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Influence of angiotensin-converting enzyme inhibition on myocardial regeneration and cardiac remodeling mediated by bone marrow-derived cells in cardiac pressure overload

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CONTENTS

1. Abbreviations

ACE	Angiotensin converting enzyme
Ac-SDKP	N-acetyl-Ser-Asp-Lys-Pro
Ang-(1-7)	Angiotensin (1-7)
BMSC	Bone marrow-derived stem cells
BM-SHAM	Bone marrow-transplanted sham-operated mice
BM-SHAM+RAM	Bone marrow-transplanted sham-operated mice treated with ramipril
BM-TAC	Bone marrow-transplanted mice after transaortic constriction
BM-TAC+RAM	Bone marrow-transplanted mice after transaortic constriction treated with ramipril
BMT	Bone marrow transplantation
BSA	Bovine serum-albumin
CSC	Cardiac stem cells
EPC	Endothelial progenitor cells
EPO	Erythropoietin
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorter
Fig.	Figure
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte/macrophage colony stimulating factor
GFP	Green fluorescent protein
H&E	Hematoxylin-eosin staining
HIF-1α	Hypoxia-inducible factor 1 alpha
HRP	Horse radish peroxidase
LV	Left ventricle
LVEF	Left ventricular ejection fraction
LVH	Left ventricular hypertrophy
MEF-2	Myocyte enhancer factor-2
MI	Myocardial infarction
MSC	Mesenchymal stem cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1

RAAS	Renin-angiotensin-aldosterone system
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
Sca-1	Stem cell antigen 1
SEM	Standard error of the mean
SHAM	Sham-operated animals
1dSHAM	One-day sham-operated animals
SHAM+RAM	Sham-operated animals treated with ramipril
1dSHAM+RAM	One-day sham-operated animals treated with ramipril
SSC	Sodium chloride Sodium citrate
TAC	Transverse aortic constriction and animals with transverse aortic constriction
1dTAC	One-day TAC-operated animals
TAC+RAM	Animals undergoing transverse aortic constriction and treated with ramipril
1dTAC+RAM	One-day TAC-operated animals treated with ramipril
TRITC	Tetramethyl rhodamine isothiocyanate
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
VSELs	Very small embryonic-like stem cells

2. Summary

Introduction: Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. For this reason stimulation of heart regeneration would be of paramount importance. In this study the short- and long-term effects of the angiotensin converting enzyme inhibitor ramipril on myocardial regeneration and cardiac remodeling in increased cardiac afterload were investigated using the mouse model of transverse aortic constriction for one day and five weeks. To study the impact of bone marrow-derived cells on cardiac remodeling and regeneration the mouse model of bone marrow transplantation was utilized.

Methods: For experiments ten-week-old male wild-type mice (C57/Bl6 and FVB/NJ) were used. Bone marrow for bone marrow transplantation (BMT) was obtained from six-week-old mice: C57Bl/6-Tg(ACTbEGFP)1Osb (express green fluorescent protein (GFP) ubiquitary) and Tg(Tie-2GFP)287Sato (express GFP only in endothelial cells). Ramipril (5 mg per body weight per day) or vehicle administration started 3 days before surgery and continued until the end of each set of experiments. Transverse aortic constriction (TAC) or sham operation was performed, after which animals were separated into for groups: two experimental groups (SHAM+RAMIPRIL and TAC+RAMIPRIL) and two control groups (TAC and SHAM). Blood pressure measurement was carried out in all animals on the tail artery during the 5 last days of each set of experiments, whereupon they were anaesthetized and left ventricular (LV)-pressure was measured. Then hearts were rapidly excised, partly fixed in formalin, partly snap frozen in liquid nitrogen. Blood and bone marrow from animals without bone marrow transplantation were collected to perform fluorescence-activated cell sorter (FACS) analysis of endothelial progenitor cells (EPC´s). The migration capacity of spleen-derived EPC´s was evaluated by modified Boyden chamber. Extracardiac neoangiogenesis was investigated by a disc angiogenesis model. Expression and protein level of HIF-1α in the left ventricle were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blot respectively. Collagen content in the myocardium was estimated by picrosirius red staining. The measurement of cardiomyocyte short-axis diameter was performed using sections stained with Ehrlich`s hematoxylin and eosin. Immunostaining on cardiac paraffin sections was carried out to identify distinct types of myocardial cells. The apoptosis detection in heart sections was performed by the ApopTag Peroxidase In Situ Oligo Ligation Assay.

Results: As expected, TAC increased left ventricular systolic pressure in both types of experiments and diminished systolic and diastolic peripheral blood pressure in the 5-week experiment. Despite the fact that ramipril did not affect both ventricular and peripheral blood pressure, it ameliorated pressure overload-induced cardiac remodeling by diminishing left ventricular hypertrophy (LVH) and cardiac fibrosis. Cardiac cell turnover (apoptosis and proliferation of cardiomyocytes and non-cardiomyocytes and apoptosis rate in endothelial cells) was increased in the TAC mice and in all groups of BMT mice. Ramipril normalized cell turnover in the pressure-overloaded heart by reducing the number of apoptotic cardiomyocytes, endothelial cells and the number of proliferating cardiomyocytes. In the myocardium of ramipril-treated BMT mice these effects were supplemented by a decrease of apoptosis rate in non-cardiomyocytes. Pressure overload for 5 weeks impaired myocardial capillarisation by reducing the density of endothelial cells, but promoted extracardiac capillary growth. In contrast, ramipril stimulated both extracardiac and cardiac capillary growth in all groups that manifested itself in the heart as an increase of the density of endothelial cells, the ratio of endothelial cells to cardiomyocytes and the ratio of capillaries to cardiomyocytes. This enhanced neoangiogenesis can be partly explained by a ramipril-induced increase of EPC numbers in the bone marrow and their augmented mobilization in the blood observed in the short-term experiment. These effects, however, disappeared after 5 weeks of ramipril treatment. While one-day TAC increased EPC level only in the blood, pressure overload for 5 weeks raised their numbers both in the blood and in the bone marrow. In both short- and long-term experiments ramipril ameliorated migratory capacity of EPC´s. Pressure overload of the left ventricle for 5 weeks increased the number of bone marrow-derived CD31 cells, but reduced the density of bone marrow-derived Tie-2-positive cells therein. By contrast, ramipril enhanced both parameters in the TAC mice promoting thereby myocardial capillary growth. Treatment with ramipril in both short- and long-term experiments decreased myocardial mRNA and protein level of HIF-1α in all groups. Increased cardiac afterload and treatment with ramipril for 5 weeks upregulated the number of bone marrow-derived myocyte progenitor cells in the myocardium. The combination of TAC and ramipril treatment provided no additional effect.

Conclusion: Our findings testify that aside from its antihypertensive action, ramipril has other beneficial effects on the cardiovascular system, which include amelioration of cardiac remodeling, stimulation of cardiac and extracardiac neoangiogenesis and mobilization of bone marrow-derived progenitor cells participating in myocardial regeneration

2. Zusammenfassung

Einfluss der Inhibition des Angiotensin-konvertierenden Enzyms auf Myokardregeneration und kardiales Remodeling vermittelt durch dem Knochenmark entstammende Stammzellen bei druckinduzierter Nachlasterhöhung

Einleitung: Herz-Kreislauf-Erkrankungen sind die wichtigste Ursache für Morbidität und Mortalität weltweit. Aus diesem Grund wäre eine Stimulation der Herzregeneration von höchster Bedeutung. In der vorliegenden Arbeit wurden kurz- und langfristige Effekte von Ramipril, einem Hemmer des Angiotensin-konvertierenden Enzyms, auf Myokardregeneration und kardiales Remodeling bei kardialer Nachlasterhöhung mit Hilfe des Mausmodells der Aortenligatur für 1 Tag und 5 Wochen erforscht. Der Beitrag der dem Knochenmark entstammenden Stammzellen zu Myokardregeneration und kardialem Remodeling wurde mittels des Mausmodells der Knochenmarktransplantation untersucht.

Methoden: Für die Experimente wurden 10 Wochen alte Wildtyp-Mäuse (C57/Bl6 und FVB/NJ) verwendet. Knochenmark für die Transplantation wurde 6 Wochen alten Mäusen - C57Bl/6-Tg(ACTbEGFP)1Osb (exprimieren grün fluoreszierendes Protein (GFP) überall) und Tg(Tie-2GFP)287Sato (exprimieren GFP nur in Endothelzellen) entnommen. Die Gabe von Ramipril (5 mg pro kg Körpergewicht pro Tag) wurde 3 Tage vor der Operation begonnen und bis zum Ende jeder Experimentserie fortgesetzt. Nach Durchführung von Aortenligatur (TAC) bzw. SHAM-Operation wurden die Tiere in 4 Gruppen eingeteilt: 2 experimentelle Gruppen (SHAM+RAMIPRIL und TAC+RAMIPRIL) und 2 Kontrollgruppen (SHAM und TAC). Blutdruckmessung wurde bei allen Tieren an der Schwanzarterie innerhalb der 5 letzten Tagen jeder Experimentserie gemessen, danach wurden sie anästhesiert und eine Messung des linksventrikulären Drucks wurde durchgeführt. Herzen wurden sofort entnommen und teilweise in Formalin fixiert bzw. in flüssigem Stickstoff schockgefroren. Blut und Knochenmark wurden von den nichtknochenmarktransplantierten Tieren zum Tötungszeitpunkt gewonnen, um EPC´s mittels FACS-Analyse zu quantifizieren. Die migratorische Kapazität von EPC´s aus der Milz wurde in einer modifizierten Boyden-Kammer determiniert. Extrakardiale Neoangiogenese wurde anhand von subkutan implantierten Disks untersucht. Expressions- und Proteinniveau von HIF-1α wurden mittels quantitativer RT-PCR und Western Blot bestimmt. Die Picro-Sirius-Rot gefärbten Paraffinschnitte wurden für die Auswertung des Kollagengehalts im Myokard

verwendet. Der Kardiomyozytenquerdurchmesser wurde morphometrisch anhand von Hämatoxilin-Eosin gefärbten Paraffinschnitten ermittelt. Immunfärbungen an kardialen Paraffinschnitten wurden durchgeführt, um verschiedene Zelltypen zu bestimmen. Apoptose-Detektion in Herzgewebe wurde mit Hilfe des Apop Tag Peroxidase In Situ Oligo Ligation Assays durchgeführt.

Ergebnisse: Wie zu erwarten war, erhöhte TAC wesentlich den linksventrikulären Druck in beiden Experimenttypen und senkte den systolischen und den diastolischen peripheren Blutdruck im 5-Wochen Versuch. Obwohl Ramipril weder den ventrikulären noch den peripheren Blutdruck beeinflusste, verbesserte es das druckinduzierte kardiale Remodeling durch Hemmung von linksventrikulärer Hypertrophie (LVH) und kardialer Fibrose. Der Zellumsatz im Herzen (Apoptose und Proliferation von Kardiomyozyten und Nichtkardiomyozyten und Apoptoseanteil in Endothelzellen) war bei den Aortenligatur-Mäusen und in allen Gruppen von knochenmarktransplantierten Mäusen erhöht. Ramipril normalisierte den Zellumsatz in druckbelasteten Herzen durch die Verminderung der Anzahl von apoptotischen Kardiomyozyten und Endothelzellen und der Anzahl der Ki-67-positiven Kardiomyozyten. Im Myokard von Ramipril behandelten knochenmarktransplantierten Mäusen wurden diese Effekte durch die Reduzierung des Apoptoseanteils in Nichtkardiomyozyten ergänzt. Druckinduzierte Nachlasterhöhung über 5 Wochen beeinträchtigte die Kapillarisierung des Myokards. Dies äußerte sich in einer Reduzierung der Endothelzelldichte. Aortenligatur förderte jedoch die extrakardiale Neoangiogenese. Ramipril hingegen stimulierte sowohl extrakardiales als auch kardiales Kapillarwachstum in allen Gruppen. Im Myokard erhöhte die Ramiprilbehandlung die Endothelzelldichte, das Verhältnis von Endothelzellen zu Kardiomyozyten und das Verhältnis von Kapillaren zu Kardiomyozyten. Diese verstärkte Neoangiogenese kann teilweise durch Ramipril-induzierte Steigerung der EPC-Anzahl in Knochenmark und Blut und ihre Mobilisierung in das Blut, die im kurzfristigen Versuch beobachtet wurden, erklärt werden. Diese Effekte waren allerdings nach 5 Wochen Ramiprilbehandlung nicht mehr nachzuweisen. Während Aortenligatur nach 1 Tag das EPC-Niveau nur im Blut erhöhte, führte kardiale Nachlasterhöhung für 5 Wochen zu einem Anstieg von EPC´s sowohl im peripheren Blut als auch im Knochenmark. In beiden Experimenttypen verbesserte Ramipril die migratorische Kapazität der EPC´s. Die kardiale Druckerhöhung für 5 Wochen erhöhte die Anzahl der dem Knochenmark entstammenden GFP-positiven, CD31-positiven Zellen, aber verminderte die Dichte der dem Knochenmark entstammenden Tie-2-positiven Zellen im Myokard. Dagegen kam es bei den Ramipril-behandelten Aortenligatur-Mäusen zu einer signifikanten Erhöhung dieser Parameter. Die ACE-Hemmung senkt die myokardiale mRNA und Protein Expression

von HIF-1α bei allen Tieren in beiden Experimenttypen. Die kardiale Nachlasterhöhung und die Ramiprilbehandlung über 5 Wochen, aber nicht ihre Kombination, führten zu einem Anstieg von dem Knochenmark entstammenden MEF-2-positiven Vorläuferzellen im Myokard.

Schlussfolgerung: Unsere Ergebnisse weisen darauf hin, dass Ramipril, außer seiner antihypertensiven Wirkung, auch andere günstige Effekte auf das kardiovaskuläre System ausübt, die die Verbesserung des kardialen Remodelings, die Stimulation von kardialer und extrakardialer Neoangiogenese sowie die Mobilisierung der dem Knochenmark entstammenden Vorläuferzellen, die an der Myokardregeneration beteiligt sind, umfassen.

3. Introduction

3.1. Potential of heart regeneration

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. Although their pathogenic mechanisms are different, they ultimately lead to a loss of distinct cardiac cells (cardiomyocytes, endothelial cells and smooth muscle cells of coronary vessels) and elicit cardiac fibrosis that in turn deteriorates cardiac performance resulting thereby in heart failure (139). For this reason the potential of heart regeneration has become the topic of large body of research.

It was assumed for a long time that in adult mammals, especially in human beings, all heart damages are healed by scar formation since most researchers have observed only fragmentary regenerative phenomena such as amitotic and, only very rarely, mitotic division of nuclei in muscle fibers or myogenic cells at the edge of the scar (115, 116). These myogenic cells or myoblasts were first discovered by Oppel and Anichkow (6, 7, 105). According to them, myoblasts separated from the destroyed muscular stumps and mixed with the cells of connective tissues forming "myogenic granulations" in the perinecrotic area. However, some authors detected newly formed cardiomyocytes (myoblasts) in the middle of scar tissue. In particular, Ring (124) described them in postinfarction scar in rabbit. These cells existed for a short time and were replaced by connective tissue. Detailed histological description of newly formed cardiomyocytes was presented by Polezhaev et al. (115, 116) in experiments with diathermal coagulation of a sector of myocardium in adult rats. They appeared as single cells or chains of myoblasts around 7 days after the operation in the center of the damage. Twenty days later the myoblasts were transformed into islets of muscle fibers surrounded by connective tissue scar. 45 days after the diathermal coagulation the islets disappeared and were finally replaced by connective tissue. Polezhaev speculated that they arose from cells released from muscle stumps or from undifferentiated cells of connective tissue which migrated in the damage zone and thereafter transdifferentiated into myoblasts under the influence of substances from destroyed cardiomyocytes. However, these newly formed cardiomyocytes were not found after damage of myocardium induced by burning, strong vacuum and ligation of the coronary arteries. Polezhaev concluded that "the resultant regeneration or scar formation depends of the nature of the damage, which means that there is a basis for the regeneration processes of muscle fibers in the heart" (115). The newly formed cardiomyocytes from different species shared common morphological traits: they were fine, not striated and sarcoplasma-rich. Further investigation carried out by Skuba (141) using immunostaining techniques revealed the presence of cardiac myosin in myoblasts. However, for some reason these findings were contradictory: first not all scientists

investigating heart regeneration found these cells, secondly some of them believed that they were not true cardiomyocytes, but most probably fibroblasts or smooth muscle cells (104, 115, 116, 128).

The evidence for an increase of cardiomyocyte number in heart hypertrophy was gained in the late forties of last century. In particular, Linzbach formulated a rule based on pathological studies, according to which the number of myofibers in human myocardium rises if heart weight exceeds 500 g (81, 82). Animal experiments also demonstrated that 50-60% increase of cardiac mass augmented cardiomyocytes number (65, 67) without changing mitotic rate in mature cardiomyocytes. Authors speculated, that new cardiomyocytes might originate from either non-differentiated cardiac cells (myoblasts) or from division of partly differentiated cardiomyocytes with 2-4 nuclei, however it was methodically impossible to prove it (65).

Development of new research methods allowed to better understand the natural ability of the heart to regenerate. Using cells labeled with different markers (GFP, Y chromosome, or species specific DNA sequences and antigens) the formation of new cells from bone marrow-derived and resident stem cells in adult hearts was shown. In particular, GFP-positive cardiac cells (cardiomyocytes, endothelial cells, smooth muscle cells) were found in different models of myocardial damage: transaortic constriction (35), heart infarction (89) and pulmonary hypertension (144). Furthermore, several studies on cardiac chimerism have revealed cardiomyocytes and other cell types of noncardiac origin containing a Y chromosome in sex-mismatched-transplanted female donor hearts (74, 96, 112, 120) and in hearts of women who received a gender-mismatched bone marrow transplantation (15, 31, 153). It should be stressed, that the number of these cells pointed out by different investigators is small, so the number of chimeric cardiomyocytes varied from one in 10^4-10^3 to 18% of total cardiomyocytes (15, 31, 74, 96, 112, 120, 153). However, some authors have reported that so-called newly formed cells especially cardiomyocytes could arise from fusion of labeled cells with adult cardiac cells (4) or could be products of horizontal gene transfer (22). Moreover, it is possible, that some kinds of histological artifacts mimic distinct types of newly formed cardiac cells (75). Thus, the myocardium possesses the restricted ability to regenerate itself by forming different types of new cells (cardiomyocytes, smooth muscle cells, endothelial cells) originating from bone marrow-derived stem cells or resident stem cells, however their number is not sufficient to restore cardiac performance and to prevent development of heart failure.

3.2. Proliferation of distinct cells in the heart

During embryogenesis and early postnatal period mammalian myocardium demonstrates a high proliferation rate of cardiomyocytes and other cardiac cells, however in the course of time their number significantly decreases (62, 104, 128, 130). In particular, studies carried out on biopsies obtained from transplanted adult human hearts revealed either very low number of Ki-67-positive cardiomyocytes (112) or did not find them at all while some stromal and endocardial fibroblasts and also few endothelial cells expressed proliferation markers (101). Cardiovascular diseases reverse the situation by eliciting a proliferative response of distinct cells in the heart (57, 139).

Slight abortive regeneration of cardiac fibers in different parts of myocardium (single mitosis, amitosis and cell nucleus fragmentation) was described by many authors both in animal experiments and pathological studies (6, 7, 61, 105, 115, 116). Using autoradiography with H^3-timidine (63, 103, 127, 129) in a rat model of heart infarction, predominant localization of these cells in the myocardium of both atria (40-50%) and in the subepicardial layer of left ventricle (15-35%) was shown. Myofibers located in other perinecrotic areas contained markedly fewer labeled cardiomyocytes (1-4%). Recent investigations have confirmed the presence of dividing cells with the characteristics of immature cardiomyocytes in the myocardium of patients suffering from heart infarction or cardiac hypertrophy caused by aortic stenosis (13, 160).

Myocardial growth and restoration require adequate blood supply. New blood vessels in adult organism originate from two sources. The minor source is endothelial progenitor cells from the bone marrow, the major source is the vessels of injured tissue containing adult endothelial cells capable of mitosis and resident precursors of endothelial cells (146, 181). Using immunostaining for the endothelial cell marker CD31, proliferation cell nuclear antigen (PCNA) and BrdU some authors revealed dividing endothelial cells in remodeling myocardium (46, 167). However, despite of myocardial vasculogenesis and angiogenesis, capillary number is not sufficient to maintain adequate blood supply and this, among other reasons, leads to further loss of cardiomyocytes (57, 139) and promotes enhanced proliferation of fibroblasts undergoing myofibroblast differentiation resulting in cardiac fibrosis (38). Thus, cardiac cell division in cardiovascular diseases can not completely compensate loss of mature cardiomyocytes and vascular cells.

3.3. Bone marrow stem cells for heart regeneration

As discussed above, modern investigations have confirmed the hypothesis that mature cardiac cells in adult mammalians can originate from undifferentiated bone marrow-derived cells circulating in the blood (15, 31, 35, 144, 153). In particular, Polezhaev suggested that newly formed cardiomyocytes might partly arise from circulating bone marrow stem cells, because many cardiomyocytes with H^3-timidine-labeled nuclei were found in the scar tissue near blood vessels (117). The bone marrow is known to be an excellent source of different types of stem cells: e.g. hematopoietic stem cells, endothelial progenitor cells, mesenchymal stem cells and very small embryonic-like stem cells. Hematopoietic stem cells (HSC) can be isolated from bone marrow through selective sorting for a particular set of surface receptors: $Lineage^-$, $c\text{-}kit^+$, $Sca\text{-}1^+$, $CD34^{lo}$, $CD38^{hi}$ (107, 142). Some studies have demonstrated the potential of HSC to differentiate into cardiomyocytes or vascular cells after myocardial damage in vivo (54, 106, 107).

Endothelial progenitor cells (EPC) represent a subset of hematopoietic stem cells expressing the hematopoietic markers CD133, CD34, stem-cell marker (Sca-1) and endothelial marker Flk-1 (VEGFR-2) (109). EPC can acquire an endothelial phenotype, repair injured vessels and contribute to neovascularisation of damaged tissues (10, 50, 56, 169, 173). Mesenchymal stem cells (MSC) are the population of nonhematopoietic cells that resides within the bone marrow stroma and have potential of multipotency (39, 54). Subsequent studies have shown their ability to differentiate into chondrocytes, osteoblasts, astrocytes, neurons, skeletal muscle, and, notably, cardiomyocytes (11, 86, 106, 111). MSC are positive for the antigens SH2, SH3, SH4, STRO-1, CD29, CD34 and CD14 (29).

Very small embryonic-like stem cells (VSELs) were recently identified in murine bone marrow and human umbilical cord blood (70, 71). They are very small and express markers of embryonic pluripotent stem cells such as Oct-3/4, Nanog, SSEA-1. Murine VSELs can differentiate into cell lineages from all 3 germ layers, among them mesoderm-derived cardiomyocytes (70). All types of the BMSCs are mobilized and recruited to the heart after myocardial injury (58, 87, 107, 137, 175) through secretion of different biological active substances such as colony-stimulating factors, erythropoietin, growth factors, hormones and nitric monoxide by damaged tissue (20). According to recent investigations an important role in the stem-cell mobilization and stimulation of angiogenesis in response to hypoxia and ischemia plays hypoxia-inducible factor 1, which activates expression of many genes involved in tissue protection, regeneration and vessel growth (49). Thus, bone marrow-derived stem cells

participate actively in the heart regeneration by differentiating into distinct types of cardiac cells. This presents an opportunity to use them for restoration of damaged myocardium.

3.4. Stimulation of heart regeneration

The first attempt to stimulate regeneration of damaged myocardium with extracts of animal embryo hearts was made by Törö (156). Further regular experiments on stimulation of heart regeneration were undertaken by the research group of Polezhaev (115-117). Rat and rabbit myocardium injured by diathermocoagulation or by induction of diphtherial myocarditis was treated by intraperitoneal injection of hydrolysates of cardiac and skeletal muscle, grafts of skeletal muscle, brain, and peripheral nerve to the necrotic area and intramuscular injections of cobalt-35 preparations. The aim was to recruit circulating stem cells to the injury area and/or to stimulate division of mature cardiomyocytes with the help of biological active substances from tissues. The authors observed a limited number of newly formed striated myofibers in the middle of the scar tissue. Advances in experimental biology and medicine in the 1990s created several therapeutic approaches to stimulate heart regeneration.

Biological active substances such as cytokines, growth factors and drugs can be utilized for mobilization of BMSC and activation of CSC (20). Orlic et al. (107) were the first to use a combined therapy of G-CSF and stem-cell factor in a murine model of myocardial infarction and showed a significant improvement in left ventricular remodeling, cardiac function and animal survival within 5 days of treatment. Some other studies carried out on this model demonstrated an increased amount of CSC and BMSC in the infarcted heart (2, 40). However, there are other reports that G-CSF does not significantly recruit BMSC and stimulate cardiomyocytes regeneration (76). The results of the major clinical trials conducted using G-CSF in the treatment of cardiac disease are very contradictory. Although FIRSTLINE-AMI and The Rigenera Study (51, 77) trials demonstrated mobilization of $CD34^+$ in peripheral blood and improved left ventricular ejection fraction in the G-CSF treated patients, results of STEMMI and REVIVAL-2 (125, 187) did not reveal any difference between groups.

Animal experiments described the beneficial effects of erythropoietin (EPO) in the heart. In particular, EPO significantly downregulated apoptosis of cardiomyocytes, induced myocardial angiogenesis by EPC mobilization in rats and dogs with MI (48, 94, 118). The clinical trial HEBE III is currently under way to estimate the efficacy of EPO in treatment of MI (12). There is plethora of other biological active substances whose ability to stimulate heart regeneration after MI has been proven in animal experiments: e.g. insulin-like growth factor-1, hepatocyte growth factor, vascular endothelial growth factor, stromal cell derived factor-1 and high mobility

group box 1 (1, 37, 78, 161, 166). They stimulate migration of CSC and BMSC to the infarcted area, promote their proliferation and survival and restore cardiac function by improving neovascularisation and myocardial tissue viability. Also it has been found, that some drugs - for example ACE inhibitors and statins - can mobilize EPC from bone marrow stimulating thereby myocardial neoangiogenesis (154).

Another approach to restoration of injured myocardium is to use stem-cell transplantation. Currently, various stem-cell populations have been analyzed for their ability to induce heart regeneration. In vivo studies based on heart transplantation of both murine and human ESC demonstrated their ability to contribute to a consistent number of newly formed cardiomyocytes and, therefore, highlight their therapeutic potential (59, 64, 90). However, several problems limiting ESC use must be solved before their clinical application can be possible: presence of unwanted non-cardiac cells, tumorigenicity and immunogenicity (146). For these reasons adult stem cells - especially different types of BMSCs - are widely used to stimulate cardiac regeneration. Bone marrow-derived stem cells can be relatively easy isolated from patient, expanded in vitro and transplanted into his heart without eliciting immune response. Although no methods have been established to induce a functional cardiac phenotype in BMSCs in vitro, numerous experimental and clinical studies have shown that their transplantation can improve left ventricular function and reduce adverse heart remodeling (28, 45, 158). However, as in the case of stimulation of heart regeneration with G-CSF, clinical effects of intracoronary infusion of bone marrow-derived cells in patients with MI or ischemic cardiomyopathy are contradictory. Whereas absolute increase in LVEF was demonstrated in BOOST, REPAIR-AMI and IACT trials, infarct size reduction and improvement of infarction wall movement velocity were observed only in IACT study (134, 147, 176). Two other randomized trials (55, 85) did not report improvements in the LVEF after intracoronary transplantation of bone marrow cells. Importantly, however, the study conducted by Janssens et al (55) did reveal a significant reduction in infarct size using robust cardiac magnetic resonance imaging.

Data obtained from animal experiments demonstrate different mechanisms by which transplanted cells can ameliorate cardiac function: transdifferentiation into cardiomyocytes (106), differentiation into endothelial cells to promote angiogenesis (52), fusion with different types of cardiac cells to enhance their viability (102) and secretion of biological active substances stimulating regeneration (24, 162).

The third approach to restore injured myocardium is to stimulate division of mature cardiomyocytes forcing their cell-cycle block. In particular, experimental findings have demonstrated that both inhibition of negative regulators of cell cycle such as $p21^{WAF1}$ and

$p27^{KIP1}$ and overexpression of positive regulators of cell division such as members of the retinoblastoma family or the cyclin-cyclin dependent kinases lead to persistent adult cardiomyocyte cycling (3). Thus, new experimental and clinical data on stimulation of heart regeneration demonstrate the efficacy of different approaches. However, further investigations are required before they could be introduced into routine clinical practice.

