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Datum: 14.12.2009

Mesenchymal stem cells derived from umbilical cord tissue: a therapeutic option for multiple sclerosis?

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Eindverhandeling voorgedragen tot het bekomen van de graad master in de biomedische wetenschappen klinische moleculaire wetenschappen

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Raf Donders
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List of abbreviations

7-AAD	7-amino-actinomycin D
aCD3	anti-CD3 monoclonal antibody
bFGF	basic fibroblast growth factor
BM-MSC	bone marrow-derived mesenchymal stem cells
BSA	bovine serum albumine
CFSE	carboxyfluorescein diacetate succinimidyl ester
CNS	central nervous system
CSF	cerebrospinal fluid
DMEM-GL	Dulbecco's modified Eagle's medium with GlutaMAX™-I
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ESC	embryonic stem cells
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
HLA	human leukocyte antigen
HRP	horseradish peroxidase
ICC	immunocytochemistry
IFN	interferon
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSC	mesenchymal stem cells
NCAM	neural cell adhesion molecule
NeuN	neuron specific nuclear protein
NF	neurofilament 70kD
NO	nitric oxide
NSC	neural stem cells
P/S	penicillin/streptomycin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PP-MS	primary progressive multiple sclerosis
RR-MS	relapse-remitting multiple sclerosis
SC	stem cells
SP-MS	secondary-progressive multiple sclerosis
TEM	transmission electron microscopy
TGF	transforming growth factor
UCMS	umbilical cord derived mesenchymal stem cells

Summary

Introduction: Multiple sclerosis is a chronic neurodegenerative disease of the central nervous system characterized by ongoing inflammation, demyelination, neuroaxonal damage and glial scar formation. Current immunomodulatory therapeutics limit inflammation but are not able to affect the underlying causes. A potential new therapeutic approach is stem cell transplantation. Animal studies have shown that mesenchymal stem cells (MSC) exert both immunomodulatory and neuroprotective functions in experimental autoimmune encephalomyelitis (EAE), the animal model of MS. The present study aims to investigate the use of MSC derived from umbilical cord tissue (UCMS). The human umbilical cord provides an easy accessible source of cells with potential for tissue repair. In order to test the potential of UCMS for cellular transplantation in MS, their potential to form neural cell types and their immunological properties will be investigated.

Materials and Methods: Neural differentiation of UCMS was induced by incubation with bFGF or Hyclone AdvanceSTEM^{TM} neural differentiation medium. Morphology was examined using phase contrast microscopy. Expression of specific neuronal and glial markers was assessed using immunocytochemical staining. Next, UCMS were analyzed for expression of the immunologically relevant surface molecules HLA-ABC, HLA-DR, CD80 & CD86 in the presence or absence of interferon (IFN)-γ using FACS analysis. Subsequently, alloreactivity towards UCMS was examined in co-culture with peripheral blood mononuclear cells (PBMC). In addition, their potential suppressive capacity towards activated lymphocytes was tested in similar co-cultures. In both assays, the proliferative response of $CD4^+$ & $CD8^+$ T-cells was assessed using flow cytometry. The possible mechanisms involved in both alloreactivity and suppression (e.g. cell contact or soluble molecules) were addressed in transwell cultures and co-cultures with irradiated UCMS. Cytokine production will be analyzed using ELISA.

Results: Neural differentiation did not induce morphological changes nor neuronal or glial marker expression. Phenotypic characterization of UCMS showed expression of HLA-ABC but minimal expression of HLA-DR, CD80, and CD86. The expression of HLA-ABC and -DR, but not the co-stimulatory molecules, was increased after IFN-γ treatment. In co-culture with PBMC, UCMS slightly induced T-cell proliferation at low cell numbers, compared to background proliferation. However, at higher cell numbers T-cell proliferation decreased to baseline level. Upon co-culture with PBMC containing activated T-cells, addition of low amounts of stem cells slightly increased proliferation. Further increasing stem cell numbers significantly suppressed T-cell proliferation in a dose dependent manner. Similar results were observed in transwell cultures. Analysis of supernatant showed that IL-10 is likely involved in T-cell suppression, however a co-culture with irradiated UCMS highlighted the involvement of contact dependent mechanisms as well.

