

Aus dem Center for Cardiovascular Research
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Role of estrogen receptor alpha in post-ischemic cardiac
protection mediated via bone marrow c-kit+ cells

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Svetlana Slavic

aus Zrenjanin

Gutachter/in: 1.: Prof. Dr. med. Th. Unger
2.: Prof. Dr. med. G. Steinhoff
3.: Prof. Dr. med. C. Tschöpe

Datum der Promotion: 07.09.2012

1	Introduction	5
1.1	Myocardial infarction	5
1.1.1	Etiology and pathology	5
1.1.2	Symptoms and signs	6
1.1.3	Diagnosis	6
1.1.4	Therapy	6
1.1.5	Complications	7
1.2	Stem cell-mediated repair of the adult heart	7
1.2.1	C-kit receptor	8
1.2.2	Origin of progenitor cells in the heart	9
1.2.3	Potential approaches to therapeutic application	9
1.2.4	Repair mechanisms	11
1.2.5	Progenitor cell-mediated paracrine protection	12
1.2.6	Apoptosis - potential target of paracrine signalling	13
1.2.7	Stem cells in clinical trials	15
1.3	Protective effects of estrogen signaling on cardiovascular system	16
1.3.1	Estrogen signaling	16
1.3.2	Gender and cardiovascular function	19
1.3.3	Hormone replacement therapy and cardiovascular health	19
1.3.4	ER α vs. ER β	20
1.4	Aim of the study	22
2	Methods and Materials	24
2.1	Materials	24
2.2	Methods	27
2.2.1	Rat model of myocardial infarction	27
2.2.1.1	Animals	27
2.2.1.2	Induction of myocardial infarction	27
2.2.2	Isolation and flow cytometry analysis of bone marrow c-kit ⁺ cells	28
2.2.2.1	Principle of flow cytometry	28
2.2.2.2	Cell isolation protocol	28
2.2.3	Isolation of adult cardiomyocytes	29
2.2.4	<i>In vitro</i> assessment of cardiomyocytes' apoptosis	30
2.2.4.1	Co-culture of cardiomyocytes and c-kit ⁺ cells	30
2.2.4.2	Staining protocol	30
2.2.4.3	Co-culture stimulation with 17 β -Estradiol	31
2.2.4.4	Cytokine neutralization in a co-culture	31
2.2.5	<i>In vivo</i> injection of conditioned medium produced by c-kit ⁺ cells	31
2.2.5.1	Preparation of conditioned medium and intra-myocardial injection	31
2.2.5.2	Cardiac Function Evaluation with Transthoracic Doppler Echocardiography	32
2.2.6	<i>In vitro</i> stimulation of c-kit ⁺ cells	33
2.2.7	RNA isolation and quantitative real-time polymerase chain reaction	34
2.2.7.1	RNA isolation and reverse transcription	34
2.2.8	CFSE based proliferation assay	37
2.2.9	c-kit ⁺ cells in human peripheral blood: isolation and flow cytometry analysis	38
2.2.10	Statistics	38
3	Results	39
3.1	Isolation and characterization of bone marrow (BM) c-kit ⁺ cells	39
3.2	Bone marrow c-kit ⁺ cells mediate paracrine cardioprotection	40

3.3	Estrogen receptor α (ER α): distribution and functional importance in bone marrow c-kit+ cells	41
3.4	Conditioned medium from c-kit+mER α + cells improves cardiac function <i>in vivo</i>	43
3.5	Molecular characterization of c-kit+ER α + cells	45
3.6	C-kit+ER α + cells support CM by up-regulation of IL-6	46
3.7	ER α improves impaired cytokine production in c-kit+ cells after ischemia	46
3.8	c-kit+ER α + cells in human peripheral blood.	49
4	Discussion	51
5	Conclusions	59
6	Summary	60
7	Zusammenfassung	61
8	References	63
	Abbreviations	I
	Acknowledgments/Danksagung	III

1 Introduction

1.1 Myocardial infarction

1.1.1 Etiology and pathology

Myocardial infarction (MI) is among the leading causes of mortality and disability worldwide. According to World Health Organisation (WHO) around 7.2 million people annually die due to coronary heart disease [1]. In essence, myocardial infarction represents an imbalance between demand and supply of myocardial perfusion which results in ischemia and death of cardiac myocytes. It is usually a manifestation of coronary artery disease and is caused by atherosclerotic plaque rupture, erosions, fissuring or dissection. Other, less frequent, causes of MI include coronary artery spasm, coronary embolism, arrhythmia, hypo/hypertension, stent thrombosis, and surgical procedures such as percutaneous coronary intervention (PCI).

Artery obstruction and low oxygen supply cause cardiomyocyte death by two different processes: necrosis and apoptosis [2]. Necrosis occurs due to depletion of adenosine triphosphate (ATP), which reduces the efficiency of energy-dependent ion pumps in the cell. Further, cellular Ca^{2+} and free radical accumulation together with lipase activation trigger protease activation and membrane damage. When a cell dies in a necrotic process, its cellular content is released into the surrounding tissue and this causes inflammation. Proteins released from necrotic cardiomyocytes can be detected in the blood stream. Several of them are useful biomarkers in diagnosing MI, including creatine kinase (MB-fraction), troponins (I and T), myoglobin, brain natriuretic peptide (BNP) and C-reactive protein (CRP). On the other hand, apoptosis is an energy-dependent death mechanism. Apoptosis leads to neat cell fragmentation into apoptotic bodies that are later removed by phagocytes. The wound is healed and non-viable tissue is replaced with a scar through several subsequent phases: inflammatory, proliferative and maturation stadium. The inflammatory phase is initiated by ischemia and cell necrosis and hallmarks of this phase are: neutrophils and monocytes recruitments to the heart tissue and cytokine release into the blood stream and myocardial tissue ($\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6) [3]. Further, in the proliferative phase, lost cardiomyocytes are replaced by granulation tissue consisting of newly formed blood vessels, myofibroblasts and fibroblasts. Finally, during the maturation phase, granulation tissue is replaced by collagen and scar tissue is formed. The entire process leading to a healed infarction usually takes at least 5–6 weeks [4].

1.1.2 Symptoms and signs

Patients with acute myocardial infarction report diffuse, non localised discomfort or pain in chest area, upper extremities or jaw, at rest or during exercise, which usually lasts longer than 20 minutes and could be accompanied by dyspnoea, syncope, nausea, weakness, light-headedness. However, certain conditions, i.e diabetes mellitus, can mask typical symptoms or symptoms could be absent. Death is a result of mechanical failure of the left ventricle or dysrhythmia. Both conditions compromise sufficient blood flow to the vital organs [4].

1.1.3 Diagnosis

According to the current consensus document of European Society of Cardiology (ESC), American College of Cardiology (ACC), American Heart Association (AHA) and the World Heart Federation (WHF), diagnosis of the MI is met when following criteria are fulfilled:

Detection of rise and/or fall of cardiac biomarkers (preferably troponin) with at least one value above the 99th percentile of the upper reference limit (URL) together with evidence of myocardial ischaemia with at least one of the following:

- *Symptoms of ischaemia;*
- *ECG changes indicative of new ischaemia (new ST-T changes or new left bundle branch block [LBBB]);*
- *Development of pathological Q waves in the ECG;*
- *Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality [4].*

1.1.4 Therapy

Current approaches in the treatment of acute myocardial infarction are:

- Reduction of myocardial ischemia: oxygen, nitroglycerine, aspirin, thrombolytic agents
- Opioid analgesia to relieve pain
- β -blockers to decrease sympathetic activity, dysrhythmia and cardiac work
- Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor antagonist (ARBs) to reduce cardiac work and improve cardiac efficiency
- Reperfusion therapy

Patients with AMI should be rapidly evaluated for possibility of receiving some form of reperfusion therapy, invasive strategy (PCI) or fibrinolytic agents, because clinical trials showed

that reperfusion therapy significantly decreases mortality rates and improves the outcome after acute MI. [5][6].

1.1.5 Complications

Complications of MI can be broadly classified into: arrhythmic, mechanical, inflammatory and left ventricular thrombus. Arrhythmias are common complications and occur due to increased sympathetic activity in the heart and cell membrane instability resulting in enhanced automaticity of the heart tissue. They can further jeopardize myocardial perfusion and worsen myocardial ischemia by shortening duration of diastole, inducing hypotension and increasing myocardial oxygen demand. Mechanical complications can lead to cardiogenic shock and include ventricular free wall rupture, septal rupture, papillary muscle rupture with mitral regurgitation, ventricular aneurism. Free ventricular wall rupture is the most serious complication after AMI. It is usually caused by large transmural infarctions and accounts for 15-30% of the deaths associated with acute MI due to cardiac tamponade. Inflammatory complications include: pericarditis and post-MI syndrome (Dressler syndrome). Left ventricular mural thrombus increases risk of systemic embolisation, most commonly manifested by stroke [7].

Heart failure is still a major concern and a most frequent cause of morbidity and mortality after MI. Rapidly developing field of regenerative medicine is now offering a concept of cell therapy, which is being designed and optimised to meet the clinical need for new therapeutic approaches to treat the heart after MI.

1.2 Stem cell-mediated repair of the adult heart

Research reports of the last decade dramatically change our comprehension of the heart. Previously, heart was considered as a terminally differentiated organ due to its limited functional recovery in situations where viable myocardium is lost. Today evidence indicates that heart is a dynamic organ with a certain capability of self-renewal. Indeed, several research groups confirmed that cardiomyocyte turnover exists in the adult heart albeit differences are found in the magnitude [8][9]. Existence of progenitor/stem cells in adult myocardium has been also repeatedly confirmed. These heart progenitors are found in clusters of small round cells with a big nuclei and a thin rim of cytoplasm [10-12]. They are characterized by membrane expression of markers c-kit, MDR1 and sca-1, which are shown to be markers of stem cells. In addition, cardiac progenitor cells are negative for markers of differentiated myocytes (cardiac myosin heavy chain, sarcomeric α -actin, desmin, and connexin 43), endothelial cells (CD31, factor

VIII, and vimentin), smooth-muscle cells (smooth-muscle α -actin and desmin), and fibroblasts (vimentin). These cells participate in cell turnover in the adult heart, as it was confirmed by post-mortem studies of male patients, who received hearts from female donors. Using a fluorescence in situ hybridization for Y chromosome, it was shown that cells from the recipient are involved in remodeling of the donor heart. Y chromosome was detected in primitive cells in the heart expressing c-kit, but also in differentiated cardiomyocytes, smooth muscle cells and endothelial cells [10]. A genetic fate-mapping study combined with heart injury model in mouse provided indirect evidence that stem cells might be involved in cardiomyocytes turnover after injury, but not in a healthy condition [13].

1.2.1 C-kit receptor

Stem cells are self-renewing, clonogenic and multipotent. In the heart and bone marrow, stem cells express c-kit receptor. C-kit⁺ cells isolated from neonatal hearts (postnatal day 0-5) have the capacity to differentiate into several cardiac cell types (myocytes, endothelial cells, smooth muscle cells) directed by specific stimulatory conditions [14][15]. During mouse development, c-kit expressing cells are initially found in embryonic yolk sac (gestational day 8), then in embryonic liver (peak day 15) from where they migrate to bone marrow [16].

C-kit is a transmembrane tyrosine kinase receptor type III which binds stem cell factor (SCF). Extracellular region of the receptor contains five immunoglobulin-like domains which facilitate binding of the ligand while intracellular region exerts kinase activity and serves to transduce the signal. In bone marrow, c-kit receptor is mainly expressed by undifferentiated progenitor cells and its expression is down-regulated upon cell maturation and differentiation. Mastocytes, natural killer cells and dendritic cells retain expression of c-kit receptor implicating its important role for the function of these cells. C-kit expressing cells could also be found in non-hematopoietic tissues and include melanocytes, interstitial Cajal cells, germ cells (reviewed in [17]). A natural ligand of c-kit receptor, SCF is produced from endothelial cells, fibroblasts but also CD34⁺c-kit⁺ hematopoietic progenitors [18,19] and is found both in a soluble and transmembrane form. When SCF binds to the c-kit receptor, cellular processes important for stem cells are controlled, including apoptosis, proliferation and self-renewal. Some of the activated signaling pathways downstream of c-kit receptor include mitogen-activated protein kinase (MAP) pathway and phosphatidylinositol 3-kinases (PI3) pathway [17].

Gene mutations that lead to function loss of c-kit receptor or SCF cause lethal anemia, hematopoietic defects and mast cell deficiency. Gain-of-function mutations are found in some

tumor types: mast cell neoplasms, gastrointestinal stromal tumours (GISTs), germ cell tumours and some leukemias.

In their study, Fazel and collaborators showed that c-kit receptor is critical for the myocardial repair after MI. Mice with dysfunctional c-kit receptor develop dilated cardiomyopathy and have poorer survival following myocardial ischemia in comparison to their wild-type littermates. Impaired mobilization of progenitor cells from bone marrow to the heart and impaired neo-angiogenesis in the affected myocardium seems to contribute to the poor outcome of mice with mutated c-kit receptor. [20,21].

1.2.2 Origin of progenitor cells in the heart

Identifying origin of progenitor cells in the heart is a difficult task, partly due to contradictory reports found in literature concerning co-expression of other membrane markers (*i.e.* CD45, CD34). Scientific reports consistently show increased abundance of cardiac progenitors in the human heart in pathological conditions such as myocardial infarction and heart failure [10,12,13,21]. This indicates a possibility that injury activates resident heart progenitors or induces mobilization of bone marrow progenitors. Studies which claim that heart progenitor cells lack expression of bone marrow markers favor the idea that these cells represent a population of resident progenitors within a heart [11]. However, it is possible that cells which migrate from bone marrow through systemic circulation and home to the heart lose epitopes of bone marrow origin, as part of their differentiation process. The hypothesis of mobilization is supported by rodent experiments in which MI was induced after bone marrow has been ablated and replaced with green fluorescent protein (GFP) labeled or lacZ positive bone marrow cells. Several researchers showed existence of cardiomyocytes and vessels around infarcted area in whose formation cells from transplanted bone marrow participated, as confirmed by GFP or LacZ expression [21-23]. Progenitor cells mobilized from bone marrow and homed to the myocardium, might improve cardiac function via mechanisms other than direct differentiation into cardiomyocytes, as explained in more detail in sections 1.2.4 and 1.2.5.

1.2.3 Potential approaches to therapeutic application

Endogenous cardiac repair capacity is apparently inadequate to maintain homeostasis after pathological insult such as myocardial infarction; heart pumping function weakens and ultimately leads to heart failure. To prevent this frequent consequence of MI and enhance heart regeneration, researchers in the stem cell field have been focusing their efforts on at least two directions: developing strategies which would enhance endogenous regenerative processes and

developing optimal cell therapy to replace lost cardiomyocytes. Endogenous repair could be potentially enhanced by targeting mobilization of progenitor cells from the bone marrow, their homing to the heart or activation of resident progenitors.

Mobilization of stem cells from the bone marrow is mainly mediated by signaling factors such as: granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), stem cell factor (SCF), stromal derived factor (SDF), interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF) [24]. Up till now, the therapeutic use of cytokines with an aim to enhance recruitment of progenitors from bone marrow to the heart showed limited success. Mono-therapy with G-CSF, the most widely used agent to mobilize stem cells, is safe but it neither elevates ejection fraction nor improves survival in a clinical trial [25]. Therefore, to make this approach feasible, researchers are developing strategies which would target both stem cell mobilization from bone marrow and homing to the target tissue *i.e.* heart [26].

Activation of resident progenitors is an attractive idea based on evidence that heart possesses certain regenerative ability and that the number of progenitors increases in the pathological conditions affecting the heart. However, Zaruba *et al.* challenged the idea that c-kit⁺ cells found in the adult heart have the ability to differentiate into cardiac phenotype [27]. They showed apparent cardiomyogenic activity of neonatal derived c-kit⁺ cells, but potential of these cells to form cardiomyocytes is lost during the heart development from neonatal to adult stage. Similarly, Talini and collaborators report that injury evokes re-expression of c-kit antigen in adult heart, but this was mostly associated with neo-angiogenesis and fibrosis and not with myogenesis[14]. Therefore, in order to promote endogenous cardiomyogenesis we need to elucidate mechanisms of progenitor cell senescence and signals that promote activation and differentiation of resident cardiac progenitors. Also, we need to find strategies to ameliorate adverse factors which, in pathological conditions, compromise endogenous repair.

Bone marrow cell transplantation was performed in numerous experimental studies published over the past few years. Different cell populations in various numbers were used: un-fractionated bone marrow, BM mononuclear cells (BM MNCs), lineage negative c-kit⁺ cells, lineage negative CD34⁺ cells, mesenchymal stem cells. Despite the methodological differences, many researchers reported positive effect of cellular therapy on myocardial function following MI, indicating that diverse mechanisms of action might be involved. A study that evoked excitement in scientific community dates from 2001 and for the first time claims that transplanted bone

marrow lineage negative c-kit⁺ cells abundantly replace injured heart tissue and take part in the formation of new cardiomyocytes and vessels [28]. Using genetic tools to track the fate of transplanted cells, some later studies questioned the possibility that bone marrow cells trans-differentiate into cardiac lineage [29,30]. No consensus currently exists on whether bone marrow stem cells have the capacity to cross lineages and become beating cardiomyocytes, and functional improvement observed after transplantation is attributed to other mechanisms such as improved vascularization, paracrine protection *etc.*

1.2.4 Repair mechanisms

Whether they were therapeutically applied or injury-mobilized, bone marrow cells which home to the myocardium might exert a beneficial effect on cardiac function. Experimental studies proposed several mechanisms that could be broadly divided into direct and indirect regeneration. Direct regeneration implies differentiation of transplanted/recruited cells into different cell types necessary for the functional recovery of myocardium: endothelial cells, smooth muscle cells, cardiomyocytes. On the other hand, behind indirect mechanism is a paracrine hypothesis of cardiac protection, which arose from studies where functional improvement was observed in the absence of evidences of long-term engraftment or cell differentiation.

The relative importance of direct tissue regeneration, through new tissue formation, versus indirect mechanisms, through paracrine effects, is still not completely understood. Because the rate of long term cell engraftment [31] and direct differentiation into cardiomyocyte is low, the contribution of cardiomyogenesis to the functional improvement after cell transplantation is debatable. Speaking of direct regeneration, it is more likely that transplanted or recruited bone marrow cells after homing to ischemic myocardium take part in neoangiogenesis. In several studies, bone marrow cell therapy has been associated with an improved blood flow and increased capillary density in the heart. For example, to address the question on how much fate acquisition contributes to the functional improvement, Yoon et al. designed a study in which vectors encoding suicide gene were put under control of specific promoters: for endothelial cells (endothelial nitric oxide synthase, eNOS), smooth muscle cells (SM22 α) and cardiomyocyte (α -myosin heavy chain, α -MHC). By selective elimination of lineage-committed cells, Yoon et al. could observe deterioration in contractile function when eNOS and SM22 cells were depleted, but not α -MHC, implicating that neoangiogenesis was a main contributor to the functional improvement. However, in this study, paracrine hypothesis cannot be excluded, and it is not

clear whether deterioration in heart function was due to lack of progenitors or paracrine factors secreted from the same cells [32].

1.2.5 Progenitor cell-mediated paracrine protection

Paracrine signalling is a process in which cells, by secreting signalling molecules, affect other, neighbouring cells. These molecules act as local mediators and affect cell processes such as proliferation, survival, differentiation, migration. Autocrine signalling refers to a situation where responder cells are the same cells which secreted signalling molecules. Paracrine properties were discovered in different cell types including haematopoietic bone marrow cells, mesenchymal stem cells (MSCs, multipotent bone marrow stromal cells), skeletal myoblasts and endothelial progenitor cells (EPC). It has been postulated that different signalling factors, secreted by cells that home to the myocardium, could exert beneficial effect on heart healing after MI by targeting different cell types and cellular processes: survival of cardiomyocytes, neovascularisation, immune response, post-ischemic remodelling or activation of resident cardiac progenitors as schematically presented in Figure 1.1. Uemura *et al.* showed that cardiomyocytes co-cultured with bone marrow stem cells were protected from anoxia-induced apoptosis due to increased activation of pro-survival gene-Akt [33]. Some cytokines are proposed to exert protective survival effect on cardiomyocytes: hepatocyte growth factor (HGF), insulin-like growth factor (IGF-I), adrenomedullin, interleukin-11 (IL-11), oncostatin M (OSM) thymosin β 4. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are important cytokines that both protect cardiomyocytes and improve neovascularisation. IL-10, secreted from bone marrow MNCs, was shown to prevent adverse cardiac remodeling and improve left ventricular (LV) recovery after MI [34]. Another study suggested that IL-10 promotes self-renewal of hematopoietic cells [35]. Interestingly, stem cell therapy was also beneficial in a non-ischemic model of heart failure. Intramyocardial injection of MSCs improves LV contractility in a doxorubicin-induced model of heart failure, presumably due to attenuated β -adrenergic receptor down-regulation [36]. Recently, it was demonstrated that intracoronary administration of exogenous cardiac progenitors induces paracrine activation of endogenous resident progenitors in a model of old MI [37].

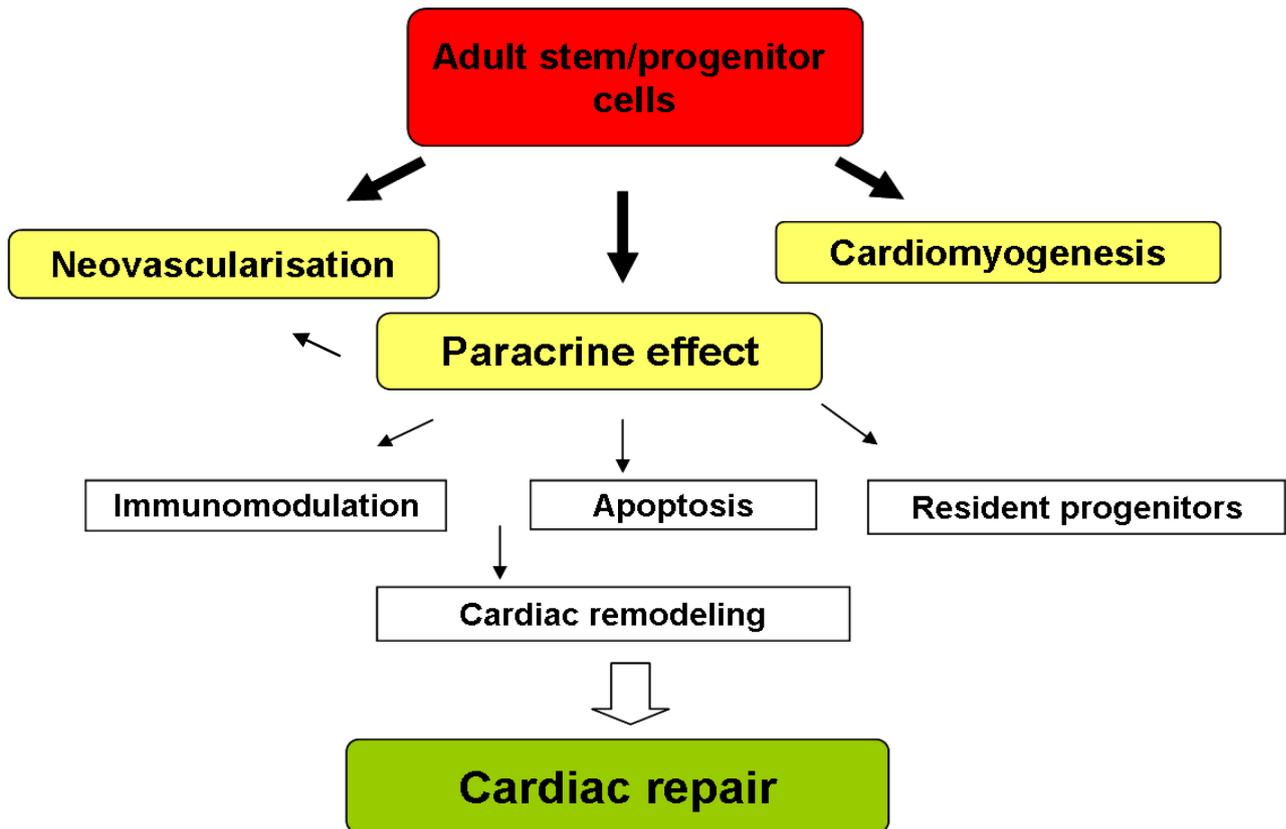


Figure 1.1. Mechanisms of stem/progenitor cell mediated heart repair. Transplanted cells directly regenerate heart tissue by taking part in the new vessel formation and trans-differentiation into cardiomyocytes. Indirectly, stem cells contribute to heart regeneration via paracrine secretion of biologically active molecules that exert their effect on various cell types and processes

We are just beginning to understand individual functions from a wide range of cytokines released from different progenitor cell types. Despite their pleiotropic properties and partially overlapping function, a complex of signaling factors, which would target several cell types, might be necessary for successful cardiac repair. Cytokine therapy might be a clinically more attractive approach compared to cell application. However, we still need to be able to pinpoint the crucial factors and signaling pathways responsible for paracrine-mediated cardiac repair before we decide to use them in practice.

