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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen: klinische en moleculaire wetenschappen

Masterproef

Characterization of stem cells derived from umbilical cord tissue

Promotor :
Prof. dr. Niels HELLINGS

Nazim Tüylüoglu

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische en moleculaire wetenschappen

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List of abbreviations

AF: Amniotic fluid

APC: Antigen presenting cell

BBB: Blood brain barrier

BM-MSC: Bone marrow mesenchymal stem cells

bFGF: basic fibroblast growth factor

CNS: Central nervous system

DC: Dendritic cell

DMEM: Dulbecco's modified Eagle's medium

EAE: Experimental autoimmune encephalomyelitis

EGF: Epidermal growth factor

ESC: Embryonic stem cells

FBS: Fetal bovine serum

HLA: Human leukocyte antigen

HSC: Hematopoeitic stem cells

HRP: Horseradish peroxidase

ICM: Inner cell mass

IVR: Intervascular region

MHC: Major histocompatibility complex

MS: Mutiple sclerosis

MSC: Mesenchymal stem cells

NSC: neural stem cells

PBS: Phosphate buffered saline

PP-MS: Primary progressive MS

P/S: Penicillin/ Streptomycin

ROS: Reactive oxygen species

RR-MS: Relapse remitting MS

(RT)-PCR: Reverse transcriptase polymerase chain reaction

SP-MS: Secondary progressive MS

SSEA: Stage specific embryonic antigen

UC: Umbilical cord

UCB: Umbilical cord blood

WJ-MSC: Wharton's jelly mesenchymal stem cells

PFA: Paraformaldehyde

PVR: Perivascular region

Abstract

Background/introduction: Multiple sclerosis (MS) is defined as a chronic, neurodegenerative, inflammatory auto-immune disease of the central nervous system (CNS). The shortages of current treatments to tackle the underlying mechanisms have caused an increased interest in cell based therapies. This, in hope to provide the injured tissue a population of functional cells and hence to restore tissue regeneration based on the large differentiation capacity, immunomodulatory and neuroprotective potential of the stem cells. The therapeutic effects of mesenchymal stem cells (MSC) have been investigated in both MS and experimental autoimmune encephalomyelitis (EAE), which resulted in contradictory findings. During this study it was aimed to characterize the Wharton's jelly mesenchymal stem cells (WJ-MSC) situated in the umbilical cord and compare their genetic expression profile with BM-MSC in order to gain sight in possible mechanisms that could attribute in their functioning in cell based therapy.

Material en methods: *In situ* staining of the umbilical cord was assessed in order to gain more insight in the immunophenotype and morphology of these WJ-MSC in their natural environment. Umbilical cords were analyzed for expression of morphological (e.g α- Tubulin, B III Tubulin, α- SMA), mesenchymal and pluripotent (e.g. CD44, CD29, CD146, Oct4) and immunological relevant markers (e.g HLA-G). In a next step the pluripotent character of low-passage and high-passage WJ-MSC was investigated. The genetic expression of different pluripotency markers such as Oct4, Sox2, Rex1 and Nanog, also known to be expressed in embryonic stem cells were assessed. Additionally the genetic profile of WJ-MSC was compared with BM-MSC. The expression of different genes involved in chemotaxis, cell adhesion, immune suppression, immune activation, pro/anti-inflammatory processes and neuroprotection were investigated. Both experiments were assessed by means of PCR.

Results*: In situ* phenotypic characterization of WJ-MSC showed expression of the mesenchymal markers CD44, CD29, CD146 and the morphological markers α- SMA, α- Tubulin and BIII Tubulin. No expression of CD14, ALDH1A1, HLA-G, Oct4 and collagen II was observed. Low-passage and high-passage WJ-MSC showed expression of Oct4, SOX2, Rex1 and Nanog. Analysis of the genetic expression profile between WJ-MSC and BM-MSC resulted besides similar expression profiles also differential expression of various genes involved in different processes.

Conclusion: Although we failed to characterize their pluripotency and immunosuppressive potential *in situ*, the results obtained during this study indicate that WJ-MSC possess stem cell characteristics in their natural environment and have a stable phenotype even after several passages of cell culture. The gene expression profile between WJ-MSC and BM-MSC suggested that WJ-MSC can be potentially used in therapy based on their immunosuppressive phenotype, their capacity to migrate towards injured tissue and their potential to produce neurotrophic factors. It would be interesting in the future to investigate the trophic factors which are expressed by these cells *in vitro* whether they are capable of introducing cell survival of injured cells and hence investigate their effect *in vivo* and asses if their functioning is the same.

Samenvatting

Introductie: Multiple sclerose (MS) wordt gedefinieerd als een chronische neurodegeneratieve inflammatoir auto-immuun aandoening van het centraal zenuwstelsel (CZS). Het gebrek aan huidige behandelingen om de onderliggende mechanismen van deze ziekte aan te pakken, heeft de interesse in cel gebaseerde therapieën opgewekt. Dit, in de hoop om het beschadigd weefsel te voorzien van een populatie functionele cellen, en als gevolg het weefsel te herstellen op basis van verschillende eigenschappen die deze cellen beschikken. Het therapeutisch effect van mesenchymale stamcellen (MSC) is reeds onderzocht in zowel MS als experimenteel auto-immuun encefalomyelitis (EAE), met tegenstrijdige bevindingen als resultaat. In de loop van deze studie werd er getracht om de "Wharton's jelly" mesenchymale stamcellen (WJ-MSC), gelokaliseerd in de navelstreng te karakteriseren en hun genetische expressie profiel te vergelijken met beenmergstamcellen (BM-MSC) om zo inzicht te hebben op mechanismen die mogelijk een rol kunnen spelen in het functioneren van deze stamcellen in cel gebaseerde therapieën.

Materiaal en methoden: *In situ* kleuringen op navelstrengweefsel werden uitgevoerd om meer inzicht te krijgen over de immunofenotypering en morfologie van deze WJ-MSC in hun natuurlijke omgeving. Navelstrengweefsels werden geanalyseerd ter detectie van morfologische (bv. α- Tubulin, B III Tubulin, α- SMA), mesenchymale en pluripotente (bv. CD44, CD29, CD146, Oct4), en immunologisch relevante (bv. HLA-G) merkers. In een volgende stap werd het pluripotent karakter van deze WJ-MSC uit/van zowel lage als hoge passages onderzocht. De genexpressie van verschillende pluripotentiemerkers zoals Oct4, Sox2, Rex1 en Nanog, waarvan ook geweten is dat deze tot expressie komen in embryonale stamcellen, werd nagegaan. Tenslotte, werd het genetisch profiel van de WJ-MSC vergeleken met de BM-MSC. De expressie van verschillende genen betrokken bij processen zoals chemotaxis, celadhesie, immuunsuppressie, immuunactivatie, pro- en anti-inflammatoire processen en neuroprotectie werd onderzocht. Beide experimenten werden beoordeeld aan de hand van PCR analyses.

Resultaten: De *in situ* fenotypering toonde aan dat de WJ-MSC de mesenchymale merkers: CD44, CD29, CD146 en de morfologische merkers: α- SMA, α- Tubulin and BIII Tubulin tot expressie brachten. De expressie van CD14, ALDH1A1, HLA-G, Oct4 en collageen II werden niet waargenomen. WJ-MSC van zowel lage als hoge passages vertoonden expressie van Oct4, SOX2, Rex1 and Nanog. Analyse van de genexpressie tussen WJ-MSC en BM-MSC toonde aan dat er naast gelijkenissen ook verschillen werden geobserveerd van genen die betrokken zijn in verscheidene processen.

Conclusie: Ondanks we er niet in geslaagd zijn het pluripotente en immunosuppresieve potentieel aan te tonen *in situ*, duiden de resultaten van de huidige studie er op dat WJ-MSC in bezit zijn van stamcel karakteristieken in hun natuurlijke omgeving en dat ze een stabiel fenotype vertonen zelf na verschillende celkweek passages. Het gen expressie profiel tussen de WJ-MSC en BM-MSC suggereerde dat WJ-MSC een therapeutisch vermogen hebben gebaseerd op hun immunosuppressieve fenotype, hun capaciteit om te migreren naar beschadigd weefsel en hun potentieel om neurotrofe factoren te produceren. Toekomstgericht zou het interessant zijn om de trofische factoren, die tot expressie gebracht worden door deze cellen, te onderzoeken in een *in vitro* studie en na te gaan of deze factoren de overlevingskansen van beschadigde cellen vergroten. Vervolgens zou een onderzoek van *in vivo* eropmoeten wijzen of hun effect dat *in vitro* plaatsvond ook *in vivo* gezien wordt.

Introduction

1. Stem cells

Generally, stem cells are defined as a population of undifferentiated cells, which have a self-renewal capacity, the ability to differentiate into multiple cell types as well as the potential to repopulate a particular tissue *in vivo* [1-3]. Different postnatal tissues (e.g. bone marrow, adipose tissue, muscle, brain) are known to be a source for these stem cells, which primarily regulate and maintain tissue homeostasis [4, 5]. Stem cells are capable of self-renewal that ensures these cells to stay in their undifferentiated state and at the same time give rise to daughter cells committed to a lineage specific differentiation [4, 6, 7]. Besides these characteristics, stem cells posses a potency hierarchy with specific proliferation and differentiation capacities [2]. Stem cells can be subdivided into totipotent, pluripotent, multipotent and unipotent stem cells regarding their differentiation potential [2]. Totipotent cells are represented by the zygote (fertilized egg) up to the 4-cell stage embryo (morula). These cells have the capacity to differentiate into all the cell types of the organism including the extra embryonic tissue [8]. Pluripotent cells represented by embryonic stem cells (ESC), are cells of the inner cell mass (ICM) of the blastocyste, which are capable of differentiating into cells of the three germlayers: mesoderm, endoderm and ectoderm [3, 8]. Unlike the totipotent cells, pluripotent cells are not able to differentiate into cells of the extra embryonic tissue, meaning that pluripotent cells by themselves cannot develop into a living organism [1, 8]. Multipotent stem cells are cells that are more restricted in their differentiation capacity compared with the totipotent and pluripotent stem cells [1]. These stem cells were traditionally believed to be committed to form cells of the tissue where they resided. However different reports have described that these cells could change their cell fate by differentiating into cells of other tissues within the same germlayer [1, 9]. Among the multipotent/adult stem cells, the hematopoetic stem cells (HSC) are the best characterized [8, 10]. However, besides HSC other adult stem cell types such as mesenchymal stem cells (MSC) and neural stem cells (NSC) also exist. These adult stem cells are mainly involved in tissue homeostasis and reside in particularly every tissue and organ of adult animals [11]. Unipotent stem cells as last, can only differentiate into one cell type [12].

Stem cells can also be classified based on a so called developmental hierarchy model, depending on the stage where they appear in during the origin and development of an organism [2]. Following this classification, stem cells can be subdivided into the earlier described pluripotent ESC, multipotent adult/somatic stem cells and the fetal stem cells [2]. Fetal stem cells are derived from the fetus or from the extra embryonic tissues such as the umbilical cord blood (UCB), amniotic fluid (AF), Wharton's jelly (WJ), placenta and amniotic membrane, which are discarded after birth [2, 13]. They

also can be isolated from other fetal tissues such as the kidney and liver [10]. These fetal stem cells represent a stem cell type between ESC and adult MSC regarding their proliferation and plasticity features. Studies have shown that these stem cells with a fetal origin express various stem cell markers that are also expressed by ESC such as Oct-4, SSEA-3, Tra 1-60 and Nanog [2, 14]. Compared with adult stem cells, fetal stem cells are known to have a bigger differentiation capacity and a lower immunogenicity, hence a greater potential in therapeutic applications [10]. In addition, the resemblance with ESC, without the ethical restrictions and the easier availability of fetal stem cells due to their localization in extra- embryonic tissue makes them also a more suitable stem cell source compared to the ESC [10].

1.1 Cell based therapies in neurodegenerative diseases

Replacing the damaged functional cells in neurological diseases such as Multiple Sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) is very difficult, because the CNS has only limited endogenous repair mechanisms which are not enough to save a whole population of cells [15]. Therefore it is important to be able to replace these damaged cells in the injured tissue by new cells or at least rescue them from cell death and hence ensure their functioning.

The main reason of interest in stem cell based transplantations is to repopulate an injured area with cells capable of differentiating by use of local biochemical cues, into cell types of the damaged tissue, which have been lost. This so called substitutive cell therapy has set the basis to investigate stem cells in therapeutic applications [4, 16]. However, other functions rather than merely the differentiation capacity and the ability to replace damaged cells gained the interest and opened perspectives in using stem cells for cell based therapies. Their ability to give trophic support and to home into sites of injury for instance and their immunomodulatory characteristics are other useful functions one can investigate for therapeutic purposes (protective cell therapy) [4].

In clinical settings, ESC would be seen as the best candidates in cell based therapies to treat disorders regarding their pluripotent phenotype, which makes them suitable for differentiating into all kinds of cell types [15, 17]. Different studies have shown the potential of ESC to differentiate into neural cell types *in vitro* [4, 17-19]. *Lam et al. 2010* investigated for instance the regulation of neural differentiation of ESC *in vitro* by growth factors such as the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), where he showed that neural cell differentiation in ESC and axonal growth was enhanced following treatment with both growth factors. [15] However, the ethical concerns regarding their use and the unregulated cell growth resulting in the formation of teratomas following *in vivo* transplantation possesses a great challenge for the safety handling of the ESC, which makes their use less convenient. As a result, ESC cannot be directly transplanted into patients.

Another source of stem cells for therapeutic use, are the adult stem cells. Although these stem cells show less plasticity with their multipotent differentiation capacity compared to ESC, a major advantage of adult stem cells is that they do not develop into teratomas following transplantation. Additionally, there are less ethical restrictions with the use of adult stem cells in comparison with ESC [4, 20]. Their presence in almost all adult tissues of the human body make them also useful for autologic stem cell transplantations, hereby avoiding immunological reactions such as the graft versus host reaction that could lead to complications [20].

