No Evidence of Transdifferentiation of Human Endothelial Progenitor Cells Into Cardiomyocytes After Coculture With Neonatal Rat Cardiomyocytes

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Background—Recent studies have suggested the differentiation of human endothelial progenitor cells (huEPCs) isolated from peripheral blood into cardiomyocytes. This study investigates whether, when cocultured, neonatal rat cardiomyocytes (NRCMs) can induce transdifferentiation of huEPCs into cardiomyocytes.

Methods and Results—Coculture experiments with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI)-labeled huEPCs and NRCMs have been performed. Cocultures have been analyzed by means of flow cytometry, 3D confocal laser microscopy, species-specific reverse transcriptase–polymerase chain reaction for the expression of human cardiac marker genes, and electron microscopy. Although fluorescence-activated cell sorting (FACS) analysis and conventional wide-field fluorescence microscopy suggested the existence of DiI+ cardiomyocytes in cocultures, no convincing evidence of cardiac differentiation of huEPCs has been obtained. Apparently, DiI+ cardiomyocytes were identified as necrotic NRCMs or NRCM-derived vesicles with high levels of autofluorescence or, alternatively, as NRCMs lying on top of or below labeled huEPCs or huEPC fragments. Accordingly, no expression of human Nkx2.5, GATA-4, or cardiac troponin I was detected.

Conclusions—No convincing evidence of transdifferentiation of huEPCs into cardiomyocytes was obtained. Although we cannot exclude that recent contrary data may be due to slightly different culture protocols, our study has revealed that recently applied standard analysis tools including FACS and wide-field fluorescence microscopy are not sufficient to demonstrate transdifferentiation in coculture settings and can lead to misinterpretation of the data obtained solely with these methods. (Circulation. 2006;113:1326-1334.)

Key Words: cells differentiation endothelium myocytes

Recent reports on cardiac differentiation of adult stem and progenitor cells raised hopes that therapeutic application of such cells may be able to restore cardiac function, eg, after myocardial infarction. Indeed, clinical studies transplanting or mobilizing bone marrow cells, subpopulations of bone marrow cells, or peripheral blood–derived endothelial progenitor cells (EPCs) demonstrated moderate improvement of cardiac function. In vitro studies reported cardiac differentiation of different types of somatic stem and progenitor cells. At present, it is controversial whether the observed functional improvements are based on transplant-related angiogenesis, paracrine effects, or transdifferentiation into cardiomyocytes. Although a series of in vitro studies reported cardiac differentiation of different types of adult somatic stem and progenitor cells, it is not yet known whether any adult stem or progenitor cells can give rise to functional cardiomyocytes. Some critical studies raised doubts about cardiac differentiation of somatic stem cells and suggested that the reported data on differentiation into cardiomyocytes may be based on cell fusion events or method-related artifacts.11–14

To corroborate a recent study of Badorff et al,15 who reported transdifferentiation of human EPCs (huEPCs) into functional cardiomyocytes, we cocultured adult human peripheral blood–derived EPCs and neonatal rat cardiomyocytes (NRCMs). Although we have obtained partially similar results, no convincing evidence of transdifferentiation of huEPCs into functional active cardiomyocytes was found. Although we cannot exclude that slightly different culture conditions may have prevented transdifferentiation in our experiments, our data point out technical limitations of fluorescence-activated cell sorting (FACS) analysis and conventional 2D immunofluorescence as well as confocal microscopy for analysis of stem cell differentiation in coculture.
settings. Data obtained with these methods should be submitted to a careful examination to prevent misinterpretation.

Methods

Coculture of Cardiomyocytes and EPCs

As standard protocol, 1,1',1’-dioctadecyl-3,3,3',3’-tetramethylindocarbocyanine (DiI)-labeled huEPCs16 cultured on slides were mixed with freshly isolated cardiomyocytes11 at different ratios (1:4 or 1:1) in DMEM, supplemented with 10% horse serum and 5% FCS, t-glutamine (2 mmol/L), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Invitrogen, Karlsruhe, Germany). The medium was changed to DMEM containing 5% horse serum after 2 days and was subsequently replaced every 2 days. As controls, huEPCs and NRCMs only were cultured as described for coculture experiments. As variations of this standard protocol, cocultivated cells were grown in DMEM supplemented with 10% FCS for 2 days, and then the medium was changed to DMEM containing 5% horse serum, or growth medium with 10% FCS was used for the whole culture period. Different lots of FCS were used for medium supplementation.

