The characterization of umbilical cord matrix stem cells in the context of multiple sclerosis

Goele Willekens

promotor :
Prof. dr. Niels HELLINGS

co-promotor :
dr. Jerome HENDRIKS

Masterproef voorgedragen tot het bekomen van de graad van
master in de biomedische wetenschappen

Universiteit Maastricht

universiteit hasselt

Acknowledgements

30 weeks ago my adventure at the immunology department of BIOMED started. Now it ends in writing this thesis. During this period, I studied the possibilities of UCMSs as a treatment of multiple sclerosis.

Since good research can only be performed in a close team, I would like to thank everyone at BIOMED.

First, I would like to thank my promotor prof. dr. Niels Hellings and co-promotor dr. Jerome Hendriks. I would like to thank you for the guidance of my experiments, for your critical look at my writings and for your constructive criticism.

Special thanks go to my supervisor drs. Raf Donders. During the past months, you shared your research experience and insights with me. Thanks for your motivation and support during the experiments. I also want to express my gratitude for revising my draft versions.

Furthermore, my appreciation goes to all the PhD students at the lab. It was nice working with you all.

I also want to thank the technical staff at BIOMED: Mrs. Igna Rutten, Mrs. Christel Bocken, Mrs. Katrien Wauterickx. When I had technical questions, you were always there to answer them.

The other master students at the lab also deserve some word of appreciation. Kathleen, Jessica, Kris, Tim, Eef, Liesbeth, Winde and Anneleen, together we had a fun time at BIOMED.

Finally, I want to thank my parents, sisters and friends on which I could always rely. Thanks for your support and trust in me.

Table of contents

List of abbreviations

BBB	blood-brain-barrier
BM-MSC	bone-marrow-derived mesenchymal stem cell
CDM	complete defined medium
CNS	central nervous system
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
EAE	experimental autoimmune encephalomyelitis
FBS	fetal bovine serum
HCS	hematopoetic stem cell
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IL	interleukin
ILT	immunoglobulin-like transcript
INF	interferon
KIR2DL4	killer cell immunoglobulin-like receptor 2DL4
LFA-1	leukocyte function associated antigen-1
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSC	mesenchymal stem cell
NK	natural killer cell
NSC	neural stem cell
NT2/D1	NTERA-2 clone D1
OCT3/4	octamer-binding transcription factor 4
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule 1
PFA	paraformaldehyde
PML	progressive multifocal leukoencephalopathy
PSGL-1	P-selectin glycoprotein ligand-1
REX1	zinc finger protein-42 (ZFP42)
SC	stem cell
SSEA-1	stage specific embryonic antigen-1
SOX2	sex-determining region Y (SRY)-box 2
UCMS	umbilical cord matrix stem cell
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-4	very late antigen 4
WJ	Wharton's jelly

Abstract

Background: Multiple sclerosis (MS) is an autoimmune disease, causing progressive neurodegeneration in the central nervous system. In search for a cure, stem cells are considered as a therapy. Most stem cell sources are difficult to access whereas umbilical cord matrix stem cells (UCMSs) can be obtained in high numbers from the umbilical cord connective tissue without difficulty. In this project, it has been evaluated whether UCMSs are suited to treat MS. Our parameters to test their suitability involved their pluripotency (OCT4, SOX2, NANOG, REX1), immunomodulatory function (HLA-G) and homing capacity (CD191, CD192, CD195, CD197, CD183, CD184). As there is discussion on the optimal growth medium, the influences of four culturing media on these parameters were also determined. Finally, the optimal method for UCMSs isolation from the umbilical cord was determined.

Methods: Cells were isolated from explants or by collagenase digestion of the umbilical cord and were cultivated in four different media. Presence of HLA-G and the pluripotent stem cell markers was evaluated with PCR and flow cytometry. Chemokine receptors and adhesion molecules were only studied by flow cytometry, together with the expression of MSC markers.

Results: PCR revealed the presence of OCT4, SOX2 and REX1 transcripts at early passages. However, flow cytometry could not detect the OCT4, SOX2 and NANOG proteins. Both MSC marker expression and HLA-G surface expression were influenced by the culturing medium. Flow cytometry described the presence of CD29, CD183 and CD184, while no detectable levels of CD191, CD192, CD195, CD197, CD15 and CD162 were found. Cells isolated by collagenase digestion of the umbilical cord were showed to be less fibroblastic with lower levels of MSC maker expression.

Conclusion: To obtain a proper and phenotypic stable stem cell population, UCMSs better are obtained from explants and cultivated in the KO-DMEM/F12 medium. UCMSs are non-pluripotent, mesenchymal stem cells that express a limited set of chemokine receptors and adhesion molecules, while HLA-G was only expressed under certain conditions. The data obtained indicate a limited migratory capacity and immunomodulatory function.

Samenvatting

Achtergrond: Multiple sclerose (MS) is een auto-immuun ziekte die progressieve neurodegeneratie van het centrale zenuwstelsel veroorzaakt. In de zoektocht naar een genezende behandeling wordt recent ook het gebruik van stamcellen overwogen. Helaas zijn vele stamcellen moeilijk te isoleren. Dit in contrast met navelstreng stamcellen die in hoge aantallen geïsoleerd kunnen worden uit het navelstrengbindweefsel. In dit project werden deze stamcellen geëvalueerd op hun geschiktheid in de behandeling van MS. Onze parameters omvatten hun pluripotentie, immunomodulatoire functie en homing capaciteiten. Als laatste werd de optimale methode voor isolatie van deze stamcellen bepaald.

Methoden: De stamcellen worden geïsoleerd uit explants of met behulp van collagenase digestie van de navelstreng. Vervolgens worden deze stamcellen opgekweekt in 4 verschillende media. Aanwezigheid van HLA-G en pluripotente stamcelmerkers werden bestudeerd met zowel PCR als flow cytometrie. De expressie van chemokinereceptoren en adhesiemoleculen, samen met de MSC merker expressie, werden enkel bestudeerd met flow cytometrie.

Resultaten: OCT4, SOX2 en REX1 RNA werden gedetecteerd met behulp van PCR hoewel OCT4, SOX2 en NANOG proteïnen niet teruggevonden konden worden. Expressie van MSC merkers en HLA-G werd beïnvloed door de kweekomstandigheden. Flow cytometrie beschrijft de aanwezigheid van CD29, CD183 en CD184 terwijl CD191, CD192, CD195, CD197, CD15 en CD162 niet detecteerbaar aanwezig waren. Cellen die geïsoleerd worden met behulp van collagenase zijn minder fibroblastisch met een lagere MSC merker expressie.

Conclusie: Om een goed en phenotypisch stabiele populatie te bekomen, kunnen navelstrengstamcellen beter geïsoleerd worden via migratie uit explants en gekweekt worden in het KO-DMEM/F12 medium. Navelstrengstamcellen blijken niet-pluripotente, mesenchymale stamcellen te zijn, die een gelimiteerde set aan chemokine receptoren en adhesie molecule tot expressie brengen. Onder bepaalde condities werd het HLA-G protein teruggevonden. Deze data indiceren een gelimiteerde migrerende capaciteit en immunomodulerende functie.

1 Introduction

"Why this deliberate, slow-moving malignity? Perhaps it is a punishment for the impudence of my desires. I wanted everything so I get nothing... I am not offering up my life willingly - it is being taken from me piece by piece, while I watch the pilfering with lamentable eyes." (W. N. P. Barbellion)

Bruce Frederick Cummings was one of the first to describe multiple sclerosis (MS) from the patient's perspective. In 1919, he published his diary 'The journal of a disappointed man' using the pseudonym W(ilhelm) N(ero) P(ilate) Barbellion. His journal illustrates his suffering from a neurological disease and his desperate search for a diagnosis. It took 10 years before he was diagnosed with 'disseminated sclerosis', currently known as MS. In spite of a diagnosis, Cummings could not be treated as no treatment was available. Unfortunately, still no curative therapy is on hand these days, leading to more stories similar to the one of Cummings [1].

1.1 Multiple sclerosis

MS is a chronic, multifocal autoimmune disease of the central nervous system (CNS). It is a heterogeneous disease, in which autoimmune T cells become activated in the periphery where after they migrate across the blood-brain-barrier (BBB). Once arrived, they are reactivated by the microglia and astrocytes. A local inflammatory environment arises, which stimulates the recruitment of more immune cells from both the innate and adaptive immune system. The present immune cells attack both the myelin sheath and underlying axons, causing demyelination and neurodegeneration. However, oligodendrocytes are also targeted by the T cells. Therefore, little remyelination can occur and the naked axons are even more prone to damage. Permanent neuronal loss will appear, leading to neurological dysfunction. In general, the disease manifests itself by visual and sensory disturbances, limb weakness, gait problems and bladder and bowel problems.

MS is one of the most common neurodegenerative diseases, which makes the need for a treatment more pressing. The incidence in Europe is estimated on 3.8 cases per 100,000 citizens with a prevalence of 80 in 100,000 [2]. Women are more prone to develop MS than men and that between 20 and 40 years of age. However, in spite of the research done, the trigger of this disease is still not fully identified. There is consensus that MS is a multifactorial disease, in which both environmental and genetic factors are involved. It is found that the risk to develop MS is higher in people with the HLA-DR15 and -DQ6 haplotypes. In addition, the newly described MS susceptibility genes interleukin 17 alpha receptor (IL17RA) and IL2RA may be involved. The environment also plays

a role, as is demonstrated by migration studies and latitude-influence fluctuations in prevalence. Finally, there are indications that infections such as the Epstein-Barr viral infection may have a causative role in the development of MS [3, 4].

Thus, many years will pass before the trigger of this disease is completely unraveled. However, what is known, is that MS is a progressive disease, and the neurological problems will accumulate, leading to a decrease in the quality of life. Although, there is no cure, the disease progression can be slowed down using a drug treatment. It must be remarked that treatment in an early phase is important to limit the extent of irreversible damage. This can only be accomplished by a rapid and differential diagnosis. Unfortunately, the diagnosis of MS remains difficult, as no differential diagnostic tests are available. Therefore, the International Panel on the Diagnosis of Multiple Sclerosis presented new diagnostic criteria in 2001, followed by a revision in 2005. The core requirements for MS diagnosis are dissemination of the lesions in time and space [5]. In other words, multiple lesions must be present in the CNS, originating from multiple attacks. To assess the current number of lesions, an MRI will be performed. Once the diagnosis is made, a treatment plan will be proposed to the patient. Interferon β (IFNβ), glatiramer acetate, mitoxantrone and Natalizumab are the most common therapies. However, none of them are curative. There is discussion on their ability to slow down disability progression and the lack of treatment effects in some patients. Furthermore, reports describe major side effects associated with most of these drugs, including Natalizumab. The use of this α4-integrin directed monoclonal antibody is accompanied with progressive multifocal leukoencephalopathy (PML), appearance of (opportunistic) infections, hepatoxicity and melanomas [6].

1.2 Stem cells in multiple sclerosis

As presented in the previous section, none of the marketed therapies can cure MS. Therefore, much energy is put into the search for a curative treatment. To enhance the chances for success, researchers are not only evaluating drug therapies but also cell-based therapies. This new approach seems to have potential, as studies on the effects of stem cells in experimental autoimmune encephalomyelitis (EAE), the animal model for MS, yield promising results. Stem cells (SCs) are unspecialized cells characterized by their unlimited self-renewal and the ability to differentiate into one or more specialized cell types. Neural stem cells (NSCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) gained most attention within the scope of MS. Currently, there are several phase I and II clinical trials using MSCs, NSCs and HSCs as a treatment for MS [7]. The following section gives a brief introduction on these types of stem cells.

NSCs are multipotent SCs located inside the brain. More specifically, these cells reside in the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus. By differentiation into neurons, astrocytes and oligodendrocytes, NSCs can stimulate cell replacement and remyelination. Secondly, after systemic injection, NSCs migrate to both the CNS and the secondary lymphoid organs, where they exert immunomodulatory function. Thus, NSCs certainly have beneficial properties. However, NSCs are only present in low numbers and the isolation procedure is very invasive, which makes them less suited for widespread therapeutic use [8]. Next to NSCs, HSCs are considered as a therapy for MS. HSCs are isolated from the bone marrow and can be used for either autologous or allogeneic transplantation. Instead of enhancing repair or acting immunomodulatory, HSCs are used for the reconstitution of the immune system. The patient undergoes conditioning followed by a HSC transplantation. During the 3 years following the treatment, in 60%-70% of the patients no disease progression was observed. However, due to the 3.3% mortality risk, the treatment is only justified when the benefits are worth the risk. This high mortality risk is caused by (opportunistic) infections in immune compromised patients. Therefore, the treatment is only advised for patients with a very poor prognosis [9]. Next to HSCs and NSCs, MSCs are considered as a therapy for MS. These stem cells display a whole repertoire of beneficial effects. Although the results are ambiguous, there is some indication that MSCs can differentiate into neurons. Therefore, they could be suitable for cell replacement in neurodegenerative diseases. Secondly, MSCs have an inhibitory effect on NK cells, T cells, B cells and dendritic cells (DCs). Due to this immunomodulatory function, they can be used in both organ transplantations and autoimmune diseases. MSCs can also stimulate the proliferation of oligodendrocyte progenitor cells and therefore have a beneficial effect on the remyelination of axons. MSCs can be isolated from numerous tissues including the bone marrow (BM-MSCs), adipose tissue, dental pulp of teeth, ... As the concentration is the highest in the bone marrow, this source is most studied and used. However, the isolation of MSCs from bone marrow is a painful and invasive procedure. Furthermore, aging is accompanied with fat deposition in the bone marrow, making MSC isolation more difficult [10].

