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.

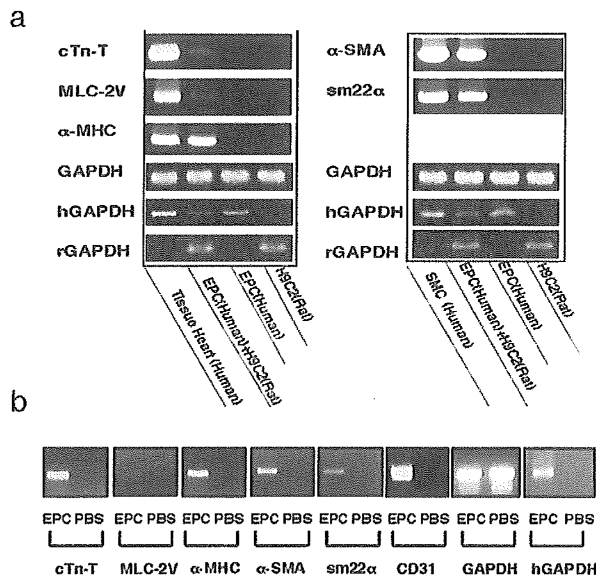

Figure 1. Expression of human-specific cardiac and smooth muscle markers in coculture system and in EPC-injected rat heart by RT-PCR. RNA samples from various culture conditions were analyzed by RT-PCR. cTn-T, MLC-2V, and α-MHC are specific markers for cardiomyocyte, and α-SMA and sm22α are specific 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.

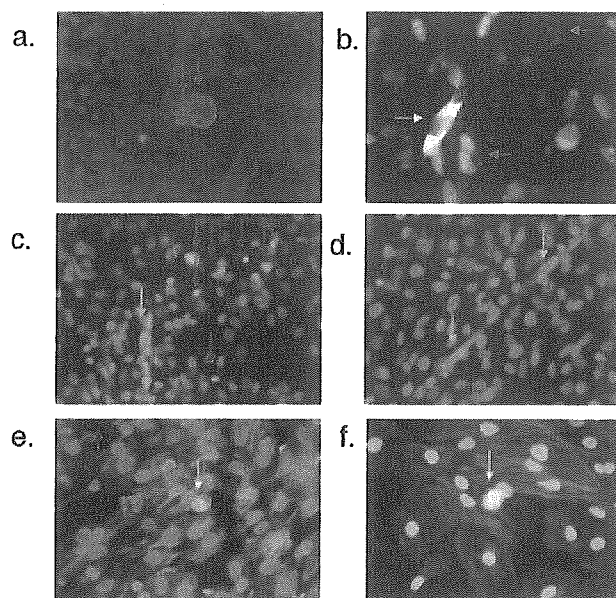

Figure 2. Expression of cardiac smooth muscle and endothelial lineage markers in coculture system by cytoimmunochemistry. After fixation of coculture cells (human EPC and rat H9C2), these cells were stained with human-specific cardiac antibody (α/β-ventricular MHC) and α-SMA antibody. a, Positive cells 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

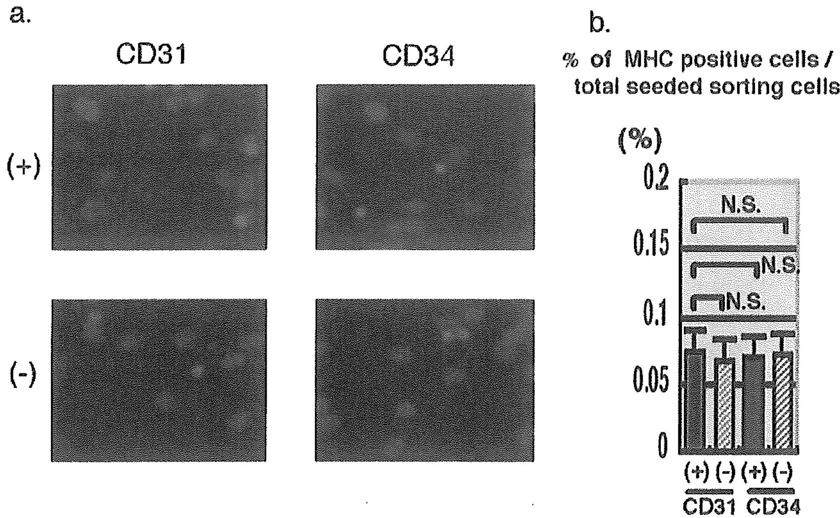

Figure 3. Evaluation of cardiac lineage differentiation frequency in coculture with H9C2 and CD31 or CD34 sorted cells from ex vivo expanded endothelial progenitor cells. After fixation of coculture cells (rat H9C2 with CD31-positive cells, CD31-negative cells, CD34-positive cells, and CD34-negative 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