Flow Cytometry

After 2 and 6 days of cocultivation, cardiomyocytes were labeled with a mouse IgG1 monoclonal antibody EA-53 to sarcomeric α-actinin (diluted 1:500; Sigma, Tautkirchen, Germany), with the use of the BD Cytofix/CytoPerm Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. After incubation with Cy3labeled donkey anti-mouse IgG (diluted 1:500; Dianova, Hamburg, Germany) as secondary antibody, cells were analyzed by flow cytometry with a FACS Calibur cell analyzer (BD Biosciences). Data were further processed with the use of WinMDI 2.8 software. Monocultures of cardiomyocytes or EPCs served as controls.

Immunohistological Staining

After 6 or 10 days of cocultivation, the cells were fixed for immunocytochemistry in paraformaldehyde. The following primary antibodies were used: MF20 anti-sarcomeric myosin (hybridoma cells were obtained from Developmental Studies Hybridoma Bank, Iowa City, Iowa), CT3 anti-troponin T (Developmental Studies Hybridoma Bank), MAB1553 anti-titin (Chemicon, Temecula, Calif), 9D10 anti-titin (Developmental Studies Hybridoma Bank), and EA-53 anti-sarcoceric α-actinin (Sigma).

The cells were then incubated with a secondary antibody, Cy3labeled donkey anti-mouse IgG (Dianova). The cells were labeled with DAPI (Sigma); images were collected with a fluorescence or confocal laser scanning microscope.

A detailed description of the methodology and data analysis is provided in an expanded Methods section, which can be found in the online-only Data Supplement.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Characterization of Isolated huEPCs and NRCMs

Preparations and cultures of huEPCs were performed as described by Badorff et al15: Biocoll-gradient purification was applied to isolate peripheral blood mononuclear cells (PBMCs) containing B cells, T cells, natural killer (NK) cells, monocytes, EPCs, and, to a certain extent, contaminating erythrocytes and thrombocytes. Subsequent cultivation on gelatin/fibronectin and washing resulted in further purification of adherent cells including monocytes/macrophages and EPCs and loss of cell debris and nonattached cells, such as lymphocytes, NK cells, erythrocytes, and thrombocytes. To characterize huEPC preparations, cells were incubated with Dil-conjugated acetylated LDL (AcLDL), which is commonly used to label and purify cells of the endothelial lineage, and with Ulex europaeus lectin type 1 (UEA-1). After 3 days of cultivation (this was the typical culture period before labeling and coculture with NRCMs), nearly 100% of the adherent mononuclear cells stained positive for Dil-AcLDL and lectin UEA-1 (Figure 1). This was to a large extent independent of the culture conditions including coating of culture flasks and composition of the culture medium used. After 3 to 7 days in endothelial growth medium, many cells displayed an elongated shape similar to cultured nonconfluent human endothelial cells.

Overall, our data are in accordance with recent findings17,19,20 showing that the majority of EPCs isolated by gradient density centrifugation with subsequent adherence-based purification as described by Dimmeler et al15,16 are AcLDLpos/UEA-1pos after 3 days of culture in endothelial growth medium, similar to mature endothelial cells. However, the majority of PBMCs initially adhering to plastic surfaces represent monocytes/macrophages,21 which have also been found to be AcLDLpos/UEA-1pos. Despite the monocyte/macrophage-like phenotype of the majority of the cells, PBMC preparations after 3 or 7 days of adherent culture in endothelial cell growth medium will henceforth be referred to as EPC preparations.

