Das regenerative Potential der CD117^{+}AT2R stimulierten Zellpopulation *in vitro* und *in vivo*

Kumulative Dissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch – Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von:

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Rostock, Mai 2014

urn:nbn:de:gbv:28-diss2015-0146-3

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to obtain

the academic degree

doctor rerum naturalium (Dr. rer. nat.)

at the Faculty of Mathematics, Physics and Natural Sciences

University of Rostock

submitted by:

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Rostock, May 2014

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Datum der Einreichung: 15.05.2014

Datum der Verteidigung: 05.06.2015

Die vorliegende kumulative Dissertation entstand in der Zeit von Mai 2010 bis April 2014 in den Forschungslaboratorien für kardialen Gewebe- und Organersatz (FKGO), am Referenz und Translationszentrum für kardiale Stammzelltherapie (RTC), an der Klinik und Poliklinik für Herzchirurgie und am Oscar Langendorff Institut für Physiologie am Universitätsklinikum Rostock.

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Abbreviations

ACE	Angiotensin converting enzymes
AcLDL	Acetylated low density lipoprotein
Ang II	Angiotensin II
AP	Action potential
ARB	Angiotensin receptor blocking agents
ASC	Adult stem cells
AT1/2R	Angiotensin II type-1/2 receptor
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMNC	Bone marrow mononuclear cells
BMSC	Bone marrow stem cells
CD	Cluster of Differentiation
CFU	Colony forming unit
Co21	Compound 21
CSC	Cardiac stem cells
CXCR	CXC chemokine receptor
EGFP	Enhanced Green Fluorescent Protein
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence activated cell sorting
EPC	Endothelial progenitor cells
ERK 1/2	extracellular signal-regulated kinase 1/2
ESC	Embryonic stem cells
HIF-1	Hypoxia-inducible-factor 1
(h)MSC	(human) Mesenchymal stem cells
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
iPSC	Induced pluripotent stem cells
JAK/STAT	Janus kinase/signal transducer and activator of transcription
Lin	Lineage
LSK	$Lin^{-}c\text{-}kit^{+}Sca\text{-}I^{+}$
LV	Left Ventricle
Los	Losartan potassium
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
Mmp 2/9	Matrix metallopeptidase 2/9
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PV-loop	Pressure/volume loop
qRT-PCR	Quantitative real-time polymerase chain reaction
RAS	Renin-Angiotensin system
SC	Stem cells
Sca-1	Stem cell antigen-1
SCF	Stem cell factor

Shh	Sonic hedgehog
SLAM	Signaling lymphocyte activation molecule
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
Wnt	Wingless-type

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Zusammenfassung

Der weltweite Anstieg koronarer Herzerkrankungen, insbesondere des akuten Myokardinfarkts (MI), welcher durch einen fortschreitenden Verschluss der Koronararterien hervorgerufen wird, stellt heutzutage eine der größten medizinischen Herausforderungen dar. Neue regenerative Ansätze, wie die vielversprechende Stammzelltherapie, werden benötigt, um die verminderten kardialen Funktionen des nekrotischen Myokards signifikant verbessern zu können. Insbesondere Stammzellen aus dem Knochenmark zeigen einen positiven Effekt auf die funktionelle Regeneration. Jedoch ist ihr Wirkmechanismus noch immer ungeklärt, wobei Transdifferenzierung, Fusion mit Kardiomyozyten oder parakrine Effekte diskutiert werden.

Transmembran Rezeptor Tyrosinkinase CD117 (c-Kit) exprimierende Stammzellen (SZ) kommen sowohl im Knochenmark als auch im Herzen vor. Ihre Injektion in das infarzierte Herz verbesserte die kardialen Funktionen im Tiermodell. Als Reaktion auf eine ischämische Schädigung werden darüber hinaus $CD117^+$ SZ aus dem Knochenmark zum Herzen rekrutiert. In murinen Tiermodellen erhöhten die infiltrierenden und kardialen $CD117^+$ SZ die Expression des vaskulären endothelialen Wachstumsfaktors (VEGF) und etablierten somit ein pro-angiogenes Milieu in der Infarktrandzone. Andere präklinische Studien mit Tiermodellen weisen auf ein Transdifferenzierungspotential der $CD117^+$ SZ in Kardiomyozyten hin. Dennoch ist die Expression kardialer Marker kein ausreichender Nachweis für eine Differenzierung in einen kardialen Zelltyp. Der Nachweis sollte durch die Detektion und Messung funktionaler und zellspezifischer Ionenkanäle erbracht werden. Dieser Arbeit wurde die elektrophysiologische Charakterisierung von $CD117^+$ SZ zu Grunde gelegt, da sie entscheidend für die Sicherheit der implantierten Zellen (Vermeidung von Arrythmien) und deren elektrophysiologische Kopplung an das aufnehmende Myokard ist. Trotz der vielfachen Verwendung von murinen $CD117^+$ SZ in *in vivo* Studien, sind ihre Ionenkanäle noch unzureichend erforscht. Wir passten in dieser Arbeit die Patch-Clamp Methodik für $CD117^+$ SZ an und konnten somit ihre Differenzierung in einen endothel-ähnlichen Phänotyp zeigen und gleichzeitig eine Differenzierung in vaskuläre glatte Muskelzellen oder Kardiomyozyten ausschließen. Im Mausmodell bewirkte die intramyokardiale Applikation von $CD117^+$ SZ eine signifikante Verbesserung der Kapillardichte, der Kollagenablagerung und der Links-Ventrikulären Funktionen drei Wochen nach induziertem MI.

Ein weiterer Schwerpunkt der Arbeit lag beim Renin-Angiotensin System, insbesondere beim Angiotensin II Typ-2 Rezeptor (AT2R), der Signale des aktiven Metaboliten Angiotensin II

weiterleitet und dem Angiotensin II Typ-1 Rezeptor (AT1R) entgegenarbeitet. In der kardialen Regeneration bewirkte eine Stimulation des AT2R eine Kardioprotektion durch die Inhibierung von Inflammation und Apoptose und verbesserte das myokardiale Remodeling in verschiedenen Studien mit Tiermodellen

Die indirekte Stimulation muriner $CD117^+$ SZ für den AT2R ermöglichte uns, Effekte auf die kardiale Regeneration *in vitro* und *in vivo* zu erforschen. Einen wesentlichen Beitrag der Stimulation konnten wir durch eine vermehrte Zellanordnung und einem erhöhten Länge-zu-Breite Verhältnis in einem 2-D Angiogenese Experiment beobachten. Ionenkanäle und gemessene Ströme änderten sich nur geringfügig, was besonders durch einen gesteigerten einwärts-gleichrichtenden Kaliumkanal und dessen erhöhter mRNA Expression deutlich wurde. Die Ergebnisse der ausgeführten *in vivo* Studie wurden durch eine AT2R Stimulation nicht beeinflusst. Wir vermuten, dass die sehr komplexe Regulation des AT2R und seine Interaktion mit dem entgegenwirkenden AT1R eine tragende Rolle spielt.

Zusammenfassend kann das Regenerationspotential der $CD117^+$ SZ parakrinen Effekten, wie der Ausschüttung von VEGF und der Differenzierung in einen endothel-ähnlichen Phänotyp zugeschrieben werden. Eine Transdifferenzierung der Zellpopulation in Kardiomyozyten muss auf Grundlage der gewonnenen Erkenntnisse ausgeschlossen werden. Dennoch konnten wir am Kleintiermodell zeigen, dass die intramyokardiale Gabe der $CD117^+$ SZ eine sichere Applikationsmethode ist und diese Zellpopulation eine therapeutische Wirksamkeit im Sinne einer funktionellen Verbesserung des geschädigten Herzens ausübt.

Summary

The worldwide increasing incidence of coronary heart diseases, particularly acute myocardial infarction, evoked by progressive coronary atherosclerosis, is one of the major medical challenges nowadays.

New regenerative approaches are needed to restore the loss of cardiac function in the necrotic myocardium. Stem cell therapy is one of those promising novel approaches. Numerous studies exist that involve varying delivery sites and methods as well as different types of stem cells. Especially bone marrow stem cells (BMSC) showed a beneficial effect on functional regeneration, but their mechanism of action is still debated and ranges from transdifferentiation to fusion or paracrine effects. Stem cells (SC) expressing the transmembrane receptor tyrosine kinase CD117 (c-Kit) were found in the bone marrow and in the heart. Their implantation improved cardiac functions after myocardial infarction in animal models. In response to cardiac damage $CD117^+$ SC are also recruited from the BMSC pool towards the infarcted heart. In murine animal studies, infiltrating and resident $CD117^+$ SC increased the expression of vascular endothelial growth factor and in this way established a pro-angiogenic milieu in the infarct border zone. Other animal studies claim a transdifferentiation potential of $CD117^+$ SC into cardiomyocytes. Nevertheless, the protein expression of cardiac markers, often shown to verify the differentiation of cells into a specific cell type, does not prove a functional differentiation. This should rather be assessed by the expression of functional and appropriate ion channels. We assessed that electrophysiological properties of implanted cells are crucial in terms of safety and to predict the electrical coupling to host cells. Interestingly, ion channels of freshly isolated murine $CD117^+$ BMSC, especially prior to implantation still lack proper descriptions. In the work presented here we show the differentiation of murine $CD117^+$ SC into an endothelial-like (nonexcitable) phenotype. We could clearly distinguish them from vascular smooth muscle cells or cardiomyocytes. Results were obtained using whole-cell patch-clamp and a range of molecular biological techniques. *In vivo*, the intramyocardial application of $CD117^+$ SC significantly improved capillary density, collagen deposition and left ventricular functions three weeks after experimentally induced myocardial infarction.

A further interest of this work was the renin-angiotensin system, particularly the angiotensin II type-2 receptor (AT2R), which mediates actions of the active metabolite angiotensin II and opposes effects of the angiotensin II type-1 receptor. In cardiac regeneration, the AT2R acts cardioprotective, displayed by anti-inflammatory and anti-apoptotic effects and improved

myocardial remodeling in animal studies. The finding of a cell population positive for AT2R and CD117 provides a potential option for new stem cell therapy. In this project we indirectly stimulated murine CD117 BMSC for the AT2R to dissect beneficial effects *in vitro* and *in vivo*. One major contribution was seen in the enhanced cell alignment and increased elongation ratio in a 2-D angiogenesis assay. Ion channels and assessed currents changed only moderately under AT2R stimulation, which was revealed by an increased inward rectifying potassium current (I_{Kir}) and increased I_{Kir} mRNA expression. Therefore, electrophysiological and molecular properties of control and AT2R-stimulated cells point to a differentiation to vascular endothelial cells. *In vivo* outcomes were not challenged by an additional stimulation of $CD117^+$ SC, which might be due to the complex regulation of AT2R and the interplay with its opposing receptor.

In summary, a regenerative potential of the $CD117^+$ SC population might be attributed to enhanced paracrine effects, such as the excretion of VEGF and their differentiation into an endothelial-like phenotype. The transdifferentiation of $CD117^+$ SC has to be excluded here. However, this work using a small animal model shows that the implantation of those cells is safe and contributes to functional improvements of the damaged heart.

Introduction

1 Incidence and pathophysiology of myocardial infarction

One of the challenges of the twenty-first century is the increasing burden of organ failure in an aging population, especially caused by cardiac diseases. Despite substantial progress in the treatment of coronary heart diseases, 7.3 million people died of heart failure in 2008, a number, which will continuously rise especially in low-income countries [1]. The most common cause of acute myocardial infarction (MI) is a coronary occlusion due to progressive coronary atherosclerosis and thrombus formation. Coronary occlusion results in an insufficient supply of oxygen and nutrients and a subsequent death of myocytes in the affected area of the heart. In most cases the left ventricle is affected, since its muscle layer is thicker than the right ventricle and energy consumption is higher. The infarcted and the unaffected myocardial regions undergo progressive changes, which are determined by the magnitude of myocytes loss, the stimulation of the sympathetic nervous system, the stimulation of the renin-angiotensin-aldosterone system and the release of natriuretic peptides [2]. The subsequent phases of cardiac wound healing in the humans are 1) myocytes cell death due to necrosis and apoptosis 2) acute inflammation 3) granulation tissue and scar formation and 4) remodeling of the left ventricle. Myocyte cell death usually develops in the subendocardium and broadens towards the epicardium until it spans the entire ventricular wall. If blood flow can be restored in time, reversibly damaged areas at the edges of the infarcted area (stunned myocardium) can recover even without an inflammatory reaction [3]. In irreversibly damaged areas, the inflammatory response is the prerequisite for healing and scar formation [4]. Following myocyte death and the subsequent release of cytokines, inflammatory cells, especially phagocytic neutrophils, enter the infarct area. Infiltrating granulocytes, lymphocytes and macrophages follow, which release cytokines, growth factors and proteases [5]. Moreover, macrophages and fibroblasts engulf the necrotic tissue and make up the granulation tissue [6]. Maturing of the granulation tissue is associated with scar formation including fibroblast proliferation and collagen deposition. The developed scar tissue stabilizes the infarcted area, but cannot actively contribute to the pumping of the heart. This phenomenon negatively influences the mechanical stress of the intact tissue leading to adaptive compensatory changes in affected cardiac cells termed hypertrophy. It is characterized by an enlargement of myocytes to compensate for the diminished cardiac

functions and to attenuate progressive dilatation [7]. In the long run hypertrophy decreases resilience of cardiomyocytes, provokes progressive dilation of the ventricle and deterioration of contractile functions. This remodeling process alters the ventricular architecture as the heart aims to normalize the increased wall stress. Together, the mechanism of hypertrophy, the increased fibrosis as well as collagen deposition and the loss of contractile tissue lead to ventricular stiffness and dysfunction. Eventually, the chronic usage of compensatory processes leads to chronic heart insufficiency and cannot save the infarcted heart.

