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

「体外培養の増幅血管内皮前駆細胞移殖による虚血性疾患治療に関する基礎・臨床研究」

平成17年度～19年度　総合研究報告書

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Ⅰ　総合研究報告書

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）

総合研究報告書

体外培養の増幅血管内皮前駆細胞移殖による虚血性疾患治療に関する基礎・臨床研究

主任研究者　浅原孝之　先端医療センター　研究所副所長

研究要旨　虚血性疾患患者を対象とした血管内皮前駆細胞(EPC)による移植療法において、自己EPCを末梢血から採取後、患部に移植する治療法が開発され、臨床応用されているが、採取EPCの質／量には限界がある。この研究プロジェクトでは、EPCを体外で培養増幅し、数・質の改善を図った上で移植治療する臨床研究の確立を目指す。前期研究では 、EPCの体外培養方法の確立に努め。中期に体外培養で得られた細胞群の安全性と効果性の評価、後期で細胞培養法の細胞培養センター（CPC）での標準手順書作製および臨床プロトコール作製を進めた。

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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) EPC体外培養増幅技術の確立

量および質を高める血管内皮前駆細胞（EPC）の培養方法を確立し、安全性・効果性を確認する。

(2) 培養細胞移植の技術開発

A) 培養血管内皮前駆細胞の移植方法・移植細胞数などの検討。

(3) CPCでのEPC培養加工技術の確立

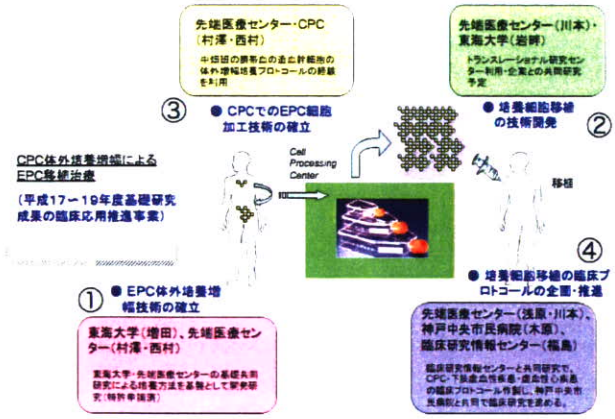
細胞培養センター（CPC）での細胞培養加工技術を、作業手順書として作製し、細胞の製造・品質管理を可能にする。

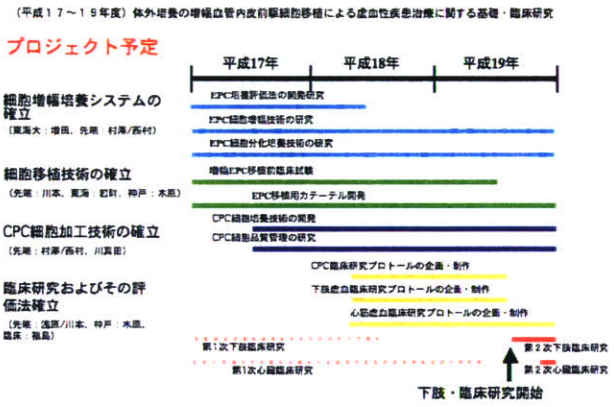
(4) 培養細胞移植の臨床プロトコールの企画・推進

CPCを用いた培養細胞移植治療のための臨床研究プロトコールの作製とともに、細胞培養・医療チームを形成する。

2. 研究方法

研究計画は、以下の４部門で遂行された。





(1) EPC 体外培養増幅技術の確立

A) 効率的な EPC 増幅法の開発：末梢血液中の血管内皮前駆細胞数は少量（0.1%以下）で不十分なため、本計画における臨床治療研究では G-CSF 製剤を５日間投与（10 μg/kg/day）し、血管内皮前駆細胞を骨髄から末梢血に強制動員させる。これで前駆細胞数は約 10 倍に増加するとされているが、より効果的な血管内皮前駆細胞増殖法をまず in vitro で確立した。

(2) 培養細胞移植の技術開発

培養細胞の移植方法を、経静脈的・経皮経管的筋注的アプローチで移植。移植細胞数の検討などを前臨床試験として進めた。下肢虚血性疾患・心筋梗塞モデルなどの動物実験で確認した。

(3) CPC での EPC 培養加工技術の確立

ＣＰＣを用いた治療用の細胞の製造が可能となる施設の要件を記した衛生管理基準書とその付随標準作業手順書（SOP）、製造に関する要件を記した製造管理基準書とその付随 SOP、と品質管理に関する品質管理基準書とその付随 SOP などの管理文書類を作成し、今後の細胞製剤製造手順の手引き書作製した。

(4) 培養細胞移植の臨床プロトコールの企画・推進

従来および本研究データに基づいた下肢虚血性疾患、続いて虚血性心疾患の臨床プロトコールの作製を計画した。この作製には臨床研究情報センター（TRI）の協力を得た。

本研究は、薬物治療・血管形成術・バイパス手術などの既存の治療に抵抗性、あるいはそれらの適応にならない慢性の虚血性心疾患患者および下肢虚血患者を対象として計画される。GCSF 製剤および apheresis による有害事象を可能な限り防止するため、日本造血細胞移植学会・日本輸血学会のガイドラインに準拠して、症例の適格基準・GCSF の用量調節規準・apheresis 施行手順・有害事象発生時の試験中止規準等を厳格に設定した。

（倫理面への配慮）

上記の臨床試験は、先端医療センター再生医療審査委員会・神戸市立中央市民病院倫理委員会から実施の承認を得た後に、厚生労働省での審査を受ける予定である。

被験者から同意を得て開始される。

3. 研究結果および考察

(1) EPC 体外培養増幅技術の確立

A) 効率的な EPC 増幅法の開発：

臍帯血あるいは骨髄・末梢血 CD34 陽性細胞あるいは CD133 陽性細胞を用いて、培養条件を広く検索した結果、hflt-3, hVEGF, hSCF, hTPO, hIL-6 無血清培地に、５％低酸素条件を組み合わせると、培養細胞中の血管内皮前駆細胞（EPC）の量・質を適切に保てることが判明した。他の因子も多数探索され、良い結果を得られるものもあったが（SDF-1, CTGF, Angiopoietin-1、など）、臨床的に安全且つ効果的な