Identity of NRCMs was confirmed with anti-sarcomeric α-actinin, anti-sarcomeric myosin, anti-titin, and anti–troponin T antibody labeling. Contaminating cell types consisted predominantly of fibroblasts, as determined by immunocytochemistry with the use of a mouse IgG1 monoclonal anti-rat prolyl-4-hydroxylase antibody (Acris, Hiddenhausen, Germany). The proportion of titinpos cardiomyocytes was 68% to 71% immediately after isolation (data not shown). Changes of cardiomyocyte content during culture were not determined.

Both cocultured EPCs and EPCs in Monoculture Increased in Length and Surface Area but Were Significantly Smaller Than Cardiomyocytes

To investigate potential transdifferentiation of huEPCs into cardiomyocytes after coculture with NRCMs, we...
analyzed the morphological changes of the EPCs. We observed that a number of EPCs changed morphology and size over time within the cocultures, namely, cell flattening and the formation of pseudopodia were observed. To evaluate whether such increase in size may correspond to cardiac differentiation as suggested by Badorff et al., the average cell length and surface of huEPCs in coculture as well as huEPCs in monoculture were assessed with microscopy after CM-DiI or DiI-AcLDL staining. Within the cocultures, the average cell length and surface area of Dil™ EPCs increased moderately (Figure 2). However, we observed a similar change in size of EPCs cultured without NRCMs (Figure 2). We also determined the size of individual NRCMs positive for anti-sarcomeric α-actinin staining: cell length (59±14 μm) as well as surface area (855±266 μm²) was significantly greater than that of EPCs (cell length, 22±10 μm; surface area, 159±87 μm²) within the cocultures (Figure 2).

A Fraction of Apparently Dil™-Positive Sarcomeric α-Actinin™ Cells as Detected by FACS Analysis Does Not Correspond to huEPC-Derived Cardiomyocytes but to Autofluorescent NRCMs

To further confirm the identity of potential huEPC-derived cardiomyocytes, cocultures of Dil-labeled huEPCs and NRCMs were analyzed with flow cytometry. The number of apparently both sarcomeric α-actinin™ (Cy™5) and Dil™ (red) cells within the cocultures increased significantly from day 2 to day 6 (Figure 3), in a manner similar to that reported by Badorff et al. However, we also observed a considerable increase of the proportion of sarcomeric α-actinin™ cells with red fluorescence when analyzing NRCMs cultured without EPCs (Figure 3), suggesting that at least a fraction of sarcomeric α-actinin™ cardiomyocytes displaying red fluorescence do not represent huEPC-derived cardiomyocytes.

Figure 2. Alterations in cell size and cell surface are similar for huEPCs in monoculture and in coculture with NRCMs. The size of cultured NRCMs is significantly greater. Cell length (A) and surface area (B) of Dil-labeled huEPCs/cardiomyocytes stained for sarcomeric α-actinin were determined microscopically with the use of ImageJ 1.30v software. Numbers of analyzed cells per group are indicated.

Figure 3. Conventional FACS analysis is not suitable to identify Dil™-positive human cardiomyocytes within cocultures of huEPCs and NRCMs. FACS analyses of cocultures (E, F) as well as of monocultures of huEPCs (A, B) and NRCMs (C, D) at day 2 (A, C, E) and day 6 (B, D, F) demonstrate a significant increase in sarcomeric α-actinin™ cardiomyocytes displaying red Dil-like fluorescence.
Immunofluorescence Microscopy Is Not Appropriate to Definitely Identify Transdifferentiation of huEPCs After Coculture With NRCMs

We have further investigated, in cocultures, the identity of the double-positive cardiomyocytes and the potential for transdifferentiation of huEPCs. All experimental procedures with regard to huEPC and NRCM isolation, cell labeling, and coculture were performed as described by Badorff et al.15 Additionally, a series of coculture experiments with experimental variations, as described in Table 1, were performed to reduce the probability of false-negative results due to inappropriate culture conditions.

