Regenerative medicine in cardiovascular disease: from tissue engineering to tissue regeneration
Grauss, R.W.

Citation

Version: Corrected Publisher’s Version
License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from: https://hdl.handle.net/1887/12556

Note: To cite this publication please use the final published version (if applicable).
CHAPTER 7

PREervation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart

E.M. Winter
R.W. Grauss
B. Hogers
J. van Tuyn
R. van der Geest
H. Lie-Venema
R. Vicente Steijn
S. Maas
M.C. DeRuiter
A.A.F. deVries
P. Steendijk
P.A. Doevedans
A. van der Laarse
R.E. Poelmann
M.J. Schalij
D.E. Atsma
A.C. Gittenberger-de Groot

Circulation 2007;116:917-27
ABSTRACT

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 $4 \times 10^5$ eGFP-labelled EPDCs (survival 2 weeks: n = 22, 6 weeks: n = 15) or culture medium (n = 24 and n = 18 respectively). Left ventricular function was assessed using a 9.4T animal-MRI. 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 volume 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, together with an increased wall thickness which might be explained by augmented DNA-damage repair activity in the infarcted area. Immunohistochemical analysis showed massive engraftment of injected EPDCs at 2 weeks, expressing α-smooth muscle actin, von Willebrand Factor, sarcoplasmic reticulum Ca$^{2+}$-ATPase and 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.
INTRODUCTION

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. As therapeutic use of embryonic stem cells is ethically intricate and technically difficult in relation to possible rejection of the cells and tumor formation, use of adult stem cells seems 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 partly been unraveled (for review see [1]). Most of the cell types used are not known to be of importance during normal cardiogenesis. We chose to transplant epicardium-derived cells (EPDCs), as these cells are known to be crucial for cardiac development, both because of their physical contribution [2] as well as their modulatory role [3,4]. During embryogenesis, epicardium migrates from the extracardiac proepicardium to cover the premature heart consisting by that time of only myocardium and endocardium with cardiac jelly in between [5]. A subset of the epicardial cells undergoes epithelial-mesenchymal transformation (EMT). These cells are called EPDCs [2]. EPDCs migrate into the myocardium to differentiate into interstitial cardiac fibroblasts, subendocardial and atrioventricular cushion mesenchymal cells, and into coronary smooth muscle cells and adventitial fibroblasts [2,6-8]. Besides 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 [11,12,13], and Purkinje fiber cell differentiation [2,14] are dependent on EPDC regulation. If epicardial outgrowth is inhibited completely, the myocardium remains thin and normal septation does not take place, leading to early embryonic death [3]. Besides 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 spontaneously undergo EMT, observed by a transformation from cobble stone into spindle shape morphology, while losing their b-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 function [20], 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]. Based on the fact 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 positively modifying the surrounding myocardium.

In this 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].
MATERIALS AND METHODS

See the online data supplement (page 169) for an expanded Materials and Methods section.

Primary Cultured Human Adult EPDCs

EPDCs were cultured from human adult epicardium which was separated mechanically from atrial appendages. Spindle shaped cells (see online supplement Figure 1) from passage two to four were used for transplantation experiments after transduction with a human vector expressing the enhanced green fluorescent protein (eGFP) gene, enabling cell tracing. An adenoviral and a lentiviral vector were used for short term (2 weeks survival) and long term (6 weeks 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 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). For short term experiments, the main left anterior descending coronary artery (LAD) was permanently ligated. For long term experiments only the branch supplying the ventral wall of the left ventricle was ligated, since extended survival until 6 weeks is not possible with the entire left ventricle infarcted.

Immediately after ligation, transplantation of $4 \times 10^5$ hEPDCs suspended in M199 (hEPDC-group, survival 2 weeks: $n=22$, 6 weeks: $n=15$), or cell-free M199 (medium-group, survival 2 weeks: $n=24$, 6 weeks: $n=18$) was performed into the ischemic myocardium. 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. 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 contrast-enhanced and cine MRI images (9.4 Tesla). Images were analyzed by manual delineation of endocardial and epicardial borders (hEPDC-group $n=17$, medium-group $n=14$, sham-group $n=13$) using dedicated software (see online supplement for detailed information). Left ventricular (LV) infarcted area, LV end-diastolic volume (EDV), LV end-systolic volume (ESV),
LV stroke volume (SV), and LV ejection fraction (EF) were computed automatically [24]. Infarcted area measurements were used to correct functional parameters for potential differences in initial infarct size.