1.2.6 Apoptosis - potential target of paracrine signalling

Apoptosis is a cell death type where intracellular death program is activated and cell dies in a controlled way. The process ensures neat removal of those cells which are no longer needed, damaged or potentially dangerous, without triggering any inflammatory reaction [38]. Tissue homeostasis is preserved owing to well controlled balance between death and proliferation. However, excessive or insufficient apoptosis might lead to disease. For example, it is believed that, apart from necrosis, apoptosis contributes to a loss of functionally contracting cardiac cells

after myocardial infarction. Apoptosis occurs as early as 2-4 hours in the myocardial area affected by artery occlusion [39] but also in a remote non-infarcted myocardium [40].

Cells undergoing apoptosis display characteristic morphological and biochemical changes. They shrink and condense, nuclear membrane disassembles and chromatin condenses and breaks up into fragments, membrane blebs and sometimes apoptotic bodies are formed. Besides DNA fragmentation, a hallmark of the apoptosis is exposure of phosphatidylserine on the outer side of the phospholipids' membrane which sends a phagocytosis signal and inhibits inflammation during phagocytosis [38].

Final executors in the apoptosis are cysteinyl aspartate proteases called caspases which can be activated by extrinsic or intrinsic signals. Extrinsic pathway is initiated through activation of death receptors, which belong to a tumor necrosis factor receptor family such as Fas and TNF-1. Binding of the ligand to these receptors activates a caspase cascade, initially caspase-8 followed by other executor caspases (-3, -6, 7), depending of the cell type affected. Lack of nutrients, oxygen or survival signals and DNA damage are some elicitors of the intrinsic pathway. A crucial event in the caspase activation of the intrinsic pathway is the release of a protein cytochrome c from mitochondria into the cytoplasm. The intrinsic pathway is well controlled by Bcl-2 family of proteins, whose pro-apoptotic and anti-apoptotic proteins control cell death by controlling cytochrome c release from mitochondria. Pro-apoptotic family members BAK and BAX are crucial for permeabilization of mitochondrial outer membrane, while anti-apoptotic family members such as BCL-2 and BCL-XL inhibit BAK and BAX. The third group of Bcl-2 proteins are the so called BH3-only proteins. Members of this group, for instance, PUMA, BID and BIM, induce apoptosis by primarily inhibiting anti-apoptotic proteins. Released cytochrome c leads to formation of apoptosome - heptameric complex of apoptotic protease activating factor (APAF1) monomers bound to cytochrome c and subsequently to caspase-9 activation. In addition, other proteins such as DIABLO are released from mitochondria and activate caspases independently from APAF1. The molecular mechanism by which BAK and BAX activate caspases has still not been clarified [38][41].

Developing strategies that would target cardiomyocyte apoptosis could potentially reduce infarct size and preserve heart contractile function. For example, heart restricted *bcl-2* over-expression reduced infarct size by 30% in an ischemia-reperfusion model [42]. Also, caspase inhibition was found to preserve left ventricular systolic function after myocardial infarction (MI) by reducing apoptosis and left ventricular remodelling [43]. Use of stem cells in therapy of MI was shown to

reduce infarct size by paracrine regulation of apoptosis in cardiomyocytes. For instance, mesenchymal stem cells (MSCs) reduce cardiomyocytes apoptosis and infarct size in a paracrine manner by activating Akt signalling in cardiomyocytes [33]. In another study, infarct size reduction and functional improvement were observed as early as 72h after an insult and cell application. Such a rapid improvement can not be explained by differentiation of injected MSCs and is probably a result of paracrine secretion of protective factors by transplanted cells [44].

1.2.7 Stem cells in clinical trials

Despite the contradicting data on mechanisms by which stem cells improve heart function, data obtained from experimental studies inspired several clinical trials which confirmed the safety of cell application but showed different measures of success [45-47]. Most of the trials used bone marrow mononuclear cells (BM MNCs), which comprise mixed populations of hematopoietic cells, mesenchymal stem cells and monocytes. In the biggest randomized, controlled trial performed to date, Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI), 204 patients were assigned to BM MNCs or placebo (serum) treatment. All patients received state-of-the-art reperfusion and pharmacological therapy. After 4 months, significant improvement in LVEF was observed among patients receiving BM MNCs compared to placebo group, and a reduction in 2-year incidence of major adverse cardiovascular events [45,48]. However, other studies, although showing safety of the cell therapy, were not as successful in achieving the desired outcome. For example, HEBE trial included 200 patients randomised to either intracoronary infusion of mononuclear BM cells, mononuclear peripheral blood cells or standard therapy (without placebo infusion). The primary endpoint, improvement in regional myocardial function in dysfunctional segments measured by MRI 4 months after acute myocardial infarction, did not significantly differ between the groups. Authors suggest several factors which might have contributed to the failure of the study to show a benefit from cell therapy: timing of the cell therapy, non-standardised number of delivered cells, short follow-up period [49]. Other clinical trials, designed to solve some crucial dilemmas (timing, the optimal cell type, indications for the cell therapy) are on the way. For example, Swiss Multicenter Intracoronary Stem cells Study in Acute Myocardial Infarction (SWISS-AMI) trial is designed with an aim to define an optimal time point of cell delivery. BM-MNC cell delivery will be performed at 5 to 7 days or 3 to 4 weeks after the initial event. The primary end point is the change in global left ventricular (LV) ejection fraction by cardiac magnetic resonance imaging at 4 months as compared to baseline [50]. Another study, the COMPARE-

AMI trial, is designed to investigate the effect of a selected population of the bone marrow stem cells (CD133+ cells) on cardiac recovery of infarcted myocardium [51].

Experience from experimental and clinical studies implicates that more effort should be invested in understanding stem cells: their biology, differentiating potential, existence of tissue progenitors and ways to activate them.

1.3 Protective effects of estrogen signaling on cardiovascular system

1.3.1 Estrogen signaling

Estrogen hormones, 17- β estradiol (E_2), estrone (E_1), and estriol (E_3) influence many physiologic functions besides the reproductive one, in which they play a central role. Among others, estrogen signaling impacts bone metabolism, lipid metabolism in the liver, growth of several cancer types, and the immune and cardiovascular systems.

All estrogen actions are mediated by two estrogen receptors (ER): ER α and ER β . They are encoded by distinct genes localized on two different chromosomes. Both receptors have similar structural organization consisting of several domains: N-terminal region which is a transcriptional activation domain (A/B domain), DNA-binding domain (C domain), region involved in receptor dimerization (D domain) and c-terminal end with a ligand-binding domain and transcriptional activation region (E/F domain) as shown in Figure 1.2 [52].

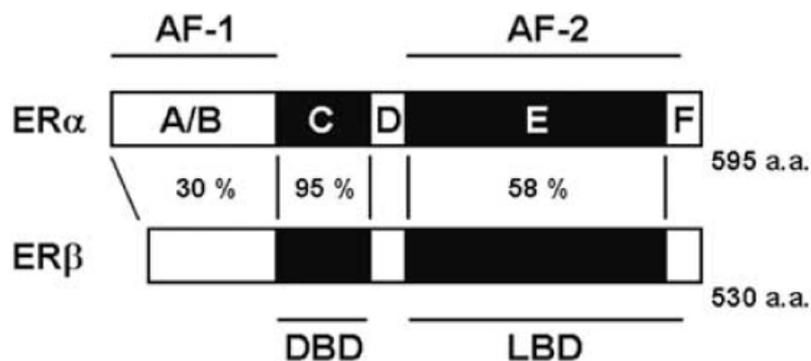


Figure 1.2. Domain organizations of human ER α and ER β with percentages indicating homology between the two receptors. Taken from [52] and modified

ERs have been long considered as ligand activated transcriptional factors. This view has been challenged and for more than a decade it is recognized that estrogen signaling is much more complex and includes both genomic and rapid membrane-initiated estrogen signaling, sometimes referred as ‘non-genomic’ signaling. Estrogen-mediated genomic signaling starts

after estrogen diffuses into the target cell and binds to the ERs. This causes conformational change and dimerization of the receptors. Complex of ER dimer and estrogen regulates gene transcription by binding to the DNA sequences in the promoter region of the responsive genes, known as estrogen responsive elements (ERE). Alternatively, estrogen-ERs complex binds to other transcriptional factors, thereby influencing expression of genes without estrogen responsive elements known as a transcriptional cross talk (*reviewed by* [53]).

Some cellular effects of estrogens cannot be explained by changes in gene transcription because they are too rapid. More than 4 decades ago, it was shown that intravenous administration of 17- β estradiol in physiological doses to the ovariectomised rat initiates rapid increase of cyclic adenosine monophosphate (cAMP) in the uterus with a time lag of only 15 seconds [54]. Non-genomic estrogen signaling is a term to describe rapid events occurring in the cell after binding of estrogen to its membrane receptors. Since discovery of estrogen binding sites in membranes of endometrial cells [55], numerous reports using different methodological approaches confirmed the existence of ER in the cell membrane [56-58]. Signaling pathways which are typically activated by rapid estrogen signaling include phosphatidyl inositol 3 kinase (PI3/AKT), cAMP/protein kinase A (PKA), phospholipase C (PLC)/protein kinase C (PKC) and MAPK signaling pathway [52]. In addition, rapid estrogen signaling also potentially affects gene transcription by activation of intracellular protein kinases which in turn regulate the activity of different transcriptional factors (Figure 1.3.).

The nature of membrane located ER is however still debatable. Initially, it was suggested that plasma membrane ERs are a subpopulation of classical receptors of 66kDa [56-58]. Mass spectrometry analysis revealed that estrogen binding proteins isolated from nuclear and membrane fractions of MCF7 cells are identical and represent classical ER α . The same study shows that estrogen stimulation does not elicit rapid signaling in ER null cell. Thus, it is not likely that E2-mediated rapid signaling in breast cancer cells is due to E2 binding to membrane/cytoplasmic proteins other than classical ER [58]. However, in different cell types, truncated forms of ERs (46kDa and 36 kDa proteins) have also been described [59,60]. In the human endometrial cancer cell line, E2 activates membrane and cytoplasmic 36 kDa ER α and induces proliferation via the protein kinase C δ pathway [60].

Because estrogen receptors do not possess a trans-membrane domain, a question arises regarding the cellular mechanisms that enable trafficking of the receptor to the plasma membrane. Cys447 in the ligand binding domain (E domain) of the receptor was shown to mediate trafficking and

maintenance of the receptor in plasma membrane. Post-translation palmytoylation of this residue is indispensable for E2 initiated rapid signaling [61]. Also, it has been reported that membrane ER exists in calveolar domains of plasma membrane. In this sense, Ser552 residue within E domain of the ER is identified as being important for the receptor association with calveolin-1 [62]. Despite the fact that 100% of ER contain palmytoylation motif, only 5-10% of endogenous ER pool are detected in plasma membranes [63]. Additional research is needed to fully understand this phenomenon and to dissect mechanisms which enable/impair receptor trafficking to the membrane.

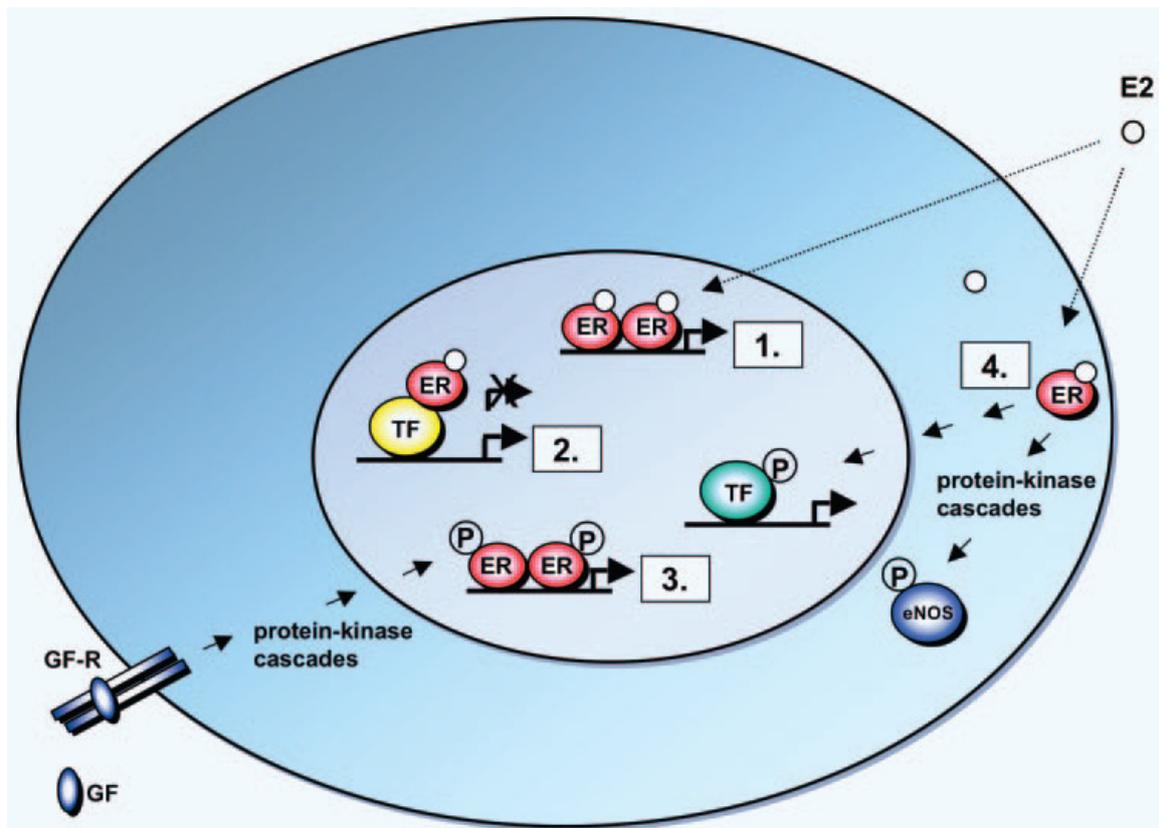


Figure 1.3 Multiple mechanisms of estrogen action: 1. ligand-activated ER dimers regulate gene transcription after binding to ERE. 2. activated ER-estrogen complex binds to transcriptional factor and regulates transcription of genes without ERE. 3. ligand-independent activation of ERs via activation of growth factor receptors. 4. Rapid estrogen signaling alters function of cytoplasmic proteins or activity of transcriptional factors. Taken and modified from the [53]

1.3.2 Gender and cardiovascular function

Female gender has been associated with reduced risk and delayed onset of cardiovascular diseases before menopause [64,65]. Protective effects of estrogen (E2) on the cardiovascular system are complex and mediated by its receptors estrogen receptor alpha (ER α) and beta (ER β) in both vasculature and myocardium.

In vessels, estrogen mediates nitric oxide (NO) dependent artery dilatation in a rapid non-genomic manner and prevents atherosclerosis by targeting genes in endothelial and smooth muscle vascular cells [66]. Anti-atherogenic protective effects of E2 are partly due to changes in lipid metabolism. Hormone replacement therapy during menopause decreases plasma levels of low density lipoproteins (LDL) and elevates plasma levels of high density lipoproteins (HDL) and also paradoxically triglycerides [67]. E2 contributes to atherosclerosis protection also via ER α dependent increase in production of prostaglandin PGI₂. PGI₂ is synthesized from cyclooxygenase 2 (COX-2) in vascular endothelial cells and, among other mechanisms, mediates protection by inhibiting platelet activation, vascular smooth muscle contraction and proliferation [68].

In patients with end-stage heart failure, a lower myocyte death rate is documented in female than in male hearts and is associated with a later onset of cardiac decompensation [69]. Mechanisms of anti-apoptotic estrogen action include activation of survival pathway PI3 kinase/Akt signaling [70] and reduction of oxidative stress [71]. Also, clinical and experimental data show gender-related differences in response to pressure overload. In a model of aortic stenosis, male rats have a depressed contractile reserve compared to female accompanied with altered gene expression in the myocardium. mRNA levels of atrial natriuretic factor (ANF) and beta-myosin heavy chain are increased and sarcoplasmic reticulum Ca²⁺-adenosine triphosphatase (SERCA2a) is decreased in males in comparison to females in response to pressure overload [72].

1.3.3 Hormone replacement therapy and cardiovascular health

Natural menopause occurs in women at average age of 51 due to diminished hormonal secretion from ovaries. Menopausal women are at increased risk of coronary heart disease, stroke, osteoporosis, bone fracture and dementia compared to aged matched cycling women. Taking this and numerous other research reports as well as results from observational studies into account, it is easy to understand the high expectations concerning risk reduction through the hormone replacement therapy in post-menopausal women. The Women Health Initiative (WHI) included two large randomized placebo controlled trials designed to investigate potential risk

and benefits of hormone replacement therapy (estrogen-progestin or estrogen only) in post-menopausal healthy women. Contrary to expectations, the study ended prematurely because risk of cardiovascular events (coronary heart disease, stroke and venous thromboembolism) outweighed the benefits (reduced risk of fracture and colon cancer) [67]. Results of WHI emphasize the importance of factors which possibly influence the effect of hormonal therapy: hormonal formulation, route of administration, pattern of administration (cyclic vs. continuous) and timing of therapy initiation. Later, the interval from menopause to hormone therapy seems to be of highest importance because irreversible alterations in the vessel wall change the way the vessel respond to hormones. It is thought that vessels with advanced atherosclerotic lesions are more susceptible to inflammatory reactions in response to late initiation of hormone therapy [73]. Therefore, additional research is required and a better understanding of cellular and molecular effect of hormonal action and receptor activation will hopefully resolve the controversy of hormonal therapy in post-menopausal women.

1.3.4 ER α vs. ER β

Contribution of ER α vs. ER β to cardioprotection is still controversial, partly due to methodological discrepancies found in literature. It is further complicated by the fact that ERs can form heterodimers to stimulate downstream target genes [74]. Also, estrogen-initiated tissue effects are not only dependent on ER α and ER β expression, but are also the result of expression and activation status of cell-specific co-activator and co-repressor proteins [73]. Several studies provide evidence that ER β is cardioprotective in cardiac ischemic injury. In their study on ER α and ER β knock-out mice (α ERKO and β ERKO, respectively), Babiker *et al.* could show that ER β reduces infarct size, but increases heart remodeling and mortality after experimental MI [75]. Similarly, contractile function after brief ischemia was more severely impaired in β ERKO than in α ERKO and WT female mice in ischemia/reperfusion injury [76]. Conversely, Booth *et al.* showed a reduction in infarct size after administration of ER α agonist 4,4',4''-[4-propyl-(1H)-pyra-zole-1,3,5-triyl]tris-phenol (PPT) before ischemic injury in a rabbit. In the same study, activation of ER β by administration of specific agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) does not influence infarct size [77]. Our group previously showed that protein levels of ER α , but not ER β are increased in peri-ischemic region of a rat myocardium after experimental myocardial infarction. Immunofluorescent staining of heart sections showed that ER α is mainly induced in peri-infarct areas in the progenitor cells- small proliferating cells, characterized by expression of c-kit and sca-1 antigens [78].

The contribution of ER α vs. ER β to the proliferation and apoptosis was studied in transiently transfected HeLa cells. E2 initiated rapid signaling and induced proliferation in ER α -transfected cells. In contrast, E2 stimulation of ER β -transiently transfected cells initiated growth arrest and apoptosis [79].

Altogether, these studies indicate that probably both estrogen receptors, ER α and ER β , up to certain degree, contribute to the protection of the cardiovascular system by targeting different cells and cellular processes.

1.4 Aim of the study

Despite the advances in diagnosis and treatment, myocardial infarction remains a major public health concern. Quest for new treatment options has turned the wind towards cell therapy, as a promising option aimed to improve cardiac function after ischemia or during heart failure. While no consensus has been reached on potential of bone marrow cells to directly regenerate cardiomyocytes, most researchers agree on their role in neoangiogenesis and paracrine cardioprotection.

A growing body of evidence emphasizes that estrogen signaling plays an important role in biology of progenitor cells. Gender differences have been found in relation to the presence and competence of progenitor c-kit⁺ cells in the human heart. Not only a larger pool of c-kit⁺ cells, but also longer telomerase, higher fraction of cycling and lower fraction of senescent cardiac progenitors are found in female compared to male hearts [9]. Importance of estrogen signaling in progenitor cell populations has been so far most extensively studied on endothelial progenitors (EPCs). It was demonstrated that in the setting of acute MI in mice estrogen via its receptors mediates mobilization, migration, proliferation and homing of EPCs. In addition, estrogen receptor α (ER α) is shown to be more relevant than estrogen receptor β for the function of EPCs [80,81].

However, the role of E2 signaling in the paracrine secretion of bone marrow progenitor cells has not been thoroughly investigated until now. E2 stimulation of mesenchymal stem cells (MSCs) enhanced the production of vascular endothelial growth factor and hearts infused with E2-treated MSCs show greater post-ischemic recovery than hearts infused with untreated MSCs [82]. Our group was able to show that conditioning media collected from cardiac c-kit⁺ cells after ER α stimulation induce myoblasts proliferation. In contrast, media collected from cardiac c-kit⁻ cells under the same stimulatory conditions do not elicit myoblast proliferation [78]. These findings emphasize the paracrine potential of cardiac c-kit⁺ progenitors and the importance of ER α in its regulation.

The aim of this study was to further investigate paracrine mechanisms in c-kit⁺ cells upon ER α activation and in ischemic conditions. For this purpose, we developed a method to isolate c-kit⁺ cells from the rat bone marrow 7 days following MI/sham operation. Our aim to evaluate the ER α expression pattern and its relevance in the paracrine function in this cell population. Furthermore, we hypothesized that bone marrow c-kit⁺ cells support cardiomyocytes (CM) in a paracrine manner and that the paracrine function of c-kit⁺ cells is influenced by ER α . Therefore,

using *in vitro* and *in vivo* approach, we aimed to investigate the relation of membrane expression of ER α in c-kit⁺ cells to their cardio-protective capacity. Finally, we aimed to pinpoint potentially cardioprotective cytokines secreted by c-kit⁺ cells under control of ER α .

