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DISSERTATION

Human placenta derived cells for myocardial cell therapy

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Rajika Roy

Jamshedpur, India

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1.1 Abstract

Introduction: Autologous adult stem cells used for cellular therapy of ischemic heart disease have yielded modest results, partly because patient age and disease state affect the functionality of the transplanted cells. Neonatal donor cells may represent an attractive alternative for use in cardiac cell therapy, and the hypothesis that such cell products exert beneficial effects in a mouse model of ischemic heart disease was tested. In addition, the impact of heart failure-related humoral factors on neonatal progenitor cell behavior was studied to assess whether they would be affected by transplantation into a diseased recipient.

Methods: Naïve amnion epithelial cells (AEC) and AEC induced to undergo epithelial-mesenchymal-transition induced (EMT-AEC) were evaluated in vitro for gene and protein expression profile, migration potential, cytokine secretion profile and gelatinase activity. The cardioprotective potential of AEC, EMT-AEC and clinical-grade placenta derived stromal cells (PLX-PAD) was evaluated in a mouse model of myocardial infarction. After four weeks cardiac function was evaluated by cardiac MRI, echocardiography and cardiac structure by histology and immunohistochemistry. To study the behavior of neonatal cells exposed to human heart failure (HF)-associated soluble factors, cord blood mesenchymal stromal cells (CB-MSC) were cultured in medium supplemented with HF serum instead of fetal bovine serum and analyzed for changes in growth kinetics, activation of stress signaling pathways and stimulation of apoptosis.

Results: Transplantation of naïve AEC resulted in neither functional improvements nor a decrease in infarct size compared to infarcted control hearts. EMT-AEC displayed an MSC-like phenotype, enhanced migratory potential and extracellular matrix modulatory potential and upregulation of survival factors compared to AEC, in vitro. In vivo, transplantation of EMT-AEC resulted not only in functional improvements but also in decreased infarct size, independent of the support of angiogenesis and arteriogenesis. Transplantation of equivalent MSC-like clinical grade PLX-PAD cells also led to improved contractile function, decreased infarct size and aided in angiogenesis. However, HF patient serum depressed the proliferation kinetics of neonatal MSC and also activated stress signaling and pro-apoptotic signaling pathways.

Conclusion: Progenitor cells from neonatal tissue sources exert cardioprotective effects in a mouse model of myocardial infarction, provided they display mesenchymal characteristics. Upon

future transplantation in patients with heart failure, depression of neonatal progenitor cell function must be expected.

1.2 Zusammenfassung (deutsch)

Hintergrund: Die Verwendung autologer adulter Stammzellen für die Zelltherapie ischämischer Herzerkrankungen ergab bisher nur mäßige Funktionsverbesserungen, unter anderem weil Spenderalter und Krankheitszustand die Funktionalität der transplantierten Zellen beeinträchtigen. Neonatale Spenderzellen, unbeeinflusst von Alterungs- und Krankheitsprozessen, könnten eine interessante Alternative darstellen. Es wurde daher untersucht, ob neonatale Zellen positive Effekte in einem Mausmodell der ischämischen Herzerkrankung ausüben und ob humorale Faktoren im Serum von Patienten mit Herzinsuffizienz das Verhalten neonataler Vorläuferzellen beeinflussen.

Methode: Native Amnionepithelzellen (AEC) und AEC, welche zur epithelial-mesenchymalen Transition angeregt wurden (EMT-AEC), wurden in vitro auf ihr Gen- und Proteinexpressionsprofil, Migrationspotential, Zytokin-Sekretionsprofil und ihre Gelatinase-Aktivität hin getestet. Das kardioprotektive Potential von AEC, EMT-AEC und klinisch verwendbaren mesenchymalen Stromazellen der Plazenta (PLX-PAD) wurde in einem Mausmodell für Myokardinfarkt untersucht. Nach vier Wochen wurde die Herzfunktion durch kardiale MRT und Echokardiographie gemessen, und die kardiale Struktur durch Histologie und Immunohistochemie evaluiert. Darüber hinaus wurden mesenchymale Stromazellen aus Nabelschnurblut (CB-MSC) unter Verwendung von Serum von Patienten mit schwerer Herzinsuffizienz anstelle von fötalem Kälberserum kultiviert und auf veränderte Wachstumskinetik, Aktivierung von Stress-Signalwegen und Apoptose-Stimulierung hin untersucht.

Ergebnis: Transplantation von nativen AEC hatte gegenüber Kontroll-Herzen mit Infarkt weder funktionelle Verbesserungen noch eine verkleinerte Infarktgröße zur Folge. EMT-AEC zeigten einen MSC-ähnlichen Phänotyp, erhöhtes Migrationspotential, erhöhtes Modulierungspotential der extrazellulären Matrix und Hochregulation von Überlebensfaktoren im Vergleich zu AEC in vitro. In vivo führte Transplantation von EMT-AEC nicht nur zu funktionellen Verbesserungen,

sondern auch zu einer reduzierten Infarktgröße, unabhängig von der Unterstützung der Angiogenese und Arteriogenese. Transplantation von äquivalenten, MSC-ähnlichen, klinisch verwendbaren PLX-PAD-Zellen führte auch zu einer verbesserten kontraktilen Funktion, einer reduzierten Infarktgröße und unterstützte die Angiogenese. HF-Patientenserum reduzierte jedoch die Proliferationskinetiken von neonatalen MSC und aktivierte auch Stress- und proapoptotische Signalwege.