Unlike Badorff et al,15 huEPCs were labeled for 24 hours instead of for 60 minutes to allow for a lower concentration of DiI-AcLDL. Control experiments demonstrated that both protocols resulted in comparable DiI-AcLDL staining of essentially all cells (data not shown). Additional experiments with an alternative labeling approach were performed with the use of the DiI derivative Vybrant CM-DiI. In a variety of experiments (Table 1), cocultures with ratios of 1:1 to 1:4 of huEPC/NRCM were set up. After 6 or 10 days, cocultures were fixed with paraformaldehyde and labeled with DAPI (blue) and myocyte-specific antibodies, followed by fluorescence detection with the use of a CyTM2-conjugated secondary antibody (green).

Within our cocultures, we observed contracting CM-DiI/DiI-AcLDL–stained cells. Thorough observation revealed that these cells were the result of physical coupling of labeled EPCs and contracting NRCMs (data not shown).

Figure 4 shows representative cells positive for sarcomeric α-actinin. Similar images were obtained with cells labeled with muscle-specific antibodies including MF20, anti–troponin T, or anti-titin. Within the cocultures, several apparently dual-labeled cells were analyzed and assigned different categories, as reported in Table 2.

We could not decide for ~12% of the seemingly double-stained cells (category III, Figure 4, Table 2) whether the obvious double staining was due to cardiomyocytes lying on top of or around labeled EPCs or, alternatively, was due to transdifferentiation or cell fusion. Of these cells, images were acquired with confocal microscopy, and the 3D digital reconstruction was analyzed with Zeiss LSM Image Browser. Using this technique, we were able to discriminate between transdifferentiation/cell fusion and NRCMs lying above or around labeled huEPCs for the vast majority of category III events. No evidence of transdifferentiation or cell fusion was obtained. Instead, NRCMs frequently grew above or enclosed labeled huEPCs or EPC fragments. Figure 5 shows a representative image. A careful examination revealed that in some cases, because of the complexity of the images, even a 3D confocal microscopy data set did not allow us to discriminate between transdifferentiation/cell fusion and NRCMs lying above or around labeled huEPCs/huEPC fragments (Figure 6A; original stack file available in Figure I in the online-only Data Supplement). To investigate whether such

**TABLE 1. Variations in Culture, Coculture, and Antibody Labeling**

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<thead>
<tr>
<th>Parameter</th>
<th>Variations</th>
<th>Variations</th>
<th>Variations</th>
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<tbody>
<tr>
<td>Coating</td>
<td>None</td>
<td>Gelatin</td>
<td>Gelatin/fibronectin</td>
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<tr>
<td>EPC medium</td>
<td>Endothelial cell growth medium</td>
<td>EGM (Cell Systems)</td>
<td>+10% or 20% of 8 different lots of FCS</td>
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<tr>
<td>EPC labeling</td>
<td>CM-DiI</td>
<td>DiI-AcLDL</td>
<td></td>
</tr>
<tr>
<td>Ratio EPC/NRCM</td>
<td>1:1</td>
<td>1:4</td>
<td></td>
</tr>
<tr>
<td>Coculture medium</td>
<td>For 2 days: DMEM + 5% FCS + 10% horse serum; thereafter: DMEM + 5% horse serum</td>
<td>For 2 days: DMEM + 10% FCS; thereafter: DMEM + 5% horse serum</td>
<td>DMEM + 10% FCS</td>
</tr>
<tr>
<td>Antibody labeling</td>
<td>Anti–sarcomeric α-actinin</td>
<td>Anti–sarcomeric myosin</td>
<td>Anti–troponin T</td>
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<tr>
<td></td>
<td>Anti–sarcomeric myosin</td>
<td>Anti–troponin T</td>
<td>Anti–titin</td>
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Different combinations of the listed variations in culture, coculture, and antibody labeling were applied in a variety of experiments.
Figure 5. The majority of apparently double-stained cardiomyocytes do not represent transdifferentiated huEPCs or huEPCs after fusion with NRCMs but NRCMs growing above or surrounding labeled huEPCs. 3D data collected with confocal microscopy were analyzed with the use of Zeiss LSM Image Browser software. In a 2D representation, all levels of the third dimension have been projected about each other on this level (A). In the 3D evaluation of the same image, the representation of slices in the third dimension level of the red or green line is shown above the image and on the far right (B). The blue lines indicate the focal plane, which is represented in the large illustration. In the 3D illustration, a NRCM is clearly recognizable that has grown around DiI-labeled huEPCs (or EPC fragments). Anti–sarcomeric α-actinin–labeled cardiomyocytes are green; Dil-AcLDL–labeled EPCs or EPC-fragments, red.

