Cell transplantation with human mesenchymal or embryonic stem cells to the heart: experimental, molecular, immunological and echocardiographic studies
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Karl-Henrik Grinnemo

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Cell transplantation with human mesenchymal or embryonic stem cells to the heart: experimental, molecular, immunological and echocardiographic studies
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It is possible to fail in many ways... while to succeed is possible only in one way.

Aristotle

To Malin, Felix and Emilia
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PAPER I-V
ABSTRACT

Background: Human mesenchymal stem cells (hMSC), human embryonic stem cells (HESC) and human fetal cardiomyocyte progenitor cells (HFCP) all represent possible candidate cells for regeneration of damaged myocardium. Although they represent cell types with many positive attributes, their capacity to engraft, to differentiate into mature cardiomyocytes, to improve myocardial function and their immunological properties are the focus of these studies.

Methods and Results: hMSC were rejected from the myocardium of immunocompetent rats even if immunosuppression with Tacrolimus was used. Re-exposure of hMSC to lymphocytes from rats previously exposed to hMSC caused a significant proliferation indicating a sensitization reaction. The hMSC survived in the myocardium of athymic T-cell deficient rats and in an ischemic rat model the survival of the hMSC increased when Tacrolimus was added. Even though the hMSC survived in the ischemic myocardium they did not differentiate into cardiomyocytes or improve the myocardial function.

HESC have the ability to differentiate into cardiomyocytes, but a major obstacle to clinical application is their immunological characteristics. We have shown that HESC express HLA class I, no HLA class II and low levels of costimulatory molecules. HESC are immunologically inert and do not inhibit immune responses during direct or indirect antigen presentation and HESC were acutely rejected when transplanted over the xenogeneic barrier into mice.

In order to study the possibility to induce tolerance towards in vivo differentiating HESC, these were implanted into the testis and heart of immunocompetent mice treated with costimulation blockade. The HESC developed into teratoma in the testis in all mice and induced regulatory T-cells to undifferentiated HESC when transplanted into the heart. When costimulation blockade was repeated HESC engrafted in the myocardium in one of five mice.

Islet-1 positive cells were identified in the atria and outflow tracts of aborted human fetal hearts. We succeeded to isolate and culture the Islet-1 positive cells and their progeny in a reproducible manner, forming spontaneously beating cardiospheres and monolayers. Implantation of the HFCP formed stable engraftments in the myocardium of SCID mice.

Conclusions: hMSC are immunogenic in xenogeneic settings and they do not improve myocardial function or differentiate into cardiomyocytes in a rat ischemia model. HESC are also immunogenic in allogeneic and xenogeneic settings. Costimulation blockade is sufficiently robust to induce tolerance to HESC in an immune-privileged environment like testis. HESC transplanted into the myocardium of immunocompetent mice induced regulatory T-cells and when the costimulation blockade was repeated the success of transplantation was similar to that seen in SCID mice. The cardiomyocyte origin of the HFCP, their capacity to grow in culture and their formation of stable engraftments in the myocardium make the HFCP a putative candidate for cardiomyoplasty.

Key words: Human mesenchymal stem cells, human embryonic stem cells, human cardiomyocyte, Islet-1, Nkx2.5, stem cell, xenotransplantation, rejection, FoxP3+ T-cells, tolerance, CTLA4Ig, anti-CD40L, LFA-1.
LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, which will be referred to by their Roman numerals.


# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ahMSC</td>
<td>adult human mesenchymal stem cells</td>
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<tr>
<td>APC</td>
<td>antigen presenting cells</td>
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<td>APD</td>
<td>action potential duration</td>
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<tr>
<td>CABG</td>
<td>coronary artery bypass grafting</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CDC</td>
<td>cardiosphere-derived cells</td>
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<tr>
<td>c-Kit</td>
<td>tyrosine kinase receptor, the receptor for stem cell factor</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CS</td>
<td>cardiosphere</td>
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<tr>
<td>CTLA4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CTLA4Ig</td>
<td>cytotoxic T-lymphocyte antigen 4 immunoglobulin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>END2</td>
<td>mouse visceral endoderm-like cells</td>
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<tr>
<td>FAC</td>
<td>fractional area change</td>
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<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
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<tr>
<td>FITC</td>
<td>fluorescein iso-thiocyanate</td>
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<tr>
<td>FP</td>
<td>field potential</td>
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<td>HBSS</td>
<td>Hanks balanced salt solution</td>
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<td>HESC</td>
<td>human embryonic stem cells</td>
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<td>HFCP</td>
<td>human fetal cardiomyocyte progenitor cells</td>
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<td>HFib</td>
<td>human fibroblasts</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>hMSC</td>
<td>human mesenchymal stem cells</td>
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<tr>
<td>IB</td>
<td>inter-beat interval</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>ICD</td>
<td>implantable cardioverter defibrillator</td>
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IFN-γ interferon-γ
IL interleukin
Islet-1 LIM homeodomain transcription factor
LFA lymphocyte functional-associated antigen
Lin− negative for blood lineage markers
LVEDD left ventricular end-diastolic dimension
MAPC multipotent adult progenitor cells
MEA multi-electrode array
MHC major histocompatibility complex
mHC minor histocompatibility complex
MLR mixed leukocyte reaction
MSC mesenchymal stem cells
OCT octamer-binding transcription factor
PBL peripheral blood lymphocytes
PBMC peripheral blood mononuclear cells
PBS phosphate-buffered saline
RNU Rowett nude rats
RPMI Roswell Park Memorial Institute
RS recycling cells
Sca-1 stem cell antigen
SCID severe combined immunodeficient mouse
SD Sprague Dawley rats
SP side population
SR serum replacement
SSEA stage specific embryonic antigen
TCR T-cell receptor
TRA tumor-related antigen
TRITC tetramethyl rhodamine iso-thiocyanate
INTRODUCTION

Stem cells and progenitor cells for cardiac repair

According to an old paradigm, the pool of terminally differentiated cardiomyocytes is established shortly after birth and any injury to the heart will induce a loss of cardiomyocytes eventually leading to heart failure (Fuchs et al. 2000; Hawke et al. 2001; Weissman et al. 2000). This paradigm is now being challenged by evidence that suggests that the heart is capable of limited regeneration presumably through the activation of resident progenitor cells or the recruitment of stem cells from extra-cardiac compartments (Lu et al. 2002; Jackson et al. 2001; Laflamme et al. 2002, Quaini et al. 2002). In an effort to replace cardiomyocytes lost due to ischemia, transplantation of stem cells has been explored as a potential therapy to repair injured myocardium. This therapy is called cardiomyoplasty (Kao et al. 1997).

Skeletal myoblasts opened the door to cardiac cell therapy. In the early 1990s, the first skeletal myoblasts were isolated, expanded and successfully transplanted to an injured heart (Chiu et al. 1995). Since this pioneering work several animal studies have provided evidence for the possibility of intra-myocardial myoblast grafting (Taylor et al. 1998; Dowell et al. 2003; He et al. 2005; van den Bos et al. 2005; McConnel et al. 2005). The mechanisms that underlie the positive effects on heart function is still a subject of debate. The implanted myoblasts do not differentiate into cardiomyocytes but instead develop into myotubes. They do not form gap junctions with the neighboring cardiomyocytes, resulting in an inability of the grafted myoblasts to beat in synchrony with the surrounding myocardium (Leobon et al. 2003; Rubart et al. 2004). A more likely mechanism for the positive effects seen after skeletal myoblast transplantation seems to be contraction of the myoblasts in response to traction or mechanical buttressing of the infarcted area (McConnel et al. 2005).

Myoblasts were the first cells to be used for cardiomyoplasty in clinical phase I studies (Menasche et al. 2001; Hagege et al. 2003) where myoblast implantation was performed concomitantly to coronary artery bypass surgery (CABG). These studies was followed by three other adjunct-to-CABG studies, reporting similar positive effects on the myocardial function (Gavira et al. 2006; Dib et al. 2005; Siminiak et al. 2004). Even though in these phase I non-controlled studies, the patients seemed to be symptomatically improved there were also reports of episodes of ventricular arrythmia necessitating the implantation of implantable cardioverter-defibrillators (ICD).

Taking these negative issues into account, we choose to explore if other stem cells could be utilised to repair the heart after ischemic damage. Human mesenchymal stem cells (hMSC) and human embryonic stem cells
(HESC), at the beginning of the year 2000 were still relatively unexplored cell types with many positive attributes. Furthermore, the new insight that the human heart is not a purely post-mitotic organ, raised my interest for the possibility of identifying a cardiac progenitor cell that could be used for repairing heart injury.

**Human mesenchymal stem cells**

**Sources and characteristics**

The adult bone marrow contains several populations of multipotent stem cells one of which is mesenchymal stem cells (MSC or hMSC for human MSC). Their multipotency has been described in several reports where the MSC have differentiated into osteoblasts, adipocytes, chondrocytes, muscle cells and stromal fibroblasts (Friedenstein et al. 1968; Caplan et al.1997; Prockop et al. 1997; Pittenger et al. 1999). Even if MSC are present as a rare population of cells in bone marrow, representing 0,001-0,01% of the nucleated cells, MSC feature several clinically relevant advantages like; ease of isolation, high expansion potential with stable gene expression and reproducible characteristics between different laboratories. Different surface markers have been used to characterize MSC, but a putative marker specific to MSC has not been identified, making the nomenclature disgruntling. Nevertheless, the hMSC should not express the hematopoietic stem cell markers CD34 and CD45 (Pittenger et al. 2004), but some MSC isolated from bone marrow express these markers during the initial phase of the culturing process (Jones et al. 2002). Even within microenvironments of the bone marrow there may be identical cells expressing different surface markers and still fulfilling the requirements for MSC. Furthermore there are reports of different subpopulations of MSC, like; multipotent adult progenitor cells (MAPC) (Jiang et al. 2002; Reyes et al. 2002; Verfaillie et al. 2003), adipose progenitor cells (Halvorsen et al. 2000; Gronthos et al. 2001; Zuk et al. 2002) and finally recycling stem cells (RS) (Prockop et al. 2003, 2001). This means that the MSC are a heterogeneous population of cells with some similar characteristics

**Immunological features**

An interesting feature of MSC are their immuno-modulating effects which might be advantageous when used in transplantation studies, since it can theoretically reduce the incidence of immune rejection. The hMSC express major histocompatibility complex (MHC) class I, lymphocyte function-associated antigen (LFA)-3 antigens, while MHC class II and intercellular adhesion molecule (ICAM)-1 antigens are expressed upon interferon (IFN)-γ stimulation. The hMSC do not express the costimulatory molecules CD80 and CD86 even after IFN-γ stimulation, which means that hMSC do not fulfil the requirements of being professional antigen presenting cells (APC). MSC from rodents, baboons and humans fail to elicit allogeneic lymphocyte proliferations in mixed leukocyte reaction tests (MLR) and they also inhibit the proliferative response induced by third party antigens (Le Blanc et al. 2003; Bartholomew et al. 2002; Di Nicola et al. 2002; Tse et al. 2003; Krampera et al. 2003). The immunosuppressive effect is more pronounced if the MSC are added early to the allogeneic immune response and this inhibitory effect is in part mediated by soluble factors (Di Nicola et al. 2002; Tse et al. 2003; Rasmusson et al. 2003). In accordance with MLR data, in vivo administration of MSC also prolonged the survival of allogeneic skin grafts transplanted to baboons (Bartholomew et al. 2002). Human fetal liver-derived MSC have also been successfully transplanted in utero to a fetus with severe osteogenesis imperfecta (Le Blanc et al. 2005). The transplanted MSC had engrafted in the bone and alloreactivity towards the implanted MSC was not detected in MLRs performed during the follow-up period.
Even if the immunological properties of hMSC in allogeneic settings are well characterized, their immuno-modulating effect in xenogeneic environments needed to be further investigated and this issue was the starting point of my thesis.

Cardiomyoplasty
Mesenchymal stem cells seem to have a special ability to home to sites of inflammation. When MSC from mice were injected intravenously to rats subjected to cycles of ischemia/reperfusion, significant amounts of MSC were seen in the circulation and subsequently in the infarcted region of the heart (Saito et al. 2002). The majority of the engrafted cells were found to express cardiomyocyte markers indicating that the MSC have developed into cardiomyocytes. In a similar study these observations were confirmed using rat MSC in a rat ischemia model (Martin et al. 2002). The window of opportunity for the homing of the MSC to the infarcted area was found to be limited if the administration of MSC was delayed more than 2 weeks, indicating that in the therapeutic situation the MSC should be transplanted in the early phase after the myocardial infarction.

The ability of homing is of course advantageous in stem cell therapy, but how the MSC exert their effects is still a puzzle. Toma and co-workers injected hMSC into the left ventricle of severe combined immunocompetent (SCID) mice and followed the fate of the lacZ labelled cells (Toma et al. 2002). A limited number of the cells survived more than one week but those that did survive more than 30 days had differentiated into cardiomyocyte like cells. In co-culture experiments, hMSC were cultured together with human cardiomyocytes and some of the hMSC differentiated towards a cardiomyogenic phenotype (Rangappa et al. 2003). In large animal studies allogeneic and autologous MSC were shown to inhibit the remodelling process and restoring myocardial function after infarction (Amado et al. 2005; Shake et al. 2002). Even if these studies were promising, they did not demonstrate the mechanism of action of MSC in cardiomyoplasty. This encouraged me to go on with the infarction studies with hMSC in rats.

Human embryonic stem cells
Isolation and culturing conditions
Human embryonic stem cells (HESC) are pluripotent stem cells that are isolated from the inner cell mass of the human blastocyst. Thomson et al and Reubinoff et al were pioneers in culturing permanent cell lines and started an era that might come to revolutionize conventional medicine, making it possible to cure diseases that until now have been beyond treatment (Thomson et al. 1998; Reubinoff et al. 2000). There are currently differentiation protocols for many cell types, such as cardiomyocytes (Mummery et al. 2003; Xu et al. 2002), hepatocytes (Rambhatla et al. 2003), hematopoetic cells (Chadwick et al. 2003; Kaufman et al. 2001), neurons (Johnson et al. 2007; Carpenter et al. 2001; Reubinoff et al. 2001; Zhang et al. 2001; Schulz et al. 2003) and endothelium (Levenberg et al. 2002). This implies that HESC have the potential to not only replace solid organ transplantation with cell therapy, but can also be used to treat progressive neurological diseases such as Parkinson’s disease (Rodriguez-Gomez et al. 2007) and a multitude of other diseases.

