Development of Serum-Free Quality and Quantity Control Culture of Colony-Forming Endothelial Progenitor Cell for Vasculogenesis

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ABSTRACT
Quantitative and qualitative impairment of endothelial progenitor cells (EPCs) limits the efficacy of autologous cell therapy in patients with cardiovascular diseases. Here, we developed a serum-free quality and quantity control culture system for colony-forming EPCs to enhance their regenerative potential. A culture with serum-free medium containing stem cell factor, thrombopoietin, vascular endothelial growth factor, interleukin-6, and Flt-3 ligand was determined as optimal quality and quantity culture (QQc) in terms of the most vasculogenic colony-forming EPC expansion, evaluated by the newly established EPC colony formation assay. The QQc of umbilical cord blood-CD34+ cells for 7 days produced a 52.9-fold increase in total cell number and 3.28-fold frequency in definitive EPC colony development, resulting in a 203.9-fold increase in estimated total definitive EPC colony number in vitro. Pre- or post-QQc cells were intramyocardially transplanted into nude rats with myocardial infarction (MI). Echocardiographic and micromanometer-tipped conductance cather examinations 28 days post-MI revealed significant preservation of left ventricular (LV) function in rats receiving pre- or post-QQc cells compared with those receiving phosphate-buffered saline. Assessments of global LV contractility indicated a dose-dependent effect of pre- or post-QQc cells and the superior potency of post-QQc cells over pre-QQc cells. Furthermore, immunohistochemistry showed more abundant formation of both human and rat endothelial cells and cardiomyocytes in the infarcted myocardium following transplantation of post-QQc cells compared with pre-QQc cells. Our optimal serum-free quality and quantity culture may enhance the therapeutic potential of EPCs in both quantitative and qualitative aspects for cardiovascular regeneration.

INTRODUCTION
Several translational researchers have demonstrated that CD34+ or CD133+ cells in umbilical cord blood (CB), bone marrow (BM), or peripheral blood (PB), previously defined as hematopoietic stem/progenitor cells, also serve as the enriched source of endothelial progenitor cells (EPCs) [1, 2], inducing neovascularization for functional recovery from ischemic injury [3–10]. Particularly autologous CD34+ or CD133+ stem/progenitor cells have been therapeutically transplanted in patients with severe ischemic heart or limb diseases, and these initial clinical experiences indicate the safety and feasibility as well as the effectiveness of cell-based therapy [3, 11–19].

However, a limitation of stem/progenitor cell therapy has been reported. EPC-enriched populations (CD34+, CD133+, CD34+/vascular endothelial growth factor receptor 2+ [VEGFR-2+], or CD133+/VEGFR-2+) are scarce even in the BM and PB. Moreover, the EPC fraction numerically and functionally declines in patients with aging [20] or cardiovascular risk factors [21, 22]. These reports suggest that poor responses to cellular therapy are due to quantitative and/or qualitative impairment of the EPC fraction. To further augment the efficacy of EPC transplantation, several methodological approaches to enhance EPC bioactivities are currently being developed [5, 23–28].

Initially, the EPC expansion culture has been established in terms of an increase in attached endothelial lineage cells following the conditioning of PB or BM mononuclear cells with endothelial growth factor-supplemented medium [4, 29–31]. Although high performances for vasculogenesis are represented in animal models, the quality and quantity culture control for medical
EPCs is limited by less proliferative activity and progressive senescence during the culture in attached EPC phenotype. In this regard, the several culture systems of suspended EPC phenotype have been developed and demonstrated the significance of EPC expansions ex vivo and the efficiency of cultured CD34<sup>+</sup> or CD133<sup>+</sup> cell transplant for neovascularization in ischemic animal models [5, 23–28].

However, despite the effective results for therapeutic strategy in many researches, EPCs remain a controversial topic among researchers as there is no definitive delineation of EPCs, no clear differentiation hierarchy, and no unambiguously defined isolation protocol. Therefore, the methodological development of EPC culture has lacked the definitive EPC evaluation methodology.

