SIXTH FRAMEWORK PROGRAMME

Specific Targeted Research Program

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SC&CR

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APPLICATION AND PROCESS OPTIMIZATION OF STEM CELL PRODUCTS FOR MYOCARDIAL REPAIR

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PROJECT EXECUTION

MAIN OBJECTIVES

Myocardial infarction is an irreversible injury where a sudden interruption of blood flow caused by the occlusion of an artery, leads quickly to cardiac myocytes death, myocardial tissue loss and scar formation. Recent findings in animal models have provided evidences that somatic stem cells from different tissues can be phenotypically reprogrammed to the cardiac lineage and propagated in exogenous stem cell niches. This has suggested that stem cell-based therapies might be applied to induce myocardial regeneration after acute injuries as infarction or chronic diseases as heart failure.
The project arose as a joined effort of European Scientists and Clinicians to provide a comprehensive and multidisciplinary approach to the study of cardiac myocyte differentiation of stem cells and to develop valid strategies for myocardial repair.

The main specific aims of the project were the following:
1) To perform phase 1 clinical trials to test the safety of autologous administration of bone marrow derived cells in patients suffering chronic ischemic disease.
2) To analyse the physiology and to assess the functional properties of stem cell-derived cardiac myocytes.
3) To analyse the signals triggering stem cell proliferation/survival and recruitment into the infarct area and to test the efficacy of stem cells transplantation in myocardial regeneration by the use of pre-clinical mouse model of heart infarction.
4) To characterize the cellular events and the molecular pathways involved in cardiac differentiation in order to establish protocols for the derivation of functional cardiac myocytes from somatic stem cells.

Contractors

Participant role	***Partner no***	***Participant name***	***Participant short name***	***Country***	***Responsible***
CO	1	Istituto Dermopatico dell'Immacolata	IDI	Italy	M. C. Capogrossi
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CR	4	The Jagiellonian University	JU MC	Poland	Dembinska-Kiec
CR	5	Inst. of Plant genetics and crop plant research	IPK	Germany	A. M. Wobus
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CR	8	European Molecular Biology Laboratory	EMBL	Italy	N. Rosenthal
CR	9	Areta International	ARETA	Italy	M.L. Nolli
CR	10	Ludwig-Maximilians Universitaet Munchen	GENE CENTER	Germany	M. Hallec

WORK PERFOMED AND RESULTS OBTAINED.

1) To perform phase 1 clinical trials to test the safety of autologous administration of bone marrow derived cells in patients suffering chronic ischemic disease.

Autologous transplantation of bone marrow stem cells into infarcted myocardium is a novel and promising strategy for the therapy of heart failure due to ischemic heart disease. Data in the literature suggest that bone marrow-derived cells transplantation into infarcted or chronically ischemic myocardium could be remarkably effective in wound repair in terms of heart muscle regeneration and improvement of coronary blood flow. However, few studies directly address the role and efficacy of human stem cells in myocardium repair and many questions regarding safety and effectiveness of their therapeutic application remain open.

Clinical application of autologous BM-derived $CD133^+$ cells for induction of angiogenesis in patients affected by ischemic heart disease and refractrory angina.

To address the above issues, Partner #2 performed a clinical trial based on bone-marrow-derived cell transplantation in patients with refractory ischemia unsuitable for conventional revascularization. Eight patients have been enrolled from December 2001 to June 2008. Patients had severe chronic angina despite maximally tolerated medical therapy and evidence of anterior, antero-lateral, or inferior ischemia on nuclear imaging. They were judged ineligible for percutaneous or surgical revascularization as assessed by coronary angiography.
Bone marrow-derived CD133+ stem cells were the elected choise because they represent a population of early precursors highly enriched for multipotent stem cells with strong angiogenic capacity. Cells were purified by immmuno-magnetic techniques and delivered via a direct intra-myocardial injection. No deaths or major complications were observed postoperatively. Periprocedural laboratory evaluations demonstrated an absence of inflammation or myocardial injury. No ventricular arrhythmias were recorded during hospitalization. Post-procedural pericardial effusion was excluded in all cases by echocardiography.
All patients experienced a significant improvement of angina class. The frequency of angina episodes per day was decreased and quality of life also improved. The 24-hour Holter recordings 1, 3, and 6 months after injection did not reveal ventricular arrhythmias.
At 6 months of follow-up, the number of segments with stress-inducible ischemia per patient decreased. Perfusion at rest also improved while the mean ejection fraction did not change significantly from baseline. An improvement of myocardial blush into the injected area was evident in 3 patients.
Were applicable, the late outcome resulted in no deaths, myocardial infarction, malignant ventricular arrhythmias or new ventricular masses after an average of 36,5 (range 8-62) months of follow-up. Improvements of angina status obtained at 6 months were found unchanged in most patients.
These results show that that direct minimally invasive inoculation of autologous bone marrow-derived CD133+ cells into the ischemic myocardium of patients with refractory angina pectoris may safely reduce anginal symptoms and improve myocardial perfusion.

Administration of autologous bone marrow-derived cells in patients affected by peripheral arterial disease.

Partner #4 performed a phase I clinical trial of autologous bone marrow-derived stem cells intramuscular delivery in 20 patients affected by peripheral artery obliterative disease (PAOD) and evaluated the improvement of vascularisation and limiting of ischemic necrosis of injected limbs. Total mononuclear or, alternatively, CD133+ purified bone marrow-derived cells were used for transplantation.
Projected observation time was 6 months. In 9 cases during the observation time (up to three months) the cellular treatment was not successful and amputation of ischemic leg was necessary. In 11 cases out of 20 , the ischemic limb was preserved over 6 months following the main observation period. The therapy however was associated with the transient, significant reduction of pain expressed by subsequent marked reduction of analgesics use and correlated with the improving psychological and clinical status due to the improvement of the overnight sleep. Hemodynamic parameters were also improved, with most striking changes over first 3-4 weeks after intervention. No changes were observed in contralateral, non injected limbs.
This study has to be considered as a pilot trial. The tendency to the ischemia reduction observed after intervention was partial and in some cases (n=11) seems to prevent major amputation. Results however do not support the suggested long-lasting beneficial effect of the bone marrow-derived cells in the treatment of the PAOD.

