Myoblast Sheet Transplantation for Treatment of Heart Failure after Myocardial Infarction

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Academic Dissertation

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Original publications

This thesis is based on the following original publications:

I	Kitabayashi K, Siltanen A, Pätilä T, Mahar A, Tikkanen I, Koponen J, Ono M, Sawa Y, Kankuri E, and Harjula A. Bcl-2 expression enhances myoblast sheet transplantation therapy for acute myocardial infarction. *Cell Transplantation* 2010;19:573-588.
II	Siltanen A, Kitabayashi K, Pätilä T, Ono M, Tikkanen I, Sawa Y, Kankuri E, and Harjula A. Bcl-2 improves myoblast sheet therapy in rat chronic heart failure. *Tissue Engineering Part A* 2011;17:115-125.
III	Siltanen A, Kitabayashi K, Lakkisto P, Mäkelä J, Pätilä T, Ono M, Tikkanen I, Sawa Y, Kankuri E, and Harjula A. hHGF overexpression in myoblast sheets enhances their angiogenic potential in rat chronic heart failure. *PLoS One* 2011;6:e19161.
IV	Siltanen A, Nuutila K, Uenaka H, Mäkelä J, Pätilä T, Vento A, Sawa Y, Harjula A, and Kankuri E. Skeletal myoblasts protect cardiomyocytes against oxidative stress via c-Met and EGFR-dependent paracrine pathways. Submitted.

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Main abbreviations

ACE	Angiotensin-converting enzyme
Ang	Angiotensin
AT1	Angiotensin receptor type 1
CABG	Coronary artery bypass grafting
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EF	Ejection fraction
EGM	Endothelial cell growth medium
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
HGF	Hepatocyte growth factor
HF	Heart failure
HUVEC	Human umbilical vein endothelial cells
L6-Bcl2	L6 myoblast cell line with constitutive Bcl2-expression
L6-HGF	L6 myoblast cell line with constitutive HGF-expression
LAD	Left anterior descending coronary artery
LVAD	Left ventricular assist device
MI	Myocardial infarction
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NYHA	The New York Heart Association
PCI	Percutaneous coronary intervention
PIPAAm	Poly(N-isopropylacrylamide)
PlGF	Placental growth factor
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SMA	Smooth muscle actin
TGF-β1	Transforming growth factor beta 1
TMRE	tetramethylrhodamine ethyl ester
TNF-α	Tumor necrosis factor alpha
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

// Abstract

Heart failure is a common, severe, and progressive condition associated with high mortality and morbidity. On average 1 to 2% of the western population is estimated to suffer from it, and the poor prognosis of the condition makes it the number one cause of death and a substantial health care burden in the developed countries. Because of population-aging in the coming decades, heart failure is estimated to reach epidemic proportions. Moreover, the increasing popularity of the western diet is leading to an increased heart failure incidence in developing countries as well. Current medical and surgical treatments have reduced mortality, but the prognosis for patients has remained poor. Transplantation of skeletal myoblasts has raised hope of regenerating the failing heart and compensating for lost cardiac contractile tissue.

In the present work, we studied epicardial transplantation of tissue-engineered myoblast sheets for treatment of heart failure. We employed a rat model of myocardial infarction-induced acute and chronic heart failure by left anterior descending coronary artery ligation. We then transplanted myoblast sheets genetically modified to resist cell death after transplantation by expressing antiapoptotic gene *bcl2*. In addition, we evaluated the regenerative capacity of myoblast sheets expressing the cardioprotective cytokine hepatocyte growth factor in a rat chronic heart failure model. Furthermore, we utilized *in vitro* cardiomyocyte and endothelial cell culture models as well as microarray gene expression analysis to elucidate molecular mechanisms mediating the therapeutic effects of myoblast sheet transplantation.

Our results demonstrate that Bcl2-expression prolonged myoblast sheet survival in rat hearts after transplantation and induced secretion of cardioprotective, proangiogenic cytokines. After acute myocardial infarction, these sheets attenuated left ventricular dysfunction and myocardial damage, and they induced therapeutic angiogenesis. In the chronic heart failure model, inhibition of graft apoptosis by Bcl-2 improved cardiac function, supported survival of cardiomyocytes in the infarcted area, and induced angiogenesis in a vascular endothelial growth factor receptor 1- and 2-dependent mechanism. Hepatocyte growth factor-secreting myoblast sheets further enhanced the angiogenic efficacy of myoblast sheet therapy. Moreover, myoblast-secreted paracrine factors protected cardiomyocytes against oxidative stress in an epidermal growth factor receptor- and c-Met–dependent manner. This protection was associated with induction of antioxidative genes and activation of the unfolded protein response.

Our results provide evidence that inhibiting myoblast sheet apoptosis can enhance the sheets' efficacy for treating heart failure after acute and chronic myocardial infarction. Furthermore, we show that myoblast sheets can serve as vehicles for delivery of growth factors, and induce therapeutic angiogenesis in the chronically ischemic heart. Finally, myoblasts induce, in a paracine manner, a cardiomyocyte-protective response against oxidative stress. Our study elucidates novel mechanisms of myoblast transplantation therapy, and suggests effective means to improve this therapy for the benefit of the heart failure patient.

1. Introduction

Treatment of myocardial infarction (MI) has advanced tremendously during the past few decades. Development of medication and interventional cardiology techniques such as percutaneous coronary intervention (PCI) have significantly reduced mortality after MI. As a result of increased survival, more people are subject to the remodeling process that takes place after MI as a compensatory mechanism (McMurray and Pfeffer, 2005). Life-style changes and medical interventions, such as smoking cessation and pharmacological inhibition of the renin-angiotensin system (RAS), can slow down the remodeling process (McMurray and Pfeffer, 2005). Despite these current treatments, in an increasing numbers of patients, the initially beneficial remodeling proceeds to pathological remodeling. Pathological remodeling, over time, leads to the inability of the circulatory system to provide sufficient perfusion for the body's requirements (Sutton, 2000), *i.e.* heart failure (HF).

Extensive research efforts have led to the availability of drugs that reduce mortality and enhance the well-being of patients suffering from HF (Dickstein *et al*, 2008). Still, 5-year mortality of end-stage HF patients is estimated to be 60 to 70% (Cowie *et al*, 2000), demonstrating the requirement for more efficacious treatment modalities. In addition, medical devices such as the left ventricular assist device (LVAD) are under rigorous development but are not yet sufficiently evolved to benefit large numbers of patients without causing serious complications (Slaughter *et al*, 2009). For some patients, heart transplantation can provide a substantial improvement in prognosis and quality of life. The shortage of organ donors, however, limits transplantation from becoming more widespread (Lloyd-Jones *et al*, 2009).

Because the underlying cause of MI-induced HF is the loss of contractile cells, cardiomyocytes, it has been postulated that the lost cardiomyocytes could be replaced by either stem cell-derived cardiomyocytes or by adult differentiated contractile cells such as skeletal myoblasts (Menasche, 2003). Because of the inherent safety problems associated at the moment with stem cells (Jeong *et al*, 2011; Zhang *et al*, 2011), skeletal myoblast transplantation is the most appealing and clinically straightforward modality to pursue replacement of cardiac contractile tissue. Myoblast transplantation has been experimentally evaluated for nearly 20 years (Marelli *et al*, 1992). Moreover, accumulating clinical evidence has shown the potential but also revealed hurdles in the development of this innovative therapy. These hurdles, such as potential arrhythmogenicity and poor initial cell survival after transplantation, have been and are addressed in experimental models at the moment and can be translated to clinical

benefit in the future (Menasche, 2011a). One innovative developmental step in myoblast transplantation has been the introduction of tissue-engineered scaffoldless cell sheets. These sheets can be manufactured without the use of biomaterials, and they preserve the intercellular and extracellular structures essential for cell function during transplantation (Memon *et al*, 2005; Villet *et al*, 2011). Furthermore, such sheets implanted epicardially enable delivery of the whole therapeutic cell population into the target tissue without cell loss by leakage through the injection channel, washout to the circulation, or apoptosis by failure to adhere (anoikis). In the present study, we enhanced the properties of these myoblast sheets by inhibiting their apoptosis and by promoting their therapeutic paracrine profile by use of gene therapy. We then evaluated the therapeutic effect of these enhanced sheets in acute and chronic experimental HF models.

2. Review of the literature

2.1 Myocardial infarction

MI is defined by the European Society of Cardiology, the American College of Cardiology, the American Heart Association, and the World Heart Federation as myocardial cell necrosis caused by significant and sustained ischemia (Thygesen *et al*, 2007). It may lead to sudden death or, in surviving patients, to severe myocardial injury. Those patients that survive the initial MI are later more prone to undergo recurrent events. MI is typically initiated by a rupture of an atherosclerotic plaque or thrombosis in a coronary artery, and less often by other mechanisms such as vasospasm. In an unstable coronary artery disease, MI may be initiated during a period of inflammation in the vascular wall. The occlusion of the coronary blood flow leads initially to necrotic death of cardiomyocytes, and an inflammatory response characterized by infiltration of macrophages and monocytes to the infarct region (Thygesen *et al*, 2007).

Typical clinical symptoms of MI include diffuse pain in the chest, upper limb, jaw, and/or the epigastric area. The MI-associated pain is typically not localized or affected by movement of the area. The pain is usually sustained for at least 20 minutes and may be associated with dyspnoea, diaphoresis, nausea, or syncope. MI can be diagnosed by several methods including detection of circulating biomarkers released from the damaged myocytes such as troponin (Jaffe *et al*, 2006), by electrocardiogram (Zimetbaum *et al*, 2003), or imaging techniques such as echocardiography (Korosoglou *et al*, 2004) or magnetic resonance imaging (Lima, 2003).

MI incidence has been studied in different age groups and with varying diagnostic criteria since the 1970s (Roger, 2007). The Olmsted County Study reported an overall MI incidence of 205 per 100,000 (Arciero *et al*, 2004) and the Worchester Heart Attack Study 244 per 100,000 of (Goldberg *et al*, 1999). Re-evaluation of the diagnostic criteria (the European Society of Cardiology and the American College of Cardiology, 2000) has been estimated to enhance sensitivity in detecting MI. In a recent incidence study that included 46,086 patients aged over 30 between 1999 and 2008, a peak incidence of 287 cases per 100,000 in 1999 was reported which then decreased annually to 208 cases per 100,000 in 2008 (Yeh *et* al, 2010). In Finland, according to the Finnish Cardiovascular Disease Register, an incidence of 796 to 889 for men and 373 to 396 for women between the years 1994 and 2002 has occurred (Lehto *et al*, 2007). In that study, a declining trend was also reported suggesting that MI incidence may be slightly decreasing. Because improved treatment has significantly reduced mortality after MI, an increasing number of patients survive the initial myocardial damage. This post-MI

damage is followed by myocardial remodeling, which can lead to a highly morbid and disabling condition: HF.

2.2 Heart failure

HF is one of the most severe health conditions in terms of mortality, morbidity, and economic costs. The condition is defined by the hearts' inability to supply sufficient perfusion to the peripheral tissues. HF can occur rapidly after an acute cardiac event such as MI or, more commonly, be a chronic condition. Moreover, the condition can present in an acute-to-chronic manner. In such cases, periods of worsening symptoms are followed by deterioration in cardiac function (McMurray and Pfeffer, 2005). The most typical symptoms associated with HF are breathlessness and fatigue, which can occur during rest or exercise. In addition, swelling of the peripheral tissues, typically the ankles, is an indicative sign of HF. These accompanying symptoms are, however, not specific for HF (Siefkin, 1985), and can be caused by several other medical conditions such as venous insufficiency, chronic lung disease, anemia, renal dysfunction, and hypothyroidism. Further, these common symptoms can also be due to medication prescribed for another disease such as calcium channel blockers or glitazones (Thomas *et al*, 2002). Due to the low specificity of these symptoms, they are only suggestive for HF (Khunti *et al*, 2000) and can result in false-positive diagnosis in primary health care (Remes *et al*, 1991). For more objective clinical criteria, the Boston Clinical Criteria (Marantz *et al*, 1988) have been used. These criteria have been validated with ventricular ejection fraction, pulmonary capillary wedge pressure measurement, and echocardiography.

A widely used classification of HF by The New York Heart Association (NYHA) classifies the condition based on the severity of symptoms into four levels (NYHA I-IV) (Dickstein *et al*, 2008). To supplement the NYHA classification, the American College of Cardiology and the American Heart Association published another classification that similarly identifies four stages in the development of HF (Dickstein *et al*, 2008). These stages are based on symptoms, patient history, and clinical criteria (Table 1).

Table 1. Classification of stages of heart failure. Data from Dickstein *et al*, 2008.

NYHA	Description
I	No symptoms and no limitations in ordinary physical activity.
II	Mild symptoms and slight limitation during ordinary activity.
III	Marked limitation in activity due to symptoms. Comfortable only at rest.
IV	Severe limitations. Experience of symptoms even while at rest.
ACC/AHA	
A	Patients at high risk. No structural or functional abnormalities.
B	Structural heart disease. No signs or symptoms of HF.
C	Symptoms of heart disease with underlying structural heart disease.
D	Advanced structural heart disease with marked symptoms at rest.

The New York Heart Association (NYHA), The American College of Cardiology (ACC), the American Heart Association (AHA)

Evaluation of the risk factors associated with HF has been challenging because of variance in study settings such as diagnostic criteria, study end-points, and patient population. The First National Health and Nutrition Examination Survey (NHANES I) epidemiological follow-up study concluded that male sex, less education, physical inactivity, cigarette smoking, overweight, obesity, hypertension, valvular heart disease, and coronary heart disease are all independent risk factors for development of HF (He *et al*, 2001). Of these factors, coronary heart disease presented the highest risk factor of 62% for HF. Other significant risk factors were cigarette smoking 16%, hypertension 10%, obesity 8%, diabetes 3%, and valvular heart disease 2%. During the past decades, coronary heart disease has become by far the most common risk factor for HF, whereas hypertension has become less significant (Gheorghiade and Bonow, 1998). Of those patients that suffer MI and survive, up to 51% have been estimated to develop HF (Lichstein *et al*, 1990; Emanuelsson *et al*, 1994; Persson *et al*, 1995). MI is thus one of the most important causes of HF and its prevalence is estimated to increase in the future with the aging population and with a decreasing number of MI-related deaths (Stewart *et al*, 2003).

In the developed countries, between 1 and 2% of the adult population suffers from HF. The prevalence increases sharply with age and in those aged over 65, the prevalence is estimated to be 6 to 10% (McMurray and Pfeffer, 2005). Although during the past 20 years the age-adjusted incidence of HF has not increased (Levy *et al*, 2002), the prevalence of HF is predicted to increase with the general aging of the population (Stewart *et al*, 2003).

HF is one of the most common causes of death, as the mortality rate is 30 to 40% within one year from diagnosis, and 60 to 70% within 5 years (Cowie *et al*, 2000). In addition to the high mortality, HF is also a highly disabling disease. Patients experience impairment in the quality of life both physically and mentally. Furthermore, the physical health impairment experienced by HF patients is greater than in other serious chronic disorders (Hobbs *et al*, 2002). Because of its high prevalence, mortality, and morbidity, HF presents a heavy and increasing burden on society, accounting for approximately 2% of health-care costs (Stewart *et al*, 2003).

2.3 Pathogenesis of heart failure after myocardial infarction

The pathophysiology of HF after MI can be arbitrarily divided into two phases: the early phase representing the pathological changes during the first 72 hours, and the late phase after that. The early phase is characterized by necrotic death of cardiomyocytes due to non-existent or limited perfusion that severely impairs the cardiac energy metabolism. This lack of perfusion subjects the cardiomyocytes to severe hypoxia or anoxia, as well as to a limited availability of nutrients. If the coronary artery is reperfused quickly, formation of reactive oxygen species (ROS) occurs without a significant inflammatory response or sustained myocardial damage, thus leading to functional recovery of the myocardium (Bolli and Marban, 1999). However, if the ischemia is prolonged, cardiomyocytes and endothelial cells undergo necrosis accompanied by an inflammatory response characterized by infiltration of monocytes, macrophages, and neutrophils. During myocardial necrosis, intracellular membrane components are released into the surrounding tissue, where they bind and thereby activate the early acting components C1-C4 of the complement system (Pinckard *et al*, 1975). The complement system then recruits monocytes to the site of injury by a C5a-dependent mechanism (Birdsall, *et al*, 1997). Moreover, suppressing the activation of the component system can be beneficial to limit myocardial necrosis (Weisman *et al*, 1990). In addition to the activated complement system, alterations in myocardial oxygen balance after MI lead to formation of ROS that further contributes to inflammation (Giordano, 2005). ROS promotes inflammation by inducing expression of proinflammatory chemokines in endothelial cells (Lakshminarayanan *et al*, 1997). These chemokines then promote inflammation by inducing leukocyte chemotaxis and neutrophil binding to endothelial cells via ICAM-1 (Sellak *et al*, 1994).

Following MI, mast cells undergo degranulation and release the proinflammatory mediator tumor necrosis factor alpha (TNF-α) to the extracellular space. TNF-α is suggested to be an upstream regulator of the cytokine-mediated inflammatory response

by induction of downstream inflammatory mediators such as IL-6 (Frangogiannis *et al*, 1998). Furthermore, TNF-α is an activator of nuclear factor kappa B (NF-κB) (Schütze *et al*, 1992), a key transcription factor in mediating the cytokine-mediated inflammatory response after MI. By binding DNA, NF-κB induces expression of a number of genes that are associated with the inflammatory response (Stancovski and Baltomore, 1997). Representing the importance of TNF-α and NF-κB in mediating inflammation after MI, neutralization of either TNF-α (Frangogiannis *et al*, 1998) or NF-κB can impair proinflammatory cytokine expression (Morishita *et al*, 1997).

