peripheral blood mononuclear cells (PB-MNCs) disclosed consistent gene expression of ER α as well as downregulated gene expressions of ER β. Under the physiological concentrations of estrogen (17β-estradiol, E2), proliferation and migration were stimulated, whereas apoptosis was inhibited on day 7 cultured EPCs. These estrogen-induced activities were blocked by the receptor antagonist, ICI182,780 (ICI). In BM transplanted (BMT) mice with ovariectomy (OVX) from transgenic mice overexpressing β-galactosidase (lacZ) regulated by an endothelial specific Tie-2 promoter (Tie-2/lacZ/BM), the uterus demonstrated a significant increase in BM-derived EPCs (lacZ expressing cells) incorporated into neovasculatures detected by CD31 immunohistochemistry after E2 administration. The BM-derived EPCs that were incorporated into the uterus dominantly expressed ER α, rather than ER β in BMT mice from BM of transgenic mice overexpressing EGFP regulated by Tie-2 promoter with OVX (Tie-2/EGFP/BMT/OVX) by ERs fluorescence immunohistochemistry. An in vitro assay for colony forming activity as well as flow cytometry for CD133, CD34, KDR, and VE-cadherin, using human PB-MNCs at 5 stages of the female menstrual-cycle (early-proliferative, pre-ovulatory, post-ovulatory, mid-luteal, late-luteal), revealed cycle-specific regulation of EPC kinetics. These findings demonstrate that physiological postnatal vasculogenesis involves cyclic, E2-regulated bioactivity of BM-derived EPCs, predominantly through the ERα. (*Circ Res.* **2007;101:598-606.**)

Key Words: estrogen ■ endothelial progenitor cell ■ estrogen receptor ■ physiological postnatal vasculogenesis

In the female reproductive system, neovascularization is a recurring phenomemnon controlled by cyclic development of transient structure and cyclical repair of damaged tissue.[1] The ovarian sex steroid hormones, estrogen and progesterone, are primarily uterotropic and control the cyclical patterns of uterine cell proliferation and vascular growth that occur throughout the nonpregnant menstrual cycle. Given the synchronized nature of neovascularization in this cyclical mannter, it is assumed that angiogenic growth factor expression is induced by steroid hormones and regulates blood vessel formation in reproductive organogenesis.[2-5]

Despite clinical evidence for the significant role of steroid hormones in endometrial neovascularization, further investigation using in vitro and in vivo experiments have yielded inconclusive results regarding pathophysiological mechanisms in angiogenesis.[6-10] Moreover, estrogen has been shown to exhibit an inhibitory effect on certain hematopoietic kinetics, including lymphocytes and monocytes, both in terms of number and function.[11-14] Endometrial vascularization has formerly been considered to develop via "angiogenesis", ie, proliferation and migration of fully differentiated endothelial cells (ECs) from preexisting "parent" vessels.[15] However, circulating EPCs have been shown to incorporate into foci of neovascularization in adult species,[16] consistent with the notion of postnatal "vasculogenesis".[17] EPCs comprise of undifferentiated blood MNCs which are mobilized from BM by ischemic stimuli and angiogenic/hematopoietic factors,[17] and subsequently home to, differentiate, and proliferate in foci of neovascularization.[18]

Original received November 3, 2006; revision received June 11, 2007; accepted July 16, 2007.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.106.144006

Given this new understanding of adult neovascularization, it is also possible that vasculogenesis could also be responsible for ovarian hormonal regulation of endometrial neovascularization. Recently, 2 groups have demonstrated promotive effect of estrogen on reendothelialization after vascular injury via EPC incorporation. Iwakura et al disclosed an NO-dependent estrogen effect on EPCs using eNOS KO mice,[19,20] and Strehlow et al indicated an estrogen dependent antiapoptotic effect on EPC biology. Hamada et al demonstrated the functional importance of ER expression by EPCs.[21] Together, these reports suggest a therapeutic application of altering estrogen levels directly, or its receptor agonists, for vascular repair.

Therefore, in the current study, we investigated the hypothesis that E2 regulates physiological neovascularization of the endometrium by modulating the biology and kinetics of BM-derived EPCs.

Methods

EPC Culture of Human and Mouse EPCs

Human and mouse EPCs were cultured by using a modified protocol that has previously been reported.[18,22,23] Phenol-red free (PRF) endothelial basal media (EBM, Clonetics) was used to delete estrogenic effect, as described in Supplementary Method (SM)-I, available online at http://circres.ahajournals.org.[24,25]

Effects of Estrogen on EPCs: Differentiation, Proliferation, Migration, and Antiapoptosis In Vitro

The assays for EPC bioactivity effected by E2 were performed, according to the detailed description in SM-II, as previously reported.[19,26]

RT-PCR for Endothelial Gene Expression in Cultured Human EPCs

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

Real Time PCR Assay for Gene Expression of Estrogen Receptors in Cultured Human EPCs

The protocol of real time PCR assay was described in SM-IV.

Mouse Cultured EPC Assay

The protocol of mouse cultured EPC assay was described in SM-V.[18,22,23]

Mouse Cornea Neovascularization Assay

The effect of E2-induced EPC kinetics on neovascularization was studied by E2 pretreated OVX mice for 4 days as described in SM-6.[18,22,27]

Study Design of BMT Animal Experiments

BMT animal models with endogenous sex hormone depletion were developed as follows: female nude SCID mice (NIHS-bg-nu-xid, Taconic, Albany, NY; 4 weeks) were lethally irradiated and received BM cells from age-matched female Tie-2 transgenic mice overexpressing β-galactosidase by Tie-2 promoter (FVB/N-TgN[TIE2LacZ]182Sato, Jackson Laboratory, Bar Harbor, Me).[17,22,28] The protocol of BMT animal experiments was depicted in SM-VII.