Depending on the localization of the thrombus and on the duration of the occlusion, MI, which causes heart failure, may wipe out up to 25% of the 2-4 billion cardioymyocytes of the human left ventricle [8]. In contrast to zebrafish or some urodele amphibians, such as newts, humans are incapable of true cardiac regeneration after a major injury. Thus, state-of the art therapies range from drugs, such as antiplatelet drugs, angiotensin-converting enzyme inhibitor or beta-adrenergic blocking agents to surgical procedures providing reperfusion (e.g. Angioblasty, Coronary Artery Bypass Graft or Coronary stenting) and eventually to heart transplantation. However, organ supply is limited to a very small minority of patients. Available drugs and invasive therapies need to be supported or superseded by novel therapeutic approaches, like tissue engineering or cell therapies that restore tissue function and are available for the majority of patients. Due to ethical and practical reasons it is out of question to test new diagnostics and therapeutic approaches in human beings. Approaches for 2D and 3D-cell culture models exist, but they lack the reproduction of microenvironment, especially the complex immune response [9]. Currently, experimental MI in animal models is indispensible to simulate the pathophysiology of an infarcted heart and to subsequently develop new successful therapies. Yet, it has to be taken into account that species and gender of the laboratory animal influences the pathophysiology of induced MI. Certain limitations, such as the small size of the heart and structural differences compared to the human heart should be considered when using mouse models and transferring results to human. Nevertheless, major advantages include the existence of transgenic and knockout mouse strains, ease of genetic modifications, handling and breeding [10]. To gain further insights into the pathophysiology of post-MI and into its molecular basics, mouse models are an ideal and established tool [11, 12].

The small animal model of MI can be evaluated by determining changes in cardiac functions *in vivo* using catheter-conductance method [13]. The application of an indwelling catheter in the left ventricle (LV) of mice offers the possibility to generate pressure-volume relationships, which are independent of the LV chamber geometry and highly accurate despite the rapid

heartbeat [14]. Important parameters are 1) the ejection fraction, which gives the percentage of change in ventricular volume in one heartbeat; 2) the cardiac output, which is the volume of blood (stroke volume) the heart pumps per minute (heart rate) and 3) the developed maximum pressure or the first derivative of intraventricular pressure (dp/dt) [15].

2 Growth and remodeling of blood vessels

The adaptation of blood vessels is a component of myocardial remodeling and a potential target of therapeutic strategies. The functional restoration of damaged tissue is dependent on the re-establishment of collateral networks and subsequent supply of the energy demanding myocytes with oxygen and nutrients. Tissue ischemia and dilatation of existent blood vessels induce the upregulation of angiogenic growth factors and the mobilization of circulating cells that stimulate cardiac vessel growth. Favorable vessel formation in tissue ischemia involves the three main processes of neovascularization: vasculogenesis, angiogenesis and arteriogenesis [16, 17]. Vasculogenesis broadly comprises the *de novo* formation of blood vessels and is responsible for earliest blood vessel formation during embryonic development. Endothelial progenitor cells (EPC) migrate towards the region of vascularization and differentiate into mature endothelial cells (EC) that form the primary vascular plexus and the later vascular network [18]. Current research demonstrated that vasculogenesis also contributes to the vascular remodeling process elicited by pathological conditions, such as wound healing or ischemic injury, in the adult organism. In this regard, increasing numbers of circulating EPC were detected shortly after MI in the peripheral blood [19].

Angiogenesis is defined as the sprouting of new capillaries from pre-existent blood vessels by EC proliferation and migration resulting in new capillary networks [20, 21]. The development of new capillaries either divides a vessel into two parts or forms new branches and is driven by a growth factor gradient. The major stimulus of angiogenesis is hypoxia leading to the upregulation and accumulation of hypoxia-inducible-factor 1 (HIF 1), a transcription factor that regulates oxygen homeostasis [22]. HIF-1 regulates the expression of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin, which in turn activates EC as well as vascular smooth muscle cell migration, proliferation and vessel enlargement [20, 23].

The term arteriogenesis refers to the process of maturation and outgrowth of pre-existing collateral arteries or their *de novo* growth to bypass arterial stenosis in response to

hemodynamic changes. Shear stress and the subsequent local activation of endothelium are the initial trigger of arteriogenesis, which typically occurs outside the area of ischemia and was found to be independent of the tissue oxygen level [24]. The stimulated EC release adhesion molecules and chemokines, attracting blood-derived monocytes, which secrete growth factors (VEGF, bFGF and platelet-derived growth factor), cytokines and matrix-degrading enzymes [16, 25, 26]. Arteriogenesis further involves basal membrane degradation, proliferation of vascular cells (endothelial and smooth muscle) and structural remodeling of the extracellular matrix [25].

3 Definition and properties of stem cells

Since their discovery more than a century ago, the research on stem cells (SC) has come a long way and offers many therapeutic approaches for various diseases. Their inherent characteristics make them promising candidates for extensive usage and distinguish them from somatic cells. A stem cell is defined by its ability to self-renew through cell division even after long periods of inactivity and its ability to differentiate into a range of more specialized progenitor cells that eventually give rise to functionally mature cells. Moreover, in contrast to most somatic cells, SC show telomerase activity, which enables them to proliferate for an unlimited time span [27]. The division of SC occurs either symmetrically (emerging of two uniform SC or two uniform specialized cells) or asymmetrically (emerging of one stem cell and one more specialized progenitor cell), a decision crucial for homeostasis and tissue maintenance.

By now, SC were detected in almost any tissue or organ. Under normal conditions, SC are needed to preserve the normal cell turnover and replenish specialized cells lost through minor injuries in tissues, such as skin, bone marrow (BM) and intestine.

Types of SC can be divided either by their potency or by their tissue of origin. The potency of a stem cell is the capacity to differentiate into specialized cell types. A totipotent cell can differentiate into embryonic and extraembryonic cell types and thus, give rise to a complete organism [28]. During embryonic development, from the morula stage onwards, cells lose their totipotency and become pluripotent. Pluripotent cells have the potential to develop into any specialized cell type of the adult organism and to generate all cell types of the three germ layers, except of the trophoblast (giving rise to the placenta) [29]. Pluripotent SC differentiate into multipotent progenitor cells. Those multipotent cells can develop into a number of different cell types, but are thought to be committed to a specific germ layer and produce a

closely related family of cells. Hematopoietic stem cells (HSC), for example, are considered multipotent as they are able to give rise to all types of blood cells [28, 30]. Finally, unipotent cells reflect the lowest potency. These cells are able to self-renew and replenish cells of a single lineage in the adult tissue, meaning that they are already committed to a tissue.
The origin divides SC into embryonic and adult stem cells. The possibility of reprogramming specialized adult cells into a state that closely resembles embryonic stem cells (ESC) gave rise to another stem cell type: induced pluripotent stem cells (iPSC) [31].

3.1 Adult stem cells

Adult stem cells (ASC) are present in most of the tissues of the human body and are also referred to as somatic SC. Their primary role is to maintain the tissue in which they are found, such as brain, spinal cord, digestive system, liver, pancreas or heart. Most ASC are lineage restricted and thus, multipotent. However, few types of ASC, such as cord blood stem cells are possibly pluripotent and display plasticity between the germ layers. Among others, this transdifferentiation phenomenon was even reported for adult BMSC differentiating into cardiomyocytes *in vitro* [32]. ASC reside in a specific area of each tissue, the cellular niche, which determines their survival, quiescence and activation [33]. Self-renewal and the balance between maintenance of the stem cell pool and production of progenitor cells engaged in tissue differentiation [34] is regulated by developmentally conserved signaling pathways: Notch [34], Wingless-type (Wnt) [35, 36], Sonic hedgehog (Shh) and Smad [37, 38].
The major source of ASC is BM, which harbors mesenchymal stem cells (MSC) and HSC. Recently, both stem cell types have received increasing attention concerning their potential for stem cell therapy. In contrast to ESC or iPSC, not a single incidence of oncogenic transformation has been reported for bone marrow stem cells (BMSC) [39].

3.2 Hematopoietic stem cells

The existence of SC in BM was already proven in 1963 by the pioneering studies on the blood regenerating system by Till and McCulloch [40]. Within the hematopoietic system HSC are the only cell population capable of self-renewal and multi-potency. During bone marrow transplantation studies, it was shown that HSC are crucial for long-term engraftment and reconstitution [41]. In fact, HSC have the potential to differentiate into all functional blood

cells [42]. Most of those HSC-derived blood cells arise and mature in BM. In a healthy adult, a small population of HSC produces the enormous number of 500 billion blood cells every day, demands that require strict control over hematopoietic progenitor proliferation [43].

For maintenance of the hematopoietic system, HSC must balance symmetric and asymmetric cell division, a process that requires the stem cell niche [33, 38]. HSC that lose contact to their niche progress towards a more differentiated state. The complex niche is a three-dimensional scaffold in the space between the vessels and the endosteum of the BM and consists of various cell types of which some are only recently elucidated. Non-hematopoietic (osteolineage cells, MSC, endothelial and perivascular cells and adipocytes) and hematopoietic (osteoclasts, macrophages and neutrophils) cellular components, together with cell-autonomous mechanisms, regulate HSC self-renewal, quiescence and responses to environmental signals [43]. The question how the niche directs self-renewal and differentiation of HSC remains. It was shown that niche compartments, especially osteoblasts and MSC, secrete cytokines and chemokines, such as granulocyte-conlony stimulating factor, granulocyte macrophage-colony stimulating factor, stem cell factor ligand (SCF) and CXCL12 (SDF-1), which influence HSC fate [35]. In this regard, and considering the recruitment of HSC to injured ischemic tissues, mobilization (recruitment) and homing are two mirror processes of major importance for future stem cell therapy [44]. By now it is known that the CXCR4/CXCL12 axis is a central regulator of HSC migration and retention. CXCL12 recruits CXCR4 expressing stem cells including HSC, EPC, CSC and MSC [45]. The presence of those chemokines influences if HSC leave the BM, enter the circulation, relocate to distant tissue (recruitment) or, eventually, return to the BM (homing) [46, 47]. The recruitment of stem and progenitor cells in the disease state of MI is also influenced by endothelial nitric oxide synthase (eNOS) and its generation of nitric oxide (NO) [48]. An increased eNOS expression in the tissue is associated with an enhanced migratory capacity of HSC and improved neovascularization. Moreover, eNOS expressing cells were found to contribute to the long-term effects of bone marrow cells in regenerative approaches [49]. Interestingly, mice lacking eNOS showed reduced VEGF-induced mobilization of EPC [50].

The analysis of peripheral stem cell migration using the elegant technique of intravital microscopy applied to the mouse cremaster microcirculation was recently described by our group [51]. This technique will contribute to enlighten necessary microenvironmental preconditions to enhance rolling and adhesion of intravascularly administered SC at the endothelial lining.

3.3 CD117 (c-Kit) stem cells

One marker to characterize HSC in mice and humans is the transmembrane receptor tyrosine kinase CD117 (c-Kit). In concert with its ligand SCF, CD117 plays an important role in HSC function and senescence. CD117 belongs to the type III receptor tyrosine kinase family and was highly conserved during evolution. The structure of CD117 consists of four domains: an extracellular domain, a transmembrane domain and an intracellular domain. The latter is split in a juxta-membrane domain and a tyrosine kinase domain by an insert region. The extracellular domain contains five immunoglobulin-like (Ig-like) domains. The first three Ig-like domains are binding sites for a SCF molecule and are left unaltered upon binding, while the last two domains in the membrane proximal region establish the contact between two activated CD117 receptor molecules [52]. The juxta-membrane domain interacts with the kinase domain and regulates its catalytic activity by stabilizing an inactive configuration, thus "autoinhibiting" the receptor [53]. SCF is a heavily glycosylated transmembrane protein that, following cleavage, exists in two forms with different effects on CD117 autophosphorylation: a membrane associated (sustained phosphorylation) or soluble (transient phosphorylation) form [54]. The simultaneous binding of dimeric SCF to two CD117 monomers results in receptor dimerization and subsequent activation of intrinsic tyrosine kinase activity and downstream signal transduction [55]. Following activation, tyrosine residues, primarily outside the kinase domain, become phosphorylated and function as docking sites for intracellular signal transduction molecules. Activation of the Ras/ERK1/2 pathway and the following transcription factors is of critical importance for cell division and survival as well as hematopoietic cell differentiation and migration [56]. Furthermore, the activated Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway regulates cell proliferation, differentiation and apoptosis. Direct association of CD117 and PI3K (Phosphoinositide 3'-kinase) signaling is required for most SCF-mediated responses. Among others, this pathway mediates SCF-induced chemotaxis and Ca^{2+} mobilization, adhesion, actin assembly, proliferation and also anti-apoptotic signaling via the activation of AKT [57, 58]. Moreover, CD117 activation triggers SFK pathway activation functioning in cell cycle progression, chemotaxis, adhesion, survival, and protein trafficking and plays an important role in ligand-induced internalization of the receptor [59]. Other activated pathways include the Src kinase signal transduction pathway related to cell survival, angiogenesis, proliferation, motility and migration and the PLC-γ pathway involved in cell proliferation and Ca^{2+}-release [60]. The multiple pathways influenced by CD117-SCF require negative

regulation as achieved by PKC family, the tyrosine phosphatase SHP1, the ubiquitin ligase Cbl and the phosphatase SHIP [54]. Interestingly, CD117 was found to be required for terminal cardiomyocyte differentiation [61] and is crucial for EC recruitment as well as neovascularization [62].

In humans and mice, CD117 is widely distributed and expressed on various cells, such as HSC, germ cells, mast cells, EC, CSC, cardiomyocytes, glandular breast epithelial cells and Cajal cells in the gastrointestinal tract [63]. The receptor is found on 1 - 4 % of BMSC in healthy individuals and 60-75 % of $CD34^+$ HSC [64].