**IMMUNOHISTOCHEMICAL ASSESSMENT**

Animals were sacrificed 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), phospho-histone H3, and phospho-histone H2AX. Non-fluorescent 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, being 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, (cTnI), atrial natriuretic peptide (ANP), 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 online 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 in 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 sacrifice at day 15. Pulmonary water content was estimated after sacrifice 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

MRI
The effect of hEPDC transplantation on 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 sacrificed 43 days after surgery. Excised hearts were treated as described for short term experiments, besides 3 hearts of the medium- and hEPDC- group that were frozen. To identify injected cells, non-fluorescent stainings for eGFP, human specific CD31 and human specific vWF were performed in paraffin sections, and for human nucleus in frozen sections.

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 ± 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.

The authors had full access to the data and take responsibility for its integrity. 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 cobble stone and a few spindle shaped cells were observed, while after two passages all cells had adopted a spindle shaped morphology (with loss of WT1 expression, see online supplement Figure 1). 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].

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

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 group 2 days after surgery. However, at 14 days after surgery a significantly higher LV EF and SV were observed in the hEPDC-treated group than in the medium-treated group (Figure 2a, b). 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, d). Both medium- and hEPDC-treated mice showed heart-function parameters that differed significantly from the 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-d).
Figure 2. Ejection fraction (a), stroke volume (b), end-diastolic volume (c) and end-systolic volume (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 versus medium-group, †: p < 0.05 versus sham-group.

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, b). Transplanted hEPDCs were not found in the lining of the coronary vessel wall (Figure 4a,b).

The infarcted host tissue only showed remaining cardiomyocytes in the subendocardial and, to a lesser extent, in the subepicardial position, which was not different between the hEPDC- and medium-group (Figure 3c, d). The cardiomyocytes and the intermediate fibrous wall tissue were negative for α-smooth muscle actin (Figure 3e, f). Nuclear PCNA expression was increased in the infarcted left ventricular wall (Figure 3g) and the border zone (Figure 3h) of the hEPDC-group as compared to the medium-group (Figure 3i, j). DNA damage was present in these areas of both the medium- and the hEPDC-group, as was demonstrated by phospho-histone H2AX staining (not shown). Phospho-histone 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 consisting of capillaries, veins and arterioles with a high vascular density throughout the ischemic area (Figure 4c),
but lacking the distribution of fine capillaries as 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 left ventricle of the hEPDC-group as compared to the medium-group (Figure 4f, g).

Wall thickness of the infarcted LV wall, the border zone and the interventricular septum was significantly larger in the hEPDC-treated group than in the medium-treated group (Figure 4h).

Figure 3. Photomicrograph of representative sections 2 weeks after MI demonstrating engrafted eGFP-positive hEPDCs (brown) in a hEPDC-treated heart (a), which are absent in the medium-treated heart (b). Remaining cardiomyocytes (expressing α/γ muscle actin [HHF35]) in the hEPDC- (c) and the medium-group (d) are positioned in the subendocardial and subepicardial region of the ischemic left ventricular wall. Consecutive sections show that the cardiomyocytes are negative for α-smooth muscle actin (ASMA) (e, f). The number of PCNA-positive cells (brown) is increased in the infarcted left ventricular wall (g) and border zone (h) of the hEPDC-group as compared to the infarcted area (i) and border zone (j) of the medium-group. LV: left ventricle, RV: right ventricle, VS: ventricular septum, END: endocardium, EP: epicardium. Scale bars a, b: 300 μm, scale bars c-f: 90 μm.
Figure 4. Vascular profile and wall thickness at 2 weeks survival. Two consecutive sections demonstrate that eGFP-positive hEPDCs (arrows) (a) were not incorporated into the CD31 positive murine (host) vessels (arrowheads) (b). Endothelial CD31 staining in the LV wall shows a high vascular density and a complex vascular network consisting of small capillaries (arrowheads) and irregularly shaped large vessels (arrows) in the hEPDC-group (c), which are lacking in the medium-group (d). These profiles are very different from the capillary rich myocardium in the sham-operated group (e). Quantification of endothelial density (expressed relative to septal values [dotted areas in f]) demonstrated a significant higher degree of vascularization in all parts of the left ventricle, represented by numbers 1-6 (f), in the hEPDC-group compared to the medium-group (g). Wall thickness of the border zone, infarcted area and interventricular septum was significantly larger in the hEPDC-group than in the medium-group (h). This difference is also visible in the pictures showing the LV wall of the hEPDC-group (c) and medium-group (d). Data are mean ± SEM. *: p < 0.05 versus medium-group. LV: left ventricle, RV: right ventricle, END: endocardium, EP: epicardium. Scale bars a–e: 200 μm, scale bar d: 400 μm.

