Preservation of Left Ventricular Function and Attenuation of Remodeling After Transplantation of Human Epicardium-Derived Cells Into the Infarcted Mouse Heart

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Background—Proper development of compact myocardium, coronary vessels, and Purkinje fibers depends on the presence of epicardium-derived cells (EPDCs) in embryonic myocardium. We hypothesized that adult human EPDCs might partly reactivate their embryonic program when transplanted into ischemic myocardium and improve cardiac performance after myocardial infarction.

Methods and Results—EPDCs were isolated from human adult atrial tissue. Myocardial infarction was created in immunodeficient mice, followed by intramyocardial injection of $4 \times 10^5$ enhanced green fluorescent protein–labeled EPDCs (2-week survival, n=22; 6-week survival, n=15) or culture medium (n=24 and n=18, respectively). Left ventricular function was assessed with a 9.4T animal MRI unit. Ejection fraction was similar between groups on day 2 but was significantly higher in the EPDC-injected group at 2 weeks (short term), as well as after long-term survival at 6 weeks. End-systolic and end-diastolic volumes were significantly smaller in the EPDC-injected group than in the medium-injected group at all ages evaluated. At 2 weeks, vascularization was significantly increased in the EPDC-treated group, as was wall thickness, a development that might be explained by augmented DNA-damage repair activity in the infarcted area. Immunohistochemical analysis showed massive engraftment of injected EPDCs at 2 weeks, with expression of $\alpha$-smooth muscle actin, von Willebrand factor, sarcoplasmic reticulum Ca$^{2+}$-ATPase, and voltage-gated sodium channel ($\alpha$-subunit; SCN5a). EPDCs were negative for cardiomyocyte markers. At 6-weeks survival, wall thickness was still increased, but only a few EPDCs could be detected.

Conclusions—After transplantation into ischemic myocardium, adult human EPDCs preserve cardiac function and attenuate ventricular remodeling. Autologous human EPDCs are promising candidates for clinical application in infarcted hearts. (Circulation. 2007;116:917-927.)

Key Words: myocardial infarction ■ stem cells ■ remodeling ■ magnetic resonance imaging ■ angiogenesis

Current therapy aimed at alleviating the sequelae of sustained myocardial infarction (MI) is not able to restore the function of the scarred area. Stem cell therapy poses a promising alternative therapy. Because therapeutic use of embryonic stem cells is an ethically intricate issue and is technically difficult in relation to the possible rejection of the cells and tumor formation, use of adult stem cells appears to be a more feasible option. Many different types of adult cells have been demonstrated to improve cardiac function after a MI, although the underlying mechanism has only been partially unraveled (for review, see Murry et al!). Most of the cell types used are not known to be of importance during normal cardiogenesis. We chose to transplant epicardium-derived cells (EPDCs) because these cells are known to be crucial for cardiac development, because of both their physical contribution and their modulatory role.

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During embryogenesis, epicardium migrates from the extracardiac proepicardium to cover the premature heart, which by that time consists of only myocardium and endocardium, with cardiac jelly in between. A subset of the epicardial cells undergoes epithelial-mesenchymal transformation (EMT). These cells are called EPDCs. EPDCs migrate into the...
myocardium to differentiate into interstitial cardiac fibroblasts, subendocardial and atroventricular cushion mesenchymal cells, and coronary smooth muscle cells and adventitial fibroblasts.2,6–8 In addition to this physical contribution of EPDCs to the coronary vessels and the fibrous heart skeleton, EPDCs have a modulatory role in cardiogenesis.9,10 Formation of the compact myocardium,3,4 coronary vessel formation,11–13 and Purkinje fiber cell differentiation2,14 are dependent on EPDC regulation. If epicardial outgrowth is inhibited completely, the myocardium remains thin, and normal septation does not take place, which leads to early embryonic death.3 In addition to hampered formation of the compact myocardium, vascular development is severely disturbed in partial epicardial abrogation.11,15

The role of EPDCs during postnatal cardiac growth has never been elucidated. It is unknown whether new EPDCs are generated continuously through EMT, contributing to the growing structures of the heart and regulating developmental processes. It has been shown, however, that adult rat epicardial cells are at least still able to undergo EMT and differentiate into smooth muscle cells in vitro.16 We recently demonstrated that adult human cells derived from epicardial explants (hEPDCs) can undergo EMT spontaneously, as observed by a transformation from cobblestone into spindle-shaped morphology, while losing their β-catenin expression.17 In the present study, we also used Wilms’ tumor 1 suppressor protein (WT1) expression to investigate this aspect. During cardiogenesis, expression of WT1 is observed in the proepicardium and the epicardial cells but is lost in the EPDCs soon after they have undergone EMT.18,19 Furthermore, it has been shown that adult epicardial cells positively modify cardiomyocyte phenotype and function20 and that WT1 is switched on de novo in adult coronary vessels in ischemic myocardium.21

Being relatively undifferentiated cells that can give rise to differentiated progeny of at least smooth muscle cells and fibroblasts, embryonic EPDCs have been referred to as stem cells.22 Given that adult hEPDCs can still undergo EMT and can give rise to the above-mentioned cell types,17 we consider adult hEPDCs as progenitor cells. We hypothesized that adult hEPDCs could recapitulate part of their embryonic program when transplanted into diseased myocardium, thereby possibly modifying the surrounding myocardium.

In the present study, we investigated in a mouse model whether adult hEPDCs could improve cardiac performance after MI. Adult spindle-shaped hEPDCs were transplanted into ischemic murine myocardium. Evaluation of this population revealed that these cells showed no expression pattern indicative of endothelial cells or cardiomyocytes.17

**Methods**

See the online Data Supplement for an expanded Methods section.

**Primary Cultured hEPDCs**

EPDCs were cultured from human adult epicardium, which was separated mechanically from atrial appendages. Spindle-shaped cells (see Data Supplement Figure 1) from passages 2 to 4 were used for transplantation experiments after transduction with a human vector expressing the enhanced green fluorescent protein (eGFP) gene, which enabled cell tracing. An adenoviral and a lentiviral vector were used for short-term (2-week survival) and long-term (6-week survival) experiments, respectively.