4 Stem cell therapy for cardiac regeneration

At the beginning of the 2000s the detection of cardiac stem cells (CSC), distinct from bone marrow hematopoietic stem cells, has challenged the perspective of cardiac biology [65]. The existence of SC in the heart implicated that the physiological cell turnover of cardiac cells is regulated by differentiation of CSC. Consequently, the heart can be partly considered as a self-renewing organ, in which resident SC are at least able to replace old, dying cells in the absence of injury [66]. Moreover, it was proven recently that genesis of cardiomyocytes occurs at a low rate (only up to 1% per year in young adults) by the division of pre-existing cardiomyocytes. Cardiomyocyte cell-cycle activity was detectable during normal ageing and injury [67, 68]. However, the potential of resident SC and cardiomyocyte turnover for regeneration is not clinically relevant and cannot prevent the adverse remodeling processes of the heart emerging from MI. Moreover, the loss of cardiac function in the necrotic myocardium cannot be restored with conventional therapeutical options [69]. Therefore, the field of regenerative medicine and stem cell therapy aims to improve regeneration of injured tissue by the delivery of SC.

Regenerative approaches aiming at a robust therapy to regenerate lost myocardium are numerous and involve varying delivery sites and methods as well as different types of SC, thought to possess progenitor features. Stem cell populations delivered include adult progenitor cells, such as bone marrow mononuclear cells (BMNC) [70-72], CD133+ cells [73, 74], MSC [75], resident CSC [76-78] and skeletal myoblasts [79, 80] as well as ESC [81] and iPSC [82, 83]. Interestingly, any cell type proved to have at least some beneficial effect on regeneration - a finding that complicates the revealing of the underlying mechanisms [8]. By now it is controversially discussed if BMSC are able to truly transdifferentiate into mature,

functional cardiomyocytes that electromechanically couple to host myocytes *in vivo*. However, expecting a paracrine effect of these cells in terms of increasing and provoking neovascularization in the injured heart makes them promising candidates for patient-specific cell therapy [84-86].

Other studies challenge the clinical safety and point out the need for further sound investigations. MSC transplanted into the heart developed bone structures [87] and implantation of skeletal myoblasts or MSC might lead to the development of arrhythmias [80]. Even though electromechanical coupling of implanted cells is not the prerequisite for an improved function, the possibility of misdirected electrical signaling has to be investigated prior to clinical testing.

4.1 $CD117^+$ stem cells for cardiac regeneration

The many identified cardiac stem and progenitor cell types include CSC positive for $CD117^+$ cell surface antigen. In terms of feasibility, the isolation of $CD117^+$ SC from the BM is superior to cardiac $CD117^+$ retrieval. A certain regenerative potential of $CD117^+$ (mice, human) or c-Kit^+ (rats) SC has been demonstrated *in vivo*, but the underlying mechanisms are still debated. In response to cardiac damage, the $CD117^+$ cells in the heart are not only resident CSC, but are additionally recruited from the BM stem cell pool [47, 88]. After being recruited to the infarcted heart, infiltrating and resident $CD117^+$ SC increase the expression of VEGF and, in this way, establish a pro-angiogenic milieu in the infarct border zone. The mobilization of $CD117^+$ SC in infarcted adult heart was reported to result in extensive angiogenesis, but they appeared incapable of cardiac myogenesis [89]. Various cell transplantation experiments also claim a differentiation potential for those BM-derived SC into “cardiomyocytes,” followed by improved cardiac functions [76, 90]. However, the findings of newly formed “cardiomyocytes” often turned out to be too optimistic, as those “cardiomyocytes” are either immature or eventually died by apoptosis [78]. Tallini et al. (2009) provided evidence that the $CD117^+$ SC from murine neonatal heart can differentiate into cells of 3 cardiovascular lineages [91]. Of note, their study showed that CD117 re-expression in the injured adult heart is associated with reduced cardiac injury, but not myogenesis, and is solely activated by local conditions, such as hypoxia and inflammatory cytokines. Xaymardan et al. (2009) showed that HSC, but not MSC, have the ability to form beating clusters [92]. The group also reported the potential of $CD117^+$ BMSC to induce myogenic differentiation in short-term culture. However, the formation of a mature

cardiomyocyte phenotype was not achieved with $CD117^+$ cells under those culture conditions. Unfortunately, impressive *in vitro* results, claiming a transdifferentiation of those cells, are often not reproducible using *in vivo* settings [85, 93]. Several studies were conducted that employed rodents as the animal model of choice showing a functional improvement, which does not necessarily correlate with increased regeneration. It could be rather due to reduced inflammation and further protection against adverse remodeling. The direct comparison and translation to humans is hampered, because of their distinct nature and hydrodynamics. Another debated point is the possible mechanism of action that either changes a $CD117^+$ CSC or BMSC into a (mature) cardiomyocyte or that provokes improved cardiac functions. Possible mechanisms include transdifferentiation [32, 94], cell fusion with mature cardiomyocytes [93, 95], or paracrine effects, specifically the stimulation of angiogenesis and myogenesis in the infarcted region [85, 88, 96-98]. Owing to the existent controversies on $CD117^+$ SC, this study first of all, aimed to investigate the behavior of those cells in terms of differentiation, angiogenesis and electrophysiological potential *in vitro*.

The previously reported phenomenon of transdifferentiation of adult cells remains questionable mostly due to methodological problems. Especially the process of cell labeling and tracking is discussed to produce false-positive or false-negative detection of implanted cells. Errors might occur by dilution of cell staining dye during cell division or cell-to-cell transfer of either staining dye (e.g. through gap junctions) or of transfected vectors inducing expression of detectable proteins [99]. To overcome these problems, sex-mismatched transplantation, the usage of transgenic animals or advanced imaging technologies such as position emission tomography have been described [100]. Moreover, the detection of transdifferentiation might be caused by the fusion of host adult cells with transplanted SC. In the present work, the challenge of cell detection was approached by using transgenic mice, expressing EGFP in all nucleated cells as cell donor. The apparent problem of low cell retention after intramyocardial cell injection was taken into account by using BD Matrigel™ as an extracellular matrix. BD Matrigel™ is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. It is liquid at four degrees and solidifies quickly after implantation. However, the injection of Matrigel™ is restricted to animal models.

5 The Renin-Angiotensin System and its receptors

The renin–angiotensin system (RAS) interferes with inflammation and acute cardiac remodeling processes during cardiac injury. This endocrine system consists of a cascade of enzymes and hormones, involved in the regulation of blood pressure and electrolyte re-absorption. The actions of two important enzymes, renin and angiotensin converting enzyme (ACE), contribute to produce the active metabolite angiotensin II (Ang II). Ang II can bind to its distinct receptors (AT1R, AT2R) with the same affinity [101]. Several pharmacological agents are available to interact with this complex peptidergic system, for instance, ACE inhibitors (e.g. captopril) and AT1 receptor blockers (ARB) (e.g. Losartan potassium). It was shown that myocardial hypoxia is associated with an increased activation of RAS, increased Ang II concentration and an increased expression of Ang II receptors [102-104].

The octapeptide Ang II is one of the most important regulators of physiological and pathological processes in the cardiovascular system. Two Ang II receptor subtypes, namely AT1R and AT2R, have been characterized so far. Although both receptors have long since been identified [105], it was generally speculated that the AT2R fulfills only minor functions that complement the AT1R-mediated actions. This notion was challenged by the recent findings of hidden opposing functions of the AT2R.

The two receptor subtypes share only 34% homology, and belong to the seven transmembrane G-protein coupled receptor family [101, 106]. Moreover, both receptor subtypes show a very distinct expression and regulation pattern as well as different signaling pathways. Activation of the AT1R by Ang II triggers vasoconstriction and sodium re-absorption and influences cell growth and apoptosis. Importantly, stimulation of AT1R is associated with cardiomyocyte hypertrophy, tissue damage, inflammation, and negative tissue remodelling [101, 107]. Until now, it was generally accepted that AT2R is highly expressed in fetal tissue. In adult human, mice and rats, expression seems to be restricted to certain cell types and tissues. The low AT2R expression in adults can be re-expressed under pathological conditions, such as stroke or myocardial infarction [101, 108-111]. Thus, the distinct tissue distribution and expression pattern of AT2R point to its potential role in development and differentiation, as well as tissue regeneration. Interestingly, this common assumption is challenged by the finding of Gao et al. (2012), who describe a higher expression of AT2R in adult than in fetus in contrast to AT1R [112]. They argue that the accepted dogma was solely based on observations in the skin and not verified for other organs. In their study, they discussed protein expression and validated

their findings in mice and rat [113]. If their results can be reproduced the current view on AT2R regulation, maturation and function has to be re-evaluated.

Research on the AT2R is conducted mainly indirectly, and previous investigations are based on transgenic animals or AT1R and AT2R antagonists [114, 115]. In 2004, a selective nonpeptide, Compound21 (Co21), was developed as the first AT2R agonist [116]. Co21 is assumed to have selective affinity, excluding AT1R activation, and allowing researchers to explore AT2R mediated actions [116]. The obtained results pointed to a very complex and often unconventional receptor signaling, implying constitutive receptor activation [117, 118]. AT2R pathways are either mediated by G-proteins or are G-protein independent. The activation of Src homology region 2 domain-containing phosphatase 1 is a G-protein independent signaling pathway and involves the physical association to AT2R [119, 120]. The activation of such phosphatases and the subsequent inactivation of kinases seem to be the main effects of AT2R signaling, which influence the actions of growth factors, cytokines, and the AT1R. AT2R controls proliferation and the inflammatory actions of kinases, such as MAPK or JAK in certain cell lines [119]. Anti-inflammatory effects of AT2R are mediated through inhibition of the phosphorylation and translocation of STAT by nuclear factor-kB [121]. In addition, AT2R regulates vasodilation via the activation of bradykinin, NO, and subsequently, cyclic guanosine 3′,5′ in the vasculature [122, 123]. Vascular relaxation is mediated by AT2R via the PI3K/Akt pathway, which stimulates NO production in vascular smooth muscle cells (VSMC) [124].

As controversies exist on the specificity of the AT2R agonist Co21 [125, 126], current research continues to use an indirect AT2R stimulation approach [127, 128]. While Ang II binds to both receptors with nearly the same affinity, AT1R antagonists are used to allow investigations on AT2R. In this work, the AT1R antagonist Losartan potassium (2-butyl-4-chloro-1-[p-(o-1H-tet-razol-5-ylphenyl)benzyl]imidazole-5-methanol mono potassium salt) (Los) was used followed by Ang II treatment. This provoked a more than ten-fold increase in AT2R mRNA expression level compared to untreated and AT2R-inhibited (AT2R inhibitor PD123319) $CD117^{+}$ SC (Fig. 1). An AT1R/AT2R functional crosstalk was already reported by Volpe et al. (2003) [129]. They described that an increase of AT2R mRNA expression occurred during treatment with AT1R antagonists. Moreover, Maseo et al. (1996) showed an inhibitory effect of AT1R on AT2R that was reversible with the AT1R antagonist Losartan potassium [130].

5.1 The AT2R in cardiac regeneration

Major actions of AT2R in the injured organism involve anti-inflammation and anti-proliferation processes. Besides, the high expectations for AT2R in cardiac regeneration were based on its expression during development of the fetus, and its re-expression during pathological conditions. Even though, the described regulation of AT2R protein expression is now questioned, recent research confirms a potential role for tissue regeneration. A non-cytotoxic $CD8^+AT2R^+$ T cell population was identified mediating anti-inflammatory (via NF-kB) and cardioprotective actions against ischemic heart injury [121, 131]. The anti-inflammatory effects of AT2R signaling involve serine/threonine and tyrosine protein phosphatases. The blockade of those phosphatases results in reduced TNF-α-induced IL-6 expression and decreased TNF-α mRNA levels. This was reported for a murine model using Co21 and also for human umbilical vein endothelial cells [121].

It was recently reported that AT2R contributes to cardioprotective cellular mechanisms by modulating both T cell and progenitor cell subpopulations in a rat model of MI [110, 131]. In studies using transgenic mice that overexpress AT2R in cardiac tissue, a cardioprotective effect was mediated via the NO pathway. Moreover, AT2R overexpression was associated with improved contractile function in adjacent non-infarcted myocardium [132, 133].

On the other hand, a negative effect of AT2R expression on cardiac remodeling was described in a mouse model. Loss of AT2R signaling (AT2R-knockout mice) was related with suppressed tissue fibrosis and abolished hypertrophic responses [134].

Still, the functions of human cardiac AT2R have not been addressed, and the translational relevance of the above mentioned results remains undefined. Thus, the first step to further investigate molecular and cellular mechanisms of AT2R actions and their therapeutic potential is to reproduce and strengthen findings from a rat model in another animal model.

6 Beneficial combination of CD117 and AT2R for regeneration

A positive role for cardiac repair is ascribed to the CD117 and the AT2R in independent research studies. Important signals that increase the regenerative potential of $CD117^+$ SC via paracrine signaling are still unknown. At the same time, the paracrine effects of AT2R are not fully understood. Whether the two receptors will find their way into new therapeutic approaches might be influenced by their successful interplay. It was recently shown that a

$CD117^+AT2R^+$ cell population exists, and increases in response to MI in rats [110]. The rat $CD117^+AT2R^+$ cell population showed a significantly increased expression of genes associated with self-renewal (c-Myc, Akt) and cardiac differentiation (GATA-4, Nkx-2.5). Moreover, these cells attenuate apoptosis of co-cultured cardiomyocytes by phosphorylation of STAT-3 and Akt. The above mentioned study was conducted in a single research group in a rat model. Thus, the question arises whether the findings are reproducible in a different animal model and if the (indirect) stimulation of AT2R on $CD117^+$ cells has an influence on their potential for regeneration. An improved function of the injured heart would reveal beneficial effects of implanted cells. In this context, the promotion of angiogenesis should be considered. Stimulation of angiogenesis was shown for $CD117^+$ SC and might be their main mechanism of action [135]. As for AT2R, a beneficial and an inhibiting influence on angiogenesis were reported [136, 137]. Hence, this work addressed the question of the inherent properties of $CD117^+$ SC with or without AT2R stimulation in regard of a possible *in vivo* application.