組み合わせは現時点で上記組み合わせと判断された。

この培養により、各細胞群培養で以下の通りの結果が得られた。

・臍帯血 CD34/133 陽性細胞を１週間で 50 倍、２週間で 500 倍 EPC コロニー形成能。

・骨髄 CD34/133 陽性細胞を１週間で 20 倍、２週間で 200 倍 EPC コロニー形成能。

・末梢血 CD34/133 陽性細胞を１週間で 10 倍、２週間で 100 倍 EPC コロニー形成能。

・１週間培養 EPC の移植後の再生力は、増幅前と同等である。

・２週間培養 EPC は、増幅率は高いが、再生能力低下傾向が見られる。

これらの結果から、CPC での EPC の体外培養治療を想定して、EPC 培養増殖を試みたところ、７日間の無血清培養により、G-CSF で増幅された末梢血 CD34 陽性細胞の総細胞数は、平均２５倍程度に増加することが判明した。また、未分化 EPC コロニー数は５０倍に増加した。適切な成長因子、サイトカインの組み合わせを考慮することにより無血清培養条件下において未分化 EPC 及び分化過程 EPC の増幅が可能であるあることが示唆された。

この in vitro の結果は、動物実験で下肢虚血・心筋虚血モデルで血管再生治療効果として判定された。

(2) 培養細胞移植の技術開発

ヌードマウス下肢虚血モデルに対するヒト末梢血 EPC（CD34+細胞）の移植実験を実施した。下記の各治療群間で下肢温存率、下肢血流改善度を比較した。

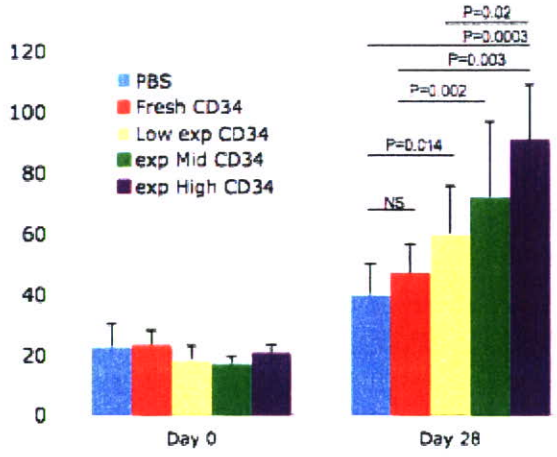
１）PBS 群

２）非培養 CD34+細胞（2X10E4 個）

３）培養 CD34+細胞（2X10E4 個、Low）

４）培養 CD34+細胞（2X10E5 個、Mid）

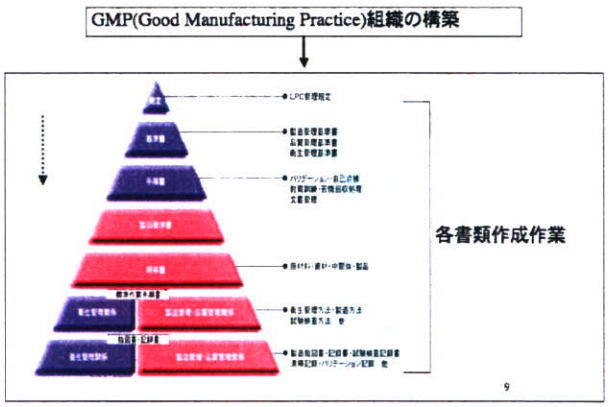
５）培養 CD34+細胞（2X10E6 個、Hi）



その結果、培養増幅後の EPC、特に高用量群で血流改善効果が顕著であった。移植方法は、局所投与で有効であったら、静脈投与による治療の効果も確認できた。

(3) CPC での EPC 培養加工技術の確立

ＥＰＣの細胞培養を通じて下記のＣＰＣ製造・品質・衛生管理文書類が策定された。それぞれの項目に対して、詳細な記述が加えられている。



衛生管理基準書

手順書

浮遊菌測定手順書
付着菌測定手順書
防虫防鼠管理手順書
搬入搬出手順書
更衣手順書
入退室手順書
入室者健康状態チェック表
海外渡航者健康記録　健康チェック表

健康状態チェック表
清掃・消毒作業手順書
清浄度管理手順書
作業手洗い作業手順書

作業衛生管理基準書
衛生管理教育訓練手順書
バリデーション手順書
苦情回収処理手順書
自己点検手順書
教育訓練手順書

バリデーションマスタープラン

製造管理基準書
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
製造指図書・記録書発行手順書
製造工程手順書
工程ラベル発行手順書
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
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

(4) 培養細胞移植の臨床プロトコールの企画・推進

本プロジェクトによって得られた結果から、本治療の臨床適用に際しては、基本的な５種類の成長因子を用いた無血清下培養を５％低酸素条件下で７日間実施することに決定していたが、CPC を使用した細胞培養増幅が必須であるため、それに対応した製品標準書・製造管理基準書・衛生管理基準書・品質管理基準書などの作成が行われ、臨床研究プロトコールの基礎となった。

臨床試験プロトコルの作成は、上記の基礎・前臨床研究成果を基に移植細胞の至適用量を明確にすることができた。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。以上の検討を元に、最終的な臨床研究プロトコールが作製されている。

4. 結論

臨床と同じ末梢血由来 EPC で無血清培養による分化・増幅法による移植細胞の体外培養法が完成した。細胞の質及び量は十分であり、安全性及び効果性は前臨床試験的に確認された。CPC の標準手順書、臨床試験全体のプロトコールの作成は予想以上に作業が難航したがほぼ終了し、臨床研究として医療応用に一歩近づける事が出来たと考える。

