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Myocyte-specific overexpression of stromal cell-derived Factor 1 facilitates cardiac regeneration and improves myocardial function after infarction in mice.

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MYOCYTE-SPECIFIC OVEREXPRESSION OF STROMAL CELL-DERIVED

FACTOR 1 FACILITATES CARDIAC REGENERATION AND IMPROVES

MYOCARDIAL FUNCTION AFTER INFARCTION IN MICE

By

Detlef Ernst-Rudolf Obal, M.D., M.S., D.E.S.A.

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A Dissertation Approved on

April, 15th 2014

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DEDICATION

This dissertation is dedicated to

my wife, Ruth Obal, MBA, CAPM,

my parents, Friedegard and Gunter Obal,

and my brother, Michael Obal and his family,

who have supported me during this exciting endeavor.

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I would like to thank Dr. Gregg Rokosh, Ph.D. for his guidance and help making this enterprise possible for a faculty colleague challenged by the combination of significant clinical work and exciting new experimental approaches. I would also like to thank the committee members, Drs. Aruni Bhatnagar, Ph.D. and William Wead, Ph.D. and Roberto Bolli, M.D. for their comments and assistance of the past five years. I would also like to express my thanks to my wife, Ruth, for her understanding and patience during the difficult times and frustrations after long hours in the laboratory. Special thanks are directed towards my clinical Chair in the Department of Anesthesiology and Perioperative Medicine, Dr. Mark Boswell, Ph.D., MBA, who supported my research and provided the framework for my success in research. Last but not least, I would express my gratefulness to the faculty within the Department of Anesthesiology who supported my research and allowed me to be in the laboratory to finish my Ph.D. thesis.

ABSTRACT

MYOCYTE-SPECIFIC OVEREXPRESSION OF STROMAL CELL-DERIVED FACTOR 1 FACILITATES CARDIAC REGENERATION AND IMPROVES MYOCARDIAL FUNCTION AFTER MYOCARDIAL INFARCTION IN MICE

Detlef Ernst-Rudolf Obal

April 15, 2014

Background: Interruption of cardiac stromal cell-derived factor 1 (SDF1)-CXCR4 axis by chronic AMD3100 administration increased myocardial injury after permanent coronary artery ligation, demonstrating the important role of this chemokine in cardiac regeneration.

Hypothesis: Cardiomyocyte-specific conditional overexpression of SDF prevents heart failure after permanent coronary ligation and facilitates cardiac regeneration.

Methods and Results: Tetracycline-controlled, α–myosine heavy chain promoter directed overexpression of cardiac SDF resulted in a significant increase of SDF expression (SDF: 8.1 ng / g protein) compared with littermate WT mice (0.02 ng / g protein) four weeks after doxycycline withdrawal. SDF overexpression increased AKT and casein kinase 1 levels in the heart. Although there was no difference in cardiac function and scar

size one week after infarction, SDF overexpression improved left ventricular (LV) ejection fraction (47±5% vs. 29±4%, p<0.05) decreased end-diastolic volume (78±10 vs. 158±30, p<0.05) and reduced infarct size measured by trichrome staining (SDF): 13±3% vs. WT (n=15): 23±3% of LV wall, p<0.05) four weeks after permanent ligation. Bromodeoxyuridine (BrdU) staining revealed increased regeneration indicated by a 5-fold increase in $BrdU^+$ / α-sarcomeric $actin^+$-cells in the border zone of the infarct (22±3% cardiomyocyte (CM) nuclei vs. 5±1% CM nuclei, p<0.01). Increased proliferation in SDF mice was confirmed by a higher number of $Ki67^+$ cells compared to WT mice. Cardiomyocyte cross sectional area in the border zone was significantly reduced in SDF mice (375±13 μm^2 vs. 434±10 μm^2, p<0.001) while capillary density remained unchanged (2348±151 / mm^2 vs. 2498±153 / mm^2) compared to WT mice.

Conclusion: This study demonstrates that cardiac-specific overexpression of SDF increases myocardial regeneration and improves LV function after permanent coronary ligation.

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CHAPTER 1

INTRODUCTION

Coronary artery disease resulting in myocardial infarction or ischemic cardiomyopathy presently remains one of the major causes of death. In the US one in four patients who suffered from myocardial infarction, dies due to heart failure. Heart failure in this context is the consequence of replacement of contractile myocytes with either fibrotic or scar tissue, resulting in an irreversible loss of functional tissue.

More than 10 years ago a major shift occurred in our understanding of cardiac physiology: Dr. Pierro Anversa has provided evidence to support the concept that the heart is not a post-mitotic organ and that myocyte turnover continues with the formation of new myocytes beyond the neonatal age in mammals.[1,2] These initial observations suggests that the myocardium might have the potential to regenerate after ischemic injury by either activation of endogenous repair mechanisms or transplantation of stem cells. The vast number of patients suffering from heart failure after myocardial infarction demonstrates that endogenous repair mechanisms are obviously not sufficient to repair or regenerate lost myocardium, thus laying the groundwork for further exogenous therapeutic treatment to increase effectiveness. Stem cell therapy utilizing different types of stem cells, combinations of different cells, and different routes of stem cell administration have been

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reported recently.[3,4] However, the most significant increase in myocardial ejection fraction (i.e. the most important variable of myocardial function) is achieved by transplantation of autologous c-kit$^+$ cardiac stem cells in patients with ischemic cardiomyopathy.[5] Nevertheless, transplantation of stem cells of any kind includes several caveats:

a) The number of cells remaining in the heart after transplantation is minimal. Using a highly sensitive RT-PCR method for detection of remaining cardiac stem cells after intra-myocardial injection, revealed that only 43% of those cells stayed in the heart five minutes after injection, and that this number further decreased to eight percent (one day) and one percent (35 days) after injection, respectively. Unpublished data show similar numbers after intracoronary infusion (Dr. Kyong Hong, Ph.D., Institute of Molecular Cardiology, University of Louisville, KY, USA), suggesting that the route of delivery did not affect the fate of the cells within the heart. Considering the positive results reported for stem cell therapy and the probably lack of cells that form new myocytes based on these studies, it appears more likely that it is not cells, but factors released by them that are responsible for the improved function seen in clinical trials.[6] Therefore, it remains controversial whether cells directly participate in cardiac regeneration or whether they are facilitators of the regeneration process by releasing paracrine factors.

b) Although compelling clinical data demonstrate safety of stem cell therapy, autologous stem cell transplantation remains a logistic challenge requiring isolation, culture, and purification of stem cells suitable for transplantation. In particular, culture and purification of different types of stem cells can result in an insufficient number of cells suitable for therapy.

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c) A recent meta-analysis reveals that even with the most commonly used stem cell, bone marrow-derived mononuclear cells (BMCs), an average improvement of left ventricular (LV) ejection fraction is only 4%[7] and with a reduction in infarct size of 4%.[8]

Therefore, our laboratory focuses its research on stromal cell-derived factor 1α (SDF), one of the factors released from the myocardium or from injected cells after cardiac transplantation. Originally thought to play an important role in maintaining the stem cell niche microenvironment within the bone marrow (BM), it became clear that SDF by itself protects the heart when given prior to myocardial ischemia reperfusion.[9] This contribution was further supported in studies where cardiac injury was exacerbated when signaling through SDF's receptor CXCR4 was blocked by chronic administration of AMD3100, demonstrating an important role of CXCR4 in cardiac homeostasis after myocardial infarction.[10]

Pre-clinical and clinical studies documented that SDF is up-regulated after myocardial infarction,[9,11-15] as part of the acute inflammatory response. This induction is mediated by hypoxia induced factor-1α (HIF-1α),[16] which has also shown to be responsible for up-regulation of CXCR4 on progenitor cells, promoting the recruitment of these cells towards myocardial injury.[17] However, the induction of SDF is transient. It peaks 48 to 72 hours after myocardial infarction then returning quickly to low baseline levels, reflecting its tight control by hypoxic signaling and proteolytic proteins such as elastase, cathepsin G, CD26 / dipeptidylpeptidase IV (DPPIV), and metalloproteinases 2 and 9. Therefore, we asked whether chronic overexpression of SDF in the heart would accelerate cardiac repair beyond the acute inflammatory phase and produce a sustained state of augmented regeneration after myocardial infarction.

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Previous work from our laboratory has shown that SDF down-regulates casein kinase 1 alpha (CK1α) in cardiac stem / progenitor cells (CSPC), which played an important role in CSPC cell cycle regulation, driving these cells towards a quiescent, non-dividing state. As CSPCs are potentially important components during cardiac repair, factors affecting their ability to regenerate damaged myocardium are crucial components to consider. In addition, CK1α is constitutively expressed in many tissues including cardiomyocytes, raising the question whether SDF overexpression will also affect cardiomyocyte cell cycle through CK1α.

Therefore, we created a transgenic mouse model in which cardiomyocyte-specific overexpression of SDF was induced four weeks before myocardial infarction and maintained throughout the entire recovery period, allowing us to not only study the acute effect on the heart but also its ability to facilitate cardiac regeneration.

This dissertation was therefore focused on

a)   the effect of elevated cardiac SDF on heart, peripheral blood, and bone marrow homeostasis,

b)   on its regulation of CK1α as potential important regulator of cardiac progenitor and cardiomyocyte cell cycle, and

c)   on SDF's potential to regenerate the heart after permanent coronary ligation.

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BACKGROUND AND LITERATURE REVIEW

Myocardial infarction as a consequence of coronary artery disease remains one of the major causes of death. After a critical loss of myocardium in the aftermath of an infarct, the remaining myocardium is unable to compensate for the reduced contractile mass resulting in increased myocardial stress. Without normalization, this increased myocardial stress leads to a progressive decline in function and profound neuroendocrine dysfunction ultimately leading to heart failure. There is increasing evidence that stem-cell mobilization to the heart and differentiation into cardiac myocytes is a naturally occurring process. However, with the potential loss of large areas of myocardium after myocardial infarction (MI) the extent of regeneration is too limited and takes place too slowly to be meaningful for the recovery of LV function. In a landmark publication, Dr. Pierro Anversa suggests that mammalian cardiomyocytes re-enter the cell cycle and subsequently undergo karyokinesis and cytokinesis.[18,19] However, the number of newly generated cardiomyocytes differs significantly from 1.5%[20] to 40%[21] dependent on the publishing group as well as the method used. Despite these differences, it now seems clear that the heart maintains its capacity to regenerate, albeit, a very limited capacity. In retaining the functionality of stem cells to regenerate damaged myocardium, the therapeutic approach for patients with ischemic cardiomyopathy has dramatically changed: Current strategies acknowledge the loss of contractile mass and aim to replace the scar tissue with new viable, contractile myocardium. The release of factors that act in a paracrine function either directly from the injured myocardium or after stem cell therapy might be an important determinant of the extent of regeneration. SDF is one of the major factors released by injured myocytes after an ischemic insult. It binds to its receptors CXCR4 and CXCR7,

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which regulate cell homing, survival, proliferation, retention of stem cells in hematopoietic, lymphopoietic organs, and tissues affected by inflammation.[22] Recent studies suggest that SDF also plays a significant role in myocardial ischemia reperfusion[9,10] and cardiac regeneration.

CLASSICAL ROLE OF SDF: HOMING FACTOR AND REGULATOR OF BONE MARROW HOMEOSTASIS

Chemokines are chemotactic cytokines that play an essential role in directing leukocyte movement during homeostasis, organ development, and inflammatory conditions – short, these molecules constitute the host defense against organ damage.[23] More than 50 different chemokines and 20 different chemokine receptors have been cloned so far. The CXC chemokine, stromal cell-derived factor 1 (SDF or CXCL12), produced by multiple BM stromal cell types and by epithelial cells in many organs,[24-29] mediates its action through the seven-transmembrane span G-protein coupled receptors CXCR4 and CXCR7 expressed on a variety of cell types. Knockouts of CXCR4, CXCR7, or SDF result in a diminished colonization of embryonic BM by hematopoietic stem cells and defect in the development of the heart, brain, and large vessels.[30-35]

As SDF has been shown to play a key role in several functions in homeostasis and injury, regulation of its expression and secretion are important. SDF expression has been found to be responsive to hypoxia. In a gradient hypoxia model Gurtner and colleagues show that SDF secretion increases in endothelial cells by hypoxia and as is a consequence of HIF-1α induction.[16] Specificity of this action was demonstrated by the presence of HIF-1α binding sites in the SDF promoter.[16] Not surprising, SDF is increased in hypoxic /

damaged tissues which serve as a chemoattractant for hematopoietic and other cells expressing CXCR4 (i.e. stem / progenitor cells). However, hypoxia can also result in up-regulation of CXCR4 as its promoter also contains consensus binding sites for HIF-1α, suggesting autocrine activation of intracellular signaling pathways.[36]

SDF AND CARDIOPROTECTION

In addition to the long-known properties of SDF in BM homeostasis, recent evidence suggests that SDF also plays an important role in cardioprotection and regeneration of injured myocardium. Several studies demonstrate SDF1 / XCR4 mediated mobilization of BM (BM)-derived stem cells to sides of ischemic injury.[12,13,24,37] Hu[9] demonstrated for the first time that ischemic preconditioning resulted in up-regulation of SDF in cardiomyocytes but not in fibroblasts and that administration of SDF prior to myocardial ischemia / reperfusion injury reduced the infarct size *in vivo*. SDF administration 25 minutes prior to global LV ischemia increases the recovery of the LV function after 40 minutes of reperfusion.[38] Increased activation by SDF of signal transducer and activator of transcription (STAT3) after ischemia reperfusion injury and an abolished protection in STAT3 knockout mice suggest that STAT3 contributes to the SDF effect on ischemia reperfusion injury.[38] Administration of SDF mediated survival in cardiac myocytes by activating extracellular regulated kinase (ERK) and AKT as we demonstrated *in vitro* and *in vivo*.[9] Besides the effect on ischemia / reperfusion injury, the importance of SDF / CXCR4 during myocardial ischemic injury was elucidated in a permanent coronary artery ligation model,[10] in which blockade of CXCR4 by chronic administration of AMD3100 increased myocardial injury and reduced LV function.[10] However, CXCR4 blockade increased the number of c-kit+ progenitor cells in the area at risk four weeks after

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myocardial infarction, suggesting regulation of cardiac progenitor / stem cell proliferation by SDF / CXCR4.

For activation of SDF / CXCR4 after myocardial infarction to become a therapeutic option the above mentioned limitations (i.e. short half-life, rapid cleavage) need be resolved, and different groups tested four different approaches:

Direct delivery of SDF

A direct delivery approach was used by Saxena who injected SDF directly into the peri-infarct zone of mice that had undergone coronary ligation and found a reduction in apoptosis and increased neovascularization.[39] To take advantage of both restoration strategies (i.e. cell therapy and delivery of SDF), Schuh injected in a rat model endothelial progenitor cells and SDF simultaneously into the border zone of the infarct four weeks after permanent coronary ligation. This approach increases LV function and increases vascularization two months after treatment.[40] Finally, Abbott developed a SDF-AdV, which was injected into the peri-infarct zone causing a 2.5-fold increase in cardiac SDF concentration compared to a control virus 48 hours after injection and resulting in high recruitment of mesenchymal stem cells towards the infarct.[13]

Transplantation of SDF overexpressing cells

Several groups used genetically modified cells overexpressing SDF to increase the local SDF concentration, using either SDF overexpressing *cardiac fibroblasts* in combination with G-CSF stimulated mobilization of $CD117^+$ and $CD34^+$ cells,[12] or *mesenchymal stem cells* transfected with a SDF expression vector.[41,42] LV function and vascular density in the risk region increase using this approach.

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Inhibition of proteolytic cleavage of SDF

To overcome the rapid degradation of SDF, Segers developed a bioengineered SDF-fusion protein resistant to MMP2 and CD26 / DPPIV cleavage, which increases recruitment of stem cells and improves LV function after intra-myocardial delivery.[43] Zaruba and colleagues followed the same approach by pharmacologically inhibiting DPPIV and therefore maintaining a higher SDF level after myocardial infarction. In combination with G-CSF[44] mediated stem cell mobilization these authors detect an increased angiogenesis and a decreased cardiac remodeling resulting in an improved LV function.

Up-regulation of CXCR4

To increase the recruitment of stem / progenitor cells to the site of injury the last approach utilized retroviral-transduced[45] or physiologically (i.e. hypoxic preconditioning) up-regulated[17] CXCR4 expression in mesenchymal- or cardiosphere-derived stem cells to accelerate the recruitment to the damaged myocardium. Both approaches result in increased recruitment of intravenous transfused cells and increase LV function.

In summary, most of the above listed studies focused on the chemoattractant properties of SDF and aimed to increase the recruitment of different types of stem / progenitor cells towards the injury site.

SDF AS REGULATOR OF CELL CYCLE BY CASEIN KINASE 1 α (CK1α)

In addition to well known effects of SDF / CXCR4 on cell migration and survival, SDF has also been shown to play a role in regulating proliferation of subpopulations of hematopoietic progenitors and CSPC. In this side, SDF drives cells to withdraw from the

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cell cycle at the G_0 / G_1 interface, resulting in quiescence of these cells.[46] In the context of stem / progenitor homeostasis, this important mechanism preserves the cell population by limiting the number of times these cells divide. Studies in isolated CSPCs demonstrate that SDF established a quiescent, non-proliferative state that was dependent on CXCR4.[46] These studies show down-regulation of CK1α, which is well known for its role in stablilizing β-catenin, and increase GSK3β activation resulted in an inhibition of cell proliferation through cycling D1 and Bmi-1 dependent mechanisms. These data confirmed previous work in hematopoietic stem cells, which required SDF or CXCR4 to maintain quiescent and provide mechanistic insight into cell cycle regulation. These findings also provide a mechanism which helps to explain the longevity of these cells in the BM niche to provide a stable stem cell pool.

PHYSIOLOGICAL ROLE OF CK1α

The protein kinase, CK1, has first been described in lactating mammary glands which determined the name of the kinase. However, CK1 is a monomeric serine / threonine protein kinase responsible for the regulation of several organ functions including membrane trafficking, cell cycle progression, chromosome segregation apoptosis, and cellular differentiation. Several isoforms (i.e. α, β, γ1, γ2, γ3, δ, and ε)[47-52] in addition to several splice variants, differing in their kinase activity, have been described.[53] CK1α is constitutively active but treatment with insulin, viral transformation, and treatment of the cell with topoisomerase inhibitors increase the activity.[54] Phosphotadylinositol-4, 5-biphosphate (PIP2) reduces CK1α activity in erythrocytes and neuronal cells and a RNA helicase DDX3 regulates CK1.[55] Furthermore, CK1 activity is inhibited by auto-phosphorylation of the c-terminal domain, which serves as a target of cellular phosphatases

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increasing CK1 activity.[56] CK1α has been linked to cell cycle progression, spindle dynamics, and chromosome segregation. The studies in **Aim II** will expand on the previous findings in isolated CSPCs and extend our knowledge to include differentiated cardiomyocytes.

STROMAL CELL-DERIVED FACTOR 1 TRANSGENIC MICE / GENOTYPING AND BREEDING

Cardiac specific conditional transgenic mice were generated on the C57BL / mouse strain. To temporarily control the SDF overexpression we used a tetracycline (tet) transactivator tet-off controlled transgenic mouse line in which myocyte-specific SDF overexpression was driven by the α-myosin heavy chain promoter[57] **(Figure 1)**. This binary inducible transgenic model requiring mice that contain both of the following transgenes:

(1) A heterologous α-myosin heavy chain (α-MHC)-cardiac-specific promoter driving the Tet-controlled trans-activator (tTA) sequence, consisting of a primer of a minimal activation domains derived from VP16 (FFF)[58] and a rTetR, selected from a yeast screen for optimized sensitivity and inductive range, placed into a cardiac-specific α-myosin heavy chain-specific promoter construct;

(2) A full-length α-MHC promoter in which three GATA sites and two tetracycline response elements (TRE) were ablated and the remaining cis-acting regions important for cardiac-specific expression were left intact. The Tet responsive element consists of seven repeats of the TetO sequence adjacent to the TATA box.[57]
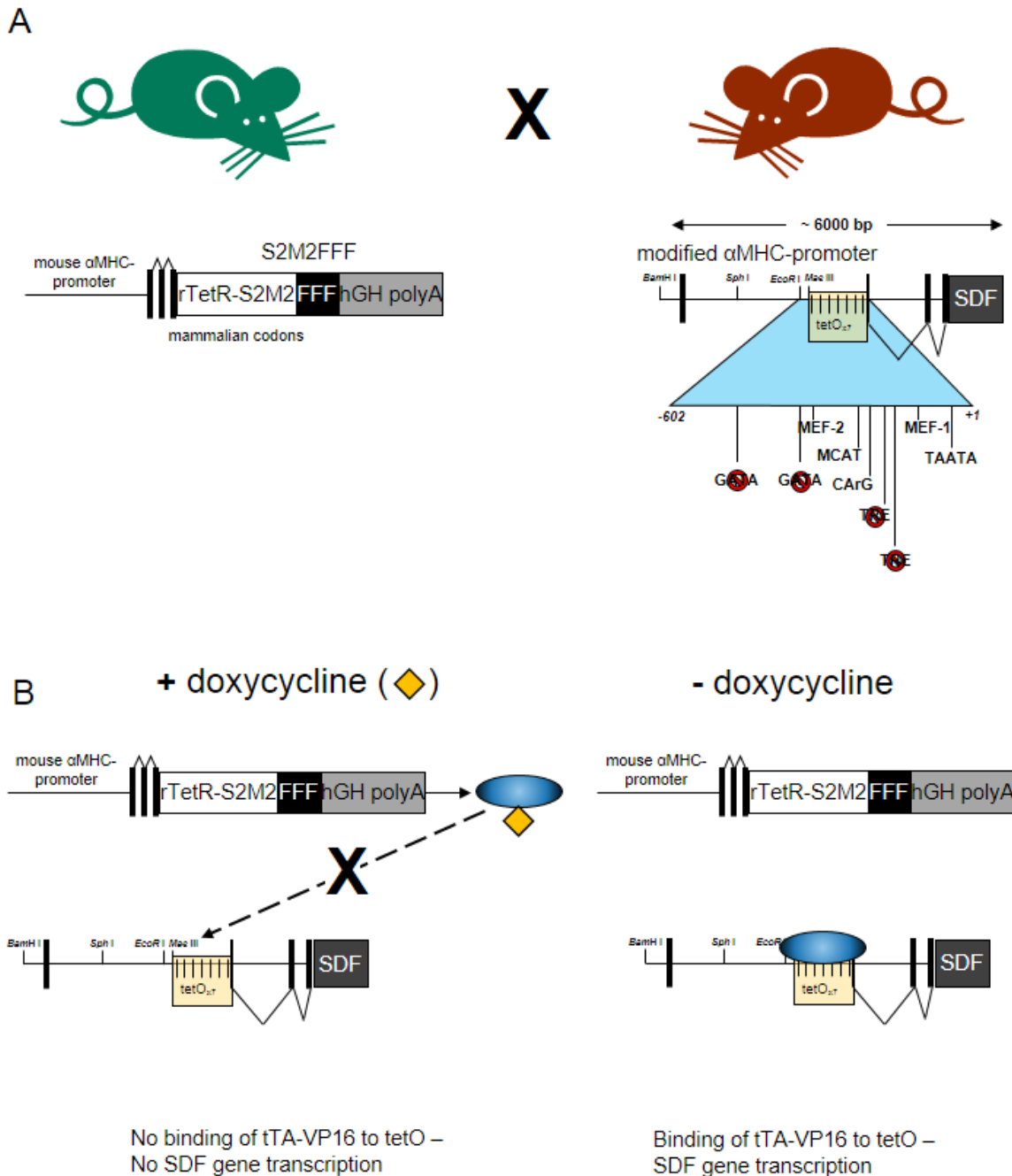
Figure I Characterization of the α-MHC specific -Tet "off" SDF transgenic model
SDF was overexpressed in double transgenic mice containing a modified (**A**, left side) α-MHC driven tetracycline activator gene and an α-MHC modified tetracycline response element directing the expression of the SDF1α gene. (**A**, right side)
In the presence of doxycycline the transcription of SDF is driven by a minimal promoter sequence and "switched off" (**B**, left side). However, in the absence of doxycycline tTA (blue cycle) can bind to the tetO binding site and accelerate SDF expression.

This chimeric promoter is ligated to the SDF gene, which is then conditionally controlled: In the presence of tetracycline or its analogue doxycycline, the transcription of SDF is only driven by the very low basal activity of the minimal promoter and "switched off" while in its absence tTA can bind to the TetO, allowing the VP16 transactivator to increase the expression of SDF, driven by the heterologous α-MHC promoter, and therefore "switched on". The tet-off model allowed us to determine the time of gene overexpression, and we removed doxycycline from the drinking water at eight weeks of age when mice become adolescent. We detected increased gene-overexpression three weeks after doxycycline withdrawal determined by PCR and western blot analysis **(Figure 2)**, and therefore performed our experiments in 12-14 weeks old mice with a one week "safety margin".
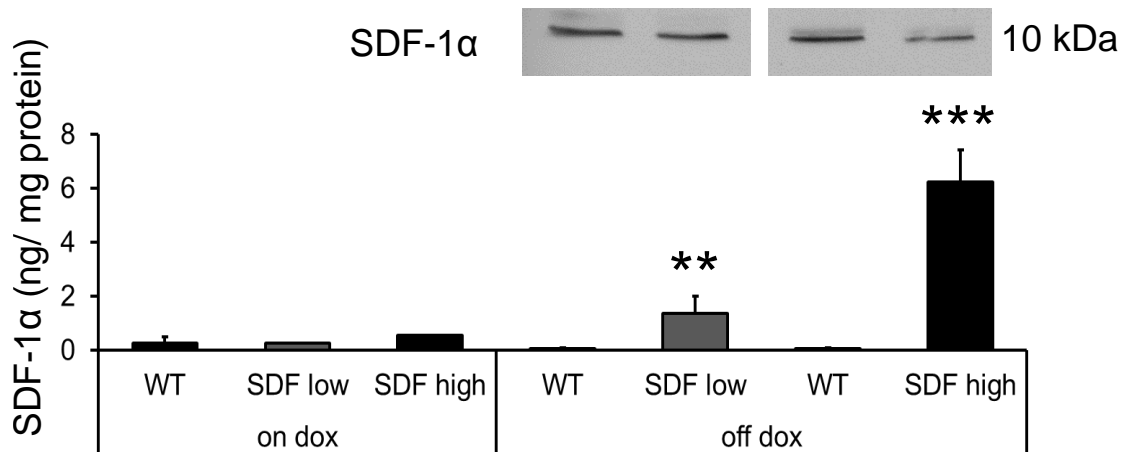
Figure II Myocardial SDF concentration before and after activation of the transgene

Myocardial SDF concentration in wild-type (WT) and in "low" **(SDF low)** and "high" **(SDF high)** expressing transgenic mice. Mice were euthanized while receiving doxycycline ("on dox") and three weeks after induction of cardiomyocyte-specific SDF overexpression ("off dox"). (N=3-7. **, $P < 0.01$, *** $p < 0.001$, Student's t-test).

Assuming a Mendelian pattern of inheritance, one in four offspring would carry both transgenes. We used exclusively male mice for the ligation experiments to avoid any estrogen cycle dependent impact on the size of injury. Therefore, 12.5% of all offspring mice were available for ligation experiments **(Table 1)**, resulting in a total of 725 mice assuming a 75-80% survival rate after one week post-ligation and four weeks post-ligation, respectively.