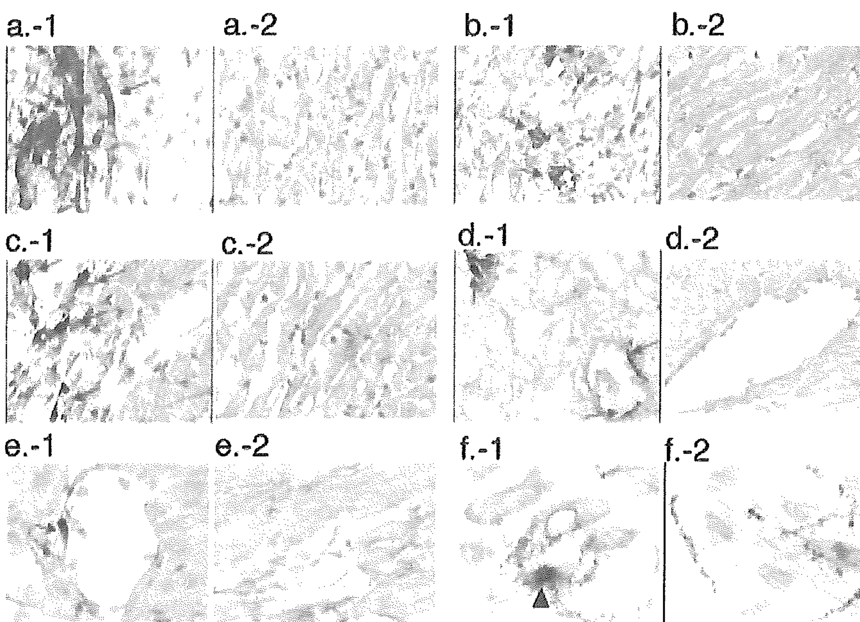

Figure 4. Expression of human-specific cardiac and smooth muscle markers in EPC-injected rat heart by immunohistochemistry. After fixation of ischemic nude rat heart injected with human EPCs or PBS, samples were stained with human-specific cardiac antibodies (a, α/β-ventricular MHC; b, cTn-I; c, BNP), 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.

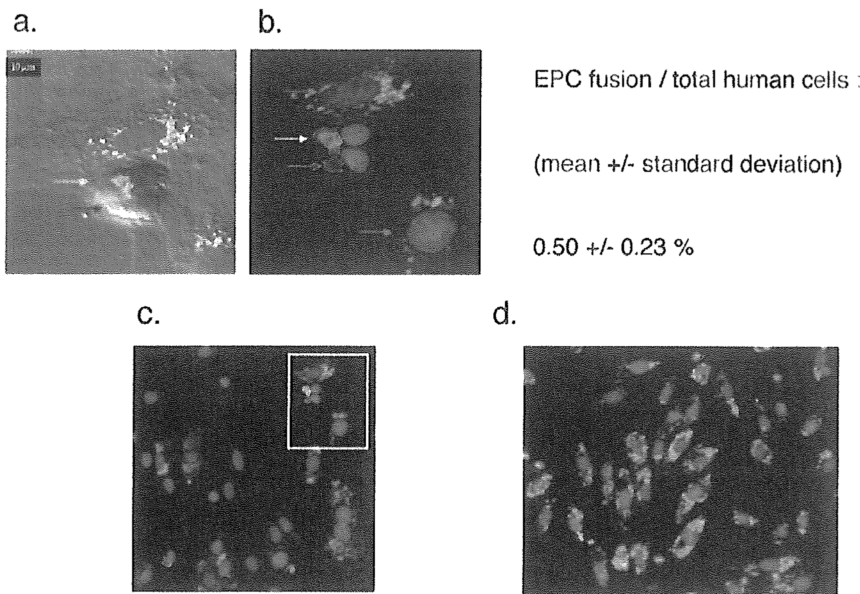

Figure 5. Evaluation of frequency of cell fusion in coculture system. We performed coculture using Qtracker (Quantum Dot Corp), which is a nanocrystal labeling marker incorporated in the vesicles of the cytoplasm. EPCs were labeled with the Qtracker 565(red arrow) and H9C2 cells were labeled with Qtracker 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 $\approx$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 ($0.50 \pm 0.23\%$) 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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遺伝子治療による血管新生療法 1