The first derivations of HESC lines were carried out by using immuno-surgery, involving mouse antibodies and guinea pig complement for isolation of the inner cell mass. Fetal calf serum (FCS) was a constituent of the culture medium and mouse fibroblast cells were used as feeder cells (Thomson et al. 1998; Reubinoff et al. 2000). All animal products that are used in the derivation pro-
cess constitute a risk of transmitting infection and the HESC might take up xenogeneic antigens such as immunogenic non-human sialoproteins and express them to the host, augmenting rejection (Martin et al. 2005). To minimise the amount of animal components absorbed during the derivation process, multiple steps have to be implemented. HESC can be cultured on postnatal human skin fibroblasts as feeder cells (Hovatta et al. 2003) and medium containing serum replacement (SR) instead of FCS can be used (Koivisto et al. 2004; Inzunza et al. 2005). Mechanical isolation of the inner cell mass instead of immuno-surgery can be used to further eliminate animal-derived substances (Ström et al. 2007). The next step towards achieving clinical quality HESC would be the combination of mechanical isolation of the inner cell mass, the use of human feeder cells and culture of the HESC in a defined medium, without SR, which also contains small amounts of animal proteins.

**The immunogenicity of human embryonic stem cells**

Review of the literature demonstrates little concern for potential immunological problems associated with HESC transplantation. In some studies it is not even specifically addressed if immunosuppression has been used. It is still not clear if HESC are immune-privileged or if immunological rejection of transplanted cells is something that needs to be addressed.

HESC express low levels of HLA class I, which is upregulated by IFN-γ stimulation or after differentiation into embryoid bodies or teratoma formation (Drukker et al. 2002; Li et al. 2004). HLA class II molecules, however, were not expressed under these circumstances. It has also been demonstrated that the expression of costimulatory molecules (B7.1, B7.2 and CD40) was low or absent on undifferentiated HESC (Li et al. 2004). This implies that the HESC lack the two important prerequisites to function as professional APCs, namely high levels of HLA class II and costimulatory molecules.

To test the immune response evoked in a xenogeneic setting, Drukker and co-workers transplanted the HESC under the kidney capsule of different immunodeficient mouse strains (Drukker et al. 2006). Normal teratoma growth was only seen in the T-cell deficient mice which suggested that xenograft rejection of HESC is a T-cell mediated immune process. In order to study the outcome in an allogeneic setting (Drukker et al. 2006) a trimeric mouse model was created where the bone marrow was reconstituted with human peripheral blood mononuclear cells (human T-, B- and NK cells). Transplantation of human skin grafts or B-cell lymphoma grafts caused immune rejection in contrast to HESC, which developed into teratoma. In an allogeneic in vitro test, specific human cytotoxic T-cells were generated directed towards HLA-A2 antigens expressed on the HESC (Drukker et al. 2006). The cytotoxic T-cells were stimulated by coculturing them with irradiated peripheral blood mononuclear cells expressing HLA-A2 antigens and loaded with influenza virus type A peptide. The sensitized cytotoxic T-cells did not cause lysis of the HESC loaded with influenza virus type A peptide, not even after IFN-γ stimulation of the HESC. Next Drukker and co-workers tested influenza virus type A infection of the HESC and again no lysis of the HESC was recognized. However, when the HESC were infected and treated with IFN-γ, effective cytotoxic T-cell mediated lysis was noted. These data indicate that HESC have little potential to activate a direct allospecific immune response. Li and co-workers (Li et al. 2004) obtained similar results from in vitro studies. In a MLR, HESC failed to induce proliferation of human peripheral blood mononuclear cells. This lack of allogeneic immune response was also seen when T-cell enriched peripheral blood lymphocytes were used. Li and co-workers also concluded that there was an active
inhibition of the allogeneic T-cell response when addition of HESC caused a decrease of proliferation of the third party immune cells induced by allogeneic dendritic cells (i.e. T-cells, HESC and dendritic cells are all from a different origin). In a xenogeneic setting, HESC were injected into the quadriceps muscle of immunocompetent mice where they were identified after 48 hours without any sign of granulocyte infiltration (Li et al. 2004).

Based on these findings these groups have previously suggested that HESC are immune-privileged. Our experience, working with similar stem cell lines, was that HESC are immunogenic and this encouraged us to study the immunogenicity of different human embryonic stem cell lines.

Cardiogenic potential
The first reports which described the differentiation of HESC into cardiomyocytes was based on the formation of embryoid bodies (Kehat et al. 2001; Xu et al. 2002). The conversion of HESC into cardiomyocytes is a process with low efficiency and that efficiency is in part dependent on the cell line used and which signalling pathways that were activated. In the first protocols, the conversion of HESC into beating embryoid bodies were reported to vary between 8 to 25% (Xu et al. 2002; Kehat et al. 2001; He et al. 2003). However, counting beating embryoid bodies is not a good method to measure the success of the conversion of HESC into cardiomyocytes, since the beating embryoid bodies contain different numbers of cardiac cells. In an attempt to increase the yield of cardiomyocytes, Mummery and co-workers started to culture the HESC on mouse visceral endoderm-like cells (END2) and by applying a serum-free culturing system about 20-25% of the HESC did convert into cardiomyocytes (Mummery et al. 2002, 2003; Passier et al. 2005).

Laflamme and co-workers have shown that HESC might be used in the future for cardiomyoplasty (Laflamme et al. 2005). As a first step they differentiated HESC into cardiac-enriched HESC progeny, where 10 to 15% of the cells expressed cardiac markers. These cells were subsequently injected into the left ventricular wall of nude rats. Although the initial grafts were predominantly epithelial, noncardiac elements were lost over time and at the end (4 weeks) the grafts were reported to mainly consist of cardiomyocytes. The grafted cells continued to proliferate during the whole follow-up period of four weeks and the graft size increased sevenfold. Interestingly, the grafted cells differentiated into capillaries and induced angiogenesis from the surrounding rat myocardium. In another study, HESC-derived beating cardiomyocytes formed stable engraftment and paced the hearts of swine with complete heart block, generating a biological pacemaker (Kehat et al. 2004). These studies indicate that HESC can differentiate into cardiomyocytes and form electromechanical coupling with the surrounding cardiomyocytes of the host, which is an important prerequisite if these cells are to be used in the reparation of damaged myocardium.

In the studies by Kehat et al and Laflamme et al the injected cardiac-enriched HESC progeny was not reported to develop into teratoma in the hearts (Laflamme et al. 2005; Kehat et al. 2004). This is in contrast to what was seen when non-purified mouse embryonic stem cell-derived cardiomyocytes were used, which formed teratoma in the majority of the hearts (Kolossov et al. 2006). The tumorgenicity of HESC transplanted into the heart is another important aspect that needs to be resolved before the HESC can be used in clinical studies.

Progenitor cells for cardiomyoplasty
The adult human heart seems to have a limited regenerative capacity, although in older age and during pathological conditions the balance is altered and the myocyte
formation is overtaken by cell death (Nadal-Ginard et al. 2003). Much of this knowledge comes from transplantation studies where sex mismatched cardiac transplants were used. In these studies the transplanted hearts were colonized by host cells that had differentiated into myocytes and vessels (Quanini et al. 2002; Muller et al. 2002). These findings opened up a new field searching for the optimal cardiac stem cell that could be used in cardiomyoplasty. So far four different cardiac stem cell populations have been identified: side population cells (SP) expressing Abcg2 (Martin et al. 2004), Lin/c-Kit+ (Beltrami et al. 2003; Urbanek et al. 2005), stem cell antigen 1 (Sca-1) (Pfister et al. 2005; Oh et al. 2003) and finally Islet-1 positive cells (Cai et al. 2003; Laugwitz et al. 2005).

The side population cells are identified by their capacity to efflux a dye named Hoechst 33342 on the basis of the high expression of membrane pumps encoded by the multiple drug-resistance genes (Goodell et al. 1996) and one of these transporters is Abcg2. Martin and co-workers have identified the Abcg2-expressing SP-cells during embryogenesis and latter stages of the developing murine heart (Martin et al. 2004). These cells were found to in part differentiate into α-actinin expressing cells after co-culture with primary cardiomyocytes. Among these SP-cells there seem to be a population of stem cells with increased capability to differentiate into cardiomyocytes. Pfister and co-workers have identified a population of CD31/-Sca-1+ cells which are more prone to both biochemically and functionally differentiate into cardiomyocytes (Pfister et al. 2005). Altogether this implies that the SP-population seem to be a heterogenous population of cells and their ability to differentiate into functionally active cardiomyocytes needs to be evaluated further.

Another set of cardiac stem cells have been reported by Beltrami and co-workers (Beltrami et al. 2003). They identified Lin/c-Kit+ cells in the myocardium of rats. In culture these cells were shown to be multipotent generating cardiac myocytes, smooth muscle cells and endothelial cells. When transplanted into the ischemic myocardium of rats, the Lin/c-Kit+ cells seemed to mature into cells expressing sarcomeric structures and the arteriolar and capillary density increased. From these data the Lin/c-Kit+ cells behave like multipotent cells capable of maturing in the ischemic myocardium.

Cardiac stem cells have been cultured from adult human myocardial specimen (Messina et al. 2004). These cardiac explants developed into multicellular clusters dubbed cardiospheres (CS), which express c-kit in roughly 30% of the cells. Messina and coworkers demonstrated that the cells in each CS were clonally derived, differentiating into cells expressing both cardiac and endothelial cell markers (Messina et al. 2004). In another study, percutaneous endomyocardial biopsy specimens were used to form multicellular cardiospheres and cardiosphere-derived cells (CDC) (Smith et al. 2007). Again the cardiospheres consistently expressed c-Kit. Even though these CDC did not beat spontaneously, they seemed to be able to differentiate into cardiomyocytes in co-culture with rat cardiomyocytes. Altogether this means that even the adult human heart seems to have progenitor cells that can to some extent differentiate into cardiomyocytes. These progenitor cells do not seem to be specific cardiomyocyte progenitors, since they also differentiate into endothelial cells and fibroblasts (Messina et al. 2004; Smith et al. 2007).

The previously described stem cells are more multipotent in their character. In contrast the LIM homeodomain transcription factor Islet-1 marks a cell population that constitutes the second or anterior heart field (Cai et al. 2003) and thus represent a true endogenous cardiac progenitor cell. According to the work by Cai and coworkers, Islet-1 positive progenitor cells seem to reside in the anterior
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posterior extent of the splanchic mesenchyme dorsal to the heart and migrate to give rise to the outflow tract, the majority of the cells in the right ventricle, the atria as well as a portion of cells in the left ventricle (Cai et al. 2003). The Islet-1 positive cells are different from the previously described stem cells even though they express the cardiogenic precursor markers Nkx2.5, GATA 4 but they do not express Sca-1, CD31 and c-Kit. Cai and coworkers suggest that the transcription factor Islet-1 is required for the survival, proliferation as well as migration into the developing heart and the expression of Islet-1 is lost as soon as the progenitor cells differentiate into cardiomyocytes (Cai et al. 2003). The Islet-1 positive cells have so far only been isolated and cultured from embryonic rodents and identified in the myocardium of humans in the early postnatal period. The number of progenitor cells seems to decrease very rapidly after birth (Laugwitz et al. 2005) and therefore these cells are difficult to identify in adult patients, if any can be found at all. Since these cells are known to mainly differentiate into cardiomyocytes, the establishment of an exogenous source of human Islet-1 positive cells seemed very attempting and stimulated me to isolate and culture these cells from fetal specimens.

Immune response against transplanted cells and tissues

Regenerative medicine, where stem cells and progenitor cells are being used to regenerate damaged tissues, has opened up a whole new field with the potential to treat different degenerative and pathological disorders. A major obstacle to their implementation into clinic will be the immune response towards the transplanted cells. Tissues and organs transplanted between non-identical individuals will inevitably succumb to rejection due to activation of the innate and adaptive immune systems. Rejection occurs due to differences of a multitude of antigens, which can be divided into three major groups. They are Human Leukocyte Antigen/ Major Histocompatibility Complex (HLA/MHC), ABO blood-group antigens and Minor Histocompatibility Complex (mHC) (Bradley et al. 2002).

The ABO antigens consist of structurally highly polymorphic carbohydrate residues bound to cell-surface glycolipids and are expressed on almost every cell in the body (Claussen et al. 1989; Ito et al. 1992). Transplanting vascularized organs between ABO incompatible individuals will cause hyperacute rejection, mediated by preformed antibodies directed towards the ABO-antigens, which activates the complement cascade (Paul et al. 1987; Cooper et al. 1990).

HLA antigens are responsible for robust immunological rejection. They can be divided into HLA/ MHC class I (HLA-A, -B, -C) and HLA/ MHC class II (HLA-DR, -DQ and –DP) (Bradley et al. 2002). These antigens are highly polymorphic cell membrane expressed polypeptide chains that determine the outcome of alloantigen specific T-cell responses in vitro and in vivo (Janeway et al. 1999). Most human cells express HLA class I molecules but the expression of HLA class II is restricted to antigen presenting cells like dendritic cells (DC), B-lymphocytes and macrophages (Viret et al. 1999).

The mHC antigens are cell-derived peptides presented at the cell surface by MHC molecules. Snell and co-workers, who first identified these antigens, demonstrated that inbred mice were able to reject tumors and skin grafts from MHC identical donors (Snell et al. 1964). The graft rejection was slower than for MHC disparate mice and therefore these antigens were designated mHC. Although the mHC are not as immunogeneic as MHC and
ABO antigens, they can nonetheless induce immune rejection (Simpson et al. 2001).

The immunological mechanisms that cause rejection of a cellular transplant can be divided into the direct (Fig. 1a) and indirect T-cell allore cognition pathways (Fig. 1b) (Caballero et al. 2006). Under normal conditions a T-cell receptor (TCR) recognises a self-MHC molecule plus a foreign peptide which leads to T-cell activation. In direct recognition, the TCR recognises the complex of peptide bound by an allogeneic MHC molecule and mistakes it for self-MHC with foreign peptide. In other words, an allogeneic MHC molecule with a bound peptide can mimic the determinant formed by a self-MHC molecule plus a particular foreign peptide subsequently also inducing T-cell activation. A prerequisite to function as an APC is the expression of MHC class II and the expression of costimulatory molecules (Rogers et al. 2001). After transplantation, the APC from the donor tissue migrate to the regional lymph nodes, where they encounter and stimulate T-cells. The proliferation and differentiation of T-cells into activated T-cells require signals from both the TCR and from costimulatory molecules (Fig. 2a).

The B7 molecules are the classical costimulatory molecules expressed on the surface of APC. These ligands bind to CD28 on the surface of T-cells and provide the second signal, which is a necessity for activating the T-cell. Stimulation of naive T-cells by the TCR without concomitant costimulation leads to a state of unresponsiveness called anergy (Frasca et al. 1998). This is a state where a T-cell not only is unresponsive on first encounter but also remains so upon re-exposure. Some of these anergic T-cells, in an unknown manner, develop into regulatory T-cells. These regulatory T-cells have the ability to inhibit new generations of naive T-cells with affinity for the same antigens. In this manner the immune system is capable of learning and remembering. Previously activated effector and memory T-cells are less dependent on costimulation than naive cells. Memory T-cells are responsible for the enhanced and accelerated immune response seen upon re-exposure to an antigen. The mechanism of memory T-cell generation and survival is a major field of investigation and tolerance induction in these cells could very well be the major obstacle to clinical tolerance induction (Kaech et al. 2002).