Considering the necessity of creating a defined assay, a novel adhesive clonogenic assay for the quantitative and qualitative analysis of EPCs based on differentiation hierarchy has been developed recently [32–37]. The new EPC colony-forming assay (EPC-CFA) enabled the distinction and the definition of two different types of EPC-colony-forming units (EPC-CFUs), that is, primitive and definitive EPC-CFUs, composed of small and large cells, respectively. Primitive EPC-CFUs (PEPC-CFUs) revealed a predominant potential for vasculogenesis. In the present study, we optimized the growth factor/cytokine combination (Comb) in a serum-free, quality and quantity culture (QQc) of CB-CD133<sup>+</sup> cells as an EPC-enriched population for vasculogenesis using EPC-CFA and further evaluated the therapeutic potential of the optimal QQc cells for cardiac repair post-myocardial infarction (MI) versus CB-CD133<sup>+</sup> cells in pre-QQc.

### MATERIALS AND METHODS

#### Preparation of CB-CD133<sup>+</sup> Cells by Magnetic Cell Sorting

CB was used for isolation of CB-CD133<sup>+</sup> cells under the approval of the ethical committees of the Cord Blood Bank and Clinical Investigation of the Tokai University School of Medicine. The protocol of CB-CD133<sup>+</sup> cell isolation is described in supplemental online Methods 1.

#### Serum-Free Culture Trials of CB-CD133<sup>+</sup> Cells

At first, 10,000 CB-CD133<sup>+</sup> cells in 500 µl of medium were plated into each well of a 24-well tissue culture dish (Primaria, BD Biosciences, San Diego, http://www.bdbiosciences.com) and cultured in a suspension manner using serum-free culture medium (StemSpan SFEM; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) containing six Combs of several growth factors/cytokines for 7 days (Table 1). Furthermore, CB-CD133<sup>+</sup> cells were similarly cultured by Comb 4 medium, named QQc medium, for 2, 4, or 7 days (Table 2).

#### EPC-CFA

To investigate the vasculogenic potential of pre- or post-QQc cells, we quantified adhesive EPC colonies by EPC-CFA using semi-solid culture medium (MethoCult SF<sup>®</sup>; StemCell Technologies) with pro-angiogenic growth factors in 35-mm Primaria dishes (BD Biosciences), as described in supplemental online Methods 2 [32–35, 37]. Eighteen days after initiation of the culture, the number of adherent colonies per dish was measured using a gridded scoring dish (StemCell Technologies) under light microscopy.

| Table 1. Investigated combinations of growth factors/cytokines for quality and quantity culture |
|-----------------------------------------------|
| **Comb** | Comb 1 | Comb 2 | Comb 3 | Comb 4 | Comb 5 | Comb 6 |
| TPO/VEGF | – | + | + | + | + | + |
| SCF | + | – | + | + | + | + |
| Flt-3 ligand | + | + | – | + | + | + |
| IL3 | + | + | + | – | + | + |
| IL6 | + | + | + | – | + | + |

Abbreviations: Comb, combination; IL, interleukin; SCF, stem cell factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor.

| Table 2. Endothelial progenitor cell (EPC) CFU production of CB-CD133<sup>+</sup> cells in QQc |
|-----------------------------------------------|
| **EPC-CFU no.** | **Day 0** | **Day 2** | **Day 4** | **Day 7** |
| PEPC-CFU (no.) | 18.9 ± 1.0 | 19.9 ± 0.9 | 14.6 ± 0.6 | 11.0 ± 0.7 |
| DEPC-CFU (no.) | 5.8 ± 1.0 | 15.3 ± 0.9 | 21.2 ± 1.6 | 19.0 ± 1.7 |
| TEP-CFU (no.) | 24.7 ± 1.5 | 35.2 ± 1.5 | 35.8 ± 1.6 | 30.0 ± 1.6 |
| Per 1 × 10<sup>6</sup> CB-CD133<sup>+</sup> cells |
| PEPC-CFU (no.) | 503.3 ± 45.7 | 608.6 ± 55.3 | 3,083.2 ± 200.1 | 12,396.1 ± 981.4 |
| DEPC-CFU (no.) | 116.7 ± 24.8 | 455.1 ± 48.0 | 3,012.6 ± 200.1 | 23,793.2 ± 2,399 |
| TEP-CFU (no.) | 620.0 ± 65.6 | 1063.7 ± 91.8 | 6,095.8 ± 38.0 | 36,189.3 ± 2,117 |

The top three rows are counted EPC-CFU numbers per 500 QQc cells in each period. The bottom three rows are estimated EPC-CFU numbers per whole QQc cell at each period from 1 × 10<sup>6</sup> pre-QQc cells (CB-CD133<sup>+</sup> cells) at day 0. The values indicated are mean ± SE in 3 dishes per each time point per sample × 6 CB samples.