Figure 6. Dil<sup>pos</sup> cardiomyocytes can be detected in cocultures of NRCMs with terminally differentiated DiI-labeled type II pneumocyte-like MLE-12 cells similar to cocultures with Dil-labeled huEPCs. 3D confocal microscopy images were analyzed with the use of Zeiss LSM Image Browser software. In cases as shown in A, even 3D confocal microscopy is not able to discriminate between EPC transdifferentiation on the one hand and fusion of Dil<sup>pos</sup> huEPC/EPC fragments with NRCMs or adhesion of Dil<sup>pos</sup> EPC fragments to NRCMs on the other hand. The fact that similar observations have been made with noncardiogenic cell types such as MLE-12 cells (B) demonstrates that mechanisms other than transdifferentiation can also account for the detected Dil-stained cardiomyocytes. The blue lines indicate the focal plane, which is represented in the large illustration. Anti–sarcomeric α-actinin–labeled cardiomyocytes are green; Dil-AcLDL–labeled EPCs or EPC-fragments, red; and DAPI, blue. High-resolution files allowing for navigation through 3D image data sets are available in the online-only Data Supplement.

Dying NRCMs Within Cocultures Display High Levels of Dil-like Red Autofluorescence

Beside apparently double-stained cells with typical morphology of cultured NRCMs, both cocultures with Dil-labeled huEPCs and NRCM monocultures contained a multitude of sarcomeric α-actinin<sup>pos</sup> small cells or vesicles displaying red fluorescence, particularly surrounding aggregations of NRCMs (Figure 7A, 7B). The majority of these cells/vesicles had a condensed nucleus (c) or even no nucleus (d) as demonstrated with the use of DAPI staining. A terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed to investigate whether these autofluorescent cells/vesicles may represent apoptotic NRCMs. This assay identified a minority of those cells/vesicles as apoptotic (Figure III in the online-only Data Supplement).

Ultrastructural analyses were performed to determine whether the observed majority of TUNEL<sup>neg</sup> autofluorescent cells/vesicles may represent necrotic rat cardiomyocytes. Indeed, electron microscopy images of day 6 NRCM cultures confirmed the existence of considerable numbers of dying NRCMs with remnants of muscle-specific filaments surrounding aggregations of viable NRCMs (Figure 7C, 7D). These cardiomyocytes displayed typical features of necrotic cells including disintegrated cell membranes.

No Evidence of Expression of Human Cardiac Transcription Factors in Cocultures of EPCs and NRCMs

To further investigate potential transdifferentiation of huEPCs into cardiomyocytes on a molecular level, we analyzed the expression of human cardiac genes with highly