Generation of antibodies against surface proteins of MSCs and set up of protocols for ex-vivo expansion of human MSCs.

In addition to stem cells of the hematopietic lineage such as the CD133+ cells, the bone marrow includes other cells endowed with a certain degree of plasticity and which have the ability to transdifferentiate and acquire different phenotypes. Such cells, referred to as mesenchymal stem cells (MSCs), belong to the stromal compartment of the bone marrow and data in the literature suggests that they could be valid candidates for regenerative cell therapy.

To generate an antibody against surface proteins of mesenchymal stem cells may help to understand where these cells are localized and how and when they are recruited to damaged tissues. In addition high affinity antibodies may allow ultra-efficient purification procedures from a variety of biological samples and sources. One of Partner #9 tasks was based on the production of antibodies specifically recognizing MSCs. Human mesenchymal cells were expanded in bio-reactor to rapidly increase the number of cells. Monoclonal antibodies were generated by in vivo injection of positive hybridomas in mouse peritoneum. After several rounds of immunizations spleens were removed, homogenated and fused to mouse myeloma cells. After cloning and testing for specific immunoglobulins production 49 positive clones were selected of which 33 were positive on mesenchymal cells while 2 recognized cells induced to differentiate into bone precursors. After subcloning 27 clones were selected for further characterization.
Immunofluorescence analysis revealed that 3 clones (M6, M20, M27) recognize a cell surface antigen. These clones were selected for further rounds of subcloning. Each clones generated about ten different single-cell subclones which antibody production was confirmed by a new ELISA test.

Contrarily to other stem cells types, MSCs can be easily obtained and expanded in vitro. However, for transplantation in humans, GMP culture conditions have to be devised. Current methods for culture and propagation of mesenchymal stem cells require the use of media containing fetal bovine serum (FBS), which represents a potential safety concern in clinical application. In order to improve the method of culture of mesenchymal cells in clinical grade conditions, Partner #9 developed a culture medium in which FBS is substituted with human platelet lysate (PL).

2) To analyse the physiology and to assess the functional properties of stem cell-derived cardiac myocytes.

Recent investigations have called for caution concerning the ability of somatic stem cells to terminally differentiate into cardiac myocytes and to acquire their functional properties. In addition, several evidences indicate that embryonic stem cell-derived cardiac myocytes are potentially arrythmogen, thus resulting unsafe for myocardial cell therapy. One specific aim of the project was to analyse the physiology of mouse ES and somatic adult stem cell-derived cardiac myocytes and in particular to assess the functional properties of stem-cell derived cardiogenic cells.

Influence of cell fusion on differentiation of human cord blood (UCB)-derived $CD34^+$ cells in contact with cardiac myocytes.

Partner #2 performed studies on human cord blood-derived CD34+ cells and showed that when co-cultured with neonatal cardiomyocytes these cells acquire excitation and contraction properties similar to those of immature cardiac myocytes. In addition they analysed the mechanism underlying CD34+ cells conversion to a cardiac phenotype. The results indicate that such conversion does not rely on a cell-autonomous transdifferentiation event and suggest that fusion with pre-determined cardiac myocytes may play a major role in the acquisition of a cardiac phenotype by blood-borne stem cells.

Molecular and functional characterization of Cardiac Progenitor Cells.

Functional competence of differentiated SC and their homogeneity with the surrounding myocardium are crucial issues in tissue repair. Therefore it is important to determine the extent to which functional differentiation of SC can be achieved, how it can be affected by the conditions under which replication/differentiation occurs and how it correlates with expression of gene products. Recent findings identify the heart as a source of progenitor cells. Partner # 3 (in collaboration with Prof. Giacomello, Università La sapienza, Roma) developed a differentiation system based on the use of cardiac progenitor cells (CPCs). CPCs were isolated as primary culture from myocardial specimens. The obtained cell population can be expanded on fibronectin as a monolayer of adherent cells. However suspension culture of these cells results in the formation of multicellular clusters dubbed “cardiospheres” (CSs). CSs include cells with stemness features and elements acquiring spontaneous contractile activity and cardiac-specific molecular markers.
The first characterization revealed the presence of molecular markers for replication, stemness and cardiac differentiation during the maturation of CSs. However, the presence of specific differentiation markers does not imply the acquisition of tissue specific function; moreover, a quantitative estimate of differentiation frequency is essential for the evaluation of differentiation protocols. Striated muscle is endowed with a specific mechanism of Ca2+ release from intracellular stores based on channels named Ryanodine Receptors (RyR), which can be opened by exposure to caffeine. Therefore, the identification of caffeine-induced (i.e. RyR-mediated) Ca2+ release has been used as a marker of functional differentiation toward a muscle phenotype. Discrimination between skeletal and cardiac muscle was then carried out by immunohystochemical demonstration of expression of the cardiac isoform of RyR channels (RyR2).

Results show that the muscle-speficic (RyR-mediated) Ca signals are rare in early cardiac precursors (pre-CS cells) and become frequent in CS-derived cells. This indicates that in the differentiation system tested, clustering of CPCs in a three-dimensional structure to form CSs may be essential to induce cardiogenic differentiation. However the kinetics of caffeine-induced Ca transients are slower in post-CS cells than in adult cardiac myocytes. This suggests that although

adequate to cause visually detectable mechanical activity, the level of EC-coupling differentiation achieved within CSs may be rather primitive.