Conclusion: The results of the present study indicate that UCMS show only limited alloreactivity and can suppress the proliferative response of (activated) T-cells at high cell numbers. This suggests that UCMS have immunomodulatory functions and thus may be useful in allogeneic transplantation. However, differentiation towards neural cell types failed. Nevertheless, this result does not indicate a lack of differentiation capacity. In the future, it would be interesting to further characterize the mechanism(s) involved in suppression and to investigate whether the UCMS show immunomodulatory properties in vivo (EAE). Additionally, their neural differentiation potential should be further investigated.

Introduction

1. Stem cells

Stem cells (SC) are undifferentiated cells that are believed to reside in practically all tissues of the body. Throughout our lives we rely on these SC, to maintain tissue homeostasis and the natural turnover of cells. In this regard, stem cells are specialized cells that continually generate progeny for tissue formation and maintenance (1). As such, they are defined by three characteristics: (a) ability to self-renew, (b) capacity to differentiate into specialized cell types and (c) ability to repopulate a tissue in vivo (2, 3). Stem cells can renew themselves indefinitely and maintain their undifferentiated state while producing cell progeny that mature into more specialized organ-specific cells. This is accomplished by continuous asymmetrical cell division (Figure 1A). One of the two daughter cells retains the stem cell characteristics while the other is destined to form specific cells. The ability to repopulate a tissue in vivo is well established for haematopoietic stem cells. Repopulation by tissue specific stem cells has only recently been shown and not yet for cells (3).

There are different types of stem cells, which vary in terms of cell-specific differentiation capacity and tissue localization (Figure 1B). Based on their potency to differentiate into different cell types, stem cells can be classified as totipotent, pluripotent, multipotent, and unipotent. The only known totipotent stem cells are the fertilized egg and blastomers of the early morula. They can give rise to all the cells and tissues of the developing embryo. Pluripotent stem cells, like embryonic stem cells, are derived from totipotent SC and are able to form all cell types of the three germ layers (endo-, meso- and ectoderm). Multipotent (e.g. hematopoietic SC) can differentiate into a number of cells from a closely related family. Unipotent SC can produce only one cell type but still have the property to self-renew (4).

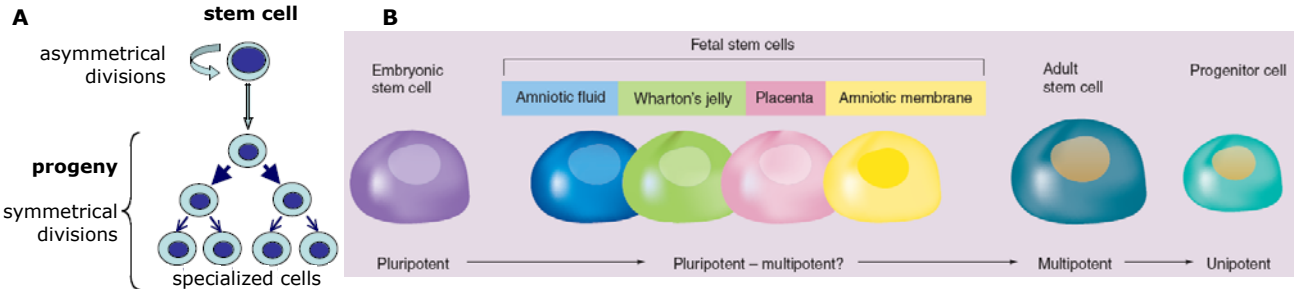

Figure 1: Current model for stem cell division and developmental/potency hierarchy. (A) Stem cells divide asymmetrically, generating a new stem cell and a progenitor daughter cell that continues to divide symmetrically into specialized cell types. (B) Stem cell lineage hierarchy is depicted ranging from pluripotent embryonic SC to the unipotent adult progenitor cell. A number of stem cells derived from fetal tissues are positioned between the embryonic and adult SC. Regarding their potency, these cells are believed to share features from both SC-types. (Figure adapted from Pappa et al. 2009 (4)).