Abbreviations: CB, umbilical cord blood; CFU, colony-forming unit; DEPC, definitive endothelial progenitor cell; PEPC, primitive endothelial progenitor cell; QQc, quality and quantity culture; TEP, total endothelial progenitor cell (PEPC-CFU plus DEPC-CFU).
microscopy. PEPC-CFUs and DEPC-CFUs were counted separately [32–35, 37].

Characterization of EPC Colonies
To confirm the endothelial characterization of the colonized cells, we measured biochemical binding with *Ulex europaeus* agglutinin I-conjugated fluorescein isothiocyanate (UEA-I-FITC), (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and uptake of acetylated low-density lipoprotein-conjugated 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (acLDL-DiI) (Biomedical Technologies, Inc., Stoughton, MA, http://www.btiinc.com), and we also examined immunocytochemistry for endothelial cell (EC)-specific markers, as described in supplemental online Methods 3.

Flow Cytometry Analysis
To characterize pre- and post-QQc cells, cells were analyzed by flow cytometry using a FACSCalibur flow cytometry system (BD Biosciences) after staining with mouse anti-human antibodies against surface markers listed in the supplemental online Material List. The data were analyzed by FlowJo, a flow cytometry analysis software (Tomy Digital Biology Co., Ltd., Tokyo, http://www.digital-biology.co.jp).

Real-Time Polymerase Chain Reaction Assay
The gene expression of proangiogenic growth factors and a cell proliferation marker, Ki67, in pre- and post-QQc cells was quantitatively analyzed by real-time polymerase chain reaction (PCR) assay as described in supplemental online Methods 4.

In Vitro Tube Formation and Incorporation Assay in Matrigel
To investigate the functional contribution of pre- or post-QQc cells to neovascular formation, the cells were applied to a tube formation and incorporation assay by coculturing with human umbilical vein endothelial cells (HUVECs) on Matrigel, as described in supplemental online Methods 5.

In Vitro Sprouting Assay in Matrigel
A colony cell fraction of PEPC- or DEPC-CFUs from pre- or post-QQc cells was isolated, and then each colony cell fraction was applied to Matrigel assay as described in supplemental online Methods 6.

In Vitro Induction of Functional Cardiomyocytes
The methods used to investigate the trans-differentiation of post-QQc cells into functional cardiomyocytes (CMCs) are detailed in supplemental online Methods 7.

In Vivo Assessment of Vascular and Cardiac Repair by Transplanted Pre- or Post-QQc Cells in the Rat Myocardial Ischemic Model
Vascular and cardiac repair by transplantation of pre- or post-QQc cells was investigated in rat myocardial ischemic model, as described in supplemental online Methods 8.

Statistical Analysis
The results were statistically analyzed as described in supplemental online Methods 9.

RESULTS

Optimization of Growth Factor/Cytokine Comb for QQc
To identify the QQc, we evaluated EPC colony producing potential among the six kinds of growth factor/cytokine Combs using EPC-CFA (Table 1). The cell numbers postculture for 7 days in six kinds of Combs varied from 26.0-fold in Comb 2 to 95.8-fold in Comb 6, compared with precultured cells (1 × 10⁴ CB-CD133⁺ cells) (Fig. 1A).

Of note, stem cell factor (SCF) exhibited the most potent factor to increase cell number because of the comparison of Comb 2 versus Comb 6. In EPC-CFA of cultured cells in all Combs, as previously indicated in mouse or human EPC-CFA [32–37], two types of EPC colonies were morphologically detected: PEPC-CFU, formed of small, round cells, and DEPC-CFU, formed of large, spindle-like cells (supplemental online Fig. 1A). These colonies showed biological features of endothelial lineage—that is, intracellular uptake of acLDL-DiI, cell surface binding of UEA-I-FITC (supplemental online Fig. 1B), and immunohistochemical positivity of endothelial antigens, such as endothelial nitric oxide synthase, VEGFR-2, and vascular endothelial (VE) cadherin (supplemental online Fig. 1C) [37].