心筋虚血における血管新生療法

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● はじめに

近年，心血管領域での再生医学に関する研究が進んでいる。心筋虚血に対して遺伝子導入あるいは細胞移植をすることにより血管再生を促す，いわゆる血管新生療法がこれらの研究の礎となっている。ボストン・タフツ大学のIsner教授らがVEGF(vascular endothelial growth factor)プラスミドの遺伝子治療を末梢動脈閉塞症患者に試みて血管再生による効果を報告して以来，再生医療と遺伝子治療が密接な関連をもつことが示唆されるようになった。その後，心筋虚血に対しても，遺伝子治療による血管新生療法の効果が示されている。本稿では心筋虚血における最近の遺伝子治療の動向について，主に基礎研究の成果を中心に記載をする。

● 遺伝子治療による血管新生療法

1　遺伝子治療の候補因子

a　成長因子　血管新生を促す成長因子として最初に同定されたのは，VEGFとFGF（fibroblast growth factor)である。VEGFにはVEGF-A, -B, -C, -Dが存在し，VEGF-A, -Bは内皮細胞に発現するVEGF受容体1および2を介して内皮細胞の増殖，遊走に関与する。VEGF-C, -DはVEGF受容体2および3を介してリンパ管性血管新生（lymphangiogenesis）を促進することが知られている。最近では別のVEGFファミリーとして，PlGF（placenta growth factor）がVEGF受容体1および2を介して血管新生に関与することが示された[1]。一方，FGFにもいくつかのファミリーが同定されており，このうちとくにFGF-1，FGF-2，およびFGF-4が高い血管新生作用を有することが知られている。そのほかHGF(hepatocyte growth factor)，アンジオポエチン（ANGPT)-1，エリスロポエチン，IGF-1（insulin-like growth factor）なども，血管新生を促進する遺伝子治療の候補にあげられている。一方，胎児発生の過程で重要な因子の一つとして知られるhedgehog（Hh）蛋白ファミリーの中でもsonic hedgehog（Shh）は虚血における血管新生を促す因子の一つとして報告されている[2,3]。

b　ケモカイン　単球系の細胞に働いて血管新生を促進するものとして，monocyte chemoattractant protein-1(MCP-1)がある[4]。またstromal cell-derived factor-1（SDF-1）は虚血組織において血管内皮前駆細胞（endothelial progenitor cell：EPC）を集積し，血管再生を促すことが動物モデルを使った実験で明らかにされている[5]。

c　転写因子　血管新生に関与する遺伝子を標的遺伝子とする転写因子として，hypoxia-inducible factor 1 (HIF-1) が候補となっている。HIF-1は低酸素，虚血状態の組織においてVEGFを賦活化することが知られており，HIF-1の強制発現がANGPT-1，ANGPT-2，PlGF,

platelet-derived growth factor-B（PDGF-B）を修飾することが明らかになっている[6]。

d　細胞外マトリックス　細胞外マトリックス蛋白の一つ Del-1 はインテグリン依存性に血管新生を促進することが知られている[7]。

2　遺伝子治療の問題点

ベクターの選択，遺伝子導入効率について検討の余地があることはいうまでもないが，一方動物実験レベルで確認される血管新生治療効果が，重症冠動脈疾患患者で十分に発揮されないことが，これまでのデータから示唆されている。Ruel らは[8]，局所の NO（nitric oxide）が血管新生に関与するという基礎研究の結果をもとにして，高コレステロール食による内皮障害ミニブタと通常食によるミニブタに対して，それぞれ慢性心筋虚血を作製し，FGF-2 蛋白を投与して心筋の血流改善を比較したところ，内皮障害モデルにおいて明らかに FGF-2 に対する血管再生の反応が障害されているという結果が得られた。したがって，臨床のプロトコールにおいて，より有効な方法を工夫することが今後の課題となっている。

