MECHANISMS BEHIND STEM CELL THERAPY IN ACUTE MYOCARDIAL INFARCTION

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ACTA UNIVERSITATIS OLUENSIS
D Medica 1378

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Academic Dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 1 of Oulu University Hospital (Kajaanintie 50), on 23 September 2016, at 12 noon

UNIVERSITY OF OULU, OULU 2016
Kujanpää, Kirsi, Mechanisms behind stem cell therapy in acute myocardial infarction.
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**Abstract**

Ischemic heart disease is one of the leading cause of death in the Western world. There is convincing evidence that stem cell therapy improves cardiac function and reduces the scar formation following an acute myocardial infarction (AMI). The mechanisms involved in the recovery remain partly unknown.

Direct injection of stem cells into myocardium is a widely used transplantation technique though there are few details available about the behavior of cells after transplantation. A cardiac explant culture model simulating tissue stress was developed in this study to examine in detail the properties of the stem cells after their transplantation. The migration range in myocardium and the number of adherent stem cells increased with time. *In vitro* and *in vivo* studies revealed that after their administration, the stem cells became localized in the slit-like spaces, such as in the capillaries.

Even though the study outcomes regarding the impact of stem cell therapy in recovery after AMI have been largely promising, the results of the clinical studies have proved to be more controversial. If one wishes to evaluate the true contribution of the stem cell therapy to the recovery, it is essential to devise a reliable study method for cell targeting. Here, iron labeled stem cells in combination with magnetic resonance imaging (MRI) were used. The MRI data corresponded to the histological results. Thus, it is concluded that MRI is a feasible method for monitoring the effectiveness of cell targeting.

Stem cell treatment was shown to increase cardiac function at three weeks after AMI. If there was a high number of stem cells in cardiac tissue after transplantation, this predicted a greater improvement in cardiac function. Improper stem cell injection may lead to leakage of the stem cells out of the myocardium, leading to un reproducible study results.

Inflammation modulating factors secreted by the stem cells are considered as key mechanisms in the recovery after AMI. There were differences in the cytokine levels between the stem cell treated and control groups in a clinical and *in vivo* animal study i.e. stem cell therapy exerted a balancing effect on the inflammatory process, a crucial component in the optimal recovery after AMI.

The present study reveals many properties of stem cells, importance of cell targeting and the influence of stem cell therapy on cytokine levels after AMI.

**Keywords:** cardiac remodeling, cell labeling, cell targeting, cytokine, magnetic resonance imaging, myocardial infarction, stem cell transplantation, stem cells
Kujanpää, Kirsi, Kantasoluhoidon vaikutusmekanismit akuutissa sydäninfarktissa.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Iskeeminen sydänsairaus on yksi yleisimmistä kuolinsyistä länsimaisissa. Tutkimusten mukaan kantasoluterapia parantaa sydämen toimintakykyä ja pienentää akuutin sydäninfarktin jälkeen sydämen muodostuvan arpiHUDoksken määrää. Paranemiseen liittyvät mekanismit ovat edelleen osittain tuntemattomia.


Kantasoluhoidon osoitettiin parantavan sydämen toimintakykyä kolme viikkoa akuutin sydäninfarktinsa jälkeen. Suuri kantasolumäärä sydänkudoksessa siirron jälkeen ennusti parempaa toipumista. Puutteellisesti suoritetun kantasoluruiskutuksen voi johtaa kantasolujen vuotamiseen pois sydänkudoksesta aiheuttaen vaihtelevuutta tutkimustuloksiin.

Kantasolujen erittäimiä tulehdusta säätäviltiä tekijöitä pidetään tärkeinä mekanismina paranemisprosesseissa. Tutkimus osoitti eroavaisuuksia kantasoluhoidetun ja kontrolleröimän välillä. Kliinisessä ja koe-eläintutkimuksessa kantasolusiirrolla todettiin tulehdusreaktiota tasapainottava vaikutus, mikä on tärkeää optimaalisen sydänlihaskudoksen paranemisen kannalta akuutin sydäninfarktinsa jälkeen.

Tutkimus toi esiin monia kantasolujen ominaisuuksia, solujen paikantamisen tärkeyden ja kantasoluhoidon vaikutuksen sytokiiniympäristöön aiheuttavien akutin sydäninfarktinsa jälkeen.

Asiasanat: kantasolu, kantasolusiirto, magnetikuvantaminen, solujen paikallistaminen, soluleimaus, sydämen uudelleen muotoutuminen, sydänlihasinfarkti, sytokiini
To my family
Acknowledgements

This work has been conducted in the Cardiothoracic Research Laboratory of the Department of Surgery, Oulu University Hospital, during the years 2007-2015.

I express my warmest thanks to my three supervisors; Professors Tatu Juvonen, Petri Lehenkari and Roberto Blanco Sequeiros, with whom I have had the honor to work. I’m deeply grateful to Professor Lehenkari who has always been there for me and answered the phone around the clock, designing the protocols and resolving many problems during this work with his innovative and positive thinking. In addition to concrete guidance and help with this project, he has become a good friend with whom I have had many encouraging, interesting and mind-opening conversations in many sections of life. I am most thankful to Professor Juvonen who recruited me to his well-established group of experimental surgeons and gave me the privilege to work with inspiring people. I wish to thank Professor Blanco Sequeiros for his positive attitude throughout this process and continuous help and support that has been crucial to this work.

I am most grateful to Docent Esko Kankuri PhD and professor Tuula Heinonen PhD for reviewing the present manuscript. Their enlightening and constructive comments greatly improved this work.

Without the concrete effort and advice of senior researchers and my co-authors Elisa Lappi-Blanco PhD, MD in pathology and Siri Lehtonen PhD in biochemistry this thesis couldn’t have been completed. You are great scientists to whom I express my warmest thanks for support, encouragement, friendship and for always making time for consultations.

My special thanks go to my senior colleagues in our experimental group Jussi Mäkelä, Hanna Jensen, Eija Rimpläinen for introducing me to the basic work in lab and science, to the amazing crew in the laboratory Tuomas Mäkelä, Fredrik Yannopoulos, Oiva Arvola, Henri Haapanen, and to my co-workers Riikka Korpi, Olli Vuolteenaho and Johanna Miettinen. Together we have had so much fun although we have also encountered difficulties. I will never forget you or your major contributions to this project.

I’d like to sincerely thank cardiologist Docent Kari Ylitalo, Docent Vesa Anttila, and Timo Väisänen PhD for their expertise and help in revising the papers. Biostatisticians Pasi Oltonen and Marianne Haapea are also recognized for their co-operation and help with the data. Seija Seljänperä, Minna Savilampi, Lissu Hukkanen, Mirja Vahera, Erja Tomperi, Riitta Vuento and Docent Hanna-Maria
Voipio deserve my deepest gratitude for their skillful assistance and technical support.

I’m very fortunate to have many good friends with whom I can spend my leisure time, have fun and share my life. I’m truly grateful for your support and trust at every turn in my life.

I’m most grateful to my family, parents Hellevi and Matti Alestalo, my brother Teemu and sister-in-law Jaana and to my parents-in-law Riitta and Lassi Kujanpää. There are not enough of words to express my love and gratitude - you have always been there for me, ready to help, listen and encourage. Your unquestioning love has raised me up when I was down and spurred me to making realities of my dreams. All of you have helped me in so many ways. In particular, I’d like to thank my brother Teemu for his technical help and advice with the figures of the present work.

Most of all, I’d like to thank my amazing husband Tero for his concrete help and support for this project. You are clever, gentle and faithful. Your love has made me who I am.

This work was financially supported by the Finnish Foundation for Cardiovascular Research, the Finnish Medical Foundation, the Maud Kuistila Foundation and Oskar Öflund’s Foundation.

Kokkola, December 2015
Abbreviations

2D two-dimensional
3D three-dimensional
α-SMA Alpha-smooth muscle actin
AMI Acute myocardial infarction
ATP Adenosine triphosphate
BMMMC Bone marrow-derived mononuclear stem cell
CABG Coronary artery bypass grafting
CHD Coronary heart disease
CX Circumflex coronary artery
CXCR C-C-X chemokine receptor
DE Delayed enhancement image
DiI Fluorescent Vybrant CM-DiI
ECG Electrocardiography
EF Ejection fraction
EGF Epidermal growth factor
ES Embryonic stem cell
FGF Fibroblast growth factor
FS Fractional shortening
FSE Fast spin echo
G-CSF Granulocyte colony stimulating factor
HGF Hepatocyte growth factor
H&E Hematoxylin and eosin stain
HSC Hematopoietic stem cell
IFNγ Interferon-γ
IL Interleukin
iNOS Inducible nitric oxide synthase
iPS Induced pluripotent stem
IV Intravenously
LAD Left anterior descending artery
LV Left ventricle
MCP-1 Monocyte chemoattractant protein-1
MMP Matrix metalloproteinase
MRI Magnetic resonance imaging
MSC Mesenchymal stem cell
PBS Phosphate-buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intention</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PTAH</td>
<td>Phosphotungstic acid hematoxylin</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell derived factor-1</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>SPIO</td>
<td>Super paramagnetic iron oxide</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computer tomography</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-elevation myocardial infarction</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I chain</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VUSPIO</td>
<td>Very small super paramagnetic iron oxides</td>
</tr>
</tbody>
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List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:


Author`s contribution

Study I

The author was responsible for the design of the study and participated in all laboratory work (anesthesia, cell isolation, culture, labeling and transplantation, cardiac tissue preparation and cardiac explant culture), measurements (histological analyses, computer assisted microscope measurements, fluorescence microscopy) and the data analysis performed. In addition, the author was the main writer of the manuscript.

Study II

The author contributed to the performance of the practical laboratory work (surgical animal work, anesthesia, cell isolation and labeling, preparation and division of the hearts into segments), measurements (histological analysis) and the data analysis performed. The author was also the main writer of the manuscript in conjunction with the first author.

Study III

The author participated in the implementation of the practical laboratory work (anesthesia, hemodynamic measurements, surgical animal work, cell isolation and transplantation, collection of blood samples, sacrifice of the experimental animals, preparation and division of the hearts into segments), measurements (histological analyses, computer assisted microscope measurements, hemodynamic measurements, spectrophotometry) and the data analysis. The author was the main writer of the manuscript along with the second author.

Study IV

The author was responsible for the design of the study and the data analysis. The author was the main writer of the manuscript along with the second author.

The contributions of the co-authors have been significant in all of the studies.
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1 Introduction

The rate of coronary heart disease mortality in Finland has been the highest in the world. Nowadays, 20 000 Finns require hospital care due to ischemic heart disease and in addition, 6 000 patients die every year at home or on the way to the hospital. The most common reason for acute myocardial infarction (AMI) is after rupturing of the atherosclerotic plaque, the released thrombus causes an occlusion of the coronary vein, resulting hypoperfusion and hypoxia in the related region of the myocardium. Permanent myocardial apoptosis and necrosis are followed by hypoxia, leading to myocardial inflammation and the triggering of the remodeling processes. The change in the myocardial architecture causes a contractile dysfunction and an impairment of cardiac function.

The established treatments for AMI are intended to achieve revascularization to reverse the injury, prevent complications, as well as improving the cardiac function and overall prognosis. The transplantation of stem cells after revascularization has been proposed as a novel treatment for AMI. The aim of the stem cell therapy is to renovate the damaged tissue. The bone marrow mononuclear cell (BMMC) has been the most commonly used cell type in non-clinical and clinical studies but skeletal myoblasts, cardiac stem cells and embryonic stem cells have also been exploited. The multipotent adult stem cells possess a differentiation potency and are known to exert specific paracrine properties through which they can mediate the healing effect at the site of injury or inflammation, such as encountered in myocardial infarction. Before clinical use in patients, there are still however, many important issues that need to be resolved.

The non-clinical in vitro models employed in stem cell research utilize organ explants, organotypic cultures and 2D or 3D dissociated cell cultures to study cell properties, behavior, labeling since this can devise novel techniques to improve stem cell therapy. Animal models are also used in non-clinical experiments; typically pigs or rats have been the experimental animals in these acute myocardial infarction models. These tests are used to measure the effect of stem cell transplantation on cardiac function and remodeling. The pig is a widely utilized large experimental animal, since pigs display many similarities in their cardiovascular system and cardiac repair processes with humans.

At present, the intramyocardial injection of stem cells is the most widely used transplantation method but the detailed information about survival and adaptation of transplanted stem cells in their new microenvironment has remained unclear. European regulations demand adherence to strict and accurate rules and there are
requirements to provide detailed information about the cell viability and potency of cell based medicinal products. Therefore, the mechanism of action of stem cells to repair infarcted heart tissue has to be clarified before this approach can be applied as a safe clinical treatment for AMI. However, it is challenging to monitor the detailed mechanisms of stem cells after their transplantation. The availability of reliable \textit{in vitro} models would be beneficial for conducting detailed mechanistic studies and to study localization of cells in the myocardium but at present, there is a paucity of these kinds of \textit{in vitro} models.

The non-clinical study results from stem cell therapy in the treatment of AMI have been largely promising and recorded improvements in cardiac function and reductions in the extent of scar formation. However, there are large variations in scar recovery and the results of non-clinical studies have been partly conflicting. One reason for these large variations might be the poor targeting of stem cells into the infarction area. Therefore, it would be advantageous to develop a reliable method for cell targeting. This could be applied to ensure homing of the transplanted stem cells to the infarction area and thus enabling proper recovery. Iron labeled stem cells in combination with the magnetic resonance image (MRI) have been used in non-clinical studies in cell targeting but the reliability of the method has been criticized. Thus, the feasibility of using an MRI-based approach as a method for cell targeting requires further studies.

Although the stem cell therapy improves healing after AMI, the mechanism leading to recovery has remained a mystery. The positive effect of transplanted cells has been postulated not only to be a consequence of stem cell proliferation and differentiation into functional cells but also to a great extent due to paracrine effects. The cytokines and growth factors secreted by the stem cells may modulate the inflammation reaction provoked by the injury, and these modulatory effects are important in inhibiting scar formation and improve cardiac function. However, the cytokine network is complex and it is challenging to unravel the overlapping and complicated cytokine network. It is especially important to clarify which cytokines are involved in myocardial inflammation and to determine how they influence the recovery process. In addition, the administration of stem cells may attenuate the post-infarction remodeling process via cytokines and trophic factors. However, more detailed information is needed about the individual cytokines and changes in the cytokine network after stem cell transplantation.

The goal of this thesis was to examine the use of bone marrow mononuclear cells and mesenchymal stem cells as a treatment for AMI. The survival and behavior of stem cells after direct injection were widely explored using both \textit{in vitro}
and *in vivo* experimental models. Moreover, the reliability of MRI as a method for cell targeting was evaluated, revealing the crucial nature of cell targeting. Interestingly, the changes in the cytokine levels after AMI with or without bone marrow mononuclear cell transplantation were examined as well as conducting an exploration of the effects on cardiac function. Basically, the focus of this thesis was on resolving current issues in the field of stem cell treatment such as attempting to develop improved stem cell therapy protocols as well as clarifying the mechanisms through which stem cell therapy can lead to recovery.
2  Review of the literature

2.1  The human heart

The human heart is an indefatigable muscle that pumps blood to the tissues every second of an individual’s lifetime. The heart consists of four cavities: two on the left side and two on the right side. The sino-atrial node, which is situated in the right atrial wall, is responsible for the initial contraction impulse and coordination of the contraction of the heart. Blood flows from the right atrium into the right ventricle from where it continues to the lungs. From the left atrium, blood flows to the left ventricle (LV) and this compartment pumps blood to the whole body. The blood supply to the heart is arranged by the right coronary artery and the left coronary artery that further separates into the left anterior descending artery (LAD) and the circumflex coronary artery (CX). When viewed in a microscope, one can observe the three basic layers of the heart wall; the endocardium, which is a thin layer in a ventricle, the myocardium and the visceral pericardium i.e. the epicardium. Individual cardiac muscle cells have a central nucleus and they are linked to each other by intercalated disks, forming an interconnecting network. The presence of gap junctions in the intercalated disks between the connected cardiac muscle cells allows free diffusion of ions and the rapid spread of the action potential from cell to cell.

2.2  Myocardial infarction

Ischemic heart disease is the leading cause of death in the developed countries and it carries significant morbidity (Lloyd-Jones et al. 2010, Rashid et al. 2015). Plaque rupture or inadequate blood flow are the basic reasons for myocardial infarction. The heart has only a limited capacity for self-renewal after AMI. Consequently, immediate reperfusion is the only way to rescue myocardium in a situation where there is the loss of more and more myocardial tissue with every passing minute (Reddy et al. 2015). There are invasive and non-invasive interventions for AMI treatment. Thrombolytic agents, such as tissue plasminogen activators or streptokinase, have been used as a treatment for AMI with ST-segment elevation. Percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG) are two alternative strategies to achieve revascularization. Placing a coronary stent during PCI improves the safety and durability of PCI. Nonetheless,
in-stent restenosis and thrombosis are risks associated with stenting. There are several studies that have compared the benefits of CABG to PCI, many of them favoring CABG due to its lower mortality rate and risk of re-intervention (Bundhun et al. 2016, Carey et al. 2009). PCI is cost-effective in the short term but in the long term, the benefits of CABG may exceed those of stenting (Birim et al. 2012, Bundhun et al. 2016). The established treatments decrease the mortality rate in AMI but they lack any effect on the remodeling process that contributes to scar formation and impaired cardiac function.

Nonetheless, it has been speculated that stem cell therapy may have many advantages over the established treatments. Briefly, the concept of clinical stem cell therapy is to transplant stem cells into the infarcted myocardium after thrombolysis and to achieve revascularization in patients after an AMI. There are major variations in the protocols that have been applied in stem cell therapy in the pioneering clinical studies. Basically, stem cell transplantation can be conducted during or after revascularization of AMI and by using either an intracoronary or an intramyocardial transplantation technique (Schachinger et al. 2009, Yerebakan C et al. 2011). It has been postulated that one way to increase the efficacy of stem cell therapy would be to improve cell retention and survival and thus enhance the engraftment of transplanted cells (Li et al. 2016).

### 2.2.1 Clinical diagnosis

The clinical diagnosis of acute myocardial infarction is always a sum of several diagnostic criteria. The clinical diagnosis is based on the typical symptoms, electrocardiography (ECG) signs and on the presence of biochemical biomarkers such as the release of troponin (Reddy et al. 2015). Usually, pain in the chest that may radiate to the neck, jaw or left arm is a typical symptom of an acute myocardial infarction. There may also be nausea, dizziness, sweating, fainting and trouble with breathing. Women and patients with diabetes mellitus may exhibit different signs and symptoms that challenge the diagnostics. Within the first few hours, there is a change in the ECG ST-segment/W-wave or left bundle branch block. The appearance of the Q wave may represent a sign of an old myocardial infarction. Myocardial necrosis causes an increase in the plasma concentration of troponin I at between 4 to 48 hours after infarction and creatine kinase from 6 to 12 hours after the onset. Also new loss of viable myocardium or regional wall motion abnormality identified by an imaging procedure or identification of intracoronary thrombus by angiography or autopsy way help to definition of AMI. There are also some well
known risk factors such as hypercholesterolemia, diabetes mellitus, smoking, age, heredity and sex that have to be kept in mind when assessing AMI.

2.2.2 Pathogenesis and pathophysiology of myocardial infarction

In order to produce the contraction that pumps blood throughout the body, the heart consumes much aerobic energy. Inadequate blood flow leads to a lack of oxygen, ischemic conditions and the death of cardiomyocytes. The most common reason for myocardial infarction is a rupture of an atherosclerotic plaque and platelet aggregation leading to coronary artery occlusion and an insufficient blood supply (Boudoulas & Hatzopoulos 2009, Bundhun et al. 2016). Hypoperfusion of the heart also occurs in disorders such as aortic stenosis or hemorrhagic shock or hypoperfusion during the course of the cardiopulmonary bypass procedure can lead to myocardial infarction. Severe ischemia results in the death of cardiomyocytes within several minutes (Krijnen et al. 2002). Toxic agents and reactive oxygen species flood into the site of tissue injury and induce cell death (Boudoulas & Hatzopoulos 2009, Bundhun et al. 2016)(Fig.1). The surviving myocytes become elongated and undergo hypertrophy. This is a compensatory process to maintain stroke volume after the loss of contractile tissue (Cohn et al. 2000).

Death of myocytes

The myocytes are dependent on aerobic energy production which is provided by adenosine triphosphate (ATP). An insufficient blood supply is rapidly fatal to the cardiomyocytes and depletion of ATP leads to a failure of the sodium pump, an influx of sodium, loss of potassium and cell swelling (Seropian IM et al. 2014). Necrosis is commonly regarded as the cause of cell death in myocardial infarction but there are some studies indicating that apoptosis also plays a role in this process (Hort 2001, Krijnen et al. 2002). It is believed that the severity and duration of ATP depletion determines the mechanism of cell death. Should the ATP concentration decline below a critical threshold, then there is the triggering of necrosis whereas an ATP value above that value favours apoptosis (Lieberthal et al. 1998, Shiraishi et al. 2001). This proposal is supported by the fact that apoptosis is a programmed cell death requiring energy and it is subjected to stringent regulation. Consequently, the absence of intracellular ATP may prevent the occurrence of apoptosis. In contrast, necrosis is a rapid loss of cellular homeostasis and it evokes rapid cellular swelling and disruption of cellular organelles (Krijnen et al. 2002). In clinical
studies, apoptosis has been detected not only in the border zone of the ischemic region but also in areas remote from the ischemic region (Piro et al. 2000, Saraste et al. 1997).