1.3 Umbilical cord matrix stem cells

A common disadvantage of the above-mentioned stem cells is their difficult harvesting. Therefore, the umbilical cord is presented as an alternative stem cell source as isolation is easy and harmless. During the embryogenesis, totipotent stem cells migrate from the placenta to the embryo. However, not all cells reach the embryo. Several stem cells reside in the stroma of the umbilical cord, called Wharton's jelly. After birth, these stem cells then can be isolated from the jelly. These isolated stem

cells are called umbilical cord matrix stem cells (UCMSs) and are fetal, multipotent cells with a fibroblast-like appearance [11].

Next to easy isolation, UCMSs have more properties that arouse the interest in them. UCMSs can be harvested in high numbers. Therefore, no extensive replication is needed and epigenetic damage can be avoided. Their primitive nature is reflected by their shorter doubling times and larger number of passages till senescence when compared to adult MSCs. Unlike embryonic stem cells (ESCs), there are no ethical issues involved with the use of UCMSs as umbilical cords are discarded after birth. Next to their differentiation into bone, cartilage and adipose tissue, UCMSs are reported to differentiate into neurons [11, 12]. These UCMSs avoid an allogeneic immune response by expression of MHC class I together with the absence of MHC class II. Therefore, killing by NK cells is avoided while being unrecognisable for T cells. Their low immunogenitcity enables broad application of these stem cells. Furthermore, UCMSs posses an immunomodulatory cytokine expression profile [13]. Finally, it is proven that UCMSs are beneficial in Parkinson's disease indicating its effectiveness in other neurodegenerative diseases [14].

In conclusion, UCMSs are primitive, easy attainable and low immunogenic stem cells, which show potential for treatment of neurodegenerative diseases. Therefore, this study will be focused on the suitability of UCMSs in the most common neurodegenerative diseases: MS.

1.4 Research topic

UCMSs look like a promising cell type for use in MS or other conditions. However, not all of their properties are evaluated yet. More specifically, not much is known about their pluripotency, HLA-G expression and migratory capacity. Therefore, we state that UCMS can migrate and express both HLA-G and the pluripotency markers.

For most cell-based therapies, stem cells have to differentiate into the appropriate cell type, which depends on the disease of interest. Therefore, stem cells with a high differentiation capacity are best suited as it enables broad application. In this project, the pluripotency of UCMSs will be evaluated by means of octamer-binding transcription factor 4 (OCT4), sex-determining region Y (SRY)-box 2 (SOX2), homeobox Nanog and zinc finger protein-42 (ZFP42/REX1) expression. These transcription factors are specific for pluripotent stem cells and therefore serve as markers for the detection of it. They activate the genes involved in maintaining pluripotency while repressing the ones involved in cell differentiation. The best known cell type which expresses these transcription factors are ESCs. However, Carlin and colleagues found these markers expressed in porcine UCMSs, indicating the pluripotency of these stem cells [15]. For human UCMSs it is still not known whether these transcription factors are present. Therefore, their expression will be examined in this project.

The second topic of this project encompasses the HLA-G expression on UCMSs. HLA-G is a non-classical HLA class I molecule. It differs from the other HLA class I molecules by its lower number of polymorphisms, restricted tissue distribution and alternative splicing. This alternative splicing leads to 7 different isoforms: HLA-G1 up to HLA-G4 are membrane-bound while the HLA-G5, -G6 and -G7 are soluble. HLA-G suppresses NK cells, T cells, B cells, DCs, monocytes and macrophages by binding on the ILT-2 (CD58), ILT-4 (CD85d) and KIR2DL4 (CD185d) receptors. HLA-G thus leads to immune tolerance, which is beneficial in allograft acceptance and autoimmune diseases [16, 17]. In MS patients undergoing a relapse, HLA-G becomes up-regulated on the local macrophages, microglia and endothelial cells. Using different mechanisms, these HLA-G^+ cells exert an anti-inflammatory effect, implicating their role in remission of the disease [18, 19]. This indicates a possible role for HLA-G in curing MS. Perhaps HLA-G^+ stem cells could do the job. If UCMSs would express HLA-G, they could act immunosuppressive and prevent the destructive inflammatory reactions going on in MS patients. Several reports note the presence of HLA-G transcripts in human UCMSs [13, 20]. However, the protein expression is never studied. Due to the clinical importance, the HLA-G expression on UCMSs will be addressed in this study.

The final focus of this project is on the migratory capacity of UCMSs. As MS is a multifocal disease, injection of stem cells direct into the brain lesions is no option. Another method is needed to get the stem cells inside the brain lesions. This study will examine if UCMSs are able to migrate to the plaques, as is proven for other SCs. MSCs and NSCs can migrate to the multiple inflammatory regions inside the brains of EAE animals after injection into the CSF or bloodstream. The migratory capacity of UCMSs towards the brain lesions in EAE is not yet determined. Although, a study performed by Rachakatla et al. indicates that these SC possess at least some migratory capacity. His study presents that in SCID mice with lung tumours, injected UCMSs migrate selectively to these lung tumours and not to the healthy tissue [21]. In conclusion, UCMSs possess a definite capacity to migrate, although the extension of it is not yet determined.

Successful migration of these stem cells can only be accomplished by the membrane expression of the correct adhesion molecules and chemokine receptors. During inflammation, the inflammatory cells present at the site of inflammation, secrete a repertoire of chemokines. In turn, these chemokines bind to the corresponding chemokine receptors present on the target cells, and guide these cells, most often immune cells, to the site of inflammation. In this project, we will study if UCMSs can be attracted in a similar matter. In function of the chemokine secretion profile of MS/EAE, the expression of the corresponding chemokine receptors on BM-MSCs is studied in the literature [22, 23]. As UCMs meet the criteria for MSCs, their chemokine receptor expression profile probably resembles that of BM-MSCs [24]. Therefore, our focus will be on the presence of these chemokine receptors on UCMSs (table 1).

Table 1. Chemokines secreted in EAE lesions and their corresponding receptors situated on the target cells.

MCP, monocyte chemoattractant protein; MCAF, monocyte chemotactic and activating factor; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T-cell expressed and secreted; Mig, monokine induced by γ-interferon; IP, γ-interferon inducible protein; SDF, stomal-cell derived factor. NK cells, natural killer cells; DC, dendritic cells.

Chemokine	Alternative name	Chemokine receptor	Target cells
CCL2	MCP-1, MCAF	CCR2	T, NK and B cells, monocytes, DC
CCL3	MIP-1α	CCR1, CCR5	T and B cells, monocytes, neutrophils, eosinophils
CCL4	MIP-1β	CCR1, CCR5	CD8+ cells
CCL5	RANTES	CCR1, CCR5	T cells, monocytes, eosinophils, DC
CCL7	MCP-3	CCR1, CCR2	T, NK and B cells, monocytes, eosinophils, and DC
CCL19	MIP-3β, ELC, ckβ11	CCR7	DC, T cells, activated B cells
CCL21	6Ckine, SLC, ckβ1	CCR7	DC, T cells, activated B cells, NK cells
CXCL9	Mig	CXCR3	T and NK cells
CXCL10	IP-10	CXCR3	T and NK cells
CXCL12	SDF-1	CXCR4	B and T cells, monocytes, DC

Once the BBB is reached, these cells are recruited from the blood into the inflamed, extravascular tissue. Extravasation of the cells is a multistep process, dependent on the adhesion molecules present on both the stem cells and endothelial cells. The mechanism is best studied for leukocytes. However, classic MSCs use a similar mechanism. Therefore, the method used by UCMSs for extravastion probably will be alike.

In summary, leukocytes first start rolling over the endothelium due to contact of the Sialyl-Lewis X-modified proteins on the leukocytes with the P-selectin and E-selectin on the endothelium. Then the leukocytes become activated and up-regulate the molecules needed for firm adhesion: leukocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4).These molecules interact with the intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on the activated endothelium for firm adhesion. Transmigration is performed with the aid of platelet endothelial cell adhesion molecule 1 (PECAM-1, also called CD31), which is expressed on both the endothelium and leukocytes [25-27]. For UCMS, the absence of CD31 is reported, together with LFA-1 expression. Therefore, presence of the other molecules will be evaluated in this project. CD15 and CD162, both Lewis X proteins are studied, together with CD29 (member of VLA-4)).

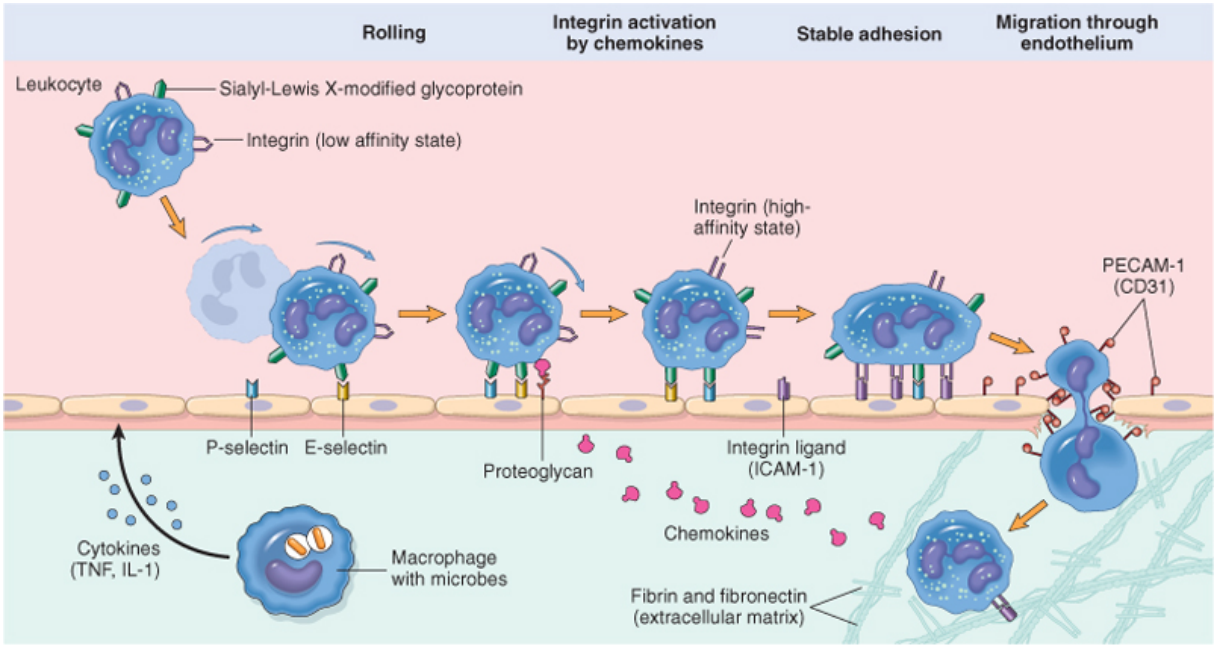


Figure 1. Endothelial and leukocyte adhesion molecules involved in the migration through the blood vessels.

The transendothelial migration is a multistep process similar to that of leukocytes and haematopoietic stem cells (HSC). In a first phase, the MSC starts rolling, then it becomes activated and a firm adhesion to the endothelium takes place. A final step is the transmigration across the endothelium. It is a process dependent on a series of adhesion molecules, each playing a role in a different phase. Intercellular adhesion molecule, ICAM-1; Tumour necrosis factor, TNF; Interleukin 1, IL-1; Platelet endothelial adhesion molecule 1, PECAM-1 [27].

In conclusion, the pluripotency, HLA-G expression and migratory capacity will be studied as it influences the suitability of these stem cells in MS and other diseases. We state that UCMS can migrate and express both HLA-G and the pluripotency markers. This hypothesis will be tested by studying the RNA and protein level of both HLA-G and the pluripotence markers. To clarify the UCMS migratory capacity, expression patterns of the adhesion molecules and the chemokine receptors will be studied using FACS analysis.

2 Materials & Methods

This study focuses on the characterization of UCMSs in the context of MS. Based on their pluripotency, homing capacity, and immunogenicity, the suitability of UCMSs for treating MS will be evaluated. The used techniques are described below.