Chemokine stimuli, ROS, and the activated complement system induce infiltration of inflammatory cells to the site of injury. One of the cell types recruited to infiltrate the tissue after MI is the neutrophil. These cells exaggerate the infarction injury by blocking the microcirculature in the heart (Engler *et al*, 1986), and by secreting agents that directly injure the myocardium (Jaeschke *et al*, 1997). Importantly, animals depleted of neutrophils have significantly smaller infarct sizes after coronary occlusion (Romson *et al*, 1983), suggesting that the infiltrated neutrophils have a detrimental effect on healing after MI. In contrast to neutrophils, other inflammatory cells such as monocyte/macrophages and mast cells are suggested to contribute to infarct healing. After MI, monocytes are recruited to the infarct site by C5a, transforming growth factor beta 1 (TGF-β1), and monocyte chemoattractant protein (MCP-1) (Birdsall *et al*, 1997). After infiltration, these cells differentiate into macrophages, and their cardiac reparative action is suggested to be mediated via secretion of growth factors (Weihrauch *et al*, 1995) and modulation of the extracellular matrix (ECM) (Ganz, 1993). These reparative processes associated with monocyte and mast cell infiltration are suggested to be prominently mediated by Il-10 mediated suppression of inflammation (Yang *et al*, 2000).

In addition to promoting inflammation, the infiltration of leukocytes, especially macrophages, induces angiogenesis. Macrophages secrete angiogenic factors, such as fibroblast growth factor 1/2 and platelet-derived growth factor that induce formation of new capillaries and vessels, ones that replace those lost by necrosis early after infarction (Kuwabara *et al*, 1995). Independent of the leukocyte-mediated angiogenesis, hypoxic conditions in the myocardium promote hypoxia-inducible factor 1 mRNA stabilization in endothelial cells. Hypoxia-inducible factor 1, in turn, induces vascular endothelial growth factor (VEGF) production that also contributes to infarction-related angiogenesis (Shima *et al*, 1995). The inflammatory and angiogenic responses peak between 1 and 2 weeks after MI. The inflammatory cells undergo apoptosis around 2 to

3 weeks after MI, and angiogenesis in the heart recedes once the healing process is completed (Sun, 2010).

In addition to the inflammatory response, the early phase after MI is characterized by infarct expansion. MI induces activation of matrix metalloproteinases (MMP) early post-MI. This activation, together with serine peptidase activity, causes degradation of collagen, and collagen struts that support the surrounding cardiomyocytes (Whittaker *et al*, 1991). Due to this ECM-degradation, the surviving cardiomyocytes undergo movement relative to each other, a phenomenon known as 'slippage'(Whittaker *et al*, 1991). Together with slippage, an increase in cell number in the myocardium, as well as cell stretching by increased sarcomeric length, contribute to infarct expansion characterized by ventricular wall thinning and cavity dilation (Gajarsa and Kloner, 2011). The infarct expansion takes place within hours of MI and results in ventricular wall stress during both systole and diastole (Sutton, 2000).

The healing process after MI constitutes a fibrotic component as well. After the initial collagen degradation promoting infarct expansion, fibrosis substitutes for the lost parenchymal tissue with a collagen scar. The first step of fibrosis following MI is the activation of TGF-β1, which is expressed primarily by the inflammatory cells (Sun *et al*, 1998). TGF-β1 is suggested to be the key mediator that induces transdifferentiation of cardiac fibroblasts, macrophages, and pericytes, as well as circulating bone marrow-derived progenitor cells, into myofibroblasts (Desmouliere *et al*, 1993). Myofibroblasts then undergo rapid proliferation and secrete collagen type I and III that is then deposited and forms the scar tissue (Cleutjens *et al*, 1995). The first detectable collagen fibers appear one week after MI. During the second week the fibers undergo organization by myofibroblasts to form scar tissue. The accumulation of this scar tissue may then continue for several weeks after the infarction. Furthermore, MI leads to upregulation of TGF-β1 and deposition of collagen also in the non-infarcted area (Cleutjens *et al*, 1995). Such interstitial fibrosis is suggested to be derived from cardiac fibroblasts, but not from myofibroblasts. Interstitial fibrosis stiffens the myocardium and can thereby promote ventricular dysfunction (Sun, 2010). Finally, as the collagen deposition and scar formation recedes, the myofibroblasts undergo apoptosis (Sun, 2010).

Following the first days after MI, cardiomyocyte death, inflammation, and infarct expansion recede. The biological processes gradually change to those associated with late remodeling including activation of RAS, ventricular dilation, and hypertrophy. Progression of these processes leads over time to HF (Sutton, 2000).

A major regulator of cardiovascular physiological and pathological homeostasis is the RAS. The circulating RAS is regulated, first, by conversion of circulation angiotensinogen to AngI by the enzyme renin, and second, and by conversion of AngI to AngII by angiotensin-converting enzyme (ACE). AngII is the most potent product of RAS and it acts to increase blood pressure by enhancing vascular smooth muscle cell constriction, and myocardial contractility, and raising sympathetic nervous system activity among other actions. These functions are facilitated by the AngII type 1 receptor (AT1). Another AngII receptor with less significance, AT2, mediates vasodilatory effects (Ducharme and Schriffin, 2010). In addition to the circulating RAS, a local cardiac RAS exists as well. Following MI, renin expression is induced in the myocardium primarily by myofibroblasts and macrophages (Ducharme and Schriffin, 2010). ACE, which is primarily expressed by endothelial cells, and the AT1 receptor, primarly expressed by macrophages, myofibroblasts, and vascular smooth muscle cells, are also upregulated in the myocardium after infarction and lead to AngII expression (Sun *et al*, 1996). At the early stage after MI, AngII is suggested to contribute to infarct repair by three mechanisms. First, by enhancing generation of ROS and thus promoting the inflammatory response (Lu *et al*, 2004), second, by contributing to the angiogenic response (Toko *et al*, 2004), and third, by promoting scar formation via induction of TGF-β in myofibroblasts and promoting their differentiation, proliferation, and synthesis of collagen (Sun *et* al, 1998). RAS activation thus contributes to infarct repair in the short term. Over time, however, continued RAS activation contributes to the pathogenesis of HF. AngII-mediated AT1 signaling contributes to the increased peripheral resistance, hypertrophy and apoptosis of cardiomyocytes, myocardial fibrosis, and activation of the sympathetic nervous system as well as oxidative stress (Werner *et al*, 2008).

After the acute stage of MI, the heart undergoes hypertrophy to adapt to the changed physiological and biochemical environment. The hypertrophic signaling includes an increase in hemodynamic load and in the biomechanical stress associated with it, and increases in paracrine activation of G protein-mediated signaling pathways, fatty acid oxidation-mediated activation of the peroxisome proliferator, and activation of phosphoinositide 3-kinase by Akt signaling (Frey *et al*, 2004). The cardiomyocyte can undergo an up to 70% growth in cell volume in transverse and longitudinal directions. As an adaptive response, hypertrophy counteracts progressive dilation of the ventricle and helps stabilize contractile function (Anversa *et al*, 1985). After the initial compensatory adaptation, hypertrophy contributes to cardiac dysfunction and development of HF. Hypertrophy can be divided into concentric and eccentric hypertrophy. Of these, eccentric hypertrophy that is mediated by volume overload and

characterized by increased myocyte length and thinning of the ventricle walls usually occurs after MI (Sutton, 2000).

Cardiomyocyte hypertrophy may occur through several molecular mechanisms that all lead to increased protein synthesis and subsequent increase in cardiomyocyte size. In G protein-mediated pathways, activation of AT1, endothelin 1 receptor, and α-adrenergic receptor couples with the $G_{\alpha q}$ pathway that leads to hypertrophic growth (Maruyama *et al,* 2002; Gupta *et al*, 2007). In addition to the $G_{\alpha q}$ signaling, the β-adrenergic receptor couples with two other G protein subtypes, G_s and G_i, which also mediate hypertrophic signaling (Daaka *et al*, 1997). Moreover, AngII and endothelin 1 utilize small G protein receptors to mediate hypertrophic actions. Other activators, mediators, and signaling pathways regulating hypertrophic signaling include RAS, TGF-β1, phosphoinositide 3-kinase, Akt, calcineurin, mitogen-activated protein kinase, protein kinase C, myotrophin, and NF-κB (Gupta *et al*, 2007).

Cell death early after MI is characterized by necrosis due to sudden anoxia and nutrient deprivation. As the remodeling progresses, apoptosis becomes the predominant form of cell death that then contributes to the transition from compensatory hypertrophy to HF (van Empel *et al*, 2005). Cardiomyocytes undergoing apoptosis are detectable in post-mortem biopsies from patients that suffered from HF (Narula *et al*, 1996). Further, animal models establish that a fairly low rate of cardiomyocyte apoptosis can lead to severe cardiac dysfunction (Wencker *et al*, 2003). Apoptosis of cardiomyocytes takes place by three mechanisms. First, the intrinsic pathway of apoptosis occurs through the mitochondrial-dependent mechanism. The mitochondrial membrane transition pore opens, leading to loss of the mitochondrial membrane potential and leakage of cytochrome c from the mitochondria into cytosol and subsequent activation of effector caspases that degrade intracellular structures. This pathway is induced by such stimuli as hypoxia, ischemia-reperfusion, and oxidative stress (Weiss *et al*, 2003). Second, the Fas receptor and the TNF-α receptor activate the extrinsic pathway of apoptosis that is then facilitated by effector caspase-8 activation (Muzio *et al*, 1998). Third, cardiomyocyte apoptosis may occur by the endoplasmic reticulum (ER)-dependent mechanism. Prolonged ER stress activates ER resident caspase-12 and contributes to apoptosis in the failing heart (Okada *et al*, 2004). Because of the prominent role apoptosis plays in development of HF, strategies counteracting myocardial apoptosis would be beneficial. Interestingly, blocking AngII and norepinephrine signaling can inhibit cardiomyocyte apoptosis. Both these molecules are important mediators in development of HF as well as potent inducers of cardiomyocyte apoptosis (Kajstura *et al*, 1997; Sabbah *et al*, 2000). Prolonged use of ACE inhibitors and β-blockers can

inhibit cardiomyocyte apoptosis (Lapointe *et al*, 2003; Zeng *et al*, 2003). Moreover, inhibiting apoptosis by targeting the effector caspases may provide benefit in inhibition of HF development (Yarbrough *et al*, 2003).

ROS, such as hydrogen peroxide (H_2O_2) and super oxide anion (O_2^-) are produced in the myocardium as byproducts of metabolism as well as during inflammation. In addition to extrinsic antioxidants such as vitamin C, the cells harbor intrinsic antioxidative mechanisms that neutralize ROS. If ROS-neutralization mechanisms are inadequate, oxidative stress occurs, and oxidation of lipids, proteins, and nucleic acids impairs cellular functions and promotes apoptosis (Sawyer, 2011). Cellular damage by oxidative stress has been reported in patients suffering from HF (Canton *et al*, 2011; Tang *et al*, 2006). Moreover, patients with HF show enhanced cardiac expression of antioxidative enzymes (Dietrich *et al*, 2000) suggesting that the endogenous antioxidative defences in HF patients are insufficient.

Under physiological conditions, glutathione and manganese superoxide dismutase (MnSOD) are important antioxidative defence mechanisms (Assem *et al*, 1997). In addition, the cardiomyocytes harbor several other antioxidative mechanisms. Of these, cystathionase gamma-lyase (CTH) is an important alleviator of oxidative stress after MI. CTH functions by catalyzing L-cysteine to produce the gaseous messenger H_2S that neutralizes ROS (Elrod *et al*, 2007). Other major cardioprotective antioxidative enzymes include thioredoxin, heme oxygenase 1 and glutathione reductase (Sawyer, 2011). Oxidative stress affects a plethora of processes in the cardiomyocytes that are associated with HF development including hypertrophy, apoptosis, alterations in gene expression, and contractility due to abnormal Ca^{2+} transients (Sawyer, 2011). In experimental HF models, neutralization of oxidative stress by administration of dietary antoxidants such as vitamin E has shown benefits (Hamblin *et al*, 2007). In clinical trials, however, vitamin E may even be associated with increased risk for HF (Marchioli *et al*, 2006), suggesting that some ROS is beneficial for the failing heart and that new strategies to alleviate pathological ROS are necessary. Therefore, a more focused approach was designed to inhibit the major ROS-generating enzyme in the myocardium, xanthine oxidase. Targeting this enzyme has shown some benefit for HF patients in a clinical trial (Hare *et al*, 2008).

Another major contributor to development of HF that has emerged during recent years is ER stress. The imbalance between a cellular need for correctly folded proteins and the inability of the ER to produce the required amount of folded proteins leads to ER stress and an unfolded protein response (UPR) as an adaptive response to correct the

imbalance (Dickhout *et al*, 2011). ER stress is suggested to mediate cardiomyocyte hypertrophy, because inducers of ER stress such as AngII or tunicamycin also induce hypertrophy (Dickhout *et al*, 2011). In the long term, AngII-induced ER stress promotes cardiomyocyte apoptosis in addition to hypertrophy, suggesting that ER stress may contribute to AngII-mediated loss of myocytes in HF (Okada *et al,* 2004). Moreover, ER stress appears to play a major role in the cardiac hypertrophy resulting from high consumption of alcohol (Li and Ren, 2008). Importantly, UPR has been shown to counteract development of HF (Cook *et al*, 2009); its effects in the long term may, however, be adverse. The cardioprotective mechanisms of UPR may be dependent on expression of atrial natriuretic peptide and B-type natriuretic peptide because expression of these cardioprotective mediators is enhanced by UPR in an X-box binding protein 1-dependent mechanism. Furthermore, because B-type natriuretic peptide reduces blood volumes through natriuresis (Yoshimura *et al*, 2001), UPR may be beneficial for HF patients. Finally, MI induces ER stress, and inhibition of UPR further exaggerates the infarct damage (Martindale *et al*, 2006). Therefore, strategies to alleviate ER stress by inducing UPR may provide benefit for patients suffering from HF. The possible protective role UPR plays after MI and in HF warrants further investigation.

2.4 Treatments for heart failure

2.4.1 Pharmacotherapy

ACE inhibitors are a family of drugs that block the conversion of AngI to AngII by inhibiting ACE activity. ACE inhibitors thereby limit the pathological effects of AngII in HF development. According to the European Society of Cardiology guidelines for treatment of HF, ACE inhibitors are recommended for all patients with HF and an ejection fraction of $\leq$ 40% unless contraindicated or not tolerated (Dickstein *et al*, 2008). ACE inhibitors can affect development of HF by reducing hypertension, by affecting AngII-mediated remodeling after MI, and by reducing mortality and morbidity in patients who have already developed HF. By counteracting AngII-mediated hypertension, ACE inhibitors have proven to be valuable tools as first-line drugs to inhibit arterial hypertension and endothelial dysfunction and thereby reduce risk for developing HF (Mancia *et al*, 2007). ACE inhibitors have also shown benefit for patients with myocardial disease without HF. Furthermore, ACE inhibition has, in high-risk patients, been shown to inhibit MI, and left ventricular hypertrophy and to reduce mortality (Yusuf *et al*, 2000). After acute MI, ACE inhibition can be utilized to inhibit the RAS and thus prevent early remodeling (Hoshida *et al*, 2001). Furthermore, in the Optimal Trial in Myocardial Infarction with the Angiotensin II Antagonist Losartan (OPTIMAAL) trial, the ACE inhibitor captopril reduced mortality after MI and acute

HF (Dickstein *et al*, 2002). In patients with vascular disease who have not yet developed HF, ACE inhibitors reduce mortality and morbidity (ONTARGET investigators, 2008). Because of the essential role of the RAS in HF, inhibition of this system is essential to patients suffering from HF. It is widely established that ACE inhibitors reduce mortality and morbidity in patients with HF (CONSENSUS trial study group, 1987; Pfeffer *et al*, 1992).

Despite the undisputed advantages of ACE inhibitors for HF patients, some do not benefit from this medication. A population of patients on long-term ACE inhibitor use, show elevated plasma AngII levels. This elevation is independent of ACE activity (van de Wal *et al*, 2006).

Another widely used medication targeting the RAS system for treatment of HF comprises the AT1 receptor antagonists. They function by blocking the actions of AngII on its receptor AT1 that mediates most of the pathologies associated with AngII signaling. Because AT1 receptor antagonists do not block the AT2 receptor, they thus allow signaling, for example, for vasodilation via AT2 (Werner *et al*, 2008). Similar to ACE inhibitors, also AT1 receptor antagonism lowers blood pressure, thus affecting the major risk factor for developing HF (Mancia *et al*, 2007). Importantly, a combination of ACE inhibition and AT1 receptor antagonism results in a more complete inhibition of the RAS as well as in increased bradykinin accumulation leading to increased bioavailability of the vasodilatory mediator nitric oxide (Unger and Stoppelhaar, 2007). Indeed, a combination of ACE and AT1 inhibition is suggested to be more beneficial than monotherapy for treating hypertension in patients at high risk for developing HF (Doulton *et al*, 2005). In addition to reducing hypertension, AT1 receptor antagonists improve endothelial dysfunction, another risk factor for HF (Schmieder, 2006). The beneficial effects of AT1 receptor antagonists for treatment of established cardiac damage, such as that after MI, or overt HF are in line with those of ACE inhibitors. In the The Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET) study that enrolled 25,620 patients aged over 55 with coronary heart disease but not HF, both ACE and AT1 receptor inhibition had similar beneficial effects on mortality, MI, stroke, and hospitalization due to HF. In that study, combination of the two therapies resulted in an increase in adverse effects, and no additive beneficial effect occurred (ONTARGET investigators, 2008).

