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DISSERTATION

Molecular mechanisms of cardioprotection by cord blood mesenchymal stromal cells

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Table of contents

Summary……………………………………………………………………………. 3

Abstract / Zusammenfassung………………………………………………...…. 3

1. Introduction…………………………………………….…………………….…… 5

2. Methods………………………………………………….……….................. 7

3. Results……………………………………………….……………….………. 11

3.1 Secretory profile of "ischemic" CBMSCs……………………………... 11

3.2 Paracrine effects of CBMSCs on cardiomyocytes……………………... 11

3.3 Paracrine effects of CBMSCs on endothelial cells…………………….. 14

3.4 Hypoxic preconditioning of CBMSCs……………………………………. 16

4. Discussion……………………………………………………………….……… 19

5. References……………………………………………………………………… 22

Affidavit…………………………………………………………………………………… 23

Declaration of selected publications…………………………………………………... 24

Publication 1: Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells……………………………………………….. 25

Publication 2: Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling…………………………….. 36

Publication 3: Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*……………………………………………………………………... 48

Curriculum vitae………………………………………………………………………. 65

Complete list of publications…………………………………………………………… 68

Acknowledgements……………………………………………………………………… 70

Abstract / Zusammenfassung

Objective: Mesenchymal stromal cells (MSC) are currently being evaluated for supportive treatment of ischemic heart disease. The aim of the present work was to determine the paracrine effects of human umbilical cord blood-derived MSCs (CBMSC) on ischemic cardiomyocytes and endothelial cells and to characterize the relevant intracellular signaling pathways. Furthermore, hypoxic preconditioning (HP) was evaluated as a strategy to optimize the performance of CBMSCs under ischemic conditions.

Methods: CBMSCs were incubated in glucose/serum-free medium at 1% O_2 to simulate ischemic conditions *in vitro*. The thus conditioned medium was collected and analyzed for secreted pro-survival proteins. Murine HL-1 cardiomyocytes and human umbilical vein endothelial cells (HUVEC) were subjected to individually adapted *in vitro* ischemia protocols, where the effects of CBMSC conditioned medium on apoptosis, viability and proliferation were tested. Phosphorylation of AKT, ERK and STAT3 was determined, and its functional relevance for cytoprotection was confirmed by small-molecule inhibitor experiments. The MSC specificity of effects was assessed by use of medium conditioned by human fibroblasts. Finally, CBMSCs were hypoxically preconditioned prior to the actual “ischemic” challenge, and the anti-apoptotic and pro-angiogenic effects of HP were investigated.

Results: CBMSC conditioned medium contained HGF, VEGF, EGF, FGF-basic, angiopoetin-2 and IL-6 and counteracted apoptosis of HL-1 cardiomyocytes and HUVECs subjected to ischemia-like conditions. In HL-1 cardiomyocytes, survival signals were transmitted by the kinases AKT and ERK in a compensatory manner. This effect was in part specific to CBMSCs. In HUVECs, the beneficial effects were mediated by STAT3. Here, fibroblast conditioned medium possessed similar anti-apoptotic and pro-mitotic potency. Hypoxic preconditioning increased the survival and pro-angiogenic action of CBMSCs under ischemia-like conditions. This was associated with anti-apoptotic AKT signaling and expression of BCL-XL and BAG.

Conclusion: CBMSC released factors protect cardiac cells from ischemic damage by activating distinct anti-apoptotic signaling pathways, but this effect is only in part MSC-specific. HP improves the performance of CBMSCs in an ischemic environment and might thus be a useful strategy to enhance the efficacy of MSCs in cardiac cell therapy in a readily translatable fashion.

Zielsetzung: Mesenchymale Stromazellen (MSC) werden gegenwärtig zur unterstützenden Behandlung von ischämischer Herzkrankheit evaluiert. Ziel der vorliegenden Arbeit war es, die parakrinen Effekte von humanen MSCs aus Nabelschnurblut (CBMSC) auf ischämische Kardiomyozyten und Endothelzellen zu bestimmen und die relevanten intrazellulären Signalwege zu identifizieren. Zudem sollte evaluiert werden, ob Überleben und Wirksamkeit von CBMSCs unter ischämischen Bedingungen durch hypoxische Präkonditionierung (HP) gesteigert werden können.

Methoden: CBMSCs wurden in Glukose/Serum-freien Medium bei 1% O_2 inkubiert um ischämische Bedingungen *in vitro* zu simulieren. Das so konditionierte Zellkulturmedium wurde abgenommen und auf sezernierte Überlebensfaktoren untersucht. Murine HL-1 Kardiomyozyten und humane Endothelzellen aus der Nabelschnurvene (HUVEC) wurden individuell angepassten Ischämieprotokollen unterzogen, in denen die Effekte des CBMSC-konditionierten Mediums auf Apoptose, Viabilität und Proliferation untersucht wurden. Die Phosphorylierung von AKT, ERK und STAT3 wurde bestimmt und die funktionelle Relevanz dieser Signalwege für den jeweiligen Zelltyp mittels Small-Molecule-Inhibitoren bestätigt. Durch zusätzliche Verwendung von Fibroblasten-konditioniertem Medium wurde die MSC-Spezifität der Effekte bestimmt. Abschließend wurden CBMSCs vor der simulierten Ischämie hypoxisch präkonditioniert und die anti-apoptotischen und pro-angiogenen Effekte der Präkonditionierung untersucht.

Ergebnisse: CBMSC-konditioniertes Medium enthielt HGF, VEGF, EGF, FGF-basic, angiopoetin-2 und IL-6 und wirkte der Apoptose von HL-1 Kardiomyozyten und HUVECs unter Ischämie-ähnlichen Bedingungen entgegen. In HL-1 Kardiomyozyten vermittelten die Kinasen AKT und ERK in kompensatorischer Weise den Überlebensstimulus; dieser Effekt war teilweise CBMSC-spezifisch. In HUVECs wurden die positiven Effekte durch STAT3 vermittelt, hier zeigte Fibroblasten-konditioniertes Medium vergleichbare anti-apoptotische und pro-mitotische Wirkung. HP steigerte das Überleben und die pro-angiogene Wirkung von CBMSCs unter Ischämie-ähnlichen Bedingungen. Diese Effekte waren mit anti-apoptotischem AKT-Signalling und der Expression von BCL-XL und BAG assoziiert.

Schlussfolgerung: CBMSC-sezernierte Faktoren beschützen kardiale Zellen vor ischämischer Schädigung durch Aktivierung von anti-apoptotischen Signalwegen; dieser Effekt ist jedoch nur teilweise CBMSC-spezifisch. HP verbessert die Wirksamkeit von CBMSCs in ischämischer Umgebung und könnte daher eine nützliche Strategie sein, die Effizienz kardialer Zelltherapie zu steigern.