Table 1 Breeding requirements to fullfil the proposed study

Treatment groups	WT sham	WT ligated	Tg_{low} sham	Tg_{low} ligated	Tg_{high} sham	Tg_{high} ligated
Group size						
1 wk	5	8			5	8
4 wks	8	18	8	18	8	18
estimated survival rate (%)						
1 wk	95	80			95	80
4 wks	95	70	95	70	95	70
estimated mice needed for experiments						
1 wk	5.3	10.0			5.3	10.0
4 wks	8.4	25.7	8.4	25.7	8.4	25.7
Male double transgenic mice (yield assuming Mendelian pattern of inheritance) (%)	87.5	87.5	12.5	12.5	12.5	12.5
Breeding effort:						
1 wk	6.0	11.4			42.1	80.0
4 wks	9.6	29.4	67.4	205.7	67.4	205.7
	15.6	40.8	67.4	205.7	109.5	285.7
Total number of mice needed to perform ligation experiments:	724.7					

CHAPTER 2

UNDERLYING PROBLEM

Myocardial infarction is a prevalent consequence of cardiovascular disease (CVD) contributing to one in four deaths in the US. Unfortunately, there is no therapy to reverse effects of lost myocardium due to myocardial infarction, only therapies to deal with the consequences. Although stem cell therapy seems to be promising to regenerate damaged myocardium, current approaches lack efficiency. It is essential that a better understanding of mechanisms associated with regeneration is obtained and then utilized to develop more effective therapeutic approaches to alleviate the significant burden of CVD and improve patient quality of life.

PROPOSED EXPERIMENTS

This thesis will evaluate the effect of chronic SDF overexpression on cardiac regeneration after permanent coronary ligation. In particular, we will focus on the effect of SDF on the myocardium and its regeneration potential and how BM homeostasis is affected. One of the key challenges in the proposed studies will be to demonstrate changes in infarct size that are a consequence of myocardial regeneration. We will use two indices to assess infarct size, scar size, and viable myocardium and accompany these with immunofluorescent detection of new myocytes by BrdU incorporation. We will further

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evaluate whether CK1α plays an important role in cardiac regeneration and whether it serves as a mediator of SDF induced cardiac regeneration.

HYPOTHESIS

Cardiomyocyte-specific overexpression of SDF accelerates cardiac regeneration after permanent coronary ligation by increasing CK1α mediated cardiomyocyte division.

SPECIFIC AIMS

I) To determine the effect of chronic cardiomyocyte-specific overexpression of SDF on cardiac and bone marrow homeostasis.

II) To determine whether casein kinase 1α mediates cardiac stem / progenitor cells and neonatal cardiomyocyte proliferation.

III) To determine whether cardiomyocyte-specific overexpression of SDF increases myocardial regeneration after permanent coronary ligation.

CHAPTER 3

AIM I – TO DETERMINE THE EFFECT OF CHRONIC CARDIOMYOCYTE-SPECIFIC OVEREXPRESSION OF SDF ON CARDIAC AND BONE MARROW HOMEOSTASIS

BRIEF PREVIEW

Background

The proposed studies focus on changes in the heart (i.e. cardiomyocytes) and in the BM as a major source of progenitor cells. As a chemoattractant, BM derived SDF helps to retain diverse population of hematopoietic precursors including CD34^{+} stem / progenitor cells (HSCs / HPCs) in the BM and serves as an "anchor" for leukocytes within the BM niche. Stressors or injury that alter the homeostatic influence of SDF would impact this balance leading to decreased retention and mobilization of these cells, leading to increased circulating levels. Therefore, we asked whether an "imbalance" due to myocardial overexpression of SDF would shift the homing preference of circulating HSPCs / endothelial progenitor cells (EPCs) towards the heart and subsequently affect the circulating stem cell pool. SDF / CXCR4 interaction promotes neovascularization in different types of tissue injury by recruiting endothelial progenitor cells.[59,60] For that reason, we measured the number of leukocytes in the peripheral blood and endothelial progenitor cells (EPCs, Sca-1^{+} / Flk-1^{+} cells) in the peripheral blood and the BM.

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Since recently published data demonstrates that SDF regulates the cardiac stem / progenitor cells' (CSPC, c-kit^{+} cells) cell cycle through CK1α[46], we also determined the expression of cardiac CK1α and the number of CSPCs in WT and SDF transgenic (Tg) mice. Previously we demonstrated that SDF administration was associated with an increase in ERK and AKT.[9] Therefore, we determined AKT under baseline condition in WT and SDF overexpressing mice.

Methods

Two lines of cardiomyocyte-specific SDF Tg mice expressing "low" (SDF low) and "high" (SDF high) levels of SDF were used in the described studies. Cardiac SDF levels were determined by ELISA before and three weeks after activation of the transgene in "high" and "low" SDF overexpressing mice. Protein levels of CK1α and AKT were determined by western blot analyses using standard protocols. The number of leukocytes was determined by an automatic hematology analyzer five and 30 weeks after initiation of cardiac SDF overexpression and the number of circulating EPCs detected by FACS analysis.

Results

Interestingly, the number of leukocytes decreased in the peripheral blood five and 30 weeks after the removal of doxycycline from the drinking water and induction of SDF overexpression in the heart while there was no change in the number of platelets and red blood cells in the peripheral blood. To verify this unexpected result we performed a second set of experiments to determine the time course of these hematological changes, confirming lower numbers of leukocytes in the peripheral blood of SDF Tg mice compared to WT

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mice. The number of EPCs was lower in SDF Tg mice in the BM and the peripheral blood. While we saw an increase in EPCs 72 hours after permanent coronary ligation in SDF Tg mice, numbers in WT mice remained unchanged.

Overexpression of SDF resulted in a concentration dependent increase of cardiac CK1α and phosphorylation of AKT and an increase in CSPCs determined by immunopathology. Interestingly, BrdU staining indicated a reduced number of dividing CSPCs.

Conclusion

Although SDF overexpression was cardiomyocyte-specific, the level of SDF was sufficient to affect the periphery and reduce circulating leukocytes and diminish the number of EPCs in the peripheral blood and the BM. CK1α increased in SDF overexpressing mice, affecting proliferation of CSPCs.

MATERIAL AND METHODS

Animals

SDF Tg mice were bred, maintained, and used in described studies at the University of Louisville under protocols approved by the Animal Care and Use Committee and adhered strictly with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996). For the proposed experiments in **Aim I** female mice were used from SDF Tg lines overexpressing low and high SDF concentrations (as outlined above) and their WT littermates served as age matched controls. A diurnal 12 hour day / night cycle was maintained. At the age of eight weeks, removal of doxycycline from the drinking water successfully induced SDF overexpression **(Figure 2)**. A second set of

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experiments was performed in elderly mice (60 weeks of age) to determine the effect of chronic SDF overexpression (i.e. at least 30 weeks) on the number of circulating leukocytes.

Hematology

Two sets of experiments were performed to establish the effect of SDF on hematopoiesis: In a first set of experiments, blood samples were taken from mice of different ages (15 and 60 weeks old) and the number of leukocytes (WBCs), red blood cells (RBCs) and platelets was determined. In a second set of experiments, we established a five-week time-course of individual changes in WBC, RBC, and platelet numbers in the peripheral blood before and after the removal of doxycycline from the drinking water. Blood samples were taken at time "0" (eight week old mice receiving doxycycline) and eight, 16, 24, and 32 days after doxycycline removal. Blood was collected by puncturing the retrobulbar plexus and collecting ~150 µl of blood into a microvette EDTA coated tube (Sarstedt Inc., Newton, NC, USA) stored on ice. The daytime of the blood collection was kept constant throughout the entire experimental period to avoid circadian changes in cell numbers. The corpuscular blood composition was determined within 30 minutes using an automatic hematology analyzer (Cell DYN 3500, Abbott Diagnostics, Princeton, NJ, USA).

Flow cytometry / FACS analysis

The number of circulating and resident BM EPCs was determined from whole blood samples collected, as described above. After automatic cell counting the samples were treated with an erythrocyte lysis buffer (Roche) for one minute at room temperature.

Samples were then washed twice to remove the cell debris and transferred to a staining buffer (PBS supplemented with 5% fetal bovine serum (FBS), 1% bovine serum albumin (BSA)). Cells were stained 45 minutes in the dark for stem cell antigen (Sca-1, Antibody, FITC conjugated) as a hematopoietic stem cell marker and for fetal liver kinase 1 (Flk-1, APC Rat anti-mouse, BD Pharmingen), the receptor for vascular endothelial growth factor as an endothelial cell marker. After two subsequent washing steps, FACS analysis was performed (Accuri C6, Becton Dickinson Bioscience, San José, CA, USA).

Bone marrow collection

Mice were euthanized by CO_2 inhalation followed by cervical dislocation, then both femurs were removed and placed in sterile phosphate buffered saline (PBS) solution. Soft tissue was removed from the bone with a fine forceps to avoid the contamination of the BM sample. The bone shaft was then flushed with PBS using a 23-gauge needle and a ten milliliter syringe. Cells were further dissociated by careful repeated passage of the flushed BM through the 23-gauge needle. The cell suspension was filtered through a 70-µm nylon mesh filter to remove clumps, counted with a hemocytometer and then transferred to a FACS staining buffer for further processing, as described above.

Coronary ligation

All animal procedures were approved by the University of Louisville IACUC in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85-23, rev. 1996). After inhalational induction of anesthesia (3-5 Vol.% Isoflurane (Abbott Laboratories, Abbott Park, IL, USA) in an induction chamber, the trachea was intubated with a 22-gauge intravenous catheter. The successful intubation

was verified by bilateral chest movement after connection to a small rodent ventilator (Harvard Apparatus, Holliston, MA, USA). The lung was volume controlled ventilated at a rate of 150-200 strokes / minute and a tidal volume of 200 µl. Having shaved the chest with an electric razor, the mice were placed on an adjustable temperature (TCAT-2LV controller, Physiotemp, Clifton, NJ, USA) heating pad with a 37°C setup. All procedures were performed under a surgical microscope (MZ95, Leica Microsystems, Buffalo Grove, IL USA), using a sterile surgical technique. After opening the thoracic cavity between the fourth intercostal space and dissection of the pericardium, the left descending coronary artery was identified and subsequently ligated by a single stich ligation with an 8.0 suture (Nylon, AA-0145, Surgical Specialties Corp, Vancouver, BC, Canada) about 2-3 mm below the aortic root. The successful ligation was confirmed by epicardial cyanosis and bulging of the myocardium during systole. The chest was subsequently closed in layers, using a 5.0 Vicryl suture (PC-1, 1855G, Ethicon, Cincinnati, OH, USA). After extubation the mice recovered for 30 minutes to one hour in an oxygen chamber ($FiO_2=0.8$) before they returned to an animal cage, being placed on a heading pad overnight.

Confocal microscopy

One limitation of conventional microscopy for achieving high-resolution images is based on the physical properties of the emitted light:
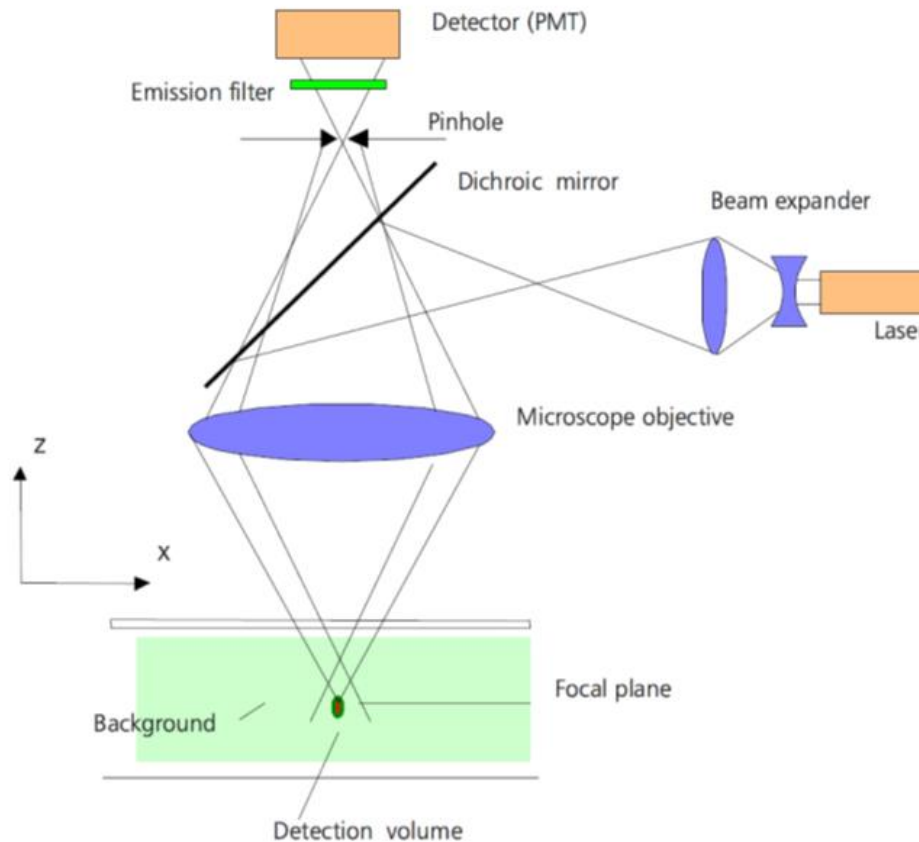
Copyright Carl Zeiss, Germany.

Figure III Confocal microscopy scheme
A microscope objective is used to focus a laser beam onto the specimen, where it excites fluorescence. The fluorescent radiation is collected by the objective and efficiently directed onto the detector via a dichroic beamsplitter. The interesting wavelength range of the fluorescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pinhole is arranged in front of the detector on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out of focus when it hits the pinhole, so most of it cannot pass the pinhole and therefore does not contribute to forming the image (*Copyright* Carl Zeiss, Jena, Germany).

In thick tissue sections, like the myocardium, the focus area is always greater than the wave-optical depth of the specific sample. Therefore, the in-focus information of the image is mixed with surrounding out of focus image information, reducing the contrast of the image. Using multiple fluorescence dyes result in a mixture of signals into different color channels, limiting conclusions about co-localization of the labeled structures. In contrast, confocal imaging uses an optical aperture, which only collects data within the focal plane while rejecting outside reflections from the sample. Using LASER (Light Amplification by Stimulated Emission of Radiation) light featuring a high spatial and temporal coherence, confocal microscopes are capable of scanning tissue samples with a single color (wavelength) and a high spatial resolution. In addition, sample images can be acquired in a single plane (i.e. slice), which result in a stack of images when moved along the z-axis in defined increments, allowing to assemble a 3-dimensional reflection of the specimen. **(Figure 3).** The Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany) is equipped with three different LASER types: An Argon / Krypton laser (emitting wavelength: 458 / 488 / 514 nm), an UV laser (405 nm), and a Helium laser (633 nm). The laser light is directed towards a dichroic mirror through a specific objective onto the specimen where specific fluorophores are excited and subsequently emit light of a different wavelength back towards a dichroic beamsplitter, which allows the passage of light of a define wavelength towards a detector via a precisely aligned pinhole. In our experiments, we used up to four different fluorophores to identify and detect co-localization of our antigen-targets **(Table 2)**.

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Table 2   Fluorophores used for detection of different antigens by confocal microscopy

Fluorophores	Excitation wavelength (nm)	Emission wavelength (nm)	Spectrum
Fluorescein (FITC)	495	519	Green
Tetramethylrhodamine (TRITC)	547	572	Red
Cyan (Cy5)	649	670	Cyan
4',6-diamidino-2-phenylindole (DAPI), nuclear stain	345	455	Blue

Preparation for pathological specimens

At the end of each recovery period (i.e. one or four weeks), the mice were re-anesthetized, and the heart was arrested in diastole by injecting a filtered saturated KCl (3 M) and cadmium (Cd, 0.1 M, Sigma Aldrich, St. Louis, MO, USA) solution through the apex into the LV cavity. Thereafter, the aorta was cannulated, and the heart retrograde perfused at a rate of 0.5-1 ml / minute with 37°C warm PBS. After five to ten minutes and complete blood removal, the perfusion was switched to 10% buffered formalin solution. The solution was infused under constant pressure (80 mmHg) for an additional 10-15 minutes. The LV cavity pressure during the fixation period was kept constant at 13 mmHg by infusing formalin through a 26-gauge needle inserted into the cavity through the LV apex. After subsequent fixation in 10% buffered formalin for up to seven days atrial appendices were removed, hearts were weighted and cut into two millimeter thick cross-sectional slices in a mouse heart tissue slicer (Zivic Instruments, Pittsburgh, PA, USA).

After tissue processing for paraffin embedding (Tissue-Tek VIP, Miles Scientific), tissue samples were cut (rotary microtome RM2155, Leica Microsystems, Buffalo Grove, IL, USA) into four micrometer thick sections for histological and immunofluorescent staining. Sections were stored at 4°C until their final usage. Prior to histological staining, sections were de-paraffinized and rehydrated to distilled water using a standard graded xylene-ethanol series.

Immunofluorescence staining

Formalin containing fixatives cause cross-linking of proteins and mask antigen sites in tissue sections, making those unavailable for detection by antibodies. Therefore, we used a basic EDTA buffer (pH 8.0) in which tissue sections were boiled for 15 minutes in a microwave oven, followed by a 30 minutes cool-down period. Thereafter, sections were equilibrated in TN buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20) for five minutes, followed by quenching of endogenous peroxidase with 3% H_2O_2 for 10 minutes at room temperature. After threefold washing in TN buffer, sections were blocked with TB buffer containing 0.5% blocking agent (FP1020, PerkinElmer Inc., Waltham, MS, USA) and subsequently incubated overnight with a c-kit antibody (1:100, mouse stem cell factor receptor / c-kit, AF1356, R&D systems, Minneapolis, MN, USA). To develop the final signal, sections were incubated with bovine anti-goat horseradish peroxidase (HRP) antibody (1:200, Cat.# 805-035-180, Jackson Immunoresearch, West Grove, PA, USA) for one hour at room temperature followed by five washing steps and four minutes incubation in amplification working buffer (1:50 diluted TRITC fluorophore in amplification buffer, TSA Plus TMR kit, PerkinElmer, Inc.). To exclude hematopoietic, c-kit$^+$ cells from our analysis, sections were incubated with a CD45 antibody (CD 45, 1:50 in 1% BSA and 10%

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donkey serum, cat.# 610265, BD transduction Laboratories) for 30 minutes at 37°C and then stained with a donkey-anti-mouse IgG FITC conjugated secondary antibody 1:100, Cat.# 715-095-151, Jackson Immunoresearch, West Grove, PA, USA). Finally, a 15 minutes incubation in a DAPI solution [1:1000 in Dulbecco's PBS (DPBS)] marked the nuclei. Prior to mounting cover glasses, tissue auto-fluorescence was blocked by a 30 minutes incubation in Sudan black B solution (Cat# 199664, 1 mg / ml in 70% ethanol, Sigma Aldrich, St. Louis, MO, USA,). CSPC proliferation was determined in a second set of tissue sections, measuring BrdU incorporation (see page 52). After c-kit staining, these sections were incubated with an anti-BrdU primary antibody (1:50, clone BMG 6H8 IgG) for two hours at 37°C, washed five times for six minutes in DPBS, and stained with a FITC conjugated secondary anti-mouse IgG antibody (5-Bromo-2'deoxy-uridine labeling and detection kit I, Roche Diagnostics GmbH, Mannheim, Germany).

Western blot analysis

The mice were euthanized as previously described, and their hearts were quickly removed and washed in ice-cold PBS. Half of the tissue was used for western blot analysis performed as previously described.[9] Briefly, the tissue was immediately homogenized in lysis buffer containing (in mmol / L): 25 Tris-HCl, pH 7.5, 100 NaCl, 0.5 EDTA, 0.5 EGTA, 1% phosphatase inhibitor cocktail 2 (P5726, Sigma Aldrich), 1% phosphatase inhibitor 3 (P0044, Sigma Aldrich), 0.1% Triton X100, and 2% protease inhibitor cocktail (P8340, Sigma Aldrich). Thereafter, the tissue homogenate was cleared by centrifugation at 14,000 x g for ten minutes, followed by determination of the protein concentration in the supernatant (Biorad Protein Assay, Bio-rad, Hercules, CA, USA). Sample aliquots were stored at -80°C until used or were denaturated for three minutes at 95°C for immediate

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usage. Gels for SDF PAGE were loaded with equal amounts of protein per lane, followed by a transfer to the Hybond ECL membrane (GE Healthcare Life Science, Piscataway, NJ, USA). After overnight incubation with primary antibodies at 4°C, proteins were detected with HRP-conjugated anti-mouse or anti-rabbit antibodies (1: 10000, Cell Signaling Technology, Beverly, MA, USA) and the ECL plus chemiluminescent detection system (GE Healthcare Life Science, Pittsburgh, PA, USA).

The following primary antibodies were used: CK1α (Cell signaling, 1:1000), AKT (Cell signaling, 1:1000), phosphor-AKT (Cell signaling, 1:1000), and GAPDH (Cell signaling, 1:1000). Densitometry analysis was performed using a Typhoon scanner (GE Healthcare Life Science, Pittsburgh, PA, USA).

SDF ELISA

Tissue homogenates of "high" and "low" SDF overexpressing mice were collected before and three weeks after induction of the transgene. Samples were prepared as described above and further handled according to the manufacturer's recommendation (Cat. No. McX120, mouse CXCL12 / SDF-1α immunoassay, RD systems). Optical density was measured at 450 nm with a wavelength correction at 570 nm. Results were calculated from a standard curve generated by dilutions of known amount of recombinant SDF protein, as previously described.[9] Levels of SDF were normalized to total protein and expressed in pg / 100 ug protein (mean±SEM).

Statistical Analysis

All data are expressed as mean±SEM. Differences were analyzed using unpaired Student's t-test or two-way ANOVA [group effect (i.e. genotype) and time effect (time

after initiating SDF overexpression)] with Bonferroni's correction for multiple comparisons. The statistical significance was defined as $P<0.05$ or in case of multiple comparisons adjusted by dividing 0.05 by the number of comparisons. The statistical analyses were performed using PRISM software (Vers. 6.0e for MAC OSX, Graphpad Software, La Jolla, CA, USA).

RESULTS

Cardiac specific overexpression of SDF decreases leukocytes in the peripheral blood and the BM.

Blood samples from mice overexpressing cardiac SDF for five or 30 weeks, respectively, revealed a reduction in WBCs after five weeks (WT: 9.8 ± 1.3 x 10^3 / ml vs. SDF: 6.2 ± 0.7 x 10^3 / ml, $P<0.05$) while the number of RBCs and platelets did not change **(Figure 4)**. In contrast, all three lineages of hematopoiesis (i.e. erythropoiesis, lymphopoesis and myelopoiesis) were depressed after long- term overexpression of SDF in the peripheral blood (**WBCs,** WT: 5.8 ± 0.7 x 10^3 / ml vs. SDF high: 3.9 ± 0.5 x 10^3 / ml, $P=0.08$; **RBCs**, WT: 8.9 ± 0.3 x 10^3 / ml vs. SDF high: 8 ± 0.3 x 10^3 / ml, $P<0.05$; **platelets,** WT: 1147 ± 62 x 10^3 / ml vs. SDF high: 900 ± 109 x 10^3 / ml, $P=0.05$). Serial experiments with repeated blood collection per mouse confirmed the WBC data. Although the data did not reach statistical significance at a specific time point, the two-way ANOVA indicated significant influence of genotype and time on WBC numbers with a reduction in WBC in the SDF Tg mice. **(Figure 5)**. Interestingly, we did not find any difference in RBC and platelet count in this set of experiments.

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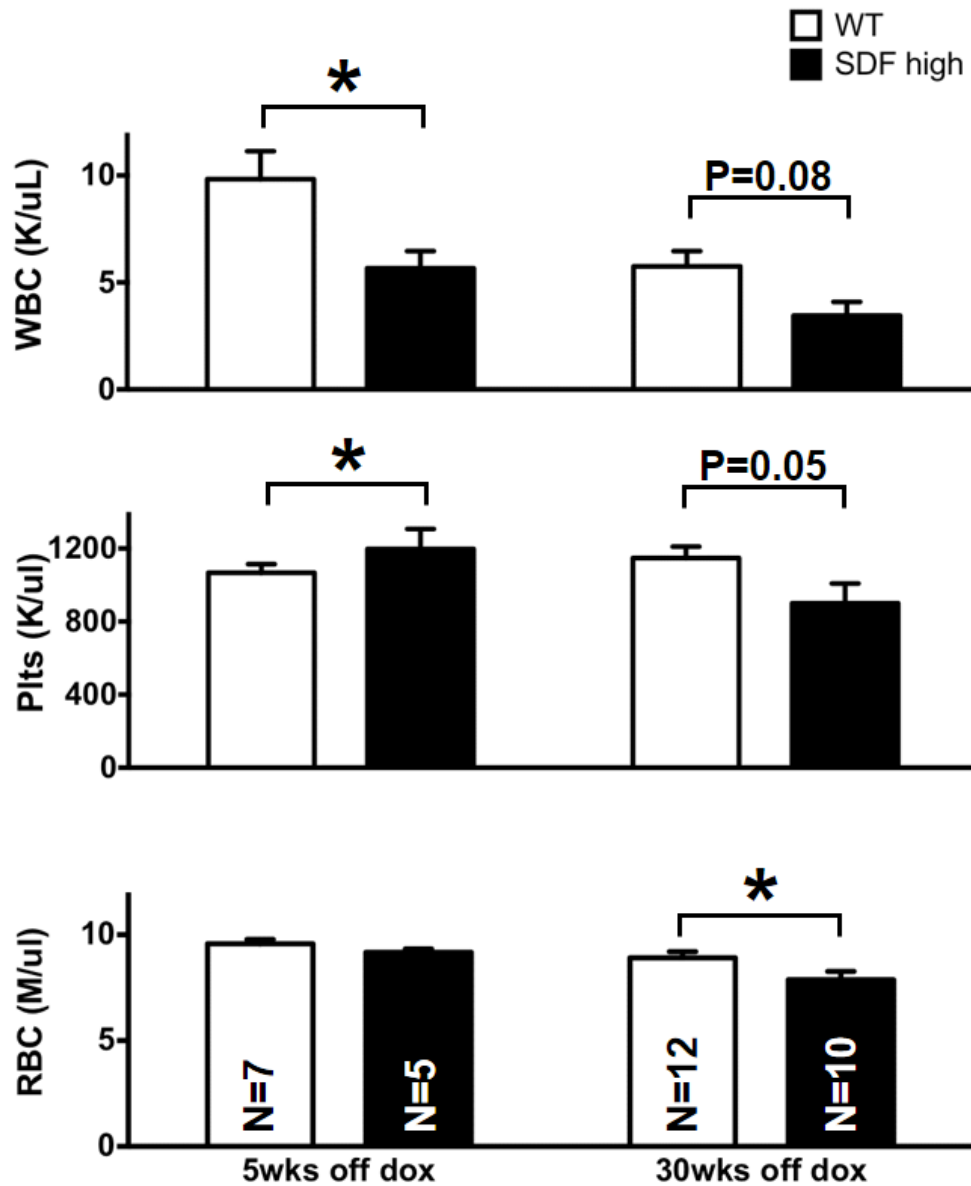
Figure IV WBCs, platelets and RBCs in the peripheral blood after five and 30 weeks of cardiomyocyte-specific SDF overexpression

Barplot represents data from WT (n=7) and high expressing (SDF high) transgenic mice 5 weeks (left side) and 30 weeks (right side) after initiating transgene expression. SDF overexpression depressed WBCs counts at both time points while the number of RBCs was only diminished in the elderly mice. Interestingly, platelet count was initially higher in the transgenic mouse, but became depressed after 30 weeks of SDF expression. Samples were taken at a single time point. Comparisons are done as unpaired t-test, *P<0.05.