2.1 UCMSs isolation and cultivation

Full term umbilical cords from both sexes were received after caesarean section with informed consent of the mother. The umbilical cord blood was removed and 5-10 cm segments were aseptically stored in a transport solution (PBS (Lonza, Belgium), 1% Pen/Strep (Invitrogen, Belgium), 0,2 % fungizone (Invitrogen)) at 4°C. Before starting the isolation procedure, the umbilical cord was rinsed several times with sterile PBS. The isolation procedure was performed within 24h after birth using 2 separate isolation techniques.

For the *explant procedure*, 1 cm segments were created from which the blood vessels were removed manually. Next, the tissue was diced into 1-2 mm^3 pieces and transferred to a 6-well plate containing 3 ml medium per well. ½ medium change was performed every 2-3 days, starting after attachment of the umbilical cord tissue to the bottom of the plate.

A second technique for UCMSs isolation involves enzymatic digestion with *collagenase type I*. The umbilical cord, with or without its blood vessels, was minced into pieces of 4-5 mm^3. The tissue was transferred to a sterile container and incubated for at least 6h on a shaker with collagenase I (2mg/ml) (Invitrogen) and 3mM $CaCl_2$. To remove the undigested pieces, the suspension was diluted with PBS and filtered employing a 70 µm pore filter. The cells were washed twice by centrifugation for 10 minutes at 1000g. Subsequently, the resuspended cells were counted and cultured. First medium change was performed after 7 days. When much cell debris was present, cells were washed with PBS before adding fresh medium. From then on, ½ medium change took place every 2-3 days.

UCMSs were cultured in the following four media: Dulbecco's modified Eagle's medium with 1g/l D-glucose and GlutaMAX (DMEM-LG) (Invitrogen), KnockOut DMEM (KO-DMEM) with 4,5g/l glucose and 2mM L-glutamine (Invitrogen) and KO-DMEM/F12 with 2mM L-glutamine. All these 3 media were supplemented with 10% FBS (Biochrom, Germany) and 1% Pen/Strep. For KO-DMEM, KnockOut Serum Replacement (Invitrogen) was tested as a replacement for FBS. A fourth medium, called 'complete defined media' was used [28]. The media consists out of 56% low glucose DMEM, 37% MCDB 201 (Sigma, Belgium), 2% FBS, 1 X insulin-transferrin-selinium X (Invitrogen), 1 X ALBU-Max (Invitrogen), 1 X Pen/Strep, 0.1 mM dexamethasone (Sigma), 100 µM ascorbic acid 2-phosphate (Sigma), 10 ng/ml epidermal growth factor (R&D systems, Belgium), 10 ng/ml platelet-derived growth factor (R&D systems). All cells were incubated in a humidified incubator at 37°C and 5%

carbon dioxide. When reaching 80-90% confluence, the cells were lifted with StemPro Accutase (Invitrogen), counted by trypan blue exclusion and subcultured.

2.2 Cultivation of JEG-3 and NT2/D1 cell lines

2.2.1 JEG-3 cell line

The JEG-3 cell line (ATCC) was cultivated in RPMI (Invitrogen) with 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% Pen/Strep and 10% FBS (Hyclone, Belgium). At confluence, the cells were detached with 0.25% trypsin, counted by trypan blue exclusion and subcultered with 20,000 cells/cm^2. The cells were cultivated in a humidified incubator at 37°C with 5% carbon dioxide. Medium change took place 3 times a week.

2.2.2 NTERA-2 cl.D1 cell line

NTERA-2 cl. D1 (NT2/D1) (ATCC) cells were cultivated in high glucose DMEM (Invitrogen) with 2 mM L-glutamin, 10% FBS (Hyclone) and 1% Pen/Strep. At confluence, the cells were detached by scraping and counted by trypan blue exclusion. The cells were subcultured at a density of $5x10^6$ cells per 75 sq cm flask. The cells were cultivated in a humidified incubator at 37°C with 5% carbon dioxide. Medium change took place 3 times a week.

2.3 Growth kinetics

To analyse long-term growth kinetics, growth curves were determined together with the number of population doublings per passage and the population doubling time. These parameters were calculated for each of the four culturing media. For the long-term growth curves, the total cell number at each passage is calculated as the ratio of the total number of cells harvested to the total number of cells seeded multiplied by the total number of cells from the previous passage [29]. The number of population doublings is obtained by taking the 2log of the number of cells harvested divided by the number of cells seeded. The population doubling time in hours is calculated by the ratio of the number of hours in passage to the number of population doublings in that passage.

2.4 RNA isolation and cDNA synthesis

RNA was isolated using the High Pure RNA Isolation Kit (Roche, Belgium) following the manufacturer's instructions. Briefly, cell pellets were resuspended in PBS and lysed. After lysation, the cells were transferred to a filter tube and centrifuged. Subsequently, DNases were removed with

a DNase solution. After several washing steps, the RNA was eluted with MilliQ. Purity and concentration of the samples were evaluated with the NanoDrop-100 Spectrophotometer (Isogen Life Science, Belgium).

The Reverse Transcription System (Promega, the Netherlands) was used for cDNA synthesis. Procedure was performed according to the manufacturer's protocol: the RNA samples were heated to 70°C for 10 minutes and placed on ice. A reaction mix consisting out of $MgCl_2$, RTase buffer, dNTPs, RNasin, AMV RTase, oligo(dT) primers, MilliQ and RNA was used. Next, the following PCR protocol was performed: 60' at 42°C, 5' at 95°C and 5' at 4°C. This PCR was performed with a Thermal Cycler (BIO RAD, Belgium).

The cDNA was further purificated by adding destilled water, fenol and chloroform/isoamylethanol. The suspension was vortexed and centrifuged (2 min at 1300g). Subsequently, the supernatant was transferred to a new reaction tube containing choloroform/isoamylalcohol. After vortexing and centrifuging, the supernatant was incubated with 3M sodium acetate and 100% ethanol for 1 hour at -80°C or overnight at -20°C. Afterwards, the solution was centrifuged for 15 minutes at 1300g at 4°C. The pellet was resuspended with 70% ethanol followed by another centrifugation step. Next, the pellet was dried and resuspended in MilliQ. cDNA quantity and purity was verified with the NanoDrop Spectrophotometer.

2.5 Polymerase chain reaction

The PCR was performed, using Taq Polymerase (Roche, Belgium) and other standard PCR products. The used primers (Eurogentec, Belgium) are represented in table 2. The PCR protocol for HLA-G consisted out of 30 cycles of 1' at 94°C, 1' at 61°C and 2' at 72°C. In these PCR reactions, JEG-3 was used as a positive control. The OCT4, SOX2 and REX1 pluripotent stem cell markers were detected with specific primers for each marker in the following PCR protocol: 35 cycles with 30'' on 94°C, 30'' on 56.1°C and 60'' on 72°C. Here, the NT2/D1 cell line served as a positive control. The PCR products were visualized using a 2% agarose gel containing 0.1% ethidium bromide.

Table2. List of primers used in the present study

For all primer sets, product size were given together with the optimal annealing temperature and sequence

Name	Product size (bp)	Ta (°C)	Forward primer	Reverse primer
OCT4	456	56.1	5'-AGCCCTCATTTCACCAGGC-3'C	5'-TGGGACTCCTCCGGGTTTTG-3'
SOX2	570	56.1	5'-AGAAGCGGCCGTTCATCGAC-3'	5'-TGCTGATCATGTCCCGGAGGT-3'
REX1	554	56.1	5'-TGAAAGCCCACATCCTAACG-3'	5'-CAAGCTATCCTCCTGCTTTGG-3'
HLA-G	1063	61	5'-CAAGGATGGTGGTCATGGCG-3'	5'-CAGCTGTTTCACATTGCAGCCTG-3'

2.6 Purification and sequencing

PCR products were separated on a 0.6% agarose gel and the bands were excised. cDNA was extracted from the gel by transferring the excised pieces on a sephadex column (GE Healthcare) and centrifuging for 2 min at 700g. Concentration and purity of the cDNA containing flow through was verified with the NanoDrop-100 Spectrophotometer. Subsequently, a sequencing PCR was performed with the BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Belgium) following the manufacturer's instructions. Shortly, a Mastermix was made containing the following products: BigDye v1.1 Terminator Sequencing Buffer (5X), BigDye Sequencing Ready Reaction mix, 4pmol/µl Fw primer and 50 ng cDNA. The PCR program consisted out of 30 seconds at 96°C followed by 25 cycli of 10'' on 96°C, 5'' on 50°C and 4' at 60°C. Afterwards, the pure PCR products were purified using sephadex, evaporated and resolved in HiDi formamide sequencing buffer (Applied Biosystems). The prepared samples were analysed with the ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were examined with the basic local alignment tool (BLAST).

2.7 Phenotypic analysis by flow cytometry

Surface expression of mesenchymal stem cell markers, adhesion molecules and chemokine receptors was analyzed using flow cytometry. Following labelled mouse/rat anti-human antibodies were used: HLA-ABC-PE, HLA-DR-PE (both from ImmunoTools, Germany); HLA-G-PE, CD10-PE, CD13-FITC, CD29-FITC, CD44-FITC, CD105-PE, CD117-PE, CD195-PE, CD197-PE (all from eBioscience); CD15-FITC, CD73-PE, CD90-FITC, CD95-FITC, CD146-PE, CD162-PE, CD183-Alexa Fluor 488 nm, CD184-PE-Cy7, CD146-PE (BD, Belgium), CD191-PE, CD192-PerCP (R&D systems).

Cells were detached by accutase. Approximately, 50,000 to 100,000 cells for each sample were transferred to a V-bottom 96-well plate. Cells were washed with FACS buffer (10% PBS, 2% FBS,

sodium azide) by centrifuging the well plate twice at 300g for 5 minutes. Subsequently, the cells were dissolved in 100µl FACS buffer and 5 µl of antibody or isotype control was added. Protected from light, the cells were incubated for 30 minutes at 4°C where after they were washed twice with FACS buffer. Afterwards, the cells were resuspended, transferred to FACS tubes and analyzed with the FacsCalibur Flow Cytometer (BD Biosciences, USA). About 20,000 cells per sample were analysed. Data were processed with the Cellquest software

2.8 Intra-cellular flow cytometry for detection of pluripotency

Detection of the pluripotency markers OCT4, SOX2 and NANOG was performed with the BD human pluripotent stem cell transcription factor analysis kit (BD Biosciences). The procedure was performed according to the manufacturer's instructions. In summary, the cells were lifted with accutase and counted. Approximately, $0.5x10^6$ to $1x10^6$ cells per sample were used. After washing with PBS, the cells were fixated with cytofix fixation buffer containing 4% PFA. After 20 minutes incubation, the cells were washed twice. Subsequently, the cells were permeabilized by incubating the cells during 10 minutes with perm/wash buffer. Next, the cells were incubated for 30 minutes with the Nanog-PE, Sox2-Alexa Fluor 647nm and Oct3/4-PerCP-Cy5.5 antibodies or the isotype controls. Finally, the cells were washed, resuspended in FACS buffer to concentration of 1-3 X 10^6 cells/ml and analyzed with the FACS Aria-II Sorter (BD Biosciences, USA) using the FACS diva 6.1 software.

2.9 Statistical analysis

Results were statistically analyzed with the GraphPad Prism 4 software. Values were expressed as mean ± SEM and were analyzed by a t-test or a one-way analysis of variance (ANOVA), followed by Bonferroni test. P-values < 0.05 were considered statistically significant. *P <0.05, **P<0.01, ***P<0.001.

3 Results

Based on several parameters, the utility of UCMSs as a treatment was evaluated. In overview, growth characteristics were calculated and pluripotency was studied. Furthermore, HLA-G expression was determined together with the expression patterns of chemokine receptors and adhesion molecules. As the optimal growth medium was not determined yet, the influences of four different culturing media on these parameters were studied.

3.1 Growth characteristics

Growth characteristics are an important parameter for the determination of the optimal growth medium for long-term cultivation and expansion of UCMSs. UCMSs were isolated using the explant technique and allowed to attach in DMEM-LG. After 1-3 passages, the cell number was sufficient to plate the UCMSs in the four different media (DMEM-LG, CDM, KO-DMEM and KO-DMEM/F12). The proliferation potential was evaluated according to the long-term growth curves, the number of population doublings and the population doubling time.