1. Introduction

Ischemic heart disease (IHD) is the leading cause of death in the world. In 2012, 13.2% of all deaths globally (7.4 million individuals) were due to IHD [1]. Mainly caused by arteriosclerotic changes of the coronary arteries, chronic IHD is characterized by a chronically deficient myocardial blood supply and the resulting lack of oxygen and nutrients. In the course of the disease, acute ischemic death of cardiomyocytes and chronic remodeling processes lead to a progressive loss of contractile myocardium and result in chronic heart failure (CHF). Revascularization procedures such as percutaneous transluminal coronary angioplasty or coronary artery bypass grafting are, in addition to pharmacological therapy, the standard treatments for chronic IHD. For more than a decade, transplantation of somatic stem/progenitor cells in the diseased heart has been under evaluation as a complementary treatment option for IHD and CHF. Several randomized controlled trials investigating the safety and efficacy of this novel therapy have been performed and are still ongoing. The cell populations investigated so far for cardiac cell therapy include mononuclear cells from bone marrow, unsorted or enriched for $CD133^+$, $CD34^+$ or aldehyde dehydrogenase$^+$ stem/progenitor cells as well as bone marrow- or adipose tissue-derived multipotent mesenchymal stromal cells (MSC) [2]. Human MSCs are spindle-shaped cells that are defined by the International Society for Cellular Therapy by their adherence to plastic culture surfaces, their expression (≥ 95%) of CD105, CD73 and CD90 and lack of expression (≤ 2%) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR as well as their potential to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* [3]. In addition to adult bone marrow, adipose tissue and peripheral blood, MSCs can also be isolated from neonatal tissues like the placenta, the umbilical cord and umbilical cord blood, and these juvenile cells have been attributed with a particularly high proliferative and functional capacity [4]. Numerous pre-clinical studies have demonstrated beneficial effects of adult MSCs on the injured myocardium and investigated various concepts of regeneration and repair. Since improvement of cardiac function was observed even without trans-differentiation of the transplanted cells, MSCs were proposed to mediate their beneficial action via secreted factors. These paracrine effects range from a favorable influence on myocardial remodeling to pro-angiogenic activity and protection of ischemically damaged cardiac cells [5]. However, the improvement in heart function observed in human patients has not been as pronounced as animal experiments have suggested. Although some clinical trials

with bone marrow MSCs yielded encouraging results, the need for further improvement has also become evident [6].

In order to improve the efficacy of cardiac cell therapy, a more detailed understanding of the underlying molecular mechanisms is required. Therefore this work was dedicated to the issue of how MSC secreted factors confer protection in myocardial ischemia. In a cell-free approach, using only MSC conditioned medium, the beneficial effects on the two cardiac cell types with immediate functional relevance, cardiomyocytes and endothelial cells, were investigated and relevant intracellular signaling pathways were to be identified. For this purpose, specific *in vitro* models of simulated ischemia were established using the murine cardiac muscle cell line HL-1 and human umbilical vein endothelial cells (HUVEC). Cyropreserved MSCs derived from human umbilical cord blood (CBMSC) were chosen for the experiments, because they are juvenile and easily available and their low alloreactivity makes them suitable for allogeneic applications. To assess the MSC-specificity of effects, also the response of "ischemic" cardiac cells to factors released by juvenile fibroblasts was investigated. A limiting factor for the efficacy of cardiac cell therapy is the poor survival of transplanted cells in the ischemic heart. MSCs themselves suffer ischemic damage, and enhancing their tolerance against ischemia might be a useful strategy to improve their performance in clinical applications. Hypoxic preconditioning is an approach known for its cytoprotective effect on several cell types [7], its favorable effects on "ischemic" CBMSCs and the molecular mechanisms involved in this phenomenon were therefore also investigated. To this end, a preconditioning protocol tailored to CBMSCs was to be designed.

2. Methods

Cell culture: Cryopreserved human CBMSCs were provided by Karen Bieback, who isolated and characterized them according to a previously published protocol [8]. Cord blood was obtained with informed consent of the mother, according to the principles outlined in the Declaration of Helsinki and with approval of the local ethics committees in Mannheim and Heidelberg. CBMSCs were expanded to the fourth passage and cultured in Dubecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Commercially available cryopreserved human foreskin fibroblasts were cultured in Iscove’s Modified Eagle Medium supplemented with 10% FBS and 1% P/S. Cryopreserved murine HL-1 cardiomyocytes were provided by William C. Claycomb, who established this cell line from transgenic mice, in which expression of the SV40 large T-antigen was targeted to atrial cardiomyocytes via the atrial natriuretic factor promoter [9]. HL-1 cardiomyocytes were cultured in gelatin/fibronectin-coated vessels in Claycomb medium supplemented with 10% FBS, 1% P/S, 2 mM L-glutamine and 100 µM norepinephrine. Commercially available HUVECs were cultured in gelatin-coated vessels in endothelial growth medium-2 with 10% FBS. Co-culture experiments were performed in Transwell®-96 Tissue Culture Systems with 1 µm pore size with HUVECs plated in the lower compartment and CBMSCs in the upper compartment.

Preparation of conditioned medium: CBMSCs or fibroblasts were cultured in glucose-free DMEM without FBS in an atmosphere of 1% O_2 and 5% CO_2, achieved by replacing O_2 with N_2 in a multi-gas incubator. After six days, conditioned medium was collected and dead cells were removed by centrifugation. Equivalent medium kept under the same conditions in vessels without cells was prepared as non-conditioned control medium.

Analysis of conditioned medium: For quantification of growth factors and cytokines in six days conditioned medium a Luminex® Screening Assay for epidermal growth factor (EGF), fibroblast growth factor (FGF)-basic, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, and angiopoetin-2 was performed. VEGF concentration after the first, second and third day was determined with the QuantiGlo® ELISA Human VEGF Immunoassay. Assays were performed in duplicate according to the manufacturer’s instructions.

***In vitro* ischemia/hypoxia models:** Hypoxic preconditioning: CBMSCs were subjected to a hypoxic (1% O_2 / 5% CO_2) atmosphere while kept in their standard medium. Simulated ischemia: Cells were subjected to a hypoxic (1% O_2 / 5% CO_2) atmosphere in conditioned or non-conditioned glucose/FBS-free DMEM. Glucose/serum deprivation: HUVECs were kept in conditioned or non-conditioned glucose/FBS-free DMEM in a normoxic (21% O_2 / 5% CO_2) atmosphere. When varying combinations of oxygen, glucose and serum withdrawal were tested, cells were kept in DMEM or glucose-free DMEM with or without 10% FBS under hypoxic or normoxic conditions.

Signaling pathway inhibition: Cells were treated with specific small-molecule inhibitors 1 h prior to and during simulated ischemia or glucose/serum deprivation. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor Wortmannin, the mitogen-activated protein kinase kinase (MEK)1/2 inhibitor UO126 and the signal transducer and activator of transcription (STAT)3 inhibitor Stattic were diluted in dimethyl sulfoxide and an equivalent amount of solvent was used for controls. The efficiency of Wortmannin, UO126 and Stattic in suppressing the phosphorylation of protein kinase B (AKT), extracellular-signal-regulated kinase (ERK)1/2 and STAT3 was confirmed by western blot.

Determination of apoptosis and necrosis: For high content imaging based detection, cells were incubated with the poly-caspase substrate sulforhodamine-Val-Ala-Asp-fluoromethylketone or with annexin V (AnnV)-fluorescein isothiocyanate (FITC) and ethidium homodimer (EthD)-III. Cells were fixed with 4% formaldehyde and nuclei were stained with 4',6-diamidino-2-phenylindole or Hoechst 33342. Poly-caspase activity, phosphatidylserine exposure, loss of plasma membrane integrity, nuclear shrinking and fragmentation as well as the total cell number were quantified using the high content imaging system Operetta® and Harmony® software. The nuclear fragmentation index was defined as the coefficient of variation of nuclear stain fluorescence intensity. For calculation of proportions of detached dead cells and adherent poly-caspase-active and non-active cells, data were normalized to the mean cell number in plates not subjected to simulated ischemia. Detached cells were subjected to trypan blue exclusion and proved to be 100% non-viable. Caspase-3/7 activity was measured using the Caspase-Glo® 3/7 Assay. After removal of detached cells by a medium exchange, the assay was performed according to the manufacturer's instructions. Data were normalized to the cell number determined in simultaneously performed experiments with the Operetta® system. The

number of cells showing morphological signs of apoptosis (shrinkage, rounding, and/or membrane blebbing) was determined by manual enumeration from light microscopy images. All assays were performed in triplicate.

Determination of viability/metabolic activity: Conversion of the tetrazolium compounds 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and water soluble tetrazolium (WST)-8 to their respective formazan by metabolically active cells was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay and the Cell Counting Kit-8, respectively. Assays were performed in triplicate according to the manufacturer's instructions.

