ON LINE SUPPLEMENT

Short, Long Term and Paracrine Effect of Human Umbilical Cord-derived Stem Cells on Lung Injury Prevention and Repair in Experimental BPD

Maria Pierro[1,2], Lavinia Ionescu[1], Tiziana Montemurro[3], Arul Vadivel[1], Gaia Weissmann[2], Gavin Oudit[4], Derek Emery[5], Sreedhar Bodiga[4], Farah Eaton[1], Bruno Péault[6], Fabio Mosca[2], Lorenza Lazzari[3], Bernard Thébaud[1]

[1]Department of Pediatrics, School of Human Development, Women and Children's Health Research Institute, Cardiovascular Research Center and Pulmonary Research Group, University of Alberta, Edmonton, Canada

[2]Department of Maternal and Pediatric Sciences, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy

[3]Cell Factory, Department of Regenerative Medicine, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[4]Division of Cardiology, Department of Medicine, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Canada

[5]Department of Radiology and Diagnostic Imaging, University of Alberta, Edmonton, Canada

[6]Department of Orthopaedic Surgery, Cellular & Molecular Pathology, David Geffen School of Medicine at UCLA, Los Angeles, CA

Correspondence:
Dr. Bernard Thébaud
University of Alberta
3-020 Katz Center
Edmonton, Alberta, T6G 2E1, Canada
Phone: 780-492-7130, Fax: 780-492-9753
E-mail: bthebaud@ualberta.ca

Methods

Human umbilical cord isolation and culture of pericytes. Pericytes (PCs) were isolated from the umbilical cords after parental consent as previously described (Supplemental Figure).[1] Briefly, human umbilical cords were dissected longitudinally to expose the vein and the two arteries and digested with 1mg/mL collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C, for a maximum of 18 hours. The cell suspension was washed and the cell pellet was resuspended and cultured in EGM2 medium (Lonza, Walkersville, MD, USA) on a pre-coated gelatin layer (Sigma-Aldrich; St. Louis, MO, USA). After 1 week, the medium was replaced with DMEM high-glucose (Invitrogen, Carlsdad, CA, USA), supplemented with 20% fetal bovine serum (FBS; Biochrom, AG, Berlin, Germany) and 1% penicillin/streptomycin (P/S, Sigma-Aldrich) and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Adherent PCs, 80% confluent, were passaged by treatment with trypsin-EDTA (Gibco, Grand Island, NY, USA), and split 1:3 in uncoated plates in the same culture conditions. Medium was changed every 3 days.

Human umbilical cord blood isolation and culture of mesenchymal stem cells (MSCs). Human cord blood (CB) was collected from newborns after parental consent and MSC isolation was performed within 12 hours as previously described (Supplemental Figure).[2] First, CB was centrifuged and plasma discarded. An enrichment protocol was performed by a negative immunodepletion of CD3+, CD14+, CD38+, CD19+, glycophorin A and CD66b+ using a commercial kit (RosetteSep Mesenchymal Stem Cell, StemCell Technologies, Vancouver, BC, Canada), and followed by a density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare, Amersham Place, UK). After washing, cells were cultured in Modified Eagle alpha-medium (Invitrogen) supplemented with 20% FBS (Biochrom) and 2mM L-glutammine (Gibco). Cultures were maintained at 37°C in humidified atmosphere containing 5% CO2. After overnight incubation, non-adherent cells were removed

and fresh medium was added; culture medium was changed every 3 days.

Generation of PCs and MSC-derived CdM. Cells were grown in 75t flask up to 90% confluence (MSC 1.500.000 cells/ fask, PCs 1.000.000 cells/flask). Then cells were rinsed 3 times with PBS and serum free media was added. After 24 hours the supernatant was harvested and centrifuged at 4000 RPM for 40 minutes in ultrafiltration tubes (Millipore, US) to obtain a 25 times concentrated CdM.[3] CdM was also obtained from human neonatal dermal fibroblasts (HNDF, ATCC, Manassas, VA, USA) and cultured in Fibroblast Basal Medium supplemented with FGM bulletkit (Lonza, Basel, Switzerland).

Animal model of O_2-arrested lung growth. Rat pups were exposed to normoxia (21% O_2, control group) or hyperoxia (95% O_2, BPD-group) from birth to P14 in sealed Plexiglas chambers (BioSpherix, Redfield, NY) with continuous O_2 monitoring.[4,5] Dams were switched every 48 hours between the hyperoxic and normoxic chambers to prevent damage to their lungs and provide equal nutrition to each litter. Litter size was adjusted to 12 pups to control for effects of litter size on nutrition and growth. Rat pups were euthanized at various time points with intraperitoneal pentobarbital and lungs and heart were processed, according to the performed experiments.