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-c) and vWF (Figure 5d-f). A number of the hEPDCs was positive for SERCA2a and SCN5a (Figure 5g-l). The transplanted cells did not express the (cardio)myocyte markers ANP, cTnI, sarcomeric myosin, and α/γ muscle actin (not shown).
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, 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.
GENERAL HEALTH AND SURVIVAL

Only a minor decrease in body weight between day of surgery and day of sacrifice (day 15) was observed in the hEPDC-treated group (-0.5 ± 0.6 g), which was not statistically different from that of the sham-operated group (+0.1 ± 0.3 g). In contrast, decrease in body weight was significantly larger in the medium-treated group (-2.7 ± 1.0 g) than in the sham-operated group and the hEPDC-treated group (Figure 6a). Decrease in body mass relative to the original weight amounted 10 ± 4% in the medium-group, 2 ± 2% in the hEPDC-group and 0 ± 1% in the sham-group.

![Graph a](image1)  ![Graph b](image2)

Figure 6. Decrease in body weight (a) and amount of lung edema (b) in three groups of mice, being medium-, hEPDC-, and sham-group at 2 weeks after surgery. Data are mean ± SEM. ‡: p < 0.05 versus hEPDC-group, †: p < 0.05 versus sham-group.

Similarly, no significant difference at day 15 in amount of lung fluid, corrected for body weight, was observed between the sham-operated and hEPDC-treated group, while 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).

![Graph](image3)

Figure 7. Survival proportions of sham-operated, hEPDC-treated and medium-treated mice until day 15, by which time all animals were sacrificed. Significantly more animals died in the medium-group than in the sham-group. †: p < 0.05 versus sham-group.
**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 higher 6 weeks after creation of MI in the hEPDC-group compared to the medium-group (Figure 8a, b). Similarly, LV EDV and ESV were significantly smaller in the hEPDC-group than in the medium-group (Figure 8c, d). Sham-operated animals showed values for these functional parameters that were all significantly different from those of the medium- and hEPDC-operated animals, except for SV (Figure 8a-d). No death was observed in the hEPDC- and sham-group, in contrast to four 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, g). 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 to the medium-group (Figure 8h). Properties of the scar itself were not significantly different between hEPDC- and medium-treated animals. The remaining cardiomyocytes as observed in the scar area at week 2 had disappeared. The infarcted area consisted in both groups of mainly 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 our study are that adult human EPDCs i) can be isolated and cultured, during which they undergo EMT, ii) engraft and survive in ischemic murine myocardium for at least 2 weeks while only few cells could be detected at 6 weeks, and iii) preserve LV function and attenuate post-ischemic 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 [2,3,10]. 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 [17], in cardiac regeneration therapy, applying them to possibly trigger embryological developmental processes which might restore or preserve cardiac function.

IMMUNOLOGICAL 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, besides interstitial and adventitial coronary fibroblasts [2]. 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 [25]. Staining for α/γ muscle actin (clone HHF35), normally expressed by almost all muscle cells [26], was negative in transplanted hEPDCs, suggesting that engrafted cells did not fully differentiate into a smooth muscle cell phenotype.

The injected hEPDCs did not acquire a cardiomyocyte phenotype, as the engrafted hEPDCs did not express any of the cardiomyocyte markers ANP, sarcomeric myosin or cTnI. This is consistent with the finding that EPDCs do not differentiate into cardiomyocytes during embryonic heart development [2,6]. Remarkably, immunostaining for SCN5a was positive in some engrafted hEPDCs. SCN5a is mainly expressed in cardiomyocytes, and has recently also been described in human gastrointestinal smooth muscle [27]. 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 part of them was positive for the endothelial cell marker vWF at 2 weeks after MI. It is interesting that the engrafted adult hEPDCs expressed vWF, as the possible contribution of embryonic EPDCs to coronary endothelium is still a subject of debate [13,28,29]. Relevant to these findings are reports
describing that endothelial markers can also be expressed by non-endothelial cells, such as certain skeletal muscle cells [30].

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, hEPDC in vitro contain only α-smooth muscle actin mRNA, while mRNA for SCN5a and SERCA2a is not observed. Moreover, vWF staining is negative in cultured hEPDCs in vitro [17]. This implies that the expression pattern of hEPDCs changes in reaction to the surrounding, ischemic tissue, resulting in relatively undifferentiated engrafted cells with a myo-endothelial phenotype [30]. The engraftment of the hEPDCs is temporary, since only few hEPDCs could be detected at week 6. These cells did not express vWF, indicating that the myo-endothelial 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 few vessels that were spread irregularly throughout the ischemic area. However, the high density of capillaries as observed in healthy myocardium was lacking in the hEPDC-group. The vessels must be of mouse origin, since no hEPDCs were observed 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, as (i) hEPDCs were not found in vessel linings, and (ii) vessels were found throughout the entire LV wall.
wall of the hEPDC-group, and not only in areas with a high density of engrafted hEPDCs. At week 6 differences in vascular profiling had disappeared, suggesting a transitional effect of hEPDCs on the vessels.