2 Methods and Materials

2.1 Materials

Chemical reagents

Reagent	Company, Town, Country
Chlorophorm	Merck KGaA, Darmstadt, Germany
Collagenase type II	Sigma, Steinheim, Germany
Cytofix/Cytoperm™	BD Biosciences, Heidelberg, Germany
EDTA	Sigma, Steinheim, Germany
17β Estradiol (water soluble)	Sigma-Aldrich, Germany
Ethanol 100%	Merck KGaA, Darmstadt, Germany
Isopropanol	
Lymphocyte separation medium PAA	Laboratories GmbH, Cölbe, Germany
Propyl pyrazole triol (PPT)	Tocris, Germany
Trizol	Invitrogen, Karlsruhe, Germany
Ultra-pure water	Biochrom GmbH, Berlin, Germany

Staining solutions

Acridine Orange (AO)	Sigma, Steinheim, Germany
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	MolecularProbes Invitrogen, Karlsruhe, Germany
4'6'-diamidino-2-phenylindole dihydrochloride (DAPI)	Invitrogen Karlsruhe, Germany
Ethidium bromide (1% m/v)	Roth, Karlsruhe, Germany
Propidium iodide	Becton Dickinson, Heidelberg, Germany

Cell Culture Media, Supplements and additional material

Calcium and Magnesium free-Hank's balanced salt solution (CMF-HBSS)	PAN Biotech, Aidenbach, Germany
Dulbecco's modified Eagle Medium (DMEM, mit 4,5 g/l Glucose; 0,58 g/l L-Glutamine; 3,7 g/l NaHCO ₃)	Gibco BRL, Karlsruhe, Germany
DMEM/F12	PAN Biotech, Aidenbach, Germany
Fenol free DMEM/F12	Gibco BRL, Karlsruhe, Germany

Penicillin/Streptomycin	Biochrom AG, Berlin, Germany
Phosphate buffered saline (PBS)	Gibco BRL, Karlsruhe, Germany
Trypsin/EDTA (1x)	PAA, Austria
<i>Sera</i>	
Bovine serum albumin (BSA)	Sigma, Taufkirchen, Germany
Donkey serum	Jackson ImmunoResearch, Hamburg, Germany
Fetal bovine serum (FBS)	Gibco BRL, Karlsruhe, Germany
<i>Additional material:</i>	
Petri dish	Nunc, Roskilde, Denmark
96 well cell culture plate	Becton Dickinson, USA
Syringe filters 0.2µm	Albet LabScience, Dassel, Germany
Syringes various sizes	Becton Dickinson, Fraga, Spain
Cell Strainer 100µm	Becton Dickinson, Erembodegem, Belgium
Anopore 8-well Strip (cell culture insert pore size 0.2µm)	Nunc, Roskilde, Denmark
MS separation columns	Miltenyi Biotec, Bergisch Gladbach, Germany

Antibodies

Primary antibodies

<i>Antigen</i>	<i>Source</i>	<i>Company</i>
c-kit (rat, human)	goat polyclonal	Santa Cruz, Heidelberg, Germany
CD31- phycoerythrin (PE) conjugated (rat)	mouse monoclonal	Becton Dickinson, Heidelberg, Germany
CD34 (rat)	mouse monoclonal	Santa Cruz, Heidelberg, Germany
CD45 (rat)	mouse polyclonal	AbD Serotec, Düsseldorf, Germany
ER α (rat, human)	rabbit polyclonal	Santa Cruz, Heidelberg, Germany
ER β (rat)	rabbit polyclonal	Santa Cruz, Heidelberg, Germany
IL-6 (human)	mouse monoclonal	Becton Dickinson, Heidelberg, Germany
IL-6 (rat)	goat monoclonal	Peptotech, Rocky Hill, NJ
IL-10 (rat)	rabbit monoclonal	Peptotech, Rocky Hill, NJ
Goat IgG (isotope control)		Santa Cruz, Heidelberg, Germany
Rabbit IgG (isotype control)		Santa Cruz, Heidelberg, Germany

Secondary antibodies and beads

Allophycocyanin (APC) anti-goat IgG	Becton Dickinson, Heidelberg, Germany
Alexa-488 anti-rabbit IgG	Jackson ImmunoResearch, Hamburg, Germany
Anti-APC multisort microBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Fluorescein isothiocyanate (FITC) anti-mouse IgG	Jackson ImmunoResearch, Germany

Nucleic acids analysis

DNase digestion kit	Promega Corp., Madison WI, USA
M-MLV Transcription kit	Promega Corp., Madison WI, USA
Oligo dNTPs	Promega Corp., Madison WI, USA
Pico pure RNA isolation kit	Arcturus, MDS Analytical technology
Random Primers	Promega Corp., Madison WI, USA
RNase Inhibitor	Promega Corp., Madison WI, USA
SuperScript® II Reverse Transcriptase	Invitrogen, Carlsbad, CA
SYBR Green® Power Universal Master Mix	Applied Biosystem, Darmstadt, Germany
96-well Multiply® PCR plate	Sarstedt, Nümbrecht, Germany

Various devices

Centrifuges/Rotors

Universal 32R	Hettich Zentrifugen, Tuttlingen, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany

Microscopes

Light microscope Leica DMIL	Leica, Wetzlar GmbH, Germany
Fluorescent microscope Leica DMIRE2	Leica, Wetzlar GmbH, Germany

Nuclear acid analysis

NanoDrop 1000	Peqlab, Erlangen, Germany
Mx3000P™ Real-Time PCR System	Stratagene, Amsterdam, Nederland

Other devices

Cell culture laminar	Holten, Allerød, Denmark
Cell Culture Incubator HeraCell®150	Heraeus/TermoScientific
Flow cytometer- cell sorter FACSAria®	Becton Dickinson, CA, USA
Flow cytometer- analyzer FACSCalibur®	Becton Dickinson, CA, USA

Thermo mixer Comfort	Eppendorf, Hamburg, Germany
Vortex Genie2	Scientific Industries, NY, USA
PH-Meter CG 840	Schott, Germany
Echocardiography-Vevo 770	VisualSonics Inc, Toronto Ontario, Canada

Surgical material

Surgical scissors and forceps

Suture:

Prolene 17mm (polypropylene) not absorbable	Ethicon GmbH, Norderstedt, Germany
Ethibond excel 13mm (polyester) not absorbable	Ethicon GmbH, Norderstedt, Germany
Ethibond excel 3.5 Ph (polyester)	Ethicon GmbH, Norderstedt, Germany
Terylene USP4/0	Serag Wiessner, Germany

2.2 Methods

2.2.1 Rat model of myocardial infarction

2.2.1.1 Animals

Male normotensive Wistar rats (200-220g, HARLAN Winkelmann, Borchon, Germany) were kept in a SPF (specific pathogen free) barrier under standardized conditions with respect to temperature and humidity, and were housed on a 12h light/12h dark cycle in groups of 5 with food and water ad libitum. Animal housing, care, and applications of experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* of the State Government of Berlin, Germany.

2.2.1.2 Induction of myocardial infarction

Rats were anesthetized with ketamine/xylazine (Sigma) 80mg/10mg/kg i.p., intubated and ventilated with a small-animal ventilator (Starling Ideal Ventilator, Harvard Apparatus) with room air at a rate of 75 cycles per minutes and a tidal volume of 3.5 ml.

A left lateral thoracotomy in the fourth or fifth intercostal space was performed and a 6-0 silk suture was tightened around the proximal left anterior descending coronary artery. Thorax, the muscle layer and skin were closed separately and the rats were allowed to recover. Sham-operated rats underwent the same surgical procedure except for the coronary ligation, and served as a control. Seven days after surgery, rats were euthanized and their hearts and bone marrow were harvested for further analysis.

2.2.2 Isolation and flow cytometry analysis of bone marrow c-kit⁺ cells

2.2.2.1 Principle of flow cytometry

Flow cytometry and cell sorting are important techniques which make it possible to analyse or to separate specific cell types from a mixed cell suspension. In a *flow cytometer* device, cells flow individually in a suspension in front of a light source (laser). Scattered light and fluorescence signals are detected for each passing particle by photo-detectors and optical filters, processed and stored on a computer. While scattered light provides useful information about cell morphology (size and granularity), using a fluorescent probes or fluorescently labeled antibodies enables researchers to analyze cellular viability, physiology (i.e. division) or expression of different intracellular or membrane proteins. Some devices possess also a sorting capacity, which means that they are able to collect only particles of interest from a mixed population by diverting them from the fluid stream into a collection tube. This is achieved by application of electric charge to the fluid drops containing particle of interest (selected by light scattering and fluorescent properties) which enables them to be collected while they pass through an electric field, while other, uncharged particles are disposed. Sorting usually has little or no effect on cell viability or function and provides a pure population of cells with desired characteristics which can be further cultured.

2.2.2.2 Cell isolation protocol

Bone marrow was harvested from femurs and tibias 7 days after surgical myocardial infarction (MI) induction by flushing bones with 22 gauge needle and PBS+10%FBS. Single cell suspension was obtained by passing the medium with bone marrow through 23G needle and syringe several times. Mononuclear cells (MNCs) were isolated using density gradient centrifugation. Cell suspension was carefully added on a layer of lymphocyte separation media and centrifuged 20 minutes at 800g and 24°C. After centrifugation, mononuclear cells formed a white ring on the interphase between separation media and PBS. MNCs were collected and washed with staining buffer (2mM EDTA, 0,5% BSA in 1x PBS) 300g \times 4. Mononuclear cells were resuspended in staining buffer and further fluorescently stained in order to enrich the population of c-kit⁺ cells using a magnetic activated cell sorting technique (MACS). Cell pellets were first incubated with primary goat anti-rat c-kit antibody and rabbit anti-rat estrogen receptor alpha (ER α) antibody and blocked against unspecific binding of secondary antibody with donkey serum (1:50 v/v). Incubation was performed for 30 minutes at 4°C in darkness. Cell pellets were further washed with washing buffer and stained with secondary antibodies: anti-goat APC IgG and anti-rabbit Alexa-488 IgG (1:50 v/v) for 30 min at 4°C in darkness. After

washing out secondary antibody, anti-APC microbeads were added and incubated for 20 minutes at 4°C. Cells were again washed from the unbound microbeads and subjected to MACS in order to enrich the c-kit⁺ cell population. For this purpose separation columns were placed on separators which contain strong permanent magnets and induce a magnetic field within the separation columns. Cell suspension with labeled cells was applied on the top of the column. Labeled cells (c-kit⁺ cells) attached to the column while unlabeled cell passed through the column into collection tube. After the column was washed several times with a staining buffer, it was removed from the magnetic field and c-kit⁺ cells were flushed out using a plunger supplied with a column. Purity of c-kit⁺ population after MACS isolation was around 75%, as confirmed with flow cytometry. Enriched c-kit⁺ population was further purified using cell sorting (FACS Aria®, Becton Dickinson, Mountain View, CA, USA) and two fractions were obtained: c-kit⁺ERα⁺ and c-kit⁺ERα⁻. Alternatively, MACS-enriched c-kit⁺ cells were stained as described above and characterized using flow cytometry analysis (FACSCalibur®). The following antibodies were used: mouse anti-rat CD34, mouse anti-rat CD45, indirectly labelled with FITC anti-mouse secondary antibody all in concentration 1:50 and primary labelled mouse anti-CD31-PE in concentration 1:10. For intracellular antigen detection, prior to staining, cells were fixed and permeabilised using a Cytotfix/Cytoperm™ for 20 minutes on ice. Further staining steps were performed in saponin-containing permeabilisation buffer. For flow cytometry analysis of c-kit⁺ cells, at least 1x10⁴ events in the c-kit cell region were acquired for each sample. The data were analyzed using FlowJo software (Tree Star, USA).

2.2.3 Isolation of adult cardiomyocytes

Adult cardiomyocytes (CM) were isolated using a modified protocol developed by Worthington Biochemical Co (Katharinen, Germany). Heart was removed from the animal and kept in cold calcium and magnesium free-Hanks balanced salt solution (CMF-HBSS) until further processing. The heart was perfused with a cold PBS in a petri dish using a syringe and a 22G needle in order to remove remaining blood from the chambers. Cardiac tissue was then minced into 1-2mm² pieces and kept in calcium and magnesium free HBSS containing 0.025% trypsin solution at 37°C for 30 minutes. Trypsin was then inhibited and in the following step tissue was further digested with collagenase (type II) at 37°C for 30 minutes and final concentration 0.5 mg/ml. Following tissue digestion, collagenase was inhibited by adding cold HBSS and cardiac cells were dispersed from tissue fragments by gentle trituration using a 25 ml pipette. Suspension was then passed through a nylon cell strainer containing 100µm pores in order to separate single cell suspension from the remaining undigested tissue. Cardiac cells were then

washed five to six times until clear population of CM was obtained. Following every washing step centrifugation speed was gradually decreased (from 75g to 45g for 5min) and the content of Ca^{2+} containing medium was gradually increased. Due to cell size, low speed centrifugation (down to 45g) of cardiac cell suspension yielded a pellet of CM while other cardiac cells were found in suspension and discarded. Live CM were counted and plated in a 96 well flat-bottom cell culture dish at density a of 15,000 per well in a low serum DMEM medium (1% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) under standard culture conditions (37°C, 5% CO_2 and 20% humidity).

2.2.4 *In vitro* assessment of cardiomyocytes' apoptosis

2.2.4.1 Co-culture of cardiomyocytes and c-kit⁺ cells

Cardiomyocytes (CM) and c-kit⁺ cells were freshly isolated according to the protocols described above. A co-culture system was designed to investigate potential paracrine influence of c-kit⁺ cells on *in vitro* CM apoptosis. CM were cultured alone or with c-kit⁺ cells plated on a semi-permeable membrane in a ratio of 1:2. Pore diameter (0.2 μm) of semi-permeable membrane prevented cell contact but allowed normal medium exchange between CM and c-kit⁺ cells. Moreover, it enabled CM to be selectively collected after culture period for further molecular analysis. Alternatively, subpopulation of c-kit⁺ cells obtained by FACS sorting was co-cultured with CM, c-kit⁺ER α ⁺ cells or c-kit⁺ER α ⁻ cells. This experiment was performed with the aim of investigating the importance of the membrane estrogen receptor (mER α) in paracrine function of c-kit⁺ cells. CM cultured alone served as a control.

2.2.4.2 Staining protocol

CM apoptosis was examined after 7 days of co-culture using a modified ethidium bromide/acridine orange (EB/AO) method [83-85]. The plate with seeded cells was centrifuged at 1000 rpm (129xg) for 5 minutes to bring down all the cells and avoid loss of dying cells which were less adherent to the plate. CM were then carefully washed with PBS to avoid high background staining and then incubated with a dye mix of acridine orange solution (AO) and ethidium bromide (EB) at 37°C for 90 min (200 μL in PBS). AO permeates all cells and makes the nuclei appear green. EB is an intercalating agent commonly used as a fluorescent tag for nucleic acids. Contrary to AO, EB only penetrate cells which lost cytoplasmic membrane integrity. EB stains the nuclei of apoptotic and necrotic cells red and dominates over AO. At the end of incubation, dye solution was discarded and CM were carefully washed 3-4 times with PBS. Pictures were acquired using a Leica DMIRE2 fluorescent microscope. Minimum of 300

CM per well were counted using an ImageJ software and mean was calculated from at least three wells per condition. Results are presented as a mean from three independent experiments. The percentage of apoptotic CM was calculated using the formula $(\text{apoptotic} + \text{necrotic CM}) \times 100 / (\text{total CM})$ and normalized to the apoptotic rate of CM cultured alone.

2.2.4.3 Co-culture stimulation with 17 β -Estradiol

Freshly isolated CM and c-kit⁺ cells were co-cultured as described above. The estradiol stimulation protocol was designed to address the question whether estrogen receptors (ER) influence paracrine function of c-kit⁺ cells. Stimulation with fresh low serum phenol free DMEM F12 medium containing 1nM water soluble 17 β -estradiol (E2) was performed on a single culture of CM and co-culture of CM and c-kit⁺ cells. The culture was stimulated one day after cell isolation and stimulation was repeated after 3 days. Un-stimulated CM in single and co-culture served as a control. Apoptosis was evaluated after 7 days of culture using EB/AO method described above.

2.2.4.4 Cytokine neutralization in a co-culture

To define crucial protective cytokines secreted from c-kit⁺ER α ⁺ cells, the neutralisation approach was applied. CM were cultured alone or in presence of c-kit⁺ER α ⁺ cells as described above. Neutralising antibodies against IL-10 and IL-6 were added to co-culture in a final concentration of 5 $\mu\text{g}/\text{mL}$ on the first and third day of culture. Apoptosis was evaluated on the fifth day of culture as described above.

2.2.5 *In vivo* injection of conditioned medium produced by c-kit⁺ cells

2.2.5.1 Preparation of conditioned medium and intra-myocardial injection

c-kit⁺ER α ⁻ and c-kit⁺ER α ⁺ cells were isolated from bone marrow of male sham Wistar rats according to protocol described in chapter 2.2.2. Conditioned medium was generated by culturing these cells in a fresh DMEM F12 culture medium supplemented with 5% fetal bovine serum. Cells were cultured at approximately 75% density (65.000 cells per well) in a 96-well culture plate for 72 hours. After this period, medium was collected into separates tubes and centrifuged at 10,000g for 5 minutes in order to remove cellular debris. Fresh medium prepared in this manner was immediately used for intra-myocardial injections avoiding freezing-thawing cycles.

Myocardial infarction was induced as previously described (chapter 2.2.1). After ligature was tightened around left descendent coronary artery, a pale area of ischemic myocardium could be

observed. In the area surrounding the ischemic myocardium, fresh conditioned medium prepared from c-kit+ER α - cells or c-kit+ER α + cells was injected in several places in total amount of 100 μ L using a 1mL syringe and 30 gauge needles. Thorax, muscle layers and skin were closed following the usual procedure.

2.2.5.2 Cardiac Function Evaluation with Transthoracic Doppler Echocardiography

Ultrasound imaging is a valuable non-invasive imaging method, most appropriate for visualizing soft tissues. We used ultrasound imaging to visualize the heart and quantify cardiac function (echocardiography). It utilizes the interaction of ultrasound waves (sound waves with frequencies greater than 20,000 Hz) with the living tissue to produce an image of the tissue. Ultrasound waves are differentially transmitted through the various soft tissues, relative to their acoustic impedance and density. At the border of two tissues with different acoustic impedances an acoustic impedance mismatch occurs and some of the sound waves are reflected and returned to the transducer. The signal from reflected ultrasound waves is transformed into electrical currents, processed and an image is created and displayed [86]. Doppler ultrasound is used to non-invasively determine the hemodynamics of the heart and big arteries. Its principle is based on the Doppler shift- change in the frequency of the ultrasound wave that is reflected by a moving target, such as red blood cells. We used Doppler ultrasound to assess the diastolic function of the heart by measuring the mitral inflow.

Trans-thoracic Doppler echocardiography was performed on day 6 after MI to assess left ventricular function. Rats were anesthetized with 2% to 3% isoflurane in oxygen through a face mask. Rats were placed on a heated platform in supine position with all legs taped to ECG electrodes for heart rate monitoring and the chest was shaved and depilated. A high-resolution imaging system Vevo 770 equipped with a 25MHz single-crystal transducer with a focal length of 15mm, a frame rate of 40Hz and the maximum field of view of 2D imaging of 21x21mm was used. All data were transferred to a computer for offline analysis. A minimum of five cardiac cycles were averaged for every animal.

M-mode recordings of LV in the short axis view were obtained from a left parasternal acoustic window at the level of papillary muscles. Dimensions of the left ventricle (LV) in systole and diastole were measured through the largest diameter of the ventricle (Figure 2.1). Systolic parameters, ejection fraction (EF) and fractional shortening (FS), were calculated according to following formulas: $FS(\%) = (LVIDd - LVIDs) / LVIDd \times 100$ and $EF(\%) = (LV Vol_d - LV Vol_s) / LV Vol_d \times 100$ where LVIDd is the left ventricular diameter in diastole, and LVIDs is the left

ventricular diameter in systole, LV Vol_d and LV Vol_s end-diastolic and end systolic left ventricular volumes.

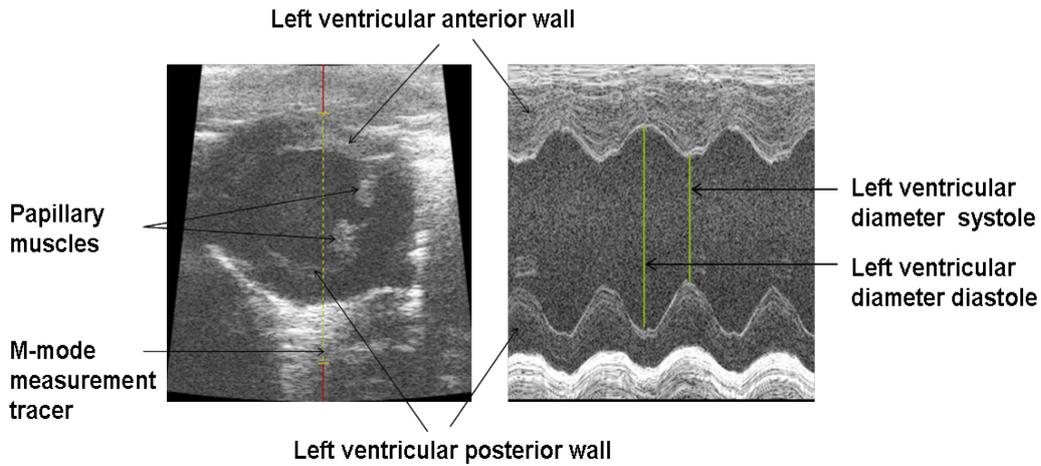


Figure 2.1 Left: Preview window shows LV in two dimensional short axis view and M-mode measurement tracer. Right: M-mode recording of the LV

Diastolic LV function was evaluated using pulsed wave Doppler recording of trans-mitral blood flow velocities in apical 4 chamber view of the LV. Velocities of E and A wave, E/A ratio were averaged from minimum 4 cardiac cycles (Figure 2.2) where E wave represents the velocity of the early diastolic filling and A wave velocity of the LV filling during atrial contraction.

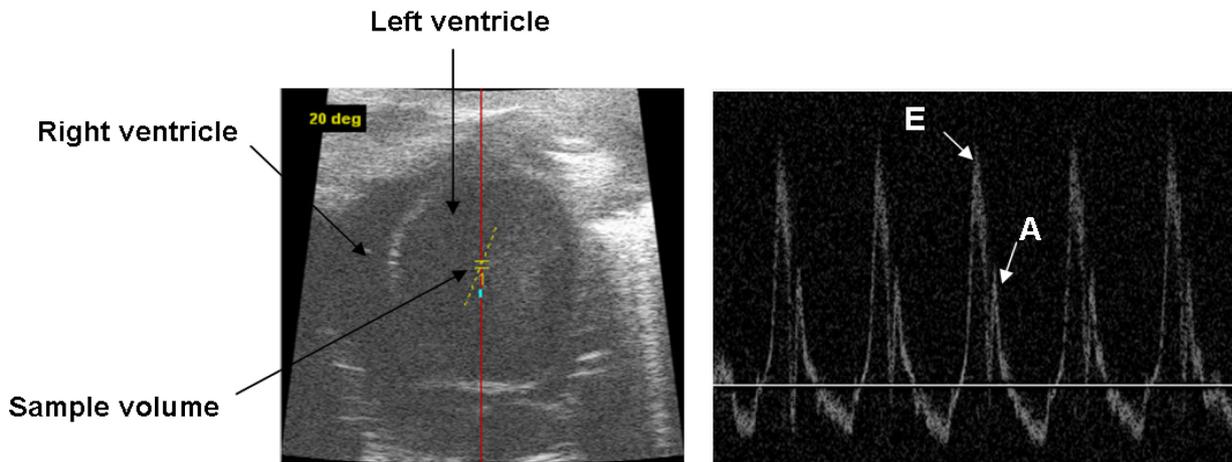


Figure 2.2. Left: apical 4 chamber view of the left ventricle and sample volume; Right: Doppler spectra of blood velocities at the level of mitral valve obtained from 4 chamber view

2.2.6 *In vitro* stimulation of c-kit⁺ cells

Following MACS isolation and FACS purification from rat or human samples, c-kit⁺ cells were cultured in phenol-free DMEM F12 medium supplemented with 10% fetal bovine serum (FBS),

100 U/ml penicillin, and 100 µg/ml streptomycin in a flat-bottom 96-well plate at density of 20,000 per well in standard culture conditions (37°C, 5% CO₂ and 20% humidity). After 24 hours, cells were washed with phosphate buffer saline (PBS) and stimulated in phenol-free DMEM F12 medium containing 5% FBS. To investigate the effect of ER stimulation on cytokine production cells were exposed to 0.1nM estradiol (E2, Sigma-Aldrich) or 1 nM ERα selective agonist propyl pyrazole triol (PPT; Tocris) for 24 hours. Finally, at the end of stimulation cells were washed and harvested for RNA analysis.

2.2.7 RNA isolation and quantitative real-time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a method used to analyze relative or absolute gene expression level. In principle, PCR is a technique which allows the DNA from selected region to be amplified several billion-fold. Gene expression analysis starts with transcription of mRNA into DNA and by use of gene specific primer pairs, expression of gene of interest can be compared between different conditions. Real-time qPCR involves the use of a fluorescent reporter molecule to monitor the progress of the amplification reaction. Fluorescence is measured at each amplification cycle; it increases step-wise and is proportional to the amplicon concentration. The greater the amount of initial DNA template in the sample, the lower is the number of cycles necessary to reach fluorescence threshold (Ct value). In this study, reaction mixture containing SYBR green fluorescent probe was used and relative gene expression was calculated using $-\Delta\Delta C_t$ method [87].

2.2.7.1 RNA isolation and reverse transcription

RNA was isolated from cardiomyocytes using a Trizol® reagent according to the manufacturer’s protocol. 1µg of total RNA was first subjected to DNase digestion to remove possible genomic DNA contamination (Table 1). Purified RNA was further transcribed to cDNA using a reverse transcription kit (Promega) for one hour at 37°C (table 2). RNA and cDNA were kept at -80°C until use. RNAs from small samples of rat and human c-kit+ cells was isolated using PicoPure isolation kit (Arcturus) according to the manufacturer’s protocol. 0.5µg of pure high quality RNA was transcribed into cDNA using SuperScript™ II reverse transcriptase according to the protocol provided by the manufacturer (table 3).