3　遺伝子のコンビネーション

血管再生効果を増幅する目的で，複数の遺伝子のコンビネーションによる遺伝子治療が心筋虚血動物モデルを用いて行われ，その効果が報告されている。Siddiqui らは，ANGPT-1＋VEGF のコンビネーション[9]，また Hao らは，PDGF-BB＋VEGF のコンビネーションでそれぞれ単独の遺伝子治療よりも優れた血管新生効果を報告している[10]。

● 次世代の遺伝子治療（細胞移植とのコンビネーション）

遺伝子治療とともに，再生医学において細胞移植療法が注目を浴びている。1997 年にわれわれのグループは，成人の末梢血中に EPC が存在することを明らかにし[11]，この細胞を用いた細胞移植による虚血性疾患への応用を実現するために，基礎研究，および小動物，大動物を用いた細胞移植実験を積み重ねてきた。ここでは細胞治療に関する詳細については他稿に譲ることとする。これらの基礎検討によって，動物レベルでの血管新生療法に EPC が有効であることは証明されたが，実際に EPC のマーカーの 1 つである CD34 陽性細胞は通常，末梢血中に 1％以下しか存在していない。そこで実際に臨床応用するためには，細胞の数を確保するとともに，細胞の質を高める工夫が必要になってくる。この点を明らかにするため，EPC の細胞移植と遺伝子治療を組み合わせた方法の臨床応用への検討が注目を浴びている。われわれが留学していた米国の施設では，重症心虚血，下肢動脈硬化症の患者に遺伝子治療による血管新生療法が続けられてきたが，その最大の問題点は，いかに安全に効率よく患部で遺伝子発現できるかにあった。

EPC による遺伝子治療は，細胞移植単独よりもさらに有効な治療法であることが動物モデルを用いた実験で明らかになってきた。具体的には虚血部位，あるいは障害動脈部位に VEGF や老化の遅延に関連した因子の遺伝子治療を施した EPC を投与することが可能と考えられる。これまでの研究で，ヒト EPC に VEGF 遺伝子を強制発現させ，細胞治療よりはるかに少ない量の細胞を投与したところ，血管新生・血流改善の面で単独細胞治療をしのぐ治療効果が確認されている[12]。最近われわれは，一般にヒト体細胞の老化に伴いテロメラーゼ活性が低下することに注目し，テロメラーゼを活性化させるテロメラーゼ逆転写酵素（TERT）の遺伝子導入を EPC に施すことにより，分裂寿命の延長を誘導することを明らかにし，報告してきた。すなわちコントロールの細胞群に比べて TERT を導入した細胞群では継代に伴うテロメラーゼ活性低下は抑制されており，実際に老化（senescence）に移行する細胞の割合も減少していた。興味深いことに TERT を導入した細胞群においてのみ内皮への分化を示唆するコロニー形成が認められた。細胞機能の評価では TERT の導入群で増殖，遊走がともに増強し，アポトーシスは抑制されていた。動物実験において，TERT を強制発現させた EPC を下肢虚血動物モデルに投与したところ，VEGF の場合と同様，単独