The changes in hematopoiesis were further investigated, and we measured the number of EPCs in the BM and peripheral blood. SDF overexpression resulted in a trend towards decreased circulating and BM resident EPCs under baseline conditions compared to that in WT mice (**Figure 6**). The number of EPCs almost doubled in the peripheral blood 72 hours after permanent coronary ligation with no change in numbers in the BM, demonstrating a reversal of impact of MI on mobilization of EPCs. However, based on the limited number of experiments (n=3) we did not reach statistical significance limiting the value of this finding.
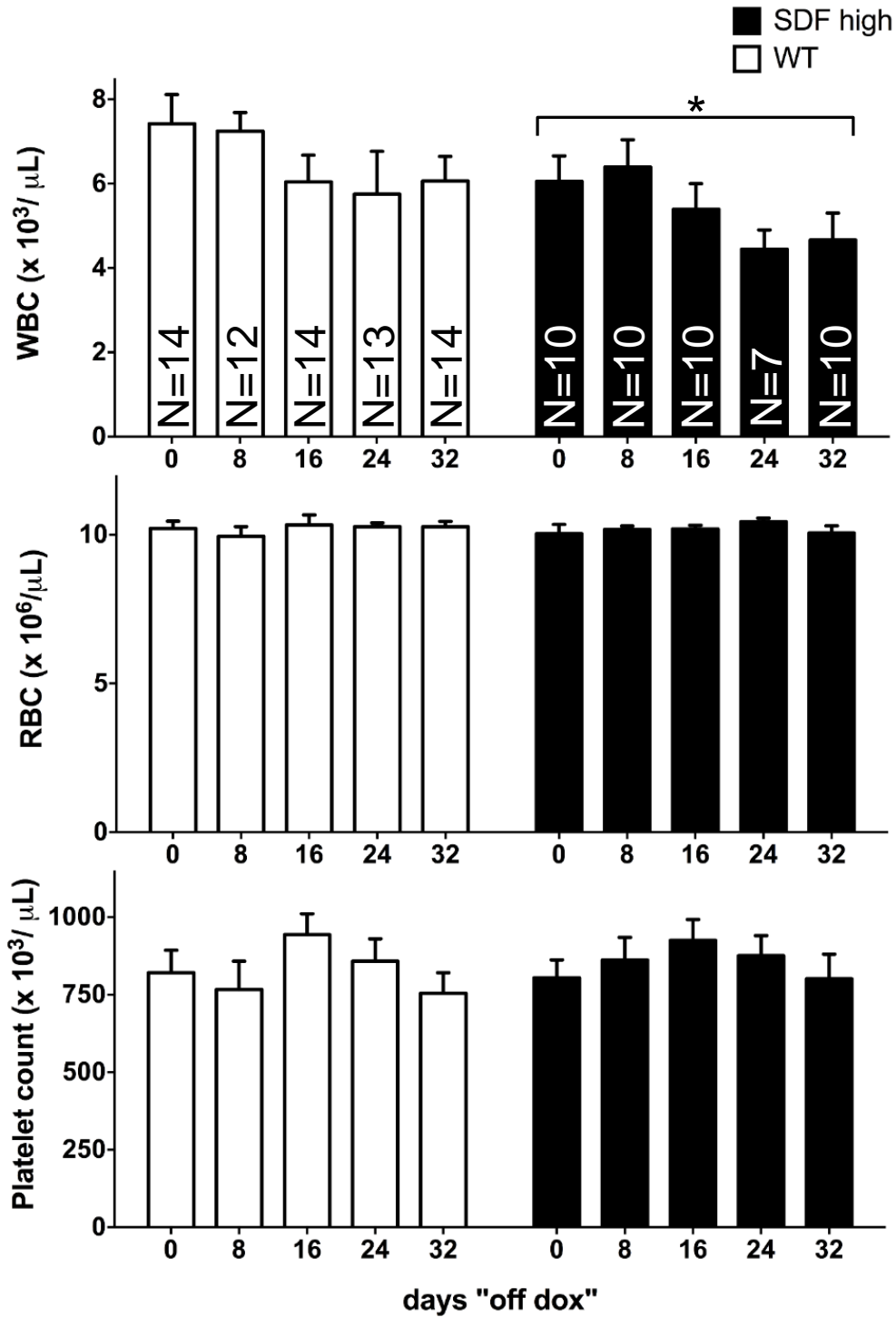
Figure V Time course of hematological changes after initation of SDF expression in WT and SDF high transgenic mice
Doxycycline was removed from the drinking water and blood samples were subsequently collected in WT and SDF high mice.
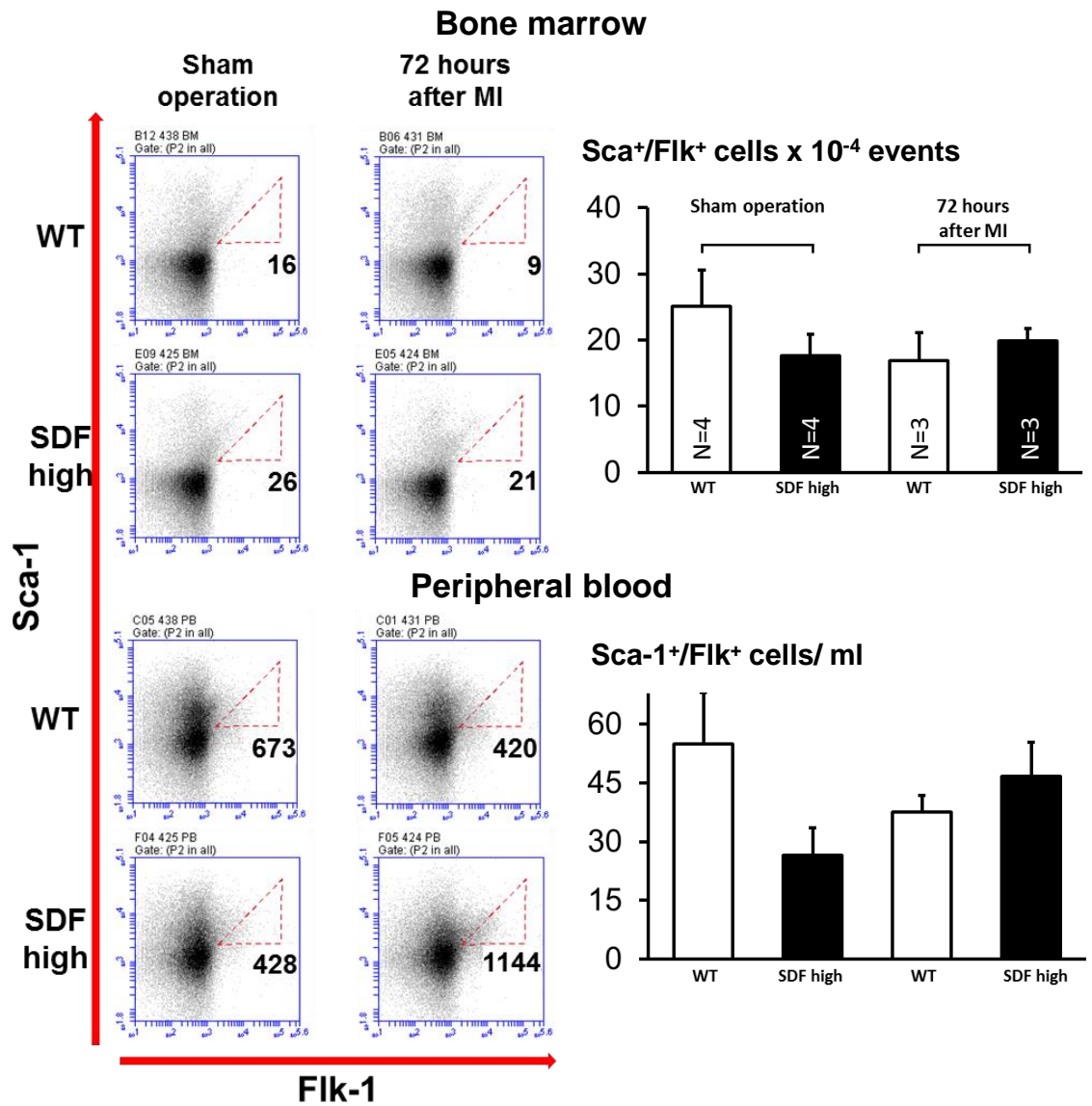
Figure VI Bone marrow resident and circulating EPCs in WT and Tg high mice
14 weeks old mice underwent sham operation or permanent coronary ligation. Blood and BM samples were collected 72 hours later. Samples were stained for Sca-1 and Flk-1 and measured by FACS analysis.

Cardiomyocyte-specific overexpression of SDF increases gene-copy dependent CK1α
expression and AKT phosphorylation in the heart.

We determined the expression of CK1α in whole heart homogenates and found that
CK1α was gene-copy number dependent increased. (**Figure 7 right side**). Similarly, total
AKT increased parallel to the SDF expression level in the heart, while phosphorylation of
AKT did not change. (**Figure 7 left side**).
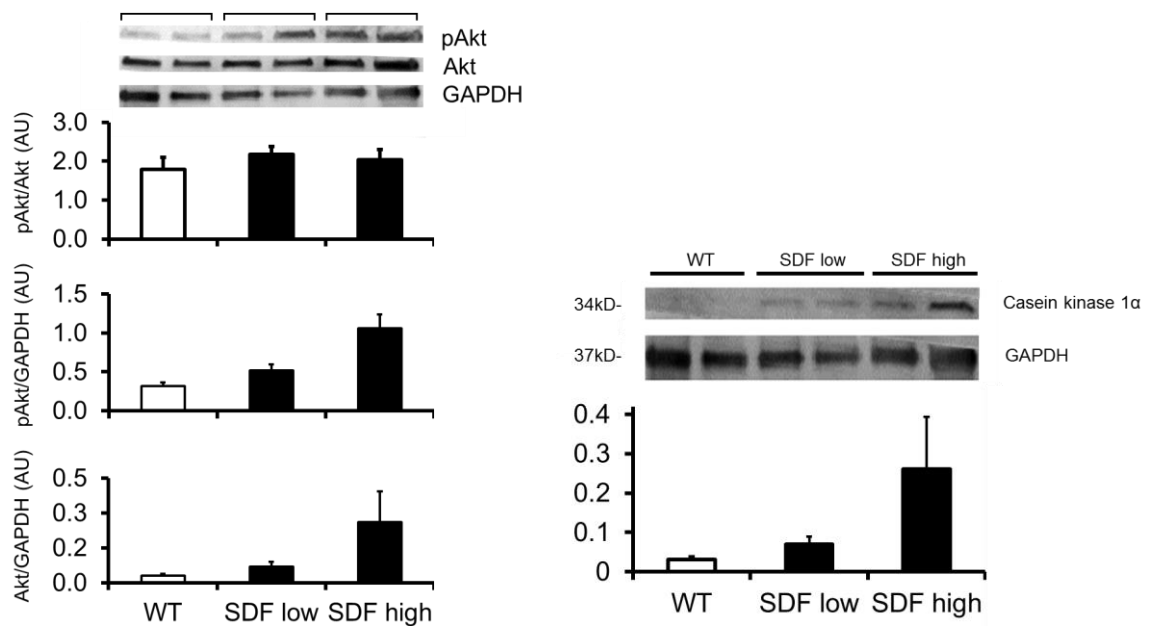
Figure VII Gene-copy dependent protein expression of AKT and CK1α
SDF overexpression resulted in gene-copy dependent increase in total AKT (left panel)
while pAKT was unchanged. SDF overexpression increases CK1α expression gene-dose
dependent. Total heart protein was collected five weeks after initiation of SDF
overexpression. The data represent two independent experiments demonstrating similar
results.

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Cardiomyocyte-specific overexpression of SDF resulted in an increased number of c-kit[+]-cells.

Sections of sham-operated WT and SDF high mice were stained for the stem cell factor receptor c-kit. The number of c-kit^{+} positive cells in the LV wall was higher in SDF overexpressing mice four weeks after sham operation, while the number of c-kit[+] / BrdU^{+} cells was similar to WT mice. Interestingly, the proportion of c-kit^{+} cells which were BrdU[+] was lower in SDF high hearts (one third) than in WT mice (one half). With the current number of experiments, these differences were not statistical significant, but might indicate a decreased proliferation of c-kit^{+} cells under the influence of SDF overexpression.
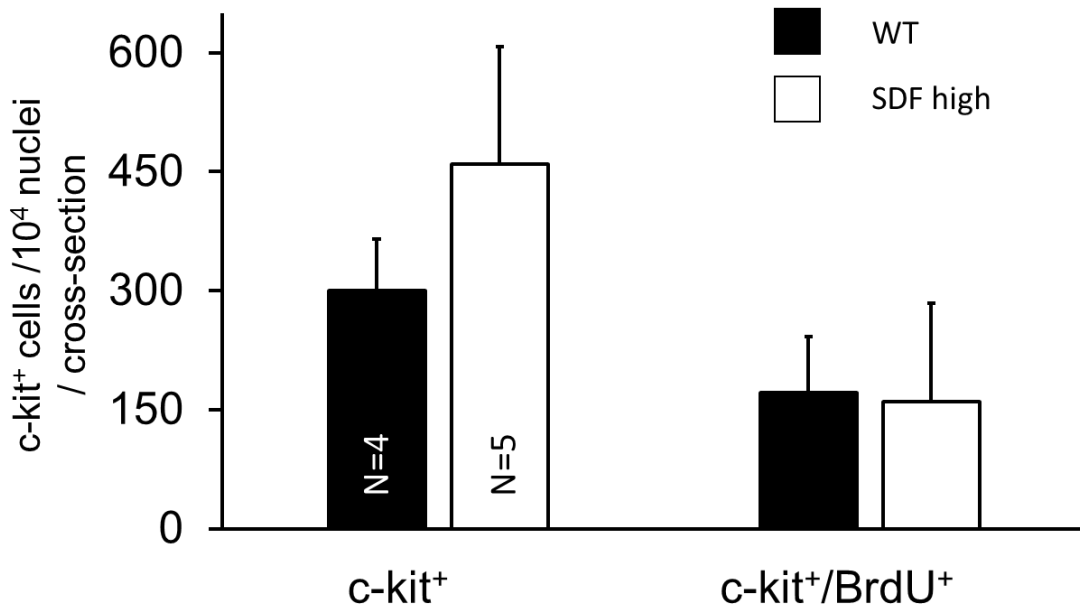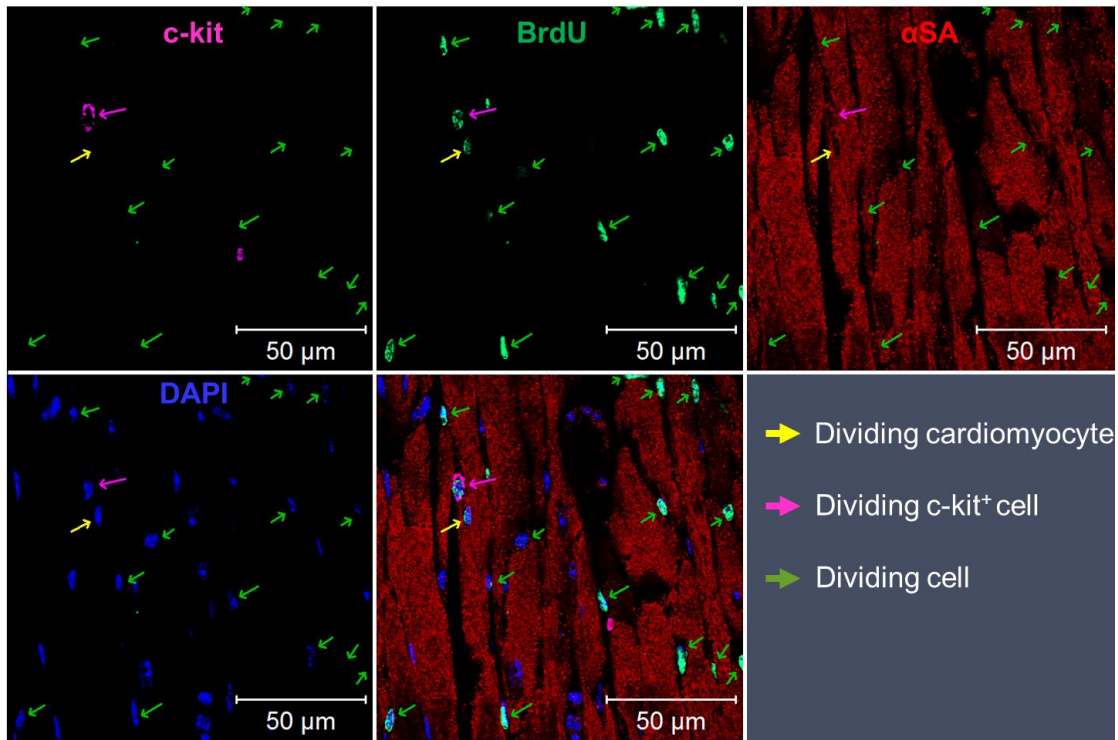
Figure VIII c-kit$^+$ cell in WT and SDF high expressing transgenic mice
The upper panel presents an example of a c-kit$^+$ CSPC, while the lower panel presents on the left side the total number of c-kit$^+$ cells and the right side the number of BrdU$^+$ / kit$^+$ cells. With the current number of experiments, there was no statistical significance between WT and SDF high mice.

CONCLUSION

Our data indicate for the first time, that chronic cardiomyocyte-specific SDF overexpression affects BM derived hematopoiesis as we found a reduction in circulating leukocytes accompanied lower, but not statistical significant, numbers of EPCs both in the peripheral blood and the BM. These results are surprising, considering a relatively short half-life of SDF when released into the peripheral blood. CD26[61,62], and serum proteases[63] quickly cleave SDF, which is reflected by failure to detect SDF in the serum of "high" level SDF expressing mice (data not shown). In fact, others also failed to detect SDF in the blood plasma[64] in a model using a RSV-promoter to drive SDF expression in all or most tissues, presumably reaching higher SDF levels than in our model. However, the authors describe an enhanced progenitor cell cycling, resulting in higher absolute numbers of CFU-GM, BFU-E, and CFU-GEMM in the spleen, and to a lesser extent in the BM, where SDF significantly increase GM-CSF-responsive CFU-GM and M-CSF-responsive CFU-M.[64] These data stand in contrast to our findings as we rather see a decrease in leukocytes.

Our data are limited considering the total number of experiments and animals included taking the variation in WBC numbers into account, even under physiological conditions. The lower number of leukocytes and EPCs in the peripheral blood can be interpreted as a change in homeostatic equilibrium between the BM and the periphery, which would diminish the ability of the BM to participate as cell source in a cardiac regeneration process.

SDF overexpression leads to an increase in CK1α. CK1α has been suggested to be constitutively active, widely expressed and without significant regulation.[53] Data from our laboratory suggest that the SDF-mediated decrease in CK1α decreases CSPCs cell

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proliferation.[46] According to this concept we found less c-kit[+] / BrdU^{+} cells in SDF high mice compared to WT mice, suggesting a more quiescent status of CSPC.

In combination with the increased phosphorylation of AKT known to provide a powerful survival signal for the heart, we conclude that cardiac overexpression of SDF might provide a cardioprotective phenotype, including an increase pool of non-cycling CSPCs.

The implications of our data are limited based on the small number of experiments and the lack of statistical significance. Therefore, these data should be interpreted with caution until confirmed by additional experiments.

CHAPTER 4

AIM II – TO DETERMINE WHETHER CASEIN KINASE 1A MEDIATES CARDIAC STEM / PROGENITOR CELLS AND NEONATAL CARDIOMYOCYTE PROLIFERATION

BRIEF PREVIEW

Background

Although SDF induces quiescence in hematopoietic progenitors and stem cells,[65,66] SDF has been shown to increase proliferation under specific experimental conditions. In the presence of thrombopoietin, SDF induces megakaryocyte progenitor cells (CFU-MK) proliferation[67] and in human circulating $CD34^+$ / $CXCR4^+$ cells, 48 hour incubation with SDF increased the percentage of cells in S / G_2 / M phases compared to untreated controls.[68] The later effect was augmented in the presence of cytokines.[68] Previous data from our group suggested that SDF facilitates CSPCs quiescence by blocking cell cycle progression at the G_0 to G_1 transition.[46] In CSPCs, we showed that SDF decreased the expression of CK1α, a protein kinase considered constitutively active in multiple tissues and to this point without any known regulation. The gene dose dependent increase in CK1 expression in the SDF Tg hearts was in contrast to that seen in CSPCs. To determine whether increased CK1α expression in CSPCs and cardiac myocytes increased proliferation CK1α was overexpressed and proliferation measured in vitro.

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Methods

The effect of CK1α overexpression on un-differentiated and differentiated cells was determined in human CSPCs [kindly provided by Dr. Roberto Bolli, M.D. (Institute of Molecular Cardiology, University of Louisville)] and cultured rat neonatal cardiomyocytes, respectively. Cells were cultured and transfected with pCMV-CK1α. CK1α protein expression was measured 48 hours later with western analysis. Control treated cells were transfected with the equivalent of pCMV-mCherry. Cell proliferation was determined by measuring BrdU incorporation 48 hours after labeling in BrdU containing medium.

Results

Transfection of human CSPCs and neonatal rat cardiomyocytes with pCMV-CK1α resulted in increased expression of CK1α that was dependent on the amount of input vector. Importantly, increased CK1α expression correlated with increased proliferation in both human CSPCs and myocytes, and proliferation increased with increasing CK1α expression.

Conclusion

These experiments indicated that the cell cycle was regulated by CK1α. Previous data suggested that SDF regulates CSPCs cell cycle through down-regulation of CK1α. The current experiments provided evidence that up-regulation of CK1α caused the reverse effect and accelerated cell proliferation in un-differentiated and differentiated cells. Therefore, our data established CK1α as an important regulator of cardiomyocyte cell cycle and its up-regulation after myocardial infarction would accelerate cardiac regeneration.

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MATERIAL AND METHODS

Isolation of human cardiac stem / progenitor cells

Human cardiac stem cells were isolated during the SCIPIO trial and stored in liquid nitrogen until used. For our experiments cells were thawed, plated, and cultured in supplemented stem cell medium containing (DMEM / F12, LIF, ITS, bFGF, Glutamine) up to 4 passages in T75 flasks. An adequate purity of the cell culture was achieved when FACS analysis indicated more than 80% c-kit$^+$ cells in the cell preparation.

Isolation of neonatal cardiomyocytes

Neonatal cardiomyocytes were obtained from Sprague-Dawley rats on the day of birth and about 10 pups were used for each cardiomyocyte preparation. After cleaning the rat with 70% ethanol, a quick decapitation was performed and the thorax was opened with scissors. The hearts were removed and quickly placed in calcium- and bicarbonate-free Hanks with Hepes buffer containing in mM (138 NaCl, 5.8 KCl, 0.8 Mg, 0.8 PO_4, 5.5 Dextrose, 20.06 HEPES, pH 7.4) and heparin (0.15 ml) for subsequent dissection. After incubation in trypsin and DNAse for 20 minutes, the tissue was slowly further dissected towards a single cell suspension. Cells were then transferred to MEM Eagles with Hanks containing penicillin, B12 and 5% calf serum medium, filtered through a cell strainer, and pre-plated for 30 minutes at 37°C to remove non-myocytes. Cells from the supernatant were then plated in 96 well plates in MEM Eagles with Hanks containing B12, 5% calf serum and BrdU (0.1 mM) for 48 hours.

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Transfection of neonatal cardiomyocytes and human CSPCs with CK1α containing plasmid

Human CSPCs and neonatal cardiomyocytes (2×10^4 cells / well) were transferred to 96 well plates and transfected using a cationic liposome formulation (Lipofectamine 2000 with plus reagent, Invitrogen / LifeTechnologies, Grand Island, NY, USA) and 0.5 µL lipofectamine LTX reagent to transfect cells with different plasmid DNA concentrations (i.e 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 µg / µl). CK1α protein expression was measured after 48 hours by western blot analysis.

Measurement of cell proliferation

The cell proliferation was measured using a commercially available BrdU detection kit (Calbiochem, QIA 58). The transfected cells were cultured in BrdU (1:2000) containing opti-Mem medium for 48 hours, and the extent of BrdU incorporation was reflected by the intensity of the absorbance in a final staining reaction: After 48 hours the cells were fixed and the DNA denaturated. After adding anti-BrdU antibody (1:100), the cells were incubated for one hour at room temperature. After a subsequent washing step a secondary HRP-conjugated antibody was added and incubated for 30 minutes at room temperature. The plates were washed again and 200 µL of substrate added to each well. After a 15 minute incubation in the dark stop solution was added. The supernatant of each well was transferred to a different 96 well plate, and the absorbance measured by platereader (Synergy 4, Biotek, Winooski, VT, USA) at dual wavelengths (450 nm and 595 nm).

RESULTS

CK1α induces proliferation of human CSPCs

After 48 hours of incubation, western blot analysis revealed a concentration dependent increase in CK1α expression in transfected CSPCs. CK1α also increased concentration dependent BrdU incorporation indicating accelerated proliferation after administration of CK1α **(Figure 9A).**

CK1α induces neonatal cardiomyocyte proliferation

The western blot analysis of protein samples from neonatal cardiomyocytes harvested 48 hours after transfection revealed a similar increase (data not shown) as seen in CSPCs. Furthermore, we detected a concentration dependent augmentation of BrdU incorporation, suggesting increasing proliferation with increasing CK1α expression **(Figure 9B).**

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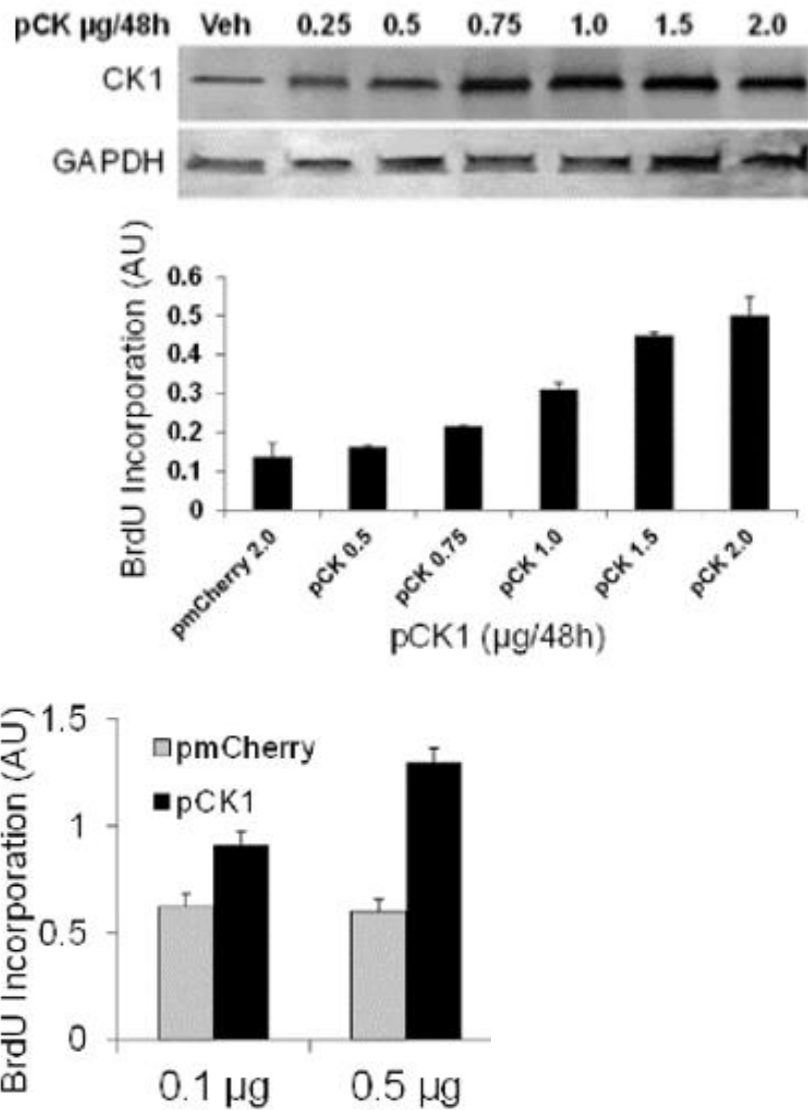
Figure IX Proliferation of human CSPC and neonatal rat cardiomyocytes after transfection with CMV-CK1α vector
9A: Proliferation of human CSPCs and **9B**, proliferation of rat neonatal cardiomyocytes. Each panel reflects the average of two experiments.