Partner #7 performed an electrophysiological characterisation of fetal human cardiac progenitor cells. Human fetal CPCs were isolated based on the expression of Sca-1. Undifferentiated CPCs expressed Cx40, 43 and 45, albeit mostly intracellular localized. To induce differentiation 5-Azacytidine and TGFβ was used. After 3 weeks CPCs differentiated into spontaneously beating cells (60-90%) and display electrophysiological characteristics more mature than that of differentiated human embryonic stem cell derived cardiomyocytes. To test whether hyperpolarization of CPCs might contribute to differentiation, undifferentiated CPCs were cultured at low potassium concentration (LowK). Such treatment induced the expression of cardiac specific markers and a spontaneous electrical activity.

Intercellular communication between mature and immature cardiomyocytes.

A characteristic feature of immature, stem cell-derived cardiac-like cells is spontaneous impulse formation and restricted electrical coupling. In order to evaluate the possible the arrithmogenic potential of these cells, Partner #7 analysed the intercellular communication between mature and immature cardiomyocytes. Their interesting results indicate that spontaneously active immature cardiomyocytes are in fact able to trigger mature cardiomyocytes depending on their level of electrical coupling and the on the amount of coupled immature myocytes. Moreover, by co-culturing non-cardiac Kir2.1 expressing cells with spontaneously active rat cardiomyocytes they show that the electrotonic coupling between the two cell types results in hyperpolarization of the cardiomyocyte membrane potential and silencing of spontaneous activity. These results demonstrate the power of electrotonic coupling for the application of specific ion currents into an engineered cellular construct such as a biological pacemaker.

Development of biopolymers matrices to embed stem cells into a 3D environment and evaluation of electrical and contactile activity of stem cells into 3D structures.

The inclusion of stem cells in three-dimensional structures before transplantation could help in order to maximize their proliferation/survival and to facilitate their cardiac differentiation program.

Partner #6 activity has been focused on the preparation and characterization of collagen I based assemblies to be used as injectable matrices. In a previous study it was shown that the addition of heparin to fibril forming collagen solutions affects the rheological behaviour of the scaffolds in such a way that the viscosity of a collagen gel decreases if heparin is added. The further characterization of the biohybrid gels necessitated their compositional analysis. Therefore, the heparin content and the morphological characteristics of different collagen based assemblies have been evaluated. Moreover, a protocol to grow 3T3 fibroblasts inside solidified collagen gels has been devised. Further analysis, focused on testing and characterizing the effects of heparin addition to fibril forming collagen solutions show that the specific heparin-collagen interaction determines the assembly process via predominantly electrostatic interaction. Lateral accretion of the prenuclear microfibrillar material leads to build-up of the collagen-heparin cofibrils with a stabilising effect due to intercalated heparin. The reconstituted cofibrils with their unusual morphology and GAG intercalation – a phenomenon not reported *in vivo* – can be expected to exhibit interesting mechanical and biochemical behaviour as a biomaterial for extracellular matrix scaffolds. Further detailed studies will address these issues and elucidate the exact binding of heparin inside the collagen fibrils.

Partner #2 set up conditions in order to culture stem cells into a collagen I-based hydrogel. During the second reporting period, the main activity was to analyze stem cells interaction within the 3D environment and their potential response to hypoxic conditions. The results show that 3D culture does not affect the phenotype of stem cells and also that culture into a 3D environment in the presence of cytokines improves survival under hypoxia conditions of CD34+ endothelial precursors. These observations indicate that collagen I-based hydrogel is a polymer that might be used as an injection medium for cellular therapy of the infarcted myocardium using endothelial progenitor cells.

Partner #7 performed studies on the electrical behaviour of neonatal rat cardiomyocytes as well as undifferentiated and differentiated cardiac progenitor cells embedded in different type of 3D collagen matrices. Different methods for introducing cells into a scaffold material were also tested. Such studies demonstrate that despite filling of 3D matrices with cardiac cells is sub-optimal, electrical coupling is adequate. They also show that filling is more efficient with cardiac precursors that can proliferate inside the matrix compared to differentiated cells.

3) To analyse the signals triggering stem cell proliferation/survival and recruitment into the infarct area and to test the efficacy of stem cells transplantation in myocardial regeneration by the use of pre-clinical mouse model of heart infarction.

Analysis of the effects of HMGB1 on Cardiac Stem Cells (CSCs).

High Mobility Box 1 Protein (HMGB-1) is a non-histone chromatin protein which modulates gene transcription. Several recent evidences, however, indicate that HMGB1 is released by dead cells, and diffuses to the membrane of nearby cells, where it activates specific receptors. In this way, HMGB1 acts as a signal of tissue damage, and could potentially have a role in inflammation, stem cell recruitment, and tissue repair and remodeling.
In order to test such hypothesis, Partner 1 investigated on the possible involvment of HMGB1 in heart repair and demonstrated that HMGB-1 administration into the mouse heart during acute myocardial infarction resulted in cardiac tissue regeneration by stimulating proliferation of resident cKit+ CSCs and significantly improved ventricular function. Subsequently, the effect of administration of HMGB1 directly to CSCs was analysed. Such experiments showed that HMGB1 has no direct effect on CSCs proliferation while it induces CSCs migration. Therefore, it was examined the hypothesis that cardiac fibroblasts (cFbs) exposed to HMGB-1 may exert a paracrine effect on CSCs. cFbs were chosen as they represent the most abundant cell population in the heart and they are particularly resistant to ischemia-induced apoptosis. By a multiplex bead-based immunoassay it was found that HMGB1 promotes growth factors and cytokines release by cFbs, The Conditioned Medium (CM) obtained from untreated- and HMGB1-treated cultured cFbs was then used to stimulate the activation of murine CSCs obtained from the non-myocytes population by magnetic selection for the antigen c-Kit. CM from HMGB1-treated cFbs significantly improved CSC migration compared to CM from untreated cFbs and to basal medium with and without HMGB1. Finally CM from HMGB-1 treated cFbs stimulated CSC proliferation, evaluated by cell counting and BrdU incorporation assay.

Cardiac regeneration in mIGF-1 transgenic mice .