In the indirect T-cell allore cognition pathway, foreign peptides and allogeneic MHC molecules from the graft are processed and presented by a recipient APC. Processed MHC

![Figure 1. Antigen presentation.](image)

**a. Direct antigen presentation.** Solid organ transplants contain donor derived antigen presenting cells (APC) which upon transplantation emigrate to the nearest lymph node where recipient T-cells are residing. These T-cells interpret donor MHC/peptide complex on the APC as self-MHC with foreign peptide. This induces T-cell activation and subsequently acute rejection.

**b. Indirect antigen presentation.** After implantation of grafts, transplant antigens like mismatched MHC molecules from the graft will be released in the local environment and subsequently processed by recipient APC. These APC present donor-derived antigens on the cell surface together with MHC class II to recipient T-cells with affinity for the peptide/MHC complex. TCR; T-cell receptor.
molecules will be recognised by T-cells like other conventional foreign protein antigens (Caballero et al. 2006). Indirect presentation results in allorecognition by CD4+ T-cells (helper T-cells) where alloantigen is acquired by host APC through the endosomal vesicular pathway (phagocytosis) and is presented by MHC class II molecules. Some antigens of phagocytosed graft material also enter the class I MHC pathway of antigen presentation and are recognised by CD8+ T-cells (cytotoxic T-cells) in a process called cross-presentation. Because MHC molecules are the most polymorphic proteins in the genome, each allogeneic MHC molecule may give rise to multiple epitopes, each recognised by different T-cells (Fairchild et al. 1998).

Tolerance induction

Ever since Medawar’s pioneering experiments (Billingham et al. 1953), the Holy Grail of the transplant community has been to induce tolerance to transplanted tissues and organs. Patients receiving a solid organ transplant to relieve end-stage organ failure face life-long immunosuppression, which substantially increases the likelihood of developing opportunistic infections, cancer and morbidity secondary to chronic drug toxicity. The induction of tolerance to the transplant would circumvent these risks and allow the recipient to have an intact immune system while maintaining graft function.

Tolerance is a state where non-reactivity to an antigen is maintained in a host. In a healthy individual this state is maintained to all the tissues in the body at a number of different checkpoints. The most crucial being the selection of T-cells in the thymus where self-reactive T-cell clones are eliminated. Although highly effective, this step is not full proof and therefore there remains a necessity to be able to re-educate T-cells even after they have graduated from the thymus. This is
achieved by the phenomena of anergy where a T cell, which binds to a peptide/MHC complex in the absence of costimulation, does not become an effector cell capable of attacking the self-tissue, but instead enters a state of active hibernation (Frasca et al. 1998). Some T cells activated under such conditions develop into regulatory T-cells (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) with the capacity to inhibit naive T-cells with similar anti-self reactivity. In this way new generations of thymic emigrants inherit host non-reactivity. Exploiting this knowledge, this state of tolerance is transferable to foreign antigens, achieved by inhibiting the signalling of costimulatory molecules at the time of transplantation.

Tolerance can be successfully achieved in rodents through a number of different strategies which all have in common the targeting of T-cell activation and donor antigen interaction. The strategy that has attracted the greatest attention targets the costimulatory molecules CD40L expressed on activated T-cells and the B7 molecules expressed on dendritic cells under inflammatory circumstances (Fig. 2b). These costimulatory molecules are expressed at the time of transplantation and in conjunction with the new antigen. This offers a temporal window in which tolerisation specific to donor antigen can be instilled. CTLA4Ig is a fusion protein consisting of the extra cellular domain of CTLA4 and a human IgG1. CTLA4Ig binds to the B7 family of costimulatory molecules expressed on dendritic cells during activation. By binding these molecules, reacting T-cells do not receive the necessary secondary signals to initiate T-cell activation which leads to anergy and apoptosis of reacting T-cells (Chen et al. 1994; Weaver et al. 1990; Jenkins et al. 1987). Anti-CD40L binds to CD40L, a molecule expressed on T-cells which is a central regulatory signal in the immune system when initiating an immune response. Inhibition of this signal leads to prevention of T-cell memory development and effector cell activation, B-cell differentiation and isotype switching as well as macrophage activation. (Banchereau et al. 1994). Treatment with costimulation blockade is given only during the first week after transplantation. The targeting of these pathways has lead to the indefinite acceptance of vascularized allogeneic and xenogeneic rat cardiac transplants in mice (Larsen et al. 1996; Elwood et al. 1998).

Depending on the type of tissue to be transplanted and the strain of mouse recipients, the outcome of tolerance induction with anti-CD40L/CTLA4Ig has been variable (Trambley et al. 1998). This is due to the presence of CD8\(^+\) T-cells in some strains of mice, which are resistant to costimulation blockade (Trambley et al. 1998; Corbascio et al. 2002). By targeting LFA-1, an important adhesion and costimulatory molecule for CD8\(^+\) T-cells, CD40L independent immune activation and cytotoxicity can be inhibited. Adding anti-LFA-1 treatment to anti-CD40L/CTLA4Ig therapy has been shown to synergistically improve graft quality and function in discordant dopaminergic porcine xenografts transplanted in to the brain of mice (Larsson et al. 2003). This therapy has also been shown to induce permanent acceptance of porcine islet grafts transplanted into wild-type diabetic mice (Kumagai-Braesch et al. 2007). By combining these three substances at the time of transplantation, tolerance seems to be induced to allogeneic and xenogeneic tissues and organs. This state of tolerance seems to be mediated partially by regulatory T-cells that inhibit naive T-cells directed towards the grafted cells. Even if it will be possible to transplant organs between individual by inducing tolerance, It is still an open question if stem cells, which change their phenotype after transplantation, respect the same rules.
AIMS OF THE STUDY

• To study the immunogenicity of ahMSC transplanted over the xenogeneic barrier into the myocardium of immunocompetent rats and to study if this immune response can be down-regulated with the calcineurin inhibitor Tacrolimus.

• To study the capability of ahMSC to engraft, differentiate into cardiomyocytes and improve myocardial function in a rat ischemia model.

• To study the antigen expression of HESC and the immunogenicity induced in allogeneic and xenogeneic settings.

• To study the ability of costimulation blockade to induce long-term acceptance of HESC implanted into immunocompetent mice. To study the difference in engraftment between testis and the myocardium.

• To investigate the existence of Islet-1 positive cells in the human fetal heart. To isolate human Islet-1 positive cells and their progeny. To culture and characterize them in vitro and finally study the engraftment of these cells in the myocardium of SCID mice.
MATERIALS AND METHODS

Animals and ethics

Male Sprague-Dawley (SD) rats, male Fischer rats (B&K Universal AB, Sollentuna, Sweden), 400 to 450g and male RNU rats (Charles River Deutschland Inc, Sulzfeld, Germany), 250 to 300g were used in paper I and II. Eight week old male C57BL/6 (B and K Universal AB, Sollentuna, Sweden) were used in paper III, IV and male Balb/c mice (B and K Universal AB, Sollentuna, Sweden) were used in paper III. SCID/beige males (C.B.-17/GbmsTac-scid-bgDF N7; M and B, Denmark) were used in paper III-V. The ethical committee affiliated to the Karolinska University Hospital approved all animal experiments together with the derivation and expansion of human mesenchymal stem cells, human embryonic stem cells and progenitor cells from aborted material.

Processing, isolation and expansion of stem cells and progenitor cells

Adult human mesenchymal stem cells- Paper I and II

To isolate ahMSC, bone marrow aspirates of 5-10 ml were taken from the sternum of patients undergoing a cardiac operation. MSC were isolated and cultured as previously reported (LeBlanc et al. 2003). Mononuclear cells recovered from the interface of Percoll separated bone marrow were washed and resuspended in human MSC medium consisting of Dulbecco’s Modified Eagles Medium-Low Glucose (DMEM-LG, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich Corp., St.Louis, MO), 1% antibiotic-antimycotic solution (Life Technologies, Gaithersburg, MD) and plated at 3x10^7 cells / 185 cm^2 in Nunclon Solo flasks (Fisher Scientific, Pittsburgh, PA, USA). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. When the cultures were near confluence, the cells were detached by treatment with trypsin and EDTA (GibcoBRL, Grand Island, NY) and replated (passaged) at a density of 1x10^6 cells per 185 cm^2 flask. After three passages, cells were harvested by treatment with 0.05% trypsin-EDTA and washed with phosphate-buffered saline (PBS).

By flow cytometry, cultured MSC were uniformly positive for CD166, CD105, CD44, CD29, SH3 and SH4 and negative for hematopoietic markers CD14, CD34 and CD45. On induction, the cells differentiated to osteoblasts, adipocytes and chondrocytes, as previously described (LeBlanc et al. 2003).

Human embryonic stem cells- Paper III and IV

HESC lines HS237, HS293, HS306, HS346, HS362, HS363, HS368 and HS401 were
derived and cultured at the Fertility Unit, Department of Gynaecology, Karolinska University Hospital (Hovatta et al. 2003; Inzunza et al. 2005). The HESC lines were derived from supernumerary blastocysts donated by couples undergoing fertility treatment. The HESC were cultured on postnatal human skin fibroblast feeder cells in serum replacement medium (Hovatta et al. 2003). The colonies were split mechanically without the use of enzymes and replated onto new human fibroblast feeder cells every 5-7 days. All these cell lines expressed markers typical of undifferentiated HESC namely alkaline phosphatase, stage-specific embryonic antigen (SSEA-4), tumour-related antigen (TRA 1-60, TRA 1-81) and octamer-binding transcription factor-4 (OCT-4) and form teratoma when injected into severe combined immunodeficient (SCID) mice.

Human fetal cardiomyocyte progenitor cells- Paper V

The abortion was performed according to technique as previously been described in detail (Westgren et al. 1994). The aborted material (gestational week 5 to 10) was transported directly from the operating room to the dissection room where the heart was the first organ to be identified to reduce the time of ischemia. For tissue culture the cardiac material was minced into small pieces and digested in 3-5 ml of collagenase solution (Collagenase NB4 0.02-0.05 U/ml, Serva Electrophoresis GmbH, Heidelberg, Germany) during continuous stirring for 5 minutes at 37°C in an incubator at humidified atmosphere containing 5% CO₂. The first fraction of cells was discarded while the remaining tissue fragments in the supernatant were repeatedly digested in fresh collagenase solution. The supernatant was saved every 15 minutes till all pieces were completely dissociated. The obtained cells were washed by centrifugation at 140 g for 5 minutes and resuspended in culture medium (Knock-Out Dulbecco’s Modified Eagle’s medium (Knock-Out DMEM) (Invitrogen, UK), non-essential amino acids (GTF, Sweden), Primocin 100µl/ 50 ml medium (Amaxa Inc, USA), 0.1 mmol/l β-mercaptoethanol (Invitrogen) glutamine (Invitrogen) and Insulin-Transferrin-Selenium (Invitrogen)). The cells were seeded directly onto tissue plastic plates (Techno Plastic Products AG, Switzerland), cultured at 37°C in a humidified atmosphere containing 5% CO₂ and medium was changed two times a week. By culturing the cells during these conditions ie. without adding serum to the culture medium, the growth of the fibroblasts was prohibited which benefit the growth of the cardiomyocyte progenitor cells. We have named these cells Human Fetal Cardiomyocyte Progenitor cells (HFCP) though they are shown to be cardiomyocyte progenitors and the name fetal is used since they are isolated from aborted fetal material. When the cultures were near confluence (average of 1 month) the cells were detached by treatment with TrypLE™ Select (GibcoBRL) diluted in phosphate-buffered saline solution (PAA Laboratories GmbH, Austria) in a ratio of 1:3 and the cells from each well were replated in three coated wells. Some self-adherent clusters (termed cardiospheres, CS) could be transferred manually onto new wells by using narrow-tipped glass pipettes.

Freezing of the cultured HFCP was performed by detaching the cells with TrypLE™ Select (GibcoBRL) diluted 1:2 with PBS. The cell suspension was then centrifuged at 140g and resuspended in 0.5ml freezing medium (20% Knock-Out serum replacement medium (Invitrogen), 10% Dimethylsulphoxide (Sigma-Aldrich) in Knock-Out DMEM (Invitrogen)). The cells were frozen gradually (-1°C per minute) down to -70°C
and stored at -180°C. When the frozen cells were recultured, they were quickly thawed to 37°C, washed and re-cultured on plastic plates pre-coated with human collagen IV (Sigma-Aldrich).

Animal surgery and implantation of stem cells and progenitor cells

Induction of myocardial infarction and implantation of ahMSC into the myocardium of rats-Paper I and II

The rats were anaesthetized with a subcutaneous injection of Midazolam (Dormicum, 5mg/kg) (F. Hoffmann-La Roche Ltd, Switzerland), Medetomidin hydrochloride (Domitor vet, 0.1mg/kg) (Orion Corp., Espoo, Finland), Fentanyl (0,3mg/kg) (B.Braun Medical AG, Seesatz, Switzerland) and subsequently endotracheally intubated. Positive-pressure ventilation was maintained at a rate of 110 cycles per minute with a tidal volume of 1.5 ml with room air supplemented by oxygen (2 l/min) using a Zoovent ventilator (Model CWC600AP, BK Universal, U.K.). The anaesthesia was reversed by an intramuscular injection of Flumazenil (Lanexat, 0.1 mg/kg) (F. Hoffmann-La Roche Ltd, Switzerland), Atipamezol hydrochloride (Antisedan vet 5mg/kg) (Orion Corp., Espoo, Finland) and Buprenorphin hydrochloride (Temgesic, 0,004mg/kg) (Schering-Plough Corp., Kenilworth, UK).

During general anaesthesia a left lateral thoracotomy was performed. The left anterior descending artery (LAD) was identified by retraction of the pericardium and was subsequently ligated with a 7-0 polypropylene suture. The thoracotomy was closed with running sutures of vicryl.

Paper I

One week after LAD-ligation the rats underwent a re-thoracotomy and the infarcted area was identified. The ahMSC was suspended in 100 µl PBS and subsequently injected into the myocardium surrounding the infarcted scar. The control rats were injected with 100 µl PBS without any ahMSC. The thoracotomy was closed in the same manner as earlier described and the anesthesia was reversed. The RNU rats were operated under sterile conditions in a ventilated chamber. The SD-rats were operated under clean but non-sterile conditions.

The rats were divided into three groups with four rats in each. Three rats in each group received ahMSC and the remaining rat functioned as a control and received 100 µl PBS. Group one was SD-rats who were not immunosuppressed, while group two was SD-rats immunosuppressed with Tacrolimus (0.1 mg/kg/day) (Fujisawa Healthcare, Inc, Deerfield, IL) given intramuscularly. These two groups received 3.5x10⁶ ahMSC. Group three was athymic RNU rats, without the need of immunosuppression, who received 2.5x10⁶ ahMSC.