The long-term growth curves are represented for each of the four media in figure 2A. Cells cultivated in DMEM-LG or CDM expanded less exponentially than the cells cultivated in KO-DMEM or KO-DMEM/F12. The number of cells cultivated in DMEM-LG or CDM remained relatively stable over most passages and started to decrease from passage 7. Past passage 5, the cells cultivated in KO-DMEM started to die. For cells cultivated in KO-DMEM/F12, this process started from passage 6. When looking at the number of population doublings, the cells cultivated in DMEM-LG showed the lowest number compared to the cells cultivated in the other media (Fig 2B). However, the number of population doublings remained stable over both early and late passages. At early passages, the cells cultivated in the three other media doubled twice per passage. This number is 25% higher compared to the cells cultivated in DMEM-LG. At later passages, CDM cultivated cells presented a decrease in the number of population doublings/passage while for the other two media no change was observed. When comparing the mean population doubling times for each medium at both early and late passages, no significant differences were seen. This indicates similar mean population doubling times for each medium (Fig 2C). Evaluation of the morphology revealed some medium-influenced differences (Fig 2D). As presented in de figures below, cells cultured in CDM showed a less fibroblastic morphology. In addition, when reaching confluence, these cells did not arrange themselves as expected but lied unorganized over each other. The cells cultivated in the three other media showed a normal fibroblastic morphology and all cells arranged themselves as they reached confluence. However, only the cells cultivated in KO-DMEM or KO-DMEM/F12 tended to form colonies. This occurrence was not seen for the cells cultivated in the other media. Finally, the effects

of the KO-SR supplement on morphology and growth was tested. Use of this supplement would avoid exposition to growth factors and hormones, which is accompanied with the use of FBS. However, UCMSs clearly showed their preference as cells cultivated in KO-DMEM with KO-SR died (Fig 2D).

Based on the cell kinetics and morphology, cells cultivated in KO-DMEM and KO-DMEM/F12 show the highest levels of stemness.

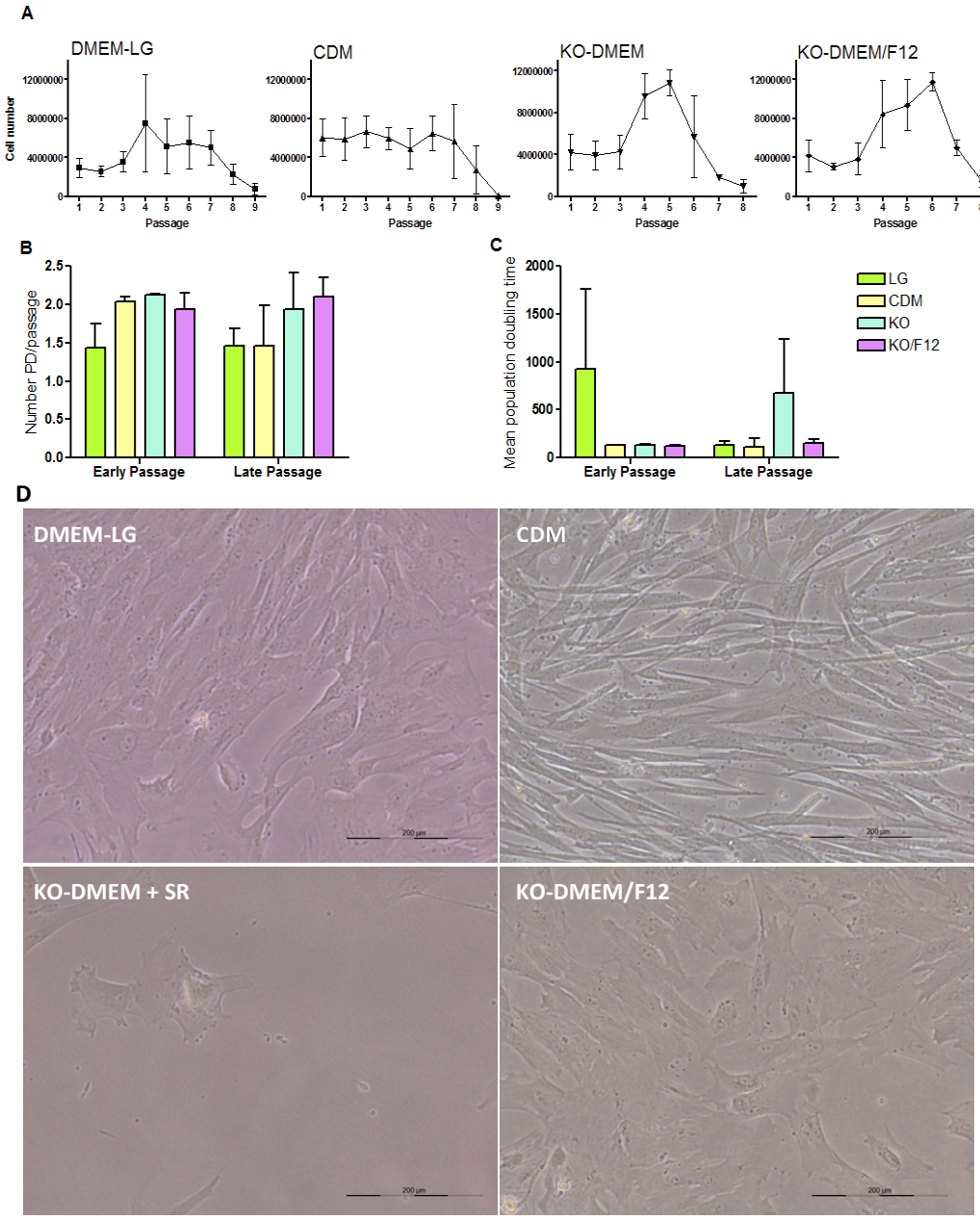


Figure 2. Comparison of the four culturing media based on growth kinetics and morphology

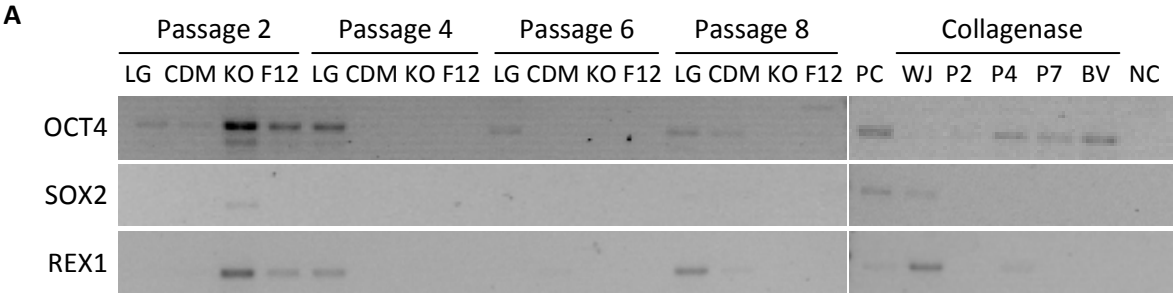
The expansion potential of the UCMSs in time are represented for all 4 culturing media (**A**). The mean number of population doublings and mean population doubling time of the cells cultured in the 4 different media are represented for both early and late passages. Early passages are defined as passage 1 to 5 while the later passages circumvent passage 5 to 9 (**B, C**). Micrographs were taken from passage 1 UCMSs obtained from the same umbilical cord and cultivated in 4 different media: DMEM-LG, CDM, KO-DMEM with SR and KO-DMEM/F12 (**D**). LG: DMEM-LG; CDM: complete defined medium; KO: KO-DMEM; F12: KO- DMEM/F12; PD: population doubling; SR: serum replacement .

3.2 Pluripotent stem cell markers were detected in UCMSs

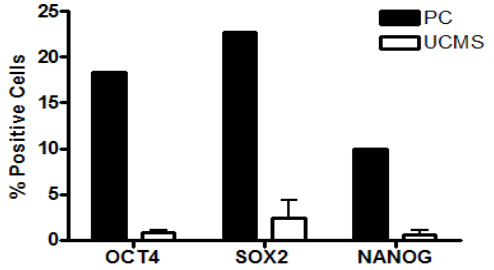
To gain more insight into the pluripotent properties of the Wharton's jelly stem cells, the expression of pluripotent stem cell markers was evaluated. The presence of OCT4, SOX2 and REX1 transcripts was studied using PCR while flow cytometry was performed for detection of the OCT4, SOX2 and NANOG proteins.

At passage 2, 4, 6 and 8, cDNA was obtained from explant-derived UCMSs cultivated in each of the four culturing media. Subsequently, a PCR was performed using this cDNA (Fig 3A). At passage 2, OCT4 was detected in samples from all four culturing media. Cells cultivated in KO-DMEM or KO-DMEM/F12 demonstrated high OCT4 expression at passage 2 but lost it before reaching passage 4. At later passages, OCT4 was only detected on UCMSs cultivated in DMEM-LG. SOX2 was detected in cells cultivated in KO-DMEM at passage 2 while none of the other samples expressed SOX2. REX1 was present at passage 2 in cells cultivated in KO-DMEM and KO-DMEM/F12. While at passage 8 it was also seen for cells cultivated in DMEM-LG.

A



B



	% Positive Cells
OCT4	0.78 (0.318)
SOX2	2.34 (1.995)
NANOG	0.62 (0.495)

Figure 3. Detection of pluripotent stem cell markers

A RT-PCR was performed at passage 2, 4, 6 and 8 with RNA obtained from UCMSs derived from explants and cultivated in 4 different media. Collagenase isolated stem cells cultivated in DMEM-LG were evaluated at passage 2, 4, and 7. In addition, cells isolated by collagenase from the Wharton's Jelly or the blood vessels of the umbilical cord were evaluated at passage 0. NT2/D1 cell line served as a positive control. Primers specific for detection of OCT4 (456 bp), SOX2 (570 bp) and REX1 (554 bp) were used (**A**). Intracellulair staining with antibodies directed against OCT4, SOX2 or NANOG, followed by flow cytometry. Cells were isolated by collagenase digestion of the umbilical cord and compared to the pluripotent NT2/D1 cell line. n=5 (**B**). Data are presented as mean ± SEM. LG: DMEM-LG; CDM: complete defined medium; KO: KO- DMEM; F12: KO-DMEM/F12; PC: positive control; WJ: Wharton's Jelly; BV: blood vessels; p: passage; NC: negative control.

To compare the explant and collagenase isolation methods, collagenase isolated UCMSs were cultivated in DMEM-LG and tested at passage 0, 2, 4 and 7 (Fig3A). At passage 2, no bands were visible while at passage 4 both OCT4 and REX 1 were detected. At passage 7, only OCT4 could be detected. Furthermore, a PCR was performed at passage 0 on both digested vessel-free Wharton's Jelly (WJ) and digested umbilical cord blood vessels (BV). The Wharton's Jelly derived cells expressed SOX2 together with REX1 while in the blood vessels only OCT4 could be detected. The presence of the pluripotent stem cell markers in UCMSs was further evaluated at the protein level using specific antibodies against OCT4, SOX2 and NANOG. However, none of the proteins was significantly expressed by UCMSs at p0 (Fig. 3B) or at later passages (data not shown). At passage 0, OCT4 is found on only 0.78 % of the cells, SOX2 on 2.34% of the cells and NANOG on 0.62% of the cells.

In conclusion, transcripts of the pluripotent stem cell markers were found at early passages. Although, these data could not be confirmed at the protein level.

3.3 The MSC characteristics of UCMSs are influenced by the culturing medium

Both explant and collagenase isolation procedures lead to heterogeneous cell populations. Therefore, presence of morphologically similar fibroblasts is realistic. To ensure a proper assessment of the stem cell population, expression of MSC markers was studied. In addition, the effects of both passaging and culturing medium on UCMSs were evaluated since in vitro cultivation may influence the maintenance of the undifferentiated state of these stem cells. Possible differentiation may be detected by a decreased expression of these markers. The key markers for identifying a stem cell as a mesenchymal stem cell are CD73, CD90 and CD105 [24]. Other MSC markers evaluated are the peptidases CD10 and CD13, the stem cell factor receptor CD117, the progenitor marker CD146 and the adhesion molecule CD44. Expression of these markers was evaluated by flow cytometry at passage 3, 5, 7 and 9 on explant-derived cells cultivated in each of the four culturing media. In addition, expression of MSC markers was compared between cell populations obtained by either explants or collagenase.

Figures 4A, B, C and D represent the data on UCMSs obtained from explants and evaluated at passage 3, 5, 7 and 9. At early passages, no differences in CD10 expression were seen between the 4 different culturing media. However, at passage 7, CD10 was significantly lower expressed on CDM cultured UCMSs compared to UCMSs cultured in DMEM-LG ($p<0.05$). When evaluating CD13 expression, no significant differences were found between the four culturing media. CD44, CD73 and CD90 were expressed on almost all the counted cells without any influences of culturing media or passaging. CD105, on the contrary showed a different expression pattern. When evaluating cells

cultivated in CDM, CD105 was present on a significant lower number of cells (passage 7: $p<0.001$) compared to the cells cultivated in DMEM-LG, KO-DMEM or KO-DMEM/F12. Significant differences were also found when comparing CD146 expression between the four culturing media. Cells cultivated in DMEM-LG express significant higher levels of CD146 compared to cells cultivated in CDM, KO-DMEM of KO-DMEM/F12 ($p<0.01$). Finally, none of the cells at early passages did express CD117. Although, CD117 seems up-regulated in time. At passage 7, CD117 is significantly up-regulated on DMEM-LG cultivated cells compared to cell cultivated in CDM and KO-DMEM ($p<0.05$). At passage 9, the cells in CDM and KO-DMEM also seem to up-regulate CD117.