7 Electrophysiological investigations

7.1 Molecular basis of patch-clamp recordings

The electrical properties of membranes are valid in all animal cells. They are based on the asymmetrical distribution of ions between the cytoplasm and the extracellular space, which are separated by the plasma membrane, an effective barrier for charged particles. In general, the membrane forms an insulator between two conductors and all three components together form a capacitor capable of storing charge. Regarding a biological system, it should be kept in mind that 1) the capacitance is proportional to the membrane surface area 2) current is proportional to the driving force of ions and 3) current is inversely proportional to the resistance of the membrane. The difference of the positive charge outside and the negative charge inside of the biological membrane is known as the resting potential of a cell. By convention, the reference for potentials in a cell system is the extracellular medium, creating a negative resting potential. In animal cells this charge difference is largely driven by passive ion movements through ion channels and maintained by electrogenic pumps, such as the Na^+/K^+ pump or Ca^{2+}/Na^+ exchanger. Ions, namely K^+, Na^+, Cl^- or Ca^{2+} move down their concentration gradient, a movement opposed by an electrical field or the present membrane

potential. Any cell establishes an individual resting potential, depending on its ion concentration, after an equilibrium condition between electrical gradient (voltage gradient) and the ion concentration gradient is reached. The resting potential of most cells is determined by the K^+ gradient and thus, driven towards the K^+ equilibrium potential (-89 mV; no net flow of K^+). However, any ion can significantly influence the membrane potential if the membrane's permeability for a certain ion changes [138]. The potential at which a certain ion gradient is in equilibrium is described by the *Nernst* equation. Following this equation and considering each ion species at a time, the approximate equilibrium potentials are: E(Na)=+60 mV; E(Ca)=+130 mV; E(K)=-89 mV; E(Cl)=-80 mV [139].

Ion channels function on the basis of the above mentioned mechanisms and completely differ from membrane pumps. They form aqueous pores that connect the intracellular medium with the extracellular solution and perform passive transport coupled to the electrochemical gradient [140]. Moreover, ion channel types can be distinguished by ion selectivity and gating. Opening of a channel is transiently achieved either by a change in membrane potential (voltage-dependent), by the binding of a ligand (ligand-dependent; intracellular or extracellular), activated by a second-messenger (Ca^{2+} or cyclic nucleotides), activated by mechanical tension (stretch-activated) or by membrane-bound G-proteins [139]. The repertoire of functioning channels in each cell is enormous and adapted to the special role each cell plays in the body. The mechanism of gating corresponds to the channel protein confirmation in an open, closed or inactivated state and is a parameter to categorize ion channels into families. The two major potassium channel families are voltage-gated potassium channels (e.g. delayed rectifier or Ca^{2+}-dependent K^+ channels) and inward rectifiers (I_{Kir}), which are voltage-independent. Rectification refers to the property of channels that biases the preferred direction of current flow to either the inward or outward direction.

7.2 Patch-clamp technique

In 1991 the Nobel prize was awarded to Erwin Neher and Bert Sakman for developing the patch-clamp technique on basis of the one-electrode voltage clamp [141]. According to the research question, the cell type and the ion channel(s) under investigation, different patch-clamp configurations, such as inside-out, outside-out, cell-attached or whole-cell recording exist. Patch-clamp is now the basis for most electrophysiological investigations, as it enables to record currents through a single open channel. In general, it allows the measurement of

currents across the cell membranes, which are mediated by channels and carriers, while controlling the membrane voltage. The principle of patch-clamp is to form an extremely high-resistance seal (up to several Giga Ohm; Gigaseal) by close contact between a previously pulled glass capillary (patch-pipette) and the cell membrane. The formed Gigaseal prevents 1) leak currents between the pipette and the electrode and 2) the flooding of the cell with extracellular bath solution. The cell current can be influenced either by an exchange of the surrounding solution or by modulation of the internal capillary solution, which diffuses inside the cell. To reach the whole-cell patch-clamp configuration used in this work, the approximated membrane patch should be broken by suction or high-voltage pulses after the formation of a Gigaseal, making the pipette continuous with the cell. Afterwards, programs of specific voltage-steps allow analyzing voltage- and time-dependent changes in membrane conductance and the measurement of whole-cell current. The command voltage programs that elicit a certain cell current can be adjusted and made to vary over time to study specific channel properties and are constantly recorded. The whole-cell current is the product of the number of functional channels in the cell membrane, the probability that the "average" channel is open and the single-channel current [139]. The current itself is composed of ion-specific-, leak- and capacitive current [142]. To distinguish the functional ion channel types in a cell, ionic current components can be separated from total current, which was described by Hodgkin and Huxley and became essential for their description of the course of an action potential [143]. Specific inhibitors and enhancers of the ion pathways through the membrane are known for most of the investigated ion channels. For instance, K^+ channels are inhibited by tetraethylammonium or cesium, while Na^+ channels can be inhibited by tetrodotoxin extracted from puffer fish. Another successful possibility to isolate ion currents is varying (or removing) a specific ion concentration in the extracellular or intracellular solution. However, whole-cell patch-clamp has its limitations. One problem is the rundown of currents or channel activities owing to the wash-out or diffusion of regulatory components, which is preventable with the addition of appropriate agents (e.g. ATP; BAY K 8644) [144].

7.3 Excitable cells

Heartbeat, muscle contraction or the processing of light and color are regulated by specific ion channels of excitable cells and the subsequent change of electrical properties. All excitable cells, such as neurons, muscle and endocrine cells of animals and some plant cells are capable

of generating an electrical signal or action potential in response to stimuli of sufficient magnitude. An action potential (AP) can be generally defined as a timed cellular event, in which the membrane potential of a cell rapidly rises and falls and herein follows a consistent trajectory [139]. Moreover, the generation of an action potential is an "all-or-none" event and its strength an intrinsic property of each cell. Thus, the intensity of an incoming stimulus can only be regulated by the frequency of action potentials fired. In the heart, the APs differ from region to region, reflecting the different roles played by the different cell types (compare table 1). The first description of AP and its generation and propagation was achieved by Alan Hodgkin and Andrew Huxley using squid axon [143]. The molecular basis of APs lies in the presence of ion channels, specifically voltage-gated ion channels [145]. If a stimulus (e.g. mechanical or a neurotransmitter) induces sufficient depolarization of the membrane by making the membrane potential more positive, those voltage-gated ion channels open. In many excitable cells, voltage-gated Na^+ channels activate and allow Na^+ to enter the cell down its electrochemical gradient. This creates a positive feedback loop as the further depolarization of the membrane activates more Na^+ channels and enhances depolarization. The AP sweeps along the cell as adjacent membrane portions become depolarized. The local electrical potential of the excited cell thus shifts from about -70 mV towards the Na^+ equilibrium potential (~+50 mV) and reaches a peak phase. Two following mechanisms repolarize the excited cell and save it from permanent electrical spasm. Firstly, the automatic inactivation of Na^+ channels, which are consequently unable to re-open, until the cell returns to the initial negative membrane potential. And secondly, voltage-gated K^+ channels open due to depolarization, but with slower kinetics than voltage-gated Na^+ channels [139, 142]. Once those delayed voltage-gated K^+ channels are activated, the K^+ efflux outruns Na^+ influx and drives the membrane potential back to the initial value (a condition termed hyperpolarization). Additional K^+ efflux further hyperpolarizes the cell and leads to a so-called refractory phase, in which no AP can be evoked. The refractory phase is the reason for the unidirectional propagation of AP in neurons. Just as voltage-gated Na^+ channels, K^+ channels inactivate automatically. After a cell-specific time span, the initial resting potential is reached and the channels can be activated again. Basically, there are two types of APs; either induced by voltage-gated Na^+ channels or by voltage-gated Ca^{2+} channels. To precisely investigate the possible differentiation of SC into excitable cells, it is necessary to distinguish the ionic basis of cardiac and smooth muscle APs and mechanism of contraction (Table 1) [146].

Table 1 Comparison of action potential (AP) properties in smooth muscle and different types of cardiac muscle [139].

Feature	Smooth muscle	Cardiac muscle – SA node/pacemaker	Cardiac muscle – ventricles
Upstroke of AP	Inward Ca^{2+} current; Ca^{2+} release	Inward Ca^{2+} current Slow upstroke	Inward Na^{+} current Fast upstroke
Duration of AP	10 msec	150 msec	250-300 msec
Ion channels	Ca^{2+}-activated K^{+} channels (BKCa); L-type and T-type Ca^{2+} channels; Voltage-gated K^{+} channels	Slow Na^{+} channels; L-type and T-type Ca^{2+} channels; Hyperpolarization-activated cyclic nucleotide-gated cation channels; Delayed rectifier K^{+} channels	Fast Na^{+} channels; L-type Ca^{2+} channels; Inward rectifying K^{+} channels; Delayed rectifier K^{+} channels

7.4 Nonexcitable cells

For a long time, nonexcitable cells, such as most of the blood cells, epithelial cells or EC, were defined as to lack functional voltage-gated Ca^{2+} channels and fast activated Na^{+} channels [139]. Consequently, they are unable to evoke regenerative action potentials. Nevertheless, the regulation of cytosolic Ca^{2+} levels is the most important response to specific stimuli and calcium-dependent K^{+} channels were found in most nonexcitable cells. Thorough analysis proved that nonexcitable cells possess nearly all kinds of channels with functional importance under physiological conditions. Ion channels found on nonexcitable cells include among others: Ca^{2+}-activated K^{+} channels, Ca^{2+}-activated nonselective cation channels, Ca^{2+}-activated Cl^{-} channels [147], inwardly rectifying K^{+} channels and mechanically activated ion channels [139]. Moreover, nonexcitable cells do express voltage-gated ion channels, but do not fire action potentials. The functional properties of ion channels in nonexcitable cells comprise volume regulation [148], vectorial secretion and reabsorption (e.g. sodium or chloride) and regulation of intracellular Ca^{2+} levels [149]. Ca^{2+} channels have a special importance because Ca^{2+} entering the cell often plays the role of a chemical messenger to activate exocytosis, or secretion, contraction, gating of other channels, ciliary reorientation, metabolic pathways as well as gene expression.

7.5 Electrophysiological properties of CD117$^+$ stem cells

As mentioned above, electrophysiological properties of implanted cells are crucial in terms of safety and to predict electrical coupling to host cells. Knowledge of the initial electrophysiological properties of the short-term cultured and CD117$^+$ AT2R stimulated stem cell population is necessary to draw conclusions on their expectable effects *in vivo*. Especially, as the protein expression of cardiac markers, which is often used to verify the differentiation of cells into a specific cell type, does not prove a functional differentiation. Differentiation of CD117$^+$ SC to cardiomyocytes or smooth muscle cells would yield detectable alterations in their electrophysiological properties (compare table 1). Acquiring the phenotype of cardiomyocytes should result in the detection of Ca^{2+} and Na^+ currents [150], while the differentiation to smooth muscle cells should develop detectable Ca^{2+} and K^+ currents [151]. Besides these two types of muscle cells that form cardiac tissue, the differentiation into cardiac or smooth muscle pacemaker or vascular endothelial cells might be possible. Electrophysiological investigations of SC were often neglected prior to cell transplantation. Few studies report electrophysiological properties of human mesenchymal stem cells (hMSC), which were shown to improve cardiac functions upon implantation [152-154]. In these studies, delayed rectifying K^+ currents (I_{Kdr}), transient outward K^+ currents (I_{to}) and calcium-activated K^+ channels (I_{KCa}) were described. Inward currents, such as Na^+ currents were mostly absent.

Previous studies of functional ion channels in mouse cells were designed as a co-culture system of murine CD117$^+$ SC and rat cardiomyocytes or focused on murine cardiac CD117$^+$ SC [155, 156]. Contrary to mouse MSC, cardiac CD117$^+$ SC do not express I_{KCa} [157]. I_{Kdr}, inward rectifying K^+ currents (I_{Kir}), and volume-sensitive Cl^- currents were found, but Ca^{2+} and Na^+ currents were absent in both cell types. The co-cultured CD117$^+$ cells expressed cardiac markers and Na^+ and Ca^{2+} voltage-gated ion channels, but functional channels could not be detected with patch-clamp recordings. Nevertheless, the ability to develop cytosolic Ca^{2+} transients upon membrane depolarization is an essential characteristic of cardiomyocytes. In this regard, studies conducted so far exclude the transdifferentiation into cardiac myocytes in culture [156, 158]. Interestingly, ion channels of freshly isolated murine bone marrow CD117$^+$ SC, especially prior to implantation, lack proper descriptions.

Aim of the study

Understanding the stem cell mechanism of action contributing to cardiac regeneration and exploring stem cells for a safe implantation is crucial for the development of novel successful stem cell therapies.

This work demonstrates an assessment of the functional characteristics of $CD117^+$ SC under normal and AT2R stimulated conditions. The purpose was to thoroughly analyze their *in vitro* and *in vivo* capacities with regard to cardiac regeneration. Stem cell effects were investigated in previous studies, but lack consistent descriptions especially on a functional level. To the best of our knowledge, electrophysiological properties of $CD117^+$ BMSC were investigated for the first time. This aimed to prove differentiation on a functional level and to estimate risks following implantation. Supporting *in vitro* experiments endeavored to characterize $CD117^+$ SC in terms of a potential differentiation to cardiomyocytes, smooth muscle cells or endothelial cells. Molecular biological techniques were used to observe changes on a transcriptional (qRT-PCR) and functional (FACS, 2-D Matrigel assay, Immunocytochemistry, acLDL assay) level and to detect influences of $CD117^+$ SC on cardiomyocytes survival *in vitro*. Finally, a mouse myocardial infarction model was the method of choice to detect effects of $CD117^+$ SC implantation and the impact of their AT2R preconditioning on cardiac regeneration.