One week after intracardiac injection of ahMSC the hearts were removed and paraffin embedded. 3 µm thick sections were prepared using a microtom (Microm, HM 355S, Microm Laborgeräte GmbH, Walldorf, Germany).

Paper II

The hMSC (1-2 x10⁶) were suspended in 50 µl Perfadex (Vitrolife Sweden AB, Kungsbacka, Sweden) and subsequently injected into the myocardium surrounding the infarcted scar. The control rats were injected with 50 µl Perfadex (Vitrolife). The rats were randomly divided into three groups. In group I (direct, RNU, without Tacrolimus); the hMSC were injected directly after LAD ligation in sixteen RNU rats, with six controls. In group II (3 days, RNU, with/without Tacrolimus) and III (3 days, Fischer, with Tacrolimus); both groups underwent injection with cells
three days after LAD ligation. Eleven RNU rats (five rats in IIa and six rats in IIb), and fourteen Fischer rats (III) were injected with hMSC through a re-thoracotomy, with seven control rats in II and seven in III. All animals in IIb (3 days, RNU, with Tacrolimus) and III (3 days, Fischer, with Tacrolimus) were treated with daily intramuscular injections of Tacrolimus (0.1 mg/kg/day) (Fujisawa Healthcare). The stem cells were injected in a standardized way at several sites and there was no difference in the injection technique when injecting in the acute or sub-acute phase of the myocardial infarction. The hearts of the euthanized rats were paraffin embedded and 3 µm thick sections were prepared using a microtom (Microm, HM 355S).

**Implantation of HESC and HFCP into mice- Paper III-V**

The mice were anaesthetized with Midazolam (5 mg/kg) (F. Hoffmann- La Roche Ltd), Medetomidine (0.1 mg/kg) (Orion Corp.), Fentanyl (0.3 mg/kg) (B. Braun Medical AG) and subsequently tracheotomized. The ventilation was maintained using a Zoovent ventilator (Model CWC600AP). During general anaesthesia the heart was exposed through a left lateral thoracotomy. After injection the thoracotomy was closed by running suture of vicryl.

In case of injection of HESC into the testis, the anaesthesia was induced and reversed as previously described but the ventilation was not supported.

**Paper III**

The HESC (200,000 HESC suspended in 15 µl Perfadex (Vitrolife) were injected into the free wall of the left ventricle (stem cell treated group, n=24 C57BL/6 and positive control group, n=2 SCID/ beige) or with 15 µl Perfadex (Vitrolife) (negative control group, n=24). Two mice from both the stem cell treated group and the negative control group were euthanized at 24 hours, 3 days, 5 days, 7 days and 10 days postoperatively, while one positive control mouse (SCID/ beige) was euthanized day 5 and examined immunohistochemically. To trace the surviving HESC, two mice from both the stem cell treated group and the negative control group were euthanized at day 1 to 5 and thereafter at day 7 and 10. One positive control mouse (SCID/beige) was euthanized at day 7 to be sure that environmental factors did not influence the survival of the HESC. The hearts of the mice used for detection of transplanted HESC were paraffin embedded and sectioned (3 µm thick sections) while the other hearts were freeze sectioned (5 µm thick sections). For the MLR, six extra C57BL/6 mice were transplanted with HESC into the myocardium. The mice were euthanized and their spleens were harvested after 3 weeks.

**Paper IV**

In these experiments the mice were treated with either active costimulation blockade or its isotype control reagents. The active costimulation blocking reagents used were anti-LFA-1 (clone M17/5.2), anti-CD40L (clone MR1), CTLA4Ig and their respective isotype control antibodies: rat IgG2b (clone 9A2), hamster IgG1 and human IgG1, all purchased from Bioexpress (West Lebanon, New Hampshire, USA). The C57BL/6 mice treated with active costimulation blockade (costimulation blockade treated group), received 0.5 mg anti-CD40L, 0.2 mg anti-LFA-1 and 0.5 mg CTLA4-Ig given ip. every other day for 8 days. The control mice received corresponding amount of the isotype control reagents (isotype control treated group).

Through an incision in the distal part of the abdominal wall, through the linea alba, the right testis was exposed and 200,000 HESC were injected under the testis capsule of SCID
mice (n=6) and C57BL/6 mice, which were randomized to receive either costimulation blockade (n=6) or isotype control reagents (n=6). The mice were euthanized after 8 weeks and the testis was harvested and analysed histochemically.

The testis is described to be an immune-privileged organ (Nasr et al. 2005). Although the HESC survive in a testis from an immunocompetent mouse treated with costimulation blockade, the result might be different if the HESC are transplanted to the heart. To further study this hypothesis, we injected another set of mice with the same amount of HESC through a left thoracotomy into the myocardium of SCID mice (n=5) and C57BL/6 mice which were again randomized to costimulation blockade (n=8) or isotype control treatment (n=8). Two mice from both the costimulation blockade treated and the isotype control treated groups were euthanized after 4 weeks, while the rest of the mice were euthanized after 8 weeks (ie. SCID n=5, C57BL/6 costimulation blockade treated n=6; C57BL/6 isotype control treated n=6).

Because of failure to induce teratoma and the appearance of cellular infiltrates at one month a second set of transplants was performed. Costimulation blockade or their isotype controls were administered both at the time of transplantation and three weeks later (n=5 in each group). We named these groups costimulation blockade x2 and isotype control x2 respectively. The mice were euthanized after 8 weeks. All the explanted testis and hearts were freeze sectioned (5 μm thick sections).

**Paper V**

Ten cardiospheres in 10 μl culture medium were injected into myocardium of the left ventricle of SCID mice (n=2) which were euthanized after two weeks. The hearts were freeze sectioned (5 μm thick sections).

**Detection of implanted ahMSC, HESC and HFCP**

The sections were subsequently prepared for hematoxylin-eosin staining to identify the site of injection and thereby the area where to find the implanted cells. It was reasoned that detection of DNA sequences specific for the human genome would provide unequivocal genetic evidence for identification of grafted cells. The fluorescent in situ hybridization technique (FISH) was used allowing the detection of specific nucleic acid sequences in histological sections. The FISH procedure has been previously described (Scorsin et al. 1997) and in brief our modified version involves the following steps. When paraffin slides were used, these were baked at 60°C for 2h, the paraffin was removed, and a rehydration process was performed using 95%, 70%, and 50% ethanol respectively. In case of freeze sections, these were fixated in 4% formaldehyde and washed in PBS and destilled water. The paraffin and freeze sectioned slides were then microwave treated in citrate buffer (10mM, pH 6.0) for 1,5 minutes at 750W followed by 4.5 minutes at 50W. A mixture of 0.5 ml pepsin (Sigma p7012) and 50 ml HCl (150 μg/ml) was applied to each slide and incubated at 37°C for 15-20 minutes (10 minutes for freeze sections) and in 2x sodium citrate-sodium chloride buffer (SSC) with 0.1% Igepal (Sigma-Aldrich Corp.) for 10 minutes. The slides were then incubated in 1% formaldehyd/ 50mM MgCl₂/ PBS for 10 minutes in 20°C and in PBS for another 5 minutes, followed by dehydration. Then 3 μl FISH probe cocktail (Vysis Inc, Downers Grove, IL), was applied to each section. The next step consisted of simultaneous denaturation of probe and target DNA at 74°C for 10 minutes. The slides were subsequently incubated overnight in a humidified chamber at 39°C to allow hybridization of probe and target DNA. After repeated washes, the remaining probe molecules were stained with antifade
containing 4,6-diamidino-2-phenylindole (DAPI). Slides were then examined using an Olympus DP10 fluorescent microscope (Olympus BX60, Olympus Optical CO, Ltd, Tokyo, Japan) where the nuclei of the transplanted cells stained red.

**Immunohistochemical analysis**

**Detection for differentiation and integration of implanted cells**

**Paper I and II**

Slides with FISH positive cells were incubated with primary antibodies directed towards cardiac specific antigens. The sections were rinsed in PBS and blocked by incubating the sections in 5% rabbit serum (X0902, Dako Cytomation, Glostrup, Denmark) for 30 minutes. The slides were incubated overnight at room temperature with mouse monoclonal antibodies specific for desmin (clone DE-R-11, Dako Cytomation), sarcomeric actin (clone 5C5, Sigma-Aldrich), myosin (clone NOQ7.5.4D, Sigma-Aldrich Corp.), tropomyosin (T9283 clone CH1, Sigma-Aldrich Corp.) and connexin 43 (clone CXN-6, Sigma-Aldrich Corp.). The sections were incubated with fluorescence-labelled (fluorescein isothiocyanate-[FITC-] labelled, F0313, Dako Cytomation) rabbit anti-mouse antibodies in the blocking solution at the dilution 1:10 and visualized by fluorescence microscope.

**Paper IV**

Sections with surviving HESC-derived cells in the heart were further stained for the cardiomyocyte markers α-actinin (clone EA-53, Sigma-Aldrich) and Desmin (clone DE-R-11, Dako Cytomation) together with TRA 1-60 (clone TRA-1-60, Chemicon International Inc, Temecula, USA) which is a marker for undifferentiated HESC. After fixation with 4% formaldehyde and blocking with 5% rabbit serum (X0902, Dako Cytomation) for 30 minutes, the slides were incubated with the respective primary antibody over night in a humidified chamber. The sections were then incubated with fluorescence-labelled rabbit anti-mouse immunoglobulins/ FITC (Dako Cytomation) and visualized in the fluorescence microscope. As positive controls we used human heart (Desmin and α-actinin) and HESC in the myocardium of SCID/beige mice which were euthanized and freeze-sectioned directly after injection (TRA 1-60).

**Paper V**

Sections close to the FISH positive slides were used for further immunohistochemical analysis to identify the degree of cardiac differentiation and integration of the transplanted cells. The chosen sections were blocked and incubated with primary antibodies specific for Nkx 2.5 (clone 259416, R&D Systems, Minneapolis, USA), Troponin T (ab10224, Abcam plc, Cambridge, UK) and Connexin 43 (clone CXN-6, Sigma-Aldrich Corp.). The sections were incubated with different fluorescence labelled secondary antibodies: Troponin T-NL557 conjugated donkey anti-rabbit IgG (R&D Systems) and for the other primary antibodies we used polyclonal rabbit anti-mouse immunoglobulins/TRITC or FITC (Dako Cytomation).

For detection of apoptosis, monoclonal anti-human Annexin V/ FITC (clone VAA-33, Bender MedSystems GmbH, Vienna, Austria) diluted 1:10 in PBS with 0,1% Triton was used, with human fetal heart as positive control.

**Immunohistochemical staining of cardiospheres, monolayer cells and whole heart sections**

**Paper V**

The beating cardiospheres were mechanically
detached with a micro-pipette, embedded in OCT (Histolab Products Ltd, Gothenburg, Sweden), frozen in liquid nitrogen and sliced into 5 µm thick sections. For staining of the monolayer cells, these were detached with TrypLE™ Select (GibcoBRL) and cultured on human collagen IV (Sigma-Aldrich Corp.) coated SuperFrost®Plus glasses (Menzel GmbH&Co KG, Braunschweig, Germany). The monolayer cells could be grown on the glasses by using flexiPERM Micro 12 chambers (Sigma-Aldrich Corp.). Before the immunohistochemical staining, the slides were fixed in 4% formaldehyde, blocked and incubated with the following primary antibodies: Islet 1/2 (guinea pig Islet 1/2, a kind gift from Thomas Jessel, Colombia University, New York), Sca-1 (clone E13-161.7, BD Biosciences, USA), CD117/ c-Kit/ SCF-receptor Ab-3 (clone K45, Thermo Fisher Scientific, UK), Nkx 2.5 (clone 259416, R&D Systems), c-Kit (CD117, clone K45, MS289-P, Labvision/ NeoMarkers, Fremont, USA), Desmin (clone DE-R-11, DakoCytomation), α-actinin (clone EA-53, Sigma-Aldrich), Troponin T (ab10224, Abcam plc), Ki67 (clone MIB-1, Dako Cytomation). For identification of cardiomyocyte progenitors, proliferation and gap junctions in the fetal hearts, these were freeze sectioned (5 µm thick) from the apex to the outflow tracts and stained for Islet-1, c-Kit, Sca-1, Nkx 2.5, Ki67 and Connexin 43 as previously described.

**Multi-electrode assay**

*Paper V*

Spontaneously beating cardiospheres were picked up with a micro-pipette and planted onto multi-electrode array plates (MEA) (Multi Channel Systems, Reutlingen, Germany) pre-coated with human collagen IV (Sigma-Aldrich). After two days they had attached to the plate, integrated mechanically and electrically forming a large simultaneously beating cluster that covered several electrodes.

Extracellular recordings were performed to characterize basal electrical activity of the beating cardiospheres. Each MEA had 60 flat, round, substrate integrated Titanium Nitride electrodes in an 8 x 8 grid and an integrated reference electrode (electrode diameter 30µm and inter-electrode distance 200 µm). The MEA was connected to an amplifier with a heated stage (Multi Channel Systems, Reutlingen, Germany). All recordings were carried out at 37°C with culture medium gassed with 5% CO₂. Medium was changed and recordings carried out twice before the recording used for analysis. This was to establish the stability of the preparation.

Data was acquired and analysed with the MC Rack software (also Multi Channel Systems). Recordings were carried out at a sampling frequency of 1 kHz and lasted for 2
minutes. The resulting field potentials (FPs), were characterized regarding inter-beat interval and waveform properties such as $FP_{MIN}$, $FP_{MAX}$ and $FP_{DUR}$. 30 successive FPs were averaged to reduce background noise and facilitate manual recognition of $FP_{MAX}$.

Immunohistochemical characterization of immune response towards grafted cells

**Paper I**

Cellular infiltration was detected by hematoxylin-eosin staining. To further characterize the inflammatory response, macrophage staining was used. The paraffin was removed in xylene, and the slides were rehydrated. The slides were then treated in citrate buffer (10mM, pH6) in the microwave at 750W for 1 minute and then for 5 minutes at 50W. The sections were incubated in 2xSSC with 0.1% Igepal for 10 minutes in 37°C before incubated over night in 20°C with the primary antibody (mouse anti-rat ED1MCA341R, Serotec, Oxford, England), diluted 1:40 in permeabilisation buffer (Dako Cytomation). After washing in 1xPBS, the slides were incubated with the secondary fluorescence-labelled rabbit anti-mouse antibody, diluted 1:20 in permeabilisation buffer (FITC-labelled, F0313, Dako Cytomation) for 2 hours and then visualized in the fluorescence microscope.