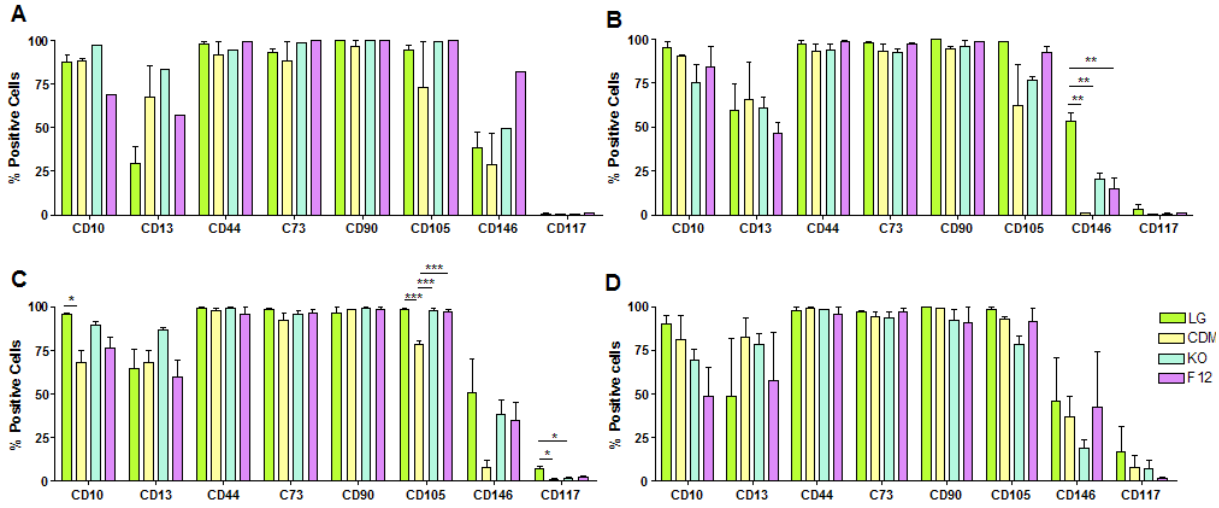


Figure 4. UCMSs derived from explants display MSC markers.

UCMss were stained with the appropriate antibodies and analyzed with flow cytometry. Data are shown for all four culturing media. Flow cytometry was performed at passage 3 (A), 5 (B), 7 (C) and 9 (D) for evaluation of long-term culturing effects. Data are presented as mean ± SEM. LG: DMEM-LG; CDM: complete defined medium; KO: KO- DMEM; F12: KO-DMEM/F12.

In figure 5, the overall mean of all passages is presented to obtain a general view on the differences in MSC marker expression caused by the four culturing media. CD10 was significantly lower expressed on cells cultivated in KO-DMEM/F12 compared to cells cultivated in DMEM-LG ($p<0.05$). UCMSs cultivated in KO-DMEM expressed significantly higher levels of CD13 compared to ones cultivated in DMEM-LG ($p<0.05$). Furthermore, the CDM population displayed a significant lower expression of CD105 and CD146 compared to cells cultivated in DMEM-LG ($p<0.05$). Finally, no significant differences in CD117 expression were seen.

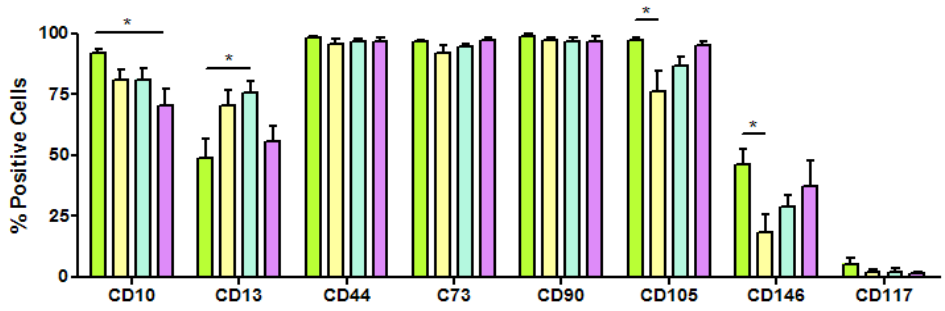


Figure 5. Mean MSC marker expression for the 4 different media.

For each maker, the mean expression is represented here per medium. The values were calculated as the mean percentage positive cells evaluated at passage 3, 5, 7 and 9.

For evaluation of the collagenase and explant isolation procedures, expression of MSC markers was compared between both cell populations (Fig 6). Explant cells showed a significant higher expression of the peptidase CD13. Furthermore, CD10 tended to be higher expressed on this population together with a decreased CD146 expression. For CD44, CD73, CD90, CD105 and CD117, no significant differences in expression were seen between both cell populations.

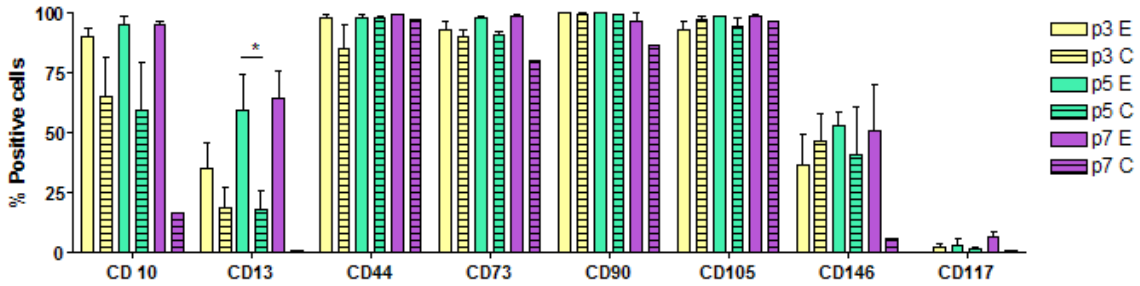


Figure 6. UCMSs isolated by collagenase digestion of the umbilical cord display differences in MSC marker expression compared to UCMSs derived from explants

UCMss were stained with the appropriate antibodies and analyzed with flow cytometry. Data are shown for both collagenase and explants-derived UCMSs cultivated in DMEM-LG. Flow cytometry was performed at passage 3, 5 and 7. Data are presented as mean ± SEM

In conclusion, MSC markers CD44, CD73 and CD90 were present on all cells without significant differences caused by passaging or culturing medium. In contrast, CD10, CD13, CD105 and CD146 expression were influenced by culturing conditions. The cells cultured in CDM expressed the lowest levels of MSC markers. Furthermore, explant-derived UCMSs displayed significant higher marker levels compared to the cell populations isolated by collagenase.

3.4 HLA-G is up-regulated on UCMSs under certain conditions

HLA-G, an atypical MHC class I molecule, is known for its immunomodulatory properties. Functional expression of this molecule would greatly enhance the suitability of UCMSs in numerous disorders. Therefore, expression of HLA-G was studied on both the RNA and protein level. In addition, the expression of HLA-ABC, HLA-DR and CD95 proteins was evaluated by flow cytometry in order to obtain a clearer view on the immunogenicity of the UCMSs.

For the detection of HLA-G transcripts, a PCR was performed. Primers were designed to amplify four splice variants of HLA-G: HLA-G1, HLA-G2, HLA-G3 and HLA-G4. cDNA was obtained at passage 2, 4, 6 and 8 from explant-derived UCMS cultivated in each of the four media. Additionally, at passage 2, 4 and 7, cDNA was obtained from collagenase isolated, DMEM-LG cultivated UCMSs. At passage 0, cDNA was obtained from both vessel-free Wharton's Jelly and umbilical cord blood vessels. The explant isolated cells are represented left of the 100bp ladder (M) (figure 7). At passage 2, no HLA-G transcripts were found. When reaching passage 4, HLA-G transcription was performed in cells cultivated in DMEM-LG and CDM. The same results were seen at passage 6. At passage 8, KO-DMEM cultivated cells also started to transcribe the HLA-G gene. However, no bands were visible for the samples cultivated in KO-DMEM/F12. The same results were seen for the cells isolated by enzymatic digestion. Collagenase isolated cells could not express HLA-G at passage 0, independent of its source: Wharton's Jelly or umbilical cord blood vessels. However, at later passages HLA-G was actively transcribed in these cells.

The used primer set was expected to yield a 1063 bp amplification product corresponding to HLA-G1, a 792 bp product corresponding to HLA-G2, a 787 bp product corresponding to HLA-G4 and a 517 bp product corresponding to HLA-G3. There were amplification products seen at the expected heights, indicating the presence of the four splice variants. However, after sequencing, only the PCR product with a length of approximately 1063 bp corresponded with the HLA-G1 transcript. The other amplification products did not match with any of the other HLA-G splice variants. Therefore, the presence of only the HLA-G1 transcript could be confirmed (Supplement 2).

Subsequently, expression of HLA-G proteins was evaluated together with the protein expression of HLA-ABC, HLA-DR and CD95. At each passage, HLA-G proteins were detected on cells cultivated in DMEM-LG. On cells cultivated in CDM, KO-DMEM or KO-DMEM/F12, no HLA-G proteins were consistently detected. Cells isolated with collagenase expressed lower levels of HLA-G although cultivated in DMEM-LG (Fig 7F). Furthermore, MHC class I was expressed on all cells, independent of the culturing medium. For the cells cultivated in DMEM-LG a significant linear trend ($p<0.05$) was visible. Cells cultivated in this medium up-regulated HLA-ABC at later passages. In contrast, the cells isolated with collagenase presented a downward trend of HLA-ABC expression. For all cells,

independent of passaging, culturing media or isolation method, no significant HLA-DR expression was seen. Finally, when focussing on CD95 expression, a significant linear trend was seen for the populations cultivated in DMEM-LG ($p<0.05$). For the explant-derived cells, CD95 was upregulated while the cells obtained by collagenase digestion tended to down-regulate CD95 upon in vitro cultivation. The UCMSs cultivated in the three other media showed no significant trends. Furthermore, no significant differences in expression levels were seen between the four culturing media.

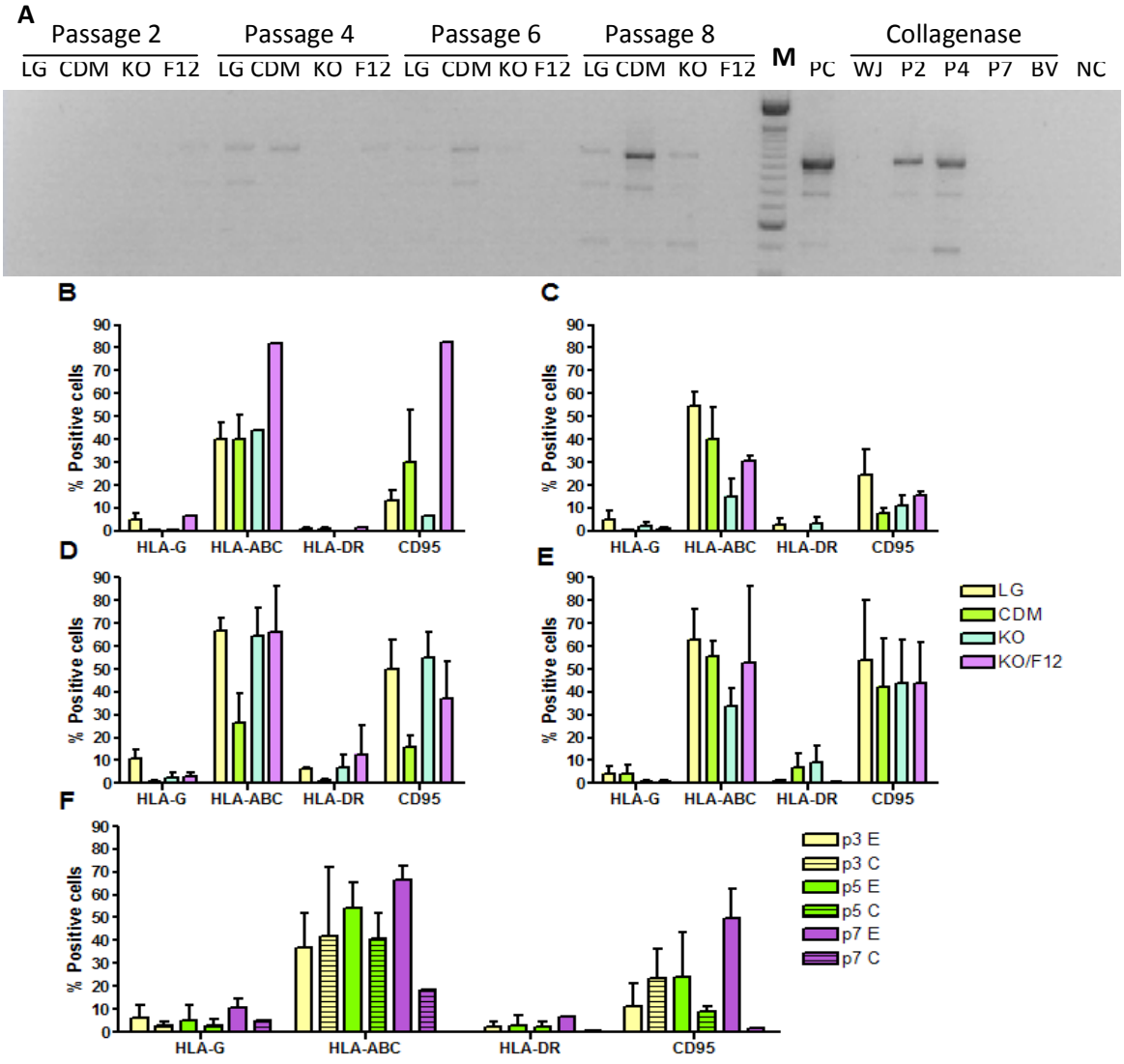


Figure 7. The immune-phenotype of UCMSs

A RT-PCR was performed at passage 2, 4, 6 and 8 with RNA obtained from UCMSs derived from explants and cultivated in 4 different media. Collagenase isolated stem cells cultivated in DMEM-LG were evaluated at passage 2, 4, and 7. In addition, cells isolated by collagenase from the Wharton's Jelly or the blood vessels of the umbilical cord were evaluated at passage 0. The JEG-3 cell line served as a positive control. Primers specific for detection of HLA-G1 (1063 bp), HLA-G2 (793 bp), HLA-G3 (517 bp) and HLA-G4 (787 bp) were used (**A**). UCMss were stained with the appropriate antibodies followed by flow cytometry. Data are shown for all four culturing media. Flow cytometry was performed at passage 3 (**B**), 5 (**C**), 7 (**D**) and 9 (**E**) for evaluation of long-term culturing effects. Comparison of collagenase and explants-derived UCMSs cultivated in