CONCLUSION

The cellular function of CK1α is not well understood. Here we provide evidence

that CK1α facilitates concentration dependent cell proliferation in human CSPCs and in

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neonatal cardiomyocytes. These data confirmed our previous finding[46] and suggested CK1α as a novel regulator of cell cycle in the CSPCs and cardiomyocytes.

The presented data reflect the results of two independent experiments in two different kind of tissues (human CSPCs and rat neonatal cardiomyocytes) suggesting the same effect of CK1α. Despite the lack of statistical significance caused by the limited number of experiments we feel confident that CK1α is a novel regulator of cell cycle in cardiomyocytes and CSPCs.

CHAPTER 5

AIM III – TO DETERMINE WHETHER CARDIOMYOCYTE-SPECIFIC OVEREXPRESSION OF STROMAL CELL-DERIVED FACTOR 1 ACCELERATES MYOCARDIAL REGENERATION AFTER PERMANENT CORONARY LIGATION

BRIEF PREVIEW

Background

In addition to other investigators, our group has previously shown that SDF, given before myocardial ischemia / reperfusion, protected the heart in a preconditioning-like manner[9] and that blockade of CXCR4 by AMD3100 accelerated myocardial injury.[10] Several previous studies have shown that increasing SDF in the heart by either preventing protease mediated cleavage[43,69] or increasing SDF concentration through transplantation transfected fibroblasts[12] or SDF overexpressing mesenchymal stem cells[13] improves myocardial function. Whether SDF facilitated myocardial regeneration was not assessed in any of these models.

Methods

The regeneration potential in SDF and WT mice was evaluated at different time points after permanent coronary ligation. The earliest infarct size measurement was

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performed three days after permanent coronary ligation to ensure an equivalent degree of injury between the mouse strains. At this early time point, we stained hearts with triphenyltetrazolium chloride (TTC) to determine the infarct size. Subsequently, we sacrificed mice after seven and 30 days to assess the LV remodeling process. At these time points, Masson's trichrome staining was utilized for infarct size assessment. During the recovery period, mice received 5-bromo-2 deoxycytidine (BrdC) allowing us to determine the amount of proliferation within the myocardium through detection of incorporated BrdU derived from BrdC. These data were verified by Ki67 staining, a marker of cell mitosis, reflecting the amount of cells undergoing cell division at the point of sacrifice. In addition to the regenerative aspect of remodeling, we determined the apoptosis rate by deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) staining. Furthermore, we determined the capillary density in the border zone and the remote area as indicators of neovascularization. Staining for the stem cell factor receptor c-kit determined the number of CSPCs.

To verify the long-term effect of SDF overexpression on cardiac remodeling, elderly mice underwent the same permanent ligation / 30 days recovery protocol.

All morphological analyses were supported by echocardiographic determination of LV function. The assessments were performed before and during the recovery period after two, 14, and 30 days, respectively. Regional myocardial function was measured with the help of speckle-tracking strain analysis.

Results

Permanent coronary ligation resulted in an equivalent injury in WT and SDF overexpressing mice. However, the outcome of cardiomyocyte-specific SDF overexpression was a reduction in infarct size and improved LV ejection fraction four weeks after myocardial infarction. This protection sustained when SDF was overexpressed for a period of at least 30 weeks (elderly mice). SDF overexpression markedly accelerated myocardial remodeling indicated by increased numbers of BrdU$^+$ cardiomyocytes in the border- and scar area of the LV and an augmented apoptosis rate. Interestingly, we did not detect any capillary density difference in WT and SDF Tg mice after sham operation or permanent coronary ligation.

Conclusion

Our data indicated that cardiomyocyte-specific overexpression of SDF facilitates myocardial regeneration after permanent coronary ligation. Surprisingly, this effect was not related to an increase in vascularization but rather to a direct effect on cardiomyocyte proliferation. The number of new cardiomyocytes appears to be too small to explain the improvement in LV function detected by echocardiography; therefore, it is likely that other paracrine effects of SDF contributed to the increase in ejection fraction.

MATERIAL AND METHODS

Experimental protocol

To investigate the early and late effect of chronic SDF overexpression on myocardial regeneration we performed two sets of permanent ligation experiments,

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gathering hearts for pathology evaluation after one and four weeks, respectively (as described below, **Figure 10**).
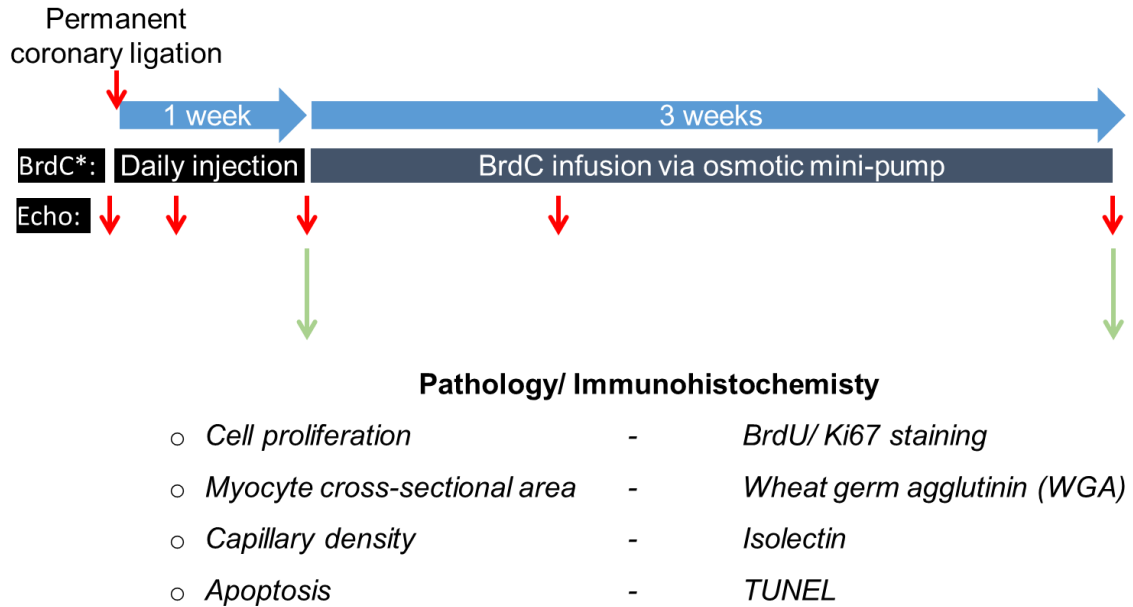
Figure X Experimental protocol of permanent coronary ligation experiments
C57 / B6 WT, SDF low, and SDF high mice (12 weeks old / four weeks off dox) were used to perform the permanent ligation experiments. Mice were followed for either one or four weeks after permanent coronary ligation prior to pathological examination. **BrdC**, 5-Bromodeoxycytidine was used as DNA labeling agent, which is deaminated prior to incorporation into DNA as BrdU, because its greater solubility in PBS and a slower rate of metabolism in rats and mice resulting in larger intracellular supply. **Echo,** Echocardiography performed before ligation, at day 2, 7, 14, and 30, respectively.

WT and "high" expressing SDF (SDF high) Tg mice were monitored during both periods, while experiments in "low" expressing SDF Tg mice (SDF low) were only performed with a four-week recovery period. To confirm whether the duration of SDF overexpression will affect the size of injury, we performed an additional set of experiments in SDF high mice for more than 30 weeks (i.e. mice of 45-50 weeks of age).

TRIPHENYLTETRAZOLIUM-CHLORIDE- (TTC) STAINING

Triphenyltetrazolium staining was used to identify viable and dead myocardium. The staining principle is based on the presence of membrane bound diaphorases that use NADH (NADPH) as electron donor to reduce tetrazolium chloride to a red formazan pigment, allowing the distinction between viable and dead tissue.[70] We assessed the initial injury 72 hours after permanent coronary ligation: Mice were anesthetized with pentobarbital (0.4 mg i.p.), the chest was opened and hearts were arrested in diastole by injection of 0.1 ml Cd-KCl (0.1M Cd, 3 M KCl) into the LV cavity. The heart was removed and the aorta was cannulated with a 14-gauge blunted needle and perfused retrograde using the Langendorff method to remove intracoronary blood. The coronary bed was perfused with heparin (100 IU) containing Krebs-Henseleit buffer at a constant rate (1 ml / minute) for ten minutes. Thereafter, the perfusion solution was changed to 10% Evans blue (E2129, Sigma Aldrich, St. Louis, MO, USA) solution to stain the remote area. After infusing one to two milliliters at a constant rate (0.5 ml / minute), excessive blue stain was removed by washing the hearts in PBS. Subsequently, hearts were transferred to a heart slicer and cut into one millimeter cross-sections. The staining was finalized by incubating the sections in 1% triphenyltetrazolium solution for 15 minutes at 37°C, demarking viable myocardium (red) from damaged myocardium (pale, unstained). To intensify the staining results, tissue sections were fixed in 4% paraformaldehyde for 20 minutes. The staining results were digitalized by a camera mounted on a surgical microscope. The infarct size was then measured by planimetry, taking both sides of each slice into account, and expressed as percentage of dry LV weight.

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TRICHROME STAINING

The sections were de-paraffinized and rehydrated to distilled water using a standard graded xylene-ethanol series before staining. The infarct size was measured after Masson's trichrome (HT15-1KT, Sigma Aldrich, St. Louis, MO, USA) staining, based on the interaction of ionized acid dyes reacting with ionized basic tissue and distinguishing collagen, scar, and muscle fibers. After an overnight incubation of the tissue sections in Bouin's solution to intensify the final coloration, the nuclei were stained with Weigert's iron hematoxylin, and the cytoplasm and the muscle with Bierbrich scarlet-acid fuchsin solution (Sigma Aldrich, HT15-1). A subsequent treatment with phosphotugstic (Sigma Aldrich, HT15-2) and phosphomolybdic acid (Sigma Aldrich, HT15-3) removed the plasma stain (Bierbrich scarlet-acid fuchsin) from collagen, which then appeared paler than the muscle stain. Then an aniline blue solution, which has a molecular weight between the plasma stain and the poly-acids, was applied to mark collagen. After 15 minutes of incubation, collagen stained blue without affecting the plasma stain. Subsequently, the sections were rinsed in acetic acid, rendering the shades of color more delicate and transparent.[71] Photos of the stained sections were digitally acquired on an inverted microscope equipped with a digital camera. The LV planimetry was performed using publicly available software (NIH Image J, 1.48s ver.) and the LV area, risk region, remote region, border zone, LV cavity area, and infarct zone were determined as previously described.[3] To quantify the degree of LV dilatation and the degree of infarct wall thinning, the LV expansion index was calculated using a modification of the method of Hochman and Choo:[72]

$$Expansion\ index = \frac{LV\ cavity}{Total\ area}\ x\ \frac{Non-infarcted\ region\ wall\ thickness}{Risk\ region\ wall\ thickness}$$

Equation 1   LV expansion index

Measurement of cardiac regeneration by chronic administration of BrdC

To measure the cardiac regeneration 5-bromo-2 deoxycytidine (BrdC, MP Biomedicals, Solon, OH, USA) was injected i.p. daily for seven days, and afterwards continuously infused until the end of the experiment. BrdC is rapidly deaminated prior to incorporation into DNA as 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue frequently used to detect proliferation of cells that can be passed to daughter cells upon replication.[73] The physical properties of BrdC (higher solubility in PBS and its slower metabolism in mice) made it more suitable for intracellular BrdU supply for DNA labeling in our experiments. During the first seven days after coronary ligation, BrdC (2 ug / g / h i.p.) was injected daily. After surviving the initial recovery phase, the mice were re-anesthetized and an osmotic minipump (Alzet type 2004, Alzet, Cupertino, CA, USA) was implanted subcutaneously between the scapulae to deliver BrdC (0.7 ug / g / h) until the end of the experiment.[74] Incorporated BrdU was determined by immunohistochemistry methods (see below).

BrdU incorporation as an indicator of cell proliferation has been criticized because cells undergoing DNA repair might also incorporate BrdU into their DNA.[75] However, the level of BrdU incorporation with DNA damage is orders of magnitude less than that during DNA synthesis and replication.[76] The final number of BrdU positive cells reflected the entire amount of cells undergoing DNA repair and / or cell division. To provide additional support for DNA synthesis and cell division sections were also stained for Ki67 (VP-RM-

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4, Vector labs, Burlingame, CA, USA): The nuclear antigen Ki67 is expressed in all phases of the cell cycle except for G_0.[77] Ki67 is not involved in DNA repair[78] and therefore a specific marker for cells undergoing mitosis as mainly apparent in the late S phase and increased in G_2 until pro- and metaphase. Measuring Ki67 positive cells in our samples allowed us to determine the number of dividing cells at the time of sacrifice.

Detection of Apoptosis

Heart failure as a consequence of myocardial infarction is characterized by progressive cell loss.[79] Previous data suggest that SDF might abolish programmed cell death.[80] Therefore, TUNEL staining was used to identify double-stranded DNA fragmentation within the scar, border, and remote zone after myocardial infarction.

Echocardiography

Echocardiographic studies were performed before the ligation experiment (baseline) and 72 hours, 14 days, and 30 days after the infarct to assess the initial injury and the progression to heart failure (14 days and 30 days, respectively) using a VEVO2100 echocardiography system (FUJIFILM VisualSonics, Toronto, Ontario, Canada) equipped with a MS400 (18-38 MHz) cardiovascular ultra-high frequency linear-array transducer probe. To ensure good quality images for speckle-tracking based strain analyses, all images were acquired at a rate of more than 200 frames / second, providing an optimal temporal resolution and minimizing artifacts. The mice were anesthetized with isoflurane (2-3 Vol.%) and the anterior chest was shaved. The mice were placed on a heated pad in the supine position and the body temperature was measured by a rectal probe to maintain 37°C. Throughout the study, the anesthesia depth was adjusted with isoflurane (~1 Vol%) to

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achieve a heart rate between 450-500 bpm. Animals with a heart rate out of the target range were excluded from the analysis. Standard parasternal long- and short-axis views were performed to obtain two-dimensional, M-mode, and speckle-tracking analysis images.[81] An average of five cardiac cycles was used for each analysis.

<u>Strain analysis</u>

The strain is a dimensionless variable reflecting the deformation of the heart in circumferential, radial, and longitudinal dimension. As an angle-independent method the quantification is less operator dependent. The method is based on the estimation of velocity vectors by tracking segmental tissue motions in multiple planes and axes serially over the cardiac cycle, providing detailed information about global and regional LV function. Thereby, a negative strain reflects shortening (or thinning) while a positive strain reflects elongation (or thickening) of a LV wall segment. Based on the anatomic orientation of LV fibers the systolic radial strain results in positive values under physiological conditions, whereas the systolic longitudinal strain results in negative values. In addition, the temporal derivative of the strain (i.e. the strain rate) reflects the rate of deformation similar to the shortening velocity per fiber length.[82]

$$\varepsilon = \frac{\Delta l}{l} \approx \frac{v(r) - v(\Delta r + r)}{\Delta r} \, \Delta t = \text{SR}\Delta t$$

Equation 2   LV strain rate

The strain rate was measured in six different areas of the LV wall: The posterior and anterior wall were divided into a basal, middle, and apical segments. For each segment the radial and longitudinal strain rate was measured, allowing us to determine precise areas of increased or decreased contractility.

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The LV mass=1.05 x ([anterior wall thickness + LVEDD + posterior wall thickness]^3 – LVEDD^3) was calculated by M-mode cubic method where the specific gravity of cardiac muscle is 1.05 g / L.[83]

Definition of border, remote zone, and scar area

Three different areas of each cross section were evaluated: The border zone was defined as an area reaching one millimeter from the end of the scar towards "healthy tissue" in one direction and "scar tissue" in the opposite one. The remote zone represents the area of the none-infarcted ventricular wall between the border zones. The scar area is defined as the fibrotic area between the edges of the border zone (**Figure 11**).

Immunuofluorescent staining

As previously described, the sections were prepared except for the following modifications: The antigen retrieval was modified dependent on the target: For staining of c-kit and surface markers like CD45 we used a basic EDTA buffer (pH 8.0), as this buffer is suitable for detection of rare and sensitive antigens. In all other cases, we used citrate buffer (sodium citrate buffer, citrate buffer, pH 6.0). Staining for c-kit$^+$ CSPCs and BrdU was performed as described above. Cardiomyocyte cross section area was determined after staining with a FITC-labeled wheat germ agglutinin antibody (WGA, Molecular Probes, Eugene, OR, USA). To quantify cardiomyocyte size, images were captured with a 40X objective and only myocytes captured in cross-sections were used for measurement.

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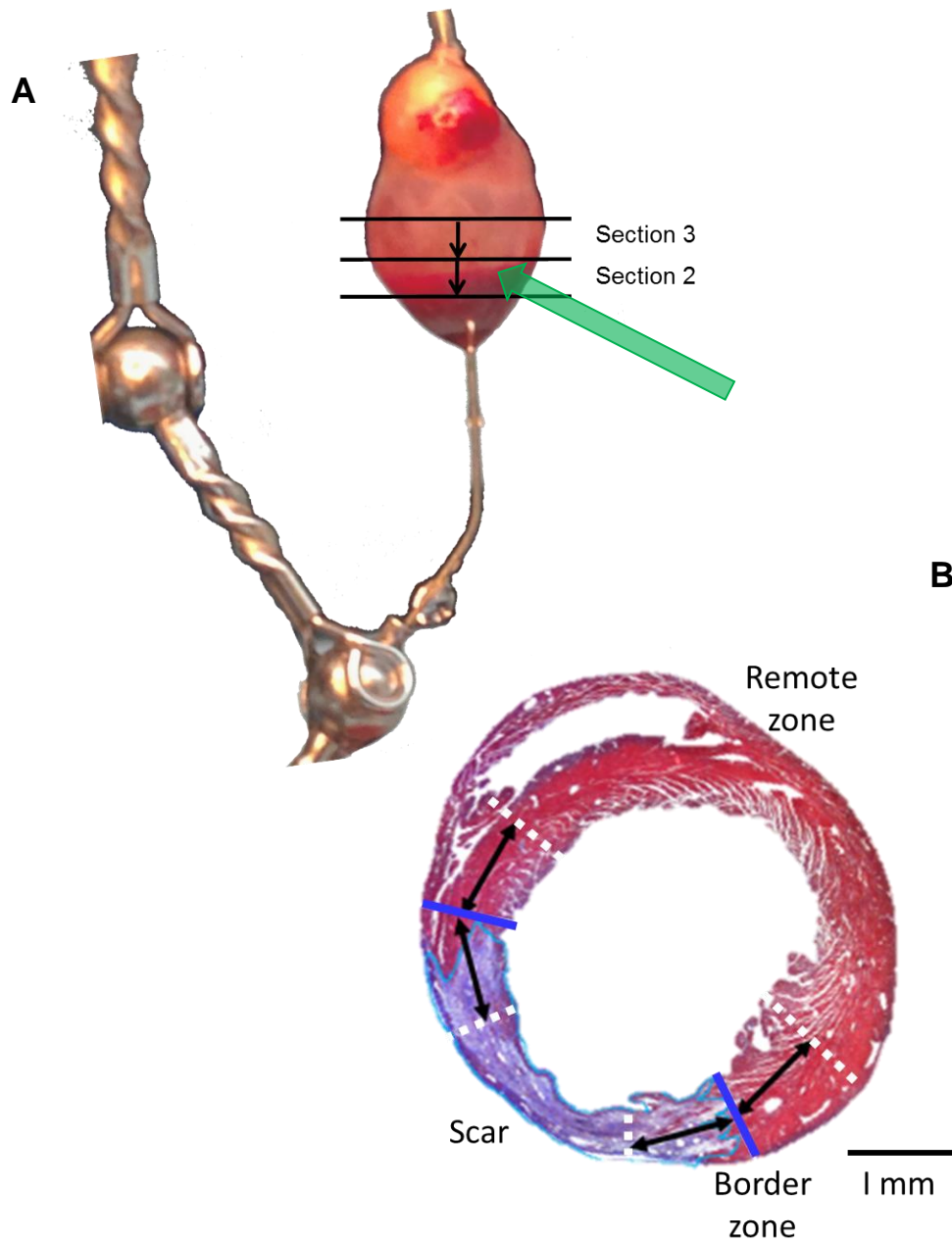
Figure XI Example of ligated WT heart and cardiac-cross section
A: Heart of a WT mouse four weeks after permanent ligation mounted on a perfusion cannula. The green arrow indicates the level of remaining blood visible through the thin, infarcted anterior wall. Black bars indicate sectional area of four total sections of which section two and three were used for analysis. **B:** Typical cross section after trichrome staining. The blue marked area reflects the scar region. The area including the scar between the two blue bars marked the area at risk. The area between the which dotted lines was considered as border zone.

Myocytes with nuclei of round morphology were selected to ensure those with true cross sectional orientation were included and those at oblique angles which would skew data were excluded. The capillary density in the border and remote zone was determined after staining with the endothelium-specific, FITC conjugated isolectin B4 antibody (FL1201, Vector labs, Burlingame, CA, USA). The number of apoptotic nuclei was detected after TUNEL staining (DeadEnd TUNEL Fluorometric System, Promega, Madison, MI, USA). Cardiomyocytes were counterstained with α-sarcomeric actin (Sigma, St. Louis, MO, USA).

Statistical analysis

All data were expressed as mean±SEM and the differences were analyzed using unpaired Student's t-test or a two-way ANOVA [group effect (i.e. genotype) and time effect (time after initiating SDF overexpression)], facilitating Bonferroni's correction for multiple comparisons. The statistical significance was defined as $P<0.05$ or in cases of multiple comparisons adjusted by dividing 0.05 by the number of comparisons. The statistical analyses were performed using PRISM (Vers. 6.0e for MAC OSX, Graphpad Software, La Jolla, CA, USA). The immunopathology assessment was performed in one to two LV cross sections by confocal microscopy. The data represented in the analysis reflecting results of five to 15 images taken at 63X magnification for each area as described above. Per treatment group, the number of animals included in each staining step reached seven to 18 mice.

RESULTS

Exclusions

From the pool of mice undergoing ligation or sham operation, 18% died before the proposed end of the experiment and 13% were excluded due to technical problems (i.e. missed ligation, anesthesia complications) resulting in an overall survival rate of 69%.

Reduction of myocardial injury

Following the American Heart Association's[84] expert committee guidelines we measured the infarct 72 hours after permanent coronary ligation to ensure that the initial injury was consistent between the mouse strains **(Figure 12)**. The LV weight of SDF high was significantly smaller compared to WT mice; nevertheless, we did not detect a difference in infarct size (WT: 36±3% vs. SDF high: 42±2% of the area at risk, P>0.05), suggesting that the initial injury was equivalent between both groups. Re-evaluation of the infarct size after one week revealed the same result [i.e. no difference in infarct size between WT (59±7%) and SDF high: 53±5% of the area at risk, P>0.05)] while the risk area was similar (WT: 49±8% vs. SDF high: 55±1% of the LV, **Figure 13**). In contrast, four weeks after permanent ligation the infarct size (percentage of LV) was significantly reduced in SDF high (13±2%) compared to WT (23±3%), P<0.01) and SDF low mice (23±5%, P<0.05), respectively. Despite the presence of the same scar size the infarct expansion index was almost significantly reduced in SDF low (1.7±0.2) compared to WT (2.4±0.4, P=0.07), reflecting an increased anterior (SDF low: 1.1±0.1 mm vs. WT: 0.8±0.1 mm, P<0.05). Similar results were obtained in elderly mice overexpressing SDF for more

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than 30 weeks: The infarct size was reduced from 24±4% (WT, n=5) to 8±2% of the LV