Stem cells exhibit a developmental hierarchy broadly classified into 3 categories: the embryonic stem cells (ESC), adult stem cells and the more recently described fetal stem cells (Figure 1B). Embryonic stem cells are obtained from the inner cell mass of day 5-8 blastocysts created by in vitro fertilization. They are termed pluripotent, since they can give rise to all tissue types of the adult body (1, 3). Adult stem cells, also termed tissue-specific or somatic stem cells, are undifferentiated cells found in a given tissue or organ. These cells are termed multipotent as they

are able to generate the mature cell types within this particular tissue or organ, to safeguard its homeostasis. Adult stem cell have been found in several organ that have a continuous turnover of cells (e.g. blood, skin and gut). They are also found in other less regenerative organs such as the brain. Fetal stem cells are mainly derived the extra embryonic tissues of fetal origin (e.g. placenta, amniotic fluid, umbilical cord blood,…). The combination of being discarded after birth, extra-embryonic (meaning more related to ESC), without ethical reservations, and easy accessible and widely available renders these tissues ideal stem cell sources compared with ESC (4).

1.1 The promise of stem cells in central nervous system disorders

The potential of SC to differentiate into various cell types is the principal reason they are being explored as treatments for degenerative diseases (e.g. Parkinson's disease, multiple sclerosis). In recent years an increasing knowledge of their biology revealed that stem cells also possess other properties besides differentiation into specialized cells. SC show for example immunomodulatory properties, are ability to home to sites of pathology and have the potential to be genetically manipulated (5). In this setting, instead of solely replacing cells, stem cell could be of far greater therapeutic use (1). A wide range of stem cells is currently being investigated for their potential as a stem cell-based therapy in CNS disorders.

Because of their pluripotency ESC are ideal candidates for central nervous system (CNS) transplantation. Yet a great deal of challenges remain that prevent them to be used in practice (e.g. uncontrolled proliferation or ethical considerations) (3).
Adult stem cells, which have the capacity to differentiate into tissue-specific cell types, represent a more feasible source for therapeutic transplantation. They could for instance be used for autologous transplantation therapies, as such eliminating the immunological complications associated with allogeneic cell transplantation. They also bypass the ethical concerns associated with ESC. However, unlike ESC, somatic stem cells are believed to be only capable of differentiating into cell types of their tissue of origin. Nevertheless, recent reports on trans-differentiation of bone marrow-derived mesenchymal stem cells (BM-MSC) into neural cells and the development of induced pluripotent stem cells, are challenging such notions (1-3, 6-8). Among somatic stem cells, neural stem cells (NSC) and mesenchymal stem cells (MSC) are possible candidates for CNS repair.
NSC are found within the olfactory bulb and hippocampus in adults. They can be produced from ESC or isolated from neurogenic regions in fetal and adult CNS (e.g. the subventricular zone). Pluchino and coworkers demonstrated that mouse NSC promote multifocale remyelinisation and functional recovery after intravenous or intrathecal injection in a chronic model of multiple sclerosis (9). These observations were confirmed by other groups (10, 11), yet the mechanisms involved were not fully understood. Functional recovery was rather due to neuroprotective effects of the grafted cells than cellular replacement. In the human setting however, it is important to keep in mind that isolation of NSC from adult patients is a difficult and dangerous procedure. Other adult stem cells, like MSC, are a more accessible (e.g. BM-MSC).
MSC are identified as adherent, fibroblast-like cells which have the capacity to differentiate into bone, fat and cartilage in vitro. It has been suggested that cells with mesenchymal stem cell characteristics reside in virtually all postnatal organs and tissues (5). Several reports indicate a

trans-differentiation of MSC into neural cell types. Differentiation is mainly induced via exposure to growth factors or cell culture-conditioned media and confirmed based on morphological differences and specific neural marker expression. Some, but not all reports addressed neural functionality after differentiation (see Table 1). Kassis et al postulated that BM-MSC induced neuroprotection in the animal model of MS via transdifferentiation and immunomodulation (12). Indeed, MSC display several interesting immunological characteristic. They appear to be lowly immunogenic (described as human leukocyte antigen (HLA) class I^+ , HLA class II^- $CD80^-$ and $CD86^-$ (5, 13). In addition, MSC are able to actively modulate or suppress immune cells (mainly T-cells) through several mechanisms (13, 14), summarized in Table 2.

