

Original Paper

# Cord Blood Mesenchymal Stromal Cell-Conditioned Medium Protects Endothelial Cells via STAT3 Signaling

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## Key Words

Cell therapy • Endothelial cell • Ischemia • Stem cell • Cord blood

## Abstract

**Background/Aims:** Cell-based therapies may be useful for treating ischemic diseases, but the underlying mechanisms are incompletely understood. We investigated the impact of cord blood mesenchymal stromal cell (CBMSC)- or fibroblast (FB)-secreted factors on starved endothelial cells and determined the relevant intracellular signaling pathways. **Methods:** HUVECs were subjected to glucose/serum deprivation (GSD) in hypoxia or normoxia, in presence of CBMSC- or FB-conditioned medium (CM). Viability and proliferation were determined via WST-8 conversion and BrdU incorporation. Apoptosis was quantified by annexin V/ethidium homodimer-III staining, nuclear fragmentation and cell morphology. mRNA expression and protein phosphorylation were determined by real-time qPCR and western blot. Experiments were repeated in presence of small-molecule inhibitors. **Results:** The negative impact of GSD was most pronounced at 21% O<sub>2</sub>. Here, medium of CBMSCs and FBs increased viability and proliferation and reduced apoptosis of HUVECs. This was associated with increased STAT3 and ERK1/2 phosphorylation and BCL-2 expression. Under STAT3 inhibition, the beneficial effect of CBMSC-CM on viability and BCL-2 expression was abolished. **Conclusion:** Factors released by CBMSCs protect endothelial cells from the deleterious impact of GSD by activation of the STAT3 survival pathway. However, this phenomenon is not CBMSC-specific and can be reproduced using juvenile fibroblasts.

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## Introduction

Mesenchymal stromal cells (MSC) and their subpopulations have been shown to ameliorate ischemic tissue injury in numerous pre-clinical studies. Clinical trials using MSCs for treatment of ischemic diseases gave promising results but also demonstrated the need for further improvement [1-3]. The beneficial MSC effects range from improved vascularization and perfusion to the protection of ischemically damaged cells and favorable influences on tissue remodeling [4, 5], notably in cardiac and skeletal muscle [6-8]. The impact on capillary density and collateral flow has been attributed to secreted factors [8, 9], but little is known about how these factors act on vascular cells in the ischemic area. We therefore established an *in vitro* model of "simulated ischemia" using human umbilical vein endothelial cells (HUVEC) and studied whether factors released from MSCs provide protective stimuli. We used MSCs from umbilical cord blood (CBMSC) because they are a uniform cell population with low immunogenicity, ready availability and robust culture and expansion behavior, and clinical trials have demonstrated their therapeutic potential for the treatment of ischemic diseases [1, 10]. To assess the MSC-specificity of these effects, we also investigated the response of endothelial cells to medium conditioned by juvenile fibroblasts (FB). We believe that a better understanding of the underlying molecular mechanisms may ultimately facilitate their more successful clinical application.

## Materials and Methods

### *Cells and cell culture*

Cryopreserved HUVECs were purchased from Life Technologies, Carlsbad, California, USA. Cells were maintained in endothelial basal medium (EBM)-2 supplemented with endothelial growth medium (EGM)-2 growth factors, cytokines and supplements (Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, California, USA), and cultured in 0.1% gelatin coated flasks and plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37 °C and 5% CO<sub>2</sub> in a humid atmosphere. All experiments were performed with HUVECs between passage seven and nine, 24 h after plating.

Cryopreserved human CBMSCs were provided by Karen Bieback, who isolated, expanded and characterized them according to a previously published protocol [11]. 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 ethical committees in Mannheim and Heidelberg (Ref. 48/05 reconfirmed in 2009). With a seeding density of 700 – 1000 cells/cm<sup>2</sup> and harvesting at subconfluency, CBMSCs were expanded to the fourth passage and cultured in Dulbecco's Modified Eagle Medium (DMEM) 21885 (low glucose), supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% CO<sub>2</sub> in a humid atmosphere (all reagents from Life Technologies, Carlsbad, California, USA). The phenotype of the cultured MSCs as well as their ability to differentiate into non-hematopoietic cell types were previously confirmed [12, 13]. CRL-2429™ fibroblasts from human foreskin were purchased in passage four from LGC Standards, Wesel, Germany. With a seeding density of 10 000 cells/cm<sup>2</sup> and harvesting at 95-100% confluency, cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) 31980, supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% CO<sub>2</sub> in a humid atmosphere (all reagents from Life Technologies, Carlsbad, California, USA).

### *Preparation of conditioned medium*

Conditioned medium was prepared using CBMSCs in passage four and FBs between passages ten and twelve. Cells were plated at 10 000 cells/cm<sup>2</sup>. After 6±1 (CBMSC) or 3±1 (FB) days, at 80–90% confluency, cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) and covered with DMEM 11966 (no glucose), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all reagents from Life Technologies, Carlsbad, California, USA). Subsequently, cells were subjected to a hypoxic atmosphere (1% O<sub>2</sub>, 5% CO<sub>2</sub>) by replacing oxygen with nitrogen in an O<sub>2</sub> and CO<sub>2</sub> controlled multi gas incubator (CB 150, Binder, Tuttlingen, Germany). After six days' incubation, conditioned medium was collected and centrifuged for 10 min at 300 x g to remove detached cells. Equivalent medium kept under the same conditions in culture flasks

without cells was prepared as control. For depletion of extracellular microvesicles, conditioned medium was filtered through a 0.2- $\mu$ m filter and subsequently centrifuged for 90 min at 100 000 x g, according to a published protocol [14]. Concentration of the medium by volume reduction was not performed. CBMSC/fibroblast viability after conditioned medium preparation was verified as previously described [12].