Cellular Identification of LacZ Expressing Cells in Uterus or Cornea of Tie-2/LacZ/BMT/OVX Mice

Uterus Experiments

The uterus of mice euthanized at day 2, day 4, and day 7 after subcutaneous E2 pellet implantation was processed for CD31 immunohistochemistry as well as LacZ staining,[17] as described in SM-VIII. LacZ positive cells in whole area, or localized in vascular wall or stroma per uterus tissue section were counted. The percentage of LacZ positive cells localized in each part versus whole area was assayed.

Cornea Experiments

Six days after making the cornea model, the cornea was observed, after staining eye balls with LacZ solution. LacZ stained tissues embedded in paraffin were processed to CD31 immunohistochemistry.

Investigation of ER α and ER β Expression by BM-Derived EPCs Incorporated Into the Uterus of Tie-2/EGFP/BMT/OVX Mice

The protocol was described in SM-IX.

EPC Culture Assay and Flow Cytometry in Menstrual Cycle of Premenopausal Women

EPC culture assay and flow cytometrical analysis were performed, using PB-MNCs of 6 healthy premenopausal females (aged 20 to 40) at 5 separate stages of the menstrual cycle: T1=early proliferative, T2=preovulatory, T3=postovulatory, T4=mid luteal, and T5=late luteal, as previously described.[29] Flow cytometrical analysis (FACS) was performed on a FACStar flow cytometer (Becton Dickinson) and a Cell Quest software (Becton Dickinson), as described in SM-X.

EPC Colony Forming Activity of PB in Menstrual Cycle of Premenopausal Women

EPC colony forming activity was also assessed by SM-XI and XII.

Notice on Experiments in Animal and Human Subjects

Notice on experiments in animal and human subjects was described in SM-XIII.

Statistical Analysis

All results are expressed as mean±SE. Statistical significance was evaluated using unpaired Student *t* test for the comparison between 2 groups and ANOVA followed by Fisher post hoc test for the comparison among multiple groups. A probability value less than 0.05 was interpreted to denote statistical significance.

Results

Quantitative Real Time PCR Assay of ER α and ER β Gene Expressions in Cultured Human EPCs

In day 7 cultured human EPCs, endothelial gene expressions of von Willbrand Factor (vWF) and CD31 were detected (Figure 1A). To establish the potential for a direct effect of E2 on EPCs, mRNA expression of ER α and ER β was assessed by quantitative real time PCR assay. The gene expressions of ER α and ER β in cultured EPCs varied during the culture period. The expression of ER α did not change between day 4 and day 7, whereas the expression of ER β was remarkably downregulated at day 7 to the level of 0.12 fold relative to day 4 (Figure 1B).

Receptor Mediated E2 Effects on EPC Activity

EPC bioactivities upregulated by E2 were deleted in the presence of ICI, suggesting the ER mediated bioactivities, as described in supplemental Figure (SF)-I.

Upregulation of EPC Kinetics After E2 Administration

To explore the systemic effects of E2 on EPC kinetics, we administered E2 to OVX female and CST male mice. The

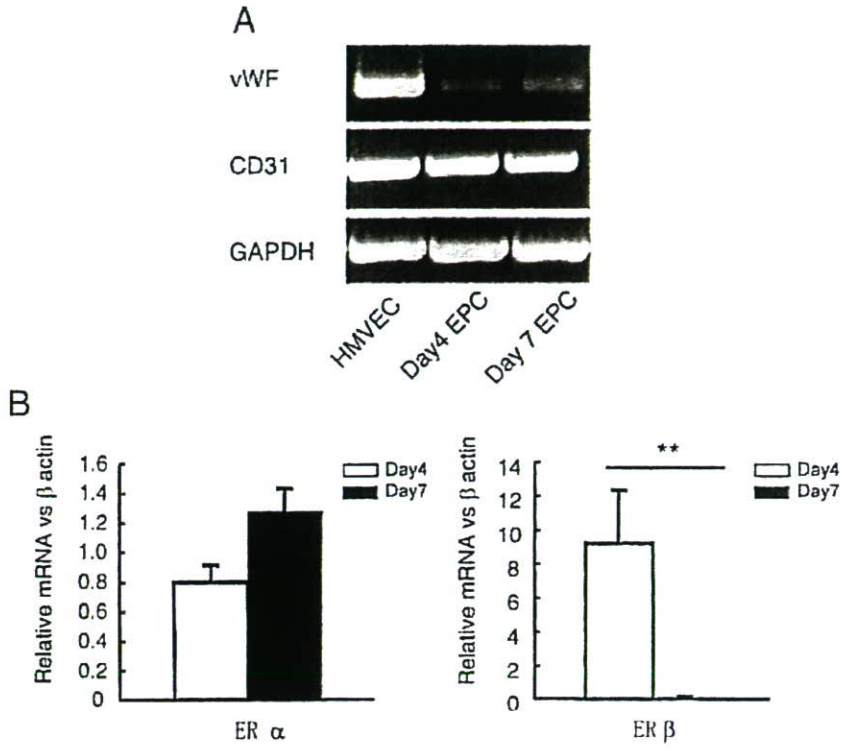

Figure 1. Quantitative real-time PCR assay of ERs gene expressions in cultured human EPCs. A, Endothelial gene expressions of vWF and CD31 in cultured human EPCs supplied for in vitro experiments. HMVECs, human microvascular ECs served as a positive control. B, Quantitative real-time PCR assay of ERs gene expressions in cultured human EPCs. The assay was performed on 3 human subjects. The analysis was performed using ddCt method. dCt values of ERs vs β-actin from each Ct value were acquired at each time point. ddCt values at each time point on each ER were calculated from each dCt value at day 4 or day 7. Relative mRNA vs β-actin presents $2^{(-ddCt)}$, n=3, $^{**}p<0.01$.

