INFARCT CELL THERAPY Report Summary

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Final Report Summary - INFARCT CELL THERAPY (Therapy after heart infarct: prevention of reperfusion injury and repair by stem cell transfer)

A. PROJECT FINAL REPORT

A.1. FINAL PUBLISHABLE SUMMARY REPORT:

A.1.1. EXECUTIVE SUMMARY

A key problem in repair and functional regeneration following myocardial infarction is the obvious inability of heart muscle tissue to regenerate itself and appropriate vascularization under conditions of increased strain caused by the reduced contractibility of the damaged heart. This frequently leads to continuous loss of functional cells, further increase of the infarct area and finally complete loss of heart function.

In this project we therefore explored possibilities for cell therapy after myocardial infarction using different procedures and sources of stem and progenitor cells. We investigated factors stimulatory for stem/progenitor cell release and activation and evaluated adoptive transfer of stem/progenitor cells of different sources, from bone marrow, from adult and cord blood, from adipose tissue and from heart tissue itself. In addition, the use of ex vivo cultured and differentiated cells including murine embryonic stem cells and human induced pluripotent stem cells was tested. Furthermore, genetic modification of these cells for improved differentiation and tissue repair was evaluated. Central to this project was further the use of an unique artificial scaffold material as a slow release device for factors and as a structural support material for the application of different cell preparations to the damaged areas. This scaffold was also used for tissue engineering in vitro followed by insertion of artificial tissue onto the infarct area.

The following main results were obtained:

In the comparison of different stem/progenitor cell types it was shown that transplantation of stem cells from bone marrow (MAPCs and MSCs) and adipose tissue (ADSCs) induces a functional benefit in cardiac tissue despite a limited degree of long-term engraftment in animal models. Some clinical data on MSCs are already available. The mechanisms by which these cells contribute to cardiac repair are obviously to a large extent related to the release of paracrine factors. The best cells in this regard may be MSCs isolated from epicardial fat.

Transplantation of circulating endothelial progenitors (ECFCs/BOECs) led to the best observed long-term engraftment of cells in a porcine model of myocardial ischemia (MI), contributed to neovascularization and improved perfusion. As increased perfusion is the precondition for any repair and regrowth of myocardium, we propose that intracoronary transfusion of ECFCs could become a first step therapy to improve perfusion, which then could be followed or combined with therapies designed to induce cardiomyocyte growth. Based on this concept we have developed a protocol for a follow-up clinical phaseI/IIa trial to test autologous ECFCs in MI patients.

Importantly, we have also defined factors which seem to be responsible for the provascularization functions of ECFCs. We believe that these could be responsible for part of the positive effects of ECFCs and could possibly be used to substitute the...
cells or to increase their functionality when added with the infused cells.

Data using different biomaterials have further shown that transplantation of cells with biomaterials increase engraftment and functional improvement. A major outcome of this project is therefore also the development of a protocol for the combined application of the best performing cells, i.e. ADSCs and ECFCs, with a nanoparticle formulation of alginate biomaterial constituting a slow release form for two specific growth factors.

In regard of ex vivo generation of cardiomyocytes and preformed vascularized heart muscle patches significant progress could be achieved. Ex vivo cardiomyocytes could only be generated from embryonic or induced pluripotent stem cells but not from any adult stem cells. Despite efficient protocols for the ex vivo generation of cardiomyocytes have been developed, their use is still hampered by the low incorporation rate observed after transplantation and the need for correct electrophysiological coupling to the endogenous heart muscle which will have to be resolved. In addition, ethical and/or safety concerns prevent their application in clinical trials at the moment.

Significant hopes for future therapies are based on the further development of prevascularized patches of heart muscle grown ex vivo from mixtures of stem and/or differentiated cells, for which we have made significant progress through engineering alginate scaffolds for improved cell-matrix interactions and the application of various electric and magnetic stimulation patterns during cell growth.

A.1.2. SUMMARY DESCRIPTION OF PROJECT CONTEXT AND MAIN OBJECTIVES

Myocardial infarction is the leading cause of congestive heart failure and death in developed countries. It affects approximately 10 million patients in Europe and the United States, with about 800,000 new cases per year. Current treatment options are very limited in preventing ventricular remodeling and usually fail to achieve repair and replacement of damaged myocardium. The capacity of the heart muscle to regenerate itself is apparently very limited and usually a connective tissue-containing scar forms in the infarct area, which has reduced contraction capabilities. Because of this scar the heartbeat can become irregular, the still healthy muscle is further stressed and more cells die. This prevents healing and frequently leads to a further increase in the infarct area and finally complete loss of heart function. Given the high morbidity and mortality rates associated with congestive heart failure and the extremely limited availability of suitable donor hearts for transplantation, which remains the only therapeutic option, novel treatment options are clearly in demand.

The general objective of this project was therefore to evaluate selected factors, cell preparations and application procedures to promote tissue repair after heart infarct and chronic ischemic heart disease. It included i) the identification of key regulatory proteins of stem cell release and activation, ii) the testing of different adult and embryonic as well as induced pluripotent stem cell preparations, iii) their genetic modulation by vectors expressing key regulatory transcription factors or cytokines and iv) the use of scaffolds to improve cell transfer and to achieve tissue engineering in vitro.

The specific objectives have been:
1) to study natural regulators of stem cell release, heart neovascularization and growth of myocardium
2) to improve methods for adult and embryonic/induced pluripotent stem cell isolation, growth and differentiation
3) to develop the means and tools for the efficient delivery of therapeutic molecules and cells to the infarct zone using polymeric scaffolds
4) to use the scaffolds for tissue engineering in vitro
5) to evaluate in preclinical models selected factors as well as therapies based on transfer of stem cells and engineered tissue
6) to prepare the basis for corresponding clinical studies incorporating the best improved preclinical protocol developed in this project

The final goal of this project was i) the preclinical evaluation of selected molecules and procedures in animal models of heart ischemia and ii) the preparation of protocols for further clinical stem cell studies based on methodologies and procedures developed largely in the course of the project. In performing this project we first gained additional novel basic knowledge on
important pathways and regulatory molecules in stem cell release as well as stem cell differentiation to endothelial cells and cardiomyocytes. Second, we used this knowledge to improve procedures to promote the repair of damaged tissue by adoptive stem cell transfer including factors released from biomaterials and to develop methods of cell and tissue engineering.

A.1.3. SUMMARY DESCRIPTION OF THE MAIN S&T RESULTS / FOREGROUNDS

In WP1 Partner 2 has investigated mobilization of bone marrow precursors in murine models and patients. Experiments in mice with genetic loss of plasminogen activator inhibitor-1 and α2-antiplasmin have highlighted a novel mechanism whereby plasmin augments hematopoietic progenitor cell mobilization following GCSF. From these models, he adapted strategies to reproduce the biological changes using pharmacological interventions in wild type mice. By doing so, Partner 2 demonstrated that TNK or μPli were capable of enhancing HPC mobilization after G-CSF, which opens interesting therapeutic perspectives. He then investigated in a small group of patients receiving thrombolytic therapy the mobilization of hematopoietic progenitor cells. In line with mouse experiments performed in the first period, the clinical data show that thrombolytic agents (i.e. TNK or staphilokinase) enhance the mobilization of hematopoietic progenitors in humans and will potentially warrant their therapeutic use in individuals who mobilize poorly in response to GCSF. However, in the course of this project it has become clear from data of the participants and the results of others that in addition to hematopoietic stem cells other progenitor cell types originating from bone marrow or other tissues may carry a great and perhaps better therapeutic potential in ischemic disease. In this regard Partner 2 has, based on promising data from the first period in the evaluation of the therapeutic potential of endothelial progenitors in animal models, focused to a large degree on these cells in part in collaboration with Partner 1. A specific form of circulating endothelial progenitors called endothelial colony-forming cells (ECFCs) or late blood outgrowth endothelial cells (BOECs), which express significantly VEGF receptor-2 on their surface, have been shown to contribute to neovascularization of ischemic tissues and repair of injured endothelium. These cells differ from monocytic early outgrowth endothelial progenitors, which do not directly contribute to vascularization, in that they are not derived from the bone marrow but rather originate from the vessel wall of microvessels. Partner 2 has shown that intracoronary transfusion of autologous ECFCs/BOECs in a porcine model of myocardial ischemia conferred greater myocardial recovery than in controls or after infusion of mesenchymal stem cells. They were able to confirm that ECFCs/BOECs contributed to neovascularization in an autocrine fashion by incorporating into the endothelial cell layer of neovessels in the infarct border zone. Complementary MRI analysis showed that improved perfusion was capable of reducing LV dilatation over time to a significantly greater extent than in control animals or in pigs which received mesenchymal stem cells (see Dubois et al. JACC 2010). Partner 2 has then continued to investigate whether these cells could be isolated from the blood of MI patients and how they would functionally behave in comparison to the cells from healthy individuals. They found that these cells, in contrast to BM-derived early outgrowth endothelial progenitors, displayed an in vitro vasculogenic potential comparable for patients and healthy individuals. Taken together these data on ECFCs/BOECs may be the most important outcome of this project with immediate relevance for clinical therapies. This has prompted Partner 2 to develop a protocol as a basis for a follow-up phase I/IIa clinical trial which he is planning to perform in the near future enrolling 21 patients with chronic ischemic cardiomyopathy (see Deliverable D10.6 - Protocols as basis for clinical trials).

The results obtained by Partner 3 in WP2 during the first period of the grant indicated that transplantation of stem cells derived from the bone marrow or the adipose tissue induce a functional benefit in cardiac tissue despite a limited degree of long term engraftment (less that 2% of the transplanted cells remain in the myocardium after 3 months). The mechanism by which these cells contribute to cardiac repair are obviously related to the release of growth factors and chemokines that are able to induce a favorable remodeling of the heart and an improvement in systolic and diastolic function. Based on these results in the second part of the grant Partner 3 has undertaken 3 different approaches in order to improve the efficacy of these cell based therapies:
1. Tissue engineering approach: while cells injected directly into the heart do not engraft significantly providing them with a scaffold could result in a significant improvement in cell engraftment and hopefully in efficacy. Using a collagen based membranes of specific conditions we have demonstrated that our hypothesis is true. Transplantation of cellularized membranes was associated with an increase in engraftment and a functional improvement

2. Mechanism based approach: the use of growth factors for cardiac regeneration is hampered by several limitations including the short half life of the protein. On the other hand stem/progenitor cell-related beneficial effects are for the most part attributed to the release of cytokines and growth factors. In order to exploit both possibilities Partner 3 has developed a controlled release system based on the use of PLGA particles combined with cytokines endowed with cardiac potential. These formulations have been tested in vitro and in in vivo models of MI and demonstrate to contribute to improve cardiac function after MI

3. Ideal stem cell source: the development of the iPS technology has the potential to provide a new source of pluripotent stem cells which will be patient specific and lacking ethical concerns. During the second period of the grant Partner 3 has derived mice and human iPS cell lines and characterized their cardiac potential in vitro and in vivo, to determine whether this source of stem cells may have significant advantages over adult tissue-derived stem cells

In WP3, supported by the encouraging data of Partner 2 in the porcine MI model described above, Partner 1 has investigated ECFCs/BOECs isolated from cord blood. This was done in close collaboration with Partner 8 and Partner 9. The basic idea in using cord blood as a source of these cells is, that previous data had indicated that endothelial progenitors isolated from MI patients would have reduced functionality. In this situation and due to the availability of large cord blood banks, the generation of these cells with reparative function from cord blood would have important advantages. These are large HLA-typed collections of cord blood, which should make the selection of appropriate HLA-matched donors possible. Furthermore, there is evidence that cord blood-derived progenitors are superior in function, i.e. proliferative and migratory capacity in comparison to cells obtained from aged people. Partner 1 has followed the characterization of key regulators of differentiation and function of EPCs and has developed procedures to genetically modify their differentiation and function. In this regard factors were found to characterize the progenitor status of ECFCs/BOECs and to be at least in part responsible for their proliferative and migratory capacity. Partner 1 has followed the hypothesis that high expression of a certain transcription factor, which has been shown to modulate the Notch pathway (K.Lipnik et al, submitted) might be a functional determinant providing these cells with the reparative capacity displayed in the porcine experiment of Partner 2.