The numerical assessment of EPC-CFA revealed a different pattern of EPC colony-forming potentials among the Comb groups from cultured cell numbers (Fig. 1B, 1C). The amount of PEPC-CFU, DEPC-CFU, or total EPC-CFU per 500 post-QQc cells was significantly higher in Comb 4 compared with the other Combs (Fig. 1B). In particular, the production of DEPC-CFU, indicating further vasculogenic capability superior to that of PEPC-CFU, was highest in Comb 4 (from 1.65-fold vs. Comb 3 to 2.65-fold vs. Comb 6). The estimated number of DEPC-CFUs produced from postculture of 1 × 10⁴ CB-CD133⁺ cells in Comb 4 significantly increased by 1.77-fold (vs. Comb 6) to 4.44-fold (vs. Comb 2) (Fig. 1C).

Together, EPC-CFA disclosed that Comb 4 (SCF, thrombopoietin [TPO], Flt3-ligand, interleukin-6 [IL-6], and vascular endothelial growth factor [VEGFR]) provides the QQc system, compared with the other Comb candidates, based on vasculogenic potential of EPC colony production. On the basis of this, the following experimental data were found under the QQc.

EPC Quality and Quantity During and Post-QQc
The total cell expansion during QQc was measured. The cell number in culture did not increase by day 2 but gradually increased, from 7.4-fold at day 4 post-QQc up to 52.9-fold at day 7 post-QQc (Fig. 2A). To evaluate the quality of vasculogenic potential during QQc, we performed EPC-CFA at each time point (Fig. 2B–2D; Table 2).

Total EPC-CFU number post-QQc did not exhibit a significant difference versus pre-QQc at day 7, although it was slightly increased at days 2 and 4 versus pre-QQc (Fig. 2B). On the other hand, the frequency of PEPC-CFU per 500 cells at each time point gradually decreased from day 2, finally reaching 0.58-fold of pre-QQc at day 7 post-QQc. Inversely, DEPC-CFU increased and reached a peak level of 3.66-fold at day 4 and 3.28-fold at day 7 post-QQc, compared with pre-QQc (Fig. 2B). Further, the ratio of DEPC-CFU number in total EPC-CFU number increased gradually (Fig. 2C).

On the basis of the expansion ratio at each time point of QQc, the estimated quantity of EPC-CFUs produced from pre-QQc was...
calculated. Numbers of PEPC-CFU, DEPC-CFU, and total EPC-CFU increased drastically and reached a maximal level at day 7 post-QQc (24.6-fold for PEPC-CFU, 203.9-fold for DEPC-CFU, and 58.4-fold for total EPC-CFU) (Fig. 2D). These findings indicate that QQc is superior at the quantity control culture to increase in EPC number, and at the quality control culture to potentiate EPC vasculogenic capability in terms of preferential increase in DEPC-CFUs.

Characterization of Pre- and Post-QQc Cells In Vitro

Flow cytometry was performed to estimate the positivities for endothelial lineage markers in pre- and post-QQc cells. The percentage of positivity for endothelial-specific markers of VEGFR-2, CD146 (P1H12), or von Willebrand factor (vWF) increased in post-QQc cells versus pre-QQc cells, although CD33/H11001 cells decreased (Fig. 3A). Concomitantly, the different positive ratio of the other hematopoietic lineage marker antigens between pre- and post-QQc cells could be detected (supplemental online Figure 2). T- or B-lymphocytes (CD3 or CD19) or dendritic cells (CD83) were rather predominant in pre-QQc cells, compared with post-QQc cells. On the other hand, the number of monocytes (CD14) or macrophages (CD68) was higher in post-QQc cells than in pre-QQc cells, concurrent with endothelial-specific
markers. Above all, the augmented frequency for VEGFR-2 or vWF was considerably higher in post-QQC cells compared with monocyte/macrophages (CD14) or macrophages (CD68). These findings indicate that QQC predominantly promotes the commitment and differentiation of CD133+ stem cells into EPCs.