5. 研究発表

１）国内

口頭発表・講演：20 件

２）海外

口頭発表・講演：40 件

原著論文：42 件

そのうち主なもの

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6. 知的所有権の出願・取得状況（予定を含む）

特許出願

1．出願番号　特願 2005-47816

発明の名称　血管内皮前駆細胞の生体外増殖方法

2．出願番号　特願 2005-47422

発明の名称　血管内皮前駆細胞分化動態解析方法

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Ⅱ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Murasawa S, Kawamoto A, Horii M, Nakamori S, Asahara T.	Niche-Dependent Translineage ommitment of Endothelial Progenitor Cells, Not cell Fusion in General, Into Myocardial Lineage Cells	*Arterioscler Thromb Vasc Biol.*	25(7)	1388-1394	2005
Iwasaki H, Kawamoto A, Ishikawa M, Oyamada A, Nakamori S, Nishimura H, Sadamoto K, Horii M, Matsumoto T, Murasawa S, Shibata T, Suehiro S, Asahara T,	Dose-Dependent Contribution of CD34-positive Cell Transplantation to Concurrent Vasculogenesis and Cardiomyogenesis for Functional Regenerative Recovery After Myocardial Infarction.	*Circlation*	113	1311-1325	2006
Kawamoto A, Iwasaki H, Kusano K, Murayama T, Oyamada A, Silver M, Hulbert C, Gavin M, Hanley A, Ma H, Kearney M, Zak V, Asahara T, Losordo DW.	CD34-positive cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction compared with total mononuclear cells	*Circulation*	114 (20)	2163-2169	2006
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

Masuda H, Kalka C, Takahashi T, Yoshida M, Wada M, Kobori M, Itoh R, Iwaguro H, Eguchi M, Iwami Y, Tanaka R, Nakagawa Y, Sugimoto A, Ninomiya S, Hayashi S, Kato S, Asahara T.	Estrogen-Mediated Endothelial Progenitor Cell Biology and Kinetic For Physiological Postnatal Vasculogenesis	*Circ Res*	101 (6)	598-606	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

Ⅲ　研究成果の刊行物・別冊

Niche-Dependent Translineage Commitment of Endothelial Progenitor Cells, Not Cell Fusion in General, Into Myocardial Lineage Cells

Satoshi Murasawa, Atsuhiko Kawamoto, Miki Horii, Shuko Nakamori, Takayuki Asahara

Objective—Previous studies from our laboratory have shown therapeutic potential of ex vivo expanded endothelial progenitor cells (EPCs) for myocardial ischemia. Our purpose was to investigate the mechanisms regulating EPC contribution to myocardial regeneration.

Methods and Results—To evaluate niche-dependent expression profiles of EPCs in vitro, we performed coculture using cultured EPCs derived from human peripheral blood and rat cardiac myoblast cell line (H9C2). Reverse-transcription polymerase chain reaction (PCR) disclosed the expression of human-specific cardiac markers as well as human-specific smooth muscle markers. Cytoimmunochemistry presented several cocultured cells stained with human specific cardiac antibody. To prove this translineage differentiation in vivo, human cultured EPCs were injected into nude rat myocardial infarction model. Reverse-transcription PCR as well as immunohistochemistry of rat myocardial samples demonstrated the expression of human specific cardiac, vascular smooth muscle, and endothelial markers. We observed the distribution of colors (Qtracker; Quantum Dot Corp) in coculture to detect the fused cells, and the frequency of cell fusion was <1%.

Conclusions—EPCs can contribute to not only vasculogenesis but also myogenesis in the ischemic myocardium in vivo. Transdifferentiation, not cell fusion, is dominant for EPCs commitment to myocardial lineage cells. Ex vivo expanded EPCs transplantation might have enhanced therapeutic potential for myocardial regeneration. (*Arterioscler Thromb Vasc Biol.* 2005;25:1388-1394.)

Key Words: cardiovascular diseases ■ endothelial ■ myocardium ■ regeneration ■ stem cells ■ vasculogenesis

Somatic stem and progenitor cells have recently demonstrated the flexibility in lineage commitment for tissue regeneration. Although bone marrow cells presented multiple lineage potential, hematopoietic stem cell demonstrated translineage commitment into other lineage cells, such as vascular cell,[1,2] neural cell,[3,4] hepatic cell,[5,6], and mesenchymal cell lineages.[2] Neural stem cell has also shown the adaptability for another lineages.[7,8] These were followed by reports that differentiated endothelial cells, either freshly isolated from mouse dorsal aorta at embryonic day 9 or established as homogenous cells in culture, differentiate into cardiomyocytes, and express cardiac markers when cocultured with neonatal rat cardiomyocytes or when injected into postischemic adult mouse heart. They also demonstrated that human umbilical vein endothelial cells also differentiate into cardiomyocytes.[9]

Bone marrow-derived endothelial progenitor cells (EPCs)[10,11] have shown the regenerative potential in myocardial ischemic animal model[1,12] via ex vivo expansion and incorporation into foci of neovascularization. The study from our laboratory[1] has demonstrated ex vivo expanded EPCs transplantation into ischemic hearts resulted in enhanced myocardial neovasculalization, as well as improved cardiac function (such as reduction in left ventricular dilatation). Histological findings supported that there occurred not only vascular regeneration but also myocardial regeneration, contributing to favorable effects of EPCs on cardiac function. Given these results, we believe EPC, which is considered the cell source for vascular regeneration, might reveal favorable potential in heart tissue regeneration, such as cardiomyocyte and vascular smooth muscle cell lineages.

Recently, Badorff et al have reported transdifferentiation of EPC into cardiomyocytes.[13] The results encourage the possibility of EPC translineage commitment into cardiomyocyte for the treatment of myocardial ischemic disease. However, they lack the in vivo evidence of this translineage commitment for organogenesis by EPC transplantation to ischemic disease patients. Furthermore, we consider the necessity of

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pursuing not only myocardial but smooth muscle lineage commitment, which is required for the stabilization of newly formed vasculatures by EPCs themselves. Very recently, Yeh et al have reported transdifferentiation of $CD34^+$-enriched cell into cardiomyocyte and smooth muscle cell in vivo.[14] Their results have shown transdifferentiation of human peripheral blood $CD34^+$ cell into cardiomyocyte was enhanced in the injured heart compared with in the heart without injury, although they did not indicate any functional significance of transdifferentiation.