NSC is one type of adult stem cells which are investigated for treating neurological diseases. NSC can be found near the ventricles (subventricular zone) of adult animals [21] or can be generated from ESC [22]. The *in vitro* differentiation of NSC into various types of neural cells such as neurons, astrocytes, oligodendrocytes has been reported various times [4, 23, 24]. The *in vivo* transplantations of NSC in neurological disease animal models have also shown different degrees of effectiveness [4]. Although *in vivo* transplantations of NSC have shown minor cases of tumor formation, the functional recovery following administration seems to be the result of neuroprotective effects of the transplanted NSC rather than the replacement of damaged neurons. The isolation of NSC, which seem to be a great source of stem cells for autologic stem cell transplantations, is however a very dangerous and invasive procedure.

For this reason other sources of adult stem cells such as MSC can be considered for transplantation purposes. Bone marrow derived mesenchymal stem cell (BM-MSC) transplantations in the CNS have been studied, resulting in contradictory findings. As a start, the plasticity capacity is a major topic where a lot of debate is going on. Although there is evidence that MSC can differentiate into neural cells [25-27] by a phenomena called transdifferentiation, others dispute these findings by calling this process the result of cell fusion [28]. In addition however, it is shown that these cells produce neurotrophic growth factors, which can promote cell survival of the damaged cells at the site of injury [29]. Studies have also indicated that MSC can suppress lymphocyte proliferation, which helps them in escaping immune recognition [30].

1.2 Wharton's jelly mesenchymal stem cells

The human umbilical cord (UC), one of the extra embryonic tissues as mentioned above is an interesting source for cells with stem cell properties. The UC is composed of 2 arteries and 1 vein surrounded by a primitive mucoid connective tissue, known as the Wharton's jelly (WJ) which is mainly comprised of proteoglycans and different isoforms of collagens [2, 14]. The main function of the WJ is to prevent compression and bending of the umbilical cord vessels, which are important structures for the delivery of oxygen, glucose, amino acids to and removal of waste products from the developing fetus [14]. The WJ can be subdivided into 3 regions, from where MSC can be isolated: the perivascular region, the intervascular region and the subamnion (Figure 1) [26]. The fact that the umbilical cord is discarded after birth, makes harvesting stem cells for therapeutic purposes from this and other extra embryonic tissues a relatively safe and non-invasive procedure [14].

Cells located in the WJ are identified as primitive MSC believed to be trapped in the connective tissue matrix during embryogenesis. It is postulated that during embryogenesis, migration of early HSC and MSC takes place trough the umbilical cord to the fetal liver and bone marrow. During this migration it is believed that the MSC stick in the WJ and become embedded in this connective tissue [14, 31]. Hence, due to this formation and entrapment in early development, these primitive cells show resemblance to ESC but also show equivalence to the more adult MSC found in the bone marrow [31]. The Wharton's jelly cells have been shown to display characteristics of MSC as defined by the International Society for Cellular Therapy [14, 32]. For instance, they have a plastic adherence capacity, which means that they grow as adherent cells in culture with mesenchymal morphology. Secondly, they express MSC specific cell surface markers (e.g. CD 105, CD 73, CD 90) and HLA-A,B,C (MHC class I), lack the expression of CD 45, CD 34 and HLA-DR (MHC class II) and have the ability *in vitro* to differentiate along chondrogenic, adipogenic and osteogenic lineages. Furthermore as mentioned earlier, these cells also exhibit properties attributed to ESC. They express the pluripotency markers Oct4, Sox2, and Nanog and however on a lower level compared with ESC, express also ESC markers such as stage specific embryonic antigen-1 (SSEA-1), SSEA-4, Tra-1-60 and Tra-1-81 [14, 26, 33-35]. Additionally, they are also known to form embryoid bodies *in vitro* [33].

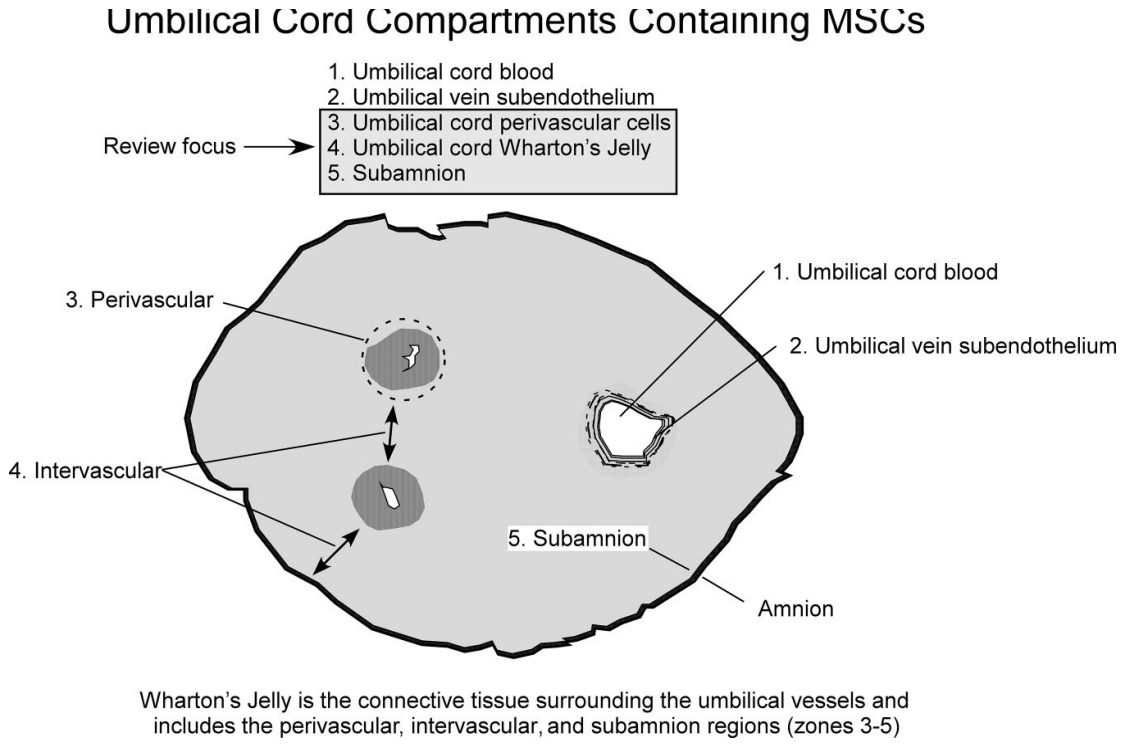

Figure 1: Umbilical cord compartments. Five different regions in the umbilical cord are known to contain mesenchymal stromal cells: (1) the umbilical cord blood, (2) the umbilical vein subendothelium, (3) the perivascular region, (4) the intervascular space and (5) the subamnion. The Wharton's jelly can be subdivided into 3 distinct regions, ranging from region 3 to 5. (*Troyer et al., 2008)* [26].

1.3 Advantages of Wharton's jelly mesenchymal over other stem cell types

Despite, the resemblance between the fetal Wharton's jelly MSC (WJ-MSC) and ESC or adult MSC, WJ-MSC also exhibit some advantages over the other two stem cell types. Compared with the adult MSC, the fetal WJ-MSC has a larger expansion capacity *in vitro [26, 36]*. Another difference is also the slower doubling time in adult MSC, which may be due to their shorter telomere lengths [36]. The fetal WJ-MSC also lack the human leukocyte antigens class II (HLA). However, on their part they synthesize HLA-G, a non-classical immunomodulatory HLA class I molecule which appears to lack in adult MSC [26]. In addition, compared with the invasive isolation procedure to obtain BM-MSC, the umbilical cord tissue is an easily accessible source for the enrichment of these cell populations with MSC-like properties, and the portion of BM-MSC in the mononuclear cell fraction is smaller compared with the richer source of WJ-MSC [2, 14].

The pluripotent ESC that seem to have a bigger advantage over the WJ-MSC due to their larger differentiation capacity are also related with different drawbacks making their use in therapeutic settings difficult. On one side, the use of ESC is prevented due to major ethical concerns regarding their origination and hence termination of the development of a potential human life [14, 20, 37]. On the other hand it is shown that transplantation of ESC results in teratoma formation [17].

2. Multiple Sclerosis

Multiple sclerosis (MS) is defined as a chronic, inflammatory, neurodegenerative autoimmune disease of the central nervous system (CNS) [38]. MS is believed to affect 2,5 million people worldwide with an onset of disease at an early age [38-40]. MS can be divided into different forms with a varying course. Relapse remitting MS (RR-MS) characterized by phases with neurological deficiency (relapses) followed by periods with some degree (complete or incomplete) of recovery is the most common form (80-90%). The majority of RR-MS cases progresses to secondary progressive MS (SP-MS) characterized by periods with an increased number of relapses. Another form, which is less represented, is known as primary progressive MS (PP-MS). During this phase the disease is progressive from onset, without the relapsing phase. PP-MS shows a gradual increase in neurodegeneration and reduced inflammation [41, 42].

The classification of MS as an autoimmune disease is largely based on the pivotal role of myelin reactive T lymphocytes in the inflammatory immunopathological processes active in MS [43]. However, besides myelin reactive T lymphocytes other immune cells such as macrophages, microglia and B cells are also believed to play an important role in MS pathology [42, 44]. Contribution to this autoimmune based view of the disease also comes from the association of the major histocompatibility complex (MHC) class 2 DR2 allele with an increased susceptibility for developing MS [44].

The diverse clinical presentations of MS can be related to an underlying diversity in the pathogenesis [44]. It is believed that myelin auto reactive T cells in the periphery are activated in MS patients [42, 44]. This activation can be preceded by different activation signals. Although the aetiology of MS is not fully understood, it is believed that different triggers can play a role in triggering the disease [41]. Reports suggest that genetic susceptibility (HLA locus on chromosome 6p21) plays an important role [41]. Additionally it is also assumed that environmental factors such as pathogens (e.g. Epstein-Barr virus) and lowered vitamin D intake (sunlight) can increase the risk for disease development [38, 40, 45, 46]. Infectious agents with sequence homology with auto antigens could trigger MS and activate auto reactive CD4+ T cells by a phenomena called molecularly mimicry [47]. The Epstein Barr virus [38, 47] could for instance express epitopes, showing similarities with self-antigens (MBP), hereby activating self-reactive T cells. Together these examples show that the contribution of genetic and environmental factors could be important for MS development.

2.1 Pathogenesis

The early phase of MS is characterized by inflammatory infiltrates that catalyze MS pathogenesis, while the progressive phase is characterized by non-inflammatory neurodegenerative processes [38, 42]. It has been implicated that MS is mostly mediated by CD4+ (Th 1 cell) responses [48]. However, there are studies that report a dominant contribution of CD8+ T cells [42]. As depicted in the figure below (Figure 2), the immune pathogenesis of MS is proposed as following. After activation of the autoreactive T cells mediated by antigen presenting cells (APC's) such as dendritic cells (DC), B-cells and macrophages [49], different processes such as adhesion to the vessel wall, extravasation, chemotaxis, cytokine production and changes in extracellular matrix integrity enable the T cells to cross the blood-brain barrier (BBB) and reach the CNS. Locally in the brain the autoreactive T cells become activated by local APCs such as microglia and astrocytes presenting myelin peptides. A variety of myelin-associated proteins including myelin basic protein (MBP) myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) are considered to be relevant for autoreactive T cell activation in MS and EAE [48]. Once activated, these autoreactive T cells will expand and secrete inflammatory and chemotactic mediators. Whereas inflammatory cytokines and reactive oxygen species (ROS) can directly damage the myelin sheath, chemokines will attract yet other immune cells, such as macrophages, which also will contribute to the cellular damage [38, 48].

The evidence for the possible autoimmune nature of MS is mostly based on experimental studies performed on the animal model of MS, namely experimental autoimmune encephalitis (EAE) [38, 42, 44, 46]. Although EAE is a useful model in the struggle to understand MS [46] there are certainly differences in the inflammatory response between MS patients and EAE affected animals [42, 49].

2.2 Treatment

Current treatments consists of administration of anti-inflammatory, immunosuppressive or immunomodulatory agents, which are partly effective in the early stages, but show limited effects in the progressive phase. An effective treatment to cure MS is not found yet [41, 42, 45].

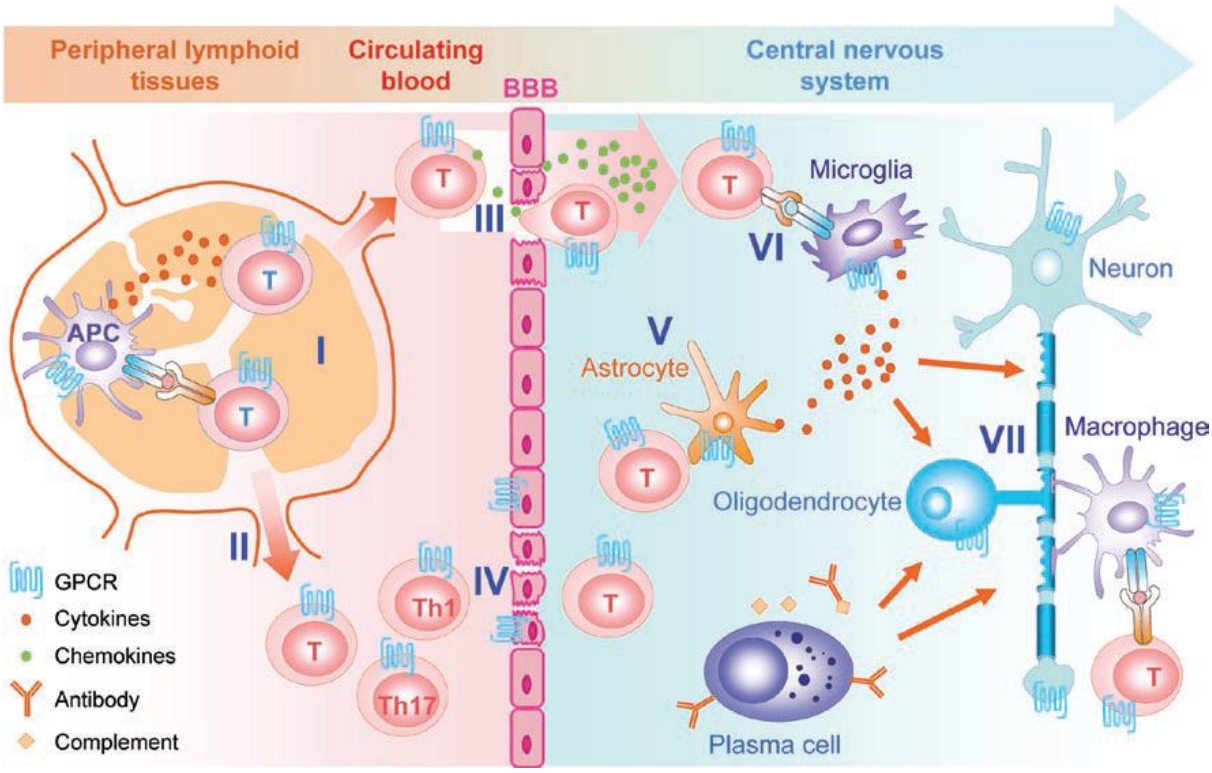

Figure 2: Representation of Multiple sclerosis pathogenesis. Following peripheral activation of auto-reactive T cells, these cells cross the blood brain barrier and become reactivated in the brain by local antigen presenting cells. This reactivation causes T cells to damage brain tissue directly by producing inflammatory mediators, or indirectly by attracting other immune cells. (Du et al.,2012) [50].

