A New Direction for Cardiac Regeneration Therapy
Application of Synergistically Acting Epicardium-Derived Cells and Cardiomyocyte Progenitor Cells

Elizabeth M. Winter, MD, PhD; Angelique A.M. van Oorschot, MSc; Bianca Hogers, PhD; Linda M. van der Graaf, BSc; Pieter A. Doevendans, MD, PhD; Robert E. Poelmann, PhD; Douwe E. Atsma, MD, PhD; Adriana C. Gittenberger-de Groot, PhD; Marie Jose Goumans, PhD

Background—Adult human epicardium-derived cells (EPDCs), transplanted into the infarcted heart, are known to improve cardiac function, mainly through paracrine protection of the surrounding tissue. We hypothesized that this effect might be further improved if these supportive EPDCs were combined with cells that could possibly supply the ischemic heart with new cardiomyocytes. Therefore, we transplanted EPDCs together with cardiomyocyte progenitor cells that can generate mature cardiomyocytes in vitro.

Methods and Results—EPDCs and cardiomyocyte progenitor cells were isolated from human adult atrial appendages, expanded in culture, and transplanted separately or together into the infarcted mouse myocardium (total cell number, 4 × 10^5). Cardiac function was determined 6 weeks later (9.4T MRI). Coculturing increased proliferation rate and production of several growth factors, indicating a mutual effect. Cotransplantation resulted in further improvement of cardiac function compared with single cell-type recipients (P < 0.05), which themselves demonstrated better function than vehicle-injected controls (P < 0.05). However, in contrast to our hypothesis, no graft-derived cardiomyocytes were observed within the 6-week survival, supporting that not only EPDCs but also cardiomyocyte progenitor cells acted in a paracrine manner. Because injected cell number and degree of engraftment were similar between groups, the additional functional improvement in the cotransplantation group cannot be explained by an increased amount of secreted factors but rather by an altered type of secretion.

Conclusion—EPDCs and cardiomyocyte progenitor cells synergistically improve cardiac function after myocardial infarction, probably instigated by complementary paracrine actions. Our results demonstrate for the first time that synergistically acting cells hold great promise for future clinical regeneration therapy. (Circ Heart Fail. 2009;2:643-653.)

Key Words: cells ■ myocardial infarction ■ transplantation ■ epicardium ■ synergism

Considering the complexity of different activated pathways in an infarcted heart, it might seem reasonable to expect that transplantation of 2 complementary types of stem or progenitor cell population to treat the ischemic heart could be superior to single cell-type injection. It has been shown that different stem and progenitor cell populations, either resident in the heart or derived from an extracardiac source, improve left ventricular (LV) function when transplanted into the infarcted heart,1,2 probably instigated by a variety of yet unknown mechanisms. Outcome of current stem cell therapy might be further improved by combined transplantation of different cells with complementary properties,3-5 such as cells that support the surrounding host tissue in a paracrine way together with cells that supply the injured heart with new cardiomyocytes.

Clinical Perspective on p 653

We recently demonstrated that human adult epicardium-derived cells (EPDCs) can support and stimulate the surrounding resident tissue of the ischemic heart when transplanted into the infarcted mouse myocardium. This resulted in preservation of LV function and attenuation of LV remodeling.6 A possible paracrine protective effect of the EPDCs on the surrounding host tissue could be explained by recapitulation of their embryonic program, which is comprehensive.7-9 During embryonic development, EPDCs, which give rise to a variety of cells, including fibroblasts and smooth muscle cells10-12 (and some studies claim that epicardial progenitors differentiate into endothelial cells and cardiomyocytes,14 but this is still a subject of debate), have a crucial modulatory role. They regulate the formation of the...
compact myocardium and the development of the Purkinje fiber system, and they substantially contribute to coronary vessel formation.

Goumans and coworkers recently published that from the human adult heart, cardiomyocyte progenitor cells (CMPCs) can be isolated that have promising properties in vitro. In culture, they are able to differentiate into functional mature cardiomyocytes without the need of being cocultured with neonatal cardiomyocytes. These cells are mainly detected in the atrium, and they can be easily isolated from human adult atrial appendages by clonogenic expansion or, using their ability to cross-react with the mouse stem cell antigen-1 (Sca-1) antibody, by magnetic cell sorting. Because they are clonogenic, have self-renewal and multiple differentiation capacity, together with telomerase activity, and have a high nucleus to cytoplasm ratio, these CMPCs were considered true progenitors.

