

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Transdifferentiation of Blood-Derived Human Adult Endothelial Progenitor Cells Into Functionally Active Cardiomyocytes

Cornel Badorff, Ralf P. Brandes, Rüdiger Popp, Stefan Rupp, Carmen Urbich, Alexandra Aicher, Ingrid Fleming, Rudi Busse, Andreas M. Zeiher and Stefanie Dimmeler

Circulation published online Feb 3, 2003;

DOI: 10.1161/01.CIR.0000051460.85800.BB

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 72514

Copyright © 2003 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circ.ahajournals.org>

Subscriptions: Information about subscribing to *Circulation* is online at
<http://circ.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Transdifferentiation of Blood-Derived Human Adult Endothelial Progenitor Cells Into Functionally Active Cardiomyocytes

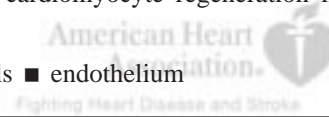
Cornel Badorff, MD; Ralf P. Brandes, MD; Rüdiger Popp, PhD; Stefan Rupp, MD; Carmen Urbich, PhD; Alexandra Aicher, MD; Ingrid Fleming, MD; Rudi Busse, PhD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Further to promoting angiogenesis, cell therapy may be an approach for cardiac regeneration. Recent studies suggest that progenitor cells can transdifferentiate into other lineages. However, the transdifferentiation potential of endothelial progenitor cells (EPCs) is unknown.

Methods and Results—EPCs were obtained from peripheral blood mononuclear cells of healthy adults or coronary artery disease (CAD) patients by cultivating with endothelial cell medium and growth factors. After 3 days, >95% of adherent cells were functionally and phenotypically EPCs. Diacetylated LDL-labeled EPCs were then cocultivated with rat cardiomyocytes for 6 days, resulting in significant increases of EPC cell length and size to a cardiomyocyte-like morphology. Biochemically, $9.94 \pm 1.39\%$ and $5.04 \pm 1.09\%$ of EPCs from healthy adults ($n=15$) or CAD patients ($n=14$, $P<0.01$ versus healthy adults), respectively, expressed α -sarcomeric actinin as measured by flow cytometry. Immunocytochemistry showed that human EPCs expressed α -sarcomeric actinin, cardiac troponin I (both with partial sarcomeric organization), atrial natriuretic peptide, and myocyte enhancer factor 2. Fluo 4 imaging demonstrated calcium transients synchronized with adjacent rat cardiomyocytes in transdifferentiated human EPCs. Single-cell microinjection of Lucifer yellow and calcein-AM labeling of cardiomyocytes demonstrated gap junctional communication between $51 \pm 7\%$ of EPCs (16 hours after labeling, $n=4$) and cardiomyocytes. EPC transdifferentiation into cardiomyocytes was not observed with conditioned medium but in coculture with paraformaldehyde-fixed cardiomyocytes.

Conclusions—EPCs from healthy volunteers and CAD patients can transdifferentiate in vitro into functionally active cardiomyocytes when cocultivated with rat cardiomyocytes. Cell-to-cell contact but not cellular fusion mediates EPC transdifferentiation. The therapeutic use of autologous EPCs may aid cardiomyocyte regeneration in patients with ischemic heart disease. (*Circulation*. 2003;107:1024-1032.)

Key Words: myocytes ■ physiology ■ cells ■ endothelium



A number of cardiovascular diseases, such as myocardial infarction, lead to cardiomyocyte loss and consequently deterioration of cardiac function. Because cardiomyocytes have a severely limited capacity to divide and thus replace damaged tissue, the use of a cardiovascular cell therapy is a promising option to regenerate cardiac tissue. Furthermore, the transplantation of healthy stem cells may correct cellular dysfunction due to mutated genes.¹ Different types of stem or progenitor cells have been shown to improve cardiac function in animal models of myocardial infarction. Human hematopoietic CD34⁺ progenitor cells or murine c-kit⁺/sca-1⁺ bone marrow cells have been used with similar beneficial effects.^{2,3} In addition, endothelial progenitor cells (EPCs) injected systemically have been reported to increase the neovascularization of ischemic tissue.⁴⁻⁶

Bone marrow-derived EPCs in the peripheral blood were first described by Asahara et al⁷ in 1997. These cells can be expanded ex vivo from mononuclear cells^{4,6,8,9} or obtained by culturing CD34⁺ or CD133⁺ hematopoietic progenitor cells.^{7,10-12} The differentiation of EPCs out of mononuclear cells or hematopoietic progenitor cells was promoted by the addition of endothelial growth factors, such as vascular endothelial growth factor.^{7,11} EPCs are characterized by their expression of the vascular endothelial growth factor receptor 2 (KDR), a marker for the angioblast lineage, and further endothelial marker proteins (such as von Willebrand factor, vascular endothelial cadherin, and endothelial nitric oxide synthase), uptake of diacetylated LDL (DiI-acLDL), and lectin binding.^{7,11,13,14} Blood-derived angioblasts or EPCs expanded ex vivo have been reported to integrate into blood vessels and improve neovascularization of ischemic hind

Received September 9, 2002; revision received November 11, 2002; accepted November 11, 2002.

From Molecular Cardiology, Department of Internal Medicine IV (C.B., S.R., C.U., A.A., A.M.Z., S.D.), and the Institute for Cardiovascular Physiology (R.P.B., R.P., I.F., R.B), University of Frankfurt, Frankfurt, Germany.

Correspondence to Stefanie Dimmeler, Department of Molecular Cardiology, University of Frankfurt, Theodor Stern-Kai 7, 60590 Frankfurt, Germany. E-mail dimmeler@em.uni-frankfurt.de

© 2003 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000051460.85800.BB

limbs and hearts in animals.^{4,6,8,15} The improvement of cardiac function by EPC transplantation was attributed to their angiogenic potential. So far, the capacity of adult EPCs to transdifferentiate into cardiomyocytes is unknown.

A recent study demonstrated that murine embryonic endothelial cells can transdifferentiate into cardiac myocytes *in vitro* and *in vivo*.¹⁶ Likewise, mouse and human embryonic stem cells or human mesenchymal stromal cells can differentiate into cardiac myocytes, which display structural and functional activity.^{17–19} However, the use of allogenic embryonic stem cells in patients raises substantial concerns. Therefore, we investigated the potential of adult circulating EPCs to transdifferentiate into cardiac myocytes.

The results of the present study demonstrate that human EPCs isolated from the peripheral blood of adults are capable of transdifferentiating into cardiac myocytes when cocultured with neonatal rat cardiomyocytes. This transdifferentiation was evidenced by phenotypic and functional parameters. Moreover, intercellular gap junctional coupling between rat cardiomyocytes and human EPCs was demonstrated.

Methods

Patients and Control Subjects

Healthy adult volunteers had neither a history of coronary artery disease (CAD) nor any clinical signs or symptoms of myocardial ischemia.