Real-time PCR revealed upregulated gene expression of proangiogenic growth factors VEGF (8.3-fold) and hepatocyte

Figure 2. Profile of EPC-CFU production from CB-CD133+ cells in QQC. (A): Fold increase of cell numbers during QQC in six CB samples. The value at each QQC period indicates the fold increase of QQC cells versus CB-CD133+ cells pre-QQC. (B): Frequency of EPC-CFU production per 500 cells at each time point of QQC. (C): Percentage of each EPC-CFU in produced total EPC-CFU. The adjacent values of shaded columns indicate the averaged percentages at each time point. (D): Estimated EPC-CFU numbers produced from an initial 10,000 CB-CD133+ cells. (B–D): *, p < .05; **, p < .01; ***, p < .001. n = 3 dishes per CB sample × 6 CB samples. Abbreviations: CB, umbilical cord blood; CFU, colony-forming unit; DEPC, definitive endothelial progenitor cell; EPC, endothelial progenitor cell; PEPC, primitive endothelial progenitor cell; QQC, quality and quantity culture.
growth factor (HGF) (14.1-fold) in post-QQc cells compared with pre-QQc cells. On the other hand, angiopoietin-1 did not show statistical significance, although it tended to decline. Alternatively, angiopoietin-2 remained at a very low expression level (Fig. 3B). These data indicate that post-QQc cells are more angiogenic cells, which predominantly express VEGF or HGF, compared with pre-QQc cells.

In vitro Matrigel assay revealed that post-QQc cells cocultured with HUVECs predominantly promoted tube formation 12 hours after cell seeding by 2.28-fold over HUVECs alone and 1.45-fold over pre-QQc cells cocultured with HUVECs (Fig. 3C). These findings showed that post-QQc cells preferentially possess the capability of augmenting angiogenesis compared with those pre-QQc cells.

**Figure 3.** Profile of pre- and post-QQc cells. (A): Flow cytometry of pre- and post-QQc cells. n = 4 umbilical cord blood (CB) samples. (B): Relative gene expression ratio of proangiogenic growth factors versus 18S rRNA in pre- or post-QQc cells assessed by quantitative real-time polymerase chain reaction. n = 6 CB samples. (C): Matrigel tube formation assay of HUVECs by coculturing with pre- and post-QQc cells. The top panels show representative features of tube formation by HUVECs in the presence or absence of pre- or post-QQc cells (magnification, x4). The graph indicates the numbers of tube formation in each group. n = 10 wells per group. (B, C): *, p < .05; **, p < .01; ***, p < .001. Abbreviations: Ang, angiopoietin; APC, allophycocyanin; FL, fluorescence; HGF, hepatocyte growth factor; HPF, high power field; HUVEC, human umbilical vein endothelial cell; PE, phycoerythrin; QQc, quality and quantity culture; VEGF, vascular endothelial growth factor.

**Therapeutic Evaluation of Post-QQc Cells Following Cell Transplantation into Infarcted Myocardium**

We transplanted pre- and post-QQc cells into myocardial ischemia animal models in vivo and then evaluated microvascular density (MVD) in the infarcted myocardium assessed by *Griffonia (Bandeiraea) simplicifolia* lectin I (isolectin B4; Vector Laboratories) staining. MVD was significantly greater in rats receiving high
doses of pre-QQc and both doses of post-QQc, compared with phosphate-buffered saline (PBS) (PBS, 501.7/11006 32.2; low dose of pre-QQc, 601.1/11006 21.4; high dose of pre-QQc, 820.6/11006 23.0; low dose of post-QQc, 770.0/11006 15.9; high dose of post-QQc, 889.1/11006 28.9/mm2; \( p < .001 \)). Also, MVD increased dose-dependently in pre- and post-QQc (\( p < .01 \) for pre-QQc and \( p < .05 \) for post-QQc) (Fig. 4A, 4C).