Determination of proliferation: 5-bromo-2-deoxyuridine (BrdU) incorporation by DNA-synthesizing cells was determined using the Cell Proliferation ELISA BrdU according to the manufacturer's instructions. Experiments were performed in triplicate.

Protein analysis by western blot: Cells were lysed in buffer containing proteinase and phosphatase inhibitors. Protein concentration was quantified and 30 – 35 µg denatured protein was resolved in 12% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes by use of semi-dry or tank blot systems. Membranes were blocked and incubated with monoclonal mouse anti-total protein and rabbit anti-phospho-protein primary antibodies overnight at 4 °C: AKT, β-Actin, BCL2-associated agonist of cell death (BAD), ERK1/2, STAT3, phospho-AKT (Ser473), phospho-BAD (Ser136), phospho-ERK1/2 (Thr202/Tyr204) and phospho-STAT3 (Tyr705). Membranes were incubated with IRDye®- or horseradish peroxidase-conjugated secondary antibodies and infrared or chemiluminescent protein detection was performed.

RNA analysis by real time quantitative PCR (RT-qPCR): Total RNA was purified from cells using the RNeasy® Mini Kit. cDNA was synthesized from DNase-treated RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR with random hexamers as reaction primers. RT-qPCR was performed using 2.5 ng template (7.5 ng for VEGF) in 25 µl reaction volume with 2 x Power SYBR® Green PCR Master Mix and the primers listed in table 1.

Gene	Forward primer 5‘ – 3‘	Reverse primer 5‘ – 3‘	Temp.
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	56 / 60 °C
Bag-1	TGAGAAGCACGACCTTCATGT	GGAACCCCTATGACCTCTTCA	56 °C
Bcl-2	GAACTGGGGGAGGATTGTGG	GCCGGTTCAGGTACTCAGTC	60 °C
Bcl-XL	GAGCTGGTGGTTGACTTTCTC	TCCATCTCCGATTCAGTCCCT	56 °C
VEGF	AGACACACCCACCCACATAC	TGCCAGAGTCTCTCATCTCC	58 °C

Table 1: RT-qPCR primers. Bag-1: Bcl2-associated anthanogene-1, Bcl-2: B-cell lymphoma-2, Bcl-XL: B-cell lymphoma-extra-large, VEGF: vascular endothelial growth factor.

Amplification conditions were as follows: 95 °C for 10 min followed by 45 cycles consisting of 95 °C for 15 s, the respective primer annealing temperature for 30 s, 68 °C for 60 s. All measurements were carried out in triplicate. Gene-of-interest expression (E) was calculated as $E = PE^{(-Ct)}$, where PE is primer efficiency and Ct is the number of cycles at which the fluorescence exceeds the threshold, and normalized to β-actin expression. Primer efficiency was determined by means of calibration curves using the formula: $PE = 10^{(-1/slope)}$.

Statistics: Results are expressed as mean ± standard error of the mean. Intergroup differences between more than two groups were analyzed by one-way analysis of variance (ANOVA) with post-hoc test: Bonferroni for comparisons between all groups or Dunnett’s t-test when treatment groups were only compared to the control group. When only two groups were compared, a two-tailed Student’s t-test was applied. For intergroup comparisons of data obtained on consecutive days, repeated-measures ANOVA with Bonferroni correction was applied. Time-related changes within only one group were analyzed by univariate ANOVA with Bonferroni correction. IBM SPSS statistics 18 - 20 was used for data analysis. A P-value < 0.05 was considered significant. For the exact number of replicates of the respective assays please refer to the attached publications.

3. Results

3.1 Secretory profile of “ischemic” CBMSCs

CBMSCs were incubated in glucose/serum-free medium in a hypoxic (1% O_2) atmosphere to imitate ischemic conditions *in vitro*. The culture medium of the cells was collected, probed for known growth factors and cytokines and applied as a cell-free carrier solution of these factors in “ischemia” experiments with cardiomyocytes (3.2) and endothelial cells (3.3). To assess the CBMSC-specificity of effects, conditioned medium (CM) was also prepared from juvenile fibroblasts. CBMSCs secreted HGF, VEGF, EGF, FGF-basic, angiopoetin-2 and IL-6. The concentrations of VEGF, EGF, and angiopoetin-2 were similar in CBMSC- and fibroblast-CM. However, fibroblast-CM contained less HGF, more FGF-basic and more IL-6. IL-10 and TNFα were not detectable in either medium (Fig. 1).

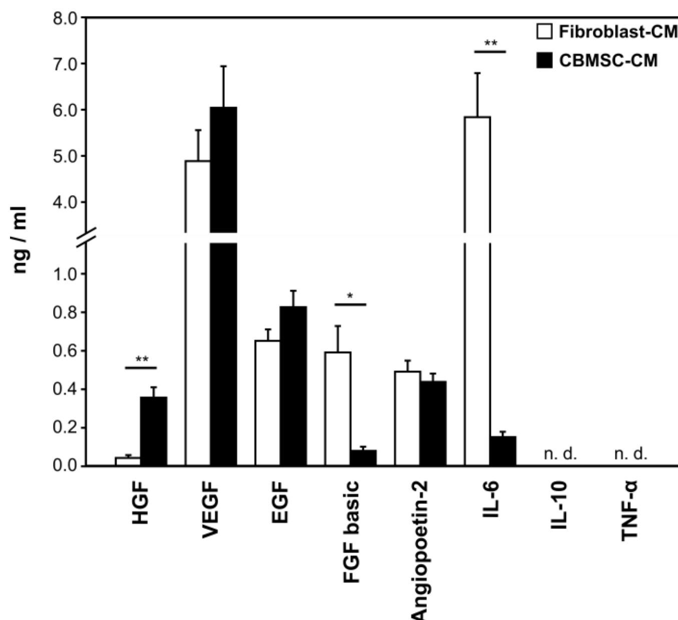

Figure 1: Growth factor and cytokine secretion by CBMSCs and fibroblasts. Growth factors and cytokines in the culture medium of CBMSCs and fibroblasts after 6 days of simulated ischemia (n=6); * $P<0.05$, ** $P<0.001$. Modified from figure 2 of Bader et al., Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells, European Journal of Cardiothoracic Surgery, 2014, 45(6):983-92, by permission of Oxford University Press.

3.2 Paracrine effects of CBMSCs on cardiomyocytes

3.2.1 *In vitro* ischemia model for HL-1 cardiomyocytes

To determine the most suitable *in vitro* ischemia model for HL-1 cardiomyocytes, cells were subjected to varying combinations of oxygen, glucose and serum withdrawal. Poly-caspase activity, cell loss and nuclear fragmentation were highest after combined withdrawal of all three factors (Fig. 2A-C); hence this condition was applied and referred to as “simulated ischemia” in subsequent experiments with HL-1 cardiomyocytes (sections 3.2.2 – 3.2.4).

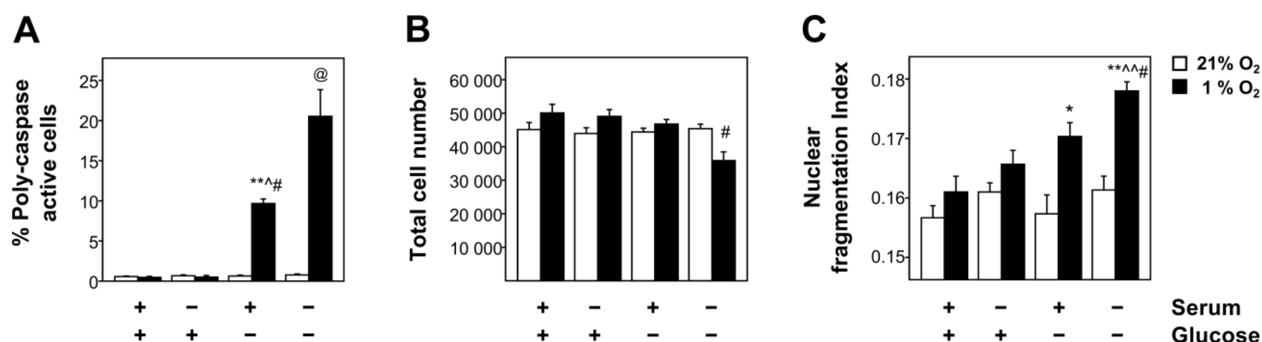

Figure 2: Effect of oxygen, glucose and serum withdrawal on HL-1 cardiomyocytes. (**A**) Poly-caspase activity, (**B**) remaining cell number and (**C**) nuclear fragmentation in response to 5 h of single and combined oxygen, glucose and serum withdrawal (n=3); @ P<0.001 vs. all other conditions; * p<0.05, ** p<0.01 vs. corresponding medium in normoxia; ^ P<0.01, ^^ P<0.001 vs. full medium in normoxia; # P<0.01 vs. full medium in hypoxia. Modified from figure 1 of Bader et al., Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells, European Journal of Cardiothoracic Surgery, 2014, 45(6):983-92, by permission of Oxford University Press.