It is suggested that the adult human EPDCs that are transplanted into the infarcted mouse heart positively influence the ischemic host myocardium not only by protecting the existing myocardium but also by stimulating migration and proliferation of resident cardiac progenitor cells, similar to their effect during cardiogenesis. Conversely, embryonic and adult cardiomyocytes, and probably CMPCs in the adult heart, are dependent on interaction with EPDCs.

Regarding this mutual dependency and because CMPCs and EPDCs have complementary functions in cardiogenesis, we hypothesized that the demonstrated positive supportive effect of adult human EPDCs on the infarcted heart might further increase when the pool of resident cardiac progenitor cells is replenished through transplantation of adult human CMPCs at the same time.

**Methods**

Details about the materials and methods are shown in the online-only Data Supplement. EPDCs and CMPCs were isolated and cultured from human adult auricles as described previously and cultured separately or together (1:1) for in vitro and in vivo experiments (Figure 1).

Proliferation and migration were studied under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions in different groups: EPDCs, CMPCs, a mixture of EPDCs and CMPCs (1:1) (mix culture), EPDCs cultured in conditioned medium (CM) of CMPCs (EPDC+CMc), and CMPCs cultured in CM of EPDCs (CMPC+CMc). Proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and by Ki67 expression. Migration was assessed in a scratch assay and in Boyden chamber experiments.

Matrix metalloproteinase (MMP) expression was determined by zymography. mRNA and protein production of several growth factors were evaluated by real-time polymerase chain reaction and enzyme-linked immunosorbent assays, respectively.

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). Myocardial infarction was created in nonobese diabetic/severe combined immunodeficient mice, after which a total number of $4 \times 10^7$ CMPCs (CMPC group, n = 13), EPDCs (EPDC group, n = 20), mixed CMPCs and EPDCs (cotransplantation or CoT group, n = 14), or control vehicle (medium group, n = 17) were injected. Each group received the same number of cells. Sham-operated animals (sham group, n = 3) were operated on similarly, but the LAD was not occluded nor was anything injected. LV function was assessed with a 9.4T animal MRI 6 weeks later.

**Results**

In vitro experiments were performed to gain more insight into the influence that CMPCs and EPDCs may have on each other’s behavior after they have been transplanted into the infarcted myocardium, considering that the in vivo situation is too complex to distinguish between factors secreted by host tissue or engrafted cells.

**Proliferation and Migration In Vitro**

Growth rate of isolated CMPCs and EPDCs was determined and compared with that of the mixed cell culture. Proliferation was significantly increased when CMPCs and EPDCs were cultured together under hypoxia (cultured for 4 days in 20% oxygen followed by 3 days of 1% oxygen) compared with the average of separate cultures (Figure 2A). EPDCs could be considered mainly responsible for this effect because, under similar hypoxic conditions, proliferation of EPDCs cultured in conditioned medium of CMPCs was significantly higher than that of EPDCs cultured in regular medium, whereas proliferation of CMPCs was not influenced by conditioned medium of EPDCs (Figure 2B). Ki67 analysis of cocultured cells confirmed that EPDCs are stimulated in their growth in the presence of CMPCs (Figure 2C; supplemental Figure 1A).

In case of hypoxia, CMPCs and EPDCs, as well as their conditioned medium, negatively influenced the migration of the other cell type in a scratch assay. This effect was not observed when the cells were cultured in a normoxic envi-
When cultured together, CMPCs (red) are significantly more motile compared with EPDCs (green), regardless of oxygen level (supplemental Figure IB). Interestingly, conditioned medium from EPDCs grown under hypoxic conditions induced a chemotactic response in both cell types as assessed by a Boyden chamber assay (Figure 2E; supplemental Figure IC). Thus, in case of hypoxia, prolifer-ation is increased and cell mobility is decreased in the mix culture compared with average of single cell-type cultures, but EPDCs can induce a chemotactic response.