**Creation of the MI and Cell Transplantation**

To avoid rejection of transplanted human cells, nonobese diabetic–severe combined immunodeficient (NOD/scid) mice were used.23 All animal procedures were approved by the Animal Ethics Committee of Leiden University and conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996). For short-term experiments, the main left anterior descending coronary artery was permanently ligated. For long-term experiments, only the branch supplying the ventral wall of the left ventricle (LV) was ligated, because extended survival until 6 weeks is not possible with the entire LV infarcted. Immediately after ligation, transplantation of 4×10^6 hEPDCs suspended in M199 (hEPDC group; 2-week survival, n = 22; 6-week survival, n = 15) or cell-free M199 (medium group; 2-week survival, n = 24; 6-week survival, n = 18) was performed into the ischemic myocardium. Sham-operated animals (2-week survival, n = 16; 6-week survival, n = 3) were operated on similarly but without ligation of the main left anterior descending coronary artery and without fluid injection. Animals were randomized to treatment.

**Short-Term Experiments**

**Magnetic Resonance Imaging**

Infarct size (2 days after surgery) and cardiac function (2 and 14 days after surgery) were assessed with contrast-enhanced and cine MRI images (9.4T). Images were analyzed by manual delineation of endocardial and epicardial borders (hEPDC group, n = 17; medium group, n = 14; sham group, n = 13) with dedicated software (see Data Supplement for detailed information). LV infarcted area, end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), and ejection fraction (EF) were computed automatically.24 Infarct area measurements were used to correct functional parameters for potential differences in initial infarct size.

**Immunohistochemical Assessment**

Animals were euthanized 15 days after surgery. Paraffin sections of the hearts (n = 5 per group) were used for immunohistochemical analysis. To investigate host tissue properties, serial sections were stained for CD31, α/γ-muscle actin, α-smooth muscle actin, proliferating cell nuclear antigen (PCNA), phosphohistone H3, and phosphohistone H2AX. Nonfluorescent anti-eGFP staining was performed to visualize the injected hEPDCs because the strong and irregular autofluorescence of the heart disturbs assessment of spontaneous eGFP fluorescence to detect engrafted cells.

To identify which proteins were expressed by the injected hEPDCs, double stainings were performed for eGFP and other proteins, including α-smooth muscle actin, von Willebrand factor (vWF), sarcoplasmic or endoplasmic reticulum Ca^2+^-ATPase (SERCA2a), voltage-gated sodium channel (α-subunit; SCN5a), cardiac troponin I, atrial natriuretic peptide, α/γ-muscle actin (clone HHF35), and sarcomeric myosin (clone MF20). A red Qdot conjugated goat anti-rabbit antibody was used to visualize eGFP staining. An appropriate biotinylated secondary antibody in combination with yellow Qdot conjugated to streptavidin was used to visualize the other proteins. For details, see the Data Supplement.

**LV Vascular Profile and Wall Thickness**

To evaluate the angiogenic effect of hEPDC transplantation, the cumulative area of CD31-stained vessel lining per total LV area was determined in the hEPDC and medium group (n = 5 for each group). LV wall thickness was measured in the hearts of the hEPDC and medium group (n = 5 for each group) by an observer blinded to treatment. Wall thickness was measured in both border zones of the infarcted wall, in the mid-infarcted area between the border zones, and in the middle of the interventricular septum.
general health and survival

Body weight was determined just before surgery at day 0 and before euthanasia at day 15. Pulmonary water content was estimated after euthanasia by subtracting dry weight from wet weight of the lungs. To correct for differences in body mass, the amount of lung fluid was expressed relative to body weight. Survival proportions were assessed.

long-term experiments

magnetic resonance imaging

The effect of hEPDC transplantation in the long term was evaluated by cine MRI images 42 days after surgery only (hEPDC group, n=15; medium group, n=14; sham group, n=3) because of the high risk of mortality during repeated imaging procedures.

immunohistochemical assessment

Animals were euthanized 43 days after surgery. Excised hearts were treated as described for short-term experiments, except for 3 hearts in the medium and hEPDC groups that were frozen. To identify injected cells, nonfluorescent stainings were performed in paraffin sections for eGFP, human-specific CD31, and human-specific vWF and in frozen sections for human nucleus.

LV vascular profile and wall thickness

Anti-CD31 and Sirius red staining and wall thickness measurements were used for host tissue investigation.

Survival

Survival proportions were assessed.

statistical analysis

Data are presented as mean±SEM. Data were analyzed with SPSS software (SPSS Inc, Chicago, Ill). For comparisons of more than 2 groups, a 1-way-ANOVA was performed (or the nonparametric Kruskal-Wallis test for the dependent variable lung weight). If the omnibus tests among groups were significantly different, post hoc tests between groups with t tests (and Mann-Whitney test for lung weight) were performed. Infarct size was used as a covariate in an ANOVA/ANCOVA of the functional data to correct for baseline differences in infarct size among groups. Differences in mortality were evaluated with the Breslow test. A level of P<0.05 was considered to represent a significant difference.

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

results

cultured adult hEPDCs

Within a few days, hEPDCs migrated from the epicardium dissected from the atrial appendages. In the first passage, mainly cobblestone and a few spindle-shaped cells were observed, whereas after 2 passages, all cells had adopted a spindle-shaped morphology (with loss of WT1 expression; Data Supplement Figure I). Cells could easily be kept in culture, with a constant doubling time between day 10 and day 50, after which they went into senescence (Figure 1). For a more extensive characterization of in vitro cultured hEPDCs, see Van Tuyn et al.17

short-term experiments

LV function

LV EF and SV decreased significantly after onset of MI. LV EF and SV were similar in the medium- and hEPDC-treated groups 2 days after surgery. However, at 14 days after surgery, significantly greater LV EF and SV were observed in the hEPDC-treated group than in the medium-treated group (Figure 2a and 2b). LV EDV and LV ESV were significantly smaller in the hEPDC-treated group than in the medium-treated group, both at 2 and 14 days after surgery (Figure 2c and 2d). Both medium- and hEPDC-treated mice showed heart-function parameters that differed significantly from the

Figure 1. Growth curves of adult hEPDCs in culture. Growth velocity is similar for different cultures.