All CAD patients had angiographically documented stenoses of $\geq 50\%$ in any coronary vessel. None of the CAD patients had a myocardial infarction within 7 days before blood sampling. Patients with significant comorbidity (infectious diseases, immunosuppressive therapy, cancer, hemodialysis) were excluded. All of the CAD patients were on standard medical therapy except for an HMG-CoA reductase inhibitor. A risk factor score (including diabetes mellitus, smoking, arterial hypertension, hypercholesterolemia, and familial disposition) was calculated as described previously.⁹

Informed consent was obtained from all subjects before blood sampling. The study protocol was approved by the local ethics committee of the University of Frankfurt.⁹

Cell Culture Experiments

Neonatal ventricular cardiomyocytes were isolated from 1- to 2-day-old Sprague-Dawley rats as described previously.²⁰ Noncardiomyocytes (primarily cardiac fibroblasts) were separated from the cardiomyocytes by differential plating onto plastic dishes. During this step, 2 $\mu\text{g}/\text{mL}$ mitomycin C (Sigma) was added. Cardiomyocytes were plated at a density of $1.2 \times 10^5/\text{cm}^2$ on gelatin-coated wells. Cardiac fibroblasts (first passage) were plated at a density of $10^5/\text{cm}^2$ for the experiments. For immunocytochemistry, cells were plated onto chamber slides.

EPCs were isolated from the peripheral blood as described previously.¹¹ After 3 days in culture, adherent EPCs were labeled with 2.5 $\mu\text{g}/\text{mL}$ Dil-acLDL (Cell Systems) for 60 minutes at 37°C followed by 3 washes with PBS. The nontransferable dye Dil-acLDL was used to allow live monitoring of the human EPCs during cocultivation. EPCs were then mixed with freshly isolated cardiomyocytes at a ratio of 1:4 (10% horse serum, 5% FCS, Invitrogen). After 2 days, the medium was changed to 5% horse serum and renewed every 2 days.

Adult human CD34⁺ cells were mobilized to and isolated from the peripheral blood as described previously.¹¹ CD34⁺ cells were cocultured with rat cardiomyocytes as above.

For some experiments, cardiomyocytes were fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. After 3 washes with PBS, EPCs were added.

Cell Size Measurements and Immunofluorescence Staining

Cells were stained for cardiac troponin I myocyte enhancer factor 2 (MEF-2) using an antibody that is broadly reactive with MEF-2 family members including MEF-2c (both Santa Cruz), pancadherin, α -sarcomeric actinin (both Sigma), or atrial natriuretic peptide (Peninsula Laboratories), followed a biotinylated secondary antibody and streptavidin-FITC (both Vector Laboratories). Human cells were identified with a phycoerythrin-conjugated anti-human HLA-DR antibody (Caltag). Nuclei were stained by DAPI (Sigma).²⁰

Cells were imaged and maximal cell length and surface area were calculated by use of an Axiovert 100 inverted microscope equipped with an AxioCam and AxioVision software (all Zeiss).

Detection of Transdifferentiation by Fluorescence-Activated Cell Sorting

After 6 days in coculture, human EPCs and rat cardiomyocytes were labeled with a phycoerythrin-conjugated human-specific anti-HLA-DR antibody (Caltag), permeabilized by use of the Cytofix/Cytoperm kit (BD Pharmingen), and stained with a FITC-conjugated (Pierce) anti- α -sarcomeric actinin antibody (clone EA-53, Sigma). Twenty thousand cells were analyzed on a FACScalibur cell sorter (BD Biosciences).

Calcium Transient Measurements

Cardiomyocytes and EPCs were cocultured on glass coverslips. After 6 days, cells were washed with phosphate-free Tyrode's solution (in mmol/L: NaCl 132, KCl 4, CaCl₂ 1.6, MgCl₂ 1, NaHCO₃ 12, and glucose 10, at a pH of 7.4, at 37°C and 5% CO₂) and incubated at 37°C (5% CO₂) with 5 $\mu\text{mol}/\text{L}$ Fluo 4-AM (Molecular Probes). After 30 minutes, cells were transferred to a heated microscope chamber superfused with HEPES-modified Tyrode's solution (in mmol/L: NaCl 140, KCl 4.7, CaCl₂ 1.3, MgCl₂ 1, HEPES 10, and glucose 5, at a pH of 7.4, at 37°C, in room air). Two platinum electrodes connected to a Grass stimulator (Grass Instruments) were used for electrical stimulation (1.64 Hz, 5 ms, 5 V). Images were obtained with a FITC filter set (excitation 488 nm, emission 512 nm) and a CCD camera (Orca-II, Hamatsu Instruments) connected to an imaging system (Openlab, Improvision). Calcium transients were averaged by time-matched superimposition of individual frames.

Dye Transfer Experiments

Cardiomyocytes were labeled with calcein-AM (2.5 $\mu\text{mol}/\text{L}$; Calbiochem) for 60 minutes at 37°C. EPCs were labeled separately with Dil-acLDL and added to the cardiomyocytes at a ratio of 1:4. Cells were then cocultured for the times indicated. Dye transfer was measured by flow cytometry in native, unfixed cells by the transfer of calcein to Dil-acLDL-labeled EPCs.

Single-Cell Microinjection

Sharp microelectrodes were loaded with the fluorescent tracer Lucifer yellow (4% in 100 mmol/L lithium chloride). Glass coverslips containing cocultured EPCs and rat cardiomyocytes were superfused with modified Tyrode's solution (in mmol/L: NaCl 132, KCl 4, CaCl₂ 1.6, MgCl₂ 0.98, NaHCO₃ 20, NaH₂PO₄ 0.36, glucose 10, and Ca²⁺-EDTA 0.05). Cells were impaled using a micromanipulator (5171, Eppendorf). Dye was injected iontophoretically. After 60 seconds, fluorescence was recorded with a fluorescein filter set (excitation 488, emission 512) and a CCD camera (Zeiss Attoflor Ratio Vision) to assess dye coupling.

Statistical Analysis

Data are expressed as mean \pm SEM. An unpaired, 2-tailed *t* test was used for the comparison of continuous variables. ANOVA was used for multiple comparisons. The risk factor score was considered to be an ordinal variable, and the Spearman rank correlation coefficient was calculated. For comparison of nominal variables, the Mann-Whitney test was used.