(SDF high, n=5, p<0.01, **Figure 14**).
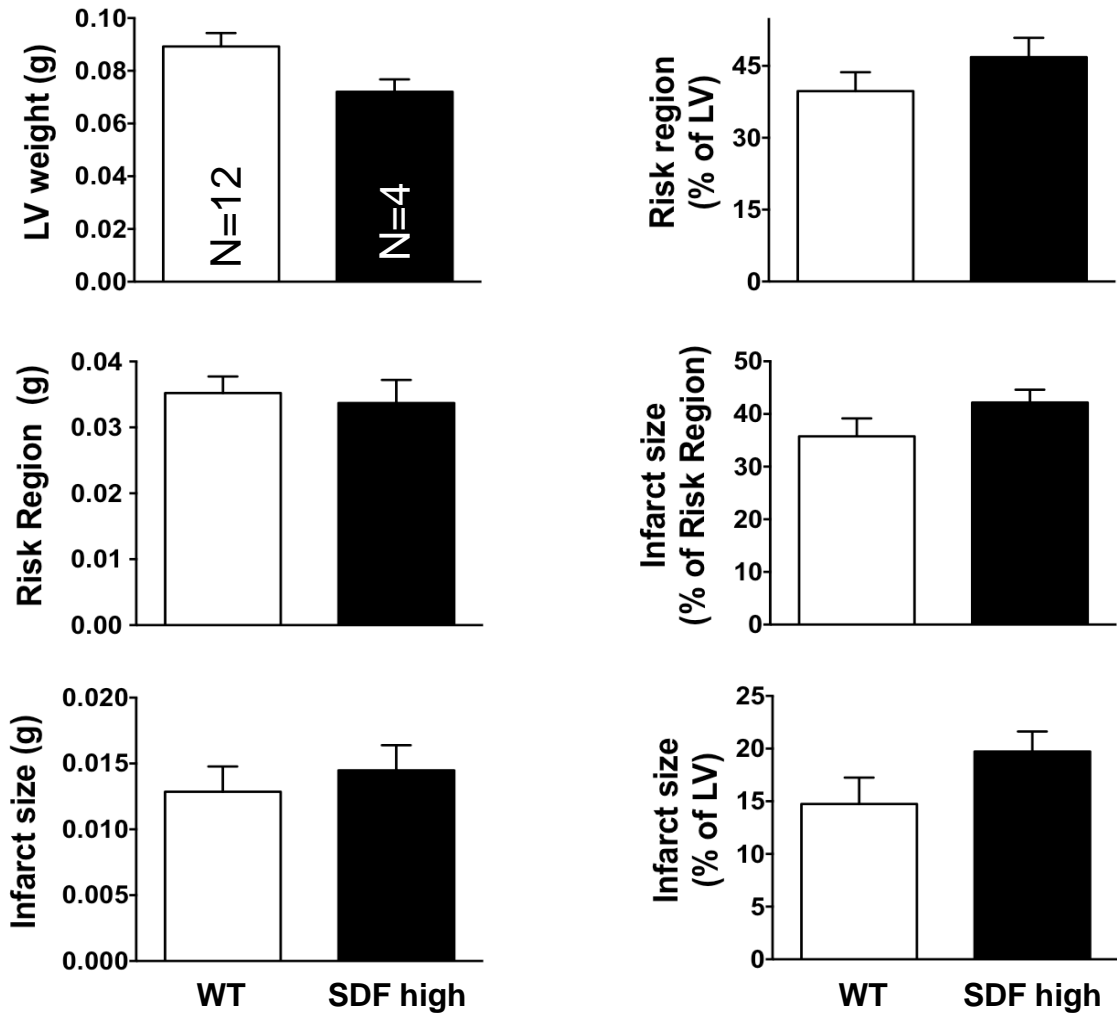
Figure XII Morphometry in WT and SDF high 72 hours after permanent ligation
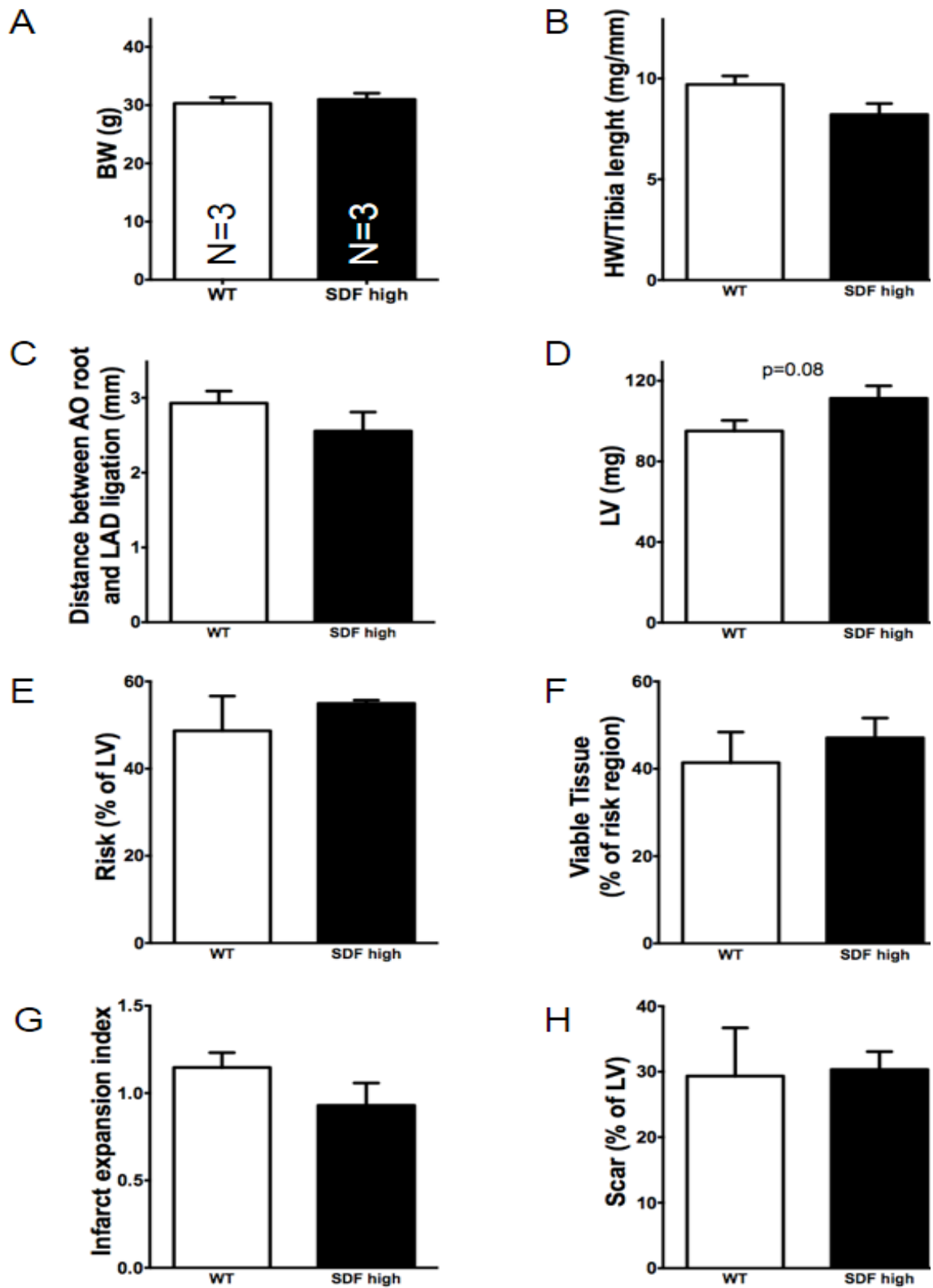
Figure XIII Morphometry of WT and SDF high mice one week after permanent ligation
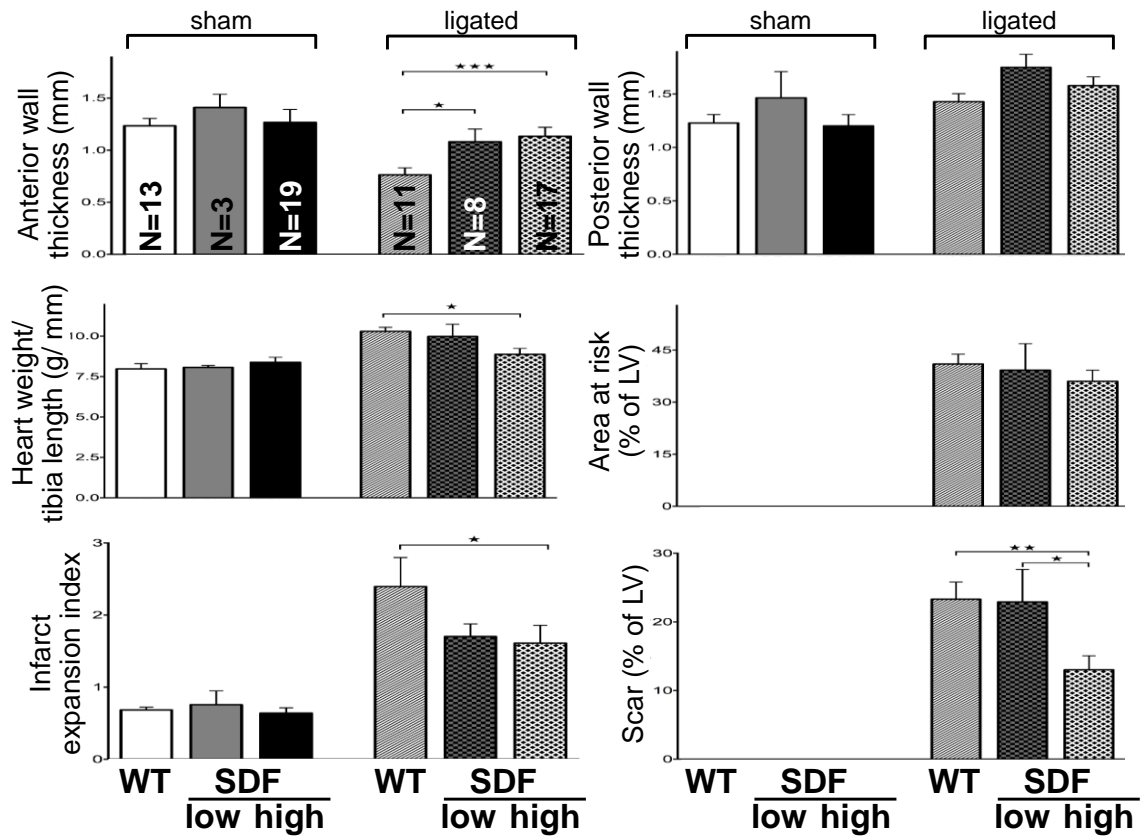
Figure XIV Morphometry in WT, SDF low and SDF high mice four weeks after permanent coronary ligation
The permanent ligation resulted in significant reduction in anterior wall thickness in WT mice compared to SDF low and SDF high, and a significant reduction of infarct size and infarct expansion index in SDF high compared to WT mice.

Improvement of LV function

The echocardiographic evaluation in young and elderly mice revealed that expression in SDF low and SDF high mice neither changed the regional wall (data not shown) nor the global LV function (**Figure 15**). However, in contrast to a progressive reduction in the LV function in WT mice, SDF mice maintained the cardiac function on the level reached two days after infarction, resulting in a significant difference in the

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ejection fraction (WT: 29±4% vs. SDF high: 47±5%, P<0.05) at the end of the experimental period. The regional wall motion was improved indicated by a trend towards an increased wall thickening fraction of the anterior wall (**Figure 16**). In addition, regional changes within the LV were analyzed by strain rate **(Figure 17)** indicating increased longitudinal and radial strain rate of the mid and apical wall sections of the anterior, infarcted wall (**Figure 18**).
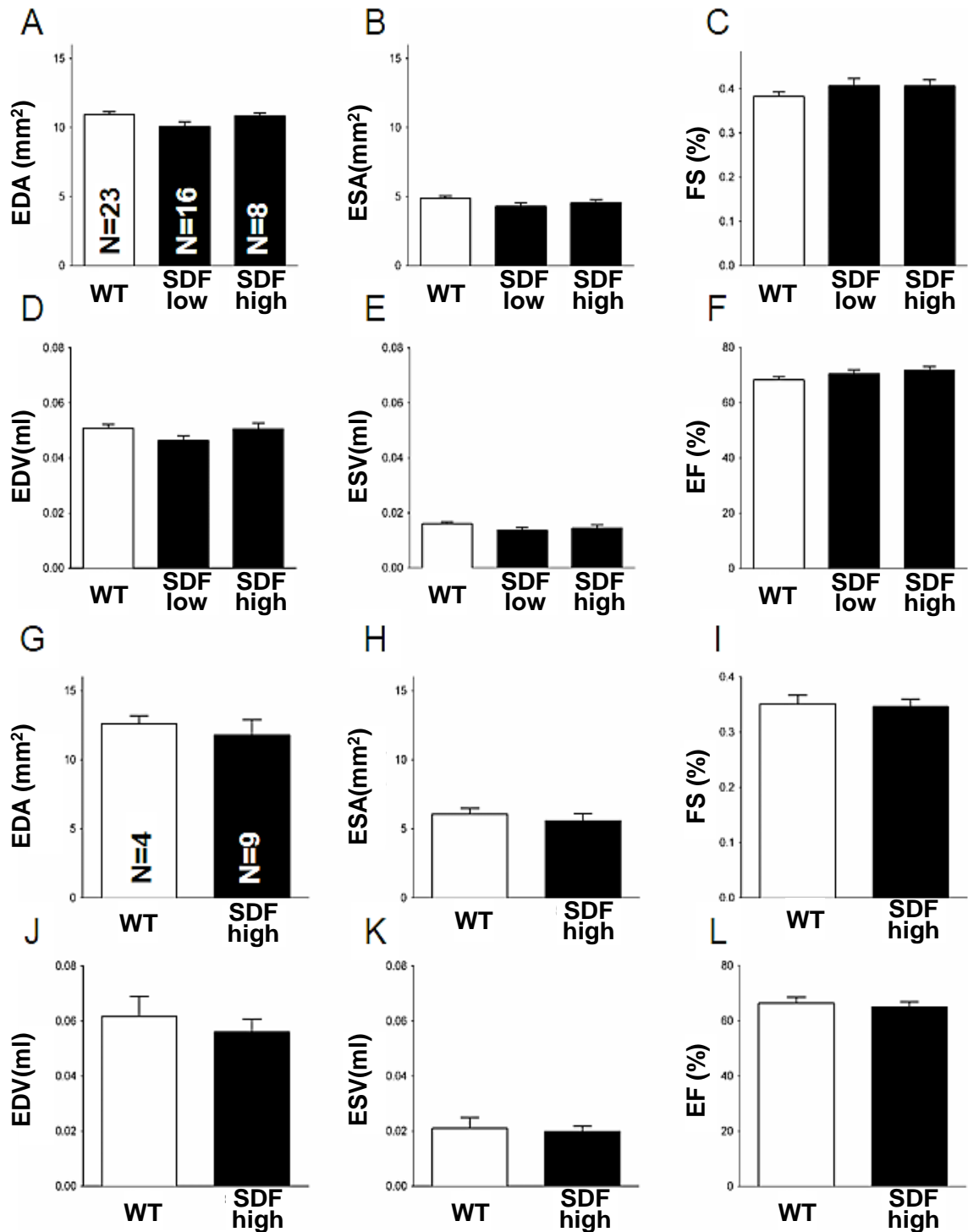
Figure XV Baseline cardiac function in mice overexpressing SDF at five weeks and more than 30 weeks

A-F: LV function variables in WT and SDF high mice overexpressing SDF for five weeks. **G-L:** LV function variables WT and SDF high mice overexpressing SDF for more than 30 weeks. **EDA:** end-diastolic area, **ESA:** end-systolic area, **EDV:** end-diastolic volume; **ESV:** end-systolic volume, **FS:** fractional shortening, **EF:** ejection fraction

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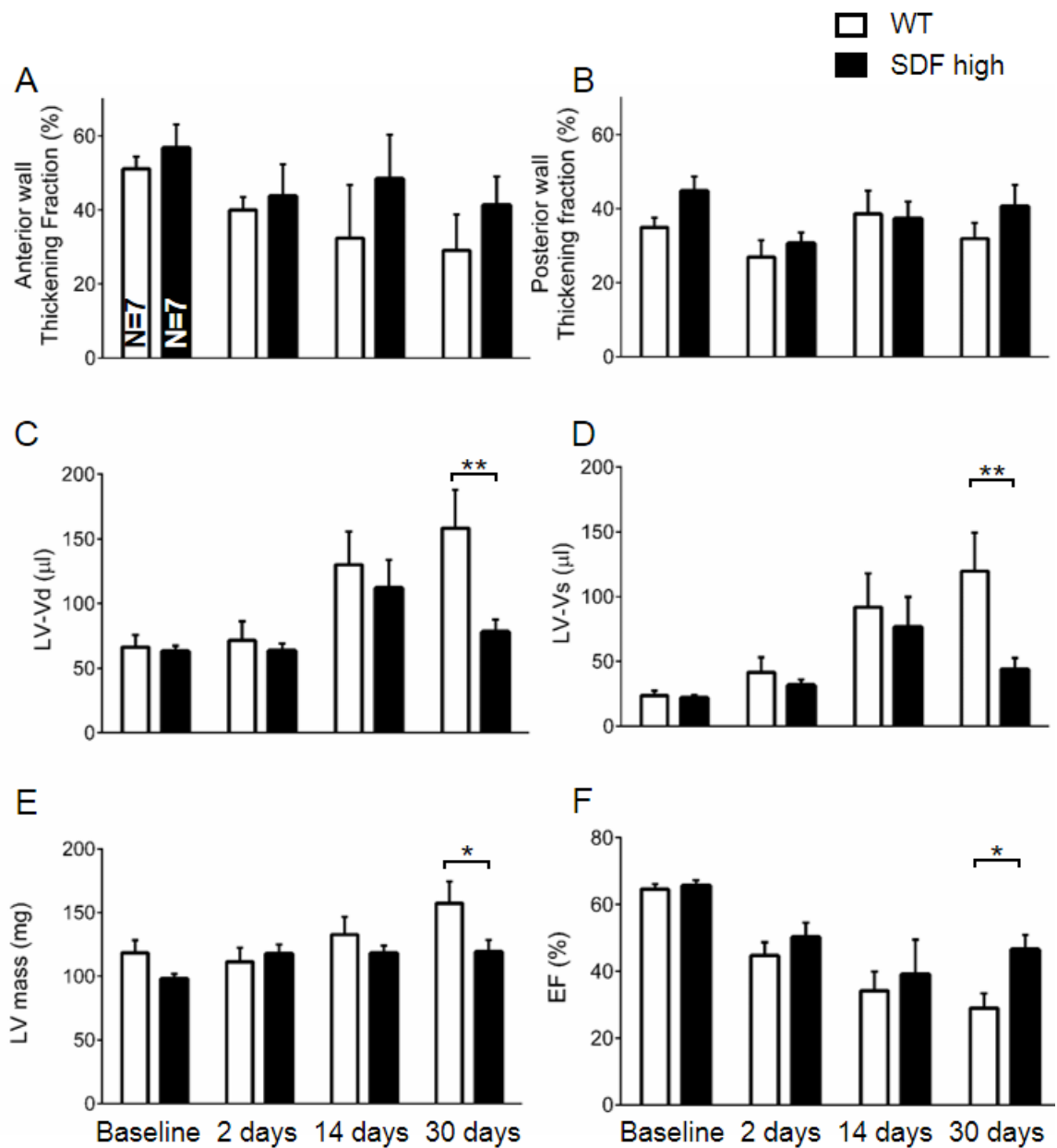
Figure XVI LV function in WT and SDF high mice before and at different time points after permanent coronary ligation

LV-Vd: end-diastolic LV volume, **LV-Vs:** end-systolic LV volume, **EF:** left-ventricular ejection fraction. *, p<0.05, ** p<0.01, Two-way ANOVA with Bonferroni correction for multiple comparisons.
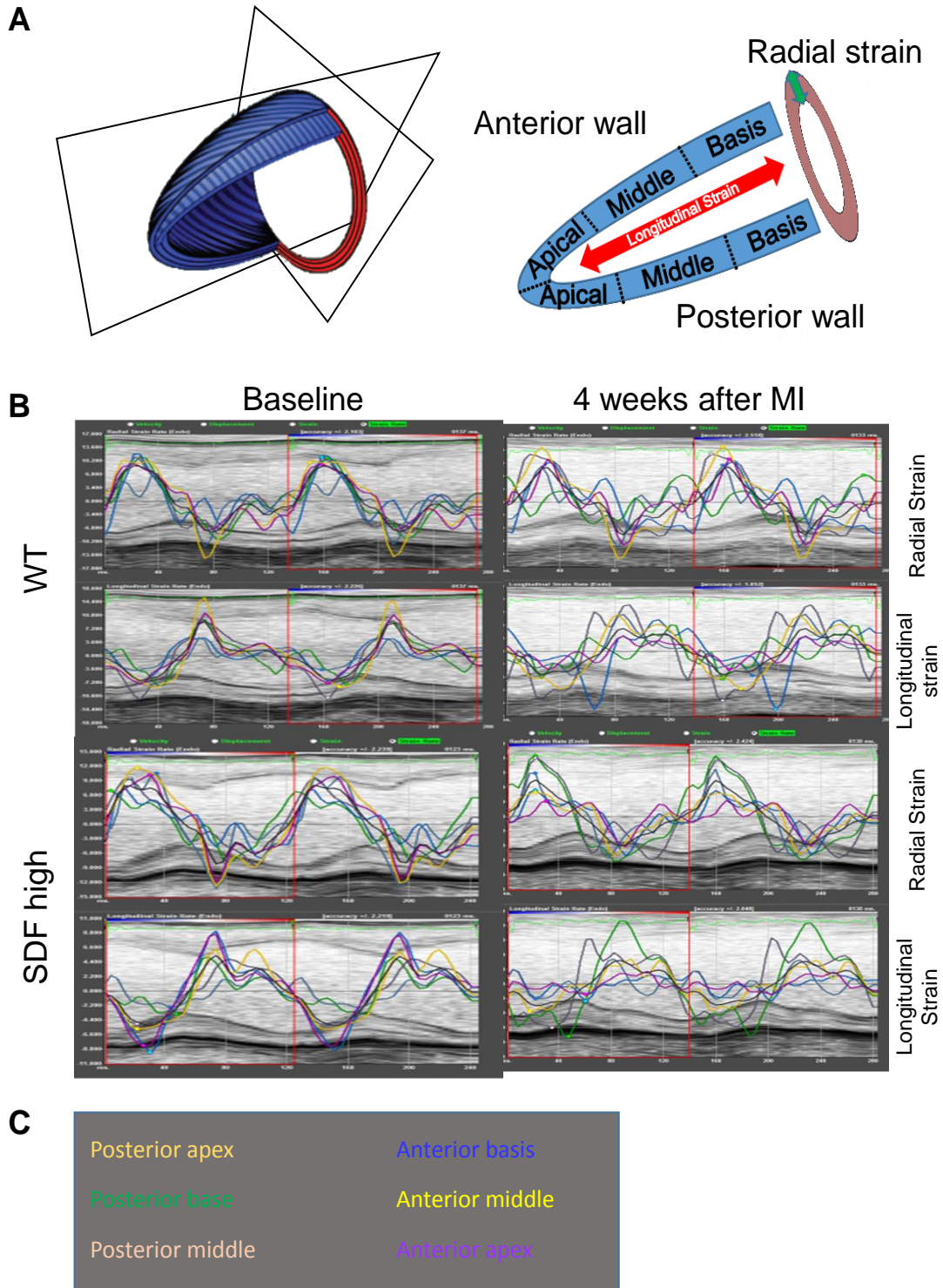
Figure XVII Examples of radial and longitudinal strain in WT and SDF overexpressing mice before and four weeks after coronary artery ligation
A: Six different areas were used for longitudinal and radial strain rate analysis. **B:** Example of radial and longitudinal strain rate of a WT mouse before **(left)** and four weeks after coronary ligation **(right)**, which resulted in asynchronous contraction. **C:** Color code used in *B*, indicating different areas of the left ventricle.
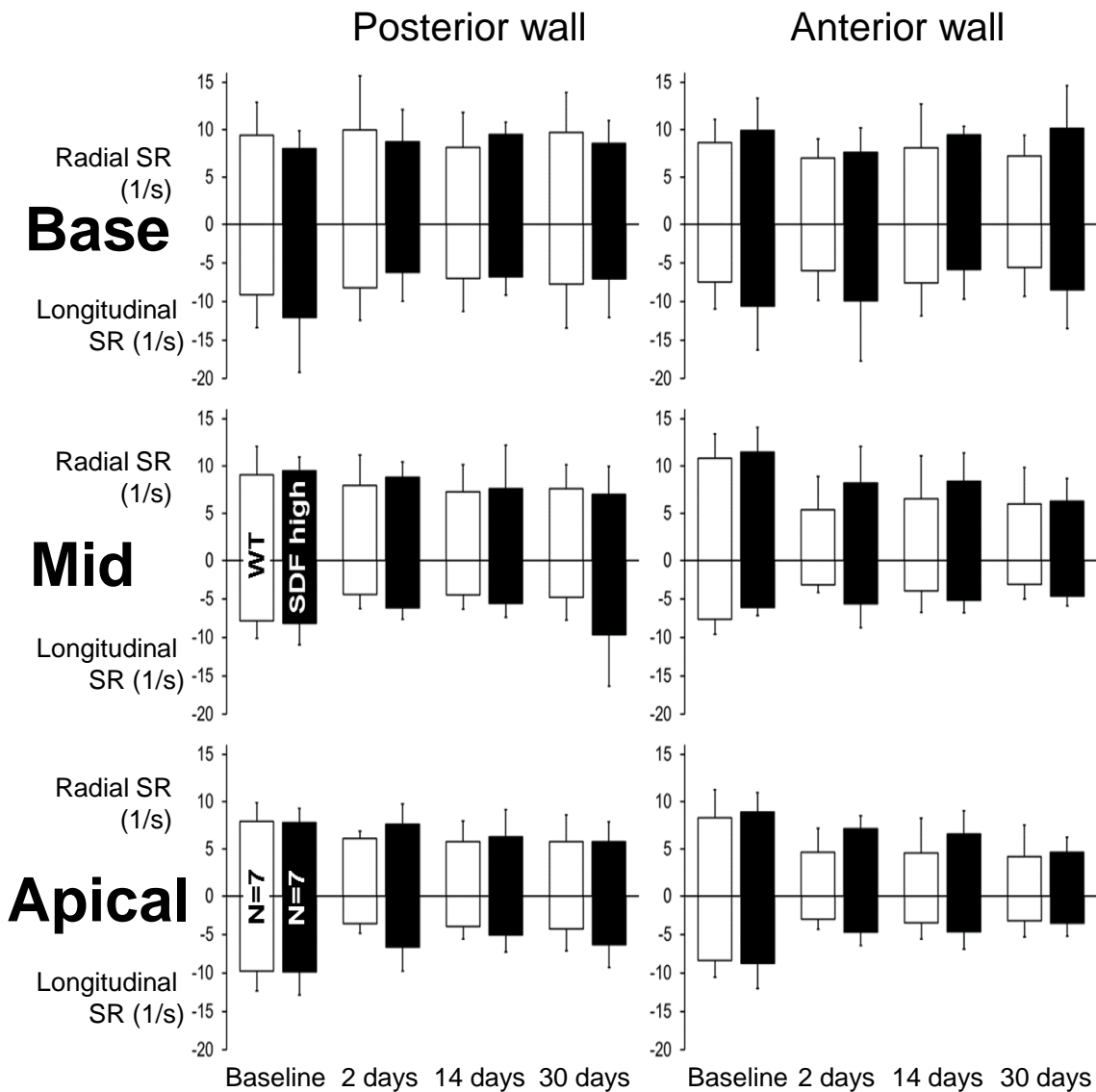
Figure XVIII Regional wall function measured by speckle trecking
Longitudinal and radial strain rate (SR) in apical, middle (mid) and base section of anterior and posterior wall in WT and SDF high mice at baseline and at different time points after permanent coronary ligation.