β-blockers function by antagonizing the beta adrenergic receptor and thereby inhibiting the effects of endogenous catecholamines, particularly epinephrine and norepinephrine. They inhibit the β1 receptor in the heart, and reduce renin secretion in the kidney, thus

downregulating the RAS (Pritchett and Redfield, 2002). β-blockers thus significantly reduce sudden death after MI, and are effective in reducing mortality and morbidity in long-term secondary prevention (Freemantle *et al*, 1999). For treatment of HF, these drugs are recommended for all clinically stable patients with EF $\leq$ 40%, and NYHA class II-IV. Furthermore, over 90% of patients using β-blockers are also using ACE inhibitors, AT1 receptor antagonists, or both (Dickstein *et al*, 2008). In the Carvedilol Prospective Randomized Cumulative Survival (COPERNICUS) trial, β-blocker medication was shown to reduce mortality and hospital admissions due to HF, as well as enhance patients' self-reported well-being. All these benefits seen with β-blockers were additional to those achieved by conventional ACE inhibitor treatment (Packer *et al*, 2002). In addition, the Study of the Effects of Nebivolol Intervention on Outcomes and Rehospitalisation in Seniors with Heart Failure (SENIORS) trial reported that β-blockers effectively reduced mortality and hospital admissions due to cardiovascular reasons in elderly patients of over 70 years old (Flather *et al*, 2005). Contraindications for β-blockers according to the European Society of Cardiology are asthma, second- or third-degree heart block, sick sinus syndrome, and sinus bradycardia. The potential adverse effects include: hypotension, worsening of HF, and extensive bradycardia (Dickstein *et al*, 2008).

Aldosterone antagonists are diuretics which antagonize the mineralocorticoid receptors, and thereby inhibit aldosterone activity. The diuretic action of these antagonists is mediated by inhibition of sodium resorption in the collecting duct of the kidney. In patients with HF, aldosterone antagonists reduce cardiac workload and edema. The European Society of Cardiology recommends aldosterone antagonists for patients with severe symptomatic HF accompanied by EF less than 35%. Moreover, such patients should have no severe renal dysfunction or hyperkalemia (Dickstein *et al*, 2008). The Randomized Aldactone Evaluation Study (RALES) trial evaluated spirolactone in treatment of HF. Patients with NYHA class III and EF $\leq$ 35% received spirolactone in addition to their existing medication (95% of patients receiving ACE inhibitors, but only 11% receiving β-blockers). Similar to β-blockers, spirolactone reduced mortality and hospital admissions and improved NYHA class. These positive effects were additive to existing treatment (Pitt *et al*, 1999). Contraindications to aldosterone antagonist treatment include serum potassium concentration of over 5.0 mmol/l, serum creatinine over 220 μmol/l, and combination of ACE inhibitor and AT1 receptor antagonist treatments. The potential adverse effects are, except for cough, similar to those of ACE inhibitors (Dickstein *et al*, 2008).

Digitalis is a cardiac glycoside that exerts its action in the heart through increased contraction, and a decrease in conduction via the atrioventricular node. Digoxin inhibits the sodium-potassium adenosine triphosphatase (Na^+ -K^+ -ATPase) and thus interferes with transport of these ions across plasmamembrane. This decreased conduction is mediated via inhibition of automaticity (Currie *et al*, 2011). Digoxin is recommended by the European Society of Cardiology for patients with symptomatic HF (NYHA class II-IV), atrial fibrillation, and EF $\leq$ 40% to reduce a rapid heart rate. In addition, digoxin can effectively control heart rate in patients who are using β-blockers (Dickstein *et al*, 2008). A large, randomized controlled clinical trial, The Digitalis Investigation Group (DIG), studied the added effects of digoxin on HF patients receiving ACE inhibitors and diuretics. Digoxin did not affect mortality but reduced hospitalization due to worsening HF. The contraindications of digoxin include second- to third-degree heart block, and caution is necessary if sick sinus syndrome is suspected. Potential adverse effects include atrial and ventricular arrhythmias, and signs of toxicity such as nausea, confusion, and anorexia (Dickstein *et al*, 2008).

Diuretics such as furosemide inhibit reabsorption of sodium from the urine in the nephrons, subsequently leading to retention of water in the urine and thereby reducing blood volume and blood pressure. Diuretics are recommended for treating hypertension and HF patients who have moderate to severe clinical signs and symptoms of congestion (Dickstein *et al*, 2008). Diuretics are known to cause RAS activation and therefore require concomitant use of ACE inhibitors or AT1 receptor antagonists. In addition to these most commonly used medications for HF, other medicines used to treat HF or HF-related co-morbidity include: hydralazine and isosorbide dinitrate, anticoagulation agents and the vitamin K-antagonist warfarin, antiplatelet agents, and statins (Dickstein *et al*, 2008).

2.4.2 Surgery and implantable devices

The proposed mechanism of surgical revascularization therapy for the ischemically failing heart has been the reperfusion of hibernating underperfused myocardium (Shanmugam and Legare, 2008). Most patients with ischemic ventricular dysfunction possess viable myocardium that could benefit from revascularization (Bax *et al*, 2004). Clear indications for patients with coronary heart disease include revascularization by coronary artery bypass grafting (CAGB) or PCI. These indications are, however, limited to patients with angina and significant coronary heart disease (Eagle *et al*, 2004). The evaluation of benefits of CABG has been difficult, however, because of the lack of randomized controlled trials (Shanmugamand and Legare, 2008). However, reduced mortality may result from CABG (Phillips *et al*, 2007). Furthermore, CABG improves

symptoms of HF in 59 to 92% of patients (Baker *et al*, 1994). Finally, as compared to medical treatment, CABG may improve survival in patients with ischemic ventricular dysfunction and low EF (O'Connor *et al*, 2002; Smith *et al*, 2006). The Alberta Provincial Project for Outcomes Assessment in Coronary Heart Disease (APPROACH) study enrolled 2538 patients diagnosed with HF (Tsuyuki *et al*, 2006). This large population-based study suggests that both CABG and PCI showed survival benefits. In addition, CABG provided increased survival over PCI (Tsuyuki *et al*, 2006). Inconclusive evidence thus suggests that patients with ischemic HF may substantially benefit from CABG and PCI revascularization.

Cardiac resynchronization therapy can improve cardiac output by synchronizing intra- and interventricular contraction. The European Society of Cardiology recommends this therapy for patients with NYHA class III-IV and EF of less than 35% (Dickstein *et al*. 2008). The Comparison of Medical Therapy, Pacing, and Defibrillation in Chronic Heart Failure (COMPANION) study reported a decrease of approximately 20% in mortality and hospitalization from all causes (Bristow *et al*, 2004). The Cardiac Resynchronization in Heart Failure (CARE-HF) trial reported that cardiac resynchronization therapy reduced total mortality by 36% as compared to medical therapy alone. Moreover, this study reported significantly reduced hospitalization (Cleland *et al*, 2005). For those patients who have suffered ventricular fibrillation or have unstable ventricular tachycardia with <40% EF, the European Society of Cardiology recommend an implantable cardiac defibrillator (Dickstein *et al*, 2008). The Sudden Cardiac Death in Heart Failure (SCD-HeFT) trial enrolling patients with dilated cardiomyopathy as well as ischemic LV dysfunction reported that defibrillator therapy reduced mortality by 23% (Bardy *et al*, 2005). Moreover, a meta-analysis suggested a mortality reduction of 25% in patients with dilated cardiomyopathy (Desai *et al*, 2004).

Ventricular remodeling and dilation in patients with HF is often associated with mitral valve regurgitation. The American College of Cardiology and The American Heart Association guidelines suggest mitral valve repair or replacement mitral regurgitation grade 3 and 4 for symptomatic patients, or grade 3 and 4 for asymptomatic patients with left ventricular dysfunction (Feldman and Glower, 2008). Global ventricular as well as individual cardiomyocyte function can be improved when mitral regurgitation is corrected with surgery (Hussaini and Kar, 2010). Mitral valve surgeries are, however, associated with significant morbidity (Feldman *et al*, 2011).

Transplantation of an allogenic heart provides a treatment option for those end-stage HF patients who do not benefit from other treatments. The 5-year survival after heart

transplantation is 72% for male and 67% female patients (Lloyd-Jones *et al*, 2009) as compared to 30 to 40% in patients receiving no transplant (Cowie *et al*, 2000). Despite the lack of controlled trials, transplantation is thought to improve survival, exercise capacity, and quality of life compared to conventional treatments. Although the individual patients benefit greatly from transplantation, few receive this treatment due to the severe donor shortage (Dickstein *et al*, 2008). Left ventricular assist devices (LVAD) have raised hopes of compensating for this shortage of organs (Cowger *et al*, 2011). The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) trial evaluated additional benefits of LVAD implantation compared to standard medical treatment. For those patients receiving LVAD implantation, survival at 1 year was 52% compared to 25% in the control group (Rose *et al*, 2001). In the HeartMate II Destination Therapy trial, continuous-flow LVAD further improved 1-year survival to 68% (Slaughter *et al*, 2009). In addition to these surgical and device-mediated therapies for treatment of HF, ultrafiltration and remote monitoring are clinical treatments that provide benefit for patients suffering from HF (Dickstein *et al*, 2008).

2.4.3 Cardiac regeneration

Within a few hours, MI can permanently destroy 25% of the 2 to 4 billion cardiomyocytes in the adult human heart (Murry *et al*, 2006). Further, cardiac pathologies and aging result in an accumulating loss of cardiomyocytes over several years (Laflamme and Murry, 2011). Cardiac regeneration has therefore been designed to replace the lost cardiomyocytes with new contractile tissue either through stimulation of endogenous regenerative mechanisms or cell transplantation (Laflamme and Murry, 2011). From the middle of the 19th century to the early part of the 20th, the heart was generally perceived as incapable of regeneration (Carvalho and Carvalho, 2010). Since 1937, indirect evidence started to accumulate suggesting that human cardiomyocytes can undergo mitosis, first in children (Macmahon, 1937), and later in adults (Lintzbach, 1960). It was not, however, until the 1990's that direct evidence of cardiomyocyte mitosis emerged (Carvalho and Carvalho, 2010). The rate of mitosis is, however, very slow and cannot account for macroscopic regeneration of the human heart (Laflamme and Murry, 2011). It is estimated that the 1% rate of cardiomyocyte renewal in 20-year-olds gradually decreases, annually reaching 0.4% in 75-year-olds (Bergmann *et al*, 2009). The source of these new myocytes in humans is currently unknown. They may originate from mitosis of already-existing cardiomyocytes (Engel *et al*, 2006), from cardiac stem- or progenitor cells (Smith *et al*, 2007), from circulating bone-marrow cells (Kuramochi *et al*, 2003), or from a combination of these.

Contrary to humans, in amphibians, cardiac regeneration is well documented (Oberpriller and Oberpriller, 1974) as it is also in zebrafish (Poss *et al*, 2002). After surgical removal of up to 20% of the apex of its heart, a zebrafish is fully able to regenerate the lost tissue. This regeneration occurs via initial formation of a fibrin clot that seals the wound. Lost tissue is then replaced by extensive mitotic division of the existing cardiomyocytes that undergo dedifferentiation and proliferation, thus providing the main contribution to regeneration. The fraction of mitotic cardiomyocytes, as evaluated by BrdU-labeling, is 20 to 30% at 2 to 4 weeks post-amputation (Poss *et al*, 2002). Undifferentiated progenitor cells make little or no contribution to this regeneration (Jopling *et al*, 2010; Kikuchi *et al*, 2010). In contrast to the remarkable regenerative potential of the zebrafish heart, mammalian hearts possess far less capacity to regenerate. The proportion of cardiomyocytes undergoing mitosis in the left ventricle of a healthy adult mouse heart is estimated to be 0.0005% (Soonpaa and Field, 1997). In the border zone of injured mouse hearts, 0.0083% of cardiomyocytes are mitotic, demonstrating that cardiomyocyte self-renewal in adult mice is a very rare event (Soonpaa and Field, 1997). As a proof of concept, adult mouse cardiomyocytes can be induced to proliferate and repair MI-induced injury by overexpression of cyclin D, an activator of the cell cycle (Hassink *et al*, 2008). Furthermore, in a more clinically relevant approach, cardiomyocyte mitosis and repair after MI in the mammalian heart can be induced by growth factors such as neuregulin 1 (Bersell *et al*, 2009) and fibroblast growth factor 1 (Engel *et al*, 2006). In contrast to the zebrafish cardiac repair mechanism, it is suggested that the very low regenerative capacity of adult mice is due to a contribution from cardiac progenitor cells rather than to mitosis of differentiated cardiomyocytes (Hsieh *et al*, 2007). However, 1-day-old mice possess a cardiac regenerative capacity similar to that of zebrafish. This regenerative capability is lost early, as 7-day-old mice are incapable of cardiac regeneration. Surgical resection has been regenerated in these 7-day-old mice by existing cardiomyocyte proliferation in the absence of any progenitor cell contribution, fibrosis, or hypertrophy (Porrello *et al*, 2011).

2.5 Myocardial infarction-induced heart failure models

Animal models for studying HF can be induced by genetic modification, pressure and volume overload, sustained tachycardia, or toxic drugs. In addition to these, ischemic models are essential for studying HF, because the most important causes of HF are coronary artery disease and MI (Abarbanell *et al*, 2010). Ischemic damage to the myocardium can be achieved by left-descending coronary artery (LAD) ligation. The LAD is typically ligated through a left thoracotomy by a suture or occlusive device to

temporarily or permanently occlude the LAD or a branch of it (Klocke *et al*, 2007). Model animals for LAD occlusion include the mouse, rat, pig, dog, and monkey (Abarbanell *et al*, 2010). Ischemia-reperfusion injury is achieved by temporary LAD ligation for a varying time, after which the LAD is reperfused. Permanent ligation induces an MI. These LAD occlusion models induce cardiomyocyte death, decrease in cardiac function, hemodynamic changes, and formation of a collagenous scar among other phenomena. The extent of myocardial damage depends on species, site of occlusion, and duration of ligation (Abarbanell *et al*, 2010).

2.6 Myoblast transplantation for heart failure

2.6.1 Transplantation of skeletal muscle

In the 1980's the first clinical case of skeletal muscle transplantation was performed to aid the failing heart. (Carpentier and Chachques, 1985). In this cardiomyoplasty procedure, the latissimus dorsi muscle is transplanted around the failing heart and stimulated electrically for synchronized beating with the myocardium and to provide functional benefit for the failing heart (Overgoor *et al*, 2003). Although over 600 procedures have been performed clinically, the results have varied, and necrosis of the latissimus dorsi is suggested to hamper the long-term efficacy of this approach (Overgoor *et al*, 2003). Therefore, during the recent years transplantation of skeletal muscle-derived cells instead of autologous muscle tissue has attracted more interest (Chacques *et al*, 2002).

2.6.2 Skeletal myoblasts

Satellite cells were originally identified in 1961 as precursor cells committed to the skeletal muscle lineage (Mauro, 1961). These quiescent mononuclear cells are located between muscle fibers and the endomycium, the thin layer of connective tissue surrounding each fiber. Satellite cells are activated by skeletal muscle damage; they proliferate rapidly and migrate to the site of injury where they undergo differentiation and fusion to regenerate new muscle fibers. Since the original description, several subpopulations of satellite cells have been identified with different characteristics including muscle stem cells and myogenic progenitors. In contrast to the hearts' limited regeneration potential, satellite cells are able to repair significant tissue damage (Tedesco *et al*, 2010). The activated satellite cells, also known as myoblasts, can be isolated from muscle biopsies and be cultured for extended periods *in vitro* without loss of their ability to differentiate (Yaffe, 1968). Myoblasts can readily be expanded in culture, acquire a contractile phenotype after differentiation, and are able to regenerate muscle tissue. Furthermore, they are resistant to multiple stress factors such as hypoxia.

This is an important feature considering the target tissue, a hypoxic infarct scar. For these reasons, myoblasts have been considered as potential cell population for cardiac cell therapy to replace lost contractile tissue and aid the failing heart (Menasche, 2003).

2.6.3 Experimental studies

Myoblasts were first transplanted to cryoinjured canine hearts in 1992 and were shown to form areas of striated of muscle tissue (Marelli *et al*, 1992). Since then, extensive experimental and clinical research has been carried out to verify feasibility and safety, and to improve the efficacy of myoblast transplantation. The experimental studies that followed Marelli's pioneering work in small and large animal models verified that transplanted myoblasts survive, are incorporated into the infarcts, differentiate into contractile myotubes, inhibit remodeling, and improve cardiac function in hearts injured by cryoinfarction (Murry *et al*, 1996; Taylor *et al*, 1998), cardiotoxins (Rajnoch *et al*, 2001), ischemia-reperfusion (Jain *et al*, 2001), and MI (Pouzet *et al*, 2001;Ghostine *et al*, 2002) in a dose-dependent manner (Pouzet *et al*, 2001). These studies also present data from injury models in which myoblast transplantation was administered at different time-points. Myoblasts improved cardiac function early after damage as well as later when cardiac remodeling had already taken place. These studies raised high expectations for myoblast transplantation to benefit HF patients.