It had been previously demonstrated by Partner #8 that the local isoform of mIGF-1 may induce hematopoietic stem cell mobilization and tissue repair. As a starting point in this project,

Partner #8 performed a detailed analysis on different transgenic mice expressing an IGF1 isoform in a myocardium specific manner using the α-MyHC cardiac-specific promoter. Postnatal mIGF-1 transgenic hearts displayed accelerated cardiomyocyte hypertrophy, due to a significant increase in cell size compared to wild-type hearts. Although systolic and diastolic components of the cardiac function were mildly affected, the hearts were not dilated and cardiac output and chamber diameters remained at normal levels throughout postnatal stages.

In parallel, two different methodologies for heart injury were established: LCA (Left Coronary Artery) ligation technique and cardiotoxin (CTX) injection in the heart wall of the left ventricle. Both methods were used to test the ability of a locally acting Insulin-like Growth Factor-1 isoform (mIGF-1) to regenerate the heart. Results show that induction of myocardial infarction in transgenic mice carrying the mIGF-1 isoform driven by a cardiac specific promoter produced localized damage, cell death and massive inflammation, but mIGF-1 transgenic animals rapidly resolved these initial responses and achieve complete repair of the injured heart without scar formation.

To determine whether myocardial tissue restoration observed in mIGF-1 transgenic hearts involved cell proliferation, the nuclear incorporation of continuously administered bromodeoxyuridine (BrdU), a marker of DNA synthesis, were assessed. At 1 month after infarct induction, frequent incorporation of BrdU in cardiomyocyte nuclei was seen in both cardiac tissue and individual cells after injury, although abundant non-muscle cells of diverse morphologies were also labeled in the vessels and surrounding myocardial tissue of mIGF-1 transgenic hearts. Although the origin and fate of these cells is still under investigation, the significant increase of proliferate activity in mIGF-1 transgenic hearts at late stages of regeneration is likely to contribute to their remarkable myocardial reconstitution in response to tissue damage.

Furthermore, by using a mouse model of enhanced regeneration of skeletal muscle expressing the mIGF-1 isoform, Partner #8 found that improved bone marrow contribution to rebuilding damaged muscle could be attributed to the myeloid/macrophage lineage. To test the effects of IGF-1 on efficiency and efficacy of cell contribution to regenerating tissue, bone marrow cells was harvested from wildtype or transgenic mice carrying a visible transgenic marker encoding human placental alkaline phosphatase (HAP). These cells were administered intravenously into mdx or mdx/mIGF-1 mice, in which the muscular dystrophic phenotype provides a milieu of continuous degeneration/ regeneration necessary for systemic cell uptake. Myocytes to which circulating cells had fused expressed the HAP marker on their membranes and were abundant only in those mdx expressing the mIGF-1 transgene, suggesting that IGF-1 induced appropriate homing signals for the incorporation of exogenous cells into the damaged muscle bed.

Finally, to test whether muscle effective regeneration requires the local polarization of macrophages towards an anti-inflammatory, or M2 phenotype, Partner #8 collaborated with the Nerlov lab to exploit a genetic blockade in the induction of C/EBPb transcription factor in response to injury, which is essential for macrophage polarization. Mice carrying a targeted deletion of two CREB binding sites in the Cebpb promoter (βΔCre mice) were analysed for their response to skeletal muscle injury. βΔCre macrophages infiltrated injured muscle normally, but failed to up-regulate Cebpb and Arg1, indicating lack of Cebpb induction and defective M2 polarization as a basis for impaired muscle regeneration. Persistence of inflammatory M1 macrophages in damaged muscle of these mice was insufficient for effective regeneration, which was impaired in the absence of M2 polarization.

These observations highlight the role of infiltrating immune cells in tissue repair and provide the first direct genetic link between the action of M2 macrophage polarisation and muscle regeneration. These studies will be extended to cardiac injury, by challenging βΔCre mice with myocardial infarction, and documenting potential decrements in cardiac repair.

4) To characterize the cellular events and the molecular pathways involved in cardiac differentiation in order to establish protocols for the derivation of functional cardiac myocytes from somatic stem cells.

Analisys of factors and genes that are involved in the differentiation pathway of stem cells into cardiac myocytes.

The development of stem cell-based therapeutic interventions aimed at reducing the devastating effects of myocardium loss by ischemic disease or infarction, requires a deep comprehension of the molecular events linked to differentiation of stem cells into cardiac myocyte lineage. Despite recent advances have shown that stem cells have the ability to generate cardiac myocytes *in vivo*, there is still a gap of information on the mechanisms underlying cell trans-differentiation toward cardiogenic lineage. Therefore, main objective of the project was to clarify, at a molecular level, the events linked to cardiac myocyte differentiation of stem cell of different origin. The obtained results give us new insights into the process of cardiac cell differentiation and specialization.

Partner #5 performed a characterization of the phenotypic plasticity of cord-blood-derived $CD133^+$ cells. First, they demonstrated that $CD133^+$ cells have a degree of non-hematopoietic potential and express transcripts of pluripotency markers including Oct-4, Sox-2, Rex-1 and leukaemia inhibitory factor (LIF) receptor, as well as markers of progenitor cells, such as HoxB4, brachyury and nestin.

Having shown by transcriptome analysis that the mouse embryonic fibroblast (MEF) cells routinely used to maintain pluripotent embryonic stem cells express transcripts of the WNT/ BMP families of signalling factors, they have assessed the effects on proliferation and differentiation of $CD133^+$ cells by Wnt signalling. These studies demonstrated that in vitro conditioning of CD133+ cells by media containing specific WNT signalling factors influences the non-hematopoietic potential of CD133+ cells and dynamically alters the expression of the neural stem/progenitor cell marker nestin and the endothelial-related cell surface markers CD31 and von Willebrand factor. They also showed that some WNT signalling molecules (specifically Wnt3a) affected the proliferation of CD133+ cells, whereas other Wnt molecules (i.e. Wnt5) influence the differentiation. They further demonstrated that prominin-1, the murine orthologue of CD133, is expressed in pluripotent ES cells and in neural progenitors later in development, which may affect their use in cell replacement studies

An additional set of studies demonstrated that Suramin exerts a ventralizing activity which specifically affects the normal pattern of cardiac phenotypes differentiating from mouse ES cells. Specifically, Suramin induces the formation of sinusnodal-like cells as a consequence of inhibitory and/or activating mechanisms that regulate early cardiac-specific genes and transcription factors.