3.5. Effects of the ACE inhibitor Ramipril on cardiac remodeling

Both experimental and clinical studies demonstrated beneficial effects of treatment with ACE inhibitors in the cardiovascular diseases (172). This is because the RAAS is involved at all stages of the cardiovascular continuum and promotes such parts of cardiac remodeling as: hypertrophy, fibrosis, apoptosis and reduction in capillary number (33). Ramipril is one of the most widely used ACE inhibitors in the routine clinical practice. For this reason its efficacy in the treatment of cardiovascular diseases has been tested in some clinical trials. Results of the HOPE study have demonstrated that ramipril decreases the development and causes regression of LVH independent of blood pressure reduction, and these changes are associated with reduced risk of death, myocardial infarction, stroke, and congestive heart failure in a high-risk cohort of patients with previous cardiovascular diseases and diabetes (30, 88). Furthermore, as it was shown in the MITRA PLUS trial, ramipril has advantages over other ACE inhibitors in reduction of hospital mortality and of nonfatal major adverse coronary and cerebrovascular events in patients with ST-elevation acute myocardial infarction (174). Experimental findings also demonstrated complete prevention or regression of LVH and cardiac fibrosis under the influence of both antihypertensive and non-antihypertensive ramipril doses in different animal models (79, 80). Cardiomyocyte loss through apoptosis in pressure overload and in essential hypertension also could be prevented by treatment with ramipril (98). As discussed above, inadequate blood supply of myocardium contributes to cardiac remodeling. Ramipril significantly increased myocardial capillary length density in spontaneously hypertensive rats and stroke prone rats independently of its antihypertensive and antihypertrophic actions (184). Thus, both clinical and experimental data demonstrate the ability of the ACE inhibitor ramipril to reduce or even to prevent cardiac remodeling.

4. Aims of the study

Stimulation of heart regeneration and prevention of cardiac remodeling in cardiovascular diseases could be reached through the mobilization of BMSC by different biological active substances. At the same time, some drugs, in particular the ACE inhibitor ramipril, possess the ability to protect myocardium against remodeling by reducing LVH and cardiac fibrosis, diminishing cardiomyocyte apoptosis and stimulating capillary growth. This raises the question whether this protection is partly caused by mobilization of BMSC, enhancement of their migratory capacity and impact of these cells on heart regeneration. Transverse aortic constriction (TAC) is a widely used model for investigation of cardiac remodeling in pressure overload. On the other hand, it was shown that TAC also enhances expression of ACE in LV and therefore is suitable to study different effects of ACE inhibitors (183). For this reasons, it was chosen for the experiments.

On this basis, we have formulated the aims of our study:

1. to investigate short-term and long-term effects of ramipril on left ventricular remodeling in pressure overload, using a mouse transverse aortic constriction model
2. to characterize effects of ramipril on turnover of cardiac cells in LVH
3. to investigate the influence of ramipril on cardiac and extracardiac capillary growth as well as on bone marrow-derived EPC's
4. to explore the impact of BMSC on cardiomyogenesis in hypertrophied adult heart and the opportunity to enhance heart regeneration with ramipril
5. to clarify HIF-1α involvement in EPC mobilization and angiogenesis in pressure overload and to elucidate the influence of ramipril thereon

5. Materials and Methods

5.1. Materials

5.1.1. Animals

Ten-week-old male wild-type mice (C57/Bl6 and FVB/N), purchased from Charles River Laboratories, Sulzfeld, Germany, were used in our experiments. For the bone marrow transplantations GFP-expressing bone marrow cells were obtained from six-week-old C57Bl/6-Tg(ACTbEGFP)1Osb mice (expressing GFP ubiquitary) and from six-week-old Tg(Tie-2GFP)287Sato mice (express GFP under the control of the Tie-2 promoter). Both mice lines were from Jackson laboratory. The study was approved by the animal ethics committee of the Universität des Saarlandes. Mice were housed under standard controlled conditions.

5.1.2. Chemicals

30% Acrylamide solution; Bio-Rad Laboratories, Germany
Albumin, Bovine, Fraction V; Sigma-Aldrich, Germany
Ammonium persulfate (APS); Serva, Germany
Amplific Diluent; PerkinElmer Life Sciences, Inc., USA
Ampuwa distillate and deionized water; Fresenius Kabi, Germany
Aprotinin; Sigma-Aldrich, Germany
Bromphenol blue; Sigma-Aldrich, Germany
ß-mercaptoethanol; Sigma-Aldrich, Germany
ß-glycerolphosphate; Calbiochem, USA
Citraconic acid anhydride; Sigma-Aldrich, Germany
Citric acid monohydrate; Merck, Germany
4',6-diamidino-2-phenylindole (DAPI); Calbiochem, Germany
Diethylpirocarbonat (DEPC); Sigma-Aldrich, Germany
Di-sodium hydrogenphosphat; Merck, Germany
DiLDL; Cell Systems, Germany
Dimethylsulfoxid (DMSO); Sigma-Aldrich, Germany
Dithiothreitol (DTT); Sigma-Aldrich, Germany
DNAse I; Roche, Germany
Dry milk powder; TSI , Germany
Ethylene glycol tetraacetic acid (EGTA); Sigma-Aldrich, Germany
Ethylene diamin tetraacetic acid (EDTA); Sigma-Aldrich, Germany
EBM- Endothelial Cell Basal Medium; Lonza, USA
ECM- Endothelial Cell Medium; Lonza, USA
Enthellan; Merck, Germany
Eosin; Merck, Germany
E-64; Uptima, France
Ethanol; Merck, Germany
Ficoll (ρ= 1.077 g/ml); Biochrom, Germany
Fetal Bovine Serum; PAA Laboratories, Germany
Full Range Rainbow recombinant protein molecular weight marker; GE Healthcare, USA

Glycerol; Sigma-Aldrich, Germany
Glycine; WVR, Germany
Goat Serum; Sigma-Aldrich, Germany
Hematoxilin; Merck, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); Merck, Germany
Hydrochloric acid 37%; Merck, Germany
Hydrogen peroxide; Merck, Germany
Immerse oil; Merck, Germany
Isotonic sodium chloride solution; B.Braun, Germany
Kliniplast paraffin-based medium for tissue embedding; Klinika Medical, Germany
Leupeptin; Sigma-Aldrich, Germany
Liquid nitrogen; Messer, Germany
Lysing Buffer for FACS; BD Bioscience, Germany
Methanol; Merck, Germany
Microcystin; Alexis Biochemicals, Switzerland
Normal Mouse Serum; Dianova, Germany
Normal Rat Serum; Dianova, Germany
Paraformaldehyd; Merck, Germany
10x PCR buffer; Roche, Germany
peqGOLD RNA Pure; Peqlab Biotechnology, Germany
Phenylmethanesulphonylfluoride (PMSF); Sigma-Aldrich, Germany
Picric acid; Sigma-Aldrich, Germany
Ponçeau S solution for electrophoresis; Serva, Germany
Potassium chlorid; Merck, Germany
Potassium dihydrogenphosphat; Merck, Germany
Rabbit Serum; Sigma-Aldrich, Germany
Random primer; Roche, Germany
RNasin; Promega, USA
Sirius Red; Polysciences Inc., Germany
Sodium azide; Sigma-Aldrich, Germany
Sodium chloride; Merck, Germany
Sodium dodecyl sulfate (SDS); Serva, Germany
Sodium hydroxide; Merck, Germany
Sodium orthovanadate; Sigma-Aldrich, Germany
Streptavidin-TRITC; Dianova, Germany
Tetramethylethylenediamine (TEMED); Sigma-Aldrich, Germany
Tris(hydroxymethyl)aminomethane (Tris); Sigma-Aldrich, Germany
Tri-sodium citrate-dihydrat; Merck, Germany
Triton X-100; Sigma-Aldrich, Germany
Trypsin; Sigma-Aldrich, Germany
Tyramide biotinylated; PerkinElmer Life Sciences, Inc., USA
Tween 20 (Polyoxyethyenesorbitan); Sigma-Aldrich, Germany
Vectashield Mounting Medium for Fluorescence; Linaris, Germany
Western Blocking Reagent; Roche, Germany
Xylen; Merck, Germany

5.1.3. Antibodies

Antibodies used in the study are listed below in the tables 1 and 2.

Table 1 Primary antibodies

Antibody	Host	Ig-fraction	Ig-concentration	Ig-dilution	Producer	Catalogue number
Immunostaining						
monoclonal anti-alpha-sarcomeric actin	Mouse	IgM	1.00 mg/ml	1:100	Sigma-Aldrich, Germany	A2172
monoclonal anti-alpha-smooth muscle actin	Mouse	IgG2a	4.5 mg/ml	1:100	Sigma-Aldrich, Germany	A2547
polyclonal anti-PECAM-1 (CD31)	Goat	IgG	0.2 mg/ml	1:15	Santa-Cruz Biotechnology, USA	sc-1506
polyclonal anti-Ki67	Rabbit	IgG	12.0-30.0 g/l	1:150	Novocastra Laboratories LTD, UK	NCL-Ki67p
polyclonal anti-MEF-2	Rabbit	IgG	0.2 mg/ml	1:30	Santa-Cruz Biotechnology, USA	sc-313
polyclonal anti-podocalyxin	Goat	IgG	0.2 mg/ml	1:100	R&D Systems, Germany	AF1556
polyclonal anti-GFP	Rabbit	IgG	whole serum	1:300	abcam, UK	ab290
polyclonal anti-GFP	Goat	IgG	1.15 mg/ml	1:100	abcam, UK	ab6673
FACS						
anti-Sca-1-FITC	Rat	IgG2a, k	0.5 mg/ml	1:20	BD Bioscience, Germany	553335
anti-VEGFR-2	Rat	IgG2a, k	0.2 mg/ml	1:20	BD Bioscience, Germany	555308
Anti-CD16/CD32 Mouse BD Fc Block™	Rat	IgG2b	0.5 mg/ml	1:6	BD Bioscience, Germany	553142
PE Rat IgG2a, k Isotype Control	Rat	IgG2a, k	0.2 mg/ml	1:20	BD Bioscience, Germany	553930
PE Rat IgG2a, k Isotype Control	Rat	IgG2a, k	0.5 mg/ml	1:20	BD Bioscience, Germany	553929
Western Blot						
monoclonal anti-HIF-1α	Mouse	IgG2b	4.0 mg/ml	1:200	Novus Biologicals, USA	NB100-105
monoclonal anti-β-tubulin	Rabbit	IgG	1.12 mg/ml	1:1000	abcam, UK	ab52623

Table 2 Secondary antibodies

Antibody	Conjugated with	Host	Producer	Concentration	Catalogue number
anti-mouse IgM	FITC	Goat	Dianova, Germany	1.5 mg/ml	115-095-020
anti-mouse IgM	TRITC	Goat	Dianova, Germany	1.3 mg/ml	115-025-044
anti-rabbit IgG	FITC	Donkey	Dianova, Germany	1.5 mg/ml	711-095-152
anti-goat IgG	FITC	Donkey	Dianova, Germany	1.5 mg/ml	705-095-147
anti-rabbit IgG	HRP	Goat	Sigma-Aldrich, Germany	1.1 mg/ml	A6154
anti-goat IgG	HRP	Rabbit	Sigma-Aldrich, Germany	7.6 mg/ml	A5420
anti-rabbit IgG	Biotin	Donkey	Dianova, Germany	1.2 mg/ml	711-065-152

5.1.4. Assays and kits

ApopTag Peroxidase In Situ Oligo Ligation Kit; Millipore Chemicon, USA
DC protein assay; Bio-Rad Laboratories, Germany
Enhanced chemiluminescence kit; GE Healthcare, USA
Omniscript RT Kit; Qiagen, Germany
TaqMan Gene expression assay for Hif-1α; Applied Biosystems, USA
TaqMan Gene expression assay for 18 sRNA; Applied Biosystems, USA
TaqMan gene expression master mix; Applied Biosystems, USA

5.1.5. Drugs

Ramipril; Sanofi-Aventis, Germany
Ketamine; Pfizer, USA
Xylazine; Bayer, Germany

5.1.6. Solutions

10xPBS: (pH 7.4)	80.0 g NaCl 2.0 g KCl 2.0 g KH_2PO_4 6.149 g Na_2HPO_4 per 1 liter Ampuwa
1xPBS: (pH 7.4)	100 ml 10xPBS 900 ml Ampuwa and 100 ml Tween 20 for 1xPBS+0.1%Tween
20xSSC: (pH 7.0)	175.32 g NaCl 100.5 g Tri-sodium citrate-dihydrate per 1 liter Ampuwa
4xSSC: (pH 7.0)	200 ml 20xSSC 800 ml Ampuwa and 100 ml Tween 20 for 4xSSC+0.1%Tween
0.05% Citraconic acid anhydride buffer: (pH 7.4)	50 µl Citraconic acid anhydride per 100 ml Ampuwa

4% Bovine serum-albumin blocking solution:	4.0 g Albumin, bovine, Fraction V per 100 ml 4xSSC
5% Milk blocking solution:	5.0 g Dry milk powder per 100 ml 4xSSC
4%PBS-buffered formalin: (pH 7.4)	4.0 g Paraformaldehyde per 100 ml 1xPBS
1mM EDTA –solution: (pH7.4)	0.29 g EDTA per 1 liter 1xPBS
EDTA-Buffer:	100 ml 10xPBS 10 ml Fetal bovine serum 40 µl 0,5M EDTA add Ampuwa to 1 liter
FACS-Buffer- BSA:	500 ml 1xPBS 25 ml Fetal bovine serum (filter through a 70 µm cell strainer before use) 2.5 g Albumin, Bovine, Fraction V 3.5 ml NaN_3
Picrosirius red:	12 g Picric acid dissolve in 1 liter Ampuwa, mixing strongly 0.5 g Sirius Red dissolve in 500 ml Picric acid solution, mixing strongly during 1-4 hours Let ripen the solution 1 week at room temperature in the dark
DEPC-water:	2 ml Diethylpyrocarbonat 2 l Ampuwa Mix for at least 6 hours with magnetic stirrer and then autoclave
Protein isolation buffer pro 1 ml:	40 µl 20mM, pH7.4 HEPES 20 µl 2mM EGTA 100 µl 1mM DTT 50 µl Sodium orthovanadate 10 µl Triton X-100 100 µl Glycerol 0.2 µl 2 µM Leupeptin 4 µl 0.4 mM PMSF 20 µl 10 µg/ml Aprotinin 2 µl 2 µM Microcystin 0.8 µl E-64 100 µl 50 µM ß-Glycerolphosphate The buffer must be prepared directly before usage

8%-SDS-polyacrylamide separating gel pro 20 ml:	9.3 ml Aqua distillate 5.3 ml 30% Acrylamide 5.0 ml 1.5 M Tris pH 8.0 0.2 ml 10% SDS 0.2 10% APS 0.012 µl TEMED
5%-SDS-polyacrilamide stacking gel pro 20 ml:	6.8 ml Aqua distillate 1.66 ml 30% Acrylamide 1.26 ml 1.0M Tris pH 8.0 0.1 ml 10% SDS 0.1 10% APS 0.01 µl TEMED
Loading Buffer for Western Blotting stocking solution:	2.0 ml Glycerol 2.0 ml 10% SDS 0.25 mg Bromphenol blue 2.5 ml Stacking gel puffer 9.5 ml Ampuwa Before usage add to 950 ml of the loading buffer 50 µl of β-Mercaptoethanol
Stacking gel puffer: (pH 6.8)	6.06 g Tris 4.0 ml 10% SDS per 100 ml Ampuwa
10x Electrophoresis buffer:	720 g Glycine 151.5 g Tris 50 g SDS 5 l Aqua distillate Dilute 1:10 before usage with Aqua distillate
Transfer buffer pro 1 l: (pH 8.3)	2.9 g Tris 14.5 g Glycine 200 ml Methanol Add aqua distillate to 1 liter

5.1.7. Instruments

Analytical balance ALC-110.4; Acculab Sartorius group, Germany
Blood Pressure Analysis System BP-2000; Visitech Systems, USA
Cell counter; The Denominator Company, USA
Cell incubator; Heraeus, Germany
Cell strainer, 70 µm; BD Bioscience, USA
Cell strainer, 100 µm; BD Bioscience, USA
Centrifuge 5415C; Eppendorf, Germany
Centrifuge; Heraeus, Germany
Confocal microscope unit QLC100; VisiTech, UK
Counting chamber Neubauer improved, depth 0.1 mm; Roth, Germany
Cover Glass; WVR, Germany
FACS Calibur; BD Bioscience, Germany
FACS tubes; BD Bioscience, Germany

Filter paper; Bio-Rad Laboratories, Germany
Film processor; Curix 60, Agfa-Gevaert AG, Germany
Fluorescent microspheres; Invitrogen, Germany
Hellendahl staining troughs; Roth, Germany
Homogenizer; B-Braun Melsungen AG, Germany
Hot plate; Labotech, Germany
Hybridisation oven Herahybrid 6; Heraeus, Germany
Ice machine MF22; Scotsman Ice Systems, USA
Improved Neubauer chamber; Roth, Germany
Inverted microscope; Olympus, Japan
Laminar-flow hood; Heraeus, Germany
Magnetic stirrer Ikamag RCT; Ika-Labortechnik, Germany
Microcentrifuge tubes 0.5; 1.5; 2.0 ml; Greiner bio-one, Germany
Micropipettes; Eppendorf, Germany
Microscope Nikon E600; Nikon, Japan
Microtom; Leica, Germany
Microwave oven; Sharp, Germany
Migration Assay Cell Culture Inserts for 24-well plates, 8.0 µm; BD Bioscience, USA
Millar Mikro-Tip 1.4 Fr Pressure Transducer Catheters; Millar instruments, USA
Mini-PROTEAN II Electrophoresis Cell; Bio-Rad Laboratories, Germany
Needles for injections; BD Drogheda, Ireland
Nitrocellulose cell impermeable filters; Millipore, Germany
Nitrocellulose transfer membrane 0.2 µm pore size; Whatman, Germany
Optical adhesive film PCR compatible; Applied Biosystems, USA
pH-meter 526; WTW, Germany
Pipettes tips; Greiner bio-one, Germany
Polysine microscope slides; Menzel-Glaser, Germany
Polyvinyl alcohol sponge; Rippey, Germany
PP-Test tubes, 15 ml; Greiner bio-one, Germany
PP-Test tubes, 50 ml; Greiner bio-one, Germany
Precesion balance VIC-212; Acculab Sartorius group, Germany
Prolene 7-0; Ethicon, Germany
Refrigerator; Liebherr, Germany
Rotilabo X-ray cassette; Roth, Germany
Serological pipettes for cell cultures; Sarstedt, Germany
Shaking platform Polynax 1040; Heidolph, Germany
Slide boxes; Roth, Germany
Stericup Vacuum driven disposable filtration system; Millipore, Germany
Surgical instruments for aortic banding; B-Braun, Germany
Syringe; Dispomed, Germany
TaqMan Real-Time PCR system AB Step One Plus; Applied Biosystems, USA
Thermomixer comfort; Eppendorf, Germany
Thermostat; Heraeus, Germany
Tissue culture dishes; TRP, Switzerland
TC-plates 6-well culture; Greiner bio-one, Germany
Trans-Blot SD semi-dry transfer cell; Bio-Rad Laboratories, Germany
UV/Vis Spectrophotometer DU 730, Beckman Coulter, USA
Volume-cycled rodent ventilator; Harvard Apparatus, USA
Vortex Mixer; neoLab, Germany
Water bath; GFL, Germany
96-Well Optical reaction plate (0.1 ml) MicroAmp; Applied Biosystems, USA
24-Well cell culture plate; Greiner bio-one, Germany

X-ray film; GE Healthcare, USA

5.1.8. Computer software

Lab Works; UVP Inc., Canada
Lucia G Software, version 4.81; Nikon, Japan

5.2. Methods

5.2.1. Experimental groups

According to the aim of the study to investigate short-term and long-term effects of ramipril on heart remodeling under increased afterload conditions two types of experiments were performed: transverse aortic constriction for 24h (1-day TAC) and for 5 weeks (5-week TAC). All animals were separated into four groups: two groups received ramipril in their diet (TAC+RAM and SHAM+RAM) and the other two groups (TAC and SHAM) received vehicle. Ramipril (5 mg per body weight per day) or vehicle administration started 3 days before surgery and continued until the end of each set of experiments. To study the potential role of bone marrow-derived progenitor cells in heart regeneration under the effect of ramipril, transplantation of GFP-expressing bone marrow cells into wild type mice was performed. Just as the other animals they were separated into four groups (Table 3).

Table 3 Number of mice in the experimental groups

Groups	Ramipril		Placebo	
	SHAM	TAC	SHAM	TAC
WT 5-week experiment	8	25	19	23
WT 1-day experiment	4	5	9	6
WT 5 week-experiment with GFP bone marrow transplantation	8	10	19	31
WT 5-week experiment with Tie-2-GFP bone marrow transplantation	3	3	4	9

5.2.2. Transverse aortic constriction

For transverse aortic constriction animals were anaesthetized with ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg, i.p.). After orotracheal intubation using a 20-gauge catheter, the tube was connected to a volume-cycled rodent ventilator (Harvard Apparatus, USA) on supplemental oxygen with a tidal volume of 0.2 ml and a respiratory rate of 110 per min. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision and aortic constriction was performed by tying a 7-0 nylon suture ligature against a 27-gauge needle (Prolene; Ethicon, Germany) to yield a narrowing 360 μm in diameter and a

transverse aortic constriction of 65–70%. Sham operation was identical except that the aorta was not ligated. At the end of each set of experiments (after 24 h and after 5 weeks) animals were anaesthetized as it was described above and LV pressure was measured by 1.4 Fr pressure-transducing catheter (Millar instruments, USA). Immediately after measurements hearts were rapidly excised and partly snap frozen, partly fixed in PBS-buffered 4% formalin followed by embedding in paraffin 48 h later. Blood and bone marrow were sampled, as well as the spleen for further preparation.

5.2.3. Bone marrow transplantation

Six-week-old C57Bl/6-Tg (ACTbEGFP)1Osb and six-week-old Tg(Tie-2GFP)287Sato mice (Jackson laboratory) were killed and marrow was obtained from the long bones. Unfractionated GFP-expressing bone marrow cells (1-2x10^7) were transplanted into ten-week-old, male, lethally irradiated (total dose 9 Gy) wild-type recipient mice (C57/Bl6 and FVB/N respectively), by injection into the tail vein 5 hours after irradiation. Four weeks after bone marrow transplantation, transaortic constriction or sham operation was performed as described above.

5.2.4. Fluorescence-Activated Cell Sorter Analysis

Blood and bone marrow from at least 13 respectively 8 animals per group were analyzed. The viable lymphocyte population was analyzed for stem cell antigen 1 (Sca-1) and vascular endothelial growth factor receptor 2 (VEGFR-2). Isotype-identical antibodies served as controls in every experiment. All incubations at +4°C were performed in the dark in a refrigerator.

5.2.4.1. FACS-Immunostaining of peripheral blood cells

In FACS-tubes (BD Bioscience, Germany) with 100 µl of EDTA-blood were added 3 ml of Lysing Buffer diluted 1:10 in Ampuwa to destroy erythrocytes. The tubes were vortexed and then left for 10 min at RT, followed by centrifugation at 1000 rpm for 10 min. To prevent unspecific binding of antibodies the pellet was resuspended in 100 µl of FACS-Buffer-BSA with 10 µl of Fc-Block and incubated at +4°C for 10 min. After incubation 10 µl of antibody were added to each tube. Then the tubes were vortexed and placed at +4°C for 30 min. To wash the cells 2 ml of FACS-Buffer-BSA were added to the suspension after which the tubes were centrifuged at 1750 rpm for 10 min. This procedure was repeated twice. After removal of the supernatant, the pellet was resuspended in 0.2 ml of 1xPBS by vortexing. The resulting cell suspension was examined by flow cytometry.

5.2.4.2. FACS-Immunostaining of bone marrow cells

To obtain bone marrow cells mouse femur and tibia were flushed out several times with 5 ml of FACS-Buffer-BSA using syringe with needle. The resulting cell suspension was filtered through a 100 µm cell strainer (BD Bioscience, USA) in 15 ml conical tubes (PP-Test tubes; Greiner bio-one, Germany), and afterwards centrifuged at 1000 rpm, +4°C for 10 min with brakes switched off. Supernatant was aspirated with care because of the high vulnerability of bone marrow cells. Once 1 ml of Lysing Buffer was added to the pellet, the tubes were placed on ice. After the lapse of 5 minutes cell-washing step was performed. For this purpose 2 ml of FACS-Buffer-BSA were added to the suspension and the cells were spun down at 1000 rpm, +4°C for 10 min. The pellet was resuspended in 1 ml of FACS-Buffer-BSA. 50 µl of the cell suspension were transferred in FACS-tubes and mixed with 10 µl of Fc-Block, after which the tubes were placed at +4°C for 10 min. Then 10 µl of antibody were added to the mixture, the tubes were vortexed and placed at +4°C for 30 min. After incubation the cell-washing step was carried out twice. The pellet was resuspended in 0.5 ml of 1xPBS whereupon the resulting cell suspension was examined by flow cytometry (FACS Calibur; BD Bioscience, Germany).

5.2.5. Migration assay of spleen-derived EPC´s

In mice, the spleen functions as a hematopoietic organ, therefore EPC´s could be isolated from there for determination of their migration capacity by a modified Boyden chamber (Migration Assay Cell Culture Inserts for 24-well plates, 8.0 µm; BD Bioscience, USA).

The spleen cells from at least 6 animals per group were resuspended in the EDTA-buffer filtered in 50ml conical tubes (PP-Test tubes; Greiner bio-one, Germany) through 70 µm cell strainer (BD Bioscience, USA), whereupon they were layered slowly on 15 ml ficoll (Biochrom, Germany) in 50ml conical tubes. To isolate spleen mononuclear cells single-step gradient method was used (121). To do this the tubes were centrifuged at 2400 rpm, at +25°C for 20 min, with brakes switched off, then the cell suspension from the interface was collected with a serological pipette, transferred in 50 ml conical tubes, diluted with 1xPBS to 40 ml and spun down at 2400 rpm for 10 min. The obtained pellet was resuspended in 5 ml of EBM and transferred into 6-well culture plates (Greiner bio-one, Germany) which were placed in a gas-controlled cell incubator (Heraeus, Germany) with water vapor saturated air containing 5% CO_2 at +37°C.

After 4 days in culture, the cells were trypsinized (500 µl of EBM with trypsin and 500 µl of 1mM EDTA per well) for 5 min in the cell incubator, whereupon they were transferred into 2.0 ml microcentrifuge tubes (Greiner bio-one, Germany) filled with 1.0 ml EBM to inactivate

trypsin and then spun down at 2000 rpm for 10 min. Modified Boyden chambers were placed in the wells of 24-well culture plates (Greiner bio-one, Germany) filled with 750 µl EBM, contained 10 µl SDF-1. After the centrifugation the pellet of the spleen-derived cells was resuspended in 500 µl EBM. Once the cells were counted, using improved Neubauer chamber (Roth, Germany), 500 cells from each probe were resuspended in 1 ml EBM and transferred into the upper part of a modified Boyden chamber. The 24-well culture plates with the modified Boyden chambers were placed in gas-controlled cell incubator. After 24 hours the undersides of the modified Boyden chambers (filters) were washed two times for 5 min each with 1xPBS, whereupon they were put in the wells containing 300 µl EBM with 5 µl DiLDL and incubated for 2 h in the cell incubator, after which the washing step was repeated. The filters were cut out with a scalpel, placed on slides, embedded in Vectashield and covered with glass coverslips. The whole filter was analyzed by fluorescence microscopy, using a filter for TRITC. All red fluorescent cells were held for positive and counted manually.

5.2.6. Disc angiogenesis model

A disc of polyvinyl alcohol sponge (Rippey, Germany 8 mm), covered with nitrocellulose cell-impermeable filters (Millipore, Germany), was used allowing capillaries to grow only through the rim of the disc. The discs were implanted subcutaneously at the time of TAC or SHAM surgery. After 5 weeks, space-filling fluorescent microspheres (0.2 µm; Invitrogen, Germany) were injected into the left ventricle to deliver them to the systemic microvasculature. The area of the disc invested by fibrovascular growth was assessed with Lucia Measurement version 4.6 software.

5.2.7. Blood pressure measurement

Blood pressure measurement was performed in all animals on the tail artery with the help of a blood pressure analysis system BP-2000 (Visitech Systems, USA) according to manufacturer's instructions during the 5 last days of each set of experiment, using at least 20 repeating measurements per day. The mean of the measurements carried out within 3 last days was taken for further analysis.

5.2.8. Histology

5.2.8.1. Preparation of paraffin sections and deparaffinization

Because of superior preservation of morphology formalin-fixed paraffin-embedded tissue was used. For histological analysis and immunostaining 3µm paraffin sections were cut with a microtome (Leica, Germany) and mounted on poly-L-lysine-coated glass slides (Menzel-Glaser, Germany) after which they were dried at +55°C in a thermostat (Heraeus, Germany) for 2 days. To deparaffinize the specimens the slides were placed onto a hot plate (Labotech, Germany) at +70°C for 30 min and then washed for 3 x 10 min in xylene bathes. Thereafter the sections were successively rehydrated in graded ethanol series (100%; 85%; 70%) and Ampuwa for 5 minutes in each solution.

5.2.8.2. Hematoxylin-eosin staining

The measurement of cardiomyocyte short-axis diameter was performed using sections stained with Ehrlich`s hematoxylin and eosin. For H&E –staining the specimens were treated as follows: first the slides were immersed in hematoxylin-solution for 20 min, washed in Ampuwa for 5 minutes, then they were stained in eosin-solution for 5 min and washed in Ampuwa for 5 min. On staining, the sections were dehydrated in graded ethanol series (70%; 85%; 100%) for 5 min in each solution, cleared in xylene for 2x3 min, embedded in enthellan and covered with cover glasses (WVR, Germany).