***In Vivo* Cells Administration.** We performed short-term experiments using a prevention and a rescue approach. For the prevention studies, newborn rat pups were randomized into seven groups: (1) room air control (RA Ctrl), (2) room air+MSCs (RA MSCs), (3) room air+PCs (RA PCs), (4) hyperoxia (O_2 Ctrl, injury model), (5) hyperoxia+HNDF, (6) hyperoxia+MSCs (O_2 MSCs), and (7) hyperoxia+PCs (O_2 PCs). For these prevention studies, rat pups received 300.000 cells in 20µl at P4 via an i.t. injection and harvested at P22.

For subsequent rescue experiments, the control cell group (HNDF) was deleted because HNDFs had no effect. For the same reason, we also deleted the room air+MSC and room air+PC groups. Thus, for rescue studies, newborn rat pups were randomized into 4 groups: (1) room air control (RA Ctrl), (2) hyperoxia (O_2 Ctrl, injury model), (3) hyperoxia+MSCs (O_2

MSCs), and (4) hyperoxia+PCs (O_2 PCs). For these rescue studies, rat pups received 600.000 in 40µl at P14 and harvested at P35.

The cell dose was adjusted to animal weight and based on the literature.[6]

We also performed long-term studies to assess the effect of stem cell administration at 6 months. In these experiments animals were treated at P4 and harvested at 6 months. Animals were randomized into 6 groups: (1) room air control (RA Ctrl), (2) room air+MSCs (RA MSCs), (3) room air+PCs (RA PCs), (4) hyperoxia (O_2 Ctrl, injury model), (5) hyperoxia+MSCs (O_2 MSCs), and (6) hyperoxia+PCs (O_2 PCs).

***In Vivo* CdM Administration.** We performed short-term experiments using a prevention and a rescue approach. In the prevention studies, newborn rat pups were randomized into six groups: (1) room air control (RA Ctrl), (2) RA+MSC CdM, (3) RA+PC CdM, (4) hyperoxia control (O_2 Ctrl), (5) O_2+MSC CdM, and (6) O_2+PC CdM. In these prevention studies, CdM was administered daily IP at the dose of 7 µl/g from P4 to P21 and animals were harvested at P22 (prevention studies).

In the rescue studies, newborn rat pups were randomized into 4 groups: (1) room air control (RA Ctrl), (2) hyperoxia control (O_2 Ctrl), (3) O_2+MSC CdM, and (4) O_2+PC CdM. In these rescue studies, CdM was administered daily IP at the dose of 7 µl/g from P14 to P28 and animals were harvested at P35. The dose of the CdM was based on Aslam et al. [7]

Long-term study animals were treated from P4 to P21 and harvested at 6 months.

Lung function tests. Animals were anesthetized using ketamine (10 mg/kg i.p) and xylazin (5 mg/kg i.p) mixture and paralyzed using a pancuronium bromide injection (1 mg/kg i.p). Tracheostomy was performed and lung function was assessed using Flexivent (Scireq, Montreal, QC, Canada).

Lung Morphometry. Lungs were inflated and fixed via the trachea with zinc formalin

solution at a constant pressure of 20 cm H_2O.[4,5] Lungs were paraffin embedded and cut into 4-µm-thick serial sections, and lungsections were stained with hematoxylin and eosin. Alveolar structures were quantified using the mean linear intercept as described.[4,5] Six lungs/group, three sections/lung and 100 high-power fields/section were counted.

Barium-gelatin angiograms and vessel density counts. A barium-gelatin mixture (60ºC) was infused in the main pulmonary artery until surface filling of vessels with barium was seen uniformly over the surface of the lung as previously described.[4,5] Four to five lungs/group, five sections/lung and ten high-power fields/section were counted. Barium-injected lung vasculature was imaged with a rodent SPECT-CT (FLEX Pre-clinical platform) using Amira software package (Gamma Medica, Northridge, CA).

Right ventricular hypertrophy (RVH) and pulmonary artery remodeling. The right ventricle free wall was separated from the left ventricle and the septal wall. The tissue was dried overnight and weighed the next day to determine the right ventricle to left ventricle+septum ratio (RV/LV+S) as an index of RVH.[5] Pulmonary artery remodeling was quantified by the medial wall thickness (MWT).[4,5] Five pups/group, three sections/lung and ten high-power fields/section were counted.