Partner 4 in WP 4 has investigated endogenous cardiac progenitor cells (CPCs) and compared various sources of mesenchymal stromal cells (MSCs). Major outcomes of the work are:
First, Partner 4 investigated what would be the best source of mesenchymal cells for heart repair. Based on the in vitro studies of Parner 4 it appears that human epicardial fat provides the best source of mesenchymal stromal cells (MSCs) with unique reparative properties for the heart. Compared with pericardial and peripheral fat, these cells have many similarities to those of atrial-derived cardiac progenitors (CPCs) plus improved regenerative potential. This is important, as in comparison to the isolation of atrial-derived CPCs this avoids the need for myocardial biopsies.
Second, these MSCs could be directed to differentiate into myogenic cells in vitro, by the demethylating agent 5-aza, and in vivo by transplantation into the heart.
Third, in the next series of experiments, Partner 4 showed that the origin of MSCs and cardiac progenitor cells (CPCs) dictates their reparative and immuno-modulatory properties. Surprisingly, in a rat model of MI, human MSCs derived from subcutaneous fat first emerged as the best cell to improve left ventricular (LV) remodeling and function. However, this could be explained by an immune reaction of the rat immune system against the human cells by. In the course of these studies Partner 4 discovered that cytokines secreted by the implanted cells are a major determinant of adverse cardiac remodeling and dysfunction, suggesting that appropriate regulation of inflammation is a key mechanism in cell therapy for infarct repair. Finally, with Partner 6, Partner 4 identified injectable biomaterials, such as alginate with growth factors, that can be used to improve cell retention and survival. They showed that CPCs could be stimulated in situ: The sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial, without added cells, improved myocardial rejuvenation.
In WP5 Partner 5 has improved incorporation of cardiomyocytes generated from embryonic and induced pluripotent stem cell into the hearts of a murine MI model. In order to improve engraftment, persistence and survival of the transplanted cardiomyocytes, multiple strategies were tested. First Partner 5, as a reference model, has used fetal mouse cardiomyocytes and could demonstrate an appropriate time-course of electrophysiological maturation and integration of the transplanted fetal cells. Second cardiomyocytes generated from embryonic and induced pluripotent stem cells were used. During the first period of this project Partner 5 has shown that transplantation of the cells alone leads only to very low incorporation rates of the cells. Now cotransplantation with various biomaterials and with MSCs was tested. When in vitro grown clusters of embryonic stem cell-derived cardiomyocytes with adult mesenchymal bone marrow cells were evaluated, the massive early loss of cells was prevented. Transplantation of these in vitro grown clusters seems to significantly slow down the loss. Similar results were obtained with iPS cell-derived cardiomyocytes. The most substantial improvement achieved with these strategies now leads to at least a few percent of the transplanted cell number persisting and surviving longer than 24 hours. This will still need additional substantial improvements when transplantation of iPS cell-derived cardiomyocytes should become a clinical reality.

During the 2nd period Partner 6 in WP6 developed scaffolds for efficient delivery of therapeutic cells to the infarcted zone and bioengineering of vascularized cardiac tissue. In addition, as part of WP10, this partner worked in collaboration with Partners 2 and 3 on the design, characterization and protocol preparation of multiple growth-factor delivery platforms to be combined with adult stem cell preparations in a large animal study (pigs) to improve cell survival, engraftment and function (WP10). Several concepts and strategies, and their combinations for efficient delivery of cells and for tissue bioengineering were investigated during the period. These included integration of multiple cell-matrix interactions into alginate scaffolds, along with application of various stimulation patterns (electrical or magnetic). Furthermore, Partner 6 investigated the integration of multiple cell types to improve cardiac cell organization and function. All this is aimed to improve cardiac cell organization and function and to promote vascularization, in order to create a platform for efficient cell delivery and cardiac tissue bioengineering, meeting the goals of milestone M6.2 and deliverable D6.3. Especially the integration of ECM-derived signals in alginate scaffolds and the addition of electrical or magnetic stimulation as in vitro maturation step showed promising results in terms of cell organization, tissue maturation and vascularization. Furthermore, Partner 6 in collaboration with Partner 4 was able to show that the combination of IGF with HGF in a slow release biomaterial-bound form improved regeneration of damaged heart muscle without added cells in a small animal model (Ruvino et al., 2011). This important finding was taken in WP10 to design a final evaluation of a combination of ECFCs with IGF and HGF to stimulate neovascularization as well as heart muscle regeneration. Importantly, Partner 6 found that the interactions between alginate-sulfate and the proteins resulted in spontaneous formation of nanoparticulate structures and that the bioconjugates of alginate-sulfate and the growth factors protected the proteins from proteolytic digestion.

Partner 7 in WP7 has studied in two- and three dimensional in vitro models with MSCs and ADSCs the influence of short and long term stimulation upon differentiation with VEGF into the endothelial direction. VEGF had little stimulatory effect alone, but serum starvation and stimulation with an endothelial cocktail of growth factors could stimulate the cells partially towards endothelial cells. There were no major differences in cell characteristics between MSCs and ADSCs isolated from healthy donors and patients with chronic ischemic heart disease. Partner 7 within a different parallel project has shown that treatment of patients with ischemic heart disease with mesenchymal stromal cells (MSCs) from the bone marrow seems to be safe and with a beneficial treatment effect (Friis et al., 2011; Haack-Sorensen et al., 2012). However, the treatment effect can demonstrate huge variations in clinical effect between different patients. This could be due to different homing capacity of the cells after injection into the heart. Therefore, clinical methods are needed to track stem cells after delivery to the heart to relate a clinical effect to the homing and efficacy of the cells. Therefore, within this project partner 7 has developed SOPs for isolation and culture expansion of MSCs and ADSCs for clinical use and has designed a protocol for labeling of MSCs with iron-oxido for clinical tracking studies after intramyocardial injection has been developed (see WP10 and D10.6). This will be tested in a follow-up clinical trials first with BM-derived MSCs and then with ADSCs, as this project has supported beneficial effects of MSCs from adipose tissue (see WP2 and 4).
Partner 8 (EuBioSci) in WP8 has produced 9 different bio-active growth factors, has established bio-assays for the evaluation of these factors, and has delivered the factors to the collaborators within this project. Furthermore, EuBioSci has lyophilized four combinations of growth factors as cocktail compositions and provide these to the partners of this project. These ready-to-use growth factor cocktails following evaluation have been used by the collaborators. An important role for Partner 8 in the second period was that he produced the large amounts of growth factors, in particular IGF-1 and HGF, needed for the final evaluation of the best combination therapy (see WP10).

Partner 9 in WP9 has collaborated until month 6 of the second period to provide sufficient cord blood stem cells and ECFCs/BOECs for the work of Partner 1 and has also supported in part Partner 2 by producing the cells. He has improved protocols for large scale generation of the cells and established a supply line for the cells. Unfortunately, during the second term of this grant, a major private financier of P9 (VivoCell) has withdrawn his support of the company. As a consequence the research/development part of the company was shut down and P9 could not further work on the project and remained solely a supplier of cord blood samples. This started about 6 months into the second period and resulted in a more or less complete reduction of activities from 9 months within the second period. All activities planned for P9 in the generation and further analysis of ECFCs/BOECs were then taken over by P1 besides the large scale cultivation and differentiation technology which was initiated by P9, but not finally developed.

In WP10 Partners P1, P2, P3, P4, P6 and P7 have collaborated, each group focusing on its special expertise. P3 in collaboration with P4 and P7 have evaluated different adult stem cells from bone marrow, adipose or cardiac tissue, P1 in collaboration with P2 have tested partially differentiated endothelial progenitor cells (ECFCs/BOECs), P1 has developed methods to genetically modify endothelial progenitors to make them more efficient, P4 and P6 have collaborated to evaluate engineered tissue and P2 collaborating with P3 have performed the final analysis of combination of cells and reagents in a porcine model. Clinical partners P2 and P7 have prepared protocols as basis for planned follow-up clinical trials using autologous ECFCs/BOECs and MSCs.

This work has highlighted on the one hand clear beneficial effects of MSCs, either from bone marrow or adipose tissue, in animal MI models, which are presumably due to paracrine secretion of factors. The available evidence supports that adipose tissue-derived stem cells, especially those from human epicardial fat, may be the best cells as they expressed the highest levels of trophic, angiogenic and immunomodulatory factors. On the other hand, endothelial progenitor cells (ECFCs/BOECs) gave exciting results in a porcine MI model improving vascularization, perfusion and function. These cells, in contrast to MSCs/ADSCs also displayed some long-term incorporation into neovessels. P4 and P6 have evaluated engineered prevascularized cardiac muscle grown on modified alginate scaffolds with encouraging results. Based on these results first a final evaluation of the combination of either ADSCs or ECFCs/BOECs together with the application of IGF and HGF in a nanoparticle formulation with a modified alginate biomaterial was started in a porcine MI model and has indicated initial promising effects, although the experiment is still ongoing and will need final evaluation. Second P2 and P7 have designed protocols for two follow-up clinical trials, one designed to track MSCs/ADSCs after injection in patients, the other to build on the most promising finding coming out of this project, the use of autologous ECFCs/BOECs for improving vascularization and perfusion, which will be the precondition for any further additional therapies to be developed with the aim to also regenerate damaged myocardium.

A.2. DETAILED MAIN S&T RESULTS / FOREGROUNDS OF INDIVIDUAL WORK PACKAGES

A.2.1. WP1 (P2 - S. JANSSENS/P. CARMELIET - VIB.Leuven)
Task 1: Genetically modified mice with altered mobilization capacities for BM precursors
Our laboratory has focused on unraveling the molecular mechanisms of bone marrow (BM) retention and mobilization of angiogenic precursors following tissue ischemia, which may critically contribute to neovessel formation. Our findings have provided additional insights in the mechanisms controlling mobilization of endogeneous stem cells by studying a variety of gene-manipulated mouse lines (uPAR, Plg, uPA, tPA, PAI-1, AP2) and in identifying potential therapeutic targets. We have first focused on the role of membrane-bound and soluble uPAR in BM precursor cell retention and mobilization. We analyzed mobilization of hematopoietic progenitor cells (HPCs) in response to G-CSF in mice lacking PAI-1 (PAI-1-/-) or α2-antiplasmin (AP-/-), the primary inhibitors of plasminogen activators and plasmin, respectively.

No genotypic differences in circulating HPCs were detectable in steady-state conditions. After G-CSF treatment (200 μg/kg/d s.c. for 5 days), circulating HPCs were detectable in the peripheral blood of the respective wild type (WT) controls. Importantly, PAI-1-/- or AP-/- mice mobilized ~1.4-fold and ~2.2-fold more HPCs in peripheral blood than respective WT littermates, indicating that genetic loss of PAI-1 or AP enhances HPC mobilization after G-CSF.