Fazit: Vorläuferzellen aus neonatalem Gewebe zeigen kardioprotektive Effekte in einem Mausmodell für Herzinfarkt, unter der Voraussetzung dass sie mesenchymale Charakteristiken aufweisen. Bei zukünftiger Transplantation in Patienten mit Herzinsuffizienz muss jedoch eine Unterdrückung der Funktion neonataler Vorläuferzellen durch Serumfaktoren erwartet werden.

2. Introduction

As summarized in the 2013 report of the American Heart Association(1), the incidence of heart failure (HF) approaches 10 per 1000 people older than 65 years (*Framingham Heart Study* data), and approximately 50% of those will die within 5 years after diagnosis (*Olmsted County* Study). The postnatal mammalian heart has been considered incapable of any degree of regeneration in the event of damage, as cardiomyocytes exit the cell cycle after birth, but recent reports have shown that the adult myocardium has some propensity towards regeneration from resident myocardial progenitor cells (2). However, those can obviously not completely restore the structure and function of damaged myocardium. Therefore, cell therapy to repair the diseased heart has been an area of intense research in the last two decades, but, after evaluating several cell populations, the search for an ideal cell product still continues. The modest outcomes of clinical cell therapy trials have been partly attributed to the age and disease related loss of function of the transplanted autologous cells (3). Neonatal progenitor cells represent an attractive cell population as they are available in abundance. Their use does not involve ethical concerns and they exhibit extremely low immunogenicity (4), making them suitable for allogeneic transplantations. Neonatal progenitor cells are formed during the initial stages of embryogenesis. The initiation and gradual development of tissues harboring neonatal progenitor cells occur simultaneously with the early stage development of the fetus. The amniotic membrane of the

placenta is comprised of a single layer of epithelial cells derived from the epiblast. After isolation they retain their epithelial morphology and display varying degree of stemness, as they are not exposed to differentiation signals during embryogenesis (5). As they also possess characteristics of mature epithelial cells, their migration potential is restricted due to the presence of tight junctions and very close cell-to-cell interactions. Epithelial-mesenchymal-transition (EMT) renders cells with increased mobility and extracellular matrix modulatory properties, phenomena that might be helpful for cell-based regeneration processes (6). Therefore we hypothesize that inducing epithelial-mesenchymal-transition in amniotic epithelial cells would enhance their cardioprotective potential and tested this hypothesis in a mouse model of myocardial infarction.

Evaluation of cell products for clinical trials requires them to be isolated and expanded under good manufacturing practice (GMP) conditions. Amongst neonatal cells a population of placental stromal cells, named PLX-PAD, have been identified that can be obtained in large numbers and expanded under GMP conditions. As these clinical grade cells display low immunogenicity and have been shown to aid in angiogenesis *in vivo* (7), we investigated their cardioprotective potential also in a mouse model of myocardial infarction.

Evaluation of autologous adult cells in rodent models of myocardial infarction showed that these cells indeed display a cardioprotective effect. However, results from human clinical trials reveal that there are disparities between the effects of cell transplantation as observed in small rodent models and in human clinical trials. Upon investigation it was reported that the transplanted autologous cells are adversely affected by humoral factors present in the sera of patients with HF (8). To evaluate the potential of neonatal progenitor cells as candidates for cardiac cell therapy, we investigated if neonatal progenitor cells would also be adversely affected after transplantation in patients with HF since they have been shown to be superior to adult cells (9). Also, it was important for us to evaluate this effect under syngeneic conditions as we studied the cardioprotective effects of neonatal cells in wild type rodents under xenogeneic conditions. Therefore, we sought to evaluate the impact of HF patient serum on human neonatal MSC behavior.

3. Materials and methods

All studies were done in accordance with the Declaration of Helsinki, with approval of the ethics committees of Charité University Medicine Berlin and Seoul National University. Full-term human placentas were obtained from healthy donors who gave informed consent for using the material for research purposes. Blood samples were collected from patients with chronic HF and healthy control subjects with informed consent of all patients and volunteers. Patients were excluded if they were not clinically stable, had cancer or any active infection.

3.1 *In vitro* experiments

Cell culture: Human AEC were isolated and cultured as previously described (10).

Cryopreserved human CB-MSC were isolated and expanded according to a previously published protocol (11). Cells used for experimental purpose were first seeded in medium supplemented with 10% FCS. After cell attachment, FCS was replaced by 10% protein-normalized human serum. Serum from healthy donors served as experimental control (human control). All experiments were also performed with cells maintained under standard culture conditions (FCS).

The details of the PLX-PAD production process have been described in the publication attached (12) . Cryopreserved PLX-PAD were thawed and injected *in vivo* without further cultivation steps.

Induction of epithelial-mesenchymal-transition (EMT): AEC were treated with 25ng/ml Transforming Growth Factor-β1 (TGF-β1) for 6 days. The cells were monitored for morphological changes every 24 hours by light microscopy.