At least three mechanisms of action have been proposed for the beneficial effects of myoblast transplantation. None of these has been decisively excluded (Menasche, 2003; Seidel *et al*, 2009). First, the transplanted cells may improve the elastic properties and strengthen the ventricular wall at the site of transplantation (Menasche, 2003). This mechanism could also improve survival of the transplanted cells. Support for this hypothesis comes from studies where, in addition to myoblasts, also non-contractile cells such as fibroblasts (Hutcheson *et al*, 2000) and bone marrow-derived cells (Agbulut *et al*, 2004) improve regional diastolic function after transplantation.

Second, the myoblasts may, after differentiation, contribute to cardiac systolic function via their contractile phenotype. In support of this hypothesis, myoblast transplantation improved both systolic and diastolic function, whereas non-contractile fibroblasts improved only diastolic function (Hutcheson *et al*, 2000). For the myoblasts to assist in cardiac contraction, electromechanical coupling between host cardiomyocytes and transplanted myoblasts is essential. Cultured myoblasts express the major adhesion and gap junction proteins N-cadherin and connexin 43 (Reinecke *et al*, 2000) that are important for electromechanical coupling and signal transduction between contractile cells (Li *et al*, 2006). However, after transplantation to the injured heart, these factors

were downregulated in differentiated myotubes, and their expression was undetectable *in vivo*. In contrast, *in vitro* findings suggest that cardiomyocytes and myotubes can contract synchronously under optimal conditions (Reinecke *et al*, 2000). Indeed, the transplanted myoblasts were electrically isolated from the host cardiomyocytes (Leobon *et al*, 2003). This shortcoming has been addressed in experimental models by overexpression of connexin 43 in myoblasts. In addition to enhanced gap junction formation, these cells differentiated more rapidly (Suzuki *et al*, 2001) and formed gap junctions with cardiomyocytes *in vivo* (Reinecke *et al*, 2004a). In addition to being electronically isolated, the myoblasts did not transdifferentiate towards a cardiomyocyte phenotype as assessed by expression of α-myosin heavy chain, atrial natriuretic peptide, and cardiac troponin I (Reinecke *et al*, 2002). Myoblasts did, however, continue to express skeletal muscle markers. Although evidence does not suggest myoblast electrical coupling or transdifferentiation, cell fusion between myoblasts and host cardiomyocytes is possible (Reinecke *et al*, 2004b). Although this mechanism could in part explain the beneficial effects of myoblast transplantation, further evidence to support this hypothesis is lacking.

Third, the mechanisms observed may be due to paracrine factors secreted by the transplanted myoblasts to stimulate the host myocardium. Some studies have detected no induction of angiogenesis after transplantation, leading to the conclusion that the paracrine mechanism is not essential (Menasche, 2003). Other studies have, however, reported induction of angiogenesis following transplantation (Suzuki *et al*, 2001). Furthermore, myoblasts secrete MMP2 as well as tissue inhibitor of MMP (TIMP) 4, soluble factors associated with antifibrotic actions of myoblast transplantation (Shintani *et al*, 2009). Moreover, the myoblasts secrete a high number of other mediators that are associated with angiogenesis – such as VEGF, placental growth factor (PlGF), hepatocyte growth factor (HGF) – and inhibition of remodeling, such as MMP7 and MMP10 (Perez-Ilzarbe *et al*, 2008). These factors correlate with the myocardial responses observed following myoblast transplantation. Finally, Gnecchi convincingly demonstrated with another cell type (bone marrow mesenchymal cells) that injection of conditioned medium alone without cells produced results comparable to those achieved with injection of cell suspension, providing strong support for the paracrine hypothesis (Gnecchi *et al*, 2006). Similar evidence with myoblasts is, however, still lacking. Due to these data, the paradigm of the mechanisms of cell transplantation therapy has in recent years shifted towards the paracrine hypothesis (Menasche, 2011b).

2.6.4 Clinical trials

Encouraged by the positive results from experimental trials in the late 90s, myoblast transplantation was translated to the first clinical trial in 2000 (Menasche *et al*, 2001). In that trial, 10 patients with severe ischemic left ventricular dysfunction (35% ≤ EF), a nonviable post-infarction scar, and an indication for CABG received injections of myoblasts as a single-cell suspension into the post-ischemic but viable areas in the myocardium. After an average follow-up of 52 months, in the myoblast group, both the EF and the NYHA class improved. A low rate of hospitalization and few cases of sustained tachycardia were detected. Moreover, engrafted myotubes were detectable in one patient who died during follow-up (Hagege *et al*, 2006). This first trial with encouraging results was followed by three other safety and feasibility trials. Gavira *et al* (2006) enrolled 12 patients with previous MI and ischemic coronary artery disease. These patients received intramyocardial injections of autologous myoblasts during CABG. Left ventricular ejection fraction, regional contractility, and perfusion improved significantly in the myoblast group at the one-year follow-up with no adverse affects detectable. Siminiak *et al* (2004) reported an intramyocardial myoblast injection study of 10 patients with prior MI who were scheduled for CABG. After a one-year follow-up, this study too, reported a significant increase in EF in the myoblast group but also four cases of sustained tachycardia. Dib *et al* (2005) reported on 24 patients with prior MI who underwent CABG and intramyocardial injection of myoblasts. Another six patients waiting for heart transplantation underwent LVAD implantation and myoblast injection. Results showed an improved EF, as well as induced metabolic activity at injection sites, with no adverse effects detectable. Furthermore, in four out of the six hearts removed because of transplantation, myoblasts were detectable (Dib *et al*, 2005). These early non-randomized phase I trials thus reported encouraging results for the clinical application of myoblast transplantation therapy. They provide evidence of feasibility along with a threat of arrhythmias, although the small patient groups and lack of controls allow no decisive conclusions.

Based on these relatively positive findings, a large, multicenter, randomized, placebo-controlled trial began (Menasche *et al*, 2008). The Myoblast Autologous Grafting in Ishemic Cardiomyopathy (MAGIC) trial involved 21 European centers and enrolled patients with severe left ventricular dysfunction, a nonviable post-infarction scar, and an indication for CABG. Patients received injections of 400 or 800 million myoblasts or placebo. The cells were cultured in two core laboratories for three weeks per sample. Again, injections were performed during a CABG, and each patient was also implanted with a defibrillator. A total of 120 patients were randomized, of whom 97 were effectively treated. No improvement occurred in global or regional left ventricular

function in the myoblast group. However, those patients receiving the higher dose of myoblasts showed a significant difference in remodeling that was presented as decreased end systolic and end diastolic volumes. Although the MAGIC trial provided some positive results, the lack of functional cardiac improvement was highly disappointing, given the results from earlier phase I trials. Although no significant differences in arrhythmias occurred in the myoblast groups as compared to the placebo group, the myoblast groups showed a tendency for early post-operative arrhythmias.

In addition to these CABG-associated trials, three studies have delivered myoblasts via a catheter-based system. In the Percutaneous Trans-coronary-venous Transplantation of Autologous Skeletal Myoblasts in the Treatment of Post-infarction Myocardial Contractility Impairment (POZNAN) trial (Siminiak *et al*, 2005), myoblasts were delivered via the coronary sinus with a TransAccess^{tm} catheter incorporated with intravascular ultrasound for guidance. This study included 10 patients with previous MI and areas of non-viable myocardium. The results showed improvement in NYHA class for all patients and improvement in EF for six of nine patients. Biagini *et al* (2006) reported on a trial with ten patients suffering from ischemic dilated cardiomyopathy receiving endocardial injections of myoblasts by catheter. Results showed significant improvement in NYHA class and EF, and a decrease in end-systolic volumes. A smaller six-patient trial delivering myoblasts via a catheter also reported improvement in EF (Ince *et al*, 2004). Finally, an open-label, randomized, prospective Safety and Effects of Implanted (Autologous) Skeletal Myoblasts (MyoCell) using an Injection Catheter (SEISMIC) trial evaluated the safety and feasibility of percutaneous myoblast transplantation in HF patients implanted with a cardioverter-defibrillator (Duckers *et al*, 2011). That study compared myoblast transplantation with the optimal medical treatment, and 26 patients received myoblast therapy with 14 randomized as controls. The results showed a trend towards improvement in NYHA classification but no improvement in EF.

2.6.5 Arrhythmias

Although the MAGIC trial showed no statistically significant associations between intramyocardial injections of myoblasts and arrhythmias, serious concerns arose because of the indication in MAGIC and other trials of the lack of safety in myoblast transplantation. To answer the question whether injected myoblasts induce arrhythmias, several experimental studies began. Abraham *et al*. (2005) showed in an *in vitro* coculture model that myoblasts seeded on top of a cardiomyocyte monolayer induced reentrant arrhythmias similar to the ventricular tachycardias detected in patients after myoblast injection. Again in this study, overexpression of connexin 43 reduced

arrhythmogenicity in this model system. Thereafter, two studies showed in a rat MI injury model that myoblast injections induced ventricular arrhythmias significantly more than did vehicle or bone marrow cell injections (Fernandes *et al*, 2006; Mills *et al*, 2007). Another study using a rat MI model showed no significant differences in arrhythmogenicity by myoblast injection, although in the healthy myocardium, injections resulted in abnormal pulse propagation (Fouts *et al*, 2006).

Although in part conflicting, these studies provide evidence that myoblast injections can induce arrhythmias. Importantly, Fukushima *et al* (2007) demonstrated with other types of cells (bone marrow mononuclear) that delivery route may determine arrhythmogenicity of cell transplantation and that needle injection, by disrupting the myocardial architecture and induction of inflammatory responses, may be the cause of the adverse effects detected. Fukushima went on to show that the early arrhythmias can be circumvented by intracoronary administration of myoblasts without affecting their efficacy. That study, however, revealed a small number of premature ventricular contractions, ones occurring also with intracoronary delivery (Fukushima *et al*, 2008). Finally, in addition to the previously demonstrated downregulation of connexin 43 in myoblasts after transplantation (Reinecke *et al*, 2000), the late-phase arrhythmogenicity was associated with connexin 43 downregulation in the host cardiomyocytes as well (Coppen *et al*, 2008). These data indicate that myoblast transplantation may have an arrhythmogenic potential and that it may be reduced by choice of cell-delivery route.

2.6.6 Routes of delivery

Cardiac delivery of myoblasts or other cells can be by several routes of administration. These include, but are not restricted to, intramyocardial (transepicardial and transendocardial), and intracoronary routes (Dib *et al*, 2011). When compared to intracoronary administration, delivery by intramyocardial injection results in relatively high retention of donor cells by the myocardium. Although effective, intramyocardial injections produce less consistent results (Hou *et al*, 2005), and the number of cells retained is approximated to be no more than 10% of the original injected population due to mechanical leakage (Hudson *et al*, 2007). In addition, the death-promoting environment of the post-MI myocardial scar and border zone subject the transplanted cells to inflammatory stimuli, hypoxia, and anoikis (Hudson *et al*, 2007). As a result, retention less than 1% of the originally transplanted cells has been documented (Zeng *et al*, 2007).

As mentioned, the adverse effects associated with intramyocardial injections are induction of arrhythmias and inflammatory responses (Fukushima *et al*, 2007).

Intracoronary administration can be via the coronary artery either during normal flow or during interrupted flow caused by balloon occlusion (Dib *et al*, 2011). Cells can be delivered through a catheter, which allows transplantation also during PCI after MI (Dib *et al*, 2011). Cell retention in the myocardium is dependent on cell migration into the myocardium across the endothelial barrier. Ischemia-reperfusion can enhance this migration (Fukushima *et al*, 2011), suggesting that intracoronary delivery may be the optimal delivery route when accompanied by PCI. In a rat model of MI, both intramyocardial and intracoronary myoblast delivery methods resulted in similar retention and therapeutic efficacy, but the lower rate of arrhythmias favored the intracoronary route (Fukushima *et al*, 2008). Finally, in recent years, epicardial transplantation of scaffoldless tissue-engineered cell sheets has emerged as a method for myoblast transplantation (Memon *et al*, 2005)

2.6.7 Myoblast sheet transplantation

The use of self-aggregating scaffoldless cell sheets as a mean of myoblast delivery to the dysfunctional heart has gained attention recently. Because scaffolds as cell carriers can result in inflammatory responses, fibrous tissue formation, and abnormal cellular function in the absence of cell-cell junctions (Villet *et al*, 2011), scaffoldless constructs formulated only of donor cells and the cell-derived ECM could therefore form an attractive strategy. Such scaffoldless constructs can be manufactured by several techniques.

For example, Stevens *et al* (2009) described a method using Teflon-coated low-adhesive cell culture dishes. A single-cell suspension was then added to the dishes and incubated with an orbital shaker. During this incubation the cells self-aggregated to form a cell sheet approximately 300 to 600 μm in thickness. These relatively thick sheets, however, underwent necrosis due to hypoxia in their center. Another method utilized fibrin-coated dishes that allowed cell attachment. Proteolytic enzymes secreted by the seeded cells then degraded the coating and allowed collection of intact cell sheets for transplantation by mechanical scraping (Itabashi *et al*, 2005).

The method most used for engineering scaffoldless cell sheets involves temperature-responsive Poly(N-isopropylacrylamide) (PIPAAm)-coated culture dishes. PIPAAm is a polymer that is hydrophobic at temperatures over 32º C, allowing adherence and proliferation of various cell populations (Okano *et al*, 1993). At temperatures below 32º C, however, PIPAAm becomes hydrophilic, undergoing rapid hydration which allows spontaneous detachment of cells as intact sheets. Importantly, the cells in such sheets deposit extracellular matrix on their basal surface, as well as form cell-cell junctions

that are retained after sheet detachment (Kushida *et al*, 1999). Furthermore, cell viability in these sheets remains comparable to that of standard cell culture conditions (Okano *et al*, 1993). Cells can be grown on PIPAAM-grafted surfaces for harvesting of sheets consisting of a single monolayer of cells (Shimizu *et al*, 2001). Moreover, multilayered sheet structures can be constructed by layering multiple monolayer sheets. This approach allows engineering of hybrid structures combining myoblasts with other cell types such as endothelial cells to achieve more efficient vasculature for the graft, post-transplantation (Sasagawa *et al*, 2010).

Memon *et al* (2005) compared the efficacy of myoblast sheet transplantation to that of intramyocardial injections into rat hearts after MI. They transplanted 10^7 myoblasts either by intracardiac injection of single-cell suspension or by epicardiac deposition of two sheets formed on PIPAAm-grafted culture dishes. They demonstrated that myoblast sheets significantly improved cardiac function as compared to vehicle or myoblast injection groups until the study-end at 8 weeks. Moreover, the anterior wall was significantly thicker and fibrosis was significantly less in the hearts transplanted with sheets. These beneficial effects were associated with increased number of cells in the myocardium characterized by the markers c-Kit, Sca-1, and CD34. Finally, the authors reported an increased myocardial expression of cardioprotective growth factors: stromal cell-derived factor 1, HGF, and VEGF, thus providing the first evidence of added benefit from myoblast transplantation as epicardial sheets instead of as intramyocardial injections.

During the next year, two other studies demonstrated the efficacy of myoblast sheet transplantation in differing injury models. Kondoh *et al* (2006) used a dilated cardiomyopathy model, in which myoblasts were transplanted to 27-week-old BIO TO-2 hamsters with moderate cardiomyopathy in their left and right ventricles. Results showed that in those animals receiving myoblast sheet therapy, further dilation of the ventricles, as measured by end-diastolic and end-systolic diameters, was inhibited, and cardiac function stabilized. In contrast, in their vehicle and myoblast injection groups, pathological remodeling proceeded and cardiac function deteriorated. Moreover, sheet therapy inhibited accumulation of fibrotic collagen. Importantly, myoblast sheet transplantation significantly prolonged the life-span of those cardiomyopathic animals. The authors discussed that the beneficial effect was possibly due to paracrine factors secreted by the myoblasts. These data provided evidence that in non-ischemic dilated cardiomyopathy, a condition in which myoblast transplantation had been associated with poor results (Pouly *et al*, 2004), myoblast sheets can also counteract remodeling and aid cardiac function and survival. Another study published in 2006 (Hata *et al*),

continued the validation of myoblast sheet therapy in a large animal model. HF was induced in dogs by 4 weeks of rapid ventricular pacing after which myoblast sheets were implanted or the heart was sham-operated. Myoblast sheet implantation improved cardiac function and led to increased ventricular wall thickness as compared to that of the sham group. Histology revealed myoblast sheet survival after transplantation, reduced fibrosis, and induction of cell proliferation in the myocardium. Although this second study involved no myoblast injection with which to compare myoblast sheets, it did provide preclinical evidence in a large-animal model of the efficacy of sheet therapy.