Because plasmin augments G-CSF-mediated mobilization, we next investigated whether thrombolytic agents enhance HPC mobilization in mice.
For the mouse study, we used recombinant human tPA (rtPA), tenecteplase (TNK; a rtPA mutant which has a prolonged half-life), and microplasmin (μPlI; a plasmin variant lacking the five aminoterminal kringle domains, which has an improved safety profile (less bleeding)). In steady-state conditions, administration of the thrombolytic compounds TNK (daily intraperitoneal bolus injection at 100 mg/kg) or μPlI (100 μg/day, continuously over a period of 5 days via osmotic minipumps) failed to induce HPC mobilization in WT mice, raising the question whether these agents might only enhance HPC mobilization in conjunction with G-CSF therapy. Indeed, when co-administered with G-CSF (200 μg/kg/d s.c. for 5 days), TNK enhanced the mobilization of HPCs with CFU-C and CFU-S by ~1.7-fold and ~2.6-fold respectively, compared to G-CSF alone. Co-administration of G-CSF and μPlI and G-CSF also stimulated mobilization of HPCs with CFU-C and CFU-S, by ~1.5-fold and ~2.8-fold respectively. These cells were capable of reconstituting hematopoiesis, as transplantation of these cells significantly increased the survival of lethally irradiated WT recipient mice.

Experiments in mice with genetic loss of plasminogen activator inhibitor-1 and α2-antiplasmin have highlighted a novel mechanism whereby plasmin augments hematopoietic progenitor cell mobilization following GCSF. From these models, we adapted strategies to reproduce the biological changes using pharmacological interventions in wild type mice. By doing so, we demonstrated that TNK or μPlI were capable of enhancing HPC mobilization after G-CSF, which opens interesting therapeutic perspectives.

Task 2: Reagents for mobilization of Bone Marrow precursors: thrombolytic agents enhance hpc mobilization in humans
Based on our findings in transgenic mice (cfr Deliverable D1.1) we evaluated whether thrombolytic compounds, that generate or increase plasmin, and which are currently being used in the clinic (tenecteplase, recombinant human tPA) or are in clinical development (microplasmin/ocriplasmin, staphylokinase) might be useful to stimulate HPC mobilization. Our mouse results indicate that thrombolytic agents only enhance HPC mobilization, when these cells are already primed by another mobilization stimulus (such as G-CSF). However, ethical reasons precluded us from testing whether thrombolytic agents enhance HPC mobilization in response to G-CSF in healthy volunteers. We therefore studied the effect of thrombolytic agents in the setting of acute myocardial ischemia, as this is a well-known stimulus for HPC mobilization. Subsequently we also studied mobilization of vasculogenic progenitor cells from bone marrow under different clinical conditions.

As a first part of these clinical studies, peripheral blood samples were collected before and 24 hours after thrombolytic treatment (TNK or staphylokinase) of patients admitted to the Coronary Care Unit with ST-segment elevation myocardial infarction (STEMI). As control group, blood samples were collected from STEMI patients who received primary percutaneous mechanical reperfusion therapy (PCI). Mobilization of HPCs was determined by quantifying CFU-Cs using methylcellulose
culture assays and the percentage of CD34+ cells using flow cytometry. A pilot group of 11 patients was analyzed: 6 received first-line PCI, while 5 received thrombolytic therapy. At the time of admission (i.e. before onset of treatment), both the PCI and Thrombolysis groups had comparable numbers of circulating HPCs. PCI treatment failed to mobilize HPCs but when compared to the pre-treatment levels, corresponding values after thrombolytic therapy showed a 690±230% increase in colony forming units (P<0.05) and a 385±75% increase in the number of CD34+ cells (P<0.005). Taken together, these findings show that thrombolytic agents enhance the mobilization of HPCs in humans and warrant further investigation of their therapeutic potential in individuals who mobilize poorly in response to GCSF.

Task 3: Evaluation of the therapeutic potential of circulating endothelial progenitors
Since our initial observations of enhanced HSC mobilization using G-CSF, it has become clear that in addition to HSC, other cell types from the bone marrow may carry a great and perhaps better therapeutic potential in ischemic disease. Most notably we have observed that endothelial progenitor cells are being recruited from bone marrow into the circulation and that a subset of these cells carry a very potent neovascularization capacity.

Circulating endothelial progenitor cells (EPCs), expressing KDR/VEGF-R2 on their surface, contribute to neovascularization of ischemic tissues and repair of injured endothelium. These cells differ from the monocyctic early outgrowth EPCs which have no direct vasculogenic phenotype but can indirectly effect angiogenesis via release of paracrine factors. We studied endogenous mobilization and homing of late outgrowth, vasculogenic EPCs in green fluorescent protein bone marrow chimera mice exposed to chronic hypoxia, a common hallmark of patients with advanced cardiac and pulmonary disease and secondary pulmonary hypertension. Despite increased peripheral mobilization, as shown by flow cytometry and EPC culture, bone marrow-derived endothelial cell recruitment in remodeling lung vessels was limited. Moreover, transfer of vascular endothelial growth factor receptor-2+/Sca-1+/CXCR-4+ cultured EPCs failed to reverse PH, suggesting hypoxia-induced functional impairment of transferred EPCs. Chronic hypoxia decreased migration to stromal cell-derived factor-1alpha, adhesion to fibronectin, incorporation into a vascular network, and nitric oxide production (-41%, -29%, -30%, and -32%, respectively, vs. normoxic EPCs; p < .05 for all). The dysfunctional phenotype of hypoxic EPCs significantly impaired their neovascularization capacity in chronic hind limb ischemia. In contrast, normoxic EPCs cultured in identical conditions were much better capable of vascular tube formation in vitro and migrated better towards SDF-1 in matrigel. Mechanisms contributing to EPC dysfunction include reduced integrin αv and β1 expression, decreased mitochondrial membrane potential, and enhanced senescence. Novel insights from chronic hypoxia-induced EPC dysfunction may provide important cues for improved future cell repair strategies.

Following up on these murine studies we have tested the hypothesis that in a large preclinical model of myocardial ischemia, induced by transient coronary artery occlusion, intracoronary transfusion of autologous late outgrowth endothelial progenitor cells conferred greater myocardial recovery than in control conditions or after infusion of mesenchymal stem cells. Phenotypic markers of EPCs in the porcine and murine model showed striking similarities and in vitro studies clearly highlighted a potent paracrine release of angiogenic growth factors and cytokines, most notably placental growth factor and hepatocyte growth factor. After in vivo infusion of genetically labeled cells, we were able to confirm that EPCs contributed to neovascularization in an autocrine fashion by clearly incorporating in the endothelial cell layer of neovessels in the infarct border zone. Complementary MRI analysis showed that improved perfusion was capable of reducing LV dilatation over time to a significantly greater extent than in control animals or in pigs which received mesenchymal stem cells (see Dubois et al. JACC 2010). Taken together, these additional experiments performed in the second part of this grant highlight the great therapeutic potential of these cells in patients with ischemic cardiomyopathy. Therefore, we have started to test whether these cells can be amplified from peripheral blood of these patients and how they would functionally behave. To our surprise, we have in preliminary experiments observed that in contrast to early outgrowth EPC, these cells maintain their vasculogenic properties in vitro, are capable of in vitro expansion for 5-10 passages without signs of senescence and have similar migration responses towards SDF-1αs EPCs cultured from normal volunteers. At present we are testing the potency of these patient-derived cells in vivo nude mice models of peripheral leg ischemia. Initial results confirm in vivo survival of labeled human cells in mice and a beneficial effect on leg perfusion.

A.2.2. WP2 (P3 - F. PROSPER - U.Navarra)
Throughout the duration of the project we have extensively tested the role of adult stem cells in models of cardiovascular diseases, mainly myocardial infarction and peripheral limb ischemia in order to address the 3 specific aims of our WP.

Task 1 involved the comparison of MAPC and ADSC in models of MI and limb ischemia. In order to achieve this aim different studies were planned and carried out using mice, rats and pigs.

Transplantation of MAPC in a model of acute MI
We compared the effect of transplantation of murine multipotent adult progenitor cells (MAPCs), a population of adult bone marrow-derived cells that differentiate into cells of mesodermal, endodermal and ectodermal origin, with murine bone marrow cells (BMCs) or fibroblasts on post-infarct cardiac function by peri-infarct injection after coronary artery ligation in mice. We demonstrate that, in contrast to the other cell populations, transplantation of MAPCs significantly improved LV contractile function for at least 8 weeks posttransplantation and, although BMCs reduced infarct size, the decrease in scar size was substantially greater in MAPC-treated hearts. As neither MAPCs nor BMCs were present beyond 1 week, the beneficial effect was not due to differentiation and direct contribution of MAPCs to the vascular or cardiomyocyte compartment. Significantly more inflammatory cells were present in MAPC- than BMC-treated hearts at 1 week, which was accompanied by increased vascularity 8 weeks posttransplantation. We hypothesize that MAPCs indirectly contributed to these effects, by secreting inflammatory [monocyte chemoattractant protein-1 (MCP)-1], and vascular growth factors [vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-BB, and transforming growth factor (TGF-β1), and others, resulting in increased angiogenesis and cardioprotection.

Transplantation of adipose derived cells in a chronic model of MI in rats
We compared the effect of transplantation of undifferentiated and cardiac pre-differentiated adipose stem cells compared with bone marrow mononuclear cells (BM-MNC) in a chronic model of myocardial infarction. Sprague–Dawley rats underwent left coronary artery ligation and after 1 month received by direct intramyocardial injection either adipose derived stem cells (ADSC), cardiomyogenic cells (AD-CMG) or BM-MNC from enhanced-Green Fluorescent Protein (eGFP) mice. The control group was treated with culture medium. Heart function was assessed by echocardiography and 18F-FDG microPET. Cell engraftment, differentiation, angiogenesis and fibrosis in the scar tissue were also evaluated by (immuno)histochemistry and immunofluorescence. One month after cell transplantation, ADSC induced a significant improvement in heart function (LVEF 46.3±9.6% versus 27.7± 8% pre-transplant) and tissue viability (64.78±7.2% versus 55.89±6.3% pre-transplant). An increase in the degree of angiogenesis and a decrease in fibrosis were also detected. Although transplantation of AD-CMG or BM-MNC also had a positive, albeit smaller, effect on angiogenesis and fibrosis in the infarcted hearts, this benefit did not translate into a significant improvement in heart function or tissue viability. These results indicate that transplantation of adipose derived cells in chronic infarct provides a superior benefit to cardiac predifferentiated ADSC and BM-MNC.

Transplantation of SVF cells in a chronic model of MI in rats
Freshly adipose-derived cells have been shown to be effective in the treatment of acute myocardial infarction (MI), but their role in the chronic setting is unknown. We sought to determine the long-term effect of the adipose derived-stromal vascular fraction (SVF) cell transplantation in a rat model of chronic MI. MI was induced in 87 rats by permanent coronary artery ligation and 5 weeks later, rats were allocated to receive intra-myocardial injection of 107 GFP-expressing fresh SVF-cells or culture media as control. Heart function and tissue metabolism, determined by echocardiography and 18F-FDG-microPET respectively, were performed together with histological studies for up to 3 months after transplantation. Transplantation of SVF induced a statistically significant long-lasting (3 months) improvement in cardiac function and tissue metabolism that was associated with an increased revascularization degree and a positive heart remodeling with a significantly smaller infarct size, thicker infarct wall, lower scar fibrosis and lower cardiac hypertrophy. Importantly, injected cells engrafted and were detected in the treated hearts for at least 3 months, directly contributing to the vasculature and to a lesser extent to myofibroblasts and
cardiomyocytes. Furthermore, SVF-release of angiogenic (VEGF and HGF) and pro-inflammatory (MCP-1) cytokines, as well as TIMP1 and TIMP4 was demonstrated in vitro and in vivo, strongly suggesting their trophic effect. These results show the potential of SVF to contribute to the regeneration of the ischemic tissue and to provide a long-term functional benefit in a rat model of chronic MI by both direct and indirect mechanisms.