EPC culture assay[22] revealed a significant increase in endothelial lineage cells in cultures of PB-MNCs isolated at 2 to 4 days after subcutaneous implantation of E2 pellet in both OVX and CST mice. EPCs, identified by acLDL-DiI uptake and BS-1 lectin- FITC reactivity, consisted principally of spindle-shaped cells, often forming colonies. The number of cultured EPCs decreased to or below the level of pre-implantation by day 7 (Figure 2A and 2B). In controls, pellet implant did not increase EPC numbers significantly. These results thus provide quantitative evidence that E2 mobilizes EPCs from BM into the peripheral circulation.

Enhanced Cornea Neovascularization After E2 Administration

Examination of the cornea in E2- or P-treated mice established the extent of vascular development induced by implantation of a VEGF-containing pellet in the mouse cornea, presented in SF-II.

Identification of BM-Derived EPCs Within the Endometrium of Tie-2/LacZ/BMT/OVX Mice After E2 Administration

Before pellet implantation, macroscopic examination of the uterus of Tie-2/LacZ/BMT/OVX mice from both groups

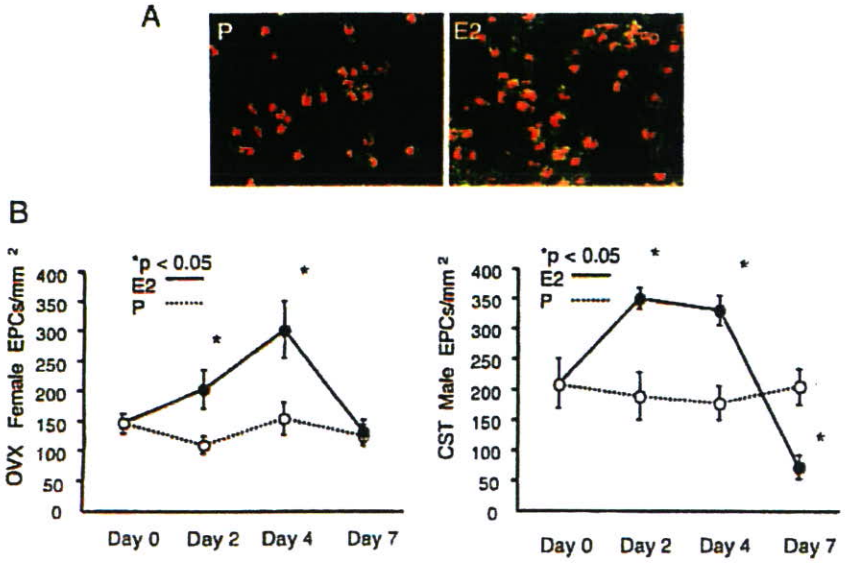

Figure 2. EPC culture assay in mice after E2 administration. A, Representative fluorescent photomicrographs of cultured EPCs merged acLDL- DiI with BS-1 lectin-FITC at day 4 in P and E2 treated OVX female mouse. ×10 magnification. B, Time course of EPC frequency in culture of PB-MNCs from E2- or P- treated mice. The results are shown for both OVX and CST mice, n=6 mice /each group.