Results and Discussion

1 Electrophysiological properties indicate an endothelial cell-like differentiation

The proposed transdifferentiation of $CD117^+$ SC into cardiomyocytes requires an investigation of their functionality. Besides the expression of cardiac markers found with immunohistochemistry, functional ion channels are a major prerequisite to truly prove differentiation. Whole-cell patch-clamp technique was utilized to determine the electrophysiological properties of isolated murine $CD117^+$ BMSC with and without stimulation of AT2R for 3 days [127, 159]. At potentials between -80 mV and +10 mV, current recordings did rarely exhibit time-dependence and currents recorded after the capacitive current did not show visible channel openings. At more positive potentials, slow activation of currents was observed, which fully established after 300–400 ms. At very positive potentials, large outward current deflections, probably opening BKCa channels, were detectable in a small fraction of $CD117^+$ SC. The most dominant current component discovered in $CD117^+$ SC was an inwardly rectifying K^+ current (I_{Kir}). The size of this current could be greatly increased by a fast exchange of extracellular solution (to K^+-rich and Na^+ low), which changed the reversal potential of the total current from -31.9±6.8 mV to +2.5±6.2 mV (n = 9) indicating a major influence of K^+ on total current (Fig. 1; taken from [127]). Of note, a previously reported delayed rectifying K^+ channel in CD117 cardiac SC was absent [155]. Outward rectification was less expressed and no major chloride channel was detectable. Blockade of hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN) with ZD7288 partly reduced the inwardly directed current (control: 45.7± 14.8 pA/pF; ZD 7288, 10 µM: 28.5±10.9 pA/pF) [127]. The current flowing through HCN channels is also called funny current or pacemaker current (I_f) and plays a key role in cardiac pacemaker cells. Stimulation of AT2R did not alter electrophysiological properties of $CD117^+$ SC. Even though few studies reported an influence
of Ang II on BKCa channels via the activation of arachidonic acid, it is imaginable that those channels are not sufficiently expressed on the investigated $CD117^+$ SC [160, 161]. The presented results deliver high evidence that $CD117^+$ BMSC will not differentiate into cardiomyocytes under the given culture conditions. The sufficient increase of detected time-dependent inward currents *in vivo* and the development of APs are unlikely.

A further aim was to evaluate the *per se* properties of $CD117^+$ SC in respect to Ca^{2+} current activation and in comparison to vascular smooth muscle cells (VSMC) of the portal vein of guinea pig. The species difference has no influence on the obtained results, since cell type specific ion and channel properties are conserved between species. We could prove that inward Ca^{2+} currents in $CD117^+$ SC were at least ten times smaller than those of VSMC and the absolute size of current flow 30 times larger in VSMC (Fig. 2; taken from [159]).

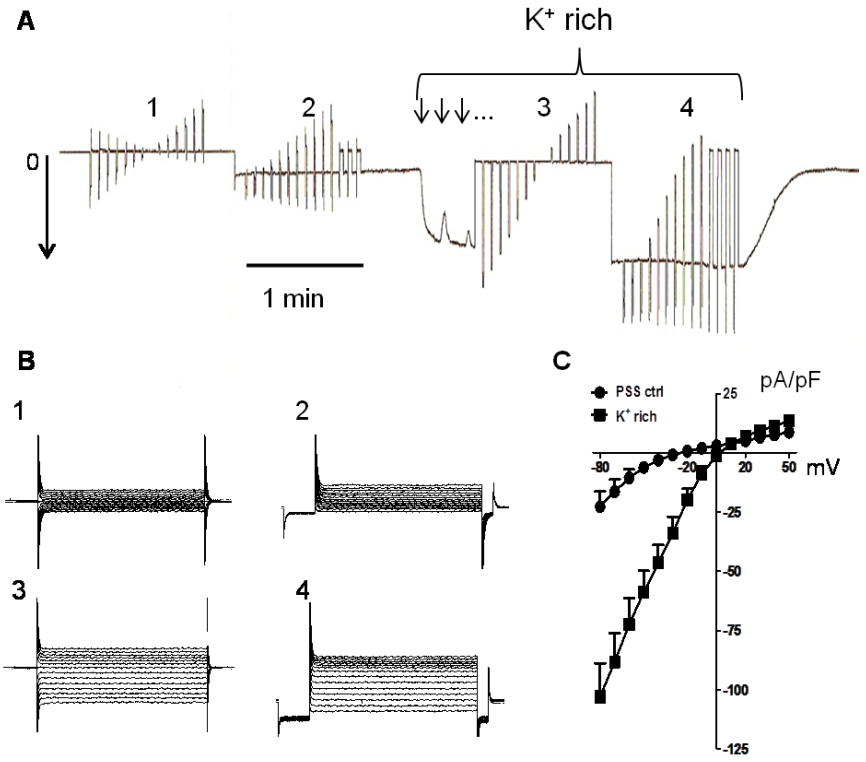

Figure 1 Current recordings during fast exchange of the external solution.
Replay of a current trace recorded in CD117+ SC for two different holding potentials and exchange of the external solution to high K+ and low Na+. The influence of the K+-rich solution on the holding current (arrows under the bracket show start of 10-s ejection pulses) and the elicited currents are presented. The arrow at the left side indicates zero current and gives a size of 100 pA/pF. Washout of K+-rich solution returns the holding current to control levels (A). Superposition of the current traces (500 ms) elicited by the 10 mV increments of the test potential (starting at -80 mV). The numbers near the traces correspond to the traces in A (B). Current–voltage relationship investigated with the same paradigm. Data given as mean ± SEM; n = 9 (C).

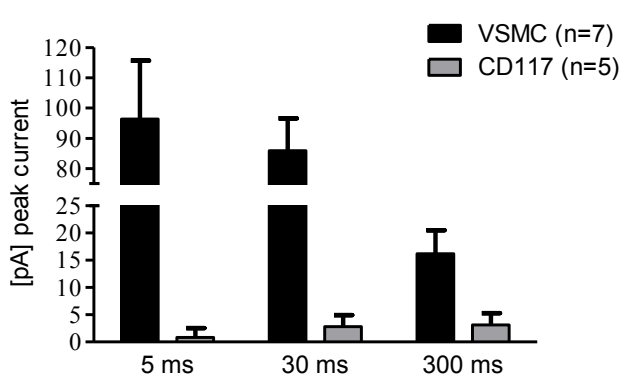

Figure 2 Comparison of inward currents of VSMC and $CD117^+$ SC.
Peak inward currents of the individual I-V curves were determined at individual potentials. Analyzed time points were 5, 30 and 300 ms of each evaluated cell. Data given as mean ± SEM.

Moreover, other depolarizing currents (e.g. Na^+ currents) were absent. The application of barium instead of Ca^{2+} -as charge carrier for current through voltage-dependent Ca^{2+} channels- evoked no increase in the time-dependent currents, which were determined at different time points. In $CD117^+$ SC, the time-dependent inactivation was slower and the voltage-dependent activation was also reduced in respect to time (Fig. 3; taken from [159])

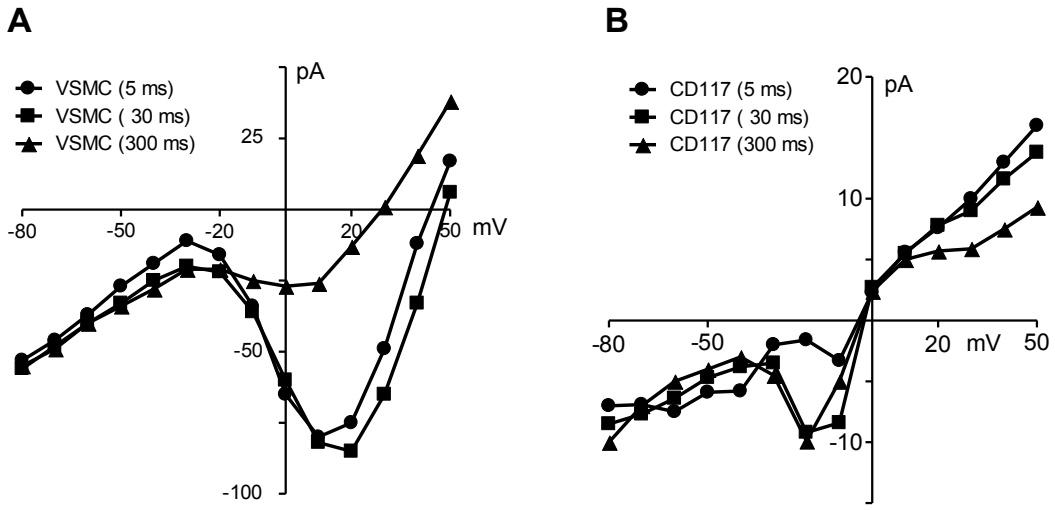

Figure 3 Representative current-voltage relationships of a VSCM (A) and a $CD117^+$ stem cell (B).
In both examples the holding potential was -60 mV and test potentials followed a pre-pulse to -90 mV. Current size was evaluated after 5 ms (square), 30 ms (triangle) and 300 ms (circle) after the depolarizing pulse. In the SC, current activation was slower than in VSMC and rapid activating Na^+ current was absent. Typical activation threshold of -30 mV was present in both cell types indicating the existence of L-type Ca^{2+} channels.

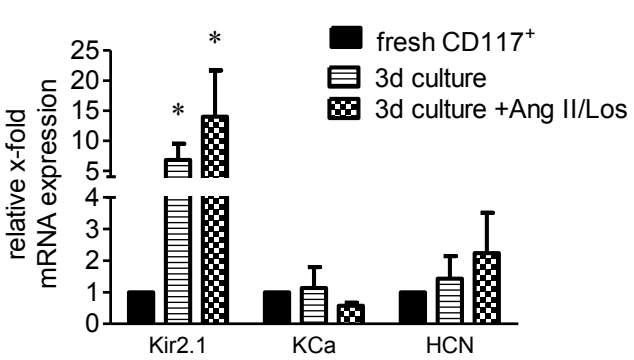

Figure 4 Ion channel mRNA expression of $CD117^+$ SC.
Kir2.1, HCN and KCa channel mRNA expression in freshly isolated and 3-day cultured $CD117^+$ SC ± AT2R stimulation (Ang II/Los). Kir2.1 mRNA expression was significantly increased after 3d. The presence of KCa and HCN mRNA is not altered. Expression levels were normalized to the expression of GAPDH. Data given as mean ± SEM; n = 5; *p < 0.05.

Additionally, analysis of mRNA expression of selected ion channels was done to refine patch-clamp results and to possibly show changes provoked by AT2R stimulation on the transcriptional level (Fig. 4; taken from [127]). The I_{Kir} 2.1, which is especially expressed on ventricular myocytes, was significantly increased in $CD117^+$ SC in culture if compared to freshly isolated cells. HCN and calcium-dependent K^+ channels were present (I_{Kv}), but their expression was not altered following culture (Fig. 4). No significant changes following stimulation were found for the analyzed channels.

In the two patch-clamp studies, to our knowledge we provide the first thorough investigation of $CD117^+$ BMSC prior to implantation. The combined results indicate a development of $CD117^+$ SC into an endothelial-like phenotype. This in turn would point to the hypothesis of paracrine effects supporting angiogenesis and improving myocardial functions. Our results further indicate that $CD117^+$ SC implantation is safe regarding misguided electrotonic coupling or signaling and that those cells are unlikely to produce arrhythmias *in vivo*. The findings of basic investigations *in vitro* and the known difficulties of implantation studies eventually suggest a low incorporation of those cells in the murine heart. However, regarding the obtained *in vitro* results, $CD117^+$ SC might physically contribute to the maintenance of cardiac function and improve neovascularization.

2 CD117$^+$ stem cells develop into an endothelial cell-like phenotype *in vitro*

Many lines of investigation point to a differentiation of CD117$^+$ SC to an endothelial-like phenotype *in vitro* [96, 135, 162]. This supports the hypothesis of paracrine signaling upon implantation. In the present work, CD117$^+$ SC cultivated for three days, with or without AT2R stimulation, were compared to freshly isolated control cells. Influences of the AT2R on CD117$^+$ SC were investigated. The efficiency of AT2R stimulation was verified by qRT-PCR, showing an 11.38 ± 2.71-fold increase in AT2R mRNA expression compared to unstimulated control cells (Fig. 5; taken from [163]).

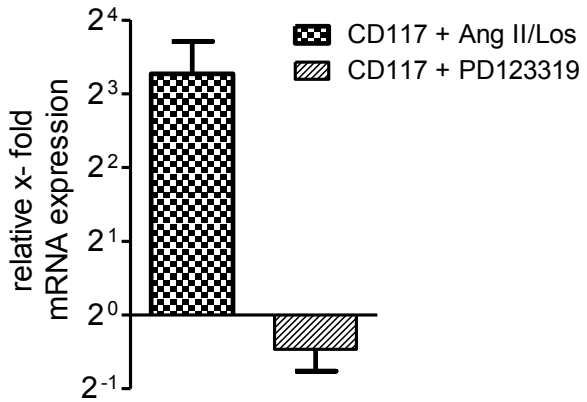

Figure 5 Quantitative real-time PCR analysis of AT2R mRNA .
CD117$^+$ SC were treated with Ang II/Los or PD123319 for 24h. Only 2 out of 5 experiments showed AT2R expression after PD123319 treatment. The mean mRNA expression levels of untreated control CD117$^+$ SC were arbitrarily given a value of 2^0. Expression levels were normalized to the expression of GAPDH. Data given as mean ± SEM; n=5.