Percentage of left ventricular (LV) fibrosis area assessed by Masson-trichrome staining, in the same manner, was significantly smaller in rats receiving low and high doses of pre- or post-QQc, compared with those receiving PBS (PBS, 29.1/11006 0; low dose of pre-QQc, 16.6/11006 1.0; high dose of pre-QQc, 16.7/11006 1.0; low dose of post-QQc, 19.0/11006 1.4; high dose of post-QQc, 19.8/11006 0.9; \( p < .001 \)) (Fig. 4B, 4C). These results suggest that transplantation of pre- and post-QQc cells may have equivalent potentials for inhibiting LV fibrosis following MI.

**Autocrine and Paracrine Effects of Pre- or Post-QQc Cells for Vasculogenesis and Myocardiogenesis in the Infarcted Myocardium**

Immunohistochemistry with a human-specific marker revealed that the histological density of human ECs was significantly greater in rats receiving low and high doses of pre- or post-QQc cells than in those receiving PBS (PBS, 0/11006 0; low dose of pre-QQc, 76.3/11006 7.5; high dose of pre-QQc, 111.5/11006 13.7; low dose of post-QQc, 148.3/11006 16.0; high dose of post-QQc, 226.3/11006 16.7/mm\(^2\); \( p < .001 \)). Human EC density was significantly greater in the high dose of pre- or post-QQc than in low dose of each cell group, respectively (\( p < .05 \) for pre-QQc and \( p < .01 \) for post-QQc). Furthermore, human EC density was significantly higher in the low and high doses of post-QQc than in the same doses of pre-QQc (\( p < .001 \)). The effect of pre- or post-QQc on rat EC density was similar to that observed on human EC density (Fig. 5A, 5C).

The histological density of human CMCs was greater in the high dose of pre-QQc cells and both the low and high doses of post-QQc cells than the PBS group (PBS, 0/11006 0; low dose of pre-QQc, 17.8/11006 1.9; high dose of pre-QQc, 83.1/11006 11.6; low dose of post-QQc, 32.3/11006 4.1; high dose of post-QQc, 134.0/11006 14.3/mm\(^2\); \( p < .001 \)). Human CMC density was dose-dependently greater in pre- and post-QQc groups (\( p < .001 \)). Human CMC density was also greater in the high dose of post-QQc than in that of pre-QQc (\( p < .01 \)). The effect of CB-CD133\(^+\) cells pre- or post-QQc on rat CMC density was almost the same as that on human CMC density. (Fig. 5B, 5C).

These data demonstrate dose-dependent autocrine and paracrine effects of pre- and post-QQc from CB-CD133\(^+\) cells on vasculogenesis and cardiomyogenesis. The potency for cardiovascular regeneration seems superior in post-QQc cells over pre-QQc.
Preservation of LV Function Post-MI by Transplantation of CB-CD133 \(^*/\) Cells Pre- or Post-QQc

The invasive hemodynamic assessment of LV function in each group showed that positive pressure and its derivative (\(dP/dt\)) were significantly greater in all cell therapy groups than in the PBS group (\(p < .001\)). \(dP/dt\) was significantly greater in the high dose of pre- or post-QQc than in the low dose of each group (\(p < .001\)). \(dP/dt\) was similar in the low dose of pre- or post-QQc group and the high dose of each group. \(-dP/dt\) was significantly smaller (better preserved) in all cell therapy groups except the low dose of pre-QQc cells than in the PBS group (\(p < .001\) for high dose of pre- and post-QQc vs. PBS; \(p < .01\) for low dose of post-QQc vs. PBS). \(-dP/dt\) was significantly smaller in the high dose of post-QQc than in the low dose of post-QQc group (\(p < .001\)). \(-dP/dt\) was significantly better preserved in the high or low dose of post-QQc groups than in the same dose of pre-QQc groups (\(p < .01\) for high-dose and \(p < .05\) for low-dose groups) (Fig. 6A). Echocardiography performed 4 weeks after cell therapy demonstrated that fractional shortening (FS) was greater in all cell therapy groups than in the PBS group (\(p < .001\)). FS was greater in the high dose of pre- or post-QQc cells than in the low dose of each group (\(p < .001\)). FS was also higher in the low or high dose of post-QQc group than in the same dose of pre-QQc (\(p < .05\) for low dose and \(p < .01\) for high dose) (Fig. 6B).