3. Wharton's jelly mesenchymal stem cells as a therapy for MS

In case of MS, stem cells could be used to restore damaged tissue by replacement of the injured or lost neurons and myelin forming oligodendrocytes. However, this substitutive cell therapy approach seems rather difficult due to the drawbacks with the *in vivo* differentiation capacity following stem cell transplantation. Stem cells could also attenuate the immune response and offer neuroprotection by providing trophic support (protective cell therapy).

Based on the outcomes from preclinical studies in animal models such as EAE, MSC transplantations have been proposed and used as a possible treatment for MS. The effects observed were mainly due to the immunomodulatory and protective properties of the MSC and not all studies were successful [51, 52].

As mentioned earlier, the WJ-MSC represent a bridge between the ESC and adult stem cells and posses various advantages over these two stem cell types. These advantages such as the lack of expression of HLA-DR (MHC class II) and relatively lower expression of HLA-ABC (MHC class I) compared with BM-MSC make the WJ-MSC more suitable in therapeutic settings. *Liu et al.* found recently that the MSC of the human umbilical cord could ameliorate EAE in mice by reducing axonal injury and demyelination due to suppression of perivascular infiltrates into the CNS [53].

4. Research goal

During this internship, the origin and characterization of the fetal WJ-MSC cells will be investigated. The expression of immunophenotypical, morphological, pluripotent and immunomodulatory markers will be assessed *in situ by* immunohistochemical staining, to investigate if the WJ-MSC posses their fetal stem cell characteristics *in situ* or acquire these following *in vitro* culturing. The pluripotent phenotype of WJ-MSC of different passages will also be investigated by looking at the gene expression of pluripotency markers such as Oct4, Sox2, Rex1 and Nanog. Furthermore the expression of different genes will be assessed between the WJ-MSC and BM-MSC by means of PCR. Both stem cell types, each from 5 different donors will be compared with each other. Some of these genes include mediators which could play an important role in assisting the stem cells to the injured area and exert their neuroprotective role in MS; anti-inflammatory mediators (e.g. LIF, TGF-β, IL-10), proteins with trophic properties (e.g. CNTF, GDNF, NT) and chemokines or chemokine receptors such as CXCR3, CXCR4, CCL2, CX3CL1. Others such as HLA-G, HLA-DR and IDO function as immunosuppressive mediators, which give these cells their low immunogenic phenotype.

Material and methods

1. Isolation and cell culturing of WJ-MSC

Umbilical cords were obtained from Ziekenhuis Oost-Limburg (ZOL) following caesarian section and informed consent of the mother. The cords arrived in the laboratory packed in sterile conical tubes containing 0,01M phosphate buffered saline (PBS; Lonza, Verviers, Belgium) supplemented with 1% Penicillin/ Streptomycin (P/S; Gibco, Paisley, UK) and 0,2% Amphotericin B (Fungizone®, Gibco). Typically, the cords were processed within 24-48 hours of birth according to the explant method. First, cords were extensively rinsed with PBS to remove as much blood as possible. Subsequently, the umbilical cord was placed in a 10 cm petri dish containing PBS supplemented with P/S and Fungizone®. Here, the tissue was cut into pieces of 1,5- 2 cm and both arteries and vein were removed to avoid endothelial cell contamination. Tissue was rinsed again and cut into small pieces (2-3 mm^2) in a sterile 10 cm petri dish. The small pieces were transferred into 6 well plates (Nunc, Roskilde, Denmark) containing KnockOut™ DMEM/F-12 medium without L-Glutamine of HEPES buffer (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 1% P/S and 1% L-Glutamine (Glutamax-1, Invitrogen) for expansion. Cultures were maintained in a humidified chamber at 37°C and 5% CO_2. The medium was changed every 3 days after the explants were attached to the bottom and cell outgrowth was observed using a Zeiss Primo Vert inverted microscope. When 80% confluence was achieved the cultures were harvested following an incubation period with Stempro® Accutase® (Gibco) at 37°C and 5% CO_2. The cell suspension was centrifugated at 1250 rpm for 5 minutes and the resulting pellet was resuspended in culture medium. Subsequently, the total cell number was determined by means of trypan blue exclusion using the Fuchs-Rosenthal counting chamber. Remaining cells were seeded for further expansion in 25cm^2 or 75cm^2 culture flasks (Nunc) or were frozen.

2. RNA isolation & cDNA synthesis

Total RNA was extracted using the High Pure RNA isolation kit (Roche applied Science; Mannheim, Germany) following manufacturer's instructions. Briefly, frozen cell pellets where thawed and resuspended with sterile PBS (Lonza). Cells were lysed using a lysis/ binding buffer, resulting in the release and adherence of nucleic acids onto the surface of the glass filter fleece. Hence, following different washing steps and incubation with Dnase I, genomic DNA and other contaminants where removed resulting in purified RNA samples. RNA samples were diluted in nuclease free water and total RNA concentration and purity were verified with the Nanodrop Spectrophotometer ND-1000 (Isogen life sciences; St-Pieters-Leeuw, Belgium). RNA was stored at -80°C for later use.

cDNA was synthesized using the Reverse Transcription System (Promega; Madison, USA) following manufacturer's instructions. A cDNA- synthesis mix (25mM $MgCl_2$, 10x Reverse Transcription buffer, 10mM dNTP mixture, 40U/µl Recombinant RNasin® Ribonuclease inhibitor, 23U/µl AMV Reverse Transcriptase, 0,5µg/µl $Oligo(dt)_{15}$ primer) was added to the RNA samples (1µg), followed by a polymerase chain reaction (60' 42°C, 5' 95°C, 5' 4°C, ∞ 4°C) in a DNA thermal cycler (Biorad). cDNA samples were diluted with nuclease free water and stored at -20°C until further use.

3. RT-Polymerase chain reaction

In a total volume of 20µl, each reaction contained 19µl reaction mixture consisting of 15,84µl autoclaved Milli Q water (Millipore), 2µl 10x PCR buffer + $MgCl_2$, 0,4µl forward and reverse primers (10µM), 0,2µl dNTP's (20mM), 0,16µl Taq polymerase (5U/µl) and 1µl of cDNA sample. As a negative control 1µl of autoclaved Milli Q water (Millipore) was added instead of the cDNA samples. After initial denaturation for 1 min at 95°C, PCR was carried out for 35/40 cycles (95°C for 30s, 60°C or other melting temperature for 30s, 72°C for 1 min) followed by 1 cyle (72°C for 10min) and $4°C$ for ∞. The PCR products were separated by electrophoresis with 2% agarose gel, stained and photographed under ultraviolet light.

Table 1: Primer sequences for pluripotency markers used in the analysis of different passages of Wharton's jelly mesenchymal stem cells.

Target	Forward primer (5' →3')	Reverse primer (5' →3')
Oct 4	AGCCCTCATTTCACCAGGCC	TGGGACTCCTCCGGGTTTTG
Sox 2	AGAAGCGGCCGTTCATCGAC	TGCTGATCATGTCCCGGAGGT
Rex 1	TGAAAGCCCACATCCTAACG	CAAGCTATCCTCCTGCTTTGG
Nanog	CAGAAGGCCTCAGCACCTAC	GAATTTGGCTGGAACTGCAT

Table 2: Primer sequences for comparative RT-PCR between WJ-MSC (n=5) and BM-MSC (n=5)

Target	Forward primer (5' →3')	Reverse primer (5' →3')
IL1b	GATGAAGTGCTCCTTCCAGG	GCATCTTCCTCAGCTTGTCC
IL6	GAGGAGACTTGCCTGGTGAA	GCTCTGGCTTGTTCCTCACT
IL4	GGACACTCGCTGCCTGGGTG	GGACAGGAATTCAAGCCCGCC
IL10 (1)	GGAGGAGGTGATGCCCCAAGC	TCGATGACAGCGCCGTAGCC
IFN-γ	TGCATCGTTTTGGGTTCTCTTGGC	TCCGCTACATCTGAATGACCTGCAT
TGF-B1	GGTTGAGCCGTGGAGGGGAAAT	TGCCATGAATGGTGGCCAGGT
HGF	GCCTCTGGTTCCCCTTCAATAGCA	ACTGTTCCCTTGTAGCTGCGTCC
LIF	GCGGCAGGAGTTGTGCC	CCCTGGGCTGTGTAATAGAG
CCL2	CTCAGCCAGATGCAATCAATGCCC	TTGGGACACTTGCTGCTGGTGAT
CCL5	CCTCCCCATATTCCTCGGACACC	ACGACTGCTGGGTTGGAGCA
CCL20	CACGGCAGCTGGCCAATGAAG	CTGTTTTGGATTTGCGCACACAGAC
CXCL2	CGCCCCTGGCCACTGAACTG	TGCCCATTCTTGAGTGTGGCTATGA
CXCL10	AGAACTGTACGCTGTACCTGCATCA	ACGTGGACAAAATTGGCTTGCAGGA
CXCL12	TCGTGCTGGTCCTCGTGCTG	GCTTGACGTTGGCTCTGGCAA
CXCL13	AGCCTCTCTCCAGTCCAAGGTGT	GGACAACCATTCCCACGGGGC
CX3CL1	ACAGCACCACGGTGTGACGA	CAAGATGATTGCGCGTTTGCCG
CCR1	GGGGATGCAACTCCGTGCCA	TTGCACAAGGACCAGGACCACC
CCR2	TCAACTGGACCAAGCCACGCA	TTGCAGAAGCGCTTGGTGATGTG
CCR3	ACGTGGGCCTGCTCTGTGAAA	GCCCAAGAGGCCCACAGTGA
CCR5	CCGCCCAGTGGGACTTTGGA	CAGCATGGACGACAGCCAGGT
CCR6	CCACCGGTGCGTGGGTTTTC	GTCAGGAGCAGCATCCCGCA
CCR7	GAGCGTCATGGACCTGGGGAAA	CCACTGTGGTGTTGTCTCCGATGT
CCR9	AGGTCACCCAGACCATCGCC	CCCGGCGGAATCTCTCACCC
CCR10	GCAGGTTTCCTGGGGCCATTACTC	ACGGTCAGGGAGACACTGGGT
CXCR2	ACCACCCAACCTTGAGGCACAG	CTGACTGGGTCGCTGGGCTTT
CXCR3	TCAACGCCACCCACTGCCAA	GACCAGCAGGGGCAGCAGAA
CXCR4	CTGGGCAGTTGATGCCGTGG	GCGTGGACGATGGCCAGGTA
CXCR5	CCCCGTGGCCATCACCATGT	TTCGTCAGGAGCCGCGACAG
CXCR6	GGCTGTGTTCCTGCTGACCCA	AAGGCAGGCCCTCAGGTATGC
CXCR7	CTGCCGGTCCTTCTACCCCG	CACTGGACGCCGAGATGGCT
CX3CR1	AGGCTGGCCCTCAGTGTGAC	ACTGAGCGCCCACACAGGAC
IL-17 receptor	ACCACAGGCGGTGGCGTTTTA	TTGGGCAGGTGGTGAACGGT
COX-1 (PTGS1)	CGCCCTCGGTAGAAGAGGCG	GAGCCCAGGAAGCAGCCCAA
COX-2 (PTGS2)	TGGCGCTCAGCCATACAGCA	CCTGTCCGGGTACAATCGCACT
mPGES1 (PTGES)	GGAAGAAGGCCTTTGCCAACCC	GTTCCACGTCGGGGTCGCT
mPGES2 (PTGES2)	CCGAGGCTCTGGCGTCCTTT	GGCGGTGCCTGCTCTTGAGT
cPGES (PTGES3)	GGCCGCGACCGGAGAGAAAA	GCTGCATTGTGAACGGGGCA
iNOS	TACTCCACCAACAATGGCAA	CGATGCACAGCTGAGTGAAT
IDO-1	AGACTGCTGGTGGAGGACAT	ACAAACTCACGGACTGAGGG
IFN-γ receptor	GCCGTCCTCAGTGCCTACACC	AGGGACCTGTGGCATGATCTGGT
PD-L1	ACTGTGAAAGTCAATGCCCC	TGCTTGTCCAGATGACTTCG
ICAM-1	AGCTTCGTGTCCTGTATGGC	ACAGTCACTGATTCCCCGAT