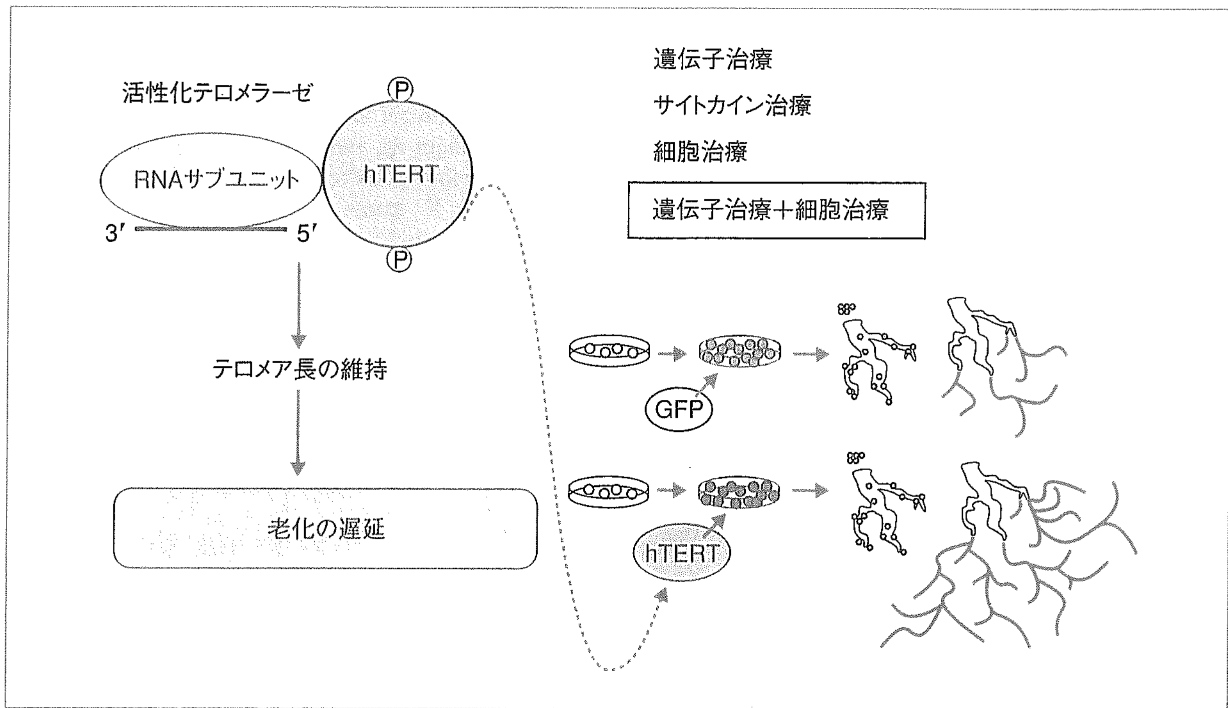

図 1　TERT 遺伝子導入後の EPC 移植

若年者から採取した EPC は内因性 TERT を発現しているが，年齢とともに発現は低下すると考えられ，動脈硬化性虚血性疾患を有する高齢者では，ほとんど発現がみられないと予想される。そこで「老化」細胞に，TERT を遺伝子導入することにより細胞を若返らせ，質の良い細胞を虚血部位に移植する方法が次世代の遺伝子治療と細胞移植のコンビネーションとして基礎検討されている。具体的には，ヒト末梢血から単核球を分離し，体外培養にて増幅後，TERT 遺伝子を導入することで個々の細胞の質を高め，虚血部位において少ない細胞数でも効率良く血管新生を誘導することを目的とする。動物実験での効果はすでに確認されており，今後は虚血性疾患患者の末梢血を用いた自己血細胞治療に遺伝子治療を併用する方法を検討している。

細胞治療をしのぐ治療効果が確認された。つまり「若返り」させた EPC が少量で虚血に対する治療効果を示したと考えられる[13]（**図 1**）。さらに別の報告では，hTERT が単に EPC の生存，増殖のみならず，パラクラインの機序で成長因子の血中への遊離を促進することが知られており，今後の併用療法の実現に向けて興味深い知見である。同時にこの研究の中で，彼らはヒト上皮細胞に hTERT を導入した系を用いて外因性テロメラーゼの誘導によって，細胞増殖に関連する遺伝子，とくに上皮成長因子受容体（EGFR）の発現が調節され，上皮細胞の増殖に関与することを示している[14]。

心筋虚血の改善に関しては，血管再生の概念とともに，最近，心筋そのものの再生の概念が生まれてきた。EPC や CD34 陽性細胞についても，血管内皮のみならず心筋に分化することが報告されており[15,16]，われわれのグループでも同様の現象を確認している[17]。ただし，臨床応用に結びつけるための効率のよい心筋再生への誘導条件は，今後さらに検討する必要があると思われる。

● おわりに

基礎研究の観点から，心血管領域における最近の遺伝子治療の動向について概要を述べた。生活習慣病の増加に伴い，動脈硬化性虚血性疾患が国民の主要疾患となりつつある。このような傾向に伴い，対象患者は必然的に高齢に偏り，血管新生を期待する患者の背景が，動脈硬化性疾患そのものや高齢によって修飾され，遺伝子治療や細胞治療の効果を妨げる可能性が示唆されている。このため，本稿の後半でも述べたように，例えば細胞老化を遅らせるような遺伝子治療と細胞治療のコンビネーションが次世代の治療として注目されている。動物実験での効果

はすでに証明されているが，今後ヒトへ応用するため，遺伝子に関してはベクターの選択，導入効率の問題，また細胞に関しては，無菌状態での無血清培地における細胞増殖技術の確立という問題を解決する必要性があり，現在これらに対して検討が続けられている。

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