Matrix Modulation

Matrix remodeling is an important determinant for the degree of cardiac dilatation. Therefore, the levels of several proteases present in the different cultures were determined for various conditions according to the in vivo experiments using zymography (Figure 3A). Coculturing CMPCs and EPDCs together for 4 or 7 days under normoxic conditions increased the total amount of MMP-2, but pro-MMP2 and active MMP2 had not changed (Figure 3B through 3D; supplemental Figure II). Coculturing under hypoxic conditions for 7 days did not influence secretion of MMP-2 (Figure 3; supplemental Figure II) or MMP-9 (supplemental Figures III and IV).

Trombospondin-1 (TSP-1) mRNA expression in the mix culture significantly increased after 7 days of hypoxia compared with the average of single CMPC and EPDC culture (Figure 4A; supplemental Figure VA), whereas TSP-2 was higher in the mix culture after 4 days normoxia but not different after 7 days of culturing under any condition (Figure 4B; supplemental Figure VB). Tissue inhibitor of metallopro-

![Figure 2. Coculturing and hypoxia influence proliferation rate and migratory capacity.](http://circheartfailure.ahajournals.org/)
teinase (TIMP)-1 mRNA was significantly higher in the mix culture at days 4 and 7 in case of 1% oxygen (Figure 4C; supplemental Figure VC). Significantly less TIMP-2 mRNA was produced in the mix culture at day 7 (hypoxia) compared with the average of single cell-type cultures (Figure 4D; supplemental Figure VD). MMP-14 mRNA expression was significantly higher in the mix culture at day 7 in case of normoxia but not different for hypoxia (Figure 4E; supplemental Figure VE). Hence, culturing CMPCs and EPDCs as a mixture under hypoxic conditions does not change their production of active MMP-2 and -9, but it does influence mRNA expression of the indirect matrix modulators TSP-1 and TSP-2 and TIMP-1 and -2.

Paracrine Factors Secreted by Different Cultures
In general, vascular endothelial growth factor (VEGF)-A mRNA expression is increased during culturing under hypoxic conditions. VEGF-A mRNA expression in the mix culture is higher than that of the average of single cell-type cultures, although not significantly different after 7 days of hypoxia (Figure 5A; supplemental Figure VIA). In contrast, coculturing and hypoxia decrease VEGF-D mRNA (Figure 5B; supplemental Figure VIB). Placental growth factor mRNA expression is increased in case of hypoxia, with levels significantly higher in the mix culture compared with the average of single ones (Figure 5C; supplemental Figure VIC). Platelet-derived growth factor (PDGF)-BB expression is generally increased by hypoxia (Figure 5D; supplemental Figure VD). Under standard conditions, the mix culture expresses higher levels of heparin-binding epidermal growth factor-like growth factor after 7 days of culturing than the average of single cell-type cultures, but this effect is not seen in case of hypoxia (Figure 5E; supplemental Figure VIE). The increase in mRNA expression observed for VEGF, placental growth factor, and PDGF resulted in increased growth factor concentrations in the medium (supplemental Figures VII and VIII). Overall, combined culture of CMPCs and EPDCs under hypoxic conditions results in increased production of some angiogenic factors such as VEGF and PDGF-BB.

Cardiac Function After Cell Transplantation
LV function declined significantly after the onset of myocardial infarction, represented by a significantly smaller ejection fraction and stroke volume and a significantly larger end-systolic volume and end-diastolic volume in the medium group compared with the sham group (Figure 6A through 6D). Transplantation of EPDCs or CMPCs reduced this process: ejection fraction was significantly improved in both the EPDC and the CMPC group (Figure 6A), and end-systolic volume and end-diastolic volume were significantly decreased in comparison with the medium group (Figure 6C and 6D). CMPCs were slightly less potent than the EPDCs: stroke volume of the EPDC group was significantly larger than that of the medium group, whereas differences between the CMPC and the medium group did not reach statistical significance (Figure 6B). When EPDCs and CMPCs were transplanted as a mixture into the infarcted heart (CoT group), LV function was preserved even further. Ejection fraction and stroke volume were significantly higher in the CoT group not only when compared with the medium group but also when compared with the CMPC and EPDC groups (Figure 6A and 6B). Moreover, end-systolic volume and end-diastolic vol-
ume of the CoT group were comparable with noninfarcted hearts (sham group), whereas LV volumes of single cell-type recipients were significantly larger than values of the sham group (Figure 6C and 6D). Thus, cotransplantation of CMPCs and EPDCs resulted in an extra improvement of LV function and attenuation of remodeling on top of the effect of these cell types when applied separately.