Migration pattern: In AEC culture plates two perpendicular scratches were made and cell culture medium with TGF-β1or without (control) was added. Cells were observed every two hours for 24 hours for changes in migration pattern by light microscopy. AEC were seeded onto 8µm pore size transwell inserts with or without TGF-β1. Assays were done in duplicates with transwell inserts being examined on the 3rd and the 5th day. For quantification, cells on the surface of transwell were fixed and stained using Crystal violet solution. Cells in the lower compartment were visualized using phase contrast light microscopy.

Protein expression profile: AEC with and without TGF-β1 were fixed, blocked and incubated with anti-human E-cadherin, anti-human β-actin, anti-human N-cadherin, anti-human periostin and anti-human pan-Cytokeratin primary antibody. Anti-mouse secondary antibody conjugated to Alexa 488 for E-cadherin, pan-Cytokeratin and β-actin, or Alexa 555 for N-cadherin and periostin was used for fluorescence detection. Nuclei were counterstained with DAPI. Images were acquired using the OPERETTA high content screener system.

Protein from AEC cultured with and without TGF-β1 was isolated and quantified. 25µg of protein was loaded on a 10% polyacrylamide gel. A semi-dry transfer system was used to transfer the proteins onto a membrane. The membrane was incubated overnight at 4ºC with antibodies against E-cadherin, occludin, Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, and β-actin. Membranes were then washed, incubated with IRDye® conjugated secondary antibodies, and analyzed using the infrared imaging system.

For protein isolated from the three CB-MSC groups, 15-20 µg was separated and blotted onto membranes. Membranes were blocked and incubated with Bax, alpha Tubulin, p21, p16, p38, Caspase 3, GAPDH, pp38, CDK1, Cyclin E2, Cyclin D1, Cyclin B1, p27, JNK, pJNK, ERK1/2, pERK1/2, CDK4, CDK2 and pp53 primary antibodies and stained with HRP- or IRDye-labeled secondary antibodies. Chemiluminescent and infrared protein detection was performed. Band quantification was performed by ImageJ.

Flow Cytometric Analysis: The immunophenotype of AEC and EMT-AEC was analyzed by flow cytometry by incubating cells with the following antibodies conjugated to fluorophores: CD34 FITC, CD45 Vioblue, CD133 PE, HLA-DR PECy7, CD14 APCCy7, CD90 APC, CD73 PE and CD105 FITC. Cells were gated in FSC and SSC and dead cells were excluded based on DAPI staining.

All the three CB-MSC groups were stained for the following markers: CD14-FITC, CD73-PE, CD34-PE, CD45-FITC, CD90-APC, CD105-FITC, and HLA-DR-FITC.

CB-MSC cell harvests and supernatants were used to determine apoptosis by AnnexinV FITC Apoptosis Detection Kit I. Necrotic cells were excluded by PI counterstaining.

CB-MSC from all three groups were fixed, washed in PBS and stained with PI solution. Cellular DNA content was assessed through PI fluorescence for cell cycle analysis.

All flow cytometry data were analyzed using FlowJo software.

CFU-F clonal assay: Please refer to the attached publication for the detailed protocol (13).

Genechip array hybridization: Samples from AEC and EMT-AEC were hybridized to an Affymetrix Human GeneChip Array U133A 2.0. Arrays were scanned and raw data were analyzed with Affymetrix Operating Software (GCOS 1.2). A Scatter plot was constructed using data analysis software, Chipster and genes with a change of more than 2-fold were selected for constructing heat maps.

Cytokine secretion profile: Conditioned medium from AEC and EMT-AEC were analyzed with the chemiluminescent Human Cytokine ELISA Plate Array according to the manufacturer's instructions. The assay was done in triplicates and the difference in luminescence intensity between AEC and EMT-AEC was calculated in percent.

To ascertain that sera were indeed characteristic for patients with chronic HF, human serum samples were thawed and protein was normalized immediately before IL-6 and TNF-α present in the sera were analyzed.

Gelatin zymography: Conditioned medium from AEC and EMT-AEC were resolved in a 10% Novex zymogram gel containing gelatin. Gels were incubated in 1X zymogram renaturing buffer followed by equilibration in 1X zymogram developing buffer. Gels were then processed overnight in 1X zymogram developing buffer and stained using coomassie brilliant blue staining solution followed by destaining. Band intensity was quantified using ImageJ.

MTS metabolic activity assay and BrdU proliferation assay: The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay and the colorimetric BrdU Cell Proliferation ELISA were performed with the three CB-MSC experimental groups according to the manufacturer's instructions on days 1, 3 and 5 on triplicate wells.

DAPI-based direct cell counting: Subsequent to MTS assay, CB-MSC from three groups were fixed and stained with DAPI. The Operetta high content screener was used to capture images that covered the entire culture surface.

Trilineage differentiation potential: Please refer to the attached publication for a detailed differentiation protocol (13).

Quantitative Real-Time PCR: Please refer to the attached publication for details (13).

3.2 *In vivo*

Induction of myocardial infarction: Please refer to the attached publication for a detailed protocol the for induction of myocardial infarction (12). 2.5 million PLX-PAD cells (n=10), 1 million AEC (n=10), 1 million EMT-AEC (n=10) in 25µl PBS or PBS only (infarct control, n=10) were transplanted by intramyocardial injection. Six mice underwent a sham operation with thoracotomy but no manipulation of the heart. After surgery, mice were given Flunixin meglumine for pain relief and were allowed to survive for 4 weeks.