Figure 2. EF (a), SV (b), EDV (c), and ESV (d) in hEPDC-treated, medium-treated, and sham-operated mice, 2 and 14 days after surgery. Please note that no intermediate time points were measured. Data are mean±SEM. *P<0.05 vs medium group; †P<0.05 vs sham group.
sham-operated animals. However, with respect to EDV on day 2 and SV on day 14, the hEPDC-treated group resembled the sham-operated group (Figure 2a through 2d).

LV Wall Structure
Histological evaluation showed that at day 15, transplanted hEPDCs had engrafted mainly in the ischemic LV wall and formed layers of cells (Figure 3a and 3b). Transplanted hEPDCs were not found in the lining of the coronary vessel wall (Figure 4a and 4b).

The infarcted host tissue only showed the remaining cardiomyocytes in the subendocardial and, to a lesser extent, subepicardial position, which was not different between the hEPDC and medium groups (Figure 3c and 3d). The cardiomyocytes and the intermediate fibrous wall tissue were negative for α-smooth muscle actin (Figure 3e and 3f). Nuclear PCNA expression was increased in the infarcted LV wall (Figure 3g) and the border zone (Figure 3h) of the hEPDC group compared with the medium group (Figure 3i and 3j). DNA damage was present in these areas of both the medium and hEPDC groups, as demonstrated by phospho-histone H2AX staining (not shown). Phosphohistone H3 staining revealed that the low number of mitotic figures was not different between groups (not shown).

Hearts from the hEPDC group showed a complex vascular network that consisted of capillaries, veins, and arterioles with a high vascular density throughout the ischemic area (Figure 4c) but that lacked the distribution of fine capillaries observed in healthy myocardium (Figure 4e). Medium-injected infarcted hearts were poorly vascularized, with small capillaries unequally dispersed throughout the ventricular wall (Figure 4d). When determined quantitatively, free-wall endothelial density, expressed relative to septal endothelial density, was significantly higher in all parts of the LV of the hEPDC group than in the medium group (Figure 4f and 4g). Wall thickness of the infarcted LV wall, the border zone, and the interventricular septum was significantly greater in the hEPDC-treated group than in the medium-treated group (Figure 4h).

By double-staining techniques, we were able to investigate a number of differentiation markers in the engrafted hEPDCs. Almost all hEPDCs expressed α-smooth muscle actin (Figure 5a through 5c) and vWF (Figure 5d through 5f). A number of hEPDCs were positive for SERCA2a and SCN5a (Figure 5g through 5l). The transplanted cells did not express the (cardio)myocyte markers atrial natriuretic peptide, cardiac troponin I, sarcomeric myosin, or α/γ-smooth muscle actin (not shown).

General Health and Survival
Only a minor decrease in body weight between the day of surgery and the day of euthanasia (day 15) was observed in the hEPDC-treated group (−0.5±0.6 g), which was not statistically different from that in the sham-operated group (0.1±0.3 g). In contrast, the decrease in body weight was significantly larger in the medium-treated group (−2.7±1.0 g) than in the sham-operated group or the hEPDC-treated group (Figure 6a). The decrease in body mass relative to the original weight amounted to 10±4% in the medium group, 2±2% in the hEPDC group, and 0±1% in the sham group. Similarly, no significant difference at day 15 in the amount of lung fluid, corrected for body weight, was observed between the sham-operated and hEPDC-treated group, whereas lung edema was present in the medium-treated group (Figure 6b).

Cumulative survival of medium-treated mice over the 2-week time period was significantly lower than that of the sham-
operated mice. Survival of hEPDC-treated mice was not different from survival of sham-operated animals (Figure 7).

**Long-Term Effect of hEPDC Transplantation**

The effect of hEPDC transplantation on cardiac function 6 weeks after MI was investigated in separate experiments. LV EF and SV were significantly greater 6 weeks after creation of MI in the hEPDC group than in the medium-treated group (Figure 8a and 8b). Similarly, LV EDV and ESV were significantly smaller in the hEPDC group than in the medium group (Figure 8c and 8d). Sham-operated animals showed values for these functional parameters that were all significantly different from those of the medium- and hEPDC-treated animals, except for SV (Figure 8a through 8d). No deaths were observed in the hEPDC or sham group, in contrast to 4 deaths in the medium group (Figure 8e).

Anti-eGFP staining only demonstrated a few engrafted hEPDCs, which were not embedded in the vessel lining (Figure 8f and 8g). No cells positive for human-specific CD31 or vWF were observed in sections consecutive to the eGFP-stained sections. In the frozen sections of the hEPDC group, no cells were detected that stained positive for human nucleus (not shown).

Wall thickness of infarcted area and border zone was increased in the hEPDC group compared with the medium group (Figure 8h). Properties of the scar itself were not significantly different between hEPDC- and medium-treated animals. The remaining cardiomyocytes observed in the scar area at week 2 had disappeared. The infarcted area in both groups consisted mainly of fibrous tissue (Figure 8i), with many vessels situated in the border zone (Figure 8j) and few vessels in the scar area (Figure 8k).

**Discussion**

The main findings of the present study are that adult human EPDCs (1) can be isolated and cultured, during which they
undergo EMT; (2) engraft and survive in ischemic murine myocardium for at least 2 weeks, whereas only a few cells can be detected at 6 weeks; and (3) preserve LV function and attenuate postischemic remodeling until 6 weeks after MI. Embryonic EPDCs are known to be of crucial importance during cardiogenesis. They are essential for proper myocardial architecture and coronary vessel formation, both through their physical contribution and through regulation of these developmental processes. Little is known about the role of adult EPDCs in the normal and diseased adult heart. We describe for the first time the use of adult hEPDCs, cells grown from human adult epicardial explants after EMT, in cardiac regeneration therapy, applying them to possibly trigger embryological developmental processes that might restore or preserve cardiac function.