Partner #7 performed experiments in which thy investigated the regulation of Connexin 43 expression during cardiac differentiation of embryonal carcinoma cells and demonstrated that the transcriptional repressor Snail negatively modulate Connexin 43 expression during the process of epithelial to mesenchymal transition which preceeds the appearance of mature cardiac myocytes.

Development of vectors to genetically modify stem cells.

For combining cell and gene therapy efficient and safe gene transfer systems are need. One mission involving Partner #2 and #10 has been to collaborate to the generation of vectors able to infect with high efficiency stem cells and to express genes potentially acting on the stem cell differentiation program.

Reports on the use of AAV vectors for modification of $CD34^+$ and, more in general, hematopoietic stem cells, presented conflicting results. Since different protocols for cell isolation and cultivation, as well as for vector production and purification had been used, a direct comparison of results was impossible. Initial transduction experiments resulted in inefficient transduction efficiencies. Thus, partner #10 performed experiments aimed at identifying critical steps and factors involved in rAAV-mediated transduction of human HSCs and to develop a protocol to achieve efficient and reliable transduction efficiencies. Results have shown that rAAV2 based vectors are versatile tools for $CD34^+$ cell transduction if transient transgene expression is sufficient or desirable. Moreover, several critical steps and factors were identified: First, CD34 cells have to be pre-expanded to induce cell surface expression of rAAV2 cell entry receptors. Furthermore, transgenes have to be encoded in a self-complementary vector genome conformation. rAAV2 treatment does not hamper $CD34^+$ cell differentiation, but the commonly used CMV promoter seems to be inadequate since promoter silencing was observed in our differentiation experiments. Nevertheless, in short-term cultures independent from the source good transduction efficiencies were obtained, which could be further enhanced if transcription-activating drugs were applied in parallel.
In conclusion, partner #9 has established the conditions for the rAAV-mediated gene transfer and will proceed to analyze the influence of different genes on cardiac differentiation of $CD34^+$ cells in collaboration with partner #2

Study of the effect of hemodynamic forces (shear stress) on the activation of cardiac specific genes in stem cells.

Heart development depends on a dynamic interaction between genetic and epigenetic factors and, at least in zebrafish, intra-cardiac hemodynamics is a key epigenetic factor regulating heart development. Several *in vitro* studies have shown that vascular endothelial cells can both sense and transduce biomechanical stimuli such as wall shear stress (SS) caused by blood flow. However, very little is known about the morphogenetic role of SS during embryogenesis and the development of the heart. One of the objective included in the project plan was to characterize the effect of mechanical flow stimulation on the activation of cardiac specific genes in stem cells.

Experiments performed by partner #1 have shown that a series of cardiovascular markers (e.g. Vascular Endothelial Growth Factor Receptor-2, Smooth Muscle Actin, α-sarcomeric Actin) have been found upregulated in ES cells that were prompted to spontaneous differentiation and successively exposed to SS in comparison with control cells kept in static conditions. Remarkably, in the same experimental conditions, neuronal as well as skeletal muscle markers did not appear. Changes in histone modifications have been also observed in ES cells that underwent SS treatment in comparison with static control cells. Specifically, an increase in phosphorylation and acetylation of histone H3 was detected, indicating that a global transcriptionally active chromatin characterizes SS-treated ES cells. In addition, it was found that MEF-2C and Smad4 transcription factors, involved in cardiac and vascular development respectively, were activated in ES exposed to SS and formed macromolecular transcriptional complexes, as they associated with histone Acetyltransferases. On the contrary, no DNA binding was observed in static ES.
Finally cardiovascular commitment of ES cells was abrogated if Nitric Oxide (NO) synthesis was inhibited, as revealed by immunofluorescence showing no staining for endothelial and smooth muscle markers in ES treated with NO synthases inhibitors and exposed to SS.
All together these results show that 1) SS anticipates the onset of cardiovascular markers in ES. 2) SS induces chromatin remodelling and activates the formation of transcriptional complexes involved during vascular and cardiac development. 3) SS-dependent effects on the cardiovascular differentiation of ES are mediated by NO.

DISSEMINATION AND USE

Public health aims: Myocardial infarction is one of the major cause of mortality and morbidity in Europe and western countries. Therefore the results of the present study will have an obvious and strong impact on the public health. Correct prevention and correct management of such disease may have profound impact on quality of life and health costs in all western countries.
The main exploitable achievements of the project include:
1) The characterization of the functional properties of stem cell-derived cardiac myocytes.
2) An increased knowledge on the signals triggering stem cell proliferation/survival and recruitment into the infarct area and on the molecular pathways involved in cardiac differentiation
3) The optimization of protocols to obtain functional cardiac myocytes from somatic stem cells.
4) The determination of the safety of autologous administration of bone marrow derived cells in patients suffering chronic ischemic disease.
Educational aims: The project coordinator and each partner will participate public events involving medical- school students to present the results of this and other studies, in order to improve the students consciousness of these issues, very often too far from their every-day routine. The use o stem-cells is one of the major promises of the near future to manage and cure severe diseases.
Commercial aims: the use of stem-cells in cardiac diseases, as well as the development of novel and effective protocols to isolate and expand stem cells, represent currently major goals of pharmaceutical industries. The potential market is enormous, considering the frequency of myocardial diseases and considering the ongoing ageing of the world-population.