<table>
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<tr>
<th>Labeling Approach</th>
<th>Category I Events&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Category II Events&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Category III Events&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Total No. of Events</th>
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</thead>
<tbody>
<tr>
<td>CM-Dil</td>
<td>668 (75.6)</td>
<td>141 (16.0)</td>
<td>75 (8.5)</td>
<td>884</td>
</tr>
<tr>
<td>Dil-AcLDL</td>
<td>251 (59.0)</td>
<td>96 (22.6)</td>
<td>78 (18.4)</td>
<td>425</td>
</tr>
<tr>
<td>Total</td>
<td>919 (70.2)</td>
<td>237 (18.1)</td>
<td>153 (11.7)</td>
<td>1309</td>
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*Category I: It can be clearly recognized that the red fluorescent particle is not located within the cardiomyocyte.
†Category II: It is not possible to totally exclude that a red staining is located within the cardiomyocyte, but the image is probably due to a cardiomyocyte lying on top or around a huEPC or EPC fragment.
‡Category III: Conventional 2D immunofluorescence microscopy definitely does not allow us to discriminate between transdifferentiation/cell fusion and cardiomyocytes lying above or around labeled EPCs.
sensitive species-specific reverse transcriptase–polymerase chain reaction (RT-PCR). We chose the transcription factors Nkx2.5, GATA-4, and FOG-2 as well as the myofibrillar protein cardiac troponin I as cardiac marker genes. No expression of Nkx2.5, GATA-4, FOG-2, or cardiac troponin I could be detected in huEPC preparations despite a high sensitivity (detection limit: 10 to 100 copies per PCR), and no cross-reactions with the rat homologues could be detected in NRCMs (Figure 8). Cultures of NRCMs and huEPCs, which had initially been grown in endothelial cell growth medium without cardiomyocytes for 3 or 7 days, were tested after 6 and 10 days of coculture. To address the issue that certain components of FCS might be essential for differentiation of huEPCs into cardiomyocytes, we analyzed the RNA prepared from cells cocultured in medium with 8 different lots of FCS, and 10 days of coculture. To address the issue that certain components of FCS might be essential for differentiation of huEPCs into cardiomyocytes, we analyzed the RNA prepared from cells cocultured in medium with 8 different lots of FCS, including those resulting in efficient differentiation of cardiomyocytes from mouse embryonic stem cells (data not shown). The integrity of NRCM RNA was confirmed with species-specific rat β-actin and rat GATA-4 RT-PCR. Although RT-PCR specific for the human housekeeping gene β-actin, which has a sensitivity similar to that of the transcription factor/cardiac troponin I–specific RT-PCRs, demonstrated the presence of human RNA, no expression of human Nkx2.5, human GATA-4, or human cardiac troponin I was detected in our cocultures. Figure 8 and Figure IV in the online-only Data Supplement show representative results of our expression analyses. In some cases, very low expression of human FOG-2, which is also expressed in other cell types such as lymphocytes, could be detected in the cocultures of huEPCs and NRCMs (Figure 8).

Discussion

A variety of cardiac diseases, including ischemic heart injury, are still poorly treated, and cell transplantation approaches are now envisaged as a potential therapeutic option and may eventually replace conventional therapies. Because adult cardiomyocytes do not have significant potential for proliferation, other cell sources, such as stem cells, are currently under intense investigation as a source for transplantable cardiomyocytes. For clinical cellular cardiomyoplasty, the cardiac differentiation of adult human peripheral blood–derived EPCs would be of highest significance because those cells can be easily isolated in considerable amounts without the need for major surgical interventions. In addition, it may be possible to achieve ex vivo cell propagation (and optional differentiation) of those cells within a few days after myocardial infarction. Finally, differentiation of huEPCs into endothelial cells, which has been demonstrated by a series of studies, could contribute to the revascularization and regeneration of infarction zones.

Although controversial, the term endothelial progenitor cells is still commonly used for the PBMC fraction isolated by plastic adherence in endothelial cell growth medium. In accordance with our results (Figure 1), several recent studies demonstrated that those cells mainly comprise nonproliferating AcLDL+/−/UEA-1− monocyte/macrophage-derived cells able to release proangiogenic factors such as vascular endothelial growth factor or hepatocyte growth factor. Nevertheless, these cells contain a small proportion of “late outgrowth endothelial cells” representing proliferating EPCs.

Recently, the “efficient transdifferentiation of human adult endothelial progenitor cells into functionally active cardiomyocytes” was reported. In this study, human PBMCs were isolated by density gradient centrifugation and cultured for 3 days in endothelial growth medium before cell labeling and coculture with NRCMs.

