A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells.

Winter: synergistic effect of cells from the adult heart

Elizabeth M. Winter1, MD, Angelique A.M. van Oorschot2, MSc, Bianca Hogers1, PhD, Linda M. van der Graaf1, BSc, Pieter Dovendans4, PhD, Robert E. Poelmann1, PhD, Douwe E. Atsma3, PhD, Adriana C. Gittenberger-de Groot*1, PhD, Marie Jose Goumans*2, PhD

1Dept. of Anatomy & Embryology, 2Dept. of Molecular Cell Biology, 3Dept. of Cardiology, LUMC; 4Dept. of Cardiology, UM CU; the Netherlands

*: authors contributed equally

Correspondence to: Prof Dr Adriana C. Gittenberger-de Groot, Department of Anatomy & Embryology, Leiden University Medical Center, Einthovenweg 20, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Tel: +31 71 526 9301. Fax: +31 71 526 8289. E-mail: acgitten@lumc.nl

Word Count: 7086

Subject Codes: [4] Acute myocardial infarction

[151] Ischemic biology – basic studies
**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. We therefore transplanted EPDCs together with cardiomyocyte progenitor cells (CMPCs) that can generate mature cardiomyocytes in vitro.

**Methods and Results:** EPDCs and CMPCs were isolated from human adult atrial appendages, expanded in culture, and transplanted separately or together into the infarcted mouse myocardium (total cell number: 4x10^5). Cardiac function was determined six weeks later (9.4T MRI).

Indicating a mutual effect, co-culturing increased proliferation rate and production of several growth factors. Co-transplantation resulted in further improvement of cardiac function compared to 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 6 weeks survival, supporting that not only EPDCs, but also CMPCs acted in a paracrine manner. Since injected cell number and degree of engraftment were similar between groups, the additional functional improvement in the co-transplantation group can not be explained by an increased amount of secreted factors but rather by an altered type of secretion.

**Conclusion**

EPDCs and CMPCs synergistically improve cardiac function after myocardial infarction, probably instigated by complementary paracrine actions. Our results demonstrate for the first time that synergetically acting cells hold great promise for future clinical regeneration therapy.

**Keywords:** myocardial infarction, cells, transplantation, synergism, epicardium
Introduction

Considering the complexity of different activated pathways in an infarcted heart, it might seem reasonable to expect that transplantation of two complementary types of stem or progenitor cell populations 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, probably instigated by a variety of yet unknown mechanisms. Outcome of current stem cell therapy might be further improved by combined transplantation of different cell types with complementary properties (Bonaros, 2006; Guarita-Souza, 2006; Nakanishi, 2008), like cells that support the surrounding host tissue in a paracrine way together with cells that supply the injured heart with new cardiomyocytes.

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. 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. During embryonic development, EPDCs, which give rise to a variety of cells including fibroblasts and smooth muscle cells (and some studies claim that epicardial progenitors differentiate into endothelial cells and cardiomyocytes), have a crucial modulatory role. They regulate the formation of the compact myocardium, 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 co-cultured 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 (MACS) \(^{21,22}\). Because they are clonogenic, and since they have self-renewal and multiple differentiation capacity, together with telomerase activity, and a high nucleus to cytoplasm ratio, these CMPCs were considered true progenitors \(^{22}\).

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 \(^{6,25,26}\). Conversely, embryonic \(^{7,8}\) and adult \(^{27}\) cardiomyocytes, and probably also CMPCs in the adult heart \(^{24}\), 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.

**Materials and Methods**

Details about the materials and methods are published in the online section. EPDCs and CMPCs were isolated and cultured from human adult auricles as described before \(^{6,28}\) \(^{21-23}\) and cultured separately or together (1:1) for *in vitro* and *in vivo* experiments (see 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 (CM) medium of CMPCs (EPDC+CMc), and CMPCs cultured in CM of EPDCs (CMPC+CMe). Proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and by Ki67 expression \(^{22}\). Migration was assessed in a scratch assay as well as in Boyden chamber experiments \(^{29}\).
Matrix metalloproteinase (MMP) expression was determined by zymography. mRNA as well as protein production of several growth factors was evaluated by real time polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) 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 (MI) was created in non-obese diabetic severe combined immunodeficient (NOD/scid) mice, after which a total number of 4x10^5 CMPCs, EPDCs, mixed CMPCs and EPDCs (Co-transplantation or CoT group), or control vehicle (Medium group) were injected. Please note that each group received the same number of cells. Left ventricular function was assessed with a 9.4T animal MRI six weeks later. Vascular density and wall thickness were evaluated in each group, as were the vessel characteristics and properties of the engrafted cells using several antibody stainings. The absolute volumes of the entire human grafts were determined and compared between groups.