3.2.2 Protective effects of CBMSC conditioned medium on HL-1 cardiomyocytes

To investigate whether CBMSC released factors are able to attenuate the “ischemic” damage, HL-1 cardiomyocytes were subjected to simulated ischemia in CBMSC-CM. The overall proportion of detached dead HL-1 cardiomyocytes was lower in the presence of CBMSC-CM, but the proportion of poly-caspase active cells remained unchanged (Fig. 3A). The rate of phosphatidylserine exposing cells with lost plasma membrane integrity (late apoptosis) was reduced, yet EthD-III permeable cells without phosphatidylserine exposure (necrosis) were slightly more frequent (Fig. 3B). To determine whether protective effects are CBMSC-specific, a viability assay was performed also with fibroblast-CM. Post-“ischemic” viability of HL-1 cardiomyocytes was enhanced in CBMSC-CM, whereas there was no significant difference in fibroblast-CM (Fig. 3C).

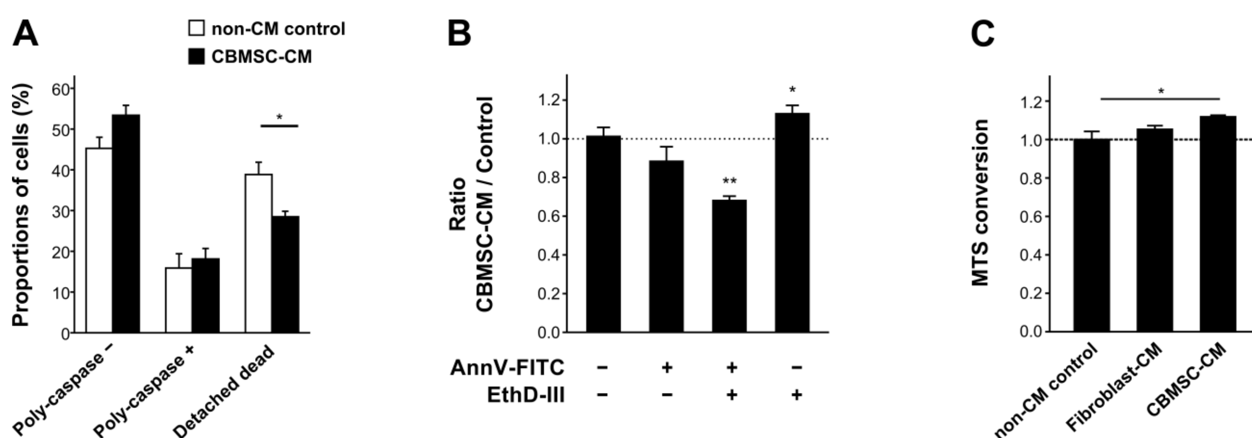

Figure 3: Protection of “ischemic” HL-1 cardiomyocytes by CBMSC conditioned medium. (**A**) Proportions of detached dead cells and adherent poly-caspase active and non-active cells (n=5), (**B)** phosphatidylserine exposure (AnnV-FITC +) and loss of plasma membrane integrity (EthD-III +) of adherent cells (n=4) and (**C**) viability relative to non-conditioned medium (n≥9), all after 5 h of simulated ischemia; * P<0.05, ** P<0.001 vs. non-conditioned control medium. Modified from figures 3, 4 and 5 of Bader et al., Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells, European Journal of Cardiothoracic Surgery, 2014, 45(6):983-92, by permission of Oxford University Press.

3.2.3 Survival signaling pathway activation in HL-1 cardiomyocytes by CBMSC secreted factors

Next, activation of different survival pathway checkpoints was examined in the “ischemic” HL-1 cardiomyocytes. In the presence of CBMSC-CM, AKT, ERK1/2 and STAT3 phosphorylation was greater than in non-conditioned control medium. Again, the CBMSC-specificity was tested using fibroblast-CM. Here, phosphorylation of AKT, ERK1/2 and STAT3 appeared mildly enhanced but was not significantly different to that in control medium. Albeit, STAT3 phosphorylation was less pronounced than in CBMSC-CM (Fig. 4A).

3.2.4 Functional relevance of activated survival signaling pathways in HL-1 cardiomyocytes

To determine the functional relevance of the survival signaling pathways activated by CBMSC released factors, cellular viability was examined after “ischemic” incubation in the presence of specific small-molecule inhibitors targeting AKT, ERK1/2 and STAT3 phosphorylation. In CBMSC-CM, viability of HL-1 cardiomyocytes was greater than in non-conditioned control medium. Individual inhibition of the AKT, ERK1/2 or STAT3 pathways did not lower the beneficial effect of CBMSC-CM. When phosphorylation of STAT3 was inhibited in combination with AKT or ERK1/2, the beneficial effect was also not affected. Only combined blocking of AKT and ERK1/2 signaling extinguished the higher viability in CBMSC-CM (Fig. 4B).

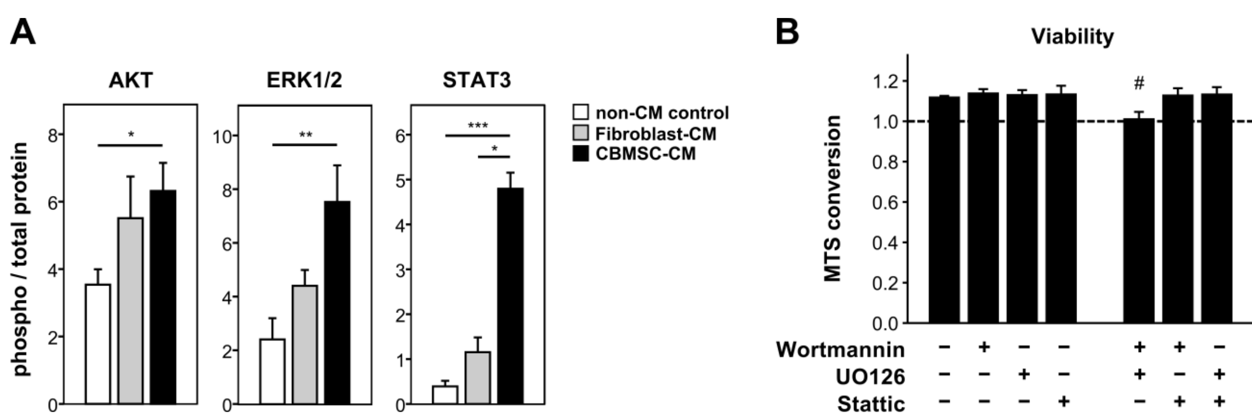

Figure 4: CBMSC conditioned medium triggered survival signaling and its functional relevance in HL-1 cardiomyocytes (**A**) Phosphorylation of AKT, ERK1/2 and STAT3, all relative to non-ischemia, after 3 h of simulated ischemia in conditioned or non-conditioned medium (n≥4); * P<0.05, ** P<0.01, *** P<0.001. (**B)** Viability after 5 h of simulated ischemia in CBMSC-CM with small-molecule inhibitors of AKT (Wortmannin), ERK1/2 (UO126) and STAT3 (Stattic) phosphorylation, relative to non-conditioned medium (n≥4); # P<0.05 vs. CBMSC-CM solvent control. Modified from figures 6 and 7 of Bader et al., Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells, European Journal of Cardiothoracic Surgery, 2014, 45(6):983-92, by permission of Oxford University Press.