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Analyses of DiI-labeled huEPCs cocultured with NRCMs with flow cytometry were performed to further investigate potential differentiation of huEPCs into cardiomyocytes. Myocyte-specific staining was performed with the use of a monoclonal antibody against sarcomeric α-actinin. In contrast to Badorff et al, we did not identify the human cells using a phycoerythrine-coupled anti–HLA-DR (major histocompatibility class II) antibody because class II major histocompatibility class antigens are not expressed by cardiomyocytes of human or rat origin. Instead, huEPCs were labeled with the red fluorescent membrane dye DiI before coculture with NRCMs.

Similar to Badorff et al., the proportion of sarcomeric α-actinin cardiomyocytes with clearly detectable red fluorescence significantly increased from day 2 to day 6 of coculture (Figure 3). However, an analysis of the monocultures of NRCMs also revealed an increasing fraction of sarcomeric α-actinin cardiomyocytes with red DiI-like autofluorescence (Figure 3) as described by others, for example, by Gao et al. These results suggested that at least a considerable proportion of double-positive cells did not represent transdifferentiated huEPCs.

In addition, when standard 2-color FACS analysis is used, other mechanisms, including dye transfer from labeled huEPCs, fusion of NRCMs with labeled huEPC/EPC fragments, or simply incomplete trypsinization of the cocultures before FACS analysis, could account for the detection of double-positive events.

To clarify the identity of the observed autofluorescent cells, cocultures of huEPCs labeled with DiI-AcLDL and NRCMs were analyzed microscopically. According to the manufacturers’ information (Molecular Probes Inc; Cell Systems), the DiI component of DiI-AcLDL is not covalently bound but only associated with red fluorescent membrane dye DiI before coculture with NRCMs. Therefore, additional alternative staining was performed with the DiI derivative Vybrant CM-DiI. This DiI derivative is not taken up via specific scavenger receptors like DiI-associated AcLDL but binds covalently to membrane components during the labeling process, thereby theoretically reducing the likelihood of dye transfer toward rat cardiomyocytes.

Remarkably, the obtained results of apparently DiI⁺⁺ cardiomyocytes are very similar to the data reported by Badorff et al. However, critical evaluation revealed that the majority of the images did not represent DiI⁺⁺ huEPC-derived cardiomyocytes but NRCMs lying above or under DiI-labelled huEPCs or EPC fragments. Such images were assigned to categories I and II, as depicted in Figure 4 and Table 2. The limitations of conventional 2D immunofluorescence microscopy became obvious for a fraction of apparently double-stained cardiomyocytes (category III, Figure 4, Table 2). Although in our setting, 3D confocal microscopy would not be able to discriminate between differentiated huEPC-derived cardiomyocytes and cardiomyocytes fused with labeled huEPCs, confocal microscopy demonstrated that in the vast majority of apparently double-stained cardiomyocytes, NRCMs simply lay above or under DiI-labeled huEPCs or EPC fragments (Figure 5).

For a minority of such cells, even 3D confocal microscopy did not allow for an unequivocal interpretation of the image (Figure 6A), thereby underscoring the difficulties in analyzing differentiation within cocultures with the use of microscopic approaches. The fact that similar observations have been made in cocultures...
of NRCMs and other somatic cell types such as type II pneumocyte-like cells (Figure 6B), which are not assumed to have cardiac differentiation potential, demonstrates that mechanisms other than transdifferentiation can account for the detected DiI-stained cardiomyocytes. In control experiments, cardiomyocytes were cultured in conditioned medium from CM-DiI-labeled EPCs for 6 days. No CM-DiI transfer via the culture medium toward cardiomyocytes was detected by FACS (data not shown). Although macrophages, which account for the majority of the so-called EPC preparations, represent one of the most fusogenic cell types,29 fusion of viable huEPCs with NRCMs appears not to have a major relevance in our setting because in this case we would expect induction of cardiac gene expression,30 which could not be demonstrated (Figure 8). Instead, the fusion of DiI-labeled “EPC” fragments with NRCMs, the adherence of such fragments to NRCMs, or CM-DiI transfer via contact of cell membranes seems to be the more likely explanation.