Reperfusion injury

Reperfusion is the only way to rescue the ischemic heart by achieving a restoration of blood flow and oxygen supply in order to restrict the infarction size and improve the clinical outcome (Cannon et al. 2000, Hausenloy & Yellon 2016). However, reperfusion can even increase the extent of myocardial cell dysfunction and cell necrosis. During the acute phase, neutrophils become activated and contribute to the acute reperfusion injury (Wu et al. 2011).
Fig. 1. Pathology of acute myocardial infarction. Occlusion of the circumflex artery (CX) leads to hypoxia induced death of the myocytes. Inflammatory cells appear in the area of injury and launch the myocardial remodeling process that leads to fibrosis and the formation of a scar. The lack of contractility of the injured myocytes decreases ejection fraction (EF) and impairs the cardiac function.
2.2.3 Pathology of myocardial infarction

The first signs of myocardial infarction are pyknotic cells and hypereosinophilic changes in the sarcoplasm of the myocytes that can be detected with the hematoxylin and eosin (H&E) stain and microscopy already after 6 hours of onset (Hort 2001, Pasotti et al. 2006). Within hours, swollen cardiomyocytes and mitochondria appear. Sarcolemma disruption leads to the release of many intracellular proteins such as creatine kinase into the extracellular space. Coagulative necrosis appears as a loss of the cell nuclei after 12 hours and neutrophilic infiltration, hemorrhagia and interstitial edema emerge within 1-3 days (Francis Stuart et al. 2016) (Fig. 1). Subsequently, myocyte striation becomes less prominent and myocyte fragmentation occurs. The leukocyte response abates and only a few leukocytes remain in the damaged tissue. The repair process begins at the peripheral area of the infarct and extends toward the center. Lymphocytes and macrophages appear and start to undertake phagocytosis in order to clear away the cellular debris and matrix degradation products from the border zone at between 3-7 days after AMI. After one week, the injury site starts to fill with granulation tissue that is composed of enlarged capillaries, macrophages and myofibroblasts. This granulation tissue evolves through loose and progressively dense collagen deposition during the subsequent 3-8 weeks. Collagen and other extracellular matrix proteins create a dense scar that has a decreased contraction potential. Consequently, myocardial ischemia leads to a decreased ejection fraction (EF) and dysfunction of the heart (Zamilpa et al. 2014). At that point, the inflammatory infiltrate gradually recedes and neovascularization becomes evident. The acellular and collagen dense scar is completed by the second month (Hort 2001, Pasotti et al. 2006).

2.2.4 Remodeling process

The myocardial remodeling initially referred to the changes in the LV after myocardial injury. Nowadays, myocardial remodeling describes a variety of changes in biophysiology and the composition of cardiomyocytes and non-cardiomyocytes as well as changes to the architecture of the LV. Remodeling has its clinical manifestation in the changes in the shape, size and function of the heart after the infarct injury (Cohn et al. 2000, Francis Stuart et al. 2016). Remodeling is a necessary response to a cardiac injury in order to rearrange the tissue of the ventricle. An ischemic condition induces the release of pro-inflammatory cytokines
that trigger an acute inflammation response. In basic terms, the remodelling process consists of cellular apoptosis, collagen degradation, fibroblast proliferation and changes in myocytes, such as hypertrophy or elongated shape (Fig.1). This leads to a dilatation of LV, an increase of end-systolic volume index and a decrease in EF. The shape of the heart becomes less elliptical and more spherical.

**Inflammatory response**

The inflammation after a cardiac injury is closely involved in the remodeling process and it has been widely studied (Frangogiannis 2014, Seropian IM et al. 2014). Several inflammatory mediators activate the innate immune system and attract the inflammatory cells to the injury site. Myocardial necrosis results in the release of components of the complement cascade.

During necrosis or apoptosis of myocytes, the stressed myocardial cells release cytokines and several kinds of damage-associated molecular patterns that activate the immune system and complement cascade.

Mononuclear cells present in the blood become attracted to the infarcted myocardium by several chemoattractants (Frangogiannis 2014). Mononuclear cells mature and differentiate into mature macrophages in the infarction site where they have multiple roles in the healing process. Macrophages release cytokines and growth factors regulating fibroblast growth and angiogenesis and they contribute to the remodeling of the extracellular matrix by producing matrix metalloproteinases (MMPs) and their inhibitors. The macrophages are also responsible for the phagocytosis of dead cells and debris as well as the clearance of apoptotic neutrophils and cardiomyocytes. The macrophages secrete high levels of many pro-inflammatory cytokines and chemokines, such as interleukin-1 (IL), IL-6, IL-8, NO and tumor necrosis factor-α (TNFα). They create a pro-inflammatory environment and attract neutrophils to the damaged area.

Complement fragment C5a, reactive oxygen species, Toll-like receptor pathways and pro-inflammatory cytokines such as TNF-α, -IL-6 and IL-1 activate neutrophils and attract these cells to the infarction area in the acute phase of the reperfusion injury (Frangogiannis 2008, Seropian IM et al. 2014, Wu et al. 2011). Neutrophils become located into the post-capillary venules and upregulate the adhesion molecules and interact with endothelial cells (Arslan et al. 2008). They release oxidants that harm the myocytes while undertaking the clearance of dead cells and debris (Duilio et al. 2001, Frangogiannis 2008, Frangogiannis 2014). Platelets are other cells that play versatile roles in wound healing in several ways,
e.g. by aggregating in areas of injury, by contributing to the formation of a fibrin-based provisional matrix and by releasing a variety of chemokines, cytokines and growth factors (Gawaz et al. 2005). The role of mast cells in the inflammatory response to myocardial infarction is rather speculative. These cells are located close to the vessels and they release several inflammatory and pro-fibrotic mediators. Mast cells seem to participate in the proliferative phase of healing since they accumulate in areas of collagen deposition and cell proliferation (Frangogiannis et al. 1998). In addition, myofibroblasts as well as vascular cells and pericytes have been proposed to be a part of the inflammatory process; they are involved in the altering the collagen matrix and/or in angiogenesis (Howard & Baudino 2014, Yong et al. 2015).

Cytokines

In AMI, an inadequate blood flow and hypoxia induce the activation of a cytokine cascade. This is a complex process that is not fully understood, but it is known that several cytokines regulate each other’s production. Consequently, cytokines have a crucial role in regulating the remodeling process, contributing to the scar formation, cardiac recovery and function after AMI. Since the cytokines participate in a complex network, they should not be studied individually but instead investigated as a unity.

Tissue stress and myocardial ischemia induce the upregulation of several pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Moro et al. 2007, Shrivastava et al. 2015). These pro-inflammatory cytokines upregulate the release of other cytokines that participate in the inflammatory process. In particular, the levels of TNF-α, IL-6 and the anti-inflammatory cytokine IL-10 become elevated during the early phase, even within a few hours of AMI according to both clinical or non-clinical studies (Frangogiannis et al. 2000, Moro et al. 2007, Seropian IM et al. 2014). The property of TNF-α, and IL-6 to decrease the contractility of the myocytes that may be a compensatory mechanism to reduce the demand for energy (Francis Stuart et al. 2016, Nian et al. 2004). TNF-α also activates MMPs that lead to matrix degradation. However, knock-out of TNF receptors resulted in a larger infarct size and more apoptosis of cardiomyocytes. Consequently, it is thought that the exact effect of TNFα depends not only on its concentration but also on the TNF receptor to which it binds (Kleinbongard et al. 2011, Kurrelmeyer et al. 2000, Francis Stuart et al. 2016). There are several anti-inflammatory cytokines that are able to suppress the intensity of the inflammation. IL-10 is an anti-inflammatory
cytokine that inhibits apoptosis and MMP9 activation and as a consequence, it reduces the size of the infarction area and it attenuates fibrosis (Frangogiannis 2014, Krishnamurthy et al. 2009). IL-10 also upregulates the production of vascular endothelial growth factor (VEGF) that increases angiogenesis in conjunction with platelet-derived growth factor (PDGF). Interestingly, patients with advanced chronic heart failure exhibit a low IL-10 concentration and a low IL-10:TNF-α ratio in blood (Stumpf et al. 2003). A novel therapeutic option has emerged i.e. reducing AMI related inflammation by administering an anti-cytokine therapy. However the results of large clinical studies have been somewhat disappointing with either neutral or negative findings (Chung et al. 2003, Mann et al. 2004). In this regard, further research is needed to explore the role of inflammation pathways and to clarify the complex cytokine network.

Alterations to the cardiac matrix

Pro-inflammatory mediators and leukocyte infiltration have a crucial role in the phagocytic removal of dead cells and matrix debris after myocardial injury. (Dewald et al. 2004, Frangogiannis 2014). The clearance of the granulocytic infiltrate and apoptotic cells by macrophages is necessary for the resolution of the post-infarction inflammatory response.

Fibroblasts are responsible for collagen production during the formation of granulation tissue. The debate over the origin of fibroblasts has a long history and numerous studies have been performed (Krenning et al. 2010, Yong et al. 2015). Fibroblasts may be differentiated leukocytes from circulation or fibroblasts from either the site of origin or recruited from blood (Camelliti et al. 2005, Ogawa et al. 2006). Nonetheless, even today, there is no consensus regarding the origin of the fibroblasts. However, it is recognized that fibroblasts infiltrate the infarction area and since they express α-SMA, this is considered as a sign of differentiation into myofibroblasts. However, again the modulation of fibroblast differentiation into myofibroblasts is poorly understood. Differentiated myofibroblasts become localized in the border zone and exhibit intense proliferative activity (Virag & Murry 2003). During the normal condition of the heart, fibroblast cells maintain the extracellular matrix network but they are capable of secreting chemokines and cytokines if stimulated by inflammatory mediators (Lafontant et al. 2006). During the proliferative phase of myocardial healing, the appearance of a signal such as fibroblast growth factor-2 (FGF-2) and angiotensin II may induce fibroblast

2.3 Stem cells

The stem cell is a unique cell type in the body. These are undifferentiated cells that have the ability to self-renew, to clone and differentiate into multiple tissues (Paxinos & Katritsis 2008). In general, stem cells are divided to adult stem cells and embryonic stem cells, according to their origin (Fig.2).

Embryonic stem cells are immortal, pluripotent cells derived from the inner cell mass of the pre-implantation blastocyst (Psaltis et al. 2014). Since it is a pluripotent cell, the embryonic stem cell can differentiate into any cell type in the adult body.

Adult stem cells (progenitor cells) are subdivided into hematopoietic stem cells (HSCs) and stromal cells, such as BMMCs (Nunez Garcia et al. 2015). Stromal cells consist of several cell types, such as mesenchymal stem cells, skeletal myoblasts, endothelial stem cells and cardiac stem cells which are present in adult tissues.

Pluripotent stem cells can also be created from the adult cells. Induced pluripotent stem (iPS) cells are derived from somatic cell reprogramming, such as fibroblasts, via overexpression of exogenous transcription factors. However, the protocols to produce cardiomyocytes from iPS cells are still under development. There is a clear need for improvements in the differentiation protocols since there are huge variations between the current techniques (Mummery et al. 2012). The two major approaches for achieving the differentiation of human pluripotent stem cells into cardiomyocytes are monolayer culture and inductive co-culture. Co-culture is simple, rapid and requires very few cells.
2.3.1 Stem cells in the body

Tissues undergo constant renewal; in this renewal process, the old or damaged cells are replaced by new healthy cells. In some tissues, such as skin and intestine, the recycling activity is rather fast whereas some other tissues, such as myocardium have a relatively limited renewal ability. Stem cells have a crucial role in cell renewal after tissue injury, illness or ageing since they produce the cell types that exist in their anatomical surroundings and thus they can develop into different cellular types and regenerate damaged tissue. In adults, stem cells are present in every tissue but their number is relatively small. Adult stem cells are multipotent cells with a limited capability to differentiate into only a few cell lineages.

The stem cell niche is the microenvironment where stem cells reside. The stem cell niche provides spatial and temporal cues to support and coordinate stem cell
activity. The niche protects stem cells from outside damage and ensures their responsiveness to a physiological need for tissue repair and replacement of damaged cells. It is thought that self-renewal occurs in all tissues, since stem cell niches have been identified in bone marrow, heart, arteries, gonads, veins, neural tissue, intestine and epidermal tissue.

2.3.2 Historical aspects of stem cells

Already in 1970, Friedenstein and colleagues demonstrated that bone marrow contains a population of HSCs and a rare population of plastic-adherent stromal cells (Friedenstein et al. 1970). Plastic adherent cells were initially referred to as stromal cells but are now commonly called mesenchymal stem cells (MSCs). Friedenstein (1974) demonstrated the ability of these MSCs to differentiate into mesoderm-derived tissue and determined their importance in controlling the niche. During 1980s, the ability of MSCs to differentiate into osteoblasts, chondrocytes and adipocytes was discovered (Piersma et al. 1985). During the early 21st century, in vivo studies demonstrated the trans-differentiation of MSCs into endoderm-derived cells and cardiomyocytes (Toma et al. 2002). In vitro co-culturing of ventricular myocytes with MSCs has induced the trans-differentiation into a cardiomyocyte phenotype (Xu et al. 2004).

2.3.3 Mesenchymal stem cells

Mesenchymal stem cells are self-renewing, plastic-adherent, multipotent and fibroblast-like cells. They possess the ability to differentiate into adipocytes, osteoblasts and chondroblasts. Consequently, MSCs have potential applications in epithelium, central nervous system, bone and cartilage repair as well as in heart and vessel diseases.

MSCs have been found to be useful in research due to their rapid proliferation in culture, easy access and non-immunogenic features. Safety issues inherent in the long-term culture of MSCs have been raised. However, MSCs cultured for a normal duration and reaching <25 population doublings have never been observed to undergo a malignant transformation (Bernardo et al. 2007). Given that long-term effects of MSC delivery into the heart have not been studied to any significant extent, it is worthwhile noting that clinical studies have been underway since 1995 and no significant adverse events have been reported.
**Definition of an MSC**

The International Society of Cellular Therapy defines MSCs by three main characteristics: I) adhesion to plastic II) expression of a specific set of membrane molecules (CD73, CD90, CD105) together with a lack of expression of the hematopoietic markers CD11b, CD14, CD 19, CD, 79, CD34 and CD45 and human leucocyte antigen-DR (HLA-DR) and III) they have the ability to differentiate within three main pathways: osteoblastic, chondrogenic and adipocytic (Kim & Cho 2015).

In addition to hematopoietic stem cells, bone marrow houses also a niche or stroma that is responsible for the maintenance of hematopoietic stem cells. The niche is formed by the stromal cells that are uncharacterised cells, such as reticular cells, fibroblasts, osteoprogenitors, which are the primary cellular components of the marrow stroma. Bone marrow was the first source of MSCs (Sousa et al. 2014) but subsequently, MSCs have been successfully isolated from other sources. Because there is no single-cell marker that specifically defines an MSC, this term refers to a highly heterogeneous subset of stromal stem cells (Neofytou et al. 2015).

There are some exceptions to the definition; these tend to depend on the tissue of origin. For example, adipose-tissue-derived MSCs express the membrane antigens CD34 and CD54 that should not be expressed according to the definition (De Ugarte et al. 2003, Konala et al. 2016). In addition to marker expression and behaviour, the murine MSCs differ from their human counterparts (Peister et al. 2004). Interestingly, MSCs isolated from various adult tissue sources display significantly different morphologies, differentiation capabilities and gene expression profiles.

**Isolation of MSC**

MSCs have been isolated from bone marrow adipose tissue, synovial tissue, lung, liver, synovium, amniotic fluid, dental pulp, skeletal muscle, umbilical cord blood and peripheral blood (Kobolak et al. 2015). The phenotypic potential of MSCs is wider than anticipated since they have the capacity to differentiate towards all three lineages as ecto-, meso- and endodermal tissues. MSCs have been isolated from numerous species. In adult mice, MSCs have been isolated from nearly every tissue type (brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, aorta, vena cava, pancreas) suggesting that MSCs may reside in all postnatal organs (da Silva Meirelles et al. 2006). Most of the studies evaluating MSC therapy for cardiac
repair have been performed using bone marrow-derived MSCs. Pigs, rats and mice models have been used to conduct experimental AMI. In a non-clinical study comparing bone marrow and adipose derived MSC transplantation after AMI, bone marrow derived MSCs seemed to exert a better preservation of LV contraction ability and lead to a smaller infarction area (Karpov et al. 2013). Bone marrow is a major source of HSCs; this represents a continually renewing source of red blood cells, platelets, monocytes and granulocytes. Since under physiological conditions MSCs do not migrate readily in the peripheral blood and available protocols are not very successful in inducing the translocation of MSC from bone marrow to the periphery, one needs to undertake isolation and culture expansions for therapeutic purposes. There are 3 critical steps to be undertaken when MSCs are isolated from the bone marrow. First, there is the separation of nucleated cells from non-nucleated red blood cells using density gradient centrifugation (Ficoll or Percoll). Secondly, the ability of MSCs to adhere to plastic, and finally one has to separate monocytes from MSCs by trypsinization.

Mechanism of action

After a myocardial infarction, the stromal cell derived factor-1/CXCR4 (SDF-1/CXCR4) signaling pathway directs the homing of stem cells to the infarct area or to the border zone (Reddy et al. 2015). The reparative effect of MSCs after an AMI encompasses an attenuation of the infarct size, a normalization of myocardial metabolo-energetic, a diminution of cardiomyocyte apoptosis as well as improvements in the indices of LV contractile and diastolic function (Psaltis et al. 2014).

In in vivo studies, human MSCs have demonstrated that they possess the ability to differentiate into cardiomyocytes in adult mice hearts (Zamilpa et al. 2014). Rat and human bone marrow-derived MSCs are able to differentiate into multinucleated myotubes, consistent with a myocyte lineage by exposing them to a special culturing protocol, (Wakitani et al. 1995, Xu et al. 2004). They express β-myosin heavy chain, desmin and α-cardiac actin. In addition, co-culture of MSCs with cardiac myocytes has shown that MSCs can differentiate into cells with a cardiac phenotype (Li et al. 2007). MSCs exhibited synchronous contractions with native myocytes in addition to α-actin positivity and gap junction formation when co-cultured with rat ventricular myocytes. Several molecules have been shown to be involved in regulating MSC differentiation such as epidermal growth factor (EGF), PDGF and fibroblast growth factor (FGF). Many of these growth factors
are thought to interact with the Wnt and TGF-β pathways in the regulation of MSC differentiation (Augello & De Bari 2010).

Due to their multi-differentiation potential and participation into wound healing and neovascularization processes, MSCs have been widely studied. In addition to their differentiation potential, MSCs exert an immunosuppressive effect in vitro and in vivo (Fig. 3). The innate immune cells are responsible for the nonspecific defence to infection (Kim & Cho 2015, van den Akker et al. 2013). MSCs seem to suppress most of these inflammatory cells. Neutrophils play a major role in the early immune response. Neutrophils develop in the bone marrow until they are activated by inflammatory chemotactic signals, such as IL-1 and IL-8 which induce their release from bone marrow and homing to the injured area. The triggering of a respiratory burst is one way by which the neutrophils try to combat an infection during the acute phase (Zamilpa et al. 2014). MSCs dampen the respiratory burst process by releasing IL-6. Furthermore, MSCs prolong the lifespan of the neutrophils and modulate their tissue migration.

In addition to neutrophils, macrophages are another cell type that play a major role in cardiac inflammation (Zamilpa et al. 2014). During the acute phase, M1 macrophages secrete many cytokines, such as IL-1β, IL-6, TNFα and interferon-γ (IFNγ) that create a strong pro-inflammatory environment, resulting in chemoattraction of more immune cells and additional damage to the myocardium. The switch to the M2 macrophages changes the cytokine palate and healing is promoted. Interestingly, co-culture of macrophages with MSCs triggers the macrophages to move towards the anti-inflammatory M2 phenotype. Apparently, the MSC-derived cytokine, IL-10, is one of the mechanisms involved in this process. Moreover, MSCs reduce the secretion of several pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα and IFN-γ by macrophages while anti-inflammatory cytokine secretion especially that of IL-10, is increased. T and B lymphocytes are components of the adaptive immune system. MSCs inhibit T and B cell proliferation through several stimuli. MSCs also modulate B cell migration, differentiation into plasma cells and thus immunoglobulin production (Sensebe et al. 2010, Williams & Hare 2011, Kim & Cho 2015).
Fig. 3. Immunomodulation by MSC. MSCs modulate the inflammation process at multiple levels by modulating the functions of the inflammatory cells, such as B and T-cells, macrophages and neutrophils.

In addition, MSCs exert trophic effects mediated by the growth factors and cytokines that they produce. MSCs secrete cytokines and growth factors that suppress the immune system, inhibit fibrosis and apoptosis, enhance angiogenesis and stimulate the differentiation of tissue-specific stem cells (Table 1) (Caplan & Dennis 2006, Kinnaird et al. 2004, Rowart et al. 2015, Zamilpa et al. 2014). The possible paracrine effect of MSCs has been demonstrated in an experimental infarction model where myocyte hypertrophy and apoptosis were attenuated in MSC treated animals (Zhao et al. 2012).
Table 1. Influence of the paracrine factors secreted by MSCs.