Transplantation of MAPCs in hind limb ischemia models in mice

We compared the vascularization and tissue regeneration potential of murine and human undifferentiated multipotent adult progenitor cells (mMAPC-U and hMAPC-U), murine MAPC-derived vascular progenitors (mMAPC-VP), and unselected murine BM cells (mBMCs) in mice with moderate limb ischemia, reminiscent of intermittent claudication in human patients. mMAPC-U durably restored blood flow and muscle function and stimulated muscle regeneration, by direct and trophic contribution to vascular and skeletal muscle growth. This was in contrast to mBMCs and mMAPC-VP, which did not affect muscle regeneration and provided only limited and transient improvement. Moreover, mBMCs participated in a sustained inflammatory response in the lower limb, associated with progressive deterioration in muscle function. Importantly, mMAPC-U and hMAPC-U also remedied vascular and muscular deficiency in severe limb ischemia, representative of critical limb ischemia in humans. Thus, unlike BMCs or vascular-committed progenitors, undifferentiated multipotent adult progenitor cells offer the potential to durably repair ischemic damage in peripheral vascular disease patients.

We also compared human AC133+ cells and multipotent adult progenitor cells (hMAPC) in a mouse model reminiscent of critical limb ischemia. hMAPC or hAC133+ cell transplantation induced a significant improvement in tissue perfusion (measured by microPET) 15 days posttransplantation compared to controls. This improvement persisted for 30 days in hMAPC-treated but not in hAC133+-injected animals. While transplantation of hAC133+ cells promoted capillary growth, hMAPC transplantation also induced collateral expansion, decreased muscle necrosis/fibrosis, and improved muscle regeneration. Incorporation of differentiated hAC133+ or hMAPC progeny into new vessels was limited; however, a paracrine angio/arteriogenic effect was demonstrated in animals treated with hMAPC. Accordingly, hMAPC-conditioned, but not hAC133+-conditioned, media stimulated vascular cell proliferation and prevented myoblast, endothelial, and smooth muscle cell apoptosis in vitro. Our study suggests that although hAC133+ cell and hMAPC transplantation both contribute to vascular regeneration in ischemic limbs, hMAPC exert a more robust effect through trophic mechanisms, which translated into collateral and muscle fiber regeneration. This, in turn, conferred tissue protection and regeneration with longer term functional improvement.

Conclusions: bone marrow and adipose derived stem cells contribute to functional improvement in models of cardiovascular diseases. In both cases, paracrine mechanisms and not direct contribution of transplanted cells seem to be the cause of tissue repair as the level of engraftment is extremely low. This information has constituted the bases for follow up studies. Based on practical issues such as easiness of procurement and cost, ADSC represent a favorable population of stem cells for large animal models, at least in the cardiac models.

Task 2: Once the potential of the two populations was determined, the SC population that gave a better improvement of the cardiac function was used in a large animal model.

Transplantation of ADSC in a pig model of MI

The aim of this work was to determine the long-term effect of transplantation of adipose-derived stromal cells (ADSCs) in a preclinical model of ischemia/reperfusion (I/R). The study was supported by our previous results in rats where we found a significant benefit in animals treated with ADSC and SVF in models of MI. I/R was induced in 28 Goettingen minipigs by 120 minutes coronary artery occlusion followed by reperfusion. Nine days later, surviving animals were allocated to receive trans-endocardial injection of a mean of 213.6±41.78 million green fluorescent protein (GFP)-expressing ADSCs (n=7) or culture medium as control (n=9). Heart function, cell engraftment and histological analysis were performed 3 months after transplantation. Transplantation of ADSCs induced a statistically significant long-lasting (3 months) improvement in cardiac function and geometry in comparison with control animals. Functional improvement was associated with an increase in
angiogenesis and vasculogenesis and a positive effect on heart remodeling with a decrease in fibrosis and cardiac hypertrophy in animals treated with ADSCs. Despite the lack of cell engraftment after 3 months, ADSC transplantation induced changes in the ratio between MMP/TIMP. Our results indicate that transplantation of ADSCs, despite the lack of long-term significant cell engraftment, increases vessel density and prevents adverse remodeling in a clinically relevant model of myocardial infarction, strongly suggesting a paracrine mediated effect. ADSCs thus constitute an attractive candidate for the treatment of myocardial infarction.

Although no directly described in the initial proposal, 2 further studies related to large animal models and to improvement in efficacy by increasing engraftment of cells were performed.

Sequential injection of skeletal myoblast in a pig model of MI
Although transplantation of skeletal myoblast (SkM) in models of chronic myocardial infarction (MI) induces an improvement in cardiac function, the limited engraftment remains a major limitation. We analyse in a pre-clinical model whether the sequential transplantation of autologous SkM by percutaneous delivery was associated with increased cell engraftment and functional benefit. Chronically infarcted Goettingen minipigs (n=20) were divided in four groups that received either media control or one, two, or three doses of SkM (mean of 329.6x10^6 cells per dose) at intervals of 6 weeks and were followed for a total of 7 months. At the time of sacrifice, cardiac function was significantly better in animals treated with SkM in comparison with the control group. A significantly greater increase in the DLVEF was detected in animals that received three doses vs. a single dose of SkM. A correlation between the total number of transplanted cells and the improvement in LVEF and DLVEF was found (P<0.05). Skeletal myoblast transplant was associated with an increase in tissue vasculogenesis and decreased fibrosis (collagen vascular fraction) and these effects were greater in animals receiving three doses of cells. Repeated injection of SkM in a model of chronic MI is feasible and safe and induces a significant improvement in cardiac function.

Tissue engineering approaches to improve cardiac performance of stem cells
Using collagen based membranes we have assess the therapeutic potential of the adipose-derived stem cell population (ADSC) transplanted on biocompatible and biodegradable collagen-scaffolds in a rat model of chronic myocardial infarction (MI). Biocompatibility of the collagen scaffold (CS) with the ADSC was confirmed by in vitro experiments of cell adhesion, proliferation and survival. Cellularized-collagen patches (CS-ADSC) were implanted in a rat model of chronic MI and induced a significant long-term functional improvement not observed when cells were directly injected into the heart. Also, a similar mechanical behavior than in the healthy hearts was detected only in the hearts transplanted with the CS-ADSC. This improvement was associated with a significant decrease in tissue fibrosis and an increase in tissue revascularization. None of these positive effects were detected when animals were treated with the non-cellularized CS or the injected ADSC. Finally, a significant greater engraftment of the cells transplanted with than without the CS was confirmed. This study has confirmed the beneficial effect of ADSC transplantation when previously seeded on a collagen scaffold, which improves graft survival and ultimately, promotes a greater cell paracrine effect that reflects in a functional improvement and positive remodeling of the ischemic heart.

Conclusions: results in large animal models mimick our findings in small animals and support testing of this cells in human clinical trials. Furthermore, repeated injections or scaffolds may represent simple systems to increase efficacy of this therapies

Task 3: to address some of the potential mechanisms by which SC contributes to tissue regeneration

From the previous studies, it was clear that the release of growth factors from the cells was implicated in tissue repair. So in the final task we have exploited those results in order to design new strategies that allow delivery of the candidate cytokines involve in cardiac repair

Use of controlled release systems for cardiac regeneration
We have used PLGA particles loaded with growth factors and have completed 2 different studies assessing the role of VEGF,
The use of pro-angiogenic growth factors in ischemia models has been associated with limited success in the clinical setting, in part owing to the short lived effect of the injected cytokine. The use of a microparticle system could allow localized and sustained cytokine release and consequently a prolonged biological effect with induction of tissue revascularization. To assess the potential of VEGF165 administered as continuous release in ischemic disease, we compared the effect of delivery of poly(lactic-co-glycolic acid) (PLGA) microparticles (MP) loaded with VEGF165 with free-VEGF or control empty microparticles in a rat model of ischemia-reperfusion. VEGF165 loaded microparticles could be detected in the myocardium of the infarcted animals for more than a month after transplant and provided sustained delivery of active protein in vitro and in vivo. One month after treatment, an increase in angiogenesis (small caliber caveolin-1 positive vessels) and arteriogenesis (α-SMA-positive vessels) was observed in animals treated with VEGF microparticles (p<0.05) but not in the empty microparticles or free-VEGF groups. Correlating with this data, a positive remodeling of the heart was also detected in the VEGF-microparticle group with a significantly greater LV wall thickness (p<0.01). In conclusion, PLGA microparticle is a feasible and promising cytokine delivery system for treatment of myocardial ischemia. This strategy could be scaled up and explored in pre-clinical and clinical studies.

In a second study either Nrg1 or FGF1 loaded particles were used in a similar model. Acidic fibroblast growth factor (FGF-1) and neuregulin-1 (NRG-1) have been identified as factors involved in cardiac repair after MI. However, the therapeutic value of these growth factors has important limitations in vivo, related to their short-lived effect and high instability after systemic administration. To circumvent these limitations, FGF-1 and NRG-1 were encapsulated into poly-lactide-co-glycolide (PLGA) microparticles (MP), which released the bioactive growth factors in a sustained manner for up to 28 days in vitro. The ability of FGF-1 MP and/or NRG-1 MP to promote cardiac regeneration was evaluated in a rat model of MI. Three months after treatment, a cardiac function improvement was detected in the rats treated with FGF1-MP (16.7 ± 4.9%, P<0.05) NRG1-MP (18.0 ± 5.7%, P<0.05) or FGF1/NRG1-MP (13.0 ± 1.9%, P<0.05) in comparison with the non-loaded (NL-MP) control group (1.1 ± 3.6%). In addition, a positive cardiac remodeling with a smaller infarct size and lower fibrosis degree, an induction of tissue revascularization and cardiomyocyte proliferation was detected. Also, recruitment of c-Kit+ CD45- progenitor cells towards the ischemic myocardium under stimulation of FGF-1 and NRG-1 delivered from the MP was detected. Collectively, these results demonstrate the ability of PLGA-MP to deliver efficiently FGF-1 and NRG-1, which promoted myocardial regeneration by distinct mechanisms of cardiac repair after MI.

Conclusions: controlled delivery of proteins implicated in mechanisms of cardiovascular repair may represent a very attractive therapy.

A.2.3. WP3 (P1 - E. HOFER - MU.VIENNA)

Description of main S & T results/foreground

Task 1: Differentiation and isolation protocols for circulating endothelial progenitor cells
Partner 1 has established in collaboration with partner 9 (VivoCell) a regular weekly supply of cord blood for the generation of circulating endothelial progenitor cells (termed ECFC, endothelial colony forming cells or BOECs, late blood outgrowth endothelial cells). The procedures were optimized and large numbers of cells were regularly obtained after outgrowth for 1 to 6 weeks. Cells seem to possess unlimited proliferative capacity over several months. We have characterized these cells in collaboration with P9 during early and late times of outgrowth by FACS analysis and realtime RT-PCR. The stem cell marker CD133 was found to be significantly expressed at early time points (1 week) and to gradually decrease over time to low or undetectable levels 6-8 weeks after culture initiation supporting the stem cell origin of these cells. Following several weeks of culture these cells expressed the endothelial markers CD31, VE-cadherin and von Willebrand factor at levels somewhat lower or comparable to amounts characteristic of mature human umbilical vein endothelial cells (HUVEC) (for further details see...
Task 2: Gene profiling of ECFCs in comparison to mature endothelial cells and other stem/progenitor cells
We have performed gene profiling from early and late stages of ECFCs from cord blood and adult peripheral blood in comparison to HUVEC and adult venous endothelial cells, respectively. Furthermore, gene profiling data obtained by P3 for MAPC (multipotent adult progenitor cells) and ADSC (adipose derived SC) and of P7 for MSC in various stages of differentiation were used for comparison. ECFCs from cord blood and adult peripheral blood showed a similar transcriptional profile which was distinct for a small number of genes from mature endothelial cells. MAPC, ADSC and MSC from P3 and P7 were largely different from ECFCs and no clear preference for analyzing specific genes of this group of cells could be delineated. This was in contrast to the data obtained from the comparison of ECFC and mature endothelial cells, which showed distinct expression only for a small number of genes which likely constitute genes responsible for the progenitor status of ECFC. We therefore concentrated on the analysis of these genes. Especially a transcription factor displaying most preferential expression was further analyzed.