Magnetic resonance imaging (MRI): Please refer to the attached publication for a detailed procedure of MRI (12).

Echocardiography: Please refer to the attached publication for a detailed procedure of echocardiography (12).

Histology: After echocardiography mice were sacrificed by cervical dislocation and hearts were excised, fixed and embedded in paraffin. 5µm sections were prepared for histological analysis.

Infarct size: Deparaffinized and rehydrated sections were stained with Masson's trichrome stain according to the manufacturer's protocol. The area of infarcted and non-infarcted myocardium was quantified using ImageJ.

Blood vessel density: After unmasking antigens and blocking, sections were incubated with anti-CD31 antibody followed by incubation with biotinylated anti-rabbit secondary antibody. The secondary antibody was conjugated to horse radish peroxidase which was developed by diamino benzidine (DAB). Nuclei were counterstained with Meyer's hematoxylin and slides were analyzed with a light microscope.

Caveolin-1 staining: Capillary vessels were also detected using a monoclonal antibody against caveolin-1 and visualized by an Alexa Fluor 488-conjugated secondary goat anti-mouse antibody. Nuclei were counterstained with DAPI and slides were analyzed by a fluorescence microscope.

TUNEL staining: Apoptotic cells were detected in deparaffinized sections by end-labeling of fragmented DNA using the DeadEnd™ Colorimetric TUNEL System according to the manufacturer's instructions. Nuclei were counterstained with DAPI and slides were analyzed by a fluorescence microscope.

Periostin expression: Following deparaffinization and rehydration, antigens were unmasked. After blocking, sections were incubated with anti-periostin antibody. Secondary anti-rabbit antibody conjugated to Alexa Fluor 647 was used for visualization by fluorescence microscopy.

Detection of transplanted cells: Deparaffinized sections were incubated with mouse anti-human nuclear antigen (hNuc) primary antibody followed by incubation with goat anti-mouse Alexa 647 conjugated secondary antibody. Nuclei were counterstained with DAPI, and sections were analyzed by fluorescence microscopy.

Detection of plasma interleukin 10 (IL-10): At the time of sacrifice, 500µl of blood was collected from the heart from PLX-PAD and control mice. The concentration of IL-10 in undiluted plasma was determined by ELISA according to manufacturer's instructions.

Statistics: For AEC, EMT-AEC and PLX transplantations intergroup differences between more than two groups were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's (equal variance given) or Dunnett's T3 (unequal variance) post-hoc test. Data between two groups were compared using two-tailed, unpaired students's t-test. For hemodynamic data Kruskal-Wallis test with pairwise comparison was used for intergroup comparison of the data distribution. A value of $p < 0.05$ was considered statistically significant.

For CB-MSC experiments ANOVA with Bonferroni's post-hoc test was done, otherwise the Kruskal-Wallis-Test or Mann-Whitney U-Test were applied to test for differences between groups. A general linear model was constructed to test the impact of several clinical variables on CB-MSC behavior within the HF patient cohort.

4. Results

4.1 AEC (*in vitro*)

Cell morphology: AEC displayed typical cobblestone morphology of epithelial cells, characterized by tight junctions.

Surface marker expression: Flow cytometric analysis of AEC revealed that they were negative for all hematopoietic lineage markers (CD45, CD34, CD133 and CD14), but were positive for stemness-associated surface markers (CD90, CD105 and CD73).

Immunofluorescence: AEC expressed very low levels of N-cadherin and showed a typical surface expression of E-cadherin localized at cell junctions. Expression of periostin was perinuclear in AEC and β-actin expression was consistent with typical epithelial cell cytoskeleton arrangement.

Western Blot: Western blot analysis revealed a strong expression of E-cadherin and occludin in AEC. Expression of total Akt was also observed though expression of phosphorylated Akt was very low. Total ERK1/2 and phosphorylated ERK1/2 expression was also prominent in AEC.

Gene expression: Microarray analysis of AEC revealed expression of typical epithelial genes. Of note were, *tight junction protein 1, keratin 6a, keratin 13, keratin 18, keratin 23, keratin 24, E-cadherin, Occludin, plakophillin 2, desmoglein, epithelial membrane protein, claudin 4, claudin 7* and *epithelial cell adhesion molecule***.** Cell cycle-related genes (i.e. *cyclin A2, B1, B2, D3*) were also predominantly expressed in AEC.

Cell mobility: In the scratch wound healing assay AEC maintained their epithelial confluence and narrowed the scratch wound only slightly during the observation period. They maintained their epithelial morphology on the surface of transwell inserts and did not migrate through the transwell to the lower compartment.

Cytokine secretion profile: Conditioned medium obtained from AEC contained the following cytokines VEGF, IL-8, IP-10, IL-1a, IFN-g, IGF-1, b-NGF, IL-10 and IL-6.

Gelatinase activity: Zymograms of AEC conditioned medium showed that gelatinase activity was higher in AEC medium than in cell free control medium evident by a higher band intensity compared to cell free control medium.