DMEM-LG. Flow cytometry was performed at passage 3, 5 and 7 (**F**). Data are presented as mean ± SEM. LG: DMEM-LG; CDM: complete defined medium; KO: KO- DMEM; F12: KO- DMEM/F12; PC: positive control; WJ: Wharton's Jelly; BV: blood vessels; p: passage; NC: negative control; E: explants; C: collagenase; M: marker.

In conclusion, HLA-DR was absent on all cells. CD95, HLA-ABC and HLA-G expression were influenced by the DMEM-LG medium. For the other media, no fluctuations in the expression patterns of these three molecules were detected. Cells isolated from explants expressed higher levels of CD95, HLA-ABC and HLA-G compared to the cells obtained from enzymatic digestion of the umbilical cord.

3.5 UCMSs express a limited set of chemokine receptors and adhesion molecules

A final topic encompasses the UCMSs membrane expression of chemokine receptors and adhesion molecules relevant for autonomous migration to MS brain lesions. The chemokine receptors considered most relevant within the scope of MS are CD191, CD192, CD195, CD197, CD183 and CD184. These receptors were studied, together with the adhesion molecules CD29, CD15 and CD162 using flow cytometry.

At passage 3, CD191, CD192, CD195 and CD197 were absent on all cells observed. However, CD191, CD192 and CD195 tended to be up-regulated in time on the UCMSs cultivated in DMEM-LG, CDM and KO-DMEM. CD183 and CD184 were expressed on a significant number of cells for all culturing media without any significant influences of passaging or culturing medium.

The chemokines secreted by the inflammatory cells direct the cells towards the brain lesions. However, once arrived at the BBB, extravasation is needed to actually reach the inflamed lesions. Therefore, expression of the correct adhesion molecules is necessary. The adhesion molecules studied here, are CD15 (SSEA-1), CD29 (VLA-4 subunit) and CD162 (PSGL-1). Data demonstrate the presence of CD29 on all cells counted by the flow cytometer without effects of passaging or culture medium. On the contrary, CD15 and CD162 were both absent on all cells at early passages. With increasing passage, these adhesion molecules tended to be up-regulated on the cells cultivated in DMEM-LG, CDM and KO-DMEM.

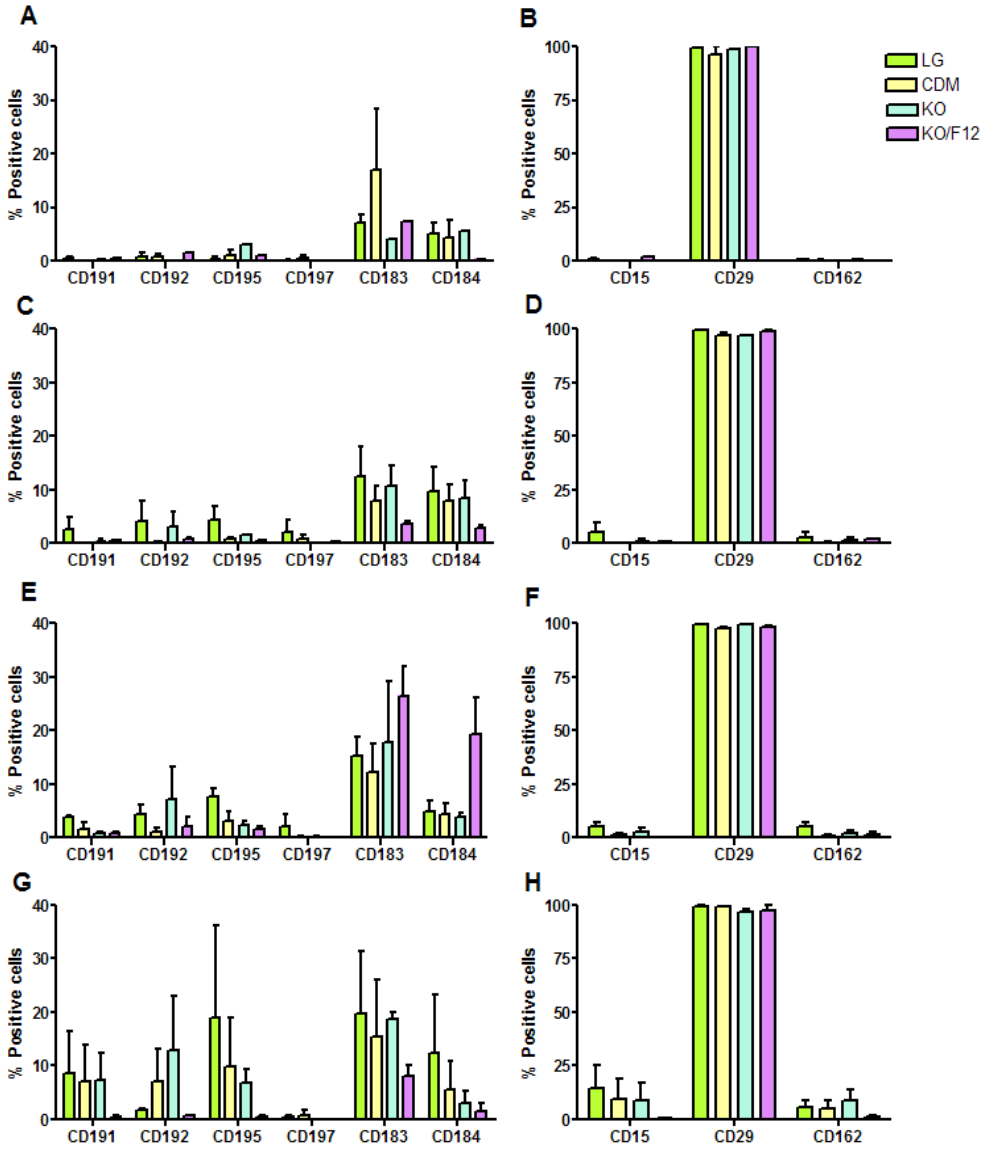


Figure 8. Expression patterns of the chemokine receptors and adhesion molecules on UCMSs

UCMss were stained with the appropriate antibodies followed by flow cytometry. Data are shown for all four culturing media. Expression of chemokine receptors was evaluated at passage 3 (**A**), 5 (**C**), 7 (**E**) and 9 (**G**). Presence of adhesion molecules was determined at passage 3(**B**), 5(**D**), 7(**F**) and 9(**H**). Data are presented as mean ± SEM.

Again, the differences in expression patterns between explant and collagenase derived stem cells was compared. CD191, CD192 and CD195 were not or less up-regulated on cells derived by enzymatic digestion compared to explant-derived stem cells. CD183 and CD184 were expressed on both cell populations. When observing the adhesion molecules, no differences were found: CD15 and CD162 were not significantly expressed on both cell populations while CD29 was highly expressed.

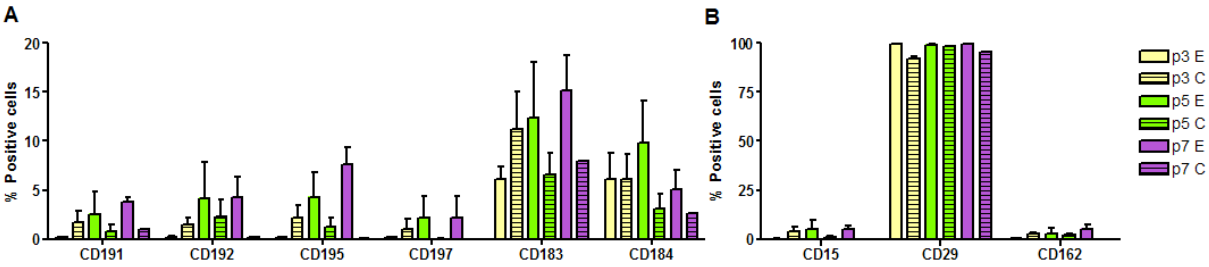


Figure 9. Expression patterns of the chemokine receptors and adhesion molecules on UCMSs isolated by collagenase

UCMss were stained with the appropriate antibodies followed by flow cytometry. Data are shown for both explants and collagenase isolation. Flow cytometry was performed at passage 3, 5 and 7 for evaluation of long-term culturing effects. Data are presented as mean ± SEM.

In summary, the chemokine receptors CD183 and CD184 were expressed on UCMSs together with the CD29 adhesion molecule. In contrast, no significant expression of CD191, CD192, CD195, CD197, CD15 and CD162 was detected. These results were seen on both explant and collagenase-derived populations.

4 Discussion

For this project, we characterized hUCMSs in the context of MS. Although the knowledge on these stem cells is rather limited, UCMSs already show promising advantages when compared to other types of stem cells. These stem cells have a more primitive nature than adult stem cells, which may enhance their applicatory opportunities [30]. Furthermore, they can be isolated in high numbers from the umbilical cord without ethical considerations. Next to the absence of tumorigenicity *in vivo*, these cells secrete both growth and angiogenic factors. In this project, several parameters were evaluated to determine their suitability in MS. Their long-term behavior, pluripotency, immunomodulatory actions and migratory capacity were more closely examined. As the most optimal growth medium for cultivation of UCMSs is not defined yet, the effects of different culturing media was also evaluated.

UCMSs are fetal stem cells and due to their primitive origin they could possess pluripotent properties. To evaluate the pluripotent character of UCMS, the presence of markers specific for pluripotency was determined. OCT4 is the most important marker, which is together with the downstream factors SOX2, NANOG and REX1 (ZFP42) involved in the up-regulation of genes involved in maintenance of the undifferentiated state, while suppressing the tissue-specific genes. Expression of these markers was evaluated on both the RNA and protein level. For detection of the transcripts, specific primers were designed against the OCT4, SOX2 and REX1 cDNA. OCT4 transcripts can undergo alternative splicing, giving rise to OCT4A (alternative names: OCT4, OCT3/4, POU5F1) as well as to OCT4B. OCT4A, localized in the nucleus, is involved in pluripotency while OCT4B is located in the cytoplasm and plays no role in maintaining the undifferentiated state. Next, there is the existence of many non-functional pseudogenes. Amplification of the splice variants or pseudogenes may lead false positive results. To overcome this problem, we used specific primers designed by Liedtke and colleagues [31]. These primers are directed against the OCT4A RNA and avoid amplification of all other transcripts. The forward primer binds in a region in exon 1, which is not homologous for the now known pseudogenes. As exon 1 is absent in OCT4B, amplification of this splice variant is also avoided. The reverse primer spans the exon-exon boundaries, which disables amplificiation of genomic DNA. Primers for the SOX2 RNA were designed at BIOMED and checked with multiple software programs for aspecific binding. No pseudogenes exist for SOX2, therefore chances for false positive results are relatively small. NANOG, another pluripotency marker, has many pseudogenes as well [32]. No primers specific for NANOG are available and amplification of unwanted transcripts cannot be excluded. Therefore, REX1, a transcription factor located downstream of NANOG was chosen for evaluation. REX1 primers applicated by Izadpanah et al. were verified and used in this study [33]. Our PCR results indicate the presence of pluripotent markers at early passages although

ex vivo cultivation lead to a rapid decrease in the transcript levels. In contrast, the flow cytometric data cannot confirm these results since no pluripotent stem cell markers were detected at the protein level. Even at passage 0, there were no detectable levels of OCT4, SOX2 or NANOG. Revising the literature yielded several articles demonstrating the presence of pluripotent stem cell markers in hUMCS by PCR, flow cytometry and immunostainings [14, 20, 29, 34]. However, some results are questioning. For example, Montemurro demonstrates the presence of OCT4 mRNA together with the absence of SOX2 and REX1 transcripts [34]. A quick control of the used OCT4 primers with BLAST revealed its binding sequences. These primers bind to sequences not located in exon 1. Therefore, it is realistic that these primers co-amplificate OCT4B, not to mention the pseudogenes, leading to false positive results. In addition to the expression of stem cell markers, the pluripotency of UCMSs has been tested by teratoma formation [35]. As Wharton's jelly stem cells do not meet this criterion, chances are small that these stem cells are true pluripotent stem cells. However, since there is no concuss on the pluripotency in UCMSs, our results cannot be confirmed neither disproved with high accuracy. We found the absence of the pluripotent transcription factors at the protein level, which may indicate the absence of pluripotency. These results may be explained by the more mature state of the fetal UCMSs compared to the pluripotent ESCs. However, differentiation assays and chimeric constructs may give a definite answer on this issue.