VCAM-1	AATGTTGCCCCCAGAGATACAACCG	GAGCTGCCTGCTCCACAGGA
HLA-ABC	TGGGAGCTGTCTTCCCAGCCC	CCACATCACGGCAGCGACCA
HLA-DR	AGACAAGTTCACCCCACCAG	AGCATCAAACTCCCAGTGCT
HLA-G (1)	GGCCCACGCACAGACTGACAGAAT	CTGGAGGGTGTGAGAACTGGCCTCG
HLA-E	TGAGGCGGAGCACCAGAGAGC	TTGGGGGCTCCAGGTGAAGCAG
CD40	TGCGACCCCAACCTAGGGCTT	AAAGCCGGGCGAGCATGAGC
CD80	CCTGCTCCCATCCTGGGCCATT	GGGCAAAGCAGTAGGTCAGGCAG
CD86	CGACGTTTCCATCAGCTTGTCTGT	GCCGCGTCTTGTCAGTTTCCA
CD31	ATTGCAGTGGTTATCATCGGAGTG	CTCGTTGTTGGAGTTCAGAAGTGG
CD44	AGCCTGGGGACTCTGCCTCG	GCGGCCTCCGTCCGAGAGAT
Adam 15	GCCCTGAGCTGGCGGGAATC	ACATCCCGCCTCCGGCGAAT
PSGL-1	TGCCTGATGGGGGTGAGGGG	TCACGGTCCTCCCTGGGCTC
CTLA-4	TGGATTTCAGCGGCACAAGGCT	CTGGGCCACGTGCATTGCTTTG
TSG-6	ATATGGCTTGAACGAGCAGC	GCAGCACAGACATGAAATCC
CD146	CTGCTGAGTGAACCACAGGA	CACCTGGCCTGTCTCTTCTC
SULF1	CCACGATCCTGGATATTGCT	TTCGAAACCTGTTACCTGGC
ALDH1A1	TGTTAGCTGATGCCGACTTGG	TTCTTAGCCCGCTCAACACT
TLR-4	CCAGGTGTGAAATCCAGACA	CACAGCCACCAGCTTCTGTA
LUM	CTTCAATCAGATAGCCAGACTGC	AGCCAGTTCGTTGTGAGATAAAC
TDO2	GGTTCCTCAGGCTATCACTACC	CAGTGTCGGGGAATCAGGT
IDO2	GCAGTGCCATTGTCTTTGGA	CTCAGCAGGGGCATCTTGTC
DSG2	GATGAGCCCAATACCCTGAA	CAACCATCCCTTCAAGCACT
DSC3	CCCTCGTGATCTTCAGTCGT	ACTTGACCGGATGAGGTCTG
NTF3	GAAACGCGATGTAAGGAAGC	TTTCTCGACAAGGCACACAC
NTF4	TGACAGGTGCTCCGAGAGAT	AGAGAAGGTCCCACTCAGGG
CNTF	CCTATGTGAAGCATCAGGGC	ATGGAAGTCACCTTCGGTTG
GDNF	GGTCTGGGCTATGAAACCAA	ACATCCACACCTTTTAGCGG
CD200	CACCGTCTATGTACAGCCCA	GGTAACAGACGTGGTCCCAT
CXCR3	GCTTTGACCGCTACCTGAAC	TGTGGGAAGTTGTATTGGCA
HO-1	AACATCCAGCTCTTTGAGGAGT	GAGTGTAAGGACCCATCGGA
CX3CR1	GACGGTTGCATTTAGCCATT	TGCTCAGAACACTTCCATGC
IL-10 (2)	AGAACCAAGACCCAGACATCAA	AATAAGGTTTCTCAAGGGGCT
Nanog	CAGAAGGCCTCAGCACCTAC	GAATTTGGCTGGAACTGCAT
GAPDH	GCTCTCCAGAACATCATCCCTGCC	CGTTGTCATACCAGGAAATGAGCTT
HLA-G (2)	CAAGGATGGTGGTCATGGCG	CAGCTGTTTCACATTGCAGCCTG
Oct3/4	AGCCCTCATTTCACCAGGCC	TGGGACTCCTCCGGGTTTTG
Sox2	AGAAGCGGCCGTTCATCGAC	TGCTGATCATGTCCCGGAGGT
Rex1	TGAAAGCCCACATCCTAACG	CAAGCTATCCTCCTGCTTTGG
VEGF	ATCTTCAAGCCATCCTGTGTGC	TCACCGCCTCGGCTTGTCACAT

4. Tissue processing

Fresh cut umbilical cord tissues from different donors were fixated for 24 hours in 4% paraformaldehyde (PFA; Merck Millipore, Darmstadt, Germany). After this period, 70% ethanol was added to optimize fixation. Next, the umbilical cords were embedded consecutively in a 5% and 30% sucrose solution overnight (cryoprotection) and subsequently embedded in paraffin wax after several dehydration steps. Paraffin embedded umbilical cord tissues were cut using a Leitz WETZLAR 1512 microtome (Leica) at a thickness of 5 micrometer (µm) by horizontal sectioning. Next, the paraffin slices were placed on a water surface on Superfrost® Plus microscope slides (Thermo Scientific Menzel-Gläser; Braunschweig, Germany) and put on a hotplate (45°C) to allow sections to flatten properly and to expand to their original dimensions. When sections were properly stretched, excessive water was removed and slides were placed in an oven at 37°C to dry at overnight.

5. Immunohistochemistry: *In situ* analysis

For phenotypical and structural analysis of the WJ-MSC, paraffin embedded umbilical cord tissues were stained for different markers.

Immunohistochemical stainings were performed using the Dako Envision®+ System-HRP (DAB) kit (Dako, Glostrup, Denmark). First, the paraffin embedded umbilical cord tissues were deparaffinized in Xylene and rehydrated trough alcohol baths with decreasing alcohol concentration (100%, 100%, 95%, 80%, 75%) followed by an incubation in distilled water and permeabilization with 0,05% Tween®20 (VWR® BDH Problabo®; Fontenay-sous-bois, France) or 0,01% Triton™ X-100 (Sigma-Aldrich; Saint Louis, USA). Next, depending on the used primary antibody, an antigen retrieval step (microwave method) was included. Prior to labeling with the primary antibody, endogenous peroxidase activity was blocked with Peroxidase block (Dako Envision®+ System-HRP (DAB) kit), sections were washed and non-specific binding sites were blocked with 10% normal goat serum (Millipore, Billerica, USA), normal donkey serum (Millipore) or normal rabbit serum (Millipore) depending on the secondary antibody. Subsequently, tissues were incubated with primary antibodies (Table 3) for 2 hours/overnight, washed and incubated with the horseradish peroxidase (HRP) conjugated secondary antibody. As secondary antibody, the HRP-conjugated goat anti-mouse, goat-anti rabbit antibody from the Dako Envision kit was used or the HRP-conjugated Rabbit anti-goat. Following different washing steps the 3,3'-diaminobenzidine (DAB) chromogen solution was added to the sections to visualize the bound antibodies. The Mayer's hematoxylin nuclear staining or the Nuclear Fast Red nuclear staining was used as a counterstaining. Umbilical cord tissues were dehydrated trough alcohol baths with increasing alcohol concentration (75%, 80%, 95%, 100%, 100%) and Xylene. Slices were mounted with DPX (Merck, Darmstadt, Germany) and covered with coverslips. Stained slides were examined with the Mirax Desk (Zeis) and Mirax viewer software.

Table 3: Overview of the primary antibodies used for IHC staining.

Primary antibody	Dilution	Species	Isotype	Company (clone)
α- SMA	1:100	Mouse monoclonal	IgG2a	Novocastra™ (αsm-1)
Oct 4	1:250	Rabbit polyclonal	IgG	Abcam
HLA-G	1:200	Mouse monoclonal	IgG1	Abcam(MEM-G/1)
B tubulin III	1:150	Mouse monoclonal	IgG2a	Sigma (2G10)
α- tubulin	1:500	Mouse monoclonal	IgG1	Abcam (DM1A)
CD 146	1:250	Rabbit monoclonal	IgG	Abcam (EPR3208)
ALDH1A1	1µg/ml	Rabbit polyclonal	IgG	Abcam
Collagen II	1:100	Mouse monoclonal	IgG2a	Abcam (5B2.5)
CD29	1:35	Mouse monoclonal	IgG1	Abcam (4B7R)
CD44	1:200	Mouse monoclonal	IgG2a	Abcam (F10-44-2)
CD14	5µg/ml	Goat polyconal	IgG	Novus biologicals

Results

1. Phenotypic and structural characteristics of Wharton's jelly mesenchymal stem cells *in situ*

Analysis of human umbilical cord tissue sections was done by immunohistochemistry.

The umbilical cord is composed of 2 arteries and 1 vein surrounded by the stromal WJ-MSC embedded in collagen fibers of the amorphous ground substance. The WJ can be subdivided into 3 regions from where the WJ-MSC can be isolated: the subamnion, the intervascular space and perivascular space (Figure 3A). The stromal cells showed both a round and a flattened stellate shaped appearance (Figure 3B-E). The cellular density in areas around the umbilical cord vessels (Figure 3B, E) was higher than in the peripheral and subamniotic regions which were characterized by more loosely arranged cells (Figure 3C,D).

A better view of the shape of the cells in the WJ is depicted in figure 4. The fibroblast–like cells were particularly stellate shaped with long cytoplasmatic processes. Their nuclei were oval or elongated; the visualization of the cell membrane however was difficult.

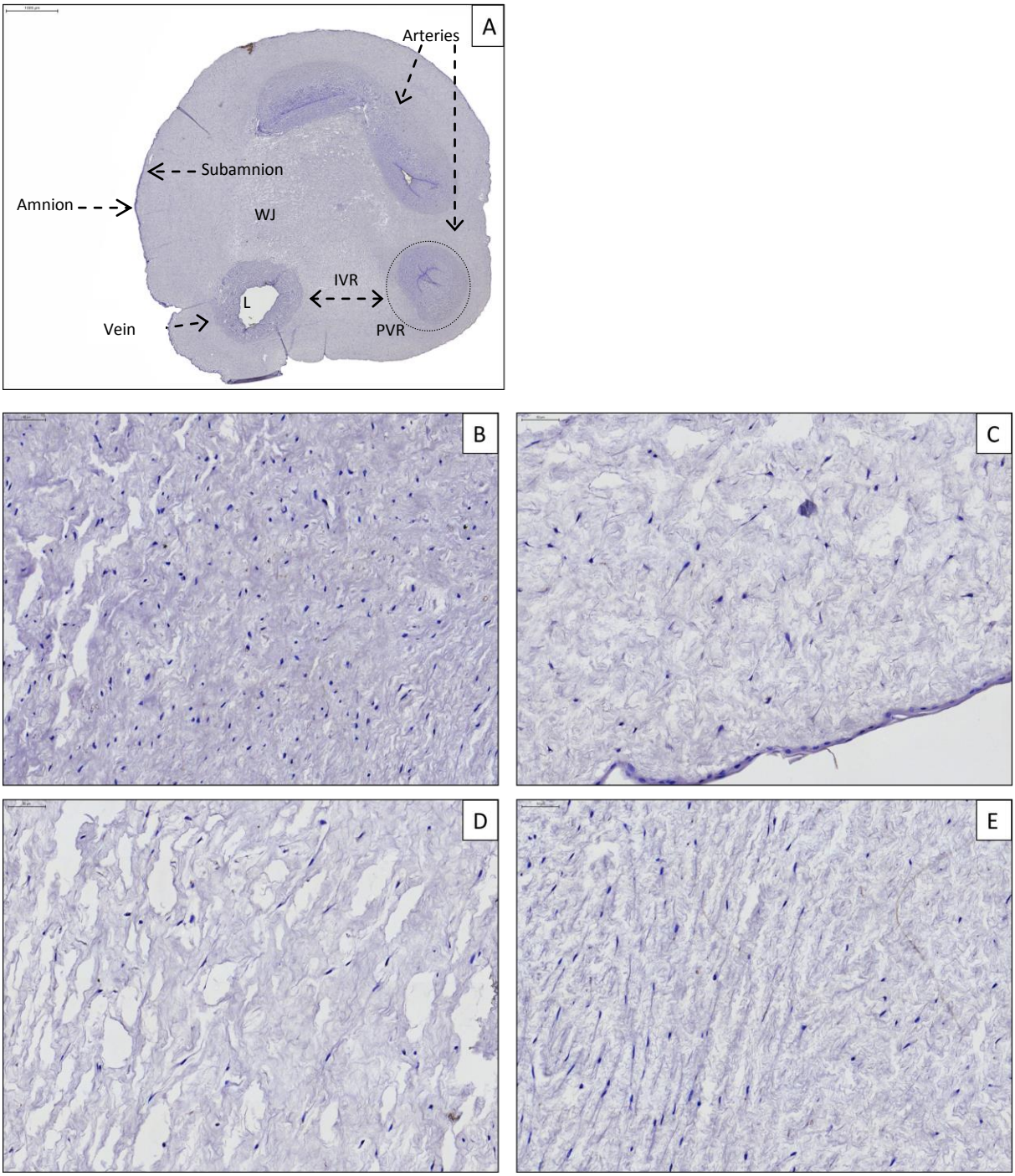

Figure 3: Light microscopic view of umbilical cord. (A) Whole umbilical cord tissue subdivided into the different regions with the presence of both arteries and vein. (B) Intervascular region (IVR) of the umbilical cord with tightly packed cells. (C) Less dense umbilical cord lining/subamnion. (D) Bulk Wharton's jelly (WJ). (E) Perivascular region (PVR). (A: magnification = fit, B-E: 40x magnification), (A: Scale bar = 1000µm, B-E: Scale bar = 50µm). L: Lumen; PVR: Perivascular region; IVR: Intervascular region; WJ: Wharton's jelly.