4.2 AEC (*in vivo*)

Heart function: As measured by transthoracic echocardiography transplantation of AEC did not result in a significantly higher left ventricular ejection fraction (LVEF) (43% [35-54%]) than that in infarct control hearts (33% [28-41%]). Speckle tracking echocardiography showed similar results where strain rate in the area of interest (AOI) did not show improvements in the AEC transplanted hearts (AEC 29 [21-46]; infarct control 23 [4-37]; sham 54 [50-59], $p>0.05$). LVEF measured by cardiac MRI also revealed similar results (LVEF: AEC 32% [23-42%], infarct control 27% [17-36%], sham 60% [48-64%], $p>0.05$).

Infarct size: Hearts treated with AEC (10.6±2%) seemed to have smaller infarcts than infarct controls (14 ± 2%), but statistical analysis indicated merely a trend ($p=0.1$).

Apoptosis: Number of apoptotic cardiomyocytes in AEC treated hearts (6.2 ± 1.5) were significantly lower than infarct controls (12.4 ± 3.5),($p=0.02$).

Cardiac extracellular matrix remodeling: Periostin protein was mainly located in the extracellular matrix and was particularly pronounced in the peri-infarct area. Semi-quantification of immunohistochemistry images revealed lower expression of periostin in AEC treated hearts compared to infarct control hearts.

Arteriogenesis and microvessel density: The number of large vessels was higher in infarcted hearts treated with AEC (6.4 ± 0.4) compared to infarct control hearts (4.2 ± 0.64), ($p=0.03$). Microvessel density was also higher in AEC treated hearts (12.4 ± 1.1) than infarct control hearts (8.5 ± 0.85), ($p=0.01$).

Cell retention: Four weeks after transplantation, AEC could not be detected in the hearts.

4.3 EMT-AEC (*in vitro*)

Cell morphology: Upon exposure to TGF-β1, AEC underwent a dramatic change in morphology. The typical cobblestone morphology was disrupted and cells displayed a spindle-

like, fibroblastoid shape. An increase in cell size was also observed through the course of the transition.

Surface marker expression: Upon completion of EMT under the influence of TGF-β1, CD90 surface expression remained unchanged, CD105 nearly completely disappeared, and CD73 was markedly reduced but still present on 33% of the EMT-AEC.

Immunofluorescence: After TGF-β1 treatment the expression of N-cadherin increased and was highest upon completion of EMT with cells acquiring a fibroblast-like morphology. Expression of E-cadherin was significantly reduced with the progression of EMT. Periostin immunostaining indicated a translocation from perinuclear to a diffuse cytoplasmic distribution. Immunostaining for β-actin demonstrated the rearrangement of the cytoskeleton consistent with EMT.

Western Blot: Upon completion of EMT, expression of E-cadherin and occludin decreased. Expression levels of total Akt and phosphorylated ERK1/2 remained unchanged but total ERK1/2 and phosphorylated Akt increased upon induction of EMT.

Gene expression: EMT completion resulted in 700 genes being differentially expressed. Of note, functional annotation and principle component analysis revealed that EMT induced activation of signaling cascades associated with TGF-β signaling (i.e. *SMAD 6/7*), regulation of the actin cytoskeleton (*transgelin*), cell migration, focal adhesion and cell-matrix interactions (*versican, tensin*). Upregulation of *N-cadherin* was also observed.

Cell mobility: In contrast to AEC, EMT-AEC rapidly migrated into the scratch wound as single cells and in multidirectional fashion. In the transwell migration assay, the change in morphology of cells plated on the transwell inserts and treated with TGF-β1 was consistent with that of standard cell culture flask surface conditions. During the observation period, EMT-AEC migrated through the membrane pores and were found in the lower compartment.

Cytokine secretion profile: Cytokine secretion profile obtained from EMT-AEC conditioned medium reflected the presence of VEGF, IL-8, IP-10, IL-1a, IFN-g, IGF-1, b-NGF,IL-10 and IL-6. The most obvious differences related to an increase in IL-8, IP-10, IL-1a, IFN-g, IGF-1, b-NGF and decrease in IL-10 and IL-6 were associated with EMT. Of note with respect to our findings regarding angiogenesis, VEGF secretion did not change.

Gelatinase activity: Gelatinase activity was highest in the conditioned medium from EMT-AEC compared to AEC and cell free control medium as indicated by additional bands and higher band intensity.

4.4 EMT-AEC (*in vivo*)

Heart function: Global left ventricular function expressed as left ventricular ejection fraction (LVEF) as measured by transthoracic echocardiography was best preserved in animals that received EMT-AEC (LVEF, 57% [43-77%]), than that in infarct control hearts (33% [28-41%]), ($p<0.001$). For comparison, sham-operated animals had a median LVEF of 70% (59-78%). Speckle tracking echocardiography confirmed these results, because the systolic strain rate in the AOI was maintained best in EMT-AEC-treated infarct tissue (EMT-AEC 45 [36-58]; infarct control 23 [4-37]; sham 54 [50-59], $p<0.05$). By cardiac MRI, we also observed that LV function was preserved in EMT-AEC-treated hearts (LVEF: EMT-AEC 44% [39-49%], infarct control 27% [17-36%], sham 60% [48-64%], $p<0.05$).