As all of these three studies were performed at the Osaka University Graduate School of Medicine, Osaka, Japan, it was therefore important that researchers from another university (Université Paris Descartes, Paris, France) performed a study comparing transplantation of myoblast sheets to myoblast delivery by injection or by biomaterial scaffold carrier (Hamdi *et al*, 2009). This study employed a chronic rat MI model in which myoblast transplantion was performed 4 weeks after LAD ligation. Myoblast sheets, intramyocardial injections, myoblast-containing collagen sponges, or PBS injections were administered and results analyzed 4 weeks after treatment. Analysis of cardiac function revealed that myoblast sheets and myoblast scaffolds significantly enhanced EF at 4 weeks with a slight but statistically non-significant advantage in favor of the myoblast sheets. In addition, both these therapies also significantly induced angiogenesis in the failing myocardium. In terms of fibrosis, again, both myoblast sheet and scaffold therapies - but not injection - reduced scar tissue. This study further verified the superiority of myoblast sheet transplantation and indicated that myoblast therapy is effective with and without a scaffold carrier.

Also in 2009, researchers from Osaka University published another demonstration of myoblast sheet efficacy with a rat model of pressure overload-induced right ventricular failure (Hoashi *et al*, 2009). Right ventricular injury was induced with pulmonary artery banding. Four weeks later, myoblast sheets were implanted, and the results were analyzed after another 4 weeks compared to those of a sham-operated group. The myoblast sheet-treated hearts showed improved systolic function as well as increased expression of HGF and VEGF as compared to those sham-operated. Moreover, myoblast sheets inhibited ventricular fibrosis and caused increased capillary density. No differences, however, were observable in systolic function, or in right ventricular shape and cell size. These experimental studies in different myocardial injury models demonstrate the efficacy of myoblast sheet therapy as superior to injections.

Sekiya *et al* (2009) then addressed the question whether myoblast sheet therapy has a dose-dependent therapeutic effect in the damaged heart. They employed a rat MI model and epicardially transplanted one, three, or five myoblast sheets layered on top of each other, revealing that cardiac function was significantly improved in animals receiving three or five sheets, but not only one. Although non-significant, a trend emerged towards better function in animals with five as compared to three sheets. A dose-dependent increase in HGF and stromal cell-derived factor 1 expression, anterior wall thickness, and vascular density was detectable as well as a decrease in fibrosis and hypertrophy. This study provided evidence that myoblast sheets have a dose-dependent effect, and that implantation of five sheets clearly improves efficacy as compared to one sheet.

Miyagawa *et al* (2010) conducted a preclinical study using a porcine ischemic cardiomyopathy model with a 6-month follow-up. Ischemia in these hearts was induced by an ameroid constrictor for 4 weeks, after which came myoblast sheet implantation or a sham operation. Even though the numbers of animals in these study groups were small (n=5), analysis showed significant improvement in cardiac systolic and diastolic function 3 and 6 months after therapy. Further, hypertrophy and fibrosis were markedly reduced, and vascular density was higher in the myoblast sheet group. Thus, this preclinical, as well as other experimental studies with various heart disease models, have shown promising indications for the clinical application of sheet transplantation.

2.6.8 Strategies to enhance myoblast transplantation

The failure of the randomized, double-blind, placebo-controlled MAGIC trial to show any functional benefit in humans suggests that novel strategies are needed to enhance the efficacy of myoblast transplantation in order to translate the promising experimental results clinically. Issues concerning cell delivery have already been discussed. Current evidence comparing myoblast sheets and injections indicates an advantage for myoblast sheets. In addition, utilization of sheets provides means to deliver cells into the myocardium with minimal loss due to leakage or anoikis. Further studies must elucidate the long-term survival benefit for the transplanted cells and incorporation of myoblasts into the host myocardium. Moreover, strategies to enhance myoblast functional integration with the host myocardium by upregulating connexin 43 have already been discussed as well as benefits and problems related to biomaterials as cell carriers.

Among strategies to enhance cell survival after transplantation, heat shock and cryopreservation treatments have been evaluated (Suzuki *et al*, 2000; Maurel *et al*, 2005). These reports present somewhat contradictory data, possibly because of differing

heat shock protocols. Heat or cold shock might be able to provide a safe pretransplantation modification to enhance myoblast therapy via upregulation of prosurvival and antioxidative proteins. Another approach to reduce early cell loss after myoblast transplantation is to utilize agents to suppress the apoptosis-promoting local immune response. For example, concomitant administration of myoblasts and Il-1β antibodies has enhanced graft survival (Suzuki *et al*, 2004). In addition to inflammation-derived stress, in transplantation to the MI-environment, the transplanted cells as well as the host cardiomyocytes are subjected to severe hypoxia that promotes graft apoptosis. For this reason, several strategies have been established in experimental models to promote perfusion in the heart. For example, myoblasts can be transplanted with recombinant angiogenic factors or can be modified to express proangiogenic genes. These include but are not restricted to VEGF (Askari *et al*, 2004), fibroblast growth factor 4 (Bialas *et al*, 2011), HGF (Tambara *et al*, 2005), and angiopoietin (Ye *et al*, 2007). Moreover, transfection of the hypoxia-inducible transcription factor 1α that controls the expression of several growth factors including VEGF augmented myoblast transplantation (Azarnoush *et al*, 2005). In addition to angiogenic strategies, overexpression of antiapoptotic genes such as *bcl2* has served to promote myoblasts graft survival after transplantation. Kutschka *et al* (2006) demonstrated that *bcl2*-expression improved survival and efficacy of cardiomyoblast therapy. Such antiapoptotic genetic modification is, however, far beyond the reach of clinical application because of the obvious tumor risks inherent in inhibition of apoptosis and possibly to the viral vectors used for gene transfection.

3. Aims of the study:

The aim of the present study was to investigate the effects of myoblast sheet therapy combined with antiapoptotic and cardioprotective growth factor gene therapy in rat acute and chronic MI models and to elucidate the molecular mechanisms associated with the response.

The specific aims of the individual studies were **to determine**:

I The therapeutic effect of myoblast sheet transplantation, and to evaluate the ability of antiapoptotic Bcl2-expression in myoblasts to prevent graft apoptosis and enhance the efficacy of therapy in a rat acute MI-induced HF model.

II In a rat chronic MI-induced HF model whether inhibition of graft apoptosis by Bcl2-expression can enhance myoblast sheet transplantation therapy, and to determine the molecular pathways mediating the host tissue angiogenic response to sheet transplantation therapy.

III The ability of cardioprotective growth factor HGF expression in myoblast sheets to promote regeneration in rat chronic MI-induced experimental HF.

IV Whether paracrine factors secreted by myoblasts can protect cardiomyocytes against oxidative stress and to elucidate the molecular mechanisms of cardioprotection mediated by myoblast-derived paracrine factors.

4. Materials and methods

4.1 Animal care and ethical statement

All experimental procedures were conducted according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and were approved by the ethics committee of the Helsinki University Central Hospital, Helsinki, Finland.

4.2 Animal treatment and anesthesia

Wistar Han rats (250-400 g, Harlan Laboratories, Rossdorf, Germany) were used in all experiments. A total of 233 animals underwent surgery or sham operation. Of these, 180 (77%) survived and were used for analysis. Rats were anesthesized with 0.4 mg/kg medetomidine s.c. (Orion Pharma, Turku, Finland) and 60 mg/kg ketamine (Parke-Davis, Barcelona, Spain). After the surgery, we administered 1.0 mg/kg atipamezole hydrochloride s.c. (Orion Pharma) to antagonize anesthesia, and 0.05 mg/kg buprenorphine hydrocholoride s.c. (Reckitt and Colman Ltd, Hull, UK) twice per day for 3 days for post-operative analgesia.

4.3 LAD ligation and sheet implantation

The animals were intubated and anesthetized, and respiration was maintained with a ventilator during surgery. Body temperature was maintained with a thermal plate. Hearts were exteriorized rapidly through a left thoracotomy and pericardiotomy, and LAD was ligated with a suture 3 mm from its origin. In the acute MI model, two circular myoblast sheets approximately 25 mm in diameter and consisting of $6x10^6$ cells were implanted on the left ventricular anterior wall immediately after LAD ligation. In the chronic MI model, 4 weeks after ligation of the LAD, all animals underwent re-thoracotomy. Two myoblast sheets were then transplanted onto the anterior left ventricular wall. After ligation and sheet implantation, hearts were returned to their normal position, and covered with pericardium to avoid adhesion to the lung and to the chest wall. Thus, those animals randomized to sheet therapy groups were grafted with a total of 1.2×10^7 cells. Control animals underwent the same procedures without sheet implantation, and sham-operated animals were without LAD ligation and sheet implantation. In the acute MI model, all animals were euthanized at 4 weeks after the thoracotomy. In the chronic MI model, animals were euthanized 4 weeks after the second thoracotomy (Figure 1).

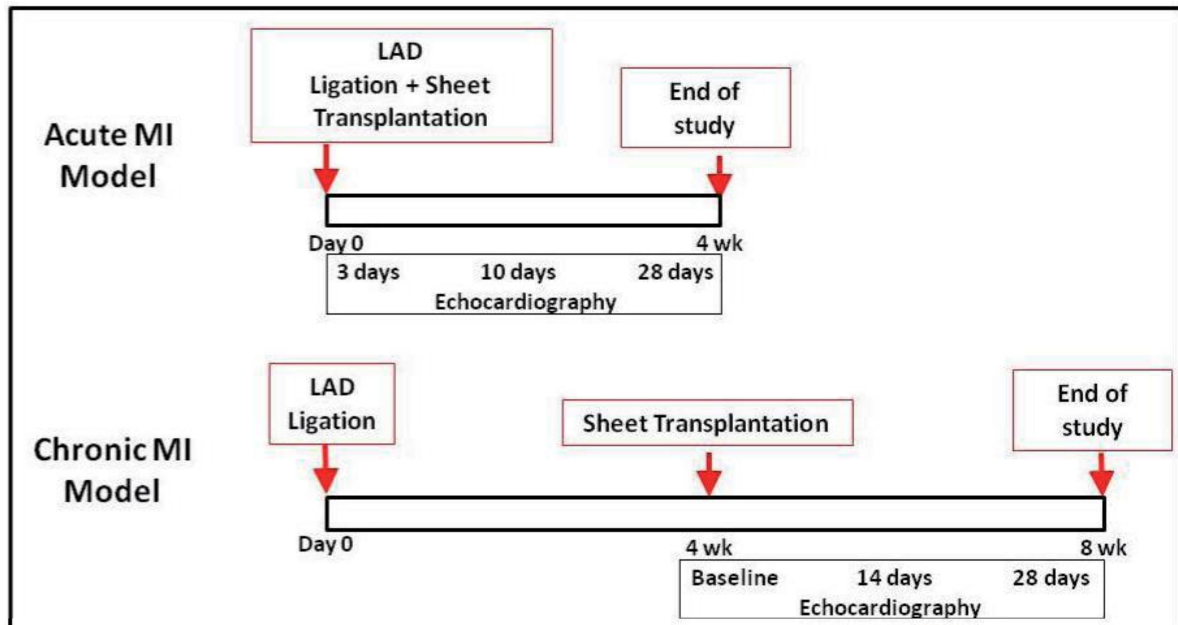

Figure 1. Schematic presentation of the animal study protocols. In the acute myocardial infarction (MI) model, left anterior coronary artery (LAD) was ligated to induce MI in rats, and myoblast sheets were transplanted. Four weeks later, results were analyzed and cardiac function was determined with echocardiography 3, 10, and 28 days following MI and sheet transplantation. In the chronic MI model, myoblast sheets were transplanted 4 weeks after LAD ligation. Results were analyzed 4 weeks after sheet transplantation. Cardiac function was evaluated before sheet therapy as well as 14 and 28 days following transplantation.

4.4 Echocardiography

In the acute MI model, all animals underwent echocardiography under anesthesia at 3 days (baseline), as well as at 10 days, and then 4 weeks after the surgery. In the chronic MI model, baseline echocardiography was performed one day before as well as 2 weeks and 4 weeks after the second surgery. The echocardiographic measurements were performed with a 7.5 MHz transducer (MyLab®25, Esaote SpA, Genoa, Italy). Anterior and posterior wall thickness were measured in the diastolic phase (AWTd, PWTd), and left ventricular diameter in both the diastolic (LVDd) and systolic (LVDs) phases in the short-axis right parasternal projection just below the mitral valves. Data were collected from three systolic cycles and averaged. We used LVDd and LVDs to calculate left ventricular fraction shortening (LVFS) and ejection fraction (LVEF) by the following formulas:

LVFS (%) = (LVDd-LVDs)/LVDd

LVEF (%) = $(LVDd^3-LVDs^3)/LVDd^3$

4.5 Histology and immunostaining

For histological and immunohistological analysis, the hearts were excised and cut into four equal transverse parts. The two middle parts were fixed in 10% neutral-buffered formalin for 48 hours, embedded in paraffin, and cut into 4-μm-thick sections. Immunohistochemistry was performed with automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Cell proliferation was evaluated by use of anti-Ki67 antibody (RM-9106, Labvision Inc, Fremont, CA, USA). Cell proliferation was assessed by staining the Ki67 proliferation-associated antigen, and proliferating cardiomyocytes were detected by double-staining with anti-Ki67 and anti-tropomyosin antibody (MS-1256, Labvision Inc). To observe vascular density, endothelial cells were stained with an antibody against von Willebrand Factor (vWF) (RB-281; Labvision Inc, Fremont, CA, USA). Cell apoptosis was detected by an antibody specifically recognizing active cleaved caspase-3 (CST #9664; Cell Signaling Technology Inc, Danvers, MA, USA) and c-Kit-positive cells with anti-c-Kit (RA14132, Neuromics, Northfield, MN, USA). Six fields from a single section were anlyzed (two images from the infarct area, border area, and remote area). In Study III, vascular structures were stained with antibody for vWF and smooth muscle actin (SMA) and used a AlexaFluor 488 and 596 secondary antibodies (Invitrogen, Carlsbad, CA, USA). Images were acquired by fluorescent microscopy (Olympus, Tokyo, Japan) and the proportion of positive cells was evaluated with ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij) software. The amount of fibrosis was evaluated from Sirius Red-stained paraffin-embedded sections. The Sirius Red-positive area was divided by the whole section area to acquire a relative amount of cardiac fibrosis. For analysis, scanned images of stained tissue sections were used and fibrotic area was evaluated with Photoshop 7.0 (Adobe Systems Inc, San Jose, CA, USA).

4.6 Ventricle dilatation

The dilation of the left and right ventricles was analyzed from histological sections. ImageJ software was used to determine the circumference of the ventricles from hematoxylin/eosin-stained paraffin-embedded sections. Papillary muscles were omitted from analysis.

4.7 Cell culture and sheets

The L6 rat skeletal myoblast cell line was from the American Type Culture Collection (CRL-1458, Manassas, VA, USA). Cells were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 10% fetal calf serum and antibiotics), passaged three times weekly, and kept at 60% confluency to

retain their differentiation potential. Passages 5 to 15 served in the experiments. Myoblast cell sheets were engineered utilizing temperature-responsive cell culture dishes (UpCell, CellSeed, Tokyo, Japan). These dishes are grafted with a temperature-responsive PIPAAm polymer that at room temperature allows spontaneous cell detachment without the use of enzyme digestion or mechanical force. We plated $6x10^6$ myoblasts on such 30 mm dishes, where sheets formed during a16-hour incubation. The sheets were washed and harvested after spontaneous detachment for cell transplantation.

To establish cultures that contain all major cell types of the myocardium, hearts of fetal Wistar rats (E17.5) were excised, minced, and enzymatically digested with trypsin (Sigma-Aldrich,St Louis, MO, USA) and collagenase IV (Worthington Biomedical, Lakewood, NJ, USA). After a 30-minute enzyme digestion with shaking in a water bath at 37 ºC, the supernatant with cells was collected, and the remaining minced tissue was subjected to another digestion. This cycle was repeated until all tissue was digested. Thereafter, we plated the collected supernatants in DMEM containing 10% horse serum, 5% fetal bovine serum, and antibiotics on 24-well cell culture dishes pretreated with 0.2% gelatin (Sigma-Aldrich, St Louis, MO, USA) to promote cell adherence for the cardiac cell migration assay. Alternatively, digested cardiac tissue was plated on cell culture dishes for 90 minutes to allow attachment of non-myocyte cells. The cardiomyocyte-enriched supernatant was then collected and plated for experiments.

To establish cultures of cardiac fibroblasts, an adherent cell population from the 90-minute plating step was extensively washed to ascertain removal of myocytes from the culture. This early-adherent cell population was passaged for six times to allow overgrowth and enrichment of cardiac fibroblasts. These cells were plated on 24-well plates for migration experiments, or on 96-well plates for the fibrosis assay, or collected the conditioned medium for experiments.

4.8 Cardiac cell migration *in vitro*

After plating, the cardiac cell cultures were incubated for 48 hours to allow proper attachment and then medium was changed to be serum-free for a period of 24 hours. After this serum deprivation, the cultures were washed and scratch-wounded with a pipette tip. To determine the ability of myoblast sheet-derived paracrine factors and forced expression of *hgf* to promote migration of cardiac cells, the serum-free DMEM was substituted with 24-hour conditioned medium derived from wild type (L6-WT), or *hgf*-expressing (L6-HGF) myoblast sheets; 24 hours later, these cultures were fixed with 4% paraformaldehyde, and were perfused the cells with Triton-X. Immunofluorescence

staining for vWF (rabbit polyclonal, Millipore, Billerica, MA, USA) and alfa-SMA (mouse monoclonal, DAKO Cytomation, Glostrup, Denmark) was used to identify and evaluate migrating endothelial and smooth muscle cells. Secondary antibodies were anti-mouse Alexa Fluor 488 and anti-donkey Alexa fluor 596 (Invitrogen, Carlsbad, CA, USA). Imuunofluorescence images were acquired of the denuded area with a Olympus IX81 microscope, DP30BW camera, and Cell F 2.7 software (Olympus, Tokyo, Japan). The number of vWF- and SMA-positive cells migrating into the denuded area was evaluated with Photoshop 7.0 (Adobe Systems Inc, San Jose, USA). Phase contrast images were acquired from the cardiac fibroblast cultures and their migration was evaluated with Photoshop 7.0.