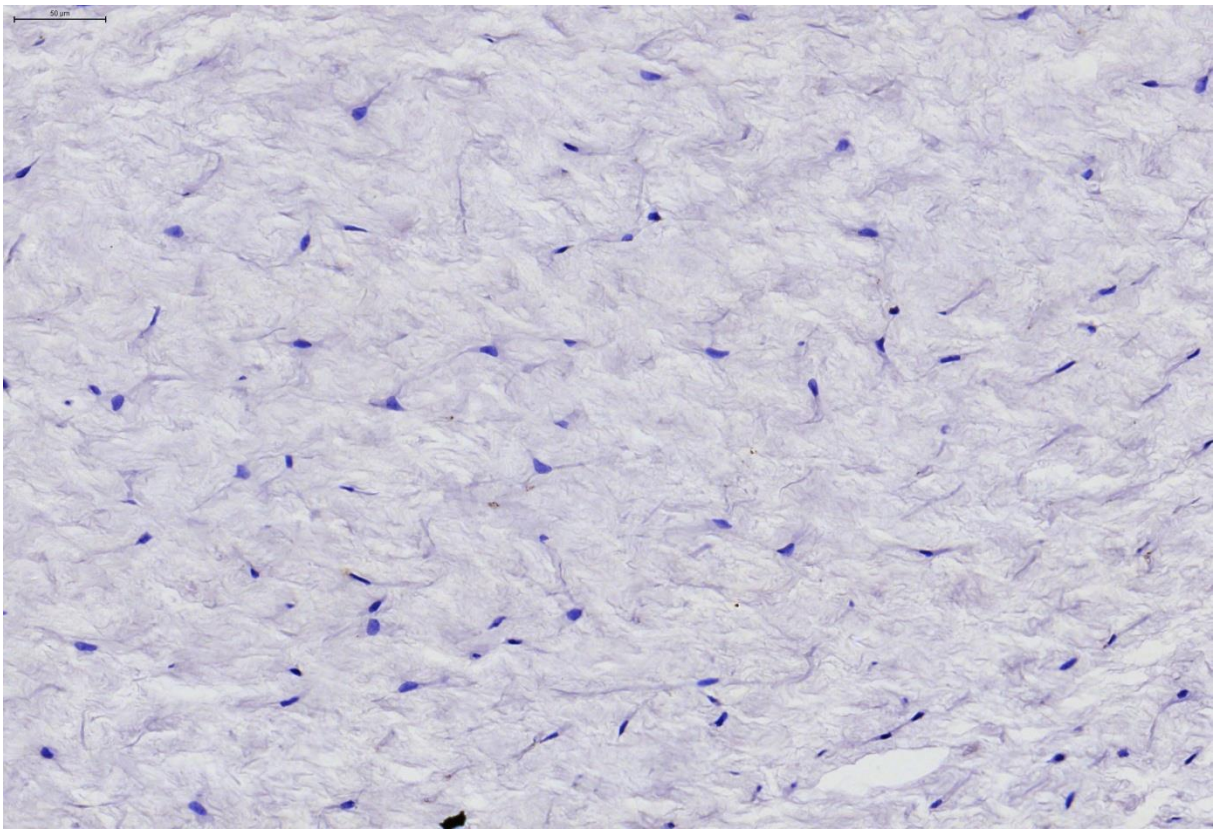

Figure 4: The Wharton's jelly stained with hematoxyline. The fibroblast like cells are embedded in a stroma consisting of collagen fibers and show a stellate shape with long cytoplasmatic processes. (40x magnification; Scale bar = 50µm).

Since the WJ-MSC originate from the umbilical cord stroma, it was of interest to investigate their phenotypic and structural characteristics in their natural environment. Immunohistochemical stainings on the umbilical cord were performed to investigate the phenotype of the WJ-MSC *in situ.* Different surface markers, known to be expressed in MSC were tested to explore the mesenchymal phenotype. Also the expression of structural markers and immunological markers was investigated.

Immunohistochemical staining revealed that predominant cells showed expression for α-SMA (Figure 5A) and α-Tubulin (Figure 5B). Staining for both markers showed clearly the stellate form of the cells with long cytoplasmatic processes. BIII Tubulin expression was observed in cells located in the subamnion (Figure 5C), however no expression was noted in cells of the WJ (Figure 5D). Collagen II expression was also not observed (Figure 5E). Some cells, but not all showed expression of the MSC markers CD 44 (Figure 6A), and demonstrated weak positivity for CD 146 (Figure 6B) and CD 29 (Figure 6C). A high expression of CD146 was also detected in the vessel walls (not shown). The WJ-MSC did not express the hematopoietic marker CD14 (Figure 6D).

In order to assess the pluripotent phenotype, expression of Oct4 was investigated. Although Oct4 positive reactivity was observed (Figure 7B), the negative control showed also positive staining

(Figure 7A). The non-classical MHC class I molecule, HLA-G was not expressed by the WJ-MSC (Figure 7D) compared with the positive expression in the placenta (Figure 7C) and also no immune reactivity to ALDH1A1 was observed (Figure 7E).

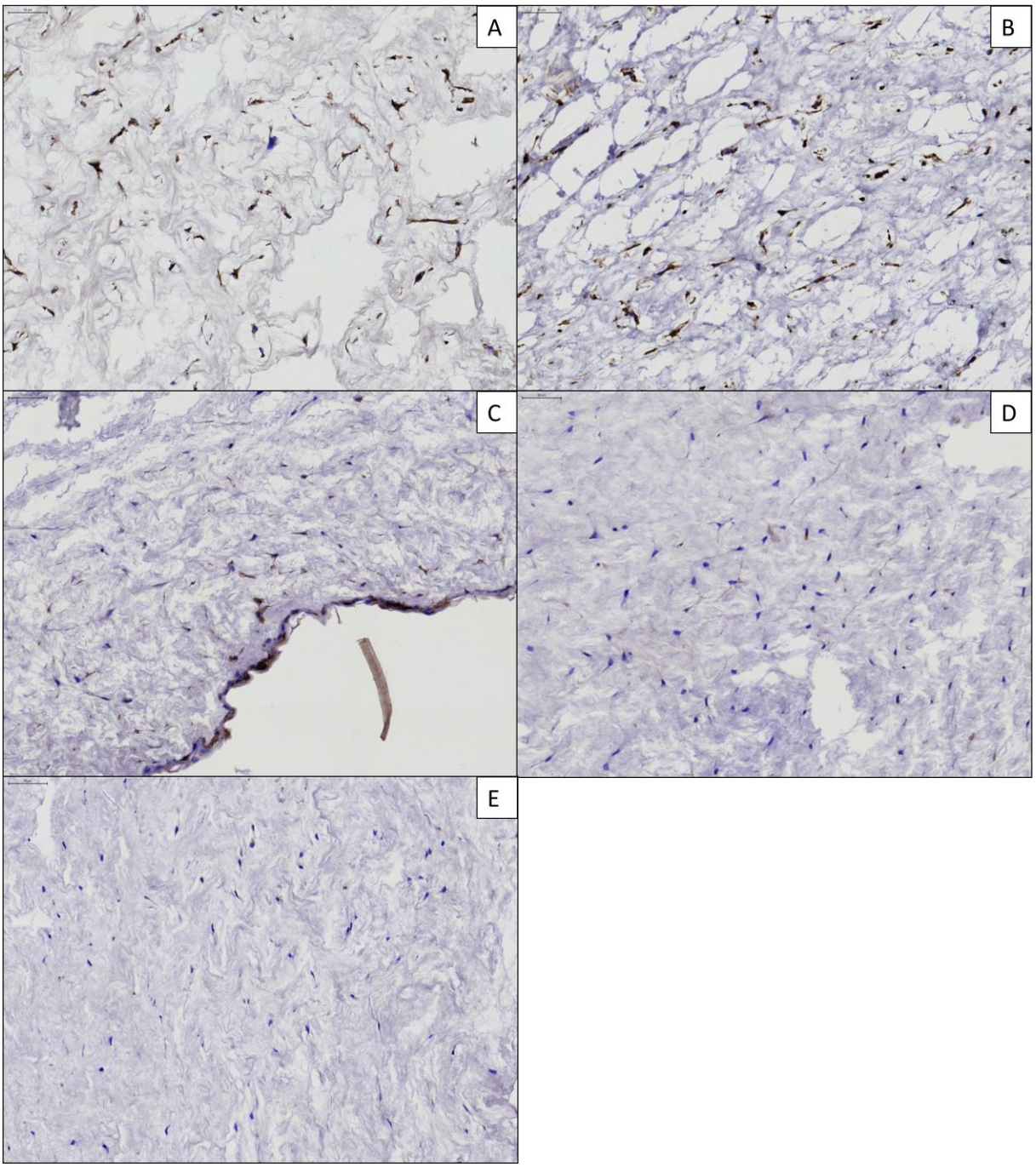

Figure 5: *In situ* expression of structural markers. All cells of the Wharton's jelly showed expression for α-SMA (A) and α- Tubulin (B). BIII Tubulin expression was present in the subamnion (C). No expression was observed in the bulk of the Wharton's jelly (D). Cells did not show expression for collagen II (E). (A-E: 40x magnification), (A-E: Scale bar = 50µm).

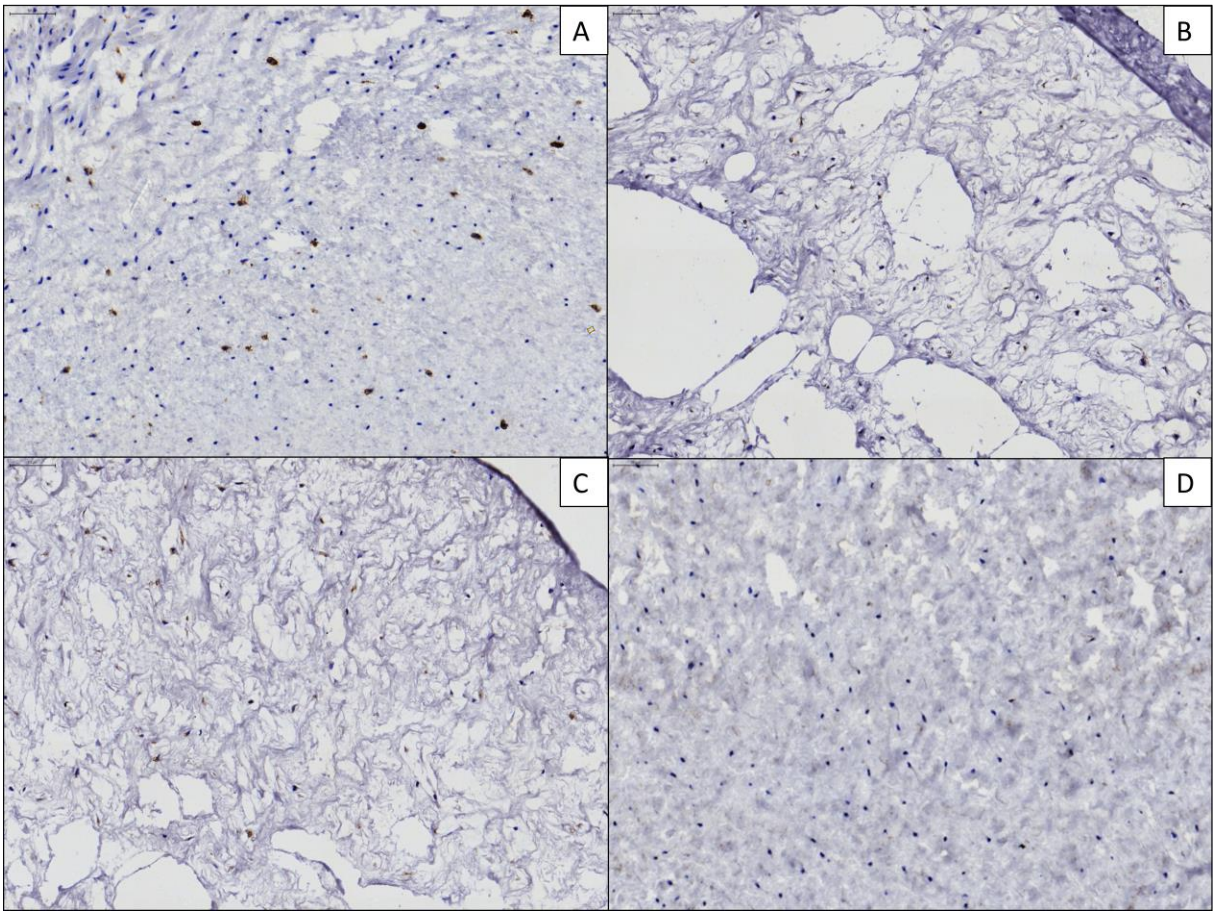

Figure 6: *In situ* expression of mesenchymal markers. A low level of expression was observed for the MSC markers CD44 (A), CD146 (B) and CD29 (C). Stem cells did not stain for the hematopoietic marker CD14 (D). (A-D: 40x magnification), (A-D: Scale bar = 50µm).

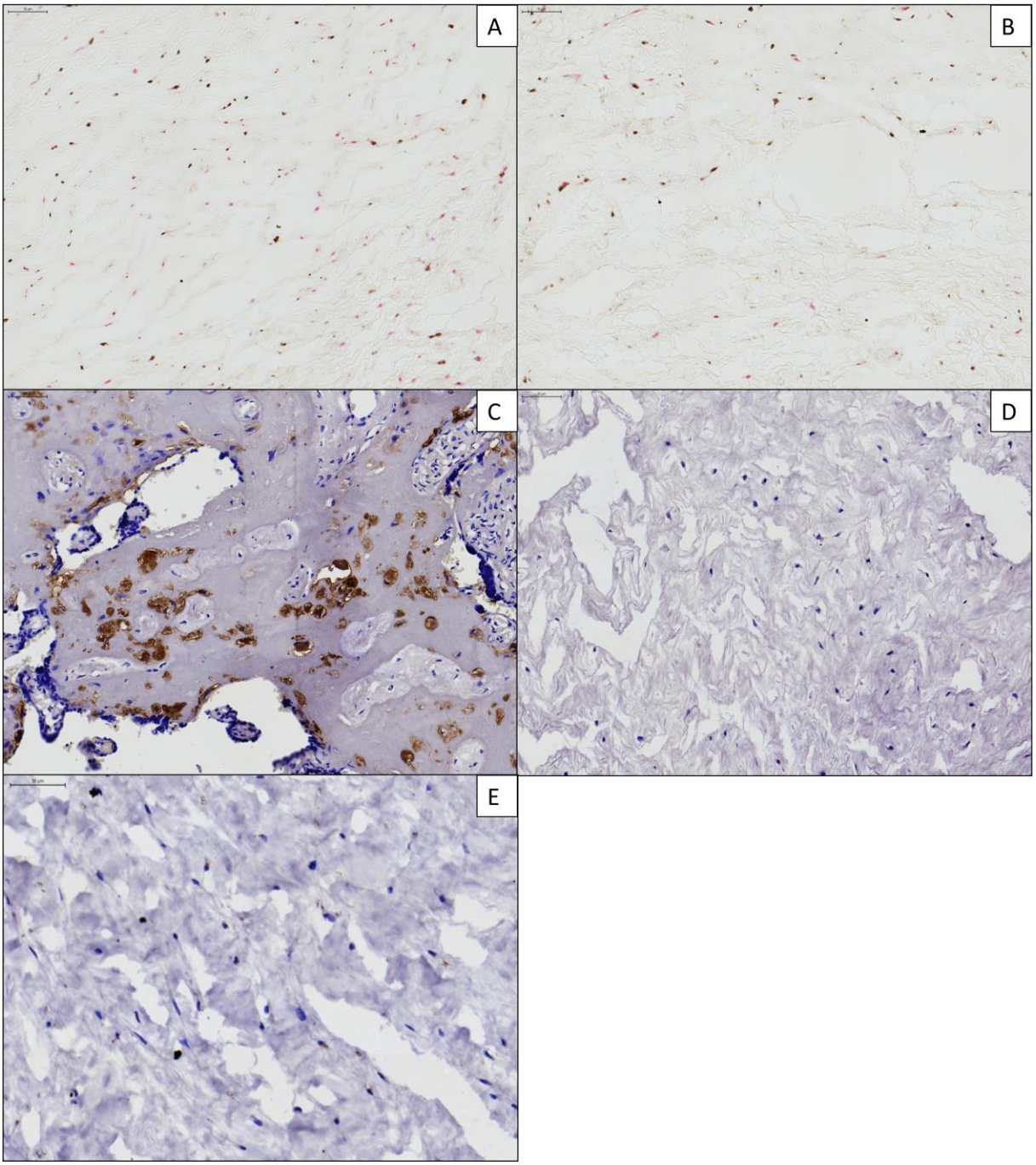

Figure 7: *In situ* examination of the pluripotent and immunosuppressive character of the Wharton's jelly mesenchymal stem cells. Although Oct4 expression seemed to be expressed by the cells in the Wharton's jelly (B), staining was also visible in the negative control (A). HLA-G expression in placenta tissue served as a positive control (C). Wharton's jelly mesenchymal stem cells did not show any staining for HLA-G (D). No expression of ALDH1A1 was observed in the Wharton's jelly (E). (A-E: 40x magnification), (A-E: Scale bar = 50µm). A&B: Nuclear fast red counterstaining.