Endogenous Murine Tissue Properties
Endothelial density in the border zone and infarcted area of the CoT group was significantly higher than that of the medium and EPDC group (Figure 7A and 7B). Values for the CMPC group were not different from any of the other groups determined (Figure 7A and 7B). Vasculature in the infarcted area (Figure 7E through 7H) was not characterized by the regular pattern of numerous capillaries as observed in normal myocardial tissue, but it consisted of an irregular pattern of capillaries and small veins (Figure 7I through 7L), some lymphatics (Figure 7M through 7P), and some arterioles (Figure 7Q through 7T) as indicated by endothelial expression of EphB4,31 lymphatic vessel endothelial hyaluronan receptor-1,32 and the smooth muscle marker α-smooth muscle actin (α-SMA), respectively. Small veins comprised the greater part of the vessels for each group. The CoT group demonstrated the highest wall thickness in the border zone and infarcted area, with values significantly different from the medium and CMPC groups (Figure 7C and 7D). Compared with the medium group, LV wall thickness was also significantly increased in these areas in the EPDC group (Figure 7C and 7D).

Graft Properties
In the medium group, no human cells could be observed as expected. Properties of the grafts observed in the CMPC, EPDC, and CoT group were comparable (Figure 8). Most
cells appeared as elongated-shaped cells engrafted in the infarcted area, dispersed along the entire longitudinal axis of the infarcted area. None of the observed engrafted cells in any group expressed the cardiomyocyte marker cardiac troponin I (Figure 8A through 8C). Although in each group a few human cells were observed incorporated in the vessel lining expressing \( \alpha \)-SMA or human CD31, most integrated CMPCs, EPDCs, and cotransplanted CMPCs and EPDCs did not express CD31 or \( \alpha \)-SMA (not shown). Cotransplantation did not alter the degree of engraftment: graft volumes were not different between groups (Figure 8D).

**Discussion**

In this study, we have demonstrated that 2 different cell populations, CMPCs and EPDCs, have their own unique pathway in which they support the infarcted heart, whereby another ratio (now used, 1:1) of these cells might be even more effective.

**CMPCs and EPDCs In Vitro**

The presence of various factors from murine and human origin in the infarcted heart having received the transplant complicates a detailed determination of the mutual effect of CMPCs and EPDCs on secreted products. Therefore, in vitro experiments were set up with conditions corresponding to the in vivo experiments.

Cocultured CMCPs and EPDCs might have been more potent than the single cell types in their capacity to support matrix remodeling in the infarcted heart. Not because of secretion of MMPs by the engrafted human cells themselves...
but rather by production of indirect matrix modulating factors. At the moment of transplantation, after 4 days of coculture, TSP-2 and TIMP-1 mRNA were significantly higher in the mix culture than in the average of single cell-type cultures. These factors are known to decrease cardiac MMP production, which might attenuate LV dilatation of the infarcted heart. TIMP-1 also stimulates cardiac fibroblast proliferation and has an antiapoptotic effect. Because TIMP-1 was still increased in the coculture after 7 days of hypoxia, TIMP-1 might have contributed to the increased wall thickness observed in the CoT group. TSP-1, which was also higher in the mix culture after 7 days of hypoxia culture, might have played a role in the observed improvement of LV function in the CoT group because TSP-1 has been demonstrated to protect the noninfarcted myocardium when expressed in the border zone of the infarct. The reduction in TIMP-2 mRNA expression in the coculture after 7 days of hypoxia suggests an attenuated inhibition of endothelial cell proliferation in the infarcted hearts of the CoT group, which is in line with the increased vessel density observed in vivo in the CoT group.

Coculturing CMPCs and EPDCs induced a significant increase in mRNA expression and protein levels of the angiogenic growth factor VEGF-A when compared with the average of the separate cultures, with a positive correlation between the hypoxia exposure time and the growth factor levels. It has been described that hypoxia-induced expression of VEGF-A can directly protect cardiomyocytes from ischemia. This suggests that the increased VEGF-A expression observed in the mix culture might have contributed not only to the increase in vessel density, together with the augmented placental growth factor and PDGF-BB production, but also to the higher wall thickness of the CoT group through enhanced tissue survival. VEGF-D, known to regulate lymphatic angiogenesis, was decreased in case of hypoxia, suggesting that lymphatic vessels, which were detected in the scar area, were not reorganized in the infarcted heart because of the human transplanted cells. No signs of increased vascular leakage or edema were observed. Furthermore, the observed chemotactic response of CMPCs and EPDCs toward conditioned medium of EPDCs grown under hypoxia might be explained by the production of VEGF-A, placental growth factor, and PDGF-BB.

