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# In vitro-microenvironment directs preconditioning of human chorion derived MSC promoting differentiation of OPC-like cells

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ABSTRACT

The loss of oligodendrocyte progenitor cells (OPC) is a hallmark of perinatal brain injury. Our aim was to develop an in vitro culture condition for human chorion-derived mesenchymal stem cells (MSC) that enhances their stem cell properties and their capability to differentiate towards OPC-like cells. MSC were grown either in serum replacement medium (SRM) or serum-containing medium (SM) and tested for their morphology, proliferation, secretome, migration, protein expression and differentiation into OPC-like cells. MSC cultured in SRM condition have distinct morphology/protein expression profile, increased cell proliferation/migration and capacity to differentiate into OPC-like cells.

## 1. Introduction

In recent years, mesenchymal stem cells (MSC) have been considered as a putative source of cells for regenerative approaches and stem cell grafting. They are capable of differentiating into cartilage, bone, adipose tissue, skin, liver, muscle and brain cells (Ullah et al., 2015). MSC have been isolated from different tissues including the placenta (Portmann-Lanz et al., 2006). The human placenta is a feto-maternal entity that consists of three layers: decidua (maternal), chorion (fetal) and amnion (fetal). MSC obtained from the placental tissue can differentiate into various lineages including neural cells (Portmann-Lanz et al., 2010a,b).

The stem cell graft's efficiency to migrate, home, integrate, survive, proliferate and differentiate into the appropriate cell types needs to be improved. The regenerative processes in cell transplantation paradigms greatly rely on the release of trophic factors that support cell activities (of either differentiated cells or resident stem cells) (Schoeberlein et al., 2011). Despite the advancements in stem cell technology, there is a high necessity to further unravel the properties of MSC and mechanisms of regeneration. Thus, optimization of stem cell techniques is essential to overcome such difficulties.

We have previously shown that the neurogenic potential of chorion MSC was higher compared to MSC from both bone marrow (BM) and amnion (Portmann-Lanz et al., 2006). In this study, human chorion MSC were exposed to different conditions (microenvironment) that were previously published, grown on serum media (SM) (Portmann-Lanz et al., 2006; Battula et al., 2007, 2008)) and in serum replacement medium (SRM)(Battula et al., 2007, 2008). In the present study, we investigated whether or not changing the microenvironment would improve MSC properties and promote differentiation into OPC like-cells.

## 2. Materials and methods

### 2.1. Isolation and culture of human chorion-derived MSC

The Institutional Review Board approved all experiments. Written consent was obtained from patients (Department of Obstetrics, The University Hospital Bern) before sampling of placental tissue. MSC were isolated from the chorion, which is the fetal part of placenta from normal term as described (Portmann-Lanz et al., 2006). MSC were cultured until passage 4 using serum medium (SM: Dulbecco's Modified Eagle's Medium (DMEM)/F12, 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 1x GlutaMAX™ [Life Technologies, Carlsbad, CA, USA]), and expanded at 37 °C, 5% $CO_2$. At passage 5, two different culture conditions were tested: $5 \times 10^5$ cells were grown in uncoated culture flasks (150cm$^2$) with 20 m l conventional culture medium (SM) or on tissue culture flasks coated with 0.1% gelatin in 20 m l human ESC medium (knockout DMEM, Sigma-Aldrich, St. Louis, MO, USA, 20% knockout serum replacement, Life Technologies; 1x GlutaMAX™, 0.1 mM β-mercaptoethanol, Sigma-Aldrich; 1% non-essential amino acids, 5 ng/ml human basic fibroblast growth factor, bFGF, PeproTech, Rocky Hill, NJ, USA) (SRM) (Battula et al., 2007; Xu et al., 2001). To keep up the bFGF levels, 2 ml of used medium (SRM)

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was replaced with fresh medium every second day. Thus, MSC were grown until passage 4, preconditioned at passage 5 and detached after reaching 90% confluency to carry out the following analyses.