Task 3: Key regulators and genes controlling differentiation and function of EPCs

Based on our and the encouraging data of P2 in WP1 using ECFCs/BOECs in a porcine model, which have shown that this endothelial progenitor cell type will be among the most promising cells coming out of this project, we have focused work in WP3 on the analysis of ECFCs/BOECs from cord blood. We have in the first period selected from gene profiling data genes/proteins preferentially expressed in the progenitor ECFCs and much less in mature endothelial cells of the vessel wall. We have now further investigated whether the expression of these genes may determine the progenitor status of these cells and their function. For this purpose we have used gain- and loss-of-function studies, i.e. overexpression of the genes by adenoviral vectors and downregulation by lentiviral expression of corresponding shRNAs. The effects of overexpression and downregulation of the genes on specific progenitor properties such as increased proliferative capacity as well as migration and sprouting potential was scored.

Selected transcription factor: One gene selected and further analyzed was a transcription factor which was the factor displaying the highest differential expression in ECFCs versus mature endothelial cells. Overexpression of the factor strongly increased the sprouting capabilities of endothelial progenitors and endothelial cells. This was apparently caused by the regulation of Notch pathway components. It specifically upregulated a Notch receptor and led to an increase of a Notch ligand at the cell surface. Vice versa downmodulation of the factor and of Notch reduced vascular sprouting. The transcription factor was further involved in the upregulation of VEGF receptor-2, which in part caused the increased sprouting. Furthermore the arterial marker ephrin B2 was upregulated, whereas the venous marker EphB4 was downmodulated. Hence these findings support a crucial role of the factor for the progenitor status, implicate the Notch pathway as a downstream mediator of its functions leading to increased capacity to induce sprouting by endothelial progenitors. It furthermore suggests the involvement of the factor and ECFCs in capillary formation and arterial specification. A manuscript on this work has been submitted.

Task 3: Cord blood progenitors with genetically altered differentiation and functional potential

We have followed the hypothesis that factors preferentially expressed by endothelial progenitors would provide, when overexpressed either by the progenitors or even by normal mature endothelial cells, these cells with the potential for improved induction of sprouting and vessel formation. As ECFCs have been shown to home to ischemic tissues and to improve vascularization, perfusion and functional parameters in MI models (see WP1), it is an attractive possibility that these cells or the factors they produce alone or in combination with the cells could be used to achieve improved effects. Therefore, it might be possible that by modulation of the transcription factor the properties of ECFCs could be improved or one might be able to generate ECFC-like functional properties by transduction of mature endothelial cells. In this regard we have evaluated adenoviral overexpression of the factor in ECFC and HUVEC. Indeed, what we observed is in line with the hypothesis that ECFC may function by integrating into the mature vessel wall and to increase sprouting. This could either be caused by the integrated progenitor cell becoming a tip cell and initiating a sprout or, even more effectively, by triggering adjacent
endothelial cells to become sprout-initiating tip cells. The capacity to do this could be provided by displaying Notch and Notch ligands to the neighbouring cell. This will be further evaluated in follow-up studies as well as the use of these genetically modified cells for improved effects in small and large animal MI models.

A.2.4. WP4 (J. Leor - U.TelAviv)

Description of main S & T results/foreground

Our general objective was to evaluate and characterize the ideal cardiac stem cells and to develop an efficient method to direct their differentiation into the myocardial lineage both in vivo and in vitro.

Our specific objectives were:
- To assess the role of donor age, gender, risk factors, or disease status on derived human CPC function.
- To define conditions for expansion and differentiation of human CPCs into cardiac cells.
- To investigate the use of various stem cell activators in activating human CSCs in the damaged heart, in situ.

To achieve these aims, we first improved the method for culturing human CPCs so that they could be obtained with a nearly 100% success rate. We then undertook to carry out a systematic study on the yield of human CPCs in correlation with clinical characteristics, age, gender and heart area from which the sample was obtained. In these studies specimens from the right atrial appendage generated the highest amount of c-kit+ cells. Interestingly, female gender and cardiac dysfunction are associated with a higher number of the stem cells. Furthermore, tissue from patients with end-stage heart failure seems to give a higher yield of CSCs. This finding supports the notion that endogenous reparative systems are stimulated in the injured heart. A surprising finding was that age did not significantly affect the number of human CSCs derived from myocardial specimens.

In the second period of the project we focused on the relative weight of CPCs, compared with MSCs from other sources. We determined the best cell source for heart repair. Based on in vitro studies, we found that human epicardial fat provides the best source of MSCs with unique reparative properties that might avoid the need for myocardial biopsy. These cells could be directed to differentiate into myogenic cells, in vitro, by the de-methylating agent 5-aza, and in vivo by transplantation into the heart. Compared with pericardial and peripheral fat, these cells have many similarities to atrial-derived CPCs and have improved regenerative potential. In the next series of experiments, we showed that the origin of MSCs and CPCs dictates their reparative and immuno-modulatory properties. Surprisingly, in a rat model of MI, MSCs derived from subcutaneous fat emerged as the best cell to improve LV remodeling and function. However, this could be explained by immune rejection of the human cells by the rat immune system. We discovered that cytokines secreted by the implanted cells are a major determinant of adverse cardiac remodeling and dysfunction, suggesting that regulation of inflammation is a key mechanism in cell therapy for infarct repair.

In addition, in a preliminary study we showed that administration of 5-Aza after MI can improve LV remodeling and infarct vascularization in rat. Our findings suggest a new epigenetic strategy for infarct repair and regeneration by reprogramming stem cells and fibroblasts in situ.

Finally, together with Smadar Cohen, we identified injectable biomaterials, such as alginate with growth factors, which can be used to improve cell implantation and survival. We have shown that CPCs could be stimulated in situ: sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial, without cells, improves myocardial rejuvenation.
Collectively, our findings on the properties of various cells could be relevant to the clinical application of cell therapy for infarct repair.

All objectives have been achieved on schedule.

A.2.5. WPS (J. HESCHELER - UKK.COLOGNE)

Description of main S & T results/foreground

Task 1: Improving engraftment, persistence and survival of transplanted cardiomyocytes

Multiple strategies to improve engraftment, persistence and survival of transplanted cardiomyocytes were tested by partner 5. So far, results have been presented at various national and international scientific meetings and manuscripts are in preparation.

a) embryonic stem cell derived cardiomyocytes (ES-CM)

Investigations on engraftment, persistence and survival of transplanted embryonic stem cell derived cardiomyocytes were extended to strategies with cotransplantation of various biomaterials or non-cardiomyocytes as supportive cells. In controls with transplantation of 300,000 ES-CM alone, 40,001±8,444 ES-CM were detected immediately after intramyocardial injection (0h) and 35,108±10,175 after 6h. Co-injection of Matrigel™ increased the numbers at 0h dose-dependently by +43.9±47.4% (M-low) and +170.9±87.3% (M-high, p vs. control < 0.01 p for trend = 0.01) but not significantly at 6h (+42.1±56.6% for M-low and +24.1±26.5% for M-high). Co-injection of alginate did not lead to significant differences as compared to ES-CM alone with -58.2±22.6% (0h) and -45.6±14.3% (6h), neither did the co-injection of fibroblasts with -42.8±30.3% (0h) and -53.2±38.1% (6h) or the co-injection of fibrin glue with -38.5±16.9% (0h) and -64.9±29.2% (6h). Therefore, neither the co-injection of any of the tested biomaterials nor of fibroblasts could prevent the massive early loss of cardiomyocytes derived from murine embryonic stem cells after intramyocardial injection. Therefore, other strategies to improve engraftment, persistence and survival of transplanted ES-CM must be identified to optimize the effectiveness of cardiac cell replacement therapy and to facilitate the possible therapeutic benefit.

Citation: Krausgrill B, et al.: Influence of co-injected biomaterials or fibroblasts on engraftment and persistence of cardiomyocytes derived from murine embryonic stem cells after intramyocardial injection. European Heart Journal 2012; 33 (Abstract Supplement): 433.

Another encouraging strategy is the transplantation of in-vitro grown mixed clusters of ES-CM and MSC. Immediately after such intramyocardial injection, we detected 20.4±9.2% of transplanted cells (i.e. ES-CM+MSC) injected and numbers decreased over time down to 11.1±6.0% at 24h and 3.7±0.9% at 48h (p=0.06 for linear trend). With selective quantification of transplanted ES-CM alone, we detected 24.7±7.8% of ES-CM injected at 0h, 10.5±5.4% at 24h and 1.0±0.4% at 48h (p<0.001 vs. 0h, p<0.01 for linear trend). Thus, the fraction of ES-CM within the detectable cells injected changed from >60% (ctrl, 0h, 24h) to <20% at 48h. Although similarly low at 48h, detection of injected ES-CM at 0h and 24h was remarkably higher than in previous studies with intramyocardial injection of similar ES-CM alone (0h: 13.3±2.8%, p=0.1 and 24h: 0.8±0.3%, p=0.05). Therefore, highly purified murine embryonic stem cell derived cardiomyocytes show remarkably enhanced engraftment but poor persistence when intramyocardially injected within in-vitro grown clusters together with adult mesenchymal bone-marrow cells. Nevertheless, transplantation of in-vitro grown clusters seems to delay or even prevent the massive early loss of transplanted single cells presumably by increasing volume, surface and adhesiveness of injected particles and by providing a beneficial milieu within the injected clusters and will therefore be further investigated.

Citation: Krausgrill B, et al.: Transplantation of highly purified murine embryonic stem cell derived cardiomyocytes in clusters together with adult mesenchymal bone-marrow cells yields enhanced engraftment but poor persistence. European Heart
b) induced pluripotent stem cell derived cardiomyocytes (iPS-CM)

Immediately after intramyocardial injection into healthy syngeneic recipient hearts, we detected 28.4±4.0% of the transplanted highly purified iPS-CM in the recipient heart. Values decreased over time to 2.6±0.9% of iPS-CM after 6h and down to 0.6±0.2% of iPS-CM after 24h. After co-transplantation with non-cardiomyocytes, the number of detectable iPS-CM at 24h was more than 2-fold with 1.4±1.1% (with cotransplanted murine embryonic fibroblasts, MEF) and 1.5±0.2% (with cotransplanted murine mesenchymal bone-marrow cells, MSC, p<0.05 vs. iPS-CM alone), respectively, corresponding to absolute numbers of approx. 4,200 or 4,600 iPS-CM as compared to approx. 1,800 iPS-CM without co-transplantation. Therefore, highly purified murine cardiomyocytes derived from iPS showed a massive cell loss already during or shortly after intramyocardial injection into syngeneic healthy recipient hearts and a poor persistence of less than 1% of the injected cells at 24h. Since this number was enhanced 2-fold by co-transplantation of non-cardiomyocytes (MEF or MSC), this strategy will be further investigated to optimize the effectiveness of cardiac cell replacement therapy and to facilitate the possible therapeutic benefit.