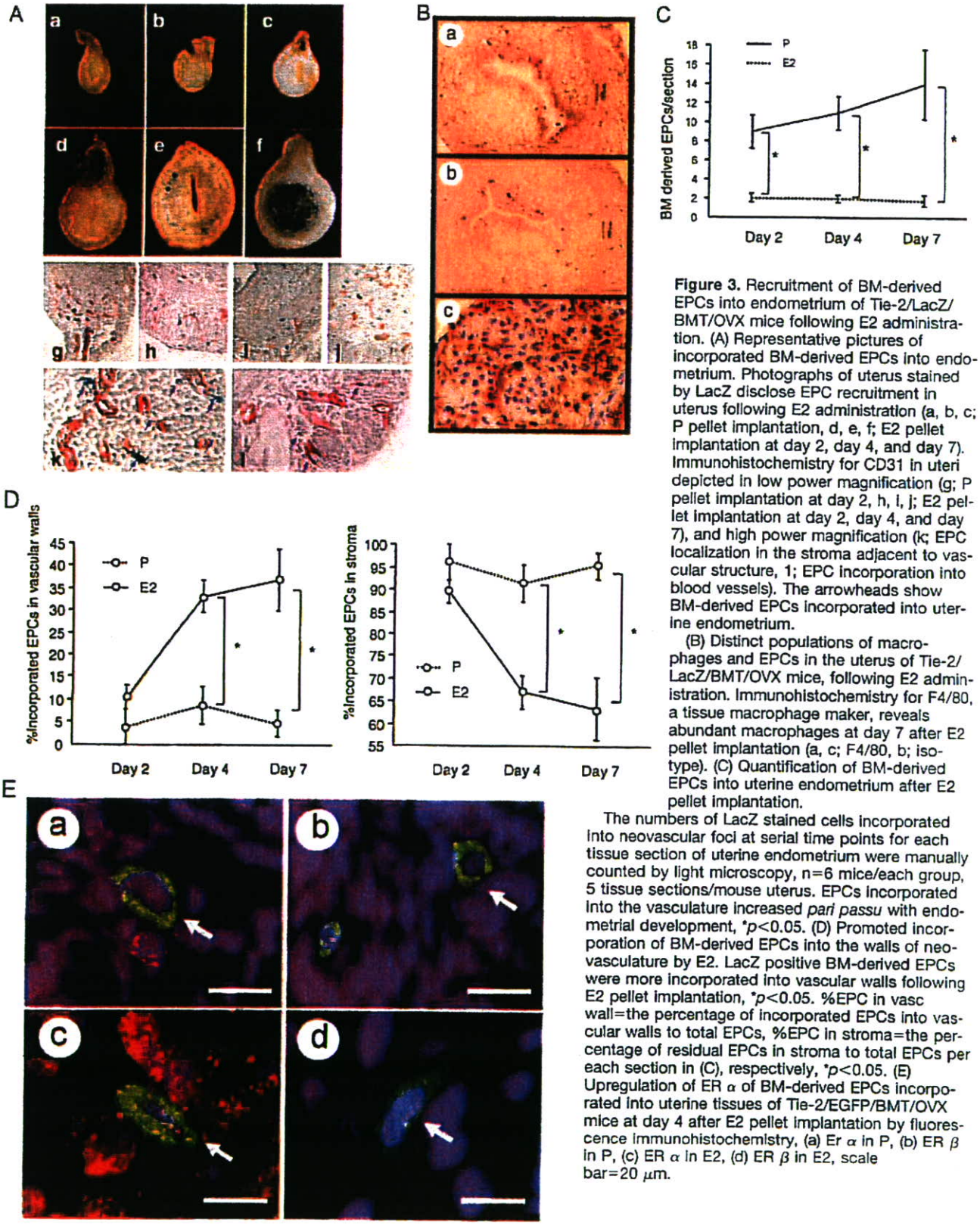

Figure 3. Recruitment of BM-derived EPCs into endometrium of Tie-2/LacZ/BMT/OVX mice following E2 administration. (A) Representative pictures of incorporated BM-derived EPCs into endometrium. Photographs of uterus stained by LacZ disclose EPC recruitment in uterus following E2 administration (a, b, c; P pellet implantation, d, e, f; E2 pellet implantation at day 2, day 4, and day 7). Immunohistochemistry for CD31 in uteri depicted in low power magnification (g; P pellet implantation at day 2, h, i, j; E2 pellet implantation at day 2, day 4, and day 7), and high power magnification (k; EPC localization in the stroma adjacent to vascular structure, 1; EPC incorporation into blood vessels). The arrowheads show BM-derived EPCs incorporated into uterine endometrium.

(B) Distinct populations of macrophages and EPCs in the uterus of Tie-2/LacZ/BMT/OVX mice, following E2 administration. Immunohistochemistry for F4/80, a tissue macrophage maker, reveals abundant macrophages at day 7 after E2 pellet implantation (a, c; F4/80, b; isotype). (C) Quantification of BM-derived EPCs into uterine endometrium after E2 pellet implantation.

The numbers of LacZ stained cells incorporated into neovascular foci at serial time points for each tissue section of uterine endometrium were manually counted by light microscopy, n=6 mice/each group, 5 tissue sections/mouse uterus. EPCs incorporated into the vasculature increased *pari passu* with endometrial development, $^*p<0.05$. (D) Promoted incorporation of BM-derived EPCs into the walls of neovasculature by E2. LacZ positive BM-derived EPCs were more incorporated into vascular walls following E2 pellet implantation, $^*p<0.05$. %EPC in vasc wall=the percentage of incorporated EPCs into vascular walls to total EPCs, %EPC in stroma=the percentage of residual EPCs in stroma to total EPCs per each section in (C), respectively, $^*p<0.05$. (E) Upregulation of ER α of BM-derived EPCs incorporated into uterine tissues of Tie-2/EGFP/BMT/OVX mice at day 4 after E2 pellet implantation by fluorescence immunohistochemistry, (a) Er α in P, (b) ER β in P, (c) ER α in E2, (d) ER β in E2, scale bar=20 μm.

disclosed occasional blue LacZ-stained cells located mainly in the mesometrium. In mice with P pellet implants, the uterus remained atrophic, and the location and frequency of LacZ-stained BM-derived EPCs did not change during observation. In contrast, Tie-2/LacZ/BMT/OVX mice with implanted E2 pellet revealed an evolving pattern of BM-derived, LacZ-positive cells within the uterus. Two days after E2 pellet implant, the frequency of LacZ-positive cells increased

throughout the uterus but remained concentrated in the outer layer of myometrium. By day 4 after implantation, EPCs continued to increase in number and were now identified in the outer and inner layers of myometrium and endometrium. EPCs finally accumulated in large numbers within the endometrium 7 days after E2 implantation. Immunohistochemical staining for CD31 in the P group demonstrated LacZ-stained CD31-positive EPCs appearing as round cells localized in the stroma adjacent to established vessels and incorporated as spindle-shaped cells into vascular walls. After E2 pellet implantation, EPCs were more frequently found to be incorporated within the vascular walls of the endometrium (Figure 3A). Counterstaining with antibody F4/80, as a tissue macrophage marker, and LacZ staining, revealed that the macrophages did not express β-gal; thereby indicating that they do not express Tie-2 and are therefore a completely separate population from the EPCs identified by Tie-2 promoter driven LacZ expression (Figure 3B). EPCs incorporation into foci of uterine neovascularization increased significantly by approximately 4.5-, 5.5-, or 7.8-fold at day 2, day 4, or day 7 after E2 pellet implantation versus endometrial tissues harvested from P pellet implants examined at identical time points. The number of incorporated EPCs per uterine section were as follows: for E2 group, day 2=9.0±1.8, day 4=11.0±1.7, day 7=14.0±3.6; in contrast, for P group, day 2=2.1±0.4, day 4=2.0±0.3, and day 7=1.8±0.5 (Figure 3C). Also, after E2 pellet implantation, the percentage of BM-derived EPCs incorporated into the neovasculature (of the total BM-derived EPCs per uterine section) increased significantly (day 2=10.44±2.7%, day 4=33.01±3.7%, day 7=36.73±6.9%); in contrast, the percentage for P group remained low (day 2=3.85±3.8%, day 4=8.56±4.1%, and day 7=4.44±2.9%; Figure 3D). On the other hand, the percentage of BM-derived EPCs in the stroma of the E2 group decreased inversely (day 2=89.57±2.7%, day 4=66.99±3.7%, day 7=63.27±6.9%), whereas the percentage for P group remained high (day 2=96.15±3.8%, day 4=91.44±4.1%, and day 7=95.56±2.9%; Figure 3D).