2. PCR analysis of pluripotency markers trough different passages of Wharton's jelly mesenchymal stem cells.

In order to investigate at what stage the cultured WJ-MSC obtain their pluripotent character, the genetic expression of a panel of pluripotency markers such as Oct4, Sox2, Rex1 and Nanog, which are known to be expressed by ESC was assessed in WJ-MSC of different passages. The WJ-MSC were isolated and cultured from the umbilical cord using the explant method. After the explants had attached to the bottom of the culture plate and cells started to grow out, cells were harvested and cultured trough different passages. From these different passages, RNA was extracted and used for PCR.

The gene expression profiles of the different pluripotency markers are depicted in figure 8. The PCR analysis of low-passage and high-passage WJ-MSC showed that mRNA transcripts corresponding to the pluripotency markers were present in particularly all passages. Oct4 and Nanog were expressed in all passages of both low-passage as well as high-passage cells. Sox2 expression was also observed in all passages, except no expression was noted in passage 10 (P10). Compared with the other markers, Rex1 expression was only seen in 5 passages (P2, P3, P4, P7 and P8). Although Rex1 expression was also observed in the high-passage cells (P7 and P8), its expression was more prominent at low-passages (P2, P3 and P4). The housekeeper gene, GAPDH served as a loading control and the transcript of the pluripotent human testicular embryonic carcinoma celline (Ntera) served as a positive control (P).

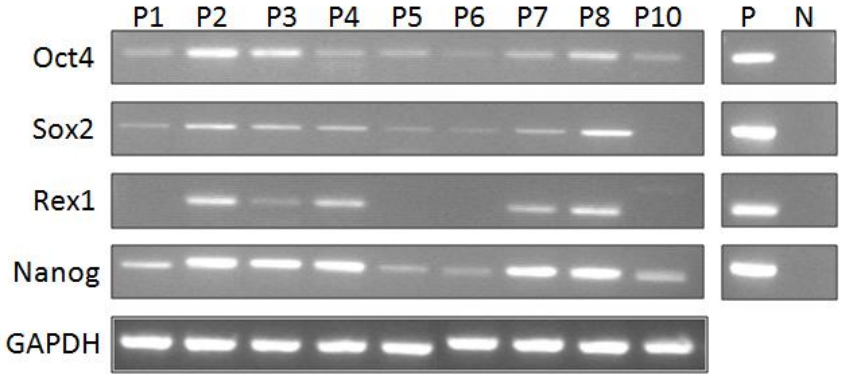

Figure 8: Reverse Transcriptase polymerase chain reaction gene expression of pluripotency markers in low-passage and high-passage Wharton's jelly mesenchymal stem cells. PCR analysis of low-passage and high-passage WJ-MSCshowed the presence of mRNA transcripts for the pluripotency markers Oct4 (row 1), Sox2 (row 2), Rex1 (row 3), Nanog (row 4). While Oct4 and Nanog were expressed in all passages, Sox2 did not show any expression in P10. Rex1 expression was observed in low-passage cells (P2, P3 and P4) showed no expression in P5 and P6 but was again present in P7 and P8. In P10 there was also no Rex1 expression just as Sox2. GAPDH (row 5) represents the loading control and Ntera was chosen as a positive control (P). The negative control, without DNA (N) showed no expression of the tested markers. (P1- P10: Passage 1- Passage 10), (P: Positive control, N: Negative control).

3. Comparison of genetic expression between WJ-MSC and BM-MSC

In order to gain more knowledge about the genetic expression profile of the WJ-MSC and the differences with the adult BM-MSC, reverse transcriptase PCR analysis was performed using RNA from 5 different donors of both stem cell types to check the expression of various genes. The differential expressed genes between the WJ-MSC (n=5) and bone-marrow mesenchymal stem cells (n=5) are shown in the figure below (Figure 9). The expression outputs of all the tested genes are given in the supplemental Figure (S1).

The analysis showed differences in the expression of genes involved in inflammatory processes, cell adhesion, chemotaxis, neuroprotection, cell trafficking, tissue regeneration, angiogenesis, pluripotency, immune regulation and modulation. IL1-B, CXCL10, ALDH1A1, DSG2 and DSC3 expression was shown in the WJ-MSC but not in the BM-MSC (Figure 9). On the contrary; CXCR5, CX3CR1, HLA-DR, CD31, Adam 15, PSGL-1, CTLA-4, TLR-4, CNTF, Oct3/4 and Rex1 gene expression was only observed in BM-MSC samples (Figure 9). Chemokines or chemokine receptors such as CCL5, CCR10, CXCR6, COX-1 and the expression of VEGF were seen in both WJ-MSC and BM-MSC. However, expression of some of these genes was hardly detectable. For instance: CCR10 (BM 1/2/4/5), CXCR6 (BM 1/2/5) and COX-1 (BM 1/2/4/5) expression showed light bands on the agarose gel of the BM-MSC samples. These genes showed also a very low level expression in the WJ-MSC samples, however expression was limited to 1 or 2 donors (Figure 9).

The other genes also involved in processes such as cell migration, chemotaxis, cell adhesion, co-stimulatory signaling, pro/anti-inflammatory processes, neuroprotection, immune activation or immune suppression which were or were not expressed in both stem cell types are listed in Figure S1.

The pro-inflammatory cytokine IL-6, genes encoding for MHC class I molecules such as HLA-ABC, HLA-G, HLA-E, chemokines such as CCL2, CCL20, CXCL2, CXCL12, cell adhesion molecules such as CD146, ICAM-1, VCAM-1, CD44 and neurotrophic factors such as NTF3, GDNF were all expressed in both the WJ-MSC as well as the BM-MSC (Figure S1). Others such as IL-10, iNOS, Nanog, IDO-1, CD86, TDO-2, IDO-2, and a panel of different chemokines and chemokine receptors (e.g. CCR1, CCR2, CCR3, CCR6, CCR7, CX3CL1,CXCL13, CXCR2, CXCR3, CXCR4) did not show any expression in the WJ-MSC nor in the BM-MSC (Figure S1).

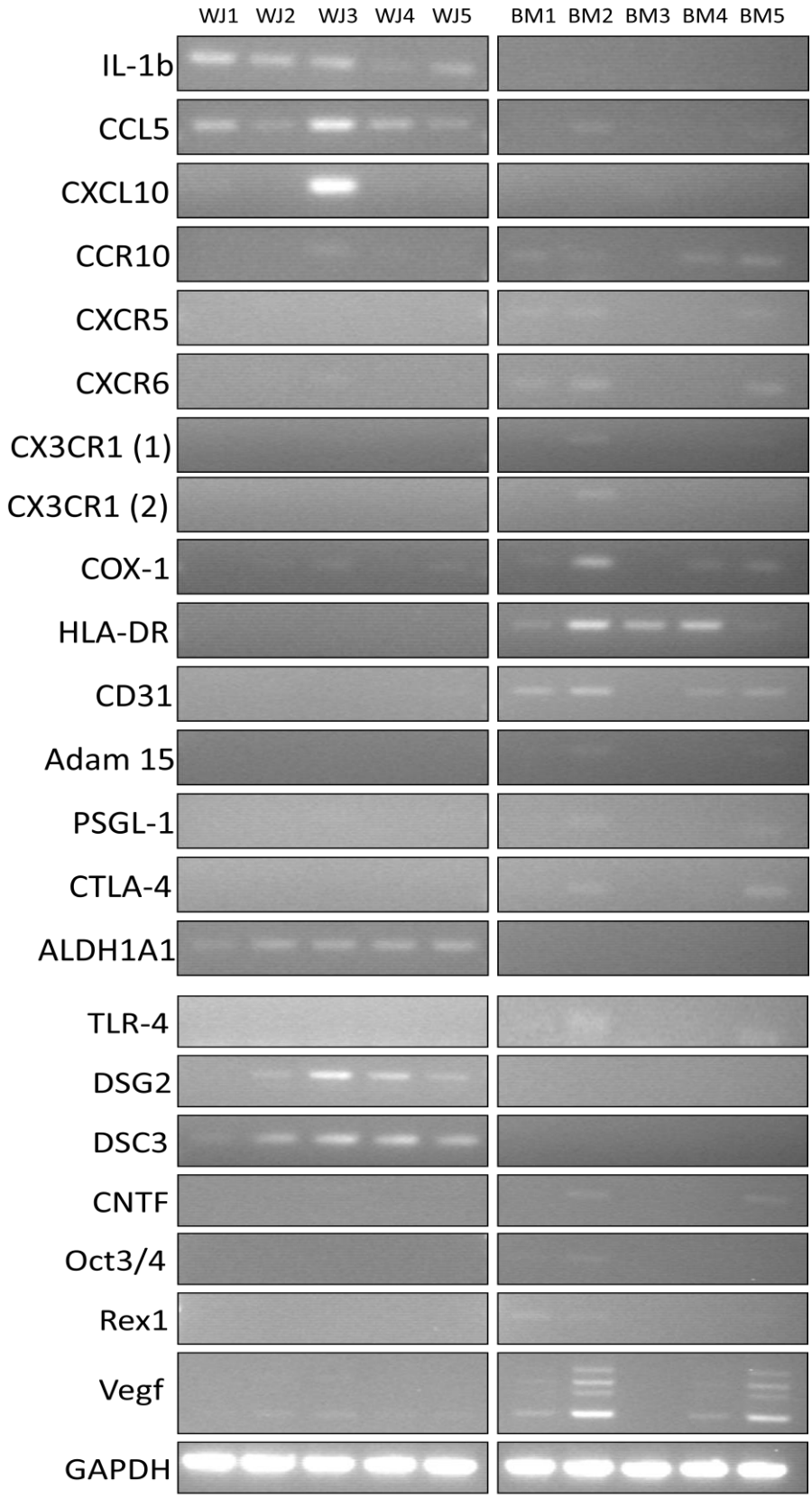

Figure 9: Gene expression profile of differentially expressed genes between Wharton's jelly mesenchymal stem cells and bone-marrow mesenchymal stem cells. Different donors of Wharton's jelly mesenchymal stem cells (WJ-MSC, n=5) and bone-marrow mesenchymal stem cells (BM-MSC, n=5) were used. GAPDH served as a positive loading control.

Discussion

As a stem cell source, a lot of research is done on WJ-MSC to reveal their therapeutic potential in cell based therapies. Their fetal phenotype, which is developmentally and operationally positioned in between the pluripotent ESC and the multipotent adult stem cells, enables them to inherit characteristics of both stem cell types. Due to the fact the cord tissue, where these cells are embedded in, is considered as mesenchymal or embryonic tissue [54], it could be postulated that these cells obtain stem cell characteristics to a certain extent.

Since no specific marker exists to identify MSC, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has set up minimal criteria to define human MSC [32, 55]. First of all, cells should possess a plastic adherence capacity in culture. Second, cells should express a panel of markers related to MSC (e.g. CD105,CD73, CD90, CD44) and lack the expression of CD45, CD34, CD 14, HLA-DR. Third, cells must show a multipotent differentiation capacity by differentiating into osteoblasts, chondroblasts and adipocytes *in vitro* conditions [32].

Various studies have reported the expression of these mesenchymal markers in cultured umbilical cord stem cells of the Wharton's jelly and have shown the above mentioned differentiation capacity, hereby proving their partially mesenchymal character [54, 55]. Others have assessed their pluripotent phenotype by investigating expression of several pluripotent markers and ESC markers such as Oct4, Nanog, SSEA-1 and SSEA-4 [33, 56]. *Schugar et al. and Margosian et al.* have analysed the mesenchymal phenotype of these WJ-MSC*in situ* [57, 58].

The first aim of this study was to characterize the WJ-MSC in their original environment and to investigate, if these cells obtain their stem cell properties *in situ* or acquire these following *in vitro* culturing. Cells located in the Wharton's jelly showed positive staining for CD44, CD146 and CD29 and lacked the expression for CD14, a marker expressed on monocytes and macrophages. These results confirmed previous findings given by *Schugar et al.* [57]. However, in this study, the 3 regions (intervascular region, perivascular region and subamnion/umbilical cord lining) which are part of the Wharton's jelly were analysed as one whole Wharton's jelly, while *Schugar et al.* and *Margossian et al.* analysed the perivascular region and Wharton's jelly separately. Schugar et al. showed that CD 146 for instance was expressed in the perivascular region and vessels, but not in the bulk Wharton's jelly. Although *Margossian et al.* provided support for the expression of CD 146 in the perivascular region they also observed weak expression of CD 146 in the WJ. CD 146 identified as an adhesion marker of endothelial cells, is also described as a MSC marker [59]. Our result regarding the CD44 staining, which showed weak expression in the WJ was also confirmed by *Schugar et al.* As last, the expression of the CD 29 marker, an integrin β-1 which is involved in cell adhesion supports the view

that the cells which reside in the WJ obtain the potential to migrate towards and home into damaged tissue.