Citation: Maass M, et al.: Cotransplantation of non-cardiomyocytes increases persistence of highly purified murine induced pluripotent stem cell derived cardiomyocytes after intramyocardial injection into syngeneic mouse hearts. European Heart Journal 2012; 33 (Abstract Supplement); 430.

Task 2: Demonstrating and maybe improving electrophysiological and structural integration and maturation of transplanted cardiomyocytes

Partner 5 extended investigations on electrophysiological and structural integration and maturation of transplanted fetal cardiomyocytes. We were able to demonstrate a time-course of the electrophysiological maturation and integration of transplanted fetal cardiomyocytes, which will be further investigated for ES- or iPS-derived cardiomyocytes, too.


Additionally, partner 5 was able to demonstrate electrophysiological integration of transplanted induced pluripotent stem cell derived cardiomyocytes – which had never been described in any publications so far.


A.2.6. WP6 (S. COHEN - BGU.BE`ERSHEVA)

Description of main S & T results/foreground

The main goal of the workpackage is to provide the means and tools for the efficient delivery of therapeutic molecules and cells to the infarct area in damaged heart. A second goal is to provide a platform technology for stem cell expansion and differentiation, which will be later used by the other partners in cell therapy of the heart. Finally, the developed platform for controlled delivery of multiple growth factors were used in the design of large animal study (pigs) aimed to evaluate the effect of several adult stem cell types on infarct repair in chronic heart failure model.

Alginate scaffolds and perfusion bioreactors as a platform for stem cell expansion and differentiation

Our first objective was to investigate in collaboration with partners the use of our bioreactor platform technology for stem cell expansion and differentiation. We tested the ability of the cultivation in perfusion bioreactor to improve cell expansion, with
possible effects on differentiation potential, when the cells are grown within alginate scaffolds. Alginate scaffolds represent an efficient tool for 3D culture of various cell types. The scaffold provides 3D environment and support for cell growth in vitro or cell in-growth in vivo, while maintaining sufficient oxygen and nutrient transfer. Combination with perfusion bioreactor further expands the use of scaffolds for cell expansion and cell constructs maturation, by improved diffusion and convective flow. Human cardiac stem cells (CSC, obtained from Partner 4) were cultivated for 2 weeks in perfusion bioreactor. Compared to static (w/o bioreactor) cultivation, we found that the cultivation in perfusion bioreactor resulted in three-fold increase in cell expansion. Concomitantly, cell progenitor state was preserved, when cultivated in perfusion bioreactor, compared with gradual decrease of progenitor marker expression under static cultivation. Furthermore, it was found that bioreactor cultivation modulates gene expression of various families (muscle, cardiac, endothelial, smooth muscle, cartilage, bone). Specifically, this type of cultivation promotes the expression of cardiovascular-related genes, and suppresses the expression of other lineages, such as bone and cartilage. Specific stainings for adipogenic and osteogenic lineages showed, that cultivation in perfusion bioreactor inhibits both types of spontaneous differentiation of CSCs. Taken together, cultivation in perfusion bioreactor was shown to be an effective tool for expansion of stem cells, in order to achieve high number of cardiovascular progenitors.

Affinity-binding alginate biomaterial for efficient delivery of multiple therapeutic proteins

Our next objective was the development of controlled release systems for the efficient delivery of therapeutic molecules to the infarcted zone. We produced controlled release systems based on affinity-binding (alginate-sulfate containing) alginate. Alginate-sulfate is a synthetic polymer, that enables specific and reversible binding of heparin-binding proteins (bio-inspired by natural mechanism of protein binding to heparin and heparan-sulfate), including various growth factors (IGF-1, VEGF, bFGF, HGF, PDGF-BB, TGF-β1 and others). The delivery system can be fabricated as 3D scaffolds, which is suitable for cell growth, implantation, defect repair or tissue replacement, or as injectable hydrogel, which is more feasible from clinical perspective due to only minimally invasive delivery techniques, for cellular or acellular delivery of therapeutic proteins. Affinity-binding alginate scaffolds were able to sequentially release three angiogenic and heparin-binding proteins (VEGF, PDGF-BB, TGFβ1) from single device. When these scaffolds were implanted subcutaneously in rats, the sequential delivery of VEGF, PDGF-BB and TGFβ1 induced the formation of mature blood vessel network.

For the production of injectable affinity-binding alginate biomaterial, alginate-sulfate-protein bioconjugates were mixed with partially cross-linked alginate solution. The resulting hydrogel increased tissue availability and retention of HGF, compared to soluble protein or protein delivered within unmodified alginate, when injected into the scar tissue immediately after acute myocardial infarction (MI) induction in rats. Moreover, such affinity-binding alginate hydrogel maximized the therapeutic effects of HGF in mice model of severe hindlimb ischemia. The effects of sequential dual (IGF-1 followed by HGF) growth factor delivery by affinity-binding alginate hydrogel on tissue repair and regeneration were tested in rat model of acute MI. This treatment prevented infarct expansion and cell apoptosis, reduced fibrosis, and induced angiogenesis, 4 weeks after injection. In addition, it showed signs of endogenous myocardial regeneration (by higher incidence of Ki-67-positive cardiomyocytes and GATA-4 positive cell clusters). Collectively, these data establish the potential of affinity-binding alginate for controlled delivery of multiple therapeutic proteins for myocardial repair. Moreover, the system represents a platform for possible combination of this approach (either scaffold or hydrogel-based) with cell transplantation, in order to improve cell survival and function after MI.

Integration of cell-matrix interactions into alginate scaffolds for efficient delivery of therapeutic cells and cardiac tissue engineering

Our next objective was the development of scaffolds for the efficient delivery of therapeutic cells to the infarct zone and cardiac tissue bioengineering. Several concepts and their combinations were investigated. These included integration of multiple cell-matrix interactions into alginate scaffolds, along with application of various stimulation patterns (electrical or magnetic). These concepts are aimed to improve cardiac cell organization and function and promote vascularization, in order to create a platform for efficient cell delivery and cardiac tissue engineering. As the regular alginate scaffold is inert to most cell types, it could be used as a “blank canvas” to investigate various signals that are important in cell adhesion, organization and function. Two ECM-derived adhesion and signaling peptides, RGD and HBP, were covalently linked to alginate, and scaffolds were prepared. Rat neonatal cardiomyocytes were seeded into
combinatorial (HBP+RGD)-, single (HBP or RGD)-peptide-modified or unmodified alginate scaffolds. The cardiac tissue developed in the HBP/RGD-attached scaffolds revealed the best features of a functional muscle tissue, as judged by all studied parameters, i.e. immunostaining of cardiac cell markers, histology, western blot of protein expressions and metabolic activity. By day 7, well developed myocardial fibers were observed in these cell constructs. At 14 days the HBP/RGD-attached constructs presented an isotropic myofiber arrangement, while no such arrangement was seen in the other constructs. The expression levels of cardiac markers sarcomeric α-actinin, N-cadherin and connexin-43 (major gap junction protein generally associated with cardiac tissue maturity), showing preservation and an increase in Connexin-43 expression with time, further supported the formation a contractile muscle tissue in the HBP/RGD-attached scaffolds.

Stimulation patterns for cardiac tissue bioengineering and vascularization
One of the major challenges in engineering thick, complex tissues such as cardiac muscle, is the need to pre-vascularize the engineered tissue in vitro to enable its efficient integration with host tissue upon implantation. Thus, we introduced endothelial cells to the cardiac cell culture to be grown within RGD-HBP-modified scaffolds. In an attempt to further improve cardiac cell organization and function, we added the concept of electrical stimulation as a tissue maturation step. Tri-culture comprising of rat neonatal cardiomyocytes, cardiac fibroblasts and endothelial cells (HMEC-2 cell line, from partner 1) was seeded in RGD-HBP-modified scaffolds and cultivated under electrical stimulation conditions using previously developed electrical stimulator. Confocal imaging showed improved cell organization and capillary-like structure formation in scaffolds grown under electrical stimulation conditions. Importantly, this stimulation increased the expression of connexin-43 and CD31 (endothelial cell marker).

In an alternative prevascularization strategy, we explored new magnetic alginate composite scaffolds to provide means of physical stimulation to cells. Magnetite-impregnated alginate scaffolds seeded with aortic endothelial cells were stimulated during the first 7 days out of a total 14 day experimental course. Immunostaining and confocal microscopy analyses supported this observation showing that on day 14 in magnetically stimulated scaffolds without supplementation of any growth factors, cellular vessel-like (loop) structures, known as indicators of vasculogenesis and angiogenesis were formed as compared to cell sheets or aggregates observed in the non-stimulated (control) scaffolds.

In summary, the integration of multiple ECM-derived signals is essential for optimal cardiac tissue organization, and was successfully achieved in alginate scaffolds. Addition of stimulation as in vitro maturation steps also showed promising results in terms of cell organization, tissue maturation and vascularization.

Active involvement in WP10
The main output of the consortium work is the preparation of a protocol for cell transplantation to the infarct heart which can serve the basis for future clinical studies. One of the main obstacles in the design of such protocol is the need to significantly improve cell survival after transplantation. For this purpose, affinity-binding alginate (based on alginate-sulfate previously developed by us under the framework of the consortium) was offered as a complimentary tool to improve cell survival, engraftment and function, by controlled delivery and presentation of pro-survival (i.e. IGF-1) and angiogenic (i.e. HGF) factors. Extensive characterization was carried out on the system of alginate-sulfate with each growth factor. After characterization step completion, formulation preparation protocol and SOP for combination of this system with adult stem cell delivery were prepared and disseminated to other involved partners for the beginning of preliminary feasibility and retention experiments in pigs.

A.2.7. WP7 (J. KASTRUP - URH.Copenhagen)

We have identified and established methods for the isolation of mononuclear cells (MNCs) and culture facilities for isolation and expansion of mesenchymal stromal cells (MSCs) from human bone marrow and adipose derived stromal cells (ADSCs) from abdominal adipose tissue. The methods are implemented with reagents and equipment approved for clinical use.

We have evaluated the stability and characteristics of MSCs during 5 weeks in vitro expansion from MNCs to MSCs by weekly studies of the cell populations phenotypic profile, immunocytochemical pattern and plasticity potential. The data indicated
that, the MSCs reach and kept their “stemness” through several weeks in culture and at hyperconfluency.

We have investigated the optimal isolation procedure of ADSCs from lipoaspirate. With the chosen procedure we achieve 5.0 x 107 ADSCs from 50 ml tissue in one week. We found that, the isolated ADSCs were morphologically similar to BM-MSCs fusiform, fibroblast-like cells and expressed the three key MSC-markers: CD105, CD90 and CD73. The viability of ADSCs and the yield ADSCs per ml tissue from stored tissue was significantly lower compared to fresh tissue.

We have compared un-stimulated and VEGF stimulated MSCs from healthy subjects and patients with chronic ischemic heart disease (IHD). Flow cytometry analysis confirmed that MSCs from healthy donors and patients with IHD expressed the same surface markers, had identical proliferation time. In both groups VEGF-stimulation significantly increased the expression of the endothelial genes thrombospondin 1, Tie-2 and vWF and induced the capacity to form ring structures, when cultivated on extracellular matrix. Therefore, our data showed that MSCs from young healthy donors and elderly patients with IHD proliferate equally well and did not differ in their baseline characteristics or endothelial differentiation capacity.

We have performed DNA-microarray analyses on un-stimulated and VEGF stimulated MSCs from healthy subjects and patients with chronic IHD using the Affymetrix Genechip Human Genome HU133 plus 2. In healthy controls a total of 637 genes were up or down-regulated significantly (p<0.05 after correction for multiple testings) and 17 angiogenese genes were upregulated, when stimulated with VEGF for one week. Patients with ischemic heart disease had less change in genes during VEGF stimulation.