PDGF-BB, necessary for maturation and stabilization of new vasculature, was increasingly secreted when CMPCs and EPDCs were cocultured under hypoxic conditions compared with the average of single cell-type cultures (supplemental Figures VII and VIII). Interestingly, in accordance with the findings that EPDC transplantation leads to transient augmented vascular density, PDGF-BB was largely produced by EPDCs under hypoxia at day 7. Although PDGF-BB production was not determined at later time points, it is not known whether this angiogenic factor also contributed to the sustained increase in vessel density as observed in the CMPC and CoT groups at week 6.

Heparin-binding epidermal growth factor-like growth factor, required for normal heart function and suggested to promote cardiomyocyte survival and stimulate cardiomyocyte contractility, was strongly downregulated in the mix culture under hypoxia, implying that a specific explanatory contribution of this growth factor to the observed differences among groups is unlikely.

**CMPCs and EPDCs Synergistically Stimulate Cardiac Function of the Infarcted Heart**

The most striking findings of this research included the significant further improvement of LV function in the group that had received the mixture of CMPCs and EPDCs compared with single cell-type recipients (although total cell number of the transplants was similar for each group).
Ejection fraction and stroke volume were significantly higher in the CoT group than in either single cell-type recipients, which themselves already improved LV function, but to a lesser extent. LV volumes were decreased in all cell transplant recipients compared with the medium group, but cotransplantation of CMPCs and EPDCs resulted in LV volumes that were, in contrast to the other groups, still larger but not significantly different from noninfarcted hearts. These results indicate a considerable additional attenuation of LV remodeling and increase of LV function caused by cotransplantation of the 2 cell types.

Morphology
In contrast to our expectations, the grafts of all 3 groups were comparable in their contents. In each group, most of the engrafted human cells were located in between murine scar fibroblasts, outside the vessel lining, and were negative for the tested endothelial and smooth muscle cell markers CD31 and α-SMA, respectively. In each group, although a few CD31- or α-SMA-expressing human cells were observed integrated in a vascular-like structure, this was extremely rare. None of the human cells expressed the cardiomyocyte marker cardiac troponin I, not even the CMPCs, which are demonstrated to easily acquire cardiomyocyte properties in vitro. A considerable contribution of new mature differentiated cells can thus not form the underlying explanation of the observed increase in cardiac function 6 weeks after cellular transplantation, connoting a paracrine mechanism.

Although proliferation rate was increased in the mix culture in case of hypoxia, the mice that received the mixture of CMPCs and EPDCs did not exhibit larger graft volumes than the single cell-type recipients, which implicates that in Figure 7. Endothelial density, expressed relative to vascular density of the interventricular septum, is significantly higher in the border zone (A) and infarcted area (B) of the CoT group compared with the medium and the EPDC groups. Values of the CMPC group are not different from any of the other groups (A and B). Wall thickness in the border zone (C) and the infarcted area (D) is significantly higher in the CoT group and the EPDC group than that in the medium group. Wall thickness of the CMPC group is significantly smaller than that of the CoT group (C and D). Differences in endothelial density and wall thickness with regard to the infarcted area are clearly visible in the representative pictures of CD31-staining within the scar (E through H). Vessel characteristics are shown in pictures of consecutive sections (I through T), which are magnifications of boxed areas in E through H. Small veins, indicated by endothelial EphB4 expression, comprise the largest part of the vasculature in the infarcted area of each group (I through L). Lymphatics can also be detected, as shown by endothelial lymphatic vessel endothelial hyaluronan receptor-1 expression (M through P). Arterioles are defined by surrounding smooth muscle cells as demonstrated by positive α-smooth muscle actin (α-SMA) staining (q through t). Scale bars in E through H represent 120 μm, and scale bars in I through T represent 30 μm. END indicates endocardium; EP, epicardium. *P<0.05.
our study, the additional effect of cotransplantation above single cell-type transplantation will not be due to an increase in the number of engrafted cells.\textsuperscript{48} Furthermore, in contrast to the results of the in vitro experiments, no signs of diminished migration of the human cells in the infarcted heart of the CoT group were observed: human cells were dispersed through the entire infarcted area in all hearts examined. We do however not know whether other factors such as the technique of transplantation\textsuperscript{49} or environmental prosurviving and -migration factors\textsuperscript{1,50} might have masked the subtle changes in migratory capacity.