5.2.8.3. Picrosirius red staining

The degree of fibrosis was determined by the use of picrosirius red staining. Slides were immersed in picrosirius red-solution for 1 hour at RT, washed in Ampuwa for 5 min. Thereafter the sections were dehydrated, treated with xylene and embedded in enthellan as it was described above. Collagen fibers were stained red, other tissue structures had yellow color. Cardiac fibrosis was quantitated morphometrically as fractional area of collagen content in percentage of myocardial content.

5.2.9. Immunostaining

5.2.9.1. Basic principle

Immunostaining was carried out to identify distinct types of myocardium cells. For this purpose different primary antibodies reacting with specific antigens of investigated cells (cardiomyocytes, smooth muscle cells, endothelial cells, progenitor cells and proliferating cells) were used. They were in turn detected by secondary antibodies with specificity for antigens of animals in which primary antibodies had been produced. The secondary antibodies were labeled with different markers (fluorochromes, biotin, horse radish peroxidase) according to the immunostaining methods used in our study.

5.2.9.2. Antigen retrieval

To reveal epitopes unmasking was performed using citraconic anhydride buffer. Slides were placed in plastic Hellendahl staining troughs (Roth, Germany) with 0.05% citraconic anhydride solution, pH 7.4 which were put in water bath at +98°C for 1 hour, whereupon they were cooled at room temperature for 30 minutes (98).

5.2.9.3. Staining methods

Because of the distinct immunoreactivity of target antigens different immunochemical staining methods (two-step indirect method for antigens with strong immunoreactivity, streptavidin-biotin method and tyramide-amplification method for antigens with weak immunoreactivity) were employed (60). In all cases sections with primary antibody were incubated in a moist chamber in a refrigerator at +4°C overnight (no less than 12 hours) followed by incubation at +37°C for 2 hours. All incubations with antibodies and reagents at +37°C were performed in a moist chamber in a water bath. Between incubation steps and after the last incubation slides were washed in 1xPBS or 4xSSC with 0.1% Tween 3 times 5 min each. 4xSSC diminishes background staining and non-specific binding of antibodies in problematical cases of immunostaining better then other wash-buffers; in contrast to this 1xPBS allows to obtain good fluorescent signal if antigen immunoreactivity is weak.

5.2.9.4. Two-step indirect method

Two-step indirect method was used to detect alpha sarcomeric actin, alpha smooth muscle actin and GFP. Before application of primary antibody all sections were incubated with 4% BSA in 4xSSC for 10 minutes at +37°C to prevent unspecific binding of antibodies. Thereafter primary

antibody was diluted (Table 1) with 4% BSA in 4xSSC and applied as described above. The next day sections were incubated with fluorochrom-labeled secondary antibody diluted 1:30 with 4xSSC at +37°C for 1 hour.

5.2.9.5. Streptavidin-biotin method

Immunostaining for Ki-67 was carried out by streptavidin-biotin method. The primary antibody was diluted with 1xPBS+0.1% Tween (Table 1). Blocking solution was not used because of the week immunoreactivity of this antigen. Incubation with primary antibody was performed as described above. The next day sections were incubated first with biotin-labeled secondary antibody diluted 1:30 with 1xPBS +0.1% Tween at +37°C for 2 hours and then with TRITC-labeled streptavidin diluted 1:50 for 30 minutes at +37°C.

5.2.9.6. Tyramide–amplification method

Antigens with the weakest immunoreactivity (PECAM-1=CD31, MEF-2, podocalyxin) were detected with the tyramide amplification-method. Primary antibody was diluted with 4xSSC +0.1% Tween and applied as described above. The next day sections were incubated first with blocking solution containing 5% milk with 4xSSC than with peroxidase-labeled secondary antibody diluted with the same solution (1:500 for CD31, podocalyxin and 1:30 for MEF-2) at RT for 30 min, after which slides were washed with 4xSSC +0.1% Tween and incubated in tyramide solution diluted 1:30 with amplification diluent for 10 min at RT. After washing with 4xSSC+0.1% Tween-solution incubation with TRITC-labeled streptavidin was performed as in the streptavidin-biotin method. To stain nuclei the sections were incubated with DAPI for 20 min in a moist chamber at RT, whereupon the slides were immersed in Ampuwa for 5 min. The sections were embedded in Vectashield and covered with cover glasses (WVR, Germany).

5.2.9.7. Control of immunostaining

To exclude false positive and false negative results control of immunostaining was carried out. Control tissue specimens were fixed, processed and embedded in the same manner as the samples from experimental animals. As positive control for proliferation marker Ki-67 sarcoma sections were used. Immunostainings for some antigens were controlled using internal tissue control, which means that examined sections contain both investigated cells and control cells: for alpha-smooth muscle actin smooth muscle cells of coronary arteries, for podocalyxin and CD31 endothelial cells of coronary arteries and for MEF-2 cardiomyocytes.

As negative control tissues were used: tubular epithelial cells of nephrones in kidney sections for alpha-sarcomeric actin, MEF-2 and alpha smooth muscle actin; LV of wild type mice without GFP-transplanted cells for GFP. To exclude cross reaction of the secondary antibody with the tissue antigens, normal (nonimmune) serum from the same species was used as the primary antibody at a protein concentration equivalent to the diluted primary antibody.

5.2.10. Apoptosis detection

Apoptosis detection in heart sections was performed using the ApopTag Peroxidase In Situ Oligo Ligation Kit (Millipore-Chemicon, USA), which unlike terminal transferase-based labeling assay (TUNEL), detects only blunt-ended or having a one base 3' overhang double-stranded breaks of DNA typical for apoptotic but not for necrotic or transiently damaged cells.

The procedure was carried out according to manufacturer's instructions (32). To determine the types of apoptotic cells the heart sections after apoptosis detection were counterstained for alpha-sarcomeric actin, CD31 and GFP. Histological examination of apoptosis indicated by brown 3-3`diaminobenzidine staining was performed by light field microscopy unit of a Nikon E 600 microscope and the type of each apoptotic cell was determined by subsequent switching to the fluorescent unit.

5.2.11. Sections examination and tissue morphometry

Histological evaluation and morphometric analyses were performed on left ventricular tissue sections with the help of a Nikon E600 microscope (Nikon, Japan) and Lucia G Software (version 4.81, Nikon, Japan). The ocular with the sampling grid was used to count numbers of CD31-positive cells, cardiomyocytes, non-cardiomyocytes, capillary profiles and cardiomyocyte profiles.

For determination of mean cardiomyocyte short-axis diameter, the short-axis diameter of at least 100 randomly chosen cross-sectioned cardiomyocytes was measured on photographs of H&E -sections obtained at 400x magnification. In each group at least 7 hearts were examined (1 section per heart).

To evaluate the degree of fibrosis ten fields in the middle third of each LV-section stained with Sirius Red were photographed at 100x magnification whereupon total interstitial fibrillar collagen content was quantified as fractional area of collagen content in percentage of myocardial tissue. In each group at least 7 hearts were examined (1 section per heart).

Sections double stained for CD31 and α-sarcomeric actin were used to determinate numbers of endothelial (CD31-positive) cells, cardiomyocytes (cross-sectioned muscle fibers

having nuclei) and non-cardiomyocytes per mm^2. For this purpose the sampling grid was randomly superimposed 20 times at 1000x magnification and both nuclei positive for endothelial or cardiomyocyte markers and non-stained nuclei lying completely within its boundaries were counted. The cell density was calculated using the formula: cell density (mm^2)=cell number in 20 fields/((0.00153664)*20), where 0.00153664 is the area of the sampling grid at 1000x magnification, 20 is the number of counted fields. Ratio of CD31-positive cells to cardiomyocytes was calculated from the obtained values.

In the same manner were estimated the densities of cross-sectioned capillary and cardiomyocyte profiles (all cross-sectioned muscle fibers with and without nuclei) at 400x magnification in tissue sections double stained for podocalyxin and α-sarcomeric actin. The profile density was calculated using the formula: cell density (mm^2)=cell number in 20 fields/((0.009604)*20), where 0.009604 is the area of the sampling grid at 400x magnification, 20 is the number of counted fields.

For determination of the mean cross-sectional area of capillary, the cross-sectional area of at least 100 randomly chosen cross-sectioned capillaries was measured on photographs of LV -sections double stained for podocalyxin and α-sarcomeric actin obtained at 400x magnification. In each group at least 7 hearts were examined (1 section per heart).

To evaluate the number of Ki-67-positive cardiomyocytes and non-cardiomyocytes, the number of GFP-positive cells, GFP-positive cells with MEF-2-positive nuclei, cells double positive for GFP and CD31, the number of bone marrow-derived Tie-2-positive cells and number of different types of apoptotic cells their respective total number was counted in sections stained for appropriate markers. Thereafter using received values and the section area measured on photographs of H&E -sections obtained at 20x magnification cell density and percentage were calculated. In each group all hearts were examined (1 section per heart). Colocalization of staining (Ki67 and α–sarcomeric actin) was controlled using a confocal microscopy unit (QLC100, VisiTech; United Kingdom on a Nikon E600 microscope).

5.2.12. Analysis of HIF-1α-expression

5.2.12.1. RNA isolation

All manipulations with nucleic acids were performed on ice to prevent their degradation. Total RNA was isolated from the LV according to the manufactures instructions and the protocol of Chomczynski, Sacchi (27) using peqGOLD RNA Pure (Peqlab Biotechnology, Germany). Thereafter DNA digestion was performed by application of DNAse I (Roche, Germany). For this purpose 10 µl of RNA probe were mixed with 6 µl of DEPC-water (DEPC Sigma-Aldrich,

Germany), 2 µl of 10x PCR buffer (Roche, Germany) and incubated for 30 min at +25°C and than at +75°C in Thermomixer comfort (Eppendorf, Germany). The RNA content was measured by UV-spectrophotometry (UV/Vis Spectrophotometer DU 730, Beckman Coulter, USA) at 260 and 280 nm. To do this 1µl of template was mixed with 49 µl of DEPC-water, as negative control 50 µl of DEPC-water were used. Employed for the RNA purity estimation the ratio 260/280 was in all samples at 1.6-1.8. The probes were stored at -80° C.

5.2.12.2. Reverse transcription

Two micrograms of isolated RNA were reverse transcripted with the help of Omniscript RT Kit (Qiagen, Germany). To do this the probes were thawed on ice, vortexed and centrifuged at top speed. Thereafter up to 2 µg RNA were transferred in microcentrifuge tubes, filled up to 10 µl with DEPC-water and mixed with 2 µl 10x Buffer RT, 2 µl dNTP Mix, 2 µl random primer (Roche, Germany), 1 µl RNasin (Promega, USA), 1 µl omniscript RT, 2 µl DEPC-water, whereupon they were incubated for 1 hour at +37°C in water bath, than for 5 min at +93°C in Thermomixer comfort followed by incubation on ice for 5 min. The probes were stored at -80° C.

5.2.12.3. Quantitative RT-PCR

Quantitative RT-PCR was performed with the TaqMan Real-Time PCR system (AB Step One Plus, Applied Biosystems, USA). The TaqMan Gene expression assays containing probes and primers for mouse genes hypoxia-inducible factor-1α (Hif-1α) and 18S ribosomal RNA were assay-on-demand purchased from Applied Biosystems. They had a FAM^{TM} reporter dye at the 5'end of the TaqMan MGB probe and a nonfluorescent quencher at the 3' end of the probe. To prepare reaction mixture, all reagents and samples were defrosted on ice and mixtured in microcentrifuge tubes in the following proportion: 2 µl cDNA, 1.25 µl gene expression assay, 12.5 µl TaqMan gene expression master mix (Applied Biosystems, USA), 9.25 µl sterile water, whereupon the tubes were vortexed and reaction mixture was transferred in the wells of a optical reaction plate (Applied Biosystems, USA). The plate was placed in the AB Step One Plus and quantitative real time polymerase chain reaction was performed according to the 2-step-manufacture protocol: 5 min at +95°C and 40 cycles (15s at +95°C for template denaturation and 1 min at +60°C for annealing of assay components and signal generation). For quantification, mRNA amount of the respective gene was normalized to the amount of 18S rRNA using the $2^{-\Delta\Delta CT}$ method (23).

5.2.13. Analysis of HIF-1α-protein content by Western blotting

5.2.13.1. Protein isolation and measurement of protein concentration

All manipulations with proteins were performed on ice to prevent their degradation. For preparation of proteins, tissue of LV was homogenized, dissolved in lysis buffer, centrifuged for 3 min, 10000 rpm, 4 °C and stored at –80 °C. Protein concentration was measured by Lowry protein assay using detergent compatible kit (DC protein assay, Bio-Rad Laboratories, Germany). For this purpose, 2 µl probe was mixed with 38 µl aqua distillate, 100 µl Reagent A, 800 µl Reagent B, vortexed and then incubated at room temperature for 15 min, whereupon the protein concentration was measured spectrophotometrically at 540 nm (UV/Vis Spectrophotometer DU 730, Beckman Coulter, USA). For negative control the same mixture was used in which 2 µl of probe were replaced with 2 µl of aqua distillate. The isolated proteins were stored at -80°C.

5.2.13.2. Gel electrophoresis and electrophoretic transfer of proteins

To carry out vertical electrophoresis in 8%-SDS-polyacrylamide gel, a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, Germany) was utilized. The gel preparation was performed according to the manufactures instructions based on the method of Laemmli (73). Samples for loading were prepared by mixing an amount of probes containing 300 µg of total protein with half of this amount of loading buffer for western blotting. The samples were incubated for 1 hour at RT, whereupon they were loaded in slots of 8%-SDS-polyacrylamide gel. Protein separation was performed at constant current of 90 V for 3 hours per gel. As protein molecular weight marker Full Range Rainbow recombinant protein molecular weight marker (GE Healthcare, USA) was used. After electrophoresis gel, filter paper (Bio-Rad Laboratories, Germany) and nitrocellulose transfer membrane (0.2 µm pore size, Whatman, Germany) were soaked in transfer puffer. For electrophoretic transfer of proteins from the gel onto a membrane a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Germany) was used and the procedure was assembled in accordance with the attached instruction manual. The transfer ran for 1 hour at 200 mA. Relative protein loading and protein transfer efficiency were evaluated with the help of the Ponçeau S solution for electrophoresis (Serva, Germany), stained for 1 min and then washed with aqua distillate. Thereafter the membrane was scanned and then washed with 1xPBS+0.1%Tween to eliminate the dye.

5.2.13.3. Immunovisualization

All washing steps and incubations with blocking solutions to reduce nonspecific antibody binding and to diminish background were executed on a shaking platform. First the membrane was immersed for 1 hour in 1% Western Blocking Reagent (Roche, Germany) diluted with 1xPBS+0.1% Tween then it was incubated with the primary antibody mouse monoclonal anti-Hif1α (Novus Biologicals, Littleton, CO, USA) and rabbit monoclonal anti-β-tubulin (abcam, UK), diluted in the same blocking solution on shaking platform for 12 hours at +4°C followed by incubation with the same primary antibody dissolved in 1xPBS+0.1% Tween for 3 hours in a hybridization oven at +37°C. After incubation the membrane was washed with 4xSSC+0.1% Tween 3 times ten minutes each and then the procedure was repeated with 4xSSC. After washing the membrane was incubated with blocking solution 5% milk+4xSSC for 1 hour at RT. Incubation with horse radish peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibody (Sigma-Aldrich, Germany) diluted 1:4000 with the same blocking solution was carried out in a hybridization oven at +37°C for 30 min. The washing step was done as described above. For the chemiluminescent detection an enhanced chemiluminescence kit (GE Healthcare, USA) was utilized and the procedure was performed in accordance with the attached instruction manual. Rotilabo X-ray cassette (Roth, Germany) was used to expose the membrane to X-ray film (GE Healthcare, USA), which was developed using film processor (Curix 60, Agfa-Gevaert AG, Germany).

5.2.13.4. Densitometry

The protein bands on developed films were evaluated with the help of the program Lab Works (UVP Inc., Canada). The amount of the HIF-1α was presented in optical density units as percentage of the amount of the control protein β-tubulin.

5.2.14. Statistical Analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed with the help of the program Statistica 6.0. One-way ANOVA with Fisher LSD post hoc test or Kruskal-Wallis test with Mann-Whitney post hoc test were used where applicable. Correlations between investigated parameters were evaluated by Spearman R-test with obligatory control of scatterplot matrix. Values of $p<0.05$ were considered significant.

6. Results

6.1. Hemodynamic data and myocardial hypertrophy

As expected, short- and long-term transverse aortic constriction significantly increased left ventricular systolic pressure (Fig. 1, 2). At the same time, treatment with ramipril did not change this parameter in all experimental groups (Fig. 1, 2). Animals after 5 weeks of aortic ligation exhibited a significant increase in the ratio of heart weight to tibia length, which, however, was substantially reduced by ramipril only in non-transplanted mice (Fig. 3).

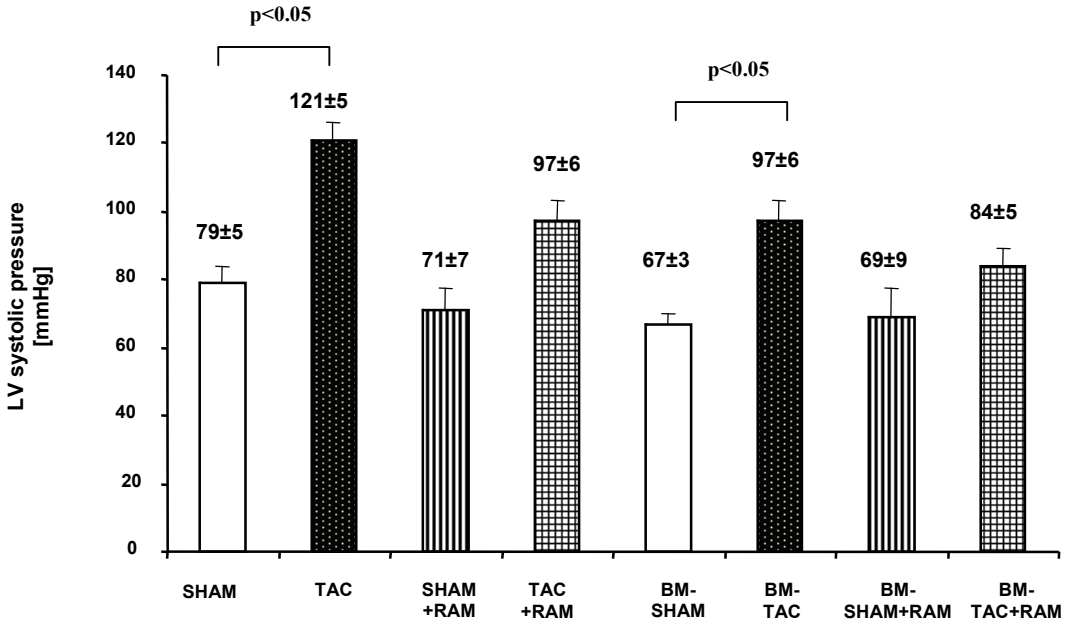


Fig. 1: LV systolic pressure in 5-week experiment. TAC significantly increased left ventricular systolic pressure. Ramipril did not influence this parameter in all groups. SHAM=sham-operated mice; TAC= transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

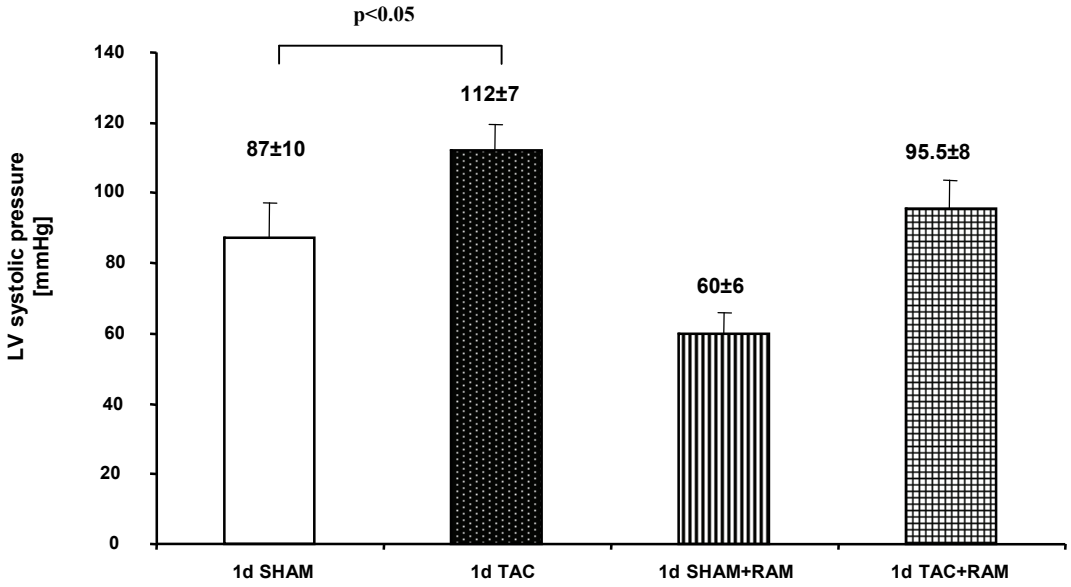


Fig. 2: LV systolic pressure in one-day experiment. TAC significantly increased left ventricular systolic pressure. Ramipril did not influence this parameter in all groups. SHAM=sham-operated mice; TAC= transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

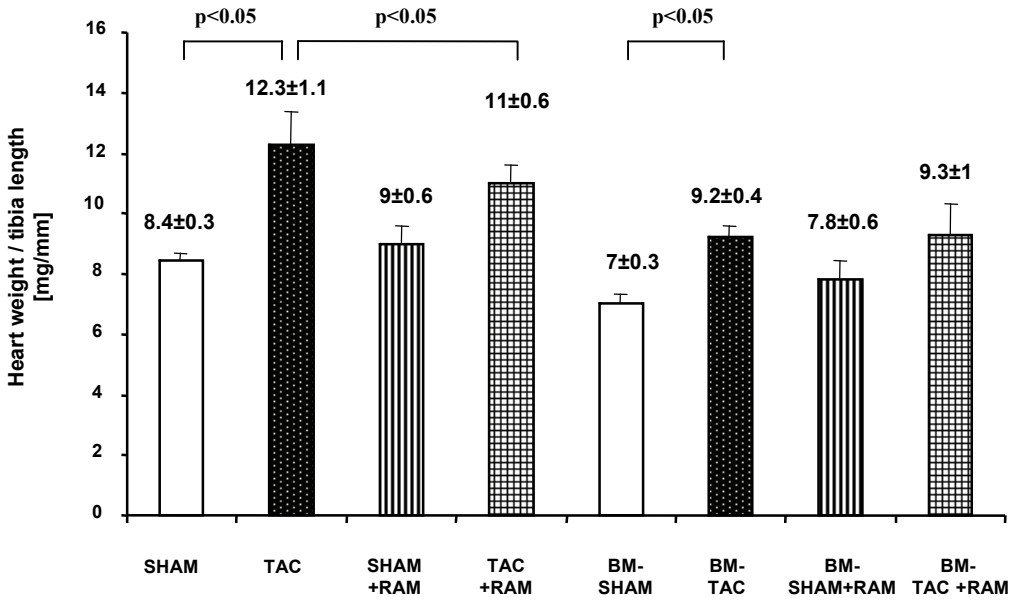


Fig. 3: Heart weight / tibia length in 5-week experiment. TAC significantly increased the ratio, which was reduced by ramipril only in non-transplanted mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

All groups of one-day-operated animals did not differ from each other concerning this ratio: 1d SHAM 5.6±0.8; 1d TAC 7.7±1.1; 1d SHAM+RAM 7.1±0.6; 1d TAC+RAM 8.4±1.1, mg/mm. Aortic ligation, but not ramipril treatment for 5 weeks decreased significantly both systolic and diastolic blood pressure of the tail artery. In mice undergoing BMT and TAC similar effects were not observed. In contrast, significant decrease of systolic blood pressure under the influence of ramipril was seen in bone marrow-transplanted but not in untransplanted TAC mice (Fig. 4, 5).

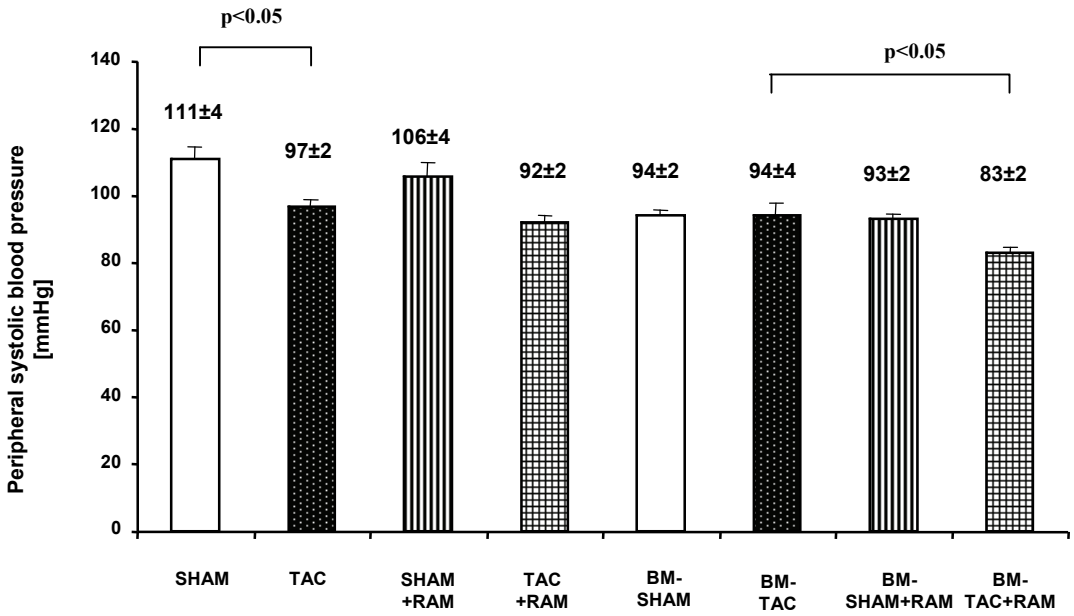


Fig. 4: Peripheral systolic blood pressure in 5-week experiment. TAC significantly decreased the systolic blood pressure only in non-transplanted animals. Ramipril reduced it in bone marrow-transplanted TAC mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

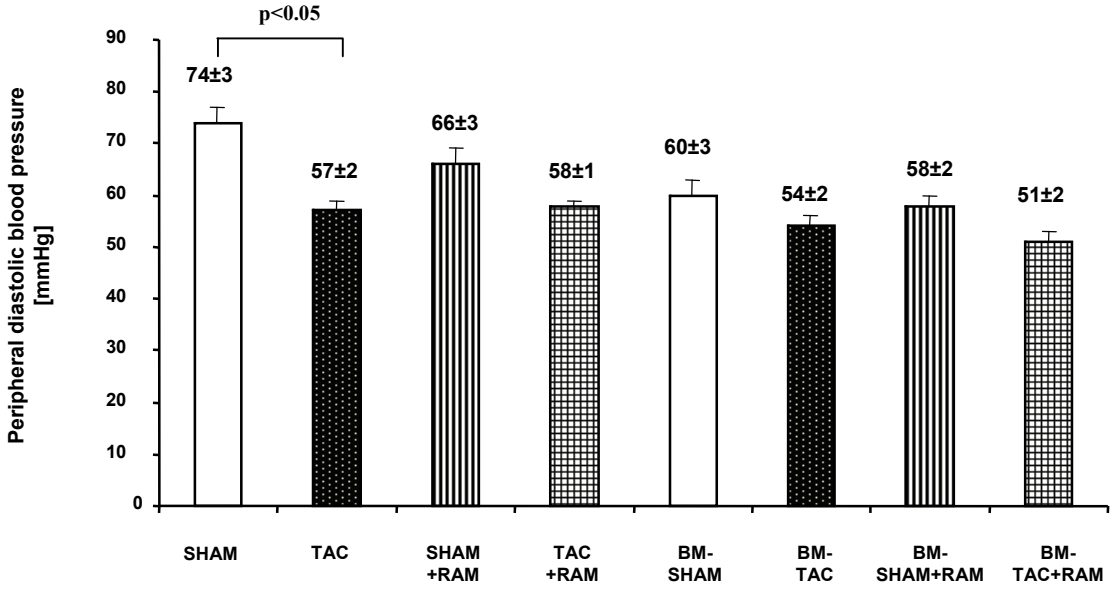


Fig. 5: Peripheral diastolic blood pressure in 5-week experiment. TAC significantly decreased the diastolic blood pressure only in non-transplanted animals. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

6.2. Heart tissue remodeling

6.2.1. Cardiomyocyte hypertrophy

To assess cardiomyocyte hypertrophy the number of cross-sectioned cardiomyocytes per mm^2 and cardiomyocyte short-axis diameter were evaluated (Fig. 7). Five-week TAC diminished the number of cross-sectioned cardiomyocytes per mm^2 and increased cardiomyocyte short-axis diameter compared to sham-operated animals (Fig. 6, 8). Ramipril in 5-week TAC mice significantly reduced cardiomyocyte short-axis diameter and augmented the number of cardiomyocytes per mm^2 (Fig. 6, 8).