We have established methods for VEGF stimulation of MSCs and ADSCs towards endothelial precursor cells. After 7 days of VEGF stimulation flow cytometry showed that the classical MSC surface markers CD105, CD90, CD73, CD13 and CD166 remained highly expressed. However, endothelial-specific antigens KDR and CD31 were not expressed in VEGF stimulated MSCs at all. PCR analyses demonstrated that the gene expression of CD105, CD90 and CD73 was significantly decreased in VEGF-stimulated MSCs. Endothelial gene markers Tie-2 and ISNIG-1 were upregulated, whereas vWF and CD31 expression was low and KDR expression was non-detectable in VEGF-stimulated MSCs. The lack of expression of chosen endothelial markers could be due to the fact that they are markers of mature endothelial cells and the present one week VEGF stimulation protocol is too short to create mature endothelial cells. In a matrigel angiogenesis assay non-stimulated MSCs had a limited capacity to form networks of capillary-like ring-structures compared to VEGF-stimulated MSCs, which formed networks of capillary-like ring-structures.

Studies with 1, 2 and 3 weeks VEGF stimulation of MSC towards endothelial precursor cells have been conducted in ADSCs from healthy controls and patients with chronic ischemic heart disease. The VEGF stimulation effect was evaluated by flow cytometry, real-time q-PCR, immunocytochemistry, angiogenesis assays. The VEGF stimulation was compared with ADSC cultured in 10% fetal bovine serum (FBS) and 2% FBS (serum starvation). The studies found evidence of a tendency for ADSC differentiation towards endothelial lineage when cultured in serum-deprivation, but VEGF stimulation alone was without any effect. VEGF stimulation seems to predispose ADSCs for differentiation towards endothelial lineage, but the cells need additional stimuli to complete the process. There were only minor differentness between healthy controls and ischemic patients, but there was a tendency toward lower proliferation time in ischemic patients.

We have in a 3-D angiogenesis models (hanging drops and co-culture) investigated the influence of 1, 2 and 3 weeks pre-treatment with VEGF for the capacity of ADSCs to sprout in the spheroid assay. We could not detect any effect of pretreatment with VEGF on sprouting of ADSCs, but there was a significant sprout of ADSCs when stimulated with a cocktail of growth factors in an enthotelial stimulation media. Co-culture of ADSCs with human vascular endothelial cells (HUVEC) demonstrated that ADSCs secreted trophic factors inducing sprouting of HUVEC.

We have used different published protocols for differentiation of MSCs to cardiomyocytes. Cardiac-specific markers Nkx-2.5 GATA 4 and BNP were not expressed at all in any of the cultures.

We have established methods for labeling of MSCs with the ion-oxide Iodex-Tat-S. The data indicate that labeling with ion-oxide does not influence MSCs growth, viability and differentiation. In addition electron microscopic evaluation of MSCs labeled from 2 to 21 hours with two different doses showed no ultrastructural changes compared to a control group.

We have performed studies with different numbers of MSCs labeled with different concentrations of Ion-oxid for different time intervals. The studies demonstrated that cells labeled for 24 hours with full dose were best detected with MRI. This concept
was confirmed in a pig study with injection of ion-oxid labeled MSCs into a pig heart.
We have compared MSCs from bone marrow, ADSC from abdominal adipose tissue, epicardial and pericardial adipose tissue and thymus. The studies demonstrated that human epicardial fat was a rich source of MSCs and that they together with pericardial and subcutaneous fat cells had many similarities with cardiac progenitor cells. Moreover, all the cells had secretion of angiogenic growth factors with more or less angiogenic capacity tube formation on a matrixgel.
We have established SOPs for isolation and culture expansion of MSCs and ADSCs for clinical use. The protocols are now tested in clinical trials. We have established ion-oxid labeling of MSCs, which can be used to track the fate of MSCs after intramyocardial injection in patients.

A.2.8. WP8 (W. Luttmann - EuBioSci)

Description of main S & T results/foreground

EuBioSci has produced bioactive growth factors for internal experiments as well as for our collaborators in the project as needed. We have started with cloning and expression studies of around 25 different growth factors requested by the partners within this project. We have cloned in different vectors with or without tags for the expression in E.coli or 293 HEK cells. Some tags were necessary for the protein purification process, but had to be removed after purification depending from the needs of bio-activity and stability. After successful cloning, we have established in most cases bioassay for measuring of specific bioactivity. Afterward, we have produced large amounts of proteins depending of the need within this project.

The growth factors were selected based on the success of the expression studies and of the need by the partners of the project. From these selected factors large amounts of protein with high quality (high purity with low endotoxin levels) were produced. For each factor we have established a bio-assay for measurement of the specific bio-activity and have measured the endotoxin level for quality control.

SDFs:
We have started with cloning and production of the methionyl form of porcine and rat stromal cell-derived factor (SDF) in E.coli. This was preferentially for the needs of our cooperation partner 3 (U.Navarra) but was also available to all other partners. We were able to produce large amounts of both cytokines from E.coli. After establishing a migration assay for both factors, the specific bioactivity has been tested and found to be as expected. Both factors have been delivered to partner 3 and others within the consortium. Later we have worked on a method to remove the methionine amino acid from the end of the molecule (all recombinant proteins produced in E.coli have methionine as the start amino acid). There, within this project we were able to provide our partner the methionyl form as well as the nonmethionyl form. Both were active in cell migration assays.

SCF and CTGF:
Together with our partners we decided in the consortial meetings to produce rat SCF (stem cell factor, kit-ligand) and CTGF (connective tissue growth factor). Comparable to SDF, we were able to produce bioactive rat SCF and human CTGF for our partners.

IGFs:
Furthermore, we have produced different lots for both isofoms, IGF-I and IGF-II, as protein with tags and have removed afterward the unwanted tag through proteolysis.

HGF:
we have first tried to produce HGF in E.coli but without success. We then got good results by transfecting HEK (293 cells), but
we got only small about of proteins after transient expression of the cells. Finally, we were able to produce HGF in a stable transfected cell line with good production rate.

VEGFs:
We have cloned different isoforms for human, mouse and rat species and produced batches of VEGF-111, VEGF-121, VEGF-165b, and VEGF-165. Each isoform has different properties during the purification process and therefore need a separate method to gain pure and endotoxinfree protein fraction.

FGF:
We have produced different lots for both isoforms, FGF-1 as well as FGF-2 and have produced large batches of FGF-1 for our partners.

Neuregulin:
We have cloned different isoforms and have produced large batch of neuregulin-1beta isoform

First we have tried to produce all growth factors in E.coli but we found that the bio-activity of some such as HGF was very low. Therefore, we have established for these growth factors transient and stable expression in the HEK293 cell line.

One of the major problems within stem cell research is that every group use growth factors from different sources with different quality, bio-activity, as well as different levels of endotoxin. Storage conditions, especially if growth factors are stored as liquid, has also influence on the the bio-activity. Furthermore, many groups use different concentration of growth factors. To overcome this problem, one aim of our work was to combine some growth factors as cocktails, and store these under different conditions.

We used different concentrations of the combinations of FGF with VEGF; HGF with IGF; EPO with EGF and L-6, SCF together with IL-7, G-CSF, GM-CSF, and IL-6. The cocktails have been distributed to the partners and tested. Our internal testing showed very good results regarding stability.

Finally, we had an extensive collaboration with partner 1 for the production of a novel factor with potential activity for improved generation of endothelial cells and vascularization. First, a stably transfected cell line producing the factor was provided from the partner 1 laboratory and a Flag-tagged version of the protein is currently produced and purified. During the second period, we have then generated different expression constructs with HIS and Flag-tags alone or in combination. Furthermore, we used proteolytic cleavage sites to remove the tag. At the end, we have produced larger amounts of this factor for partner 1 as well as for other partners within the consortium.


Description of main S & T results/foreground

Partner 9 has in the first period established and improved his standard production possibilities for stem cells from fresh and frozen cord blood samples. In the second period, Partner 9 has continued to regularly supply P1 and to some extent also P2 and P7 with cord blood and generated cord blood stem cells, i.e. P1 and P2 with ECFCs/BOECs and P7 with mesenchymal stem cells from cord blood. As a cord blood bank this partner has large collections of cord blood available and is continuously collecting cord blood through contracts with several hospitals. Cord blood is collected and stored for i) women who want to store frozen autologous cord blood cells for potential later use for their children; ii) the storage of cells in a bank for the use of HLA-matched or non-matched stem cells in various clinical therapies such as leukemia therapies or regeneration therapies for
IHD or iii) collected for research work by cooperation partners. Stem cells in part prepared by P9 were used by Partners 1 and 7 for gene profiling of ECFCs and MSCs (see WP3 and WP7). Other batches were available for Partner 2, Partner 3 and Partner 7 for evaluation, e.g. in animal MI models. The work with ECFCs, boosted by the results of P2 with autologous ECFCs in the procine model (see WP1), has resulted in the promising characterization of two factors of ECFCs which might characterize the progenitor status of the cells and could be responsible for the pro-vascularization effects of the cells in MI models. Evaluation of the human cells in immunocompromised rats have indicated their potential usefulness in the regeneration of heart tissue comparable to autologous cells. This is important as it is likely that future clinical trials employing stem cells, such as ECFCs and MSCs, to treat patients with congestive heart disease may depend in part on cells stored in cord blood banks, because these cells may not properly function when taken from the patient and cord blood may be the best way to find an appropriately HLA matched unit. This could be the case for a majority of patients, even if in future some patients will have the opportunity to use their own (autologous) stem cells that have been saved from their parents right after birth.

Partner 9 has expanded formerly frozen UCB cells by treatment with SCF (stem cell factor) and GM-CSF (granulocyte-macrophage colony stimulating factor) in the presence of IL-6 and started to evaluate whether the newly isolated peptide may be of potential use in improved cryopreservation/thawing protocols or in expansion of the stem cells.

Unfortunately, during the second term of this grant, P9 (VivoCell) would run into a problem regarding his financial basis and had to reduce collaborators by half. This was caused by the unforeseen withdrawal of a major private financier. In parallel with this the extent of research done by the company was largely reduced and P9 could not further work on the project, but remained a supplier of cord blood samples. This started about 6 months into the second period and resulted in a major reduction of activities from 12 months within the second period. All activities planned for P9 in the generation and analysis of cord blood stem cells were then taken over by P1 besides the large scale cultivation and differentiation technology which was initiated by P9, but not finally developed.

A.2.10. WP10

P1 - E. Hofer - MU.Vienna
P2 - S. Janssens - VIB.Leuven
P3 - F. Prosper - U.Navarra
P4 - J.Leor - U.TelAviv
P6 - S.Cohen - U.Be`erSheva
P7 - J. Kastrup - URH.Copenhagen

Description of main S & T results/foreground

WP10 was a cooperative effort of P1, P2, P3, P4, P6 and P7, each group focusing on its special expertise. P3 in collaboration with P4 and P7 have evaluated different adult stem cells from bone marrow, adipose or cardiac tissue, P1 in collaboration with P2 have tested partially differentiated endothelial progenitor cells (ECFCs/BOECs), P1 has developed methods to genetically modify endothelial progenitors to make them more efficient, P4 and P6 have collaborated to evaluate engineered tissue and P2 collaborating with P3 have performed the final analysis of combination of cells and reagents in a porcine model. Clinical partners P2 and P7 have prepared protocols as basis for planned follow-up clinical trials using autologous ECFCs/BOECs and MSCs.