<table>
<thead>
<tr>
<th>Target</th>
<th>Secreted Factor</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>Fibroblast growth factor-7 (FGF-7)</td>
<td>Induces endothelial cell proliferation</td>
<td>Haynesworth et al. 1996</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Monocyte chemokine protein-1 (MCP-1)</td>
<td>Induces angiogenesis; recruit monocytes</td>
<td>Wen et al. 2012</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>Induces endothelial cell proliferation, migration, tube formation</td>
<td>Haynesworth et al. 1996, Wen et al. 2012</td>
</tr>
<tr>
<td>Immunomodulatory</td>
<td>Interleukin-6 (IL-6)</td>
<td>Regulates inflammation, VEGF induction</td>
<td>Haynesworth et al. 1996, Wen et al. 2012</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Prostaglandin E2 (PGE2)</td>
<td>Inhibits inflammation</td>
<td>Liang et al. 2014, Spaggiari et al. 2008</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Inducible nitric oxide synthase (iNOS)</td>
<td>Inhibits inflammation</td>
<td>Sato et al. 2007</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Hepatocyte growth factor (HGF)</td>
<td>Inhibits CD4+ T cell proliferation</td>
<td>Gneccchi et al. 2006, Liang et al. 2014</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Heme Oxygenase-1 (HO1)</td>
<td>Inhibits T-cell proliferation</td>
<td>Chabannes et al. 2007</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Interleukin-10 (IL-10)</td>
<td>Inhibits Th-cell differentiation</td>
<td>Qu et al. 2012</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Basic fibroblast growth factor (bFGF)</td>
<td>Enhances proliferation of endothelial and smooth muscle cells</td>
<td>Kinnaird et al. 2004, Liang et al. 2014</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Granulocyte colony stimulating factor (G-CSF)</td>
<td>Increases proliferation and differentiation of neutrophils</td>
<td>Haynesworth et al. 1996</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Thymosin-β4 (Tβ4)</td>
<td>Promotes cell migration</td>
<td>Gnecchi et al. 2006, Wen et al. 2012</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Secreted frizzle-related protein-1</td>
<td>Enhances cell development</td>
<td>Dufourcq et al. 2008, Liang et al. 2014</td>
</tr>
</tbody>
</table>
### Table

<table>
<thead>
<tr>
<th>Target</th>
<th>Secreted Factor</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Secreted frizzle-related protein-2</td>
<td>Inhibits apoptosis; enhances cell development</td>
<td>Mirotsou <em>et al.</em> 2007, Liang <em>et al.</em> 2014</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Macrophage colony stimulating factor (M-CSF)</td>
<td>Increases proliferation and differentiation of monocytes</td>
<td>Haynesworth <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Cell proliferation, recruitment and survival</td>
<td>Insulin-like growth factor-1 (IGF-1)</td>
<td>Regulates cell growth and proliferation; inhibits apoptosis</td>
<td>Gnechi <em>et al.</em> 2006, Liang <em>et al.</em> 2014</td>
</tr>
<tr>
<td>Remodeling</td>
<td>Tumor necrosis factor-α (TNF-α)</td>
<td>Degrades matrix molecules; cell proliferation</td>
<td>Kinnaird <em>et al.</em> 2004, Liang <em>et al.</em> 2014</td>
</tr>
<tr>
<td>Remodeling</td>
<td>Plasminogen activator (PA)</td>
<td>Degrades matrix molecules</td>
<td>Kinnaird <em>et al.</em> 2004b</td>
</tr>
</tbody>
</table>

#### 2.4 Stem cell therapy in myocardial infarction

The ultimate aim of stem cell therapy is to achieve a renewal of damaged tissue. Stem cell therapy aims to replace cells damaged by disease or lost due to injury. This approach has been explored in the treatment of Parkinson’s disease and diabetes. It has been proposed that stem cell therapy would represent a promising way to treat myocardial infarction where damaged cardiomyocytes need to be replaced.

The current treatments for AMI are aimed at establishing revascularization (Reddy *et al.* 2015). Stem cell transplantation has been proposed as novel treatment for AMI to be applied in combination with revascularization. Stem cell therapy strives to replace the apoptotic myocytes via stem cell differentiation and regeneration (Zamilpa *et al.* 2014). Since stem cells have also the ability to modulate inflammation and regulate the formation of fibrosis and angiogenesis after myocardial infarction, these properties represent new approaches to tackling the structural remodeling of the myocardium. Nonetheless, therapeutic strategies
using stem cells are still in their infancy and will need more studies and improvements in protocols before they become a routine procedure. The optimal means of delivery and cell type to be administered as well as cell tracking are topics of intensive investigation. In addition, there are open questions about the cell survival after transplantation and the behavior of the transplanted cells in the tissue.

Stem cell therapy can be divided into 3 different approaches: stem cell transplantation, stem cell mobilization and regulation by growth factors and cytokines (Srinivas et al. 2009). In non-clinical and clinical studies, the concept has been to mobilize stem cells from the stem cell niche i.e. from bone marrow, to increase the number of stem cells in the blood flow and thus to achieve cell homing to the injured area. Stem cell transplantation is a more straightforward method in which harvested stem cells are delivered to the area of injury. It is recognized that several growth factors and cytokines regulate the actions of stem cells and make a significant contribution to the final outcome. Consequently, the regulation of growth factors and cytokines is a novel approach being examined in stem cell therapy.

The biology involved in cardiovascular regeneration is complex and incompletely understood which unfortunately limits our ability to develop effective therapeutic approaches to restore cardiac function to injured tissue. Model systems are crucial in clarifying the biological mechanisms of cardiac regeneration and in devising novel therapeutic ways to repair and restore human cardiovascular function after an AMI. It would be highly advantageous if an easily reproducible and manipulated, inexpensive model system were available. An ideal model should also be ethically acceptable and recapitulate human disease pathophysiology. Both in vivo and in vitro models have been used to study stem cells and their therapeutic effects. Computational and in vitro models are ethical, inexpensive and easy to reproduce and manipulate; the advantages of in vivo animal models, especially in large animals, are that they have more similarities to human physiology. In vivo studies can provide clinical information about the use of stem cells whereas in vitro models concentrate more on details and attempt to find novel ways to improve stem cell therapy protocols. The benefit of in vitro models is the high level of control over the experimental parameters in a biological system. In vivo models describe better the complex behavior of the heart within a living organism. Experimental studies are necessary for further development of stem cell therapy since many factors involved in the regeneration process are poorly understood. The methods applied and the results emerging from in vitro, in vivo and clinical studies will be discussed in detail in chapters 2.4.7 – 2.4.9.
2.4.1 Results of stem cell therapy

While the results originating from stem cell transplantation after AMI have been extremely intriguing and promising, they have also tended to be controversial. When evaluating the therapeutic effect of stem cell transplantation after AMI, the functional recovery of the heart is the most important clinical aspect. Many non-clinical studies have attempted to reveal the effects leading to increased functional recovery by observing the differentiation of the transplanted stem cells and their impact on the inflammatory process and the remodeling of the myocardium.

As stated, AMI induces the death of myocytes and causes scar formation that reduces cardiac function. In vivo studies have revealed the beneficial effects of stem cell therapy after AMI. In several studies, transplantation of either BMMCs or MSCs after AMI has been reported to improve EF, cardiac perfusion, vascular density and to decrease scar formation (Table 2). In clinical studies, the primary end points are the changes in LV volumes and EF in comparison with a control group over time. There are several encouraging clinical studies showing that BMMC or MSC transplantation via intracoronary infusion or intramyocardial injection can achieve with increased EF (Table 4 in chapter 2.4.9) (Wohrle et al. 2013, Gao et al. 2015, Rodrigo et al. 2013) In these studies, the follow-up times have varied from 6 to 24 months. The improvement in the EF appears to be sustained for a prolonged time. Gao et al. (2015) reported improved EF in cell therapy treated patients even after 18 months from transplantation. Despite the doubts that the stem cell therapy has recently encountered, BMMC transplantation has already achieved a similar improvement in EF as can be obtained from with established therapeutic strategies (Reffelmann et al. 2009). However, also controversial results have been reported. In a clinical study, 58 patients were randomized to undergo PCI with or without MSC transplantation (Wang et al. 2014). After 6 months of follow-up, although EF improved in the MSC groups, the change was not statistically significant.

In addition to the functional improvement after AMI, stem cell therapy has also been claimed to exert an effect on the architecture of the LV. MSC transplantation was demonstrated to reduce the infarction size in a clinical study (Wang et al. 2014). Many signalling pathways and soluble cytokines and growth factors have been implicated in mediating the different cellular effects leading to the improved recovery (Psaltis et al. 2014). These can be categorized as 1) immunomodulatory and anti-inflammatory factors, 2) anti-fibrotic factors, 3) chemoattractants or 4) trophic factors, such as anti-apoptotic, supportive of proliferation or proangiogenic.
factors. In one *in vivo* study, stem cell therapy was shown to improve cardiac function and arteriole density and reduce both the extent of apoptosis of myocytes and the infarct size (Pennella *et al.* 2016). There are only a few studies that have examined the electrophysiological remodeling in infarcted myocardium. Interestingly, MSC transplantation seems to promote angiogenesis and alter ion channel expression, leading to electrophysiological remodeling (Lai *et al.* 2013).

Despite the positive findings observed in clinical studies, the details of the key mechanisms behind the stem cell therapy are still not fully understood. The positive effect of stem cell transplantation is postulated to be not only a consequence of stem cell proliferation and differentiation of the transplanted MSCs into functional cells but also to a great extent due to significant paracrine effects. Table 2. Results of BMMC and MSC Therapy in Large-Animal Models.

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Model</th>
<th>Type of Cell</th>
<th>Route of delivery</th>
<th>Follow-Up Imaging</th>
<th>Effects of Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qi <em>et al.</em> 2008</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IC</td>
<td>MRI</td>
<td>↑EF; ↓scar size</td>
</tr>
<tr>
<td>Sheu <em>et al.</em> 2015</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>Echocardiography</td>
<td>↓scar size</td>
</tr>
<tr>
<td>Pennella <em>et al.</em></td>
<td>Rabbit</td>
<td>AMI</td>
<td>BMMC</td>
<td>IM</td>
<td>Histology</td>
<td>Neoangiogenesis</td>
</tr>
<tr>
<td>Hashemi <em>et al.</em> 2008</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td>↓scar size</td>
</tr>
<tr>
<td>Gyongyosi <em>et al.</em></td>
<td>Swine Subacute MI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td></td>
<td>↓scar size</td>
</tr>
<tr>
<td>Dakhlallah <em>et al.</em></td>
<td>Rat Subacute MI</td>
<td>AMI</td>
<td>reprogrammed IM, BMMC, microRNA</td>
<td>IM</td>
<td>Echocardiography</td>
<td>↑EF, ↓fibrosis</td>
</tr>
<tr>
<td>Amado <em>et al.</em> 2005</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td>↑EF; ↓scar size; ↑ Diastolic function</td>
</tr>
<tr>
<td>Hatzistergos <em>et al.</em> 2010</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td>↓scar size</td>
</tr>
<tr>
<td>Tao <em>et al.</em> 2015</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC, BMMC</td>
<td>IM</td>
<td>Echocardiography, SPECT</td>
<td>↓myocardial perfusion, vascular density</td>
</tr>
<tr>
<td>Hua <em>et al.</em> 2014</td>
<td>Rat</td>
<td>AMI</td>
<td>MSC+VEGF gene</td>
<td>IM</td>
<td>Echocardiography</td>
<td>↑EF; ↓capillary density</td>
</tr>
<tr>
<td>Price <em>et al.</em> 2006</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IV</td>
<td>Echocardiography</td>
<td>↑EF; ↓hypertrophy</td>
</tr>
<tr>
<td>Source</td>
<td>Species</td>
<td>Model</td>
<td>Type of Cell</td>
<td>Route of delivery</td>
<td>Follow-Up Imaging</td>
<td>Effects of Therapy</td>
</tr>
<tr>
<td>---------------</td>
<td>---------</td>
<td>--------------</td>
<td>--------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Shake et al. 2002b</td>
<td>Swine</td>
<td>Subacute MI</td>
<td>MSC</td>
<td>IM</td>
<td>Sonomicrometry crystals</td>
<td>↑ Systolic wall thickening</td>
</tr>
<tr>
<td>Hamamoto et al. 2009</td>
<td>Sheep</td>
<td>AMI</td>
<td>MSC</td>
<td>surgical?</td>
<td>Echocardiography</td>
<td>↑EF; ↑EDV</td>
</tr>
<tr>
<td>Perin et al. 2008</td>
<td>Canine</td>
<td>MI</td>
<td>MSC</td>
<td>IC/MI</td>
<td>Echocardiography</td>
<td>↑EF; ↑EDV; ↓ESV</td>
</tr>
<tr>
<td>Fan et al. 2014</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td>↑EF; ↓scar size</td>
</tr>
<tr>
<td>Quevedo et al. 2009</td>
<td>Swine</td>
<td>Chronic MI</td>
<td>MSC</td>
<td>MI</td>
<td>MRI</td>
<td>↑EF; ↓scar size; ↑ Myocardial perfusion</td>
</tr>
<tr>
<td>Schuleri et al. 2009</td>
<td>Swine</td>
<td>Chronic MI</td>
<td>MSC</td>
<td>surgical?</td>
<td>MRI</td>
<td>↑EF; ↓scar size</td>
</tr>
<tr>
<td>Zhao et al. 2014</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>MI</td>
<td>SPECT</td>
<td>↑EF; ↓scar size</td>
</tr>
<tr>
<td>Halkos et al. 2008</td>
<td>Swine</td>
<td>Chronic MI</td>
<td>MSC</td>
<td>IV</td>
<td>LV angiography</td>
<td>Preserved EF; ↑ vascular density</td>
</tr>
<tr>
<td>Makkar et al. 2005</td>
<td>Swine</td>
<td>Chronic MI</td>
<td>MSC</td>
<td>surgical</td>
<td>Echocardiography; Preserved EF LV angiography</td>
<td></td>
</tr>
<tr>
<td>Li et al. 2010</td>
<td>Swine</td>
<td>AMI</td>
<td>BMMC</td>
<td>IC</td>
<td>Echocardiography</td>
<td>↓scar size</td>
</tr>
<tr>
<td>Makela et al. 2007</td>
<td>Swine</td>
<td>AMI</td>
<td>BMMC</td>
<td>IM</td>
<td>Echocardiography</td>
<td>↑EF</td>
</tr>
<tr>
<td>Zhou et al. 2009</td>
<td>Swine</td>
<td>AMI</td>
<td>MSC</td>
<td>IM</td>
<td>MRI</td>
<td>↑EF; ↑capillary density</td>
</tr>
</tbody>
</table>

MI=myocardial infarction; IC=intracoronary infusion; IM=intramyocardial injection; MRI=magnetic resonance imaging; EDV=End-diastolic volume; ESV=End-systolic volume; hiPSC=human induced Pluripotent Stem Cell; FS=fractional shortening; SPECT=single photon emission computed tomography; ↑=increase; ↓=decrease

2.4.2 Differentiation of stem cells

The concept that adult BMMCs are able to differentiate and consequently participate in the formation of cardiomyocytes in adult human hearts was evidenced according to the chimerism detected after sex-mismatched organ transplantation (Quaini et al. 2002). Eight hearts from female donors were transplanted to male recipients. After transplantation, Y-chromosome-positive cardiomyocytes, smooth-muscle cells and endothelial cells were detected from the transplanted heart suggesting that the cells had been translocated from the host to the graft. The study revealed that translocated host cells were able to differentiate and regenerate new
myocytes and vascular cells. Since then, the differentiation potential of the stem cells has been extensively investigated using *in vitro* and *in vivo* models.

Different stem cells have different self-renewal capabilities and differentiation abilities. The current evidence suggests that growth factors, extracellular matrix and multiple metabolic pathways provide important signals for stem cell self-renewal and differentiation (Hu *et al.* 2016). MSCs have the ability to differentiate into adipocytes, osteoblasts and chondroblasts (Kobolak *et al.* 2015) (Fig.4). The fate of the new cell is governed by down-regulating the pluripotent specific genes, up-regulating the terminal specific genes and switching between different subsets of metabolic enzymes.

![Fig.4. Differentiation potential of MSCs. The differentiation towards other lineages originating from the mesoderm, such as cardiomyocytes, remains questionable.](image)

The differentiation potential has been confirmed in an *in vitro* study where MSCs were able to differentiate into myocytes with or without co-culture with myocytes (Zamilpa *et al.* 2014, Shim *et al.* 2004). In a rat model where MSCs were intramyocardially injected one week after AMI, the transplanted MSCs were able to differentiate into cardiomyocytes in the infarct zone (Huang *et al.* 2013). This is
in line with other in vivo animal studies examining the differentiation ability of MSC into cardiomyocytes (Garikipati et al. 2014, Hao et al. 2015). The delivery method (intramyocardial, intracoronary, intravenous) does not seem to influence the differentiation ability of MSCs according to work conducted with an experimental rabbit model (Guo et al. 2011, Hao et al. 2015). However, there are also studies contradicting these findings and as a consequence, there is no consensus regarding the differentiation abilities of stem cells.

2.4.3 Paracrine effects of stem cells

The cardioprotective effect of the stem cells is mediated not only by their ability to transdifferentiate into cardiomyocytes or endothelial cells but also by supplying angiogenic, anti-apoptotic and mitogenic factors (Zamilpa et al. 2014). After an AMI, pro-inflammation cytokines are secreted into the infarction area in order to trigger the inflammatory process. Anti-inflammation cytokines are cardioprotective and provide balance to the injured area; for the most part, these agents are responsible for healing processes. Stem cells modulate inflammation by secreting cytokines that can be considered as cardioprotective.

One of the anti-inflammatory cytokines, IL-10, is produced by many inflammatory cells and MSCs. IL-10 stimulates the immune cells to differentiate towards a specific subtype and suppresses the pro-inflammatory cytokine levels (van den Akker et al. 2013). In mice, treatment with IL-10 reduced both the activity of MMP-9 and the extent of fibrosis. Moreover, IL-10 significantly improved LV functions and attenuated infarct wall thinning (Krishnamurthy et al. 2009). After an intramyocardial injection of BMMCs, the IL-10 level was significantly increased and the cardiac function improved. The improvement was reported to associate with a reduction in the accumulation of T lymphocytes and the extent of myocardial collagen deposition (Burchfield et al. 2008). BMMCs and MSCs promote healing and under hypoxic conditions, such as in the infarcted area, they secrete VEGF, a growth factor that promotes angiogenesis (Kinnaird et al. 2004a, Miyahara et al. 2006, Ohnishi et al. 2007).

In addition, stem cells can modulate the inflammation process by contributing to the pro-inflammatory cytokine levels. MSC transplantation after acute myocardial infarction was reported to decrease the gene expression of several pro-inflammatory cytokines TNFα, IL-1β and IL-6 and to inhibit the deposition of type I and III collagen (Guo et al. 2007).
MSCs have been proven to secrete a wide range of cytokines and growth factors: PDGF, bFGF and VEGF that promote pro-angiogenesis, MMP1, MMP2 and MMP9 has influenced the remodeling of extracellular matrix whereas IL-6, inducible nitric oxide synthase (iNOS) and hepatocyte growth factor (HGF) are immunomodulatory cytokines (Gnecchi et al. 2006, Hou et al. 2016, Yao et al. 2015). This raises the question of whether BMMCs or MSCs are able to secrete these cytokines or growth factors also in the myocardium after an AMI. The improved understanding of the cytokine network secreted by stem cells has opened a whole new vista in stem cell therapy. As a consequence, one priority should be to explore cytokines secreted by stem cells after AMI.

2.4.4 Cell types used for myocardial infarction

The ideal cell type for cardiac cell therapy would be autologous, highly resistant to malignant transformation and yet capable of differentiation. Several cell types from different origins have been evaluated in studies but these investigations have failed to lead to any clear consensus about the best cell type. There are conflicting results from studies comparing different cell types (Karpov et al. 2013).

The majority of clinical and experimental studies of stem cell therapy for AMI have used bone marrow derived cells due to the easy access to the niche as well as safety considerations (Nunez Garcia et al. 2015). However, sources of stem cells have also been identified in other organs, such as heart, arteries, gonads, veins, neural tissue, intestine and epidermal tissue. In addition to bone marrow, peripheral blood or umbilical cord blood have been used as the source of adult stem cells in clinical studies for AMI. Furthermore, new sources of stem and progenitor cells for therapy are topics of intensive investigation.

Bone marrow is the major source of adult stem cells and the best characterized. In addition, bone marrow cells have a long history of use in therapeutic bone marrow replacement for blood diseases. BMMCs exhibit multi-potentiality and transdifferentiate so they can adopt multiple phenotypes. In addition, bone marrow cells have the ability to repopulate many non-hematopoietic tissues, such as cardiomyocytes and endothelium (Nunez Garcia et al. 2015, Lee et al. 2005). Bone marrow contains a large number of HSCs and only a minority of MSCs. MSC possess the ability to produce a variety of trophic and immunomodulatory factors that can directly promote cell survival and reduce inflammation (Faiella & Atoui 2016). The results have been mostly promising and there are no ethical concerns surrounding their use. In a rat model, the intravenous injection of BMMCs led to a
less calcifying remodeling and increased EF compared to the controls (Holinski et al. 2012). The therapeutic effect of BMMCs on cardiac function recovery has been demonstrated also in clinical studies (Ni et al. 2014, Nuri & Hafeez 2012). Furthermore, a systematic meta-analysis in patients revealed that BMMC therapy was effective at improving the LVEF (Liu et al. 2016). Since BMMCs are the treated individual’s own cells, they do not pose any safety concerns and they are readily prepared in hospitals. The autologous origin and convenience of BMMCs are the major advantages of their use in cell therapy for AMI. In addition, there is no risk of experiencing an immunological rejection if one administers autologous BMMCs. Consequently it was decided to utilize BMMCs in the present series of studies.

Cardiac stem cells can be viewed as an interesting new option, holding the potential for self-renewal and differentiation into the major specialized cell types present in the heart: vascular cells and myocytes. However, the exact origin and isolation protocol of the cardiac cells are still unclear, prohibiting their therapeutic use (Dimmeler et al. 2008). It is clear that the use of cardiac stem cells will require more studies.

Embryonic stem cells (ES) can differentiate into any cell type in the adult body but their use has encountered many obstacles, not least of which are ethical concerns (Nunez Garcia et al. 2015, Psaltis et al. 2014). In addition, the risk of forming a teratoma, which can trigger an immune response capable of destroying the transplanted ES. For this reason, the survival of ES in grafts has emerged as an open question which needs to be resolved before this approach can be used in the transplantation clinic.

Skeletal myoblasts have two significant advantages as a candidate cell source. These cells survive more readily in the hostile post-infarct environment and secondly, they more clearly retain the capacity to regenerate in vivo (Nunez Garcia et al. 2015). However, one limitation to their use is that they do not possess the same electrophysiological properties as cardiomyocytes and they are unable to synthesize gap junction proteins. These deficiencies increase the risk of life-threatening arrhythmias.

In conclusion, each of these stem cells has its own advantages and disadvantages, which are briefly summarized in Table 3 below. Only time will decide which stem cell type proves to be the optimal choice.
Table 3. Stem Cells in Treating Acute Myocardial Infarction.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Source</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow Stem Cells</td>
<td>Bone marrow</td>
<td>Feasible, safe, readily prepared in hospitals, no ethical concern, autologous, no immunological rejection</td>
<td>Controversial success in clinical studies, Pluripotency uncertain</td>
</tr>
<tr>
<td>Cardiac Stem Cells</td>
<td>Heart, Infarct border</td>
<td>Differentiation into all cardiac lineages</td>
<td>Isolation of the cardiac stem cells from an aging heart may not sufficiently improve function</td>
</tr>
<tr>
<td>Embryonic Stem Cells</td>
<td>Human embryo</td>
<td>Evolve with cardiomyocytes action potential</td>
<td>Ethical opposition, Possible teratoma formation</td>
</tr>
<tr>
<td>Skeletal Myoblasts</td>
<td>Muscle</td>
<td>Survive and differentiate in human hearts, Readily obtained, Low risk of tumor formation</td>
<td>The lack of gap junction formation, Ventricular arrhythmias</td>
</tr>
</tbody>
</table>

2.4.5 Stem cell delivery

The success or failure of stem cell therapy in treating AMI can depend on the delivery technique. Several potential delivery routes for stem cell transplantation have been examined and they all have their advantages and disadvantages (Fig. 5). In the treatment protocols, AMI stem cells have been delivered using intracoronary injection, intravenous infusion, intramyocardial injection through the epicardium or through the endocardium by a percutaneous approach and retrograde coronary venous system. All of these delivery methods have been used in both non-clinical and clinical studies. However, stem cell transplantation is still a topic of research and it is not currently used as a clinical treatment for AMI patients.