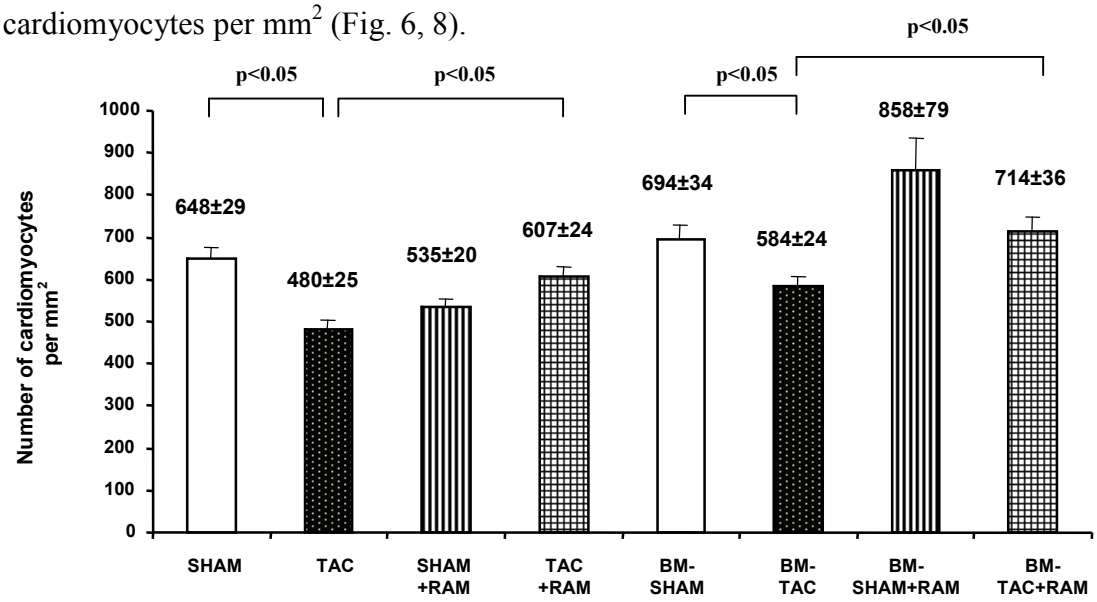


Fig. 6: Cardiomyocyte number per mm^2 in 5-week experiment mice. Five-week TAC diminished the number of cross-sectioned cardiomyocytes per mm^2. Ramipril augmented the number of cardiomyocytes per mm^2 in TAC mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

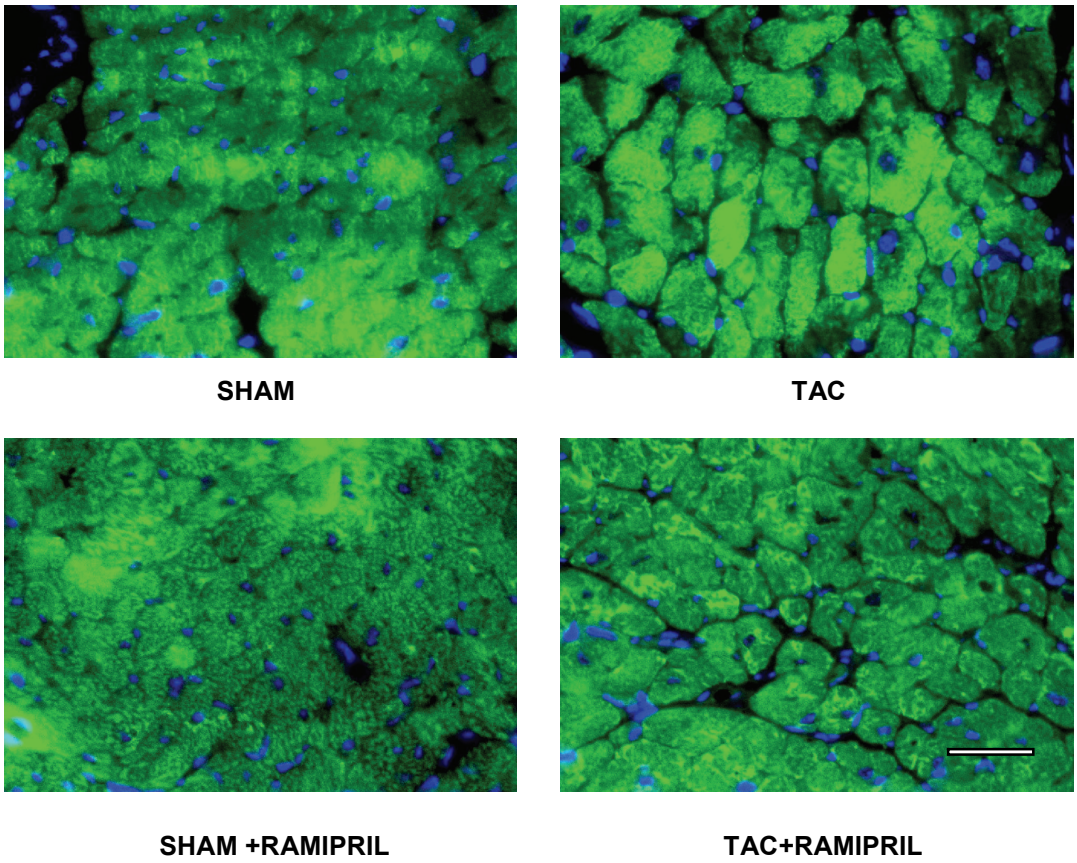


Fig. 7: Cardiomyocytes. Paraffin sections of 5-week experiment mouse hearts. Cardiomyocytes in green for α-sarcomeric actin, nuclei in blue by DAPI-staining. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction.

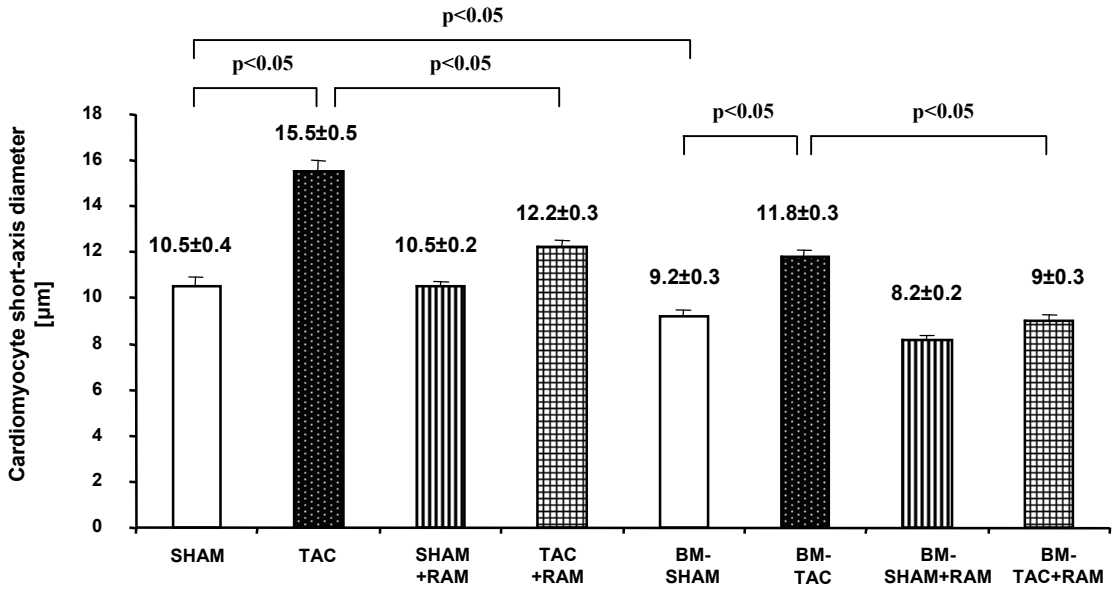


Fig. 8: Cardiomyocyte short-axis diameter in 5-week experiment mice. Five-week TAC increased cardiomyocyte short-axis diameter. Ramipril reduced it in TAC mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

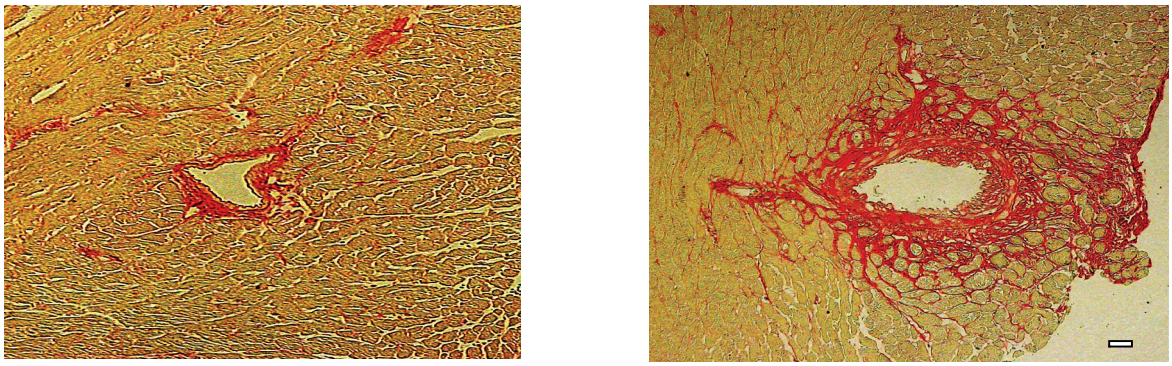
One-day TAC did not influence both parameters (Table 3).

Table 3 Cardiomyocyte number per mm^2 and cardiomyocyte short-axis diameter in one-day-operated mice

Parameters	1d SHAM	1d TAC	1d SHAM +RAM	1d TAC +RAM
Number of cardiomyocytes per mm^2	996±68	1074±75	643±60	638±25
Cardiomyocyte short-axis diameter, µm	10.0±0.6	10.2±0.2	9.7±0.1	9.9±0.2

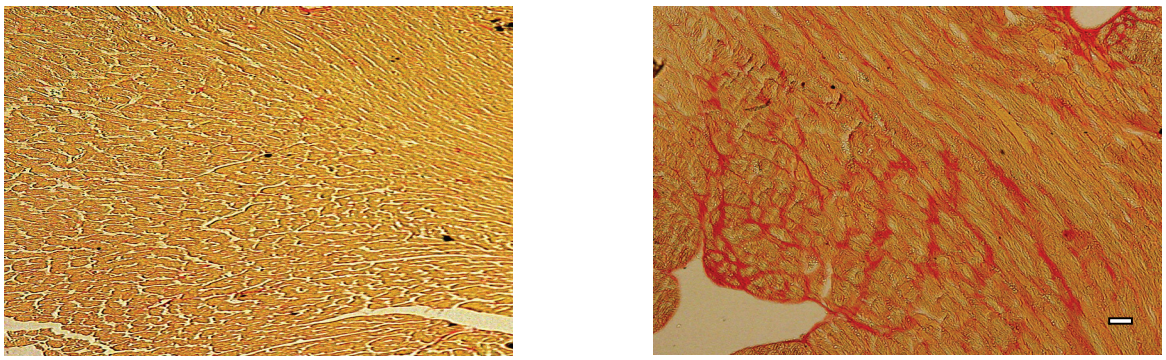
6.2.2. Cardiac fibrosis

Five-week TAC induced perivascular as well as interstitial cardiac fibrosis quantitated morphometrically as fractional area of collagen content as percentage of the myocardial content (Fig. 9, 10). Pressure overload for 5 weeks increased collagen content in the mice without BMT (Fig. 11). In bone marrow-transplanted mice marked cardiac fibrosis was found even in sham-operated animals and 5-week TAC led to its further increasing (Fig. 12).



SHAM **TAC**

Fig. 9: Sirius-Red staining for collagen (red colour). Paraffin sections of 5-week experiment mouse hearts. Perivascular fibrosis in a TAC-mouse. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction.



SHAM **TAC**

Fig. 10: Sirius-Red staining for collagen (red colour). Paraffin sections of 5-week experiment mouse hearts. Interstitial fibrosis in a TAC-mouse. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction.

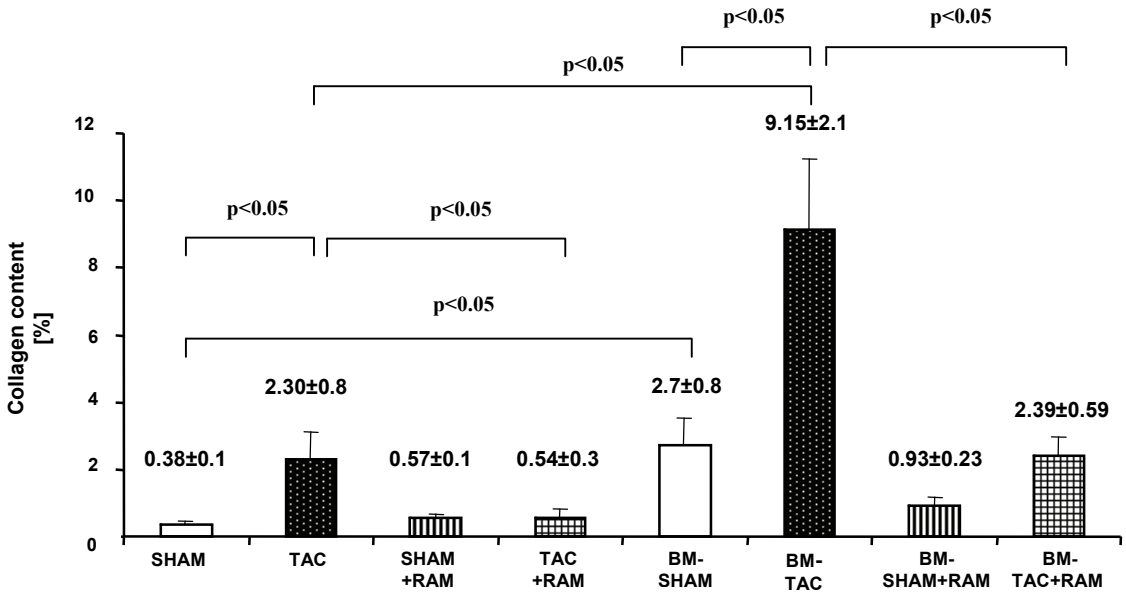


Fig. 11: Collagen content in myocardium of 5-week-operated mice. Pressure overload for 5 weeks increased collagen content in the mice without bone marrow transplantation. In bone marrow-transplanted mice marked cardiac fibrosis was found even in sham-operated animals and 5-week TAC further increased it. Ramipril significantly reduced collagen content in pressure-overloaded myocardium. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

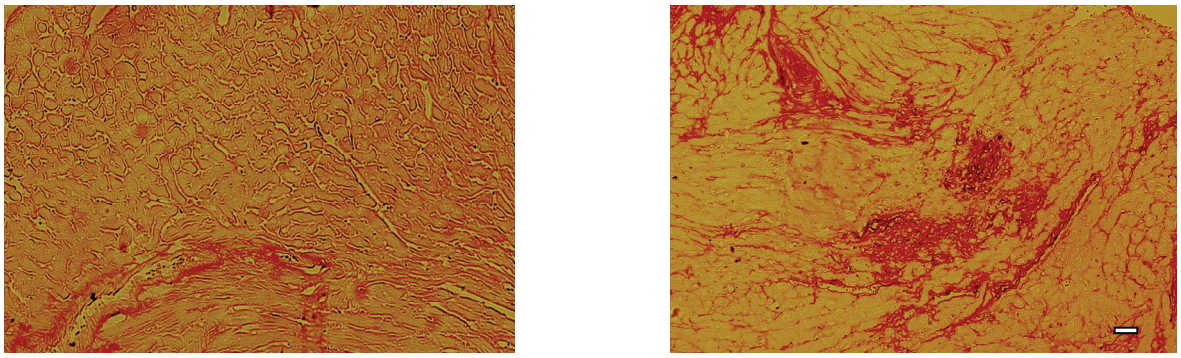


Fig. 12: Sirius-Red staining for collagen (red colour). Paraffin sections of 5-week experiment mouse hearts. Interstitial fibrosis in a BM-SHAM- and BM-TAC-mouse. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice.

In bone marrow-transplanted mice cardiac fibrosis was more pronounced than in other experimental animal. This may be due to the influence of irradiation (Fig. 11, 12). GFP-positive cells lacking both endothelial and myocardial markers massively infiltrated fibrotic areas in bone marrow-transplanted mice (Fig. 13). Ramipril significant reduced collagen content in 5-week TAC mice (Fig. 11, 14).

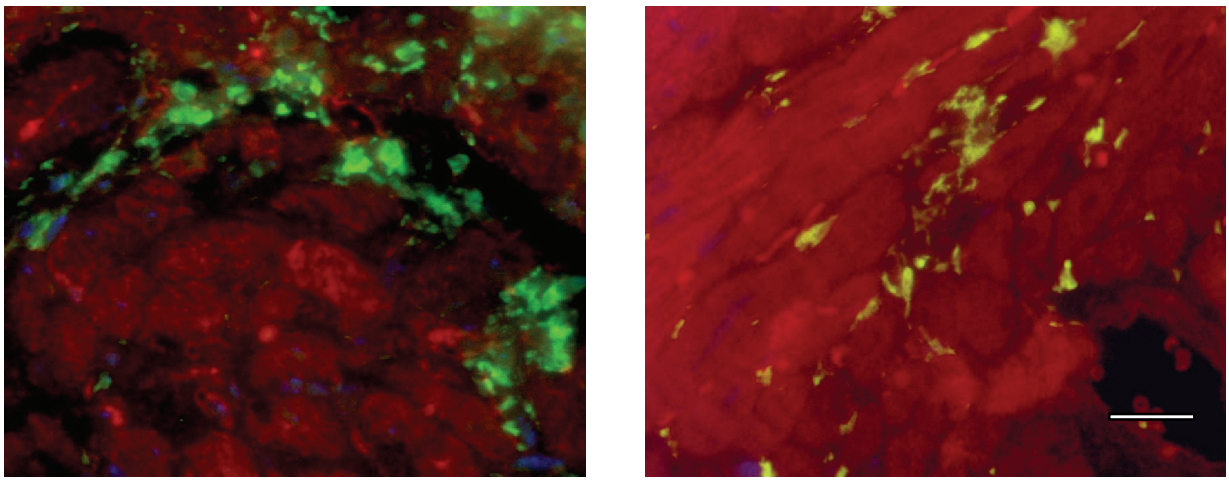
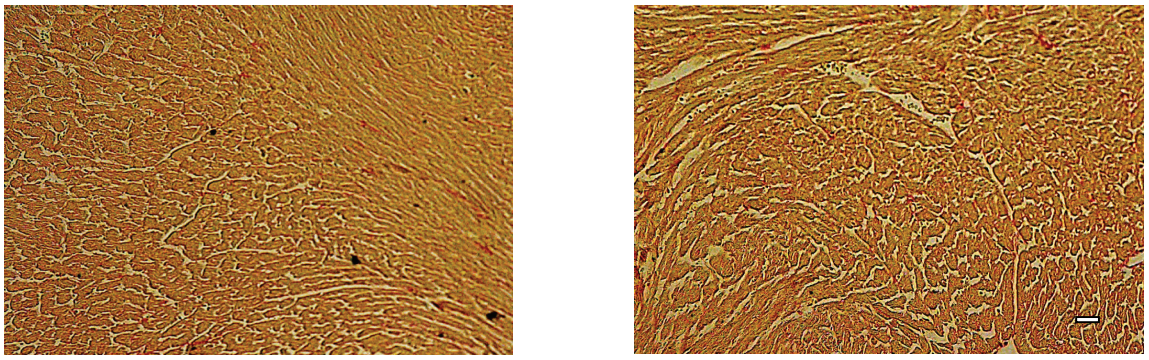


Fig. 13: GFP-positive cells lacking both endothelial and myocardial markers massively infiltrated fibrotic areas in bone marrow-transplanted mice. Paraffin sections of 5-week experiment mouse hearts. GFP in green, cardiomyocytes in red for α-sarcomeric actin, nuclei in blue by DAPI-staining. Bar = 30µm.



SHAM+RAMIPRIL **TAC+RAMIPRIL**

Fig. 14: Sirius-Red staining for collagen (red colour). Paraffin sections of 5-week experiment mouse hearts. Reduced perivascular and interstitial fibrosis in a TAC+RAM mouse. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

6.2.3. Cell turnover in the left ventricular myocardium

6.2.3.1. Cycling cardiac cells

The nuclear protein Ki67 (Fig. 15-17), which is expressed during all stages of the cell cycle excluding G_0[3], was used to measure the number of cardiac cells undergoing cell division. Pressure overload for 5 weeks significantly increased the number of Ki67-positive non-cardiomyocytes and induced synthesis of Ki67 protein in some cardiomyocytes (Fig. 18-21). Some Ki67-positive cardiomyocytes were binucleated (Fig. 17). In the bone marrow-transplanted mice Ki67-expressing cardiomyocytes were found even in sham-operated animals, although the cardiomyocyte short-axis diameter in this group was significantly lower than in

sham mice without BMT (Fig. 20, 21). Treatment with ramipril during 5-week experiment diminished significantly numbers of Ki67–positive cells in all groups (Fig. 18-21).

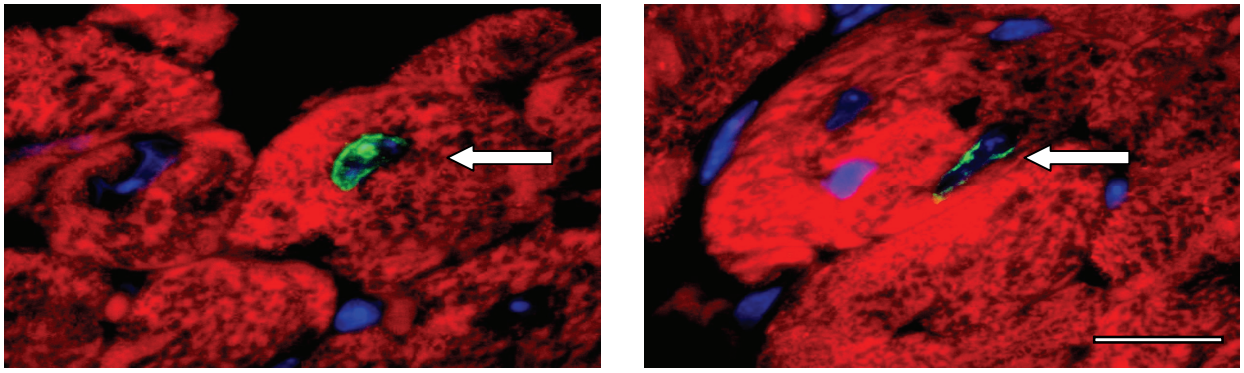


Fig. 15: Ki67-positive cardiomyocytes in TAC mice. Paraffin sections of 5-week experiment mouse hearts. Ki67 in green, cardiomyocytes in red for α-sarcomeric actin, nuclei in blue by DAPI-staining. Bar = 30µm.

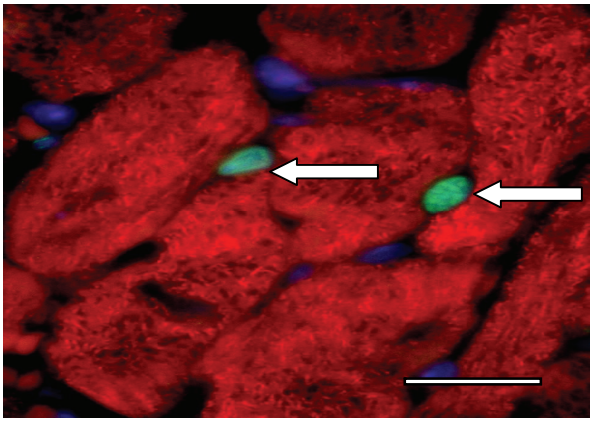


Fig. 16: Ki67-positive non-cardiomyoycytes. Paraffin section of a 5-week experiment mouse heart. Two Ki67-positive non-cardiomyoycytes in a TAC mouse. Ki67 in green, cardiomyocytes in red for α-sarcomeric actin, nuclei in blue by DAPI-staining. Bar = 30µm.

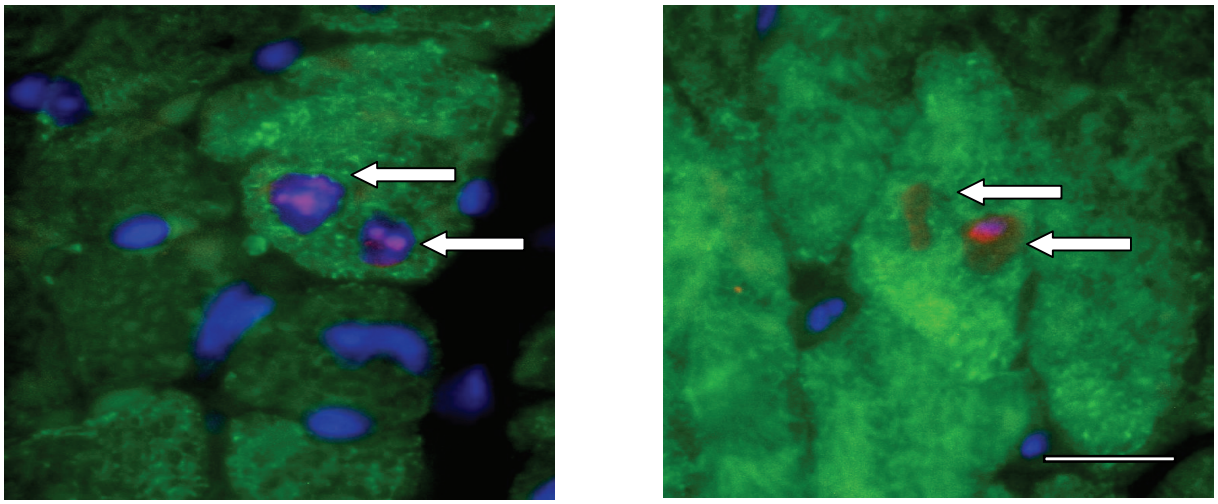


Fig. 17: Binuclear Ki67-positive cardiomyocytes in TAC mice. Paraffin sections of 5-week experiment mouse hearts. Ki67 in red, cardiomyocytes in green for α-sarcomeric actin, nuclei in blue by DAPI-staining. Bar = 30µm

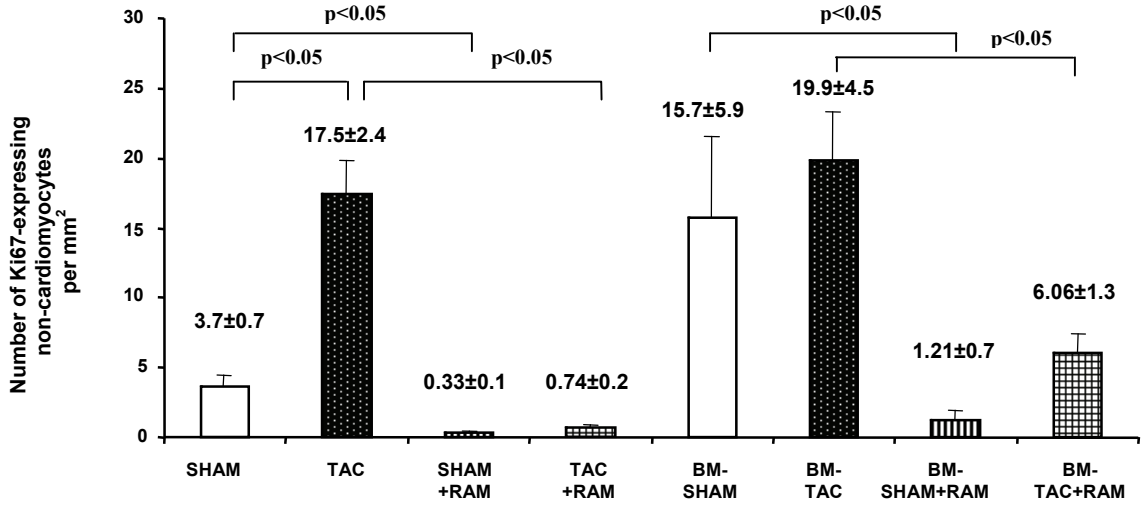


Fig. 18: Number of Ki67-expressing non-cardiomyocytes per mm^2 in 5-week experiment mice. Five-week TAC increased the number of Ki67-positive non-cardiomyocytes per mm^2. Ramipril diminished the number of Ki67-positive non-cardiomyocytes per mm^2 in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