Following AT2R inhibition with its antagonist PD123319, AT2R mRNA was detectable in only 2 out of 5 CD117$^+$ SC samples. The addition of PD123319 to CD117$^+$ SC downregulated AT2R mRNA levels to those observed in control cells and showed that increased expression of AT2R mRNA in CD117$^+$ SC is not an inherent property, but can be induced (Fig. 5). FACS analysis of stem cell marker expression showed a significant decrease of stem cell marker Sca-1 and CD34 and a significant increase of the hematopoietic marker CD45 upon cell culture, independent of stimulation for the AT2R This points to less immature cells and might be a hint for differentiation in culture (Table 2).

Table 2 Immunophenotypic analysis of CD117$^+$ SC (freshly isolated and after 3d of culture ± Ang II/Los).

Antibody	Fresh (n=8)	3 days (n=5)	3 days +Ang II/Los (n=5)
CD45	77.38 ± 1.81	99.98 ± 0.02 *	99.96 ± 0.02 *
CD34	14.65 ± 1.62	4.88 ± 1.3 #	4.48 ± 0.81 #
Sca-1	11.85 ± 1.37	3.7 ± 0.46 #	4.18 ± 0.69 #

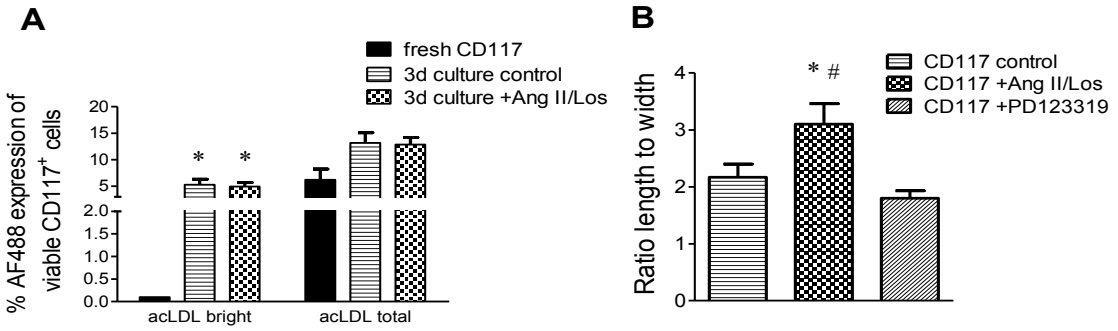

Figure 6 Analysis of endothelial properties of CD117$^+$ SC.
Quantitative analysis of acetylated low density lipoprotein (acLDL) uptake of freshly isolated and 3-day cultured CD117$^+$ SC. AcLDL (Alexa Fluor488) uptake of living cells was measured and analysed with FACS BD LSR II. Three subpopulations (negative, low and bright uptake) were distinguishable in respect to the fluorescence intensity. Total acLDL uptake is composed of the low and bright subpopulations. After 3 days CD117$^+$ SC showed a significant uptake of acLDL compared with freshly isolated cells. n=5–6; *p < 0.01 vs. fresh CD117 (**A**). The ratio length to width was analyzed as a measure of cell elongation. Five cells in each 5 random fields of view were blindly assessed in three independent experiments. The influence of AT2R treatment and inhibition was compared to control CD117$^+$ SC. n=3;*p<0.001 vs. CD117 control; #p<0.05 vs. CD117 +PD 123319 (**B**). Data given as mean ±SEM.

A differentiation into an endothelial-like cell type was first of all found by the expression of endothelial marker CD31 and VEGF in single culture (compare with [127]). Moreover, the uptake of acetylated low density lipoprotein (acLDL) is a widely used assay to analyze endothelial cell properties. The acetylation of the lysine residue of LDL prevents the binding of the LDL complex to its receptor. EC are capable of taking up this acetylated protein because of their "scavenger" receptors. Compared to freshly isolated CD117$^+$ SC, cells in culture showed an increased uptake of acLDL –independent of stimulation for the AT2R (Fig. 6A; taken from [127]) .

In addition, a 2-D Matrigel *in vitro* assay was deployed to investigate influences of stimulation on cell alignment and cell morphology. The elongation ratio (length to width) of Ang II/Los stimulated cells is significantly increased compared to control and PD123319 treated cells (Fig 6B; taken from [163]). Cell alignment and sprouting was also increased following Ang II/Los addition compared to control and PD123319 treated cells [163].

Besides those results in a single culture, a co-culture setup was used to approximate the situation *in vivo*. The co-culture experiments were largely conducted by Steffen Müller under my supervision and were published in his diploma thesis [164]. The commercially available AT-1 mouse atrial cardiomyocyte tumor lineage-derived cell line HL-1 [165] was cultured together with $CD117^+$ SC in a ratio of 1:2 with or without the stimulation of AT2R under either normoxic or hypoxic conditions. We confirmed that HL-1 cells express cardiac markers and assessed their viability under various conditions. As expected, hypoxia significantly reduced the viability of HL-1 cells. The stimulation with Ang II, Los or both had no significant effect on the investigated parameters. In contrast, Wange et al. found that the apoptotic rate of HL-1 cells increased under anoxic condition (no oxygen) following treatment with Ang II. This effect was reversed when Los was added to the HL-1 culture [166].

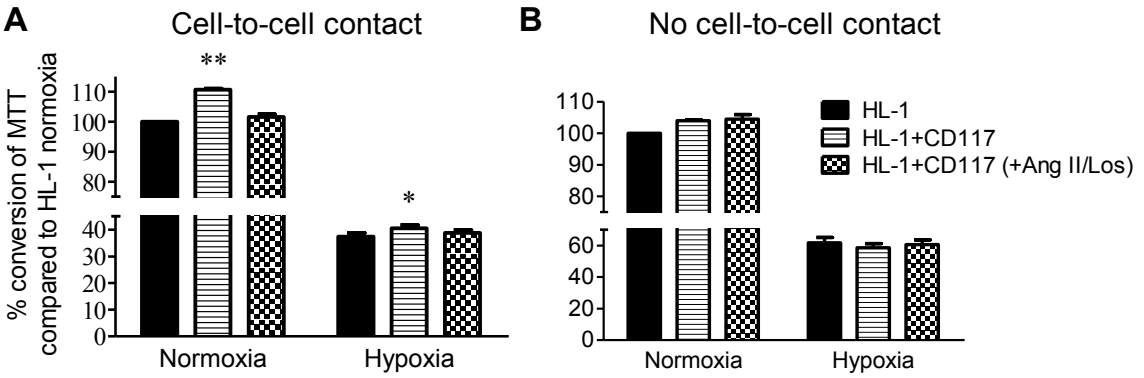

Figure 7 HL-1 vitality in single culture and co-culture with $CD117^+$ cells (±Ang I/Los).
Bar charts illustrate HL-1 vitality determined by MTT –assay after 48 hours in culture with or without the addition of $CD117^+$ SC. Hypoxia treated groups underwent oxygen deprivation for 24h. A significant increase of HL-1 vitality was detectable following CD117 addition, if cells were cultured with cell-to-cell contact (n=4, in duplicates) (A), but not if an inlet filter was used, which prevented HL-1 and $CD117^+$ SC cell-to-cell contact (n=2, in duplicates) (B). Data given as mean ± SEM; **p<0.01 vs. normoxic HL-1 group; *p<0.05 vs. hypoxic HL-1 group.

Upon co-culture, $CD117^+$ SC expressed endothelial cell surface markers VEGF and CD31 and, to a certain extent the cardiac transcription factor Mef2-B. Troponin-I or α-smooth muscle actin expression was not detectable [164].

Further, the influence of $CD117^+$ SC (± Ang II/Los) on HL-1 vitality and viability was analyzed. Vitality of HL-1, measured as the enzymatic conversion of MTT, showed a significant increase when directly co-cultured with $CD117^+$ SC under normoxic and hypoxic conditions (Fig. 7A; taken from [163]). However, additional stimulation for the AT2R attenuated this effect. Viability of Hl-1 cells was not significantly influenced by $CD117^+$ SC (± Ang II/Los) (Fig. 7A). The applied approach of subjecting a co-culture to hypoxia differs from the *in vivo* situation. In implantation studies, SC are injected after MI after the hypoxic

environment is established. Moreover, SC are delivered to the border zone of the infarct and might not be directly influenced by the prevalent lack of oxygen supply. Limitations of the present *in vitro* study are the simultaneous treatment of HL-1 and $CD117^+$ SC and the lack of further stimuli from the microenvironment. However, it appears that direct cell-to-cell contact and interaction rather than exclusive cytokine secretion is responsible for the increased vitality under hypoxia. If the two cell populations are separated by an inlet filter, but cultured in the same medium, no positive effect on HL-1 vitality under hypoxia was observed (Fig. 7B; taken from [163]) [163]. In a recent study, a co-culture of HSC with neonatal rat ventricular myocytes showed that direct cell-to-cell contact induces anti-apoptotic pathways in HSC and indicated cardioprotective effects of HSC on cardiomyocytes [167]. This is in line with our findings and points to a beneficial effect of $CD117^+$ BMSC on the survival of cardiomyocytes.

3 Implantation of CD117$^+$ stem cells significantly improved cardiac functions

Transgenic mice, with Enhanced Green Fluorescent Protein (EGFP) cDNA under the control of a chicken β-actin promoter and cytomegalovirus enhancer, were used to isolate donor cells (Charles River, Germany). In a first step, the EGFP expression of CD117$^+$ SC of BM and lung as well as mononuclear cells (MNC) isolated from spleen and thymus was analyzed with flow cytometry. Limitations of those transgenic mice, regarding their inconsistent EGFP expression, were previously described for HSC and questioned an application for transplantation studies [168]. We found that only 35.3±2.4 % of magnetic activated cell sorting-isolated CD117$^+$ BMSC expressed EGFP in contrast to 87.5±6.8 % of lung CD117$^+$ SC (n=5) [163]. MNC of thymus and spleen showed an EGFP expression of 22.5±2.7 % and 58.4±6.3 %, respectively. This analysis showed that the EGFP expression was somewhat impeded in these BMSC and ubiquitous EGFP expression was not given in the investigated transgenic mice. In a second step, CD117$^+$ BMSC were purified for their expression of EGFP with FACS Aria (BD Bioscience). The purified population (re-analysis: 85.7 % viable EGFP$^+$) was subjected to CFU colony forming assay in order to detect if cells maintain EGFP expression during culture and differentiation for 12 days. Afterwards, only 49.5±8.1 % of the viable cells (n=2) still expressed EGFP as determined by flow cytometry. Transferring these results to the *in vivo* situation makes it likely to underestimate cell retention, since EGFP negative donor cells cannot be distinguished from host cells (male donor, male recipient). Owing to the low cell yield, purifying EGFP$^+$CD117$^+$ BMSC with FACS prior to implantation was not feasible. Hence, the number of detected cells *ex vivo* had to be extrapolated from the FACS analysis and quantitatively assessed with qRT-PCR.

The implantation method was tested in advance using CFDA-SE dye stained C2C12 mouse myoblast cell line and stained cells were found in all three injection sites after 24 h. Retrieval of EGFP$^+$CD117$^+$ BMSC was tested 24 and 48 h and 3 weeks after implantation. Few cells were found after 24h, while no EGFP signal was detectable after 48 h and 3 weeks, respectively. *Ex vivo* analysis of implanted cells in cardiac tissue showed co-localization of EGFP and VEGF as well as EGFP and CD34. 24 hours after implantation, the number of retrieved cells was similar after injection into a recipient mouse with MI compared to a sham-operated animal. However, compared to the injected cell number (2x10$^{\wedge 5}$) the recovered cells found in frozen tissue sections was considerably low. Many studies point to cell retention as a major problem. If cells are injected with NaCl (0.9%), up to 95% of cells are lost during the first 25 minutes [100]. Moreover, cell retention after three weeks is limited because of the

hostile environment offered in the infarcted area, which lacks supporting cells or tissues, oxygen or nutritive substrates, but contains an excess of neutrophils and scavenger cells. Considering this and the low expression of EGFP in $CD117^+$ SC gives a reasonable explanation for the remarkable loss of implanted cells. We could show that $CD117^+$ BMSC display inconsistent EGFP expression in the investigated transgenic mice strain, which hampers cell retention analysis.

To analyze a possible improvement of cardiac functions, experimental MI was induced in C57BL/6J recipient mice and $CD117^+$ BMSC from $EGFP^+$ C57BL/6J donor mice were implanted into the infarct border zone. Despite the inconsistent EGFP expression as determined with FACS, we decided to use $EGFP^+CD117^+$ cells. In two independent experiments we found that protein expression (FACS) did not match mRNA levels. Only 50% of purified $EGFP^+CD117^+$ cells subjected to CFU still expressed EGFP protein after 12 days, but mRNA analysis showed that the relative EGFP mRNA expression was as high as in the positive control ($EGFP^+$ heart) and thus, investigation of EGFP mRNA might more closely resemble the magnitude of recovered cells. The experimental set-up is shown in table 3.

Table 3 Set-up of in vivo experiments (Permanent Ligation)

Group	Experimental Procedure
Sham	Thoracotomy and Matrigel™ implantation
MI-C	Thoracotomy, LAD ligation, Matrigel™ implantation
MI-CD117	Thoracotomy, LAD ligation, 24h incubated CD117+ SC in Matrigel™ implantation
MI-CD117 +AngII/Los	Thoracotomy, LAD ligation, 24h AT2R stimulated CD117+ SC in Matrigel™ implantation

The left ventricular remodeling process in mice is largely completed after three weeks [169] and catheter-conductance method was consequently applied to determine cardiac function via pressure and volume parameters. The pressure/volume loop measurements were recorded under normal (baseline) and stress (dobutamine) conditions after calibration (pressure and volume) and correction of the volume signal for wall conductance (parallel volume). Under both conditions, the implantation of $CD117^+$ SC seems to improve left ventricular functions (Fig. 8; taken from [163]). The ejection fraction and cardiac output were significantly increased if the two cell-injected groups were compared to the MI-C group (Fig. 8B+C). Heart weight/body weight ratio, which is a commonly used index of cardiac hypertrophy, decreased in the groups with implanted cells (Fig. 9A; taken from [163]). Mortality improved as well, but was not significantly different as determined with Log-rank (p=0.209 MI+

CD117+Ang II/Los compared to MI-C) and Gehan-Breslow-Wilcoxon test (p=0.171 MI+CD117+Ang II/Los compared to MI-C) (Fig. 9B; taken from [163]).