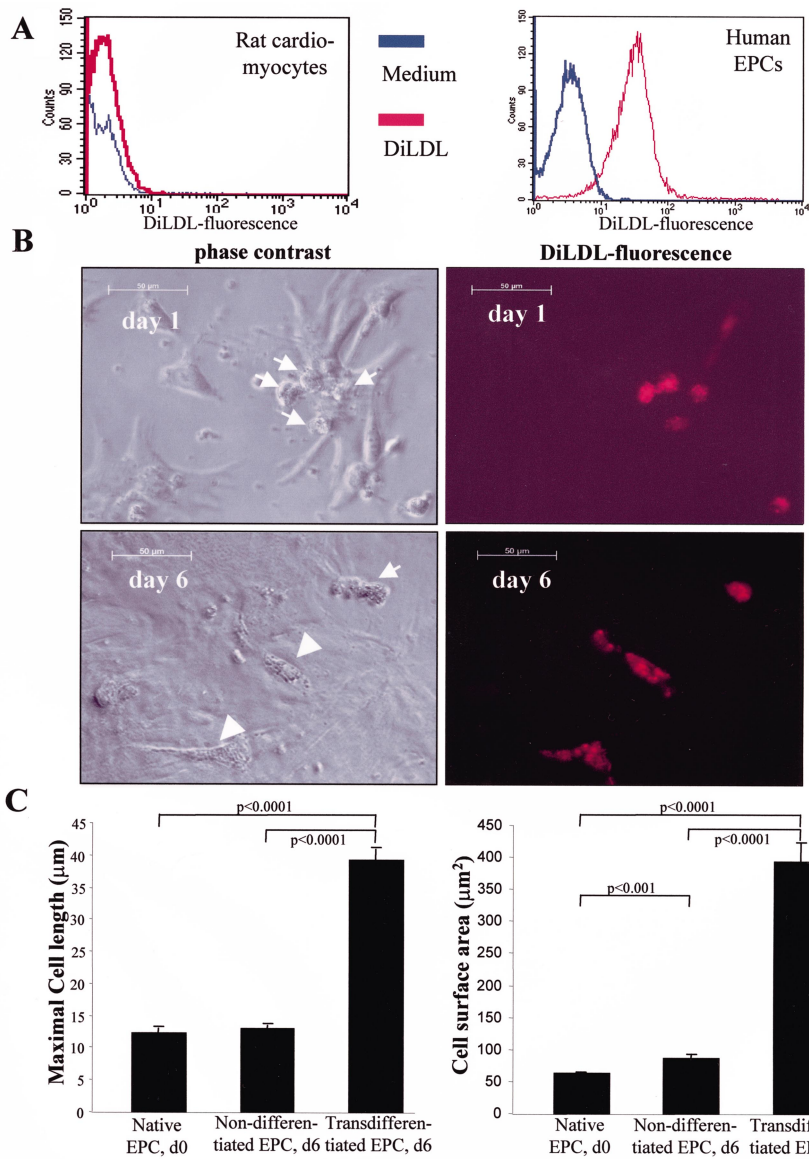


Figure 1. Morphology of human EPCs cocultured with rat cardiomyocytes. A, Rat cardiomyocytes (left) or human EPCs (right) were incubated with Dil-acLDL (red lines) or medium only (blue lines) followed by flow cytometry analysis. B, Top: Morphology after 1 day of coculture. Phase contrast (left) and Dil-acLDL fluorescence (right) of same field; magnification $\times 400$. Arrows indicate EPCs. Bottom: Morphology after 6 days of coculture. Arrowheads indicate transdifferentiated EPCs; arrow indicates an EPC without altered morphology. C, Quantification of cell length (left) and surface area (right) of native EPCs, non-transdifferentiated EPCs, and transdifferentiated EPCs (both after 6 days of coculture). Shown are mean \pm SEM of $n=12$ cells from a representative experiment per group.

Results

Human Blood-Derived EPCs Can Transdifferentiate Into Cardiomyocytes

We reported previously that human EPCs can be generated from peripheral blood mononuclear cells.^{9,11,14} After 3 days, >95% of the adherent human EPCs expressed several endothelial markers (data not shown; see References 9, 11, and 14).

For the coculture experiments, vital EPCs were separately labeled by the fluorescent tracer Dil-acLDL before the coculture. Dil-acLDL is specifically taken up by EPCs but not by cardiomyocytes (Figure 1A). Once internalized, Dil-acLDL becomes membrane-bound and cannot be transferred to adjacent cells in culture. Thus, Dil-acLDL labeling allows EPC detection in living cells. Importantly, Dil-acLDL is retained within EPCs for at least 14 days in culture (data not shown).

The potential of human EPCs to transdifferentiate into cardiomyocytes was assessed in vitro using a coculture of human EPCs with rat neonatal cardiomyocytes. Initially, EPCs from healthy adult volunteers were used.

Within 12 hours of coculture, Dil-acLDL-labeled human EPCs adhered to cardiomyocytes (Figure 1B). After 6 days of coculture, many human EPCs had integrated with the rat cardiomyocytes (Figure 1B). Compared with nonintegrated EPCs, integrated EPCs displayed significant increases of their cell length and surface area, similar to adjacent cardiomyocytes (Figure 1C).

After 6 days of coculture, several Dil-acLDL-positive and human HLA-positive EPCs expressed cardiac-specific proteins such as cardiac troponin I (data not shown) and atrial natriuretic peptide and myocyte-specific proteins such as α -sarcomeric actinin and MEF-2 (Figure 2). Furthermore, sarcomeric organization was observed in some human EPCs (Figure 2B). In contrast, under basal conditions, EPCs did not express myogenic marker proteins (Figure 3, A and E, and data not shown).

Transdifferentiation of EPCs was quantified by flow cytometry analysis (Figure 3). EPCs were identified by a human-specific HLA-DR antibody (red channel, EPC marker). (Cardio)myocytes were identified by their expression of

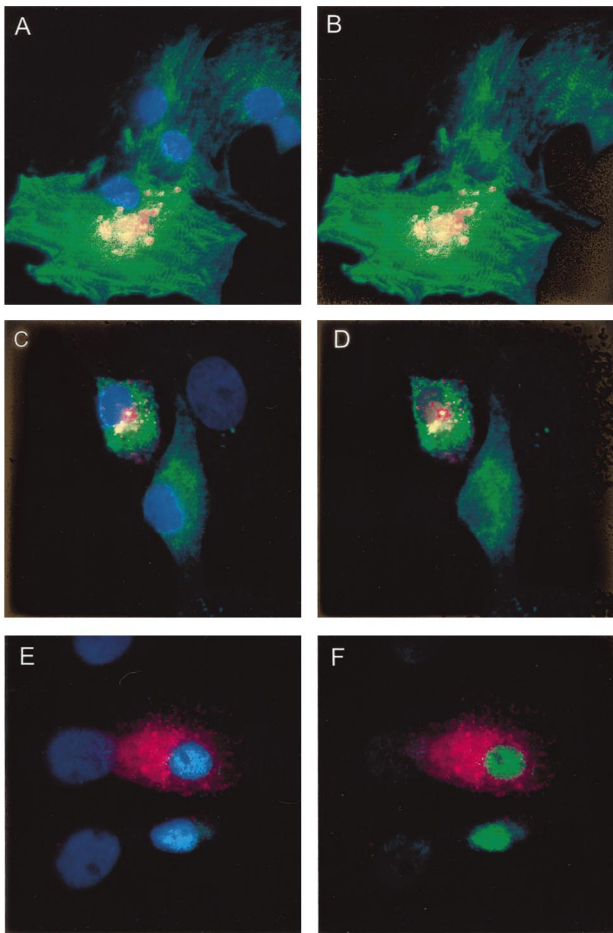


Figure 2. Expression of cardiomyocyte proteins and sarcomeric organization in human EPCs cocultured with rat neonatal cardiomyocytes. Triple labeling of EPCs after 6 days in coculture with rat cardiomyocytes. Nuclei (DAPI) are blue (A, C, E), human EPCs (Dil-acLDL and human HLA) are red, and cardiomyocyte markers are green; magnification $\times 400$. A and B, α -Sarcomeric actinin (green); C and D, atrial natriuretic peptide (green); E and F, myocyte enhancer factor-2 (nuclear expression, green). Cells without staining of cardiac marker proteins (blue nuclei, C and D) represent rat fibroblasts.