In this regard, to evaluate niche-dependent expression profiles of EPCs in vitro, we performed coculture of EPCs derived from human peripheral blood and rat cardiac myoblast cell line (H9C2). We also evaluated the frequency of cell fusion phenomenon in the coculture system. Furthermore, to prove equivalent translineage commitment in vivo, human cultured EPCs were transplanted into nude rat myocardial infarction model to sample for transcriptional and expressional evidences.

Methods

Coculture With EPC and H9C2 Cell Line

Total peripheral blood mononuclear cells were isolated from human volunteers by density gradient centrifugation. All procedures were in accordance with the institutional committee. After 4 days in culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), new media was applied, and the culture was maintained through day 7 or later. In the culture of EPC after day 7, reseeding was performed once per week.[15] Rat cardiac myoblast cell line (H9C2) was cultivated in DMEM with 10% fetal bovine serum and 5% horse serum. EPC was detached at day 7 and re-seeded onto semi-confluent H9C2 monolayer. Coculture was maintained in the feeding medium of H9C2 for 7 days with 1-time application of new media and sampled for reverse-transcription polymerase chain reaction (RT-PCR) or cytoimmunochemistry. All cells were incubated under normoxia (pO_2, 152 mm Hg) condition.

Sorting of Cultured EPCs and Coculture Subpopulation of EPCs and H9C2

We sorted cultured EPCs to determine which subpopulation of EPCs mainly contributed to cardiac lineage commitment. Briefly, we sorted day 7 cultured EPCs by CD31antibody (BD Pharmingen, San Jose, Calif) using BD FACSAria Cell-Sorting System (BD Biosciences, San Jose, Calif), or CD34 (BD Pharmingen) antibody using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and obtained positive and negative fractions, respectively. Then we performed coculture with each fraction and H9C2 on 4-chamber glass wells. Cardiac lineage commitment was evaluated by cytoimmunochemistry 7 days after coculture.

RT-PCR

Cocultured cells were lysed in RNA lysis buffer (Ambion, Austin, Tex). However, EPC or PBS-injected myocardial samples were homogenized in RNA lysis buffer. RNA was extracted using RNA extraction kit (Ambion). DNAase digestion was performed after RNA extraction. The RT-PCR was performed by a system according to the manufacture (Clontech, Palo Alto, Calif). Briefly, each primer was amplified for 35 cycles. In every case, each cycle consisted of 95°C for 30 seconds, followed by 65°C for 3 minutes. The primers for RT-PCR were designed as shown in the Table. These primers other than GAPDH and mGAPDH were designed to identify human specific expression of each target.

Cytoimmunochemistry

Day 7 coculture (human EPCs plus H9C2) on a 4-chamber slide was fixed with ice-cold 100% methanol for 7 minutes and washed with PBS 3 times. Cytoimmunochemistry was performed using cardiac antibodies, α/β-ventricular myosin heavy chain (Chemicon, Temecula, Calif), brain natriuretic protein (kindly provided from Dr Itoh, Kyoto University, Japan), cTn-I (Chemicon), smooth muscle lineage antibody, α-SMA (clone 1A4) (Sigma, Saint Louis, Mo), endothelial lineage antibody, CD31 (DAKO, Carpinteria, Calif), and human leukocyte antigen (HLA)-ABC (BD Biosciences Pharmingen) for detecting human cells. Antibodies except for cTn-I and α-SMA are reactive only for humans. We used human-specific α/β-ventricular MHC antibody to evaluate cardiac lineage commitment in coculture with sorted subpopulation of EPCs and H9C2. Proportion of cardiac lineage commitment was evaluated by counting α/β-ventricular MHC-positive cells per total seeded sorting cells in each chamber slide.

Rat Myocardial Infarction Model

Athymic nude rats (Harlan, Indianapolis, Ind) aged 7 weeks and weighing 135 to 140 grams were anesthetized with ketamine and xylazine intraperitoneally. After operatively induced myocardial ischemia,[1] the arrhythmic nude rats each received systemic (1×10^6) or intramuscular injection of 2.5×10^5 culture-expanded human EPCs in 2 sites of myocardial ischemic lesions; 2.5×10^5 EPCs were suspended in 25 μL of PBS, and only 25 μL of PBS was injected in control group. We performed EPC transplantation in 10 rats (5 for systemic injection, 5 for intramuscular injection), and injected PBS in 5 rats (control). Three weeks after operation and injection, these rats were euthanized and myocardial samples were put into OCT compound (Sakura, Torrance, Calif) for frozen tissue section (immunohistochemistry) or directly frozen in liquid nitrogen for RNA extraction (RT-PCR).

Immunohistochemistry

Frozen slides were prepared by Criostat (Microm, HM505E; Walldorf, Germany) and stained with cardiac antibodies (α/β-ventricular MHC) (Biocytex, Marseille, France), BNP (kindly provided by Dr Itoh, Kyoto University, Japan), cTn-I (Biomeda, Foster City, Calif), or smooth muscle lineage antibody, Calponin (DAKO), or endothelial lineage antibody, CD31(DAKO). Both α/β-ventricular MHC and cTn-I are different antibodies used in cytoimmunochemistry. Connexin43 (BD Biosciences Pharmingen) was used for experiment of gap junction and double-stained with HLA-ABC (BD Biosciences Pharmingen) for detecting human cells. Antibodies except for connexin 43 were active only for humans. We used DAB system (brown) for single antibody staining for visualizing the signals. Double staining was performed using DAB system for HLA antibody and VIP system (purple) for connexin 43 antibody.