In conclusion, the ability of MSC to regulate the immune response, coupled with their differentiation capacity, opens a great deal of possibilities for treating the underlying causes of immune-mediated diseases as well as repairing damaged tissue.

Table 1: Overview of several neural differentiation approaches.

source of MSC	approach	cell type	functionality test	reference
bone marrow	BDNF/EGF/NGF	neuron-like cells	/	Choong et al. 2007 (15)
umbilical cord	-NCM -SHH/FGF8	dopaminergic neuron	striatal graft into Parkinsonian rats	Fu et al. 2006 (16)
pectoralis muscle	-neurococktail (17) -EGF/ bFGF	neuron/astrocyte/ oligodendrocyte	calcium uptake	Schultz et al. 2006 (18)
$CD34^+$ HSC	ACM	neuron/astrocyte	/	Reali et al. 2006 (8)
bone marrow	bFGF	neuron	calcium imaging	Tropel et al. 2006 (19)
bone marrow	-bFGF/EGF -ACM/NCM	neuron/astrocyte		Bossolasco et al. 2005 (6)
umbilical cord	-BME/Salvia Miltiorrhiza	nerve-like cell	/	Ma et al. 2005 (20)

ACM, astrocyte conditioned medium; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; BME, β-mercaptoethanol; EGF, epidermal growth factor; FGF8, fibroblast growth factor 8; HSC, haematopoietic stem cell; NCM, neural conditioned medium; NGF, neural growth factor; SHH, sonic hedgehog.

Table 2: Summary of immunosuppressive mechanisms used by MSC.

mechanism	target	effect	reference
IDO induction in MSC and DC	T-cells	tryptophan depletion and suppression of T-cell proliferation	Matysiak et al. 2008 (21)
PGE2/HGF/TGF-β	T-cells	suppression of T-cell proliferation	Keating 2006 (13)
IL-10	T-cells	abberant DC maturation and suppression of T-cell proliferation	Beyth et al. 2005 (22, 23)
contact dependent induction of regulatory APC	T-cells	T-cell unresponsiveness	Beyth et al. 2005 (22)
NO production	T-cells	inhibition of STAT-5 phosphorylation suppression of T-cell proliferation	Sato et al. 2006 (24) Ren et al. 2008 (25)
HLA-G	T-cells NK-cells	contact dependent suppression by membrane bound and secreted HLA-G	Selmani et al. 2008 (26)

APC, antigen presenting cells; DC, dendritic cell; HGF, hepatocyte growth factor; HLA-G, human leukocyte antigen G; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin 10; NK-cells, natural killer cells; NO, nitric oxide; PGE2, prostaglandin E2; TGF-β, transforming growth factor β

2. Multiple sclerosis

Multiple sclerosis (MS) is a chronic neurodegenerative disease of the CNS and is the major cause of non-traumatic disability in young adults. The disease is characterized by inflammatory lesions within the white matter tracts of the brain and spinal cord. These lesions, or sclerotic plaques, are the result of an aberrant immune response against the myelin sheath. The continuous inflammation results in demyelination and the accompanied impairment of nerve impulse transmission. This clinically manifests in a variety of neurological deficits, such as visual and sensory disturbances, fatigue, ambulation problems, lack of coordination and cognitive defects. Symptoms are often associated with the location of the lesions which show a preference for the spinal cord, brain stem, periventricular tissue and optic nerves (27, 28). At its onset, MS can be clinically categorized into 2 major forms. In most patients (80-90%), MS develops as a relapse-remitting disease (RR-MS). The disease course is characterized by intermittent episodes of attacks and neurological dysfunction (relapse), along with periods of remission with partial or complete recovery (remission). However, 40% of RR-MS patients slowly but surely experience an increased disability because of the acquired irreversible damage over time. Many years after onset, these patients will develop secondary-progressive disease course (SP-MS), illustrated by a gradual progression of disability with or without clinical attacks superimposed (28, 29). A second group of patients (10 to 20%) displays a primary progressive disease course (PP-MS). These patients experience a gradual progression of disability from disease onset without acute attacks (27, 30).