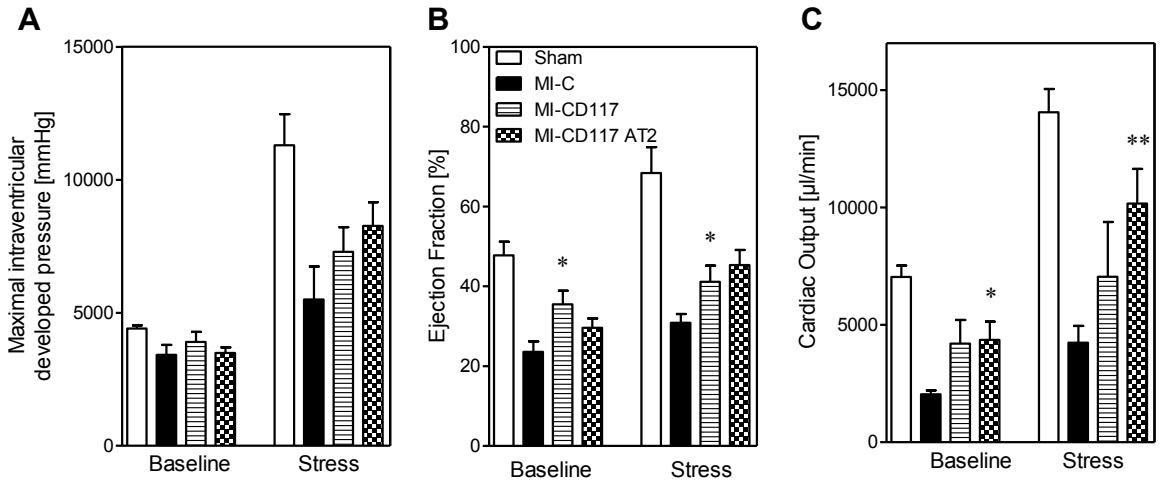

Figure 8 Cardiac functions 3 weeks after MI.
Left ventricular functions at both baseline and stress (dobutamine) conditions were assessed by catheterization. Maximal intraventricular developed pressure was increased in both SC implanted groups compard to MI-C (A). Ejection fraction and cardiac output was significantly increased in MI-CD117 and CD117 +Ang II/Los group, respectively, under both conditions (B+C). Data given as mean ± SEM; n=6; *p<0.05; **p<0.01 vs. MI-C.

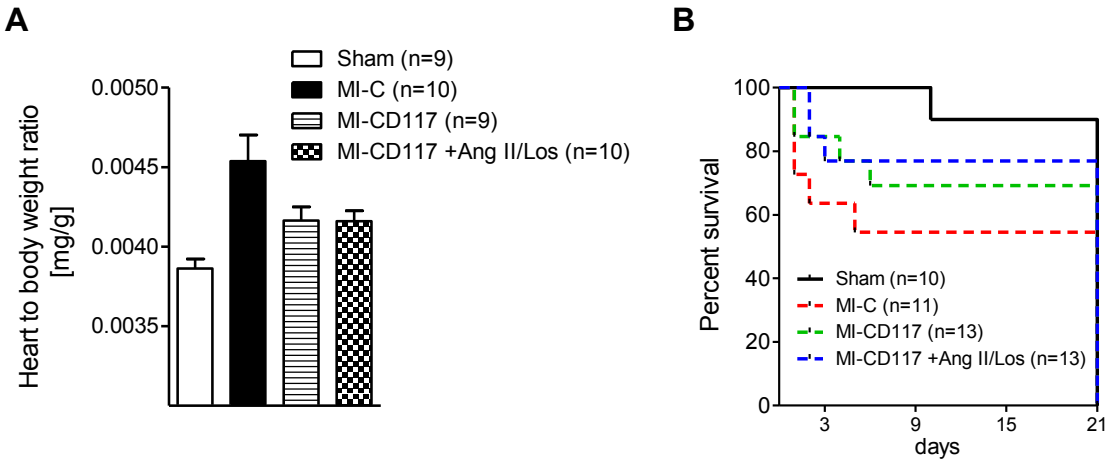

Figure 9 Improved heart to body weight ratio and mortality 3 weeks after MI.
Statistical analysis showed the tendency of improved heart to body weight ratio following SC implantation, which was however not statistical significant (Student's t-test) (A). Therapy related survival rates [%] did not significantly differ among the MI groups as determined with Log-rank and Gehan-Breslow-Wilcoxon test (B). Data given as mean ± SEM.

In addition to functional tests, hearts and lungs were explanted after termination of the experiment and frozen histological sections of hearts were prepared. Heart tissue, comprising part of the infarcted area, and the lung was used for RNA isolation and qRT-PCR analysis to assess cell retention and the escape of implanted cells from the heart towards the lung. The implantation of $CD117^{+}$ SC resulted in a significantly increased capillary density (Fig. 10; taken from [163]) and a significantly decreased collagen deposition (Fig. 11; taken from

[163]) independent of stimulation. This correlates with improved LV- functions and might be attributable to paracrine effects of $CD117^+$ SC.

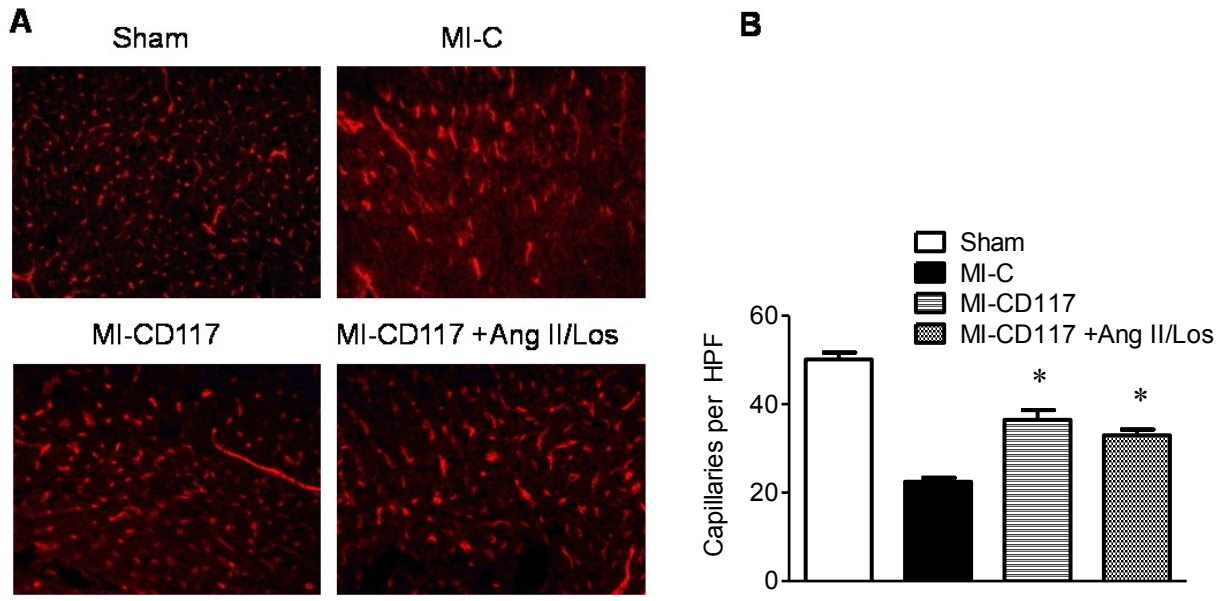

Figure 10 Increased capillary density in remote area of infarct zone.
Representative biotinylated tomato lectin perfusion staining in remote area of infarct zone of each group. Bound lectin was stained with AlexaFluor 568 secondary antibody (A). Capillary density in the remote area is significantly increased following SC implantation 3 weeks after MI (B). Data given as mean ± SEM. *p<0.01 vs. MI-C; n=5

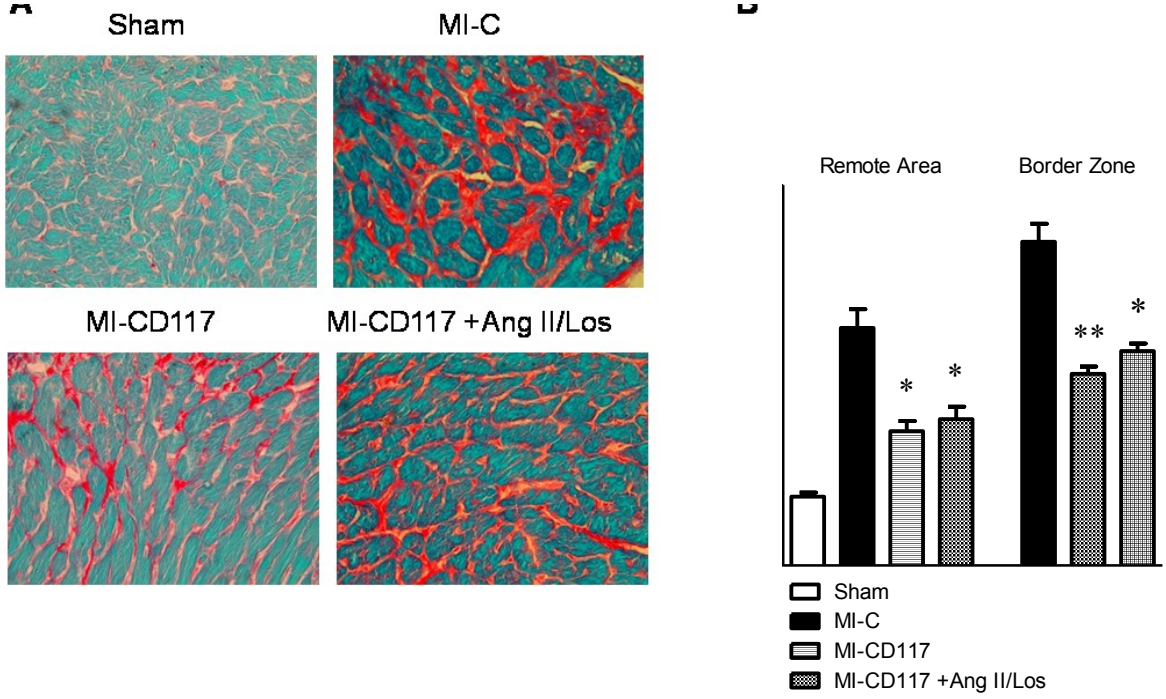

Figure 11 Collagen deposition in remote area and border zone of infarct.
Representative Fast Green FCF (myocytes)/Sirius Red (fibrosis) stainings at the border zone of the infarcted area (A). Collagen deposition is significantly decreased in the remote area and border zone of infarct in both SC treated groups 3 weeks after MI (B). Data given as mean ± SEM. *p<0.05, **p<0.01 vs. MI-C; n=6

Even though $EGFP^+$ cells were not detectable using immunohistochemistry, EGFP mRNA expression was measurable with qRT-PCR three weeks after MI (Fig. 12F). The migration of

intramyocardially implanted cells into filter organs such as the lung is an acknowledged process [170]. EGFP mRNA expression was found in the heart sections and correlates with the expression found in lungs (Fig.12F). Quantification of the total EGFP mRNA amount was impeded by the co-use of explanted hearts for cryosections and RNA analysis. However, after three weeks SC were still present in the heart, which correlates to the observed functional improvements.

Analyses of gene expression revealed influences of implanted cells in the heart three weeks after MI (Fig. 12; taken from [163]). The expression of AT2R mRNA in both SC treated groups was visibly increased compared to MI-C. Due to the high standard deviation, differences lack significance (Fig. 12A). The upregulation of eNOS expression was shown to improve the migratory capacity of bone marrow–derived stem cells [49] and eNOS was reported to be involved in increased BMSC migration following AT2R stimulation [171]. After three weeks, eNOS mRNA expression in hearts injected with CD117 +AngII/Los was significantly lower than after $CD117^{+}$ SC implantation (Fig. 12C). Regarding BMSC migration, we could not find significant differences in mRNA levels of CD34 stem cell marker (not shown) or stem cell homing factor CXCL12 (SDF-1) (Fig. 12E). We speculate that SC migration is an immediate process, which is not subjected to changes in chronic ischemia and not associated with eNOS regulation at later time points. Moreover, eNOS is required as a sensor for physiological vascular adaptation to blood flow, thus, increased eNOS expression might have contributed to increased capillary density.

MMP-2 is a key factor for micro-environmental adhesion, maturation and differentiation of stem cells [47], but also for the breakdown of extracellular matrix in normal physiological processes and tissue remodeling. Controversies exist on the regulation of MMP-2 after MI. Studies suggest that inhibition of MMP-2 activity improves the survival rate after acute MI by preventing cardiac rupture and delays post-MI remodeling through a reduction in macrophage infiltration [172]. Interestingly, blockade of both Ang II receptors resulted in increased stiffness, fibrosis and increased MMP-2 activity, which was reversed with AT1R blockers [173, 174]. This is in line with the present work, as stimulated $CD117^{+}$ SC provoked remarkably decreased MMP-2 mRNA levels (Fig. 12D). To estimate a potential pro-survival effect of implanted cells, we analyzed mRNA levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax (Fig. 12B). A reduced Bax/Bcl-2 ratio was reported to alleviate cardiomyocyte apoptosis. Increased Bcl-2 levels in the acute stage of MI and upregulation of Bax expression by chronic cellular responses of the affected myocytes against (mechanical) stress was reported previously [175]. Bax/Bcl-2 ratio was significantly decreased in the SC implanted groups,

when compared to MI-C (Fig. 12B), pointing to long-term anti-apoptotic effects after SC administration [163].