**Antigen expression and in vitro immune response**

**Mixed Leukocyte Reaction**

**Paper I**

To evaluate whether the ahMSC elicit an immune response or not a MLR was performed on peripheral blood (PBL) from SD-rats injected with $2.5 \times 10^6$ ahMSC. A separate experiment was performed with five SD-rats. Blood from three of the rats, who got ahMSC, were pooled and blood samples from the other two rats (one control) were tested separately. We also collected peripheral blood from one rat in each of the three groups described above, including one control rat who got PBS. This was done at the same time as these rats were killed and the hearts were taken out for further analysis. Lymphocytes from each sample were separated on a Ficoll-Isopaque density gradient (Lymphoprep., Nyco, Norway). The cells were washed twice and suspended in Eagle’s minimal essential medium containing HEPE buffer, 10% heat inactivated human AB serum and antibiotics. MLR was performed as previously described (LeBlanc et al. 2003). In short, $1 \times 10^5$
responder cells were co-cultured with 1x10^5 irradiated stimulator cells, which were inactivated by 20 Gy irradiation. To each culture, 0, 1x10^3 or 1x10^5 of ahMSC were added. ^3H-thymidine (Radiochemical Center, Amersham, U.K.) with a specific activity of 5 Ci/mmol/l was added to each culture 24 hours prior to harvest. The cells were harvested automatically on a glass fiber filter using a Tomtec harvesting machine (Tomtec, Orange, CT, USA) on day 6. Tritiated thymidine incorporation is expressed as counts per minute (cpm).

**Paper III**

In order to simulate the immune response induced in a xenogeneic setting, splenocytes from naive and immunized C57BL/6 mice were harvested. Spleens were homogenised and red cells were lysed by a short incubation with 1 ml dH_2O and then rescued with 20 ml Hanks Balanced Salt Solution (HBSS). CD4+ T-cells were purified with the CD4+ T-Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Non-CD4+ T-cells were indirectly magnetically labelled by using a cocktail of biotin-conjugated antibodies against CD8a, CD45R, DX5, CD11b and Ter-119, and Anti-Biotin MicroBeads. Labelled cells were removed by using a MACS Column and a MACS Separator (all from Miltenyi Biotec). The purity of CD4+ T-cells was more than 95% estimated by FACS analysis.

Dendritic cells (DC) were prepared from the bone marrow of the femurs of syngenic C57BL/6 and Balb/c mice. The mice were euthanized by cervical dislocation and the bone marrow was aspirated. The bone marrow cells were cultured for 5 days in DMEM (GibcoBRL) supplemented with 10% fetal calf serum, 2mM L-glutamine, 100μg/ml streptomycin, 100U/mL penicillin (all from GibcoBRL), 5x10^{-5} M 2-mercaptoethanol (Sigma-Aldrich Corp.) and 10 ng/ml recombinant mouse GM-CSF (R&D Systems) in humidified air containing 5% CO_2 at 37°C. The medium was changed on day 3 and 5. The DC were isolated using the CD11c (N418) Microbeads (Miltenyi Biotec).

DC (1x10^6) from C57 BL/6 mice were co-cultured with irradiated (15 Gy) HESC (3x10^5) and HFib (3x10^5) respectively in culture medium (Roswell Park Memorial Institute (RPMI-)1640 supplemented with 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/mL penicillin and 5x10^{-5} M 2-mercaptoethanol) for 24 hours. DC were then irradiated (15Gy). MLRs were performed using CD4+ T-cells (1x10^5 cells per well) from immunized/ non-immunized C57 BL/6 mice and exposing them to syngenic DC alone (2x10^4 cells per well) or syngenic DC co-cultured with irradiated HESC/ HFib or irradiated (15 Gy) allogeneic Balb/c DC (2x10^4 cells per well). In parallel, CD4+ T-cells (1 x 10^5 cells per well) from immunized and non-immunized C57 BL/6 mice were stimulated directly with irradiated donor-type HESC (2 x 10^4 cells per well) or HFib (2 x 10^4 cells per well). Five wells per culture were set up in 96-well round-bottomed micro-titre plates (25850, Corning) and incubated in humidified air containing 5% CO_2 at 37°C. Each culture was labelled with 2 μCi [^3H] thymidine (Radiochemical Center) for 20 hours prior to harvest. The proliferation of the responders was measured from day 2 till day 5 and each experiment was repeated three times. The proliferation is presented as both cpm and mean Stimulation Index (SI). This is defined as the ratio of mean cpm of CD4+ T-cells (+DC in indirect stimulation) cultured in presence of the stimulators, to mean cpm of background CD4+ T-cells (+DC in indirect stimulation) without stimulators.

To simulate an allogeneic transplantation of HESC, peripheral blood mononuclear cells (PBMC) were isolated from buffy coat from normal healthy volunteers by Ficoll-Paque density gradient centrifugation. Approximately 300x10^6 PBMC were used
for preparation of DC and the rest was frozen with 10 % DMSO (Sigma-Aldrich Corp.) and kept in liquid nitrogen until the start of MLR. For DC preparation, non-adherent cells were removed after overnight incubation in RPMI-1640 containing 10% human serum. The adherent cells were collected from the culture bottles by gently scraping with a plastic cell scraper. Cultures were prepared with a density of 1x10^6 cells/ml in the medium containing 800 U/ml GM-CSF and 500 U/ml IL-4. On day 3 and 5 fresh medium and cytokines were added. On day 8 of incubation, the DC were harvested. The DC were then co-cultured with irradiated HESC and HFib for 24 hours as described in the mouse MLR above.

The CD4^+ T-cells were prepared from the defrosted PBMC and purified by the CD4^+ T-cell Isolation Kit II (Miltenyi Biotec). Non-CD4^+ T-cells were labelled by using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD133, TCR-γ/δ and Glycophorin A, and then incubated with Anti-Biotin MicroBeads. The isolation of pure CD4^+ T-cells was achieved by depletion of magnetically labelled cells using MACS Column and MACS Separator (all from Miltenyi Biotec).

A primary MLR was performed using CD4^+ T-cells (1x10^5 cells per well) incubated with irradiated DC alone (2x10^4 cells per well) or DC co-cultured with irradiated HESC and HFib or irradiated allogeneic DC (2x10^4 cells per well). The responder proliferation, presented as mean Stimulation Index, was studied from day 4 to 7 and each experiment was repeated three times. Stimulation Index is calculated as the ratio of mean cpm of CD4^+ T-cells cultured in presence of the antigen (AutoDC+HESC, AutoDC+HFib or AlloDC), to mean cpm of background CD4^+ T-cells with syngeneic human DC.

**Paper IV**

Splenocytes from naive C57BL/6 mice, together with C57BL/6 mice from the costimulation blockade treated and the isotype control groups were used for this experiment. The spleens were homogenized and the CD4^+ T-cells were separated as previously described. CD4^+CD25^+ T-cells from C57BL/6 mice of the costimulation blockade and isotype control groups were separated according to the manufacturer’s protocol using a CD25 MicroBead Kit (Miltenyi Biotec) and the purity was estimated to 70% by FACS analysis.

The DC were prepared from the bone marrow of the femurs of syngeneic C57BL/6 mice according to the protocol previously described. The DC (1x10^6) from C57BL/6 mice were co-cultured with irradiated (15Gy) HESC (3x10^5) and human fibroblasts (HFib) (3x10^5) respectively in culture medium (RPMI-1640 supplemented with 5% heat inactivated fetal calf serum, 2 mmol/l L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin and 5x10^{-5} mol/l 2-mercaptoethanol) in humidified air containing 5% CO_2 at 37°C for 24 h. The DC were then irradiated (15Gy). The MLR was performed exposing naive CD4^+ T-cells (5x10^4 cells per well) to syngenic DC (2x10^4 cells per well) alone or syngenic DC co-cultured with HESC or HFib (2x10^4 cells per well). To study if the regulatory T-cells from mice treated with costimulation blockade or its isotype control reagents could specifically down modulate the immune response induced by HESC, the previously separated CD4^+CD25^+ T-cells (5x10^4 cells per well) were added to the wells. Five wells per culture were set up in 96-well round-bottomed microtitre plates (25850; Corning, USA) and incubated in humidified air containing 5% CO_2 at 37°C. Each culture was labelled with 2 µCi [3H] thymidine (Radiochemical Center) for 20 h prior to harvest. The proliferation of the responders was measured at day 6 as cpm and each experiment was repeated 3 times. Two mice from each group were used for each MLR.
RNA isolation and oligonucleotide microarray analysis

*Paper III*

After isolation of mRNA from all seven HESC lines with RNAeasy mini kit (Qiagen, Hilden, Germany), total RNA (50 ng for all samples) were amplified, labelled and hybridised according to the Affymetrix two-cycle GeneChip® Eukaryotic small sample target labelling assay version II (Affymetrix; Santa Clara, CA, USA). 20 µg of biotin-labelled cRNA was fragmented and hybridised to Human Genome U133 Plus 2.0 array (Affymetrix; Santa Clara, CA, USA). Arrays were stained and scanned according to Affymetrix protocols.

The gene transcript levels were determined from data images with algorithms in the GeneChip® DNA Analysis Software (Affymetrix; Santa Clara, CA, USA) and further analysis of data was performed with Kensington software (InforSense, London, UK). At the detection level, each probe set was assigned a call of present (P), absent (A), or marginal (M). A gene with detection call “present” in two biological replicates was considered to be expressed. The comparison level analysis of gene signal intensity was determined as fold change (Average Fold Change, calculated using two biological replicates) to GAPDH, a common housekeeping gene, and OCT-4, a common marker for undifferentiated human embryonic stem cells.

Flow cytometry analysis

*Paper III*

The cell-surface markers of HESC were analyzed by immunostaining and flow cytometry. Antibodies directed towards HLA-A, B, C antigen/ RPE (Clone W6/32); HLA- DP, DQ, DR antigen/ FITC (Clone CR3/43) (Dako Cytomation) and their respective isotype controls were used. Human fibroblast feeder cells (HFib) and HESC (HS237) were analyzed before and after stimulation with IFN-γ (Sigma-Aldrich Corp.), 50 U/ml for seven days. 2x10^6 cells were incubated with antibody for 30 minutes on ice, washed and resuspended in PBS. The cells were assayed in a flow cytometer (FACScan, Becton-Dickinson) and the data analyzed with Cellquest software (Becton-Dickinson). The experiment was repeated three times.

Cardiac function measurements

*Paper II*

In vivo cardiac function and dimensions were assessed by echocardiography using a Vingmed Vivid 5 (Vingmed A/S, Norway) ultrasound system equipped with a 10 MHz transducer. The rats were sedated with Medetomidin/Midazolam and examined in a supine position and subsequently anaesthesia was reversed with Flumazenil/Atipamezol hydrochloride, as previously described. The examinations were performed before LAD-ligation, one week after injection of hMSC/Perfadex and after four to six weeks. Parasternal long-and short-axis views were obtained and stored on a magnet-optic (MO) disc. From the M-mode tracings (guided by 2-D images in the parasternal long axis), measurements of the left ventricular end diastolic dimension (LVEDD) as well as the dimensions of the septum and posterior wall were performed. To assess the contractile function of the left ventricle, the fractional area change (FAC = Diastolic area –Systolic area)/ Diastolic area) was calculated by performing planimetry in the parasternal short axis view. The measurements were made at the widest obtainable part of the left ventricle in the long axis view and at the level of the papillary muscles in the short axis view.
**Statistical analysis**

**Paper I**
All MLR data are presented as mean ± SD.

**Paper II**
All histological and echocardiographic analyses were done blinded. All values are presented as mean ± SD. The data collected for treated animals and controls were compared by paired Student’s $t$ test at every time point. Data retrieved over time from echocardiography were tested with unpaired Student’s $t$ test for each group of animals and when there was a significant difference ANOVA for repeated measurements was performed. A $p<0.05$ was considered statistically significant.

**Paper IV**
MLR data are presented as mean ± SD. For statistical analysis of MLR data, Wilcoxon Signed Ranks Test was used as a non-parametric test for two related samples. A $p<0.05$ was considered as significant.
RESULTS

**Paper I: Xenoreactivity and Engraftment of Human Mesenchymal Stem Cells Transplanted into Infarcted Rat Myocardium**

**Aims**
To study the immune response induced by ahMSC transplanted over the xenogeneic barrier and if ahMSC engraft in the experimentally induced ischemic rat myocardium.

**Detection of implanted ahMSC**
LAD ligation induced a transmural infarction in a reproducible manner in each rat. The ahMSC implanted one week after myocardial infarction could only be detected by FISH in the immunoincompetent RNU rats (Fig.3). In the histological sections the implanted ahMSC were randomly dispersed without any sign of alignment, staining negative for cardiomyocyte markers and for the gap junction protein connexin 43.

**Immune response**
In immunocompetent SD rats transplanted without immunosuppression, the ahMSC induced an intense cellular immune response in the area of injection of the stem cells one week after transplantation (Fig.4). This immune response seemed to be mediated primarily by macrophages. In the SD rats immunosuppressed with Tacrolimus and in

**Figure 3.** The ahMSC (red cells) were identified by FISH in the myocardium of RNU rats one week after transplantation. In figure bar represents 200 μm.

**Figure 4.** The ahMSC induced a massive round cell infiltration in the myocardium of immunocompetent SD rats, one week after transplantation. In figure bar represents 100 μm.
RNU rats the macrophage mediated immune response was less prominent.

In the first set of MLR experiments, PBL were harvested from non-ischemic SD rats 7 days after cardiac injection of ahMSC. The PBL were mixed with 1 or 10% ahMSC and the uptake of $^{3}$H thymidine was measured and compared to that of the control rat, with no previous exposure to ahMSC. As shown in Fig.5A, significant lymphocyte proliferation ($p<0.01$) was observed when ahMSC were co-cultured with lymphocytes from SD rats previously exposed to ahMSC. No immune response was detected in animals not previously exposed to ahMSC.

To evaluate if ahMSC induced a sensitization reaction in SD rats immunosuppressed with therapeutic levels of Tacrolimus and in RNU rats, PBL from rats used in the infarction model were analyzed in MLR. Lymphocyte proliferation was not significantly increased in PBL from SD rats with immunosuppression, when exposed to ahMSC. The ahMSC did not elicit any proliferation in lymphocytes from RNU rats (Fig.5B). However, significant proliferation was observed in animals without immunosuppression post-ahMSC injection, indicating a sensitization reaction.

**Conclusions**

Adult human mesenchymal stem cells induce an immune response and are rejected when transplanted over the xenogeneic barrier into the myocardium of immunocompetent rats. Furthermore, ahMSC induce a sensitization reaction in immunocompetent rats indicated by a significant proliferation of lymphocytes from SD rats previously exposed to ahMSC. Even though re-exposure to ahMSC by lymphocytes from immunosuppressed SD rats did not cause a significant increase in proliferation, the immunosuppressive effect of Tacrolimus was not sufficient to inhibit rejection of the implanted ahMSC. Even though ahMSC are transplantable across allogeneic barriers, graft rejection can occur in a xenogeneic model.

![Figure 5](image-url)

**Figure 5.** Immunological response in vitro to ahMSC. (A) Re-exposure of ahMSC to lymphocytes from SD rats previously transplanted with ahMSC caused a significant lymphocyte proliferation as assayed by the MLR (B: pooled peripheral blood from 3 syngenic rats; C: 1 individual rat). Exposure of ahMSC to naive lymphocytes from a SD rat did not induce a significant proliferation. A: peripheral blood from a naive SD rat; Bg: background. Experiments were performed in triplicate with a significance of $p<0.01$. Mean ± SD.