α -sarcomeric actinin, a classic myogenic marker (FITC-labeled, green channel). Initially, no double-positive cells were detected (Figure 3A). In contrast, after 6 days of coculture, $9.94 \pm 1.39\%$ of the human cells expressed α -sarcomeric actinin (Figure 3, B and E). Human CD34⁺ hematopoietic progenitor cells also differentiated into actinin-positive cells with a comparable efficiency (Figure 3, C and D).

Importantly, no transdifferentiation of EPCs was observed when they were cultured with conditioned medium from rat cardiomyocytes (Figure 3E).

Calcium Transients in Transdifferentiated EPCs

To obtain proof of cardiomyocyte-like function after transdifferentiation, we measured calcium transients in living Dil-acLDL-labeled human EPCs (red fluorescence) using the calcium indicator fluo-4 (green fluorescence). Transdifferentiated EPCs displayed periodic oscillations in calcium similar to and synchronized with those in adjacent rat cardiomyo-

cytes (Figure 4). Time-matched averaging of individual calcium signals revealed that human EPCs and rat cardiomyocytes generated calcium transients of similar amplitude and duration (Figure 4D). Only transdifferentiated but not nondifferentiated human EPCs exhibited calcium transients similar to rat cardiomyocytes (Figure 4, E and F).

Gap Junctional Communication Between Human EPCs and Rat Cardiomyocytes

Next, we investigated whether human EPCs can physically interact with cardiac myocytes. Microinjection of the fluorescent dye Lucifer yellow into rat cardiomyocytes revealed coupling as determined by direct dye transfer to adjacent EPCs (Figure 5, E and F). Similarly, coupling between Dil-acLDL-labeled EPCs and rat cardiomyocytes labeled with the gap junction-permeable fluorescent dye calcein (green fluorescence) was observed (Figure 5, A through D). A time-dependent calcein transfer from the cardiomyocytes to the EPCs resulted in a significant increase in calcein-positive Dil-acLDL-labeled EPCs (Figure 5B). Because of the colocalization of red and green fluorescence, these cells appear yellow after superimposition of the fluorescent images (Figure 5B). Quantification by flow cytometry demonstrated that $\approx 60\%$ of the Dil-acLDL-labeled EPCs were positive for calcein after 24 hours of coculture (Figure 5, C and D). Interestingly, such dye transfer was not observed when rat cardiac fibroblasts were labeled with calcein and cocultured with EPCs (Figure 5D). Addition of the gap junction inhibitor phorbol 12-myristate 13-acetate (PMA) (50 nmol/L) significantly reduced the dye transfer, suggesting that cardiomyocytes are linked to EPCs by gap junctions (Figure 5B). Similarly, heptanol (1 mmol/L), another gap junction blocker, partially prevented calcein dye transfer (data not shown).

These data indicate that human EPCs are physically connected to cardiac myocytes by intercellular gap junctions.

Cell-to-Cell Contact but Not Intercellular Fusion Is Required for Transdifferentiation

Having demonstrated that EPCs and cardiomyocytes are physically connected, allowing dye transfer between these 2 distinct cell types, we investigated whether gap junctional exchange of low-molecular-weight molecules is required for transdifferentiation. Therefore, we incubated the coculture of EPCs and cardiac myocytes with the gap junction inhibitor PMA (50 nmol/L) and determined the transdifferentiation of EPCs into cardiac myocytes by fluorescence-activated cell sorting analysis. However, the addition of PMA did not inhibit transdifferentiation of EPCs ($124.9 \pm 18.3\%$ actinin-positive human cells compared with the control without PMA; $n=3$; $P=NS$).

Recent studies reported that phenotypic changes of embryonic stem cells may occur as a consequence of cell fusion.²¹ To investigate whether this mechanism underlies EPC transdifferentiation, we used paraformaldehyde-fixed cardiomyocytes, which cannot fuse with other cells but have an intact cell surface for coculture. After 6 days of coculture with fixed cardiac myocytes, EPCs displayed increases in cell length and surface area (data not shown). Moreover, after coculture with fixed cardiac myocytes, several EPCs revealed expression of