Increased cell turnover, but unchanged capillary density

The staining for BrdU showed that the number of BrdU$^+$ cardiomyocytes increased in the

remote, border, and scar area of the LV in SDF high mice (**Figures 19, 20, and 21**). The

data were confirmed by an increased number of Ki67$^+$ cardiomyocytes four weeks after

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myocardial infarction (**Figure 22**). Measurement of the capillary density indicated no difference between WT and SDF high, sham and permanent coronary ligated mice (**Figure 23).** Surprisingly, similar to the increase in cell proliferation (BrdU staining, Ki67 staining) we detected an increase in TUNEL[+] nuclei after one and four weeks in SDF high, reflecting an increase in apoptosis **(Figure 24).** However, cardiomyocyte cross-sectional area was smaller in SDF high mice than in WT controls, indicating less hypertrophy four weeks after myocardial infarction **(Figures 25 and 26).**

B BrdU⁺ cardiomyocytes (% of cardiomyocytes)

D BrdU⁺ nuclei (% of cardiomyocyte nuclei)
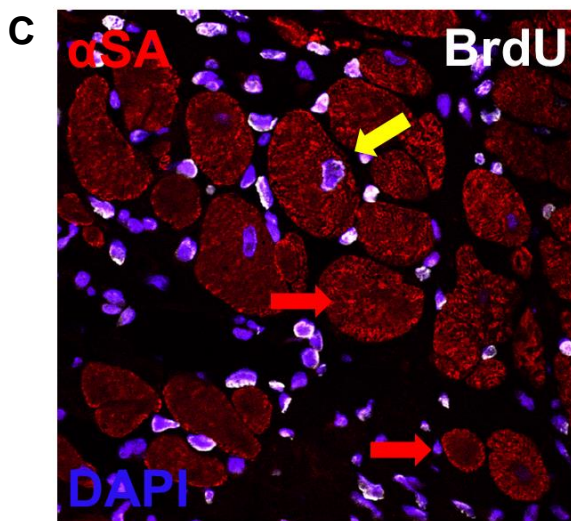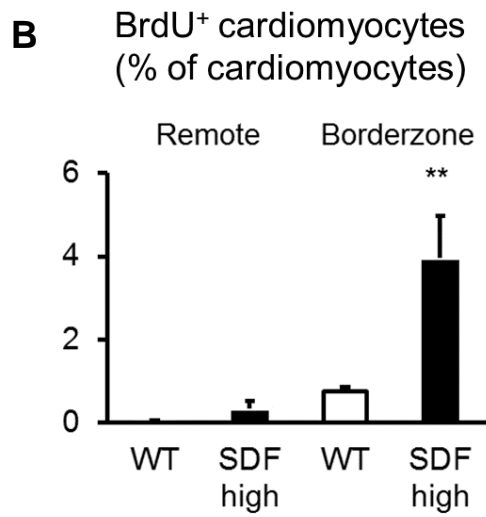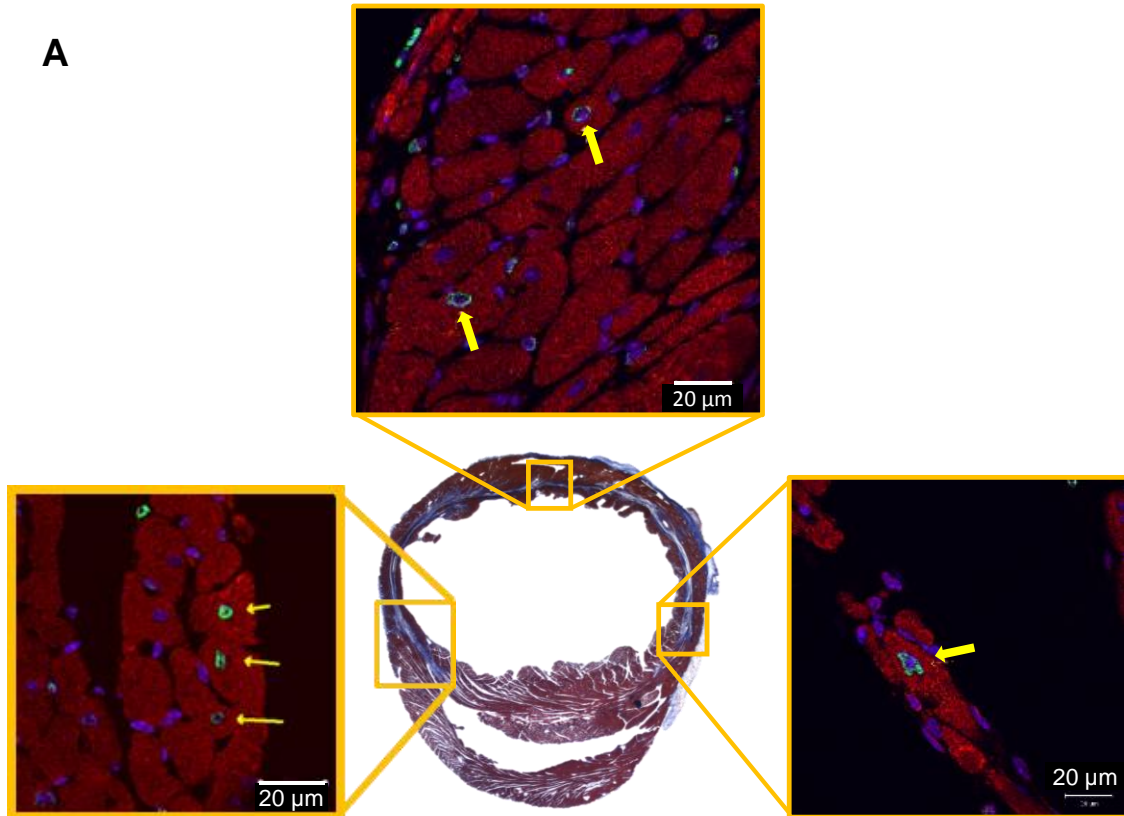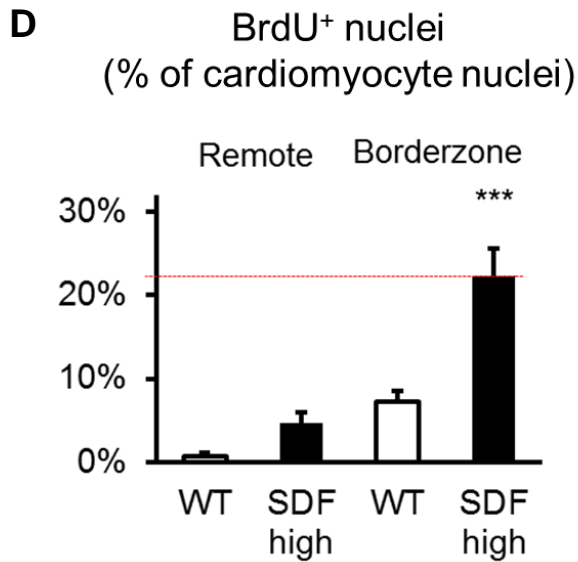
Figure XIX BrdU⁺ / α-sarcomeric actin⁺ cardiomyocytes in the border and remote zone four weeks after myocardial infarction in SDF high and WT mice

A: Representative cross-section with inserts reflecting BrdU$^+$ / α-sarcomeric actin (αSA)$^+$ cardiomyocytes in border- and scar area (yellow arrows). **B:** Number of BrdU$^+$ cardiomyocytes expressed as percent of all cardiomyocytes. **C:** typical cross-section within the border-zone indicating the presence of cardiomyocytes with (yellow arrow) and without nuclei (red arrows). **D:** BrdU$^+$ nuclei, expressed as cardiomyocyte nuclei. (BrdU$^+$: FITC=green, αSA: TRITC=red, DAPI=blue). N=10-12, **,$p<0.01$, ***, $p<0.001$, unpaired student t-test.
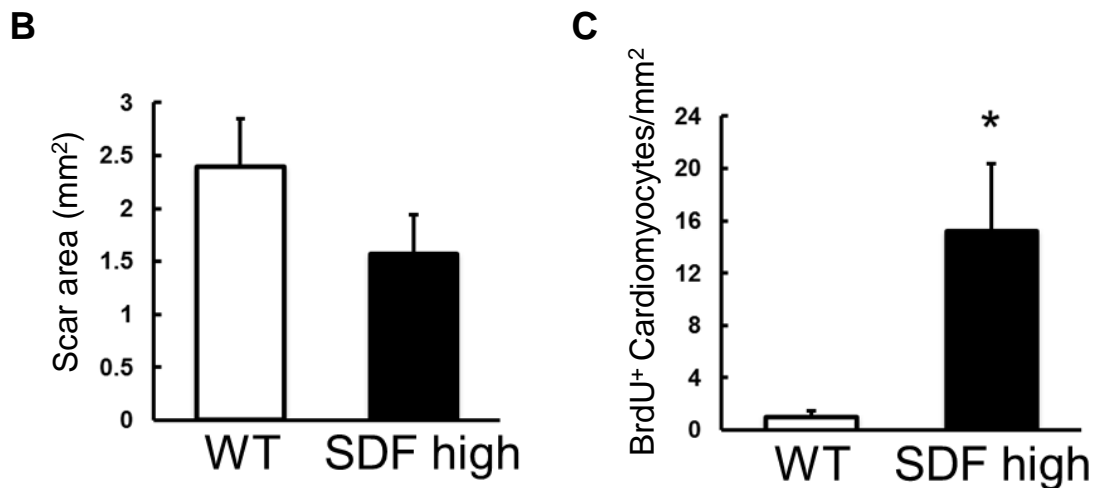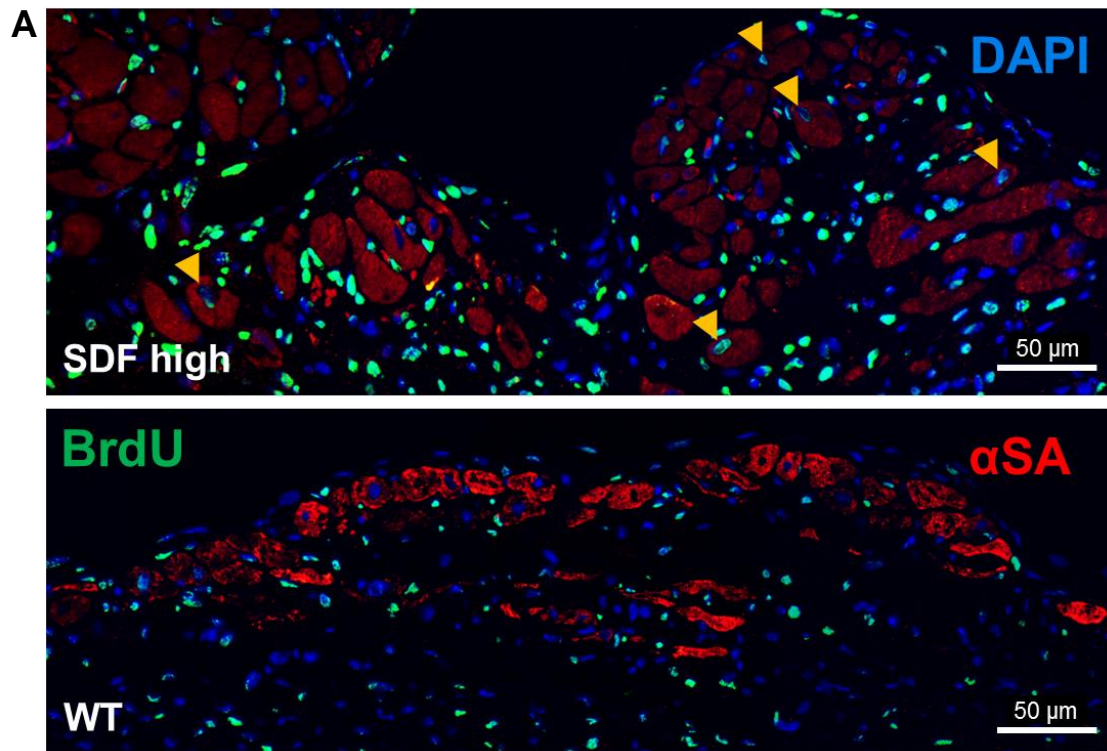
Figure XX BrdU⁺ cardiomyocytes in the scar area of WT and SDF high mice four weeks after infarction
A: representative cross-section of BrdU (green), DAPI (blue) and α-sarcomeric actin (red) stained sections SDF high mice (**A**, top panel, SDF high) and WT (**A**, lower panel) mice. **B**: Infarct size.   **C:** Number of dividing, BrdU⁺ cardiomyocytes in the scar area (N=6, *p<0.05).

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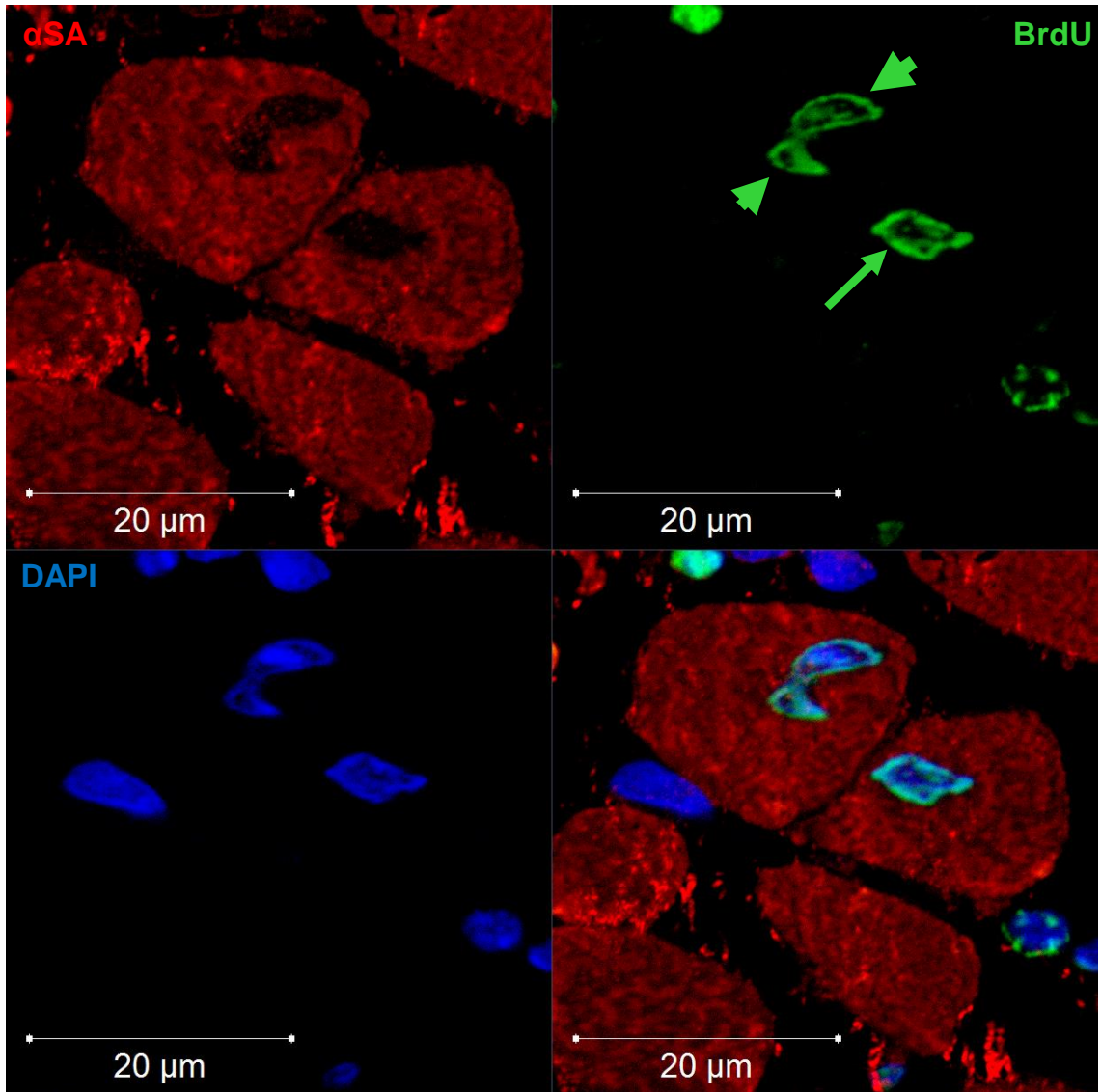
Figure XXI Dividing cardiomyocyte in the border zone of a SDF high overexpressing mouse

Arrow heads indicate nucleus undergo karyokinesis, while arrow indicate the nucleus of a daughter cell after cytokinesis.
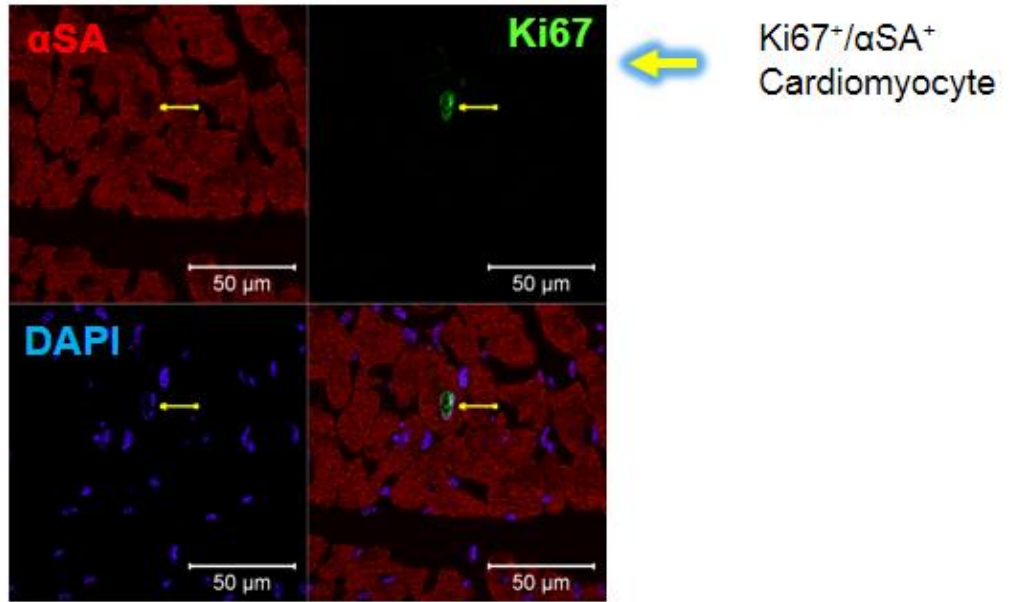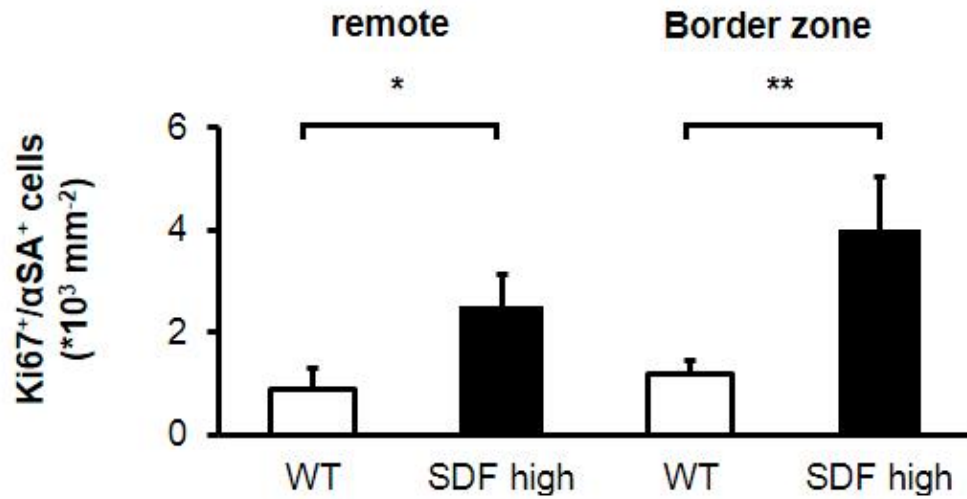
Figure XXII Ki67^{+} cardiomyocytes in the remote and border zone of WT and SDF high mice four weeks after infarction
A: representative example of an area at the border zone of a SDF high mouse, showing a dividing cardiomyocyte. **B:** Number of Ki67^{+} / α-sarcomeric actin[+] in the border and remote zone of WT and SDF high mice (N=7-9, *, $p<0.05$, **, $p<0.01$, unpaired student's t-test).

A   Sham operated
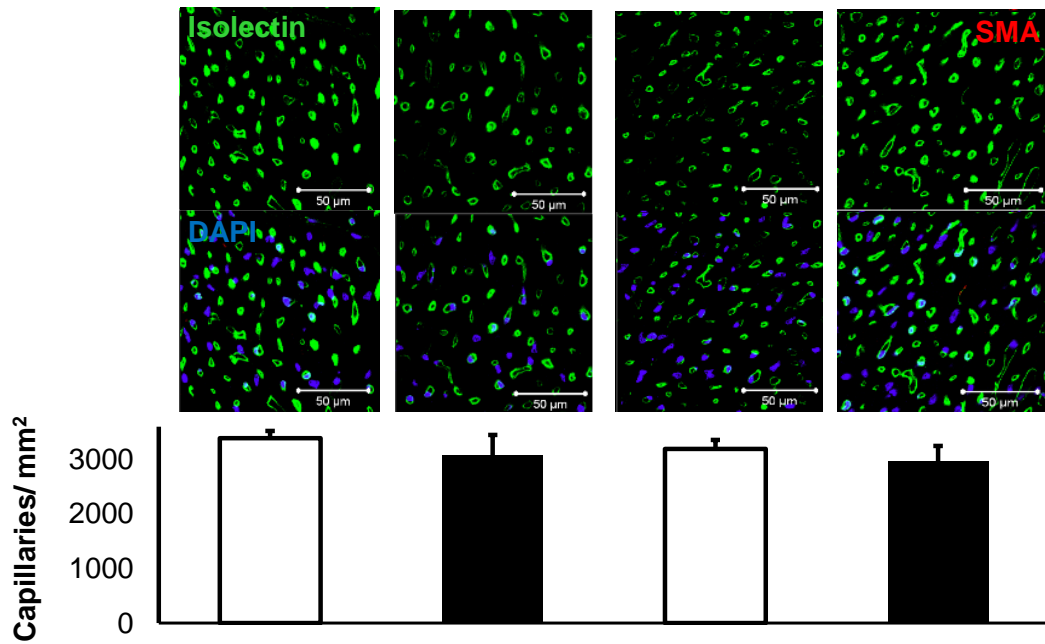
B   4 weeks after myocardial infarction
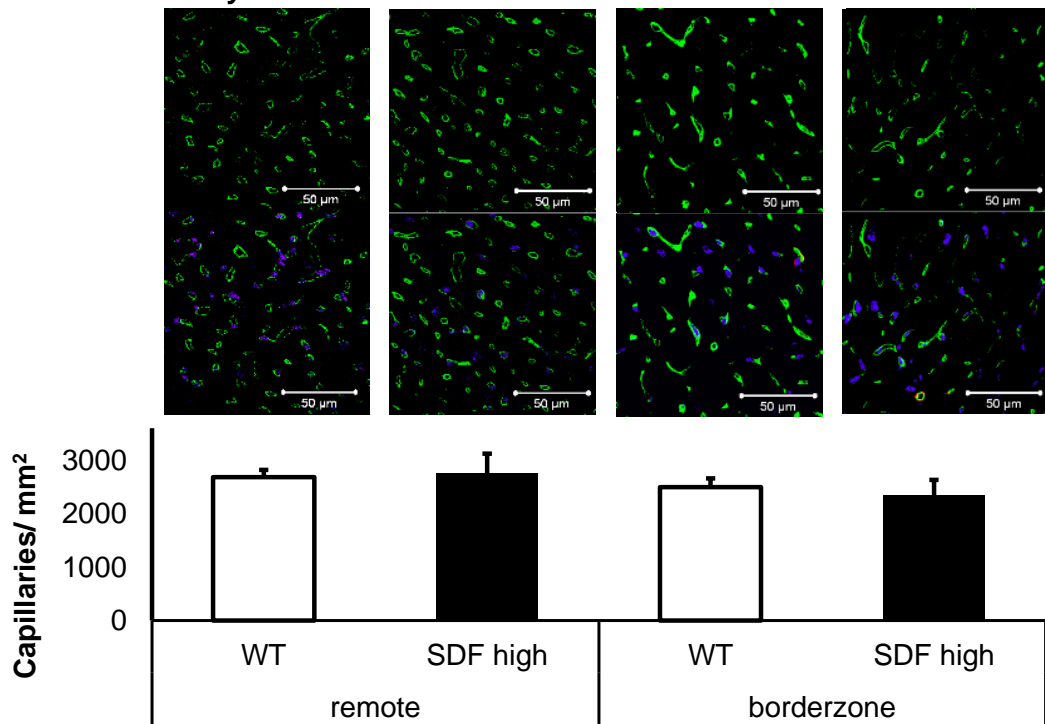
Figure XXIII Capillary density in WT and SDF high mice after sham operation and four weeks permanent ligation in the remote and border zone
A: Capillary density in sham operated animals. **B:** Capillary density in mice underwent permanent coronary ligation (N=12-14).

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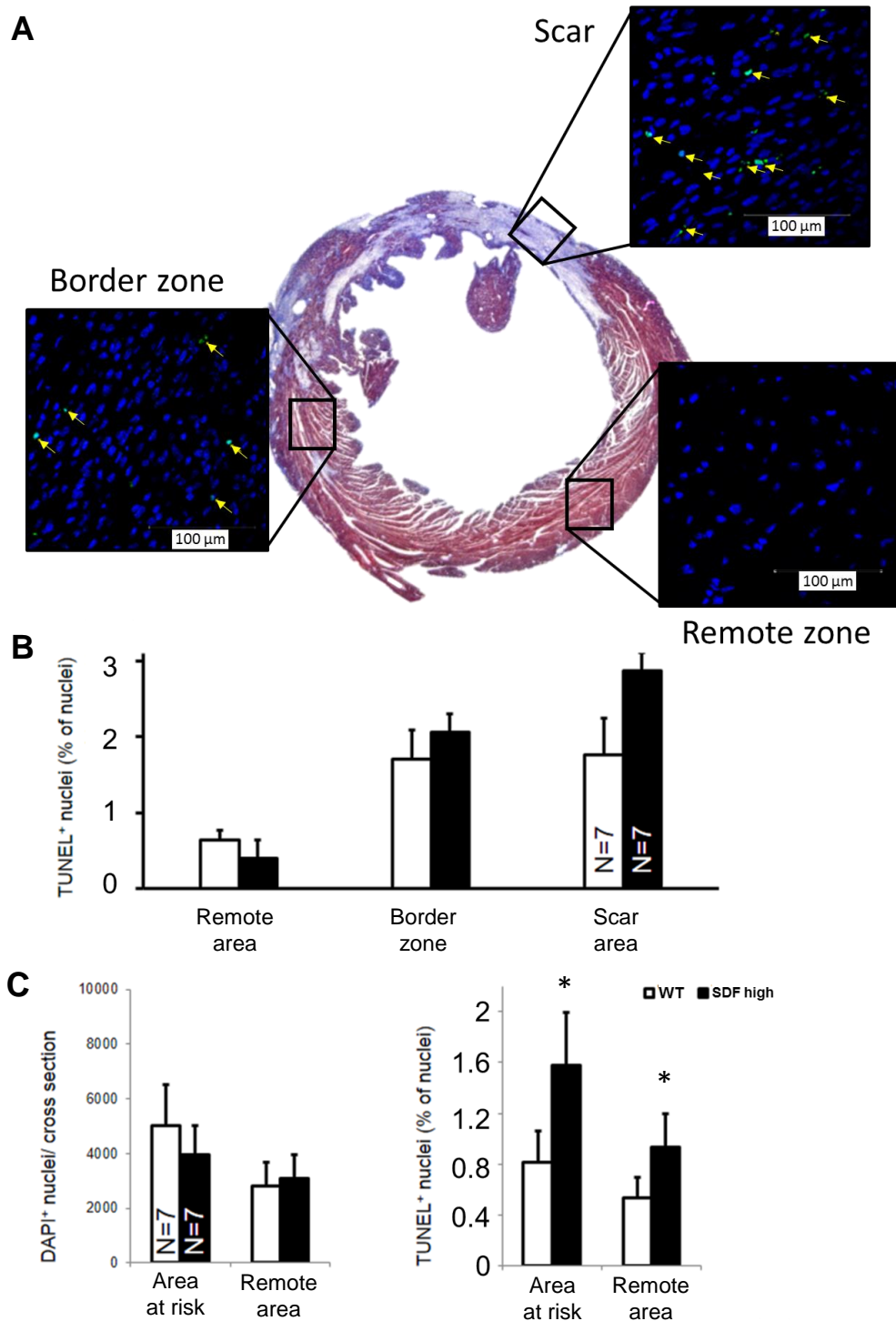
Figure XXIV TUNEL[+] nuclei one and four weeks after permanent ligation
A: Representative cross section through a SDF high heart with images of TUNEL[+] section in scar, border, and remote zone one week after permanent ligation. **B, C:** Quantitative analysis of TUNEL[+] nuclei (yellow arrows) in WT and SDF high mice in each area one **(B)** and four **(C, right)** weeks after infarct. * $p<0.05$, unpaired student's t-test.
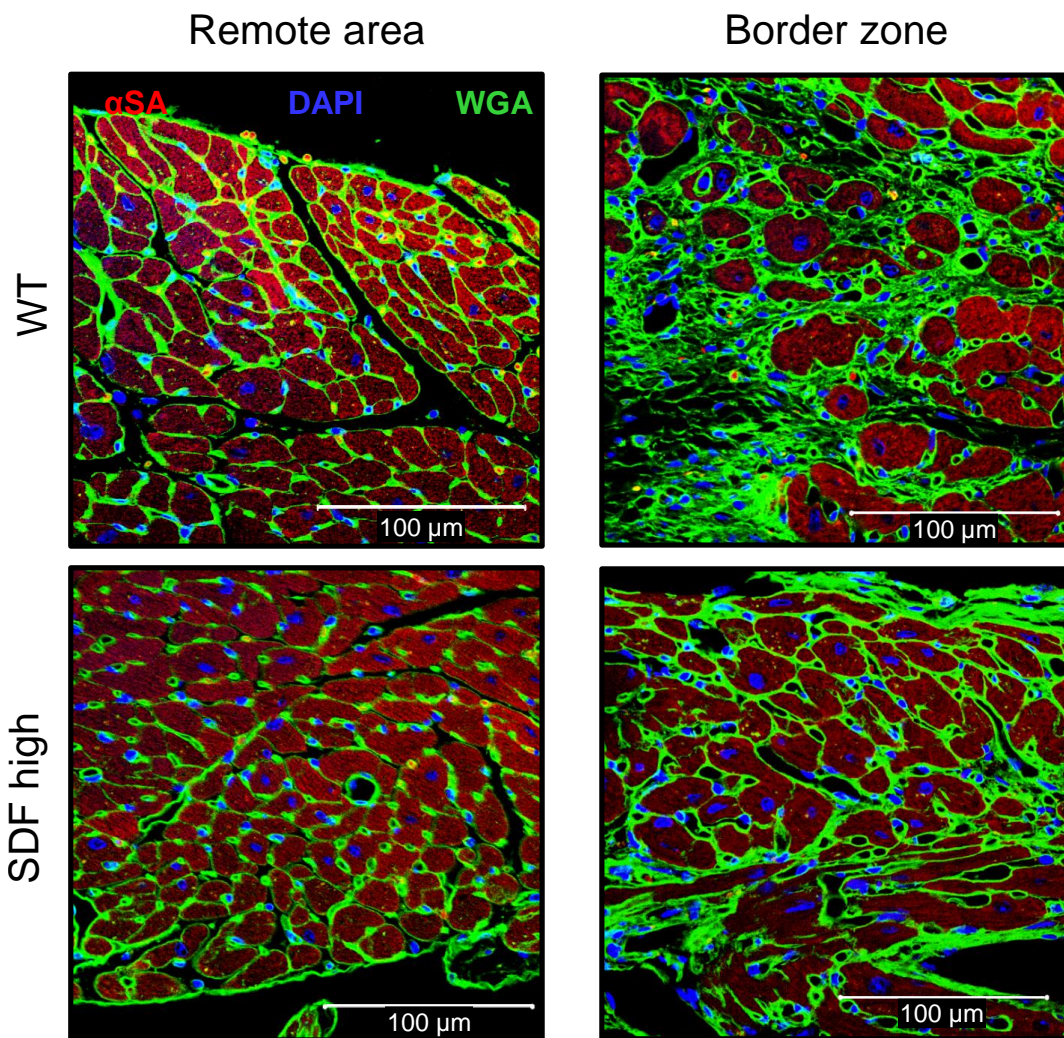
Figure XXV Wheat-germ agglutinin staining for measurement of myocytes cross-sectional area
Wheat-germ agglutinin staining of representative areas of remote (left) and border-zone (right) in WT and SDF high mice.