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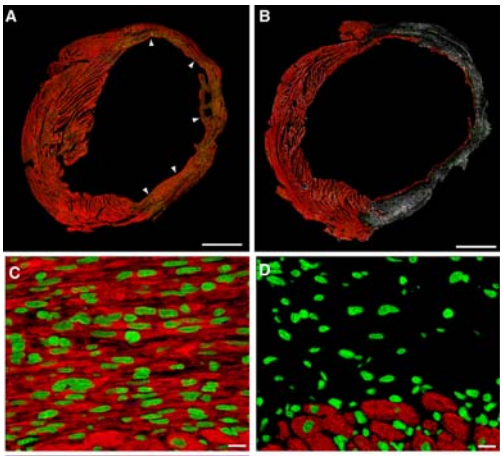
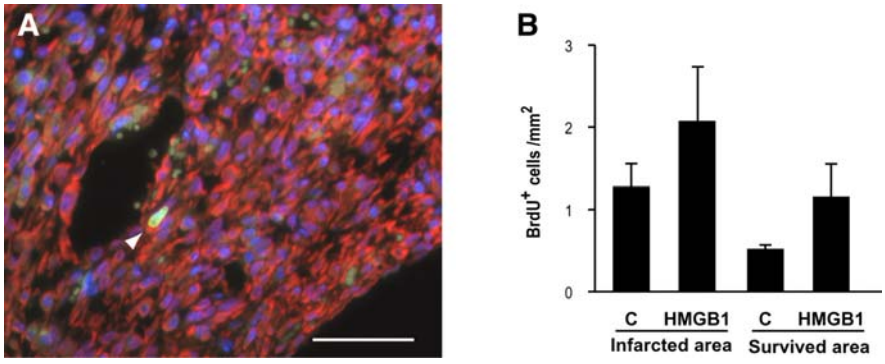
HMGB1 ON CARDIAC TISSUE REGENERATION



(A-B) Low-power view of α-sarcomeric actin immunostaining (red fluorescence) of infarcted mouse heart, treated with HMGB1**(A)** and GST **(B)** and sacrificed 1 week later. Arrowheads indicate a band of regenerated myocardium. Bar, 1mm. **(C)** α-sarcomeric actin immunostaining of infarcted HMGB1 (n=9) and **(D)** GST (n=25) treated hearts. Bar, 10 μm.



(A) Section of infarcted HMGB1-treated heart, 7 days after coronary occlusion and BM-c-kit$^+$ injection. BM-c-kit$^+$-derived cells were identified by BrdU immunostaining (green fluorescence). Arrowhead indicates a BrdU$^+$ cell. The same section was stained with α-sarcomeric actin (red fluorescence) and Hoechst to evidence nuclei (blue color). Bar 50 μm **(B)** Bar graph of the mean of circulating BM derived c-kit$^+$ cells in the infracted area and in the survived area of GST (C) and HMGB1 treated heart (n=3).

TRANSGENIC MICE OVEREXPRESSING IGF1 IN THE HEART.

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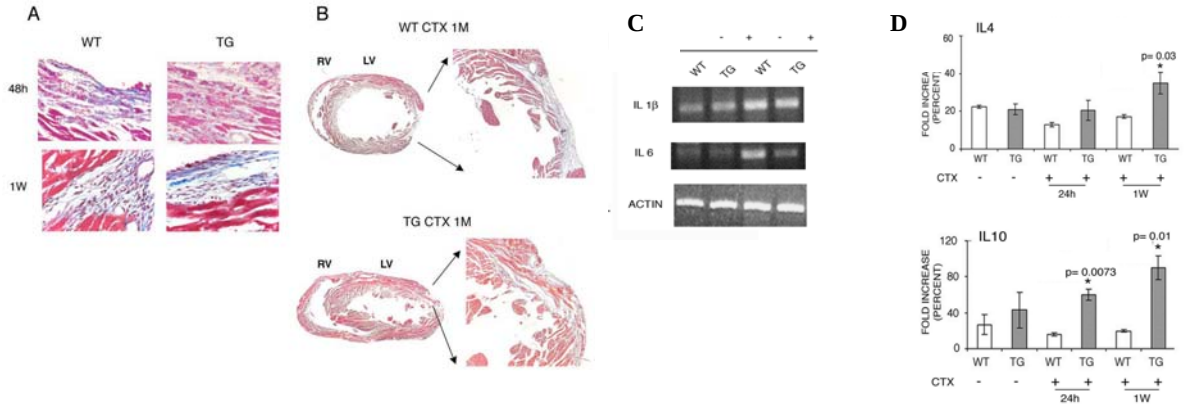
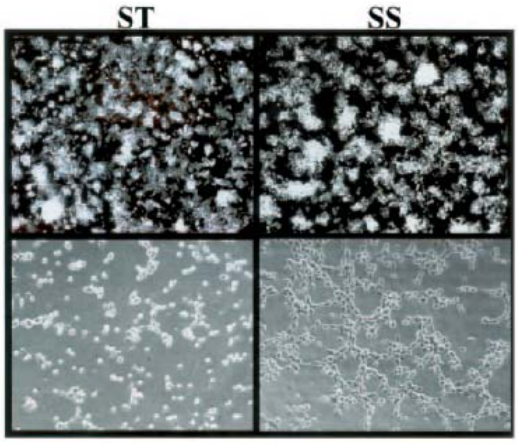
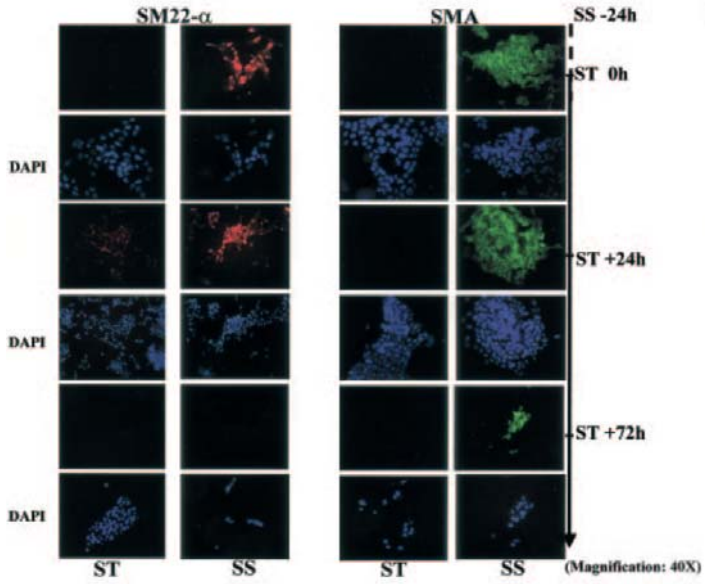