2.1 Etiology

The etiology of MS in unknown, however, it is generally assumed that inflammation is initiated in genetically susceptible individuals and requires additional environmental triggers (31). The most consistent association with MS susceptibility has been localized to the major histocompatibility complex (MHC) region (HLA class II) (32). However, large-scale gene expression studies were not able to demonstrate a major susceptibility gene and suggest that multiple loci are involved (31). Population genetics alone can not explain the distribution of MS. Equally important is the interplay between genes and environmental factors. Geographical location (temperate climates) seems to be linked to the prevalence of MS, which occurs more frequently with increasing distance from the equator (32). Also, microbial agents (viruses and bacteria) are believed to be associated with MS. It is assumed that microbial (viral) epitopes are involved in the activation of autoreactive T-cells through cross-reactivity with auto-antigens (molecular mimicry) (33).

2.2 Pathogenesis

MS is currently hypothesized as an autoimmune disease, mainly mediated by $CD4^+$ Th 1 cell responses but in some cases reported to be caused by $CD8^+$ T-cells (reviewed by Vanderlocht et al. (29)) or Th_{17} cells ($CD4^+$ $IL\text{-}17^+$ T-cells) (32). A model of the presumed immune pathogenesis is depicted in Figure 2. Following activation, T-cells cross the blood-brain barrier through a multi-step process which involves adhesion, extravasation and extracellular matrix disruption. Once inside the CNS, T-cells become reactivated upon binding to antigen presenting cells (microglia or astrocytes) and clonotypic expansion of antigen specific T-cells occurs. Upon contact with the antigen, T-cells initiate their effector functions which involve recruitment of macrophages (by $CD4^+$ T-cells) and direct cytotoxicity to HLA class I expressing cells (by $CD8^+$ T-cells). B-cells are activated in the

lymph nodes, clonotypically expand and release autoantibodies in the lesions and cerebrospinal fluid (CSF). Upon deposition, these antibodies are able to initiate complement mediated cytotoxicity and induce antibody-mediated phagocytosis by macrophages. Activated macrophages also produce of toxic mediators e.g. nitric oxide and proteases, which primarily attack oligodendrocytes (34). CNS resident immune cells (microglial cells) are activated upon myelin destruction during the acute phase of the MS. Together with astrocytes, microglial cells contribute to the ongoing inflammatory response through presentation of self-antigen and secretion of cytokines and chemokines, which attract more immune cells.