4.9 Endothelial cell proliferation, migration, and tubulogenesis

Conditioned medium was collected from myoblast sheets incubated in serum-free DMEM for 48 h to demonstrate the effect of myoblast cell sheet-derived paracrine mediators on endothelial cell proliferation, migration, and tubulogenesis. Conditioned medium was dialyzed with Slide-A-Lyzer 2K cassettes (Thermo Fisher Scientific, Waltham, MA, USA) to remove salts and metabolites. The dialysate was lyophilized and reconstituted in Endothelial Cell Growth Medium (EGM, Lonza, Basel, Switzerland) for experiments. For the proliferation experiments, human umbilical vein endotehelial cells (HUVECs) at $5x10^3$ cells/cm^2 were allowed to attach for 24 h, were washed, and were incubated in conditioned L6-WT, or L6-Bcl2-expressing sheets for 24 h. Cells were counted from phase-contrast images (40x magnification) of triplicate wells. For endothelial cell migration analysis, HUVECs were grown in EGM at an initial density of $1x10^4$ cells/cm^2 for 24 h. The monolayers were wounded with a pipette tip, incubated in myoblast sheet-conditioned medium, and photographed at 0 and 24 h post-wounding. We assessed endothelial migration by subtracting the denuded area after 24 h from the area at 0 h. Analysis was by Photoshop 7.0 (Adobe Systems Inc, San Jose, USA). For 3-dimensional cultures in collagen lattice, we used HUVECs expressing green fluorescent protein (GFP). The lentiviral vector for GFP was a kind gift from Professor Seppo Ylä-Herttuala, AIV Institute, Kuopio, Finland. HUVEC-GFP (10^4 cells) collagen lattices were made from collagen I solution (Millipore, Billerica, MA, USA), DMEM, and PBS at a 1:1:3 ratio. The final concentration of collagen was 1 mg/mL. EGM or conditioned EGM was then added to the wells. Fluorescent images were acquired at 10x magnification after 72 h with a fluorescence microscope (Olympus, Tokyo, Japan). Endothelial cell proliferation, migration, and tubulogenesis assays were carried out with or without SU5416 (VERGR1/2 inhibitor, 2 μM; Sigma-Aldrich, St Louis, MO, USA). Inhibitor toxicity was assessed at those concentrations by

lactate dehydrogenase release into the medium from HUVECs treated with the inhibitor (Cytotoxicity Detection Kit Plus; Roche, Basel, Switzerland).

4.10 Transfection

The L6 myoblasts were transfected for 24 hours in the presence of pBabepuro retroviral vector and 8 μg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA) to initiate a cell line with constitutive overexpression of *bcl2* or *hgf*. The vectors were acquired from Biomedicum Genomics, Helsinki, Finland. The transfected cells were selected with incubation in growth medium containing 2 μg/ml puromycin for 48 hours. To verify Bcl-2 protein overexpression, we performed western blotting and immunofluorescence microscopy. Verification of HGF expression was by *in situ* hybridization and ELISA.

4.11 *In situ* hybridization

We performed the *in situ* hybridization with an automated stainer (Ventana Medical Systems Inc, Tuczon, AZ, USA). We used the antisense primer sequence 5'-ATTTAGGTGACACTATACACAAGCAATCCAGAGGTACGC-3' to detect human *hgf* mRNA and sense primer sequence 5'-TAATACGACTCACTATAGGCCTCGGCTGGCCATCGGG-3' as a control sequence.

4.12 ELISA

For detection of secreted hHGF from the L6-WT- and L6-HGF-conditioned medium, we used the human HGF Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA). VEGF-A protein secretion from L6-WT and L6-Bcl2 sheets was determined from conditioned medium using a rat VEGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA). VEGF-A expression was also determined from staurosporine-treated (10 ng/ml, Sigma-Aldrich, St Louis, MO, USA) or nutrient-deprived sheets (serum-free medium).

4.13 Western blotting

Western blotting samples were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Redmond, WA, USA). Detection was with appropriate alkaline phosphatase-conjugated secondary antibodies. The primary antibodies were mouse monoclonal anti-Bcl-2 (610539, clone 7, BD Biosciences, San Jose, CA, USA), mouse monoclonal anti-myogenin (sc-12732, clone F5D, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), and mouse monoclonal anti- troponin T (T6277, clone JLT-12, Sigma-Aldrich, St Louis, MO, USA).

4.14 Immunofluorescence imaging

L6 myoblasts were grown on coverslips for 48 h prior to fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100. These coverslips were incubated with anti-Bcl-2 antibody and labeled with an appropriate Alexa Fluor secondary antibody (Invitrogen, Carlsbad, CA, USA). Their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and coverslips were mounted on microscopy slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Samples were visualized with an Olympus IX70 microscope.

4.15 Sheet survival *In vivo*

L6-WT and L6-Bcl2 myoblasts were labeled with GFP by incubation with a lentiviral vector and 8 μg/ml polybrene for 24 h. Acute MI, myoblast cell sheet formation, and transplantation were performed as described here. The animals, each receiving either L6-WT-GFP or L6-Bcl2-GFP sheets, were euthanized and their hearts excised at 3 weeks after surgery. Bright field and fluorescence images of the excised heart surface were acquired from the site of transplantation with the Leica MZ FLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). From these images, the GFP signal intensity was assessed with ImageJ software. Images were background subtracted and the fluorescence intensity measured from green channels of the RGB images.

4.16 Myoblast proliferation, apoptosis, and differentiation

L6-WT or L6-Bcl2 myoblast viability after 48-h serum deprivation or staurosporine treatment was assessed by the mitochondria-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide (MTT) to formazan (Roche, Mannheim, Germany). We added 10 μL MTT (5 mg/ml in PBS) per 96-well (initial volume 100 μl). Following a 4-h incubation period at 37°C, the formazan was dissolved in DMSO, and the amount of dye was quantified with a plate reader (540 nm/650 nm). Cell adherence was determined 48 h after treatment. Nuclei were stained with DAPI, and cells were washed thoroughly with PBS. Cell nuclei were counted with CellC software version 1.11 (Selinummi *et al* 2005). The early phase of apoptosis, characterized by phosphatidyl serine translocation, was determined 24 h after serum deprivation or staurosporine treatment by cell surface annexin V binding (Invitrogen, Carlsbad, CA, USA). Fluorescence (340 nm/460 nm) was measured with Wallac Victor2. L6-WT and L6-Bcl2 sheet apoptosis was determined by measuring caspase-3 activity after the induction of apoptosis by either serum deprivation or staurosporine for 24 h. Caspase-3 activity was measured with the EnzChek® caspase-3 assay kit

(Invitrogen, Carlsbad, CA, USA). For differentiation, sheets were deprived of serum to induce myoblast differentiation into myotubes. Western blotting samples were then collected for evaluation of troponin T and myogenin expression.

4.17 Mitochondrial membrane potential

Because loss of mitochondrial membrane polarity is an early marker of cardiomyocyte death, the ability of myoblast-secreted mediators to preserve mitochondrial membrane polarity was determined. Cardiomyocyte cultures were treated with myoblast-conditioned medium, cardiac fibroblast-conditioned medium, or fresh culture medium with 100 nM tetramethylrhodamine ethyl ester (TMRE, Sigma-Aldrich, St Louis, MO, USA) for 15 minutes and the cells were washed to remove any excess dye. TMRE diffuses to functional mitochondria where it exhibits bright fluorescence. This signal is quenched upon opening of the mitochondrial transition pore and subsequent loss of mitochondrial membrane potential. Oxidative stress was induced with H_2O_2, fluorescence was quantified with Wallac Victor2, and immunofluorescence images were acquired at 24 hours.

4.18 *In Vitro* fibrosis and apoptosis assays

To study whether paracrine factors derived from L6-HGF myoblasts can inhibit collagen deposition, such as in the case of acute MI, or can degrade collagen already deposited by fibroblasts, as in chronic MI, an *in vitro* fibrosis model was employed. To this end, human dermal fibroblasts (ATCC, CRL-2088) or rat cardiac fibroblasts were cultured in DMEM containing 10% FCS, as detailed above, to confluency. L-ascorbic acid 2-phosphate (50 mg/ml, Sigma-Aldrich, St Louis, MO, USA) was used to induce collagen deposition, while untreated wells served as controls. The cultures were then treated with fresh culture medium (control) or L6-HGF conditioned medium. At 7 days, the cultures were fixed with 10% formalin, and deposited collagen was stained with Sirius Red dye (Sigma-Aldrich, St Louis, MO, USA). Cultures were then washed thoroughly with 0.5% acetic acid to remove any unbound dye. Collagen-bound Sirius Red was dissolved in 0.1 M NaOH, and the amount of collagen was determined by measuring optical density at 540 nm. For the collagen degradation assay (mimicking an already formed fibrotic scar), collagen was allowed to accumulate for 7 days (baseline). Incubation was continued with control or with L6- HGF-conditioned medium withour ascorbic acid for another 7 days. The amount of collagen was then quantified as described above. To determine whether L6-HGF myoblasts are more resistant to apoptosis than are L6-WT myoblasts, 10,000 myoblasts were plated per well on a 96-well plate and allowed the cells to adhere for 24 hours. Cells were then treated with

staurosporine to induce apoptosis for another 24 hours. Myoblast viability was quantified with an MTT assay.

4.19 Microarray

For microarray gene expression analysis of myoblasts, myoblast sheets, cardiomyocytes, or cardiac tissue samples, total RNA was isolated with Trizol reagent or the RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples with RNA integrity number ≥ 8 were included in the studies. Further, sample processing and hybridization to the Affymetrix GeneChip Rat Genome 230 2.0 chip (Affymetrix, Santa Clara, CA, USA) or Illumina RatRef-12 BeadChips (tissue samples, Agilent, Santa Clara, CA, USA) was by Biomedicum Genomics, University of Helsinki (http://www.biomedicumgenomics.fi). All data are compliant with Minumum Information about a Microarray Experiment (MIAME) guidelines (http://www.mged.org/Workgroups/MIAME/miame.html). Data were normalized to the median with the RMA algorithm and analyzed with Genespring 11 software (Agilent, Santa Clara, CA, USA).

4.20 Quantitative real-time PCR

Isolated total RNA from cultured cardiomyocyte samples was treated with DNAse 1 (Sigma Chemical, St Louis, MO, USA) and used ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) for mRNA reverse transcription. qPCR was perfeormed with the LightCycler carousel-based system (Roche Diagnostics, Basel, Switzerland). We normalized the data to rat 18S rRNA (*rps18*) and used the following primers: activating transcripton factor 4 (*atf4*) forward AGACACCTTCGAATTAAGCACATTC, reverse CCTCGCTGCTCAGGAAGCT; tribbles homolog 3 (*trib3*) forward TCTCCTCCGCAAGGAACCT, reverse TCTCAACCAGGGATGCAAGAG and *rps18* forward ACATCCAAGGAAGGCAGCAG, reverse TTTTCGTCACTACCTCCCCG. Samples were amplified using FastStart DNA Master SYBR Green 1 (Roche Diagnostics, Basel, Switzerland).

4.21 Statistical analysis

Data are presented as mean±standard error of the mean. Differences between groups were compared using analysis of variance (ANOVA) followed by the Bonferroni post-test. In comparision between two groups, Students' t-test was applied. Statistical analyses were performed with GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA, USA).

5. Results

Table 2. Main findings from Study I with acute MI model.

Study I	L6-WT	L6-Bcl2
Cardiac function	Not improved	Improved
Angiogenesis	Induced	Strongly induced
Fibrosis	Not reduced	Reduced
Proliferation	Induced	Induced
Sheet survival	Less than 3 weeks	3 weeks

Wild type (L6-WT) and Bcl2-expressing (L6-Bcl2) myoblast sheet transplantation therapy.

Table 3. Main findings from Studies II and III with chronic MI model.

Study II and III	L6-WT	L6-Bcl2	L6-HGF
Cardiac function	Improved at 2 weeks	Improved at 4 weeks	Similar to L6-WT
Angiogenesis	Induced	Strongly induced	Very strongly induced
Fibrosis	Not reduced	Not reduced	Not reduced
Proliferation	Not induced	Induced	Not defined

Wild type (L6-WT), Bcl2-expressing (L6-Bcl2), and HGF-expressing (L6-HGF) myoblast sheet transplantation therapy.

5.1 Transfection and functionality of Bcl-2 and HGF

To evaluate the efficacy and functionality of retrovirus-mediated gene transfection, *bcl2*-expression was evaluated with western blotting using a Bcl-2-specific antibody. Bcl-2-expression was 28-fold higher in L6-Bcl2 cells than in L6-WT cells. Immunofluorescence staining revealed a markedly higher level of expression as well as granular perinuclear localization. Evaluation of the functionality of transfection revealed that L6-Bcl2 myoblasts had higher viability than did the L6-WT cells under serum starvation (0.371±0.002 vs. 0.279±0.004, $p<0.001$) and staurosporine stress (0.244±0.012 vs. 0.102±0.006, $p<0.001$). Furthermore, L6-Bcl2 myoblasts cell adherence was higher (2.0-fold, $p<0.001$) and rate of apoptosis lower (1.6-fold, $p<0.001$). L6-Bcl2 myoblasts effectively formed sheets and were resistant to caspase-3 activation under serum starvation (1.7-fold higher in L6-WT sheets, $p<0.01$) and staurosporine treatment (2.0.fold higher in L6-WT sheets, $p<0.001$). Finally, L6-Bcl2

myoblasts underwent fusion and myotube formation in sheets within 96 hours. This differentiation was associated with expression of the myocyte markers troponin T and myogenin. Following LAD ligation and transplantation to rat hearts, antiapoptotic functionality of *bcl2*-expression was evaluated *in vivo*. Analysis of GFP-labeled sheets revealed that at 3 weeks post-transplantation, L6-Bcl2 sheets had an survival benefit as compared to L6-WT sheets (2.3-fold higher green fluorescence signal, $p<0.05$). Expression of hHGF was evaluated with *in situ* hyridization using a labeled antisense RNA probe. Over 90% of myoblasts showed extensive human *hgf*-expression on the transcriptional level.

Furthermore, ELISA showed that hHGF is effectively translated in L6-HGF sheets because after 24-hour incubation the L6-HGF sheet-conditioned medium contained 61.3±6.7 ng/ml hHGF protein, whereas no hHGF secretion was detectable in L6-WT myoblast sheets. The ability of hHGF to stimulate rat cells was demonstrated by treatment of isolated cardiomyocyte cultures with conditioned medium from L6-HGF sheets. L6-HGF conditioned medium effectively induced migration of rat vWF-positive cells, demonstrating functionality of hHGF (21.8±2.1 vs. 14.0±1.6, $p<0.001$ vs. L6-WT). Moreover, rat hearts transplanted with L6-HGF sheets showed more vWF-positive cells (0.86±0.07% of whole tissue area) than did the L6-WT transplanted animals, (0.68±0.03%) confirming hHGF secretion and functionality *in vivo* ($p<0.05$).

5.2 Cardiac function

Induction of acute MI by LAD ligation effectively impaired cardiac function. Ten days after transplantation of wild-type or *bcl2*-expressing myoblast sheets, only the L6-Bcl2 group (EF 37.8±1.9%) showed significantly recovered cardiac function compared with controls (30.0±1.9%, $p<0.05$). This effect was slightly diminished by 28 days post-transplantation, although the difference remained significant from the control group ($p<0.05$). The L6-WT group also showed a similar trend. However, the difference was significant only for the L6-Bcl2 ($p<0.05$) group at 10 days; no significant difference appeared between the L6-WT and control groups (Figure 2).

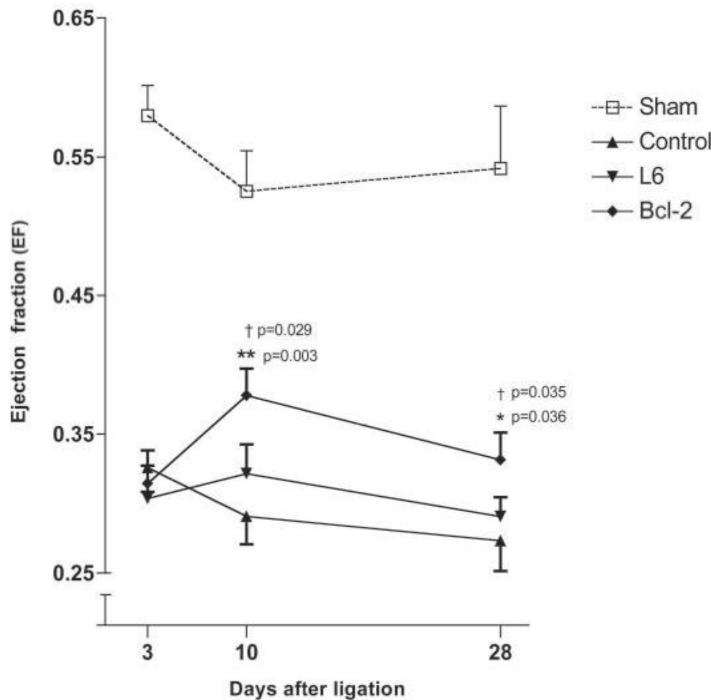

Figure 2. Cardiac function in acute myocardial infarction model (Study I). Left ventricular ejection fraction at indicated time-points after wild type (*n* = 17, L6-WT) and Bcl2-overexpressing (*n* = 20, L6-Bcl2) myoblast sheet therapy for acute myocardial infarction. Control rats (*n* = 22) underwent AMI without sheet transplantation, and sham-operated rats (*n* = 5) underwent left-sided thoracotomy. * p<0.05 and ** p<0.01 as compared to control group, † p<0.05 as compared to L6-WT group. Figure reproduced by permission from the publisher.