(A, B) Trichrome staining of 4 months old wild-type and transgenic hearts at 48 hours, 1 week, and 1 month after CTX injection in left ventricular wall. Comparable results were obtained with similar analyses on six different groups of animals. (C) Early events in mIGF-1 induced regeneration. RT-PCR of inflammatory interleukins IL6 and IL1β 24 hours after CTX injection in wild-type and transgenic hearts. PCR was normalized by β-actin content in each sample. (D) Real time PCR of the anti-inflammatory cytokines IL4 and IL10 in transgenic mIGF-1 (grey bars) and wild-type (white bars) hearts 24 hours and 1 week after CTX injection. Asterisks indicate significant relative values (p-value<0.05). The results are the average of three independent experiments.

EFFECT OF SHEAR STRESS ON STEM CELLS



In the upper panel, phase contrast microscopic shows ES before and after SS treatment. Lower panel shows that in ES exposed to SS a vascular differentiation program is activated



Expression of smooth muscle-specific markers (SMA and SM22-α), endothelial-specific markers (VEGFR-2 and PECAM) and cardiac-specific markers (MEF-2C and α□sarcomeric actin) in ES exposed for 24 hours to laminar shear stress

INTERCELLULAR COMMUNICATION BETWEEN MATURE AND IMMATURE CARDIOMYOCYTES

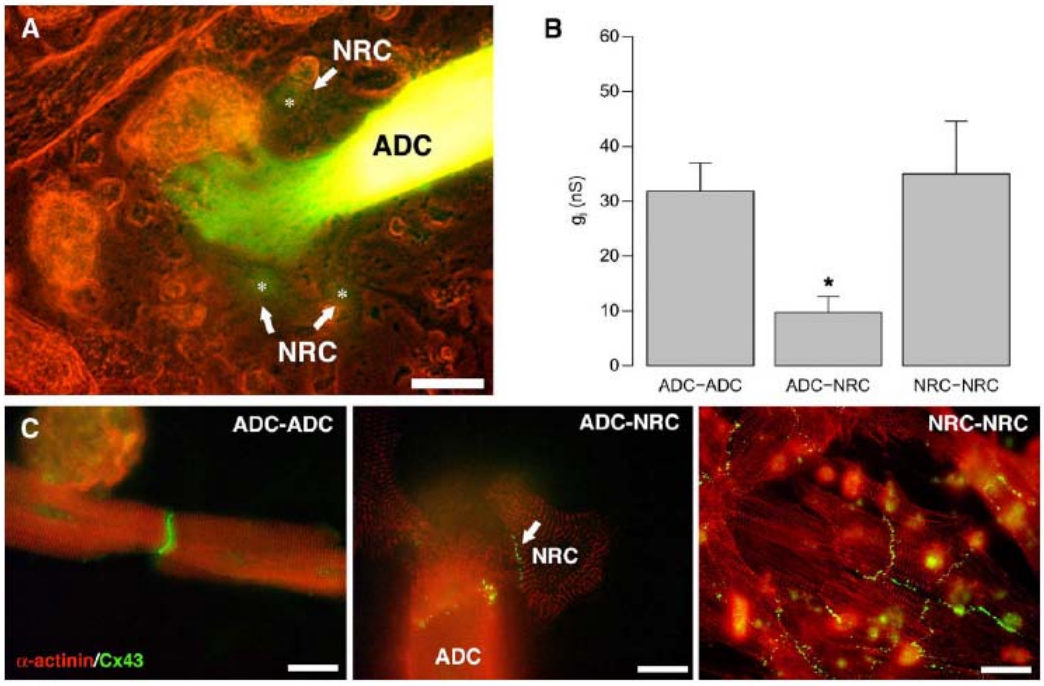


Fig. 4. A: Lucifer Yellow dye transfer from the injected ADC to neighbouring NRCs (indicated with asterisks and arrows). B: Average gap junctional conductance (g_j) between homotypic and heterotypic pairs of cardiomyocytes. Asterisk indicates that conductance between ADC–NRC is significantly lower as compared to that between ADC–ADC and NRC–NRC. C: Representative immunohistochemical labelling of α-actinin (red) and Cx43 (green) of homotypic and heterotypic interactions suggests that Cx43 levels are higher in NRC–NRC and ADC–ADC combinations as compared to the ADC–NRC combination. Scale bar=25 μm.