3.1 and 3.2 Adapted from publication 1: Bader et al., Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells, European Journal of Cardiothoracic Surgery, 2014, 45(6):983-92.

3.3 Paracrine effects of CBMSCs on endothelial cells

3.3.1 *In vitro* ischemia model for HUVECs

To establish a suitable *in vitro* ischemia model for HUVECs, glucose and serum were withdrawn from cells in hypoxia and in normoxia. Under hypoxic conditions, cell number, viability and proliferation of HUVECs were reduced, but the impact of glucose/serum deprivation was more severe in normoxia (Fig. 5 A-C). Hence, normoxic glucose/serum deprivation was applied to induce cellular damage in HUVECs in subsequent experiments (sections 3.3.2 – 3.3.4).

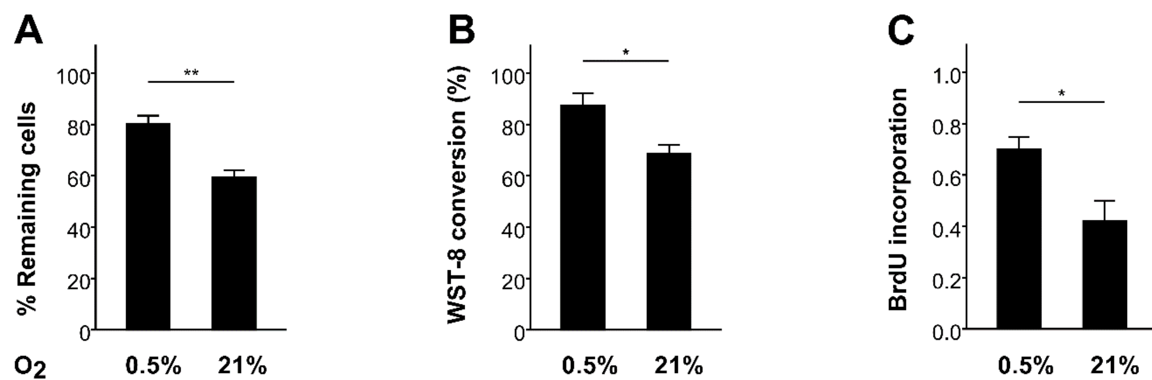

Figure 5: Effect of hypoxic and normoxic glucose/serum deprivation on HUVECs. (**A**) Cell number (n≥6), (**B**) viability (n=8) and (**C**) proliferation (n≥12), all relative to full medium, after 6 h of glucose/serum deprivation at 0.5% O_2 and 21% O_2; * $P<0.01$, ** $P<0.001$. Modified from figure 1 of Bader et al., Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling, Cellular Physiology and Biochemistry, 2014, 34(3):646-657.

3.3.2 Protective effects of CBMSC conditioned medium on HUVECs

To investigate whether factors released from CBMSCs or fibroblasts are able to lower endothelial cell damage, HUVECs were subjected to glucose/serum deprivation in the respective conditioned media. Cell number, viability and proliferation were higher in the presence of both CBMSC- and fibroblast-CM (Fig. 6 A-C). The frequency of cells showing morphological signs of apoptosis (shrinkage, rounding, and/or membrane blebbing) was lower in both conditioned media (Fig. 6 D). The rate of cells exposing phosphatidylserine with preserved plasma membrane integrity (early apoptosis) was also reduced in both conditioned media, as was the rate of non-phosphatidylserine-exposing but EthD-III permeable cells (necrosis) (Fig. 6 E).

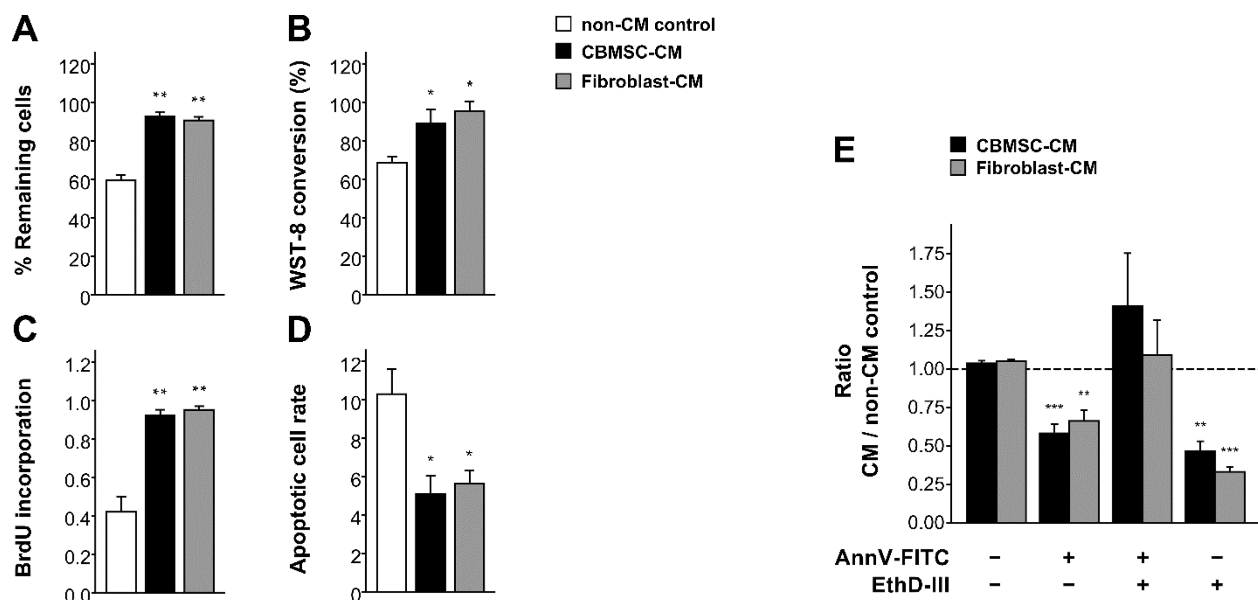

Figure 6: Protection of glucose/serum deprived HUVECs by CBMSC conditioned medium. (**A**) Cell number (n≥8), (**B**) viability (n≥5) (**C**) proliferation (n≥5) and (**D**) frequency of cells exhibiting morphological characteristics of apoptosis (n≥7), all relative to full medium, and (**F**) phosphatidylserine exposure (AnnV-FITC +) and loss of plasma membrane integrity (EthD-III +) (n=8) after 6 h of glucose/serum deprivation in CBMSC- and fibroblast-CM; * P<0.05, ** P<0.01, *** P<0.001 vs. non-conditioned medium. Modified from figures 2 and 3 of Bader et al., Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling, Cellular Physiology and Biochemistry, 2014, 34(3):646-657.

3.3.3 Survival signaling pathway activation in HUVECs by CBMSC secreted factors

Here again, activation of the three survival pathway checkpoints was examined. AKT phosphorylation of glucose/serum deprived HUVECs was decreased in the presence of CBMSC-CM and a similar but not significant reduction was observed in fibroblast-CM. Phosphorylation of STAT3 was elevated in both CBMSC- and fibroblast-CM. ERK1/2-phosphorylation was enhanced in fibroblast-CM, while in CBMSC-CM the increase did not reach statistical significance (Fig. 7 A). Furthermore, *BCL-2* mRNA expression of glucose/serum deprived HUVECs was increased in CBMSC- and fibroblast-CM (Fig. 7 B).