#### *"Simulated ischemia"*

HUVECs were subjected to combined glucose/serum deprivation (GSD) by replacing their standard culture medium with CBMSC- or FB-conditioned medium or with equivalent non-conditioned control medium. In these media HUVECs were incubated under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>) or subjected to a hypoxic environment (0.5% O<sub>2</sub>, 5% CO<sub>2</sub>) in an incubator chamber (Billups-Rothenberg, Del Mar, California, USA and BioSpherix, Lacona, New York, USA). HUVECs incubated under normoxic conditions in DMEM 31966 (high glucose) supplemented with 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin served as reference (= full medium) (all reagents from Life Technologies, Carlsbad, California, USA).

#### *Evaluation of cell damage*

Metabolic activity was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). After 6 h of GSD, HUVECs were incubated for 4 h in medium containing water soluble tetrazolium (WST)-8. Absorbance at 450 nm, resulting from the conversion of WST-8 to WST-8 formazan by metabolically active cells, and at 650 nm (reference) was measured using a standard micro plate reader. The number of HUVECs showing morphological signs of apoptosis was determined by manual observation. After 6 h of simulated ischemia, light microscopy images were taken of the center of each microplate well. Only cells exhibiting a typical apoptotic morphology (shrinkage, rounding, and/or membrane blebbing) were counted. To further characterize the mode of cell death, the Apoptotic/Necrotic Cells Detection Kit (PromoKine, Heidelberg, Germany) was used. After 6 h GSD in 96-well imaging plates (PerkinElmer, Rodgau-Jügesheim, Germany), cells were stained with annexin V (AnnV)-fluorescein isothiocyanate (FITC) and ethidium homodimer (EthD)-III, fixed with 4% paraformaldehyde (PFA) and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The total cell number and proportions of early apoptotic, late apoptotic and necrotic cells were quantified using the high content imaging system Operetta® and Harmony® software (PerkinElmer, Waltham, MA, USA). In separate experiments, nuclear fragmentation was quantified: cells were fixed with 4% PFA, nuclei were stained with DAPI and analyzed with the Operetta® system. The nuclear fragmentation index (NFI) was defined as the coefficient of variation of nuclear stain fluorescence intensity. 5-bromo-2-deoxyuridine (BrdU) uptake was determined with the Cell Proliferation ELISA, BrdU (Roche Diagnostics, Mannheim, Germany). Cells were incubated with BrdU during the final 3 h of the GSD period, and cells were processed according to the manufacturer's instructions. Absorbance at 370 nm and 492 nm (reference) was measured using a standard microplate reader.

#### *Gel electrophoresis and western blot analysis*

Cells were washed with DPBS and lysed in PRO-PREP™ Protein Extraction Solution (Intron Biotechnology, Sungnam, South Korea). Protein concentration was determined by DC™ Protein Assay (Bio-Rad, Hercules, California, USA) and denatured protein (30  $\mu$ g) was resolved in a 12% polyacrylamide sodium dodecyl sulfate (SDS) gel and transferred to nitrocellulose membranes, which were blocked and incubated with monoclonal mouse anti-total-protein and rabbit anti-phospho-protein primary antibodies overnight at 4 °C: Akt, phospho-Akt (Ser473), extracellular-signal-regulated kinase (ERK)1/2, phospho-ERK1/2 (Thr202/Tyr204), signal transducer and activator of transcription (STAT)3, phospho-STAT3 (Tyr705) (all from Cell Signaling Technology, Danvers, Massachusetts, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Merck Millipore, Darmstadt, Germany). After 1 h incubation with goat anti-mouse IgG (H+L) / goat anti-rabbit IgG (H+L) horseradish peroxidase conjugated secondary antibodies (Life Technologies, Carlsbad, California, USA), blots were analyzed using Amersham™ ECL™ Western blotting detection reagents (GE Healthcare, Little Chalfont, UK).

#### *Real time quantitative PCR (RT-qPCR)*

Cells were washed with DPBS and RNA was purified using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from DNase (Sigma-Aldrich, St. Louis, Missouri, USA) -treated total RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad,

California, USA) with random hexamers as reaction primers. RT-qPCR was performed in a Mastercycler® ep gradient S realplex<sup>2</sup> (Eppendorf, Hamburg, Germany), using 2.5 ng template in 25 µl reaction volume with 300 nM of each primer and 2 x Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, California, USA). Amplification conditions were as follows: 95 °C for 10 min followed by 35 cycles consisting of 95 °C for 15 s, 56 °C for 30 s, 68 °C for 60 s. For B-cell lymphoma-2 (BCL-2) only 150 nM primers were used and annealing temperature was set to 60 °C. The following oligonucleotides were used as forward and reverse primers respectively: 5'-CATGTACGTTGCTATCCAGGC-3' and 5'- CTCCTTAATGTCACGCACGAT-3' for beta-actin, 5'-TGAGAAGCAGCACCTTCATGT-3' and 5'-GGAACCCCTATGACCTCTTCA-3' for BCL2-associated athanogene (BAG-1), 5'- GAACTGGGGGAGGATTGTGG-3' and 5'- GCCGGTTCAGGTACTCAGTC-3' for BCL-2, 5'-GAGCTGGTGGTTGACTTTCTC-3' and 5'-TCCATCTCCGATTTCAGTCCCT-3' for B-cell lymphoma-extra-large (BCL-XL). All measurements were carried out in triplicates. Gene-of-interest expression (E) was calculated as  $E = \text{primer efficiency}^{-(C_t)}$  (where  $C_t$  is the number of cycles at which the fluorescence exceeds the threshold) and normalized to beta-actin expression.