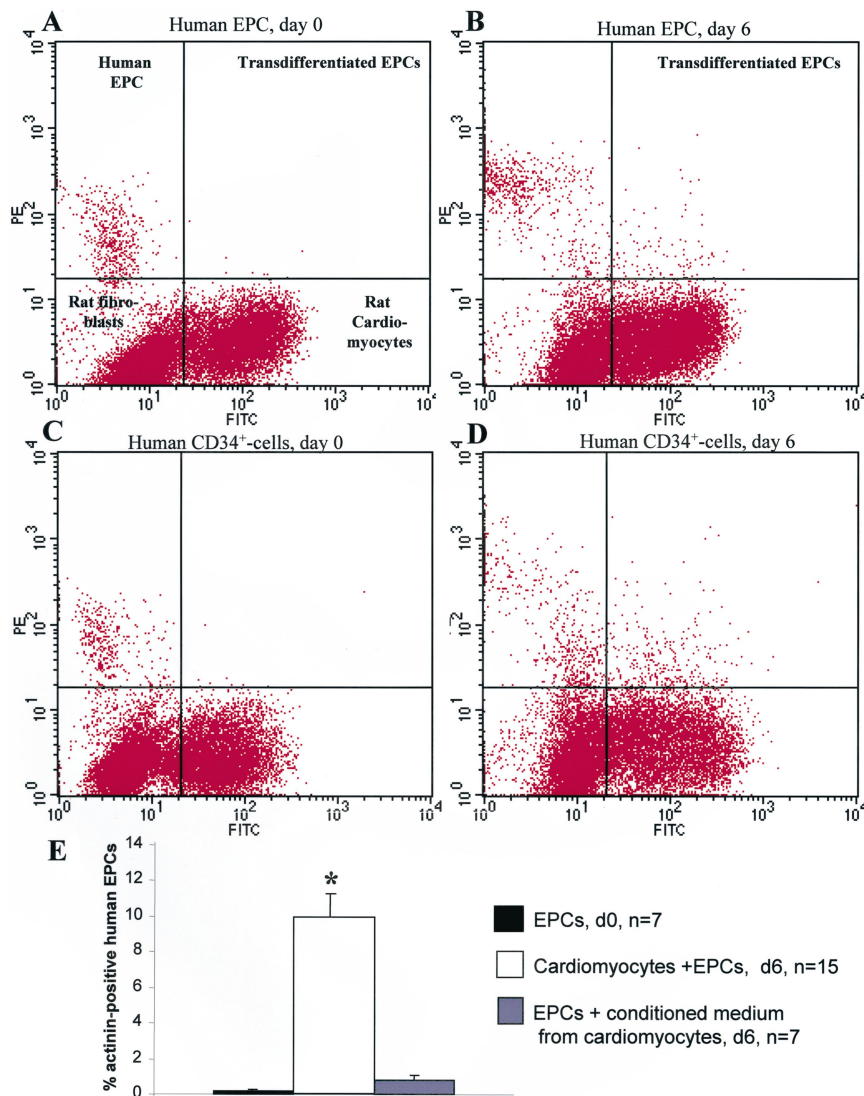


Figure 3. Quantification of EPC transdifferentiation into cardiomyocytes. A and B, Representative flow cytometry analyses of human EPCs after mixing with rat cardiomyocytes (A) or after 6 days of coculture with rat cardiomyocytes (B). Cells were stained with an anti- α -sarcomeric actinin antibody (green fluorescence) to identify myocytes; human cells are identified with a human-specific anti-HLA-DR antibody (red fluorescence). EPCs are in upper left, rat cardiac fibroblasts in lower left, and rat cardiomyocytes in lower right quadrant. Transdifferentiated EPCs expressing α -sarcomeric actinin are in upper right quadrant in B. C and D, Representative flow cytometry analysis of human CD34⁺ cells after mixing with rat cardiomyocytes (C) or after 6 days of coculture with rat cardiomyocytes (D). Cells were stained as above. E, Quantitative group data. Percentage of EPCs expressing α -sarcomeric actinin at baseline (black bar), after 6 days of coculture with rat cardiomyocytes (white bar), or after 6 days of culture with medium conditioned from rat cardiomyocytes (gray bar). Values are mean \pm SEM. * $P < 0.01$ vs baseline.

American Heart Association
 Bringing Heart Disease and Stroke

cardiac-specific proteins. As shown in Figure 6A, human EPCs (anti-HLA staining; red fluorescence) showed the expression of α -sarcomeric actinin (blue fluorescence) and nuclear MEF-2 staining (green fluorescence).

Cadherins mediate calcium-dependent cell-to-cell contact and regulate diverse signaling processes.²² To elucidate a potential role of calcium-dependent cell-to-cell contact, EPCs were incubated with the calcium chelator EDTA or EGTA. EDTA and EGTA abolished the adhesion of EPCs to fixed cardiomyocytes (Figure 6B). Moreover, cadherin staining was detected at the interface between cardiomyocytes and EPCs (Figure 6C). Taken together, these data suggest that calcium-dependent cell-to-cell communication, but not intercellular fusion or gap junctions are essential for EPC transdifferentiation.

EPCs From CAD Patients Can Also Transdifferentiate Into Cardiomyocytes

Patients with CAD and risk factors for atherosclerosis have a significantly reduced number of EPCs compared with healthy control subjects without cardiovascular risk factors. In addition,

EPCs from CAD patients are functionally impaired, as evidenced by a significantly reduced migratory activity in cell culture.⁹ Because any EPC cell therapy would clinically target CAD patients, we investigated whether EPCs isolated from the peripheral blood of CAD patients are also capable of transdifferentiating into cardiomyocytes.

Therefore, we cocultured EPCs from 14 CAD patients with rat cardiomyocytes. Morphologically, EPCs from CAD patients integrated with the rat cardiomyocytes similarly to the EPCs from healthy volunteers (data not shown). After 6 days of coculture, the percentage of actinin-positive, transdifferentiated human EPCs was quantified by flow cytometry, and the results were compared with the data obtained from 15 age- and sex-matched healthy control subjects without any evidence of CAD. The prevalence of cardiovascular risk factors tended to be higher among CAD patients, but only the higher prevalence of hypercholesterolemia reached statistical significance (Figure 6A).

The survival of human EPCs in the coculture did not differ between groups (data not shown). As shown in Figure 3, $\approx 10\%$ of EPCs from healthy adult volunteers expressed

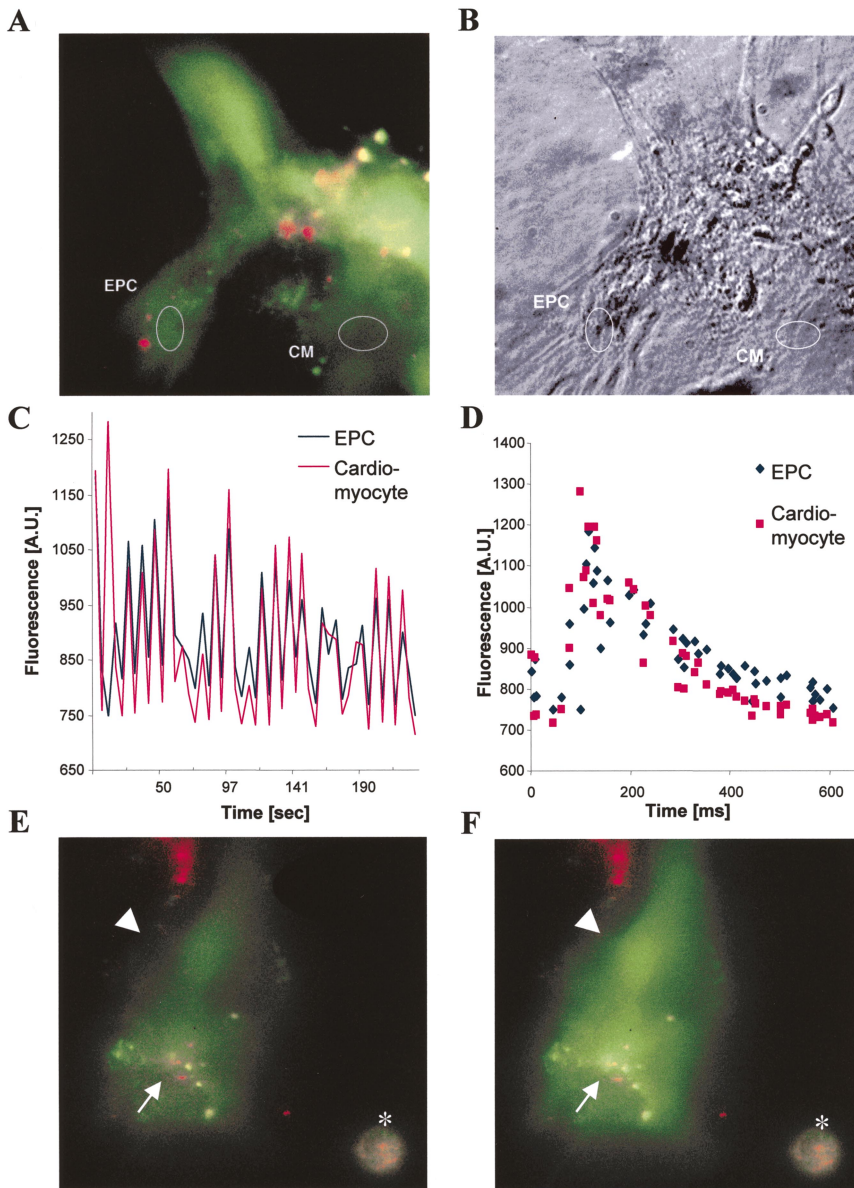
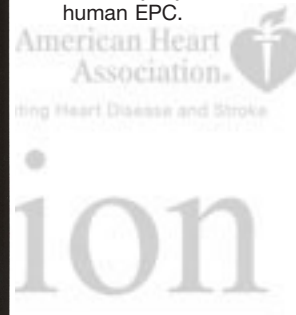