The increased PCNA expression in the infarcted area and border zone of the hEPDC-group compared to the medium-group might partly explain the significantly increased wall thickness in these areas of the hEPDC-group, both shortly and long after MI. PCNA is a central protein in both replication and DNA-damage repair [31,32,33]. Since phospho-histone H3 staining revealed only 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 [34] due to augmented repair might then have contributed to a thicker wall. Further research, however, is needed to unravel the processes underlying 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 [35], as well as increased proliferation of cardiac stem cells [36] and other host tissue cells immediately after MI (before day 14) might have affected wall thickness [34,37-39]. It seems unlikely that new cardiomyocyte formation [40] contributed to the increment in wall thickness in the hEPDC-group, since the cardiomyocytes observed in the subendocardial and subepicardial region 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 [41]. Moreover, while wall thickness at week 6 was still increased in the hEPDC-treated hearts, hardly any cardiomyocytes were observed in the ischemic area.

FUNCTIONAL IMPROVEMENT

LAD occlusion results in MIs that vary in size with associated variability in ventricular volumes [42]. In order to discern possible treatment effects, it seems mandatory to determine initial infarct size and correct functional data for any differences in infarct size. We performed this by assessing infarct size with contrast-enhanced MRI images and subsequent covariance analysis of functional parameters [43]. Functional data were acquired using MRI, which is considered to be the gold standard for ventricular function assessment in small animals [44], creating high resolution images especially at 9.4 Tesla. In contrast to one- or two-dimensional 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 induction of MI. This improvement was represented by a higher EF, larger
SV, and less lung edema in the hEPDC-group compared to medium-treated animals. Moreover, a smaller EDV in the hEPDC-group demonstrated that ventricular remodeling was reduced by hEPDC transplantation. However, EDV was still 2-3 fold higher in the hEPDC group than in the sham-group, illustrating the fact 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 already 2 days after onset of MI. It can not 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, since left ventricular dysfunction and mortality are highly correlated [45]. The animals of the hEPDC-group did not show cardiac cachexia 2 weeks after MI, defined as weight loss >7.5 % of the original weight [46], and known as a severe complication of chronic heart failure 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, illustrating 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, demonstrating decreased remodeling 6 weeks after MI. The survival benefit for the hEPDC-treated group after 6 weeks confirmed the beneficial influence of hEPDC transplantation on the long term. These data suggest that the effect of hEPDCs on cardiac function is stable, although we still can not 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 our data that the injected hEPDCs protect host tissue cells through augmented DNA-damage repair, resulting in augmented cellular survival [34] and subsequent prevention of extreme wall thinning, which will contribute to preservation of left ventricular function and reduced functional remodeling both on the short and on the long term. A paracrine effect of the transplanted cells on the host tissue is indicated since functional data demonstrate an effect of the hEPDCs already at day 2, and since 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 shape morphology, similar to embryonic EPDCs that have undergone epithelial mesenchymal transformation enabling migration into the myocardium [2]. As they preserved cardiac function and reduced remodeling after MI, both shortly and long after onset of MI, autologous EPDCs seem promising for use in cardiac regeneration therapy. Preferably, these cells should be injected intramyocardially, using catheter-based methods described previously [48]. In the current 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 using this technique as therapy in atrial fibrillation [49]. Transplantation of autologous EPDCs will not give ethical problems, and there will be little risk of rejection. This makes them ideal candidates for cell therapy. Spontaneous tumor formation is no issue, as we demonstrated that adult hEPDCs do not divide indefinitely, nor did we observe any tumor formation in the ischemic mouse heart until 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.

ACKNOWLEDGMENTS
The authors are indebted to Pieter Voigt and Robert Klautz for kindly supplying the leftover surgical atrial appendages, to Jan Lens for excellent preparation of the figures, and to the animal facility.

FUNDING SOURCES
This research was funded by grant 53.345 from the Interuniversity Cardiology Institute of the Netherlands.
REFERENCES


166


[28] Poelmann RE, Gittenberger-de Groot AC, Mentink MM, Bokenkamp R, Hogers B. Development of the
cardiac coronary vascular endothelium, studied with antiendothelial antibodies, in chicken-quail chimeras.


Jacoby C, Molojavyi A, Flogel U, Merx MW, Ding Z, Schrader J. Direct comparison of magnetic resonance imaging and conductance microcatheter in the evaluation of left ventricular function in mice. Basic Res Cardiol. 2006;101:87-95.

Gottlieb SH, Ouyang P, Gottlieb SO. Death after acute myocardial infarction: interrelation between left ventricular dysfunction, arrhythmias and ischemia. Am J Cardiol. 1988;61:7B-12B.


Gneccchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. 2006;20:661-669.


ONLINE DATA SUPPLEMENT

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 EPDCS**
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 [1]. 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.hPgtk.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 [2]. 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 x 10⁵ 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 [3].

**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 Ca2+-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 (Q1011MP, 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).

Supplemental 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.
REFERENCES