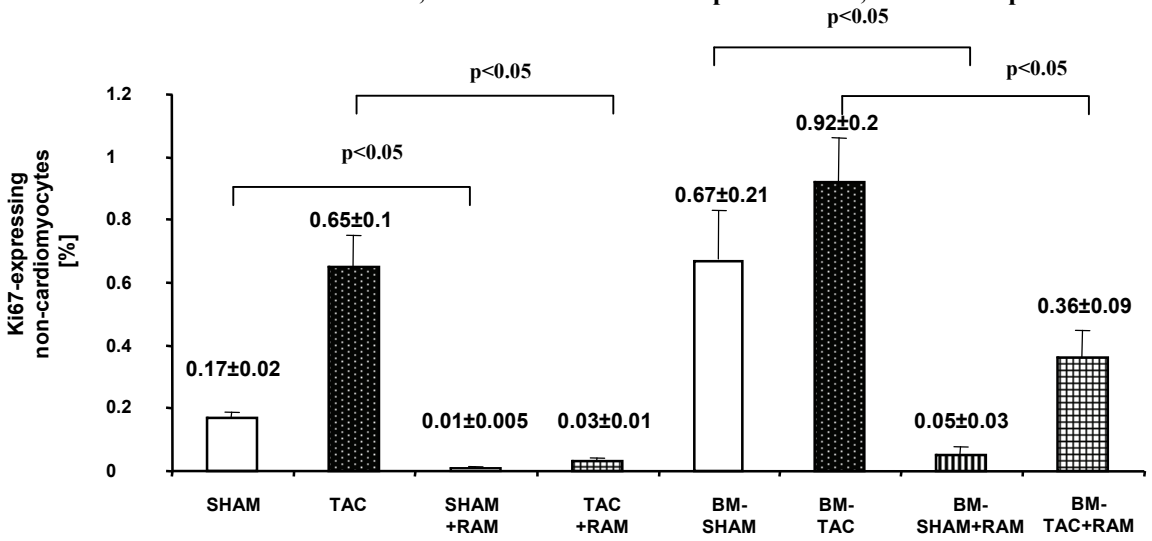


Fig. 19: Percentage of Ki67-expressing non-cardiomyocytes in 5-week experiment mice. Five-week TAC increased the percentage of Ki67-positive non-cardiomyocytes. Ramipril diminished the parameter in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

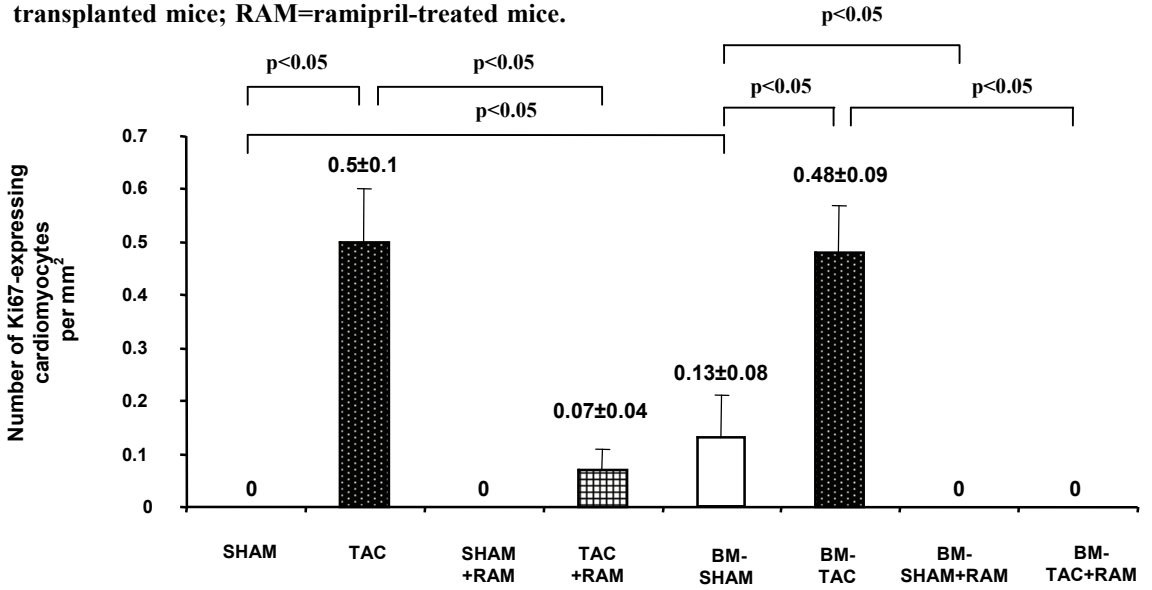


Fig. 20: Number of Ki67-expressing cardiomyocytes per mm^2 in 5-week experiment mice. Five-week TAC increased the number of Ki67-positive cardiomyocytes per mm^2. Ramipril diminished the number of Ki67-positive cardiomyocytes per mm^2 in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

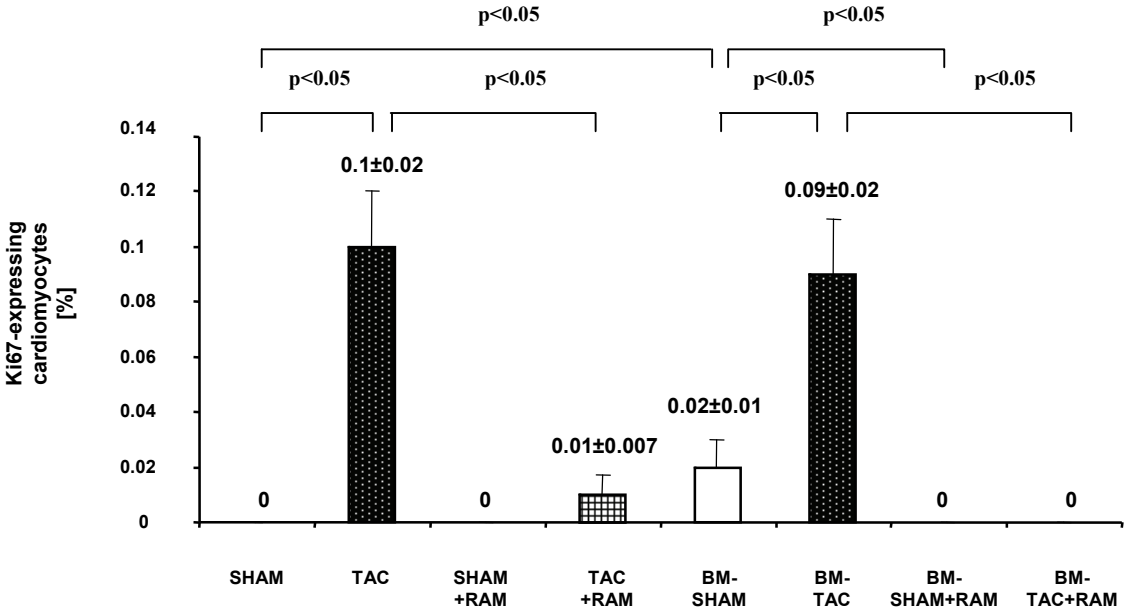


Fig. 21: Percentage of Ki67-expressing cardiomyocytes in 5-week experiment mice. Five-week TAC increased the percentage of Ki67-positive cardiomyocytes. Ramipril diminished the parameter in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

In contrast to the foregoing results, neither TAC nor ramipril influenced significantly the numbers of Ki67 –positive cardiomyocytes and non-cardiomyocytes in one-day-operated mice (Table 4).

Table 4 Ki67-positive cells in myocardium of one-day-operated mice

Parameters	1dSHAM	1dTAC	1dSHAM +RAM	1dTAC +RAM
	Ki-67-expressing non-cardiomyocytes			
Number per mm^2	1.75±1.03	1.46±0.7	1.59±1.7	0.19±0.09
%	0.06±0.03	5.89±3.01	0.057±0.05	0.046±0.02
	Ki-67-expressing cardiomyocytes			
Number per mm^2	0.23±0.15	0.37±0.2	0	0
%	0.06±0.03	0.036±0.02	0	0

6.2.3.2. Apoptosis

Transaortic constriction for 5 weeks in mice without BMT significantly enhanced apoptosis rates of cardiomyocytes, non-cardiomyocytes and $CD31^{pos}$ cells in the heart. In mice that underwent TAC, ramipril substantially decreased the percentage of apoptotic cardiomyocytes and endothelial cells (Fig. 22-26).

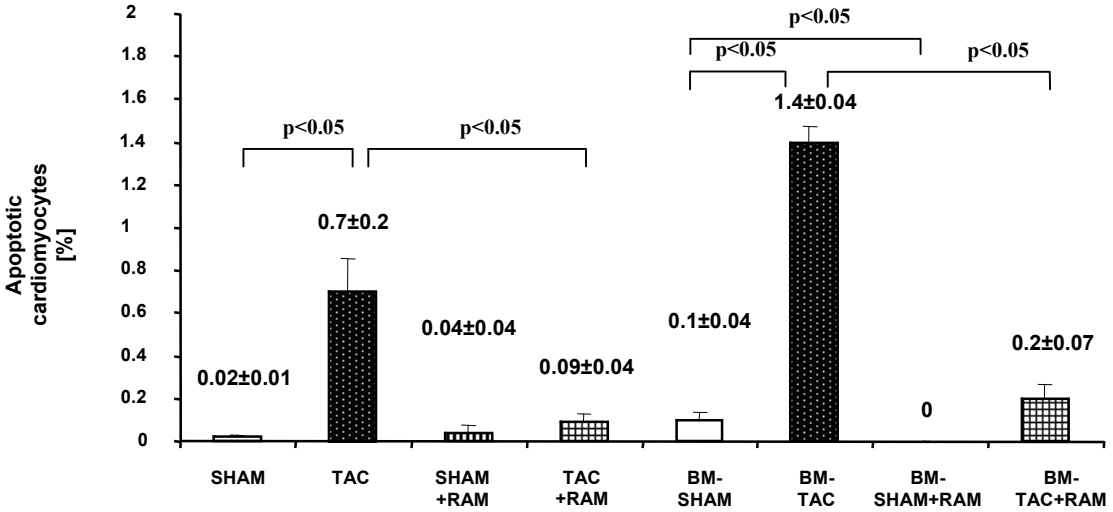


Fig. 22: Percentage of apoptotic cardiomyocytes in 5-week experiment mice. Five-week TAC increased the percentage of apoptotic cardiomyocytes. Ramipril diminished the parameter in TAC mice without BMT and in all groups of bone marrow-transplanted animals. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

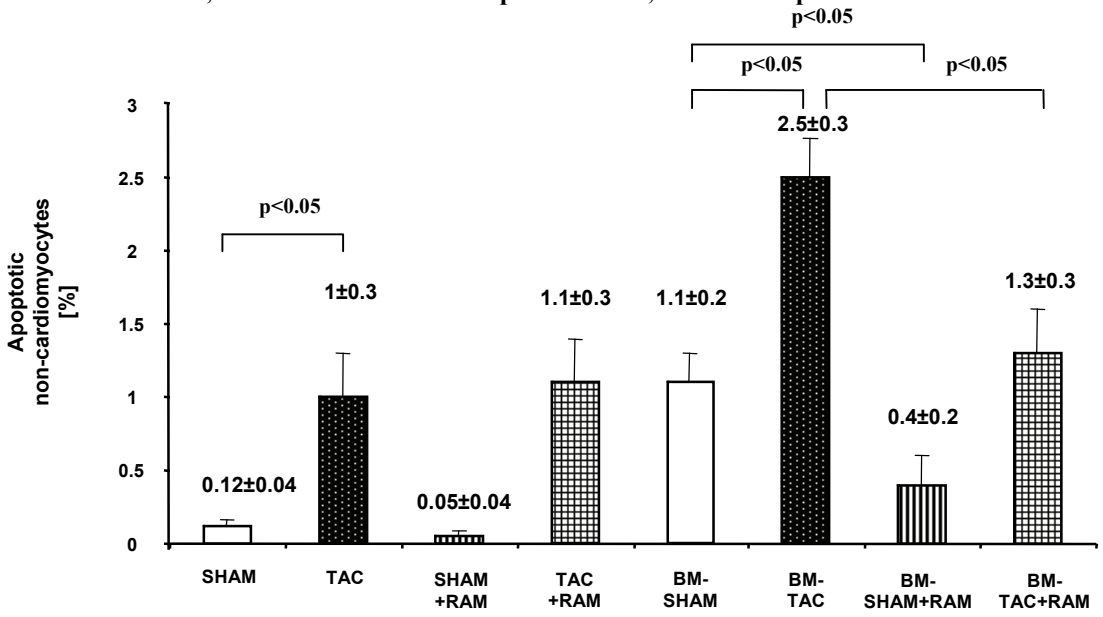


Fig. 23: Percentage of apoptotic non-cardiomyocytes in 5-week experiment mice. Five-week TAC increased the percentage of apoptotic non-cardiomyocytes. Ramipril did not influence the parameter in non-transplanted animals, but significantly reduced it in all groups of bone marrow-transplanted mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

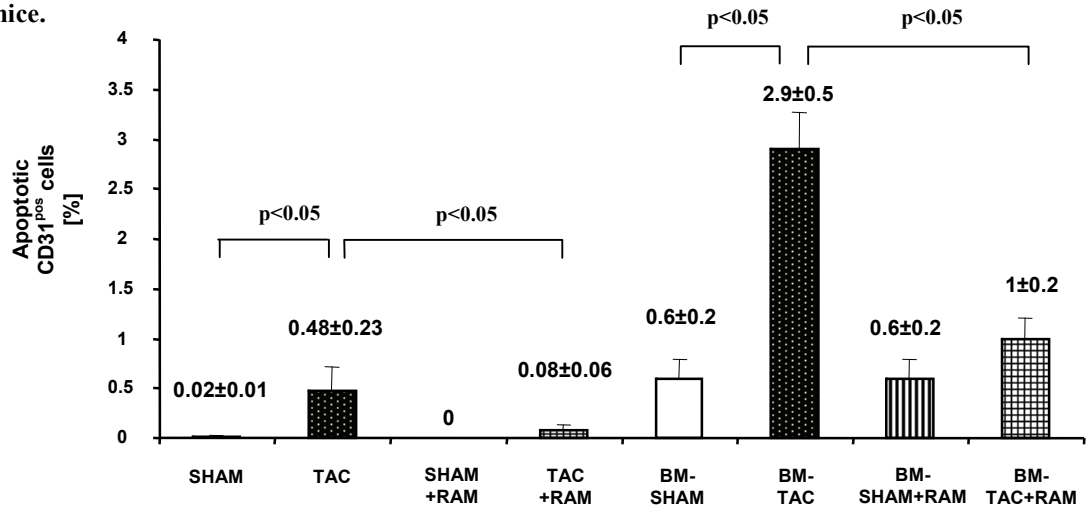
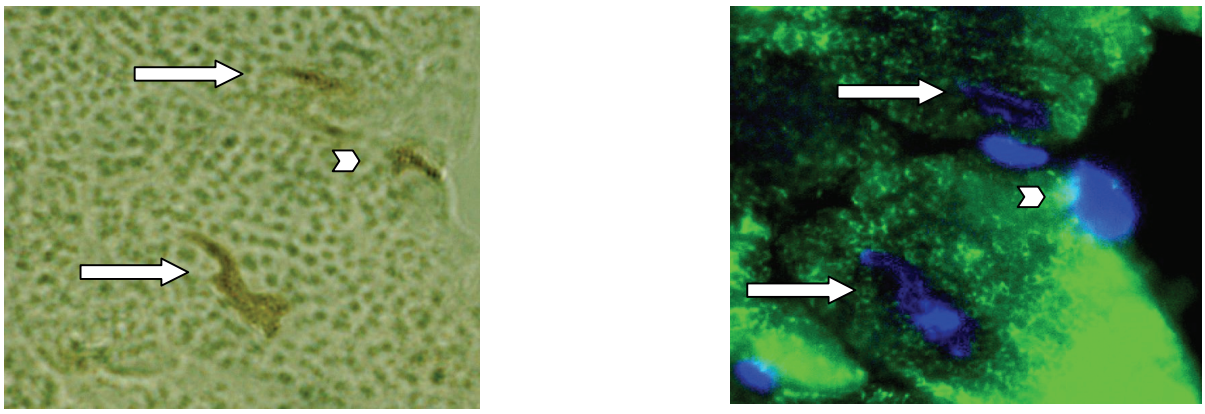
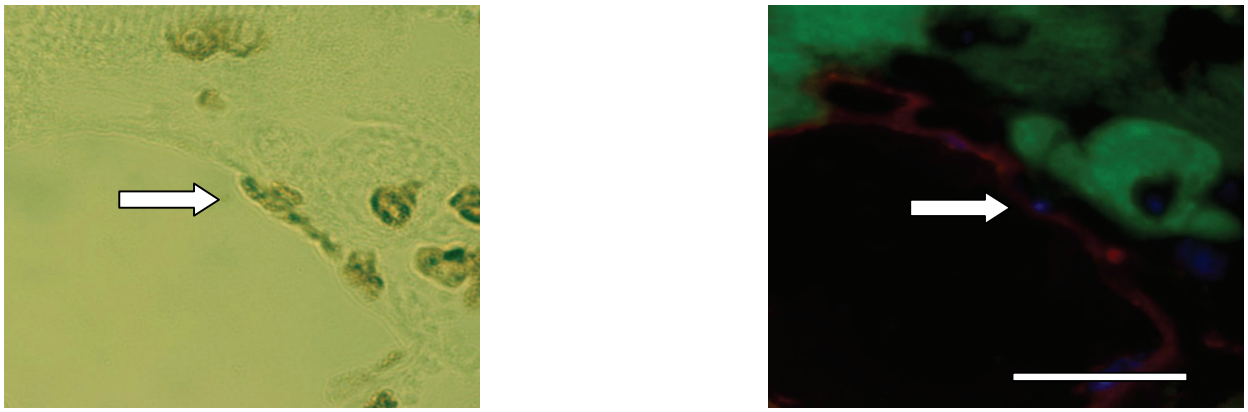


Fig. 24: Percentage of apoptotic CD31^{pos}cells in 5-week experiment mice. Five-week TAC increased the percentage of apoptotic CD31^{pos}cells. Ramipril significantly diminished the parameter in TAC mice with and without bone marrow transplantation. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.



A B

Fig. 25: Apoptotic cells. Paraffin section of a 5-week experiment mouse heart with transverse aortic constriction: (A) shows light microscopic staining for apoptosis (brown), (B) shows fluorescence microscopy for the myocyte marker α–sarcomeric actin (green). Nuclei are stained blue by DAPI. Apoptotic myocytes are marked by arrows, an apoptotic non-myocyte by arrowhead. Bar = 30µm.



A B

Fig. 26: Apoptotic CD31-positive cell. Paraffin section of a 5-week experiment mouse heart with transverse aortic constriction: (A) shows light microscopic staining for apoptosis (brown), (B) shows fluorescence microscopy for the endothelial cell marker CD31 (red). Cardiomyocytes in green for α–sarcomeric actin, nuclei in blue by DAPI. The apoptotic CD31-positive cell is marked by arrow. Bar = 30µm.

Bone marrow transplantation led to increased rates of apoptosis compared to non-transplanted mice, which were further enhanced by TAC. In contrast to mice without BMT, in bone marrow-transplanted animals ramipril significantly decreased the number of apoptotic cells- cardiomyocytes, non-cardiomyocytes and CD31 ^{pos} cells- in all groups (Fig. 22-26). There were no differences in the percentage of GFP ^{pos} cells undergoing apoptosis between the experimental groups: BM-SHAM 32±11.2; BM-TAC 29.1±7.2; BM-SHAM+RAM 33.2±7.8; BM-TAC+RAM 40.9±4.9 %.

Correlation analysis revealed close positive interrelationships between parameters characterizing remodeling of heart tissue and cell turnover parameters in 5-week experiment (Fig. 27).

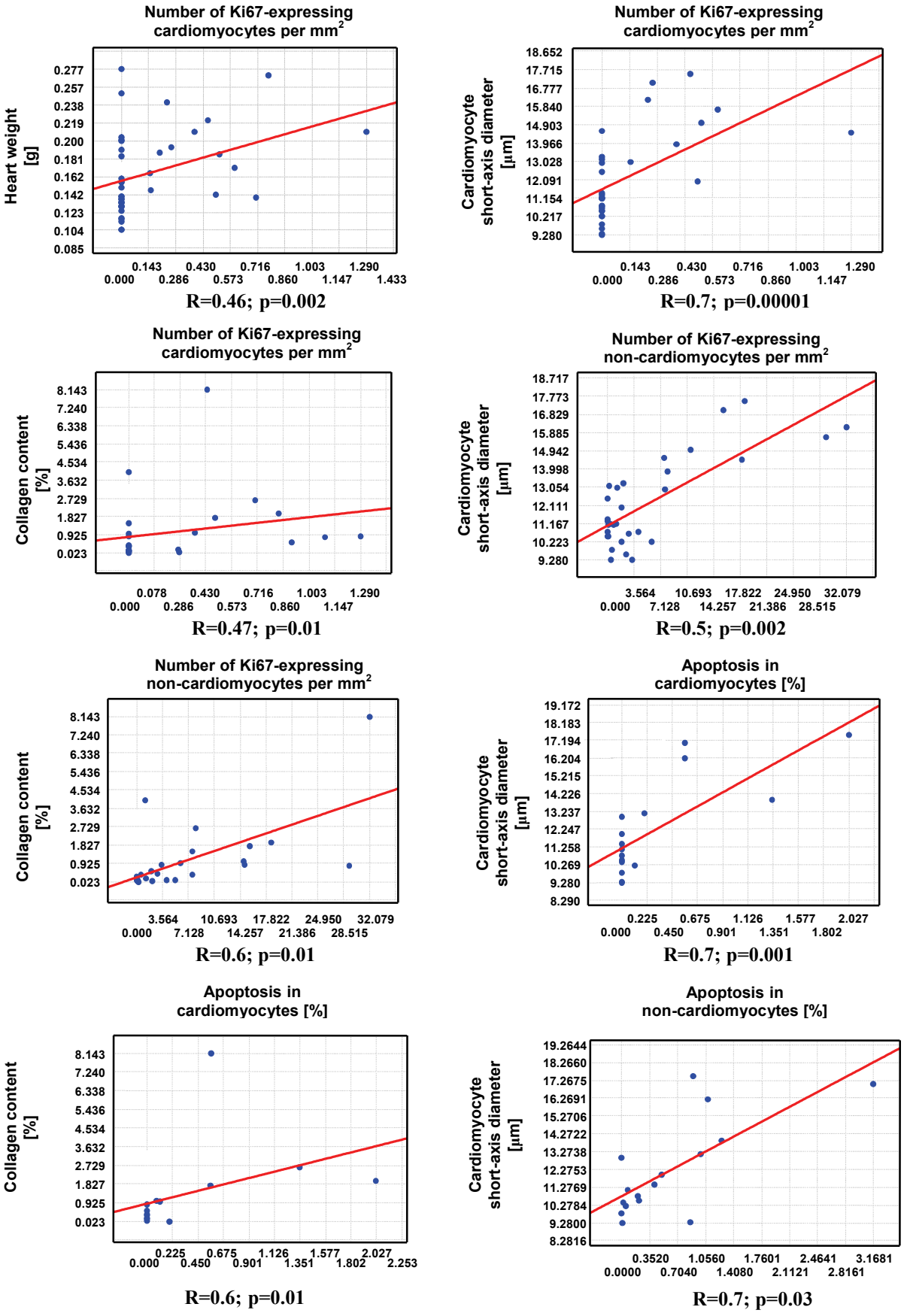


Fig. 27: Correlations between parameters characterizing remodeling of myocardium and cell turnover. Myocardial remodeling in non-transplanted mice (cardiomyocyte hypertrophy and fibrosis) was positive associated with cell turnover (proliferation and apoptosis in cardiomyocytes and non-cardiomyocytes) in myocardium.

6.3. Neoangiogenesis

6.3.1. Extracardiac neoangiogenesis

Transaortic constriction, initiated after implantation of subcutaneous polyvinyl discs (Fig. 28) (n≥5 per group), increased the area of neoangiogenesis as expected by 155±10% compared with sham-operated mice ($p<0.05$), while ramipril even extended this effect in both groups ($p<0.05$, Fig. 29).

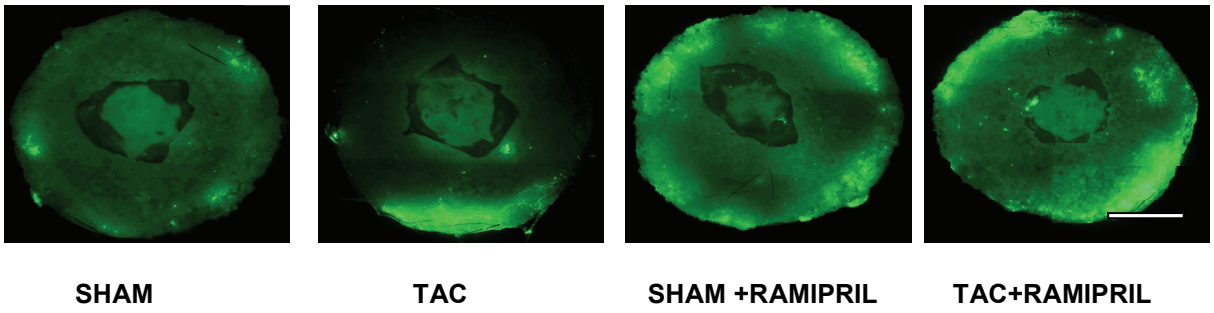


Fig. 28: Newly sprouted vessels in subcutaneously implanted polyvinyl disks after 35 days. Bar = 1mm.

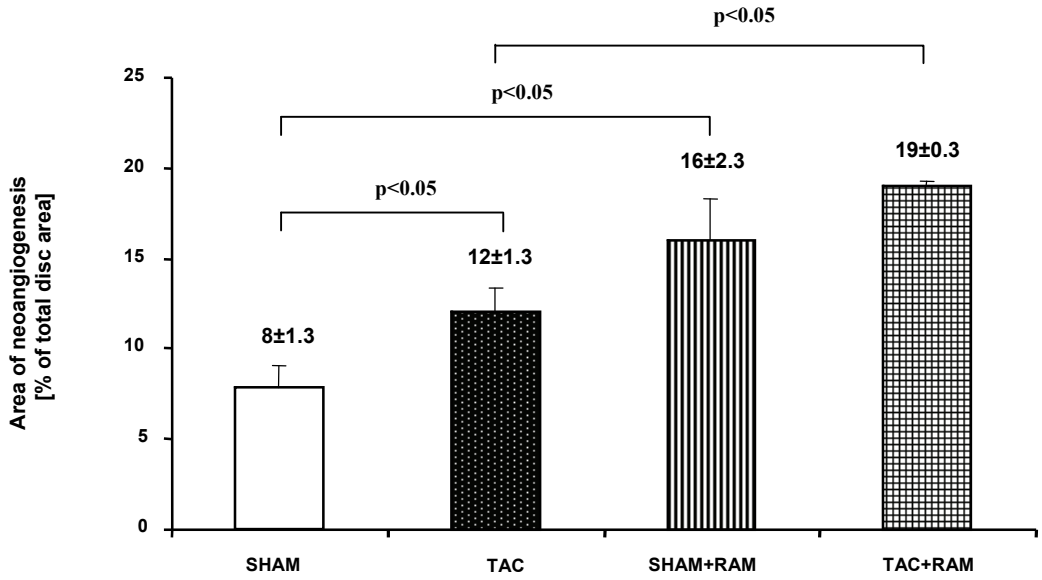


Fig. 29: Neoangiogenesis in implanted subcutaneous polyvinyl discs. TAC stimulated extracardiac capillary growth, but neoangiogenic effect of ramipril was stronger in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

6.3.2. Endothelial progenitor cells

A large body of research shows that bone marrow-derived EPC´s play a very important role in tissue remodeling and angiogenesis. Thus, the influence of transaortic constriction and treatment with ramipril on EPC mobilization and their migration capacity was investigated.

6.3.2.1. FACS analysis data

The mean EPC number in the blood and in the bone marrow in all experimental groups (Fig. 30, 31) was calculated as percentage of the one in the relevant sham groups.

One-day TAC substantially raised EPC level in the peripheral blood, but not in the bone marrow (Fig. 30). Ramipril treatment for 3 days prior to surgery significantly increased EPC numbers in both groups compared to baseline levels of sham mice (Fig. 30). Hence, contrary to aortic ligation ACE inhibition significantly augmented the number of EPC´s not only in the peripheral blood, but also in the bone marrow.