Figure 4. Calcium transients in transdifferentiated human EPCs. A, Human EPCs and rat cardiomyocytes (CM) were labeled with fluo 4 (green fluorescence) after 6 days of coculture. EPCs are identified via their Dil-acLDL labeling (red fluorescence). B, Phase-contrast image from same field as in A. Note integrated and cardiomyocyte-like morphology of human EPCs. C, Oscillation of calcium signal (fluorescence in arbitrary units, A.U.) of human EPC (blue) and rat cardiomyocyte (purple) from A over time. D, Time-matched averaging of individual calcium transients of human EPC (blue) and rat cardiomyocyte (purple) from A. E and F, Fluo 4 fluorescence at time 0 ms (E) and at peak (150 ms, F) of a nondifferentiated (*) and a morphologically transdifferentiated human EPC (arrow) together with a rat cardiomyocyte (arrowhead). Note calcium transient in transdifferentiated human EPC and rat cardiomyocyte but not nondifferentiated human EPC.



JOURNAL OF THE AMERICAN HEART ASSOCIATION

α -sarcomeric actinin after 6 days of coculture with rat cardiomyocytes. In contrast, the transdifferentiation efficiency of EPCs from CAD patients was significantly reduced compared with the control group (Figure 7, A and B). Interestingly, the EPC transdifferentiation capacity was inversely correlated with the number of cardiovascular risk factors (Figure 7C).

Taken together, EPCs from CAD patients are in principle capable of transdifferentiating into actinin-positive cells, albeit less efficiently than EPCs from healthy volunteers.

Discussion

The present study demonstrates that EPCs isolated from the peripheral blood of healthy adult volunteers and patients with CAD are capable of transdifferentiating into cardiac myocytes when cocultured with rat cardiomyocytes. After 6 days of coculture, EPCs displayed typical phenotypic and functional properties of cardiomyocytes. Human EPCs and rat

cardiomyocytes exhibited gap junctional communication, as evidenced by dye coupling experiments.

In our *in vitro* system, the transdifferentiation potential of human adult EPCs was similar to that previously described for murine embryonic endothelial cells or human semiembryonic umbilical endothelial cells.¹⁶ Thus, our results extend the identification of cells capable of transdifferentiation to circulating human adult EPCs. Significantly, human EPCs and purified CD34⁺ hematopoietic progenitor cells exhibited a comparable capacity to transdifferentiate into cardiomyocytes, as evidenced by a similar percentage of actinin-positive cells.

A pivotal functional property of cardiomyocytes is their excitation-contraction coupling. By use of fluo 4 imaging, calcium transients of similar amplitude and duration were detected in transdifferentiated human EPCs and rat cardiomyocytes. The calcium transients of the transdifferentiated human EPCs were comparable to the ones described previ-