Secondly, in order to investigate their pluripotent and low immunogenic phenotype *in situ*, immune reactivity respectively to Oct4 and HLA-G markers were tested. Results showed that the negative control (incubation with only the secondary antibody) illustrated positive staining for Oct4 expression. No conclusive conclusions can be made based on these current results regarding the pluripotent phenotype of WJ-MSC *in situ.* Further optimization of the staining protocol is necessary. In case of HLA-G expression, no immune reactivity to HLA-G was observed in umbilical cord tissue. As a positive control, placenta tissue which is known to express HLA-G [60], was included in the staining procedure. The trophoblast cells of the placenta clearly showed expression of HLG-G compared with cells in the WJ. These results could indicate that WJ-MSC acquire HLA-G expression and (as a consequence) a part of their low immunogenic character following *in vitro* culturing of these cells. The expression of ALDH1A1 (Aldehyde dehydrogenase 1 family, member A1), an enzyme functioning in retinoic acid production was also not detected in WJ-MSC residing in the umbilical cord. ALDH1A1 is also known to be highly expressed in stem cells and suggested to regulate stem cell function [61]. Various reports have described ALDH1A1 to be enriched in HSC where they regulate differentiation of these HSC [62, 63]. *Klingeman et al.* reported an increase of ALDH1A1 gene expression in cultured WJ-MSC compared with BM-MSC [62]. In accordance with this, a possible explanation for the lack of expression in umbilical cord tissue may be that the cells which reside in the cord stroma are more primitive and are in an undifferentiated state, while during *in vitro* culturing they show more potential to differentiate.

Staining for structural markers such as α-SMA, gave support to findings of previous studies [54, 64]. α-SMA showed positive expression in the vessel walls and cells of the WJ. This protein is a typical marker of smooth muscle cells, which is also known to be expressed in pericytes and myofibroblasts [65-67]. The presence of α-SMA in the cells of the WJ supports the view of their role in the contraction of the umbilical cord [66, 68]. In order to reveal the origin of umbilical cord stromal stem cells (UCSSCs), Farias and coworkers have performed a study to assess if UCSSCs were derived from the WJ and not from the vascular smooth muscle wall of the blood vessels [67]. Immunohistochemistry staining on umbilical cord tissue and cultured UCSSCs revealed that cells of the WJ and vascular smooth muscles in the umbilical cord tissue and cultured UCSSCs all expressed α-SMA. However, expression of CD10, a biomarker of B-cell lymphomas and also been reported in human fibroblasts [69] , placental and bone-marrow MSC [70] was only detected in the cultured UCSSC and cells of the WJ. No CD10 expression was observed in smooth muscle cells of the vascular

wall [67], indicating that the UCSSCs are derived from the WJ and not from the smooth muscle cells of the vascular wall.

Furthermore, although our results showed positive WJ expression of α- Tubulin, which are part of the microtubules together with β- Tubulin, surprisingly, βIII Tubulin expression was only slightly observed in some cells of the subamnion but not in the bulk of the Wharton's jelly. The stellate shape of the cells and their long cytosolic processes *in situ* were clearly visible following staining with these structural markers which confirms findings of other research groups concerning their morphological features [64, 68, 71]. It is notable that no expression for collagen II was observed, while the WJ tissue is mostly composed of collagen fibers. One possible explanation would be that the staining was not performed well. However, *Mitchell et al.* stated that the amount of collagen in the Wharton's jelly is low, which indicates the primitive state of the tissue [71].

After the initial *in situ* characterization of the WJ-MSC, their pluripotent character was assessed by means of PCR. Low-passage and high-passage WJ-MSC showed expression of different pluripotency markers such as Oct4, Sox2, Rex1 and Nanog. These markers are known to be expressed by ESC [72]. Both Oct4 and Nanog were expressed in all passages, while Sox2 lacked expression in P10. Rex1 expression was not stable compared with the other 3 genes. Its expression was shown in P2, P3, P4, P7, P8 cells but was lost in P1, P5, P6, P10. These findings support the fact that WJ-MSC posses a stable pluripotent phenotype even after several passages. The expression of Oct4 in all passages supports the fact that these cells should also obtain their pluripotent character *in situ,* which was investigated as mentioned earlier, but no conclusive results were obtained in the immuno-histochemical analysis due to positive staining of the negative control. It should be noted that repetition of this experiment with different donors is required to be able to make conclusions about differences in gene expression profiles of these pluripotency markers.

In the next step, the genetic expression profile of WJ-MSC was investigated and compared with the expression in BM-MSC. BM-MSC have been described extensively in regenerative medicine and are seen as the golden standard in cell based therapies. In addition, due to the partial resemblance with adult MSC regarding their differentiation capacity, phenotype, homing capacity and immune regulation, bone-marrow stem cells were used for this comparative study.

The expression of various genes related with different processes such as, cell migration, chemotaxis, cell adhesion, co-stimulatory signaling, pro/anti-inflammatory processes, neuroprotection, immune activation or immune suppression were assessed. Despite many resemblances between both stem cell types, they also showed differences in their gene expression profile. The WJ-MSC, but not the BM-MSC showed expression of the inflammatory cytokine IL-1B, the chemokine CXCL10, ALDH1A1

which is involved in retinoic acid production, DSG 2 and DSC3, respectively Desmoglein 2 and Desmocollin 3 which are involved in cell adhesion. On the contrary; Adam15, known to be involved in cell adhesion and a mediator of inflammation; PSGL-1, a chemotactic molecule; CTLA-4, known as an inhibitor of T cells; CD31, involved in cell adhesion and angiogenesis; HLA-DR, a MHC class II surface molecule which activates the immune system; TLR-4, important in the activation of the innate immune system; the chemokine receptors CX3CR1 and CXCR5; the neurotrophic growthfactor CNTF involved in neuron survival; the pluripotency markers, Oct4 and Rex1 were all only expressed in the BM-MSC samples. The chemokine receptors CCR10 and CXCR6; Cox-1, a regulator of angiogenesis and involved in cell signaling, and VEGF which is also involved in angiogenesis and vasculogenesis showed also expression in different samples of the BM-MSC but were only very slightly expressed in WJ-MSC. Analysis of a microarray experiment between umbilical cord mesenchymal stem cells (UC-MSC) and BM-MSC by K*lingeman et al.* supported some of our findings. DSC3, ALDH1A1, DSG2, IL-1B were all upregulated in UC-MSC as was the case in our study. Interestingly, VEGF which was also shown by Klingeman and coworkers to be upregulated in UC-MSC showed barely any expression in our WJ-MSC compared with the BM-MSC [62]. *Yoo et al.* however supported our finding regarding VEGF expression in BM-MSC [73]. Another remarkable finding was the observation of Oct4 (1 sample) and Rex1 (3 samples) expression (very light bands) in BM-MSC, while no expression was noted in WJ-MSC which are known to express these pluripotency markers [26, 33] (also shown and discussed in our previous experiment). The lack of HLA-DR expression in WJ-MSC also supported by others [26] suggests a low immunogenic character compared with BM-MSC.

Various other markers also involved in above mentioned processes showed similar expression patterns in both stem cell types. Chemokines and chemokine receptors such as CCR1, CCR2, CCR3, CCR6, CCR7, CX3CL1,CXCL13, CXCR2, CXCR3, CXCR4; the anti-inflammatory cytokine IL-10; the immune modulatory IDO-1, IDO-2 and TDO-2 showed no expression in any sample of both the WJ-MSC and BM-MSC. The same was true for iNOS, Nanog and CD86. Surprisingly Nanog expression was not shown in WJ-MSC, which was contradictory with the results obtained in the previous experiment, where was shown that Nanog expression was stable through different passages. A possible explanation for this contradiction could be the genetic variations in tissue samples of different donors. Repetition of this experiment will however give more clarity and rule out possible mistakes made during the PCR preparation process.

The expression of other genes such as the pro-inflammatory cytokine IL-6 and other pro-inflammatory mediators (e.g. cPGES, mPGES1, mPGES2, COX-1, COX-2) ; a panel of different chemokines, chemokine receptors and cytokine receptors (e.g. CCL2, CCL20, CXCL2, CXCL12, IL-17R, IFN-γ R); various cell adhesion molecules (e.g. CD146, CD44, VCAM-1, ICAM-1); growthfactors such as

GDNF, NTF3 and HGF; MHC class I molecules which play a role in immune suppression such as HLA-G and HLA-E or in immune activation such as HLA-ABC; immune suppressive mediators (CD200 and PD-L1; mediators involved in cell migration, differentiation, co stimulation (e.g. TSG-6, LUM, SULF1, LIF, CD40 and CD80) were observed in both stem cell types.

Results of this comparative study also showed that not all samples of a particular stem cell type did or did not express a gene. This indicates that genetic variation between donors and different passages of cells also determines the genetic profile and hence the usability of these stem cells in cell based therapy. To asses accurate quantification of gene expression between both stem cell types, quantitative RT-PCR should be performed which will give a better view of the up and down regulated genes. The simultaneous expression of genes promoting inflammation and immune activation together with expression of genes involved in cell migration, survival, growth and immune suppression suggests the dual character of theWJ-MSC. The expression of transctipts for chemokine receptors together with the expression of genes involved in cell adhesion and migration imply that WJ-MSC show the potential to become activated by mediators (e.g. chemokines) and migrate to and home into the site of inflammation. Expression of HLA-G, HLA-E, PD-L1, CD200 and the lack of HLA-DR expression provides support for their low immunogenic phenotype which makes these cells suitable for transplantation purposes. In addition, expression of VEGF, NTF3, GDNF and LUM suggests that following transplantation and migration into the damaged tissue, these cells could contribute to repair by promoting cell survival. It should be noted that repeating this experiment is necessary for the assuring reliability of the obtained results.

Conclusion

To conclude, the results obtained during the present study indicate that WJ-MSC in their natural environment *(in situ)*, share most of the characteristics with the adult bone-marrow mesenchymal stem cell*s,* including morphology and immunophenotype. Their pluripotent phenotype however was not assessed. This does not mean that these cells do not possess the pluripotent phenotype, because gene expression analysis of different pluripotency markers has shown that both low-passage as well as high-passage WJ-MSC show a stable expression of these markers. The same was true for HLA-G, a non classical MHC I molecule involved in immune suppression, which was not observed *in situ*, although PCR analysis showed upregulated expression of HLA-G in cultured WJ-MSC of different passages. However, upregulation in genetic expression will not necessarily mean that expression will be observed on protein level. Though, these results indicate that expression is possible and suggest that further research and repetition of these experiments is necessary to be able to make definite conclusions.

The results of the genetic expression profile between the WJ-MSC en BM-MSC illustrates that although similarities between the two stem cell types exist, they also show differences. The expression of HLA-G and lack of HLA-DR expression implies the low immunogenic character of these WJ-MSC which implies that following allogeneic transplantation, these cells could avoid the immune system. The expression of several chemokine receptors and other mediators involved in migration and adhesion support for the view that these cells can migrate to injured tissues and home there to exert their effects. As last the expression of several growth factors suggests that the WJ-MSC not only have an immunomodulatory capacity but they also exert a neuroprotective and growth stimulating function.

All together with previous findings, these results suggest that WJ-MSC are a good stem cell source with a stable phenotype that have the potential to be used in cell based therapies. However further research is required of not only their characterization in order to reveal how these stem cells now could exert their beneficial effect, or of the best culture conditions to obtain the best population useful for future cell therapies but also possible cell manipulations which need to be optimized before administration to ensure a positive effect.

In further research, the genetic expression profile of the tested genes but also other markers could be compared with protein based assays, to investigate if the findings on genetic level are also expressed on a protein level. In addition, growth factors that are shown to be expressed by these WJ-MSC could be used *in vitro* assays to look at their effect on damaged neurons and oligodendrocytes.

Subsequently, these growth factors which show a positive effect could be administered *in vivo to* investigate whether they exer*t* similar effects *in vivo.* What also would be interesting is to investigate the expression of the pluripotency markers on a larger scale of different passages to assess if the cells lose their pluripotent character or stay stable. Based on this, cells of the most optimal passages then can be used for therapeutic purposes. Moreover, it could be considered to investigate if it is possible to manipulate these WJ-MSC in order to skew them towards a more favorable phenotype for transplantation purposes such as a lower expression of pro-inflammatory markers or a higher expression of growth factors, cell adhesion molecules and immune suppressive mediators.