Table 1. DNase digestion

Reagent	Volume (µL)	Thermal Profile
1µg RNA	8	

Methods

DNase	1	
DNase buffer	1	
		30 minutes at 37°C
DNase stop solution	1	10 minutes at 65°C

Table 2. Reverse transcription protocol

Reagent	Volume (µL)	Thermal Profile
1µg RNA	12	
Random primers	1	
		5 minutes at 70°C
5x M-MLV Buffer	5	
M-MLV enzyme	0.75	
dNTPs (25mM)	0.75	
Ultra pure water	4.5	
Total Volume	25	60 minutes at 37°C

Table 3. SuperScripttmII reverse transcription protocol

Reagent	Volume (µL)	Thermal Profile
0.5µg RNA	10	
Random primers	1	
dNTPs (10mM)	1	
		5 minutes at 65°C
Add 5x first strand buffer	4	
0.1M DDT	2	
Rnase OUT	1	
		2 minutes at 25°C
Add Superscript II RT enzyme	1	
Total Volume	20	10 minutes at 25°C
		50 minutes at 42°C
		15 minutes at 70°C

Real-time PCR was carried out for 40 cycles (95°C 15 sec, 60°C 1 minute, table 4). The primers were selected to be intron spanning and sequences for human and rat primers are listed in tables 5 and 6 respectively. Specificity of primers was confirmed on following website:

<http://www.ncbi.nlm.nih.gov/BLAST> so that amplification of unwanted sequences could be excluded. Primer dimer formation and amplification of genomic DNA were excluded using melting curves at the end of the PCR run.

Results were analyzed using the MxPro™ ET QPCR software (Stratagene) and $\Delta\Delta C_t$ method. Expression levels of genes of interest were normalized to the expression of β -actin gene. A minimum of three independent experiments were included in a final statistical analysis.

Table 4. Real-time PCR reaction mixture and thermal profile

Reagent	Volume (μ L)	Thermal Profile
cDNA (50 μ g)	5	2 min at 50°C 5 min at 95°C 15 sec 95°C 60 sec 60°C 30 sec 55°C 30 sec 95°C } 40 cycles
2x SYBR GreenI® Mastermix	12.5	
Forward Primer (20 μ M)	0.125	
Reverse Primer (20 μ M)	0.125	
Ultra-pure Water	7.25	
Final Volume/reaction	25	

Table 5. Human primer sequences for SYBR green® Real-time PCR

Gene (Human)	Primer sequences for SYBR green® Real-time PCR
18s	Forward: 5-CCG CAG CTA GGA ATA ATG GAA TA-3 Reverse: 5- TCT AGC GGC GCA ATA CGA AT -3
IL-6	Forward: 5- TAC CCC CAG GAG AAG ATT CCA -3 Reverse: 5- GAG ATG CCG TCG AGG ATG TAC -3

Table 6. Rat primer sequences for SYBR green® Real-time PCR

Gene (Rat)	Primer sequences for SYBR green® Real-time PCR
Akt	Forward: 5-TCA ACA ACT TCT CAG TGG CAC AA-3 Reverse: 5-GGC AGC GGA TGA TGA AGG T-3
c-Myc	Forward: 5-GGT GGA AAA CCC GAC AGT CA-3 Reverse: 5-ACC GAG TCG TAG TCG AGG TCA T-3

β -Actin	Forward: 5-ATC GCT GAC AGG ATG CAG AGG-3 Reverse: 5-CGC TCA GGA GAA GCA ATG AT-3
Stat-3	Forward: 5-GCG ATA GCT TCC CCA TGG A-3 Reverse: 5-CTC TTT GCT GGC TGC ATA TGC-3
p53	Forward: 5-CCC ACC ATG AGC GTT GCT-3 Reverse: 5-GAT TTC CTT CCA CCC GGA TAA-3
Bcl-2	Forward: 5-GCG CTC AGC CCT GTG-3 Reverse: 5- GGT AGC GAC GAG AGA AGT CAT C-3
Bax	Forward: 5- TGG TTG CCC TCT TCT ACT TTG C-3 Reverse: 5- TGA TCA GCT CGG GCA CTT TA-3
IL-11	Forward: 5'-CCA GCT GAT GGA GAC CAC AAT-3' Reverse : 5'-GAA GCT GCA AAG ATC CCA GTGT-3'
IL-10	Forward: 5'-AAG GCA GTG GAG CAG GTG AA-3' Reverse : 5'-CGT AGG CTT CTA TGC AGT TGA TGA-3'
IL-6	Forward: 5'-ATA TGT TCT CAG GGA GAT CTT GG AA-3' Reverse: 5'-AGT GCA TCA TCG CTG TTC ATA CA-3'
ER α	Forward: 5'-TGCCTCTGGCTACCATTATGG-3' Reverse: 5'-TATGTCCTTGAATGCTTCTCTTAAAGAA-3'
Ki67	Forward: 5- CGC CTC AGA GAT TTT GGG AG -3 Reverse: 5- TGC CGT CTT AAG GTA GAA CTT GC -3

2.2.8 CFSE based proliferation assay

Proliferation of c-kit⁺ cells upon estrogen receptors stimulation was measured by flow-cytometry. The assay is based on use of a division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). CFDA-SE is highly membrane permeable and non-fluorescent due to two acetate groups. As soon as it is taken up by cells, intracellular esterases remove acetate groups and convert the molecule into a highly fluorescent form of carboxyfluorescein succinimidyl ester (CFSE). Within the cell CFSE is covalently bound to the membrane proteins and remains in the cell for a long period. The principle of the assay is the equal distribution of the dye within daughter cells and halving of the initial fluorescence in the cell division.

Before staining, freshly isolated rat c-kit⁺ cells were cultured for 24 hours in a 96-well plate in DMEM F12 supplemented with 10% FBS under standard conditions. Culture medium was removed and cells were incubated with pre-warmed staining solution containing 1x PBS, 10% FBS and CFDA-SE in a final concentration of 10 μ M for 15 minutes at 37 $^{\circ}$ C. Then, staining solution was replaced with fresh pre-warmed medium and incubated for 30 minutes at 37 $^{\circ}$ C for complete acetate hydrolysis. Finally, media was removed and cell culture was stimulated with fresh phenol free DMEM F12 medium containing 0.1nM estradiol or 1nM PPT and 5% FBS. After 96 hours of culture, cells were detached from the plate using trypsin and collected for flow cytometry. Acquisition of cell fluorescence was performed on FACSCalibur $^{\circ}$ device (Becton Dickinson) and data were processed and analysed using a FlowJo software (Tree Star). A minimum of 1x 10⁴ events in the region of c-kit⁺ cells were acquired. Quantification of proliferation was performed according to the method described by Hawkins [88].

2.2.9 c-kit⁺ cells in human peripheral blood: isolation and flow cytometry analysis

Samples of peripheral venous blood were obtained from male and female patients diagnosed with systolic or diastolic heart failure, or from controls matched by age and gender. Blood samples were collected in K-EDTA tubes and processed within 8 hours. Peripheral blood mononuclear cells were isolated by density gradient centrifugation. Blood was diluted in an equal volume of 1x phosphate buffer saline (PBS) containing 2mM EDTA (pH 7.2) and gently layered on 12 mL of lymphocyte separation medium (ρ =1.077 g/mL at 20 $^{\circ}$ C). The sample was centrifuged for 35 min at 400xg and 24 $^{\circ}$ C in a swinging rotor without brake. Mononuclear cells were collected from the interphase and washed two times with a cold staining buffer (2mM EDTA, 0.5% BSA in 1x PBS). C-kit⁺ cells were isolated by magnetic activated cell sorting technique as described in a previous section. Distribution of estrogen receptor alpha and IL-6 was analysed using a flow cytometry and a staining protocols described above. For IL-6 visualization, cells were initially fixed and permeabilised and then incubated with monoclonal PE labeled anti human IL-6 antibody in a saponin-containing permeabilisation buffer on ice (40 min, concentration 1:10).

2.2.10 Statistics

Results are expressed as mean \pm SEM (the standard error of the mean). Two-group comparisons were analysed by two-tailed Student t-test. Three or more groups were compared and analysed by one-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at value of $p < 0.05$.

3 Results

3.1 Isolation and characterization of bone marrow (BM) c-kit⁺ cells

Progenitor c-kit⁺ cells were isolated from the rat bone marrow (BM) 7 days after sham/myocardial infarction (MI) operation. To enrich the c-kit⁺ population, bone marrow mononuclear cells (MNCs) were magnetically labeled against the c-kit⁺ antigen and positively selected from the mixed cell population using magnetic activated cell sorting (MACS). C-kit⁺ cells were further characterized by their labeling against CD34, CD45 and CD31 antigens, which are commonly present in hematopoietic and endothelial progenitor cells. Using flow cytometry (FACS), frequency of c-kit⁺ cells presenting these antigens was analyzed after MI and compared to sham. MI was found to increase c-kit⁺ cell population in the rat bone marrow (Figure 3.1.A). MACS isolation yielded $1.70 \pm 0.33 \times 10^6$ c-kit⁺ cells from sham rats while $3.12 \pm 0.57 \times 10^6$ c-kit⁺ cells were isolated from MI rats (t-test, $p < 0.05$). This difference was also observed when the number of isolated c-kit⁺ cells was expressed as a percentage of obtained BM MNCs. C-kit⁺ cells represented $1.36 \pm 0.19\%$ of BM MNCs in sham animals, whereas after MI c-kit⁺ cells represented $2.98 \pm 0.62\%$ of BM MNCs (t-test, $p < 0.05$). MI operation did not influence the number of isolated BM MNCs in comparison to sham operation (t-test, $p > 0.05$).

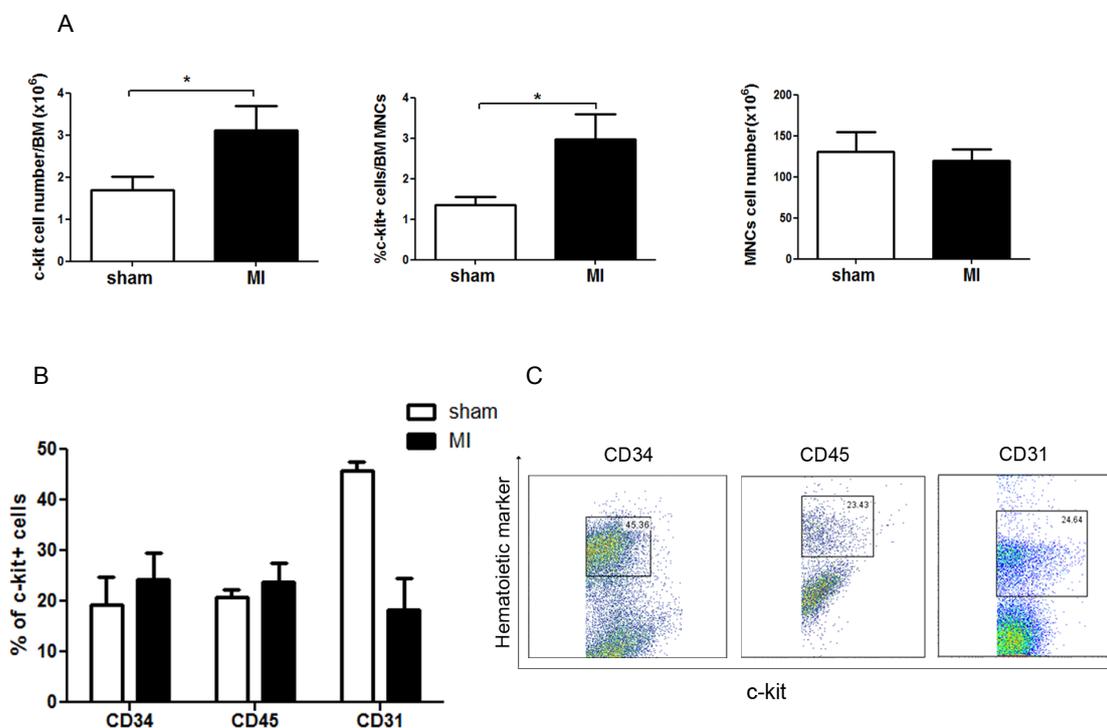


Figure 3.1 Isolation and flow cytometry analysis (FACS) of rat bone marrow (BM) c-kit⁺ cells. (A, from left to right): Absolute number of c-kit⁺ cells obtained after MACS isolation, number of c-kit⁺ cells expressed as percentage of BM MNCs and number of MNCs in sham (n=9) or MI (n=14) rats. (B): FACS analysis of CD34 (n=4), CD45 (n=4) and CD31 (n=4) expression in sham and MI c-kit⁺ cells, presented as a percentage of all c-kit⁺ cells (C): Representative FACS plots of post-infarct BM c-kit⁺ cells expressing CD34, CD45 and CD31. * $p < 0.05$ vs. sham

FACS analysis showed that c-kit⁺ cells expressed markers of hematopoietic cells, CD34 and CD45. These antigens were detected in similar frequency after sham and MI operation. According to the expression of CD31 antigen, endothelial progenitors accounted for around 45% of c-kit⁺ cells in sham. MI induced a 2.48-fold fall in percentage of CD31⁺ cells in c-kit⁺ cells population (p=0.05) (Figure 3.1.B and C).

3.2 Bone marrow c-kit⁺ cells mediate paracrine cardioprotection

Further, we have investigated whether bone marrow derived c-kit⁺ cells possess the capacity to reduce apoptosis of co-cultured cardiomyocytes (CM) in a paracrine manner. For this purpose, we designed an *in vitro* co-culture system of c-kit⁺ cells and adult ventricular CM. The rate of apoptotic CM co-cultured with c-kit⁺ cells was reduced by $16.51 \pm 3.08\%$ (p<0.05, one-way ANOVA) compared to CM cultured on their own. Notably, the reduced apoptotic rate of CM remained unaltered when c-kit⁺ cells were separated from co-cultured CM by semi-permeable membrane ($15.57 \pm 4.55\%$, p>0.05), suggesting that paracrine soluble factors released by co-cultured c-kit⁺ cells may contribute to their cardioprotective mechanisms (Figure 3.2.A). A quantitative PCR analysis of genes regulating apoptosis was performed in CM to investigate mechanisms of c-kit⁺ cells-supported survival of CM in co-culture. An anti-apoptotic gene *Bcl-2* was found up-regulated 1.85-fold (p<0.05) in CM co-cultured with c-kit⁺ cells, whereas pro-apoptotic genes *Bax* and *p53* were down-regulated 1.38-fold (p<0.01) and 1.32-fold (p<0.05), respectively, compared to CM cultured alone. The *Bcl-2/Bax* ratio, commonly used as an indicator of a pro-survival effect, was increased 2.57 fold in CM co-cultured with c-kit⁺ cells compared to CM cultured alone (Figure 3.2B and C). The mRNA level of the STAT3 gene was not regulated in our experimental setting.

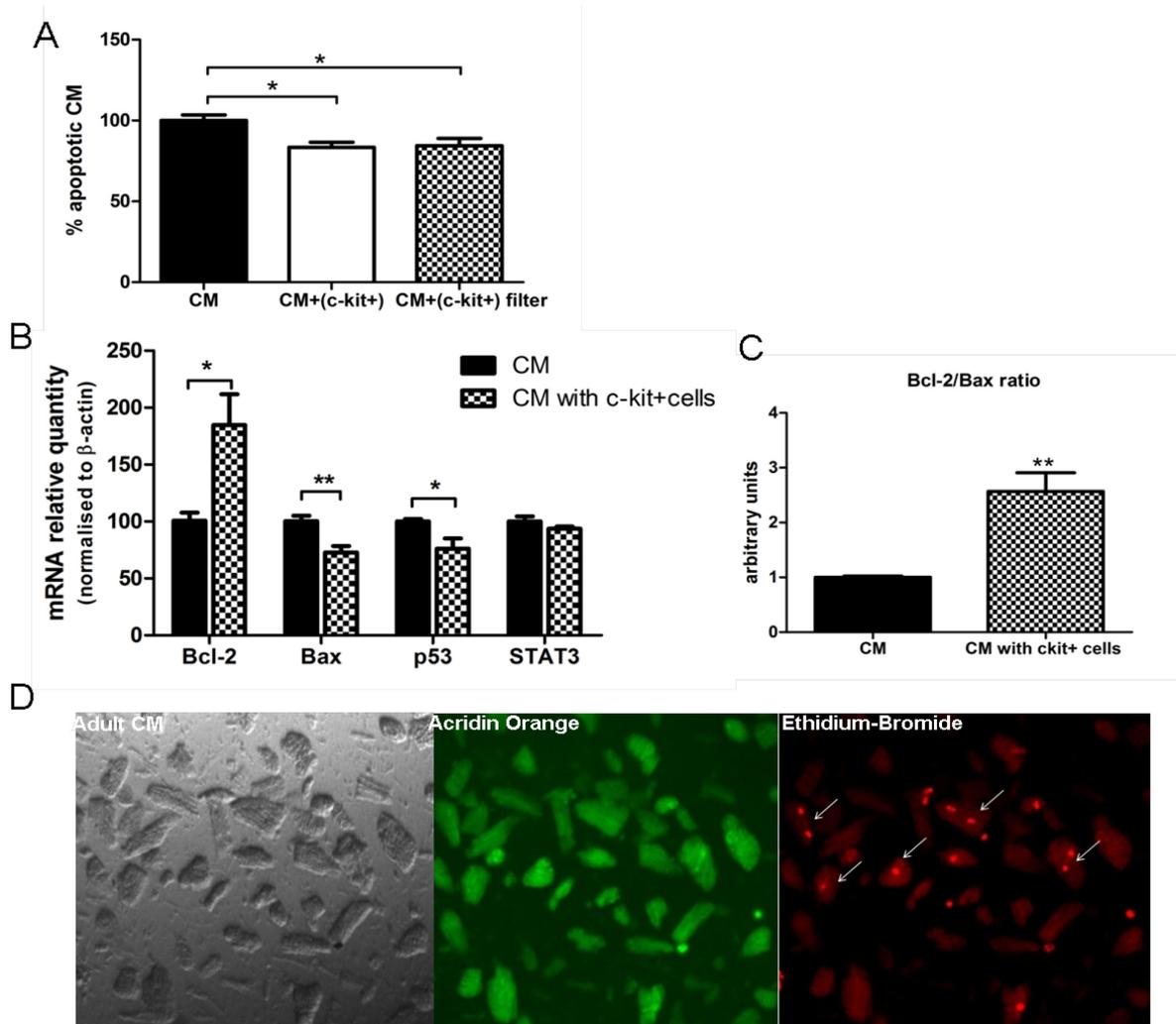


Figure 3.2 *In vitro* effect of bone marrow c-kit⁺ cells on apoptosis of adult cardiomyocytes (CM). (A): C-kit⁺ cells reduce the rate of apoptotic CM independently of cell contact. The number of apoptotic CM is expressed as a percentage of all counted CM. The apoptotic rate of CM co-cultured with c-kit⁺ cells was normalized to the apoptotic rate of CM cultured alone (n=4 for each group). (B): Expression pattern of genes Bcl-2 (n=4), Bax (n=4), p53 (n=6) and stat3 (n=3) in adult CM. (C): Bcl-2/Bax ratio is increased in CM co-cultured with c-kit⁺ cells and indicates the anti-apoptotic effect of c-kit⁺ cells. (D): CM *in vitro*: phase contrast microscopy (left); acridin orange stains all cells (green, middle); arrows shown for ethidium bromide staining indicate accumulation of ethidium-bromide in apoptotic nuclei (red, right). *p<0.05, **p<0.01

3.3 Estrogen receptor α (ER α): distribution and functional importance in bone marrow c-kit⁺ cells

Increased expression of ER α , but not ER β , has previously been observed in peri-infarcted myocardium and has mostly been detected in small proliferating c-kit⁺ cells [78]. Since c-kit⁺ cells found in the post-infarct myocardium may originate in bone marrow, here we used bone marrow cells to examine and quantify the distribution of ER α in c-kit⁺ cells by FACS analysis. When staining with primary and secondary antibodies was performed in cell-permeabilising condition, ER α was detected as a nuclear receptor in 82.79 \pm 6.27 percent of c-kit⁺ cells. MI did

not significantly influence this frequency. Notably, a surface staining of live, non-permeabilised cells showed that ER α also existed as a membrane receptor, mER α (Figure 3.3. A and B). Among c-kit⁺ cells, the percentage of those expressing mER α decreased from 40.74 ± 8.80 % in sham to 20.94 ± 1.78 in MI ($p=0.058$). Further, we investigated whether membrane ER α is relevant for paracrine function of c-kit⁺ cells. Combining MACS isolation method and subsequent FACS sorting, we isolated two distinct subpopulations of c-kit⁺ cells: c-kit⁺ER α ⁻ and c-kit⁺ER α ⁺ cell population, according to their membrane expression of ER α . In co-culture, both c-kit⁺ER α ⁻ and c-kit⁺ER α ⁺ cells exerted a protective anti-apoptotic effect on CM. However, c-kit⁺ER α ⁺ cell population was more potent in mediating cardiomyocyte protection than c-kit⁺ER α ⁻ (Figure 3.3C).

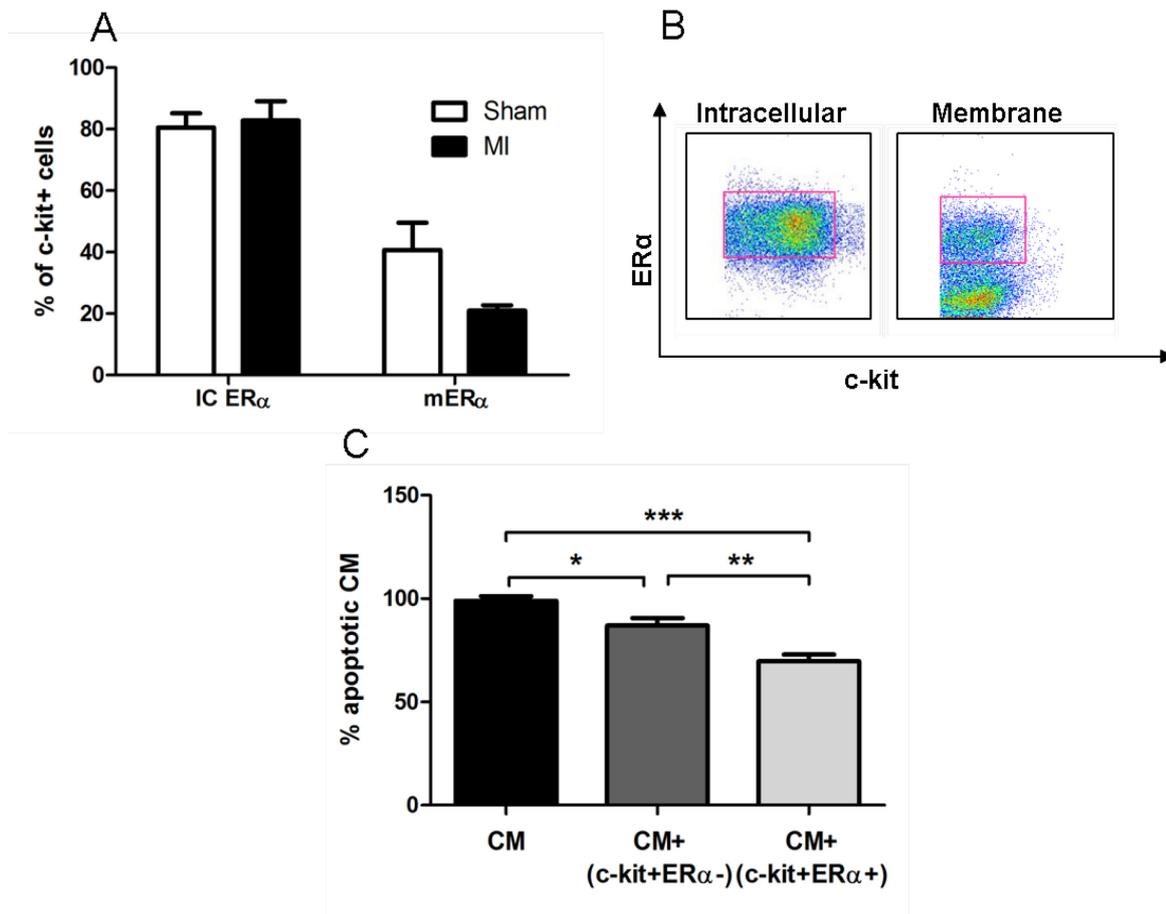


Figure 3.3 Distribution and functional characterization of ER α in c-kit⁺ cells (A): FACS quantification of ER α distribution in c-kit⁺ cells expressed as a percentage of all c-kit⁺ cells. Left: intracellular (IC) ER α in sham (n=4) and post-infarct (MI) c-kit⁺ cells (n=3); right: membrane (m) ER α distribution in sham (n=5) and MI c-kit⁺ cells (n=5). (B): Representative FACS dot plots of intracellular and membrane expression of ER α in c-kit⁺ cells. (C): apoptotic rate of CM co-cultured with c-kit⁺ER α ⁻ or with c-kit⁺ER α ⁺ normalized to the apoptotic rate of CM cultured alone (n=6 for each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.4 Conditioned medium from c-kit+mER α + cells improves cardiac function *in vivo*

Taken together, the data described above data indicate that c-kit+ cells protect CM independently of cell contact in a paracrine manner and that c-kit+ER α + cells possess greater potential to secrete cardio-protective factors. We further aimed to extend relevance of these findings to *in vivo* settings by assessing the cardiac function after injection of c-kit+cell-derived conditioned medium into peri-ischemic myocardial regions. We examined the functional effects