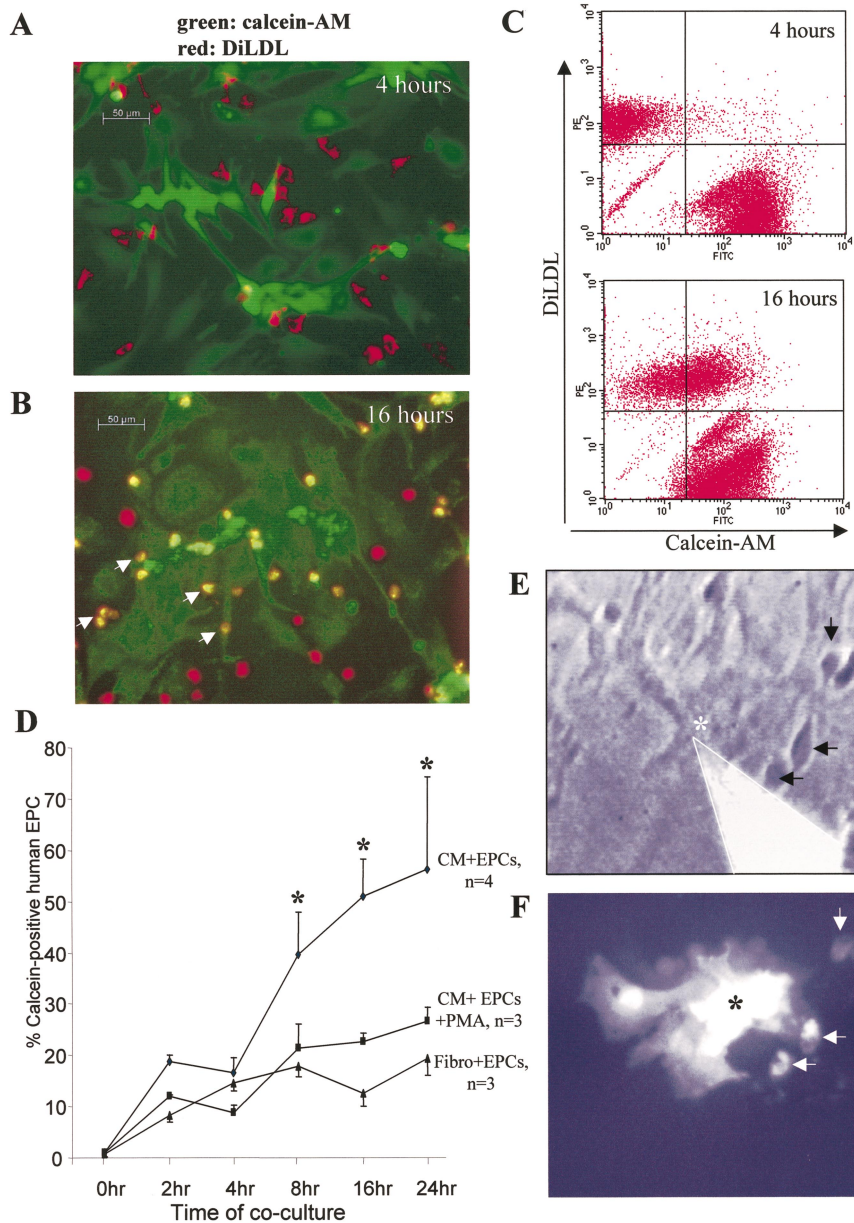


Figure 5. Gap junctional communication between EPCs and rat cardiomyocytes. A through C, Human EPCs were labeled with nontransferable DiI-acLDL (red fluorescence), and rat cardiomyocytes were labeled with gap junction-permeable calcein-AM (green fluorescence) before coculture. Superimposed images after (A) 4 hours and (B) 16 hours of coculture, both magnification $\times 200$. Arrows indicate human EPCs that have taken up calcein. Yellow color results from colocalization of red and green fluorescence. C, Representative flow cytometry analyses after 4 hours (top) or 16 hours (bottom). EPCs that have taken up calcein dye are in right upper quadrant. D, Quantitative group data for coculture of human EPCs with rat cardiomyocytes without inhibitor (rhombus) or in presence of gap junction inhibitor PMA (50 nmol/L) (square). As control, human EPCs were cocultured with rat cardiac fibroblasts (triangle). Values are mean \pm SEM from $n=3$ to 4 independent experiments per data point. * $P < 0.05$ vs coculture with inhibitor or coculture with fibroblasts. E and F, Microinjection. A single cardiomyocyte with adjacent human EPCs was microinjected with Lucifer yellow. E, Phase-contrast image of injection. F, Fluorescent image 60 sec after injection. Asterisks indicate cardiomyocyte; arrows, coupled EPCs.

ously for cardiomyocytes generated from human embryonic stem cells.²³ Together, these data demonstrate that human EPCs are capable of acquiring not only phenotypic (increase in cell length and area as well as expression and organization of sarcomeric proteins) but also functional characteristics of cardiomyocytes.

To be functionally integrated into cardiac tissue, EPCs must become electrically coupled to neighboring cardiomyocytes. These intercellular connections are typically established by gap junctions. Indeed, on coculture, human EPCs exhibited gap junctional communication with adjacent rat cardiomyocytes, as evidenced by dye transfer experiments.

Recent studies imply an important role of cell fusion for phenotypic transdifferentiation.²¹ In detail, embryonic stem cells were found to be capable of forming hybrids with differentiated cells when cocultivated, which leads to epigenetic reprogramming. Consequently, the altered phenotype of

the embryonic stem cell does not arise from direct conversion of the cell type but rather from cell fusion. However, transdifferentiation of EPCs to cardiac myocytes was also detected when paraformaldehyde-fixed, dead cardiac myocytes were used for coculture. Therefore, the present data exclude the possibility that cellular fusion is a prerequisite for the phenotypical change of EPCs to cardiomyocytes (Figure 6). However, our data also demonstrate that cell-to-cell contact is essential for EPC transdifferentiation, because conditioned medium containing soluble factors released from cardiomyocytes was not sufficient to reproduce EPC transdifferentiation in the absence of cardiomyocytes (Figure 3E). One possibility is that cadherins may mediate this crucial cell-to-cell contact. Beyond the physical interaction of cohering cells, cadherins regulate diverse signaling processes, such as differentiation, proliferation, and migration.²² Indeed, calcium depletion, which prevents cadherin-mediated cell-to-