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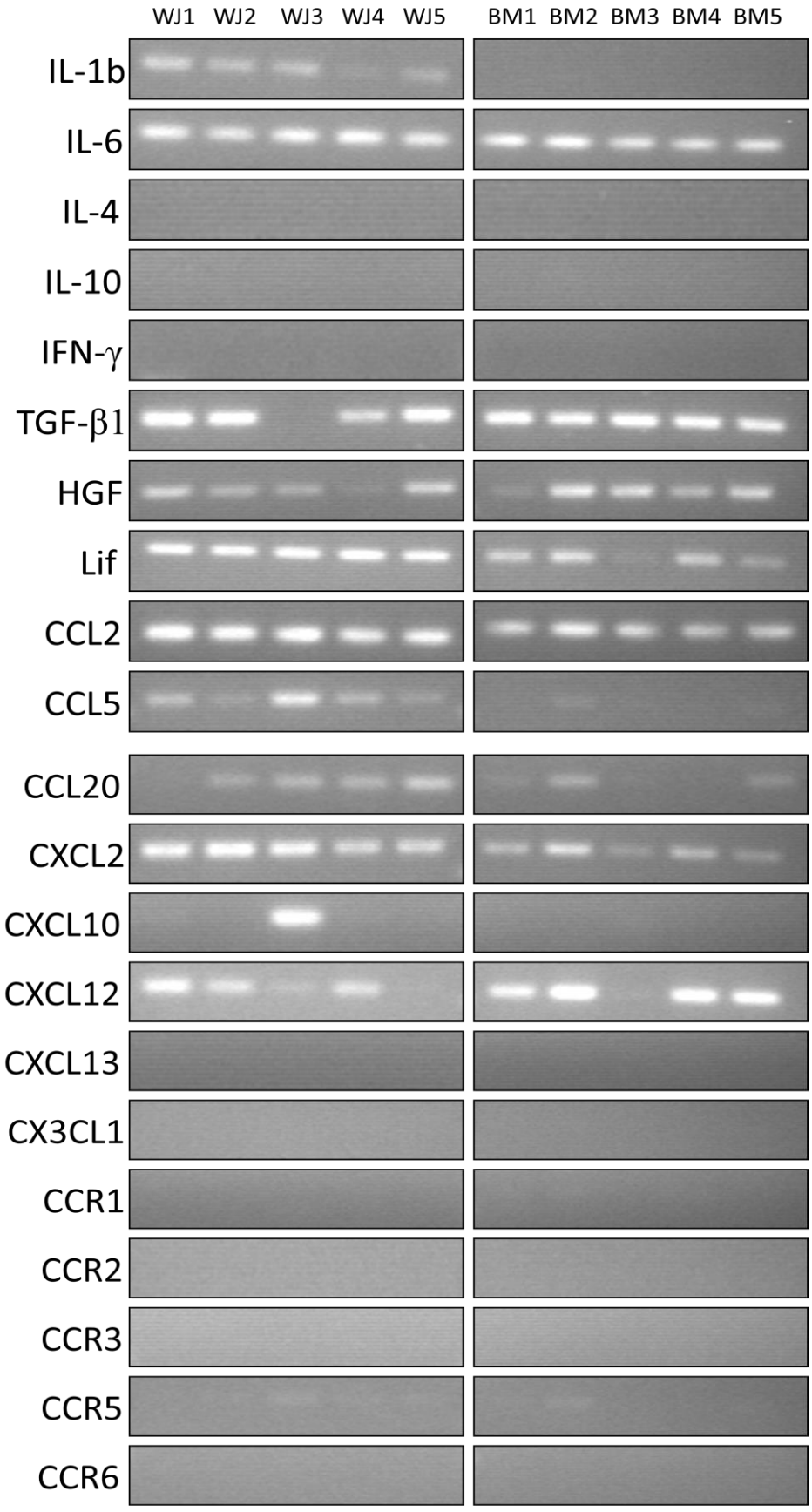
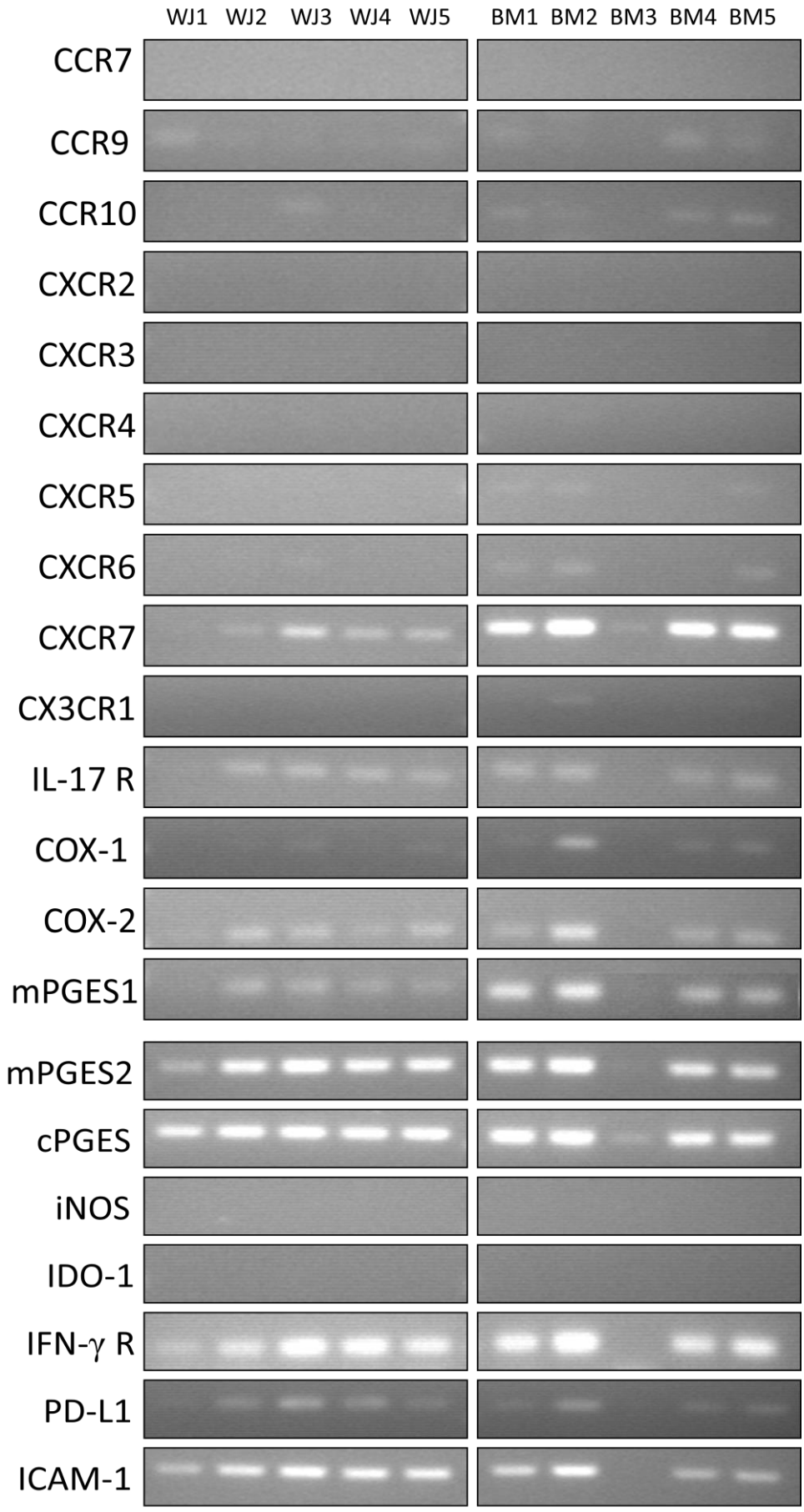
Supplemental information

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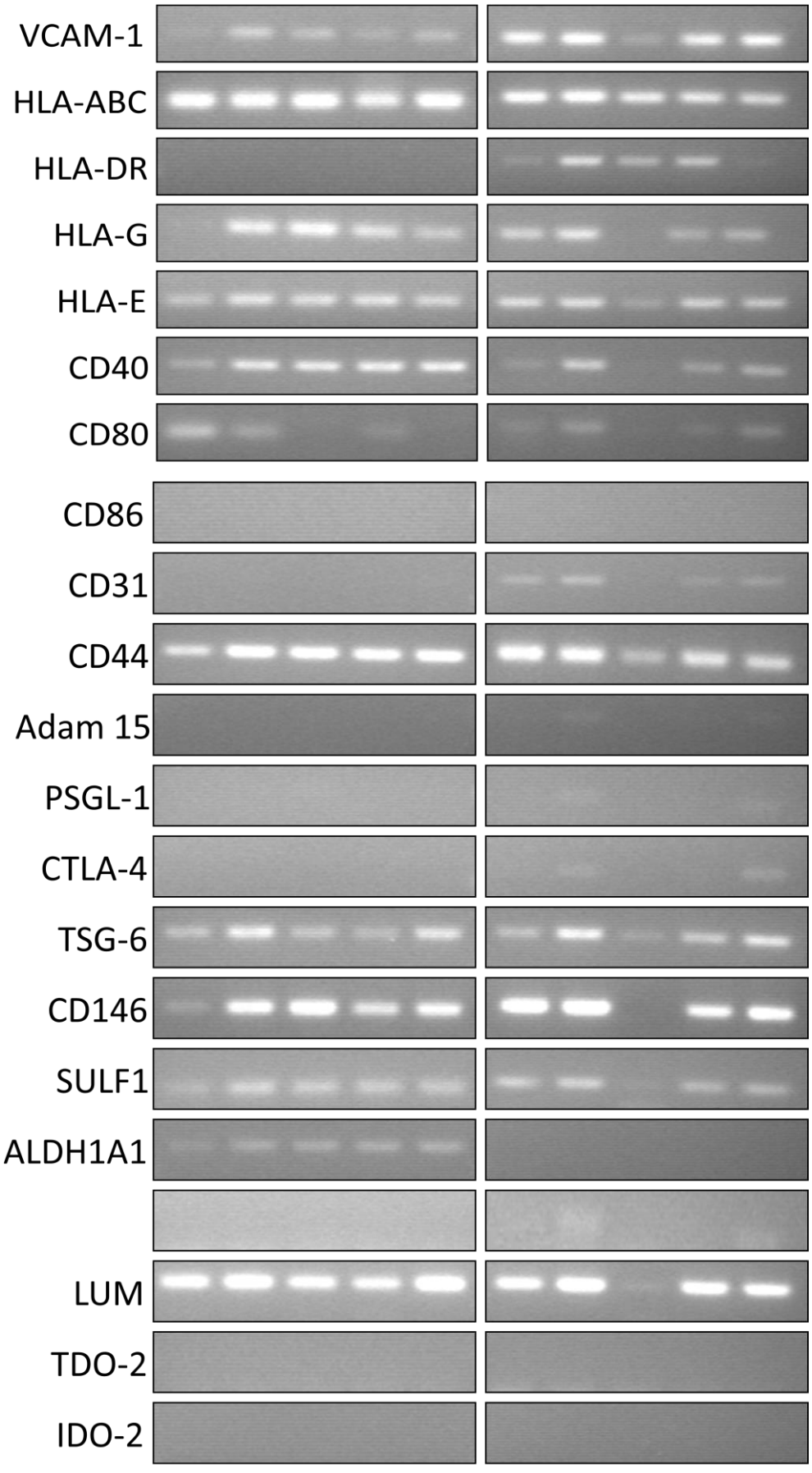
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HLA-DR
HLA-G
HLA-E
CD40
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TDO-2
IDO-2

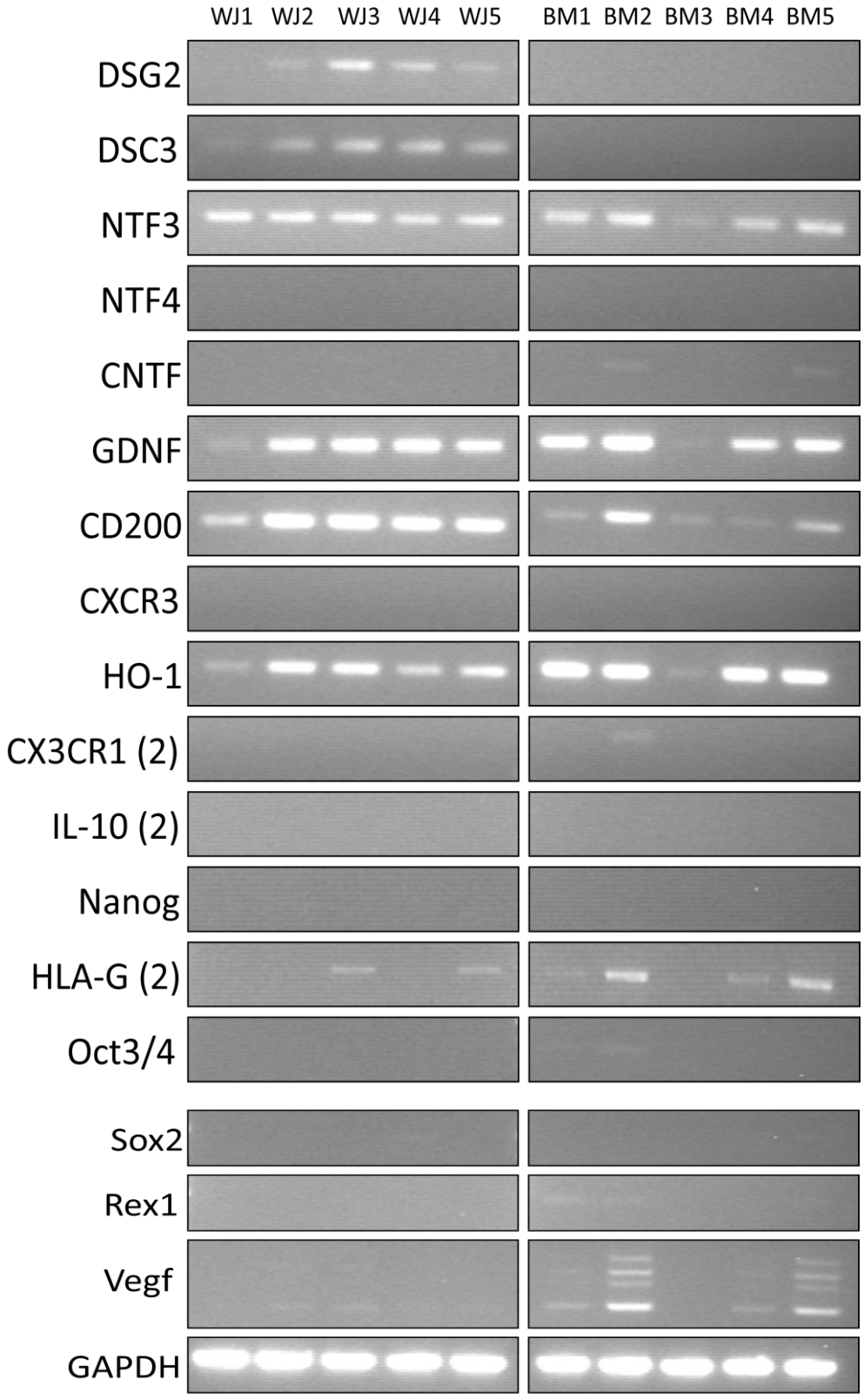

Figure S1: **Gene expression profile between Wharton's jelly mesenchymal stem cells and bone-marrow mesenchymal stem cells**. Different donors of Wharton's jelly mesenchymal stem cells (WJ-MSC, n=5) and bone-marrow mesenchymal stem cells (BM-MSC, n=5) were used. GAPDH served as a positive loading control.

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Richting: **master in de biomedische wetenschappen-klinische en moleculaire wetenschappen**
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Tüylüoglu, Nazim

Datum: **20/08/2013**