These findings indicate that BM-derived EPCs incorporate into foci of neovascularization during E2-induced endometrial maturation. This effect was restricted to E2-responsive organs: incorporated EPCs in other organs such as lung, liver, or skin,[17] could not be enhanced by E2 (data not shown). The sequence of histologic patterns observed suggests that E2 mobilizes BM-derived EPCs via the circulation (vide infra) into the myometrium from mesometrium, which precedes accumulation and incorporation into the neovasculature of the endometrium. The representative feature of BM-derived EPC incorporation into endothelial layer of vessel wall in uterine endometrium was recognized at day 7 after E2 pellet implantation by fluorescence immunohistochemistry of EGFP cellular positivity in CD31 positive endothelial layer (SF-III).

BM-derived EPCs (Tie-2/EGFP positive cells) incorporated into uterine tissues in Tie-2/EGFP/BMT/OVX mice, expressing ER α by stimulation of E2 pellet implantation for 4 days, but not ER β by fluorescence immunohistochemistry (Figure 3E). In this context, it is intriguing to note that the pattern of EPC recruitment and incorporation is identical to the previously established pattern of in situ VEGF expression in the hormone-regulated cycle of endometrial development and regression.[3]

Recruitment of BM-Derived EPCs into Cornea Neovascularization of Tie-2/LacZ/BMT/OVX Mice Following E2 Administration

Macroscopically, BM-derived EPCs stained with LacZ were observed more frequently in cornea of E2 pellet implanted mice, as compared with P pellet implanted, as presented in SF-4.

EPC Kinetics Through Human Menstrual Cycle

The morphology of cultured EPCs varied at different phases of the menstrual cycle (Figure 4A). At the preovulatory phase (T2), cultured EPCs, identified by double staining with acLDL-DiI and UEA-1-FITC, were recognized as isolated round adhesive cells, seldom forming colonies. After ovulation through the luteal phase (T3 to T5), EPCs appeared spindle-shaped, frequently exhibiting colony formation. Frequent colony formation was noted during the early proliferative phase (T1). The frequency of EPCs in culture decreased to the lowest level at the preovulatory phase (T2), increased gradually through ovulation, and remained high even during the early proliferative phase (Figure 4B).

As shown in Figure 4C, ovulation was identified between T2 and T3 by a surge of luteinizing hormone; the associated expression patterns of E2 and progesterone conform to the typical pattern of the menstrual cycle. VEGF levels were lowest at T1, then increased rapidly, reaching a peak at the T2 before slowly decreasing through the luteal phase. The pattern of VEGF expression was thus synchronized with E2. The numeric values of hormones and EPC numbers during menstrual cycle are shown in supplemental Table I.

Flow cytometrical analysis of PB-MNCs was used to determine the frequency of endothelial-specific antigen expressing cells as well as circulating immature EPCs according to the phase of the menstrual cycle. KDR was more frequently expressed by circulating cells from the preovulatory through luteal phases (Figure 4D-b). VE-cadherin antigen positive cells also increased, but the peak expression was until the later luteal phase. Differentiated ECs, positive for P1H12 antigen,[30] were identified at T1, immediately after menstruation, and did not augment, whereas KDR or VE-cadherin–positive endothelial lineage cells increased, after steroid hormone peaks (Figure 4D-b). Of note, the cell populations of CD133-positive or CD34-positive cells involving circulating immature EPCs in PB during the menstrual cycle disclosed a fluctuating pattern with significant amelioration at T3 following E2 peak (Figure 4D-c and 4D-d).

Discussion

The female reproductive system constitutes a unique exception to the quiescent vasculature of the normal healthy adult as the requirement for neovascularization recurring on a cyclic basis. Specifically, in every estrous cycle, the sequential maturation of the endometrium, as well as ovarian follicles and corpora lutea, is accompanied by concomitant development of elaborate capillary networks. Given the extent of newly forming vascular volume in endometrial