#### Signaling pathway inhibition

HUVECs were treated with specific small-molecule inhibitors 1 h prior to and during simulated ischemia. The MEK1/2 inhibitor UO126 (Cell Signaling Technology, Danvers, Massachusetts, USA) and the STAT3 inhibitor Stattic (Merck Millipore, Darmstadt, Germany) were applied at a final concentration of 5 µM and 7.5 µM, respectively. Both inhibitors were diluted in dimethyl sulfoxide (DMSO) and an equivalent amount of solvent was used for control.

#### Statistical analysis

Results are expressed as means ± SEM. All cell culture experiments and PCR analyses were performed in triplicates and the sample size (n) indicates the number of triplicates. The significance of intergroup differences was determined by one-way analysis of variance (ANOVA) with two-tailed Dunnett's t-test. When only two groups were compared, a two-tailed Student's t-test was performed. IBM SPSS statistics 20 was used for data analysis. A P-value < 0.05 was considered significant.

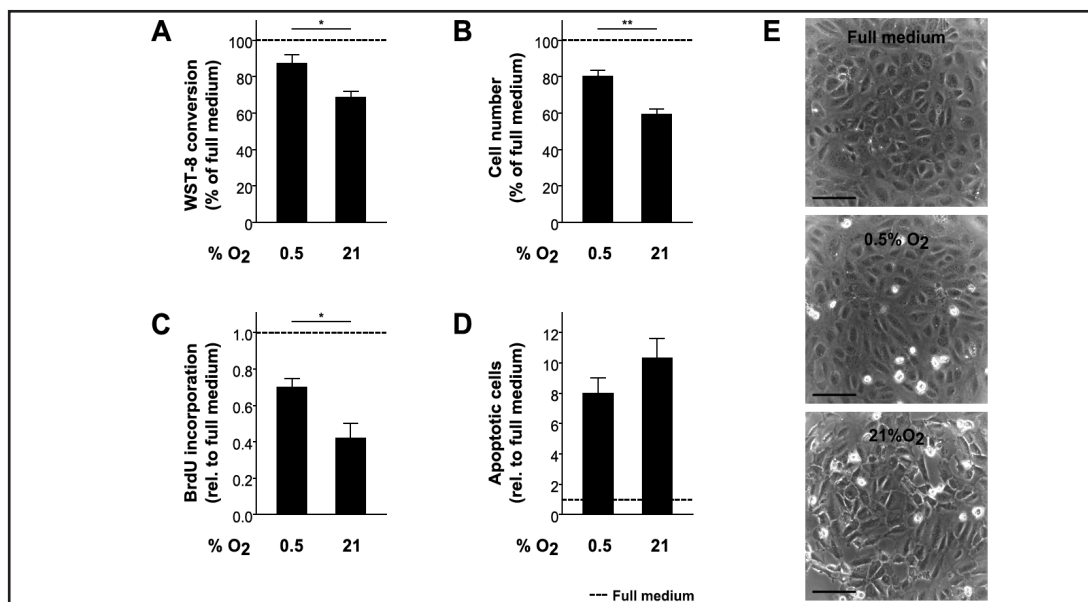
## Results

#### Validation of the "simulated ischemia" model in HUVECs

In order to mimic ischemic conditions *in vitro*, HUVECs were deprived of glucose and serum while kept in a hypoxic atmosphere (0.5% O<sub>2</sub>), and the effect of GSD was also investigated in normoxia (21% O<sub>2</sub>). Metabolic activity, total cell number, apoptosis and proliferation rate were compared to those of HUVECs supplied with full medium. After 6 h of GSD, WST-8 conversion of the cells declined to 87±5% in hypoxia and further to 69±3% in normoxia (P < 0.01 vs. hypoxia) (Fig. 1A). Accordingly, the proportion of remaining adherent cells was 80±3% in hypoxia but only 59±3% in normoxia (P < 0.001 vs. hypoxia) (Fig. 1B). BrdU uptake decreased to 0.7±0.05 (normalized to full medium data) in hypoxia, but even further to 0.42±0.08 in normoxia (P < 0.01 vs. hypoxia) (Fig. 1C). The number of cells showing an apoptotic phenotype was enhanced by factor 8±1 in hypoxia and by factor 10±1 in normoxia (Fig. 1D). In summary, withdrawal of glucose and serum for 6 hours impaired the viability and proliferation of HUVECs, but the deleterious effect was more pronounced in presence of 21% oxygen. Leaving sufficient room for both, improvement or further deterioration, normoxic GSD appeared to be more suitable and was therefore used for our conditioned medium experiments.