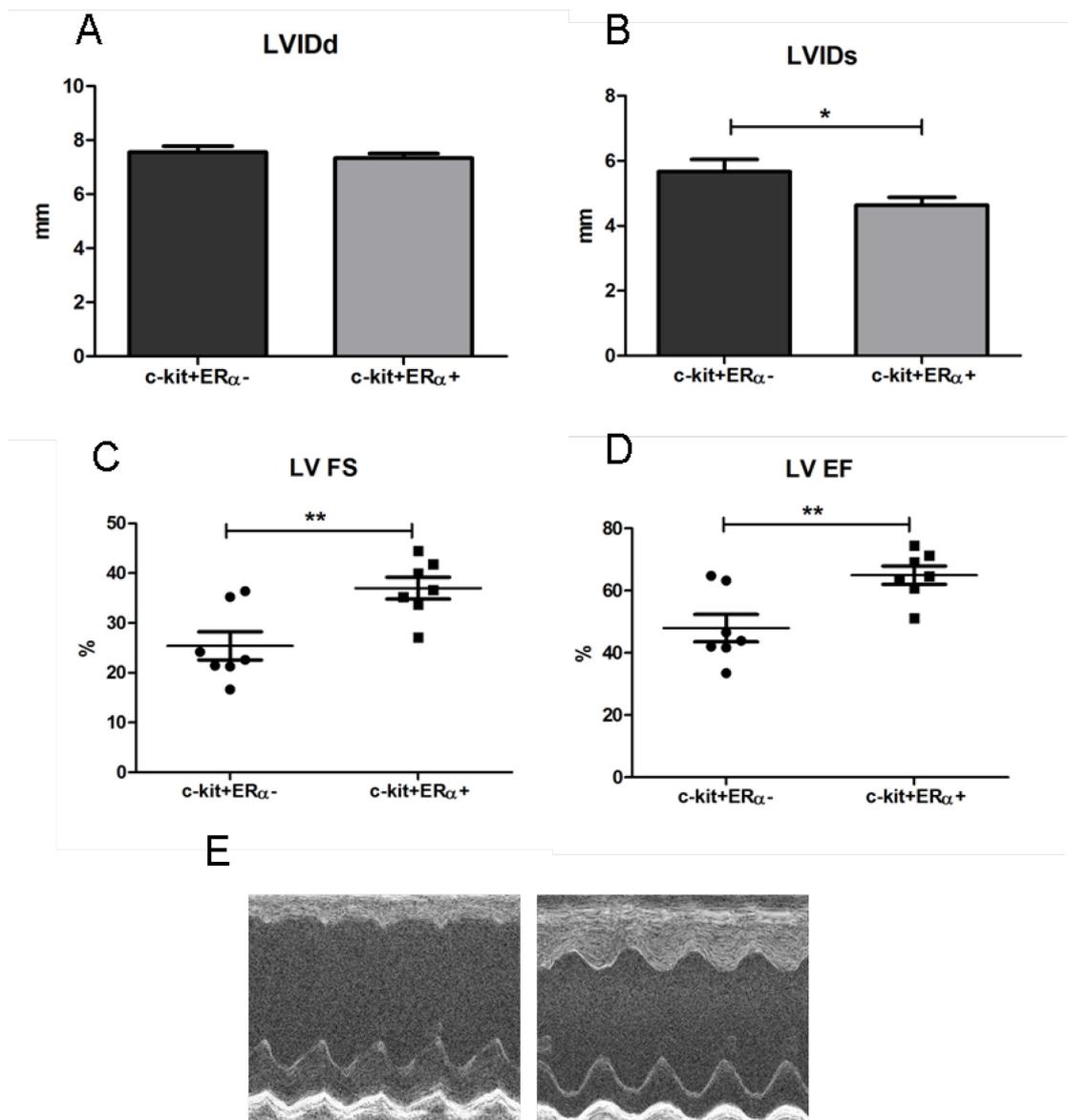


Figure 3.4 Left ventricular diameters and function measured by echocardiography. (A): Left ventricular diastolic diameter. (B): Left ventricular systolic diameter. (C): Left ventricular fractional shortening. (D): Left ventricular ejection fraction. (E) Representative m-mode echocardiograms of LV injected with conditioned media from c-kit+ER α - (left) and c-kit+ER α + (right) cells. n=7 per group. *p<0.05; **p<0.01;

of injected conditioned medium produced by c-kit+ER α - cells and c-kit+ER α + cells by means of echocardiography.

As presented in Figures 3.4. and 3.5, injection of c-kit+ER α + cell-derived conditioned medium significantly improved left ventricular (LV) systolic and diastolic function, as compared to the effect of injected c-kit+ER α - cell-derived conditioned medium. C-kit+ER α + cell-derived conditioned media reduced LV systolic diameter from 5.67 \pm 0.37mm to 4.64 \pm 0.24mm (p <0.05). Ejection fraction (EF) and fractional shortening (FS) were enhanced by 17% and 11.6% (p <0.01) respectively, by c-kit+ER α + cell-derived conditioned media compared to media derived from c-kit+ER α - cells (Figure 3.4).

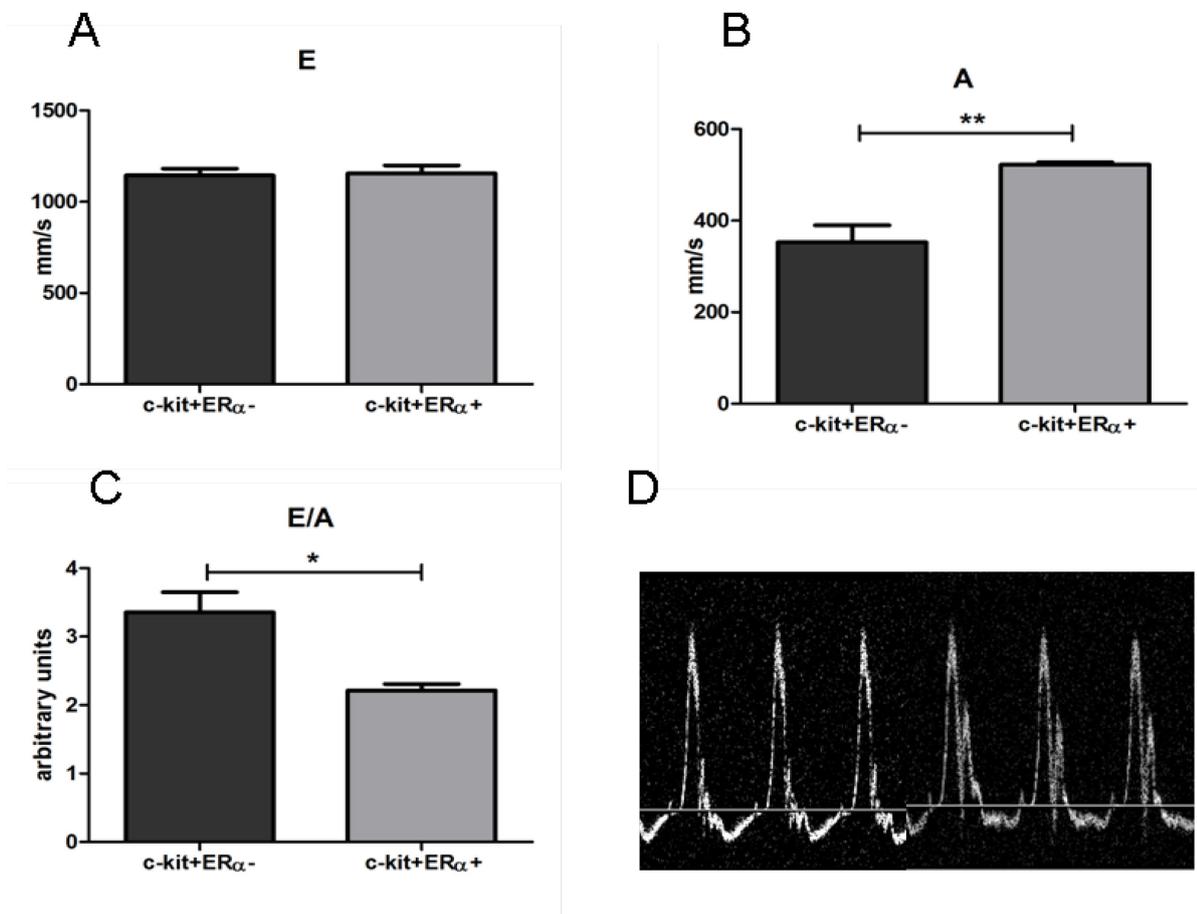


Figure 3.5 Left ventricular diastolic function assessed by pulse wave Doppler echocardiography. (A): Velocity of the early ventricular filling waves (E). (B): Velocity of the atrial contraction filling waves (A). (C): estimation of left ventricular relaxation using ratio of velocities of early ventricular filling wave (E) and atrial contraction filling wave (A). (D): representative Doppler recordings of LV filling of animals treated with conditioned media prepared from c-kit+ER α - (left, n=5) and c-kit+ER α + (right, n=4) cells. * p <0.05; ** p <0.01

Additionally, post-ischemic treatment with c-kit+ER α + cell-derived conditioned media raised velocity of the atrial contraction filling wave (A wave) from 352.9 \pm 36.89 mm/s to 522.1 \pm 4.94

mm/s ($p < 0.01$) and subsequently reduced E/A ratio 1.52-fold ($p < 0.05$) compared to c-kit+ER α - cell-derived conditioned media. Velocity of the early filling wave (E) remained unchanged (Figure 3.5). These data provided the functional evidence that post-infarct BM c-kit+ER α + progenitor cell population also mediates paracrine protection against heart injury *in vivo*.

3.5 Molecular characterization of c-kit+ER α + cells

Prompted by distinct functional significance of membrane ER α observed in *in vitro* and *in vivo* assays described above, we next dissected expression of potential cardioprotective cytokines in two subsets of c-kit+ cells, ER α - and ER α +. We investigated expression of two members of IL-6 cytokine family (*IL-6* and *IL-11*) and *IL-10* (Figure 3.6A), as these were previously shown to exert an anti-apoptotic effect on cardiomyocytes. Real-time PCR analysis showed that mRNA level of *IL-6* was highly up-regulated (20-fold) in c-kit+ ER α + cells compared to c-kit+ER α - (t-test, $p < 0.001$). *IL-10* mRNA was also significantly increased by 2.7-fold in c-kit+ER α + cells compared to c-kit+ER α - (t-test, $p < 0.05$). In contrast, *IL-11* mRNA was 1.6-fold lower in c-kit+ER α + cells than in c-kit+ER α - cells (t-test, $p < 0.01$).

We then investigated genes related to cell cycle regulation and proliferation, *Ki67* and *Myc*. Expression of both genes was down-regulated in c-kit+ER α + cells by 2.7-fold (t-test, $p < 0.001$) compared to c-kit+ER α - cells (Figure 3.6.B).

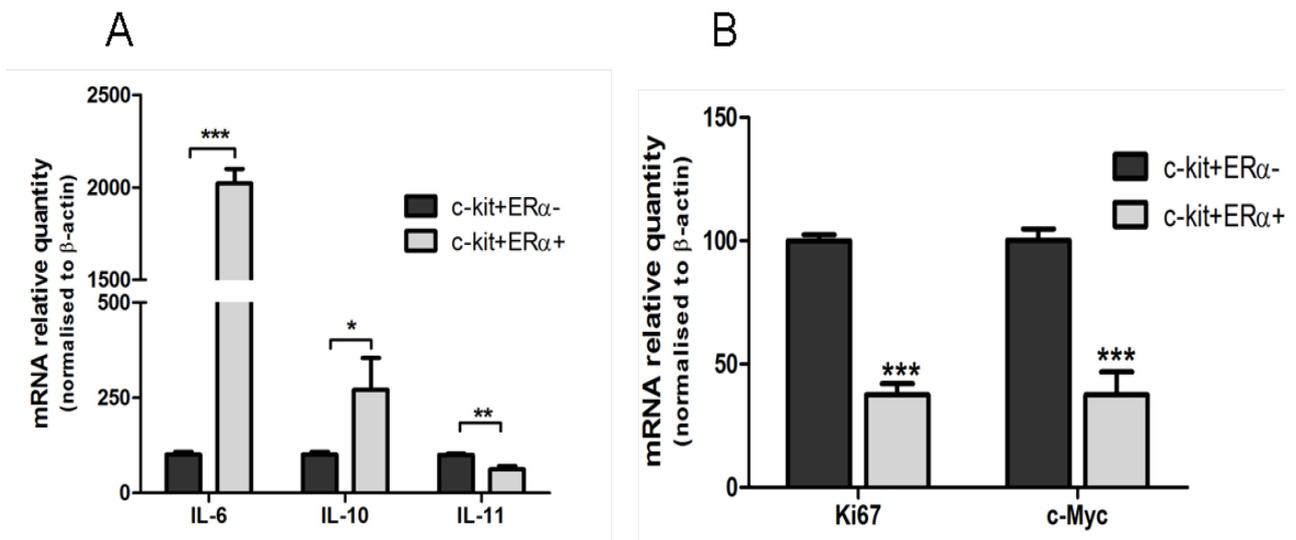


Figure 3.6 Gene expression pattern in post-infarct c-kit+ER α + cells. (A): Cytokine production *IL-6* (n=4), *IL-10* (n=5), *IL-11* (n=7) and (B): proliferation potential *Ki67* (n=5) and *c-myc* (n=4) compared between c-kit+ER α - and c-kit+ER α + cells by real-time PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Therefore, these data indicate potential importance of membrane ER α in paracrine production of IL-6 and IL-10 cytokines, rather than in stimulation of proliferation in c-kit+ cells. Increased

production of IL-6 and IL-10, but not IL-11 could account for the observed differential effect mediated by two subsets of c-kit⁺ cells.

3.6 C-kit⁺ER α ⁺ cells support CM by up-regulation of IL-6

We further investigated which cytokines secreted by c-kit⁺ER α ⁺ cells are indispensable for paracrine cytoprotective effect. Because IL-6 and IL-10 were up-regulated in the c-kit⁺ER α ⁺ subset we tested their individual contribution by adding neutralizing antibodies into co-culture of c-kit⁺ER α ⁺ cells and CM. Addition of IL-6 neutralizing antibody completely abolished paracrine protection of c-kit⁺ER α ⁺ on CM, and the rate of apoptotic CM increased to the apoptotic rate of CM cultured alone. However, addition of IL-10 antibody had no influence on the CM apoptosis rate (Figure 3.7).

These results indicate an important role of IL-6 but not IL-10 in paracrine cytoprotection mediated by c-kit⁺ER α ⁺ cells.

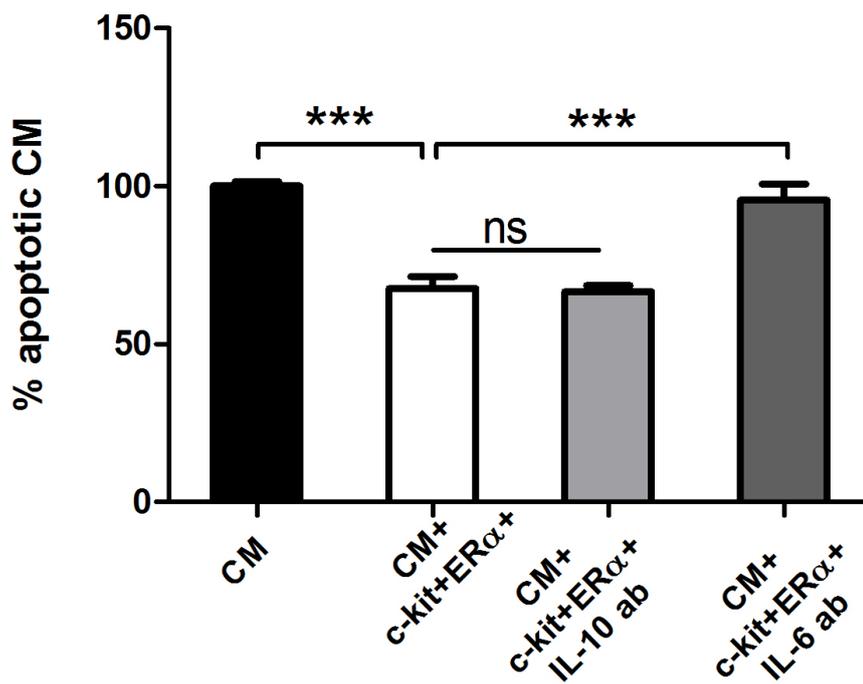


Figure 3.7 Effect of IL-10 (n=3) and IL-6 (n=7) neutralisation on CM apoptosis in a co-culture with c-kit⁺ER α ⁺ (n=6). Rate of apoptotic CM was calculated and normalised to the rate of CM cultured alone (n=5); ***p<0.001.

3.7 ER α improves impaired cytokine production in c-kit⁺ cells after ischemia

To understand whether myocardial ischemia, as a local event, has wider systemic consequences on a bone marrow progenitor population, we compared secretion and proliferation potential

Results

between c-kit⁺ cells isolated after sham and MI operation. We observed that ischemia decreased mRNA levels of cytokines IL-6 and IL-10 (4.76 and 2.42-fold respectively, $p < 0.001$) in c-kit⁺ cells in comparison to sham (Figure 3.8A). In addition, the post-ischemic c-kit⁺ cells showed significantly lower proliferative capacity compared to sham. Proliferation was measured by flow cytometry using CFSE-based assay and the result was confirmed by PCR analysis of gene *Ki67*, expressed by cells in mitosis (Figure 3.8B and C). Changes in cytokine production and proliferation potential caused by MI were accompanied by regulation of ER α mRNA levels. MI c-kit⁺ cells expressed 1.52-fold lower levels of ER α mRNA in comparison to sham c-kit⁺ cells ($p < 0.05$, Figure 3.8A).

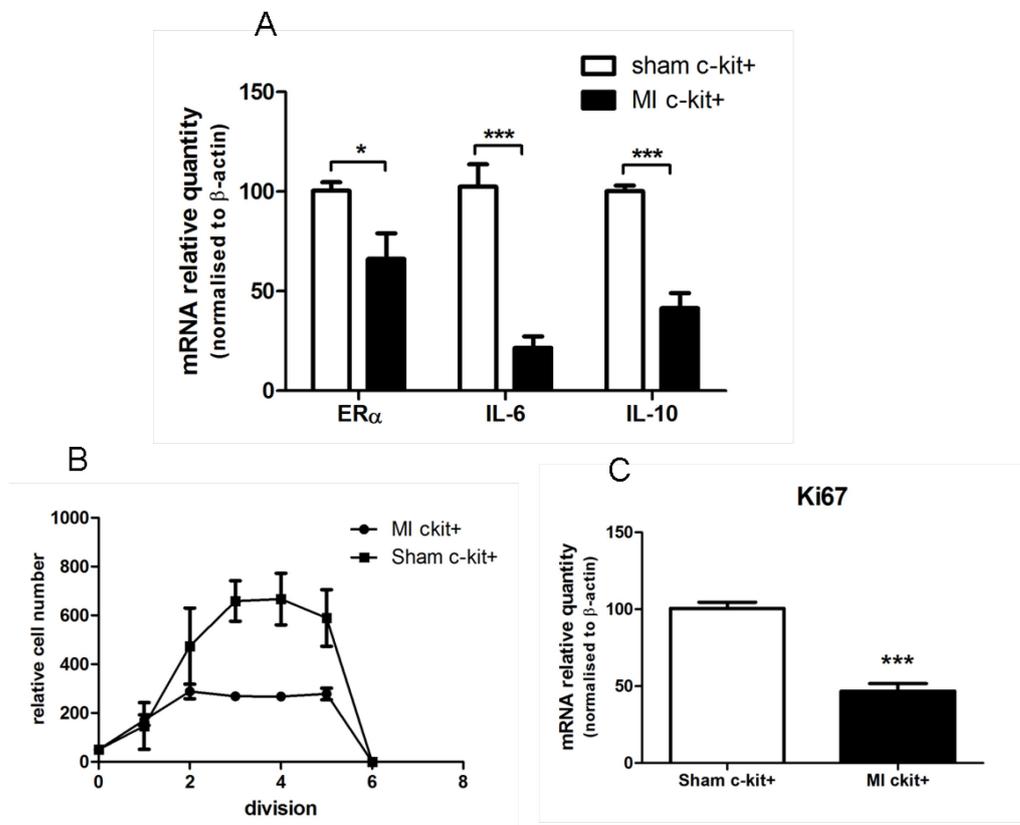


Figure 3.8 Influence of MI on BM c-kit⁺ cells (A): Expression of *ER α* (n=6), *IL-6* (n=5) and *IL-10* (n=6) genes compared with real-time PCR between sham c-kit⁺ cells and post-infarct (MI) c-kit⁺ cells. (B): Proliferation of c-kit⁺ cells upon 96h of culture measured by CFSE staining and flow cytometry. (n=2). (C): Expression of *Ki67* mRNA in sham and MI c-kit⁺ cells measured by real-time PCR (n=6). * $p < 0.05$, *** $p < 0.001$

Results

Previous findings indicated an important link between ER α expression (on mRNA and protein level) and capacity of c-kit⁺ cells to produce cytokines IL-6 and IL-10 (Figures 3.6 and 3.8). To assess whether production of cytokines in c-kit⁺ cells is under ER α control, c-kit⁺ cells were stimulated *in vitro* with 0.1 nM 17- β estradiol (E2) or with 1nM selective ER α agonist propyl pyrazole triol (PPT) for 24h. E2 stimulation increased the expression of *IL-6* and *IL-10* genes 2.6- and 3.5-fold, respectively (p<0.05). Similarly, treatment with PPT increased the expression of *IL-6* (2.3-fold, p<0.05) and *IL-10* genes (3.7-fold, p<0.01) (Figure 3.9.A). Furthermore, we investigated in a functional assay whether E2 stimulation contributes to the protection mediated by c-kit⁺ cells. CM cultured alone or co-cultured with c-kit⁺ cells were exposed to 1nM E2. E2 stimulation further decreased the apoptosis rate of CM in co-culture compared to those in unstimulated condition or single-culture CM (Figure 3.9B), possibly due to increased cytokine production. In addition, ER α stimulation in c-kit⁺ cells restored impaired proliferation capacity caused by ischemia (Figure 3.9C).

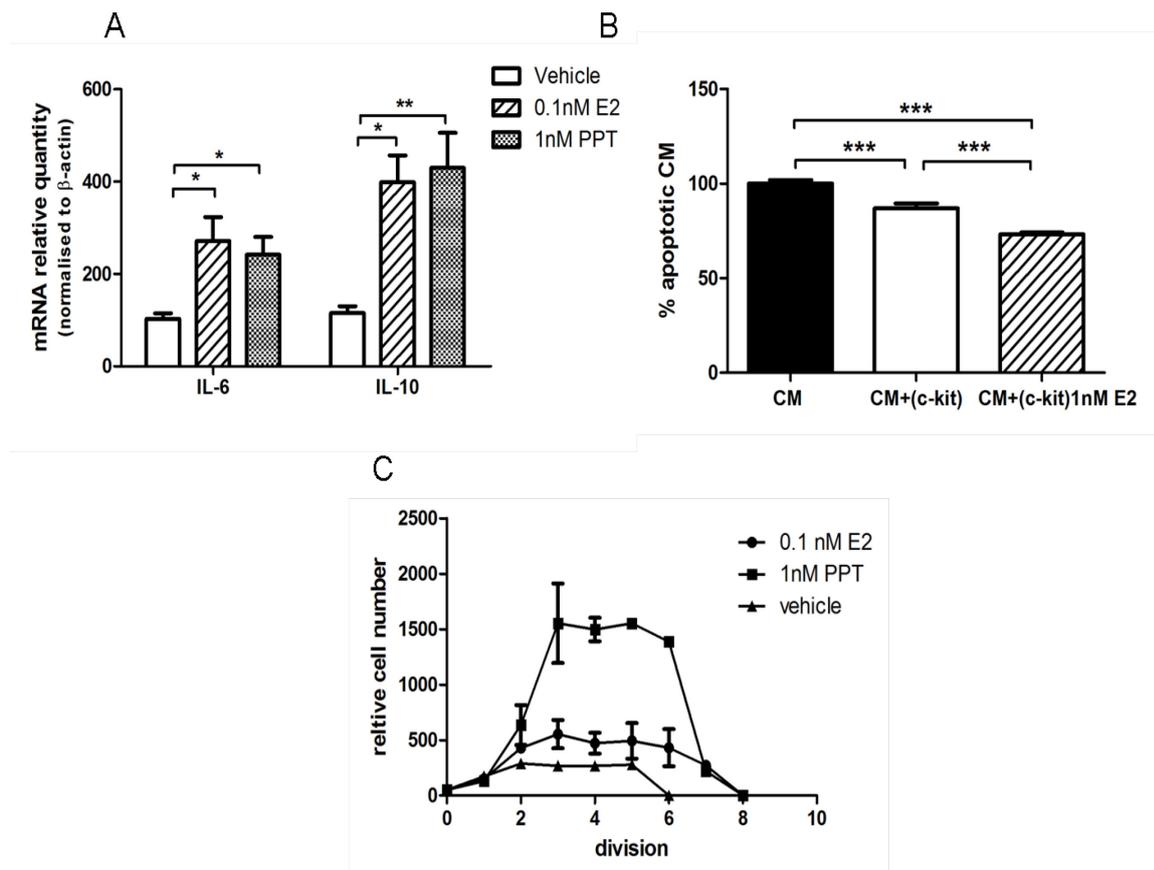


Figure 3.9 Estrogen influences cytokine production and proliferation in c-kit⁺ cells. (A): Stimulatory effect of estradiol (E2) or direct ER α agonist propyl pyrazole triol (PPT) on cytokine production of post-infarct c-kit⁺ cells (n=5) measured by real-time PCR. (B): Estradiol stimulation improves paracrine function of c-kit⁺ cells. Apoptotic rate of CM co-cultured with c-kit⁺ cells (n=9) or with estradiol stimulated c-kit⁺ cells (n=8) was calculated and normalized to the apoptotic rate of CM cultured in a dish alone (n=10). (C): Proliferation of c-kit⁺ cells upon 96h of stimulation measured by CFSE staining and flow cytometry. (n=2)

Altogether, these findings suggest an important role of estrogen signaling for the biology of c-kit⁺ cells, influencing processes such as proliferation and cytokine secretion.