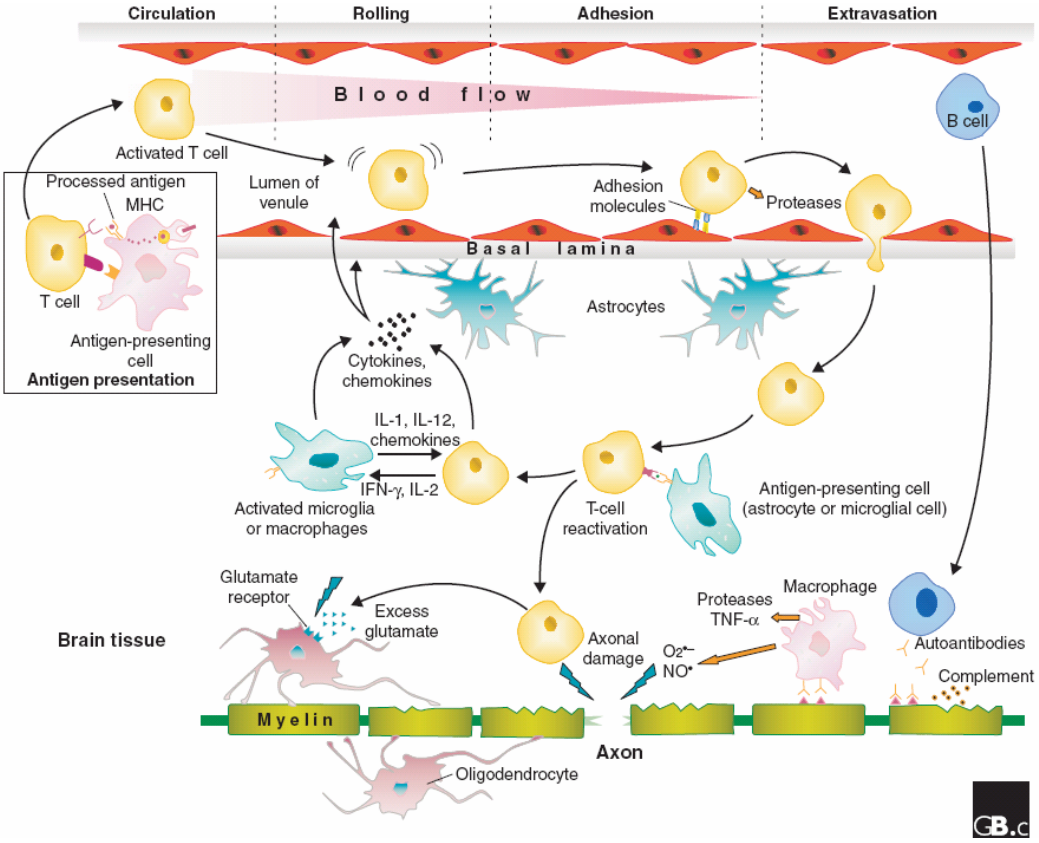

Figure 2: Schematic representation of MS pathogenesis. Upon activation in the periphery (insert), T-cells cross the blood-brain barrier and are reactivated by resident antigen-presenting cells. This second activation step triggers an immune cascade involving recruitment of new immune cells and the production of cytokines and cytotoxic mediators, which ultimately lead to demyelination and axonal damage. (Image reproduced from Baranzini et al., 2002 (31)).

Many of the current pathogenic concepts are derived from the animal model of MS, experimental autoimmune encephalomyelitis (EAE). EAE is a $CD4^+$ T-cell mediated inflammatory disease induced in animals (mainly rodents) by injection of myelin protein antigens with adjuvant. The symptoms of EAE in mice and rats are varied and mimic different clinical manifestations of human MS (35). EAE can also be induced by transfer of myelin reactive $CD4^+$ T-cells from a diseased subject into a unaffected animal (adoptive transfer), which illustrates the role of immunity in disease onset (34, 35). Although EAE does not entirely correspond with the clinical and pathologic features of human MS (32), it is a valuable tool to study disease mechanism or potential novel therapeutics.

2.3 Histopathology

The pathological hallmark of MS is the formation of sclerotic plaques which are spread throughout the CNS. Important features are inflammation, demyelination, a variable degree of remyelination, loss of oligodendrocytes, axonal damage and gliosis (formation of scar tissue) (36).