#### Protective effects of CBMSC- and FB-conditioned medium on glucose/serum deprived HUVECs

WST-8 conversion was higher in the presence of either CBMSC- or FB-conditioned medium (CBMSC-CM: 89±7% of full medium and FB-CM: 96±5%; vs. control: 69±3%, P < 0.05) (Fig. 2A) and the number of remaining adherent cells was correspondingly higher (CBMSC-CM: 93±2% of full medium and FB-CM: 91±2%; vs. control: 59±3%, P < 0.001)



**Fig. 1.** Viability, proliferation and apoptosis of glucose/serum deprived HUVECs. (A) WST-8 conversion (0.5%: n = 8; 21%: n = 8), (B) cell number (0.5%: n = 6; 21%: n = 13) (C) BrdU incorporation (0.5%: n = 12; 21%: n = 13) and (D) frequency of cells exhibiting morphological characteristics of apoptosis (0.5%: n = 7; 21%: n = 11) after 6 h glucose/serum deprivation at 0.5% O<sub>2</sub> and 21% O<sub>2</sub> (\* P < 0.01, \*\* P < 0.001; Student's t-test). All values expressed in relation to glucose/serum supplied cells cultured at 21% O<sub>2</sub> (Full medium). Data shown as mean ± SEM. (E) Representative microphotographs taken by light microscopy, scale bar = 100 μm.

(Fig. 2B). Also BrdU incorporation was preserved in conditioned medium (CBMSC-CM:  $0.92 \pm 0.03$ -fold of full medium and FB-CM:  $0.95 \pm 0.02$ -fold; vs. control  $0.42 \pm 0.08$ -fold, P < 0.001) (Fig. 2C). The frequency of cells showing morphological characteristics of apoptosis was reduced in conditioned medium (CBMSC-CM: 5±1-fold increase compared to full medium and FB-CM: 6±1-fold increase; vs. control: 10±1-fold increase, P < 0.05) (Fig. 2D), and the nuclear fragmentation index was lower (CBMSC-CM: NFI  $0.151 \pm 0.003$  and FB-CM: NFI  $0.150 \pm 0.004$ ; vs. control: NFI  $0.175 \pm 0.002$ , P < 0.001) (Fig. 2E). The rate of cells exposing phosphatidylserine with preserved plasma membrane integrity (early apoptosis) was also reduced in conditioned medium (CBMSC-CM:  $0.58 \pm 0.06$  of control, P < 0.001; FB-CM:  $0.66 \pm 0.07$  of control, P < 0.01), as was the rate of non-phosphatidylserine-exposing but EthD-III permeable cells (necrosis) (CBMSC-CM:  $0.47 \pm 0.06$  of control, P < 0.01; FB-CM:  $0.33 \pm 0.03$  of control, P < 0.001) (Fig. 3).

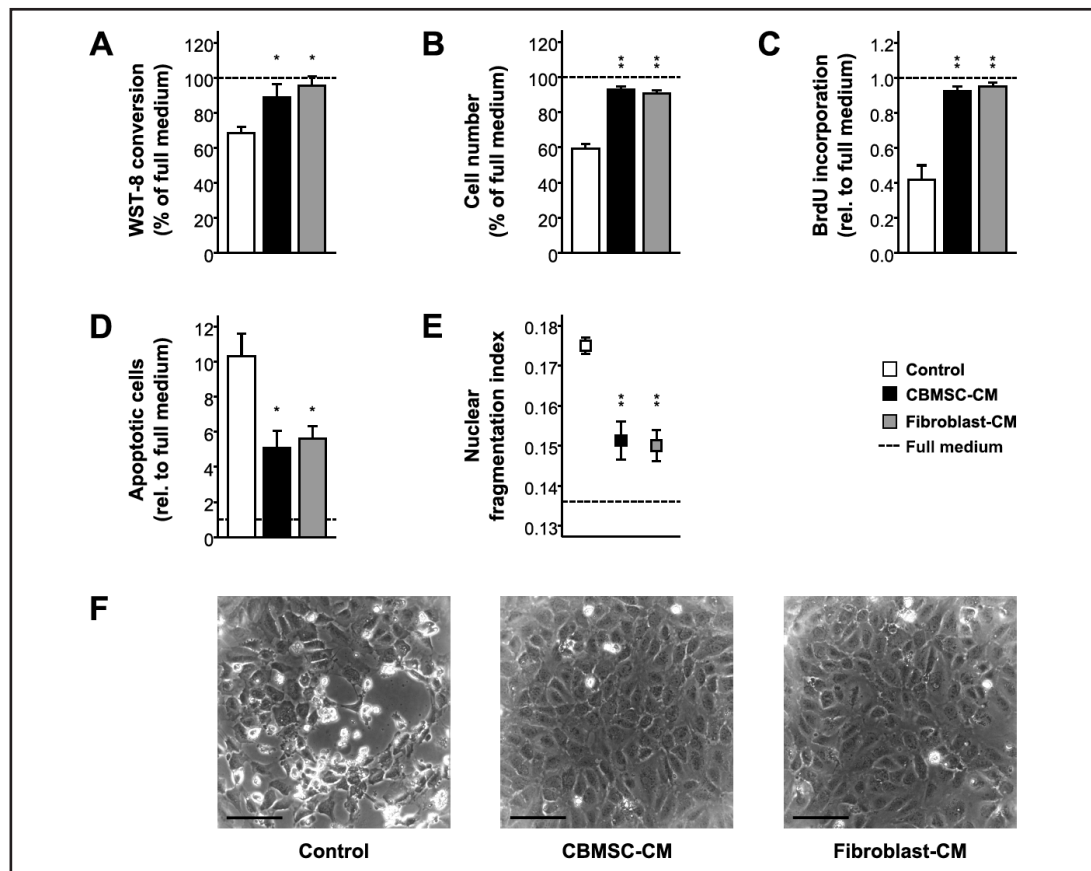
#### *Effect of soluble factors and extracellular microvesicles in CBMSC-conditioned medium*

As we have previously shown, both CBMSC- and FB-conditioned medium contain growth factors like vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) [12]. In addition to such soluble factors, protein- and RNA-containing microvesicles are also known to be released from cells in the culture medium [14]. To test whether the beneficial effects of conditioned medium were mediated by extracellular vesicles, we repeated the GSD experiment with microvesicle-depleted CBMSC-conditioned medium. Neither 0.2-μm filtration nor subsequent ultracentrifugation at 100 000 x g lowered the effect of CBMSC-conditioned medium on metabolic activity of glucose/serum deprived HUVECs (Fig. 4).