Immunologic Characterization of hEPDCs In Vivo

After 2 weeks, many hEPDCs were observed in the LV wall. They expressed the smooth muscle cell marker α-smooth muscle actin. This is in line with the fact that embryonic EPDCs express this protein when they differentiate into coronary smooth muscle cells, in addition to interstitial and adventitial coronary fibroblasts. The α-smooth muscle actin–positive hEPDCs were not observed in the vessel wall but as isolated cells located in the scar tissue, having a shape similar to that of the surrounding fibroblasts. Part of the engrafted hEPDCs also expressed the marker SERCA2a, which is expressed in smooth muscle cells, skeletal muscle cells, and cardiomyocytes. Staining for α/γ-muscle actin (clone HHF35), normally expressed by almost all muscle cells, was negative in transplanted hEPDCs, which suggests that engrafted cells did not fully differentiate into a smooth muscle cell phenotype. The injected hEPDCs did not acquire a cardiomyocyte phenotype, because the engrafted hEPDCs did not express the cardiomyocyte markers atrial natriuretic peptide, sarcomeric myosin, or cardiac troponin I. This is consistent with the finding that EPDCs do not differentiate into cardiomyocytes during embryonic heart development. Remarkably, immunostaining for SCN5a was positive in some engrafted hEP-

Figure 5. Confocal microscopic images showing differentiation markers of engrafted hEPDCs in ischemic myocardium 2 weeks after MI. Most eGFP-positive hEPDCs (red; a, d) show colocalization with α-smooth muscle actin (ASMA; yellow [b] and merge [c]) and vWF (yellow [e], merge [f]). A number of eGFP-positive hEPDCs (red; g, j) express SERCA2a (yellow [h], merge [i]), and SCN5a (yellow [k], merge [l]). Scale bars=20 μm.
DCs, SCN5a is mainly expressed in cardiomyocytes and has recently also been described in human gastrointestinal smooth muscle. Therefore, we consider the expression pattern of the transplanted hEPDCs as that of a smooth muscle cell with extraordinary features, such as SCN5a expression.

Although the engrafted cells did not integrate into the vessel wall, a large portion of them were positive for the endothelial cell marker vWF at 2 weeks after MI. It is interesting that the engrafted adult hEPDCs expressed vWF, because the possible contribution of embryonic EPDCs to coronary endothelium is still a subject of debate. Relevant to these findings are reports describing that endothelial markers can also be expressed by nonendothelial cells, such as certain skeletal muscle cells.

The expression profile of cultured hEPDCs in vitro is different from that of engrafted hEPDCs in vivo. Whereas engrafted hEPDCs in vivo stained positive for α-smooth muscle actin, vWF, SERCA2a, and SCN5a proteins, hEPDCs in vitro contained only α-smooth muscle actin mRNA, and mRNA for SCN5a and SERCA2a was not observed. Moreover, vWF staining was negative in cultured hEPDCs in vitro. This implies that the expression pattern of hEPDCs changes in reaction to the surrounding ischemic tissue, which results in relatively undifferentiated engrafted cells with a myoendothelial phenotype.

The engraftment of the hEPDCs is temporary, because only a few hEPDCs could be detected at week 6. These cells did not express vWF, which indicates that the myoendothelial phenotype is at least partly transitional.

**Histological Characteristics of the Surrounding Host Tissue**

High vascular densities were observed in all parts of the LV wall of hEPDC-injected hearts 2 weeks after MI. The highly organized vascular network in the host tissue of the hEPDC group consisted of variably sized but mainly large-diameter vessels, whereas the medium group contained only a few vessels that were spread irregularly throughout the ischemic area. However, the high density of capillaries observed in healthy myocardium was lacking in the hEPDC group. The vessels must have been of murine origin, because no hEPDCs were observed to be integrated in the vessel lining, a finding confirmed at 6 weeks’ survival. It remains to be investigated whether more vessels had survived or new murine (host) vessels were formed after hEPDC injection. An indirect paracrine stimulatory effect of hEPDCs on vessel survival or angiogenesis is suggested, because (1) hEPDCs were not found in vessel linings, and (2) vessels were found throughout the entire LV wall of the hEPDC group, not just in areas with a high density of engrafted hEPDCs. At week 6, differences in vascular profiling had disappeared, which suggests a transitional effect of hEPDCs on the vessels.

The increased PCNA expression in the infarcted area and border zone of the hEPDC group compared with the medium group might explain in part the significantly increased wall thickness in these areas of the hEPDC group, both early and late after MI. PCNA is a central protein in both replication and DNA-damage repair. Because phosphohistone H3 staining revealed only a few mitotic figures, which was not different between groups, it is likely that the PCNA-positive cells represent cells with DNA-damage repair rather than proliferating cells. This was supported by the fact that DNA damage was indeed present in both groups. Increased cellular survival due to augmented repair might then have contributed to a thicker wall. Further research, however, is needed to unravel the processes that underlie the increased PCNA activity and to determine whether the PCNA-positive cells are indeed repairing DNA damage or whether they represent activated and proliferating cells as well.

On the other hand, diminished ventricular dilatation itself, as well as increased proliferation of cardiac stem cells and other host tissue cells immediately after MI (before day 14), might have affected wall thickness. It seems unlikely that new cardiomyocyte formation contributed to the increment in wall thickness in the hEPDC group, because the cardiomyocytes observed in the subendocardial and subepicardial regions of the infarcted area of the hEPDC and medium group were negative for α-smooth muscle actin.
which is normally expressed by primitive but not by adult cardiomyocytes. Moreover, whereas wall thickness at week 6 was still increased in the hEPDC-treated hearts, hardly any cardiomyocytes were observed in the ischemic area.