Evaluation of Frequency of Cell Fusion in Coculture System

We performed coculture using Qtracker (Quantum Dot Corp, Hayward, Calif), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565 and H9C2 cells were labeled with Qtracker 655. We observed the distribution of colors to detect the fused cells by fluorescent microscopy (Olympus IX71; Tokyo, Japan). When the cells fuse, the fused cell has both colors. Because the nanocrystals are larger than organic dyes, they are not transferred between cells, so each cell type would maintain the single color until they fuse. EPC fusion ratio was detected by counting the number of fused cells that indicated yellow in cytoplasmic area out of the number of total labeled EPCs in 10 different high-power fields (×200). The data were shown as the mean±SD.

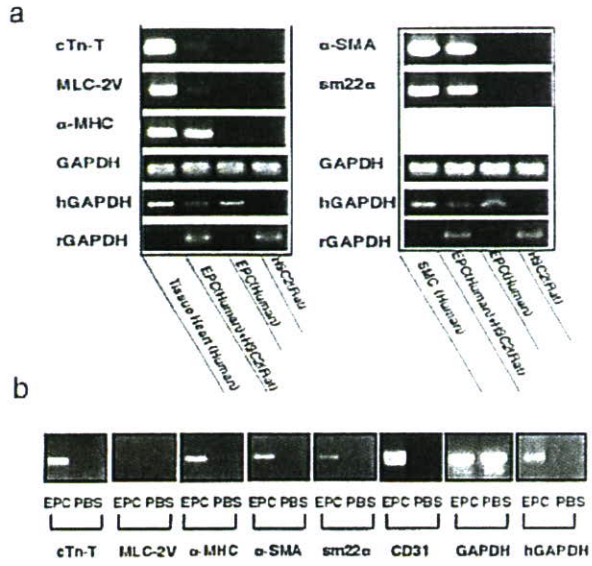

Figure 1. Expression of human-specific cardiac and smooth muscle markers in coculture system and in EPC-injected rat heart by RT-PCR. RNA samples from various culture conditions were analyzed by RT-PCR. cTn-T, MLC-2V, and α-MHC are specific markers for cardiomyocyte, and α-SMA and sm22α are specific markers for smooth muscle cells. Left lane of each column shows positive control, and right lane of each column shows negative control (only rat-derived H9C2). Human EPC did not express both cardiac and smooth muscle cell markers. After coculture with human EPC and rat H9C2, both cardiac and smooth muscle markers were observed (a). RNA samples were obtained from ischemic nude rat heart injected with human EPC or PBS. RT-PCR was performed using the same condition as used in coculture samples. Left lane of each column shows the data from EPC injected heart sample. Right lane of each column shows the data from PBS-injected heart sample (b). GAPDH served as internal standard. GAPDH recognizes both human and rat, and hGAPDH only recognized human.

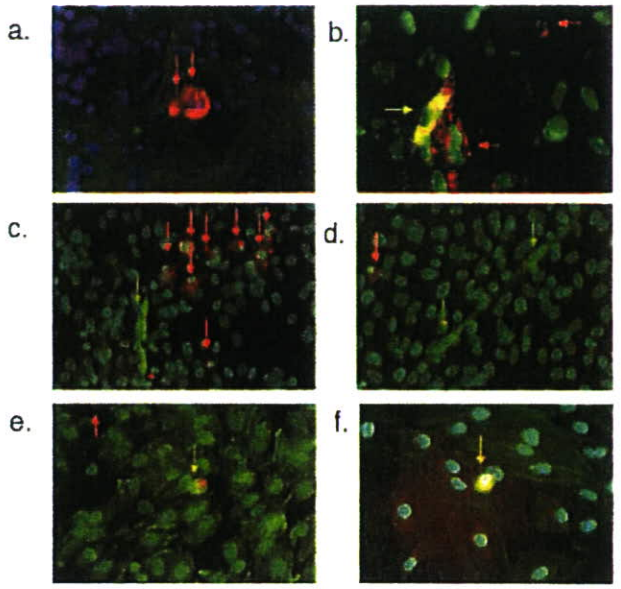

Figure 2. Expression of cardiac smooth muscle and endothelial lineage markers in coculture system by cytoimmunochemistry. After fixation of coculture cells (human EPC and rat H9C2), these cells were stained with human-specific cardiac antibody (α/β-ventricular MHC) and α-SMA antibody. a, Positive cells for cardiac marker (red; arrow). b, Positive cells for both cardiac and smooth muscle markers (yellow; arrow) and the only positive cell for cardiac marker (red; arrow). c and d, Staining with human-specific cardiac antibody (α/β-ventricular MHC) (green; arrow) and human-specific endothelial lineage antibody (CD31) (red: arrow). Coculture cells were stained with both cardiac antibody (cTn-I) (green) and human cell antibody (HLA-ABC) (red; arrow), or smooth muscle lineage marker (green) and human cell antibody (HLA-ABC). Double-stained cell shows cardiac marker-positive cell derived from human cell (yellow; arrow) (e) and smooth muscle lineage marker-positive cell derived from human cell (yellow; arrow) (f). Blue shows DAPI.

Results

Coculture of Human EPCs and Rat Cardiac Myoblasts (H9C2) Expressed Human-Specific Cardiac and Smooth Muscle Markers

We performed RT-PCR to evaluate the human specificity of the primers. Cardiac-specific markers such as cTn-T, MLC-2V, and α-MHC were expressed in human heart RNA (Clontech) but not expressed in RNA from H9C2 and human EPCs. Smooth muscle markers such as sm22α and α-SMA were expressed in RNA from human smooth muscle cells, but not expressed in RNA from rat smooth muscle cells and human EPCs. Endothelial marker such as CD31 was expressed in RNA from human umbilical vein endothelial cells and human EPCs, but not expressed in RNA from rat endothelial cells (data not shown). Also, these primers except for CD31 were not expressed in RNA from human EPCs alone. RT-PCR was performed using these primers 7 days after initiating coculture of human EPCs and H9C2. RT-PCR from coculture samples disclosed the expression of cardiac markers (cTn-T, MLC-2V, α-MHC) and smooth muscle markers (sm22α, α-SMA) (Figure 1a). These data suggested that coculture condition induced human EPCs to express cardiac and smooth muscle lineage-specific genes. We designed 3 types of GAPDH primers, human-specific, mouse-/rat-specific, and both human and rat cross-reactive, to standardize the amount of DNA in each lane.