The infusion of stem cells into the coronary arterial circulation is relatively straightforward, fast, mini-invasive and the costs are low (Wang & Jokerst 2016). Due to these properties, intracoronary infusion is the most widely used transplantation method to treat AMI in clinical studies. In addition, there is no limit to the number of cells that can be obtained, controlled randomised trials are more feasible and the delivery is straightforward to perform in a clinical setting. In brief, a standard over-the-wire balloon angioplasty catheter is placed into the target coronary artery and the balloon is inflated at a low pressure in order to block blood flow while the cells are infused through the distal lumen. The main advantage is its
direct infusion into the target and the resulting homogenous cell population in the tissue. The outcomes of intracoronary delivered stem cell transplantation as assessed via its effect on cardiac function have varied in the different studies (Choudry et al. 2016, Gao et al. 2015). Concerns have been raised about the intracoronary transplantation delivery due to the possibility of the formation of cell clusters or cell fragments that could potentially act as microemboli (Wang & Jokerst 2016). Another issue is that implantation into the coronary artery may modulate blood flow within the already damaged area of cardiac tissue.

The intramyocardial transplantation is a promising approach for stem cell delivery (Psaltis et al. 2014). Intramyocardial injection allows cell transplantation visually directly into the ischemic area, border zone or both areas. The procedure can be performed either by catheter-based or by direct surgical intramyocardial injection.

In the catheter-based method, a transendocardial injection delivers the stem cells directly into the myocardium (Psaltis et al. 2014). The catheter is navigated in the left LV with either fluoroscopic guidance or electroanatomic mapping; when it is in the desired region, the stem cells are injected via a needle deployed from the tip of a catheter. Perin et al were the first to exploit transendocardial stem cell delivery in man; they used a NOGA catheter for injection and examined the effect of bone marrow stem cells on end-stage ischemic heart disease (Perin et al. 2003). There was a sustained improvement in EF from baseline in treated patients when this was assessed at 4 months after cell delivery.
Fig 5. Schematic overview of the different delivery techniques into the injured myocardium used in the stem cell therapy.
Direct surgical intramyocardial injection is the most direct, accurate and precise approach to transplant stem cells into the infarcted myocardium (Psaltis et al. 2014). Along with the intracoronary stem cell infusion, it has been the most extensively used delivery method in stem cell research. The area of infarction can be detected preoperatively and this is a great advantage for cell homing. In in vivo animal studies, neoangiogenesis and improved EF have been reported after intramyocardial stem cell transplantation (Inaba et al. 2014, Pennella et al. 2016).

It is demanding to perform a clinical study using a direct surgical intramyocardial delivery of stem cells. The direct injection of stem cells requires attention, patience and skill. The cell distribution will vary depending on the injection protocol. High costs, a limit to the number of cells which can be delivered and a risk of transmural perforation are the disadvantages linked with the direct intramyocardial delivery method. However, there is a high degree of homing and efficient response to the cell therapy. In a clinical study, MSCs were intramyocardially injected using NOGA catheter for patients after AMI (Rodrigo et al. 2013). That study revealed that intramyocardial injection of MSCs in patients shortly after AMI was not only a safe but also a feasible method during an up to 5-year-follow-up. EF improvement was demonstrated up to 5 year after MSC transplantation but the finding was not significantly different in statistical terms compared to controls.

However, there is the risk of mechanical leakage following direct intracardiac injection. After AMI and intramyocardial injection of induced pluripotent stem cells for mice, a massive early displacement was reported from the injection site to the pulmonary circulation with subsequent lung accumulation (Martens et al. 2014). Interestingly, the retention rate was higher in a large animal (pig) group in comparison with a small animal (rat) group (Teng et al. 2006). According to that study, multiple injections into a non-beating heart increased the retention rate. Leakage and displacement from injection site may explain, at least partly, the large variation in the efficacy of stem cell treatment.

In comparisons of intracoronary and intramyocardial transplantation methods, the intramyocardial approach appears to achieve the highest retention rate of transplanted stem cells and greater impact on LV function according to the outcomes of three separate in vivo studies conducted in a pig model (George et al. 2008, Hou et al. 2005, Makela et al. 2009). The result was confirmed also in a study undertaken in rats; the retention of the transplanted stem cells in the ischemic myocardium was dependent on the cell delivery method. In comparison with tail vein and left ventricle injections, the direct myocardial injection resulted in the
highest rate of cell retention (Elhami et al. 2013). In our previous study conducted in pigs, intramyocardial injection of BMMCs was demonstrated to be superior to the intracoronary method in terms of cell homing (Makela et al. 2009). Consequently, intramyocardial transplantation can be considered at present as the optimal delivery method for stem cells at least when conducted during open chest surgery.

2.4.6 Stem cell tracking

Imaging methods for cell tracking have a crucial role in cell therapy. After isolation of transplanted cells, they are labeled with contrast agent and purified from unlabeled cells in order to obtain a cell product emitting a high signal and thus good contrast against the adjacent tissue. After delivery, stem cell tracking has proved to be a useful tool for evaluating the viability and engraftment of transplanted cells. Imaging methods provide information for optimizing the cell delivery strategy, improving protocols and for evaluating the functional outcome of cell therapy. In the published studies, either direct or non-direct labeling methods have been used for cell labelling (Wang & Jokerst 2016). In direct labelling, small molecules, nanoparticles, radioisotopes or fluorophores are added to the cell surface or the cell interior. In indirect labeling, a reporter gene is introduced into the genome of the cell of interest which expresses an enzyme, a receptor, a fluorescent or bioluminescent protein suitable for imaging the cell.

In direct cell labelling, MRI, single photon emission computer tomography (SPECT) or positron emission tomography (PET) has been used. After labelling the cells with F-fluorodeoxyglucose, PET is able to monitor cell homing and their biodistribution (Wang & Jokerst 2016). Despite the high spatial resolution, the short half lifetime is a major obstacle for long-term cell tracking. A small number of cells can be detected using SPECT imaging due to its high sensitivity. Cells can be labelled by several radioisotopes such as Technetium$^{99}$ and Idium$^{111}$ (Makela et al. 2009, Suhett et al. 2015). Radioactivity is then measured by a Gamma camera producing a 3D image. Barbash et al. was the first to apply a SPECT technique to follow in vivo stem cell activity in cardiac therapy (Barbash et al. 2003). Both clinical and non-clinical animal studies have been performed using SPECT imaging to monitor stem cells and perfusion and to assess the functional effects of transplanted stem cells. However, the possible effect of radioactivity on the transplanted cells, such as radiation-induced cell damage cannot be excluded (Wang & Jokerst 2016). In addition, the low cellular retention and signal loss due to radioactive decay are also disadvantages associated with SPECT.
Either gadolinium or super paramagnetic iron oxide (SPIO) can be used for direct labelling of stem cells for MRI but gadolinium suffers from an inherently high threshold for detectability (Cores et al. 2015)(Fig. 6). SPIOs vary in size from 50-180nm and very small super paramagnetic iron oxides (VUSPIOs) are <10nm. They can be histologically detected using Prussian blue staining (Fig.6). After incubating the cells with SPIOs, they are induced to enable endocytosis of a contrast agent that appears as a hypo-intense area using T₁/T₂ relaxation in the MRI assessment (Accomasso et al. 2016). SPIOs are composed of an iron oxide core coated with dextran, polyethylene glycol or starch containing a polymer that prevents aggregation (Cores et al. 2015). SPIOs have been widely used in both clinical and non-clinical studies in cell tracking, although the long term fate of the labelled cells after transplantation remains unclear.

In non-direct labelling, a transfer gene which is not normally expressed in target cells is incorporated into the genome of the cell prior to its transplantation (Wang & Jokerst 2016). The image tracer targets the transfer gene product if it has been expressed. The appearance of the gene product confirms the survival of the transported cells.

Although stem cell tracking using MRI and SPIOs has been widely exploited in non-clinical in vitro and in vivo studies, the reliability of the method has been criticized. No systematic detection of cells using MRI and histology has been performed to prove if there is any correlation between the methods and feasibility of MRI for cell tracking.
2.4.7 In vivo animal studies

Clinical and experimental in vivo animal studies have revealed similar positive outcomes after stem cell administration. In brief, in vivo animal studies and clinical studies have both displayed improvements in LVEF and cardiac perfusion, attenuations in contractile dysfunction, reduced infarct size and neovascularization (Amado et al. 2005, Pennella et al. 2016, Tse et al. 2003, Williams et al. 2011, Zhou et al. 2009)(table 2 in chapter 2.4.1).

In most of the experimental studies, rats, rabbits, mice or pigs have been the species used. Experimental AMI has been conducted using rather similar methods in all species i.e. ligation of the coronary arteries and allowing reperfusion after occlusion (Fan et al. 2014, Lai et al. 2013, Pennella et al. 2016). In addition, balloon occlusion of the coronary artery is a possible technique to induce AMI especially with a large animal model such as the pig (Amado et al. 2005). The use of an appropriate experiment animal is essential if one wishes to undertake a critical evaluation of stem cell therapy outcomes. For example, canine hearts have a robust collateral circulation that can make it difficult to achieve a consistent degree of ischemic injury between animals using occlusion of a coronary artery. Basically, since it is a large experiment animal, the pig exhibits more similarities between humans in terms of its cardiovascular anatomy and physiology. The metabolism and their small number of coronary collaterals in the pig heart resemble that found.
in humans. The comparable heart size of pigs to humans makes them valuable for testing replacement of heart valves or catheter-based therapies such as stent placement. However, there are financial and logistical challenges associated with the use of large pigs as experiment animals.

In addition to variations in the animal species, there have been a wide variation in the cell numbers administered in experimental *in vivo* studies. Typically a large number of stem cells (6x10^6-3x10^7) have been used in experimental cellular therapy (Fan *et al.* 2014, Ye *et al.* 2014). Although the differences in the cell numbers are rather extensive, reduced infarction size and improved EF was achieved in both studies.

In addition, the timing of the transplantation, the follow-up time and the cell type have all varied between the *in vivo* animal studies. In two separate studies in which the stem cells were transplanted either 1 day or several months after infarction, LVEF increased significantly (Ge *et al.* 2006b, Herreros *et al.* 2003). In an experiment designed to assess the optimal timing for stem cell delivery, cardiac stem cells were administered by intracoronar injection either 2 hours or 7 days after AMI (Crisostomo *et al.* 2015). The effect on cardiac function and infarct size was equal between the groups. Controversially, some studies have indicated that LVEF was not improved when transplantation occurred 9 days after AMI (Penicka *et al.* 2007).

There are numerous *in vivo* studies with multiple variations in the protocols investigating the benefits of stem cells in treating AMI. Nevertheless, many critical questions considering the optimal transplantation technique, cell tracking methods and the key mechanisms of the stem cell therapy have remained unresolved.

### 2.4.8 In vitro studies

Human and *in vivo* animal studies have revealed the beneficial effects of stem cell transplantation on cardiac function and scar formation. However, it is difficult to examine the mechanisms through which stem cells function in either clinical or *in vivo* studies. Information about the viability, structure and behaviour of stem cells is valuable in helping to understand and improve stem cell therapy and protocols. *In vitro* models represent a valuable option for answering these questions.

Several in vitro models have been used in stem cell research, e.g. single cell, cell culture, tissue, whole heart, and microfluidic models. The single cell model is a useful tool for evaluating the electromechanical properties of single cardiomyocytes and cardiac physiology. For example, pluripotent stem cells were
used in a single cell culture model to analyse myocardial contractility (Ribeiro et al. 2015). In experiments which are closer to the in vivo condition, the behaviour of the groups of cells has been studied using either 2D or 3D cell culture models. 2D culture systems have been used to assess drug-induced cardiotoxicity, molecular signalling pathways and to evaluate gene therapy approaches (Garbern et al. 2013). However, 2D cultures lack the complexities of the real cellular microenvironment and extracellular matrix. 2D cell culture systems can also be layered into 3D structures. Another option is to culture cells in 3D cultures such as scaffolds or matrices, which is a common method used in tissue engineering. Ruan et al. compared 2D and 3D cultures in their abilities to reveal the maturation and the differentiation of pluripotent stem cells (Ruan et al. 2015). The 3D culture seems to favour cardiac differentiation and mechanical stress promotes cardiomyocyte structural and functional maturation. The coculture model simulates a multicellular tissue in which the cellular cross-talk between different cell types is crucial. Multiple cell types can be cultured in different ways, for example in transwell culture systems that allow paracrine activity between the cells. The microfluidic system or “lab-on-a-chip” aims to capture the electrophysiology of a single cell mimicking the native cellular milieu by recapitulating fundamental organ-specific dynamic conditions in microfluidic cell culture devices (Garbern et al. 2013). Miniaturization to the micrometer scale confers precise control over the cellular microenvironment in order to study cell behaviour. A recent study revealed a novel technique, the 3D heart-on-a-chip microdevice, has enabled the generation of cardiac micro-tissue and its quantitative analysis and visualization under controlled conditions (Marsano et al. 2016).

A relatively small number of stem cell studies have been conducted in vitro, considering the fact that stem cell therapy is already in human phase studies. For the most part, adhesion, migration, differentiation and transmigration of stem cells have been evaluated using several kinds of collagen invasion, adhesion and differentiation assays as well as observing the cell surface proteins (Stankovich et al. 2011, Steingen et al. 2008, Ting et al. 2010). As far as is known, the fate of injected stem cells in myocardial tissue has not been studied in vitro. Therefore, it was decided to develop a cardiac explant culture model from pig heart to simulate tissue stress and to study in detail the behaviour of stem cells in the myocardial tissue microenvironment after their injection. The survival of transplanted stem cells in hypoxic and inflamed myocardial tissue is an open question that is difficult to explore in vivo. Cardiac explant culture model were used to assess the adhesion and infiltration rate of stem cells after injection to evaluate the activity of stem cells...
in myocardial tissue. The properties of stem cells exposed to inflamed conditions have been studied \textit{in vitro} by culturing MSCs with anti-inflammatory cytokines; apparently transforming growth factor beta-1 (TGF-\(\beta\)1), TGF-\(\beta\)3 and VEGF support the growth of cells (Freytes \textit{et al.} 2012).

Cell labelling is being increasingly used to monitor the fate of the transplanted cells \textit{in vivo} and in histological evaluations. \textit{In vitro} models have predominated in the development of cell labelling methods (Betzer \textit{et al.} 2015). Uncoated superparamagnetic maghemite nanoparticles are a newly synthesized MRI contrast agent which can label stem cell (Skopalik \textit{et al.} 2014). Before their use in in vivo models, the viability, proliferation, nanoparticle uptake efficiency and migration capacity of labelled cells has to be tested \textit{in vitro} using methods such as fluorescence microscopy and absorption spectroscopy.

In summary, \textit{in vitro} studies are useful for exploring the characteristics and behaviour of stem cells and to explain and improve the results of stem cell therapy and they are a good complement to the \textit{in vivo} models.

\textbf{2.4.9 Clinical studies}

As a consequence of the promising results of the non-clinical experiments, several clinical studies have been performed but the efficacy of stem cell therapy in cardiac tissue repair has remained controversial and unresolved. As shown in table 4, the protocols applied in the clinical studies have varied extensively, making it difficult to compare the results.

The major cell types used in the clinical studies have been skeletal myoblasts, unfractionated BMMC or BMMCs and other bone marrow or blood-derived cells (Table 4). Some of the first cells to be tested in clinical studies were skeletal myoblasts that had been expanded in culture from isolated satellite cell progenitors from a muscle biopsy (Menasche \textit{et al.} 2008). In this randomized, placebo-controlled and double-blind study, ninety-seven patients with depressed LV function were enrolled with sixty-seven of them receiving myoblasts. However, myoblast injections combined with coronary surgery failed to improve heart function. A more recent Phase II randomized trial in which heart failure patients received intramyocardial transplantation of myoblasts did not reveal any improvement in EF (Duckers \textit{et al.} 2011). Thus, these largely negative results do not support the initiation of more trials with myoblasts.

The majority of clinical studies have used either BMMC or MSCs as the cell type. These trials have shown that both intracoronary and intramuscular injection
of MSC are feasible and safe (Gao et al. 2015, Rodrigo et al. 2013). Increased EF was observed at 6 months after AMI in the patients treated with the intracoronary BMMC injection (Traverse et al. 2014). However, the difference was not statistically significant when compared to controls at the one-year follow-up. In contrast, improved EF was reported as long as up to 3 years after AMI and BMMC transplantation when a high number of cells (>324x10⁶) were delivered (Wohrle et al. 2013).

Thus far, three application routes have been used to transplant cells into the heart in the clinical studies (table 4). The major route utilized has been directed intracoronary injection through an inflated over-the-wire balloon catheter. In addition, transendocardial or direct transepicardial injection has been applied. The cell transplantation methods have been discussed in more detail in chapter 2.4.5.

The timing when the cells have been transplanted after the AMI has ranged from 1 day up to several months (Table 4). Despite the large variation in timing, the beneficial effects on cardiac function are clear as shown in table 4. The optimal timing for cell delivery was analyzed in a clinical study where BMMCs were injected by the intracoronary route between 24 hours to 30 days after PCI and AMI (Huang et al. 2015). An increase in EF compared to controls was measured when BMMCs were transplanted either 24 hours or 3-7 days after AMI. The optimal timing for cell transplantation seems to be 4-7 days after AMI as confirmed also by a large meta-analysis investigating over 2000 patients (Liu et al. 2016). In addition, both the age of patients and the quality of transplanted stem cells may contribute to the recovery potential after AMI (Golpanian et al. 2015, Schutt et al. 2015). In a recent study, Golpanian et al. (2015) compared the use of MSCs in treating AMI in patients either under or over 60 years old. Surprisingly, that study revealed that older individuals did not exhibit an impaired response to MSC therapy.

Due to the large variation in the study protocols and the relatively small number of patients, it is difficult to draw any firm conclusions from the available data. The largest, systematic meta-analysis enrolling 2625 patients from a total of 50 clinical studies indicated that patients treated with bone marrow cells exhibited a greater EF and a smaller infarct size (Jeevanantham et al. 2012) and the improved EF persisted beyond 12 months. Importantly, the all-cause mortality, cardiac mortality, recurrent AMI, and stent thrombosis were significantly reduced in the cell therapy group.

Clearly, larger studies will be required before embarking on the clinical use of stem cell therapy in treating AMI patients. Although the stem cell treatment seems to be beneficial, the key mechanisms underpinning this effect remains unknown.
The remodeling and the inflammation process play crucial roles in cardiac repair but their effect of stem cell transplantation require more studies.

**Table 4. Models used in clinical stem cell studies.**

<table>
<thead>
<tr>
<th>Speciality of the study</th>
<th>Number of patients</th>
<th>Follow-up time</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMMC</td>
<td>25+79</td>
<td>12 months</td>
<td>increase in EF</td>
<td>Huang et al. 2015</td>
</tr>
<tr>
<td>Peripheral blood stem cells</td>
<td>35+35</td>
<td>6 months</td>
<td>increase in EF</td>
<td>Li ZQ et al. 2007</td>
</tr>
<tr>
<td>Cardiosphere-derived cells</td>
<td>8+17</td>
<td>6 months</td>
<td>reduced scar size</td>
<td>Makkar et al. 2012</td>
</tr>
<tr>
<td>MSC</td>
<td>19+30</td>
<td>1 year</td>
<td>reduced scar size</td>
<td>Golpanian et al. 2015</td>
</tr>
<tr>
<td>Skeletal myoblast</td>
<td>67+30</td>
<td>6 months</td>
<td>No change in EF</td>
<td>Menasche et al. 2008</td>
</tr>
<tr>
<td><strong>Transplantation method</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>58+58</td>
<td>18 months</td>
<td>Increase in EF myocardial perfusion increased</td>
<td>Gao et al. 2015</td>
</tr>
<tr>
<td>IM</td>
<td>20+35</td>
<td>36 months</td>
<td>myocardial perfusion increased</td>
<td>Yerebakyan C et al. 2011</td>
</tr>
<tr>
<td>transendocardial injection</td>
<td>19+30</td>
<td>6 months</td>
<td>reduces scar size</td>
<td>Golpanian et al. 2015</td>
</tr>
<tr>
<td><strong>Timing of cell transplantation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>34+33</td>
<td>4 months</td>
<td>regional LV function improvement</td>
<td>Herbots et al. 2009</td>
</tr>
<tr>
<td>3-5 days</td>
<td>120</td>
<td>12 months</td>
<td>Infarct size decreased</td>
<td>San Roman et al. 2015</td>
</tr>
<tr>
<td>24hrs-30d &gt;4 months</td>
<td>25+79 (BMMC)+4(MSC)</td>
<td>12 months</td>
<td>EF improvement</td>
<td>Huang et al. 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regional LV function improve, reduced infarct size</td>
<td>Williams et al. 2011</td>
</tr>
<tr>
<td><strong>Analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECHO</td>
<td>30+28</td>
<td>6 months</td>
<td>LVEF improvement</td>
<td>Wang et al. 2014</td>
</tr>
<tr>
<td>CMRI</td>
<td>65+30</td>
<td>12 months</td>
<td>EF increased at 3 months</td>
<td>Traverse et al. 2014</td>
</tr>
<tr>
<td>SPECT/ECHO</td>
<td>45+9</td>
<td>5 years</td>
<td>improved EF up to 5 years,</td>
<td></td>
</tr>
<tr>
<td>Angio, ECHO, SPECT</td>
<td>10+17</td>
<td>4 months</td>
<td>No improvement in EF</td>
<td>Penicka et al. 2007</td>
</tr>
</tbody>
</table>

BMMC=Bone Marrow Mononuclear Cell; MSC=Mesenchymal Stem Cell; IC=Intracoronral; IM=Intramyocardial; EF=Ejection Fraction; LV=Left Ventricle; CMRI=Cardiac Magnetic Resonance Imaging; SPECT=Single-photon Emission Computed Photography
3 Aims of the study

The thesis was undertaken in order to obtain answers to the following research questions:

1. How do the transplanted stem cells behave in the myocardium after delivery? Although stem cell therapy is in the clinical phase of therapeutic development, the fate of the injected stem cells in their new microenvironment after transplantation has not been examined in vitro. The aim was to clarify the location and possible adaptation of the MSCs after their injection into live myocardial tissue.