**Functional Improvement**

Main left anterior descending coronary artery occlusion results in MIs that vary in size, with associated variability in ventricular volumes. To discern possible treatment effects, it appears mandatory to determine the initial infarct size and to correct functional data for any differences in infarct size. We performed this step by assessing infarct size with contrast-enhanced MRI images and subsequent covariance analysis of functional parameters. Functional data were acquired by MRI, which is considered to be the “gold standard” for ventricular function assessment in small animals, creating high-resolution images, especially at 9.4T. In contrast to 1D or 2D echocardiography and conductance catheter measurements, computation of ventricular volumes from MRI images is not based on specific geometric assumptions but on real data, which makes it a reliable method for determination of infarcted, abnormally shaped hearts.

We showed that hEPDC transplantation in the acutely infarcted myocardium improved cardiac function 2 weeks after MI. EF (a) and SV (b) were significantly higher in the hEPDC-injected group 6 weeks after MI. hEPDC transplantation resulted in significantly smaller EDV (c) and ESV (d) than with medium injection. A better survival was observed in the hEPDC group than in the medium group (P<0.057; e). Only a few eGFP-positive hEPDCs (brown) were detected in the hearts 6 weeks after hEPDC transplantation, mainly in the border zone (f, g). hEPDCs (arrows) did not integrate in the vessel lining (g). Wall thickness of the infarcted area and the border zone was significantly higher in the hEPDC group than in the medium group 6 weeks after MI (h). i, j, and k are from hEPDC-treated hearts but are also representative of medium-treated hearts. Sirius red staining demonstrates that the infarcted area consisted of mainly fibrous tissue (i). Many vessels could be observed in the border zone (j), whereas hardly any vessels were found in the infarcted area (k). Data are mean±SEM. *P<0.05. RV indicates right ventricle; END, endocardium; EP, epicardium; a, artery; v, vein. Scale bars in f and g=30 μm; scale bar in i=600 μm; scale bars in j and k=60 μm.
after induction of MI. This improvement was represented by a higher EF, larger SV, and less lung edema in the hEPDC group than in medium-treated animals. Moreover, a smaller EDV in the hEPDC group demonstrated that ventricular remodeling was reduced by hEPDC transplantation. However, EDV was still 2- to 3-fold higher in the hEPDC group than in the sham group, which illustrates the fact that hEPDC transplantation does result in less deterioration or preservation of cardiac function, not in restoration of normal function. An early protective effect of the hEPDCs was indicated by the fact that a smaller LV EDV and ESV were observed as soon as 2 days after the onset of MI. It cannot be determined whether this early effect is responsible for the observed positive influence of hEPDC transplantation after several weeks. Studies in which hEPDCs are injected a few days after MI might reveal this contribution.

The increased survival proportions in the hEPDC group might be explained by the improved cardiac function and reduced ventricular remodeling, because LV dysfunction and mortality are highly correlated. Animals in the hEPDC group did not show cardiac cachexia 2 weeks after MI, defined as weight loss $>$7.5% of the original weight, which is known to be a severe complication of chronic heart failure and which is associated with a poor prognosis. Cardiac cachexia, however, was observed in the medium-treated group, which had a relative weight loss of 10%. Mice in the hEPDC group showed less lung edema than mice in the medium group, which illustrates that the absence of weight loss in the hEPDC group was not obscured by edema.

To investigate whether the beneficial effect of hEPDC transplantation on the infarcted heart remained until a definitive scar had been formed, an additional set of experiments was performed with analysis 6 weeks after MI. We demonstrated that EF and SV were still significantly higher in the hEPDC group than in the medium group. Moreover, ESV and EDV were again significantly smaller in the hEPDC-injected group, which demonstrates decreased remodeling 6 weeks after MI. The survival benefit for the hEPDC-treated group after 6 weeks confirmed the beneficial influence of hEPDC transplantation in the long term. These data suggest that the effect of hEPDCs on cardiac function is stable, although we still cannot exclude an early paracrine effect, as has been described for other stem cells.34,47

The exact mechanism underlying the improvement in cardiac function caused by hEPDC transplantation remains to be investigated. We suggest from the present data that the injected hEPDCs protect host tissue cells through augmented DNA-damage repair, which results in augmented cellular survival and subsequent prevention of extreme wall thinning, which will contribute to preservation of LV function and reduced functional remodeling in both the short and long term. A paracrine effect of the transplanted cells on the host tissue is indicated, because functional data demonstrate an effect of the hEPDCs as early as day 2, and because histological data show differences between groups in host tissue properties rather than newly formed donor-derived tissue in the hEPDC group.

Clinical Relevance
We showed that adult hEPDCs grow easily in culture during several passages and acquire spindle-shaped morphology, similar to embryonic EPDCs that have undergone EMT, which enables migration into the myocardium.2 Because they preserved cardiac function and reduced remodeling both early and late after the onset of MI, autologous EPDCs appear promising for use in cardiac regeneration therapy. Preferably, these cells should be injected intramyocardially, by use of the catheter-based methods described previously.48 In the present study, atrial appendages were harvested during CABG procedures. For broader clinical applications, a minimally invasive cardiac surgical technique is to be preferred, such as endoscopic surgery. Atrial appendages are removed by this technique as therapy in atrial fibrillation.49 Transplantation of autologous EPDCs will not generate ethical problems, and there will be little risk of rejection. This makes them ideal candidates for cell therapy. Spontaneous tumor formation is not an issue, because we demonstrated that adult hEPDCs do not divide indefinitely, nor did we observe any tumor formation in the ischemic mouse heart up to 6 weeks after transplantation. In a clinical setting, EPDCs can probably only be transplanted in the chronically infarcted, reperfused heart. We are currently studying this aspect.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