Exercise capacity. Rats were run on a treadmill adjusting the speed according to the following protocol: 1 min at 10 meters/min, 1 min at 11 meters/min, 1 min at 12 meters/min, 2 min at 13 meters/min, 5 min at 15 meters/min, 17 meters/min until exhaustion. Exhaustion was defined by sitting on the shock panel longer than 5 seconds.

Total Body CT-Scan. Rats were anesthetized using inhaled isoflurane and 3 to 4 sections with 1028 slides/section were taken with a rodent SPECT-CT (FLEX Pre-clinical platform) using Amira software package (Gamma Medica, Northridge, CA).

Real-time PCR. Total RNA was extracted from pulverized frozen lungs using Qiagen RNeasy kit (Qiagen, Mississauga, ON). RNA was quantified using a Nanodrop system (ND-1000 ThermoFisher Scientific, Wilmington, DE) and cDNA was prepared from 1ung RNA

using random hexamers. PCR was performed on an ABI 7900 and using Taqman Universal PCR master mix (Applied Biosystems), Human Alu sequence primers and values determined from a standard curve prepared from pure pericytes and MSCs.[8] All results are expressed as a ratio of Alu sequences normalized to human 18S. Three animals/group were harvested 10 min after injection (P4), 1 day after injection (P5), 2 days after injection (P6) and at 22 days of life.

Immunofluorescence for β2-microglobulin. Immunofluorescent staining was performed on nonadjacent 5μm paraffin-embedded lung sections using rabbit anti-human β2-microglobulin (Abcam, Cambridge, MA, USA) and appropriate secondary antibodies (Invitrogen, Carlsbad, CA, USA). Nuclei were identified by DAPI staining. Five random fields of four sections per animal were analyzed by confocal microscopy.

Statistics. Values are expressed as means ± SEM. Intergroup differences were assessed using analysis of variance with post hoc test (Fisher's probable least significant difference test) (SPSS v 18). A value of $P<0.05$ was considered statistically significant. All investigators performing evaluations were blinded to the experimental groups.

References

1. Montemurro T, Andriolo G, Montelatici E, et al. Differentiation and migration properties of human fetal umbilical cord perivascular cells: potential for lung repair. *J Cell Mol Med* 2010;**15**(4)**:**796-808.
2. Morigi M, Rota C, Montemurro T, et al. Life-sparing effect of human cord blood-mesenchymal stem cells in experimental acute kidney injury. *Stem Cells*;**28**(3)**:**513-22.
3. Gnecchi M, Melo LG. Bone marrow-derived mesenchymal stem cells: isolation, expansion, characterization, viral transduction, and production of conditioned medium. *Methods Mol Biol* 2009;**482:**281-94.
4. Thebaud B, Ladha F, Michelakis ED, et al. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation* 2005;**112**(16)**:**2477-86.
5. van Haaften T, Byrne R, Bonnet S, et al. Airway Delivery of Mesenchymal Stem Cells Prevents Arrested Alveolar Growth in Neonatal Lung Injury in Rats. *Am J Respir Crit Care Med* 2009;**180**(11)**:**1131-42.
6. Jiang M, He B, Zhang Q, et al. Randomized controlled trials on the therapeutic effects of adult progenitor cells for myocardial infarction: meta-analysis. *Expert Opin Biol Ther* 2010;**10**(5)**:**667-80.
7. Aslam M, Baveja R, Liang OD, et al. Bone Marrow Stromal Cells Attenuate Lung Injury in a Murine Model of Neonatal Chronic Lung Disease. *Am J Respir Crit Care Med* 2009;**180**(1122-30).
8. McBride C, Gaupp D, Phinney DG. Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. *Cytotherapy* 2003;**5**(1)**:**7-18.

Supplemental Figure Legends

Supplemental Figure. A. Characterization of cord derived perivascular cells (UCPCs). Immunofluorescence and fluorescence intensity histograms with specific antibodies for membrane antigens (orange line) and irrelevant isotypic-matched Ab as negative control (green line). **B. Characterization of cord blood derived mesenchymal stem cells (CBMSCs).** Immunofluorescence and fluorescence intensity histograms with specific antibodies for membrane antigens (purple line) and irrelevant isotypic-matched Ab as negative control (green line).

Supplemental Figure