Infarct size: Hearts treated with EMT-AEC (3.2 ± 2%) had significantly smaller infarct size than infarct controls (14 ± 2%), ($p<0.01$).

Apoptosis: Number of apoptotic cardiomyocytes in EMT-AEC treated hearts (3.8±1.2) were significantly lower than infarct control hearts (12.4±3.5), ($p=0.001$).

Cardiac extracellular matrix remodeling: Periostin expression could be detected in all segments of the heart. Semi-quantification of immunohistochemistry images showed higher periostin expression in EMT-AEC treated hearts compared to infarct control hearts.

Arteriogenesis and microvessel density: The number of large vessels was higher in infarcted hearts treated with EMT-AEC (6.6 ± 0.41) compared to infarct controls (4.2 ± 0.64), ($p=0.007$). Microvessel density was also higher in EMT-AEC treated hearts (12.5 ± 0.87) than infarct control hearts (8.5 ± 0.85), ($p=0.03$).

Cell retention: Four weeks after transplantation, EMT-AEC could not be detected in the hearts.

4.5 PLX

Heart function: As measured by transthoracic echocardiography, in PLX-PAD-treated hearts, stroke volume was higher than in infarct control hearts (PLX-PAD, 0.09±0.009 ml vs. infarct control, 0.06±0.004 ml, p=0.01). Accordingly, fractional shortening was higher in PLX-PAD-treated hearts (PLX-PAD, 28±3 % vs. infarct control, 19±3 %, p=0.03). Analysis of regional 2D strain and strain rate, expressed as the ratio between AOI and the reference area, also demonstrated better contractile function in PLX-PAD-treated areas of infarcted hearts (strain: PLX-PAD, 47±6 vs. infarct control, 21±2, p=0.002, strain rate: PLX-PAD, 41±10 vs. infarct control, 21±5, p=0.05).

Infarct size: Infarct size was significantly smaller in PLX-PAD (5.5±2.4 %) treated hearts than in infarct control hearts (13.6 ± 3 %), (p=0.04).

Apoptosis: The number of TUNEL positive cardiomyocytes was significantly lower in PLX-PAD-treated infarcted hearts (9.8 ± 1.7) than in infarct control hearts (30.3 ± 6.8), (p=0.001).

Cardiac extracellular matrix remodelling: Semi-quantification of immunohistochemistry images showed higher periostin expression in PLX-PAD treated hearts compared to infarct control hearts.

Arteriogenesis and microvessel density: The total number of mature arterial blood vessels was higher in the PLX-PAD-treated group (6.9 ± 0.4) than the infarct controls (4.6 ± 0.7), (p=0.004). The number of microvessels in the infarct border zone was also higher in PLX-PAD-treated hearts (48 ± 6) than in infarct control hearts (37 ± 5), (p=0.01).

Detection of human cells and plasma IL-10: Transplanted human PLX-PAD cells could not be detected in the mouse hearts after four weeks. Also, no IL-10 could be detected in the plasma of PLX-PAD-treated or PBS control mice.

4.6 CB-MSC

Cell morphology: After 5 days of HF serum treatment, CB-MSC retained their spindle-shaped morphology.

Surface marker expression: Flow cytometric analysis of HF serum treated CB-MSC revealed neither qualitative nor significant quantitative differences between the three groups after 5 days of treatment. 98% of live cells expressed CD73, CD90 and CD105; less than 2% of live cells expressed CD14, CD34, CD45 and HLA-DR.

Western Blot: Amongst the cyclin dependent kinases studied, Cyclin D1 expression was elevated ($p<0.05$) and p16 remained unchanged in response to HF serum ($p=0.2$). Upregulation of p21 and p27 and a trend toward downregulation of Cyclin B1 was observed (p21, $p=0.03$; p27, $p=0.03$; Cyclin B, $p=0.2$). In response to HF serum, most CB-MSC rested in the G1 phase of the cell cycle, despite a certain pro-mitotic stimulation (Cyclin D1 upregulation). Increased levels of cleaved caspase 3 was observed ($p=0.03$). JNK total protein ($p=0.02$) and phosphorylated JNK ($p=0.03$) were significantly elevated, while phosphorylated ERK1/2 was reduced ($p<0.05$).

Gene expression upon differentiation: Differentiation induced upregulation of ALP (osteogenesis), PPARγ (adipogenesis) and SOX9 (chondrogenesis) in all groups (relative gene expression in differentiated vs. undifferentiated cells, $p=0.03$ for all groups and all markers). ALP expression in HF serum treated undifferentiated cells was elevated compared to human control serum or FCS treated undifferentiated cells, and did not increase further during osteogenic differentiation. Quantitatively, the osteogenic and chondrogenic differentiation potential did not differ between the three CB-MSC groups (ALP, $p=0.6$; SOX9, $p=0.1$). Of note, PPARγ expression in adipogenic differentiated HF serum treated cells was markedly lower compared to the FCS control ($p=0.02$).

Cytokine secretion profile: Compared to human control serum, IL-6 ($p=0.002$) and TNF-α ($p=0.002$) concentrations were higher in all heart failure patient sera. However, no correlation could be established between cytokine content and CB-MSC proliferation or apoptosis.