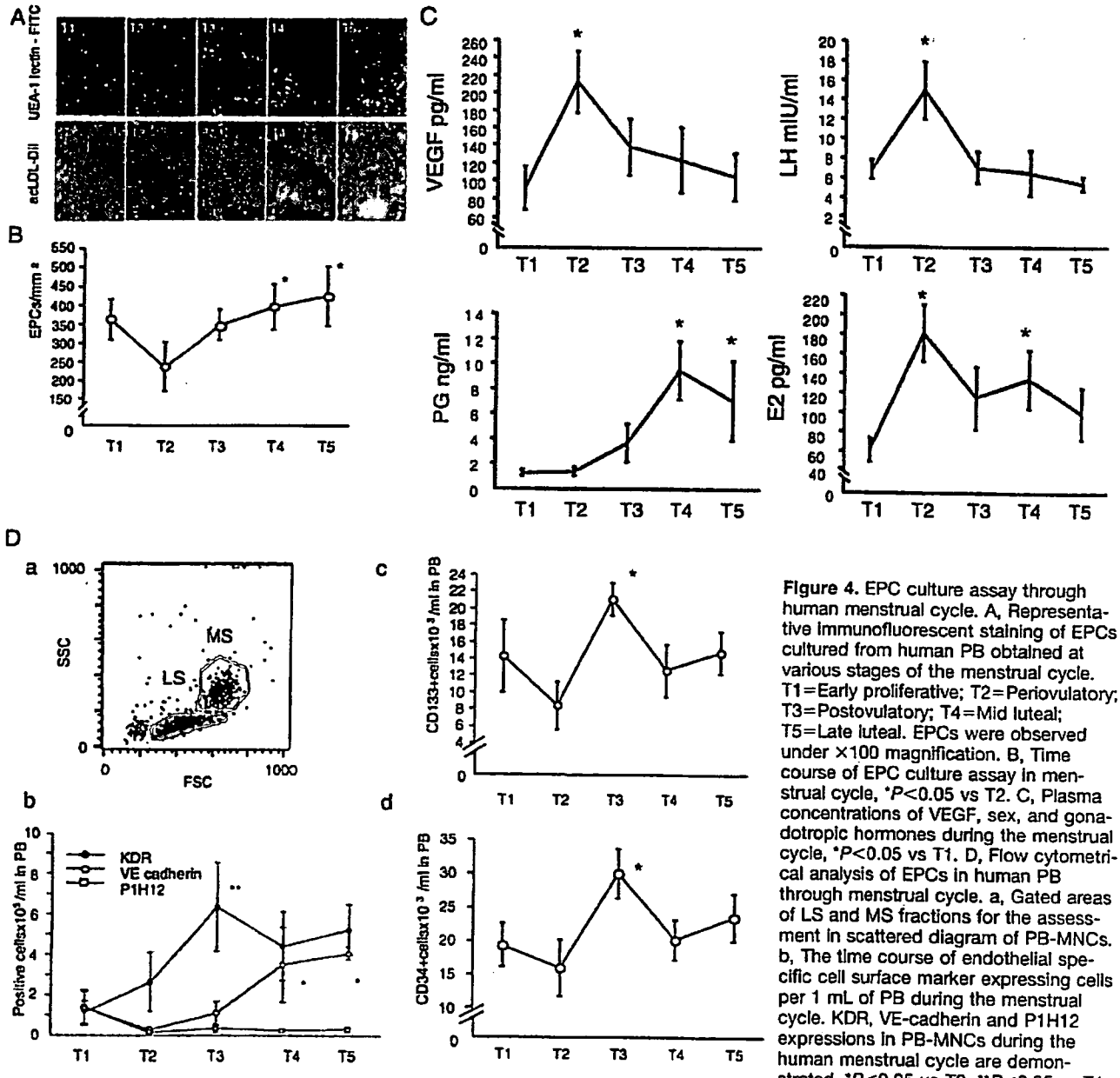

Figure 4. EPC culture assay through human menstrual cycle. A, Representative immunofluorescent staining of EPCs cultured from human PB obtained at various stages of the menstrual cycle. T1=Early proliferative; T2=Periovulatory; T3=Postovulatory; T4=Mid luteal; T5=Late luteal. EPCs were observed under ×100 magnification. B, Time course of EPC culture assay in menstrual cycle, *P<0.05 vs T2. C, Plasma concentrations of VEGF, sex, and gonadotropic hormones during the menstrual cycle, *P<0.05 vs T1. D, Flow cytometrical analysis of EPCs in human PB through menstrual cycle. a, Gated areas of LS and MS fractions for the assessment in scattered diagram of PB-MNCs. b, The time course of endothelial specific cell surface marker expressing cells per 1 mL of PB during the menstrual cycle. KDR, VE-cadherin and P1H12 expressions in PB-MNCs during the human menstrual cycle are demonstrated, *P<0.05 vs T2, **P<0.05 vs T1. c and d, The time course of immature EPC surface marker expressing cells (CD133-positive or CD34-positive cells) per 1 mL of PB, *P<0.05 vs T2.

development, it is possible that vasculogenic mechanisms may play a significant role in this cyclic organization. In this regard, we hypothesized that one of the main gender hormones, estrogen, controls EPC biology for cyclic neovascularization. The present findings provide evidence that the physiologic cycle of estrogen regulates EPC kinetics, ie, differentiation, proliferation, migration, apoptosis, mobilization, and ultimately incorporation into foci of neovascularization in the developing endometrium. Although the therapeutic potential of E2 for enhancing the contributions of EPCs for reendothelialization after vascular injury has been suggested, the physiological role of estrogen for EPC mediated vascular development has not been well established.[19,20,31]

Our EPC culture assay experiments demonstrated variations in EPC number and morphology throughout the phases of the menstrual cycle. These morphological changes are indicative of enhanced differentiation potential of circulating EPCs corresponding to cyclic hormonal changes. The peak increase in EPC number followed the peak serum concentrations of estrogen, as well as VEGF. This interval may potentially reflect a combination of estrogen effect on EPC proliferation, differentiation, and estrogen-induced mobilization from BM that has been suggested previously.[19,20,31]