To date, no therapy has been developed that repairs cardiac damage after coronary artery occlusion. Cellular transplantation therapy might improve performance of the resulting scar tissue. We introduced a novel cell type, the epicardium-derived cell (EPDC), which is present in the adult heart and which might be applicable as additional therapy for the failing infarcted human heart. EPDCs are crucial for embryonic heart development, both through their regulatory role in formation of the compact myocardium and through their physical contribution in forming the extensive population of interstitial fibroblasts, as well as the coronary smooth muscle cells and adventitial fibroblasts. The role of adult EPDCs in the adult healthy and diseased heart has never been elucidated. We have shown for the first time in vivo that adult human EPDCs (hEPDCs) are still very potent. Transplantation of hEPDCs into the infarcted mouse heart results in preservation of left ventricular function and attenuation of remodeling both early and late after myocardial infarction. The influence on surrounding tissue is similar to the embryonic situation: Wall thickness is increased, together with an augmented vessel density. Translation to a clinical setting is promising; adult hEPDCs can be isolated easily from atrial tissue, which could be harvested during a thoracoscopic procedure. After culturing, autologous hEPDCs could be injected into the infarcted heart of the patient shortly after the ischemic event. The present data show that adult hEPDCs have high potential in the diseased heart, which makes them interesting candidates for additional therapy after a myocardial infarction.
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In the version of the article, “Preservation of Left Ventricular Function and Attenuation of Remodeling After Transplantation of Human Epicardium-Derived Cells Into the Infarcted Mouse Heart,” by Winter et al that was posted online on August 6, 2007 (DOI: 10.1161/CIRCULATIONAHA.106.668178), an error occurred.

In the Figure 8 legend, lines 23 through 26, the description of panels j and k read, “Hardly any vessels could be observed in the border zone (j), whereas many vessels were found in the infarcted area (k).” The description should have read, “Many vessels could be observed in the border zone (j), whereas hardly any vessels were found in the infarcted area (k).”

This error has been corrected in the final print version of the article in the August 21, 2007, issue of the journal (Circulation. 2007;116:917–927) and in the current online version. The publisher regrets this error.

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Materials and Methods

Primary cultured human adult EPDCs

EPDCs were cultured from human adult epicardium. Epicardium was separated mechanically from human atrial myocardium, which was obtained as leftover material after coronary artery bypass graft (CABG) procedures. Epicardial tissue was cut into small pieces, placed into two 9.5 cm² culture dishes (Primaria, BD Biosciences, San Jose, CA, USA) and covered with a coverslip to prevent floating. The epicardial graft was removed immediately after the first human EPDCs (hEPDCs) migrated from the explant. Cells were cultured in medium consisting of 45% DMEM, 45% M199, and 10% FCS, to which 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen, Paisley, UK) and 2 ng/mL bFGF (BD Biosciences) was added, at 37 °C in a humidified air-5% CO₂ atmosphere incubator. When cultures reached confluency, cells were trypsinized, diluted three times and reseeded. Growth curves were determined (n=4).

Cells from passage 1 (cobble stone shape) and passage 2-4 (spindle shape) were tested for their Wilms’ Tumor 1 suppressor protein (WT1) expression. Cells were fixed with methanol 100% for 15 min. After antigen retrieval with 0.1% Triton x-100 for 30 minutes (1086031000, Merck KGaA, Darmstadt, Germany) endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 15 min. Cells were incubated overnight with WT1 antibody (sc-192, Santa Cruz, Santa Cruz, CA, USA), combined with biotinylated goat anti-rabbit IgG (BA-1000, Vector Labs, Burlingame, CA, USA). The signal was amplified with the ABC staining kit (PK-6100, Vector Labs), using 3,3’-diamino-benzidine tetrahydrochloride (D5637, Sigma-Aldrich) as substrate for horseradish peroxidase. Sections were briefly counterstained with Mayer’s hematoxilin.
Virus-mediated eGFP transduction of hEPDCs

Cells from passage two to four (spindle shape) were used for transplantation experiments. To enable cell tracing, cultured adult hEPDCs were transduced with the enhanced green fluorescent protein (eGFP) gene 72h before transplantation. For the short term experiments (2 weeks survival), cells were transduced with the adenoviral vector hAd5/F50.CMV.eGFP. Cells were incubated with 50 infectious units (IU) of hAd5/F50.CMV.eGFP per cell. Because adenoviral expression is lost within a few weeks, hEPDCs used for long term experiments (6 weeks survival) were transduced with the lentiviral vector Lv.hPgc.eGFP (12.5 HeLa transducing units/ml per cell) instead of the adenoviral vector.

Creation of the MI and cell transplantation

To avoid rejection of transplanted human cells, 8-12 weeks old male nonobese diabetic-severe combined immunodeficient (NOD/scid) mice (Charles River, Wilmington, MA, USA) with a body weight of 25-30 g were used. All animal procedures were approved by the Animal Ethics Committee of the Leiden University and conformed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, Revised 1996). Animals were anesthetized with isoflurane 5% for induction and 1.5-2% for maintenance in a gas mixture of oxygen and N₂O (1:1). Mice were placed in a supine position on a controlled heating pad, intubated and ventilated mechanically (Harvard Ventilator) with a tidal volume of 240 µL and a frequency of 200/min. The thoracic cavity was opened in the fifth intercostal space. After opening of the pericardial sac, the left anterior descending coronary artery (LAD) was visualized and permanently ligated using 7.0 suture (Prolene, Johnson and Johnson, New Brunswick, NJ, USA). For short (2 weeks)
survival experiments, the location of ligation was 1 mm caudally from the tip of the left auricle. For long (6 weeks) survival experiments only the frontal branch of the LAD was ligated, since extended survival until 6 weeks is not possible with total LAD ligation. Transplantation of $4 \times 10^5$ hEPDCs suspended in a total volume of 20 µL M199 (hEPDC-group, survival 2 weeks: n=22, 6 weeks: n=15), or 20 µL cell-free M199 (medium-group, survival 2 weeks: n=24, 6 weeks: n=18) was performed by 5 injections using a beveled needle into the ischemic myocardium of the left ventricle and the border zone, immediately after ligation of the LAD. Sham-operated animals (survival 2 weeks: n=16, 6 weeks: n=3) were operated similarly, but without ligation of the LAD and without fluid injection. After recovery, animals received food and water with antibiotics (Ciproxin and Polymixin B, 10 mg/mL) and an antimycotic (Fungizone 10 mg/mL) ad libitum. Animals were randomized to treatment