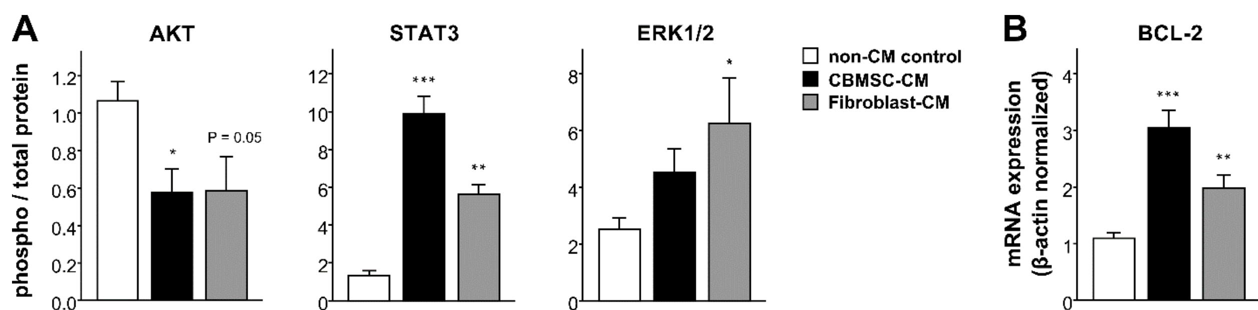

Figure 7: CBMSC conditioned medium triggered survival signaling in HUVECs. (**A**) Phosphorylation of AKT, STAT3, and ERK1/2 (n≥4) and (**B**) BCL-2 expression (n≥7), all relative to full medium, after 3 h of glucose/serum deprivation in CBMSC- and fibroblast-CM; * P<0.05, ** P<0.01, *** P<0.001 vs. non-conditioned medium. Modified from figure 5 of Bader et al., Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling, Cellular Physiology and Biochemistry, 2014, 34(3):646-657.

3.3.4 Functional relevance of activated survival signaling pathways in HUVECs

To determine the functional relevance of activated survival signaling pathways, cellular viability and expression of the anti-apoptotic gene *BCL-2* were examined after glucose/serum deprivation in the presence of specific small-molecule inhibitors. Viability of glucose/serum deprived HUVECs was increased in CBMSC-CM. This beneficial effect was completely abolished when phosphorylation of STAT3 was inhibited but not attenuated when ERK1/2 signaling was blocked (Fig. 8 A). *BCL-2* mRNA expression was enhanced in CBMSC-CM. Under STAT3 inhibition this induction of *BCL-2* was completely eliminated (Fig. 8 B).

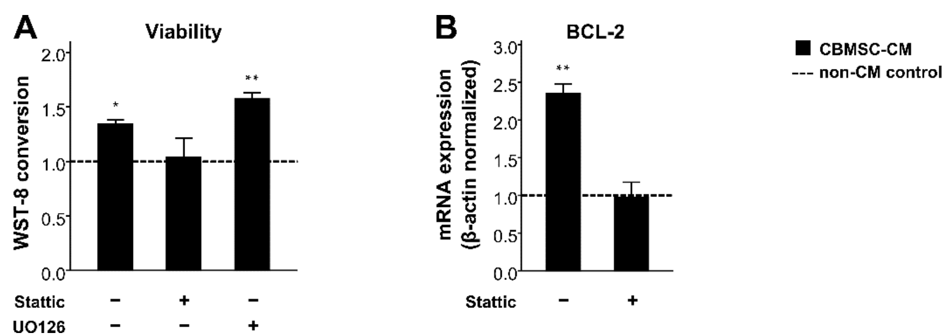

Figure 8: Functional relevance of CBMSC conditioned medium triggered survival signaling in HUVECs. (**A**) Viability (n≥8) after 6 h and (**B**) BCL-2 expression (n≥8) after 3 h of glucose/serum deprivation in CBMSC-CM with small-molecule inhibitors of STAT3 (Stattic) or ERK1/2 (UO126) phosphorylation, both relative to non-conditioned medium; * P<0.01, ** P<0.001 vs. non-conditioned medium. Modified from figure 6 of Bader et al., Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling, Cellular Physiology and Biochemistry, 2014, 34(3):646-657.

3.3 Adapted from publication 2: Bader et al., Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling, Cellular Physiology and Biochemistry, 2014, 34(3):646-657.

3.4 Hypoxic preconditioning of CBMSCs

3.4.1 Enhancement of CBMSCs' ischemic tolerance

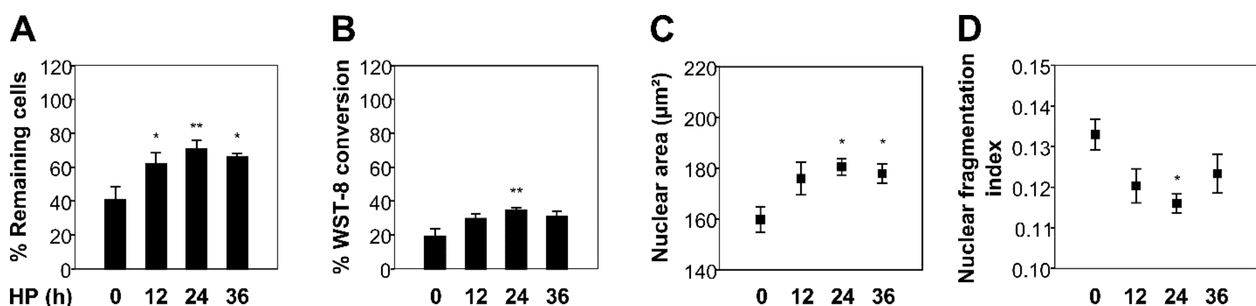

Figure 9: Reduced cell loss and damage of hypoxically preconditioned CBMSCs. (**A**) Cell number and (**B**) viability, both relative to untreated cells, (**C**) nuclear area and (**D**) nuclear fragmentation after 0, 12, 24 or 36 h of hypoxic preconditioning (HP) followed by 24 h of simulated ischemia (n≥6); * P<0.05, ** P<0.01 vs. 0 h HP. Modified from figure 1 of Bader et al., Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*, PLoS One, 2015, 10(9):e0138477.

CBMSCs themselves suffer ischemic damage, which impairs their beneficial action on other cell types. To evaluate whether their ischemic tolerance can be increased by hypoxic preconditioning, CBMSCs were incubated under hypoxic conditions in full medium prior to an “ischemic” challenge, achieved by hypoxic incubation in glucose/serum-free medium. Different time periods were tested to determine the optimal preconditioning protocol. “Ischemic” damage of CBMSCs was reduced by hypoxic preconditioning and was lowest after a preconditioning period of 24 h. Here, cell number and viability were enhanced and nuclear shrinking and fragmentation were attenuated (Fig. 9 A-D). Hence, in subsequent experiments (section 4.4.2 – 4.4.3) a hypoxic preconditioning period of 24 h was applied.