3.8 c-kit⁺ERα⁺ cells in human peripheral blood.

To translate our findings to humans, we next isolated and analyzed c-kit⁺ cells from peripheral blood of healthy controls and heart failure patients (HF). In accordance with the experimental data, human c-kit⁺ cells express ERα as a membrane and nuclear receptor, as shown in Figure 3.10A. The frequency of c-kit⁺ERα⁺ cells in peripheral blood mononuclear cells was much lower in female HF patients than in male HF patients. Moreover, female HF patients had a lower frequency of c-kit⁺ERα⁺ cells than female controls, whereas in males, frequency of c-kit⁺ERα⁺

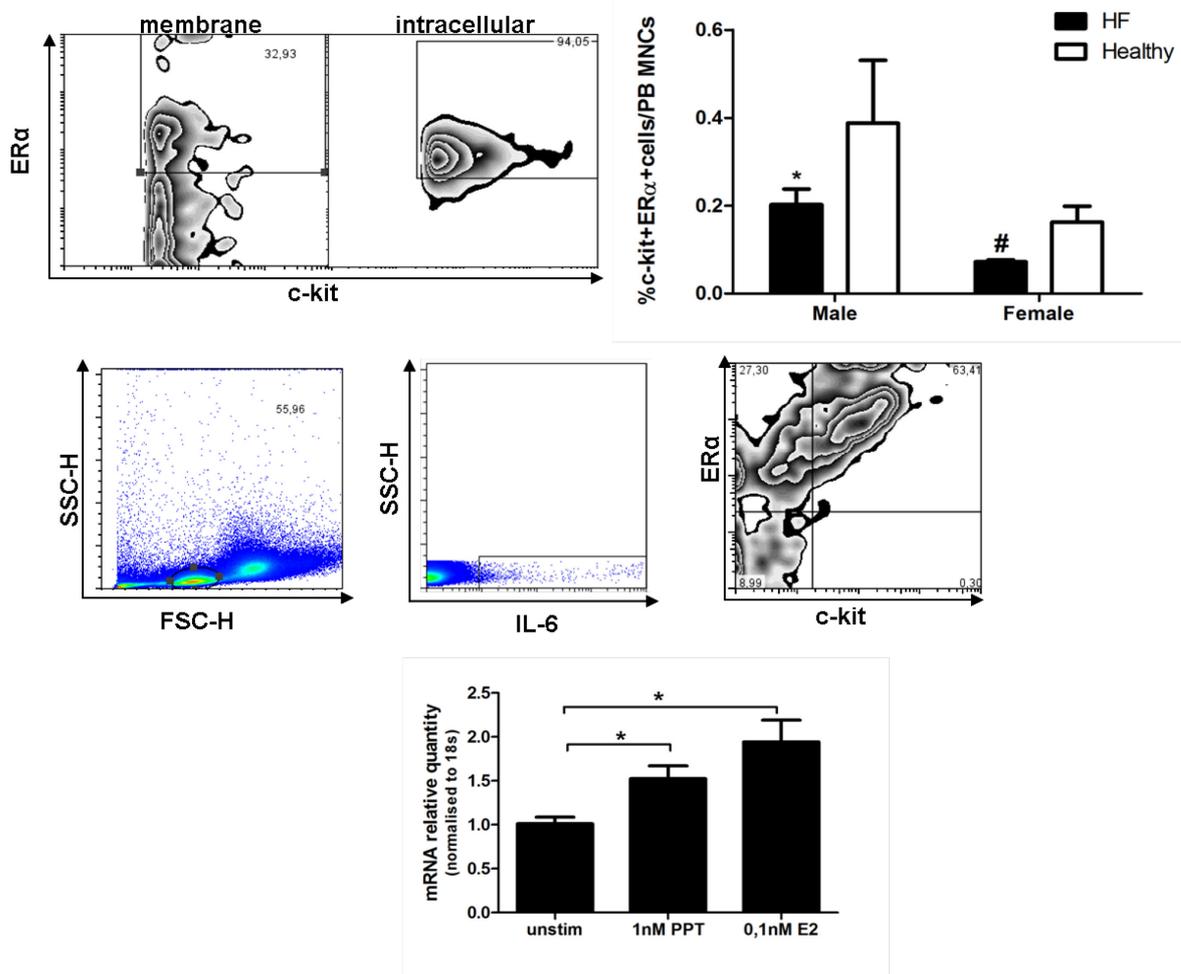


Figure 3.10 Isolation and analysis of human peripheral blood c-kit⁺ cells from heart failure patients (HF) and controls. (A): Representative FACS plots of ERα distribution in human peripheral blood c-kit⁺ cells. (B): Distribution of c-kit⁺ERα⁺ cells in peripheral blood mononuclear cells (MNCs) of heart failure patients and healthy controls. *p<0.05 male HF (n=7) vs. female HF (n=4); #p<0.05 female HF (n=4) vs. female controls (n=2); male controls (n=3); (C): Representative FACS plot of c-kit and mERα expression in IL-6 producing peripheral blood mononuclear cells in a male HF patient. All IL-6 producing c-kit⁺ cells co-express mERα. (D): Estradiol (E2) and direct ERα agonist propyl pyrazole triol (PPT) stimulate IL-6 production in c-kit⁺ cells isolated from male HF patients (n=3) as measured by real-time PCR.

cells was decreased by tendency in HF patients compared to controls. These findings indicate a potential mobilization of c-kit+ER α + from blood to heart in response to ischemic injury in patients with heart failure, especially in females (Figure 3.10B). Notably, flow cytometry analysis revealed that all IL-6 producing c-kit+ cells were c-kit+ER α + cells (Figure 3.10C). This supports our experimental finding, that mER α could be a marker of highly cytokine producing subpopulation of c-kit+ cells. We next investigated the hypothesis that IL-6 production in human c-kit+ cells is indeed under ER α regulation. For this purpose, c-kit+ cells isolated from male HF patients were stimulated *in vitro*. Both estradiol and ER α agonist PPT increased IL-6 gene expression in these c-kit+ cells, providing clinical evidence that ER α modulates paracrine secretion of IL-6 also in human c-kit+ cells.

4 Discussion

Paracrine signaling is an important mechanism by which various progenitor cells exert their protective effects. It has been postulated that it is in a paracrine manner that bone marrow progenitor cells modulate left ventricular remodeling and improve neovascularisation and LV function after myocardial infarction [21,33,89]. However, the role of estrogen receptors (ERs) in the modulation of paracrine function in progenitor cells has not been thoroughly investigated so far. The novelty of this study is, therefore, that it for the first time identifies presence of the membrane ER α (mER α) in progenitor c-kit⁺ cells and provides evidence for its functional importance. The study presented here demonstrates that the expression of mER α is accompanied by a specific pattern of cytokine production by BM c-kit⁺ cells. As a result of increased cytokine production, c-kit⁺ER α ⁺ cells provided better paracrine protection compared to c-kit⁺ER α ⁻ cells and supported survival of co-cultured cardiomyocytes (CM) *in vitro* and improved cardiac function *in vivo*. In addition, IL-6 was identified as a key mediator of the paracrine effect provided by c-kit⁺ER α ⁺ cells. C-kit⁺ cells expressing membrane and intracellular ER α were also found in human blood, and their percentage was decreased in blood from heart failure patients.

We have isolated and characterized a population of c-kit⁺ cells from a rat bone marrow using FACS analysis. By combining MACS isolation protocol and method of FACS sorting, we were able to positively select c-kit⁺ cells from other bone marrow cells and use them in further experiments. We observed that the number of isolated c-kit⁺ cells increased after myocardial ischemia. C-kit⁺ cell population in bone marrow comprises of hematopoietic and endothelial progenitors, as confirmed by analysis of CD45 and CD34 antigen expression using flow cytometry. Expression of these antigens in the c-kit⁺ cell population ranged between 19% and 24%. In addition, we observed a tendency toward lower abundance of c-kit⁺CD31⁺ cells after an ischemic insult compared to sham. This raises a possibility that, among c-kit⁺ cells, endothelial progenitors were preferentially mobilized from bone marrow after myocardial ischemia.

Paracrine protective properties of bone marrow cells have been mostly investigated on a population of mesenchymal stem cells (MSCs) [33]. Intramyocardial injection of both MSCs over-expressing Akt and conditioned media from the same cell type restores cardiac function after ischemia [44]. We have previously shown that cardiac c-kit⁺ cells under stimulatory conditions protect cardiomyocytes (CM) *in vitro* [78][85]. In this study, we have investigated

whether bone marrow c-kit⁺ cells in an un-stimulatory condition secrete protective cytokines and reduce apoptosis of co-cultured CM in a paracrine manner. Indeed, we could observe that survival of CM was supported in co-culture by c-kit⁺ cells. The fact that c-kit⁺ cells protected CM independently of cell contact suggested a paracrine protective mechanism.

We have observed, that c-kit⁺ cells were characterized by intra-cellular and membrane expression of ER α . Over the past decades, experimental and observational studies gathered a large body of evidence that favors protective effect of estrogen and female gender on cardiovascular system, which are mediated by estrogen receptors (ERs) expressed by cells in the heart, vessels and metabolic tissues. However, in placebo-controlled clinical trials, conducted by the Women's Health Initiative (WHI), estrogen-only or combined estrogen-progestin hormone replacement therapy did not reduce but increased cardiovascular risk in postmenopausal women [67]. Results of these studies prompt the scientific community to re-address critical questions concerning mechanisms of hormonal actions, especially on tissues in postmenopausal women.

ERs have long been regarded as ligand-activated transcriptional factors, and most of the benefits they mediated are attributed to the nuclear signaling and changes in transcription of responsive genes. Since membrane ERs were discovered, their origin and physiological relevance have been debated [55]. However, it has been well established that rapid estrogen (E2) signaling changes the activation status of cellular kinases and phosphatases and leads to cellular responses including proliferation, survival and migration [79,90,91].

Classical E2 signaling that activates nuclear estrogen receptors is essential for reaching the reproductive maturity. Genetic deletion of the total cellular pool of ER α results in a specific phenotype [92]. ER α KO mice exert extensive abnormalities in structure and function of reproductive organs and mammary glands: infertility due to uterus and ovary atrophy, abnormal ovary histology, increased abdominal fat etc. This phenotype could not be rescued in a mouse model which has E domain of ER α expressed only in membranes. Therefore, in the physiology of reproductive organ development, it seems that nuclear signaling plays a major role [92].

However, in the vascular system, physiological relevance of the rapid, membrane-initiated, E2 signaling has been well established. E2 modulates arterial tonus and induces vasodilatation in a rapid manner via activation of ERK/MAP kinase and phosphatidylinositol 3-kinase [93]. Recently, Chambliss *et al.* confirmed that non-nuclear estrogen signaling is physiologically

relevant for vascular tissue homeostasis [94]. Estrogen-dendrimer conjugate (EDC), which selectively stimulates membrane ERs but is excluded from the nucleus, promotes endothelial cell migration and protects against vascular injury *in vivo*. Interaction between ER α and G protein G α i subunit is a mechanism through which estrogen or EDC mediate endothelial cell proliferation and migration [94]. Importantly, EDC treatment does not exert uterotrophic or tumor growth promoting effects, which makes membrane ER an interesting target for future development of selective ER modulators [94]. In line with these findings, a down-regulation of proliferation markers such as Ki67 and c-myc was observed in BM c-kit+ER α + cells in this study. Thus, although it is established that membrane estrogen signaling induces cell proliferation, it seems that this effect depends on the cell type and availability of downstream signaling proteins. It remains to be investigated which molecules link membrane ER α and anti-proliferation response, as well as the relevance of low proliferation potential in the paracrine function of progenitor cells.

The role of membrane ER α in the function of stem/progenitor cells other than endothelial progenitors has not been thoroughly investigated until now. The discovery that membrane ER α is indeed present in c-kit+ cells led us to further investigate its importance related to paracrine protection. According to the membrane ER α expression, we isolated and functionally compared two subsets of c-kit+ cells: ER α + and ER α - c-kit+ cells. *In vitro*, c-kit+ER α + cells mediated an improved anti-apoptotic effect on CM in comparison to ckit+ER α - cells. This observation suggests that c-kit+ER α + cells have greater capacity to produce cyto-protective factors compared to c-kit+ER α - cells. We tested this hypothesis *in vivo* by injecting conditioned media prepared from c-kit+ER α - and c-kit+ER α + into rat myocardium following ischemia. In line with our *in vitro* data, intra-myocardial injection of conditioned media prepared from c-kit+ER α + improved systolic and diastolic parameters of cardiac function assessed by echocardiography. Therefore, our results imply that c-kit+ER α + cells improve post-ischemic cardiac function through paracrine reduction of CM death. To our knowledge, the link between membrane ERs and paracrine protection provided by progenitor cells has not been investigated so far. The influence of non-genomic estrogen signaling on cytokine production has only been studied in Kupffer cells and splenic macrophages, where stimulation of membrane ERs with BSA-conjugated estradiol normalizes capacity of these cells to produce IL-6, TNF α and IL-10 following trauma-hemorrhage via MAPK pathways [95].

We further demonstrated that the presence of membrane ER α in BM c-kit⁺ cell population is associated with an increased potential of cytokines IL-6 and IL-10 production, as assessed by PCR analysis. An interesting question that arises from our findings concerns the signaling pathway that could link membrane-expressed ER α and IL-6 production in c-kit⁺ cells. A growing body of evidence indicates an important role of phosphatidylinositol-3 (PI3) kinase/Akt signaling in the biology of progenitor cells, *i.e.* survival and paracrine function. Activation of the Akt pathway was shown to increase the release of putative cytoprotective factors. This is accompanied by an improved cyto-protective effect provided by mesenchymal stem cells when these are injected *in vivo* after myocardial infarction [44,96]. On the other hand, phosphorylation of Akt triggered by activated PI3 kinase is crucial for IL-6 production in dendritic cells after activation of c-kit receptor [97]. It has been repeatedly shown that rapid, membrane-initiated, estrogen signaling activates the PI3/Akt pathway. Therefore, it is tempting to speculate that the PI3/Akt pathway represents a link between plasma-membrane-expressed ER α and enhanced IL-6 production in c-kit⁺ ER α ⁺ cells shown in this study.

In this study, we identified IL-6 as an indispensable factor produced by c-kit⁺ER α ⁺ cells to support the survival of CM. Neutralization of IL-6 cytokine *in vitro* completely abolished protective effect of c-kit⁺ER α ⁺ cells on co-cultured CM, whereas IL-10 neutralization did not confer any effect.

IL-6, besides other cytokines such as IL-11, LIF, oncostatin M and ciliary neurotrophic factor belong to the IL-6 cytokine family. These cytokines are known to regulate several essential cellular functions. They activate downstream JAK/STAT1/3 or SHP-2/MEK/ERK pathways via shared a receptor, gp-130 [98].

IL-6 is a pro-inflammatory cytokine and has been suggested as a biomarker of heart failure. It has been repeatedly shown that plasma levels of IL-6 correlate with severity of ongoing inflammatory reactions and left ventricular dysfunction [99,100]. Plasma IL-6 is produced by various cell types including macrophages, granulocytes and lymphocytes but also by cells in the peripheral vascular bed (endothelial cells, smooth muscle vascular cells). Increased secretion of IL-6 in HF patients is probably stimulated by over-activated sympathetic nervous system. Positive correlation is found between plasma levels of norepinephrine and IL-6, whereas treatment with beta blockers negatively correlates with IL-6 plasma levels[99]. Interestingly, the same study shows that female gender is an independent predictor of high IL-6 plasma levels, underlining the important role of estrogen hormone in IL-6 production [99].

On the other hand, randomized clinical trials failed to show any benefit for the patients with heart failure when pro-inflammatory cytokines were pharmacologically targeted [101][102]. These studies raised a doubt of whether increased levels of IL-6 and other pro-inflammatory cytokines as TNF are a cause or consequence of heart failure.

An increasing body of evidence suggests that pro-inflammatory cytokines as TNF and IL-6 initiate protective cellular signals [102]. Binding of IL-6 to its receptor activates STAT3 signaling. In CM, activation of STAT3 signaling pathway mediates important survival and hypertrophic signals. It has been previously shown that STAT3 is activated in ischemic heart injury and mediates a pro-survival and anti-apoptotic effect [103]. In line with this, induction of IL-6 mRNA is identified in viable myocytes bordering the ischemic area one hour after reperfusion, in an ischemia-reperfusion model. It was suggested that this induction of IL-6 confers an autocrine protective effect [104]. In patients with end-stage dilated cardiomyopathy, decreased myocardial IL-6 mRNA and protein expression are accompanied by decreased myocardial STAT3 expression [105]. STAT3 activation reduces apoptosis by transcriptional regulation of anti-apoptotic gene *Bcl-2* and pro-apoptotic gene *Bax* [106]. Recently, it has been demonstrated that STAT3 also translocates to the mitochondria and inhibits opening of mitochondrial permeability transition pores and cytochrome c release [107]. Here, we demonstrated that IL-6 derived from c-kit⁺ER α ⁺ cells reduced CM apoptosis *in vitro*. The protective effect was confirmed by PCR analysis of STAT3 downstream target genes. In CM co-cultured with c-kit⁺ cells, expression of anti-apoptotic gene *Bcl-2* was increased while expression of pro-apoptotic gene *Bax* was reduced, in comparison to CM cultured without c-kit⁺ cells. In accordance with our study, *in vivo* application of IL-6 cytokine in complex with its soluble receptor (sIL-6R) was shown to inhibit apoptosis of CM and reduce infarct size in an ischemia-reperfusion model [108]. Similarly, in the brain, a rise of endogenous IL-6 levels has been shown to reduce infarct size following acute transient cerebral ischemia. Blockage of endogenous IL-6 reduces STAT3 phosphorylation, increases neuronal apoptosis and aggravates neurological function after cerebral ischemic insult [109].

These contradicting findings indicate the possibility that plasma and tissue IL-6 levels are regulated by distinct mechanisms and confer different biological responses.

IL-11 is another member of the IL-6 cytokine family but it does not induce an inflammatory reaction. It has been reported that ischemia increases secretion of IL-11 in myocardial tissue. Also, it has been suggested that increased myocardial levels of IL-11 confer an anti-apoptotic

and pro-angiogenic effect in the post-infarct myocardium [110]. In the current study, we observed decreased mRNA levels of *IL-11* in c-kit+ER α + in comparison to c-kit+ER α - cells. Therefore, it is not likely that IL-11 was responsible for superior cytoprotective effect mediated by c-kit+ER α + cells.

Besides its pro-survival effects, gp-130 signaling has an essential role in cell cycle regulation and promotion of proliferation in embryonic cardiomyocytes and hepatic cells [111,112]. It is, therefore, tempting to speculate that paracrine secretion of IL-6 *in vivo* could also mediate other beneficial effects, such as activation of resident cardiac progenitors after cardiac injury.

Reports on estrogen receptor-mediated influence on IL-6 production are inconsistent and partly conflicting. This is because estrogen regulates *IL-6* expression in a complex manner that is dependent on many factors, including experimental conditions, disease model, cell type investigated, estrogen formulation and concentration [113]. In this study, we provide evidence that *in vitro* stimulation of ER α increases production of cytokines IL-6 and IL-10 in bone marrow c-kit+ cells. Moreover, E2 stimulation improved paracrine protective effect of c-kit+ cells and further reduced apoptosis of co-cultured CM, likely due to increased IL-6 production. In line with our data, microarray analysis of gene expression in bone marrow c-kit+ cells shows that *IL-6* gene is the most strongly up-regulated gene following E2 stimulation [114]. At variance with our findings, several studies reported inhibition of *IL-6* production by E2 stimulation in other cell types [115,116]. Interestingly, using ER α KO and ER β KO models, Brown et al. observed that ERs regulate brain production of pro-inflammatory cytokines in both ligand-dependent and ligand-independent manner [115].

Cell therapy using progenitor cells derived from BM or peripheral blood from patients themselves (autologous cell therapy) has evoked hope for improving the post-ischemic cardiac performance. However, several reports indicate that cardiovascular disease itself may significantly impair the functional activity of endogenous progenitor cells. For instance, BM derived undifferentiated cells isolated from patients with HF have less potent migratory and colony-forming activity *in vitro* and a reduced neo-vascularization capacity *in vivo* [117]. Here, we provide new evidences that myocardial ischemia has a harmful impact on the functional capacity of c-kit+ cell population in bone marrow. Production of cardio-protective cytokines IL-6 and IL-10 and proliferation potential were impaired in c-kit+ cells after MI. In addition, lower ER α mRNA levels in c-kit+ cells were found after MI compared to sham. Several processes could have contributed to this observation. It seems that MI impairs ER α transcription and/or

translocation to the membrane. This could be due to systemic inflammation or other unknown mechanisms and warrants further investigation. Also, a tendency toward decreased percentage of c-kit⁺ER α ⁺ cells in bone marrow was found after MI. Therefore, preferential mobilization of highly cytokine secreting c-kit⁺ER α ⁺ subset could have attributed to the observed lower cytokine expression in c-kit⁺ cells remaining in bone marrow after MI. Regardless of the mechanisms involved, the observation that myocardial ischemia may impact the function of progenitor cells, not only in the heart but also in distant tissues such as bone marrow, could have important therapeutic implications in the process of cell therapy design. Importantly, ER α stimulation was shown to recover the paracrine production of cardio-protective IL-6 in both rat and human c-kit⁺ progenitor cells, offering a potential translational option to reverse the impaired function of stem/progenitor cells in patients with cardiovascular disease.

Human heart contains a pool of self-renewing, multipotent c-kit⁺ cells [118,119]. Increased abundance of c-kit⁺ cells was found in severely failing human hearts compared to controls. It was suggested that these c-kit⁺ cells originate from bone marrow due to their high frequency of co-expression of bone marrow marker CD45 [12]. We have identified a population of c-kit⁺ cells in mononuclear fraction of human blood samples and confirmed some of our previous findings. ER α was detected in a form of a membrane and nuclear receptor in human peripheral blood c-kit⁺ cells. Further, we found decreased abundance of c-kit⁺ER α ⁺ cells in blood samples from female heart failure (HF) patients compared to male HF patients and female controls. Based on observed gender-related differences in the frequency of c-kit⁺ER α ⁺ cells in human blood, it seems that mobilization of progenitor cells from the blood to the target tissue is more efficient in female patients with HF compared to male. This hypothesis is in line with a recent finding showing that female hearts have a larger pool of functionally competent progenitor cells than male at all ages [9]. Therefore, it appears plausible that gender not only influences replicative potential and senescence of heart c-kit⁺ cells, but also their mobilization from the blood to the heart. Further investigation is warranted to determine which local tissue or cellular factors contribute to this effect. Additionally, we have provided evidence that the relevance of ER α in human c-kit⁺ cells is linked to cytokine production. Using flow cytometry analysis, we found that within c-kit⁺ cell population, IL-6 was preferentially expressed in ER α ⁺ cells. In addition, *in vitro* stimulation of human c-kit⁺ cells confirmed that IL-6 cytokine secretion was regulated by ER α . Taken together, these findings indicate that gender and ERs are important players when it comes to heart regeneration.