Cocultured Cells Stained With Cardiac and Smooth Muscle Antibody

Cytoimmunochemistry was performed after fixation of cocultured cells. Human-specific cardiac antibody (α/β-ventricular MHC) stained human EPCs 7 days after initiating coculture with H9C2. The morphology of α/β-ventricular MHC-positive cells was round or spindle, and the frequency was ≈0.1% (Figure 2a). Several cocultured EPCs double-stained with both human-specific cardiac antibody (α/β-ventricular MHC) and α-SMA antibodies were observed (Figure 2b). The morphology of these double-stained cells was spindle and the frequency was <0.08%. We observed positive cells for human cardiac antibody besides human endothelial lineage-positive cells (Figure 2c and 2d). Based on the identification of human-derived cells by HLA antibody, Tn-I and HLA double-stained cardiac lineage-positive cells derived from human cells (Figure 2e), and α-SMA and HLA double-stained human-derived smooth muscle lineage cell (Figure 2f). These in vitro data suggested that coculture condition induced human EPCs to express both cardiac and smooth muscle lineage-specific proteins. Furthermore, we

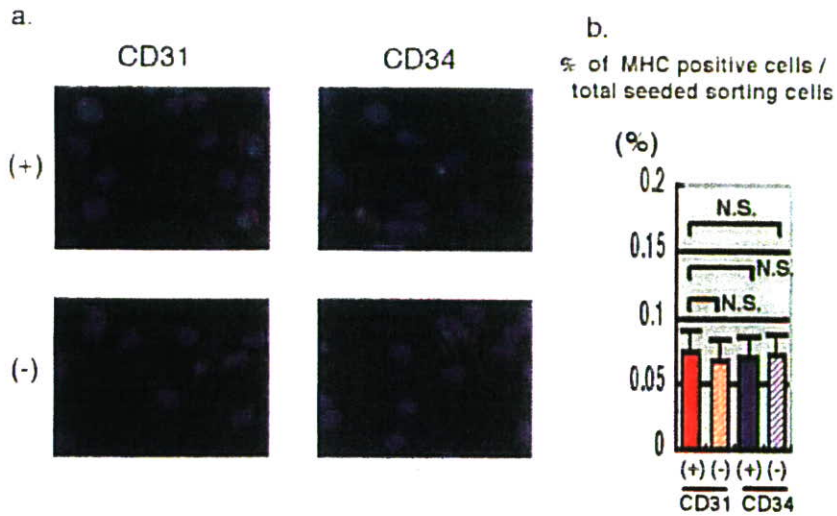

Figure 3. Evaluation of cardiac lineage differentiation frequency in coculture with H9C2 and CD31 or CD34 sorted cells from ex vivo expanded endothelial progenitor cells. After fixation of coculture cells (rat H9C2 with CD31-positive cells, CD31-negative cells, CD34-positive cells, and CD34-negative cells, respectively) (a), these cells were stained with human-specific cardiac antibody (α/β-ventricular MHC). The positive cells (red) were counted and the proportion was evaluated as percent MHC-positive cells/total seeded sorting cells (b). Blue shows DAPI.

designed coculture with subpopulation of cultured EPCs and H9C2 to define the endothelial marker subpopulation that will mainly contribute to translineage commitment. We sorted CD31 positive fraction and negative fraction, or CD34 positive fraction and negative fraction, and then cocultured with H9C2 in each fraction (Figure 3a). Cytoimmunochemistry disclosed the frequency of cardiac lineage commitment in each sorting fraction, and no difference was observed among the coculture for cardiac lineage commitment in coculture with both CD31 and CD34 fractioning (Figure 3b).

RT-PCR of EPC-Injected Myocardial Samples Demonstrated the Expression of Human-Specific Cardiac and Vascular Smooth Muscle Markers

RT-PCR using human EPC-injected rat myocardial samples disclosed the expression of cardiac (cTn-T, MLC-2V, α-MHC) and smooth muscle-specific (sm22α, α-SMA) genes. However, RT-PCR using PBS-injected rat myocardial samples did not express any cardiac and smooth muscle genes (Figure 1b). The data confirmed RT-PCR using coculture samples in vitro. We designed human-specific GAPDH to standardize the DNA amount derived from transplanted human cells.

Immunohistochemistry of EPC-Injected Myocardial Samples Demonstrated the Expression of Cardiac and Vascular Smooth Muscle Markers

Frozen sections of EPC-injected myocardial samples were stained with human-specific cardiac antibodies (α/β-ventricular MHC, cTn-I, BNP) (Figure 4a-1, 4b-1, 4c-1, respectively), human-specific smooth muscle cell antibody (Calponin) (Figure 4d-1), and human-specific endothelial marker (CD31) (Figure 4e-1). For gap junction experiment, connexin 43 and HLA antibodies were used for double staining because connexin 43 antibody had cross-reactivity with human and rat. In PBS-injected rat myocardium, connexin 43 stained gap