In the chronic MI model, cardiac function had significantly declined, and the remodeling had taken place at the time of myoblast sheet transplantation 4 weeks after induction of cardiac damage. The L6-WT group (30.8±2.0%, p<0.05) showed significantly higher EF than did the control group (25.4 ±1.7%) at 2 weeks after transplantation, but this difference was lost at 4 weeks. Moreover, the L6-Bcl2 group (31.6±1.5%, p<0.05 vs. control) showed a significant improvement at 2 weeks, but in contrast to L6-WT, this effect was sustained at the 4-week time-point (34.0 ±1.6%, p<0.05 vs. control and L6-WT). L6-HGF sheet transplantation also significantly improved cardiac function (32.2±1.1%) as compared to the control group (27.0±1.6%, p<0.05). We found, however, no additional functional benefit to that of beyond L6-WT sheet transplantation therapy (32.7±1.9%, p<0.05 vs. control). In the chronic model, both left ventricular end-systolic and end-diastolic diameters increased after LAD ligation, reflecting the remodeling process. Myoblast sheet transplantation effectively

inhibited this ventricular dilation (p<0.01 L6-WT vs. control; p<0.05 L6-HGF vs. control) (Figure 3).

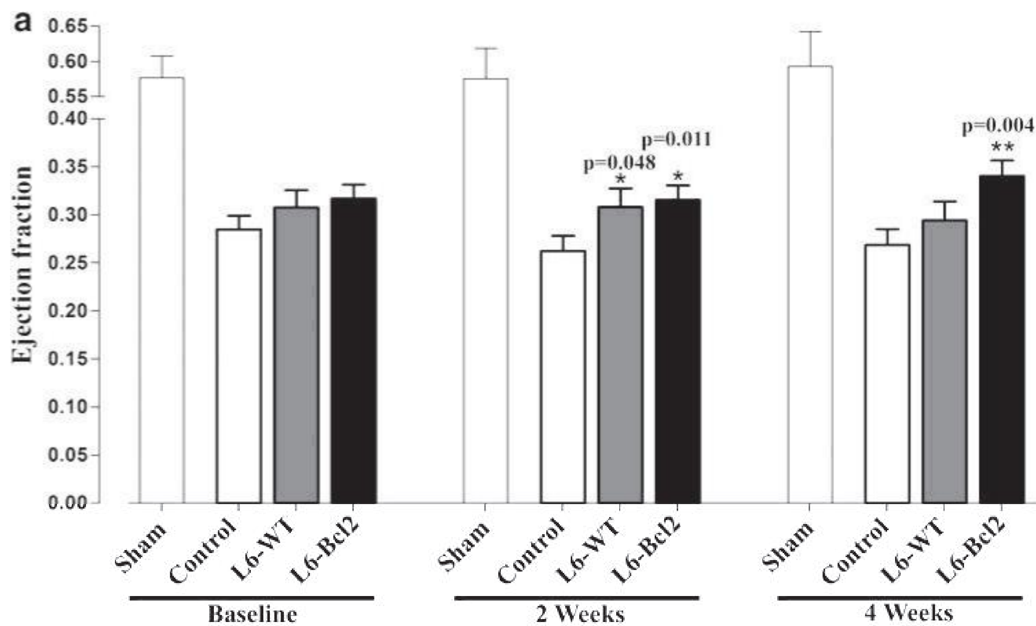

Figure 3. Analysis of cardiac function (Study II). Myocardial infarction was induced by ligation of the left anterior descending coronary artery (LAD) in all rats except for a sham group receiving thoracotomy only. All rats underwent rethoracotomy 4 weeks after ligation, and wild type (n=16, L6-WT) or Bcl-2- overexpressing (n=19, L6-Bcl2) myoblast sheets were transplanted. Control rats (n=21) and sham-operated rats (n=5) received no therapy. * p<0.05 and ** p<0.01 as compared to control group. Figure reproduced by permission from the publisher.

5.3 Angiogenesis

In both acute and chronic HF models, MI induced a potent angiogenic response as compared to that of sham-operated animals as evaluated by staining of endothelial marker vWF or smooth muscle cell marker α-SMA. In both models, myoblast sheet transplantation had an additive effect on the angiogenic response in the ischemic infarct and border regions. Suppression of graft apoptosis with Bcl-2 in the acute (percent vWF-positive cells per whole tissue: 1.53±0.11 vs. 1.04±0.08 control group, p<0.05) and chronic models (1.31±0.10 L6-Bcl2 vs. 0.99±0.07 control, p<0.05) (Figure 4), as well as expression of proangiogenic HGF in myoblast sheets in the chronic model further improved the angiogenic capacity of the myoblast sheet therapy (0.78±0.05 L6-HGF vs. 0.49±0.07 control, p<0.05). Microarray analysis revealed upregulation of the proangiogenic genes VEGF (2.2-fold) and PlGF (5.5-fold). Increased expression of VEGF from L6-Bcl2 as compared to L6-WT myoblast sheets under stress conditions

(1.8-fold higher under serum starvation, p<0.01; 1.5-fold higher under staurosporine, p<0.05) was confirmed with ELISA.

In vitro endothelial cell proliferation (L6-WT 743±26, vs. control 628± 19, p<0.05), migration (L6-WT 165900±6030, vs. control 82250±6506, p<0.001), and tubulogenesis assays revealed induction of these processes by L6-WT sheet-derived paracrine factors. In addition, paracrine factors from L6-Bcl2 sheets had a more potent effect on endothelial cells (proliferation 961±50, p<0.01 vs. control, p<0.05 vs. L6-WT; migration 196900±6502, p<0.001 vs. control, p<0.05 vs. L6-WT). These processes were significantly inhibited by the small molecule tyrosine kinase inhibitor SU5416 which inhibits the Flk1 and Flt1 receptor tyrosine kinases (also known as VEGFR1 and VEGFR2). In primary cardiac cell cultures, L6-WT myoblast sheet-derived paracrine factors induced migration of vWF- (14.0±1.6, p<0.05 for control) and SMA-positive cells (33.8±5.3, p<0.05 vs. control) but not tropomyosin-positive cells. Paracrine factors from L6-HGF sheets induced migration of these cells even more effectively (SMA 74.5±4.1 cells, p<0.001 vs. control, p<0.05 vs. L6-WT; vWF 21.8±2.1 cells, p<0.01 vs. control, p<0.05 vs. L6-WT). Further, when transplanted *in vivo*, L6-HGF sheets raised the number of vWF- (59% higher) and SMA-positive cells (109% higher) more than did L6-WT sheet transplantation (p<0.05).

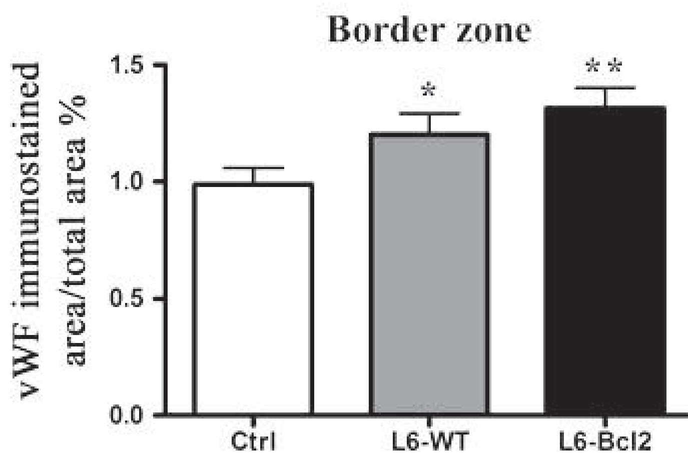

Figure 4. Quantitative evaluation of vascular density in the infarct border zone in chronic myocardial infarction model (Study II). Samples were collected 8 weeks after LAD ligation or sham operation (sham group) and 4 weeks after re-thoracotomy and therapy with or without (control and sham groups) myoblast sheets. Expression of von Willebrand factor (vWF) from paraffin-embedded myocardial tissue sections using immunohistochemical staining. vWF expression from infarct and border zones and their combination was evaluated from L6-WT- or Bcl-2-expressing (L6-Bcl2) sheet transplanted groups, and control animals. * p<0.05 and ** p<0.01 as compared to control group. Figure reproduced by permission from the publisher.

5.4 Fibrosis

The proximal LAD ligation induced severe myocardial damage in rats that was associated with extensive left ventricular fibrosis. Four weeks after LAD ligation, Sirius Red-stained cross-sectional histological samples, representing the size of the collagenous scar tissue, showed that 26% of the total myocardial cross-section was fibrotic whereas sham-operated animals showed marginal staining for collagen. Those animals transplanted with L6-Bcl2 myoblast sheets (21.2±0.8%) showed significantly less fibrosis than did the control (25.6±1.8%, $p<0.001$ vs. L6-Bcl2) or L6-WT (24.6±1.6%, $p<0.05$ vs. L6-Bcl2) sheet-transplanted animals (Figure 5). In the chronic model, analysis of fibrosis revealed that at 8 weeks after MI, control animals had the same amount of fibrosis as in the acute model. This suggests that the fibrous scar in the rat MI model develops during the first 4 weeks and that no additional fibrosis takes place between 4 and 8 weeks. The ability of myoblast sheet transplantation to reduce the amount of fibrosis after it has already developed was evaluated in the chronic HF model, showing that transplantation of L6-WT or L6-Bcl2 myoblast sheets did not affect the amount of collagen already deposited. L6-HGF sheets were then evaluated in a similar fashion. In line with L6-Bcl2 sheet results, L6-HGF sheet transplantation had no effect on the amount of fibrosis in the myocardium after chronic MI.

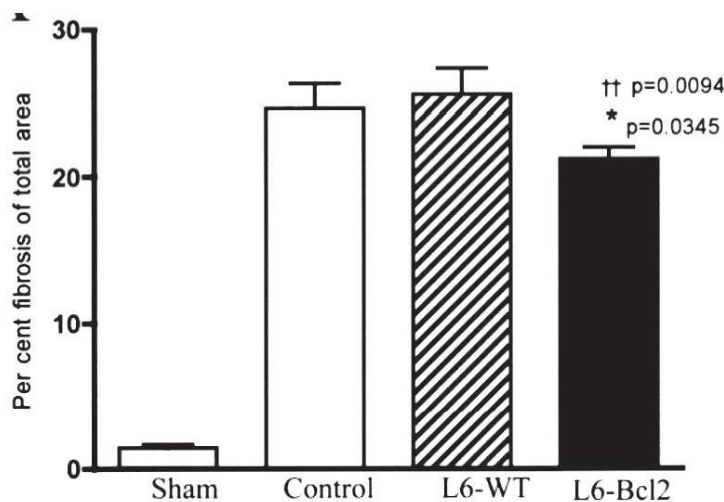

Figure 5. Quantitative evaluation of fibrosis (Study I). Four weeks after left anterior descending coronary artery ligation and wild-type (L6-WT, n=17) or Bcl2-expressing (L6-Bcl2, n=20) myoblast sheet transplantation, myocardial fibrosis was evaluated from Sirius Red-stained paraffin-embedded sections. Control animals (n=22) received no sheet transplantation and sham-operated rats (n=5) underwent only thoracotomy. * $p<0.05$ as compared to control group, †† $p<0.01$ as compared to L6-WT group. Figure reproduced by permission from the publisher.

5.5 Proliferation

The ability of myoblast sheet transplantation to induce cell proliferation in the myocardium was assessed from paraffin-embedded sections by immunostaining the nuclear proliferation-associated antigen Ki67. Analysis of Ki67 expression from myocardial sections in the acute HF model revealed that L6-WT myoblast sheet transplantation significantly induced cell proliferation in the infarct region (46.1 ± 3.9 cells, vs. control 33.8 ± 3.3, $p<0.05$), in the infarct border zone (52.2 ± 3.8 cells vs. control 40.5 ± 3.0, $p<0.05$ vs. control), but not in the non-ischemic remote myocardium. Furthermore, L6-Bcl2 myoblast sheet transplantation stimulated proliferation throughout the myocardium (infarct 52.1 ± 3.1 cells, $p<0.001$ vs. control; border 51.9 ± 4.1 cells, $p<0.05$ vs. control; remote 48.9 ± 3.3 cells, $p<0.01$ vs.control). In the non-ischemic remote area, the difference was significant also as compared to that of L6-WT sheet transplantation ($p<0.01$). The number of proliferating cells was then evaluated in the chronic HF model. At the end of the study, no differences in myocardial cell proliferation were detectable in the L6-WT group in any of the evaluated myocardial areas. In the group transplanted with L6-Bcl2 sheets, however, a significantly higher number of proliferating cells were detected throughout the myocardium in the infarct (10.6 ± 0.6 cells, $p<0.001$ vs. control; $p<0.01$ vs. L6-WT), border zone (29.0 ± 2.1 cells, $p<0.05$ vs. L6-WT), and remote areas (16.2 ± 1.2 cells, $p<0.05$ vs. control; $p<0.01$ vs. L6-WT). Double-staining of Ki67 and tropomyosin was then performed on the paraffin section to determine whether the proliferating cells were cardiomyocytes or other cells. Very few cells in the sections were identified that coexpressed both markers, indicating cardiomyocytes in mitosis. In addition, Ki67 did not appear to localize to vascular structures, suggesting that the proliferating cells are not cardiomyocytes or endothelial cells.

5.6 Cardiac repair

To elucidate the possible mechanisms of the therapeutic effect of myoblast sheet transplantation, immunohistichemical staining for the stem cell antigen c-Kit was performed to determine whether stem cell infiltration may play a role in the therapeutic effect. Control and L6-WT groups showed a slightly higher number of c-Kit-positive cells in the myocardium, as compared to the sham-operated group, but this difference was not significant. In animals transplanted with L6-Bcl2 sheets, however, a significantly increased number of c-Kit-positive cells (53.0 ± 5.9 cells, vs. control 45.1 ± 6.1 and L6-WT 45.8 ± 5.2, $p<0.01$ for both) was detected, suggesting that stem cell activation may play a role in mediating the therapeutic effect. Moreover, to determine whether myoblast sheet transplantation can protect the cardiomyocytes in the host

myocardium against ischemia-induced stress, paraffin-embedded myocardial sections were stained for tropomyosin to determine whether a higher number of myocytes survive in hearts receiving myoblast sheet transplantation. The analysis revealed that in the L6-Bcl2 group (1.6-fold more tropomyosin staining vs. control, $p<0.05$), an increased number of cardiomyocytes survived in the infarct area. Furthermore, the L6-WT group showed a higher but statistically nonsignificant increase in tropomyosin staining.

5.7 Cardiomyocyte protection

Because myoblast sheet transplantation prevented loss of cardiomyocytes in the acute MI model – as determined by the lower relative amount of fibrosis in animals receiving L6-Bcl2 sheet transplantation – and in the chronic model as determined by the increased number of tropomyosin-positive cells in the infarct region, we hypothesized that myoblasts can protect cardiomyocytes in a paracrine manner. To answer this question, the ability of myoblast-secreted paracrine factors to protect cardiomyocytes against oxidative stress was determined by using isolated rat cardiomyocytes stressed with H_2O_2. Initially, both myoblast-derived (MTT assay optical density: 0.28±0.01vs. control 0.23±0.01, $p<0.001$) and cardiac fibroblast-derived factors (0.44±0.03, $p<0.001$ for control and myoblast groups) enhanced mitochondrial function in cardiomyocyte cultures. After inducing oxidative stress, only myoblast-derived mediators protected cardiomyocyte viability as measured by MTT assay (optical density: 0.21±0.01, vs. control 0.05±0.01 and fibroblast groups 0.09±0.02, $p<0.001$ for both) (Figure 6). Moreover, myoblast- (fluorescence arbitrary units: 6831±323) but not cardiac fibroblast-conditioned medium (3690±291) sustained the mitochondrial membrane potential in cardiomyocytes ($p<0.001$). Microarray analysis of cardiomyocyte gene expression induced by myoblast-secreted factors revealed upregulation of the antioxidative gene cystathionase gamma-lyase (17.8-fold, *cth*) and tribbles homologue 3 (18.6-fold, *trib3*). Treatment with tyrosine kinase receptor inhibitors against EGFR ($p<0.001$) and c-Met ($p<0.01$), but not against VEGFR, inhibited the protective effect, suggesting that the protection involves EGFR and c-Met-dependent signaling pathways. Analysis of gene expression from myocardial tissue samples following myoblast sheet transplantation revealed upregulation of *trib3* (1.9-fold) in the myocardium. Moreover, our microarray experiment revealed upregulation of several antioxidative genes that could mediate cardioprotection. Of these factors, secreted phosphoprotein (*spp1*, also known as osteopontin) is possibly a strong candidate to mediate the protective effect.