3.4.2 Activation of anti-apoptotic mechanisms in CBMSCs

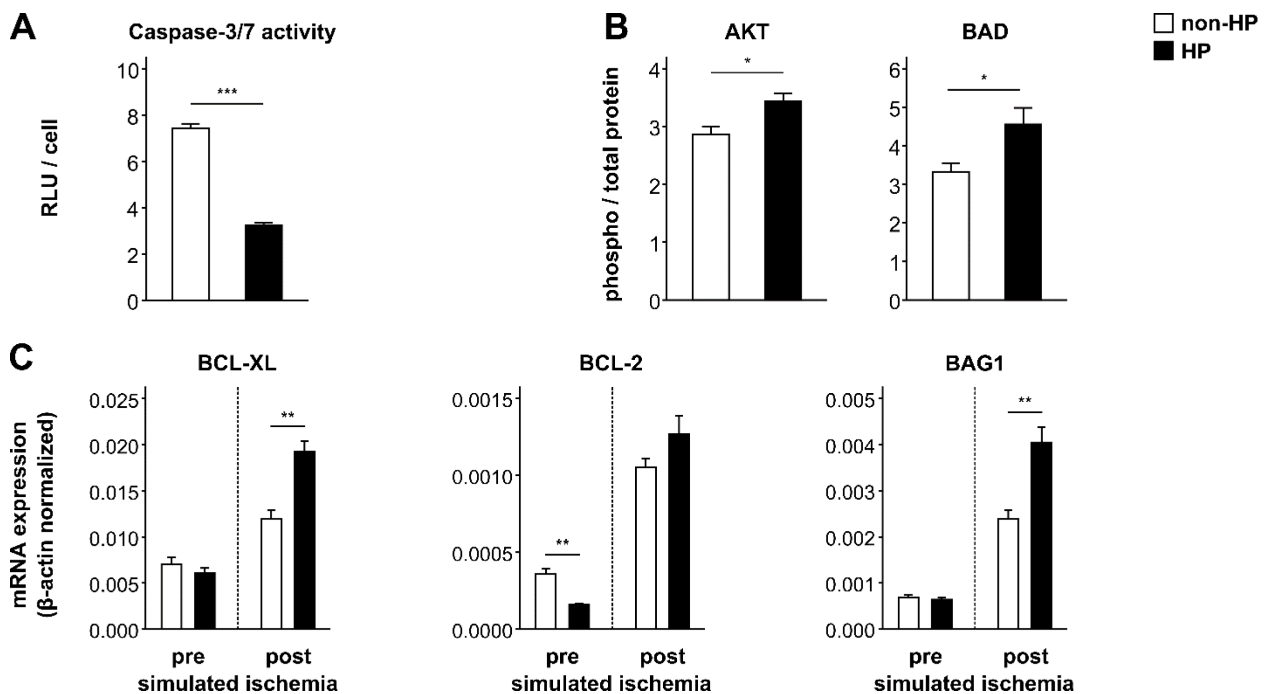

Figure 10: Anti-apoptotic effect of hypoxic preconditioning on “ischemic” CBMSCs. (**A**) Cellular caspase-3/7 activity (n=3) and (**B**) phosphorylation of AKT and BAD (n=4) after 24 h of simulated ischemia with or without preceding hypoxic preconditioning (HP) for 24 h, (**C**) expression of BCL-XL, BCL-2 and BAG 1 (n=6) prior and after 24 h of simulated ischemia with or without preceding HP for 24 h; * P<0.05, ** P<0.01, *** P<0.001. Modified from figures 3 and 4 of Bader et al., Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*, PLoS One, 2015, 10(9):e0138477.

In hypoxically preconditioned CBMSCs, caspase-3/7 activity after simulated ischemia was substantially lower than in non-preconditioned cells (Fig 10 A). AKT phosphorylation and phosphorylation (i.e. inactivation) of the pro-apoptotic AKT target protein BAD were elevated (Fig. 10 B). Before the onset of simulated ischemia, mRNA expression of anti-apoptotic factors *BCL-XL* and *BAG1* was similar in hypoxically preconditioned and non-preconditioned CBMSCs, whereas *BCL-2* expression was suppressed by hypoxic

preconditioning. Simulated ischemia augmented the mRNA expression of all three factors. However, hypoxically preconditioned cells showed significantly higher levels of *BCL-XL* and *BAG1* after simulated ischemia, and their initial loss of *BCL-2* expression was fully compensated (Fig. 10 C).

3.4.3 Enhancement of pro-angiogenic effects of CBMSCs

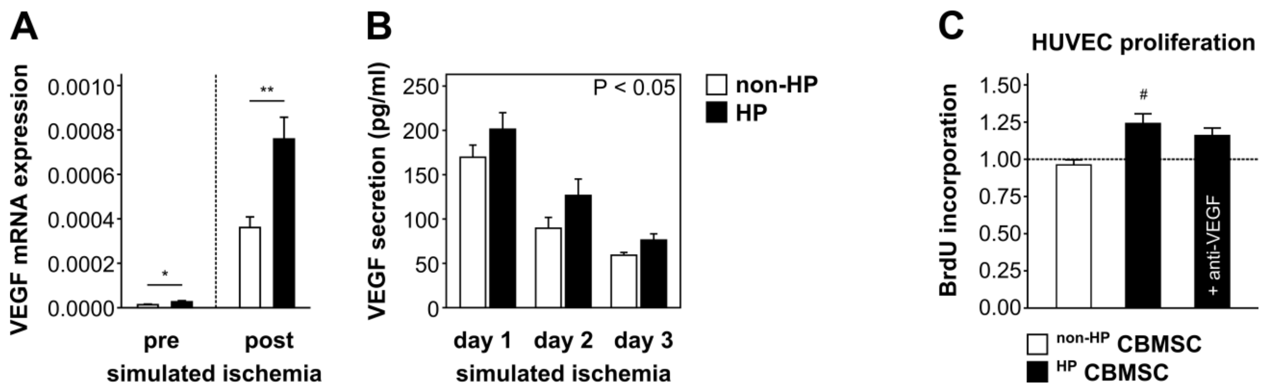

Figure 11: Pro-angiogenic effect of hypoxic preconditioning on "ischemic" CBMSCs. (**A**) VEGF expression prior and after 24 h of simulated ischemia with or without preceding hypoxic preconditioning (HP) for 24 h (n≥6); * P<0.05, ** P<0.01. (**B**) VEGF secretion on three consecutive days of simulated ischemia with or without preceding HP for 24 h (n≥11). (**C**) Proliferation of HUVECs after 24 h of simulated ischemia in coculture with hypoxically preconditioned or non-preconditioned CBMSCs and with addition of anti-VEGF antibody (n≥3). Data expressed in relation to HUVEC mono cell cultures; # P<0.05 vs. HUVEC mono cell cultures. Modified from figures 5 and 6 of Bader et al., Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*, PLoS One, 2015, 10(9):e0138477.

To evaluate whether the hypoxic preconditioning protocol also augments functional properties of CBMSCs, expression of the pro-angiogenic growth factor VEGF was quantified. Prior to the onset of simulated ischemia, VEGF mRNA expression was already higher in hypoxically preconditioned CBMSCs than in non-preconditioned cells. Although its expression markedly increased in both groups during simulated ischemia, the VEGF mRNA level was still twice as high in hypoxically preconditioned cells (Fig. 11 A). VEGF protein secretion, measured on three consecutive days of simulated ischemia, was accordingly higher in hypoxically preconditioned CBMSC (Fig. 11 B). Finally, the paracrine effect of CBMSCs on endothelial cells was studied in a transwell co-culture model. Proliferation of HUVECs under simulated ischemia was enhanced in co-culture with hypoxically preconditioned CBMSCs but not with non-preconditioned CBMSCs. This mitogenic effect was partially neutralized in the presence of an antibody against VEGF (Fig. 11 C).

3.4 Adapted from publication 3: Bader et al., Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*, PLoS One, 2015, 10(9):e0138477.

4. Discussion

Protective effects of MSCs on the ischemic heart have been demonstrated in various animal models. However, functional benefits achieved in humans have so far been modest and, therefore, the clinical relevance of cardiac cell therapy is still low. One of the major problems is that cells are transplanted into ischemic tissue. For a more successful clinical translation it is therefore necessary to better understand the impact of MSCs on cardiac cells under ischemic stress, as well as to improve the resistance of transplanted cells to ischemia. Prerequisite is a detailed understanding of the underlying molecular mechanisms and for this purpose suitable experimental models are required. As MSCs are proposed to convey cardioprotection in a paracrine manner, a cell-free approach using only conditioned medium of CBMSCs was deployed in the present work. The cardiac muscle cell line HL-1 and HUVECs were used to study the protective effects of this medium on "ischemic" cardiomyocytes and endothelial cells on a molecular level. Emphasis was placed on three well known survival signaling pathways and their central checkpoints, the kinases AKT and ERK and the transcription factor STAT3. Furthermore, hypoxic preconditioning was investigated as a strategy to further improve the survival and therapeutic potential of CBMSCs in an ischemic environment and a preconditioning protocol tailored to these cells was established.