Next, the immunogenicity of these stem cells was evaluated by means of MHC and CD95 expression. HLA-G expression by UCMSs was evaluated on both the RNA and protein level. If UCMSs would functionally express HLA-G, they could exert an anti-inflammatory and immunomodulatory function. For detection of the RNA transcripts, a PCR was performed with specific primers designed by Wiendl and colleagues [36]. As expected, HLA-G transcripts were found in UCMS. Although, the primer set would amplify splice variants HLA-G1 till -G4, sequencing could only confirm the presence of HLA-G1. When comparing our data to the literature, HLA-G mRNA expression in UCMSs is confirmed by both La Rocca and Weiss [13, 20]. It is worth to mention that HLA-G transcripts are a common phenomenon in most healthy cells. Still, this does not necessarily indicate the presence of HLA-G proteins on the cell surface. HLA-G surface expression is described for only a few restricted tissues including the thymus, cornea, nail matrix and trophoblast. In this project, HLA-G was detected with the 87G antibody, which is directed against HLA-G1 and the soluble HLA-G5. As only the cells, and not the medium was analysed in this project, conclusions can only be drawn on HLA-G1. When looking at our data, HLA-G1 proteins are only found back in low levels on UCMSs cultured in DMEM-LG. No detectable levels of HLA-G proteins were present on the UCMSs cultivated in CDM, KO-DMEM and KO-DMEM/F12. Later on, this medium-dependent difference in HLA-G expression will be addressed. However, our data obtained on the cells cultivated in CDM, KO-DMEM and KO-

DMEM/F12 are in line with the data of Sarugaser et al. Using flow cytometry, he could not detect HLA-G on human umbilical cord perivascular cells [37].

The FAS receptor CD95 was found back on UCMSs in relatively high percentages. Expression of this molecule may indicate an increased sensitivity for immune cell-induced apoptosis. In contrast, an article published by Corsini and colleagues demonstrates the benefits of CD95 expression on stem cells [38]. This article claims that stimulation of the FAS receptor has beneficial effects on stem cell survival and neuronal specifications. Accordingly to this article, up-regulation of CD95 on UMCSs may enhance its suitability as a therapy for MS. Furthermore, UCMSs show low MHC class I expression together with the absence of MHC class II, CD80 and CD86 [39]. Due to this expression pattern, UCMSs will show low immunogenicity after in vivo administration.

Low immunogenicity is not the only prerequisite to pass the 'MS-suitability test'. It would be of great advantage if UCMSs can to home to targeted tissues after systemic administration. This would be the inflamed brain lesions for MS patients, although an immunomodulatory function in the secondary lymphoid organs cannot be excluded. To reach the correct organs, expression of the suited chemokine receptors and adhesion molecules is necessary. Regarding the set of chemokines secreted by the inflammatory cells located in the MS lesions, the chemokine receptors most important in leukocyte homing are CCR1, CCR2, CCR3, CCR4, CCR7, CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5 [22]. The expression of these receptors is already evaluated for BM-MSC and it is reported that a number of receptors, including CCR1, CCR2, CCR5, CCR7, CXCR3 and CXCR4 are present on these cells [25, 40]. Therefore, the presence of these receptors on UCMSs was evaluated in this project. Only CD183 and CD184 were found to be present on our stem cells. Absence of the other chemokine receptors will be explained later on.

After homing to the BBB, cells migrate into the inflamed tissue by extravasation. This is a multi-step process dependent on a proper interaction between the molecules present on both the endothelium and stem cells. For the UCMSs, it would mean that expression of Lewis X proteins, very late antigen 4 (VLA-4), leukocyte function associated antigen 1 (LFA-1) and platelet endothelial cell adhesion molecule 1(PECAM) is needed.

Flow cytometric results indicate the absence of two lewis X proteins: PSGL-1 (CD162) and SSEA-1 (CD15). However, CD29 (ITGB1), a subunit of the VLA-4 molecule is found to be present on the UCMSs. In addition, Weiss et al. found both the ITGAL (CD11a) and ITGB1 (CD18) transcripts in UCMSs, two compounds constituting the LFA-1 molecule [14]. They also reported the absence of the adhesion molecule PECAM (CD31). Thus, these stem cells do not display the full set of adhesion molecules. However, as several molecules are present, extravastion may still be attainable. The article of Rachakatla and colleagues confirms this assumption. As it demonstrates the selective homing of UCMSs to lung tumors in SCID mice [21].

When consulting the literature, it is striking that there is still no consensus on the optimal growth medium for the cultivation of UCMSs. Therefore, another focus of this project is on the optimal growth medium for *ex vivo* expansion of UCMSs. To accomplish this aim, four culturing media were evaluated. A standard low-glucose medium (DMEM-LG) was compared with three other media: KO-DMEM, KO-DMEM/F12 and CDM. KO-DMEM and KO-DMEM/F12 are specific for cultivation of both ESCs and iPSCs. In addition, Weiss and colleagues designed a medium (here called CDM) for the cultivation of Wharton's jelly derived stem cells [28].

A parameter for comparison of the different media was the expression of MSC markers. Data demonstrate a significant decreased expression of CD10, CD105 and CD146 on cells cultivated in CDM. Those cells seem to lose their stem cell characteristics. CDM contains MCDB 201, PDGF and EGF. As these products stimulate proliferation of fibroblasts, overgrowth by these cells may account the decrease in MSC marker expression. Alternatively, UCMSs may start to differentiate when cultivated in this medium, leading to a decreased expression of the MSC markers. MSCs differentiating into osteoblasts, chondrocytes or adipocytes demonstrate a decreased expression of the MSC markers, including CD105, supporting our differentiation hypothesis [41]. However, independent of the explanatory theory, this CDM medium does not fit the criteria for cultivation of UCMSs as it does not stimulate the maintenance and growth of undifferentiated stem cells. The cells cultivated in the three other media express the three key markers for MSC (e.g. CD73, CD90, CD105) at a high level without a significant decrease in time. However, cells cultivated in DMEM-LG and KO-DMEM tend to up-regulate HLA-G, chemokine receptors and adhesion molecules in time. This phenomenon is more pronounced for the cells cultivated in DMEM-LG and starts at an earlier time point compared to UCMSs cultivated in KO-DMEM. Remarkably, this up-regulation is not accompanied with a down-regulation of the MSC marker expression. Therefore, differentiation into tissue-specific cells is not a satisfactory explanation. DMEM-LG is a basal medium without supplemental factors for maintenance of the undifferentiated state. In addition, FBS, which contains hormones and growth factors, is added. Therefore, stem cells cultivated in this medium may lose their fetal characteristics and mature into adult mesenchymal stem cells, a cell type located further down the developmental continuum [30]. Adult mesenchymal stem cells express high levels of MSC markers while possessing a whole repertoire of chemokine receptors and adhesion molecules. Furthermore, HLA-G is reported to be functionally expressed on MSCs while being absent on the more primitive ESCs [42-44]. As KO-DMEM is designed for cultivation of pluripotent stem cells, it contains factors that help to maintain the undifferentiated state of stem cells. Therefore, the maturation process may be started at a later time point compared to the cells cultivated in DMEM-LG.

An alternative explanation for the up-regulation of the molecules on cells cultivated in DMEM-LG and KO-DMEM, may be the development of tumorigenicity. Perhaps these stem cells transform to tumour cells due to the *ex vivo* culturing. Numerous tumors are known to express MSC markers, HLA-G, pluripotent stem cell markers, chemokine receptors and/or adhesion molecules. Data on UCMSs demonstrate the presence of an active telomerase together with VEGF production [12, 13]. Thus in theory, UCMSs can replicate limitless and stimulate angiogenesis. These characteristics cover two of the six hallmarks of cancer. In vitro cultivation may lead to mutations in proto-oncogenes and/or tumor suppressor genes, which directs the cell to a full tumor phenotype. However, this interpretation is less likely as most cells only reach passage 10 and thus cannot replicate endlessly. However, to test this cancer-hypothesis, abnormal expression of the onco-genes and tumor suppressor genes involved in cancer could be evaluated together with the telomerase activity.

Based on our findings, the best medium depends on the purpose of the UCMS. Cells cultivated in KO-DMEM/F12 display the most stable expression patterns compared to the UCMSs cultivated in the other media. Therefore, this medium is considered the best medium for maintaining the undifferentiated state of the UCMSs. DMEM-LG and KO-DMEM are associated with an up-regulation of chemokine receptors, adhesion molecules and HLA-G. Assuming the theory that the up-regulation is correlated with maturation of the UCMSs into adult stem cells, these media may be suited for cultivation of UCMSs for specific purposes. For example, up-regulation of chemokine receptors and adhesion molecules may be beneficial in applications were UCMSs need some migratory capacity.

Finally, the most optimal isolation procedure for UCMSs was determined. Cells isolated by enzymatic digestion of the umbilical cord expressed lower levels of CD10, CD13 and CD146 compared to the cells isolated from explants. Furthermore, CD95, HLA-ABC, CD183 and CD184 tend to be down-regulated in time on these cells. Thus, the population isolated by this procedure differs from the population obtained from explants. This was confirmed by the difference in morphology between both stem cell populations. The cells isolated by collagenase are less spindle-shaped and differ from the normal fibroblast-like morphology. In summary, morphologic and flow cytometric data demonstrated a decreased stemness together with a decreased homing and immunogenic capacity of the collagenase isolated population. Therefore, we can conclude that collagenase digestion is less suited for UCMSs isolation than explants.

In conclusion, UCMSs are non-pluripotent stem cells that express MSC markers at high levels. Therefore, UCMSs are considered as MSCs with a fetal origin. This fetal origin may be reflected by the difference in expression patterns of certain molecules compared to BM-MSCs. For example, UCMSs express CD29 together with the chemokine receptors CD183 and C184. CD191, CD192, CD195,

CD197, CD15 and CD162 are absent on these cells although the culturing conditions may lead to an up-regulation. In contrast, all these molecules are found to be present on adult BM-MSCs, which present a more mature phenotype. Although, the UCMSs express a more limited amount of homing molecules, these stem cells may still have the potential to migrate to the brain lesions. Furthermore, HLA-G transcripts are found, and HLA-G protein expression can be up-regulated under certain conditions. Although the percentage positive cells is relatively low, an immunomodulatory function cannot be excluded. Finally, the most optimal isolation procedure and culturing medium were chosen: to obtain and maintain the proper cell population, UCMSs better are obtained by migration out of explants and maintained in KO-DMEM/F12.

In the future, the functionality of the molecules evaluated in this project must be determined. The functioning of the chemokine receptors will be tested using a transwell system. To ensure the positive results obtained on HLA-G expression are not caused by aspecific binding of the antibody, a western blot will be performed. Detection of a 39kDa band will clarify our results obtained by flow cytometry and PCR. If this molecule is actual present, its functionality will be tested by suppression assays. Other future plans may involve a senescence assay and a telomerase assay to confirm the stem cell/tumor characteristics of the obtained cells. Data resulting from these tests may also further distinguish between the UCMSs cultivated in the four different media. Furthermore, the migratory capacity of UCMSs to secondary lymphoid organs can be tested *in vitro* by flow cytometry as CD62L (L-selectin) is needed for extravastion in the lymph nodes. These data may give a first indication on a possible immunomodulatory function in the secondary lymphoid organs. If these future tests give positive results, then the UCMSs can be tested in an *in vivo* (EAE) model.