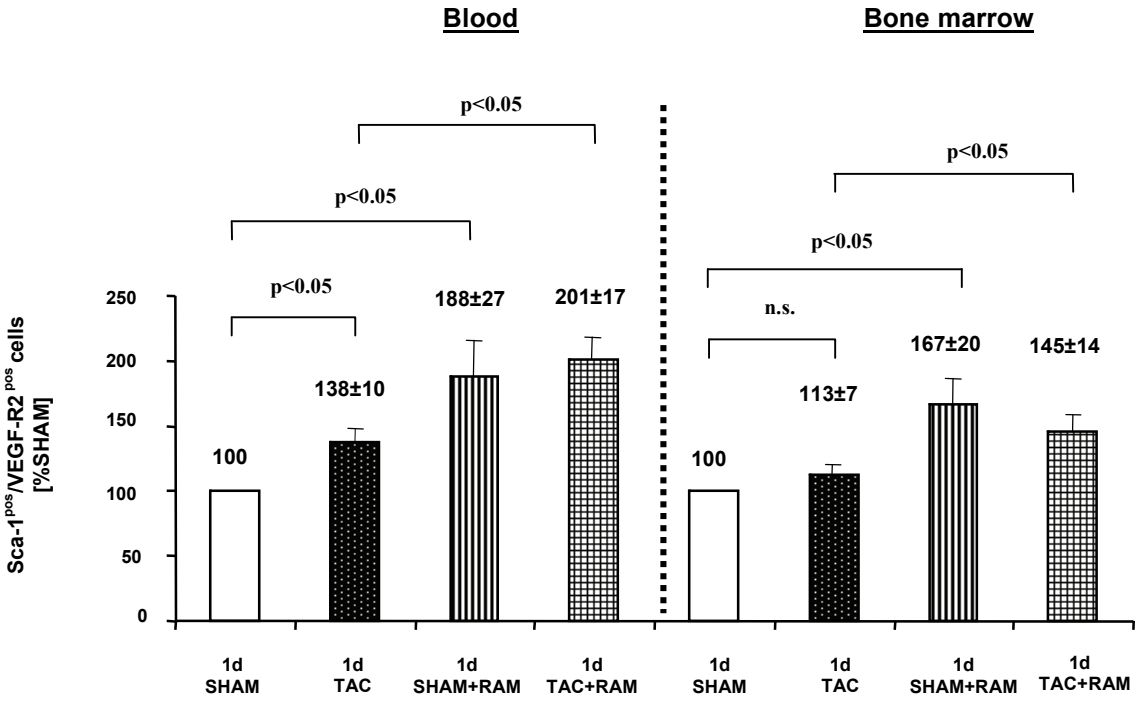


Fig. 30: EPC levels in blood and bone marrow in one-day experiment. One-day TAC substantially raised EPC level only in the peripheral blood. In contrary, ACE inhibition significantly augmented the number of EPC`s not only in the peripheral blood, but also in the bone marrow in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

Unlike one-day TAC, aortic ligation for 5 weeks led to upregulation of EPC number both in the blood and in the bone marrow (Fig. 31). The effect of ramipril was also distinct from the one in one-day experiment: EPC levels in both treated groups did not differ from baseline levels of sham mice in the peripheral blood and in the bone marrow (Fig. 31). Thus, in 5-week experiment TAC was the only group with significantly increased EPC levels in the peripheral blood and in the bone marrow as compared with the baseline levels of sham animals.

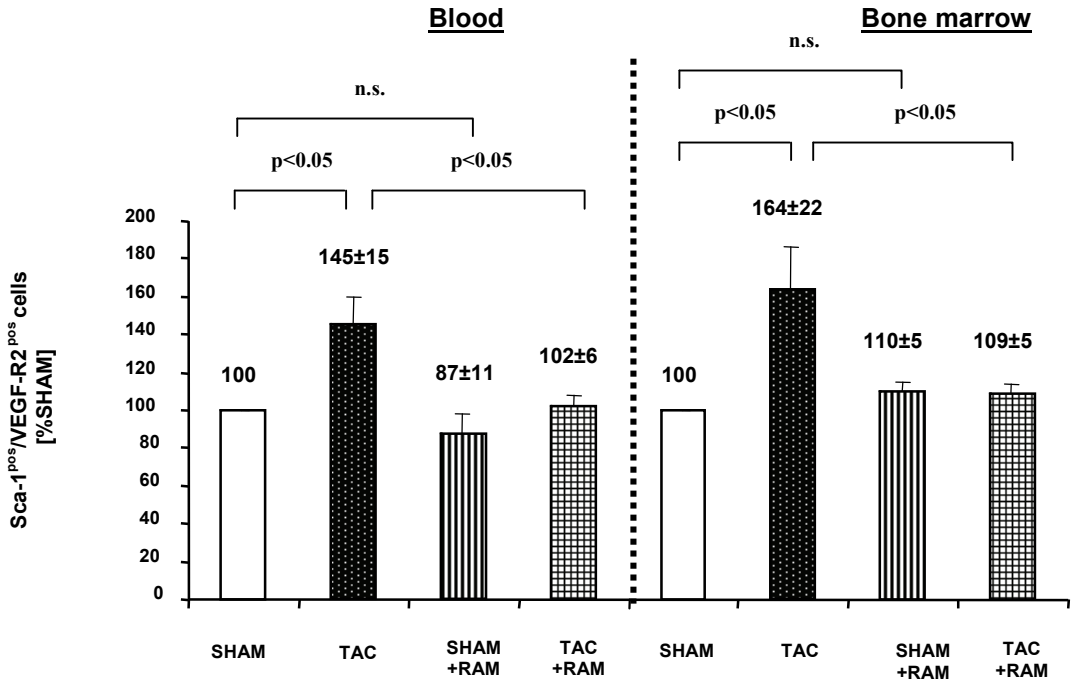


Fig. 31: EPC levels in blood and bone marrow in 5-week experiment. TAC for 5 weeks upregulated the EPC number both in the blood and in the bone marrow. In ramipril-treated groups EPC levels did not differ from baseline levels of sham mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

6.3.2.2. Migration assay data

Neither one-day nor 5-week TAC had any significant effect on the number of DiLDL^{pos} endothelial cells migrating through the mesh of a modified Boyden chamber (0.3 cm^2) compared to sham surgery. Ramipril treatment enhanced the number of migrating cells in all groups (Fig. 32).

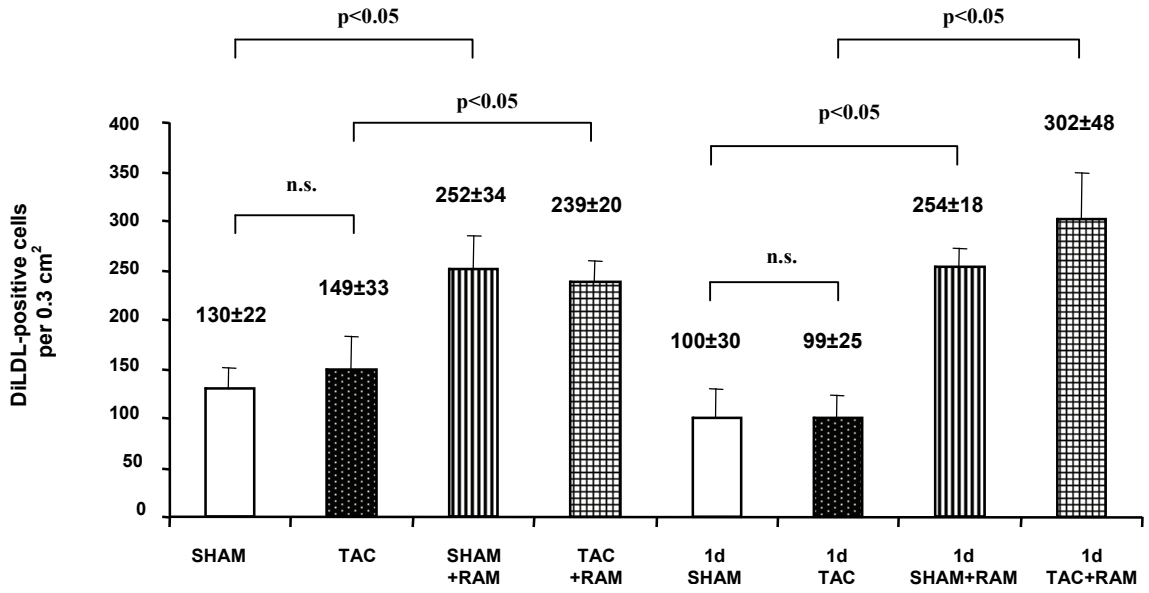


Fig. 32: Migration capacity of EPC. Ramipril increased the number of migrating cells in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

6.3.3. Capillarisation of the left ventricle

To identify endothelial cells in myocardium, immunostaining for platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) commonly used as an endothelial marker was performed (Fig. 35). Five-week TAC diminished significantly the number of CD31-positive cells per mm^2, but did not change the ratio of $CD31^{pos}$ cells to cardiomyocytes (cross-sectioned muscle fibers having nuclei) - detected by staining for α-sarcomeric actin. By contrast, one-day TAC did not affect both parameters (Fig. 33, 34). Ramipril elevated significantly the number of $CD31^{pos}$ cells per mm^2 and the ratio of $CD31^{pos}$ cells to cardiomyocytes in 5-week experiment (Fig. 33, 34).

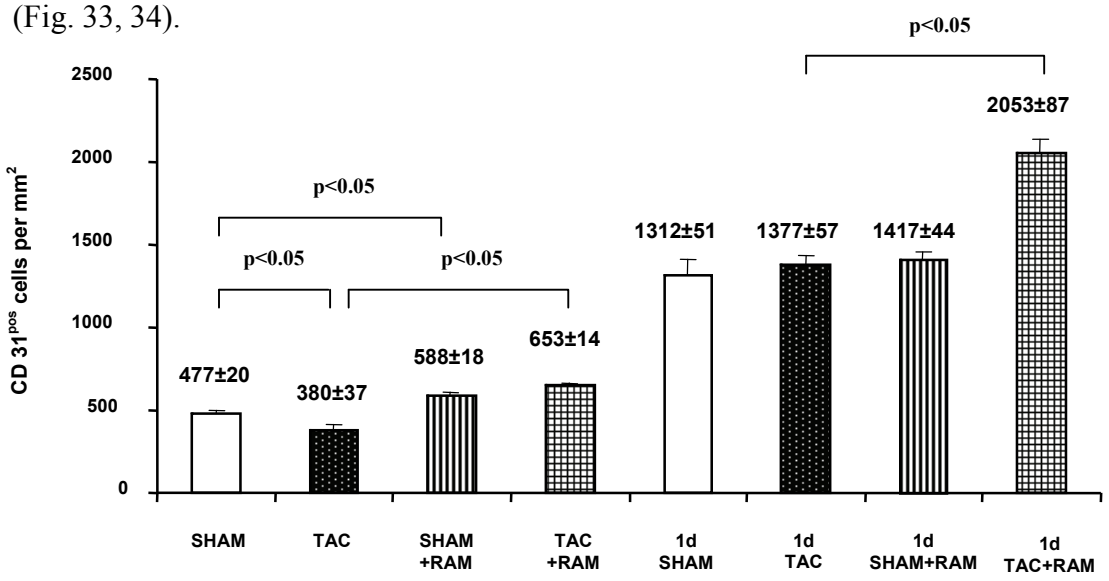


Fig. 33: Number of CD31-positive cells per mm^2. The number of CD31-positive cells per mm^2 was diminished by TAC, but increased by ramipril treatment in all groups in 5-week experiment. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

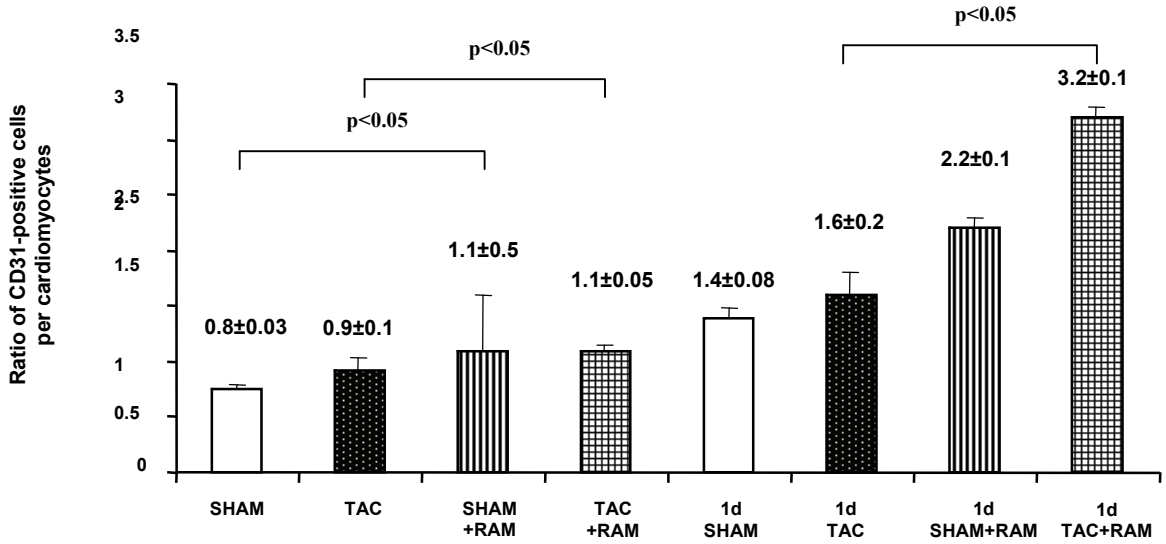


Fig. 34: Ratio of CD31-positive cells per cardiomyocytes. The ratio of CD 31^{pos} cells to cardiomyocytes was not changed by 5-week TAC, but increased by ramipril treatment in all groups in 5-week experiment and in treated aortic-ligated mice in one-day experiment. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

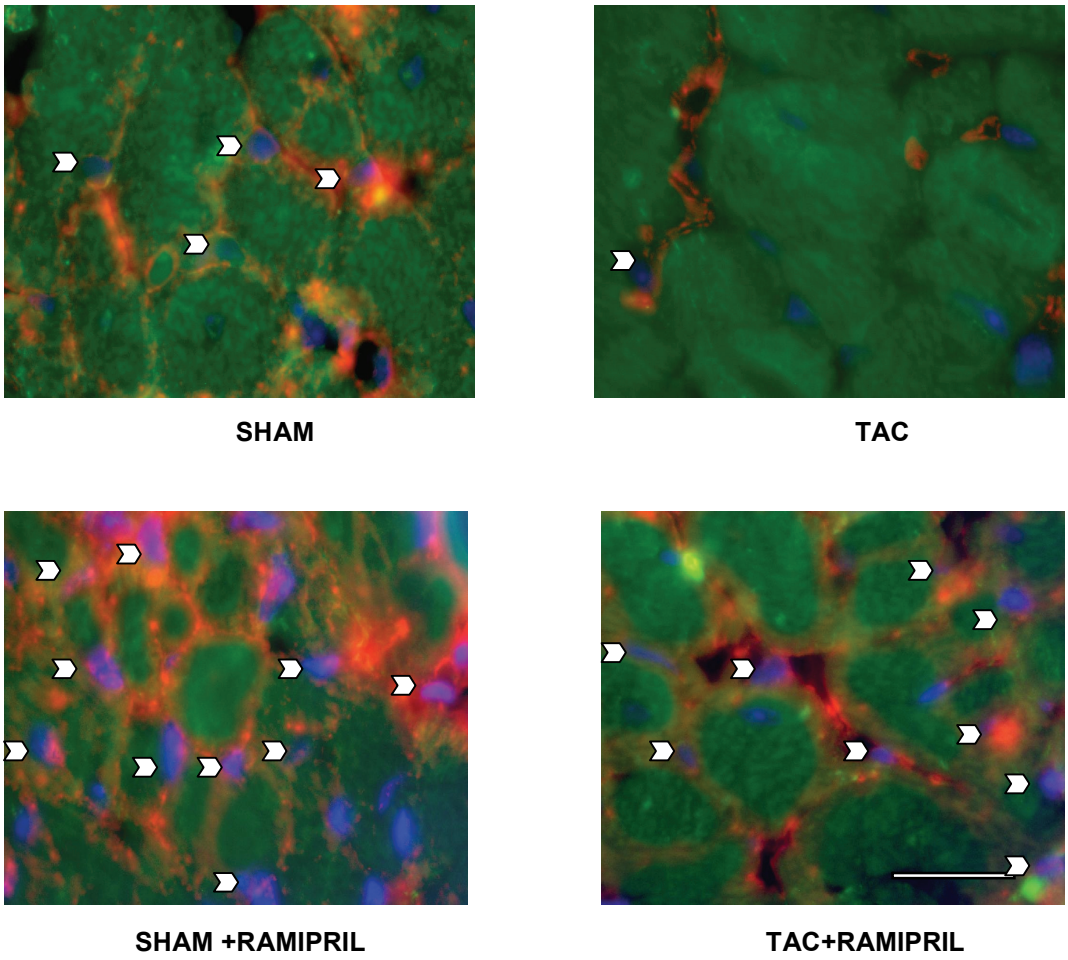


Fig. 35: CD31-positive cells in the left ventricular myocardium. Paraffin sections of 5-week experiment mouse hearts. Immunostaining for the endothelial cell marker CD31 (red). Cardiomyocytes in green (α-sarcomeric actin), nuclei in blue (DAPI). $CD31^{pos}$ cells are marked by arrowhead. TAC significantly reduced the number of $CD31^{pos}$ cells in myocardium per mm^2. Treatment with ramipril led to a significant increase of number of $CD31^{pos}$ cells per mm^2 and the ratio of $CD31^{pos}$ cells to cardiomyocytes in sham mice, but also in TAC animals. Bar = 30µm. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

In one-day experiment both parameters were substantially enhanced only in aortic-ligated animals treated with ramipril (Fig. 33, 34). Capillary density in myocardium was evaluated by immunostaining for podocalyxin - the sialoglycoprotein of endothelial cells (Fig. 36). In 5-week experiment the number of capillaries per mm^2 and the ratio of capillaries to cardiomyocyte profiles (all cross-sectioned muscle fibers with and without nuclei) were not significantly changed by TAC (Fig. 37). Ramipril treatment for 5 weeks increased the number of capillaries per mm^2 in all mice, however the difference was significant only between sham groups (Fig. 37). In contrast, the ratio of capillaries to cardiomyocyte profiles was substantially elevated in all ramipril-treated animals (Fig. 38).

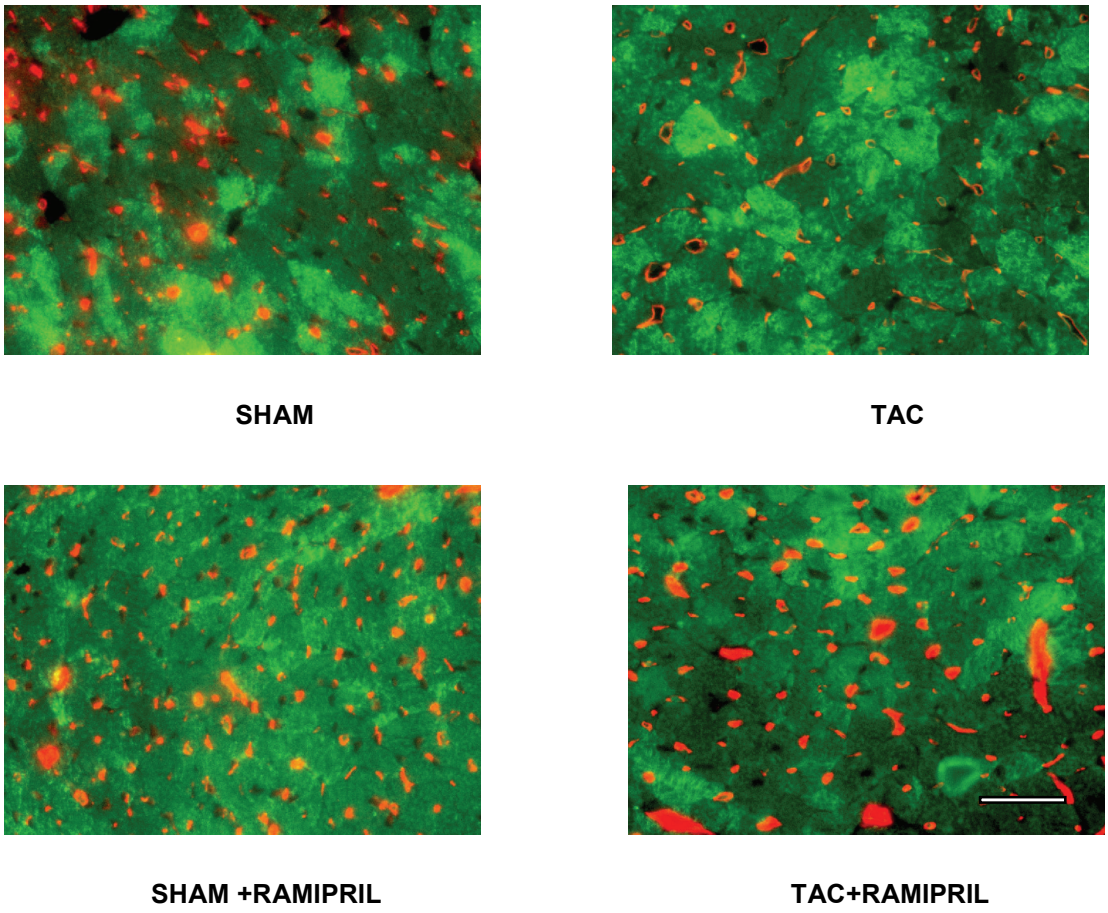


Fig. 36: Capillaries in the left ventricular myocardium. Paraffin sections of 5-week experiment mouse hearts. Immunostaining for podocalyxin. Capillaries in red (podocalyxin), cardiomyocytes in green (α-sarcomeric actin). Treatment with ramipril led to a significant increase of the ratio of capillaries to cardiomyocyte profiles in all animals compared to control. Bar=30 µm. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

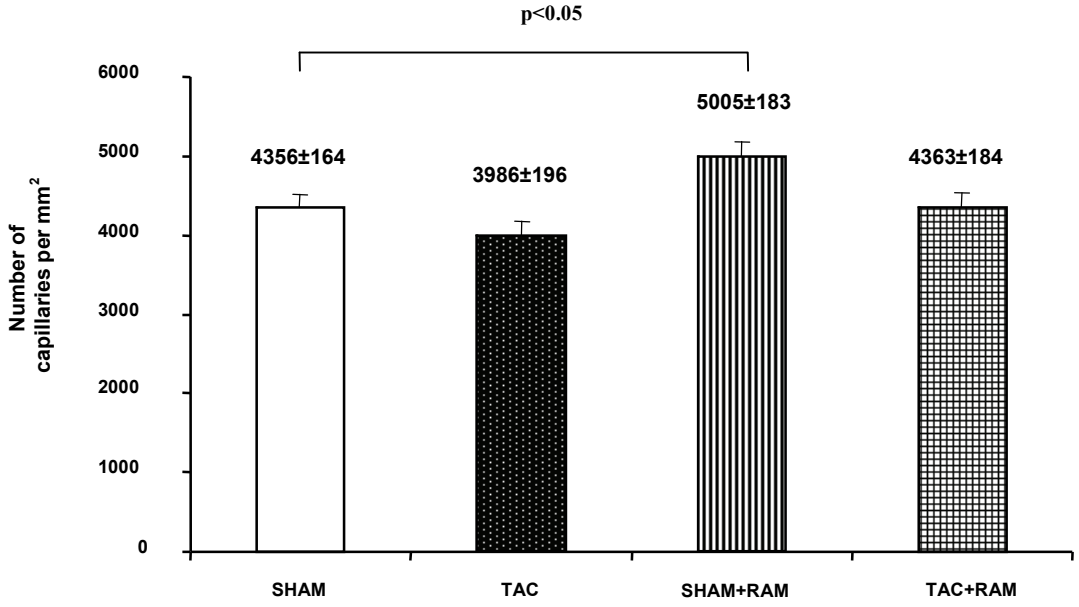


Fig. 37: Capillary density (podocalyxin) in the left ventricular myocardium of 5-week experiment mice. Ramipril treatment for 5 weeks increased the number of capillaries per mm^2 in all mice, however the difference was significant only between sham groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

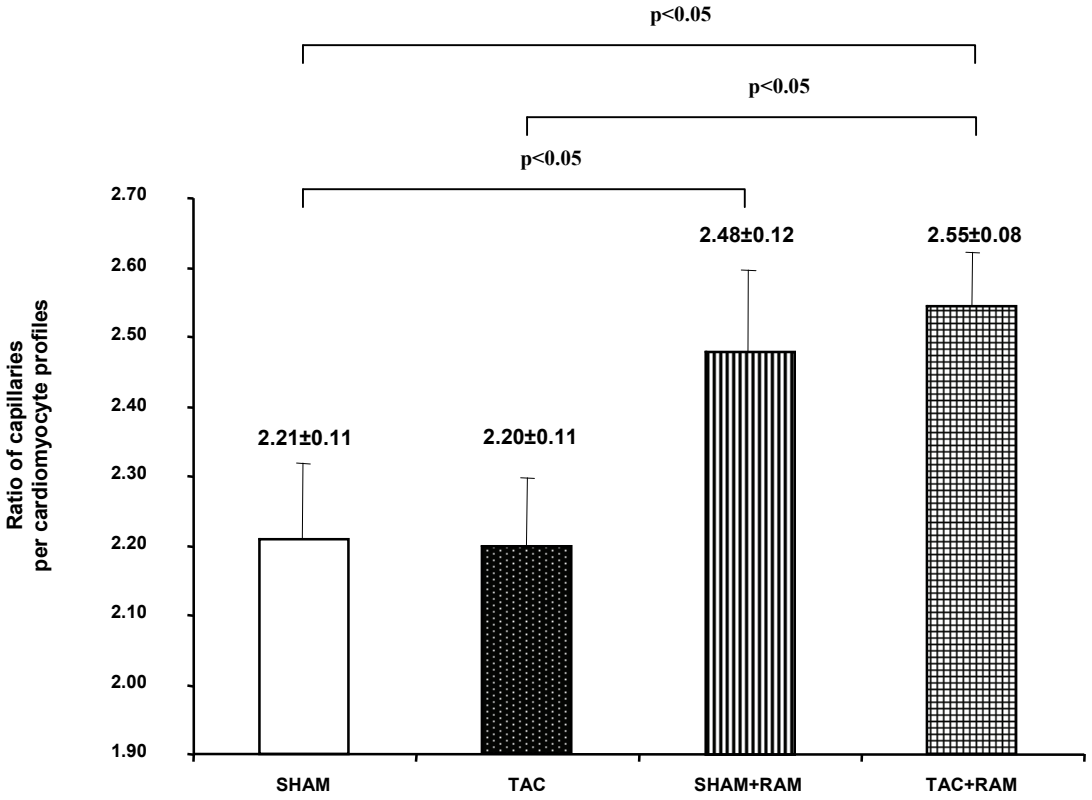


Fig. 38: Ratio of capillaries (podocalyxin) per cardiomyocyte profiles in the left ventricular myocardium of 5-week experiment mice. The ratio of capillaries to cardiomyocyte profiles was substantially elevated in all ramipril-treated animals. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

It must be underscored, that in 5-week experiment there was no distinction among groups in density of cardiomyocyte profiles (all cross-sectioned muscle fibers - with and without nuclei (Table 5). Pressure overload significantly increased the mean cross-sectional area of capillaries in 5-week experiment. Neither TAC nor ramipril treatment changed the number of capillaries per mm^2, the ratio of capillaries to cardiomyocytes, density of cardiomyocyte profiles and the mean cross-sectional area of capillaries in one-day experiment (Table 5).

Table 5 Density of cardiomyocyte profiles and mean cross-sectional area of capillaries

Parameters	TAC	SHAM	TAC +RAM	SHAM +RAM	1dTAC	1dSHAM	1dTAC +RAM	1dSHAM +RAM
Density of cardiomyocyte profiles per mm^2	1834±114	2025±145	1741±108	2045±130	2258±164	2417±139	2460±127	2308±123
Mean cross-sectional area of capillaries, mm^2	7.5±0.3 *	6.3±0.36 #	7.6±0.4 *	6.5±0.2	8.4± 0.5	7.7±0.5	8.0±0.3	8.1±0.6

*- $p<0.05$ compared to SHAM from the same group
#- $p<0.05$ compared to TAC from the same group

6.3.4. Bone marrow-derived endothelial cells

To examine to what degree bone marrow-derived cells contribute to cardiac remodeling and angiogenesis, co-staining for CD31 and GFP and immunostaining for GFP of Tie-2-positive cells were performed (Fig. 39, 40). Aortic constriction significantly increased the percentage of CD31-positive GFP cells; this effect was enhanced by ramipril in the pressure-overloaded mice but not in the sham group (Fig. 40).

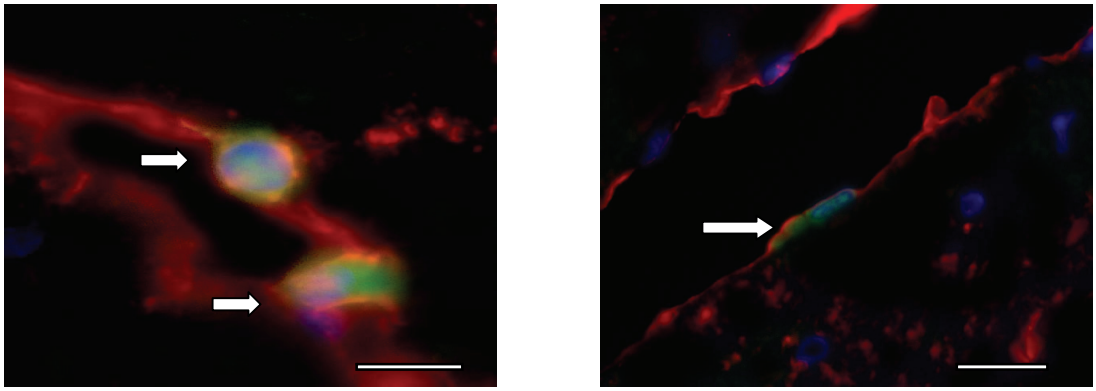


Fig. 39: CD31-positive GFP cells in the myocardium of left ventricle. Paraffin sections of 5-week experiment mouse hearts. CD31-positive GFP cells in TAC-mouse are marked by arrows. CD31 in red, GFP in green, nuclei in blue by DAPI-staining. Bar= 30 µm.