Future studies

We have observed an improvement of cardiac function after treatment of the MI hearts with conditioned media from c-kit+ER α + cells and suggested that this was due to apoptosis reduction mediated by up-regulated IL-6. However, future studies should explore if IL-6 and cytokines other than IL-6 may have contributed to the post-ischemic cardiac improvement via unrelated mechanisms, i.e., neo-vascularisation, activation of resident progenitors in the heart or modulation of immune response. For example, we detected increased levels of IL-10 mRNA in c-kit+ER α + cells. Although IL-10 did not convey a survival protective effect on CM in our study, benefits of increased IL-10 secretion may be expected in *in vivo* situation due to its anti-inflammatory properties. Indeed, it was demonstrated that IL-10 after MI decreased T-cell accumulation, collagen deposition and reactive hypertrophy but had no influence on infarct size, neutrophil accumulation or neo-vascularisation [34]. In addition, we must take into consideration the contributive protective effect of other cytokines.

5 Conclusions

Although cell therapy holds promise in the treatment of acute myocardial ischemia, the mechanisms underlying its beneficial effects remain enigmatic. There is not enough evidence that supports the hypothesis of cardiac muscle regeneration after cell therapy in patients. By secreting various factors, progenitor cells form cytokine milieu which favors the regeneration of the injured myocardium. The study presented here provides a novel view on the regulation of cytokine production by progenitor cells. It identifies an expression pattern of ER α in a rat and human progenitor c-kit⁺ cells and provides an evidence for its functional importance. The membrane ER α has been demonstrated here to be an important factor that influences paracrine secretion in c-kit⁺ cells *in vitro* and *in vivo*. We were able to pinpoint IL-6 as a crucial cytokine secreted by c-kit⁺ER α ⁺ cells to support cardiomyocyte survival. It has also been shown here that myocardial infarction impairs the paracrine function of progenitor cells in the bone marrow, which could be improved by ER α stimulation.

This study has its clinical relevance because it brings new insights to understanding the mechanisms that govern paracrine secretion by progenitor cells. This is critical for successful development of the cell therapy as a strategy aimed to regenerate human myocardium.

6 Summary

Despite the advances in diagnosis and treatment, myocardial infarction remains a major concern for the health system worldwide. Cell therapy emerged as a novel and promising treatment option aimed to improve cardiac function after ischemia. Whereas no consensus has been reached on potential of bone marrow cells to directly regenerate cardiomyocytes, paracrine secretion seems to represent an important mechanism by which progenitor cells mediate cardioprotection.

Cardioprotective actions of estrogen have been well recognized for many years. Recent studies indicate a novel role of estrogen receptors (ER) in stem/precursor cell-involved cardiac repair. C-kit is a transmembrane tyrosine kinase receptor expressed by undifferentiated progenitor cells. Taking into account that cardiac, c-kit-expressing (c-kit⁺) precursor cells are mainly recruited from bone marrow (BM) c-kit⁺ cell population, we aimed here to elucidate the functional importance of ER α in BM c-kit⁺ precursor cells after ischemic heart injury.

The c-kit⁺ cells were isolated from femurs and tibias of male wistar rats 7 days after myocardial infarction (MI) by magnetic activated cell sorting (MACS) in combination with fluorescent activated cell sorting (FACS). After MI, the percentage of c-kit⁺ cells in BM increased by 2.11 fold. BM c-kit⁺ cells, which expressed both intracellular and membrane ER α , were shown to inhibit apoptosis of co-cultured cardiomyocytes in a paracrine manner. According to the expression of membrane ER α , BM c-kit⁺ cells were further sorted using FACS into c-kit⁺ER α ⁺ and c-kit⁺ER α ⁻ cell populations. BM c-kit⁺ER α ⁺ cells were more potent in reducing apoptosis of co-cultured cardiomyocytes than BM c-kit⁺ER α ⁻ cells. In addition, the post-ischemic intra-myocardial injection of conditioned medium from c-kit⁺ER α ⁺ improved systolic and diastolic left ventricular function compared to medium collected from c-kit⁺ER α ⁻ cells. c-kit⁺ER α ⁺ cells were characterized by increased production of cytokines IL-6 and IL-10, and lower production of IL-11. MI impaired paracrine function of BM c-kit⁺ cells and stimulation with 17 β -estradiol could enhance cytokine production and the BM c-kit⁺ cell-mediated cardioprotective effect. Importantly, blocking IL-6 by neutralizing antibody abolished the effect of BM c-kit⁺ER α ⁺ cell in supporting co-cultured cardiomyocytes. Finally, ER α -mediated IL-6 production and reduced percentage of c-kit⁺ER α ⁺ cells were also observed in blood c-kit⁺ cells of patients with heart failure. Thus, this work puts forward a novel cardioprotective mechanism mediated by BM c-kit⁺ER α ⁺ cells via paracrine IL-6.

7 Zusammenfassung

Trotz des Fortschritts in der Diagnose und Therapie erregt Herzinfarkt noch großes Besorgnis für das Gesundheitssystem in der ganzen Welt. Der Einsatz von Stammzellen hat hohe Erwartungen geweckt, als eine neue potenzielle Therapiemethode die Herzfunktion nach dem Herzinfarkt verbessern zu können. Obwohl klinische und experimentelle Studien bereits einen günstigen Einfluss von Stammzellen aus dem Knochenmark auf die Herzfunktion gezeigt haben, ist deren Wirkungsmechanismus noch unklar. Die parakrine Schutzwirkung durch verschiedene Zytokine, die von Stammzellen freigesetzt werden, ist wahrscheinlicher als eine direkte Umwandlung in Herzmuskelzellen.

Die protektive Wirkung von Östrogen auf das Herz-Kreislauf-System ist seit Jahren bekannt. Neue Ergebnisse deuten darauf hin, dass Östrogenrezeptoren für die Funktion der Stammzellen, die bei der kardialen Reparatur beteiligt sind, eine wichtige Rolle spielen. Die Progenitorzellen, die im Herz zu finden sind, exprimieren den Oberflächenmarker c-kit und werden wahrscheinlich aus dem Knochenmark nach dem akuten Herzinfarkt mobilisiert.

Das Ziel dieser Arbeit war zu erklären, welche Rolle der Östrogenrezeptor alpha für die Funktion der Progenitorzellen-c-kit⁺ Zellen aus dem Knochenmark nach dem Herzinfarkt hat.

Dafür wurde ein Herzinfarkt-Versuchsmodell an männlichen Wistar Ratten angewandt und eine Methode entwickelt, die MACS (Magnetic Activated Cell Sorting) und FACS (Fluorescent Activated Cell Sorting) Technologie kombiniert, um c-kit⁺ Zellen aus dem Knochenmark zu isolieren.

Sieben Tagen nach dem Herzinfarkt stieg der prozentuelle Anteil der isolierten c-kit⁺ Zellen aus dem Knochenmark auf das 2.11fache an. Die c-kit⁺ Zellen haben in der Zellkultur durch parakrine Schutzwirkung Apoptosis von adulten Kardiomyocyten gehemmt. Weiterhin zeigten c-kit⁺ Zellen aus dem Knochenmark sowohl Intrazellulär- als auch Oberflächenexpression von Östrogenrezeptoren alpha (ER α). Mittels FACS-Technologie und auf Grund der Extrazellulärexpression von ER wurde die c-kit⁺ Zellpopulation in c-kit⁺ER α ⁻ und c-kit⁺ER α ⁺ Zellen aufgeteilt. c-kit⁺ER α ⁺ Zellen zeigten eine verbesserte Schutzwirkung auf das Überleben von adulten Kardiomyocyten in der Zellkultur als die c-kit⁺ER α ⁻. Zusätzlich wurden die Überstände von c-kit⁺ER α ⁺ oder c-kit⁺ER α ⁻ Zellen nach 48 Stunden ins periischämischen Myokard von Ratten unmittelbar nach dem Herzinfarkt eingespritzt. Im Vergleich zu dem

Effekt des Überstands von c-kit+ER α - Zellen, führte die Injektion des Überstands von c-kit+ER α + zu einer Verbesserung der systolischen und diastolischen linksventrikulären Funktion. Diese Ergebnisse weisen darauf hin, dass c-kit+ER α + Zellen ein ~~erhöhtes~~ erhöhtes Potenzial für Ausschüttung von kardioprotektiven Zytokinen haben. Dies wurde mittels real-time PCR bestätigt. c-kit+ER α + zeigten eine ~~erhöhte~~ erhöhte mRNA Expression von IL -6 und IL-10 und eine geringere mRNA Expression von IL-11. Weiterhin wurde durch Neutralisierung von IL-6 in der Zellkultur der kardioprotektive Einfluss von c-kit+ Zellen auf den Kardiomyocyten völlig aufgehoben, während die Neutralisierung von IL-10 die Apoptosis von Kardiomyocyten nicht beeinflusst hat. Außerdem war nach einem Herzinfarkt die Produktion von Zytokinen in c-kit+ Zellen vermindert, welche sich aber nach die 17 β -Östradiol Stimulierung wieder erhöhen ließ. Schließlich wurde die c-kit+ER α + Zellpopulation auch in humanem peripheren Blut entdeckt und bestätigt, dass ER -kontrollierte IL-6 Produktion von auch in humanen c-kit+ Zellen stattfindet.

Im Gesamten zeigen diese Ergebnisse einen neuartigen kardioprotektiven Mechanismus, den c-kit+ Progenitorzellen über eine ER α -abhängige Hochregulation von IL-6 vermitteln.

8 References

- [1] World Health Organisation, “Cardiovascular diseases (CVDs) Fact sheet N°317,” 2011.
- [2] Y. Lee and A.B. Gustafsson, “Role of apoptosis in cardiovascular disease.,” *Apoptosis : an international journal on programmed cell death*, vol. 14, Apr. 2009, pp. 536-48.
- [3] a Herskowitz, S. Choi, a a Ansari, and S. Wesselingh, “Cytokine mRNA expression in postischemic/reperfused myocardium.,” *The American journal of pathology*, vol. 146, Feb. 1995, pp. 419-28.
- [4] K. Thygesen, J.S. Alpert, and H.D. White, “Universal definition of myocardial infarction.,” *Journal of the American College of Cardiology*, vol. 50, Nov. 2007, pp. 2173-95.
- [5] H.P. Rang, M.M. Dale, J.M. Ritter, and P.K. Moore, *Pharmacology*, Churchill Livingstone, 2003.
- [6] E.M. Antman, D.T. Anbe, P.W. Armstrong, E.R. Bates, L.A. Green, M. Hand, J.S. Hochman, H.M. Krumholz, F.G. Kushner, G.A. Lamas, C.J. Mullany, J.P. Ornato, D.L. Pearle, M.A. Sloan, and S.C. Smith, “ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction--executive summary. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to revise the 1999),” *Journal of the American College of Cardiology*, vol. 44, Aug. 2004, pp. 671-719.
- [7] A.K. Kondur, P. Hari, and S.R. Pitta, “Complications of Myocardial Infarction,” *eMedicine WebMD*, 2008.
- [8] O. Bergmann, R.D. Bhardwaj, S. Bernard, S. Zdunek, F. Barnabé-Heider, S. Walsh, J. Zupicich, K. Alkass, B.A. Buchholz, H. Druid, S. Jovinge, and J. Frisén, “Evidence for cardiomyocyte renewal in humans.,” *Science (New York, N.Y.)*, vol. 324, Apr. 2009, pp. 98-102.
- [9] J. Kajstura, N. Gurusamy, B. Ogórek, P. Goichberg, C. Clavo-Rondon, T. Hosoda, D. D’Amario, S. Bardelli, A.P. Beltrami, D. Cesselli, R. Bussani, F. del Monte, F. Quaini, M. Rota, C. a Beltrami, B. a Buchholz, A. Leri, and P. Anversa, “Myocyte turnover in the aging human heart.,” *Circulation research*, vol. 107, Nov. 2010, pp. 1374-86.
- [10] F. Quaini, K. Urbanek, A.P. Beltrami, N. Finato, C.A. Beltrami, B. Nadal-Ginard, J. Kajstura, A. Leri, and P. Anversa, “Chimerism of the transplanted heart.,” *The New England journal of medicine*, vol. 346, Jan. 2002, pp. 5-15.
- [11] A.P. Beltrami, L. Barlucchi, D. Torella, M. Baker, F. Limana, S. Chimenti, H. Kasahara, M. Rota, E. Musso, K. Urbanek, A. Leri, J. Kajstura, B. Nadal-Ginard, and P. Anversa, “Adult cardiac stem cells are multipotent and support myocardial regeneration.,” *Cell*, vol. 114, Sep. 2003, pp. 763-76.

- [12] H. Kubo, N. Jaleel, A. Kumarapeli, R.M. Berretta, G. Bratinov, X. Shan, H. Wang, S.R. Houser, and K.B. Margulies, "Increased Cardiac Myocyte Progenitors in Failing Human Hearts," *Circulation*, 2008.
- [13] P.C.H. Hsieh, V.F.M. Segers, M.E. Davis, C. MacGillivray, J. Gannon, J.D. Molkenin, J. Robbins, and R.T. Lee, "Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury.," *Nature medicine*, vol. 13, Aug. 2007, pp. 970-4.
- [14] Y.N. Tallini, K.S. Greene, M. Craven, A. Spealman, M. Breitbart, J. Smith, P.J. Fisher, M. Steffey, M. Hesse, R.M. Doran, A. Woods, B. Singh, A. Yen, B.K. Fleischmann, and M.I. Kotlikoff, "C-Kit Expression Identifies Cardiovascular Precursors in the Neonatal Heart.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, Feb. 2009, pp. 1808-13.
- [15] M. Xaymardan, M. Cimini, S. Fazel, R.D. Weisel, W.-Y. Lu, U. Martin, R.P. Harvey, and R.-K. Li, "c-Kit function is necessary for in vitro myogenic differentiation of bone marrow hematopoietic cells.," *Stem cells (Dayton, Ohio)*, vol. 27, Aug. 2009, pp. 1911-20.
- [16] R. Palacios and S. Nishikawa, "Developmentally regulated cell surface expression and function of c-kit receptor during lymphocyte ontogeny in the embryo and adult mice.," *Development (Cambridge, England)*, vol. 115, Aug. 1992, pp. 1133-47.
- [17] V.C. Broudy, "Stem cell factor and hematopoiesis.," *Blood*, vol. 90, Aug. 1997, pp. 1345-64.
- [18] M.L. Linenberger, F.W. Jacobson, L.G. Bennett, V.C. Broudy, F.H. Martin, and J.L. Abkowitz, "Stem cell factor production by human marrow stromal fibroblasts.," *Experimental hematology*, vol. 23, Sep. 1995, pp. 1104-14.
- [19] M.Z. Ratajczak, W.I. Kuczynski, D.L. Sokol, J.S. Moore, C.H. Pletcher, and A.M. Gewirtz, "Expression and physiologic significance of Kit ligand and stem cell tyrosine kinase-1 receptor ligand in normal human CD34+, c-Kit+ marrow cells.," *Blood*, vol. 86, Sep. 1995, pp. 2161-7.
- [20] M. Cimini, S. Fazel, S. Zhuo, M. Xaymardan, H. Fujii, R.D. Weisel, and R.-K. Li, "c-kit dysfunction impairs myocardial healing after infarction.," *Circulation*, vol. 116, Sep. 2007, pp. I77-82.
- [21] S. Fazel, M. Cimini, L. Chen, S. Li, D. Angoulvant, P. Fedak, S. Verma, R.D. Weisel, A. Keating, and R.-K. Li, "Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines.," *The Journal of clinical investigation*, vol. 116, Jul. 2006, pp. 1865-77.
- [22] K.A. Jackson, S.M. Majka, H. Wang, J. Pocius, C.J. Hartley, M.W. Majesky, M.L. Entman, L.H. Michael, K.K. Hirschi, and M.A. Goodell, "Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells.," *The Journal of clinical investigation*, vol. 107, Jun. 2001, pp. 1395-402.

- [23] M. Kucia, B. Dawn, G. Hunt, Y. Guo, M. Wysoczynski, M. Majka, J. Ratajczak, F. Rezzoug, S.T. Ildstad, R. Bolli, and M.Z. Ratajczak, "Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction.," *Circulation research*, vol. 95, Dec. 2004, pp. 1191-9.
- [24] S. Vandervelde, M.J.A. van Luyn, R.A. Tio, and M.C. Harmsen, "Signaling factors in stem cell-mediated repair of infarcted myocardium.," *Journal of molecular and cellular cardiology*, vol. 39, Aug. 2005, pp. 363-76.
- [25] M.G. Engelmann, H.D. Theiss, C. Theiss, V. Henschel, A. Huber, B.J. Wintersperger, S.O. Schoenberg, G. Steinbeck, and W.-M. Franz, "G-CSF in patients suffering from late revascularised ST elevation myocardial infarction: final 1-year-results of the G-CSF-STEMI Trial.," *International journal of cardiology*, vol. 144, Oct. 2010, pp. 399-404.
- [26] M.-M. Zaruba, H.D. Theiss, M. Vallaster, U. Mehl, S. Brunner, R. David, R. Fischer, L. Krieg, E. Hirsch, B. Huber, P. Nathan, L. Israel, A. Imhof, N. Herbach, G. Assmann, R. Wanke, J. Mueller-Hoecker, G. Steinbeck, and W.-M. Franz, "Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction.," *Cell stem cell*, vol. 4, Apr. 2009, pp. 313-23.
- [27] M.-M. Zaruba, M. Soonpaa, S. Reuter, and L.J. Field, "Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts.," *Circulation*, vol. 121, May. 2010, pp. 1992-2000.
- [28] D. Orlic, J. Kajstura, S. Chimenti, I. Jakoniuk, S.M. Anderson, B. Li, J. Pickel, R. McKay, B. Nadal-Ginard, D.M. Bodine, A. Leri, and P. Anversa, "Bone marrow cells regenerate infarcted myocardium.," *Nature*, vol. 410, Apr. 2001, pp. 701-5.
- [29] L.B. Balsam, A.J. Wagers, and J.L. Christensen, "Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium.," *Nature*, vol. 428, 2004, pp. 668-673.
- [30] C.E. Murry, M.H. Soonpaa, H. Reinecke, H. Nakajima, H.O. Nakajima, M. Rubart, V. Poppa, G. Bradford, J.D. Dowell, D.A. Williams, and L.J. Field, "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts.," *Nature*, vol. 428, 2004, pp. 13-15.
- [31] J. Terrovitis, R. Lautamäki, M. Bonios, J. Fox, J.M. Engles, J. Yu, M.K. Leppo, M.G. Pomper, R.L. Wahl, J. Seidel, B.M. Tsui, F.M. Bengel, M.R. Abraham, and E. Marbán, "Noninvasive quantification and optimization of acute cell retention by in vivo positron emission tomography after intramyocardial cardiac-derived stem cell delivery.," *Journal of the American College of Cardiology*, vol. 54, Oct. 2009, pp. 1619-26.
- [32] C.-H. Yoon, M. Koyanagi, K. Iekushi, F. Seeger, C. Urbich, A.M. Zeiher, and S. Dimmeler, "Mechanism of Improved Cardiac Function After Bone Marrow Mononuclear Cell Therapy. Role of Cardiovascular Lineage Commitment.," *Circulation*, Apr. 2010.
- [33] R. Uemura, M. Xu, N. Ahmad, and M. Ashraf, "Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling.," *Circulation research*, vol. 98, Jun. 2006, pp. 1414-21.

- [34] J.S. Burchfield, M. Iwasaki, M. Koyanagi, C. Urbich, N. Rosenthal, A.M. Zeiher, and S. Dimmeler, "Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction.," *Circulation research*, vol. 103, Jul. 2008, pp. 203-11.
- [35] Y.-J. Kang, S.-J. Yang, G. Park, B. Cho, C.-K. Min, T.-Y. Kim, J.-S. Lee, and I.-H. Oh, "A novel function of interleukin-10 promoting self-renewal of hematopoietic stem cells.," *Stem cells*, vol. 25, Jul. 2007, pp. 1814-22.
- [36] S. Dhein, J. Garbade, D. Rouabah, G. Abraham, F.-R. Ungemach, K. Schneider, C. Ullmann, H. Aupperle, J.F. Gummert, and F.-W. Mohr, "Effects of autologous bone marrow stem cell transplantation on beta-adrenoceptor density and electrical activation pattern in a rabbit model of non-ischemic heart failure.," *Journal of cardiothoracic surgery*, vol. 1, Jan. 2006, p. 17.
- [37] X.-L. Tang, G. Rokosh, S.K. Sanganalmath, F. Yuan, H. Sato, J. Mu, S. Dai, C. Li, N. Chen, Y. Peng, B. Dawn, G. Hunt, A. Leri, J. Kajstura, S. Tiwari, G. Shirk, P. Anversa, and R. Bolli, "Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction.," *Circulation*, vol. 121, Jan. 2010, pp. 293-305.
- [38] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Molecular biology of the CELL*, Garland Science Taylor & Francis Group, 2007.
- [39] W. Cheng, J. Kajstura, J.A. Nitahara, B. Li, K. Reiss, Y. Liu, W.A. Clark, S. Krajewski, J.C. Reed, G. Olivetti, and P. Anversa, "Programmed myocyte cell death affects the viable myocardium after infarction in rats.," *Experimental cell research*, vol. 226, Aug. 1996, pp. 316-27.
- [40] A. Abbate, G.G.L. Biondi-Zoccai, R. Bussani, A. Dobrina, D. Camilot, F. Feroce, R. Rossiello, F. Baldi, F. Silvestri, L.M. Biasucci, and A. Baldi, "Increased myocardial apoptosis in patients with unfavorable left ventricular remodeling and early symptomatic post-infarction heart failure.," *Journal of the American College of Cardiology*, vol. 41, Mar. 2003, pp. 753-60.
- [41] R.J. Youle and A. Strasser, "The BCL-2 protein family: opposing activities that mediate cell death.," *Nature reviews. Molecular cell biology*, vol. 9, Jan. 2008, pp. 47-59.
- [42] Z. Chen, C.C. Chua, Y.S. Ho, R.C. Hamdy, and B.H. Chua, "Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice.," *American journal of physiology. Heart and circulatory physiology*, vol. 280, May. 2001, pp. H2313-20.
- [43] Y. Chandrasekhar, S. Sen, R. Anway, A. Shuros, and I. Anand, "Long-term caspase inhibition ameliorates apoptosis, reduces myocardial troponin-I cleavage, protects left ventricular function, and attenuates remodeling in rats with myocardial infarction.," *Journal of the American College of Cardiology*, vol. 43, Jan. 2004, pp. 295-301.
- [44] M. Gnechi, H. He, N. Noiseux, O.D. Liang, L. Zhang, F. Morello, H. Mu, L.G. Melo, R.E. Pratt, J.S. Ingwall, and V.J. Dzau, "Evidence supporting paracrine hypothesis for

- Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement.,” *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, vol. 20, Apr. 2006, pp. 661-9.
- [45] V. Schächinger, S. Erbs, A. Elsässer, W. Haberbosch, R. Hambrecht, H. Hölschermann, J. Yu, R. Corti, D.G. Mathey, C.W. Hamm, T. Süselbeck, B. Assmus, T. Tonn, S. Dimmeler, and A.M. Zeiher, “Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.,” *The New England journal of medicine*, vol. 355, Sep. 2006, pp. 1210-21.
- [46] M. Tendera, W. Wojakowski, W. Rużyłło, L. Chojnowska, C. Kepka, W. Tracz, P. Musiałek, W. Piwowarska, J. Nessler, P. Buszman, S. Grajek, P. Breborowicz, M. Majka, and M.Z. Ratajczak, “Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracor,” *European heart journal*, vol. 30, Jun. 2009, pp. 1313-21.
- [47] J. Wöhrle, N. Merkle, V. Mailänder, T. Nusser, P. Schauwecker, F. von Scheidt, K. Schwarz, M. Bommer, M. Wiesneth, H. Schrezenmeier, and V. Hombach, “Results of intracoronary stem cell therapy after acute myocardial infarction.,” *The American journal of cardiology*, vol. 105, Mar. 2010, pp. 804-12.
- [48] B. Assmus, A. Rolf, S. Erbs, A. Elsässer, W. Haberbosch, R. Hambrecht, H. Tillmanns, J. Yu, R. Corti, D.G. Mathey, C.W. Hamm, T. Süselbeck, T. Tonn, S. Dimmeler, T. Dill, A.M. Zeiher, and V. Schächinger, “Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction.,” *Circulation. Heart failure*, vol. 3, Jan. 2010, pp. 89-96.
- [49] A. Hirsch, R. Nijveldt, P. a van der Vleuten, J.G.P. Tijssen, W.J. van der Giessen, R. a Tio, J. Waltenberger, J.M. Ten Berg, P. a Doevendans, W.R.M. Aengevaeren, J.J. Zwaginga, B.J. Biemond, A.C. van Rossum, J.J. Piek, and F. Zijlstra, “Intracoronary infusion of mononuclear cells from bone marrow or peripheral blood compared with standard therapy in patients after acute myocardial infarction treated by primary percutaneous coronary intervention: results of the randomized controlled HEBE ,” *European heart journal*, Dec. 2010.
- [50] D. Sürder, J. Schwitter, T. Moccetti, G. Astori, K. Rufibach, S. Plein, V. Lo Cicero, S. Soncin, S. Windecker, A. Moschovitis, A. Wahl, P. Erne, P. Jamshidi, C. Auf der Maur, R. Manka, G. Soldati, I. Bühler, C. Wyss, U. Landmesser, T.F. Lüscher, and R. Corti, “Cell-based therapy for myocardial repair in patients with acute myocardial infarction: rationale and study design of the SWISS multicenter Intracoronary Stem cells Study in Acute Myocardial Infarction (SWISS-AMI).,” *American heart journal*, vol. 160, Jul. 2010, pp. 58-64.
- [51] S. Mansour, D.-C. Roy, V. Bouchard, B.K. Nguyen, L.M. Stevens, F. Gobeil, A. Rivard, G. Leclerc, F. Reeves, and N. Noiseux, “COMPARE-AMI trial: comparison of intracoronary injection of CD133+ bone marrow stem cells to placebo in patients after acute myocardial infarction and left ventricular dysfunction: study rationale and design.,” *Journal of cardiovascular translational research*, vol. 3, Apr. 2010, pp. 153-9.