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

In vitro experiments were performed to gain more inside into the influence 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 to that of the mixed cell culture. Proliferation was significantly increased when CMPCs and EPDCs were cultured together under hypoxia (4 days cultured in 20% oxygen followed by 3 days of 1% oxygen) compared to the average of separate cultures (Figure 2a). EPDCs could be considered mainly responsible for this effect, since under similar hypoxic conditions, proliferation of EPDCs cultured...
in conditioned medium of CMPCs was significantly higher than that of EPDCs cultured in regular medium, while proliferation of CMPCs was not influenced by conditioned medium of EPDCs (Figure 2b). Ki67 analysis of co-cultured 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 cells were cultured in a normoxic environment (Figure 2d). When cultured together, CMPCs (red) are significantly more motile compared to EPDCs (green) regardless of oxygen level (Supplemental figure 1b). 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 1c). Thus, in case of hypoxia proliferation is increased and cell mobility is decreased in the Mix-culture compared to 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). Co-culturing CMPCs and EPDCs together for 4 days or 7 days under normoxic conditions increased the total amount of MMP-2, but pro-MMP2 and active MMP2 had not changed (Figure 3b,c,d; Supplemental figure 2). Co-culturing under hypoxic conditions for 7 days did not influence secretion of MMP-2 (Figure 3; Supplemental figure 2) or MMP-9 (Supplemental Figure 3,4).

Trombospondin-1 (TSP-1) mRNA expression in the Mix-culture significantly increased after 7 days of hypoxia compared to the average of single CMPC and EPDC culture (Figure 4a; Supplemental figure 5a), while 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 5b). Tissue inhibitor of metalloproteinase (TIMP) -1 mRNA was significantly higher in the Mix-culture at day 4 and at day 7 in case of 1% oxygen (Figure 4c; Supplemental figure 5c). Significantly less TIMP-2
mRNA was produced in the Mix-culture at day 7 (hypoxia) compared to the average of single cell type cultures (Figure 4d; Supplemental figure 5d). 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 5e). 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, -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 6a). In contrast, co-culturing and hypoxia decrease VEGF-D mRNA (Figure 5b; Supplemental figure 6b). Placental growth factor (PIGF) mRNA expression is increased in case of hypoxia, with levels significantly higher in the Mix-culture compared to the average of single ones (Figure 5c; Supplemental figure 6c). Platelet-derived growth factor (PDGF)-BB expression is generally increased by hypoxia (Figure 5d; Supplemental figure 6d). Under standard conditions, the Mix-culture expresses higher levels of Heparin binding epidermal growth factor-like growth factor (HB-EGF) 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 6e). The increase in mRNA expression observed for VEGF, PIGF and PDGF resulted in increased growth factor concentrations in the medium (Supplemental Figure 7, 8). Overall, combined culture of CMPCs and EPDCs under hypoxic conditions results in increased production of some angiogenic factors like VEGF and PDGF-BB.