These results suggest that transplantation of CB-CD133 \(^*/\) cells either pre- or post-QQc may be beneficial for preservation of LV function post-MI. The present data also show a dose-dependent effect of each cell therapy and the superior potency of post-QQc cells over pre-QQc cells for functional...
recovery. The echocardiographic data of LV function post-MI by transplantation of pre- or post-QQc cells are shown in supplemental online Table 1 and supplemental online Figure 3.

**DISCUSSION**

In the present study, we disclosed optimal serum-free QQc for quantitative and qualitative control of colony-forming EPCs by recently defined EPC-CFA. The optimal QQc system augmented the potential of CB-CD133/H11001 cells not only by specific augmentation of provasculogenic colony-forming EPCs producing DEPC-CFUs but also by upregulating autocrine or paracrine effects of proangiogenic growth factors in produced cells. Furthermore, in vivo experiments using a rat model of MI showed predominant cardiac repair postintramyocardial transplantation of post-QQc cells compared with CB-CD133/H11001 cells as pre-QQc cells.

**Clinical Significance of the Serum-Free Quality and Quantity System Optimized in This Study**

In the hematopoietic field, serum-free expansion cultures for hematopoietic stem cells (i.e., CD133+ cells or CD34+ cells in CB) have so far been developed to reconstitute hematopoiesis following BM ablation by chemotherapy for malignancies [38, 39]. On the basis of these ex vivo hematopoietic expansion cultures, the development of EPC expansion culture has also been attempted, because EPCs share common surface markers, such as CD34 and CD133, with hematopoietic stem cells. First of all, Pesce et al. [5] expanded CB-CD34+ cells for 7 days using a serum-free cytokine cocktail of SCF, Flt-3 ligand, interleukin-3 (IL-3), and IL-6, which was also used by Bonanno et al. [40], included as Comb 1 (Table 1), and successfully increased the number of CB-CD34+ cells by 10-fold. Although the arteriole length density in ischemic adductor muscles was significantly greater following transplantation of CB-CD34+ cells postexpansion compared with saline injection, the angiogenic parameter was similar in the CB-CD34+ cell pre- and postexpansion groups. Skeletal myogenesis was also less enhanced in the CB-CD34+ cell postexpansion group than in the pre-expansion group [5]. Other groups have also developed growth factor cocktails to test EPC expansions from CB-CD34+ or CD133+ cell populations and demonstrated significant increases in total cell number and EPC marker-positive cell number [25, 41]. All studies demonstrated the efficacy of transplantation into myocardial ischemic models by the equivalent or better improvement of cardiac function or vascular incorporation compared with pre-expansion EPCs.

However, there was a crucial limitation to establish the scientific and clinical culture system by conventional EPC biological methodologies. EPC origin and differentiation biology have remained a major concern among researchers for years, as there is still no definitive concept and methodology for EPCs. In this regard, researchers have long resisted the lack of qualitative and quantitative measures of regenerative EPCs to establish the culture for EPCs.

Here we used the newly established methodology of EPC-CFA to develop the culture system to increase in number and function of colony-forming EPCs for vascular regeneration. We have recently demonstrated EPC-CFA using semisolid medium and single or bulk CD133+ or CD34+ cord blood cell exhibited the formation of two types of attaching cell colonies made of small or large cells featuring endothelial lineage potential and properties, termed small-cell EPC-CFU and large-cell EPC-CFU, respectively [37]. In vitro and in vivo assays of colony cells of each EPC-CFU revealed a differentiation hierarchy ranging from PEPC-CFU, with highly proliferative activity, to DEPC-CFU, with vasculogenic properties, respectively. In particular, the transplantation of each colony cell represented not only the therapeutic potential of both EPC-CFUs for neovascular formation in ischemic animals.