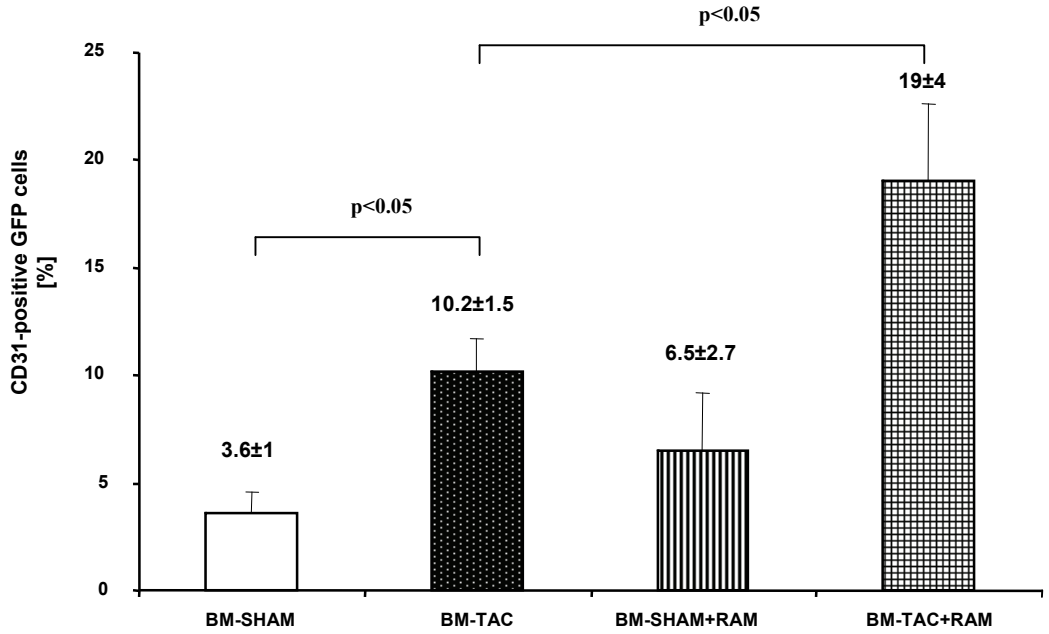


Fig. 40: Percentage of CD31-positive GFP cells in the left ventricular myocardium of 5-week experiment bone marrow-transplanted mice. TAC increased the parameter; treatment with ramipril further enhanced this effect in pressure-overloaded mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

Tie-2 is a receptor tyrosine kinase, which binds and is activated by angiopoietins - protein growth factors that promote angiogenesis. Tie-2 is expressed principally on vascular endothelium and thus can be used as reliable marker for endothelial cells (Fig. 41). Five-week TAC significantly reduced the number of bone marrow-derived Tie-2 cells per mm^2 (Fig. 42). Tie-2

TAC KMT-mice treated with ramipril exhibited substantially higher density of bone marrow-derived Tie-2 cells in myocardium compared to Tie-2 BM-TAC animals (Fig. 42). In contrast, there was no difference between sham mice receiving vehicle and sham mice treated with ramipril (Fig. 42).

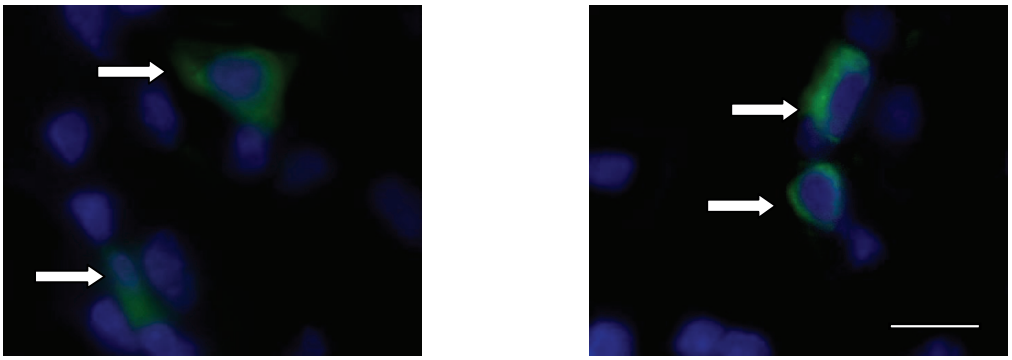


Fig. 41: Tie-2-GFP-positive cells from bone marrow in the left ventricular myocardium. Paraffin section of 5-week experiment mouse heart. GFP in green, nuclei in blue by DAPI-staining. Bar = 30µm.

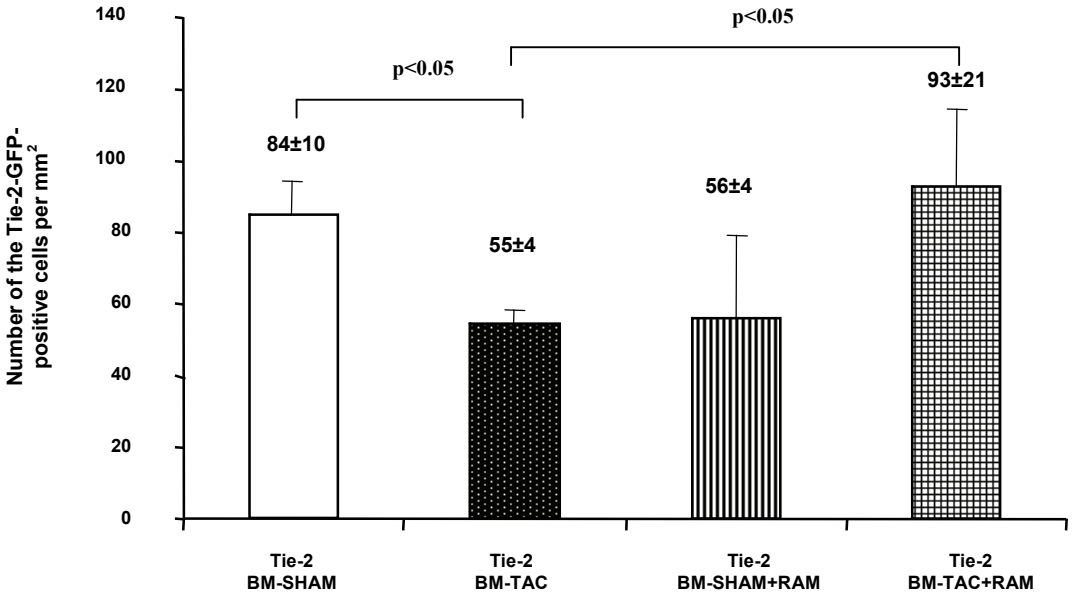


Fig. 42: Number of bone marrow-derived Tie-2-GFP-positive cells per mm^2 in 5-week experiment. TAC reduced the number of bone marrow-derived Tie-2 cells, which was substantially increased by ramipril treatment. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

A detailed knowledge of the relationship between cardiac capillaries and bone marrow-derived endothelial cells is very important for an understanding of the mechanisms of heart regeneration and for stimulation of myocardial restoration. However, the relationship could be different in distinct conditions: pressure overload, treatment with ramipril and their combination. To investigate interrelations between capillary density and number of bone marrow-derived Tie-2-positive cells per mm^2 in experimental groups, the statistical method of within-group correlations was used (Table 6).

Table 6 Within-group correlations between capillary density and number of Tie-2-positive cells per mm^2

Groups	R	p
SHAM	-0.99	0,008
TAC	-0.03	0.93
SHAM+ RAM	-0.06	0.95
TAC+ RAM	0.99	0.01

Within-group correlations demonstrated that in sham-operated animals capillary density negatively correlated with the number of bone marrow-derived Tie-2-positive cells per mm^2, however this interrelation disappeared after TAC. In contrast, treatment with ramipril caused positive relationship between these two parameters in the Tie-2 BM-TAC+RAM group, but disrupted it in Tie-2 BM-SHAM+RAM mice. Thus, application of ramipril in TAC mice led to a significant positive relationship between capillary density and density of bone marrow-derived Tie-2-positive cells in myocardium.

6.4. Bone marrow-derived myocyte progenitor cells

MEF-2 (myocyte enhancer factor 2) proteins are a family of transcription factors that play a pivotal role in the differentiation of stem cells toward a myocyte phenotype. Thus, co-staining for MEF-2 and GFP was performed to reveal bone marrow-derived myocyte progenitor cells in myocardium (Fig. 43).

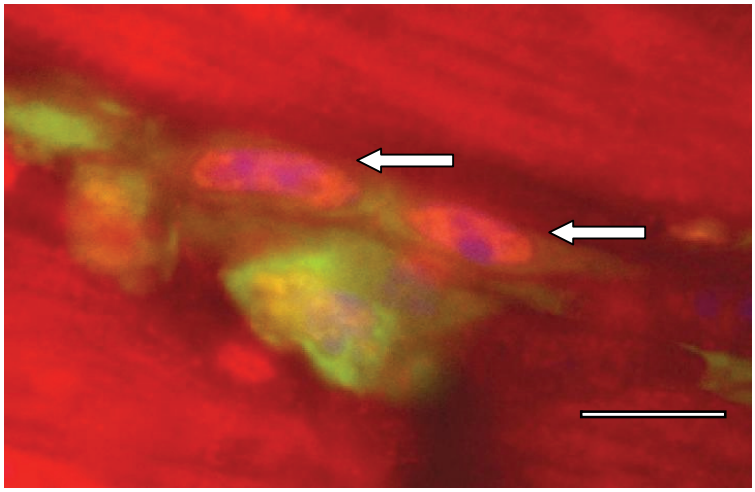


Fig. 43: MEF-2-positive GFP cells. Paraffin section of a 5-week experiment TAC mouse heart. Two MEF-2-positive GFP cells between cardiomyocytes. MEF-2 in red, GFP in green, nuclei in blue by DAPI-staining. Bar = 30µm.

Aortic constriction significantly increased the number of MEF-2^{pos} GFP ^{pos} cells per mm^2 (Fig. 44). Ramipril significantly enhanced the parameter in the sham group but not in the TAC mice (Fig. 44). These cells were not transdifferentiated into smooth muscle cells as examined by costaining of α-smooth muscle actin, also we did not find any large cardiomyocyte derived from the bone marrow by co-staining with α-sarcomeric actin and GFP.

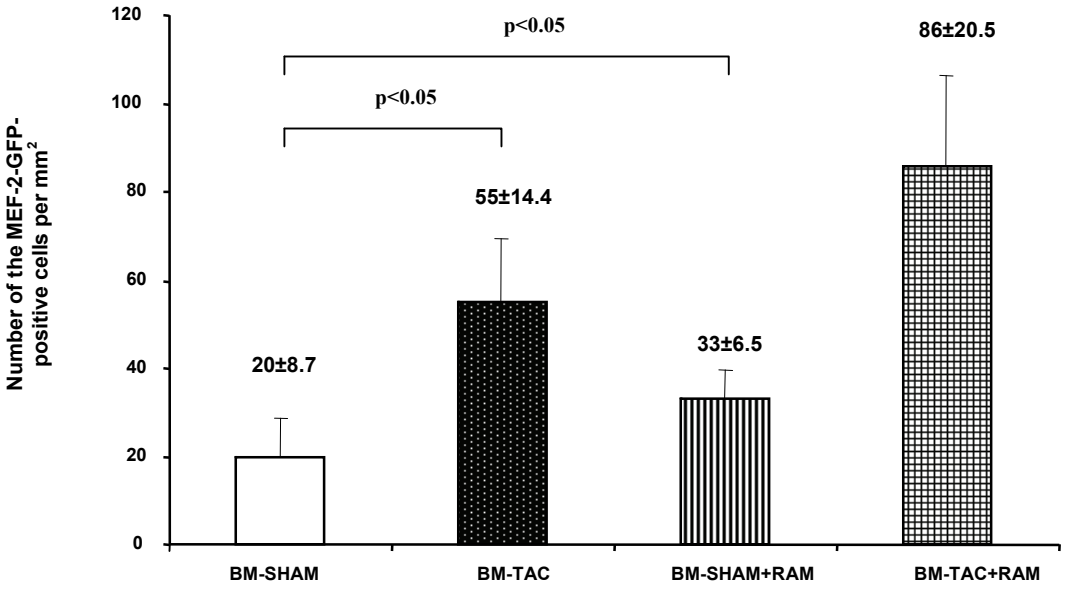


Fig. 44: Number of bone marrow-derived MEF-2-GFP-positive cells per mm^2 in 5-week experiment. TAC increased the number of bone marrow-derived MEF-2 cells. Ramipril substantially enhanced the parameter in the sham group, but not in the TAC mice. SHAM=sham-operated mice; TAC=transverse aortic constriction; BM=bone marrow-transplanted mice; RAM=ramipril-treated mice.

6.5. Hypoxia-inducible factor 1 alpha

HIF-1 is a transcription factor composed of the constitutive HIF-1β and one of the hypoxia-inducible subunits: HIF-1α, HIF-2α, HIF-3α. In hypoxia, the α/β heterodimer binds to the hypoxia response elements in target genes (171). HIF-1α plays the most important role in the regulation of hypoxic response. According to our RT-PCR and Western blot results, one-day TAC substantially increased HIF-1α content in the myocardium (Fig. 45, 46). However, after 5 weeks of aortic ligation the situation was reversed completely: HIF-1α level was significantly higher in the sham-operated animals than in the TAC mice. Ramipril enhanced degradation of HIF-1α on mRNA- and protein level in all groups in both short- and long-term experiments (Fig. 45, 46).

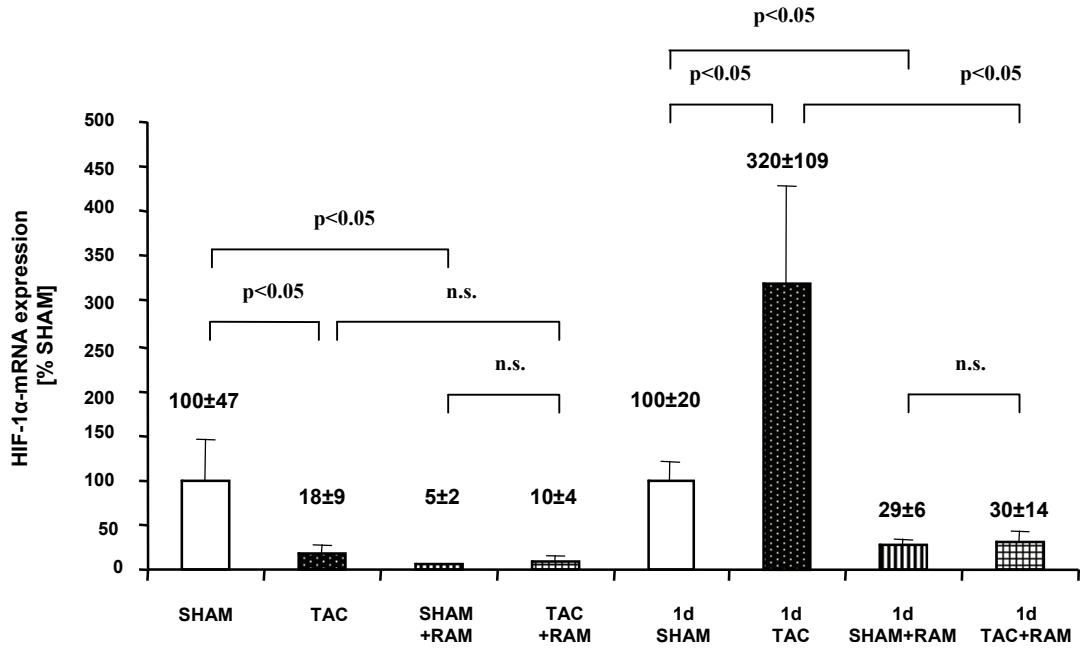


Fig. 45: Expression of HIF-1α–mRNA in the myocardium. Expression of HIF-1α–mRNA was increased by one-day TAC, but diminished by 5-week TAC. Ramipril treatment decreased HIF-1α–mRNA level in all groups. SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

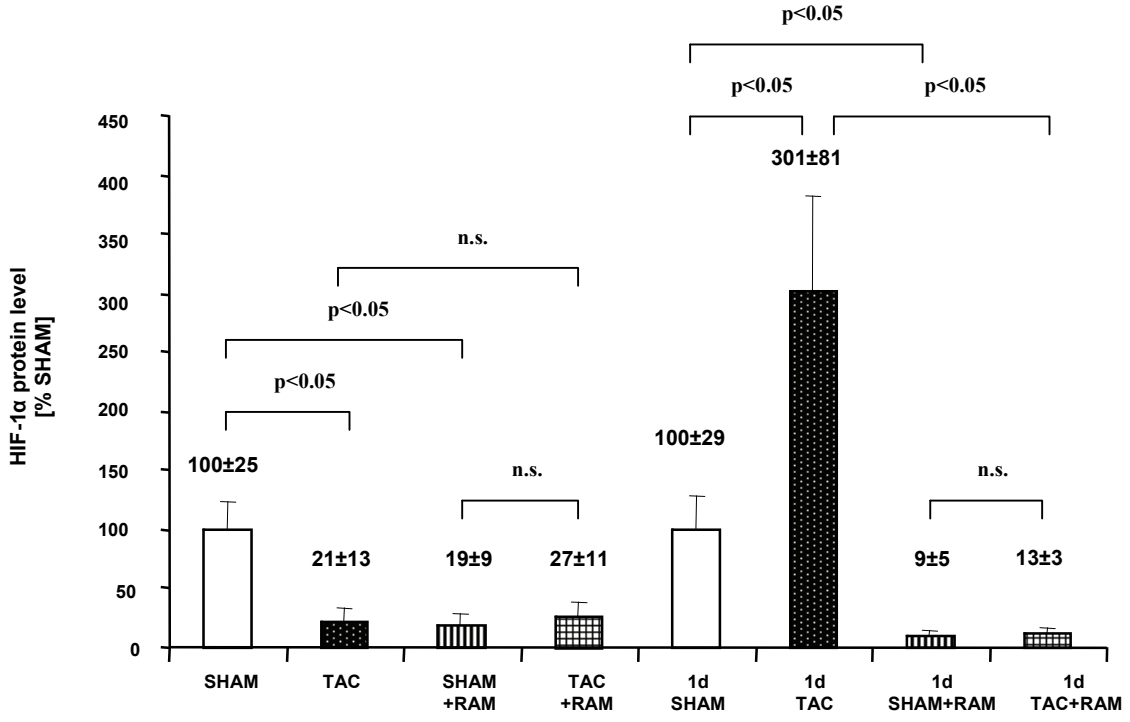


Fig. 46: Protein level of HIF-1α in the myocardium. Protein level of HIF-1α was increased by one-day TAC, but diminished by 5-week TAC. Ramipril treatment decreased protein level of HIF-1α in all groups SHAM=sham-operated mice; TAC=transverse aortic constriction; RAM=ramipril-treated mice.

7. Discussion

As demonstrated in clinical and experimental studies carried out during the past several decades, progression of adverse cardiac remodeling - comprising ventricular hypertrophy, myocardial fibrosis and loss of cardiac cells - plays a major role in the development of heart failure and should be regarded as a pivotal target for treatment. For this reason many cardiovascular drugs have been tested for their ability to improve clinical outcomes and to reduce or to prevent cardiac remodeling in patients with cardiovascular diseases. ACE inhibitors demonstrated significant amelioration of myocardial structure, reduction of death rate and prevention of congestive heart failure (43). On the other hand, there are evidences of their capacity to promote heart regeneration by mobilization of stem cells from the bone marrow (154). On this basis, influence of the ACE inhibitor ramipril on myocardial regeneration and cardiac remodeling mediated by bone marrow-derived cells in cardiac pressure overload was investigated.

7.1. Effects of ACE inhibition on cardiac remodeling in TAC

7.1.1. Transverse aortic constriction leads to pressure overload

As expected, short- and long-term TAC significantly increased left ventricular systolic pressure, which was not reduced by ramipril. Furthermore, aortic ligation but not treatment with ramipril for 5 weeks substantially diminished both systolic and diastolic peripheral blood pressure. At the same time, ramipril ameliorated LVH induced by pressure overload for 5 weeks. These facts testify the effectiveness of the chosen pressure-overload model and prove our hypothesis that the observed effects of ramipril are not caused by hemodynamic changes.

7.1.2. Amelioration of left ventricular hypertrophy and cardiac fibrosis

Cardiac hypertrophy in cardiovascular diseases has traditionally been considered an adaptive response to biomechanical stress. However, results from the Framingham Heart Study showed an association between ventricular hypertrophy and increased cardiac mortality (164), casting doubt on the validity of the wall-stress hypothesis. Furthermore, experiments on different murine models, limiting the development of hypertrophy in response to chronic pressure overload demonstrated little or no deterioration in cardiac function after long-term TAC (36, 47). Thus, the quest for new drugs capable to reduce or to prevent cardiac hypertrophy is one of the main tasks of cardiovascular research.

In our study, as expected, 5-week TAC elicited LVH, which manifested itself as an increase in both the ratio of heart weight to tibia length and cardiomyocyte short-axis diameter.

Ramipril substantially reduced these parameters in TAC mice without BMT but did not diminish significantly left ventricular systolic pressure. The ratio of heart weight to tibia length and cardiomyocyte short-axis diameter were similar in all sham groups and in bone marrow-transplanted TAC mice and were not affected by ramipril. This effect can be explained by the existence of the threshold at which ramipril shows its antihypertrophic action. Our results agree with the findings of other experimental and clinical studies which have demonstrated the ability of both antihypertensive and non-antihypertensive ramipril doses to reduce LVH (79, 80, 88). As shown in animal experiments, the main mechanism underlying this beneficial effect of ramipril is the increase of cardiac kinin levels, which in turn stimulate the B2-kinin receptor (19, 79, 100). In particular, inhibition or knockout of the B2-kinin receptor abrogates the anti-hypertrophic effect of ramipril (148, 178). However, other mechanisms are also involved in this process. So, for example, ACE inhibition increases Ang-(1-7) level in the heart which in turn reverses cardiac hypertrophy (157). At the same time, some authors did not observe reduction of LVH in rats with TAC under the influence of ramipril (92, 184). However it is necessary to underline that the dose used in these experiments was 5 times lower (1 mg per body weight per day) than in our study (92, 185). Furthermore, dose-dependent anti-hypertrophic effects of ramipril have been demonstrated in experiments with spontaneously hypertensive rats (41). Kinin receptors of various species have different affinities for their ligands and hence different doses of ramipril are needed to produce a kinin level sufficient for receptor activation (123).

Myocardial fibrosis is another component of cardiac remodeling. Accumulation of extracellular matrix in the cardiac interstitium disrupts the coordination of myocardial excitation-contraction coupling in both systole and diastole and may result in profound functional impairment. Beyond its effects on function, myocardial fibrosis also promotes arrhythmogenesis through impaired anisotropic conduction and subsequent generation of reentry circuits (57). Therefore, inhibition of excessive production of collagen in myocardium is a critical task in the treatment of cardiovascular diseases and prevention of heart failure.

Five-week TAC elicited fibrotic change in the mice without BMT, which was most pronounced in the perivascular area of coronary arteries/arterioles of LV and progressed slowly along the small arterioles into the intercellular space between cardiomyocytes. The same pattern of cardiac fibrosis in mice with TAC was observed in the study of Xia et al. (177). The peculiarities of fibrotic change in the bone marrow-transplanted mice were its presence even in sham-operated animals and predominance of interstitial fibrosis that can be explained by influence of irradiation. It is assumed, that radiation-induced myocardial fibrosis results from injury to capillary endothelial cells that cause destruction or obstruction of capillaries and compromised microcirculation (139).

As it is known, pressure overload increases level of angiotensin II in the heart which in turn stimulates fibroblast proliferation, their transformation into myofibroblasts and intensive collagen synthesis by both cell types resulting in fibrotic change (121). Recent investigations have shown that fibroblasts in myocardium originate from three major sources: resident fibroblasts, bone marrow-derived fibroblasts and fibroblasts developed through endothelial-to-mesenchymal transition (93, 145, 165, 180). In our study GFP-positive cells lacking both endothelial and myocardial markers massively infiltrated fibrotic areas. They might be inflammatory cells and fibroblasts migrated from the bone marrow and participated in cardiac remodeling.

Treatment with ramipril significantly reduced collagen content in both 5-week TAC groups and in BM-SHAM mice. Our results conform with those of Linz et. al. (79, 80) who demonstrated a potent antifibrotic effect of both antihypertensive and non-antihypertensive ramipril doses in different models of myocardial damage. Moreover, it was revealed that ramipril reduced cardiac mRNA levels of the collagens α_1(I) [col I] and α_1(III) [col III] in aortic banded-hypertensive rats 2 and 6 weeks after aortic constriction (80). In further agreement with our results Grimm et al. (44) showed that ramipril but not hydralazine attenuated interstitial and perivascular fibrosis without markedly affecting left ventricular systolic pressure in aortic banded rats. Further, ramipril in comparison to vehicle- and hydralazine-treated rats significantly blunted the induction of connective tissue proteins such as collagen I and III, laminin B and fibronectin at both the mRNA and protein levels (44). The capacity of ramipril to elevate the level of cardiac kinins also plays a role in its antifibrotic effect. In particular, it was shown that treatment of isolated coronary microvessels with ramipril enhanced nitric oxide production through a kinin-mediated mechanism (182). At the same time, the increased cardiac level of nitric oxide produced by endothelial nitric oxide synthase can protect myocardium from interstitial and perivascular fibrosis in pressure overload (126). Recently, investigations have shown also, that increases of Ac-SDKP and Ang (1-7) levels through ACE inhibition suppress cardiac fibrosis (114, 136, 157). Probably in this way the antifibrotic influence of ramipril and other ACE inhibitors is mediated through different mechanisms.

It is significant that our histological findings (LVH, cardiac fibrosis) resemble typical pathological changes found in patients with cardiovascular diseases accompanied by left ventricular pressure overload (aortic stenosis and systemic hypertension) that confirms clinical and pathological relevance of our TAC model (139). Thus, ramipril reduced LVH as well as cardiac fibrosis in pressure overload. These features are crucial for prevention of adverse cardiac remodeling.

7.1.3. Cardiac cell turnover

Physiological cell turnover plays an important role in maintaining normal heart function and architecture. This is achieved by the dynamic balance of cell proliferation and apoptosis in distinct cell types forming the heart (21). New data obtained by Bergmann et al. (14) using radiocarbon dating of DNA provide strong evidence that cardiomyocytes can repopulate in the human heart, but this process is very slow and hence most cardiomyocytes will not be exchanged over the course of a normal human life span (14). However, adverse tissue remodeling in pathological conditions can disturb renewal of cardiomyocytes and other cardiac cells. This raises the questions how far maladaptive cardiac remodeling in pressure overload impairs cell turnover and how ramipril can improve it. To answer this, immunostaining for the proliferation marker Ki67 and in situ oligo ligation assay for apoptosis detection were used.

Ki67 is a protein of approximately 395 kDa located in the nucleus. It is present during all active phases of the cell cycle (G_1, S, G_2 and mitosis), but is absent from the resting cell (G_0) (18, 135). This makes it an excellent marker for determining the so-called growth fraction of a given cell population. However, the presence of Ki67 does not allow to define whether the cell will undergo cytokinesis or only karyokinesis. Pressure overload for 5 weeks significantly increased the number of Ki67-expressing non-cardiomyocytes and induced synthesis of Ki67 protein in some cardiomyocytes. In reviewing the literature, no data was found on the quantitative immunohistochemical comparison of cell proliferation in LV of sham- and TAC-operated adult mice. However, our findings are in agreement with the results of autoradiographic experiments carried out on normal mouse LV, autoradiographic experiments and immunostaining studies performed on LV of sham- and TAC- operated adult rats. Data from animal experiments demonstrated either the absence of thymidine-H^3-, BrdU- or PCNA-labeled cardiomyocytes and non-cardiomyocytes in LV or their very low number which varied according different authors from 0.004 to 0.04% in adult mice and from 0.004 to 2.46% in adult rats (128, 142). Transverse aortic constriction in adult rats according to different sources increased their number to 0.3-29.75% (128, 142). Moreover, the present findings seem to be consistent with the immunostaining data obtained from human myocardial biopsies and autopsy hearts. Immunohistochemical investigations on proliferation rate in normal human myocardium revealed very small number of cardiomyocytes and non-cardiomyocytes expressing proliferation markers-Ki67 and PCNA, however it was significantly increased in the diseased human hearts (9).

Despite the pronounced expression of Ki67 in some cardiomyocytes after 5 weeks of TAC, they did not exhibit mitotic figures. Many of Ki67-negative cardiomyocytes had two nuclei; moreover, binuclear Ki67-expressing cardiomyocytes were found in some TAC animals,

testifying thereby that karyokinesis was not necessarily followed by cytokinesis and led to binucleation. This accords with earlier observations, which showed that most cardiomyocytes in the myocardium of adult mice are binucleated (163).