(B) Lymphocyte proliferation was not significantly increased in PBL from SD rats with immunosuppression (SD 1), when re-exposed to ahMSC. The ahMSC did not induce any proliferation in lymphocytes from RNU rats (RNU), however significant proliferation was observed in SD rats without immunosuppression (SD 2) post-ahMSC injection, indicating a sensitization reaction. K: control animals; Bg: background. Each bar depicts one experiment performed in triplicate. Mean ± SD.
**Paper II: Human mesenchymal stem cells do not differentiate into cardiac myocytes in a cardiac ischemic xenomodel**

**Aims**

To study the capability of adult human mesenchymal stem cells to engraft, differentiate into cardiomyocytes and improve myocardial function in a rat ischemia model.

**Histological analysis**

The hMSC seemed to be rejected from the myocardium of immunocompetent Fischer rats even in the group immunosuppressed with Tacrolimus. Surviving hMSC were found in half of the hearts (8/16) of RNU rats, where the hMSC were injected directly after LAD ligation, treated with immunosuppression and euthanized after four weeks. There were only few surviving cells, encapsulated by dense fibrous tissue and the stem cells had to some degree migrated from the site of injection into the infarction area (Fig.6A). hMSC were also detected after six weeks in RNU rats, injected with hMSC three days after LAD ligation and treated with immunosuppression. In these rat hearts the hMSC had migrated into the infarction area and they were arranged in a more dispersed manner, separated from the recipients’ cardiomyocytes by dense fibrous tissue (Fig.6B). Neither of the surviving hMSC expressed cardiomyocyte markers nor connexin 43.

**Cardiac function**

The LAD ligation resulted in a reproducible decrease in cardiac function with dilatation of the left ventricle in all groups (Table 1). FAC decreased (0.57 ± 0.10 vs. 0.50 ± 0.15, p=0.001) and LVEDD increased (0.65 ± 0.08 vs. 0.70 ± 0.08, p=0.0005) between baseline and 1 week in the whole material and there were no difference between the groups. However, at 1 week postoperatively FAC was significantly higher in Fischer rats, injected with hMSC three days after LAD ligation and treated with immunosuppression, compared to controls (0.54 ± 0.12 vs. 0.38 ± 0.15, p=0.026). This early difference, however, was not evident any longer at 6 weeks prior to sacrifice (0.49 ± 0.10 vs. 0.44

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*Figure 6.* (A) ahMSC (red cells) identified by FISH four weeks after implantation into the acutely infarcted myocardium of RNU rats without immunosuppression. The ahMSC are arranged in clusters surrounded by dense fibrous tissue. (B) ahMSC (red cells) injected three days after LAD ligation into the myocardium of RNU rats with immunosuppression. The ahMSC seemed to have migrated more freely into the infarction tissue. In figure bar represents 200 μm.
FAC remained unchanged between 1 week and 4 to 6 weeks in the whole population (0.49 ± 0.15 vs. 0.47 ± 0.11, p=0.2) and regardless whether stem cells had been delivered or not. Indeed, transplantation of hMSC did not seem to reverse the remodeling and dilatation, assessed by LVEDD, of the left ventricle. In contrast, the left ventricle continued to dilate between 1 and 4 to 6 weeks (0.70 vs. 0.78, P<0.001) with no significant difference between animals that received treatment compared to controls.

## Conclusions

Human mesenchymal stem cells are immunogenic in a xenogeneic model and were rejected when transplanted into the hearts of immunocompetent rats, even if immunosuppression was used. The hMSC required an implantation into immunoincompetent rats as well as immunosuppression to survive. The surviving hMSC did not differentiate into cardiomyocytes after four to six weeks in an ischemic myocardium and they did not improve the myocardial function.

### Table 1. Echocardiographic data of the left ventricular end diastolic diameter (LVEDD) and fractional area change (FAC) during the follow-up period. hMSC – human mesenchymal stem cells. Significant difference by paired Student’s t test between preop and 1 week is indicated by * and between 1 week and 4-6 weeks is indicated by †. Significant difference by unpaired Student’s t test between treated animals and controls at any time point is indicated by ‡. A p-value < 0.05 was considered a statistically significant difference.

<table>
<thead>
<tr>
<th>Group I</th>
<th>n</th>
<th>LVEDD Preop</th>
<th>LVEDD 1 week</th>
<th>LVEDD 4-6 weeks</th>
<th>FAC Preop</th>
<th>FAC 1 week</th>
<th>FAC 4-6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>(direct, RNU, without Tacrolimus) (hMSC)</td>
<td>16</td>
<td>0.63 ± 0.08</td>
<td>0.70 ± 0.08 †</td>
<td>0.79 ± 0.09 †</td>
<td>0.60 ± 0.10</td>
<td>0.52 ± 0.20</td>
<td>0.49 ± 0.10</td>
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<tr>
<td>(Control)</td>
<td>5</td>
<td>0.70 ± 0.12</td>
<td>0.70 ± 0.13 †</td>
<td>0.78 ± 0.10 †</td>
<td>0.52 ± 0.03</td>
<td>0.47 ± 0.09</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>Group II</td>
<td>a. (hMSC) (3 days, RNU, without Tacrolimus)</td>
<td>5</td>
<td>0.59 ± 0.05 †*</td>
<td>0.74 ± 0.04 †*</td>
<td>0.84 ± 0.03 †</td>
<td>0.54 ± 0.04</td>
<td>0.51 ± 0.04 †</td>
</tr>
<tr>
<td>b. (hMSC) (3 days, RNU, with Tacrolimus) (Control)</td>
<td>6</td>
<td>0.68 ± 0.06</td>
<td>0.76 ± 0.10</td>
<td>0.88 ± 0.08</td>
<td>0.49 ± 0.06</td>
<td>0.45 ± 0.11</td>
<td>0.41 ± 0.10</td>
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<tr>
<td></td>
<td>6</td>
<td>0.69 ± 0.08 ‡</td>
<td>0.76 ± 0.01</td>
<td>0.83 ± 0.06</td>
<td>0.47 ± 0.08</td>
<td>0.52 ± 0.03</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>Group III</td>
<td>(3 days, Fischer, with Tacrolimus) (hMSC)</td>
<td>14</td>
<td>0.66 ± 0.09</td>
<td>0.67 ± 0.08 †</td>
<td>0.78 ± 0.09 †</td>
<td>0.64 ± 0.07 *</td>
<td>0.54 ± 0.12 ‡</td>
</tr>
<tr>
<td>(Control)</td>
<td>7</td>
<td>0.60 ± 0.07</td>
<td>0.70 ± 0.09</td>
<td>0.77 ± 0.08</td>
<td>0.59 ± 0.12 *</td>
<td>0.37 ± 0.15 ‡</td>
<td>0.44 ± 0.17</td>
</tr>
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</table>
Paper III: Human embryonic stem cells are immunogenic in allogeneic and xenogeneic Settings

Aims
To study the antigen expression and the immunogenicity of human embryonic stem cells when transplanted across allogeneic and xenogeneic barriers.

DNA micro-array and flow cytometry analysis
Gene expression analysis in the HESC lines HS237, HS293, HS306, HS346, HS362, HS363 and HS368 was performed including HLA class I and II, co-stimulatory and adhesion molecules. Undifferentiated HESC contain mRNA for HLA class I and class II and some co-stimulatory and adhesion molecules. The gene expression of molecules known to down-modulate immune responses was also examined and there was no expression of CD95L, IL-10 and low expression of TGF-β. This indicates that if HESC are capable of down-modulating immunological reactivity, it is probably not through the expression of these molecules.

By flow cytometry, undifferentiated HESC expressed high levels of HLA class I but no HLA class II (Fig.7). Stimulation with IFN-γ for 7 days lead to a ten times increase of HLA class I expression on HESC, but no surface expression of HLA class II. This is in contrast to HFib, where IFN-γ caused an expression of HLA class II.

Histological analysis
HESC injected into the myocardium of C57BL/6 mice induced a T-cell mediated immune response which peaked at day 5-7 and declined till day 10. The lymphocyte infiltration was characterized by the presence

Figure 7. Expression of HLA class I (A, B) and HLA class II (C, D) alloantigens detected by flow cytometry. Stimulation with IFN-γ for 7 days lead to a ten times increase of HLA class I (B) expression on HESC, but no surface expression of HLA class II (D).
Figure 8. HESC induce a T-cell mediated immune response in the myocardium of C57BL/6 mice, characterized by (A) activated macrophages (CD11b+ cells) and (B) T-cells (CD3+ cells) on day 5. The T-cell response is characterized by both (C) CD4+ cells and (D) CD8+ cells. In figures, inflammatory cells stain green. In figure bar represents 100 μm.

Figure 9. The HESC (red cells) were identified by FISH in the myocardium of C57BL/6 mice at (A) day 1 and (B) day 3. The HESC were rejected at day 4. (C) In the myocardium of SCID mice the HESC were present at day 7. In figure bar represents 200 μm.
of CD11b⁺, CD3⁺, CD4⁺ and CD8⁺ cells (Fig.8). The implanted HESC were rejected by day four which was in contrast to SCID mice where HESC could be found after seven days (Fig.9).

Mixed leukocyte reaction

In a xenogeneic setting, we studied the capacity of HESC to induce an in vitro immune response both through direct and indirect antigen presentation pathways. In the direct antigen presentation pathway, CD4⁺ T-cells from C57BL/6 mice were cultured with irradiated HESC of the same cell line as the mice had been transplanted with and proliferation was compared to that induced by irradiated HFib. Naive CD4⁺ T-cells proliferated to a similar degree in response to HESC as to HFib. CD4⁺ T-cells from transplanted mice proliferated three times more than naive CD4⁺ T-cells already at day 3. This response was not as pronounced towards HFib, indicating specificity towards the immunizing antigen (Fig.10a,c). In order to study the effect of

![Figure 10](image1)

The proliferation of CD4⁺ T-cells, expressed as counts per minute (cpm) or Stimulation Index, after direct stimulation with either HESC or HFib as the only stimulators (Fig. a and c) or indirect stimulation with HESC or HFib incubated with syngeneic C57BL/6 DC or allogeneic Balb/c DC only (Fig. b and e). Stimulation Index is defined as the ratio of mean cpm of CD4⁺ T-cells (+DC in indirect stimulation) cultured in presence of the stimulators, to mean cpm of background CD4⁺ T-cells (+DC in indirect stimulation) without stimulators. The experiment was repeated three times. Fig d shows the allogeneic immune response of human CD4⁺ T-cells induced by HESC or HFib cultured with purified syngeneic human DC in comparison to allogeneic DC. Stimulation Index is calculated as the ratio of mean cpm of CD4⁺ T-cells cultured in presence of the antigen (AutoDC+HESC, AutoDC+HFib or AlloDC), to mean cpm of background CD4⁺ T-cells with syngeneic human DC. Each MLR was repeated at least three times with different cell donors.
HESC during indirect antigen presentation, C57BL/6 DC were cultured together with HESC or HFib. Again the HESC induced higher proliferation of CD4\(^+\) T-cells from immunized mice than from the naive mice already at day 3 (Fig. 10b,c). Proliferation of CD4\(^+\) T-cells is similar between HFib and HESC cultured with DC indicating that the HESC do not down-modulate DC function during exposure to naive or immunized CD4\(^+\) T-cells.

In order to imitate a clinical situation, the capacity of HESC to elicit an allogeneic immune response by indirect antigen presentation was studied. Human DC were cultured with both HESC and HFib. As shown in Fig. 10d, both HESC and HFib induced an equivalent amount of CD4\(^+\) T-cell proliferation. The proliferation of naive CD4\(^+\) T-cells stimulated by syngeneic DC cultured with HESC or HFib is four-fold less than the proliferation attained when culturing with allogeneic DC only. These results indicate that HESC have similar capability to affect human DC as HFib, but are four-fold less immunogeneic than allogeneic DC.

**Conclusions**

Human embryonic stem cells express HLA class I, no HLA class II and low levels of co- stimulatory molecules. The HESC are immunologically inert and do not inhibit immune responses during direct and indirect antigen presentation and they are rejected when transplanted over the xenogeneic barrier.
Paper IV: Costimulation blockade induces tolerance to HESC transplanted to the testis and induces regulatory T-cells to HESC transplanted into the heart

Aims
To study the ability of costimulation blockade to induce long-term acceptance of HESC implanted into immunocompetent mice. To study the difference in engraftment between testis and the myocardium.

Histological analysis
The HESC had developed into teratoma in the testis of all SCID mice and costimulation treated C57BL/6 mice when studied two months after transplantation. The cells in the teratoma were of human origin (Fig.11A) and the only inflammatory cells present in the testis of the costimulation blockade treated mice were mouse CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T-cells (Fig.11B). In the isotype control treated C57BL/6 mice no surviving HESC could be identified after two months. HESC engrafted in two of five hearts in SCID mice forming expansive teratoma-like tumors. C57BL/6 mice transplanted intracardially and treated with costimulation blockade rejected their grafts within one month, demonstrating lymphocytic infiltrates at the site of injection (Fig.12). In the isotype control mouse there was only a scar left one month after implantation of HESC. Repeated costimulation blockade three weeks after implantation of HESC into the myocardium of C57BL/6 mice caused survival of HESC-derived cells after two months (n=1/5) (Fig.13). The cells were encapsulated in the myocardium and there was no sign of inflammatory response.

Mixed leukocyte reaction
Mixed Leukocyte Reactions (MLR) were designed to mimic the clinical setting of HESC transplantation where the host DC present antigens from processed HESC to host CD4\(^+\) T-cells. As shown in figure 14, the CD4\(^+\)CD25\(^+\) T-cells separated from the costimulation blockade group significantly down-regulated naive CD4\(^+\) T-cell proliferation with a mean

Figure 11. (A) HESC-derived teratoma in the testis of a costimulation blockade treated C57BL/6 mouse two months after injection. The teratoma was of human origin since all the cells were FISH positive (red cells). (B) Close to the capsule at the interface of normal tissue and teratoma there were clusters of mouse CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T-cells (green cells). In figure bar represents A; 200 \(\mu\)m and in figure B; 100 \(\mu\)m.
Figure 12. HESC transplanted into the myocardium of costimulation blockade treated C57BL/6 mice, induced a T-cell mediated immune response one month after injection. The immune response was characterized by (A) activated macrophages (CD11b+ cells) and (B) T-cells (CD3+ cells), where both the (C) CD4+ cells and (D) CD8+ cells were present. In figures, inflammatory cells stain green. In figure bar represents 100 μm.

Figure 13. HESC transplanted into the myocardium of C57BL/6 mice, treated with costimulation blockade reagents both directly after transplantation and again three weeks later. This lead to acceptance of the HESC as demonstrated by FISH positive cells (red cells) two months after injection. In figure bar represents 200 μm.