#### *Signaling pathway regulation in glucose/serum deprived HUVECs*

Compared to cells incubated in full medium, GSD resulted in similar phosphorylation of Akt and STAT3, while ERK1/2 phosphorylation was  $2.5 \pm 0.4$ -fold increased (Fig. 5A).



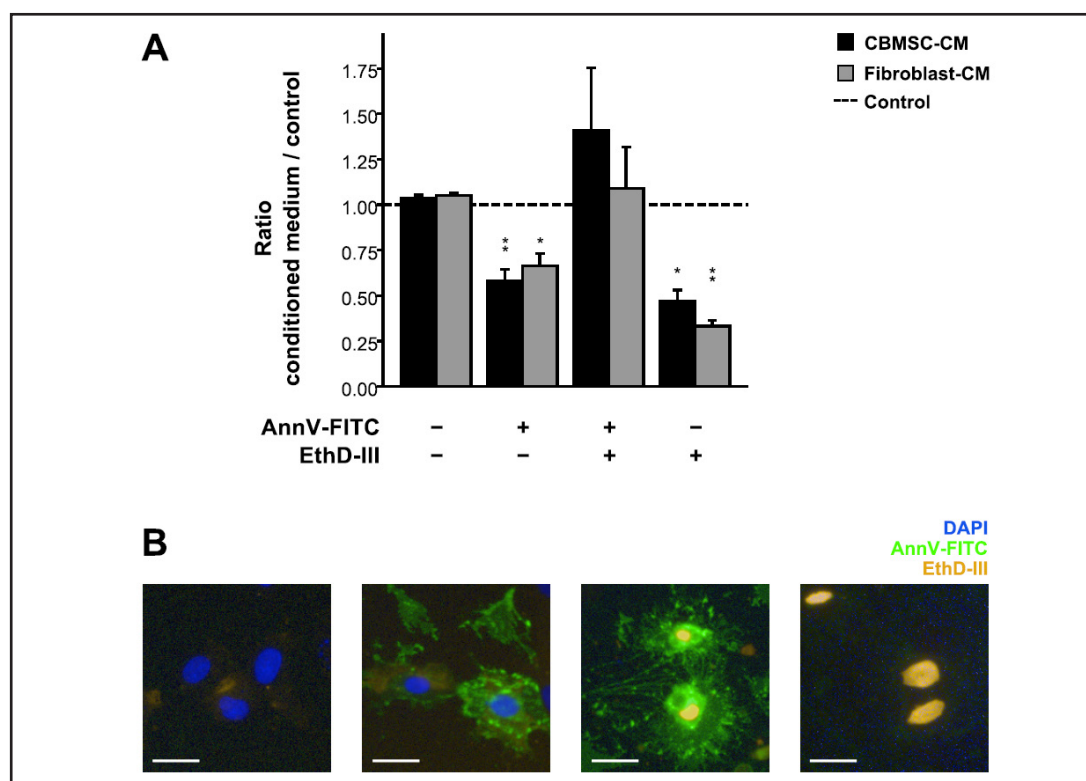


**Fig. 2.** Survival, proliferation and apoptosis of HUVECs after glucose/serum deprivation in CBMSC- and FB-conditioned medium. (A) WST-8 conversion (control: n = 8; CBMSC-CM: n = 5; FB-CM: n = 5), (B) cell number (control: n = 13; CBMSC-CM: n = 8; FB-CM: n = 8), (C) BrdU incorporation (control: n = 13; CBMSC-CM: n = 8; FB-CM: n = 5), (D) frequency of cells exhibiting morphological characteristics of apoptosis (control: n = 11; CBMSC-CM: n = 7; FB-CM: n = 8) and (E) nuclear fragmentation (control: n = 21; CBMSC-CM: n = 12; FB-CM: n = 12) after 6 h normoxic glucose/serum deprivation (\* P < 0.05, \*\* P < 0.001 vs. control; ANOVA with Dunnett's t-test). (A-D) Values expressed in relation to glucose/serum supplied cells (Full medium). Data shown as mean ± SEM. (F) Representative microphotographs taken by light microscopy, scale bar = 100 μm.

In the presence of conditioned medium, however, we detected distinct differences in the phosphorylation pattern of all three survival pathway checkpoints. Phosphorylation of Akt decreased to  $0.6 \pm 0.1$  in CBMSC-CM (P < 0.05 vs. control), and we observed a trend toward a similar reduction in FB-CM ( $0.6 \pm 0.2$ , P = 0.05 vs. control). Phosphorylation of STAT3 increased 10 ± 1-fold in CBMSC-CM (P < 0.001 vs. control) and 5.6 ± 0.5-fold in FB-CM (P < 0.01 vs. control). ERK1/2-phosphorylation was 6 ± 2-fold higher in FB-CM (P < 0.05 vs. control), while in CBMSC-CM, the average ERK1/2-phosphorylation was 4.5 ± 0.8-fold higher but did not reach statistical significance (P = 0.3 vs. control) (Fig. 5A).