Cardiac function after cell transplantation

LV function declined significantly after onset of MI, represented by a significantly smaller ejection fraction (EF) and stroke volume (SV), and a significantly larger end-systolic volume (ESV) and end-diastolic volume (EDV) in the Medium group compared to the sham group (Figure 6a-d).
Transplantation of EPDCs or CMPCs reduced this process likewise: EF was significantly improved in both the EPDC and the CMPC group (Figure 6a), and ESV and EDV were significantly decreased in comparison to the Medium group (Figure 6c, d). CMPCs were slightly less potent than the EPDCs: SV of the EPDC group was significantly larger than that of the Medium group, while 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. EF and SV were significantly higher in the CoT group not only when compared to the Medium group, but also compared to the CMPC and EPDC group (Figure 6a, b). Moreover, ESV and EDV of the CoT group were comparable to non-infarcted hearts (sham group) while LV volumes of single cell type-recipients were significantly larger than values of the sham group (Figure 6c, d). Thus, co-transplantation 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, b). Values for the CMPC group were not different from any of the other groups determined (Figure 7a, b). Vasculature in the infarcted area (Figure 7e-h) 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 as well as small veins (Figure 7i-l), some lymphatics (Figure 7m-p), and some arterioles (Figure 7q-t) as indicated by endothelial expression of EphB4 \(^{31}\), LYVE-1 \(^{32}\), and the smooth muscle marker \(\alpha\)-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 group (Figure 7c, d). Compared to the Medium group, LV wall thickness was also significantly increased in these areas in the EPDC group (Figure 7c, d).
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-c). Although in each group a few human cells were observed incorporated in the vessel lining expressing α-smooth muscle actin (α-SMA) or human CD31, most integrated CMPCs, EPDCs and co-transplanted CMPCs and EPDCs did not express CD31 or α-SMA (not shown). Co-transplantation did not alter the degree of engraftment: graft volumes were not different between groups (Figure 8d).

Discussion

In this study we have demonstrated that two different cell populations, isolated from the human adult heart, which are complementary in their function during cardiogenesis, are more powerful in their protection and stimulation of the infarcted heart than either of these cell types separately. This implicates that both 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.

Co-cultured 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 co-culture, 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 anti-apoptotic effect. Since TIMP-1 was still increased in the co-culture after 7 days
hypoxia, TIMP-1 might have contributed to the increased wall thickness and attenuated LV
remodeling 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, as TSP-1 has been demonstrated to protect the non-infarcted myocardium when
expressed in the border zone of the infarct. The reduction in TIMP-2 mRNA expression in the
co-culture after 7 days 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.

Co-culturing CMPCs and EPDCs induced a significant increase in mRNA expression and
protein levels of the angiogenic growth factor VEGF-A when compared to 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 not only have contributed to the increase in vessel density,
together with the augmented PIGF 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 due to the
human transplanted cells. No signs of increased vascular leakage or edema were observed.
Furthermore, the observed chemotactic response of CMPCs and EPDCs towards conditioned
medium of EPDCs grown under hypoxia might be explained by the production of VEGF-A, PIGF,
and PDGF-BB.

PDGF-BB, necessary for maturation and stabilization of new vasculature, was
increasingly secreted when CMPCs and EPDCs were co-cultured under hypoxic conditions
compared to the average of single cell type cultures (Supplementary Figure 7,8). Interestingly, in accordance with the findings that EPDC transplantation leads to transient augmented vascular density⁶, PDGF-BB was largely produced by EPDCs under hypoxia at day 7. Whereas PDGF-BB production was not determined at later time points, it is not known whether this angiogenic factor contributed as well to the sustained increase in vessel density as observed in the CMPC and CoT group at week 6.

HB-EGF, required for normal heart function and suggested to promote cardiomyocyte survival and stimulate cardiomyocyte contractility⁴⁴,⁴⁵, was strongly downregulated in the Mix-culture under hypoxia, implying that a specific explanatory contribution of this growth factor to the observed differences between 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 to single cell type-recipients (while total cell number of the transplants was similar for each group). EF and SV were significantly higher in the CoT group than in either single cell type-recipient, which themselves already improved LV function but to a lesser extent. LV volumes were decreased in all cell transplant-recipients compared to the Medium group, but co-transplantation of CMPCs and EPDCs resulted in LV volumes that were, in contrast to the other groups, still larger but not significantly different from non-infarcted hearts. These results indicate a considerable additional attenuation of LV remodeling and increase of LV function due to co-transplantation of the two cell types.

Morphology

In contrast to our expectations, the grafts of all three 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 though, a few CD31 or α-SMA expressing
human cells were observed integrated in a vascular like structure, but 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\textsuperscript{21,22}. 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\textsuperscript{46,47}.