Figure 14. In a MLR, CD4+CD25+ T-cells separated from the costimulation blockade group significantly down-regulated naive CD4+ T-cell proliferation induced by HESC with a mean of 22% (*, p<0.05), while CD4+CD25+ T-cells from the isotype control group did not inhibit the proliferation of naive T-cells. The down-regulatory effect of CD4+CD25+ T-cells was specific towards undifferentiated HESC, since they did not inhibit the proliferation of naive T cells towards HFib.
of 22% (p<0.05), while CD4<sup>+</sup>CD25<sup>+</sup> T-cells from the isotype control group did not inhibit proliferation of naive T-cells. Furthermore, the down-regulatory effect of the immune response mediated by CD4<sup>+</sup>CD25<sup>+</sup> T-cells was specific towards undifferentiated HESC, since they did not inhibit the proliferation of naive T cells towards HFib.

**Conclusions**

Our findings indicate that costimulation blockade is sufficiently robust to induce tolerance to HESC in the immune-privileged environment of the testis. HESC transplanted into the myocardium of costimulation blockade treated C57BL/6 mice induced specific regulatory T-cells and when the costimulation blockade was repeated, the success of transplantation was similar to that seen in SCID mice.
Paper V: Isolation, Expansion, Characterisation and Transplantation of Human Fetal Cardiomyocyte Progenitor Cells

Aims
To identify Islet-1 positive cells in the myocardium of human fetal hearts. To isolate human Islet-1 positive cells and their progeny. To culture and characterize them in vitro and finally study the engraftment of these cells in the myocardium of SCID mice.

Identification of Islet-1 and Nkx 2.5 positive cells in the fetal heart
The Nkx 2.5 positive cells were found in abundance in the ventricles and atria of the fetal heart. As the Nkx 2.5 positive cells mature, gap junctions are formed between the cells and about 15 to 20% of the cells are proliferating. The Islet-1 positive cells are clustered around the atria and outflow tracts (Fig. 15), staining negative for c-Kit and Sca-1.

Characteristics of the cultured human fetal cardiomyocyte progenitors
During the first week in culture, aggregates of mixed cells developed and from these clusters phase-bright cells gathered forming spontaneously beating cardiospheres (Fig.16A-C)). These cardiospheres were also derived from the single cell suspension. The CS were beating spontaneously between 50 to 90 beats per minute. During the second week in culture, monolayer cells started to migrate out from some beating CS, forming a spontaneously beating sheet of cells (Fig.16D,E). We have also been able to freeze and thaw the beating CS without affecting their phenotype (Fig.16F).

In immunohistochemical analysis more than 90% of the cells in the CS stained positive for Nkx 2.5 and neither of the cells expressed c-Kit or Sca-1. Islet-positive cells were found in the centre of the CS (Fig. 17A) and the CS also stained positive for Desmin and α-actinin. Troponin T positive cells were only seen in some parts of the CS (Fig.17B), while the majority of the monolayer cells expressed Troponin T together with Nkx 2.5, Desmin and α-actinin (Fig.18). The monolayer cells also stained negative for c-Kit and Sca-1.

In electron microscopy plenty of gap junctions were seen between the cells which also contained contractile elements together with mitochondria and glycogen deposits indicating metabolic active cells (Fig.17C,D). The contractile elements were not organised in the cytoplasm, a picture typically seen in cells not performing any work load.

In a multi-electrode array recordings autonomous beating frequency and estimation of action potential durations of the CS were determined (Fig.19A). The field potentials obtained from extracellular recordings have in mathematical models been related to the first derivative of the membrane potential, thus providing information on transmembrane ion currents (Regehr et al. 1989). The mean autonomous inter-beat-interval (IBI) of the cardiosphere clusters (n = 7) was 1579 ms (SD = 633, range = 923 - 2748). The IBI generally increased slightly when fresh medium was first added but was then stable throughout further medium changes.

The mean FP_DUR calculated from one representative electrode recording from each preparation (n = 7), was 172 ms (SD = 58, range = 110 – 265).

An important cardiomyocyte property is rate dependence of the action potential duration (APD). Pacing in 100 ms steps at intervals between 1000 ms and 600 ms resulted in a rate dependent decrease in FP_DUR of up to 35%, at the highest stimulation frequency, compared with the autonomous FP_DUR (Fig.19B).
Characteristics of the intra-cardially transplanted human fetal cardiomyocyte progenitor cells

The implanted HFCP formed stable grafts in the myocardium of SCID mice, preserving their cardiomyocyte phenotype two weeks after implantation (Fig. 20).

Conclusions

The Islet-1 positive cells are clustered around the atria and outflow tracts. Furthermore we have shown that it is possible to isolate and culture true cardiomyocyte progenitor cells from aborted human fetal material, forming stable engraftments in the myocardium of SCID mice.

Figure 15. The whole human fetal hearts (gestational week 5 to 10) were transected. (A) Islet-1 positive cells (red cells) were clustered around the atria and outflow tract. (B) Nkx 2.5 positive cells were seen ubiquitously in the whole myocardium, (C) showing an intense proliferation represented by the Ki67 marker (green cells). (D) As the Nkx 2.5 positive cells mature they form gap junctions (Connexin 43) with neighboring cells (red dots). In figures, the nuclei stain blue by DAPI. Bars indicate 100 μm.
Figure 16. Phase contrast microscopy of human fetal cardiomyocyte progenitor cells in culture. (A) During the first week in culture the cells formed aggregates of mixed cells. From these aggregates, phase-bright spontaneously beating clusters developed, which we named cardiospheres (CS). (B) Cardiospheres also developed directly from the single cell suspension. (C) In culture the CS grew to a size between 30 to 200 μm. (D, E) A monlayer of spindle-shaped cells developed from the beating CS, forming a sheet of contracting cells. (F) We have also been able to freeze and thaw the CS without changing their phenotype. Bars indicate 100 μm (A, B, F) and 200 μm (C, D, E).
**Figure 17.** In the beating cardiospheres (CS) (A) the Islet-1 positive cells (red nuclei) were found in the centre. The majority of the cells in the CS expressed Nkx2.5 but (B) Troponin T is only expressed in some parts of the CS (green cytoplasm). (C, D) In electron microscopy it is evident that the HFCP are metabolic active cells with mitochondria (M), glycogen deposits (G) and they contain contractile elements (S) and form gap junctions with neighboring cells (GJ). In the figure, the nuclei are stained blue by DAPI. Bars indicate 50 μm in figure A and 100 μm in figure B.

**Figure 18.** The monolayer cells expressed (A) Nkx 2.5 (green cells), (B) Desmin (red cells) and the majority of the cells also expressed (C) Troponin T (green cells). In figure the nuclei stain blue by DAPI. Bars indicate 100 μm.
Figure 19. (A) A representative field potential (FP, compiled from 30 consecutive FPs to reduce noise) recorded from a CS cultured on a multi-electrode array (MEA). Indicated are the FP components FP<sub>PRE</sub>, FP<sub>MIN</sub>, FP<sub>MAX</sub> and FP<sub>DUR</sub>. (B) This diagram shows cardiosphere action potential duration (APD) rate dependence. One cardiosphere was paced through an external electrode with a biphasic pulse in 100 ms steps. FP<sub>DUR</sub> was measured from one external recording electrode at each pacing interval. Graph shows FP<sub>DUR</sub> for each interval as percent of the autonomous FP<sub>DUR</sub>. The CS cluster exhibited FP<sub>DUR</sub> rate dependence with an up to 35% decrease in FP<sub>DUR</sub> at a stimulation interval of 600 ms.

Figure 20. The transplanted HFCP were identified by FISH, 2 weeks after implantation into the myocardium of SCID mice (red nuclei). The cardiomyocyte phenotype seem to have been preserved. In the figure, the nuclei stained blue by DAPI. Bar indicate 100 μm.
GENERAL DISCUSSION

Human mesenchymal stem cells for cardiomyoplasty

An important advantage with hMSC would be that the patients’ own stem cells could be used for cardiomyoplasty and thereby avoiding immune rejection. Whether these multipotent stem cells can differentiate into cardiomyocytes and improve myocardial function is still not consistently demonstrated. Mouse and rat MSC have been shown to home to the site of infarction and to differentiate into cardiomyocytes (Saito et al. 2002; Martin et al. 2002). In a similar manner autologous and allogeneic pig MSC have also shown to inhibit the remodeling process after myocardial infarction and increase myocardial function (Shake et al. 2002; Amado et al. 2005). When we for the first time in a controlled manner transplanted hMSC into the infarcted myocardium of rats they did not affect the remodeling process, nor did the hMSC differentiate towards a cardiomyocyte phenotype (Grinnemo et al. 2005). Instead the implanted hMSC appeared to be undifferentiated cells but they seemed to have migrated from the peri-infarct region into the area of infarction.

The lack of differentiation among implanted hMSC observed in this study may be explained by the xenogeneic setting. Differentiation stimuli between species may differ and the hMSC might differentiate into cardiomyocytes in an autologous or allogeneic setting. However, currently it is not known which factors or cell-to-cell contact characteristics that are the specific differentiation signals. Furthermore, it is not clarified if MSC can differentiate into cardiomyocytes. When hematopoietic lineage mouse Lin−/c-Kit+ bone marrow cells were injected into the peri-infarct region of mice, these cells were once thought to regenerate up to 68% of the infarcted region within 9 days and the implanted cells had transformed into cardiomyocytes (Orlic et al. 2001). In an attempt to re-evaluate these findings, Nygren and co-workers used different approaches to induce myocardial infarction and deliver transgenically marked bone marrow cells to the myocardium of mice (Nygren et al. 2004). Both the unfractioned bone marrow cells and purified populations of hematopoietic stem cells engrafted within the infarction area, but only transiently and the engrafted cells did not differentiate into cardiomyocytes. In contrast, in the myocardium outside the infarction area, some hematopoietic cells had fused with the cardiomyocytes of the host. If the mechanism of cell fusion also is applicable for hMSC is not clear, but this mechanism might explain the low frequency of cardiomyocyte differentiation seen in SCID mice (Toma et al. 2002) and during in vitro culturing with human cardiomyocytes (Rangappa et al. 2003). An alternative explanation for the discrepant results lies in the different assays used to detect cardiomyogenic differentiation. In
those studies cited above, the identification of transplanted cells relies mainly on immuno-fluorescence staining to track the cells and monitor cell differentiation. In an infarcted heart there is a high level of nonspecific autofluorescence and to detect the transplanted cells one must use methods, which produces intense signals that clearly can be separated from background signaling. In our studies we have identified the implanted cells by the FISH method where a DNA probe binds to and clearly stain total human DNA. This makes it possible to distinguish the transplanted human cells from rat cells, thereby excluding the effect of autofluorescence.

Mesenchymal stem cells are also thought to exert their effects through paracrine pathways. In Miyahara’s study, a monolayer of rat MSC was applied over the infarcted area and the engrafted cells grew to form a thick stratum that included newly formed cells, undifferentiated MSC and few cardiomyocytes (Miyahara et al. 2006). The mesenchymal sheet also acted through paracrine pathways producing angiogenic and anti-apoptotic factors like vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). The paracrine effect mediated by hMSC was not evident in our infarction model in rats (Grinnemo et al. 2005). This might be due to the xenogeneic setting. In an allogeneic setting the hMSC fail to induce alloreactivity (Le Blanc et al. 2003, 2004; Rasmussen et al. 2005) and they also act immunosuppressive and may reverse severe steroid resistant acute graft-versus-host disease after allogeneic stem cell transplantation (Le Blanc et al. 2004). In contrast when hMSC were injected into immunocompetent Sprage-Dawley rats after myocardial infarction they induced an immune rejection (Grinnemo et al. 2004). An immune reaction to the transplanted hMSC was supported both by the histological examination of sectioned hearts, where massive infiltration of immune cells were noted one week after injection, as well as by significant proliferation when murine lymphocytes, derived from animals that had received a MSC injection, were co-cultured with hMSC. This indicates that an immune reaction had occurred against the transplanted human cells. Rejection in a xenogeneic but not in an allogeneic system may be explained by the fact that although hMSC suppress the formation of CD4 and CD8 T-cells, immune responses following xenotransplantation include both acquired immunity and innate immunity, in which natural antibody, complement, NK cells and macrophages all play independent rolls (Zhang et al. 2000).

In RNU rats, engraftment was improved in animals treated with Tacrolimus (Grinnemo et al. 2005). The observation of hMSC in immunosuppressed RNU rats but not in untreated RNU rats is disgruntling. This may to some extent be explained by the fact that RNU rats seem to be capable of producing T-cells some weeks after birth. But the most likely explanation is that the cells are in a state of rejection, which is not complete at four weeks but finished after six weeks. Not all rejection needs to be mediated by T cells and therefore there are other components of the immune system, which could be the main effector component responsible for rejection in this human to rat strain combination. The low number of surviving hMSC could then be related to rejection since none of these animals were treated with immunosuppression. In allogeneic studies in vitro, hMSC has been shown to be able to down-modulate immune responses by increasing the production of interleukin-10 (IL-10) and decreasing IFN-γ production partially mediated by secretion of prostaglandin E₂ (PGE₂) (Aggarwal et al. 2005). Although hMSC clearly are immuno-modulatory in vitro in an allogeneic setting, these mechanisms may not be sufficiently robust in a discordant xenogeneic setting. The potential number of reactive T-cell clones is much greater in discordant xenogeneic
models and the incompatibility of inhibitory receptors may allow for NK-cell activation not present in the allogeneic setting.

From this point of view, it seems unlikely that the supposed effect of hMSC can be evaluated in a xenogeneic environment, where the immune response towards the grafted cells most certainly mask an eventual paracrine effect and where the differentiation signals might be species specific. Therefore, the only way to study the hMSC role in cardiomyoplasty will most certainly be in a well performed autologous or allogeneic setting.

**Are human embryonic stem cells immune-privileged or not?**

It has been consistently demonstrated that HESC express low levels of HLA class I, which is upregulated by IFN-γ stimulation, while the HESC do not express HLA class II (Grinnemo et al. 2006; Drukker et al. 2002; Li et al. 2004). The expression of costimulatory molecules (B7.1, B7.2 and CD40) is low or absent on undifferentiated HESC (Grinnemo et al. 2006; Li et al. 2004). This implies that the HESC lack the two important prerequisites to function as professional APC, namely high levels of HLA class II and costimulatory molecules. In order to ascertain if HESC express molecules known to down-modulate immune responses, the expression of TGF-β, CD95L and IL-10 was studied (Grinnemo et al. 2006). Gene micro-array analysis revealed no or very low expression of these molecules indicating that under normal circumstances HESC do not down-regulate immune responses by these well established pathways.