2. Is MRI a feasible method for tracking injected cells in the myocardium? The primary interest was to determine whether there is a correlation between the radiological and the pathological methods for monitoring cell targeting after acute myocardial infarction.

3. How do the transplanted stem cells influence the cardiac function acutely after a myocardial infarction? The main interest was to determine whether there is any correlation between the EF increase and the number of transplanted cells after injection into the infarcted myocardium.

4. What is the effect of the transplanted BMMCs on the cytokine network and on the inflammation process contributing to remodeling after AMI? This issue was studied in both the in vivo and the clinical study.
4 Materials and methods

In this section material and methods are summarized. A Roman number (I-III) indicates the studies of the thesis in which the method was used. The primary fields of interest in the separate studies are shown in Table 5.
Table 5. The primary fields of interest in the separate studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Type</th>
<th>Group</th>
<th>Cell label</th>
<th>Experiment Model</th>
<th>Follow up time</th>
<th>Cell Migration</th>
<th>Cell Adhesion</th>
<th>Cell Targeting using MRI</th>
<th>Cell Targeting using histochemistry</th>
<th>Evaluation of the heart function</th>
<th>Cytokine profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>MSC</td>
<td>Control</td>
<td>SPIO, Dil</td>
<td><em>in vitro</em></td>
<td>7d</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>BMMC</td>
<td>Control</td>
<td>SPIO</td>
<td><em>in vivo</em> animal study</td>
<td>2h</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>BMMC</td>
<td>Control</td>
<td>SPIO</td>
<td><em>in vivo</em> animal study</td>
<td>21 days</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>BMMC</td>
<td>Control</td>
<td></td>
<td>clinical study</td>
<td>6 months</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMMC=Bone Marrow Mononuclear Cell; SPIO=Super Paramagnetic Iron Oxide; MSC=Mesenchymal Stem Cell; Dil= Vybrant CM-Dil dye
4.1 Test animals (I,II,III)

The animals used in this study were provided by a nearby piggery in Oulu. The Yorkshire pigs were 8-10 weeks old and the median weight of the pigs was 26.1 kg (±1.9kg) in the stem cell group and 27.5 kg (±1.2kg) in the control group. The pigs were allowed to adjust to the new surroundings of the laboratory facilities for one week before the elective operation. Their well-being was monitored daily by the staff of the Laboratory Animal Centre and the researchers. Since the pig is a group animal, they were allowed to stay in communities before and after the operation. They had an opportunity to drink fresh and cool tap water and to eat a special food (Sianherkku, Raisio, Finland) with all the necessary vitamins and minerals. To ensure the comfortable living in the stall, the pigs had hay on the floor, a small cottage and lots of toys with which to play.

4.2 Patients (IV)

Study IV was a clinical sub-study of previously reported FINCELL study. Patients with ST-elevation AMI (STEMI) were admitted to the University Hospital of Oulu or the University Hospital of Turku in Finland between October 2001 and February 2007. A total of 522 subjects were enrolled in the trial. In order that they could be included in the study, the patients had to satisfy the following eligibility criteria: age younger than 75 years, STEMI confirmed via troponin release and electrocardiography, thrombolytic therapy initiated within 12 hours of symptoms, no cardiogenic shock, no rescue PCI due to chest pain and no need for urgent PCI immediately after thrombosis, no need for coronary bypass graft surgery, hemodynamic instability, no refusal of the patient to participate, and no severe coexisting condition that would interfere with the patient’s ability to comply with the protocol.

The study protocol conformed to the Declaration of Helsinki and was approved by the ethics committee of the Northern Ostrobothnia Hospital District. All subjects signed written informed consent for enrollment in the study and treatment.

4.3 Anesthesia (I,II,III)

The experimental animals were sedated by intramuscular injection of ketamine hydrochloride (350mg) and midazolam (45mg). For administration of anesthetic drugs and to maintain the fluid balance with Ringer acetate solution, a catheter was
inserted into a peripheral ear vein. After induction with fentanyl (25 μg/kg intravenously [IV]) (Hameln, Hameln, Germany) and pancuronium (0.2 mg/kg IV) (Hameln), the pigs were intubated and ventilated with 50% oxygen. Anesthesia was maintained by a continuous infusion of midazolam (0.25 mg/kg/h IV) (Pfizer AB, Sollentuna, Sverige), fentanyl (25 μg/kg/h IV), pancuronium (0.2 mg/kg/h IV) and inhalation anesthesia (isoflurane 0.5%) throughout the whole experiment. At anesthesia induction and during extubation, 1.5 g of cefuroxime was administered IV.

4.4 Isolation of cells (I-IV)

The mononuclear cells were harvested from the bone marrow in studies I-III, with 2 x 10 mL of bone marrow being aspirated from beneath the tibial tuberosity into a syringe containing 5000 IU of heparin with the animal under anesthesia. In study IV, 80 mL of bone marrow was aspirated from the posterior iliac crest into a heparin-treated syringe under local anesthesia on the morning of the PCI day. In all studies, the bone marrow aspirate was diluted (1:6) in phosphate-buffered saline (Sigma-Aldrich, St. Louis, Missouri, USA) and subjected to a density-gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, Chicago) to exclude granulocytes and erythrocytes. Mononuclear cells were collected from the interphase and washed with phosphate-buffered saline (Sigma-Aldrich) (studies I-III) or heparinized physiological saline (study IV).

In study IV, mononuclear cells were suspended in 10 mL of medium containing 5 mL of the patient’s own serum and heparinized physiological saline. After the filtering of the BMMC suspension, the quality-control procedures (i.e., microbial culture for sterility and flow cytometer analysis to count CD34+ cells) were performed. The BMMC separation procedure was done and the cells were administered by intracoronary injection. The placebo medium contained physiological saline. The validity of the cell preparation system was assessed as described previously (Huikuri et al. 2008a).

4.5 Culture of cells (I)

After isolation, mononuclear cells were plated for 2 days with hematopoietic with other non-adherent cells being washed away during medium changes. On the basis of adhesive properties of the MSCs, they were then collected and expanded in culture and labeled. The culturing was performed using Minimum Essential
Medium Eagle alpha modification supplemented with 10% fetal calf serum (PromoCell, Heidelberg, Germany), 100 U/mL penicillin, 0.1 g/l streptomycin, 20 mM HEPES and 2mM L-glutamine (Sigma-Aldrich). Half of the medium was replaced twice a week during culturing with the cells being passaged (passage 2-4) when they reached confluence.

4.6 **Characterization of cells (I)**

The following conjugated antibodies were used in cell surface characterization: CD44-PE (Acris Antibodies, Herford, Germany), CD 29-APC (Acris Antibodies), CD46-FITC (Acris Antibodies) and CD105-PE (Acris Antibodies). Negative surface antigens for MSCs were incubated simultaneously as a group in the same samples: CD45-PE (Acris Antibodies) and CD31-APC (Acris Antibodies). IgG1 control (FITC Mouse IgG1 kappa Isotype control, BD Biosciences, Erembodegem, Belgium) was also analyzed by incubating MSCs for 20 minutes at room temperature with the MSCs being washed and suspended in phosphate-buffered saline (Sigma-Aldrich) +0.5% bovine serum albumin (Merc, NJ, USA). The FACSCalibur (Becton Dickinson, New Jersey, USA) equipped with dual lasers emitting at 488 and 633nm was used in analyzing the samples. The fluorescence emissions were measured at 530-30nm, 585-42nm and 661-16nm. The data was compensated and analyzed with BD CellQuest TM Pro (Becton Dickinson). Positivity for an antibody was defined by negative and isotype controls.

4.7 **Labeling of cells (I,II,III)**

Either fluorescent Vybrant CM-Dil dye- (DiI) or SPIO labeling was used for cell targeting (table 6). To ensure that labeling did not affect the cells, two different labeling methods were used in study I. In studies II and III, only SPIO labeling was used.

In study I, SPIO labeling (39 mg/mL ferucarbotran corresponding to 36 mM of iron) was performed by incubating the stem cells overnight with Resovist (Bayer Healthcare, Berlin, Germany) in 37°C. After labeling, the cells were washed twice with phosphate-buffered saline (Sigma-Aldrich) and trypsinized. The total of cell amount was counted and suspended in a saline solution to a density of 10 x 10³ (group 1) or 100 x 10³ (group 2 explants) MSCs in 30µl.

The effect of SPIO labelling on cellular proliferation rate was assessed by culturing control cells and MSCs and analysing them with the 3-(4,5-
dimethylthiazol-2-yl)-2.5/diphenyltetrazolium bromide assay (tetrazole, MTT; Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, 500 cells/well were plated in six replicate wells per sample, cultured for 3, 7 and 10 days and incubated for 2 hours with MTT (0.5mg/mL). After the cells were lysed in dimethylsulfoxide, absorbance was read at 550nm against 650nm.

In study I, Dil dye labeling was conducted according to the manufacturer’s instructions (Invitrogen, Thermo Fischer Scientific, Inc.). In brief, the cells were suspended at a density of 33 000-1x10^6/ml and mixed with 5µL/ml of cell-labeling solution. The cells were incubated for 20 min at 37°C and then the sample was centrifuged and the supernatant was removed. The cells were resuspended in preheated phosphate –buffered saline (Sigma-Aldrich) with the wash being repeated three times.

In studies II and III, SPIO labelling (39 mg/mL ferucarbotran corresponding to 36 mM of iron) was performed by incubating the BMMCs in Resovist (Bayer Healthcare) at room temperature, diluted in Minimum Essential Medium Eagle alpha modification (Sigma Aldrich) for three hours. After incubation, the cells were washed three times with phosphate –buffered saline (Sigma Aldrich). Trypan Blue vital stain (Sigma Aldrich) was used in order to confirm the viability of the cells. The cells were suspended in a saline solution at density of 10^6 cells/2 ml.

In addition, a roller bottle and electroporation methods were used in labeling in study II. In the roller bottle method, the cells were incubated with 1 or 2mg of iron (Resovist®, Bayer Healthcare) in a volume of 10mL while the samples were rotated continuously to prevent any aggregation. Prior to electroporation, the cells were suspended in a sterile 600µl electroporation cuvette and mixed with 1 or 2mg of iron and electroporated with two square wave protocols (100V for duration of 10ms or 100V 5x100V for a duration of 10ms). After electroporation, the cells in the cuvettes were transferred into ice and washed with phosphate –buffered saline (Sigma Aldrich). Control cells were incubated with a basal medium without any label.
Table 6 Labeling methods used in the study.

<table>
<thead>
<tr>
<th>Label</th>
<th>Cell</th>
<th>Labelling method</th>
<th>Used in study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dil</td>
<td>MSC</td>
<td>Incubation</td>
<td>I</td>
</tr>
<tr>
<td>SPIO</td>
<td>MSC</td>
<td>Incubation</td>
<td>I</td>
</tr>
<tr>
<td>SPIO</td>
<td>BMMC</td>
<td>Incubation</td>
<td>II, III</td>
</tr>
<tr>
<td>SPIO</td>
<td>BMMC</td>
<td>Roller bottle</td>
<td>II</td>
</tr>
<tr>
<td>SPIO</td>
<td>BMMC</td>
<td>Electroporation</td>
<td>II</td>
</tr>
</tbody>
</table>

Vybrant CM-Dil=Dil; Super Paramagnetic Iron Oxide =SPIO; Mesenchymal Stem Cell= MSC
Bone Marrow Mononuclear Cell=BMMC

4.8 The cardiac explant culture model (I)

The pigs were sedated as previously described. After heparin administration (500 IU/kg), the pigs were sacrificed with an intravenous injection of pentobarbital (60 mg/kg). The hearts were removed under sterile conditions and the ventricles were placed for 15 minutes in a specific buffer that was designed to maintain the viability of the tissue. The buffer was composed of glutamine acid 70 mM, taurine 15 mM, KCl 30 mM, glucose 11 mM, KH2PO4 10mM, MgCl2 0,5mM, Hapes 10mM and Eta 0,5mM. Adjustment of the buffer to pH 7.3 was conducted with KOH. A biopsy needle (Kai Medical, Germany) was used to prepare cardiac explants from the ventricles and to standardize the size of the explants to 6x8mm (226mm³).

Two different in vitro cardiac explant culture models were developed and therefore the explants were divided into 3 groups, namely the control group, group 1 and group 2 (table 7).

In the control group and in group 1, either saline or MSCs were injected into the center of the explant using a 27G x ¾ needle and a 1mL syringe. MSCs were labeled with SPIO or Dil dye. The explants were transported into 24-well plates containing Minimum Essential Medium Eagle alpha and supplements: 2% fetal calf serum (Promo Cell), 100 U/mL penicillin, 0,1 g/l streptomycin, 10 µg/mL insulin (Novo Nordisk, Bagsvaerd, Denmark), 0.1 µM dexamethasone, 0.1 mM indomethasine and 2 mM L-glutamine (Sigma Aldrich).

Group 2 explants were set into the inserts of the Millicell-96 Cell Culture Insert Plate (Millipore, USA). A total of 100 x 10³ of Dil or SPIO labeled MSCs in a volume of 30 µl were carefully placed onto the top of the cardiac explants via a syringe. Both the explant in the insert and the culture media were set into the chamber.
Culture media were replaced daily from each explant group. At four time points (2h, 48h, 72h, 7 days), the cardiac explants were taken from the culture media and fixed in formaldehyde and embedded in paraffin. Tissue samples were sectioned (4 µm) and deparaffinised prior to histological and immunohistological assessment.

Table 7. The study groups in the cardiac explant culture model.

<table>
<thead>
<tr>
<th>Group</th>
<th>Labeling</th>
<th>Time points</th>
<th>number of explants/time point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>no stem cells</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Group I</td>
<td>SPIO, DiI</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Group II</td>
<td>SPIO, DiI</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

SPIO=Super Paramagnetic Iron Oxide; DiI= Vybrant CM-Dil

4.8.1 Histological and immunohistological staining procedures (I-III)

The histological evaluation was conducted using standard protocols with hematoxylin eosin (H&E), Prussian blue and Mallory’s phosphotungstic acid hematoxylin (PTAH) stains. In the Prussian blue staining, the sections were incubated for 30 minutes in 2% potassium ferrocyanide, which reveals the intracellular iron of the SPIO-labeled MSCs as a blue color. PTAH shows muscle striata, fibrin and nuclei in blue, and elastic fibers as a deep brownish or red color (table 8).

In the experiments aimed at clarifying the localization of the injected MSCs and specifying the different cell types, immunohistochemical stainings against α-smooth muscle actin (α-SMA)(Dako, Glostrup, Denmark), desmin (Dako) and a platelet/endothelial cell adhesion molecule (PECAM-1)(Santa Cruz Biotechnology, Inc.) were used. Myofibroblasts, vascular smooth muscle cells and the pericytes which surround the capillaries were detected by α-SMA. Desmin labels both striated (skeletal and cardiac) and smooth muscle cells. PECAM-1 was used to identify endothelial cells. Cell proliferation was assayed with Ki67 (Novocastra, Leica Microsystems, Wetzlar, Germany) immunohistochemical staining that detects a protein expressed by the cells in the proliferative phases i.e. G1, G2, M and S of the cell cycle. For α-SMA and desmin, the Dako Envision Kit (Dako) was applied for the IHC with a 30 min incubation with the primary antibody. For PECAM-1, a Goat Kit was used with a 60 min incubation for the primary antibody. The sections incubated with phosphate -buffered saline (Sigma Aldrich) instead of the primary antibody were used as the negative control. Ki-67 antibody was
detected with a polymer based system using automated Leica Bond III Tissue stainer (Leica Microsystems, Wetzlar, Germany) according to the manufacturer’s instructions.

Table 8. Histological and immunohistological stains used in the studies.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Target</th>
<th>Used in Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin eosin (H&amp;E)</td>
<td>Morphology of the tissue</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Prussian blue</td>
<td>Intracellular iron of the SPIO-labeled MSCs</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Mallory’s phosphotungstic acid hematoxylin (PTAH)</td>
<td>Muscle striata, fibrin and nuclei in blue. Elastic fibers as a deep brownish or red color.</td>
<td>I</td>
</tr>
<tr>
<td>α-smooth muscle actin (α-SMA)</td>
<td>Myofibroblasts, vascular smooth muscle cells and the pericytes</td>
<td>I</td>
</tr>
<tr>
<td>Desmin</td>
<td>Striated (skeletal and cardiac) and smooth muscle cells</td>
<td>I</td>
</tr>
<tr>
<td>Platelet/endothelial cell adhesion molecule (PECAM-1)</td>
<td>Endothelial cells</td>
<td>I</td>
</tr>
<tr>
<td>Ki67</td>
<td>Cell proliferation</td>
<td>I</td>
</tr>
</tbody>
</table>

4.8.2 Adhesion and localization of cells (I)

Prussian blue was used to detect and evaluate the localization of the SPIO-labeled stem cells. The identification of the cells was based on the color intensity, shape and size. Prussian blue was also used to evaluate the adherence of the cells to the endothelium or myocytes and possible intravascular localization of the stem cells was examined. The criteria for the adherence of a stem cell to the endothelium were defined as a positive staining reaction for Prussian blue and an elongated shape of a stem cell adjacent to an endothelial cell or a myocyte.

4.8.3 Migration of cells (I)

The infiltration range of the stem cells after injection was assessed in a light microscope. The site of the injection was defined by a star shaped cleft in the middle of the explant. Maximal infiltration range was measured from the centre of the injection site to the furthest cell at time points 24h, 48h, 72h and 7 days. A commercial computer program was used for quantification (Scientific Image Analysis, MCID_m4 3.0 Rev 1.1).
4.9 The porcine model (II, III)

A porcine model for acute myocardial infarction (AMI) had been created previously in our study group (Makela et al. 2007). In brief, CX was occluded for 90 min after thoracotomy in an experimental animal under general anesthesia. Both acute (study II) and chronic (study III) models were used to examine stem cell targeting (study II) and to evaluate efficacy of BMMC transplantation on cardiac function (study III)(table 9).

In both studies II and III, the stem cell isolation and labeling were conducted under general anesthesia before the experimental cardiac infarction. Biochemical samples were taken for analysis. Immediately after myocardial infarction and the intramyocardial BMMC transplantation delivered via a 26G needle, MRI was performed for cell targeting.

In an acute model (study II), the experimental animals were sacrificed after MRI and ex vivo MRI for the heart was conducted. After the MRI, the hearts were dissected and subdivided into 16 segments according to a standardized myocardial segmentation scheme for the histological stainings (Fig.7 in chapter 4.9.6.).

In the chronic study III, echocardiography was performed to measure cardiac function before and after myocardial infarction. In addition, MRI was conducted immediately after infarction and after 21 days to detect the transplanted BMMCs. After the animals were sacrificed, the hearts were removed for immunohistological stainings.

Table 9. Models of in vivo animal studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of pigs</th>
<th>Number of segments analysed</th>
<th>Time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study II</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Study III</td>
<td>31</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

4.9.1 Hemodynamic and temperature monitoring (II, III)

Anesthesia, cell isolation and labelling were performed as previously described. A pulmonary artery thermodilution catheter (CritiCath, 8 French [Ohmeda GmbH 6 Co. Erlangen, Germany]) was introduced via the right femoral vein into the central circulation in order for blood sampling, measuring the cardiac output, monitoring the pulmonary pressure, pulmonary capillary wedge pressure and central venous pressure as well as recording of the temperature of the blood. In order to monitor
arterial pressure and for blood sampling, an arterial line was placed into the right femoral artery. Heart function was assessed using electrocardiographic monitoring.

4.9.2 Functionality of transplanted stem cells (II)

The effect of SPIO labeling on the cellular proliferation rate was assessed by culturing control cells and labeled BMMCs and by analyzing them with the (3-(4,5-dimethylthiazol-2-yl)-2,5-)diphenyltetrazolium bromide assay (tetrazole, MTT, Sigma-Aldrich) according to the manufacturer’s instructions. Shortly, the MTT absorbance of the cells was measured at 1-7 days after labeling by spectrophotometric means.

4.9.3 Echocardiography (III)

A transthoracic echocardiography (Acuson Sequoia 512, Siemens, Munich, Germany) was performed at the baseline, 30 minutes after reflow and at 21 days. The thicknesses of the ventricular walls, EF, the LV mass and cardiac output were calculated using standard measurements.

4.9.4 Biochemical analysis (III, IV)

Systemic arterial and venous blood samples were taken at the baseline, 90 minutes after occluding CX and 30 minutes after the initiation of reperfusion. Blood gases, pH, electrolytes, serum ionized calcium, glucose, haemoglobin levels (i-STAT Analyzer; i-STAT Corporation, East Windsor, NJ), basic blood count, leukocyte differential count (Cell-Dyn analyser [Abbot, Santa Clara, CA]) and troponin I chain (TnI) (Immunoassay, Innotrac AIO, Turku, Finland) were measured. In addition, in study III the cytokine levels were determined at the baseline, after 24 hours of CX occlusion and after 21 days.

In studies III and IV, the cytokine levels were determined with Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories Inc.) according to the manufacturer’s instructions. Briefly, the sample was centrifuged (13300 g, 10 min), diluted and analysed. The results were automatically calculated with five-parameter logistic equations. High sensitivity range standard settings were utilized.
4.9.5 Myocardial infarction and cell transplantation (II, III)

The heart was exposed after left side anterolateral thoracotomy. CX was distal dissected and occluded using a silicone loop (Surg-I-Loop, Scanlan, St.Paul, Minnesota) during 90 minutes to cause myocardial ischemia. After removal of the silicone loop, papaverine (40mg/1mL) was administered into the CX to avoid vascular spasms. After setting the pleural tube in place, the chest cavity was closed. BMMCs were injected into five different locations within the infarcted area perfused by previously occluded circumflex coronary artery using a 26-gauge-needle.