Adult stem cells from BM, adipose and cardiac tissue:

P3 in collaboration with P2 and P7 has first performed a systematic study evaluating different forms and isolates of stem cells including multipotent adult progenitor cells (MAPC), adipose-derived stem cells (ADSC) and mesenchymal stem cells (MSC) in an acute model of myocardial infarct in mice and a chronic model of myocardial infarct in rats. All cells displayed beneficial effects regarding LV contractile function and infarct size. Based in part on these data and own previous experiments and clinical trials (see Haack-Sorensen et al., 2012) P7 has
prepared a protocol as a basis for a follow-up clinical trial using human autologous MSCs and the tracking of the cells after injection (see below and D10.6).

In the above mentioned experiments of P3, MAPCs were not present beyond 1 week after peri-infarct injection, but presumably contribute to improvement by secreting stimulatory factors. MSC provided also a long-term benefit associated with smaller infarct size and higher vascularization. The conclusion from these studies is that ADSC provide a source of cells which can be equal or superior to the other stem cell types for one or several parameters. ADSC improved significantly heart function, tissue viability and vessel density. They seemed to be especially advantageous as they induced a long-lasting (3 month) improvement of cardiac function, tissue metabolism, revascularization and heart remodeling displaying smaller infarct size and thicker infarct wall resulting in better systolic and diastolic functions. In conclusion, the obtained data suggest ADSC as an optimal beneficial source of stem cells on which further work should be focused. P3 further determined the long-term effects of ADSCs in a porcine model (Mazo et al., 2012) and also evaluated ADSCs transplanted on collagen scaffolds (Arana et al., 2012).

In this context P4 in collaboration with P7 has found that human epicardial fat provides the best source of MSCs with unique reparative properties (see Adutler-Lieber et al., 2012, and Naftali-Shani et al., 2012). These cells had many similarities to those of atrial-derived cardiac progenitors plus improved regenerative potential. The use of these cells could also avoid the need of cardiac biopsies.

Partially differentiated endothelial progenitors:
In terms of a more differentiated cell, the ECFCs/BOECs, P2 has obtained exciting results on the stimulation of perfusion and heart functions in a porcine model. These cells also integrated to some extent contributing to increased vascularization. As increased perfusion will be the precondition for any other cell therapy aiming at reconstituting heart muscle, a protocol as basis for a follow-up clinical trial was prepared by P2 (see below and D10.6.). P1 is further in progress to develop genetic modification protocols to increase the efficiency of the ECFCs. The overexpression of factors has been shown to increase the pro-vascularization activity of ECFCs in vitro. Corresponding experiments using MI models are underway.

Engineered tissue:
P4 and P6 have collaborated to evaluate engineered cardiac muscle and its integration with host tissue upon implantation. It included attempts to prevascularize the patch by introduction of endothelial cells to the cardiac cell culture to be grown within RGD-HBP-modified scaffolds. In addition, in order to further improve cardiac cell organization and function, electrical stimulation as a tissue maturation step was included. Tri-culture comprising of rat neonatal cardiomyocytes, cardiac fibroblasts and endothelial cells was seeded in RGD-HBP-modified scaffolds and cultivated under electrical stimulation conditions using previously developed electrical stimulator (Dvir et al., 2009; Ruvinov et al. 2012b).

Combination of cells, factors and biomaterials:
Based on the exciting data of P2 (Dubois et al., 2010) using ECFCs/BOECs in a porcine model showing improved vasculatiation and perfusion and on the encouraging data of P3 regarding ADSCs (Mazo et al., 2012) as well as on data of P4 and P6 on the delivery of IGF and HGF from an injectable alginate biomaterial (Ruvinov et al., 2012a) resulting in improved myocardial regeneration we have designed a final experiment combining both principles, stimulation of vasculatization by ECFCs or ADSCs and stimulation of cardiomyocyte repair by IGF plus HGF in a porcine MI model. Groups of 10 mini-pigs were injected with 200 ug each of IGF plus HGF in a nanoparticle formulation with alginate (protocol worked out by P6) alone or in combination with autologous ECFCs or ADSC. Initial functional analysis (echo, MR) were positive, however the endpoint of the experiment has not yet been reached and histology has also to be performed for final conclusions. We are however confident that this evaluation will lead to a step forward in cell therapy of MI.

Based on the above summarized data clinical partners P2 and P7 have so far prepared the following protocols and will perform follow-up clinical trials using MSCs and ECFCs in MI patients without other options (see deliverable D10.6). In case that the above described porcine experiment using the combination of cells, factors and alginate should give the expected results, P2 and P7 will collaborate to design corresponding follow-up trials in collaboration with P6 and P9.
A.3. SCIENTIFIC PUBLICATIONS

As a final joint effort each partner has written a review describing the state-of-the-art in the area of the cells/technologies of his specific expertise which includes summaries of the work performed in the course of this project. This will be published by Springer Verlag beginning of next year. It will include a foreword/introduction describing the InfarctCellTherapy/INELPY consortium and acknowledge the support by the EC. The reviews prepared for this compendium are attached in Deliverable D11.5. The reviews are destined to go to the publisher in December 2012 after a final editing/revision following an review in November 2012. The table of contents of the book are given below:

Book Title: Adult and pluripotent stem cells: Potential for regenerative medicine of the cardiovascular system
Editors: Jürgen Hescheler, University Hospital of Cologne, Germany, and Erhard Hofer, Medical University of Vienna, Austria
Publishing Editor: Meran Owen
Springer Verlag, Heidelberg and New York (2014)

Table of contents:
1- Cell therapy of acute myocardial infarction and ischemic cardiomyopathy:
from experimental findings to clinical trials
Stefan P. Janssens
Gasthuisberg University Hospital, KU Leuven
2- Mesenchymal stem cells for cardiac repair. Preclinical models of disease
Manuel Mazo, Miriam Araña, Beatriz Pelacho, Felipe Prosper
Navarra and Foundation for Applied Medical Research, Pamplona, Spain
3- Endothelial progenitor cells derived from cord or peripheral blood and their potential for regenerative therapies
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bVivoCell Biosolutions GmbH, Graz Austria
4- Resident cardiac progenitor cells
Ayelet Itzhaki-Alfia and Jonathan Leor
Tamman Cardiovascular Research Institute, Leviev Heart Center, Sheba Medical Center; Sheba Center of Regenerative Medicine Stem Cells and Tissue Engineering, Sheba Medical Center, Tel-Hashomer; Neufeld Cardiac Research Institute, Sackler Faculty of Medicine, Tel-Aviv University, Israel
5- Cardiac cell replacement therapy with pluripotent stem cell derived cardiomyocytes
Benjamin Krausgrill, Marcel Halbach, Jürgen Hescheler
University Hospital of Cologne, Cologne, Germany
6- Biomaterials for cardiac tissue engineering and regeneration
Emil Ruvinov and Smadar Cohen
Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva, 84105 Israel
7- Clinical gene and stem cell therapy in patients with acute and chronic myocardial ischemia.
Jens Kastrup MD DMSc, Annette Ekbland MSc PhD, Mandana Haack-Sørensen MSc PhD, Anders B. Mathiasen MD, Abbas A. Qayyum MD.
Cardiology Stem Cell Laboratory and Cardiac Catheterization Laboratory 2014, The Hearth Centre, Rigshospitalet Copenhagen University Hospital and Faculty of Health Sciences, DK-2100 Copenhagen, Denmark.
8- Adipose-derive stromal/stem cells and their differentiation potential into the endothelial lineage
PEER-REVIEWED PUBLICATIONS OF THE CONSORTIUM:

WP1 - P2 - S.JANSSENS / P.CARMELIET - VIB.Leuven


WP2 - P3 - F.PROSPER - U.Navarra


WP3 - P1 - E.HOFER - MU.Vienna


Submitted or in preparation:


WP4 - P4 - J. LEOR - U.TelAviv


*Ruvinov E, Leor J, Cohen S. The promotion of myocardial repair by the sequential delivery of igf-1 and hgf from an injectable alginate biomaterial in a model of acute myocardial infarction. Biomaterials. 2011;32:565-578

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WP5 - P5 - J.Hescheler - UKK.Cologne


Pfannkuche K, Hannes T, Khalil M, Noghabi MS, Morshed A, Hescheler J, Dröge P. Induced pluripotent stem cells: a new


WP6 - P6 - S. COHEN - U.Be`erSheva


Ruvinov E, Leor J, Cohen S. The effects of controlled HGF delivery from an affinity-binding alginate biomaterial on angiogenesis and blood perfusion in a hindlimb ischemia. Biomaterials, 2010; 31(16):4573-82


*Sapir Y, Kryukov O, Cohen S. Integration of cell-matrix interactions into alginate scaffolds for promoting cardiac tissue regeneration. Biomaterials, 2011; 32(7):1838-47


WP7 - P7 - J. KASTRUP - URH.Copenhagen


WP8 - P8 - W.Luttmann - EuBioSci

A.4. POTENTIAL IMPACT

Based on the described work the project has achieved or will achieve in the follow-up impacts at several levels:

On the basic/translational research level:
Our data have been published in numerous peer-reviewed journals (28 articles) and reported at scientific meeting. Each PI and their major collaborators have attended between 10 to 20 international scientific meetings during the course of the project and have reported results of the projects in invited talks, short communications and poster presentations. This amounts to a total of over 100 international presentations. This should have sufficiently communicated our results and should contribute to advance the respective research field. In addition we are in progress to prepare a book publication with review articles in the field with Springer Verlag (see D11.5)

On the clinical level: Based on major outcomes of the project or two clinical partners P2 and P7 have prepared protocols as basis for follow-up clinic trials. Both partners are already engaged in ongoing clinical trials using cell transplantation therapies and are European experts in this field. Our results have shown that follow-up clinical trials with fat tissue-derived mesenchymal stem cells and especially with endothelial progenitors (ECFCs) should be promising. If successful this should lead to a health benefit for IM patients. This is important as currently few or no therapeutic options exist for part of ischemic cardiomyopathy patients. In the medium turn we assume that also the combination therapy explored with cells plus slow release forms of growth factors may find its way to the clinic and in the longer term ex vivo generated tissue patches are promising.

On the company level: Based on the identification of novel factors of potential importance for tissue regeneration we will in collaboration with the two partner companies further develop these to evaluate their potential therapeutic use. Following the foundation of a start-up we will try to obtain support from the EC, from national agencies and from private investors for the further development. If this will be successful then the project may have generated an impact on the European biomedicine-related biotechnology industry.

On the public dissemination level:
At the occasion of the final meeting in Vienna we have made a press release via the Austrian Press Agency (APA) on the content and major outcome of the project (attached below). This was then published partially on the website of the “Standard” and in printed form in the “Salzburger Nachrichten” (attached below), two major quality newspapers in Austria with regular reports on scientific news.
Finally we have published with Insight Publishers a two page article on the project. Insight publishes Europe's leading publications focusing on research and development, helping to drive knowledge transfer and create real impact for research projects. With a core readership across all research disciplines and across industry, academia and science, the publication also has a carefully targeted circulation according to each edition's particular focus. The publication is supported online via the popular www.projects.eu.com website, Europe's first dedicated online community bringing those involved in research and development at every level the latest news, analysis and opinion and the chance to exchange ideas and find partners. Our article is available to the public at http://viewer.zmags.com/publication/0ba7a868#/0ba7a868/88.

As such we assume to have made an impact in the public in advertising the support of the EC for this project and the need of further research in this area.

On the educational and training level:
Finally, the project has provided opportunities for education and training of Ph.D. students and post-doctoral fellows at excellent European laboratories. It has provided opportunities for young post-doctoral fellows and students to perform work in a promising area of molecular medicine and biotechnology.

A.5. WEBSITE ADDRESS AND CONTACT DETAILS

THE WEBSITE OF THE PROJECT IS:

www.infarctcelltherapy.eu

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**Result In Brief**
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