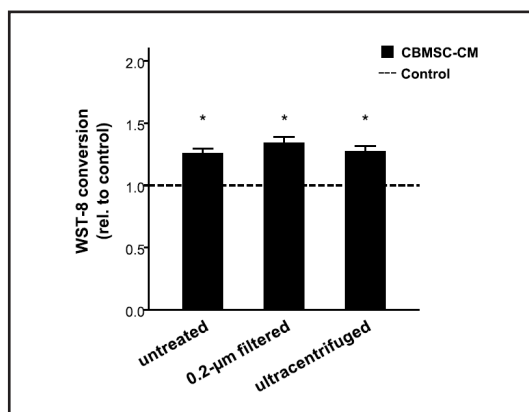
#### *Modulation of gene expression in glucose/serum deprived HUVECs*

BCL-2 mRNA expression was similar in glucose/serum-deprived cells and cell incubated in full medium, while mRNA expression of BCL-XL and BAG-1 was 1.6 ± 0.1-fold and 1.6 ± 0.2-fold higher in GSD (Fig. 5C). In the presence of CBMSC-CM, BCL-2 expression increased 3.0 ± 0.3-fold (P < 0.001 vs. control) and 2.0 ± 0.2-fold in FB-CM (P < 0.01 vs. control). Neither BCL-XL nor BAG-1 was further induced in the presence of conditioned medium (Fig. 5C).



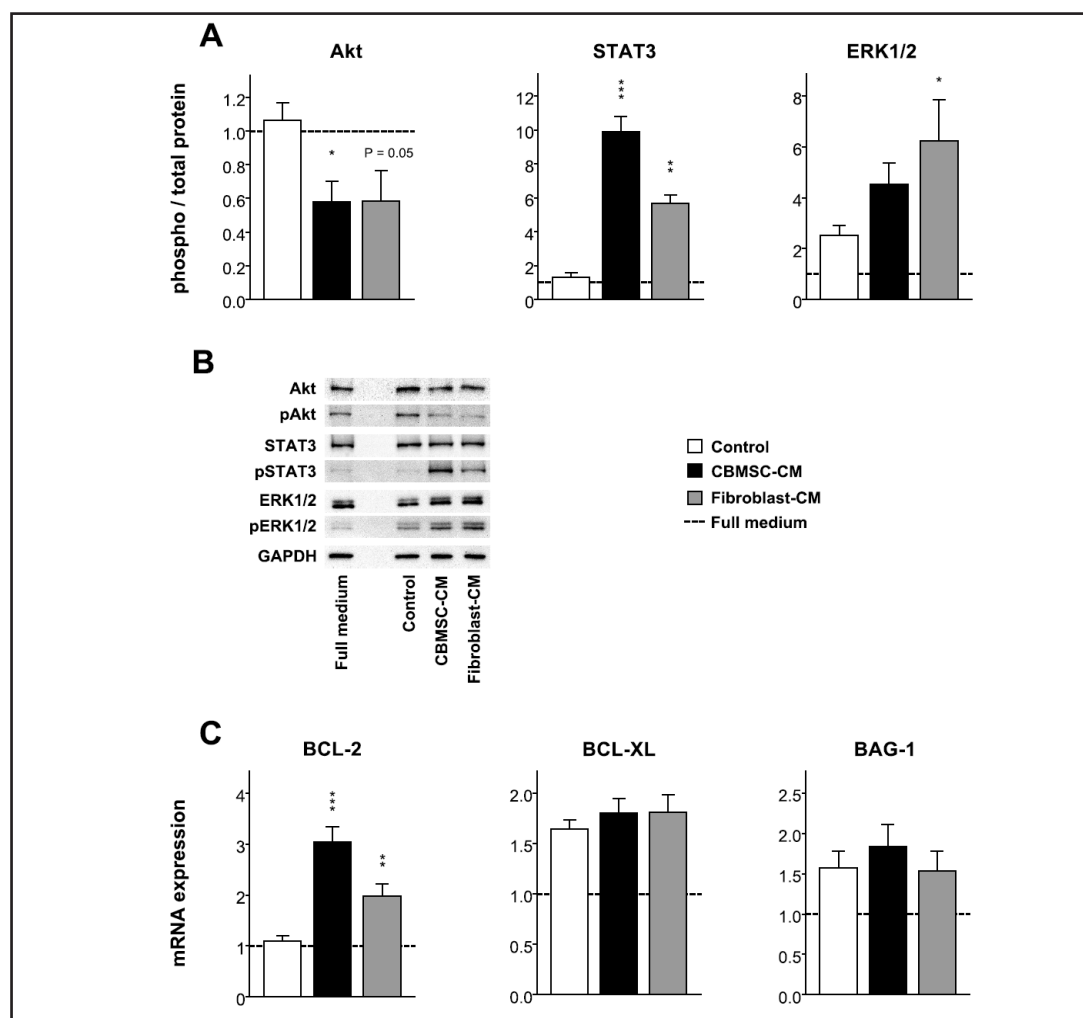
**Fig. 3.** Phosphatidylserine exposure and loss of plasma membrane integrity of HUVECs after glucose/serum deprivation in CBMSC- and FB-conditioned medium. (A) Percentage of AnnV-FITC binding and EthD-III permeable cells after 6 h normoxic glucose/serum deprivation in CBMSC-CM (n = 8) and FB-CM (n = 8), expressed in relation to non-conditioned control medium (\* P < 0.01, \*\* P < 0.001 vs. control; ANOVA with Dunnett's t-test). Data shown as mean  $\pm$  SEM. (B) Representative microphotographs of vital (AnnV-FITC -/EthD-III -), early apoptotic (AnnV-FITC +/EthD-III -), late apoptotic (AnnV-FITC +/EthD-III +) and necrotic (AnnV-FITC -/EthD-III +) HUVECs taken by Operetta® high-content imaging microscopy, scale bar = 25  $\mu$ m.