**Short term experiments**

**MRI**

Infarct size (2 days after surgery) and cardiac function (2 and 14 days after surgery) were assessed using a vertical 9.4 Tesla Wide Bore magnet (AVANCE console) (Bruker, Etlingen, Germany) with a 30-mm birdcage resonator and an actively shielded gradient set, which had a maximum gradient strength of 1 T/m and a rise time of 110 µs. Bruker ParaVision 3.02 software was used for image acquisition. Animals were anesthetized with 5% isoflurane for induction and 1.5-2% isoflurane for maintenance in a mixture of oxygen and air (1:1) with a flow of 0.6 L/min, and placed head-up into an animal holder. Electrocardiogram (ECG) electrodes (3M, Red Dot™), attached to the right forelimb and the tail, and a respiration detection cushion placed under the thorax, were used to monitor ECG and respiratory rate (Bruker BioTrig).
Gadolinium-DOTA (Dotarem, Guerbet) was injected (37.5 µmol in 150 µL) via the tail vein 40 ± 15 min before contrast-enhanced images were made.

**Contrast-enhanced images for infarct size measurement**

To visualize and measure infarct size, 18 contiguous 0.5 mm short-axis contrast-enhanced images were obtained on day 2, covering the entire heart, using an ECG- and respiratory-triggered fast gradient echo (FLASH) sequence with a flip angle of 60° to null the signal from the myocardium. A 45 ms repetition time and an echo time of 1.9 ms were used. The signal was averaged 6 times. The field of view was 25.6 x 25.6 mm, projected on a 256 x 256 matrix, resulting in a pixel size of 100 x 100 µm.

**Cine images for cardiac function**

An ECG- and respiration-triggered cine FLASH sequence with a flip angle of 15°, a repetition time of 7 ms, and an echo time of 1.9 ms was used to assess cardiac function on day 2 and 14. During one cardiac cycle 18-30 frames were acquired, dependent on heart rate. The signal was averaged 4 times. The field of view was as described above. To cover the entire left ventricle, six to nine contiguous short axis slices of 1 mm thickness were made.

**Determination of infarct size and cardiac function**

The MRI images were converted to DICOM format for analysis with the MASS for Mice software package (Leiden, the Netherlands). The endocardial and epicardial borders were delineated manually by an observer blinded to the experimental groups (hEPDC-group n=17, medium-group n=14, sham-group n=13). Subsequently, the infarcted area of the left ventricle was computed automatically (the contrast-enhanced left ventricular (LV) area divided by the total LV area), as were LV end-diastolic volume (EDV), LV end-systolic volume (ESV), LV stroke volume (SV), and LV...
ejection fraction (EF)\(^4\). Infarcted area measurements were used to correct functional parameters for potential differences in initial infarct size.

**Immunohistochemical assessment**

Mice were sacrificed 15 days after surgery. Hearts were excised and fixed by perfusion, followed by immersion during 48 h at 4°C with 4% paraformaldehyde in phosphate-buffered saline (0.1 M, pH 7.4). Subsequently, hearts (n=5 per group) were embedded in paraffin, and sectioned at 5 µm. To block endogenous peroxidase, sections were incubated with 3% hydrogen peroxide for 15 min; antigen retrieval was accomplished by microwaving for 12 min at 98°C for all sections, except for those that were stained for α-smooth muscle actin and proliferating cell nuclear antigen (no antigen retrieval) or the endothelial marker CD31 (6-min treatment with pronase at room temperature). Sections were incubated at room temperature with primary antibodies overnight, and with secondary antibodies for 60 min. To investigate host tissue properties, serial sections were stained for CD31 (clone MEC13.3, 550274, Pharmingen, San Diego, CA, USA), α/γ muscle actin (clone HHF35, M0635, DAKO, Glostrup, Denmark), α-smooth muscle actin (clone 1A4, A2547, Sigma-Aldrich, St. Louis, MO, USA), proliferating cell nuclear antigen (PCNA, clone PC10, M0879, Dako), phospho-histone H3 (06-570, Upstate, Billerica, MA, USA), and phospho-histone H2A.X (clone JBW301, Upstate) combined with an appropriate secondary antibody. Anti- eGFP (A11122, Molecular Probes, Paisley, UK) staining was performed to visualize the injected hEPDCs because the strong and irregular autofluorescence of the heart disturbs assessment of spontaneous eGFP fluorescence to detect engrafted cells. The following secondary antibodies were used: biotinylated goat anti-rat IgG (559286, Pharmingen), biotinylated goat anti-rabbit IgG (BA-1000), biotinylated horse anti-mouse IgG (BA-2000, Vector Labs), and rabbit anti-mouse
IgG conjugated to horseradish peroxidase (P0260, DAKO). For visualization of eGFP, α/γ muscle actin, PCNA, phospho-histone H3, and phospho-histone H2AX staining, the signal was amplified with the ABC staining kit (PK-6100, Vector Labs), and for CD31 staining, visualization was enforced by the CSA system (K1500, DAKO), using 3,3’-diamino-benzidine tetrahydrochloride (D5637, Sigma-Aldrich) as substrate for horseradish peroxidase. Sections were briefly counterstained with Mayer’s hematoxilin.