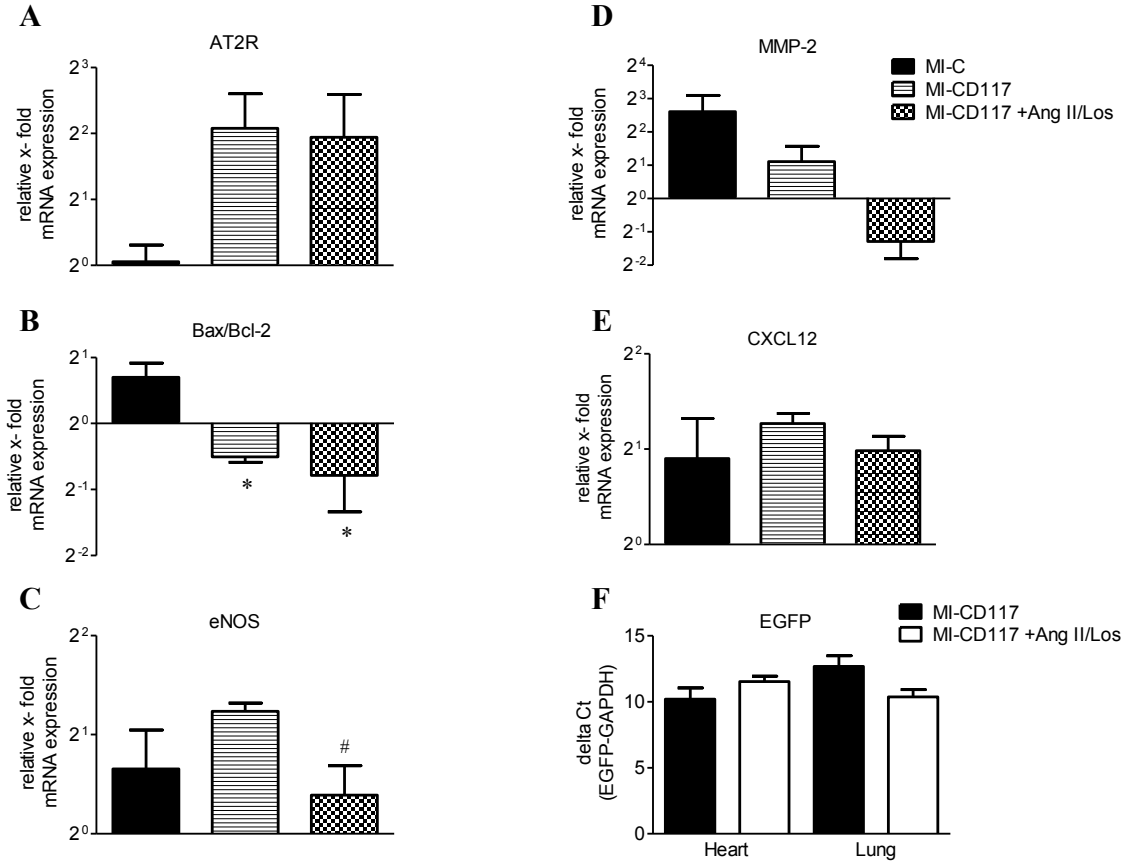

Figure 12 Quantitative real-time PCR analysis of heart sections three weeks after MI.
qRT-PCR analysis for AT2R (A), Bax/Bcl-2 (B), eNOS (C), MMP-2 (D), CXCL12 (E) and EGFP (F) genes, respectively in heart sections. SC implantation upregulates AT2R as well as eNOS and downregulates Bax/Bcl-2 ratio and MMP2 expression. EGFP gene levels are given as ΔCt (cycle threshold difference between EGFP and GAPDH) as EGFP is not expressed in Sham and MI-C. Otherwise, the average mRNA expression level in the Sham hearts was arbitrarily given a value of 1 (2^0). Expression levels were normalized to the expression of GAPDH. Data given as mean ± SEM; n=6, *p<0.05 vs. MI-C; #p<0.05 vs. MI-CD117.

Conclusions

The presented work aimed to assess the potential of $CD117^+$ BMSC to contribute to cardiac regeneration after MI. Of particular interest were the evaluation of safety and the retention of cells participating in functional improvements. With the two patch-clamp studies and supporting *in vitro* experiments, we provide the first thorough investigation of $CD117^+$ BMSC prior to implantation. The combined *in vitro* results indicate a development of $CD117^+$ SC into an endothelial-like phenotype, but exclude a transdifferentiation into cardiomyocytes or smooth muscle cells. Our results further show that $CD117^+$ SC implantation is safe regarding misguided electrotonic coupling or signaling and, that those cells are unlikely to produce arrhythmias or APs *in vivo*. Of note, it is also unlikely that $CD117^+$ SC will contribute to mechanical contraction of the heart.

The analysis of EGFP mRNA levels indicated that the implanted $CD117^+$ SC physically contributed to the maintenance of cardiac function and improved neovascularization. The implanted SC supported cardiac function via paracrine stimulation of angiogenesis and acted cardioprotective as indicated by increased eNOS and decreased Bax/Bcl-2 ratios. Importantly, the stimulation of AT2R on $CD117^+$ SC provoked no additional improvement of cardiac functions, fibrosis or capillary density. Benndorf et al. (2010) even showed a negative influence on remodeling after pharmacological AT2R stimulation in a murine infarct model [176]. In contrast, a recent study showed significantly enhanced vessel density four weeks after MI as well as reduced cardiomyocyte apoptosis and inflammation three days after the implantation of AT2R stimulated BMSC into the infarct border zone in rats [171]. Species differences, SC population and variation in experimental setups -in terms of stimulation- might contribute to the different outcomes and hamper a thorough comparison. Moreover, it has to be considered that the RAS is activated following MI and that the endogenous activation of AT1R/AT2R might cover specific effects of $CD117^+$AT2R stimulated SC leaving this experimental group without significant effects *in vivo*. The significant increase of cell alignment and cell elongation after AT2R stimulation *in vitro*, pointed to a mechanism of action that might be masked by AT1R related-effects *in vivo*. Furthermore, a recent study proposed that a beneficial or detrimental effect of AT2R during myocardial regeneration is largely dependent on expression levels [177]. A defined cardiac-selective overexpression of AT2R was previously shown to protect heart functions from ischemic injury [178]. It is hence imaginable, that in addition to SC implantation, the intramyocardial application of AT2R agonists or AT1R antagonists would yield significant improvements.

A regenerative potential of the $CD117^+$ SC population might be attributed to their differentiation into an endothelial-like phenotype and to enhanced paracrine effects, such as the secretion of VEGF. Importantly, this hypothesis was supported by patch-clamp measurements presented here. The improved cardiac function might be a result of cardioprotective actions of $CD117^+$ SC and not their electromechanically coupling to native cells. Positive effects could not be ascribed to the AT2R, as stimulation of $CD117^+$ SC might not be sufficient to completely up-regulate functions covered by the AT2R. The influence of the AT2R seems to be subjected to a complex regulation and is likely dependent on the AT1R/AT2R crosstalk [179]. Therefore, it will be crucial to analyze the mechanisms underlying AT2R function in the interplay with its opposing receptor AT1.

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Publications

I Ludwig M, Steinhoff G, Li J. The regenerative potential of angiotensin AT2 receptor in cardiac repair. Canadian Journal of Physiology and Pharmacology 2012:287-93.

II Ludwig M, Skorska A, Tölk A, Hopp HH, Patejdl R, Li J, Steinhoff G, Noack T. Characterization of ion currents of murine $CD117^{pos}$ stem cells in vitro and their modulation under AT2R stimulation. Acta Physiologica 2013; 208:274-87.

III Donndorf P, Ludwig M, Wildschütz F, Useini D, Kaminiski A, Vollmar B, Steinhoff, G. Intravital Microscopy of the Microcirculation in the Mouse Cremaster Muscle for the Analysis of Peripheral Stem Cell Migration. J Vis Exp 2013:e50485.

IV Ludwig M, Patejdl R, Steinhoff G, Noack T. Comparison of the electrophysiological properties of murine $CD117^{pos}$ stem cells with vascular smooth muscle cells (guinea-pig portal vein). Trace Elements and Electrolytes 2014.

V Skorska A, von Haehling S, Ludwig M, Lux CA, Kleiner G, Gaebel R, Klopsch C, Dong J, Curator C, Altarche-Xifró W, Slavic S, Unger Th, Steinhoff G, Li J, David R, The CD4+AT2R+ T-cell subpopulation improves post-infarction remodeling and restores cardiac function. Journal of Cellular and Molecular Medicine 2015.

VI Submitted:
Ludwig M, Tölk A, Skorska A, Maschmeier C, Gäbel R, Lux C, Steinhoff G, David R. Exploiting AT2R to improve CD117 stem cell function in vitro and in vivo – perspectives for cardiac stem cell therapy

Erklärung über den Eigenanteil an den Manuskripten

Publikation	Eigenanteil
I	Literaturrecherche und Verfassen des Manuskriptes
II	Planung und Durchführung aller Experimente (außer qRT-PCR) Auswertung aller Experimente

	Erstellen der Graphiken Wesentlicher Anteil am schriftlichen Beitrag zur Publikation
III	Beteiligung an der Durchführung der Experimente (Isolation und Färbung der Zellen; Einarbeitung des medizinischen Doktoranden)
IV	Planung und Durchführung der Experimente zu den $CD117^+$ Zellen Erstellen der Graphiken Wesentlicher Anteil am schriftlichen Beitrag zur Publikation
V	Beteiligung an der Durchführung der Experimente (Isolation, FACS) Anteil an der Verfassung des Manuskriptes
VI	Planung und Durchführung aller Experimente (außer qRT-PCR) Auswertung aller Experimente Erstellen der Graphiken Verfassen des Manuskriptes

Acknowledgements

Faculty, friends and family members have helped me to complete this dissertation. I would like to express my gratitude to all of them for their support and assistance.

First of all, I would like to express my appreciation and gratitude to my advisor, Prof. Dr. Gustav Steinhoff, Director of the Department of Cardiac Surgery for the constant guidance and scientific supervision, but also for the freedom to pursue independent work.

I would especially like to express my deep appreciation to Prof. Dr. Thomas Noack, Oscar Langendorff Institute of Physiology, University of Rostock for his guidance and support in both practical and theoretical matters. His continuous interest in my work majorly contributed to the successful completion.

I would like to thank Prof. Dr. Birgit Piechulla, Institute of Biological Sciences, University of Rostock for the kind co-supervision of my dissertation and her helpful suggestions during my mid-term presentation.

Moreover, I want to thank Prof. Dr. Hans Imboden, retired from the Institute of Cell Biology, Bern, Switzerland for the possibility of a research exchange and the lasting friendly communication and word of advice.

I sincerely thank Anita Tölk (!) and Christian Maschmeier for their excellent practical work, advice, fruitful discussions, reliability, joy and optimism inside and outside the lab. Special thanks to Anna Skorska, Ralf Gäbel and Ines Burghardt for their patience and encouragements and the good times spent together. I also thank Steffen Müller for accepting me as his diploma supervisor and really appreciate his conducted work.

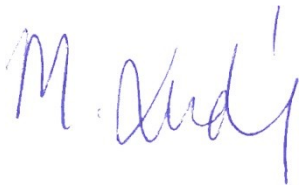
Further, I want to thank all my previous and current colleagues: Prof. Robert David, Dr. Cornelia Lux, Margit Fritsche, Prof. Jun Li, Dr. Nan Ma, Evgenya Delyagina, Yue Zhang, Anna Schade, Dr. Weiwei Wang, Madeleine Bartsch, Carolin Steps, Karina Müller, Erik Pittermann, Natalia Voronina, Frauke Hausburg, Paula Müller, Julia Jung, Dr. Christian Rimmbach and Dr. Cajetan Lang. Thanks for creating a comfortable and amiable atmosphere, for their supportive discussions and fun times in the lab.

High gratitude shall be paid to the team of the Department of Cardiac Surgery and Reference- and Translation Center for Cardiac Stem Cell Therapy: Dr. Gudrun Tiedemann, Dr. Ulrike Ruch, Dr. Jana Große, Dr. Sandra Kurzawski, Dr. Frauke Stähler, Jana Gabriel, Katrin Höfer, Dr. Christian Klopsch, Dr. Peter Donndorf and Dr. Dritan Useini.

Selbstständigkeitserklärung

Ich versichere, die vorliegende Arbeit zum Thema „The regenerative potential of the $CD117^{+}$ AT2R stimulated Cell Population *in vitro* and *in vivo*“ selbständig verfasst und keine anderen Hilfsmittel, als die angegebenen benutzt zu haben. Stellen, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen sind, habe ich in jedem einzelnen Fall durch Angabe der Quelle kenntlich gemacht.
Ich erkläre, meine wissenschaftliche Arbeit nach den Prinzipien der guten wissenschaftlichen Praxis gemäß der gültigen „Satzung der Universität Rostock zur Sicherung guter wissenschaftlicher Praxis“ angefertigt zu haben.

Rostock, 08. Mai 2014



Reprints of Publications included in this Dissertation

1 **The regenerative potential of angiotensin AT2 receptor in cardiac repair.**
Ludwig M, Steinhoff G, Li J
Canadian Journal of Physiology and Pharmacology 2012:287-93.

2 **Characterization of ion currents of murine CD117^{pos} stem cells in vitro and their modulation under AT2R stimulation.**
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Acta Physiologica 2013; 208:274-87.

3 **Comparison of the electrophysiological properties of murine CD117^{pos} stem cells with vascular smooth muscle cells (guinea-pig portal vein).**
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4 **Exploiting AT2R to improve CD117 stem cell function in vitro and in vivo – perspectives for cardiac stem cell therapy**
Ludwig M, Tölk A, Skorska A, Maschmeier C, Gäbel R, Lux C, Steinhoff G, David R
As submitted to Cellular Physiology and Biochemistry 2015.