### 2.2. Flow cytometry

Cells grown in the two experimental conditions were analyzed by flow cytometry for extracellular markers at passage 5: CD105, CD90, CD73, CD45, CD34, CD14 and HLA-DR (Portmann-Lanz et al., 2006). MSC at passage 5 were trypsinized, resuspended in DMEM/F12/10% FBS and washed with PBS, pH 7.3, 10% FBS. The cells were labeled with the primary (Oct4: Santa Cruz Biotechnology, Dallas, TX, USA; Nestin: Acris, Herford, Germany; Pax-6: Santa Cruz Biotechnology; Frizzled9, FZD9/CD349: BioLegend, San Diego, CA, USA; Musashi1: Merck Millipore, Billerica, MA, USA) and secondary antibodies (anti-mouse IgG Alexa Fluor 488; anti-rabbit IgG Alexa Fluor 488; anti-goat IgG FITC, all Life Technologies; anti-mouse IgM FITC, Merck Millipore) for 1 hour at 4 °C each and washed 3 times with FACS buffer (1% FBS in PBS) between incubation steps. For intracellular FACS staining, the cells were initially fixed with 1% paraformaldehyde (PFA) in PBS for 10 min and blocked for unspecific binding by incubation with FACS buffer for 30 min. Cells were permeabilized with 0.1% Triton™ X100 for 10 min and washed 3 times with FACS buffer before the incubation with the primary antibody. After incubation with the secondary antibody, the cells were washed 3 times, resuspended in the FACS buffer and the FACS analysis was performed. For extracellular FACS staining, the same procedure was followed excluding the PFA fixation step. Negative controls were obtained by incubating MSC with the secondary antibody only. Cells were analyzed by SORP LSR-II (BD Biosciences) and quantified with flow cytometry analysis software (FlowJo v10, Tree Star, Inc., Ashland, OR, USA).

### 2.3. qRT-PCR

Total RNA was extracted from MSC at passage 5 using the QIAshredder (Qiagen, Venlo, The Netherlands) and RNeasy Mini Kit (Qiagen). cDNA was synthesized from 5 µg of total RNA using SuperScript™ III reverse transcriptase (Life Technologies). The following primers and probes were used: OCT4/POU5F1: forward primer (fw) 5′-ACCCACACTGCAGCAGATCA-3′, reverse primer (rv) 5′-CACAC TCGGACCACATCCTTCT-3′, probe (pr) 5′-CCACATCGCCCAGCAGCT TGG-TAMRA-3′; PAX6: fw 5′-GCTTCACCATGGCAAATAACC-3′, rv 5′-GGCAGCATGCAGGAGTATGA-3′, pr 5′-CCTATGCAACCCCCAGTCCC CAG-TAMRA-3′; MSI: fw 5′-CTCCAAAACAATTGACCCTAAGGT-3′, rv 5′-GACAGCCCCCCCACAAAG-3′, pr; 5′-CGAGCACAGCCCAAGATGGTG ACTC-TAMRA-3′. TaqMan gene expression assays (Life Technologies) were used for NES (Hs00707120_s1) and FZD9 (Hs00268954_s1). Standard settings were used for qRT-PCR (7300 Real Time PCR System, Life Technologies; 45 cycles). The transcripts were normalized to a reference gene (GAPDH: fw 5′-GCTCCTCCTGTTCGACAGTCA-3′, rv 5′-ACCTTCCCCATGGTGTCTGA-3′, pr 5′-CGTCGCCAGCCGAGCCACA-TAMRA-3′) with human fetal brain RNA as the calibrator.