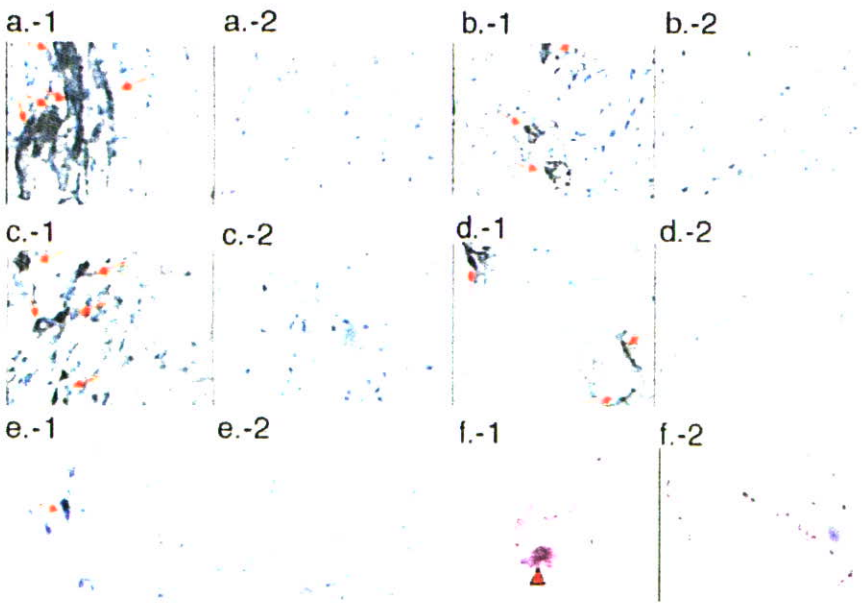

Figure 4. Expression of human-specific cardiac and smooth muscle markers in EPC-injected rat heart by immunohistochemistry. After fixation of ischemic nude rat heart injected with human EPCs or PBS, samples were stained with human-specific cardiac antibodies (a, α/β-ventricular MHC; b, cTn-I; c, BNP), human-specific smooth muscle antibody (d, calponin), and human-specific endothelial marker (e, CD31). For the experiment of gap junction, tissue sample was stained with connexin 43 in rat myocardium (f-2: connexin 43) or double-stained with connexin 43 and HLA antibodies in EPC-transplanted rat myocardium (f-1, connexin 43 and HLA, arrowhead). Left photo of each group shows rat heart with human EPCs, and right shows rat heart with PBS. The right photo of each group shows negative control for each human-specific antibody (Figure 4a-1 through 4e-1). DAB staining system was used for HLA and VIP staining system was used for connexin 43. All other immunohistochemical stainings (each staining by single antibody) were performed using chemical (DAB) method. Nuclei were stained by hematoxylin staining. Photos from (a to e) ×400 magnification. Photos (f-1 and f-2) ×1000 magnification.

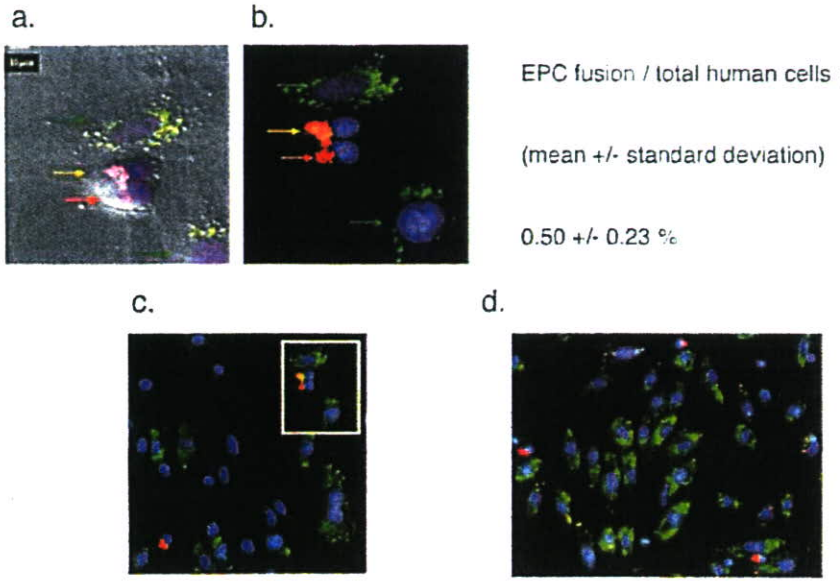

Figure 5. Evaluation of frequency of cell fusion in coculture system. We performed coculture using Qtracker (Quantum Dot Corp), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565(red arrow) and H9C2 cells were labeled with Qtracker 655 (green arrows) (a and b). Cell fusion was observed in the EPCs attached to H9C2 (yellow arrow) (a and b). Low-magnification photo was indicated (c), and the white square (c) was equivalent to (b). EPC fusion ratio was evaluated by counting fusion cells out of total human-derived cells and indicated the frequency as mean±SD (d).

junctions in cardiomyocytes (Figure 4f-2). However, double-stained cells disclosed the connection between rat cardiomyocyte and human-derived cell (Figure 4f-1, arrowhead). To test the human specificity of the antibodies, immunohistochemistry was performed using PBS-injected rat myocardium as the negative control. Each antibody did not react with rat cardiomyocytes (Figure 4a-2, 4b-2, 4c-2), rat smooth muscle cells (Figure 4d-2), and rat endothelial cells (Figure 4e-2). These in vivo data suggested that human EPCs transplantation caused multi-lineage differentiation into cardiac, smooth muscle, and endothelial lineages in the ischemic myocardium. Immunostaining by using connexin 43 and HLA revealed that transdifferentiated EPCs connected to other surviving rat derived cardiomyocytes (Figure 4f-1).

Evaluation of Frequency of Cell Fusion in Coculture System

We performed coculture using Qtracker (Quantum Dot Corp), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565 (red) and H9C2 cells were labeled with Qtracker 655 (green) (Figure 5c). Only EPCs attached to H9C2 incorporated both green and red dye markers (white square in Figure 5c and yellow arrow in Figure 5a and 5b). However, the frequency of cell fusion was very low from these data. We evaluated EPC fusion ratio by counting fusion cells out of total human-derived cells and indicated the frequency as mean±SD (0.50±0.23%) (Figure 5d). These data were equivalent to the result demonstrated by Badorff et al.[13]

Discussion

Previous studies from our and another laboratories showed therapeutic potential of ex vivo expanded EPCs for myocardial ischemia.[1,12] We hypothesized that EPCs can contribute to not only vasculogenesis but also myogenesis in the ischemic myocardium. Although differentiated endothelial cells were the candidate for therapeutic application of ischemic disease, EPCs proved themselves as much more effective by animal model experiment[16].