4.9.6 Magnetic resonance imaging and analysis (II, III)

In vitro, in vivo and ex vivo MRI imaging was performed in study II. Only in vivo MRI imaging was conducted in study III. In study II, in vitro stem cell MRI was conducted using a 9.4T Oxford NMR vertical magnet (Oxford Instruments PLC, Witney, UK) and by using the same 1.5T MR scanner (GE, Signa Twinspeed, Milwaukee, USA) that was used for later in vivo and ex vivo imaging of the myocardium. All radiological evaluations were performed independently by a radiologist and a trained researcher blinded to both histological and clinical data. A cardiac segmentation scheme based on the coronary vascular territories (Fig. 7) was used for the visual assessment of the extent of the infarcted area and detection of the BMMC label with MRI. In the in vitro MRI in study II, 10^7 of SPIO labeled BMMCs were suspended into 1mL of agarose gel. Imaging at 9.4T, spectroscopic T1, T2, and T2* relaxation times were measured using a 10mm spectroscopic probe. The calculation of relaxation times and sequences have been presented in more detail in the original article. Relaxation times acquired at 9.4T were fitted with exponential relaxation equations using an in-house MATLAB script (MATLAB, Mathworks Inc., Natick, MA) and assuming mono-exponential decay. Signal intensity in 1.5T MR images was analyzed using a public domain program. A region of interest (ROI) with a surface of 0.5cm2 was manually drawn on each sample and control specimen in order to quantitatively characterize the signal intensity change due to the SPIO label. The signal intensities of labeled specimen were reported as a percentage (%) of signal intensities of the controls.

In the acute model (study II), in vivo and ex vivo MRI were performed immediately after the experimental infarction and BMMC transplantation. In the in vivo assessment, a MRI 4-channel cardiac coil and a 1.5T scanner were combined.
with ECG and respiratory gating simulating free breathing. Fiesta 2D CINE MRI was acquired in the vertical long axis, the horizontal long axis and the short axis orientations; these are presented in more detail in the original article. T1 short axis images were used when evaluating the extent of the infarcted area and stem cell label visualization using the standardized segmentation scheme (Fig.7). The extent of the acute ischemic myocardium was recorded as a number of segments affected. The change in the segmental signal intensity caused by the iron labels was qualitatively assessed as follows: (0) no change; (1) slight; (2) moderate; and (3), strong signal intensity change. Ford radiological-pathological correlation analysis, scores one and two were combined. In the ex vivo scan, the 1.5T MRI and a T2* and T2 FSE sequences were used. The relative percentage change of signal intensity loss caused by the iron label in each segment was quantitatively calculated by using a public domain program (Osirix, version 5.5). The signal intensity of spared myocardium was used as a reference. An ROI-based method was used to identify the segmental area in each slice in the systolic and diastolic phases. In the DE images, 0.2mmol/kg gadopentetate dimeglumine contrast agent (Magnevist, Bayer Heathcare) was injected via the left subclavian vein.

In in vivo pig study III, in vivo MRI, T1 CINE MR, perfusion, and delayed enhancement (DE) sequences were used. A. T1 CINE MR images were acquired in the vertical long axis, the horizontal long axis, and the short axis orientations. A gradient-echo planar inversion – recovery, multishot, T1-weighted images were obtained using a perfusion sequence. Segmented inversion recovery fast gradient echo images were obtained using a DE sequence. A dose of 0.2 mmol/kg of gadopentetate dimeglumine (Magnevist; Schering AG) was used as the contrast agent, and injected intravenously injected immediately prior to perfusion imaging. DE imaging was conducted 10- 15 minutes after the contrast administration. The parameters of the sequences have been described in detail in the original articles.

The change in contractility was assessed using short axis image and three step scaling systems: 1, hypokinesia; 2, dyskinesia; and 3, akinesia. The change in perfusion was visually observed and graded as either normal or abnormal. DE images were used in order to evaluate the extent of the infarcted area. In the evaluation of the EF, the GE Healthcare advantage workstation AW4.2 and analysis software (MASS v.5.2, Medis Inc.) were used with the data being presented as mean values from two independent readings.

Stem cell targeting based on the hypointense area in T1-weighted short axis images. In study III, a three-scale classification based on a qualitative analysis of the positive finding and detected signal intensity change caused by the iron labeled
stem cells was used as follows: 1, slight; 2, moderate; and 3, strong. For data analysis, the first two values were combined to allow for a more direct comparison with the histological quantitative analysis.

Fig. 7 Segmentation of the left ventricle.

4.9.7 Histopathology (II, III)

After sacrifice of the experiment animal with an intravenous injection of pentobarbital (60mg/kg) and the ex vivo MRI (in study II), the hearts were fixed in 10% formaldehyde. The heart was divided according to the specific segmentation scheme based on the coronary vascular territories, similarly to the MRI evaluation. The left ventricle was divided into 16 segments that were embedded in paraffin, sectioned (5 µm) and deparaffinised prior to staining (Fig. 7 in chapter 4.9.6).

Histological evaluation with H&E and Prussian blue stains were conducted as described in chapter 4.8.1. The morphology of the myocardial tissue was evaluated using H&E staining by an experienced pathologist. The injury area on each segment was assessed as follows: 0=normal tissue, 1=granulation tissue, 2=necrotic areas and 3=fibrotic areas.
Prussian blue stain was used in the detection of SPIO labeled transplanted BMMCs as described previously in this thesis. The number of the BMMCs on each section was qualitatively evaluated with the following scoring: 0=no BMMCs 1= small amount of the BMMCs and 2= large amount of the BMMCs. In the more detailed analysis, the proportional area of BMMCs on each segment was quantified using a commercial computer program (Scientific Image Analysis, MCID_m4 3.0 Rev 1.1).

4.10 Clinical model (IV)

The behaviour of the injected MSCs in myocardium and the effect of the transplantation of the stem cells on EF and on the inflammation process was measured in studies I-III. The clinical model was used to evaluate the stem cell therapy in patients in addition to our in vitro and in vivo animal models. Study IV is a sub-study of the previously reported FINCELL study which concentrated on the evaluation of the remodeling process after AMI.

Patients with ST-elevation myocardial infarction (STEMI) were randomly assigned in a 1:1 ratio to have either BMMC or placebo treatment. Bone marrow aspiration, cell collection, and cell preparation were performed 40-77 hours after the onset of symptoms. PCI was considered as a baseline time point. The response to stem cell therapy was evaluated by measuring the changes in the cytokine profile and the improvement in cardiac function.

4.10.1 Stem cell transplantation

PCI was performed on the infarct area supplied by the culprit coronary lesion. PCI was performed with implantation of paclitaxel drug-eluting stents in all patients and the medium containing either the BMMCs or placebo medium was administered via the intracoronary route by using intermittent balloon inflation in the stent.

4.10.2 Cardiac function

Cardiac function was evaluated using the left heart ventricular angiography at baseline and 6 months later. The left ventricular volume and EF were calculated using the biplane area-length method; the left ventricular outflow tract was included in the measurements (Yli-Mayry & Huikuri 1993).
4.10.3 Biochemical analysis

Blood sampling was performed in order to analyse the increase in the TnI levels and the cytokine profile. The TnI concentration in the plasma samples was determined at 2 and 4 days after the onset of symptoms. The serum samples from baseline i.e. within PCI, 2 days post-PCI, and 4 days post-PCI were used in the cytokine measurements.

The serum was stored at -20°C until the cytokine analysis. The plasma samples were collected in ethylenediamine tetra-acidic acid tubes on ice and immediately centrifuged, and the plasma was stored at -70°C until analysis. An Innotrac Aio! analyser (Innotrac Diagnostics) was used to determine the concentration of TnI from plasma samples. The biochemical analysis of the cytokines was described in more detail in chapter 4.9.4. and in the original manuscripts.

The cytokine balance was evaluated in clinical study IV. The sum of the pro-inflammatory cytokines and anti-inflammatory cytokines was calculated at baseline and then at 2 days post-PCI, and 4 days post-PCI and the percentage change from baseline to 2 days post-PCI and from baseline to 4 days post-PCI was measured. The correlation between the change in anti-inflammatory and pro-inflammatory cytokines was evaluated with Kendall’s tau correlation coefficient.

4.11 Statistical analysis

Statistical analysis was performed using the SPSS statistical program (SPSS SmartViewer version 22.0, SPSS, Inc.) and SAS (version 9.2, SAS Institute Inc. NC). Student’s t test or Mann-Whitney U test were used to assess the distribution of variables between the study groups. The SAS procedure “Mixed” was used for repeated measurements. Cohen’s kappa coefficient was used to measure the agreement between two raters and a value of 0.6 was considered as significant finding. All results are presented as mean and standard deviation. Reported p values are as follows: p difference between the groups and p(t*g) indicates a group-time interaction. A p-value less than 0.05 was considered statistically significant. In study IV, Kendall’s tau was used to measure the correlation between two rates; a value of 0.6 was considered a significant finding.
4.12 Ethical considerations

The animals were maintained and the procedures were performed in accordance with the European Convention for the protection of vertebrate animals being used for experimental and other scientific purposes (ETS 123, Council of Europe 2006) and the European Union recommendation on the guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). The study was approved by the Protocol Authorization Committee of Finland (project license ESLH-2007 09818/YM-23).

All of the study protocols were designed according to the principles of the 3Rs: Replacement, Reduction and Refinement. These principles were developed in 1959 as a framework for humane animal research to conduct high quality science and to develop alternative approaches to the use of experimental animals (Parker & Browne 2014).

The aim of study I was to monitor the migration and behavior of the transplanted stem cells in the myocardium. A cardiac explant culture model was created to measure these issues in vitro instead of utilizing an in vivo animal model. In the model devised here, the tissues were taken from animals that were not killed solely for this purpose, which is in line with the Replacement principle. In addition, in the cardiac explant culture model, dozens of explants were obtained from the heart of one single experimental animal and this reduced the number of animals used per study.

In the in vivo animal studies II and III, well-established methods which minimized suffering and improved pig welfare were applied according to the Refinement principle. The anesthesia protocol was carefully developed to minimize the pain and suffering of the experiment animals through the protocol. The optimal anesthesia before, during and after the operation was provided by the researchers and the well-being of the experiment animals was monitored daily by the staff of the Laboratory Animal Centre. By applying the acute model in study II, it was possible to minimize any lasting harm to the animal but still to gather all of the necessary data for the purposes of this study. In addition, the living conditions of the animals were comfortable as mentioned in chapter 4.1.
5 Results

5.1 Study I

5.1.1 No signs of cardiac myocyte apoptosis in the cardiac explant culture model (I)

Both the control group and group 1 explants were transported into 24-well plates and group 2 explants were set into the inserts of the Millicell-96 Cell Culture Insert Plate.

Control group and group 1

There was no difference in the myocardial histology between the control group and group 1. The structure of the myocytes was well preserved in both groups after 7 days in the culture and PTAH staining revealed organized actin filament structures at all time points. On the contrary, after 7 days’ degeneration, swelling and apoptosis of the endothelial cells located in the center of the explants were found. At this time point, half of the biopsies in the control group and all the biopsies in group 1 were also surrounded by spindle-shaped cells which were positive for α-SMA but negative for desmin and PECAM-1. In addition, there was budding of PECAM-1-positive endothelial cells and capillaries at the marginal zone of the explants at the 7 day time point in the culture.

Group 2

In group 2, there was variable and often extensive extracellular edema and degeneration of the myocytes in the explants after 7 days in culture. There was also vacuolization, edema and degeneration of endothelial cells, even total destruction of the capillaries. No proliferation of spindle-shaped cells or capillaries was observed in the marginal zone at the 7 day time point.
5.1.2 The migration range of the injected stem cells increases over time (I)

After injection into the cardiac explant, MSCs often remained in aggregates but also single cells were observed in group one. MSCs located in slit-like spaces and some of them could be identified as capillaries using PECAM staining. Injected MSCs were found outside the initial injection site and the migration range increased over the course of time. The migration range differed significantly between the time points of 2 hours (mean 1193 µm, S.D 288µm) and 7 days (mean1384 µm, S.D 255 µm; p=0.02). The migration range of Dil labeled MSCs was not able to be measured due to technical challenges. Therefore, only SPIO labeled MSCs were used in the evaluation of the range of the migration.
In group 2, MSCs were placed on the surface of the explant. No significant migration occurred into the deeper part of the explant. After 7 days, 85% of the DiI labeled stem cells were still on the surface (Fig. 8).

5.1.3 MSCs in close contact with myocytes and endothelial cells (I)

Both SPIO and DiI labeled MSCs that were injected into the center of the cardiac explants changed their morphology. They became elongated and flattened and became localized in a parallel manner along capillary endothelial cells or myocytes already after 2 hours from the injection. The number of adherent cells increased during the follow-up period. Between the time points of 2 hours (mean 1.43 S.D
1.342) and 7 days (mean 4.00 S.D 0.816), there was a statistically significant (p < 0.05) difference in the number of the SPIO labeled stem cells in contact with the endothelium or myocytes

5.2 Study II

5.2.1 Proliferation of the transplanted BMMCs

No significant difference was detected in vitro between the transplanted SPIO labeled BMMCs and controls in the proliferation rate. However, 7 days after the initialization of the culture, a statistically significant difference (p<0.001) was detected in the proliferation rate between the cells labeled by electroporation and the control cells.

5.2.2 In vitro MRI results

In vitro MR analyses showed relaxation value alteration at 9.4 T in accordance with the iron content of the samples. In the 1.5T in vitro imaging, the T2W FSE images provided the clearest reduction in observed signal intensity between labeled cells and unlabeled controls. In the roller bottle labeled (0.2 mg Fe/mL) group, there was an 89±4% signal reduction in the T2W FSE images but only a 60±7% reduction in signal intensity was detected in the electroporation group.

5.2.3 Transplanted SPIO labelled BMMCs in histology

All the cells adhering to the plastic surface displayed significant intracellular iron granules after 1h of labeling. After transplantation, the BMMCs were mainly localized in slit-like spaces within myocyte bundles or inside vessels and remained in aggregates. Single cells were also observed, with some of them being located between the myocytes. Some of BMMCs had become elongated and flattened; these were situated along the capillary endothelial cells and/or myocytes in a parallel fashion. BMMCs were detected on the segments supplied by CX (the site of injections) in 2 to 4 segments out of 5 segments on each pig.
5.2.4 The function of the heart in an acute infarction pig model (II)

In the in vivo MRI analysis, the mean EF measured with MRI was 51.9 ±5.0 percent in all animals. Delayed enhancement and abnormal perfusion indicative of myocardial damage was detected in all pigs in 4 segments (segments 5, 6, 11, 12,) supplied by the occluded circumflex coronary artery.

5.2.5 Stem cell label visualisation in the in vivo MR images (II)

In the qualitative MR analyses, an intramural area of signal loss throughout the whole Cine MRI sequence cardiac cycle was determined to reflect the presence of SPIO labeled cells; this was detected in one to four cardiac segments supplied by the CX in each animal, corresponding to the histological evidence. The detected signal loss in the intramural area was also present in the ex vivo MRI evaluation at the same locations. In the qualitative histological analysis, all scores were observed in each animal. The qualitative MRI and histological analyses concurred in 5 out of 6 cell containing segments. In the non-concurring segment, a strong label content was observed in MRI while in histology, the amount of cells was small although it was observed in two out of the three stained sections that in terms of volume corresponded to the single MRI slice.

In the quantitative in vivo MRI analyses, 24-64% signal intensity loss was detected in the SPIO containing segments. In the segments where no label was observed in the visual MRI analysis, the measured signal intensity change varied from a 1% loss to a 3 % increase. In the histological evaluation, in the segments containing BMMCs, the percentage of the surface area covered by BMMCs varied from 0.002 to 0.927%.

5.2.6 Spatial correspondence of stem cells on MRI and histology (II)

Each of the in vivo MRI slices corresponded to three of the stained histological sections. Hence, when comparing segmental signal loss in MR image and sectional staining in histology, the highest measured histological value within the area was recorded. Both segmental quantitative and qualitative analysis of spatial correspondence between the in vivo MRI and histology showed 100% sensitivity and 100% specificity (Fig.9).
Fig 9 Spatial correspondence of SPIO labeled cells between MRI and histology. The relative change in signal intensity loss on MRI caused by Fe labelled stem cells (a) correlates with surface ratio of cells on each segment seen in histology (b). (Article II).

5.3 Study III

5.3.1 Comparability of the study groups (III)

Two pigs in a stem cell group and one pig in the control group died during the experiment and these were excluded from the analysis. The weight of the pigs did not differ between the two study groups. At the baseline, the mean diastolic pulmonary artery pressure and diastolic artery pressure differed significantly between the study groups. Otherwise, hemodynamic, temperature and metabolic baseline values were similar in all groups. At the baseline, the cardiac function was similar in the stem cell group and in the control group animals.

5.3.2 Hemodynamic and metabolic data (III)

After infarction, there was a release of troponin in both groups, indicating that there had been a critical-sized heart injury. The concentration of this heart enzyme was similar in both study groups pre- and postoperatively. During the experiment, hemodynamic and metabolic variables exhibited no significant differences between the study groups over time (table 10). During 90 minutes of CX occlusion, pulmonary artery diastolic and systolic pressures increased and mean arterial
pressures decreased in both groups. After occlusion, cardiac output decreased in stem cell and control groups.

Table 10. Hemodynamic values obtained in the in vivo pig model study. No statistical differences between the study groups. (Table from article III).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Study group</th>
<th>Weight (kg)</th>
<th>Heart rate (bpm)</th>
<th>MAP s (mmHg)</th>
<th>Cardiac output</th>
<th>PAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Control</td>
<td>26.1</td>
<td>110</td>
<td>121/79</td>
<td>3.5</td>
<td>21/13</td>
</tr>
<tr>
<td></td>
<td>Stem cell</td>
<td>27.5</td>
<td>108</td>
<td>110/66</td>
<td>3.1</td>
<td>19/11</td>
</tr>
<tr>
<td>CX occlusion</td>
<td>Control</td>
<td>115</td>
<td>108/63</td>
<td>110/63</td>
<td>3.4</td>
<td>22/13</td>
</tr>
<tr>
<td></td>
<td>Stem cell</td>
<td>118</td>
<td>103/59</td>
<td>110/63</td>
<td>3.2</td>
<td>22/12</td>
</tr>
<tr>
<td>Reflow 30min</td>
<td>Control</td>
<td>127</td>
<td>98/56</td>
<td>110/63</td>
<td>3.0</td>
<td>21/13</td>
</tr>
<tr>
<td></td>
<td>Stem cell</td>
<td>132</td>
<td>103/63</td>
<td>110/63</td>
<td>2.9</td>
<td>20/12</td>
</tr>
</tbody>
</table>

CX= arteria coronary circumflex; MAP=Mean Arterial Pressure; PAP=Pulmonary arterial pressure

5.3.3 Inflammation process and differences in the cytokine levels (III)

During the whole experiment, there were no significant differences in white blood cell counts between the study groups as a function of time: lymphocytes $p(t^*g)=0.798$, monocytes $p(t^*g)=0.229$ and neutrophils $p(t^*g)=0.346$.

The change in the cytokine levels differed between the study groups at the time points 24 hours and 21 days but the difference was not statistically significant. There were increases in the concentrations of the cytokines eotaxin, granulocyte-colony stimulating factor (G-CSF), MIP-1β, VEGF and IFNγ in the stem cell group but these declined in the control group at 24 hours of infarction. The PDGF level decreased considerably in the control group and slightly also in the BMMC group (control group mean -554 vs BMMC -36 $p=0.08$).

A major increase in the IL-1ra level appeared in the BMMC group at 21 days (mean control group +59 vs. BMMC group 211 $p=0.52$). The PDGF level increased in the BMMC group whereas the PDGF concentration decreased in the control group (mean control group -315 vs. BMMC +363 $p=0.08$). A major change in concentrations was also observed in two cytokines, G-CSF and IFNγ. The levels of these cytokines increased in BMMC group (mean G-CSF +92.0 vs control -10, IFNγ +165 vs. control group -411).
5.3.4 Morphology of the cardiac tissue after infarction (III)

In both groups, mainly fibrosis but also granulation tissue and necrosis were observed in an area of 5 segments supplied by CX. There were a large number of inflammation cells in the area with the myocardial injury. Cartilage was observed in one stem cell group animal and in one control group animal in an area of one segment.

5.3.5 Abnormal kinetics of the heart (III)

MRI revealed abnormal kinetics in segments supplied by CX in every stem cell group experimental animal at both two hours and 21 days of infarction. Myocardial hypokinesia or akinesia was detected from four to five segments (total of 5) at two hours after infarction and from 0 to 5 segments (total of 5) on the 21st postoperative day. Abnormal kinetics were compared to the results of the histological analysis of myocardial injury (granulation tissue, necrosis and fibrosis) on each segment but the abnormal kinetics did not correspond to the histological changes (Cohen’s kappa <0.5).

5.3.6 Changes in cardiac function (III)

The cardiac function was similar in both study groups at the baseline ($p=0.8$, mean EF control group 70%, SD 8 vs. mean EFBMMC 70%, SD 7). Two hours after CX occlusion and myocardial infarction, EF decreased similarly in both groups (mean control group EF=47%, SD 13 vs. BMMC group EF=52%, SD 10). There was no statistically significant difference in EF between the study groups at that time point ($p=0.30$). The mean fractional shortening (FS) in the control group was 24% SD 8 which was very similar to the value in the BMMC group, 26% SD 7 ($p=0.35$).