Colony formation pattern of CB-MSC: Quantification of clonal efficiency showed a significantly lower percentage of clonogenic cells in the HF group (human control, vs. HF, $p=0.005$; FCS, vs. HF, $p=0.05$). Notably, in the presence of HF serum CB-MSC formed more small-sized clusters and fewer large-sized clusters than in human control serum or FCS.

Impaired CB-MSC proliferation: Irrespective of the type of serum, CB-MSC grew steadily, but with differences in growth rate between the groups. Overall, CB-MSC proliferation was significantly suppressed in the HF group during the first 3 days of treatment and accelerated during the final phase of observation. In the BrdU cell proliferation assay, CB-MSC DNA synthesis levels were low after 1 and 3 days of exposure to HF serum but after 5 days approached that of control serum treated CB-MSC. The DAPI-based direct cell counting method and the MTS metabolic activity assay confirmed this observation.

Cell cycle progression in CB-MSC: Flow cytometric quantification of DNA content in cells stained with PI showed that majority (>70%) of cells were in the G0/G1 phase and the remaining cells were almost equally distributed between the S and G2/M phases. However, in the HF group there were significantly more cells in G0/G1 (human control vs. HF, $p=0.04$; FCS vs. HF, $p<0.05$) and fewer cells in the S phase (human control vs. HF, $p=0.04$; FCS vs. HF, $p=0.006$). Interestingly, the percentage of cells in G2/M phase did only differ between the HF and the FCS group (human control, vs. HF, $p=1$; FCS, vs. HF, $p=0.01$).

Apoptosis in CB-MSC: Flow cytometric analysis of AnnexinV stained cells revealed a significantly higher number of cells that had translocated phosphatidyl serine to the outer surface of their plasma membranes in the HF group (human control vs. HF, $p=0.03$; HF vs. FCS, $p=0.6$). By PI staining, the proportion of necrotic cells (PI+) was not significantly different between human control, HF serum and FCS treated cells (necrotic cells: overall $p=0.07$). While the intergroup difference in phosphorylated p53 expression indicated a trend towards increased levels ($p=0.07$), Bax protein expression did not differ between the groups ($p=0.5$).

5. Discussion

Prominent neonatal cells that have been exploited for usage in regenerative medicine are Wharton's jelly mesenchymal stromal cells (WJ-MSC), cord blood mesenchymal stromal cells (CB-MSC), amniotic epithelial cells (AEC), amniotic mesenchymal cells (AMC) and placenta - derived stromal cells (PLX-PAD). In our study we investigated the cardioprotective effects of neonatal progenitor cells, namely, AEC, EMT-AEC and PLX-PAD in vivo and the impact of heart failure patient serum on the behavior of CB-MSC *in vitro*.

AEC are derived from embryonic epiblast cells and mirror their epithelial morphology, while displaying varying degree of stemness. Flow cytometry data revealed the presence of surface markers commonly found on mesenchymal stomal cells (CD73, CD90, CD105) (14). AEC also displayed ECM modulatory properties as evident in the zymogram. Keeping these in mind the cardioprotective effects of naïve AEC were evaluated in a mouse model of myocardial infarction. Though AEC treated hearts displayed a lower number of TUNEL positive cells and a higher blood vessel and microvessel density compared to PBS controls, it did not result in improved heart function or a reduction of infarct size. This can be attributed to the limited migration potential of epithelial cells. The scratch wound assay revealed that AEC migrated at a slow rate, only as cell sheets and did not migrate at all through transwells. Therefore, to enhance the migration potential and ECM modulatory properties of AEC, we induced EMT and evaluated them *in vitro* and *in vivo*. EMT-AEC displayed all the hallmark properties associated with EMT. Of note, they displayed upregulation of N-cadherin, enhanced migration potential and higher gelatinase activty in comparison to AEC. Transplantation of EMT-AEC resulted not only in improved global heart function but also a significantly lower infarct size compared to AEC and PBS controls. Although AEC transplantation led to decreased number of TUNEL positive cells than the controls, EMT-AEC transplantation further enhanced that effect. EMT-AEC hearts displayed the least number of apoptotic cells. The significant reduction in infarct size upon EMT-AEC transplantation can be attributed not only to enhanced migration potential and enhanced ECM modulatory properties but also to anti-apoptotic effects of EMT-AEC, the upregulation of survival proteins associated with EMT and enhanced secretion of cytokines. However induction of EMT did not change the secretion of angiogenic cytokine VEGF, *in vitro*. Also, no significant differences were observed in mature blood vessel density and microvessel density in the hearts transplanted with AEC and EMT-AEC.