References

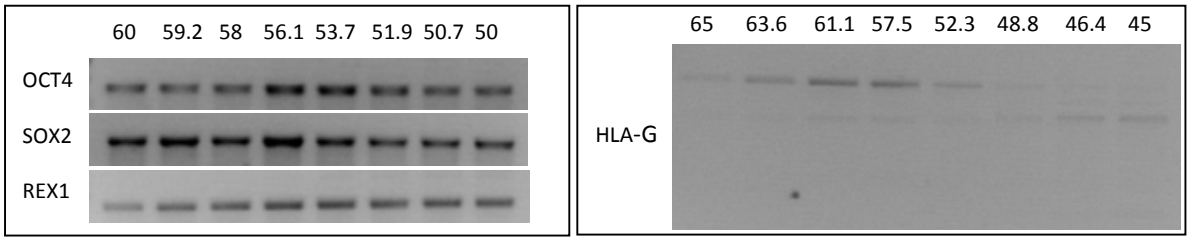
1. Murray, T.J., ed. *Multiple sclerosis: the history of a disease*. 2005, Demos medical publishing: New York.
2. WHO, *Atlas multiple sclerosis resources in the world* 2008, World Health Organization.
3. Ebers, G.C., *Environmental factors and multiple sclerosis.* Lancet Neurol, 2008. **7**(3): p. 268-77.
4. Hauser, S.L. and J.R. Oksenberg, *The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration.* Neuron, 2006. **52**(1): p. 61-76.
5. Polman, C.H., et al., *Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria".* Ann Neurol, 2005. **58**(6): p. 840-6.
6. Comi, G., *Treatment of multiple sclerosis: role of natalizumab.* Neurol Sci, 2009. **30 Suppl 2**: p. S155-8.
7. Health, U.S.N.I.o. *ClinicalTrials.gov*. 2010 [cited; Available from: www.clinicaltrials.gov.
8. Pluchino, S., et al., *Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates.* Ann Neurol, 2009. **66**(3): p. 343-54.
9. Capello, E., et al., *Autologous haematopoietic stem-cell transplantation in multiple sclerosis: benefits and risks.* Neurol Sci, 2009. **30 Suppl 2**: p. S175-7.
10. Karussis, D., et al., *Immunomodulation and neuroprotection with mesenchymal bone marrow stem cells (MSCs): a proposed treatment for multiple sclerosis and other neuroimmunological/neurodegenerative diseases.* J Neurol Sci, 2008. **265**(1-2): p. 131-5.
11. Troyer, D.L. and M.L. Weiss, *Wharton's jelly-derived cells are a primitive stromal cell population.* Stem Cells, 2008. **26**(3): p. 591-9.
12. Mitchell, K.E., et al., *Matrix cells from Wharton's jelly form neurons and glia.* Stem Cells, 2003. **21**(1): p. 50-60.
13. Weiss, M.L., et al., *Immune properties of human umbilical cord Wharton's jelly-derived cells.* Stem Cells, 2008. **26**(11): p. 2865-74.
14. Weiss, M.L., et al., *Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease.* Stem Cells, 2006. **24**(3): p. 781-92.
15. Carlin, R., et al., *Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells.* Reprod Biol Endocrinol, 2006. **4**: p. 8.
16. Menier, C., et al., *Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule.* Tissue Antigens. **75**(3): p. 201-6.
17. Carosella, E.D., et al., *HLA-G: from biology to clinical benefits.* Trends Immunol, 2008. **29**(3): p. 125-32.
18. Fainardi, E., et al., *Intrathecal synthesis of soluble HLA-G and HLA-I molecules are reciprocally associated to clinical and MRI activity in patients with multiple sclerosis.* Mult Scler, 2006. **12**(1): p. 2-12.
19. Fainardi, E., et al., *Potential role of soluble human leukocyte antigen-G molecules in multiple sclerosis.* Hum Immunol, 2009. **70**(12): p. 981-7.
20. La Rocca, G., et al., *Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers.* Histochem Cell Biol, 2009. **131**(2): p. 267-82.
21. Rachakatla, R.S., et al., *Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors.* Cancer Gene Ther, 2007. **14**(10): p. 828-35.
22. Szczucinski, A. and J. Losy, *Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies.* Acta Neurol Scand, 2007. **115**(3): p. 137-46.
23. Man, S., E.E. Ubogu, and R.M. Ransohoff, *Inflammatory cell migration into the central nervous system: a few new twists on an old tale.* Brain Pathol, 2007. **17**(2): p. 243-50.

24. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
25. Fox, J.M., et al., *Recent advances into the understanding of mesenchymal stem cell trafficking.* Br J Haematol, 2007. **137**(6): p. 491-502.
26. Ruster, B., et al., *Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells.* Blood, 2006. **108**(12): p. 3938-44.
27. Kumar, V., ed. *Robbins basic pathology*. 8th edition ed. Acute and chronic inflammation. 2007, Elsevier: Philadelphia.
28. Seshareddy, K., D. Troyer, and M.L. Weiss, *Method to isolate mesenchymal-like cells from Wharton's Jelly of umbilical cord.* Methods Cell Biol, 2008. **86**: p. 101-19.
29. Nekanti, U., et al., *Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells.* Stem Cells Dev. **19**(1): p. 117-30.
30. Pappa, K.I. and N.P. Anagnou, *Novel sources of fetal stem cells: where do they fit on the developmental continuum?* Regen Med, 2009. **4**(3): p. 423-33.
31. Liedtke, S., et al., *Oct4 and its pseudogenes confuse stem cell research.* Cell Stem Cell, 2007. **1**(4): p. 364-6.
32. Booth, H.A. and P.W. Holland, *Eleven daughters of NANOG.* Genomics, 2004. **84**(2): p. 229-38.
33. Izadpanah, R., et al., *Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue.* J Cell Biochem, 2006. **99**(5): p. 1285-97.
34. Montemurro, T., et al., *Differentiation and migration properties of human fetal umbilical cord perivascular cells: potential for lung repair.* J Cell Mol Med.
35. Fong, C.Y., et al., *Comparative growth behaviour and characterization of stem cells from human Wharton's jelly.* Reprod Biomed Online, 2007. **15**(6): p. 708-18.
36. Wiendl, H., et al., *A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape.* J Immunol, 2002. **168**(9): p. 4772-80.
37. Sarugaser, R., et al., *Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors.* Stem Cells, 2005. **23**(2): p. 220-9.
38. Corsini, N.S., et al., *The death receptor CD95 activates adult neural stem cells for working memory formation and brain repair.* Cell Stem Cell, 2009. **5**(2): p. 178-90.
39. Donders, R., *Mesenchymal stem cells derived from umbilical cord tissue: a therapeutic option for multiple sclerosis?* . 2009, Hasselt University: Diepenbeek.
40. Chamberlain, G., et al., *Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing.* Stem Cells, 2007. **25**(11): p. 2739-49.
41. Jin, H.J., et al., *Down-regulation of CD105 is associated with multi-lineage differentiation in human umbilical cord blood-derived mesenchymal stem cells.* Biochem Biophys Res Commun, 2009. **381**(4): p. 676-81.
42. Nasef, A., et al., *Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G.* Transplantation, 2007. **84**(2): p. 231-7.
43. Selmani, Z., et al., *HLA-G is a crucial immunosuppressive molecule secreted by adult human mesenchymal stem cells.* Transplantation, 2009. **87**(9 Suppl): p. S62-6.
44. Drukker, M., et al., *Characterization of the expression of MHC proteins in human embryonic stem cells.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 9864-9.

Supplemental data

S.1 PCR optimalisation

PCR was optimised for each primer set by a temperature gradient PCR performed with the positive controls. cDNA from the NT2/D1 cell line was used for optimalisation of the PCR with OCT4, SOX2, REX1 primers. For all 3 primer sets, 56.1°C was chosen as the optimal annealing temperature. The JEG-3 cell line was used for optimalisation of the HLA-G PCR. For HLA-G, 61°C was chosen as the most optimal annealing temperature.



Supplemental figure 1.PCR optimalisation

PCR optimalisation for OCT4, SOX2, REX1 and HLA-G primers by temperature gradient PCR.

S.2 Sequencing data of the amplification products resulting from a PCR with HLA-G primers.

The PCR resulted in amplification products of three different lengths: approximately 100bp, 800bp and 500 bp. The bands were excised, purificated, sequenced and analysed with BLAST(n=3). The sequences obtained after sequencing are shown here:

1000 bp sequence

- Expected to correspond to HLA-G1
- Homology found: 96%
- Sequence:
  TCCCCCCCNNCNNTTNTTTTTAACCTNCACCCAGCTACTNTTTTTGGCCCTGACCCTGACCGAGACCTG
  GGCGGGCTCCCACTCCATGNTGTATTTCAGCGCCGCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGC
  TTCATCGCCATAGGGCTACGTAGGACGACACGCAGTTCGTGCGGTTCGACAGCGACTCGGCGTGTCC
  GANGATGGAGCCGCAGGGCGCCGTGGGTGGAGCAGGAGGGGCCAGAGTATTGGGAAGAGGAGAC
  ACGGAACACCAAGGCCCACGCACAGACTGACAGAATGAACCTGCAGACCCTGCGCGGCTACTACAAC
  CAGAGCGAGGCCAGTTCTCACACCCTCCAGTGGATGATTGGCTGCGACCTGGGGTCCGACGGTCGCC
  TCCTCCGCGGGTATGAACAGTATGCCTACGATGGCAAGGATTACCTCGCCCTGAACGANGACCTGCG
  CTCCTGGACCGCAGCGGACACTGNGGCTNANATCTCCAAGCGCAAGTGTGANGCGGCCAATGTGGC
  TGAACAAANGANAGCCTACCTGGAGGCACGTGCNTGNANTGGCTCCNANATNCNNGGAAACNGNA
  NGANANCNNNNNNNNNNNNCCNNNNANANNAN

800 bp sequence:

- Expected to correspond to HLA-G2 or HLA-G4
- Homology found: 0% for both isotypes
- Sequence:
  CCCANCCCCCCCCCCGGTNANTNGGANCCANCGCGNGNTANTGGCGGGGACACGGCCGCNACCAG
  GGCGGGCNCCCACANAAANTGGAGGGGAGCGCANNCACCCGGCCGGGCCCGGCCGCGAGNAGAG
  CCCCNCCACAACNCGCACGGNCAACGAGGCACGAGAAGCAGAAACGNGCGNCNACNACAGCGAAC
  GNGGANGANCGNGGAAAANGCNACACNCGGGCCCAAGAGCGAAGNAGCNNGGGGGGGGCACNG
  AGGNNNNGNNGAAGNAGNCNACNCGGGAGNGCCCGCNCCCNAACGCACCNGGGCGCCNCGANC
  GANAGCGGGNACNCGCCCNCCGGNNGNACNNANCACCCAACGGCACANAGGCACCANCGGCGNC
  CGCNGNNNCGNGACCGANAANGCNNCNGAACCGCGACGNGCGGNGAANCACCCGGACCANCNC
  NNNGGCCGNACCANNGNCGCGCNNNACNCNNNANCCAGGCNCCCNCCGGCNNGNCCNNNNCNC
  CGCACNNCACGNGANGAGACCCCGCANCCNCCCNNCGACGCNACAAAACAGGGCGNCNCNNCGC
  ACNGCCNACACGAGNCACCCANGCGACCCGCGCGCACGANAACNNCGNNNCACNNCGCGGCACN
  ANAANCNCNACNNNCGAGGCGACACGGCGNNGCAGCGGGANNNGNNGGANNCNGG

500 bp sequence:

- Expected to correspond with HLA-G3
- Homology found: 0%
- Sequence:
  CCNCNTNCCCCCCCCCCCCCCCCTTTAATTNCACCNGCCNANCCNCCGCATNTTNTTCCCCCGCANAG
  AACCCTAGGGCANCACCTNCCNAGNNCAGGCAGGCCAGATTNANGAGGAGGCGTCCACCACACCN
  CAATAGCCANTAACAAGTAGCGCCCCAAGCNAGAAGCCCAGGAGGCCCTGACCTTNNCCTACGGAT
  CGAGCCCTGAAGGCCNCGGCCCNGAAGGCCTGGGGCGGGAAGAAGGAGAACCNGAAGGCGGCGC
  AGGCAGGAGCANGCCAAGCAGAGCCNNGGCCAACAGCCTTGNNAGGCANCGAAAGNACNCNCCC
  NANCNANANGGCCGGACGCCAGCGGCCAGCNAGNCCACANNCNCANCNANCANGCCAATCAAGA
  GNCAGAGCNCCNAANCCCCNNAANAANAAAACNGTNCCNNGCNCNANACNAGCNNNCNNCNNN
  NNCGCNCNNNCCTANNAANCCNCCCNCCCACNGNANANGNAAAANCNANACCACCCCNNCNGGC
  NANAACACCANCCCNNNCCACNAGACACANCCCCANANNNCNCGCNNCACNANANNCNCGACCA
  AGANNCNNACNAAAGCCAAANCANCGCGNGACCCANAAAANAACCANACNCGCCNNCGAAACAA
  CCGANCNAAACTCANNACNANCGACCAACNAACACAACAANATCNGCNCNAAAG

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
The characterization of umbilical cord matrix stem cells in the context of multiple sclerosis

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2010**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Willekens, Goele

Datum: **15/06/2010**