Drukker et al and Li et al have in different settings demonstrated that HESC lack the ability to induce allogeneic (Drukker et al. 2006; Li et al. 2004) and xenogeneic immune responses (Li et al. 2004). Based on these findings they suggested that HESC are immune-privileged, which is in contrast to our results working with similar stem cell lines. In our studies, injection of HESC into the myocardium of immunocompetent C57BL/6 mice evoked signs of rejection within 3 days and peaked at day 5 to 7 (Grinnemo et al. 2006). Similar results were obtained when HESC were injected into the testis of C57BL/6 mice (Grinnemo et al. 2007a). In a MLR, HESC induced mouse CD4+ T-cell proliferation equivalent to that of human fibroblasts when acting as the only APC. Lymphocytes isolated from mice transplanted with HESC showed increased reactivity towards HESC when re-exposed in vitro in a MLR, indicating that an immunological memory response has been induced. In an allogeneic setting, in MLRs with purified human CD4+ T-cells and dendritic cells from the same donor, HESC induced a similar level of proliferation as human fibroblasts, without any sign of inhibiting proliferation. According to these data HESC are immunologically inert and do not inhibit immune responses.

These contradictory results can be explained by examining the nuances in the design of the experiments. In the case of the experiments described by Drukker and co-workers (Drukker et al. 2006), the lack of rejection of injected HESC was compared with the successful rejection of transplanted allogeneic skin grafts or a B-cell lymphoma cell line. Both of these controls contained high numbers of APC, which express HLA class II and costimulatory molecules. The transplantation was performed 1 week after whole body irradiation, which relatively depletes the host of professional APC leaving the burden of antigen presentation on the transplant itself. In the case of skin transplant the graft contains high numbers of Langerhans cells which are extremely competent antigen presenting cells and in the case of the B-cell lymphoma cell line the
cell line itself is an antigen presenting cell. This allowed the transplants to be rejected by the direct pathway at a time when the host lacked antigen presenting cells of its own and was thereby incapable of inducing rejection by indirect presentation. The in vitro data (Drukker et al. 2006) indicating that HESC loaded with influenza virus type A peptide were not recognized by human cytotoxic T-cells, only supports the theory that HESC are poor antigen presenting cells. When HESC are infected with functional influenza virus type A in combination with IFN-γ, the HESC lysed by the cytotoxic T-cells. Infection of the cells with functional viral particles leads to a number of changes to the infected cell. The changes induced includes increased antigen presentation but also upregulation of adhesion molecules. These effects naturally lead to increased recognition by cytotoxic T-cells and subsequently target lysis.

There are also conflicting data in the in vitro MLR studies. In our experiments (Grinnemo et al. 2006), 95% pure populations of CD4+ T-cells were isolated and tested with syngenic dendritic cells. The only foreign antigens present in this system were derived from HESC mimicking the circumstances of transplantation of a cell transplant lacking APCs to a normal host and inducing indirect antigen presentation. The same experiment was conducted with human fibroblasts instead of HESC and yielded a similar outcome indicating that HESC have similar immunological characteristics as cultured human fibroblasts. In Li and co-workers MLR study (Li et al. 2004), the experiments were conducted with cell populations selected by their lack of adherence to plastic and gradient centrifugation making it difficult to assess which components of the immune system that were responding. Differences in HESC isolation and culture methods can also explain discrepancies achieved in different studies.

From our data it is evident that HESC are not immune-privileged and will be rejected when transplanted over the allogeneic and xenogenic barriers.

Is it possible to induce immunological tolerance towards HESC?

In a pioneering study by Fändrich and co-workers, rat embryonic stem cells (RESC) were transplanted into the portal circulation of allogeneic rats and engrafted without supplementary host conditioning (Fändrich et al. 2002). The RESC were found in the liver, spleen, mesenteric lymph nodes and thymus indicating the migratory capacity of these cells. Furthermore, the RESC-derived cell lines gave rise to B-cells and monocyte type cell populations, whereas T- and NK-cells were not found in the long-term chimeras. The RESC were also shown to be able to induce transplantation tolerance to a cardiac allograft. The authors’ conclusion was that the clonal deletion of alloreactive T-cells was due to the surface expression of Fas-ligand (FasL) on RESC which endowed them with the capacity to induce apoptosis in reactive T-cells. The HESC do not express FasL and in our previous work they are shown to induce both allogeneic and xenogeneic immune responses (Grinnemo et al. 2006).

In order to circumvent immune rejection without using chronic immunosuppression, we attempted to induce a state of immunological tolerance towards the engrafted cells. The strategy that has attracted the greatest attention targets the costimulatory molecules CD40L, B7 and LFA-1 molecules, expressed on T-cells and DC under inflammatory circumstances. By using this triple costimulation blockade (anti-CD40L/CTLA4Ig/anti-LFA-1) given during the first 8 days after transplantation, permanent acceptance of adult pig islets can be achieved in wild type diabetic mice (Kumagai-Braesch et al. 2007). Using the
same triple costimulation blockade regime, HESC were engrafted in the testis of all immunocompetent C57BL/6 mice tested, forming exophytic growing teratoma at two months after transplantation, with similar growth characteristics as in the SCID mice (Grinnemo et al. 2007a). In contrast no surviving cells were seen in the myocardium of costimulation blockade treated C57BL/6 mice. The HESC also induced an acute CD4+ T-cell mediated immune response one month after injection, causing rejection of the implanted HESC. Nonetheless CD4+CD25+ T-cells specific to undifferentiated HESC with the capacity to down-regulate naive T-cells responding to undifferentiated HESC in vitro developed in these recipients. Similar CD4+CD25+Foxp3+ T-cells were also seen in close proximity to the teratoma in the testis of costimulation blockade treated mice. This implies that the host was tolerated to undifferentiated HESC, but with differences in outcome depending on if the HESC were transplanted to the testis or the heart. There are two possible explanations for this difference in outcome. The testis is an immune-privileged environment and subsequently more permissive to transplantation (Nasr et al. 2005) and/or engraftment into the mouse myocardium is a far too strange environment for HESC.

The sertoli cells of the testis express FasL which induces apoptosis of reactive T-cells directed towards intra-testicular grafts (Bellgrau et al. 1995). This capacity when combined with costimulation blockade tips the balance from rejection to the induction of regulatory T-cells and tolerance induction (Nasr et al. 2005). In the heart, HESC differentiate, in response to signals in the environment or in response to the lack of them. This induces changes in their antigen expression and thereby they are no longer protected from rejection by the regulatory T-cells induced at the time of tolerisation. If the environment of the mouse heart is sufficiently foreign, the HESC may lack the necessary signals to justify their existence and subsequently commit suicide.

In order to study the possibility to induce tolerance towards the in vivo differentiating HESC, we repeated the costimulation blockade treatment three weeks after transplantation. In one mouse of five, the HESC-derived cells were found in the myocardium after two months, but did not develop into teratoma. Instead the engrafted cells were encapsulated in the myocardium and there was no sign of inflammatory response. In the surrounding myocardium there was no regulatory T-cells present, which is in accordance to the findings when pig islets were transplanted into mice using the same costimulation blockade protocol and where no regulatory T cells were observed locally (Kumagai-Braesch et al. 2007).

There also seems to be a difference in tumorigenicity between HESC transplanted into the heart compared to testis. HESC developed into teratoma in the testis of all SCID mice, but only in two of six hearts (Grinnemo et al. 2007a). The difference in results is probably due to factors pertaining to engraftment in the myocardium, similar to the findings when non-purified mESC-derived cardiomyocytes were injected into the heart (Kolossov et al. 2006). These findings also demonstrate that HESC are tumorigenic, not only in an immune-privileged area like testis (Nasr et al. 2005) but also in the heart. In contrast, when human embryo bodies containing beating cardiomyocytes were injected into the myocardium of T-cell deficient rats or immunosuppressed pigs, they were not reported to develop into teratoma (Laflamme et al. 2005; Kehat et al. 2004). This difference to our results might be due to that the cells in the embryo bodies are already pre-differentiated and thereby lack the ability to develop into teratoma. Another explanation might be the difference in follow-up period. In our study the mice were followed for eight weeks while in Laflamme’s study the hearts were harvested.
after four weeks (Laflamme et al. 2005), and thereby the tumor formation might be masked by the time factor.

Altogether these findings indicate that it is possible to induce tolerance towards undifferentiated HESC transplanted into the testis but not the heart of immunocompetent mice by using costimulation blockade. The peripheral tolerance seems to be mediated by regulatory T-cells. By using highly purified HESC-derived cardiomyocytes or human cardiomyocyte progenitor cells that do not change their phenotype after transplantation, it might be possible to induce peripheral tolerance and reduce the risk of tumor formation.

**Human fetal cardiomyocyte progenitor cells for cardiomyoplasty**

It would be very advantageous for regenerative medicine if it was possible to derive purified populations of pre-differentiated cardiomyocytes, since it would reduce the risk of tumor formation and it would be easier to induce immunological tolerance. From where we stand today it is not possible to use HESC-derived cardiomyocytes for cardiomyoplasty, since the derivation processes described are very inefficient and the majority of the cells are not cardiomyocytes (Xu et al. 2002; Mummery et al. 2003, 2005). According to the work of Kolossov and co-workers there seem to be an impending risk of teratoma formation if unpurified ESC-derived cardiomyocyte preparation are used for cardiomyoplasty (Kolossov et al. 2006). Another aspect is that animal products are used in the derivation process which is not acceptable for use in patients (Mummery et al. 2003, 2005).

With this in mind we decided to search for the optimal human cardiac progenitor cell. Urbanek and coworkers have identified Lin/c-Kit progenitor cells in the hearts of patients who died acutely after myocardial infarction and in explanted hearts from patients who underwent cardiac transplantation due to chronic ischemia (Urbanek et al. 2005). According to their results even the adult human heart seems to contain multipotent cardiac progenitor cells that can divide and differentiate into cardiomyocytes, smooth muscle cells and endothelial cells. The progenitor cell pool is enhanced acutely after myocardial infarction, but in case of chronic heart failure progenitor cell division is impaired. This together with increased apoptosis of the progenitor cells in the chronically ischemic myocardium probably results into a decline of functionally competent cardiac progenitor cells. This might explain why these progenitor cells cannot replace injured myocardium in case of chronic heart failure.

Cardiac stem cells have also been cultured from human myocardial specimen (Messina et al. 2004; Smith et al. 2007), but their capacity to differentiate into cardiomyocytes seemed to be limited. Another issue is that there seems to be very few c-Kit cells in the adult human heart, where the majority of these cells are of hematopoietic origin and neither of these cells stained positive for stemness markers (Pouly et al. 2007). The only true endogenous cardiac progenitor cell known to be able to specifically build up the myocardium is the Islet-1 progenitor cell (Cai et al. 2003; Laugwitz et al. 2005). These cardiac progenitor cells seem to disappear during the early postnatal period (Laugwitz et al. 2005) and therefore we focused to identify and isolate these cells from aborted fetal hearts (gestational week 5 to 10). The Islet-1 positive cells were arranged in clusters around the atria and the outflow tracts (Grinnemo et al. 2007b). In contrary, the Nkx2.5 cardiomyocyte progenitors were spread in the whole myocardium showing an intensive proliferation. These
findings support the idea (Cai et al. 2003) that during the embryogenesis of the human heart, Islet-1 positive cells migrate into the heart from the second heart field and during the differentiation process they loose their expression of Islet-1.

In culture the human fetal cardiomyocyte progenitor cells (HFCP) form spontaneously beating cardiospheres and monolayer cells (Grinnemo et al. 2007b). In the centre of the cardiospheres Islet-1 positive cells are found surrounded by cardiomyocyte progenitor cells of different maturity. The CS showed constant growth and about 10% of the cells were proliferating. The monolayer cells mainly expressed Nkx2.5 and Troponin T. This phenomenon indicates that the progenitor cells are passing through a constant maturation process from the centre of the cardiosphere, finally developing into a beating sheet of more mature cardiomyocytes. Both the cardiospheres and the monolayer cells stained negative for c-Kit and Sca-1 indicating that the HFCP are totally different from the stem cells found in the adult human heart. Furthermore, when the adult stem cells from heart specimens were cultured they formed cardiospheres and monolayers but they did not beat spontaneously (Messina et al. 2004; Smith et al. 2007). The cardiospheres also expressed c-Kit and hematopoietic markers and the cells were multipotent in their character. In co-culture with beating cardiomyocytes the cardiosphere-derived cardiomyocytes acquired biophysical features characteristic of cardiomyocytes, but they did not beat. Already in culture the HFCP formed cardiomyocytes with true sarcomeric structures, mitochondria and glycogen deposits reflecting metabolic active cells (Grinnemo et al. 2007b). In a multi-electrode array system (MEA) the autonomous beating frequency of the cardiospheres and their response to external stimuli were evaluated. The beating cardiospheres were shown to have action potentials similar to embryonic atrial-like and ventricular-like cells, with signs of a pacemaker function. Furthermore the CS exhibited the ability to adapt action potentials with increasing beating frequency, which have an important clinical implication if these cells are to be used in cardiomyoplasty.

In our cultures the majority of the cells in the cardiospheres were Nkx2.5 positive cells and the Islet-1 positive cells were only seen in the centres. We do not know to what extent the Nkx2.5 positive cells were derived from the Islet-1 positive cells or if they were from other origin. The growth of the cardiospheres might be a result of proliferation of Nkx2.5 positive cells and the Islet-1 positive cells in the centre might be quiescent. Even if this is the case, our culturing system derives pure and expanding populations of cardiomyocyte progenitor cells in contrast to the impure derivations of cardiomyocytes from HESC (Xu et al. 2002; Mummery et al. 2003, 2005).

Transplantation of beating CS into the myocardium of SCID mice formed stable engraftments, where the implanted cells continued to express Nkx2.5 and Troponin T, with no signs of apoptosis. The cardiomyocyte origin, the capacity to grow in culture make the human fetal cardiomyocyte progenitor cells putative candidates for cardiomyoplasty, but further improvements needs to be done before this cell type can be investigated clinically.
GENERAL CONCLUSIONS

- ahMSC are immunogenic in xenogeneic settings. The calcineurin inhibitor Tacrolimus does not prevent rejection of ahMSC transplanted into the myocardium of immunocompetent rats.

- ahMSC do not differentiate into cardiomyocytes and ahMSC do not improve myocardial function in an ischemic rat model.

- HESC express HLA class I, no HLA class II and low levels of costimulatory molecules. Stimulation with IFN-γ causes a 10-fold up-regulation of HLA class I, but no surface expression of HLA class II. HESC are immunologically inert and do not inhibit immune responses during direct or indirect antigen presentation and HESC were acutely rejected in a xenogeneic setting.

- Costimulation blockade is sufficiently robust to induce long-term acceptance to HESC transplanted into the testis but not the myocardium of immunocompetent mice. The peripheral tolerance seems to be mediated by regulatory T-cells.

- The Islet-1 positive cells were found in the aborted human fetal heart (gestational week 5 to 10) arranged as clusters around the atria and the outflow tracts. Furthermore we have demonstrated that it is possible to culture and expand human fetal cardiomyocyte progenitor cells from aborted fetal material and they form stable engraftments in the myocardium of SCID mice.
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