Although EMT-AEC transplantation led to significant improvements in heart structure and function their evaluation in clinical trials is limited by the fact that they are not clinical grade cells. This led to the incorporation of an equivalent MSC-like clinical grade neonatal progenitor cell product in our study that is available as an off-the-shelf product and manufactured under GMP conditions. PLX-PAD cells are derived from feto-maternal tissues and expanded under closed 3-D conditions. Their clinical grade expansion and their MHC profile makes them suitable for allogeneic transplantation (12). Their immune-privileged nature is reflected by the

fact that $2.5x10^6$ cells were used for a single dose of transplantation. However, this did not lead to deleterious adverse effects. The animals survived for four weeks and structural and functional analysis revealed a beneficial effect of cell therapy. These cells have been reported to aid in angiogenesis in hind limb ischemia model (7), which reiterates their potential for induction of angiogenesis in ischemic tissues, *in vivo*. Intramyocardial transplantation of these cells lead to a significant decrease in infarct size and an increase in mature vessel count and microvessel density compared to PBS controls. The decrease in infarct size was primarily mediated via paracrine factors that had a protective effect on cardiomyocytes and endothelial cells under ischemic conditions. This was again confirmed by the presence of less apoptotic cells in PLX-PAD treated hearts. Reports have indicated mobilization and stimulation of resident cardiac stem cells via VEGF secreted by transplanted MSC (15). Though not investigated in our studies, it cannot be completely ruled out that this phenomenon can contribute to the observed cardioprotective effects.

Treating CB-MSC with heart failure serum indicated that the serum indeed had an effect on the behavior of CB-MSC. The treatment impaired the proliferation kinetics as evident by MTS, BrdU assay and DAPI labeled cell counting. The impaired proliferation kinetics was observed only on a short term basis (until day 3). However by day 5 growth kinetics was similar to that of control serum and FCS controls. Down regulation of pERK1/2 was consistent with impaired proliferation. Also, the clonal efficiency of CB-MSC was affected with HF serum treatment giving rise to smaller colonies. HF serum treatment also triggered cellular apoptosis as evidenced by activation of caspase 3. These results reflect upon the clinical perspective that in order to achieve beneficial effects of cell transplantation, long term survival of cells is essential.

In conclusion, we observed that neonatal cells indeed display cardioprotetive effects mediated primarily via paracrine factors, migratory potential and extracellular matrix modulating properties. However, HF-associated soluble factors also affect the functionality of transplanted neonatal cells on a short term even under allogeneic conditions. Therefore, these factors need to be taken into consideration for designing an ideal cell therapy product for cardiac regeneration.

6. Limitations of the study

Our animal model of myocardial infarction incompletely reflects the clinical situation, because acute infarction by permanent occlusion of the LAD was treated by intramyocardial cell injection. In the clinical setting, every attempt is made to quickly re-open the coronary artery and reperfuse the infarcted tissue, followed by intravascular cell delivery, while intramyocardial cell injection is most often done in patients with chronic ischemic heart disease. However, this model is preferred for proof of concept studies, because it results in a more uniform infarct size and more reliable cell delivery in small rodents. Although the cell product was deposited correctly within the myocardium, variations in the efficacy of cell injection cannot be excluded because no live cell tracking techniques were employed. We were not allowed to perform baseline echocardiography measurements of LV function immediately after LAD occlusion, so all conclusions are based on the intergroup comparison at 4 weeks rather than the change in infarct size in an individual animal.

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Affidavit

I, Rajika Roy certify under penalty of perjury by my own signature that I have submitted the thesis on the topic 'Human placenta derived cells for myocardial cell therapy'. 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)" 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 (s.o) and are answered by me. 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 which I am author of 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.

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Date 04.12.2013 Signature

Declaration of any eventual publications

Rajika Roy had the following share in the following publications:

Publication 1: Impact of heart failure on the behavior of human neonatal stem cells in vitro
Kristin Klose, dipl.-ing., Rajika Roy, MSc, Andreja Brodarac, Phd, Andreas Kurtz, Phd, Andrea Ode, PhD, Kyung-Sun Kang, Prof., Karen Bieback, PhD, Yeong-Hoon Choi, MD,and Christof Stamm, MD
Journal of Translational Medicine 2013
Contribution – data interpretation, manuscript preparation

Publication 2: Cardioprotection by placenta-derived stromal cells in a murine myocardial infarction model
Rajika Roy, MSc, Andreja Brodarac, PhD, Marian Kukucka, MD, Andreas Kurtz, PhD, Peter Moritz Becher, MD, Kerstin Jülke, PhD, Yeong-Hoon Choi, MD, Lena Pinzur, MSc, Ayelet Chajut, PhD, Carsten Tschöpe, MD, Christof Stamm, MD
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Contribution – study design, animal experiments, data analysis and interpretation for in vivo experiments, manuscript preparation

Publication 3: Epithelial-to-mesenchymal transition enhances the cardioprotective capacity of human amniotic epithelial cells
Rajika Roy, MSc, Marian Kukucka, MD, Daniel Messroghli, MD, Désirée Kunkel, PhD Andreja Brodarac, PhD, Kristin Klose, dipl.-ing, Sven Geißler, PhD, Peter Moritz Becher, MD, Sung Keun Kang, PhD,Yeong-Hoon Choi, MD, Christof Stamm, MD
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Contribution - Study design, animal experiments, data analysis and interpretation, preparation of manuscript

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Publication 1

Impact of heart failure on the behavior of human neonatal stem cells *in vitro*

Kristin Klose, **Rajika Roy**, Andreja Brodarac, Andreas Kurtz, Andrea Ode, Kyung-Sun Kang, Karen Bieback, Yeong-Hoon Choi and Christof Stamm

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Publication 3

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

My curriculum vitae is not published in the electronic version of my work for privacy reasons.

COMPLETE LIST OF PUBLICATIONS

1. **Impact of heart failure on the behavior of human neonatal stem cells *in vitro***
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