**Fig. 4.** Effect of microvesicle depleted CBMSC-conditioned medium. Viability (WST-8 conversion) of HUVECs after 6 h normoxic glucose/serum deprivation in untreated (n = 6), 0.2- $\mu$ m filtered (n = 6) and 100 000 x g centrifuged (n = 6) CBMSC-CM, expressed in relation to non-conditioned control medium (\* P < 0.001 vs. control; ANOVA with Dunnett's t-test). Data shown as mean  $\pm$  SEM.



#### Functional relevance of CBMSC-conditioned medium triggered STAT3 activation

GSD experiments with CBMSC-conditioned medium were repeated in the presence of specific small-molecule inhibitors. The efficiency of Stattic and UO126 in suppressing the phosphorylation of STAT3 and ERK1/2 in glucose/serum deprived HUVECs was confirmed by western blot (Fig. 6A). The WST-8 conversion rate of glucose/serum starved HUVECs was increased in CBMSC-CM ( $1.34 \pm 0.04$ -fold of control, P < 0.01). In the presence of the STAT3 inhibitor Stattic, this beneficial effect was completely abolished ( $1.0 \pm 0.2$ -fold of control, P = 1), whereas blocking MEK/ERK signaling with UO126 did not influence the CBMSC-CM



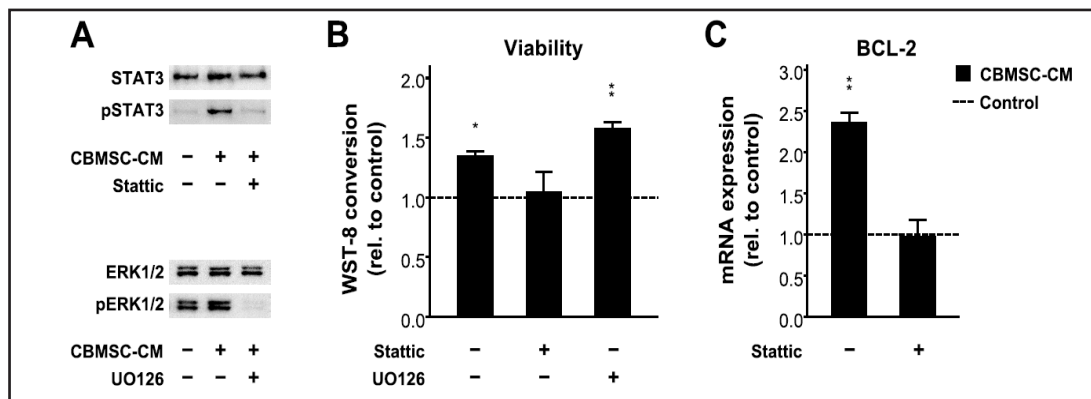
**Fig. 5.** Protein phosphorylation and gene expression in HUVECs after glucose/serum deprivation in CBMSC- and FB-conditioned medium. (A) Phosphorylation of Akt (control: n = 6; CBMSC-CM: n = 6; FB-CM: n = 4), STAT3 (control: n = 6; CBMSC-CM: n = 6; FB-CM: n = 4) and ERK1/2 (control: n = 5; CBMSC-CM: n = 5; FB-CM: n = 4) after 3 h normoxic glucose/serum deprivation, determined by western blot. (B) Representative western blot images. (C) beta-actin normalized mRNA expression of BCL-2 (control: n = 11; CBMSC-CM: n = 7; FB-CM: n = 7), BCL-XL (control: n = 11; CBMSC-CM: n = 7; FB-CM: n = 7) and BAG-1 (control: n = 11; CBMSC-CM: n = 7; FB-CM: n = 7) after 3 h normoxic glucose/serum deprivation, determined by RT-qPCR (\* P < 0.05; \*\* P < 0.01, \*\*\* P < 0.001 vs. control; ANOVA with Dunnett's t-test). All values expressed in relation to glucose/serum supplied cells. Data shown as mean  $\pm$  SEM.

effect ( $1.57 \pm 0.06$ -fold of control,  $P < 0.001$ ) (Fig. 6B). Of note, CBMSC-CM triggered induction of BCL-2 ( $2.4 \pm 0.1$ -fold of control,  $P < 0.001$ ) was completely eliminated under STAT3 inhibition ( $1.0 \pm 0.2$ -fold of control,  $P = 1$ ) (Fig. 6C).

## Discussion

We found that the deleterious impact of glucose/serum deprivation on HUVECs is more pronounced in normoxia than in hypoxia. Soluble factors secreted by umbilical cord blood MSCs act anti-apoptotic and pro-mitotic on the starved endothelial cells, but factors released





**Fig. 6.** Functional relevance of CBMSC-conditioned medium triggered STAT3 activation. (A) Protein phosphorylation and its inhibition by small-molecule inhibitors determined by western blot. (B) Viability determined via WST-8 conversion assay (solvent:  $n = 15$ ; Statistic:  $n = 8$ ; UO126:  $n = 15$ ). (C) beta-actin normalized mRNA expression of BCL-2, determined via RT-qPCR (solvent:  $n = 6$ ; Statistic:  $n = 6$ ) (\*  $P < 0.01$ , \*\*  $P < 0.001$  vs. control; ANOVA with Dunnett's t-test). Data collected on HUVECs after (A and C) 3 h or (B) 6 h normoxic glucose/serum deprivation in CBMSC-CM, CBMSC-CM with 7.5  $\mu\text{M}$  Statistic and CBMSC-CM with 5  $\mu\text{M}$  UO126. All values expressed in relation to non-conditioned control medium. Data shown as mean  $\pm$  SEM.

by fibroblasts exert similar effects. STAT3, but not ERK1/2 transmits the protective signals in endothelial cells and is needed for the induction of the anti-apoptotic BCL-2 gene.