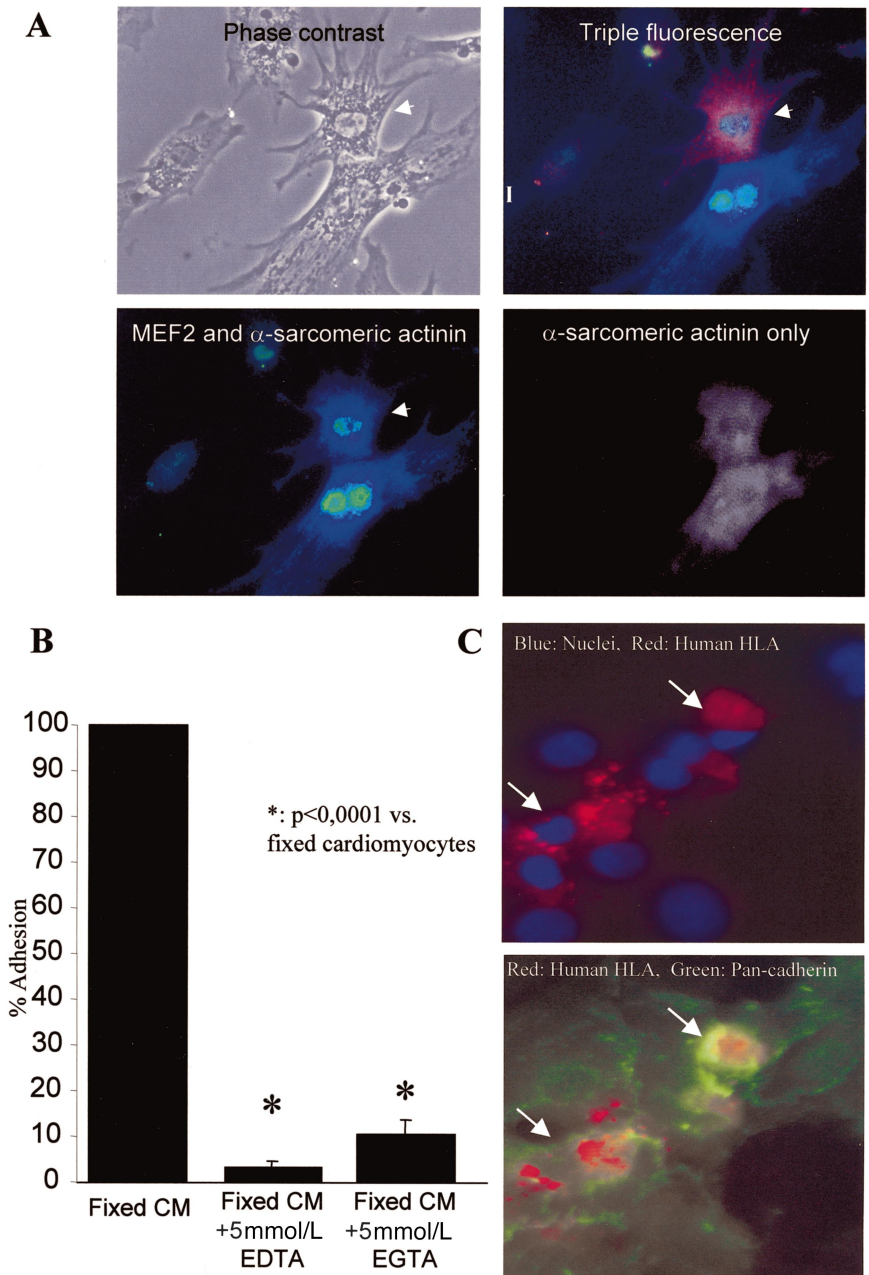


Figure 6. EPCs can transdifferentiate when cocultivated with fixed cardiomyocytes. **A**, Transdifferentiation of EPCs on paraformaldehyde-fixed cardiomyocytes. After 6 days, immunostaining against human HLA (red fluorescence), α -sarcomeric actinin (blue fluorescence), and MEF-2 (green fluorescence) was performed (n=3). Lower right, Black-and-white image for optimized visualization of striated myofilaments. Arrow points to a human cell expressing MEF-2 and α -sarcomeric actinin. **B**, Adhesion of EPCs to paraformaldehyde-fixed cardiomyocytes is calcium-dependent. Di-acLDL-labeled EPCs were incubated with fixed cardiomyocytes in presence or absence of EDTA and EGTA for 12 hours. Then, adherent EPCs were counted. Values \pm SEM (n=4) are expressed as percentage of control. **C**, Cadherin expression at interface between EPCs and cardiomyocytes. After 6 days of coculture, cells were stained for cadherins (green fluorescence); nuclei are blue and human cells are red.

cell contact, abolished the adhesion of EPCs to cardiomyocytes. In addition, cadherins are expressed in EPCs and are localized to the sites of cell-to-cell contact between EPCs and cardiomyocytes. These data support a potential role of cadherins for EPC transdifferentiation. However, further experiments are necessary to prove that concept and to identify the role of specific cadherins.

In vivo experiments in animals demonstrated that injection of EPCs improves cardiac neovascularization and function.⁵ Therefore, EPCs might be useful for cell therapy to potentially promote both neovascularization and cardiac regeneration in patients with ischemic heart disease. Importantly, EPCs isolated from the peripheral blood of CAD patients were also capable of transdifferentiating into cardiomyocyte-like cells in our cell culture model, albeit with a lower efficiency than EPCs from healthy adult volunteers. These

data are consistent with our previous observation that EPCs from CAD patients are functionally impaired in cell culture.⁹

In summary, adult blood-derived human EPCs from healthy adult volunteers and CAD patients can transdifferentiate into cardiomyocytes in vitro after coculture with neonatal rat cardiac myocytes. Ultimately, these cells might be useful for a potential cell therapy to promote cardiac regeneration in patients with ischemic heart disease.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (Di600/5-1 to Drs Dimmeler and Badorff). We thank Heike Aranda, Melanie Näher, and Christiane Mildner-Rihm for expert technical assistance and Tina Ott for secretarial assistance. We are indebted to Dr Ulrike Köhl, Department of Pediatrics, University of Frankfurt, for providing human CD34⁺ cells.

A

Parameter	No CAD	CAD patients	p value
number of patients	15	14	
Age (years, mean±SEM)	47.5±4.4	54.6±2.9	0.185
% male	60	85.7	0.390
% Diabetes	14.3	8.3	0.642
% Hypertension	21.4	50	0.134
% Smoking	28.6	50	0.272
% Hypercholesterolemia	28.6	83.3	0.006
% Familial Disposition	21.4	41.7	0.059
% transdifferentiated EPCs after 6 days co-culture (mean±SEM)	9.94±1.39	5.04±1.09	0.008

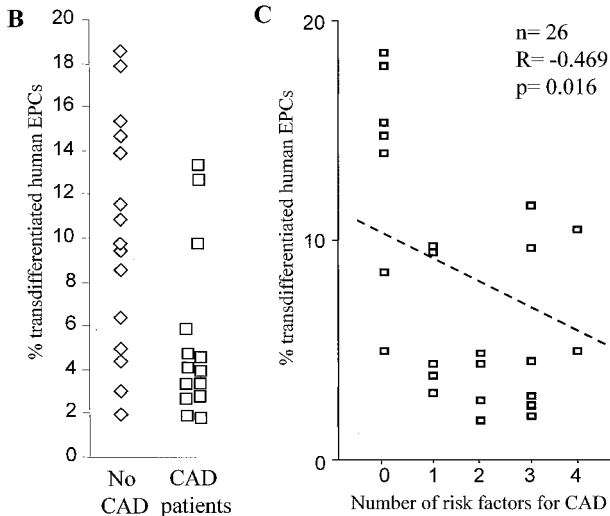


Figure 7. EPCs from CAD patients can transdifferentiate into actinin-positive cells. **A**, Clinical characteristics of control group (No CAD) and CAD patients. For metric parameters, mean±SEM is shown. **B**, Individual transdifferentiation data points for control group (No CAD) and CAD patients. **C**, EPC transdifferentiation capacity was correlated with total number of risk factors for CAD. Pooled data from control and CAD group. For 3 individuals in **A**, a risk factor score could not be calculated.

This article is dedicated to the memory of Dr Jeffrey Isner, who inspired our work.

References

- Gussoni E, Soneoka Y, Strickland CD, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature*. 1999;401:390–394.
- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430–436.

- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701–705.
- Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest*. 2000;105:1527–1536.
- Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634–637.
- Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A*. 2000;97:3422–3427.
- Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
- Schattteman GC, Hanlon HD, Jiao C, et al. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest*. 2000;106:571–578.
- Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001;89:E1–E7.
- Gehling UM, Ergun S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood*. 2000;95:3106–3112.
- Dimmeler S, Aicher A, Vasa M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest*. 2001;108:391–397.
- Bhattacharya V, McSweeney PA, Shi Q, et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34⁺ bone marrow cells. *Blood*. 2000;95:581–585.
- Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34⁺ cells identifies a population of functional endothelial precursors. *Blood*. 2000;95:952–958.
- Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation*. 2001;103:2885–2890.
- Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res*. 1999;85:221–228.
- Condorelli G, Borello U, De Angelis L, et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc Natl Acad Sci U S A*. 2001;98:10733–10738.
- Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest*. 1999;103:697–705.
- Muller M, Fleischmann BK, Selbert S, et al. Selection of ventricular-like cardiomyocytes from ES cells in vitro. *FASEB J*. 2000;14:2540–2548.
- Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest*. 2001;108:407–414.
- Badorff C, Ruetten H, Mueller S, et al. Fas receptor signaling inhibits GSK3β in cardiomyocytes and is required for pressure overload-induced hypertrophy. *J Clin Invest*. 2002;109:373–381.
- Ying Q-L, Nichols J, Evans EP, et al. Changing potency by spontaneous fusion. *Nature*. 2002;416:545–548.
- Steinberg MS, McNutt PM. Cadherins and their connections: adhesion junctions have broader functions. *Curr Opin Cell Biol*. 1999;11:554–560.
- Sauer H, Theben T, Hescheler J, et al. Characteristics of calcium sparks in cardiomyocytes derived from embryonic stem cells. *Am J Physiol*. 2001;281:H411–H421.