The increase in the number of circulating EPCs expressing KDR or VE-cadherin antigen after peak estrogen levels and the decrease after downregulation of sex hormones were demonstrated by flow cytometrical analysis. Given their

essential function in embryonic vasculogenesis,[32–35] KDR and VE-cadherin were used to detect EPCs in PB-MNC population. Similarly, Strehlow et al have shown that estrogen mobilizes BM-derived EPCs (CD34 positive/KDR positive cells) into circulation of human subjects.[31] A temporal discrepancy in expression between KDR and VE-cadherin antigen in preovulatory and postovulatory phases was observed, which may suggest a differential effect of ovulatory estrogen on EPC biology. The basis for this differential expression may be related to the former findings that the expression of Flk-1 (homologue of mouse KDR) has been considered to represent a very early endothelial lineage marker during embryogenesis, whereas VE-cadherin–positive EPCs are considered differentiated from Flk-1–positive/VE-cadherin–negative cells.[35] The initial increase in KDR-positive cells seen during pre- to post-ovulatory phases may result from mobilization of immature EPCs into circulation, followed by an increase in committing EPCs during the luteal phase.[35] In contrast to KDR and VE-cadherin, P1H12 was used as a marker for differentiated ECs and was present at lesser frequency during preovulatory to luteal phases than other markers. Thus, differentiated ECs may circulate in PB only when dislodged by physiological blood vessel regression during menstruation.

The animal experiments provide potential insights into the significance of cyclic changes in EPC kinetics observed in human subjects. Systemic E2 pretreatment enhanced EPC mobilization detected in EPC culture assay and promoted enhanced neovascularization in the mouse cornea micropocket model. These results indicate that systemic estrogen stimulates EPC kinetics in the circulation, subsequently contributing to neovascularization via vasculogenesis. BMT experiments have demonstrated recruitment and incorporation of BM-derived Tie-2 receptor expressing cells, putative EPCs, during estrogen-induced endometrial development. It is intriguing to note that this pattern of EPC recruitment and incorporation is identical to the previously established pattern of in situ VEGF expression in the hormone-regulated cycle of endometrial development and regression.[3] In addition to EPCs found have incorporated into vascular structures, other round and at times even spindle-shaped cells were frequently identified in the uterine stroma. Regarding the finding, the cells could represent tissue macrophages derived from BM incorporating into uterine stroma during estrous cycle. The existence of BM-derived cells defined as macrophages (stained by F4/80) is also important to discuss because this may identify a significant role of blood bone cells in endometrial formation, especially regarding neovascularization. The similar findings were pointed out by several publications.[36,37] Tie-2 expressing BM-derived EPCs need vasculogenic environments introduced by angiogenic cytokines secreted from BM-derived macrophages. Therefore, this balance of EPCs and macrophages might play a pivotal role in neovascularization in endometrial formation.

Although the fate of the EPCs is currently uncertain, such cells may comprise EC reservoirs for the next round of endometrial development. The concept of BM-derived progenitor cell reservoirs in normal tissues is consistent with the notion of BM-derived satellite myoblasts and mesenchymal stem cells in muscle or other normal organs.[5,38] Using the same BMT models, we have previously demonstrated similar stroma-localized EPCs in growing neoplasms, wound healing, severe ischemia, and even though more sparsely—in normal organs.[17] Flk-1–positive cells previously demonstrated in the uterine myometrium[39] may represent similar cells. We have considered that the effect of estrogen may be direct or indirect (eg, mediated via VEGF). Evidence for a direct effect was given by the fact that EPC kinetics in CST male mice, lacking reproductive organs to respond to estrogen, responded equivalently in the case of OVX female mice, leaving a potentially estrogen-responsive (ie, VEGF-producing) uterus. This finding suggests that estrogen enhances EPC kinetics by direct interaction with EPCs or associated cells, such as in the BM microenvironment.

Our in vitro assay presented E2 promotion on EPC differentiation, migration, proliferation, and apoptosis inhibition, as partly indicated previously.[19,31] The findings that enhanced biological activities, such as proliferation, migration, antiapoptosis, and differentiation stimulated by E2 were blocked by an nonselective ERs antagonist (ICI), supported the fact that these effects of estrogen on EPCs were via functional ERs which were detected by mRNA in EPCs.

The importance of ERs on EPC bioactivity, using a myocardial infarcted model of ER α and ER β knockout mice has been recently documented by Hamada et al.[21] The authors described that ER α expressed in EPCs plays a more potent role in pathological vasculogenesis, rather than ER β. The present study disclosed the higher significance of ER α versus ER β in physiological vasculogenesis as well. During the culture period of human EPCs for 7 days through 4 days, the ER α expression was remained at the high level with the downregulation of ER β expression by real time PCR assay, as shown in Figure 1B. Accordingly, the EPC bioactivities disclosed in vitro study of day 7 cultured EPCs are considered to be brought through ER α. Furthermore, in vivo study of Tie-2/EGFP/BMT/OVX mice, the incorporated EPCs in uterus via E2 stimulation disclosed the expression of ER α, but not ER β, as shown in Figure 3E.

These findings may suggest that each ER shares the roles on EPC differentiation cascade, ie, the predominant function of ER β for EPC immature stage at provasculogenic state or ER α for EPC maturing stage at vasculogenic state, although the ER β function especially has yet to be elucidated.

Given the consideration, even in pathological vasculogenesis, ie, coronary vessel formation in infracted hearts, as shown by Hamada et al[21] as well as physiological vasculogenesis, each ER may have a unique role on EPC differentiation.

The basis for organogenesis has become a seminal issue for organ transplantation or therapeutic regeneration of damaged organs. Embryogenesis as well as physiological organogenesis in adult species reveal essential elements of organogenesis, devoid of pathological stimuli, including inflammation. The physiological regenerative processes constitute natural models that indicate how organs are established and survive. Blood vessel development is clearly one of the essential processes for organogenesis. The present study demonstrates that the unique system of cyclical blood vessel development and regression during the menstrual cycle, which occurs more

than 300 times in a female life span, involves hormone-mediated in situ proliferation, incorporation, differentiation, and survival of BM-derived progenitor cells. Above all, the unique EPC kinetics during menstrual cycle provides "dogma" for EPC biology as shown in supplemental Table I. Also, these findings have important implications for the impact of estrogen on vessel formation in disease states. Further insights regarding the precise mechanisms responsible for such physiological vasculogenesis will likely contribute to advanced methods and concepts for organ development in vivo as well as ex vivo.