References

- [52] M. Marino, P. Galluzzo, and P. Ascenzi, "Estrogen signaling multiple pathways to impact gene transcription.," *Current genomics*, vol. 7, Jan. 2006, pp. 497-508.
- [53] L. Björnström and M. Sjöberg, "Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes.," *Molecular endocrinology (Baltimore, Md.)*, vol. 19, Apr. 2005, pp. 833-42.
- [54] C.M. Szego and J.S. Davis, "Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 58, Oct. 1967, pp. 1711-8.
- [55] R.J. Pietras and C.M. Szego, "Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells," *Nature*, vol. 265, 1977.
- [56] M. Razandi, "Cell Membrane and Nuclear Estrogen Receptors (ERs) Originate from a Single Transcript: Studies of ER and ER Expressed in Chinese Hamster Ovary Cells," *Molecular Endocrinology*, vol. 13, Feb. 1999, pp. 307-319.
- [57] T.C. Pappas, B. Gametchu, and C.S. Watson, "Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding.," *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, vol. 9, Mar. 1995, pp. 404-10.
- [58] A. Pedram, M. Razandi, and E.R. Levin, "Nature of functional estrogen receptors at the plasma membrane.," *Molecular endocrinology (Baltimore, Md.)*, vol. 20, Sep. 2006, pp. 1996-2009.
- [59] L. Li, M.P. Haynes, and J.R. Bender, "Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, Apr. 2003, pp. 4807-12.
- [60] Z. Wang, X. Zhang, P. Shen, B.W. Loggie, Y. Chang, and T.F. Deuel, "Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66.," *Biochemical and biophysical research communications*, vol. 336, Nov. 2005, pp. 1023-7.
- [61] F. Acconcia, P. Ascenzi, A. Bocedi, E. Spisni, V. Tomasi, A. Trentalance, P. Visca, and M. Marino, "Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol.," *Molecular biology of the cell*, vol. 16, Jan. 2005, pp. 231-7.
- [62] M. Razandi, G. Alton, A. Pedram, S. Ghonshani, P. Webb, and E.R. Levin, "Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane.," *Molecular and cellular biology*, vol. 23, Mar. 2003, pp. 1633-46.
- [63] E.R. Levin, "Plasma membrane estrogen receptors.," *Trends in endocrinology and metabolism: TEM*, vol. 20, Dec. 2009, pp. 477-82.

- [64] C.G. Isles, D.J. Hole, V.M. Hawthorne, and A.F. Lever, "Relation between coronary risk and coronary mortality in women of the Renfrew and Paisley survey: comparison with men.," *Lancet*, vol. 339, Mar. 1992, pp. 702-6.
- [65] L. Mosca, J.E. Manson, S.E. Sutherland, R.D. Langer, T. Manolio, and E. Barrett-Connor, "Cardiovascular Disease in Women : A Statement for Healthcare Professionals From the American Heart Association," *Circulation*, vol. 96, Oct. 1997, pp. 2468-2482.
- [66] M.E. Mendelsohn and R.H. Karas, "The protective effects of estrogen on the cardiovascular system.," *The New England journal of medicine*, vol. 340, Jun. 1999, pp. 1801-11.
- [67] J.E. Rossouw, G.L. Anderson, R.L. Prentice, A.Z. LaCroix, C. Kooperberg, M.L. Stefanick, R.D. Jackson, S. a a Beresford, B.V. Howard, K.C. Johnson, J.M. Kotchen, and J. Ockene, "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.," *JAMA : the journal of the American Medical Association*, vol. 288, Jul. 2002, pp. 321-33.
- [68] K.M. Egan, J.A. Lawson, S. Fries, B. Koller, D.J. Rader, E.M. Smyth, and G.A. Fitzgerald, "COX-2-derived prostacyclin confers atheroprotection on female mice.," *Science (New York, N.Y.)*, vol. 306, Dec. 2004, pp. 1954-7.
- [69] S. Guerra, A. Leri, X. Wang, N. Finato, C. Di Loreto, C.A. Beltrami, J. Kajstura, and P. Anversa, "Myocyte death in the failing human heart is gender dependent.," *Circulation research*, vol. 85, Oct. 1999, pp. 856-66.
- [70] R.D. Patten, "17 -Estradiol Reduces Cardiomyocyte Apoptosis In Vivo and In Vitro via Activation of Phospho-Inositide-3 Kinase/Akt Signaling," *Circulation Research*, vol. 95, Sep. 2004, pp. 692-699.
- [71] M. Satoh, C.M. Matter, H. Ogita, K. Takeshita, C.-Y. Wang, G.W. Dorn, and J.K. Liao, "Inhibition of apoptosis-regulated signaling kinase-1 and prevention of congestive heart failure by estrogen.," *Circulation*, vol. 115, Jun. 2007, pp. 3197-204.
- [72] E.O. Weinberg, C.D. Thienelt, S.E. Katz, J. Bartunek, M. Tajima, S. Rohrbach, P.S. Douglas, and B.H. Lorell, "Gender differences in molecular remodeling in pressure overload hypertrophy.," *Journal of the American College of Cardiology*, vol. 34, Jul. 1999, pp. 264-73.
- [73] M.E. Mendelsohn and R.H. Karas, "Molecular and cellular basis of cardiovascular gender differences.," *Science (New York, N.Y.)*, vol. 308, Jun. 2005, pp. 1583-7.
- [74] S.M. Cowley, "Estrogen Receptors alpha and beta Form Heterodimers on DNA," *Journal of Biological Chemistry*, vol. 272, Aug. 1997, pp. 19858-19862.
- [75] F.A. Babiker, D.J. Lips, E. Delvaux, P. Zandberg, B.J.A. Janssen, F. Prinzen, G. van Eys, C. Grohé, and P.A. Doevendans, "Oestrogen modulates cardiac ischaemic remodelling through oestrogen receptor-specific mechanisms.," *Acta physiologica (Oxford, England)*, vol. 189, Jan. 2007, pp. 23-31.

- [76] S.A. Gabel, V.R. Walker, R.E. London, C. Steenbergen, K.S. Korach, and E. Murphy, "Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury.," *Journal of molecular and cellular cardiology*, vol. 38, Feb. 2005, pp. 289-97.
- [77] E.A. Booth, N.R. Obeid, and B.R. Lucchesi, "Activation of estrogen receptor- α protects the in vivo rabbit heart from ischemia-reperfusion injury," *Am J Physiol Heart Circ Physiol*, 2005, pp. 2039-2047.
- [78] M. Brinckmann, E. Kaschina, W. Altarache-Xifró, C. Curato, M. Timm, A. Grzesiak, J. Dong, K. Kappert, U. Kintscher, T. Unger, and J. Li, "Estrogen receptor alpha supports cardiomyocytes indirectly through post-infarct cardiac c-kit+ cells.," *Journal of molecular and cellular cardiology*, vol. 47, Jul. 2009, pp. 66-75.
- [79] F. Acconcia, P. Totta, S. Ogawa, I. Cardillo, S. Inoue, S. Leone, A. Trentalance, M. Muramatsu, and M. Marino, "Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling.," *Journal of cellular physiology*, vol. 203, Apr. 2005, pp. 193-201.
- [80] A. Iwakura, S. Shastry, C. Luedemann, H. Hamada, A. Kawamoto, R. Kishore, Y. Zhu, G. Qin, M. Silver, T. Thorne, L. Eaton, H. Masuda, T. Asahara, and D.W. Losordo, "Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix m.," *Circulation*, vol. 113, Mar. 2006, pp. 1605-14.
- [81] H. Hamada, M.K. Kim, A. Iwakura, M. Ii, T. Thorne, G. Qin, J. Asai, Y. Tsutsumi, H. Sekiguchi, M. Silver, A. Wecker, E. Bord, Y. Zhu, R. Kishore, and D.W. Losordo, "Estrogen receptors alpha and beta mediate contribution of bone marrow-derived endothelial progenitor cells to functional recovery after myocardial infarction.," *Circulation*, vol. 114, Nov. 2006, pp. 2261-70.
- [82] G.S. Erwin, P.R. Crisostomo, Y. Wang, M. Wang, T.A. Markel, M. Guzman, I.C. Sando, R. Sharma, and D.R. Meldrum, "Estradiol-Treated Mesenchymal Stem Cells Improve Myocardial Recovery After Ischemia," *Journal of Surgical Research*, vol. 152, Apr. 2009, pp. 319-324.
- [83] D. Ribble, N.B. Goldstein, D.A. Norris, and Y.G. Shellman, "A simple technique for quantifying apoptosis in 96-well plates.," *BMC biotechnology*, vol. 5, Jan. 2005, p. 12.
- [84] C. Curato, S. Slavic, J. Dong, A. Skorska, W. Altarache-Xifró, K. Miteva, E. Kaschina, A. Thiel, H. Imboden, J. Wang, U. Steckelings, G. Steinhoff, T. Unger, and J. Li, "Identification of Noncytotoxic and IL-10-Producing CD8+AT2R+ T Cell Population in Response to Ischemic Heart Injury.," *Journal of immunology (Baltimore, Md. : 1950)*, vol. 185, Oct. 2010, pp. 6286-93.
- [85] W. Altarache-Xifró, C. Curato, E. Kaschina, A. Grzesiak, S. Slavic, J. Dong, K. Kappert, M. Steckelings, H. Imboden, T. Unger, and J. Li, "Cardiac c-kit+AT2+ cell population is increased in response to ischemic injury and supports cardiomyocyte performance.," *Stem cells (Dayton, Ohio)*, vol. 27, Oct. 2009, pp. 2488-97.

- [86] R.W. Coatney, "Ultrasound imaging: principles and applications in rodent research.," *ILAR journal / National Research Council, Institute of Laboratory Animal Resources*, vol. 42, Jan. 2001, pp. 233-47.
- [87] T.D. Schmittgen and K.J. Livak, "Analyzing real-time PCR data by the comparative C(T) method.," *Nature protocols*, vol. 3, Jan. 2008, pp. 1101-8.
- [88] E.D. Hawkins, M. Hommel, M.L. Turner, F.L. Battye, J.F. Markham, and P.D. Hodgkin, "Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data.," *Nature protocols*, vol. 2, Jan. 2007, pp. 2057-67.
- [89] H. Ebel, M. Jungblut, Y. Zhang, T. Kubin, S. Kostin, A. Technau, S. Oustanina, S. Niebrügge, J. Lehmann, K. Werdan, and T. Braun, "Cellular cardiomyoplasty: improvement of left ventricular function correlates with the release of cardioactive cytokines.," *Stem cells (Dayton, Ohio)*, vol. 25, Jan. 2007, pp. 236-44.
- [90] V.-I. Alexaki, I. Charalampopoulos, M. Kampa, H. Vassalou, P. Theodoropoulos, E.N. Stathopoulos, A. Hatzoglou, A. Gravanis, and E. Castanas, "Estrogen exerts neuroprotective effects via membrane estrogen receptors and rapid Akt/NOS activation.," *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, vol. 18, Oct. 2004, pp. 1594-6.
- [91] C. Cosentino, M. Di Domenico, A. Porcellini, C. Cuzzo, G. De Gregorio, M.R. Santillo, S. Agnese, R. Di Stasio, A. Feliciello, A. Migliaccio, and E.V. Avvedimento, "p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival.," *Oncogene*, vol. 26, Mar. 2007, pp. 2095-103.
- [92] A. Pedram, M. Razandi, J.K. Kim, F. O'Mahony, E.Y. Lee, U. Luderer, and E.R. Levin, "Developmental phenotype of a membrane only estrogen receptor alpha (MOER) mouse.," *The Journal of biological chemistry*, vol. 284, Feb. 2009, pp. 3488-95.
- [93] X. Guo, M. Razandi, A. Pedram, G. Kassab, and E.R. Levin, "Estrogen induces vascular wall dilation: mediation through kinase signaling to nitric oxide and estrogen receptors alpha and beta.," *The Journal of biological chemistry*, vol. 280, May. 2005, pp. 19704-10.
- [94] K.L. Chambliss, Q. Wu, S. Oltmann, E.S. Konanah, M. Umetani, K.S. Korach, G.D. Thomas, C. Mineo, I.S. Yuhanna, S.H. Kim, Z. Madak-Erdogan, A. Maggi, S.P. Dineen, C.L. Roland, D.Y. Hui, R.A. Brekken, J.A. Katzenellenbogen, B.S. Katzenellenbogen, and P.W. Shaul, "Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice.," *The Journal of clinical investigation*, vol. 120, Jun. 2010, pp. 2319-30.
- [95] T. Suzuki, H.-P. Yu, Y.-C. Hsieh, M.A. Choudhry, K.I. Bland, and I.H. Chaudry, "Estrogen-mediated activation of non-genomic pathway improves macrophages cytokine production following trauma-hemorrhage.," *Journal of cellular physiology*, vol. 214, Mar. 2008, pp. 662-72.
- [96] S.Y. Lim, Y.S. Kim, Y. Ahn, M.H. Jeong, M.H. Hong, S.Y. Joo, K.I. Nam, J.G. Cho, P.M. Kang, and J.C. Park, "The effects of mesenchymal stem cells transduced with Akt in

- a porcine myocardial infarction model.," *Cardiovascular research*, vol. 70, Jun. 2006, pp. 530-42.
- [97] N. Krishnamoorthy, T.B. Oriss, M. Paglia, M. Fei, M. Yarlagadda, B. Vanhaesebroeck, A. Ray, and P. Ray, "Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma.," *Nature medicine*, vol. 14, May. 2008, pp. 565-73.
- [98] P. Fischer and D. Hilfiker-Kleiner, "Role of gp130-mediated signalling pathways in the heart and its impact on potential therapeutic aspects.," *British journal of pharmacology*, vol. 153 Suppl, Mar. 2008, pp. S414-27.
- [99] T. Tsutamoto, T. Hisanaga, A. Wada, K. Maeda, M. Ohnishi, D. Fukai, N. Mabuchi, M. Sawaki, and M. Kinoshita, "Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure.," *Journal of the American College of Cardiology*, vol. 31, Feb. 1998, pp. 391-8.
- [100] E. Roig, J. Orús, C. Paré, M. Azqueta, X. Filella, F. Perez-Villa, M. Heras, and G. Sanz, "Serum interleukin-6 in congestive heart failure secondary to idiopathic dilated cardiomyopathy.," *The American journal of cardiology*, vol. 82, Sep. 1998, pp. 688-90, A8.
- [101] S.D. Anker and A.J.S. Coats, "How to RECOVER from RENAISSANCE? The significance of the results of RECOVER, RENAISSANCE, RENEWAL and ATTACH.," *International journal of cardiology*, vol. 86, Dec. 2002, pp. 123-30.
- [102] S. Lecour and R.W. James, "When are pro-inflammatory cytokines SAFE in heart failure?," *European heart journal*, vol. 32, Mar. 2011, pp. 680-5.
- [103] S. Negoro, K. Kunisada, E. Tone, M. Funamoto, H. Oh, T. Kishimoto, and K. Yamauchi-Takahara, "Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction.," *Cardiovascular research*, vol. 47, Sep. 2000, pp. 797-805.
- [104] M. Gwechenberger, L.H. Mendoza, K.A. Youker, N.G. Frangogiannis, C.W. Smith, L.H. Michael, and M.L. Entman, "Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions.," *Circulation*, vol. 99, Feb. 1999, pp. 546-51.
- [105] E.K. Podewski, D. Hilfiker-Kleiner, A. Hilfiker, H. Morawietz, A. Lichtenberg, K.C. Wollert, and H. Drexler, "Alterations in Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling in patients with end-stage dilated cardiomyopathy.," *Circulation*, vol. 107, Feb. 2003, pp. 798-802.
- [106] T. Fukada, M. Hibi, Y. Yamanaka, M. Takahashi-Tezuka, Y. Fujitani, T. Yamaguchi, K. Nakajima, and T. Hirano, "Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis.," *Immunity*, vol. 5, Nov. 1996, pp. 449-60.

- [107] K. Boengler, D. Hilfiker-Kleiner, G. Heusch, and R. Schulz, "Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion.," *Basic research in cardiology*, vol. 105, Nov. 2010, pp. 771-85.
- [108] K. Matsushita, S. Iwanaga, T. Oda, K. Kimura, M. Shimada, M. Sano, A. Umezawa, J.-ichi Hata, and S. Ogawa, "Interleukin-6/soluble interleukin-6 receptor complex reduces infarct size via inhibiting myocardial apoptosis.," *Laboratory investigation; a journal of technical methods and pathology*, vol. 85, Oct. 2005, pp. 1210-23.
- [109] T. Yamashita, K. Sawamoto, S. Suzuki, N. Suzuki, K. Adachi, T. Kawase, M. Mihara, Y. Ohsugi, K. Abe, and H. Okano, "Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of Stat3 activation in the protection of neurons.," *Journal of neurochemistry*, vol. 94, Jul. 2005, pp. 459-68.
- [110] M. Obana, M. Maeda, K. Takeda, A. Hayama, T. Mohri, T. Yamashita, Y. Nakaoka, I. Komuro, K. Takeda, G. Matsumiya, J. Azuma, and Y. Fujio, "Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction.," *Circulation*, vol. 121, Feb. 2010, pp. 684-91.
- [111] K.L. Streetz, T. Luedde, M.P. Manns, and C. Trautwein, "Interleukin 6 and liver regeneration.," *Gut*, vol. 47, Aug. 2000, pp. 309-12.
- [112] Z. Sheng, D. Pennica, W.I. Wood, and K.R. Chien, "Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival.," *Development (Cambridge, England)*, vol. 122, Feb. 1996, pp. 419-28.
- [113] R.H. Straub, "The complex role of estrogens in inflammation.," *Endocrine reviews*, vol. 28, Aug. 2007, pp. 521-74.
- [114] C. Lemieux, I. Cloutier, and J.-F. Tanguay, "Estrogen-induced gene expression in bone marrow c-kit⁺ stem cells and stromal cells: identification of specific biological processes involved in the functional organization of the stem cell niche.," *Stem cells and development*, vol. 17, Dec. 2008, pp. 1153-63.
- [115] C.M. Brown, T.A. Mulcahey, N.C. Filipek, and P.M. Wise, "Production of proinflammatory cytokines and chemokines during neuroinflammation: novel roles for estrogen receptors alpha and beta.," *Endocrinology*, vol. 151, Oct. 2010, pp. 4916-25.
- [116] E. Vegeto, S. Belcredito, S. Eteri, S. Ghisletti, A. Brusadelli, C. Meda, A. Krust, S. Dupont, P. Ciana, P. Chambon, and A. Maggi, "Estrogen receptor-alpha mediates the brain antiinflammatory activity of estradiol.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, Aug. 2003, pp. 9614-9.
- [117] C. Heeschen, R. Lehmann, J. Honold, B. Assmus, A. Aicher, D.H. Walter, H. Martin, A.M. Zeiher, and S. Dimmeler, "Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease.," *Circulation*, vol. 109, Apr. 2004, pp. 1615-22.
- [118] C. Bearzi, M. Rota, T. Hosoda, J. Tillmanns, A. Nascimbene, A. De Angelis, S. Yasuzawa-Amano, I. Trofimova, R.W. Siggins, N. Lecapitaine, S. Cascapera, A.P.

- Beltrami, D.A. D'Alessandro, E. Zias, F. Quaini, K. Urbanek, R.E. Michler, R. Bolli, J. Kajstura, A. Leri, and P. Anversa, "Human cardiac stem cells.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, Aug. 2007, pp. 14068-73.
- [119] E. Messina, L. De Angelis, G. Frati, S. Morrone, S. Chimenti, F. Fiordaliso, M. Salio, M. Battaglia, M.V.G. Latronico, M. Coletta, E. Vivarelli, L. Frati, G. Cossu, and A. Giacomello, "Isolation and expansion of adult cardiac stem cells from human and murine heart.," *Circulation research*, vol. 95, Oct. 2004, pp. 911-21.

Abbreviations

MI, myocardial infarction

IL, Interleukin

SCF, stem cell factor

CD, cluster of differentiation

BM, bone marrow

MSCs, mesenchymal stem cells

EPC, endothelial progenitor cells

LV, left ventricular

DNA, deoxyribonucleic acid

TNF, tumor necrosis factor

MNCs, mononuclear cells

E2, 17 β estradiol

ER α , estrogen receptor alpha

ER β , estrogen receptor beta

mRNA, messenger ribonucleic acid

ERKO, estrogen receptor knock-out

PPT, 4,4',4''-[4-propyl-(1H)-pyra-zole-1,3,5-triyl]tris-phenol

DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile

CM, cardiomyocyte

AO, acridine orange

EB, ethidium bromide

DMEM, Dulbecco's modified Eagle Medium

DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride

PBS, Phosphate buffered saline

BSA, Bovin serum albumin

FBS, Fetal bovine serum

APC, Allophycocyanin

FACS, fluorescent activated cell sorting

MACS, magnetetic activated cell sorting

IgG, Immunoglobulin G

v/v, volume/volume

w/v, weight/volume

U/ml, units per milliliter

HBSS, Hanks Balanced Salt Solution

EF, ejection fraction

FS, fractional shortening

LVID, left ventricular internal diameter

PCR, Polymerase Chain Reaction

CFSE, carboxyfluorescein succinimidyl ester

EDTA, ethylenediaminetetraacetic acid

PE, phycoerythrin

HF, heart failure

Acknowledgments/Danksagung

Center for Cardiovascular Research (CCR)/Institute for Pharmacology at Charité in Berlin opened its doors for me three and a half years ago. Thanks to this fact, I was able to develop and guide my scientific interest and become one step closer in reaching independence in scientific thinking and practice. For this reason, I am very grateful to Dr. Jun Li, Priv. Doz. Dr. Elena Kaschina and Prof. Dr. Thomas Unger for giving me an opportunity to become part of the team, for scientific guidance and for believing in my potentials.

As every other, my beginning in Berlin and beginning of work on my doctoral thesis was bound with unknown, uncertainties and difficulties. People whom I work with on daily basis in CCR made this beginning much easier and I thank them enormously on that. Especially, I thank my group colleges Caterina Curato, Wassim Altarche-Xifró, Anna Skorska, Kapka Miteva and Melanie Timm, I have not only learned a lot from, but also for being great friends!

Also, I thank other CCR'ers that unselfishly shared their time and knowledge to help me. For scientific discussion and support I am thankful to Priv. Doz. Dr. Kai Kappert. I also thank my colleges and friends Dr. Ludovit Paulis, Pawel Namsolleck, Veronica Valero for advices and support, and Kristin Lucht, Manuella Sommerfeld and Ulrich Rudolf Kemnitz for daily help in laboratory and animal facility. I am very thankful to Miranda Schroeder for all the help she offered. I thank GK-754 program, BCRT and Charité for financial support.

Further, I thank our collaboration partners: Prof. Dr. Gustav Steinhoff and Dr. Stephan von Haehling, without them this work would not be complete.

I thank Prof. Dr. Marija Mostarica, Prof. Dr. Zorica Ramic and Priv. Doz. Dr. Dusan Popadic from Institute of Immunology, Medical University of Belgrade for introducing me into science and supporting my wish to continue the path I started.

I especially thank my family Ksenija, Vesna, Dragan and my dear Heinz for understanding and patience and for giving me strength to go on when it gets hard.

Berlin, 30th of May, 2011

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Erklärung

„Ich, Svetlana Slavic, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Role of Estrogen Receptor alpha in Post-ischemic Cardiac Protection Mediated by Bone Marrow c-kit+ Cells, selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum
31.05.2011

Unterschrift