**Figure 6.** Improvement of left ventricular (LV) function following transplantation of pre- or post-QQc cells. (A): Hemodynamic assessment of LV function by micromanometer-tipped conductance catheter. (B): Quantitative assessment of LV function by echocardiography. *, p < .05; **, p < .01; ***, p < .001. †, p < .01 versus PBS; ‡, p < .001 versus PBS. n = 10 rats per group. Abbreviations: FS, fractional shortening; Hi, high dose in pre- or post-QQc group; Lo, low dose in pre- or post-QQc group; PBS, phosphate-buffered saline; QQc, quality and quantity culture.
but the priority of the regenerative property of DEPC-CFU compared with that of PEPC-CFU. Therefore, numerical evaluation of both EPC-CFUs generated from any stem cell sources permits estimation of their vasculogenic property. In the present study, we have tried to optimize growth factor and cytokine combinations for colony-forming EPC expansion, to detect the best combination to especially increase the frequency of DEPC-CFU and total number of both EPC-CFUs in culture.

In our recent report, analysis of EPC-CFA revealed that VEGF added to the combination of early acting growth factors (SCF, TPO, Flt-3 ligand, IL-6) [42] for hematopoietic stem cell expansion promotes the EPC commitment and differentiation of single CB-CD133+ cells [43]. Given this finding, we investigated the productivities of EPC-CFU in the several combinations, including the previously reported ones [5, 40], in comparison with the combination (VEGF, SCF, TPO, Flt-3 ligand, IL-6) named Comb 4. For example, Comb 6 (SCF, TPO, Flt-3 ligand, IL-3, IL-6, VEGF) represented the most increase in cell number after culture but resulted in a significantly lower frequency of DEPC-CFU, whereas Comb 4 demonstrated the highest total EPC-CFU frequency and, in particular, the highest DEPC-CFU frequency postculture among the groups (Fig. 1B, 1C). Of interest, Comb 4 exhibited greater EPC-CFU productivity, especially in DEPC-CFU, compared with the previously reported Comb 1 (SCF, Flt-3 ligand, IL-3, IL-6) for EPC expansion [5, 40]. Collectively, we determined that the Comb 4 growth factors are the most effective culture system for vasculogenic colony-forming EPC expansion, and we named them the QQc control system.

On the other hand, differing from a conventional culture of total mononuclear cells for adhesive EPC expansion [29, 43], the present QQc enabled suspended EPCs to robustly expand even from a scarce stem cell fraction (CB-CD133+) under serum-free conditions. The lack of requirement for either serum, with its possible risk of viral/bacterial infection, or a cellular detachment procedure using chemical substances (e.g., trypsinization) would support the safety of stem cell therapy in clinical applications.

Enhanced Vasculogenic Potential of Post-QQc Cells over Pre-QQc

The specific increase in DEPC-CFUs in EPC-CFA and enhanced endothelial expressions, such as VEGFR-2, CD146, and vWF, was demonstrated in CB-CD133+ cells post-QQc compared with those pre-QQc. These findings indicate that the QQc system may augment differentiation of CB-CD133+ cells into the EC lineage. On the other hand, the capacity of tube-like structure formation with HUVECs and sprouting capability were also enhanced in post-QQc cells, compared with pre-QQc (supplemental online Fig. 4A, 4B). Moreover, post-QQc cells exhibited the predominant potential not only of incorporation into the tubes (supplemental online Fig. 4C) but also of proliferation (supplemental online Fig. 4D). Production of critical proangiogenic growth factors, such as VEGF or HGF, was also enhanced in post-QQc cells versus pre-QQc.

These data indicate another mechanism underlying the enhanced vasculogenic potential of post-QQc cells: upregulation of proangiogenic cytokine secretion for autocrine and paracrine actions, besides the performance of post-QQc cells per se. Given this evidence, the QQc system may possess not only quantitative but also qualitative advantages in acquiring an optimal EPC resource for therapeutic applications.

Conclusions

The present study demonstrates that our novel evaluation method for serum-free expansion provides a transplantable EPC...
source with quantitative and qualitative advantages for cardiovascular regeneration in ischemic disease.

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**AUTHOR CONTRIBUTIONS**

H.M.: conception and design, collection and assembly of data, data analysis and interpretation, financial support, manuscript writing; H. Iwasaki: collection and assembly of data, data analysis and interpretation; A.K.: assembly of data, data analysis, interpretation, manuscript writing; H.A.: collection and assembly of data, data analysis, interpretation; M. Ishikawa, M. Ii, T.S., A.S., R.I., M.H., and H. Ishida: assembly of data, data analysis and interpretation; S.K.: providing cord blood, financial support; T.A.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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