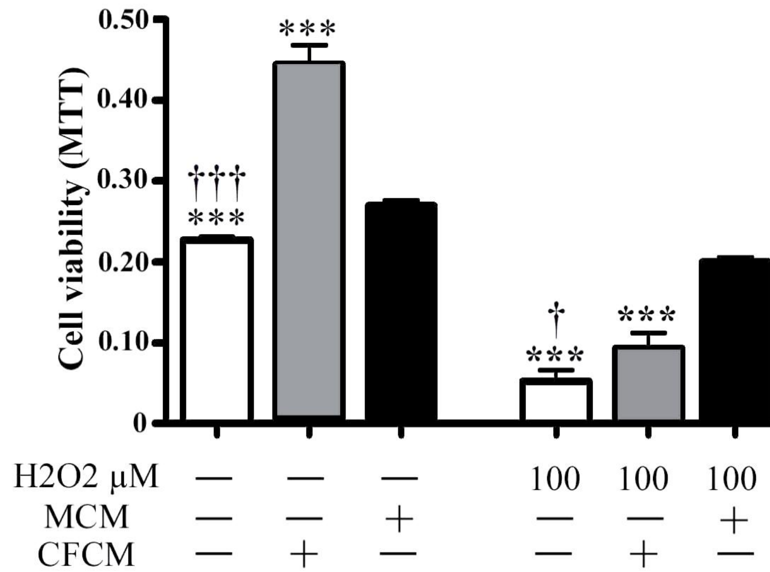

Figure 6. Paracrine factors from myoblasts protect cardiomyocytes against oxidative stress. Cardiomyocytes were isolated from fetal rat hearts and cultured for 48 hours. Cardiomyocytes were treated with conditioned medium from myoblasts (MCM) or cardiac fibroblasts (CFCM) for 24 hours. Thereafter, oxidative stress was induced in cardiomyocytes with H2O2 for 24 hours after which cardiomyocyte viability was evaluated with MTT assay. *** $p<0.001$ as compared to MCM group, ††† $p<0.001$ as compared to CFCM group, † $p<0.05$ as compared to CFCM group.

6. Discussion

During recent decades, understanding of the pathophysiology and molecular mechanisms of HF after MI has advanced tremendously. This awareness has manifested itself in the clinic as safe and effective medical treatments such as ACE inhibitors and β-blockers. These current medications have reduced mortality and improved the well-being of patients suffering from HF. In addition, the development of surgical and interventional techniques has improved the treatment of HF (Dickstein *et al*, 2008). However, neither medical nor surgical treatments address the underlying cause of MI-induced HF: the progressive loss of contractile tissue leading to cardiac dysfunction. LVAD could be the answer for many patients to increase their cardiac output, but currently this therapy is hampered by severe complications and requires years of technical development before help can be expected for large patient populations (Slaughter *et al*, 2009). Advancements in heart transplantation have made that an appealing alternative for some patients, but transplantation is doomed to remain marginal due to donor shortage (Lloyd-Jones *et al*, 2009).

The strategy to introduce new contractile cells into the myocardium to replace the lost cardiomyocytes has been an active area of research for two decades. Many cell populations from various tissue sources have undergone experimental and clinical evaluation for use in cardiac cell therapy (Menasche, 2011a). Most of these cell types, however, possess intrinsic debilities: hematopoietic stem cells lack the ability to contract (Murry *et al*, 2004), mesenchymal stem cells are few and may be tumorigenic (Satija *et al*, 2009), embryonic stem cells have limited availability, raise ethical issues, and include a risk for teratomas (Chung *et al*, 2011), and induced pluripotent stem cells require extensive *ex vivo* modification in addition to their tumorigenic potential and possible immunogenicity (Zhao *et al*, 2011). Although many of these shortcomings can be addressed in experimental settings and models, their use is far beyond the reach of today's clinicians. Skeletal myoblasts present a muscle phenotype-committed cell population that, because of many of its characteristics such as *ex vivo* expandability and hypoxia tolerance, can be considered one of the most ideal cell populations to have a foreseeable clinical future. Because the first generation of experimental and clinical myoblast transplantation trials ended with the MAGIC trial, we have the opportunity to evaluate the current critical weaknesses of this therapy approach and to develop new strategies to circumvent them.

In the present study, we have utilized myoblast transplantation therapy using self-aggregating scaffoldless cell sheets as the means to transplant myoblast to the failing

heart. The approach we used allows effective delivery of cells without early loss of cells by leakage or anoikis. Moreover, combining gene therapy with these sheets allows avoidance of early loss of cells by apoptosis. Thereby, the duration of therapy can be prolonged, and therapeutic angiogenesis induced in the ischemic myocardium to supply perfusion to the remaining cardiomyocytes as wells as to the transplanted myoblast sheets. Our results demonstrate that inhibition of graft apoptosis can enhance the efficacy of myoblast sheet transplantation therapy in rat acute and chronic HF models. Moreover, myoblast sheets can serve effectively as gene therapy vehicles to deliver, for example, cardioprotective and proangiogenic growth factors to aid the chronically failing heart. Finally, our data suggest novel mechanisms that mediate the therapeutic response to the cardiomyocytes in a paracrine manner.

6.1 Methodology

We used the rat L6 cell line as a myoblast model of transplantation. The use of this cell line instead of isolated primary myoblasts accommodates some obvious advantages. It allows transplantation of a pure population of myoblasts and therefore allows the conclusion from our studies that the effects observed are derived from myoblasts and not from contaminating cells from the primary cell isolation, such as fibroblasts. It also excludes one source of experimental variation stemming from the cell isolation step that may affect cell characteristics such as viability. The use of a cell line, however, also has some inherent issues. The L6 is an immortalized cell line, and even though these cells can differentiate and exit the cell cycle, the cell line was initiated by use of mutagens (Yaffe, 1968). These introduced mutations can affect the myoblasts' phenotype and can also modulate the myoblasts' therapeutic characteristics when transplanted to the heart. Moreover, because of the use of mutagens and the over 40-year history of this cell line (Yaffe, 1968), it may have undergone changes that evoke immune responses in the host after transplantation. Such immune responses would render these cells more like a model of allogenic than like autologous transplantation. Furthermore, because the Wistar rat from which the L6 line originates is an outbred strain, immune responses may also be derived from that source.

We used the Wistar rat MI model to study myoblast sheet transplantation therapy. Due to the rapidity of the rat metabolism as compared to that of humans, the classic processes in the heart after LAD ligation – necrosis, healing, remodeling – occur at a more rapid pace (Fishbein *et al*, 1978). The pathophysiological alterations are, however, similar to those observed in humans (Goldman and Raya, 1995). Both rats and humans have limited collateral circulation. After MI, in response to coronary occlusion, the rat

undergoes a transmural infarction whereas subendocardial infarction and papillary muscle necrosis are rare (Zornoff *et al*, 2009). The rats we used were relatively young, which may introduce differences between their rapid infarct healing and the much slower healing in humans who are typically elderly at the time of MI. The LAD ligation model is accompanied by high mortality rates within 48 hours mainly due to arrhythmias (Zornoff *et al*, 2009). In the acute model, the mortality rate of the animals was approximately 30% during infarction, sheet implantation, and follow-up. This figure is at the low end compared to rates described in the literature (Zornoff *et al*, 2009). Despite the low mortality rates, infarction was effectively induced, as measured by cardiac function and amount of fibrosis. Mortality rates after MI were even lower in our two studies using chronic infarction models with animals undergoing two surgeries: LAD ligation and sheet implantation. Furthermore, the magnitude of myocardial injury in this model varies widely due to different sites of coronary occlusion and differences in the anatomy of individual hearts (Zornoff *et al*, 2009). In our studies, this variation was effectively controlled by excluding animals with EF ≥ 40% before randomization to study groups.

Thus far, most cell therapy clinical trials have used transplantation of autologous cells. The autologous cell origin possesses clear benefits in terms of cell availability as well as of immunocompatibility. Serious drawbacks are, however, associated with the use of autologous cells. First, because of variance between patients, quality control, and evaluation of the therapeutic potential of cell products are difficult (Menasche, 2011a). Second, because patients in need of cardiac therapy are often elderly, the therapeutic functionality of the cells from these patients may also be reduced (Li *et al*, 2010). Third, if core facilities are used for culturing the autologous cells, the transportation of cells leads to additional costs and logistic difficulties. Fourth, in treating acute MI, the use of autologous cells is unfeasible because of the lack of time to culture and expand cell numbers. Therefore, only allogenic myoblasts are available to treat acute MI. Allogenic off-the-shelf cell therapy permits detailed characterization and evaluation of the therapeutic potential of the cell product. However, because of predicted immunoreactions against allogenic cells, suppression of such responses is necessary. Recently, Imanishi *et al* (2011) showed that allogenic myoblasts are cleared from the myocardium shortly after transplantation, and that selection of major histocompatibility complex-matched allogenic myoblasts, will prevent this clearance. Furthermore, the therapeutic efficacy of these matched myoblasts was superior to that of the mismatched cells, suggesting that allogenic myoblast transplantation is a vital strategy for treatment of cardiac dysfunction.

6.2 Results

In our study utilizing transplantation of either wild type or *bcl2*-expressing myoblast sheets, we found that by promoting graft survival, L6-Bcl2 therapy enhanced cardiac function and angiogenesis, and reduced fibrosis. Bcl-2 is a key modulator of apoptosis. It is localized on the outer mitochondrial membrane, where it interacts with the proapoptotic mediators Bax and Bak and inhibits permeabilization of the mitochondrial membrane, thereby inhibiting the intrinsic mitochondrial pathway of apoptosis (Brooks *et al*, 2007). In the cardiac microenvironment, Bcl-2 attenuates oxidative and inflammatory stress-induced apoptosis (Jamnicki-Abegg *et al*, 2005), as well as hypoxia-induced apoptosis (Li *et al*, 2007). Furthermore, in line with our results showing enhanced survival of myoblast sheets in the infarcted myocardium, the antiapoptotic efficacy of Bcl-2 to enhance transplanted cells' survival and transplantation effectiveness has been confirmed by other studies using cardiomyoblasts and bone marrow mesenchymal stem cells (Kutchka *et al*, 2006; Li *et al*, 2007).

The prevailing hypothesis is that the transplanted cells exert their therapeutic effects by secreting paracrine factors that benefit the diseased host myocardium (Menasche, 2011b). Supporting this hypothesis, we could detect no sheet structures from the hearts 4 weeks after transplantation, even from those hearts receiving L6-Bcl2 sheets, suggesting that the grafts do not survive and fail to integrate with the host myocardium indefinitely. It is therefore plausible that the therapeutic effects are mediated in a paracrine manner. In addition to the prolonged survival, our data revealed that the L6-Bcl2 sheets secrete higher concentrations of the proangiogenic mediators VEGF and PlGF, suggesting that Bcl-2 improves both graft survival and graft functionality. It should be noted that in these studies we were unable to exclude the possibility of immunoreactions against the L6 myoblast sheets. Such reactions could explain the discrepancy between previously documented long-term survival and incorporation of myoblasts after transplantation (Hagege *et al*, 2006) and the lack of sheet structures in our study 4 weeks after transplantation.

The mechanisms of myoblast transplantation therapy are not fully elucidated. The lack of sheets in our myocardial sections suggests that the beneficial effects were not due to added contractile force or elastic properties of myoblasts but rather to paracrine mediators released from the transplanted cells. Myoblasts secrete various paracrine growth factors and mediators (Perez-Ilzarbe *et al*, 2008). Our results demonstrate that formation of myoblast sheets induced production of proangiogenic VEGF and PlGF from these myoblast sheets. This induction was associated with enhanced myocardial

angiogenesis, a response reported also by others (Memon *et al*, 2005). We went on to show that the endothelial cell responses were mediated via the Flt1/Flk1-dependent pathway. These data suggest that the paracrine effect may in part be mediated by induction of angiogenesis in the post-MI myocardium.

In addition to endothelial cells, other cell types are also likely targets of myoblast-derived paracrine signaling. Perez-Ilzarbe *et al* (2008) reported stimulation of smooth muscle cells and HL-1 cardiomyocytes by myoblast-conditioned medium *in vitro*. Our results suggest that in acute and chronic MI models, myoblast sheet transplantation may show cardiomyocyte-protective effects *in vivo*. We therefore evaluated the cardiomyocyte-protective ability of myoblast-derived paracrine factors, showing that the mediators secreted from myoblasts induced antioxidative responses in cardiomyocytes that protected them against oxidative stress in an EGFR- and c-Met-dependent manner. This protective response was associated with induction of the antioxidative gene cystathionase gamma-lyase. Microarray analysis suggested upregulation of secreted phosphoprotein (osteopontin) in the hearts *in vivo* after sheet transplantation. Osteopontin is upregulated in the heart by ischemia-reperfusion, and is associated with remodeling, inhibition of fibrosis, and induction of angiogenesis (Denhardt *et al*, 2001). In addition, the protective response was also associated with UPR-like gene expression in the cardiomyocytes. ER stress is induced in the heart by several pathological conditions such as MI (Doroudgar *et al*, 2009), ischemia-reperfusion, and HF (Toth *et al*, 2007). As an adaptive response to ER stress, UPR is initiated in the heart, and it protects cardiomyocytes from ER stress-induced death (Groenendyk *et al*, 2010; Isodono *et al*, 2010). Further studies are necessary, however, to determine the significance of myoblast induced-UPR in cardiomyocyte protection.

Several studies have determined the role HGF plays in the heart. This growth factor inhibits cardiomyocyte death by suppressing oxidative stress (Ueda *et al*, 2001), and reduces fibrosis by inhibiting TGF-β-expression in myofibroblasts and by promoting their apoptosis (Mizuno *et al*, 2005). Moreover, HGF induces angiogenesis (Funatsu *et al*, 2002) and effectively protects the myocardium after an acute ischemic injury (Jayasankar *et al*, 2003). Because the cardioprotective ability of HGF in an acute MI setting has been extensively studied, we evaluated the ability of HGF expression to improve myoblast sheet therapy in a chronic HF model. As expected, L6-HGF therapy effectively induced angiogenesis after transplantation. We, however, could detect no additional functional benefit to L6-WT sheet therapy. This is in contrast to Tambara *et al* (2005) who reported that myoblast transplantation with recombinant HGF resulted in effective incorporation of myoblasts into the host myocardium, enhanced cardiac

function and reduced myocardial fibrosis. These differences can result from a different cell source (neonatal primary myoblasts), or use of a gelatin hydrogel as carrier.

The enhanced vascular density in our study suggests that the chronically ischemic myocardium may be perfused more effectively, although we did not fully determine the functionality of the new vasculature. In line with our observations, Perin *et al* (2011) reported that HGF transfection to a chronic HF model caused increased cardiac perfusion without any associated improvement in cardiac function. It is possible that the improved perfusion only promotes local contractility but not global function. To analyze cardiac function, we used echocardiography. What must be taken into account is the possibility that regional enhanced myocardial contractility was missed due to methodological insensitivity (echocardiography). Finally, in our chronic model, cardiomyocyte death, fibrosis, and neoangiogenesis have mostly receded, and the endogenous cardiac healing process has ended at 4 weeks post-MI. The therapeutic potential of HGF in this environment to significantly repair the damaged heart by modulating these processes may be limited to increased perfusion.

7. Summary and conclusions

We used rat acute and chronic MI models to induce cardiac dysfunction. We then evaluated the treatment of this dysfunction with tissue-engineered myoblast sheets. In addition, these sheets were modified to express antiapoptotic *bcl2* or cardioprotective *hgf* to enhance the efficacy of myoblast sheet therapy. Furthermore, we studied the mechanisms of the cardiomyocyte-protective paracrine factors secreted by myoblasts. The main findings of these studied were:

I Bcl2-expression prolonged myoblast sheet survival and increased the sheets' secretion of proangiogenic mediators. Transplantation of these sheets after acute MI improved cardiac function, reduced fibrosis, and induced therapeutic angiogenesis.

II Transplantation of apoptosis-resistant Bcl2-expressing myoblast sheets improved cardiac function of 4-week-old chronic infarctions. This improvement was associated with increased cardiomyocyte survival in the infarct region and induction of angiogenesis in a VEGFR1/2-dependent manner.

III HGF expression in the myoblast sheets induced angiogenesis in the chronic MI model and was equal to wild type sheets in improving cardiac function, and inhibiting remodeling. Neither of the sheet therapies reduced already-formed scar tissue.

IV Myoblast-derived paracrine factors induced redox- and unfolded protein response-associated gene expression in cardiomyocytes and protected them against oxidative stress by inhibiting loss of mitochondrial membrane polarity. These effects were mediated in a EGFR- and c-met-dependent manner.

Our results provide evidence that inhibition of apoptosis in myoblast sheets can enhance their efficacy for treating HF after acute and chronic myocardial infarction. Furthermore, we show that myoblast sheets can serve as vehicles to deliver growth factors and induce therapeutic angiogenesis in the chronically ischemic heart. Finally, myoblasts induce, in a paracine manner, a cardiomyocyte-protective response against oxidative stress. Our study presents novel mechanisms of myoblast transplantation therapy and means to improve this therapy for the benefit of the HF patient.

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