While 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 our study the additional effect of co-transplantation above single cell-type transplantation will not be due to an increase in the number of engrafted cells\textsuperscript{48}. Also, 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 like the technique of transplantation\textsuperscript{49} or environmental pro-surviving 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 two kinds were not marked, and because no differences were observed between the engrafted human cells of the groups regarding their expression pattern, morphology and graft size. We can therefore not rule out that one cell type had survived preferentially\textsuperscript{51}. However, since 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, since most engrafted human cells of the three 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 can not 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 group 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 group 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 co-transplantation rather than increased levels of secreted factors. We can however not 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 which can function synergistically like 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.

Acknowledgments

We are thankful to Jan Lens for his assistance in preparation of the figures, and to the Cardiothoracic Surgery department of the LUMC for their collection of the human left-over material.

Funding Sources
This research was funded by grant 05.303 from the Interuniversity Cardiology Institute of the Netherlands, and by a VIDI-grant (016.056.319, MJG) from the Netherlands Organization for Scientific Research (NWO).

Disclosures

None.
Reference List


Figure 1. Graph represents different culture conditions applied for in vitro experiments. For the proliferation assay condition A (4 days normoxia), B (7 days normoxia) and C (4 days normoxia followed by 3 days of hypoxia) were used. For the zymography, RT-PCR and enzyme-linked immunosorbent assay (ELISA) condition A, B, C and D (7 days of normoxia) were used. Condition A and C are in line with the in vivo experiments, in which cells were cultured during 4 days under standard conditions (20% oxygen) before being transplanted into the ischemic myocardium (represented by 1% oxygen culturing). Together, condition B, C and D demonstrate the effect of extended hypoxia, since absolute culture periods are similar.

Figure 2. Co-culturing and hypoxia influence proliferation rate and migratory capacity. In case of hypoxia, proliferation is significantly increased in the Mix-culture of CMPCs and EPDCs compared to the average of single cell type cultures (a). EPDCs are considered mainly responsible for the observed enhancement in proliferation rate: proliferation rate of EPDCs is significantly increased when cultured in CMPC-conditioned medium, while CMPCs are not influenced by EPDC conditioned medium (hypoxia) regarding proliferation rate (b). This was confirmed when analyzing the number of Ki67 positive nuclei per well, a marker for cell proliferation, under similar hypoxic conditions (c). Under hypoxia but not in case of normoxia, migratory capacity is significantly decreased when conditioned medium of the other cell type is applied and when CMPCs and EPDCs are cultured as a mixture (only in comparison to EPDC culture) (d). Using a boyden chamber assay, hypoxic EPDC conditioned medium chemo-attracted both CMPCs and EPDCs (e). CMPC+CMe: CMPCs cultured in conditioned medium of EPDCs, EPDC+CMc: EPDCs cultured in conditioned medium of CMPCs, MIX: Mix-culture of CMPCs and EPDCs. Non-CM: non conditioned medium. CMc: conditioned medium of CMPCs. CMe: conditioned medium of EPDCs. *: p<0.05

Figure 3. Matrix metalloproteinase (MMP) -2 production as determined by zymography. An example of a gelatin containing zymogram showing the different MMPs of medium from cells grown for 4 days 20% oxygen and 3 days 1% oxygen (a). Total MMP-2 (b) production is
significantly increased in the Mix-culture compared to the average of single cell type cultures, but
Pro-MMP-2 (c) and active MMP-2 levels are not changed (d). Values of day 4 are normalized to
culture medium. Values of day 7 are expressed relative to data for CMPC at day 7 (20% oxygen).
This difference for the subject of normalization is marked by the dotted line. The grey contour
within the black bar (average of single cell type cultures) indicates the relative contribution of
CMPCs (lower part) and EPDCs (upper part). *: p<0.05.