To develop a suitable *in vitro* model for ischemia, we subjected HUVECs to combined glucose/serum deprivation in both hypoxia and normoxia. Under hypoxic conditions survival and proliferation of the cells were impaired, while in a normoxic atmosphere cell damage was even more severe. For glucose/serum starved HUVECs, hypoxia apparently acts as protective factor, while other groups described deleterious effects of hypoxia on HUVECs and mature endothelial cells kept in normal medium [15-17]. The damage to glucose/serum starved HUVECs was alleviated by CBMSC-conditioned medium, which reduced apoptotic and necrotic cell death and increased the number of metabolically active cells with preserved proliferative capacity. Recent reports described favorable effects of MSC-derived microvesicles on endothelial cells [18, 19], but we found that the beneficial action on starved HUVECs does not depend on such structures. Anti-apoptotic and pro-mitotic effects of MSCs on endothelial cells have been described before [18, 20, 21], but the underlying mechanisms have rarely been investigated. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MEK)/ERK pathways typically transduce signals promoting survival and proliferation [22, 23]. Hung et al. reported that activation of Akt, but not ERK is responsible for beneficial effects of bone marrow MSC-conditioned medium on oxygen/serum-deprived endothelial cells [24]. Our findings confirm that MSC-triggered ERK activation is not required for protection, but CBMSC-released factors clearly suppressed Akt phosphorylation in our model. The janus kinase (JAK)/STAT pathway is also known to mediate cytoprotection [25, 26]. As we and others have shown before, MSC-secreted factors are able to activate STAT3 in skeletal and cardiac myocytes as well as in cardiac progenitor cells [12, 27-29]. However, STAT3 is not in every cell type a key mediator of the cytoprotective stem cell action [12]. In our model, STAT3 was prominently activated in HUVECs in the presence of CBMSC-conditioned medium, and when STAT3 phosphorylation was blocked, the beneficial effect was completely abolished. Induction of anti-apoptotic genes BCL-2, BCL-XL or BAG-1 is one mechanism by which these pathways can promote survival of injured cells [30-32]. While BCL-XL and BAG-1 were up-regulated in HUVECs as endogenous response to glucose/serum deprivation, BCL-2 expression was triggered by CBMSC-released factors in the medium. Confirming the crucial role of STAT3, BCL-2 induction was completely abolished when STAT3 phosphorylation was blocked. Such anti-apoptotic action of STAT3-mediated BCL-2 induction has also been described in polyamine-depleted cells [30].

To determine whether non-stem cells exert similar beneficial effects, we included juvenile foreskin fibroblasts in our experiments. As we have shown before, both cell types differ in their secretory profile [12]. While secreting comparable amounts of VEGF, EGF and Angiopoietin-2, fibroblasts release less HGF but higher amounts of bFGF and interleukin 6 (IL-6) [12]. Yet, we found that their anti-apoptotic and pro-mitotic action on endothelial cells was similar to that of CBMSCs. Although STAT3 phosphorylation and subsequent BCL-2 expression were less pronounced, activation of the STAT3/BCL-2 axis was sufficient to convey cytoprotection. Increased DNA synthesis and BCL-2 expression has previously been observed in oxygen/serum-deprived HUVECs in the presence of cardiac fibroblast-conditioned medium, which Zhao et al. attributed to activation of the MEK/ERK pathway [33]. We also found that fibroblast released factors were potent activators of MEK/ERK signaling, but MEK/ERK activity was not required for cytoprotection.

Taken together, we showed that factors released by human CBMSCs protect endothelial cells from the deleterious impact of nutrient and serum deprivation, but this is not a CBMSC-specific phenomenon and can be mimicked by using juvenile fibroblasts. STAT3 activation is the key mediator of cytoprotection and induces expression of the anti-apoptotic BCL-2. These results provide new insights into the complex intracellular processes that convey cytoprotection by paracrine factors and may help to better exploit the potential of cell therapy for ischemic diseases.

From the clinician's point of view, the paracrine effect of MSC-conditioned medium might be exploited by preparing cell-free products, as has, for instance, been suggested in the context of cerebral ischemia or liver regeneration [34, 35]. In the ischemic heart, such cytoprotective products may help reduce infarct size expansion after acute myocardial infarction, or possibly ameliorate the impact of "scheduled" ischemia during cardiac surgery [9].

### Limitations

Deprivation of glucose and serum in an ambient atmosphere of 21% O<sub>2</sub> is a situation that may not reflect the actual conditions in ischemic tissue. In fact, an O<sub>2</sub> concentration of 21% rather resembles hyperoxic conditions *in situ*. In consequence, we could not take reoxygenation caused injury into account, which is a major cause for cell damage after ischemia *in vivo*. Although our observed endpoints, survival and proliferation, are essential for neoangiogenesis, we did not directly assess endothelial cell function.

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### Disclosure Statement

None declared.

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