Acknowledgments

This paper is dedicated to Dr. Jeffrey M. Isner. We would like to gratefully acknowledge him for his inspirational leadership, friendship and encouraging support. EPC colony assay was performed following the pretrial using cord blood under the approval of the ethical committees of Cord Blood Bank and Clinical Investigation in Tokai University School of Medicine. We also thank Dr. Kiyoshi Ando in Research Center for Regenerative Medicine for the management of the research facility; Dr. Yoshinori Okada and Dr. Jobu Itoh in Teaching and Research Support Center for the technical advices and supports; members of the animal facility in Tokai University School of Medicine as well as Miss. Sachie Ota as the secretary assistant. Especially, we greatfully thank Dr. Oren Tepper in Institute of Reconstructive Plastic Surgery, New York University Medical Center to finally check English grammar and style in the revised text.

Sources of Funding

This work was supported by grants from the National Institutes of Health (HL53354, and HL57516), Bethesda, MD; the ministry of Health, Labor and Welfare (H14-trans-001, H14-trans-002, H17-014), Japan; the ministry of Education, Culture, Sports, Science and Technology (Academic Frontier Promotion Program), Japan. H.M. is supported in part by Uehara Memorial Foundation and Kanagawa Nanbyo Foundation in Japan, and C.K. by a Cologne Fortune Grant in Germany. Estrogen receptor antagonist (ICI182,780) was kindly gifted by Astra Zeneca Pharmaceutical company.

Disclosures

None.

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Synchrotron Radiation Coronary Microangiography for Morphometric and Physiological Evaluation of Myocardial Neovascularization Induced by Endothelial Progenitor Cell Transplantation

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Background—Therapeutic effect of stem cell transplantation (SCTx) for myocardial neovascularization has been evaluated by histological capillary density in small animals. However, it has been technically difficult to obtain imaging evidence of collateral formation by conventional angiography.

Methods and Results—Peripheral blood CD34+ and CD34− cells were isolated from patients with critical limb ischemia. PBS, CD34− cells, or CD34+ cells were intramyocardially transplanted after ligating LAD of nude rats. Coronary angiography of ex vivo beating hearts 5 and 28 days after the treatment was performed using the third generation synchrotron radiation microangiography (SRM), which has potential to visualize vessels as small as 20 μm in diameter. The SRM was performed pre and post sodium nitroprusside (SNP) to examine vascular physiology at each time point. Diameter of most collateral vessels was 20 to 120 μm, apparently invisible size in conventional angiography. Rentrop scores at day 28 pre and post SNP were significantly greater in CD34+ cell group than other groups ($P<0.01$). To quantify the extent of collateral formation, angiographic microvessel density (AMVD) in the occluded LAD area was analyzed. AMVD on day 28 post SNP, not pre SNP, was significantly augmented in CD34+ cell group than other groups ($P<0.05$). AMVD post SNP closely correlated with histological capillary density ($R=0.82$, $P<0.0001$).

Conclusions—The SRM, capable of visualizing microvessels, may be useful for morphometric and physiological evaluation of coronary collateral formation by SCTx. The novel imaging system may be an essential tool in future preclinical/translational research of stem cell biology. (***Arterioscler Thromb Vasc Biol.* 2007;27:1326-1333.**)

Key Words: synchrotron radiation microangiography ■ image ■ CD34+ cells ■ neovascularization ■ myocardial infarction

Stem/progenitor cell transplantation (SCTx) investigated since the early 1990s is a novel approach for vascular regeneration therapy in ischemic diseases.[1-3] One of the examples of the SCTx is transplantation of adult peripheral blood CD34+ cells that are endothelial progenitor cell (EPC)-enriched population. Transplantation of CD34+ cells prevents left ventricular (LV) dilatation and wall thinning, inhibits myocardial fibrosis and apoptosis, and preserves LV function through augmentation of myocardial neovascularization and blood flow.[4-9] Evidence of increased vascularity by therapeutic neovascularization such as CD34+ cell transplantation has been obtained by histological assessment of capillary density and physiological evaluation of tissue perfusion has been by microsphere methods in small sized animals (mice and rats) with acute MI.[10] However, the histological examination has limitation for precise assessment of vascular physiology in response to environmental stress. Though microsphere assessment was performed to evaluate physiological blood flow, it was pointed out to lack significant reproducibility in small animal models. Several research groups have utilized other approaches such as corrosion casts[11,12] and angiography[13,14] to visualize collateral vessels.

Original received November 29, 2006; final version accepted February 12, 2007.

From Stem Cell Translational Research (H.I., A.K., A.O., S.H., T.M., M.I., H.N., Y.M., M.H., T.A.), Kobe Institute of Biomedical Research and Innovation/RIKEN Center for Developmental Biology; the Department of Cardiovascular Surgery (H.I., T.S., H.H., S.S.), Osaka City University Graduate School of Medicine; the Department of Image-based Medicine (K.F.), Kobe Institute of Biomedical Research and Innovation; the Department of Radiology (K.F., K.S.), Kobe University Graduate School of Medicine; the Research & Utilization Division (K.U.), Japan Synchrotron Radiation Research Institute, SPring-8, Sayo; and the Department of Regenerative Medicine Science (T.A.), Tokai University School of Medicine, Isehara, Japan.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/ATVBAHA.106.137141