Figure 4. mRNA expression of matrix modulating factors in cultured cells. Co-culturing CMPCs
and EPDCs for 7 days under hypoxia results in significantly increased trombospordin (TSP) -1
mRNA expression compared to the average of single cell type cultures (a). In early Mix-cultures
TSP-2 mRNA expression is significantly augmented, but this effect disappears in time (b). Tissue
inhibitor of metalloproteinase (TIMP) -1 is significantly enhanced (c) but TIMP-2 is significantly
decreased (d) in the Mix-culture after 7 days of hypoxia. MMP-14 mRNA expression increases
significantly in case of co-culturing under standard conditions, but it is not altered in hypoxic
culture conditions (e). All data are expressed relative to values of CMPCs, with values of the two
groups at day 4 normalized to day 0 (not shown) and of day 7 to day 7 (marked by the dotted
line). The grey contour within the black bar (average of single cell type cultures) indicates the
relative contribution of CMPCs (lower part) and EPDCs (upper part). *: p<0.05

Figure 5. Growth factors in cultured CMPCs and EPDCs as measured with RT-PCR. Vascular
endothelial growth factor (VEGF) -A mRNA expression increases significantly in an hypoxic
environment and in case of co-culturing (a). VEGF-D mRNA expression is significantly decreased
in the (average of) single cell type culture as a result of hypoxia, but not in the Mix-culture in
which expression is already low in extended normoxia culture (b). In a hypoxic environment,
Placental growth factor (PIGF) expression is significantly higher in Mix-cultures than in the
average of single cell type cultures (c). Platelet-derived growth factor (PDGF) -BB expression is
augmented by hypoxia, but not influenced by co-culturing CMPCs and EPDCs (d). Heparin
binding epidermal growth factor-like growth factor (HB-EGF) mRNA expression is significantly
higher at day 7 (normoxia) in co-culture than in the average of single cell type cultures (e). All data are expressed relative to values of CMPCs, with values of the two groups at day 4 normalized to day 0 (not shown) and of day 7 to day 7 (marked by the dotted line). The grey contour within the black bar (average of single cell type cultures) indicates the relative contribution of CMPCs (lower part) and EPDCs (upper part). *: p<0.05

**Figure 6.** Left ventricular function at week 6 after induction of the myocardial infarction and cell transplantation. Ejection Fraction (EF) is significantly higher in the CMPC and EPDC group than in the Medium group, but a combined transplantation of these two cell types (CoT) results in an even better EF compared to single cell type recipients (p<0.05) (a). Note that the total number of transplanted cells is similar for each group (4x10^5). Stroke Volume (SV) of the CoT group is significantly higher than SV of the groups in which CMPCs or EPDCs are injected separately, among which the EPDC group is already superior to the Medium group regarding SV (b). End-systolic volume (ESV) and end-diastolic volume (EDV) of the CMPC, EPDC, and CoT group are significantly smaller than that of the Medium group. ESV and EDV of the single cell type recipients are still larger than those of the Sham group, while volumes of the CoT group are not significantly different anymore (c and d). #: p<0.05 versus all groups, *: p<0.05, ns: no significant difference.

**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 to the Medium and the EPDC group. Values of the CMPC group are not different from any of the other groups (a, 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 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-h). Vessel characteristics are shown in pictures of consecutive sections (i-t), which are magnifications of boxed areas in e-
h. Small veins, indicated by endothelial EphB4 expression, comprise the largest part of the
avasculature in the infarcted area of each group (i-l). Lymphatics can also be detected, as shown
by endothelial LYVE-1 expression (m-p). Arterioles are defined by surrounding smooth muscle
cells as demonstrated by positive α-smooth muscle actin (α-SMA) staining (q-t). *: p<0.05. END:
endocardium, EP, epicardium. Scale bars in e-h represent 120 μm, scale bars in i-t represent 60
μm

Figure 8. Graft properties. Double stainings for human specific Integrin β1 (green) and cardiac
Troponin I (red) demonstrate that none of the engrafted human cells in any of the groups
expressed this cardiac marker (a-c). Total graft volume was not different between the three
groups (d). Scale bars= 50 μm
A New Direction for Cardiac Regeneration Therapy: Application of Synergistically Acting Epicardium-Derived Cells and Cardiomyocyte Progenitor Cells.

Elizabeth M. Winter, Angelique A.M. van Oorschot, Bianca Hogers, Linda M. van der Graaf, Pieter Doevedans, Robert E. Poelmann, Douwe E. Atsma, Adriana C. Gittenberger-de Groot and Marie Jose Goumans

_Circ Heart Fail._ published online August 6, 2009;
_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/early/2009/08/06/CIRCHEARTFAILURE.108.843722