Some authors suggest that genome multiplication through polyploidy and multinucleation protects tissue from different kinds of stress and extends cell life span because it is associated with the increased expression of genes involved in protection from apoptosis, DNA damage, hypoxia and oxidative stress (5). As indirect evidence for this concept could be viewed our finding of Ki67-expressing cardiomyocytes in BM-SHAM mice whose cardiomyocyte short-axis diameters are significantly lower than those of non-transplanted sham mice. However, the influence of radiation induced this compensation mechanism. Therefore, it is possible that even though mature cardiomyocytes express Ki67, they do not necessarily divide and hence do not yield two daughter cells, their unfinished cell division leads to genome multiplication, which prolongs their life span. This might be a compensation mechanism, which saves cardiomyocytes from death and sustains organ structure and function under the pressure overload despite of insufficient cardiomyocyte renewal**.** Further investigations should elucidate interactions between two fundamental processes supporting architecture and function of the myocardium: cardiomyocyte renewal and genome multiplication in mature cardiomyocytes.

Apoptosis is another part of natural cell turnover (21), however its rate is increased in pressure overload, leading to disturbance of heart structure and function (8). To detect apoptosis the in situ oligo ligation assay, staining specifically cells that contain typical apoptotic double-stranded breaks which are blunt-ended or have a one base 3' overhang, was utilized (32). In our study 5-week TAC substantially increased the number of cardiomyocytes and non-cardiomyocytes undergoing apoptosis. Bone marrow transplantation led to augmented rate of apoptosis, which was further enhanced by TAC. At the same time, pressure overload did not influence the number of apoptotic bone marrow-derived cells. Our results are in agreement with those of Teiger et al. (152) who showed that pressure overload increased apoptosis rates of cardiomyocytes and non-cardiomyocytes in rat hearts. Moreover, data obtained from human hearts demonstrated that numbers of apoptotic cardiomyocyte nuclei varied at 0.001-0.002% in control hearts and 0.08-0.25% in various cardiac diseases (21). Correlation analysis confirmed the close positive associations between cardiomyocyte short-axis diameter, collagen content, characterizing cardiac remodeling and parameters of cardiomyocyte turnover.

Treatment with ramipril for 5 weeks significantly decreased the numbers of Ki67–positive cells in all groups independent of TAC and BMT. The reduction of apoptosis rates in all investigated cell types was observed only in the bone marrow-transplanted TAC animals. By

contrast, in non-transplanted mice, ramipril significantly diminished only the number of apoptotic cardiomyocytes and endothelial cells in pressure overload.

Although in a literature search no information was found about the influence of ACE inhibitors on cell proliferation in pressure-overloaded adult heart, some authors showed that enalapril significantly decreased cell turnover (proliferation and apoptosis of cardiomyocytes and non-cardiomyocytes) in neonatal rat heart and in infarcted heart of adult rats (26, 151). As discussed above, cell division of mature cardiomyocytes induced through stress conditions leads to polyploidy which although it enhances cell life span could be viewed as indirect evidence of tissue stress. For this reason, the antiproliferative influence of ramipril on cardiomyocytes and other cardiac cells in pressure overload in combination with its antihypertrophic and antifibrotic effects might be rather positive because it mirrors the protective role of ACE inhibition against tissue stress thereby saving structure and function of the myocardium. On the other hand, in our TAC model of pressure overload, as well as in the model of pulmonary artery banding, ramipril substantially decreased the number of cardiac cells undergoing apoptosis (17). The positive correlations between cardiomyocyte short-axis diameter, collagen content and the parameters of cardiomyocyte turnover demonstrate that the decrease of cardiomyocyte hypertrophy and cardiac fibrosis is associated with a reduction of the number of apoptotic and proliferating cardiomyocytes. It is noteworthy, that ramipril also ameliorated cell turnover in bone marrow transplanted-sham-operated animals. This demonstrates its mitigating effect on irradiation-induced damage (66, 131). Therefore, our study further confirmed the ability of ACE inhibitors, in particular of ramipril, to diminish cell turnover of cardiomyocytes and non-cardiomyocytes in the diseased heart. This must be regarded as a positive effect because along with antihypertrophic and antifibrotic effects, ACE inhibitor protects myocardial structure and function against pressure overload.

7.2. Cardiac and extracardiac neoangiogenesis

Myocardial performance depends not only on the number and size of cardiomyocytes, but also on the vascular supply of energy substrates and oxygen. However, as mentioned in the introduction, a large body of research shows that although myocardial capillary growth and recruitment of reserve capillaries occur in the compensated phase of pathological cardiac hypertrophy caused by pressure overload, they are not able, unlike capillarisation in physiological cardiac hypertrophy, to maintain an adequate blood supply for growing cardiomyocytes (170). Moreover, recent investigations show that disruption of coordinated tissue growth and angiogenesis contributes to the transition from compensated cardiac hypertrophy to

heart failure (132, 138). For this reasons, stimulation of angiogenesis and restoration of adequate blood supply in the hypertrophied myocardium are of paramount importance.

To evaluate myocardial capillary growth, immunostainings for platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) as endothelial cell marker and podocalyxin as capillary marker were used. Pressure overload for 5 weeks significantly reduced the number of $CD31^{pos}$ cells per mm^2, but did not change the ratio of $CD31^{pos}$ cells to cardiomyocytes, capillary density and the ratio of capillaries to cardiomyocytes. On the other hand, 5-week TAC substantially enhanced apoptosis rate in $CD31^{pos}$ cells and increased the mean cross-sectional area of capillaries. Thus, even though increased cardiac afterload reduced the number of endothelial cells in capillaries, the capillary density and the ratio of capillaries to cardiomyocytes remained unchanged.

At first glance it would seem that the decreased number of $CD31^{pos}$ cells per mm^2 is in contradiction with the unchanged capillary density. However, as discussed further below, the loss of endothelial cells in pressure-overloaded myocardium is compensated by development of new endothelial cells from cardiac- and bone marrow-derived stem cells and division of adult endothelial cells. They replace dead endothelial cells maintaining thereby capillary structure and density. However, increased cardiac afterload deteriorates eventually the ability of stem cells to proliferate and to differentiate into endothelial cells and the ability of adult endothelial cells to divide. This stops the replacement of dead endothelial cells and leads ultimately to a decrease of capillary density contributing thereby to the transition from compensated cardiac hypertrophy to heart failure. Moreover, as mentioned above, pressure overload in the compensated phase opens reserve capillaries and, what is more important, stimulates angiogenesis which along with the replacement of dead endothelial cells in capillaries sustains temporarily the capillary density and the ratio of capillary to cardiomyocytes. This contention is supported by our finding of new vessel formation in implanted subcutaneous polyvinyl discs in TAC animals. It is noteworthy, that although pressure overload for 5 weeks diminished the number of cardiomyocytes (cross-sectioned muscle fibers having nuclei) per mm^2, it did not change the density of cardiomyocyte profiles (all cross-sectioned muscle fibers with and without nuclei). At first glance, these facts appear to be contradictory, however, this discrepancy can be explained by the laterally growth of side branches of existing cardiomyocytes, compensating cardiomyocyte loss (21). Thus, although pressure overload leads to apoptosis in endothelial cells and cardiomyocytes thereby reducing their density, development of new endothelial cells, recruitment of reserve capillaries, myocardial neoangiogenesis and formation of cardiomyocyte side branches sustain temporarily capillary density and the ratio of capillaries to cardiomyocytes. However, as shown by ample evidence, the increased pressure overload leads eventually to disruption of coordinated cardiac

hypertrophy and angiogenesis. This in turn contributes to the transition from the compensated phase to heart failure.

Treatment with ramipril for 5 weeks significantly diminished apoptosis of endothelial cells, increased their density and the ratio of endothelial cells to cardiomyocytes in all groups. Furthermore, ACE inhibition elevated capillary density, but this enhancement was only significant in the sham-operated animals but not in the TAC group. This could be explained, as discussed above, by stimulation of new vessel formation in pressure overload, which smoothed out the difference between these TAC groups. Moreover, ramipril substantially augmented extracardiac angiogenesis in both groups. This was demonstrated in our study using subcutaneous implantation of polyvinyl discs. However, more importantly, ramipril increased the ratio of capillary to cardiomyocytes in all groups. It is noteworthy that this parameter augmentation in the group of aortic-ligated mice receiving ramipril was significant compared to both the TAC- and the sham-operated animals. At the same time, neither the mean cross-sectional area of capillaries nor the density of cardiomyocyte profiles were effected by ramipril. For these reasons, the increase of the ratio of capillaries to cardiomyocytes was really caused by capillary growth but not by changing these two parameters.

Our findings are in agreement with the results of other investigators using alternative experimental models: spontaneously hypertensive rats, hypertension in rats induced through chronic nitric oxide inhibition by L-NAME, renal hypertension, injections of angiotensin II. They showed that although pressure overload and increased production of angiotensin II stimulate angiogenesis, ACE inhibition enhances this process many-fold (149, 159, 170). Elevation of bradykinin level under the influence of ACE inhibitors is thought to be the main mechanism promoting neoangiogenesis. In particular, it was shown that both blockade and knockout of B1- and B2-kinin receptors impaired neoangiogenesis induced by ACE inhibition (42, 109, 140). However, this is not the only mechanism involved in angiogenesis promotion caused by ACE inhibitors. Increase of Ac-SDKP level exerted angiogenic effects on cultured endothelial cells and improved cardiac angiogenesis in rat myocardial infarction (84, 168). Further investigations will probably reveal new mechanisms underlying the angiogenic effects of ACE inhibitors. Thus, treatment with ramipril stimulated cardiac and extracardiac formation of new capillaries and increased the ratio of capillaries to cardiomyocytes independent of pressure overload.

7.3. Effects of ACE inhibition on the bone marrow-cardiac axis of myocardial regeneration

Bone marrow-derived stem cells, as discussed in the introduction, actively contribute to heart regeneration. Different biological active substances can enhance their mobilization and regenerative potential. Therefore to investigate the influence of ramipril on the impact of the bone marrow-derived cells on heart regeneration is of great interest and importance.

7.3.1. Endothelial progenitor cells

One-day TAC substantially increased the number of circulating EPC´s in blood, but not in bone marrow. In contrast, 5-week TAC enhanced the EPC levels in both tissues, meanwhile, neither short- nor long-term pressure overload led to amelioration of EPC migratory capacity, which was increased by ramipril treatment in all groups. Moreover in the short-term but not in the long-term experiment this effect of ramipril was accompanied by enhanced EPC levels in blood and bone marrow.

The ramipril-induced EPC mobilization led to a significant increase in numbers of bone marrow- derived endothelial cells in the TAC hearts, as demonstrated by experiments with both types of bone marrow transplantation. Meanwhile, despite enhancing capillary density, ramipril did not significantly raise the number of bone marrow-derived endothelial cells in the myocardium of sham-operated animals. As was mentioned in the introduction, the major source of endothelial cells for new vessels is the vessels of injured tissue containing adult endothelial cells capable of mitosis and resident precursors of endothelial cells (146, 181), and the minor one is the EPC´s from bone marrow (146). The data of our workgroup obtained from experiments with aortic ligation in eNOS knockout mice support this contention (97). It was expected that the number of $CD31^{pos}$ cells per mm^2 and the ratio of $CD31^{pos}$ cells to cardiomyocytes in pressure-overloaded myocardium would be significant lower in eNOS knockout mice than in wild type mice, because eNOS plays a pivotal role in EPC´s mobilization from the bone marrow (155). However, contrary to expectations, it was found that although pressure overload in eNOS knockout mice did not increase EPC numbers in blood and bone marrow, comparison of the number of $CD31^{pos}$ cells per mm^2 and the ratio of $CD31^{pos}$ cells to cardiomyocytes in eNOS knockout and wild type mice did not reveal any difference (97). Based on these findings, one can speculate that ramipril enhances capillarisation by stimulating both types of EPC´s, however, in pressure-overloaded heart resident EPC´s can not alone provide adequate neoangiogenesis because they suffer from pathological conditions in myocardium caused by pressure overload (e.g. apoptosis, endothelial-to-mesenchymal transformation) and so EPC´s from bone marrow take more active part thereby increasing significantly the number of bone marrow-derived

endothelial cells in the heart. This consideration is sustained by within-group correlations between the capillary density and the number of bone marrow-derived Tie-2-GFP-positive cells per mm^2. The histological characterization of bone marrow cells migrating into the heart can be difficult because leucocytes or pericytes lay in the vicinity of endothelial cells. Moreover, different nonangiogenic inflammatory cells such as platelets, macrophages, granulocytes, T/NK cells, lymphocytes, neutrophils and mast cells express CD31 (53). To overcome these issues, Tie-2-GFP mice as donor animals for additional bone marrow transplantation studies were used. Tie-2 is the receptor of angiopoietin and consequently all cells possessing this protein take an active part in angiogenesis (34). For this reason, Tie-2 is a more reliable marker for bone marrow-derived cells involved in angiogenesis.

Negative correlation between the capillary density and the number of bone marrow-derived Tie-2-GFP-positive cells per mm^2 in the sham group testifies that in normal condition EPC´s from bone marrow do not participate actively in capillary endothelial cell turnover. Disruption of this relationship by ramipril also argues for a relative small role of bone marrow-derived EPC´s in myocardial neoangiogenesis in sham-operated mice, because although on the one hand treatment with ramipril increased capillary density, on the other hand it diminished the number of bone marrow-derived Tie-2-positive cells per mm^2. One can assume that in this case increase in capillary density was caused by division and differentiation of resident endothelial precursor cells and division of adult endothelial cells stimulated by ramipril. Apparently, this process is slow, because neither the number of Ki67-positive non-cardiomyocytes nor the number of CD31-positive cells per mm^2 were increased in sham mice after three days of ramipril treatment. Aortic ligation for 5 weeks increased the percentage of bone marrow-derived CD31-positive cells in the TAC-hearts, but reduced the density of bone marrow-derived Tie-2-positive cells. This contradiction could be explained by the fact that, as discussed above, apart from endothelial cells different nonangiogenic inflammatory cells express CD31. For this reason, one can assume that the majority of bone marrow-derived CD31-positive cells in pressure-overloaded myocardium is nonangiogenic inflammatory cells. Hence, TAC-induced mobilization of EPC´s from bone marrow did not raise the number of Tie-2-GFP-positive cells in the heart, moreover there was no correlation between the capillary density and the number of bone marrow-derived Tie-2-positive cells per mm^2 in the TAC-mice. This fact can not be explained by apoptosis of bone marrow-derived cells because neither aortic ligation nor ramipril treatment changed apoptosis rate in GFP-positive cells. However, recently Zeisberg et al. (180) have shown that cardiac endothelial cells contribute to cardiac fibrosis in an aortic banding model through endothelial-to-mesenchymal transition elicited by TGF-β1. One can assume that bone marrow-derived EPC´s transdifferentiate into fibroblasts enhancing thereby cardiac fibrosis. The

presence of a large number of CD31-positive GFP cells and bone marrow-derived Tie-2-GFP-positive cells in fibrotic areas can be viewed as an argument in favor of this hypothesis. Hence, transformation of old and new endothelial cells in fibroblasts might be one more reason of disruption of coordinated tissue growth and angiogenesis resulting in the transition from compensated cardiac hypertrophy to heart failure.

At the same time both clinical and experimental studies revealed the ability of ramipril to reduce TGF-β1 level in the plasma and in the heart (133, 179). One further possible mechanism of ramipril`s ameliorative effect on the myocardial structure and capillarisation is the inhibition of endothelial-to-mesenchymal transition by reduction of TGF-β1 level. Treatment with ramipril of aortic-ligated mice led ultimately to the positive correlation between capillary density and the number of bone marrow-derived Tie-2-positive cells per mm^2 in the heart, demonstrating thereby participation of EPC´s from bone marrow in ramipril-induced neoangiogenesis. Thus, ramipril raised the numbers of EPC´s in blood and bone marrow, ameliorated their migratory capacity and increased the number of bone marrow-derived endothelial cells in pressure-overloaded myocardium.

7.3.2. Mechanisms of ramipril-induced EPC mobilization

Recent investigations have revealed different beneficial effects of the RAAS modulation on progenitor cells (119). Our findings on circulating EPC´s in blood are in agreement with those of Min et al. (91) who showed improvement of migration, proliferation, adhesion, *in vitro* vasculogenesis capacity and increase of EPC level in blood under the influence of ramipril in patients with stable coronary artery disease. At the same time, these effects did not correlate with plasma levels of classical angiogenic factors as VEGF, GM-CSF or tumor necrosis factor-α. However, mobilization, improvement of EPC functions and stimulation of angiogenesis could be caused by angiogenic factors in the heart itself. In particular, Sano et al. (132) demonstrated that although myocardial angiogenesis was promoted by HIF-1α-dependent induction of angiogenic factors during the first week after TAC, pressure overload for 4 weeks substantially reduced HIF-1α level despite the presence of hypoxia through accumulation of p53 that impaired vessel formation. This impairment can also be caused by p53-induced premature senescence of endothelial and stem cells that ultimately leads to apoptosis (25, 69). Similar results were obtained in our experiments: expression and protein level of HIF-1α in pressure-overloaded heart were substantially increased in short-term experiment, but diminished in the long-term one.

Morrissey et al. (95) showed reduction of p53 mRNA level in kidney under the influence of ACE inhibition in unilateral ureteral obstruction. Since induced through ureteral obstruction increase of urine pressure in the kidney resembles pressure overload in aortic ligation, one can

assume that ACE inhibition can reduce p53 expression in myocardium enhancing thereby mRNA and protein levels of HIF-1α. Thus, one can speculate that observed effects of ramipril on EPC´s and cardiac angiogenesis might be caused by increase of HIF-1α level in the heart. Contrary to expectations, ramipril significantly diminished HIF-1α level in all groups in both types of experiment. This suggests that intervention through ACE inhibition acts on EPC´s independently from HIF-1α induced angiogenic factors. Moreover, this reduction of HIF-1α level, unlike 5-week TAC with vehicle treatment, must be regarded as a positive effect of ramipril because it is accompanied by neoangiogenesis and amelioration of cardiac remodeling.
It is noteworthy that other inhibitors of ACE also promote EPC mobilization, proliferation and amelioration of their migratory capacity, which, according to different authors, are mediated not through one but through many distinct mechanisms. In particular, Thum et al. (154) showed that trandolapril increased EPC level in blood by eNOS-independent activation of MMP-9 in the bone marrow. At the same time, Kränkel et al. (68) revealed that mobilization and angiogenic action of EPC´s in injured tissues are mediated by the B2-receptor of bradykinin via a B2R/phosphoinositide 3-kinase/eNOS-pathway. This influence of ACE inhibitors on circulating EPC level is not confined to altering of activity and modification of pre-existing proteins but also includes change of gene expression. Specifically, treatment with ramipril increased the number of circulating EPC´s in patients with essential hypertension. This was accompanied by enhanced expressing of homeobox A9 in these cells, which contributes to endothelial commitment of EPC, postnatal neovascularisation and endothelium repair (113).

Similarly, different mechanisms may be involved in the reduction of EPC level in blood and bone marrow after 5 weeks of ramipril treatment. For instance, long-term ACE inhibition may exhaust the bone marrow- or peripheral blood EPC pool or may make EPC´s incapable to respond adequately to a continuously present stimulus for mobilization in terms of desensitization. For example, as detailed above, increased bradykinin level and its B2-receptor play a very important role in the realization of ACE inhibitor effects on EPC´s. Blaukat et al. (16) demonstrated downregulation of the B2-receptor in human fibroblasts during prolonged agonist exposure. One can speculate that this mechanism also underlies the observed reduction of EPC levels. Since ramipril ameliorated EPC migratory capacity and stimulated angiogenesis more effectively than pressure overload, a depletion of EPC pool may not only be due to exhausted mobilization, but may also be attributable to enhanced recruitment and improved neo-endothelial incorporation of EPC´s.

Despite the fact that change of myocardial protein level of HIF-1α in pressure overload, treatment with ramipril and their combination does not play a role in feed back regulation of EPC mobilization and neoangiogenesis, other feedback mechanisms can cause the observed

effects. At the same time considering the pleiotropic effects of ramipril, the reduction of EPC´s level in blood and bone marrow after 5 weeks of treatment might be also caused by other still unknown mechanisms. Thus, the observed influence of ramipril on EPCs is not caused by HIF-1α-dependent induction of angiogenic factors in the heart and might be mediated not through one but through different mechanisms demonstrating thereby the pleiotropic effects of ACE inhibition.

7.3.3. Myocyte progenitor cells

Stem cells expressing early cardiac markers including myocyte enhancer factor 2 (MEF-2) were recently identified in bone marrow (70, 175). Moreover, they are mobilized to the peripheral blood and chemo-attracted to the infarcted myocardium in mice. Our findings demonstrate that increased cardiac afterload upregulated MEF-2^{pos}-GFP ^{pos} cells in the myocardium. Ramipril significantly enhanced their number in the sham group but not in the TAC mice. The increase of MEF-2^{pos}-GFP ^{pos} cells in the myocardium in pressure overload and under the influence of ramipril might be mediated through two different mechanisms. Treatment of TAC mice with ramipril did not significantly increase the number of bone marrow-derived myocyte progenitor cells in comparison to the untreated TAC group.

Myocyte progenitor cells did not transdifferentiate in adult cardiomyocytes or smooth muscle cells. However, as discussed in the introduction, bone marrow-derived cardiomyocytes were found in different models of myocardial damage: transaortic constriction (35), myocardial infarction (89) and pulmonary hypertension (144). Moreover, Matsushima et al. (89) revealed that perindopril stimulated the formation of new cardiomyocytes in the infarcted mouse heart. Nevertheless, our results highlight the ability of ramipril to increase the number of bone marrow-derived myocyte progenitor cells in myocardium. Further investigations must be performed to stimulate differentiation of mobilized myocyte progenitor cells into adult cardiomyocytes furnishing thereby the opportunity to create new therapeutic approaches for cardiomyocyte repopulation in diseased heart. All discussed mechanisms of ramipril action under condition of increased cardiac afterload are depicted in figure 47.

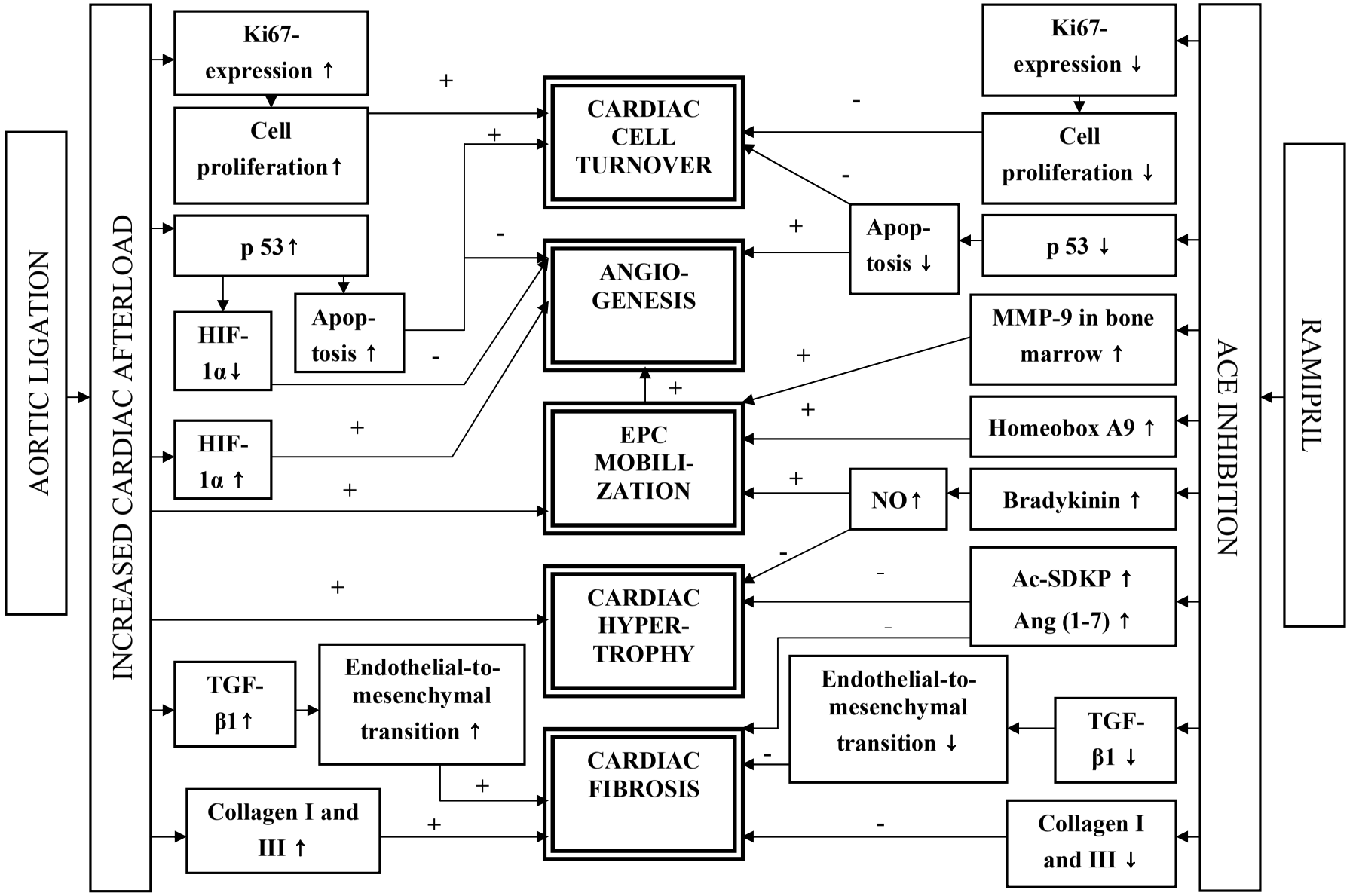


Fig. 47: Possible mechanisms of ramipril action in increased cardiac afterload

The following conclusions can be drawn from the present study.

7.4. Conclusions

1. Transverse aortic constriction for one day and five weeks is a suitable model to investigate the short- and long-term effects of ACE inhibition, which are not caused by reduction of peripheral blood pressure on cardiac remodeling under conditions of increased cardiac afterload.

2. Ramipril ameliorates cardiac remodeling by reducing LVH and cardiac fibrosis in pressure-overloaded myocardium.

3. Increased cardiac afterload for 5 weeks and radioactive radiation stimulate cell turnover in the myocardium which manifests itself as raise of numbers of proliferating and apoptotic non-cardiomyocytes, induction of Ki-67 synthesis and apoptosis in cardiomyocytes and an increase in apoptotic rates of endothelial cells. There are positive interrelationships between parameters characterizing remodeling of heart tissue and cell turnover parameters.

4. Ramipril normalizes cardiac cell turnover in pressure-overloaded myocardium by reducing apoptotic rates in cardiomyocytes and endothelial cells, diminishing the number of Ki-67-positive cardiomyocytes. Moreover, in bone marrow-transplanted animals ramipril decreases apoptosis and proliferation rates in cardiomyocytes and non-cardiomyocytes in all groups.

5. Pressure overload for 5 weeks diminishes the density of endothelial cells in the myocardium but does not change the ratio of endothelial cells to cardiomyocytes, the capillary density and the ratio of capillaries to cardiomyocytes. Ramipril significantly increases the density of endothelial cells, the ratio of endothelial cells to cardiomyocytes and the ratio of capillaries to cardiomyocytes in all groups.

6. Increased cardiac afterload and ramipril treatment in all groups promote extracardiac capillary growth.

7. In short-term experiment the EPC level in blood is increased by TAC and ramipril, in the bone marrow only by ramipril. In long-term experiment the EPC level in blood and in the bone marrow is augmented under conditions of increased cardiac afterload. In both short- and long-term experiments ramipril ameliorates migratory capacity of EPC´s.

8. Pressure overload of LV for 5 weeks raises the number of bone marrow-derived CD31 cells, but reduces the density of bone marrow-derived Tie-2-positive cells therein. By contrast, ramipril increases both parameters in the aortic-ligated group promoting thereby myocardial capillary growth.

9. Both short- and long-term treatment with ramipril decreases myocardial level of HIF-1α in all groups. The observed effects of ramipril on EPC´s and capillary growth in the myocardium are not caused by HIF-1α-dependent induction of angiogenic factors in the heart and might be mediated not through one but through different mechanisms caused by the pleiotropic effects of ACE inhibition.

10. Increased cardiac afterload and treatment with ramipril for five weeks upregulate the number of bone marrow-derived myocyte progenitor cells in the myocardium. Combination of TAC and ramipril does not enhance this effect.

Our findings testify that aside from its antihypertensive action ramipril has other ameliorative effects on the cardiovascular system, which comprise diminishment of cardiac remodeling, stimulation of cardiac and extracardiac neoangiogenesis and mobilization of bone marrow-derived stem cells participating in myocardial regeneration. Further investigations must unveil different mechanisms underlying these effects that will furnish the opportunities to create new strategies in the treatment of cardiovascular diseases and cardiovascular drug development.

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10. List of publications

Original articles

Pfeiffer P, Müller P, **Kazakov A**, Kindermann I, Böhm M (2006)
Time-dependent cardiac chimerism in gender-mismatched heart transplantation patients.
J Am Coll Cardiol; 48:843-850

Müller P*, **Kazakov A*,** Semenov A, Böhm M, Laufs U (2008)
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Clin Res Cardiol 99, Suppl 1, April 2010: P1335.

11. Curriculum Vitae

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2003- till now	Scientific assistant in the Cardiological scientific laboratory in the Department of Cardiology, Angiology and Intensive Care of University Clinic of Saarland (Homburg/Saar) (Director: Prof. Dr. M. Böhm)