After 21 days, EF had significantly improved in the BMMC group (mean control group EF=53% SD 9 vs. BMMC EF=65%, SD 14 $p=0.02$). The EF improvement in the stem cell group was 14 percentage points whereas in the control group it was only 7 percentage points ($p=0.30$). FS differed between the study groups: control group 27% SD 6 and BMMC 40% SD 14 ($p=0.018$). The difference in FS between the groups at time points of baseline vs. 21 days was statistically significant (mean control -14 SD 9.9 vs. BMMC 1 SD 14 $p=0.011$).
5.3.7 MRI corresponds with histology in BMMC targeting (III)

Histological evaluation

LV was divided into 16 segments with 5 of them being supplied by the occluded CX (segments 5, 6, 11, 12 and 16). In the histological analysis, SPIO labeled BMMCs were detected in the infarction area. In a semi-quantitative analysis, either small or large numbers of transplanted BMMCs were detected in at least two segments in each BMMC treated animal.

MRI data

At two hours after CX occlusion, MRI detected BMMCs on an area of three segments that were the same as those found in the histological evaluation (segments 6, 11, 12). Cohen’s kappa coefficient was used to estimate the correspondence between histology and MRI in targeting BMMC. In segments 6 and 12, Cohen’s kappa coefficient was 0.57. By combining three or four adjacent segments (7 combinations), the kappa coefficient was elevated to 0.667.

It was not possible to detect BMMCs on the 21st postoperative day due to changes in the tissue and insufficient visualization of the cell label.

5.3.8 Improvement in EF correlates with the number of transplanted BMMCs in the myocardium (III)

EF was measured using MRI at 2 hours and at 21 days. The absolute difference in EF between the time points was 18% and 11 percentage points that was kept as cutoff point to represent recovery. Table 11 shows the proportional area of BMMC on the different segments. The proportional area of BMMCs on the segments supplied by the CX varied from 0.0 to 60.6%, in the histological quantification. The median value of the measured segments was higher in those animals which displayed an EF increase greater than 11 units. BMMCs were localized in a wider area both in histological and in the MRI analysis (≥3 segments in histology, ≥2 segments in MRI).
Table 11. Proportional area of transplanted BMMCs on segments and correspondence to change in EF.

<table>
<thead>
<tr>
<th>EF Increase</th>
<th>Number of quantified segments</th>
<th>Seg.5</th>
<th>Seg.6</th>
<th>Seg.11</th>
<th>Seg.12</th>
<th>Seg.16</th>
<th>Seg median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above mean</td>
<td>5</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.7</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Above mean</td>
<td>5</td>
<td>60.0</td>
<td>0.3</td>
<td>6.5</td>
<td>29.1</td>
<td>0.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Under mean</td>
<td>5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.8</td>
<td>15.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Under mean</td>
<td>5</td>
<td>0.3</td>
<td>0.7</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

EF=Ejection Fraction, mean increase 18% and 11 percentage points was kept as cutoff point. (Article III)

5.4 Study IV

5.4.1 Patients

Study IV is a sub-study of the previously reported FINCELL study, in which 522 patients were assessed for eligibility and 80 patients were included into the trial (Huikuri et al. 2008b). From the original study, a total of 26 patients (control group, n=12; BMMC group, n=14) were included into study IV in which the only inclusion criterion was an equal time delay from onset of symptoms to PCI and the injection. A detailed characterisation of the patients has been provided in the original article (Huikuri et al. 2008). The mean age of the patients was 59 ±13 years in the control group and 60±10 years in the BMMC group. The mean time delay from the symptoms to PCI was 56 hours (control group, mean 59±10; BMMC group, 52±12). The mean number of mononuclear cells injected was 461x10⁶. The mean number of CD34+ cells was 3.3x10⁶. The two study groups were well matched with regard to their characteristics at baseline.

5.4.2 Troponin release increased

The release of troponin into the plasma was determined at the onset of symptoms, and at 2 and 4 days after the onset of symptoms. Mean troponin concentration increased in both study groups with no statistically significant difference being observed between the groups.
5.4.3 No difference in ejection fraction

Left ventricular angiograms for the 11 control group patients and 13 BMMC group patients were available at baseline and at 6 months. The mean absolute change of the global LVEF was individually measured for the patients. The change was higher in the BMMC group although the difference was not statistically significant (placebo 1.1 SD 10.2 vs. BMMC 9.5 SD 16.0; \( p=0.15 \))

5.4.4 Stem cell transplantation balances the inflammation process

There was no difference between the study groups in the cytokine levels at the baseline. The levels of the pro-inflammatory cytokine, such as IL-6, varied widely among the patients (Fig.10). At 2 days after PCI and injection, IL-6 levels varied from 1.12 to 11.54 pg/mL in the control group and from 0.96 to 11.42 pg/mL in the BMMC group. Similarly extensive variability was observed with respect to the concentrations of the anti-inflammatory cytokines. At 2 days after PCI and injection, IL-10 concentrations varied from 0.82 to 82.5 pg/mL in the control group and from 0.99 to 5.76 pg/mL in the BMMC group. Thus, there were huge variations in the baseline cytokine levels and thus it was not possible to observe any visible or statistically evident trend between the groups.

The correlation was measured between the percentage changes in the levels of anti-inflammatory cytokines (IL-4, IL-10, IL-13) and in pro-inflammatory cytokines (IL-6, TNF-\( \alpha \), IL-1b, IL1-ra, IFN\( \gamma \)) from baseline to 2 days after PCI and injection and from baseline to 4 days after PCI and injection. A correlation was observed in the percentage change of the anti-inflammatory and pro-inflammatory cytokine concentrations in both study groups at 2 days after PCI and injection (placebo group, Kendall’s tau 0.6, \( p=0.01 \); BMMC, Kendall’s tau 0.7, \( p=0.001 \))(Fig.12 in chapter 5.5). At 4 days after PCI and injection, there was a clear correlation between the anti-inflammatory and pro-inflammatory cytokines in the BMMC group (Kendall’s tau 0.7 \( p<0.001 \)) but not in the control group (Kendall’s tau 0.3 \( p=0.17 \)).
Fig. 10. Cytokine levels were determined at baseline, at two days and at four days. Due to the major variability in individual cytokine concentrations among patients, no apparent trend could be observed. (Figure from article IV)

5.5 Comparison of the results obtained from studies I-IV

Table 12 shows and compares the main results of studies I-IV. The mechanism behind stem cell therapy was assessed using different study models namely *in vitro*.
study, in vivo animal studies and a clinical study. It is interesting that there are many similarities in the results despite the major differences in the study approaches.

The behavior of cells after injection into myocardium was assessed using histology in in vitro study I and in the in vivo pig model. In both models, the transplanted cells remained as aggregates but also single cells were visualized. The in vitro study revealed also the increased range of migration and the higher number of cells in contact with other cells over time which was possible to be evaluated by determining more time points.

EF and troponin levels were evaluated both in the in vivo pig model (study III) and in clinical study (study IV). Troponin levels did not differ between the study groups in either the pig model or in the clinical study. The EF showed a trend to being improved in BMMC group in pig model and in clinical study. In the pig model, the difference reached statistically significance (mean control group EF=53% SD 9 vs. BMMC EF=65%, SD 14 $p=0.02$).
Table 12. Comparison of results obtained from *in vitro*, *in vivo* and clinical studies.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result in the <em>in vitro</em> study</th>
<th>Result in the <em>in vivo</em> pig study</th>
<th>Result in the clinical study</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSC migration</td>
<td>The migration range increased over time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSC adhesion</td>
<td>The number of MSCs in contact with other cells increased over time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behaviour of cells after injection to myocardium</td>
<td>Remained in aggregates, also single cells</td>
<td>Located in slit-like spaces</td>
<td></td>
</tr>
<tr>
<td>BMMCs targeting</td>
<td>Correspondence between MRI and histology</td>
<td>Sensitivity 100%</td>
<td></td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>Infarction decreased at baseline</td>
<td>Trend to improvement at 6 months in BMMC group</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMMC improved (53% vs. 65%) at 21 days</td>
<td>No statistical significance</td>
<td></td>
</tr>
<tr>
<td>Troponin</td>
<td>No difference between study group</td>
<td>No difference between study group</td>
<td></td>
</tr>
<tr>
<td>Change in anti-inflammatory cytokines</td>
<td>No difference between study groups</td>
<td>No difference between study groups</td>
<td></td>
</tr>
<tr>
<td>Change in pro-inflammatory cytokines</td>
<td>different trend in IFNγ and TNF-α</td>
<td>No difference between study groups</td>
<td></td>
</tr>
<tr>
<td>Correlation between anti-inflammatory and pro-inflammatory cytokines</td>
<td>Clear trend in BMMC group</td>
<td>Possible correlation existed in BMMC group</td>
<td></td>
</tr>
</tbody>
</table>

MSC= Mesenchymal stem cell; BMMC=Bone marrow mononuclear cell; IFNγ=Interferon-γ; TNF-α=Tumor necrosis factor-α

The cytokine profile was determined in the *in vivo* pig model (study III) and in the clinical study (study IV). In the pig model, the cytokine levels were measured immediately after AMI and cell injection (i.e. baseline) and then at 24 hours and at 21 days later (Fig.10). In clinical study, the measurement was performed at baseline and at two and four days after PCI; the time when the BMMCs were injected was
considered as baseline in this experiment. In both models, cytokine levels varied widely among the patients and the pigs. Although no statistically significant differences appeared in the pig model, there was a tendency that the absolute cytokine levels differed between the study groups. At 24 hours, the trendline of the pro-inflammatory IFNγ differed between the study groups (controls -260 SD 576 vs. BMMC +5 SD 38 (p=0.66). In the clinical model, no statistically significant difference between the study groups or any visible trend could be observed in any of the single cytokine levels at any of the time points.

The correlation between the percentage changes in anti-inflammatory cytokines (IL-4, IL-10, IL-13) and in pro-inflammatory cytokines (IL-6, TNF-α, IL-1b, IL1-ra, IFNγ) was observed in the in vivo pig model and in the clinical study as shown in figures 11 and 12. In the clinical study, a clear correlation between anti-inflammatory and pro-inflammatory cytokines was detected in the BMMC group (Kendall’s tau 0.7 p<0.001) but not in the control group (Kendall’s tau 0.3 p=0.17) at four days. A similar correlation between the anti-inflammatory and pro-inflammatory cytokines was observed in BMMC treated pigs after AMI in study III (Fig. 11). However, due to the small number of animals in this analysis, a larger study will be required to confirm this finding.
Fig. 11 Correlation between anti-inflammatory and pro-inflammatory cytokines at 24 hours in the in vivo pig model study.
Fig. 12 Correlation between anti-inflammatory cytokines at 2 days and at 4 days after cell transplantation in the clinical study IV. (Figure from article IV)
6 Discussion

6.1 The main findings of the study

The focus of this thesis was to examine the mechanisms behind the effect of the stem cell therapy on infarcted heart tissue. Different kinds of methodologies can be used when employing in vitro approaches. The strength of this study is that it has combined in vitro, in vivo and clinical studies; this diverse approach can produce more detailed information about the mechanisms functioning in stem cell therapy. The first study investigated stem cells in their microenvironment in the myocardium after their injection. Study I examined the behaviour of MSCs in myocardium after intramyocardial stem cell injection. This helped to find the optimal cell transplantation method that was used in the subsequent in vivo animal studies. The in vivo animal studies II and III concentrated on cell targeting and on the beneficial effects of stem cell therapy. Study II demonstrated that MRI was a reliable method for monitoring cell targeting in the acute phase after an AMI. The number of transplanted cells corresponded with the improvement in EF after AMI according to study III. In the clinical study IV, BMMC transplantation was associated with a preserved balance between pro- and anti-inflammatory cytokines after STEMI in PCI treated patients.

A cardiac explant culture model was created in order to elucidate the behaviour of stem cells in cardiac tissue after injection. The cardiac explants were grown in culture for up to 7 days. The model simulated tissue stress and proved to be a feasible method for studying the effects of stem cells in the myocardium in vitro. MSCs injected in the centre of the cardiac explant showed active movement and adhesion capabilities in the tissue.

Study II evaluated the reliability of MRI in targeting the injected stem cells in myocardium after AMI. In a pig model, AMI was produced by occluding the CX and the localization of SPIO labeled BM-MCs was detected with both MRI and histology. A high spatial correlation between the findings from MRI and histology was observed; this demonstrated the feasibility of using MRI in targeting labelled cells within cardiac tissue.

Study III concentrated on the beneficial effects of stem cell transplantation. In a chronic AMI pig model, BMMC injection after AMI was observed to improve cardiac function. The quantity of transplanted BM-MCs correlated with the extent of the improvement. In addition, the study detected differences in the cytokine
levels between the study groups, evidence that the beneficial effect of stem cell transplantation may be result of a paracrine effect.

Study IV was a sub-study of the previously published FINCELL study that revealed increased cardiac function in BMMC treated patients after STEMI and PCI. Here, it was demonstrated that stem cell transplantation exerted a balancing effect on the anti-inflammatory and pro-inflammatory cytokine levels and remodeling. This may partly explain the favorable effect of stem cell transplantation after AMI.

6.2 The cardiac explant culture model (I)

Direct intramyocardial injection is one of the most widely used delivery methods in stem cell therapy (Sheng et al. 2013). Even though this approach has been used in the clinic, the behaviour of injected cells has not been studied in detail in vitro. Therefore, the cardiac explant culture model was developed to obtain novel data about the localization, migration and possible adhesion of the injected MSCs in myocardium. After the optimal size of the explant was determined in pilot experiments, a suitable culture medium as well as the number and concentration of the MSCs were optimized.

The cardiac explant culture model provides mechanistic data that is difficult to obtain from human studies. The survival of myocytes in explant culture for 7 days was longer than expected. There are no similar cardiac explant culture models that would provide data revealing myocyte apoptosis; this highlights the novelty and significance of these results. It was found that the structure of myocytes was well preserved and they were no signs of apoptosis. In culture, myocytes enter into a dormant state where they remain viable. In this dormant state, the myocytes are not able to contract and this diminishes their energy consumption. This state is analogous to the in vivo myocardial tissue reaction which has been observed by MRI and ultrasound techniques (Bodi et al. 2010). Once the myocytes enter a dormant state, the endothelial cells have the highest metabolism in the myocardium. Degeneration, swelling and apoptosis of the endothelial cells were observed in the centre of the explants where there was insufficient diffusion of nutrients. However, diffusion of nutrients was adequate around the marginal zone of the explants and this was responsible for budding of endothelial cells and capillaries.

The second sign of the regeneration ability of the tissue and one of the most interesting observations in the cardiac explant culture model was the spindle-shaped cells that appeared at the explant periphery. These spindle-shaped cells were
interpreted as being myofibroblasts. Myofibroblasts are cells which are essential for wound healing and remodeling (Yong et al. 2015). According to previous in vitro studies, the extracellular matrix stiffness and mechanical strain of myocardium exert an effect on the differentiation of the cardiac fibroblasts into myofibroblasts. The preparation of cardiac explants poses a similar mechanical strain that induces the differentiation of myofibroblasts. In conclusion, cardiac explants are adaptive and can be cultured.

Although the cardiac explant culture model simulates tissue stress, it has certain limitations, such as the lack of inflammatory cells and the poor perfusion. Inflammatory cells and cytokines have a crucial role in the remodeling process in the myocardium (Frangogiannis 2014). Remodeling changes the architecture of myocardium, leading to the formation of granulation tissue and fibrosis within a few weeks (Zamilpa et al. 2014). It is likely that perfusion and contraction of myocytes would have an effect on the migration range of injected MSCs. On the other hand, the lack of perfusion and contractility highlights the true migration potential of MSCs after injection into myocardium.

As a conclusion, the cardiac explant culture model proved to be a unique model with which to study the effect of injected stem cells in their microenvironment in cardiac tissue and furthermore, it produced data that will be valuable in the future development of stem cell therapy.

6.3 Behaviour of the stem cells after injection to cardiac tissue

In study I, MSCs that were injected into the centre of the explant often remained in aggregates but also single cells were observed. The same phenomenon appeared in BMMCs after intramyocardial injection in an acute myocardial infarction pig model in study II. The cardiac explant culture model made it possible to follow-up the migration range of the injected stem cells; this would not have been possible in an in vivo model. The migration of the BMMC has not been studied previously in in vitro studies using myocardial tissue explants. It was somewhat surprising that, with time, the injected MSCs managed to migrate away from the initial injection site even though the cultured explants were lacking perfusion and myocyte contractile properties. There was a statistically significant difference in the migration range between the time points of 2 hours and 7 days. The migration of stem cells after injection into the damaged area is a crucial key step in allowing stem cells to participate to tissue repair. Stem cells injected into an infarction site need to be able to migrate throughout the infarction area and to modulate
inflammation or to transmigrate into the myocytes (Faiella & Atoui 2016). The migration of stem cells is under intensive investigation and some important regulatory factors have been described, including chemoattractants, growth factors and receptors (Li & Jiang 2011). Previously, it was noted that a considerable number of stem cells could migrate spontaneously in the absence of any migratory stimulus in the *in vitro* migration assay in support of the results of the present study (Laupheimer *et al.* 2014). That study revealed also that a combination of SDF-1 and adenosine triphosphate, that is released in response to ischemia, elicited strong migration of nucleated bone marrow cells. In a recent study, substance P was shown to mediate the migration of MSC-like ST2 cells (Dubon & Park 2016). In addition, there are reports that TNF-α and G-CSF can increase the migration and mobilization of MSCs (Fu *et al.* 2009, Hemeda *et al.* 2010, Xiao *et al.* 2012, Zhang *et al.* 2010a). In the future, it would be interesting to develop a cardiac explant culture model to study migration of stem cells after incubation with certain regulatory factors.

The ability to adhere is a sign of stem cell viability after injection to myocardium (Lee *et al.* 2015). However, the stem cell adhesion after intramyocardial injection is difficult to study in clinical settings. Thus, the cardiac explant culture model is a very useful way to study stem cell adhesion after injection and to observe the number of adherent cells over the course of time. In study I, injected MSCs were found to be in close contact with myocytes and endothelial cells already 2 hours after injection. In addition, the number of adherent stem cells increased over time. Consistent with these findings, Steingen *et al.* (2008) demonstrated that the transmigration of MSC underneath the endothelial layer occurred within 240 minutes. Furthermore, TNF-α appears to increase the number of adherent MSCs (Xiao *et al.* 2012). TNF-α is a pro-inflammatory cytokine released from injured tissues which is known to contribute to the remodeling process after a myocardial infarction. In the *in vivo* study III, the change in cytokine levels at two time points, 24 hours or 21 days, did not significantly differ between the BMMC group and the control group. However, some studies have demonstrated that MSCs can secrete TNF-α which suggests that possibly they have the ability to regulate their adhesion capabilities (Kinnaird *et al.* 2004b). Preconditioning using hypoxia, pre-treatment and the use of genetic modification to overexpress adhesion signals have been shown to increase MSC adhesion (Lee *et al.* 2015).

Behaviour, migration or adhesion of transplanted stem cells in myocardium after AMI have not been studied in detail in clinical studies, possibly due to the lack of suitable methods. The present results in pigs demonstrated that MSCs adapt to
the new microenvironment after injection into the cardiac tissue. There was an increased number of transplanted cells in contact with other cells and an extensive migration range was revealed in this cardiac explant culture model that simulates tissue stress. A similar behaviour of transplanted BMMCs was observed in myocardium after AMI also in the in vivo pig model in study II. This suggests that transplanted stem cells adapt and migrate away from the initial injection site also when used in a therapeutic manner in pigs. It is possible that transplanted stem cells would behave similarly in humans although this proposal is still somewhat speculative. Further studies will be needed to clarify how cytokines and growth factors can regulate stem cell adhesion and migration. The cardiac explant culture model would represent one feasible way to study adhesion of MSCs in myocardium, for example, after preconditioning, or following some form of pre-treatment or even the use of genetic modification of MSCs.

**6.4 Cell targeting using MRI**

An effective, non-invasive and non-toxic technique for cell tracking would be advantageous for monitoring the in vivo behaviour of implanted MSCs or BMMCs and for understanding the fate of these cells. MRI has been proposed as the gold standard for the in vivo monitoring of SPIO-labeled stem cells. SPIO shortens the relaxation time on T2 and T2*-weighted images, leading to a reduced signal intensity and thus a darkened image (Accomasso et al. 2016, Lau et al. 2010).

SPIO is a clinically approved metal oxide nanoparticle that is used in cell labelling. SPIO has the unique property of superparamagnetism that confers several advantages, such as the generation of heat in alternating magnetic fields and the ability to be guided to a specific tissue or organ by external magnetic fields. In this study, carboxydextran coated ferucarbotran was used. The benefit of using ferucarbotran (Resovist) is that there is no need to use a transfection agent and one can achieve more than 90% efficiency in cell labelling (Zhang et al. 2010a). In addition, cell viability is not significantly affected after incubation with even a high ferucarbotran concentration (Metz et al. 2004, Will et al. 2015). Exposure of SPIO-labeled human MSC to magnetic fields did not change their migration properties, viability, proliferation or differentiation capabilities (Schafer et al. 2010).

SPIO labelled BMMCs or MSCs were used in studies I, II and III in this thesis. In study II, intramyocardial BMMC injection was used in an acute porcine infarction model to evaluate the reliability of MRI in cell targeting. This study demonstrated the high spatial correlation between the SPIO labeled stem cells
detected on MR images and in the histological evaluation. The fact that no false positives appeared in the MRI data increased the feasibility of using MRI for monitoring cell targeting. In recent *in vivo* or clinical studies, the correlation between histology and MRI in cell detection has not been measured in detail although this would be one way to validate the use of MRI in cell targeting during the acute phase. In a pig study where the transplanted cells were detected at 8 weeks, MRI findings correlated with histology in targeting SPIO labeled MSCs (Yang et al. 2011). Since study II clearly demonstrated the reliability of MRI for cell targeting after AMI, it was decided to use this method in the pig model in study III.