Besides artifacts as shown in Figures 5 and 6, a considerable number of small spherical sarcomeric α-actininpositive cells “cytosomes” with red fluorescence comparable to the applied DiI compounds were observed in the vicinity of aggregations of NRCMs (Figure 7) in cocultures as well as in NRCM monolayers. The number of such cells increased with the age of the analyzed cultures, similar to the number of obviously autofluorescent cells as detected by flow cytometry (Figure 3). On the basis of our findings (Figures 3 and 7 and Figure III in the online-only Data Supplement) and the fact that dying cells usually display high levels of autofluorescence,28 we assumed that these cells or cytosomes represented apoptotic or necrotic cardiomyocytes with already disorganized contractile apparatus.

To further support our findings of the absence of cardiac transdifferentiation of huEPCs after coculture with NRCMs, species-specific RT-PCRs for human cardiac marker genes were performed. Because these PCRs were found to be highly sensitive (detection limit of PCR was at least 100 template copies per PCR), this approach should have detected even a low level of expression in a subtraction of differentiated human cells. Heterokaryons could only be detected if fusion resulted in a cardiomyocyte-like phenotype, as previously described.30

We chose Nkx2.5, GATA-4, and FOG-2 as target genes because these transcription factors are not expressed in EPCs (including the main contaminating cell types monocytes and macrophages) or in cell types known to arise from EPCs such as smooth muscle cells and differentiated endothelial cells. These transcription factors are relatively specifically expressed in all developmental stages of human cardiomyocytes. Moreover, cardiac troponin I was included as a specific marker expressed in differentiated cardiomyocytes.

All 3 transcription factors are expressed in developing and adult human cardiomyocytes but not in cells of endothelial lineage: Nkx2.5 shows the earliest embryonic expression and is already present in the cardiac crescent.31 It is expressed through all stages, including the adult heart, and was found to be essential for commitment of mesoderm into the cardiac muscle lineage in P19 embryonal carcinoma cells.32 GATA-4 can be detected at the earliest during the formation of the linear heart tube. It is expressed in cardiomyocytes as well as in the intestinal epithelium, the primitive endoderm, and the gonads.33 Expression of FOE-2, binding partner of GATA-4, arises in the looping heart. FOE-2 is predominantly expressed in the heart, nervous system, and testis but also at lower levels in lymphocytes.32 Both factors GATA-4 and FOE-2 can also be detected in the adult heart.34 Cardiac troponin I is expressed specifically in cardiomyocytes and not in smooth muscle cells or skeletal muscle.35

Although a variety of culture conditions were applied, we did not detect any expression of human Nkx2.5, GATA-4, or cardiac troponin I (Figure 8 and Figure IV in the online-only Data Supplement). However, a negligible expression of human FOE-2 was detected. This very low expression of FOE-2 appeared not to reflect the existence of human cardiomyocytes but the contamination of huEPC preparations with other cell types such as lymphocytes, which have also been shown to express FOE-2.22

In conclusion, we did not find convincing evidence of the differentiation of adult human blood–derived EPCs into cardiomyocytes after coculture with NRCMs. Although we cannot entirely exclude the possibility that slightly different culture protocols may explain the difference between our results and those of others, our study clearly demonstrates that conventional dual-labeling FACS analysis combined with 2D immunofluorescence microscopy and even 3D confocal microscopy is insufficient to confirm cell transdifferentiation in coculture experiments and that such data should be interpreted with caution.

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Disclosures

None.

References

Recent clinical studies transplanting or mobilizing bone marrow cells, subpopulations of bone marrow cells, or peripheral blood–derived endothelial progenitor cells (EPCs) demonstrated moderate improvement of cardiac function after myocardial infarction. However, it is still unknown whether paracrine mechanisms, formation of endothelial cells and endothelial outgrowth from blood. Further investigation in vitro and in vivo will be indispensable to identify mechanisms of functional improvement after transplantation of stem/progenitor cells.
No Evidence of Transdifferentiation of Human Endothelial Progenitor Cells Into Cardiomyocytes After Coculture With Neonatal Rat Cardiomyocytes

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