To identify which proteins were expressed by the injected hEPDCs, double stainings were performed for eGFP and other proteins, being α-smooth muscle actin (clone 1A4), von Willebrand Factor (vWF, 4400-5884, Biogenesis, Poole, UK), sarcoplasmic or endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a, clone 2A7-A1, MA3-919, Affinity BioReagents, Golden, CO, USA), voltage-gated sodium channel (α-subunit) (SCN5a, sc-23174, Santa Cruz), cardiac troponin I, (cTnI, clone 19C7, 4T21, HyTest, Turku, Finland), atrial natriuretic peptide (ANP, clone 23/1, CBL66, Chemicon, Temecula, CA, USA), α/γ muscle actin (clone HHF35), and sarcomeric myosin (clone MF20, Hybridoma Bank, Iowa City, IA, USA). A Red Qdot conjugated goat anti-rabbit antibody (Q11421MP, Invitrogen) was used to visualize eGFP staining. An appropriate biotinylated secondary antibody (donkey anti-goat [605-706-002, BioTrend, Cologne, Germany] or horse anti-mouse [BA-2000, Vector Labs]) in combination with Yellow Qdot conjugated to streptavidin (Q10111MP, Invitrogen) was used to visualize the other proteins. Stained sections were analyzed by light microscopy or confocal microscopy (Leica CTR 6000, Leica Confocal Software v2.6.1, excitation laser of 488 nm).
LV vascular profile and wall thickness

To evaluate the angiogenic effect of hEPDC transplantation, the cumulative area of CD31-stained vessel lining per total LV area was determined in the hEPDC- and medium-group (n=5 for each group). Measurements were performed in five different sections, which were chosen in a uniform manner, using apex and ligature for orientation. The most apical section was located 50 µm cranially from the apex, the most basal section was situated 50 µm caudally from the ligature. Three other sections were equally distributed between the apical and basal sections. Endocardial staining was excluded from quantitative analysis. Per section, two areas in the interventricular septum, and six equally displayed areas in the free wall were analyzed (Figure 4f). To normalize for differences in staining intensity among sections, values determined in the free wall areas were expressed relative to the values of the interventricular septal areas of the corresponding section. Measurements were performed by an observer blinded to treatment, using Image Pro software package (Media Cybernetics, Silver Spring, MD, USA).

LV wall thickness was measured in the hearts of the hEPDC- and medium-group (n=5 for each group) by an observer blinded to treatment. Average LV wall thickness was quantified from microscopic images of five sections per heart (same sections as described for endothelial density measurements). Wall thickness was measured in both border zones of the infarcted wall, in the mid-infarcted area in between the border zones, and in the middle of the interventricular septum. Measurements were performed perpendicular to the ventricular or septal wall.

General health and survival

Body weight was determined just before surgery at day 0 and before sacrifice at day 15. Wet and dry weights (acquired after freeze-drying) of the lungs were measured
after sacrifice. Pulmonary water content was estimated by subtracting the dry weight from the wet weight. To correct for differences in body mass, the amount of lung fluid was expressed relative to body weight. Survival proportions were assessed. Death was defined as spontaneous death before sacrifice or death during anesthesia throughout MRI experiments.

**Long term experiments**

Experiments with evaluation 6 weeks after myocardial infarction were performed to investigate the effect of hEPDC transplantation after a definitive scar had been formed.

**MRI**

Because of the high risk of mortality during repeated imaging procedures, cine MRI images were performed (methods were similar to procedures described for short term experiments) only 42 days after surgery (sham-group n=3, medium-group n=14, hEPDC-group n=15), not on day 2.

**Immunohistochemical assessment**

Animals were sacrificed 43 days after MI. Excised hearts were treated as described above. Per group 3 paraffin embedded hearts were used for immunohistochemical evaluation. To identify injected cells, single non-fluorescent antibody stainings against eGFP, human specific CD31 (clone CLB-HEC/75, M1536, Sanquin, Amsterdam, the Netherlands) and human specific vWF (4400-5884, Biogenesis) were performed. Staining methods were similar to the above described protocols, using an appropriate biotinylated secondary antibody (also biotinylated horse anti-goat IgG [BA-9500, Vector Labs]) together with the ABC kit.
To perform staining for human nucleus (clone 235-1, MAB1281, Chemicon), 3 hearts (without fixation) of the hEPDC-group and the medium-group were embedded in Tissue-Tek (OCT compound, 4583, Sakura Finetek, Zoeterwoude, the Netherlands), frozen at -80° C for 48 h, and stored at -20° C before sectioning at 8 µm. Inhibition of endogenous peroxidase was performed as described above. Sections were incubated overnight with the primary antibody against human nucleus, subsequently incubated with biotinylated horse anti-mouse IgG (BA-2000, Vector Labs), blocked with normal Horse serum, and incubated with ABC staining kit (PK-6100, Vector Labs).

**LV vascular profile and wall thickness**

Sirius Red staining and antibody staining against CD31 (clone MEC13.3, Pharmingen), together with wall thickness measurements were used for host tissue investigation.

**Survival**

Survival proportions until sacrifice at day 43 were assessed according to the methods described for short term experiments.

**Statistical analysis**

Data are presented as mean ± standard error (SEM). Data were analyzed with SPSS software (SPSS Inc, Chicago, IL, USA). For comparisons of more than two groups, a one-way-ANOVA was performed (or the non-parametric Kruskal-Wallis for the dependent variable lung weight). If the omnibus tests among groups were significantly different, post-hoc tests between groups using t-tests (and Mann-Whitney for lung weight) were performed. Infarct size was used as covariate in an analysis of (co-)variance of the functional data to correct for baseline differences in
infarct size among groups. Differences in mortality were evaluated using the Breslow test. A level of p<0.05 was considered to represent a significant difference.
Results

Cells from the first passage, mainly cobble stone shaped, demonstrated a high expression of WT1 (Supplemental Figure 1a). Transformation of these cobble stone cells into spindle shaped cells was accompanied by reduction in WT1 expression (Supplemental Figure 1b).
Reference List


Figure 1. Expression of Wilms’ Tumor 1 suppressor protein (WT1) in adult hEPDCs. High expression of WT1 is observed in the cobble stone shaped cells grown from the epicardial explant (a). Spindle shaped cells from the second passage demonstrate a decreased WT1 expression (b). Scale bars: 60 µm.