Loss of HL-1 cardiomyocytes during simulated ischemia was ameliorated by conditioned medium of CBMSCs, which was applied as a cell-free solution of their secreted factors. Although early apoptotic markers remained unchanged in the presence of CBMSC conditioned medium, late apoptosis of HL-1 cardiomyocytes was clearly attenuated and, adversely, necrosis was slightly enhanced. In the conditioning phase, CBMSCs released anti-apoptotic proteins in the medium but also diminished its nutrient (amino acids) content. With this in mind, it appears plausible that signal-regulated apoptotic cell death was counteracted, while more cardiomyocytes died passively by necrosis due to the reduced energy supply. CBMSC conditioned medium activated AKT, ERK and STAT3 signaling in HL-1 cardiomyocytes, but only AKT and ERK proved to be relevant for CBMSC mediated cytoprotection. The beneficial effect of CBMSC conditioned medium was only abolished when both PI3K/AKT and MEK/ERK signaling were simultaneously blocked. Both pathways have been shown to crosstalk distinctly, with inhibition of one pathway activating the other and vice versa [10]. Furthermore, they are proposed to converge on common protective endpoints like the suppression of pro-apoptotic BCL-2

family members [11-13]. When STAT3 signaling was inhibited, alone or in combination with AKT or ERK, CBMSC-mediated protection was not affected. Hence, in cardiomyocytes, STAT3 appears not to be a key mediator of the paracrine survival stimuli of CBMSCs. This is further corroborated by the fact that STAT3 activity alone was unable to compensate for the simultaneous loss of AKT and ERK signaling. The effect of fibroblast conditioned medium on AKT, ERK and STAT3 activation and HL-1 cardiomyocyte viability was limited and did not reach statistical significance. Although both media shared some characteristics in their growth factor and cytokine profile, there were also relevant differences. Important here is that fibroblast conditioned medium contained less HGF, which is known for its anti-apoptotic activity on cardiomyocytes [14]. Among the factors found in similar amounts was VEGF, a growth factor crucial for endothelial cell survival and proliferation. In line with this finding, the favorable effects of CBMSC and fibroblast conditioned medium on HUVECs were similarly pronounced. Both media increased the number of metabolically active and proliferating cells and reduced apoptosis of glucose/serum starved HUVECs. In contrast to cardiomyocytes, HUVECs also showed attenuation of necrotic cell death. Endothelial cells have a lower energy demand than beating cardiomyocytes [15] and also their deviant “ischemia” model here (only glucose/serum deprivation) might have accounted for this difference. CBMSC and fibroblast conditioned medium activated STAT3 and ERK signaling in HUVECs but only STAT3 proved to be relevant for CBMSC mediated cytoprotection. When STAT3 phosphorylation was inhibited, also induction of the anti-apoptotic gene *BCL-2* was abolished, which demonstrates a crucial role of the STAT3/BCL-2 axis in endothelial cells. Although activation of this pathway was less pronounced in response to fibroblast conditioned medium, the impulse was sufficient to provide equal protection. AKT signaling, clearly involved in the effects on cardiomyocytes, was not required for endothelial cell protection.

The paracrine effect of CBMSCs on endothelial cells, as well as their own ischemic tolerance, was further improved by hypoxic preconditioning. The optimal preincubation protocol for CBMSCs was determined to be 24 h at 1% O_2 in glucose/serum-containing medium, which is in line with most protocols described for bone marrow derived MSCs (for a detailed list of references please refer to attached publication #3). AKT activation and downstream BAD inhibition were detected in hypoxically preconditioned CBMSCs after 24 h of subsequent simulated ischemia, demonstrating sustained actuation of this

anti-apoptotic pathway. Also anti-apoptotic genes were induced by hypoxic preconditioning. During simulated ischemia, BCL-XL expression was amplified, as was expression of BAG1, an enhancer of the anti-apoptotic action of BCL-2. Ultimately, these mechanisms resulted in reduced apoptosis and increased survival of “ischemic” CBMSCs. Moreover, expression of VEGF was doubled by hypoxic preconditioning. Given a greater number of surviving CBMSCs and concomitantly enhanced VEGF expression, secretion of VEGF was elevated and this eventually reinforced the beneficial effects on “ischemic” endothelial cells.

Conclusions and outlook

In the present work, the molecular mechanisms that underlie cardioprotection by CBMSCs were characterized. To this end, an approach was developed that exploits the cardioprotective capacity of CBMSCs without the need of actual cells, so that clinical translation is much simplified. Moreover, a protocol was designed that improves the resistance of CBMSCs to ischemia by means of hypoxic preconditioning, so that less cell loss may be expected following transplantation into ischemic tissue. While the protective paracrine effects of CBMSCs on HL-1 cardiomyocytes in simulated ischemia were transmitted by AKT and ERK in a compensatory manner, cytoprotection of HUVECs was mainly mediated by STAT-3 signaling. Of note, only the effects on HL-1 cardiomyocytes were CBMSC-specific, while the effects on HUVECs can also be elicited by fibroblasts. The concept of CBMSC conditioned medium can be further developed into clinically translatable protocols for cell-free cytoprotection during induced cardiac surgical ischemia and possibly also for tissue salvage following myocardial infarction. If viable CBMSC cell products are to be used in situations of myocardial ischemia, their functional capacity can be enhanced by targeted hypoxic preconditioning, obviating the need for additional pharmacologic or genetic manipulation of the cells. Ultimately, these strategies may help to improve the outcome of clinical cardiac cell therapy.

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Affidavit

I, Andreas Matthäus Bader, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Molecular mechanisms of cardioprotection by cord blood mesenchymal stromal cells”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" of the ICMJE, www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (see above) and are my responsibility. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and of which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date   Signature

Declaration of selected publications

Andreas Matthäus Bader had the indicated share in the following publications:

Publication 1: Bader AM, Brodarac A, Klose K, Bieback K, Choi YH, Kurtz A and Stamm C. Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells. European Journal of Cardio-Thoracic Surgery 2014; 45(6):983-92.
Contribution in detail: Study design, collection of data, data analysis and interpretation, manuscript writing.

Publication 2: Bader AM, Brodarac A, Klose K, Bieback K, Choi YH, Kang KS, Kurtz A and Stamm C. Cord blood mesenchymal stromal cell-conditioned medium protects endothelial cells via STAT3 signaling. Cellular Physiology and Biochemistry 2014;34(3):646-657.
Contribution in detail: Study design, collection of data, data analysis and interpretation, manuscript writing.

Publication 3: Bader AM, Klose K, Bieback K, Korinth D, Schneider M, Seifert M, Choi YH, Kurtz A, Falk V and Stamm C. Hypoxic preconditioning increases survival and pro-angiogenic capacity of human cord blood mesenchymal stromal cells *in vitro*. PLoS One 2015; 10(9):e0138477.
Contribution in detail: Study design, collection of data, data analysis and interpretation, manuscript writing.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Bader AM, Brodarac A, Klose K, Bieback K, Choi YH, Kurtz A, Stamm C. Mechanisms of paracrine cardioprotection by cord blood mesenchymal stromal cells. Eur J Cardiothorac Surg 2014;45(6):983-92. http://dx.doi.org/10.1093/ejcts/ezt576

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

Complete list of publications

Full articles:

Roy R, Haase T, Ma N, **Bader A**, Becker M, Seifert M, Choi YH, Falk V, Stamm C. Decellularized amniotic membrane attenuates post-infarct LV remodeling. Journal of Surgical Research 2015; In Press.

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