Considering the flexibility of somatic stem and progenitor cells for lineage commitments,[1,8,13] we investigated whether the translineage commitment of EPCs contribute to cardiomyogenesis and vasculogenesis for functional improvement after EPC transplantation. To elucidate the mechanism of translineage commitment, we developed the detection system to differentiate target cell transcription and expression. Established coculture system detected human myocardial and smooth muscle lineage profiles from the cell population derived from human EPCs and rat cardiomyocytes without cross-reactivity between species. Rat cardiac myoblast cell line (H9C2) was cocultured with human EPCs for RT-PCR to distinguish species-specific markers. The RT-PCR system detected only human-specific cardiac and smooth muscle markers but not rat cardiac and smooth muscle markers. Using this system, translineage commitment from EPC to cardiac and smooth muscle lineages was detected precisely.

Using human-specific cardiac antibody (α/β-ventricular MHC), the percentage of positively stained EPCs was ≈0.1% among incubated EPCs by immunohistochemical determination. In addition, using both human-specific cardiac and smooth muscle antibodies, the percentage of double-positively stained EPCs was <0.08%. This indicates the phenomenon of EPC translineage commitment is not a common differentiation cascade during in vitro condition cocultured with myocardial lineage cells. Despite that we have already found the therapeutic potential of cultured EPCs in ischemic animal models, it still remains the issue which subpopulation of EPCs mainly contributes to cardiac lineage commitment. To address this point, we performed the sorting of cultured EPCs using CD34 or CD31 surface marker as one of the candidate markers for EPC and also established markers for endothelial cells. It should be noted that no specific markers are available for purifying EPCs yet, although a lot of challenges have been reported from various laboratories around the world. However, it could be possible

to compare positive and negative fractions and evaluate the tendency regarding cardiac lineage commitment. Our findings suggested that coculture in positive or negative fractions with cardiac lineage cells (H9C2) revealed no difference in cardiac lineage commitment in the case of both CD31 and CD34 fractioning as shown in Figure 3b. In this experiment, we conclude that at least both CD31 and CD34 are not key markers to determine the contribution of cardiac lineage commitment, and that the chance of contamination of mesenchymal stem cells is excluded because negative fraction that is supposed to include mesenchymal stem cells is incompetent in cardiac lineage commitment compared with positive fraction of CD31 or CD34. We will make effort to identify the precise marker for purifying EPCs in our next research endeavors.

Although we are interested in the emergence of double-lineage marker expressing (α/β-ventricular MHC and α-SMA) cell in vitro as the process of translineage commitment, in early heart development multiple smooth muscle lineage genes are reported to be expressed as regulators of muscle differentiation. α-SMA as well as sm22-α, a calponin-related protein, is expressed in cell lines derived from embryonic and adult hearts.[17] These protein detections might reflect early phase of myocardial lineage differentiation in this coculture system.

As discussed for years, we are still clueless regarding the mechanism of translineage differentiation. Along with formerly discussed transdifferentiation and de-differentiation, several groups have recently reported spontaneous cell fusion occurring in coculture between embryonic stem cells and bone marrow cells,[18] or between embryonic stem cells and brain-derived cells.[19] Cell fusion has long been known to achieve effective reprogramming of cells. Terada et al have reported that the frequency of spontaneous cell fusion was very low. Nevertheless, Lagasse et al have reported robust (30% to 50%) levels of transdifferentiation.[6] To define the frequency of cell fusion in this coculture condition, we used Qtracker system to determine the population of cell fusion. The frequency of cell fusion was rarely seen ($0.50\pm0.23\%$) though Qtracker system clearly disclosed the phenomenon of cell fusion. Transdifferentiation, but not cell fusion, is the main mechanism in our coculture system. Our finding regarding cell fusion is compatible with the data reported by Badorff et al.[13] They have concluded that cell-to-cell contact, but not cellular fusion, mediated EPC transdifferentiation. Although our data indicated lower proportion of cardiac lineage commitment, it could be the difference in methods, for example, EPC culture method, evaluation method, and antibodies used for the evaluation. Yeh et al have not investigated the cell fusion issue in their article; however, they have also suggested that phenotypic conversion of the injected $CD34^+$ cells may occur predominantly through transdifferentiation.[14]

We have expanded in vitro experiments to deduce whether this is a pathophysiological phenomenon observed in vivo. After transplantation of human EPCs to rat ischemic heart models, myocardial samples disclosed both human cardiac and smooth muscle, as well as endothelial lineage gene expressions detected by RT-PCR and immunohistochemistry. We also performed the experiment to confirm the cross-talk between ischemic rat cardiomyocyte and transplanted human-derived EPC by immunohistological staining with connexin 43, one of the gap junctional molecules. The functional connection was observed between rat cardiomyocyte and human EPC in ischemic region.

The evidence that translineage commitment of EPCs into cardiomyocyte and smooth muscle cell lineages in vivo encourages therapeutic application of EPCs for myocardial ischemic diseases. The results indicate the occurrence of niche-dependent translineage differentiation of EPCs for vasculogenesis and cardiomyogenesis for heart regeneration. Because the severely damaged myocardium requires significant heart organogenesis, the potency of EPCs to supplement myocardial and smooth muscle lineage cells is very reasonable to regenerate heart tissues. The emergence of newly formed cardiomyocyte may reconstitute destroyed myocardium and provide cross-talk signaling toward vasculogenesis. Furthermore, the occurrence of smooth muscle lineage supports the maturation and maintenance of newly formed blood vessels by original endothelial lineage cells derived from EPCs. Recent publication suggested CD34 transdifferentiation into cardiomyocytes, smooth muscle cells, and endothelial cells in ischemic rat heart.[14] These generated systemic biological cross-talk between lineages are proceleusmatic for the ischemic heart disease treatment. These data suggest that EPC transplantation therapy has beneficial effects via both blood flow improvement and myogenesis in myocardial regeneration.

However, the frequency of myogenesis observed in this study is not enough to encourage functional improvement by translineage differentiation of EPCs themselves. Further mechanistic investigation is necessary to improve the transdifferentiation ratio and apply for clinical trial.

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