### 2.4. Differentiation

MSC were differentiated into adipocytes, osteocytes and chondrocytes (StemPro differentiation kits, Life Technologies) and analyzed as described (Portmann-Lanz et al., 2006). Differentiation of MSC into oligodendrocyte progenitor (OPC)-like cells was done as follows (Fu et al., 2007; Zhang et al., 2010): The MSC (passage 5) were trypsinized (0.25% trypsin, 1 mmol/l EDTA, Life Technologies) and replated (1:2 ratio) in DMEM/F12 medium containing 10 ng/ml epidermal growth factor (EGF, BD Biosciences, Franklin Lakes, NJ, USA) and N2 supplement (1:100, Life Technologies) for 3 days. Thereafter, $1 \times 10^5$ MSC/ml were plated into ultra-low attachment cell culture flasks (Corning, Corning, NY) in neurospheres (NS) medium (neurobasal medium, 20 ng/ml bFGF, 20 ng/ml EGF, B27 1:50, Life Technologies) that lead to the formation of free-floating neurospheres. After three days in NS medium, neurospheres were plated on poly-L-lysine- and laminin-(Sigma-Aldrich) coated Lab-Tek® glass chamber slides (Sigma-Aldrich) for further differentiation (neurobasal medium, 10 ng/ml bFGF; 10 ng/ml PDGF; 1% FBS; 1 µM purmorphamine, Calbiochem, San Diego, CA).

### 2.5. Immunocytochemistry

Before immunostaining, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.3, 10 min, room temperature (RT)) and treated with 0.1% Triton-X (in PBS, 10 min, RT; only for intracellular staining). The cells were then stained with the primary (PDGF-Rα: rabbit, 1:500, Abcam, Cambridge, UK; O4: mouse, 1:100, Merck Millipore; NF200: rabbit, 1:500, Acris; O1: mouse, 1:100, Merck Millipore; GFAP: mouse, 1:100, Merck Millipore; Olig2: goat, 1.200, Santa Cruz; Vimentin: mouse, 1:500, Sigma-Aldrich) and secondary (anti-mouse IgG, Alexa Fluor-488/594, 1:200; anti-rabbit IgG, Alexa Fluor-488/594, 1:200; anti-mouse IgM, Alexa Fluor-594, 1:200, all Life Technologies; anti-goat IgM, FITC, 1:200, Jackson ImmunoResearch, West Grove, PA) antibodies for 1 hour each, followed by three washes (PBS, 10 min) after each antibody. Fluorescein isothiocyanate-conjugated phalloidin (1:500, Sigma-Aldrich) was used to visualize actin filaments. After staining, the cells were washed and visualized by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany). The surface area of MSC was analyzed with the ImageJ software (Rasband WS. U.S. National Institutes of Health, Bethesda, MD, USA) after scale bar calibration.

### 2.6. Quantitative measurement of proteins secreted by the MSC

$1 \times 10^5$ MSC grown in SM or SRM conditions were cultured with 2 ml of medium in a 6-well plate. The media were collected after 48 hours and growth factors, chemokines and cytokines released from the cells quantified using Bio-Rad Luminex 100 Bio-Plex Liquid Array Multiplexing System. The following assays were performed: Human cytokine 27-plex panel (Cat. #M50-0KCAF0Y), human cancer biomarker panel 1, 16-Plex (Cat. #171-AC500M) and human cytokine SDF-1α set (Cat. #171-B6019M). Fresh medium was used as a negative control and the value was subtracted with SM and SRM condition to avoid any background signal present before analyzing the actual measurement.

### 2.7. Cell proliferation and wound healing assays

PrestoBlue™ cell viability reagent (Life Technologies) was used to quantitatively measure cell proliferation. Cells were harvested and the cell proliferation was analyzed at 0, 6, 12, 24 and 48 hours after onset of cultivation. To analyze the cell migration, MSC (SM or SRM) were grown to confluency. A sterile 200 µl pipette-tip was used to mimic a wound by scratching and removing a discrete area of the monolayer. Then, the plate was washed gently with PBS and the cells were cultured in their respective medium without serum (to prevent cell-proliferation). Cell migration at the wound site was observed at different time points up to 60 hours with time-lapse live imaging (BioStation system, Nikon, Tokyo, Japan). The wound area was analyzed at different time points using ImageJ software.

### 2.8. Statistics

Statistical analysis was performed by analysis of variance (ANOVA) with Sigma plot™ 11.0 software (Systat Software, Inc., Chicago, IL). All experiments were conducted at least three times and the following symbols were used to show the degree of significance: "*" if $P \leq 0.05$, "**" if $P \leq 0.01$ and "***" if $P \leq 0.001$, respectively.