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Cardiomyocyte cross-sectional area (µm^2)

Figure XXVI Histogram of cardiomyocyte cross-sectional area
Upper panel shows the number of myocytes of each specific size (logarithmic scale) in the border (left side) and the frequency distribution (right side, median [range]) in the border zone. The lower panel shows the corresponding results in the remote zone. There was a slight difference between WT and SDF high mice showing smaller myocytes in the border zone. Total number of myocytes analyzed in the border zone (WT: 925, SDF high: 974) and in the remote zone (WT: 851, SDF high: 1148). *, $p < 0.05$, Mann-Whitney test.

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CONCLUSION

In conclusion, our data clearly indicate that in a permanent coronary artery ligation model, SDF overexpression fails to protect against ischemic injury. The key feature of a permanent coronary artery ligation model is the absolute lack of oxygen and nutrition support to the ischemic area. As the myocardium of mice lacks significant collateral circulation compared to other species (i.e. canine, human)[85] the survival of myocardium due to collateralization in this model is very unlikely. Importantly, we found increased myocardial regeneration already within four weeks after the injury, as indicated by BrdU and Ki67 staining. Surprisingly, regeneration was accompanied by increased apoptosis. Cardiomyocytes stained positive for BrdU were detectable as early as one week after myocardial infarction, partially featuring already an adult cardiomyocyte phenotype (**Figure 27**).

Different possible mechanisms might be responsible for this impressive regeneration potential: Previous work suggested that even adult cardiomyocytes have the potential to undergo cell division initiated by either inhibition of p38 MAP kinase[86] or GSK-3β inhibition.[87] Furthermore, circulating as well as resident c-kit^{+} progenitor cells might be differentiated towards a cardiomyocyte phenotype. Interestingly, we detected a significant increase in apoptosis in SDF overexpressing mice one and four weeks after myocardial infarction. These data contrast published work in myeloid progenitor cells[64,88] in which apoptosis was inhibited in the presence of SDF.

The overexpression of SDF prevents a decline of global and regional LV function after myocardial infarction. About 20% of all cardiomyocyte nuclei were BrdU[+], representing about 4% of all cardiomyocytes regenerated in the border zone. The volume

of newly generated cells appears to be too small to cause a significant increase in LV function, suggesting that secreted SDF also acted as a paracrine factor increasing myocardial contractility.[89] However, neither short-term (five weeks) nor long-term (more than 30 weeks) SDF overexpression affected the myocardial function in sham-operated,
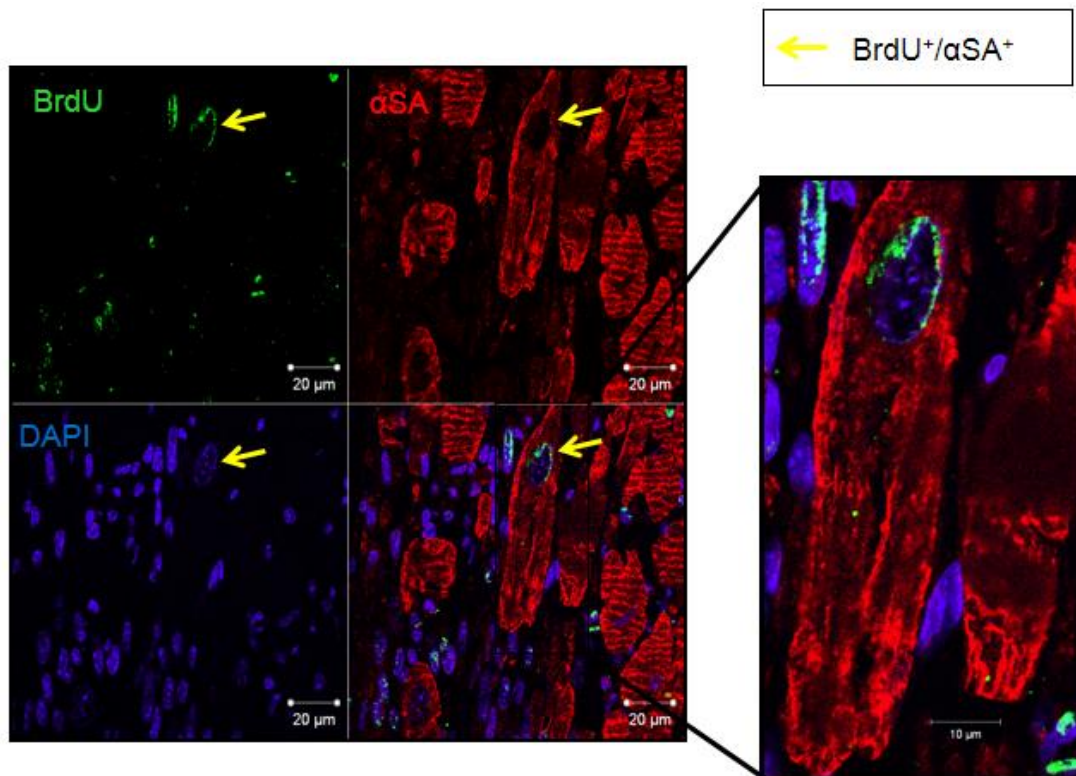
Figure XXVII Adult phenotype of new generated myocyte one week after infarction

non-infarcted, mice. This might suggest that cardiomyocytes changed their sensitivity towards SDF after myocardial infarction by accelerating cardio-protective signaling pathways.

SDF / CXCR4 was identified as a key factor in the recruitment of stem cells to areas of tissue injury in multiple organ systems.

Interestingly, our data did not confirm the overall finding that SDF / CXCR4 acts as a molecular hub modulating neo-angiogenesis.[90] SDF not only promotes revascularization by engaging with CXCR4 expressed on the vascular cells but also supports mobilization of pro-angiogentic CXCR4^{+}VEGFR1^{+} hematopoietic cells, thereby accelerating revascularization of ischemic organs. Shahin Rafii introduced the concept that, by modulating plasma SDF levels, the CXCR4 antagonist AMD3100 acutely promotes, while chronic AMD3100 treatment inhibits, mobilization of pro-angiogenic cells.

In contrast to most of the previous studies suggesting neovascularization after SDF treatment, in our model SDF was only overexpressed in cardiomyocytes and our intervention was not accompanied by simultaneous infusion of hematopoietic stem/progenitor cells or their mobilization from the bone marrow (i.e. by administration of G-CSF or AMD3100) as previous described. Therefore, our data suggested that cardiomyocytes-specific SDF overexpression might primarily act as a para- and / or intracrine factor causing cardiomyocyte proliferation.

CHAPTER 6

SUMMARY AND FUTURE RESEARCH DIRECTIONS

Several different studies have been performed using SDF as chemokine to accelerate cardiac regeneration after myocardial infarction. However, less was known about the direct effect of chronic cardiomyocyte-specific expression of SDF cardiac regeneration and regulation of cell proliferation after myocardial infarction. Our results can be summarized as follows:

- This is the first study showing that cardiomyocyte-specific overexpression of SDF results in improved LV function, decreased myocardial infarction, and increased generation of new cardiomyocytes.

- We found no difference in myocardial infarct size and LV function directly after permanent ligation (i.e. three and seven days), which suggested the same initial injury between WT and SDF overexpressing mice.

- LV function remained unchanged despite cardiomyocyte-specific overexpression of SDF for five and 30 weeks, respectively.

- SDF overexpression resulted in a gene copy-number dependent increase of CK1α and phosphorylation of AKT affect two important factors of cardiac regeneration: An increase in CSPC numbers within the heart of SDF high mice, and an increase of cardioprotective signaling.

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- However, BM function seems to be diminished as the number of leukocytes decreased after five and 30 weeks exposure to increased cardiac SDF levels, respectively. In contrast to previous published data, we did not see an increase in neo-vascularization after SDF overexpression but an increase apoptosis indicated by more TUNEL$^+$ cells.

As discussed above stem cell therapy currently appears to be the most attractive approach to generate new myocardium after an infarction. Nevertheless, some limitations apply as mentioned above, and therefore, alternatives are needed.

Here we used a model of cardiac-specific SDF overexpression and detected a marked increase in cardiac regeneration with a reduction in infarct size. Some special findings should be further annotated:

What is the origin of the new cardiomyocytes?

We detected a significant increase in cardiac regeneration including a marked increase in BrdU$^+$ or Ki67$^+$ cardiomyocytes after permanent coronary ligation. The question becomes whether these are newly generated cardiomyocytes or a sort of differentiated, cardiac linage committed progenitor cells. Unfortunately, our study does not allow us to determine the origin of these newly formed cells. As extensively reviewed by Dr. Pierro Anversa's group[91] and others, several explanations are possible:

a) Fate mapping studies using dilution of GFP labeling as indicator for newly formed cardiomyocyte, indicated 35% of the cardiomyocytes in the border zone and 28% in the remote-zone were newly generated, compared to 14% in sham operated mice.[74] In contrast to Dr. Anversa's group, Richard Lee suggests that pre-existing cardiomyocytes are

the primary source of cell replacement after injury.[76] Using in vitro experiments, Keating and colleagues providing evidence that cardiomyocyte division occurs by either p38 MAP kinase inhibition[86] or blockade of GSK-3[87] resulting in newly formed adult mammalian cardiomyocytes.

b) C-kit^{+} progenitor cells are considered as cardiac-specific stem cell source, giving rise to oligolineage progenitors, which after losing their self-renewal ability and withdrawal from cell-cycle, eventually become mature cardiomyocytes.[92,93]

c) Finally, reprogramming and trans-differentiation have been proposed as sources for new cardiomyocytes. Reprogramming of somatic cell (i.e. cardiac fibroblasts) towards cardiac lineage requires complex modulation using several transcription factors[94,95] which appears unlikely in the model described in this thesis.

As chemoattractant, SDF has the potential ability to recruit hematopoietic stem cells to the infarct area, where those cells de-differentiate to myocytes or vascular cells. Although we cannot rule out this possibility, the absence of EPC mobilization after myocardial infarction in SDF Tg mice suggests that circulating cells most likely are not responsible for the improved function and generation of new cardiomyocytes seen in our experiments.

Cell cycle control by SDF appears to be different between cardiomyocytes and CSPCs.

Previous published data suggest that SDF mediates CSPC quiescence through a CK1α dependent mechanism, as administration of CK1α resulted in decreased CSPC proliferation. The western blot analysis from SDF overexpressing hearts in this study revealed a gene-copy number dependent increase in CK1α, which was accompanied by an

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increased number of CSPC in sham-operated animals. However, the number of BrdU[+] / c-kit^{+} cells within the heart was similar to the WT controls, indicating decreased CSPC proliferation and confirming our previous in vitro work.[46] Interestingly, besides the marked increase of BrdU[+] / α-sarcomeric actin[+] cardiomyocyte numbers in the border zone after myocardial infarction, we also detected in the non-ischemic region accelerated proliferation of cardiomyocytes. Assuming that CK1α expression in this region of the heart is similar to sham-operated animals, the data would suggest that cardiomyocyte proliferation is subject to a different regulatory pathway, which might or might not include CK1α.

Besides the increase in BrdU[+] and Ki67^{+} cardiomyocytes one and four weeks after myocardial infarction, we detected a significant increase in TUNEL[+] nuclei, indicating increased apoptosis and cardiac remodeling:

Increased apoptosis

In contrast to previously published work suggesting anti-apoptotic properties of SDF, these findings came as a surprise, but might be explained by the prolonged exposure of circulating cells towards SDF in weeks prior to myocardial ischemia. Different authors suggested that circulating CD8^{+},[96] CD4^{+},[97] and specific tumor cells,[98,99] undergo apoptosis after prolonged exposure to SDF. Interestingly, even concentrations as low as 10 ng / l might induce apoptosis dependent on the duration circulating cells are exposed to the chemokine.[97] The molecular mechanism triggering the cell death is not quite understood, but Herbein[96] detects after a relative high concentration of SDF (i.e. 1 µg / l) a progressive increase of CD95 antigen expression on the cell surface as well as CD95L within the cell.

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Others describe up-regulation of TNFα or TNF-R which led to cell death after secondary exposure to SDF.

Finally, we did not find any difference in capillary density between WT and SDF overexpressing mice:

SDF overexpression did not promote neovascularization

SDF is identified to directly participate in new blood vessel formation *in vitro* by preventing EPC apoptosis[100,101] and *in vivo* by inducing vasculogenesis. Mobilization of pro-angiogenic hematopoietic cells can be increased by either elevating SDF levels or by blocking CXCR4 by AMD3100.[102,103] However, dynamics of mobilization between both methods are quite different as injection of AMD3100 rapidly mobilized hematopoietic stem cells, pro-angiogenic cells, and EPCs, while SDF mediated mobilization requires several days. Petit and colleagues suggest a "niche-dependent mobilization model", in which different "geographical microenvironments" are stimulated by both methods:[90] The easy accessible perivascular niche, readily available to release hematopoietic stem cells, will respond quickly to an CXCR4 inhibition. In contrast, SDF mediated release of hematopoietic stem cells from the stromal or osteoblastic niches within the BM require a cascade of events and therefore requires several days to release hematopoietic stem cells into the circulation.[104]

Although we did not reach the significance level, the number of EPCs was reduced in the BM sample and the peripheral blood, respectively, which would suggest a diminished pool of EPCs in the Tg mice, reflected by the absence of EPC mobilization 72 hours after permanent ligation.

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Furthermore, it has been reported that knockdown of CK1αLS inhibits vascular cell proliferation in various isolated vessels (i.e. human umbilical vein endothelial cells, human coronary artery smooth muscle cells, and human coronary artery endothelial cells).[105] As CK1αLS is thought to function antagonistically to its related cytosolic splice forms CK1α and CK1αS, which are known to negatively regulate pro-proliferative pathways,[106,107] SDF mediated overexpression of CK1α might also be responsible for diminished revascularization after myocardial infarction.

LIMITATIONS

The conclusion that SDF overexpression facilitates increased regeneration after myocardial infarction resulting in improved function was based on the fact that we found an increase in BrdU$^+$ cardiomyocytes in the border-zone and in the scar area four weeks after myocardial infarction. As with many methods, BrdU incorporation as marker of cell division is recently challenged by the fact that theoretically, the replacement of thymidine with BrdU during DNA repair might also result in positive stained nuclei which could falsely interpreted as a cell undergo karyokinesis. In a recent study Richard Lee demonstrates that ^{15}H thymidine incorporation is two magnitudes higher in cells undergoing cell division than after H_2O_2 induced DNA damage and DNA synthesis.[76]

Our results might reflect SDF mediated increase in DNA synthesis after hypoxia induced cell injury without increased cell proliferation. However, Masson's trichrome analysis after one week indicated that 30% of the LV were scar tissue, similar between WT and SDF high mice. While the amount of scar tissue was significantly diminished in SDF

high mice (13%) the scar size remained the same in WT mice four weeks, suggesting the generation of new, viable myocardium in the SDF Tg hearts.

The combination of both observations (i.e. higher magnitude of BrdU incorporation in myocytes undergo karyokinesis and a significant reduction in infarct size) lead to our conclusion that cardiomyocyte specific overexpression of SDF facilitates regeneration of myocardium after permanent coronary ligation.

FUTURE DIRECTIONS

For the first time, our experiments provide evidence that the regeneration profile of the heart is facilitated after permanent coronary ligation by overexpressing SDF in the cardiomyocytes, which resulted in newly generated myocytes, increased LV function, and reduced myocardial infarct size. Further studies are needed to determine the role of CK1α in cardiomyocyte and stem cell proliferation to determine whether it is suitable for gene therapy directly targeting myocytes renewal. The effect of chronic myocyte-specific SDF overexpression on BM homeostasis needs to be further evaluated as a depression of the BM would limit SDF overexpression as therapeutic options in the future.

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Aeba, R., Yozu, R., Yamagishi, H., Kitamura, T., Fukuda, K. & Ieda, M. Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12667-12672 (2013). *[PMID: 23861494]*

95.   Fu, J.D., Stone, N.R., Liu, L., Spencer, C.I., Qian, L., Hayashi, Y., Delgado-Olguin, P., Ding, S., Bruneau, B.G. & Srivastava, D. Direct Reprogramming of Human Fibroblasts toward a Cardiomyocyte-like State. *Stem cell reports* **1**, 235-247 (2013). *[PMID: 24319660]*

96.   Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., O'Brien, W.A. & Verdin, E. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature* **395**, 189-194 (1998). *[PMID: 9744279]*

97.   Colamussi, M.L., Secchiero, P., Gonelli, A., Marchisio, M., Zauli, G. & Capitani, S. Stromal derived factor-1 alpha (SDF-1 alpha) induces CD4+ T cell apoptosis via the functional up-regulation of the Fas (CD95)/Fas ligand (CD95L) pathway. *Journal of leukocyte biology* **69**, 263-270 (2001). *[PMID: 11272277]*

98.   Wendt, M.K., Drury, L.J., Vongsa, R.A. & Dwinell, M.B. Constitutive CXCL12 expression induces anoikis in colorectal carcinoma cells. *Gastroenterology* **135**, 508-517 (2008). *[PMID: 18558091]*

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101. Salvucci, O., Yao, L., Villalba, S., Sajewicz, A., Pittaluga, S. & Tosato, G. Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. *Blood* **99**, 2703-2711 (2002). *[PMID: 11929756]*

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103. Capoccia, B.J., Shepherd, R.M. & Link, D.C. G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism. *Blood* **108**, 2438-2445 (2006). *[PMID: 16735597]*

104. Zhong, R., Law, P., Wong, D., Merzouk, A., Salari, H. & Ball, E.D. Small peptide analogs to stromal derived factor-1 enhance chemotactic migration of human and mouse hematopoietic cells. *Experimental hematology* **32**, 470-475 (2004). *[PMID: 15145215]*

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106.   Price, M.A. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev* **20**, 399-410 (2006). *[PMID: 16481469]*

107.   Zhu, J., Shibasaki, F., Price, R., Guillemot, J.C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P. & McKeon, F. Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. *Cell* **93**, 851-861 (1998). *[PMID: 9630228]*

CURRICULUM VITAE

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Louisville, Kentucky, 40202 USA

EDUCATION:

Medical:

1992-1999   Medical School Heinrich-Heine-University of Düsseldorf, Germany

Graduate:

04/ 2014   Doctor of Philosophy (Department of Physiology & Biophysics, University of Louisville, KY, USA)

08/ 2010   Master of Science (Department of Physiology & Biophysics, University of Louisville, KY, USA)

2008-2014   Ph.D. program at the Department of Physiology & Biophysics, University of Louisville, KY, USA (Mentor: Gregg Rokosh, Ph.D., Chair: Roberto Bolli, M.D., Institute of Molecular Cardiology)

Residency

1999-2004   Residency, Department of Anesthesiology, University Hospital Düsseldorf, Germany, Chair of the Department: J. Tarnow, Professor of Anesthesiology, FRCA; since 2007: Chair: B. Pannen, Professor of Anesthesiology

Fellowships

2003   Transesophageal Echocardiography course, Hamburg, Germany

2001   Training visit at the Department of Physiology, University of Regensburg, Germany (Chair: A. Kurtz, Professor of Physiology); Education in preparation of isolated rat kidneys and measurement of renal renin regulation

1998   Four months internal medicine rotation at the General Hospital of Ashton-under-Lyne (teaching hospital of the University of Manchester, Great Britain)

1997   Department of Pediatrics, University of Pretoria, South Africa & Department of Cardiology, University of Pretoria, South Africa

1996   Department of Anesthesiology and surgical intensive care medicine, urban hospital, Düsseldorf (*Städt. Krankenhaus Gerresheim*, Düsseldorf, Germany).

| 1995 | Department of vascular surgery and renal replacement (Chair: W. Sandmann, Professor of vascular surgery) at the University Hospital of Düsseldorf & Clerkship in internal medicine at an urban hospital, Düsseldorf (St. Martinus - Krankenhaus, Düsseldorf, Germany). |

Medical Activities during medical school

| 1992-1999 | Paramedic in an urban EMS service in the city of Neuss, Germany |
| 1992-1998 | Nursing on SICU (surgical intensive care unit, University Hospital, Düsseldorf, Germany) |

ACADEMIC APPOINTMENTS:

2006-present	Assistant Professor Department of Anesthesiology and Perioperative Medicine, University of Louisville, KY, USA
05/ 2005	Diplomat of the European Society of Anesthesiology (D.E.S.A.)
1999	M.D. Thesis: "Effect of acidotic reperfusion on myocardial reperfusion injury in dogs in vivo" (Magna cum laude)

OTHER POSITIONS AND EMPOLYMENT

| 2004-2006 | Anesthesiologist at the Department of Anesthesiology and Perioperative Medicine, University of Düsseldorf, Germany |

BOARD CERTIFICATION, LICENSURE, REGISTRATION:

GREAT BRITAIN:	Specialist Register British General Medical Council (03/ 006)
KENTUCKY:	FL018 (12/ 005)
GERMANY:	Licensure to practice medicine (7/ 999)
2013	Refreshed ACLS training – valid until 12/ 015
2005	Certified Leading Emergency/ Rescue Physician
2004	Board certified for Anesthesiology (Germany)
2000	Specialist for Emergency/ Rescue medicine (EMS Physician)
1992	State-certified Paramedic (EMS)

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES:

Memberships

Since 2006	American Heart Association (AHA)
Since 2006	International Anesthesia Research Society (IARS)
Since 2006	American Society of Anesthesiology (ASA)
Since 2006	British Medical Council
2006-2007	American Medical Association (AMA)
2003-2010	National Society of Rescue Medicine, Nordrhein-Westfalen
2001-2010	European Society of Anesthesiology (ESA)
Since 2000	German Society of Anesthesiology (DGAI)

Activities Clinical Research

Since 06/ 010	Principle investigator of retrospective data-analysis "Effect of storage time of packed red blood cells on outcome after massive blood transfusion." Retrospective collection of 500 patient data files between 2005-2012.
2008-2012	Member of the NIH funded (P20 RR024489-01A1) COBRE grant (Center of Excellence in Diabetes and Obesity Research, PI: Aruni Bhatnagar, Ph.D.), 15% time effort
2007-2013	Local principal investigator of the multicenter study ENIGMA II in Louisville, KY, USA (7500 patients, 36 centers), actually ~170 patients in Louisville enrolled (~5% of total enrolled patients)
Since 2006	Member of the Outcomes Research Consortium[TM] (Head: D. Sessler, M.D.) and conductance of multiple studies evaluating the effect of anesthetics on the depth of sedation in volunteers.

Activities Basic Research

08/ 2006	Invited scholar of the Baltic Summer School: "Stem cell biology –Future perspectives and possible treatment options." 20.08.-01.09.2006 Kiel, Germany
Since 2006	Training in molecular biology at the Institute of Molecular Cardiology (Chair: R. Bolli, M.D.), Mentor: Gregg Rokosh, Ph.D.
2002-2004	Establishment of a chronic renal ischemia-reperfusion model in the rat *in vivo* (Measurement of renal function variables); Establishment of histology quantification of renal cell damage (Immune-histology, H&E sections in cooperation with the Departments of Anatomy II and Pathology, University of Düsseldorf, Germany)
2001	Establishment of an acute model of lung injury after acidic aspiration in the rat *in vivo* (Continuous measurement of blood gases with the Paratrend® monitor device, cooperation with S. Loer, M.D, outside lecturer of anesthesiology, Düsseldorf, Germany)
2000-2001	Establishment of an acute model of renal ischemia/ eperfusion experiments in the rat *in vivo* (Continuous measurement of renal blood flow, side divided urinary outflow, histological determination of cell damage)
Since 2000	Working in a ratio of 60/ 0 in the operation theatre and as scientific assistant in the Section of Experimental Anesthesiology, University of Düsseldorf, Germany (Chair: J.Tarnow, Professor of Anesthesiology, FRCA)
1999	Establishment of an acute model of ischemia-reperfusion situations in the rat heart *in vivo* (Continuous measurement of left ventricular pressure, cardiac output)
1995	Course of laboratory animal care and handling, Central Animal Research Institute, University of Düsseldorf, Germany (Chair: A. Treiber, veterinary doctor)
1995	Course for scientific statistical analysis using Windows SPSS computer software

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HONORS AND AWARDS:

2003   *First Price* - Best abstract prize competition at the annual Euroanesthesia congress, Glasgow, Scotland:

Obal D, Müllenheim J, Favoccia C, Kratz M, Dettwiler S, Preckel B, Schlack W: Anaesthetic preconditioning and reduction of reperfusion injury by sevoflurane offered additive myocardial protection mediated by opening of KATP channels. Eur J Anaesthesiol 2003; 20 (Suppl. 30), A5.