In the CoT group, we could not discriminate between engrafted CMPCs and EPDCs because the 2 kinds were not marked, and no differences were observed between the engrafted human cells of the groups regarding their expression pattern, morphology, and graft size. Therefore, we cannot rule out that 1 cell type had survived preferentially.\textsuperscript{51} However, because there was a considerable additional favorable effect of combined transplantation above single cell-type transplantation, it is suggested that both cell types were present in the graft of the CoT group, although the exact proportion could not be determined.

As mentioned above, because most engrafted human cells of the 3 different transplant groups remained in a fairly undifferentiated state, with only scarce contribution to the vascular network, if at all, the positive effect of (co-)transplantation cannot be explained by cellular differentiation into new functional endothelial cells, smooth muscle cells, or cardiomyocytes as hypothesized for the CMPCs.\textsuperscript{21,22} Rather, factors secreted by the human grafts and/or activation of cascades in the murine cardiac tissue must be responsible for the observed improvements in the infarcted hearts.\textsuperscript{46,47} In line with this, the increased number of vessels observed in the border zone and infarcted area of the CMPC and CoT groups was of mouse origin, which also indicates a paracrine effect of the transplanted cells. Regarding the vascular pattern, it must be noted that the scar area in the CMPC and CoT groups did not exhibit the regular arrangement of numerous small capillaries as is characteristic for healthy myocardial tissue, but next to an irregular pattern of capillaries, it consisted of small veins, some arterioles, and lymphatics. With comparable graft size and distribution among groups, it is suggested that distinctive, complementary paracrine pathways underlie the additional effect of cotransplantation rather than increased levels of secreted factors. However, we cannot exclude that CMPCs and EPDCs mutually stimulate secretion of certain products regardless of cell numbers.

In conclusion, these results demonstrate that CMPCs and EPDCs, which are both crucial during cardiogenesis, are complementary and act synergistically in their improvement of cardiac function of the infarcted adult heart. The favorable effect of combined transplantation is at least partly explained by stimulation of distinct paracrine cascades. Our data suggest that future research must focus on unraveling the mechanisms underlying the positive effect of various stem cells that have been demonstrated to improve cardiac function and tissue properties of the infarcted heart, with the aim to identify many complementary acting cell types that can function synergistically, as we demonstrated for CMPCs and EPDCs. A balanced cocktail of cells with complementary paracrine and differentiation properties might ultimately lead to a promising cellular treatment of the infarcted heart.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

With view to improving the degree of stem cell benefit to heart function, we introduce a promising new concept. We demonstrated that by combining 2 different cardiac cell populations with complementary properties, function of the infarcted heart improves synergistically. We introduced “protective and supportive” human adult epicardium-derived cells together with adult cardiomyocyte progenitor cells to supply the ischemic heart with new cardiomyocytes as well as scaffold. In vitro studies showed increased proliferation and inhibited migratory capacity in coculture of these cells under hypoxic conditions and beneficial secretion profiles of proteases and growth factors. Transplantation of cardiomyocyte progenitor cells or epicardium-derived cells into the infarcted mouse heart improved left ventricular function as shown before, but cotransplantation of equal numbers of cardiomyocyte progenitor cells and epicardium-derived cells without altering cell numbers transplanted enhanced cardiac performance even further. No differentiation into cardiomyocytes was observed within the time frame of 6 weeks. The synergistic effect of epicardium-derived cells and cardiomyocyte progenitor cells was mainly explained by complementary paracrine actions. The basic concept of applying a combination of complementary cell populations into the infarcted heart holds great promise for clinical regeneration therapy. By applying the increasing knowledge on the underlying mechanisms to cell transplantation studies, future research could reveal the ultimate balanced cocktail of several synergistically acting cell populations.
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