In study III, at 21 days after cell administration, it was not possible to detect BMMCs with MRI due to changes occurring in the tissues such as fibrosis and insufficient visualization of the cell label. In another *in vivo* pig study, MSCs were detected with MRI at three weeks after AMI and intracoronary transplantation (Peng et al. 2013). In contrast to the present results, the transplanted SPIO labeled cells were seen as a hypointense area which could be differentiated from the surrounding tissue. At present, long term cell engraftment and the reliability of MRI in cell targeting are still being debated (Huang et al. 2015). An *in vivo* pig model study indicated that SPIO labeled MSCs could still be detected as long as 8 weeks after their intracoronary transplantation (Yang et al. 2011). Immunohistochemistry revealed that the SPIO label was not taken up by macrophages or monocytes. However, the results of that study are controversial. In a rat study, a significant iron-dependent MRI signal was identified at 3 days as well as at 2, 3 and 4 weeks after intramyocardial injection of SPIO-labeled MSCs. Histology revealed only a few or no viable stem cells after 4 weeks, instead there were iron-containing macrophages (Ma et al. 2015). Consequently following apoptosis, SPIOs might be phagocytosed by non-stem cells such as macrophages. In a recent study, dead cells could be distinguished from the live, transplanted stem cells by applying a dual MRI contrast technique in a rat model (Ngen et al. 2015). This novel method could provide useful information on the possible death of the transplanted cells. Here, it was not possible to evaluate the presence of iron-containing macrophages due to lack of adequate methods, which is a clear limitation. Despite several attempts, macrophage specific antibodies did not function properly when applied to the myocardial tissue of the pig. However, no sign of stem cell apoptosis was detected in the histological analysis suggesting minimal leakage of iron from the labeled stem cells. There is one published study claiming that 5-15% of local macrophages can become SPIO-positive after administration of SPIO-labeled stem cells (Pawelczyk et al. 2009). Even though it was not possible to identify the numbers of iron-containing
macrophages, it is speculated that the MRI signal obtained is most likely due to the labelled MMNCs and not due to labelled macrophages.

The intensity of signals may decrease with time, possibly due to dilution of iron oxide particles after cell proliferation, migration of labeled cells and biodegradation of the iron oxide particles (Daldrup-Link et al. 2005, Huang et al. 2015). Therefore, a smaller number of SPIO labeled stem cells is detected in histological analysis after 21 days. Nevertheless, in clinical studies, histological analysis is often not possible and according to the present findings, MRI is a feasible method for monitoring cell targeting.

6.5 The influence of stem cells on cardiac function

After myocardial infarction, the loss of myocytes and scar formation leads to decreased EF and cardiac function (Zamilpa et al. 2014). Consequently, the recovery of the left ventricle EF is one of the most concrete signs of the beneficial stem cell therapy.

In the in vivo animal study III, the EF improvement was significantly higher in BMMC group 21 days after AMI. This improvement in EF encountered in the BMMC group is in line with other studies where the experimental porcine infarction model and intramyocardial injection of the stem cells were used and subsequently the cardiac function has improved (Fan et al. 2014, Zhao et al. 2014, Sheu et al. 2015).

In the clinical study IV, the improvement in EF was higher in BMMC treated patients. However, there was no statistically significant difference in EF between the study groups after 6 months even though the difference appeared in the original FINCELL study (Huikuri et al. 2008). In this sub-study, a limited number of patients were enrolled and this influences the statistical evaluation when assessing differences between the study groups in EF. A similar trend towards improved EF without statistically significance between the treatment groups was shown also in another clinical study at 6 months after AMI and MSC transplantation (Wang et al. 2014). That study examined more patients (n=58) than included in the present work.

There are several clinical studies reporting the benefits of stem cell therapy on recovery after AMI and improved cardiac function (Gao et al. 2015, Heeger et al. 2012, Rodrigo et al. 2013, Skalicka et al. 2012). In one recent clinical study, an intracoronary infusion of 4.9x10⁸ BMMCs delivered from 24 hours to 7 days after AMI and PCI increased EF and myocardial perfusion in comparison to controls at 12 months (Huang et al. 2015). That study protocol and the number of transplanted
BMMCs were rather similar to those encountered in study IV. The difference was in the longer follow-up time (12 months) in the former study. In double-blind, randomized, controlled clinical study, the improvement in EF was demonstrated to last for up to 3 years in patients treated with intramyocardial injection of BMMCs after AMI and PCI (Wohrle et al. 2013). Interestingly, the improvement was most apparent in those patients treated with a cell number higher than the median value (324x10^6 cells).

However, the results from the clinical studies are still controversial. San Roman et al. (2015) compared three different BMMC approaches for their abilities to improve cardiac function after AMI in 120 patients. Intracoronary injection of BMMCs in combination with or without G-CSF mobilization after AMI and PCI did not increase the EF compared to controls when assessed at 12 months. In two clinical studies, Grajek et al. and Traverse et al. reported a similar negative effect of stem cell therapy on the change in EF after 12 months of AMI (Grajek et al. 2010, Traverse et al. 2014).

Despite the vigorous research and several clinical studies, the small number of patients and differences in study settings, may well explain the conflicting results. Several meta-analyses have been conducted to reveal the true benefit of stem cell therapy. The short-term effect (i.e. at less than 12 months) of BMMC transplantation on cardiac function was analysed in a meta-analysis involving 17 randomized controlled clinical studies (Cong et al. 2015). It was reported that intracoronary injection of BMMCs resulted in a significant improvement in EF and reduced LV end-systolic volume and wall motion score index. A recent meta-analysis consisting of 2035 patients from 31 controlled clinical studies analysed the optimal timing for BMMC transplantation following AMI (Liu et al. 2016). The cell transplantations had been performed starting at day 1 after AMI, with the latest being delivered more than 4 months after AMI (Table 4 in chapter 2.4.9). A multiple-treatment meta-analysis indicated that the optimal timing for improvement of EF and the reduction in the incidence of major adverse cardiac events was at 4-7 days after the AMI.

One of the largest meta-analyses was published in Circulation in 2012 (Jeevanantham et al. 2012). This meta-analysis consisting of a total of 50 studies and 2625 patients reported greater EF, a smaller infarct size and improved remodeling in stem cell treated patients after AMI. In addition, these benefits persisted for more than 12 months. A systematic review of randomized controlled trials in which BMMCs had been transplanted by the intracoronary route in AMI patients consisted of a total of 8 trials and 726 patients (Zhang et al. 2010b). At the
follow-up which was equal to or longer than 12 months, the BMMC treated patients enjoyed a greater improvement in EF and exhibited a smaller infarction size. In a further analysis, it was claimed that the BMMC transplantation-induced EF increase was more significant in patients under 55 years of age and with cells transferred 6-7 days after AMI.

Here, in studies II and III conducted in pigs, the intramyocardial injection was used as a transplantation method to ensure homing of administered cells to the infarction area in order to promote the cardiac recovery. Both the histological and MRI analyses detected that the transplanted BMMCs were present in the infarction area. In a more detailed examination, the number of transplanted BMMCs in myocardium correlated with the extent of improvement in EF, a somewhat surprising but logical finding. As far as we are aware, the correlation between the numbers of stem cells actually remaining transplanted within the heart and the cardiac function recovery has not been previously demonstrated at this level in a large animal model.

Unsuccessful stem cell injection may lead to stem cell leakage during delivery and consequently to much smaller numbers of actual transplanted stem cells in the myocardium than originally intended. If the true stem cell number in myocardium is too small, this may impair the outcome of stem cell therapy. The possibility of leakage has to be considered, especially when using large animal models, beating hearts and multiple injections (Teng et al. 2006). Study III emphasized the relevance of monitoring the cell targeting after injection, since it detected a correlation between the improvement in EF and the number of actual transplanted cells in myocardium. This is in line with a previous clinical study which demonstrated that if the number of transplanted BMMCs was greater than the median value then this resulted in a greater improvement in the EF (Wohrle et al. 2013). In conclusion, cell targeting using MRI or histology is crucial to ensuring study quality, especially when intramyocardial stem cell injection is being used.

In addition to the influence of transplantation method, recovery after the stem cell therapy may be affected by the timing of transplantation and the infarction size as well as the number and origin of the transplanted cells. Apparently, a greater effect on the infarction size can be achieved when BMMCs are transplanted at 7 days after AMI (Clifford et al. 2012, Huang et al. 2015). At that stage, the pro-inflammatory phase of the inflammation process is being transformed into the anti-inflammatory direction and thus the transplanted cells have the possibility to modulate inflammation. If there is only a small number of administered cells, this could impair the functional results, possibly due to the small rate of cell retention.
The effects on the infarction size and EF appear to be greater if BMMCs are delivered at doses \( >10^8 \) (Clifford et al. 2012a). In addition, a more pronounced effect of the stem cell therapy seems to be obtained in patients with larger infarcts or a lower baseline EF. As a consequence, stem cell therapy should perhaps be considered as treatment for those patients with the poorest prognosis and the most impaired cardiac function.

Although the variation in cardiac recovery after stem cell therapy is wide, it does seem that stem cells have the potential to restore the cardiac function after AMI (Clifford et al. 2012b). The fact that different studies report such controversial results on the change in cardiac function after stem cell transplantation highlights the complexity of stem cell therapy. One may predict that the reasons for the variation in the results will only become clear when the molecular basis of the remodeling process in myocardium becomes clarified. Consequently more research is required to develop stem cell therapy further.

### 6.6 The influence of stem cells on cardiac remodeling

The loss of myocytes during AMI increases the loading stress on the heart which leads to the maladaptive myocyte hypertrophy and changes in the ventricular architecture and function. Stem cell therapy aims to modulate the cardiac remodeling process, reducing the infarction size and improving the heart function (Zamilpa et al. 2014).

The growth factors and cytokines secreted by stem cells modulate the inflammation process and can influence remodeling in the left ventricle. In study III, the change in the cytokine levels at time points 24 hours and 21 days differed between the controls and BMMC treated pigs. However, the difference was not statistically significant due to the extensive variability in cytokine levels.

The level of the anti-inflammatory cytokine, IL-10, decreased in the control group but moderately increased in the BMMC group at 24 hours in the pig model. A similar increase in IL-10 level and also improved EF was reported in clinical study after chronic coronary total occlusion and stem cell transplantation (Li et al. 2015). The present result is in line with the previous in vivo animal study where MSCs were injected into the infarcted myocardium (Cho et al. 2014). A subsequent analysis revealed that MSCs increased the IL-10 levels via macrophages by shifting the macrophage phenotype. One in vitro study similarly demonstrated that the coculture of macrophages and MSCs along with the MSC-derived IL-10 caused the macrophages to move towards displaying an anti-inflammatory phenotype (van den...
Akker et al. 2013). Consequently, it could be argued that the increase in the IL-10 level provides a favourable environment for remodeling. In fact, the treatment with IL-10 has been reported to suppress the inflammatory response as well as improving LV function, reducing the infarction size and attenuating infarct wall thinning (Krishnamurthy et al. 2009). The elevation of the IL-10 level observed here in the BMMC group is possibly due to its secretion from the BMMCs but the cytokine may also have been released by other inflammatory cells, such as macrophages (Rowart et al. 2015). However, the immunomodulatory effect of BMMCs, such as the increase in the IL-10 level, can be considered to promote cardiac recovery.

At 21 days, the differences in the change of the cytokine levels between the study groups, were more obvious in in vivo pig model (study III). The increase in the concentrations of FGFbasic, G-CSF, TNFα and PDGF levels appeared in BMMC group. The PDGF level increased slightly in controls also whereas the G-CSF level decreased. Similar results have been reported after AMI and MSC injection in a rat study that examined the paracrine factors secreted by transplanted MSCs (Yao et al. 2015). The injection of MSCs led to significantly higher levels of FGF, PDGF and TNFα. FGFbasic, G-CSF, and PDGF have been demonstrated to exert an effect on neovascularisation: in a rat model G-CSF induced collateral vessel growth and PDGF stimulated arteriolar growth after AMI (Hausenloy & Yellon 2009, Srinivas et al. 2009, Awada et al. 2015, Takano et al. 2003). Exogenous FGFbasic after experimental AMI was shown to increase angiogenesis and myocardial perfusion, leading to improved EF (Zhang et al. 2011). In a rat model, the overexpression of VEGF and PDGF was reported to be related to the improved EF after AMI (Das et al. 2009).

Two time points, 24 hours and 21 days, were chosen for taking the samples for cytokine analysis in the pig study because these time points reflect the pro-inflammatory and anti-inflammatory phases occurring in the myocardium after AMI. Inflammation appears in the myocardium within hours after the AMI (Francis Stuart et al. 2016). Therefore, in the present study, it was assumed that there would be an increase in pro-inflammatory cytokines at 24 hours. Surprisingly, no clear increase in pro-inflammatory cytokines TNF-α, IL-1β, IL-6 levels was detected in the control group. However, there was a decline in the IL-6 level in both study groups at 21 days which is in line with other reports (Kosmala et al. 2005). The more sustained change in the cytokine levels was examined at the 21 day time point. As expected, there was an increase in the levels of the anti-inflammatory cytokines at 24 hours after AMI (study III). In further studies, it would be beneficial to use
more time points to track more accurately the changes taking place in the cytokine levels. The timing for elevation and inversion of distinct cytokine levels varies from hours to days, resulting in the remodeling of the myocardium.

The wide variation in the cytokine levels between individual animals at all of the time points limits the interpretation of the results in study III. The initial power calculation for significance levels of different cytokines showed that even examining a significantly larger number of experimental animals would not overcome the large variability between the cytokine responses. The wide variation in the cytokine levels observed in study III illustrates the complexity of the cytokine networks. Cytokines communicate and regulate the levels of each other in attempts to achieve a balance between pro- and anti-inflammatory properties (Li et al. 2014). Despite the variation, the large changes in the cytokine levels and the difference in cytokine levels between the study groups in study III was interpreted as being the result of BMMC transplantation.

Cytokines were the focus of the clinical study IV. Similarly to the pig study III, the cytokine levels varied widely among the patients at each time point in both study groups i.e. control and BMMC-treated patients. Due to the broad variation in baseline cytokine levels, no evident or statistically significant trend between the groups could be confirmed. The correlations of percentage changes in anti-inflammatory cytokines (IL-4, IL-10, IL-13) and in pro-inflammatory cytokines (IL-6, TNF-α, IL-1b, IL1-ra, IFNγ) from baseline to 2 days after PCI and injection and from baseline to 4 days after PCI and injection were measured. In the further analysis, BMMC treatment after AMI seemed to maintain a balance between pro-inflammatory and anti-inflammatory cytokines at 24 hours also in a pig model study III although the number of animals available for this analysis was rather small. This was a novel approach to assess the cytokine profile, since the majority of the previous studies have concentrated on analysing the changes in only one individual cytokine. The present study detected a correlation in the percentage change of the anti-inflammatory and pro-inflammatory cytokine concentrations in both study groups at 2 days. At 4 days, there was correlation only in the BMMC treated patients. The pro-inflammatory reaction and the switch to the anti-inflammatory phase happen within hours after an AMI and it is known to be crucial for optimal recovery (Frangogiannis et al. 2000, Francis Stuart et al. 2016). If either phase is overactive or incompletely resolved, there may be adverse LV or electrophysiological remodeling, ventricular arrhythmia and sudden cardiac arrest. A prolonged pro-inflammatory phase leads to LV dilation, adverse remodeling and ventricular dysfunction (Francis Stuart et al. 2016, Frangogiannis et al. 2002). An
overactive anti-inflammatory phase can promote fibrosis outside the infarct region and an increased risk for diastolic dysfunction. Studies III and IV suggest that BMMC transplantation maintains the balance between pro-inflammatory and anti-inflammatory cytokines after AMI such that the environment is optimal cardiac repair. Since this clinical model is easily reproducible, it would be interesting to confirm this result with a larger number of patients.

Despite the doubts and criticism recently raised against cell therapy, this therapy still represents a potential valid treatment for AMI. A better understanding of the inflammatory process in the myocardium after AMI and the complex cytokine network may provide an explanation for variability in the results of cardiac function after cell therapy. After transplantation and homing to the infarction area, the cells have to survive in the inflamed microenvironment. The release of pro-inflammatory cytokines should be followed by the anti-inflammatory phase of inflammation. In this regard, the timing of cell transplantation has to be optimized. It has been shown that MSCs survive better in a culture in anti-inflammatory environment than when exposed to pro-inflammatory cytokines (Freytes et al. 2012). This indicates that the optimal timing for cell transplantation might not be immediately after infarction, but instead after a few days.

One of the characteristic features of the cytokine network is its functional pleiotropy and redundancy. An individual cytokine exhibits a wide range of biological effects on various cell types and several cytokines exert similar and overlapping actions on the same cell type. The overlapping, multifunctional and also mutually antagonistic effects of the cytokines complicate the unravelling of the cytokine network and clarification of its role in cardiac injury and repair. Consequently, the controversial results of stem cell therapy are not surprising but rather should be viewed as a challenge, emphasizing the need to resolve the current mysteries of cytokines and remodeling.

6.7 Study limitations

The main topic of this thesis project was to examine the mechanisms activated when the stem cells are administered in the treatment to AMI. Both clinical studies and animal experiments were used to investigate the benefits of stem cell transplantation after AMI. A large animal model, like the pig model, is generally considered to mimic a human patient due to the similarities in cardiovascular system and the size of the heart. However, the pigs used in this study were young
and had better potential to undergo regeneration and repair than AMI patients, who are generally old.

SPIO labeling was used in studies I-III to detect the transplanted cells using histological staining or MRI. There are indications of iron leakage to myocardium after apoptosis of the SPIO labelled stem cells and this can cause problems with interpreting the results. However, in the histological assessment conducted at 21 days, the morphology of BMMCs was typical for SPIO labeled cells. There were hardly any signs of BMMC disintegration evident in the histological analysis, suggesting that there had been minimal leakage of iron from the labeled BMMCs. One limitation of this study is that the presence of iron-containing BMMCs could not be definitely evaluated with MRI at 21 days due to multiple, mainly infarction-related, tissue issues. The infarction-related transmural necrosis and the consequent progression of scarring caused a marked reduction in the ventricular thickness and the water content leading to a much reduced MRI signal, preventing the detection of the label.

The cytokine levels were measured in the large animal pig model in study III. An obvious limitation in this study is the cytokine multiplex–assay which was used, since this had been optimized for human use i.e. the assay is not necessarily appropriate for this kind of cross-species investigation, especially when low values are being determined (Merilainen et al. 2012). However, since there is no pig specific cytokine multiplex–assay, the only option was to adopt the system for human use.

Another limitation is that storage and defrosting may cause some degradation of the cytokine serum samples. In study IV, the serum samples were defrosted only once before cytokine measurements. However, usually in these kinds of experiments, samples may well be frozen and defrosted several times and this could have a greater impact on their values. Thirdly, in studies III and IV, the measured cytokine levels were obtained from the systematic circulation and thus do not represent the cytokine concentrations present in the myocardium and infarction area. Due to this limitation, the measured cytokine levels were possibly lower in the serum samples than they would have been in myocardium. It is also possible that some smaller changes in cytokine levels may not have been detected. At present, there were no technical or ethical possibilities to obtain samples from the infarction area so that it would be possible to observe local changes in the cytokine network after AMI. Nonetheless, in both the pig and clinical studies, the cytokine levels were measured from serum, and thus they are comparable in this respect.
6.8 Future aspects

Non-clinical and clinical studies have demonstrated that stem cell therapy using BMMC or MSC transplantation is a safe and feasible way to treat AMI (Crisostomo et al. 2015, Huikuri et al. 2008, Liu et al. 2016). The bone marrow is an attractive source because it is easily accessible and contains a number of stem cells, including hematopoietic and MSCs. The future for stem cell therapy should be focused on obtaining a better understanding of the mechanism of functional improvements. More in vitro and clinical studies will be required to reveal the changes in the cytokine network after AMI and the effects of stem cell transplantation on remodeling and cytokine levels.

One of the challenges for the future will be to improve the durability and survival of stem cells in the adverse environment into which they are engrafted. The devised cardiac explant culture model offers many benefits for evaluating the biological effects on stem cells after transplantation into myocardium. The cardiac explant culture model represents an excellent tool for understanding how transplanted stem cells behave in the myocardium during the pro-inflammatory or anti-inflammatory phases and how they contribute to maintaining the inflammation balance. The cardiac explant culture model is an ideal environment to measure the impact of single cytokines on the behavior of stem cells. In addition, it would be interesting to adopt the cardiac explant culture model for evaluating the behavior of the different stem cell types or preconditioned or genetically conditioned cells and to compare their benefits or disadvantages after transplantation to myocardium. The different ways of boosting stem cells will be an intriguing and highly relevant issue for the future. Preconditioning of stem cells by exposing them to hypoxia, hyperoxia or cytokines or subjecting them to genetic modification has been postulated as potentially improving cell survival and modifying the MSC secretome and thus enhancing their therapeutic efficacy (Li et al. 2016).

In the cardiac explant culture model, the tissue of the pig myocardium was used. In the future, it would be interesting to reproduce study I using human cardiac tissue in order to obtain comparable results with respect to stem cell migration and adhesion in the human tissue. Tissue could be obtained from the atrium of the heart or from heart transplantation patients or from heart surgery in pediatric patients. MicroRNAs are thought to play critical roles in many pathological cardiac processes following AMI (Konala et al. 2016). They may have considerable potential as novel regulators in the MSC-based treatment for AMI e.g. they may modify cardiovascular cell differentiation, exert anti-arrhythmic effects and have
paracrine properties. MicroRNAs can be packaged into exosomes that are subsequently released from the MSCs by exocytosis. MicroRNAs and exosomes are being currently investigated to elucidate their potential effects on intercellular signaling and how their paracrine effects contribute to the remodeling process. In the future, proteomics offers an attractive opportunity for the analysis of the MSC secretome.
7 Conclusions

1. The cardiac explant culture model simulates ischemic myocardial tissue and is a feasible, novel method with which to study the behaviour of transplanted stem cells. The migration range and the number of adhering MSCs increased over time, evidence of the active movement of MSCs within the explant.

2. MRI offers a fast and feasible method for clinical use to detect SPIO labeled BMMCs in the myocardium.

3. BMMC injection after AMI improved EF in the in vivo pig model. The quantity of transplanted BMMCs measured in the pigs correlated with the improvement in cardiac function after AMI. BMMC injection seemed to modulate cytokine profile after AMI.

4. BMMC transplantation was associated with a preservation of the balance between pro- and anti-inflammatory cytokines after STEMI in PCI-treated patients. This may partly explain the favorable effect of stem cell transplantation after AMI.

5. BMMC transplantation after AMI achieved similar results in the in vivo pig model and in the clinical study. The improved EF and an ability to modulate and balance cytokine levels were observed after BMMC treatment. The experimental acute myocardial pig model was assessed as a valid model which can simulate cardiac infarction in humans.
References


Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S & Epstein SE (2004b) Marrow–derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94(5): 678–685.


Original publications


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