COMMITTEE ASSIGNMENTS AND ADMINISTRATIVE:

Since 2008   Departmental Research Board (Overview of financial, academic and organizational aspects of departmental research activities, guidance to clinical research fellows)

2007-2013   University of Louisville Medical Center Blood Transfusion Committee (quarterly meetings)

Since 2007   University of Louisville Medical Trauma Quality Conference (monthly meetings)

PEER REVIEWER OF MANUSCRIPTS:

Since 2013   Review editor "Frontiers in Physiology, Oxidant Physiology"

Since 2011   Annual abstract reviewer for the AHA scientific sessions, basic science abstracts "Ischemia Reperfusion Injury" section

2014   Ad hock Reviewer:

             * BMC Anesthesiology

             * TAAP – Toxicology and Applied Pharmacology

2013   Ad hoc Reviewer:

             * American Journal of Cardiology

             * TAAP – Toxicology and Applied Pharmacology

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2011	Ad hoc Reviewer:
	* American Journal of Cardiology
	* Cardiovascular Diabetology
2010	Journal of Cellular and Molecular Neurobiology
2007	British Journal of Anaesthesiology

BOARD MEMBERSHIPS:

Since 2013	Editorial Board of Frontiers in Oxidant Physiology – Review Editor
Since 2013	Editorial Board BioMed Research International - Anesthesiology

TEACHING:

Since 2006	Scheduled Classes for Medical Students and Residents
2012	ACLS lecturer for Anesthesia Residents
2012	Course Director, Initiator, Program presenter, 1st Louisville Airway Day, May 24th 2012, Louisville, KY, USA (CME accredited program)
2010	ASA Instructor of Difficult Airway Management (ASA annual meeting, San Diego, CA, USA)
Since 2009	Lectures for medical students and Ph.D. graduates in the Department of Pharmacology at the University of Louisville, KY, USA (4 hours/ year)
Since 2008	Lectures for medical students in the Department of Physiology and Biophysics at the School of Medicine, University of Louisville, KY, USA (8 hours/ semester)
Since 2006	Regular lectures for residents of the Department of Anesthesiology and Perioperative Medicine, University of Louisville, Louisville, KY, USA
2004-2005	Lecture for final year medical students in Anesthesiology, Department of Anesthesiology, University Hospital Düsseldorf, Germany
1999-2001	Clinical tutor for medical students in emergency medicine, Department of Anesthesiology, University Hospital Düsseldorf, Germany
1995-1997	Medical student tutor for cardiac and respiratory physiology, Department of Physiology, University of Düsseldorf

Predoctoral Students supervised and mentored:

Since 1999　　　Laboratory tutor for medical students during their work for the thesis to doctorate

H. Scharbatke	-	2004-2007	-	magna cum laude
F. Hoetterkes	-	2005-2008	-	magna cum laude
S. Dettwiler	-	2007-2009	-	magna cum laude
C. Favoccia	-	2007	-	pending
W. Brown (M.S.)	-	since 2009		
F. Memon (M.S.)	-	2010-2011		
T.B. Wright (M.S.)	-	2010-2013		

<u>Mentor and Advisor of clinical research fellows</u>

Ragu Govinda	(2006-2008)
Anton Grankin	(2008-2010)
Alexander Bautista	(2009-2010)
Sunitha Kanchikandadai	(2010-2011)
Sahil Chabra	(2011-2013)

<u>ABSTRACTS AND PRESENTATIONS:</u>

Oral Presentations: National/ International Meetings

1. **Obal D**: Perioperative Administration of ondansetron or dolasetron does not lengthen QT interval. Outcomes Research Meeting at the American Society of Anesthesiology Annual Meeting, Washington, DC. 10/ 7/ 012

2. **Obal D**: Fiberoptic Intubation, 1st Difficult Airway Course, Paris Simulation Center, University of Louisville, KY, May 12th, 2012

3. **Obal D**: Perioperative Use of Statins, Annual Kentucky Society of Anesthesia Spring Meeting, Marriott Cincinnati at Rivercenter, Covington, KY, March 24th, 2012

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4. **Obal D**: Effect of Storage Time on Outcome after Massive Blood Transfusion. Outcomes Research Meeting at the American Society of Anesthesiology Annual Meeting, San Diego. 10/ 7/ 010

5. **Obal D**, Schlack W: Intraoperative myocardial ischemia – what should I do ?; TED session at the annual meeting of the German society of Anesthesiology 04/ 7/ 005

6. **Obal D**: Organprotection by anesthetic preconditioning. Scientific meeting of the outcome research department, University of Louisville, KY, USA 02/ 1/ 005

7. **Obal D**: Prevention and treatment of perioperative myocardial ischemia

8. What the anesthesiologist should know. Didactic lecture for the residencies of the Department of Anesthesiology, University of Louisville, KY, USA. 02/ 7/ 005

9. **Obal D**: Perioperatives Nierenversagen; Didactic lecture 04/ 004 Department of Anesthesiology, University Hospital Düsseldorf, Germany

10. **Obal D**, Fräßdorf J, Müllenheim J, Preckel B, Schlack W: Rolle der Opiatrezeptoren bei der Morphin - induzierten späten Präkonditionierung. Anästhesiologie & Intensivmedizin 2003; 44 FV 109.6.

11. **Obal D**, Müllenheim J, Preckel B, Thämer V, Schlack W: How do administration time and concentration affect the cardioprotection by sevoflurane against reperfusion injury? Anesthesiology 2001; 95, A682.

12. **Obal D**, Müllenheim J, Scharbatke H, Thämer V, Schlack W: Two minutes of sevoflurane administration are enough to protect the heart against reperfusion injury. Eur J Anaesthesiol 2001;18:A162.

13. **Obal D**, Preckel B, Müllenheim J, Thämer V, Schlack W: Einfluss der Konzentration und Applikationsdauer von Sevofluran auf die Ausdehnung des Reperfusionsschadens des Herzens. Anästhesiologie & Intensivmedizin 2001;42:877.

14. **Obal D**, Preckel B, Hoff J, Thämer V, Schlack W: Einfluß einer Stellatumblockade auf die Ventrikelfunktion - Studie an wachen Hunden. Anästhesiol Intensivmed Notfallmed Schmerzther 1998

Oral Presentations: Local/ Regional Meetings

Grand Round presentations at the University of Louisville, Department of Anesthesiology:

- **Obal D**: Grand round, Department of Anesthesiology & Perioperative Medicine: Perioperative Lung Function; August 22nd, 2013

- **Obal D**: Grand round, Department of Anesthesiology & Perioperative Medicine: Nitrous oxide – ENIGMA trial and other mysteries; July 7th, 2013

- **Obal D**: Grand round, Department of Anesthesiology & Perioperative Medicine: QTc interval – neglected by anesthesiologists; August 3rd, 2012

- Vessel E, **Obal D**: Grand round, Department of Anesthesiology & Perioperative Medicine: Repeat Operation on a Traumatic Hemorrhage; April 27th, 2012

- **Obal D**   : Grand round Department of Anesthesiology & Perioperative Medicine: "Introduction to ROTEM" June 2011

- **Obal D**   : Grand round Department of Anesthesiology & Perioperative Medicine: "Introduction to TEG" February 2011

- Bo Hargett, **Obal D**. M&M Conference: "Why so hypotensive?" December, 13th 2010

- **Obal D**. The perioperative ischemic evaluation-2 (POISE-2) trial or "What's new in the protection against perioperative infarction in patients undergoing non-cardiac surgery?" November, 8th 2010

- **Obal D**. "Blood transfusion - do we need to change our current practice?" July 6th 2009

- **Obal D**. M&M Conference: Rush in the morning – the 5.30 am case. March 2nd, 2009

- **Obal D**, Lenhardt R, Turan A, Akca O. Research at the Department of Anesthesiology and Perioperative Medicine. May 18th, 2008

- Lenhardt R, **Obal D**. Hemorrhagic Shock & Massive Transfusion - Standard Procedure at the UofL Hospital, March 10th, 2008

- **Obal D**. Anesthesia and coagulation disorders. What the Anesthesia Provider Should Know ! April 14th, 2008

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- **Obal D**. The ENIGMA II Trail. "Nitrous Oxide Anesthesia and Cardiac Morbidity after Major Surgery". May 17th, 2007
- **Obal D**, Henson L. Perioperative and Anesthetic Care for Trauma Patients, October 9th, 2006
- **Obal D**. Anesthesia for emergency triplet A surgery. July 13th, 2006

Poster Presentations: National/ International Meetings

1. **Obal D**, Yang D, Sessler DI: Perioperative administration of ondansetron or dolasetron is not associated with QTc prolongation. ASA Annual meeting Abstract 2012, 12-A-3862-ASAHQ

2. **Obal D**, Dai S, Keith R, Dimova N, Kingery J, Zheng Y-T, Zweier J, Velayutham J, Prabhu S, Li Q, Conklin D, Bhatnagar A, Bolli R, Rokosh G: Overexpression of cardiomyocyte-specific extracellular superoxide dismutase increases nitric oxide bioavailability and reduces infarct size after ischemia/ reperfusion. Circulation 2010 AHA Annual meeting, Chicago

3. Bautista A, Brown B, Sessler D, Wadhwa A, **Obal D**: Effect of storage time of blood transfusion products on outcome in massive transfusion situations. Anesthesiology 2010, A1515

4. Kasuya Y, Govinda R, **Obal D**, Turan A, Sessler DI: Effect of Aminophylline on Loss of Consciousness, BIS, and Propofol Requirement in Human Volunteers. Anesthesiology 2007; 107: A521

5. **Obal D**, Favoccia C, Dettwiler S, Preckel B, Schlack W: Desflurane Postconditioning Reduces Renal Reperfusion Injury Anesthesiology 2005; 103: A337

6. Fräßdorf J, Weber NC, Toma O, **Obal D**, Kojda G, Preckel B, Schlack W: Opioide receptors are mediators of late cardioprotection – involvement of the nuclear transcription factor κB Cardio-Visionen - Symposium der Nordrhein-Westfälischen Akademie der Wissenschaften, 2004, 1: 149

7. Dettwiler S, **Obal D**, Favoccia C, Preckel B, Schlack W: Preconditioning induced by the volatile anaesthetic sevoflurane did not improve renal function after

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ischaemia and reperfusion. Pflügers Arch Eur J Physiol 2004, Deutscher Physiologen Kongress

8. Toma O, Weber NC, Wolter JI, **Obal D**, Preckel B, Schlack W: Die Desfluran-induzierte Präkonditionierung wird über eine zeitabhängige Aktivierung der PKC-ε und ERK-2 vermittelt. Anästhesiologie & Intensivmedizin 2004, 45: 691-718

9. Pakulla MA, **Obal D**, Seidel D, Loer SA: Salzsäure-induzierte Lungenschädigung – Effekte einer frühen partiellen Flüssigkeitsbeatmung auf den Gasaustausch und das Überleben von Ratten. Anästhesiologie & Intensivmedizin 2004, 45: 691-718

10. Weber NC, Toma O, Wolter JI, **Obal D**, Schlack W, Preckel B: Xenon induziert eine pharmakologische Präkonditionierung des Rattenherzens in vivo über eine Induktion der PKC-ε und p38 MAPK. Anästhesiologie & Intensivmedizin 2004, 45: 691-718

11. Toma O, **Obal D**, Weber NC, Dettwiler S, Preckel B, Schlack W: Zeitlicher Verlauf von PKC-ε und ERK-2 bei der Desfluran-induzierten Präkonditionierung am Rattenherz in vivo. Anästhesiol Intensivmed Notfallmed Schmerzther 2004; DAK: PO-1-3.3.

12. Ley S, **Obal D**, Dettwiler S, Preckel B, Schlack W: Effekt von Sevolfluran-induzierter Präkonditionierung auf den Ischämie-Reperfusionsschaden der Niere. Anästhesiol Intensivmed Notfallmed Schmerzther 2004; DAK: PO-1-3.2.

13. **Obal D**, Dettwiler S, Ley S, Preckel B, Schlack W: Sevoflurane induced preconditioning did not improve renal function after ischaemia/ eperfusion. Eur J Anaesthesiol 2004; ESA annual meeting

14. **Obal D**, Dettwiler S, Weber NC, Preckel B, Schlack W: Myocardial protection by preconditioning through protein kinase C activation depends on isoflurane concentration. Anesthesiology 2004; 101: A701

15. **Obal D**, Dettwiler S, Favoccia C, Preckel B, Schlack W: Renal function did not improve after Sevoflurane induced preconditioning. Anesthesiology 2004; 101: A724

16. **Obal D**, Müllenheim J, Dettwiler S, Schlack W: Effect of protein kinase c inhibition by staurosporin on isoflurane preconditioning in the rat heart in vivo. Anesthesiology 2003; 99, A1550.

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17. Toma O, Weber NC, **Obal D**, Preckel B, Schlack W: Xenon induces myocardial protection by preconditioning. Involvement of protein kinase C (PKC). Anesthesiology 2003; 99, A1540

18. Fräßdorf J, **Obal D**, Malpohl A, Preckel B, Müllenheim J, Schlack W: Morphine and LPS induced late preconditioning: Are opioidreceptors trigger, mediator or both ? Eur J Anaesthesiol 2003; 20 (Suppl. 30), A231.

19. Pakulla MA, **Obal D**, Seidel D, Loer SA: Partial liquid ventilation in acid-induced lung injury - effects of immediate and delayed initiation. Intensive Care Med 2003; 29 (9 Suppl.), 559

20. Pakulla MA, **Obal D**, Loer SA: Intraarterielles Blutgasmonitoring bei Ratten. Anästhesiologie & Intensivmedizin 2003; 44, PD 404.7

21. Fräßdorf J, **Obal D**, Preckel B, Müllenheim J, Schlack W: Rolle der Opiatrezeptoren bei der Morphin- und Lipopolysaccharid-induzierten späten Präkonditionierung. Anästhesiol Intensivmed Notfallmed Schmerzther 2003.

22. **Obal D**, Kratz M, Dettwiler S, Favoccia C, Preckel B, Müllenheim J, Schlack W: Die Gabe von Sevofluran während der Reperfusion verstärkt die Wirkung der Anästhetika induzierten Präkonditionierung. Anästhesiologie & Intensivmedizin 2003; 44, FV109.4.

23. Pakulla MA, **Obal D**, Loer SA: Continuous intra-arterial blood gas monitoring in rats. Eur J Anaesthesiol 2003; 20 (Suppl. 30), A113

24. **Obal D**, Scharbatke H, Müllenheim J, Preckel B, Schlack W: Myocardial Protection by Preconditioning with Sevoflurane Is Further Enhanced by Sevoflurane Administration during Reperfusion. Anesthesiology 2002; 96, A607

25. Preckel B, Müllenheim J, Hoff J, **Obal D**, Thämer V, Schlack W: Inotropic effects of inhalational anesthetics in dogs with severe heart failure. Anesth Analg. 2002;94:S-283.

26. Pakulla MA, **Obal D**, Loer SA: Kontinuierliche intraarterielle Blutgas-Messung bei Ratten. Intensivmedizin und Notfallmedizin 2002; 39 (Suppl. 1), I/ 6

27. **Obal D**, Preckel B, Scharbatke H, Thämer V, Schlack W: Is the protective effect of sevoflurane against myocardial reperfusion injury concentration dependent ? Eur J Anaesthesiol. 2001;18:A161

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28. Scharbatke H, **Obal D**, Müllenheim J, Preckel B, Thämer V, Schlack W: Bereits eine zweiminütige Gabe von Sevofluran schützt das Herz vor dem Reperfusionsschaden in vivo. Studie an Ratten. Anästhesiologie & Intensivmedizin 2001;472.

29. Preckel B, **Obal D**, Scharbatke H, Müllenheim J, Thämer V, Schlack W: Two minutes of sevoflurane administration are enough to protect against myocardial reperfusion injury. Minerva Anestesiol 2001;67:139.

30. Preckel B, **Obal D**, Scharbatke H, Müllenheim J, Thämer V, Schlack W: The protective effect of sevoflurane against myocardial reperfusion injury is concentration dependent. Minerva Anestesiol 2001;67:138.

31. Heiderhoff M, **Obal D**, Hoff J, Preckel B, Schlack W, Thämer V: Effect of volantile anaesthetics of frequence-dependency of myocardial contraction velocity in chronically instrumented dogs. Pflügers Arch Eur J Physiol 1999;437:P28-12.

32. Heiderhoff M, **Obal D**, Preckel B, Thämer V, Schlack W: Effect of a left stellate ganglion block on left ventricular function in chronically instrumented dogs. Br J Anaesth 1999;82:A.203.

33. **Obal D**, Heiderhoff M, Preckel B, Thämer V, Schlack W: Effect of volatile anaesthetics on frequence-dependency of myocardial contractility in chronically instrumented dogs. Eur J Anaesthesiol 1999

34. Preckel B, Schlack W, Gonzáles M, **Obal D**, Barthel H, Thämer V: Influences of Angiotensin II AT_1 receptor antagonism on ischaemia / reperfusion injury of the dog heart *in vivo*. Pflügers Arch Eur J Physiol 1998;435.

35. Gonzáles M, Preckel B, Schlack W, **Obal D**, Barthel H, Thämer V: Einfluß einer Angiotensin II AT_1 Rezeptorblockade auf den myokardialen Ischämie / Reperfusionsschaden am Hundeherzen in vivo. Z Kardiol 1998;87:FV 980.

36. Preckel B, Schlack W, **Obal D**, Heiderhoff M, Hoff J, Thämer V: Effect of a left stellate ganglion blockade on left ventricular function in conscious dogs. Pflügers Arch Eur J Physiol 1998;435.

37. **Obal D**, Preckel B, Barthel H, Ebel D, Grunert S, Schlack W, Thämer V: Effekt einer intrakoronaren pH Senkung nach Koronarokklusion auf die Infarktgrösse *in vivo*. Z Kardiol 1997;86:335.

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38. Preckel B, Schlack W, Comfère T, **Obal D**, Thämer V: Holothan reduziert den Reperfusionsschaden nach regionaler Koronarokklusion in vivo. Anästhesiol Intensivmed Notfallmed Schmerzther 1997;32:FV17.1.

39. Preckel B, Schlack W, **Obal D**, Gonzáles M, Thämer V: Wirkung verschiedener Inhalationsanästhetika auf den myokardialen Reperfusionsschaden *in vivo*. Z Kardiol 1997;86:90

40. Preckel B, Schlack W, **Obal D**, Thämer V: Different effects of Enflurane, Isoflurane, Sevoflurane, Desflurane on myocardial reperfusion injury *in vivo*. Br J Anaesth 1997;78 (Suppl. 1): A.149

41. Barthel H, Preckel B, Kojda G, Schlack W, **Obal D**, Kottenberg K, Noack E, Thämer V: Effekte verschiedener NO Donatoren auf die myokardiale Funktion am Hundeherzen *in vivo*. Anästhesiol Intensivmed Notfallmed Schmerzther 1997; 32 (Suppl. 1):S153.

42. Preckel B, Kojda G, Schlack W, Barthel H, Ebel D, **Obal D**, Grunert S, Kottenberg K, Noack E, Thämer V: Inotropic effects of different NO-donors in the dog heart *in vivo*. Pflügers Arch Eur J Physiol 1997; 433 (Suppl. 6):R 22.

43. Preckel B, Schlack W, Ebel D, Barthel H, **Obal D**, Grunert S, Meyer O, Halilovic S, Thämer V: Reperfusion with acidotic blood after coronary artery occlusion does not reduce infarct size in dogs. Pflügers Arch 1996; 431, Suppl.6:R146.

44. Preckel B, Kojda G, Schlack W, Ebel D, Barthel H, **Obal D**, Kottenberg K, Thämer V: Influence of spontaneous NO-donors and organic nitrates on regional myocardial inotropy in the dog heart *in vivo*. Eur J Anaesthesiol 1996

Poster Presentations: Local/ Regional Meetings

- **Obal D**, Hu X, Dimova N, Brittian K, Book M, Guo Y, Bhatnagar A, Bolli R, Rokosh G: Inducible, cardiomyocyte-specific transgenic stromal cell derived factor 1 expression facilitates cardiac regeneration after permanent coronary ligation in mice. The cardiovascular forum for promoting centers of excellence and young investigators. International Academy of Cardiovascular Science annual meeting, Louisville August 15-17, 2013

PATENTS:

    N/ A

RESEARCH FUNDING:

PEER Reviewed:

NIH COBRE Grant A. Bhatnagar, Ph.D. (PI)   *04/ 1/ 9-present*

Center of excellence in diabetes and obesity research (Collaborator: 15% time support)

The specific objective of the Center is to develop a basic and clinical understanding of the molecular mechanisms of diabetes and obesity and how they contribute to the burden of cardiovascular disease. In this focus I am studying the impact of hyperglycemia on cardiac reparative potential by mesenchymal and cardiac specific stem cells and whether modulation of cardiac environment increases regeneration tendency in diabetes.

CLINICAL SERVICE:

I provide general anesthesia services in the main operating room at the University of Louisville Hospital, covering general surgery, vascular surgery, neurosurgery, endovascular procedures, oncology surgery, liver surgery, ENT surgery, OB/ YN surgery and procedures, cardiovascular and thoracic surgery, trauma surgery. The University of Louisville is a level one-trauma center; therefore, orthopedic surgery and trauma surgery procedures are a major focus of our work, including regional anesthesia procedures as well as emergency procedures (rescue surgery after major trauma). The anesthesia service covers all cardiovascular resuscitations and difficult endotracheal intubations 24/ at the University Hospital. My call schedule includes six to eight 12 hour call shifts including two night/ weekend calls/ month. These call shifts require physician's in-house presence.

PUBLICATIONS, BOOK CHAPTERS, TEXTBOOK

Peer reviewed paper:

1. Houser P, Miller J, **Obal D**: A case of critical intra-operative tracheal stenosis complicated by tension pneumothorax and postobstructive pulmonary edema. Clin Anesthesia 2014; xy: xx-xy (submitted for publication)

2. **Obal D**, Yang D, Sessler DI: Perioperative doses of ondansetron or dolasetron do not lengthen the QT interval. Mayo Clin Proc 2014; 89 (1): 69-80

3. Luo J, **Obal D**, Dimova N, Tang XL, Rokosh G: Cardiac myocyte-specific transgenic ecSOD targets mitochondria to protect against Ca(2+) induced permeability transition. Front Physiol. 2013 Oct 29;4:295. doi: 10.3389/ phys.2013.00295.

4. **Obal D**, Dai S, Keith R, Dimova N, Kingery J, Zheng Y-T, Zweier J, Velayutham J, Prabhu S, Li Q, Conklin D, Bhatnagar A, Bolli R, Rokosh G: Overexpression of Cardiomyocyte-specific Extracellular Superoxide Dismutase Increases NO Bioavailability and Reduces Infarct size after Ischemia Reperfusion. Basic Res Cardiol 2012;107(6):305. doi: 10.1007/ 00395-012-0305-1. Epub 2012 Oct 26)

5. Wadhwa A, Kandadai SK, Tongpresert S, **Obal D**, Gebhard RE: Ultrasound guidance for deep peripheral nerve blocks: a brief review. Anesthesiol Res Pract. 2011; 2011:262070. Epub 2011 Jul 27.

6. Turan A, Kasuya Y, Govinda R, Orhan-Sungur M, **Obal D**, Rauch S, Dalton J, Akça O, Sessler DI: The effect of aminophylline on loss of consciousness, bispectral index, propofol requirement, and minimum alveolar concentration of desflurane in volunteers. Anesth Analg. 2010 Feb 1;110(2):449-54

7. West MB, Rokosh G, **Obal D**, Velayutham M, Xuan YT, Hill BG, Keith RJ, Schrader J, Guo Y, Conklin DJ, Prabhu SD, Zweier JL, Bolli R, Bhatnagar A: Cardiac myocyte-specific expression of inducible nitric oxide synthase protects against ischemia/ eperfusion injury by preventing mitochondrial permeability transition. Circulation. 2008 Nov 4;118(19):1970-8.

8. Preckel B, **Obal D**, Müllenheim J, Hennes J, Heiderhoff M, Thämer V, Schlack W: Effects of halothane, sevoflurane and desflurane on the force-frequency relation in the dog heart in vivo. Can J Anaesth. 2006 Nov;53(11):1118-25.

9. **Obal D**, Rascher K, Favoccia C, Dettwiler S, Schlack W: Post-conditioning by a short administration of desflurane reduced renal reperfusion injury after differing of ischaemia times in rats. Br J Anaesth. 2006 Dec;97(6):783-91.

10. **Obal D**, Dettwiler S, Favoccia C, Rascher K, Preckel B, Schlack W: Effect of sevoflurane preconditioning on ischaemia/ reperfusion injury in the rat kidney in vivo. Eur J Anaesthesiol. 2006 Apr;23(4):319-26.

11. Weber NC, Toma O, Wolter JI, **Obal D**, Müllenheim J, Preckel B, Schlack W: The noble gas xenon induces pharmacological preconditioning in the rat heart in vivo via induction of PKC-epsilon and p38 MAPK Br J Pharmacol 2005, 144(1):123-32.

12. **Obal D**, Dettwiler S, Favoccia C, Scharbatke H, Preckel B, Schlack W: The influence of mitochondrial K_{ATP}-channels in the cardioprotection of preconditioning and postconditioning by sevoflurane in the rat in vivo. Anesth Analg. 2005 Nov;101(5):1252-60

13. Fräßdorf J, Weber NC, **Obal D**, Toma O, Müllenheim J, Kojda G, Preckel B, Schlack W: Morphine induces late cardioprotection in rat hearts in vivo: the involvement of opioid receptors and nuclear transcription factor kappaB. Anesth Analg. 2005 Oct;101(4):934-41

14. **Obal D**, Weber NC, Zacharowski K, Toma O, Dettwiler S, Wolter JI, Kratz M, Müllenheim J, Preckel B, Schlack W: Role of protein kinase C-ε (PKC-ε) in isoflurane induced cardioprotection. Low, but not high concentrations of isoflurane activate PKC-ε. B J Anaesth 2005; 94(2):166-73

15. Schober P, Seidel D, Kalb R, **Obal D**, Pakulla MA, Loer SA: Partial liquid ventilation in acute salt water-induced lung injury. Eur J Anaesthesiol. 2005 Jul;22(7):536-40.

16. **Obal D**, Kindgen-Milles D, Schoebel F, Schlack W: Coronary artery angioplasty for treatment of perioperative myocardial ischaemia. Anaesthesia 2005; 60(2):194-7

17. Toma O, Weber NC, Wolter JC, **Obal D**, Preckel B, Schlack W: Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and

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extracellular regulated kinase 1 and 2 in the rat heart in vivo. Anesthesiology 2004, 101; 6:1372-1380

18. Preckel B, Müllenheim J, Hoff J, **Obal D**, Thämer V, Schlack W: Haemodynamic changes during halothane, sevoflurane and desflurane anaesthesia in dogs before and after the induction of severe heart failure. Eur J Anaesth 2004; 21(10):797-806

19. Pakulla MA, **Obal D**, Loer SA: Continuous intra-arterial blood gas monitoring in rats. Lab Anim Sci 2004;38:133-137

20. Barthel H, Ebel D, Müllenheim J, **Obal D**, Preckel B, Schlack W: Effect of lidocaine on ischemic preconditioning in the isolated rat heart. Br J Anaesth 2004;87:905-911

21. Pakulla MA, Seidel D, **Obal D**, Loer SA: Hydrochloric acid-induced lung injury: effects of early partial liquid ventilation on survival rate, gas exchange, and pulmonary neutrophil accumulation. Intensive Care Med 2004;30:2110-2119

22. **Obal D**, Scharbatke H, Barthel H, Preckel B, Müllenheim J, Schlack W: Cardioprotection against reperfusion injury is maximal with only 2 minutes of sevoflurane administration in rats. Can J Anaesth 2003; 50:940-945

23. **Obal D**, Preckel B, Scharbatke H, Müllenheim J, Höterkes F, Thämer V, Schlack W: One MAC of sevoflurane provides protection against reperfusion injury in the rat heart *in vivo.* Br J Anaesth 2001;87:905-911

24. Preckel B, Schlack W, Gonzalez M, **Obal D**, Barthel H, Thämer V: Influence of the angiotensin II AT1 receptor antagonist irbesartan on ischemia/ eperfusion injury in the dog heart. Basic Res Cardiol 2000;95:404-412.

25. Müllenheim J, Preckel B, **Obal D**, Heiderhoff M, Hoff J, Thämer V, Schlack W: Left stellate ganglion block has only small effects on left ventricular function in awake dogs before and after induction of heart failure. Anesth Analg 2000;91:787-792.

26. Preckel B, Schlack W, **Obal D**, Barthel H, Ebel D, Grunert S, Thämer V: Effect of acidotic blood reperfusion on reperfusion injury after coronary artery occlusion in the dog heart. J Cardiovasc Pharmacol 1998;31:179-186.

27. Preckel B, Schlack W, Comfère T, **Obal D**, Barthel H, Thämer V: Effects of enflurane, isoflurane, sevoflurane and desflurane on reperfusion injury after

regional myocardial ischaemia in the rabbit heart in vivo. Br J Anaesth 1998;81:905-912

28. Schlack W, Preckel B, Barthel H, **Obal D**, Thämer V: Halothane reduces reperfusion injury after regional ischaemia in the rabbit heart *in vivo*. Br J Anaesth 1997;79:88-96.

Non-Peer reviewed paper:

1. Wadhwa A, Gebhard RE, **Obal D**: Single or multiple guidance methods for peripheral nerve blockade in modern-day practice of regional anesthesia. Advances in Anesthesia 2010; 28: 187-210