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF CARDIAC PROGENITOR CELLS

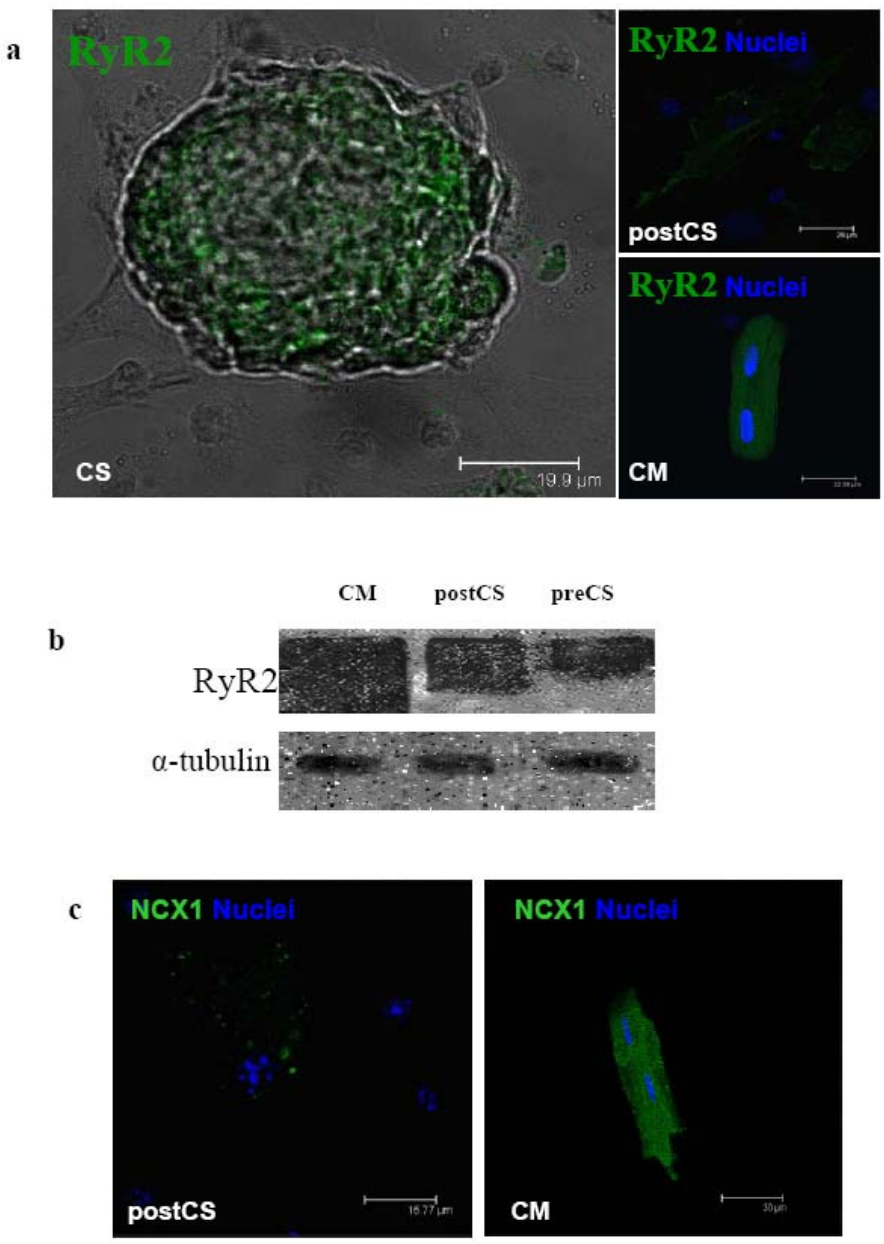


Figure 5_ Molecular detection of cardiac RyR2 and NCX1 in CPCs. a) confocal images of Hoechst nuclear (blue) and RyR2 (green) positive overlapped signals revealed the presence of cardiac RyR2 isoform in three-dimensional structure of CSs and in postCS cells; the expression of RyR2 in adult cardiac myocytes (CM) is also shown for comparison; **b**) representative western blot of RyR2 protein level in adult myocytes (CM), postCS and preCS cells. **c**) Hoechst nuclear (blue) and NCX1 (green) positive overlapped signals show the presence of NCX1 protein in postCS (left) and in cardiac myocyte (right)

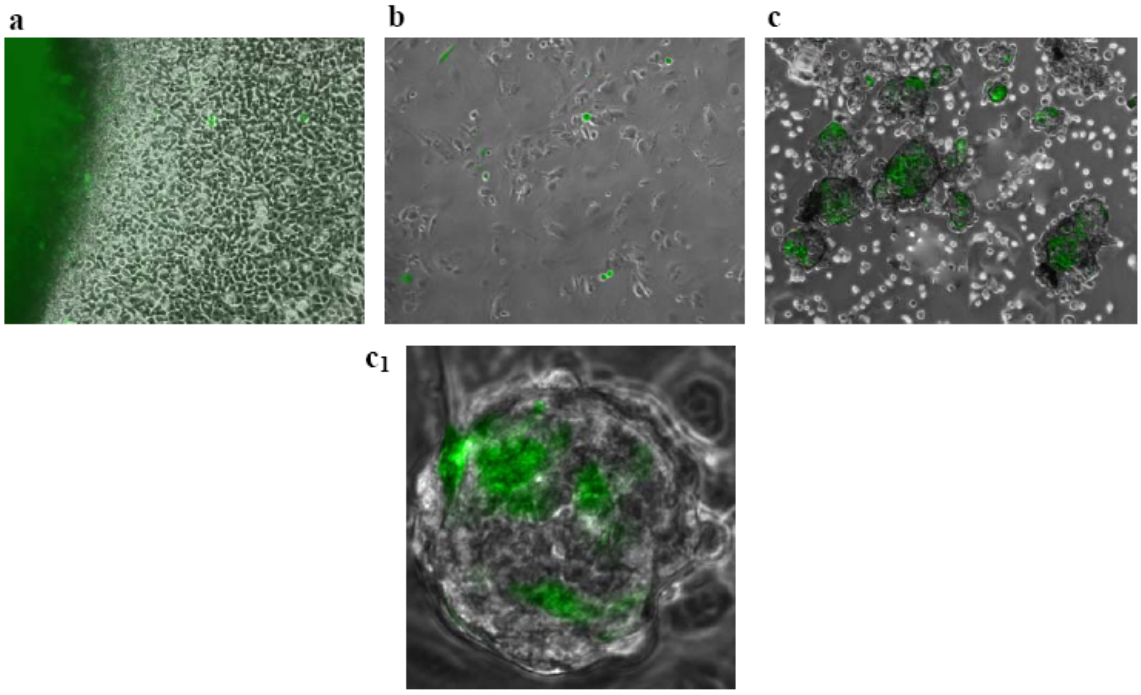


Figure 7_monitoring of cardiac marker (cTnI) expression. Fluorescence microscopy of enhanced green fluorescence protein (EGFP) expression in hEnAct_TNNI3-LVV transduced cardiac explants (fluorescence and overlapped with light transmission images). Images show the increase of reporter gene expression from explants to the mature cardiospheres (7a-c).