MS lesions may be characterized as active or inactive, defined by the inflammatory reaction or by the presence of myelin-laden macrophages. *Active lesions* consist of perivascular infiltrates of lymphocytes (mainly $CD4^+$ T-cells with occasional B-cells) and monocytes. $CD4^+$ T-cells are also found in the normal appearing white matter beyond the margin of demyelinisation. In contrast, $CD8^+$ T-cells and macrophages are most prominent in the centre of the plaque (27). Reactive astrocytes and microglia can also be found within the lesions. *Chronic active lesions* show an ongoing inflammatory activity (lymphocytes and macrophages) at the edge of the plaque. The centre of the plaque becomes hypocellular and glial scar formation by reactive astrocytes begins (27). Nevertheless, up to a certain extent within these active lesion, oligodendrocytes proliferate and start the remyelination process. In that case the plaques represent morphologically as sharply demarcated lesions in the white matter with reduced myelin content, also referred to as shadow plaques. *Chronic inactive lesions* develop in a later stage of the disease. The recurrent inflammatory attacks result in the loss of demyelinating oligodendrocytes and injury to neurons, which eventually lead to axonal degeneration and finally the formation of scar tissue by reactive astrocytes (gliosis) (32, 37).

Among MS patients, there appears to be a pathological variability within the lesions. Four different patterns have been described (38). Pattern I (20%) and II (50%) are characterized by T-cell and macrophage mediated demyelination, sharp bordered lesions and relatively preserved oligodendrocytes which extensively remyelinate after injury. Pattern II is distinct from pattern I by the deposition of immunglobulins and complement activation. Patterns III (25%) and IV (<5%) are characterized by low oligodendrocyte numbers and poor remyelinating capacity. Type III and IV lesions were highly suggestive of a disease process initiated in the CNS itself (e.g. virus-induced primary oligodendrocyte dystrophy) followed by the cascade of inflammation and demyelination.

2.4 Diagnosis and current treatments

As no specific diagnostic test exists, MS is diagnosed dependent on a detailed anamnesis including a careful neurological examination. Furthermore, supportive paraclinical tests are performed including CSF analysis (oligoclonal bands) after a lumbar puncture, evoked potentials and magnetic resonance imaging (MRI) (27, 30). The latter has become an important tool in currently used diagnostic criteria. A classic criterion is the evidence of lesions in the CNS disseminated in time and space (more than one clinical episode of neurological deficit involving lesions in more than one area of the CNS) (36). MRI allows for the sensitive detection of lesions in time and space and thus contribute to an early and more precise diagnosis (McDonald criteria).

A variety of disease-modifying agents are currently available to treat MS. For treatment of acute exacerbations, high doses of corticosteroids (e.g. methylprednisolone) are used to shorten symptom duration. Nevertheless, they do not restrain the development of relapses. Patients with RR-MS are currently treated with interferon-β (IFN-β), which is a naturally occurring cytokine. Also

the synthetic polypeptide glatiramer acetate, which resembles myelin basic protein, is used. These therapies are mainly aimed at reducing relapse rates and preventing further disability, which is effective in early stages of MS but rather ineffective in the more progressive stage of the disease (37, 39). A more recent treatment strategy implies the administration of selective adhesion molecule inhibitors (SAM inhibitors). Natalizumab is the first α_4-integrin antagonist within this class (40, 41). Natalizumab effectively reduces relapse rate and progression in patients with RR-MS (41). Patients that are in the progressive phase of the disease (SP-MS, PP-MS) receive more aggressive therapy since other treatments have little or no effect. In that case immunosuppressant or chemotherapeutic drugs (e.g. Mithoxantrone) are used (37).
Currently approved MS therapeutic only affect the symptoms of the disease and not its underlying cause(s). None of the presently available drugs selectively inhibit the autoimmune response, nor do they stimulate regeneration of damaged tissue. To fill this therapeutic gap, novel treatment strategies, like for instance cell-based therapies, are being investigated (see also section 1.1).

3. Stem cells transplantation as a therapy in MS

As already described, MS is a chronic disease of the CNS, characterized by ongoing inflammation and loss of oligodendrocytes and neurons. Treatment with stem cells could induce tissue regeneration and/or modulate the immune response. MSC have already been used to treat MS, with variable success (42). After wiping out the patient's own immune system through high-dosed chemotherapy or irradiation, BM-MSC (autologuous or HLA-matched) are transplanted to reconstitute the bone marrow and produce a novel immune system that is not autoreactive.
Accumulating evidence suggest that *MSC derived from the umbilical cord* show therapeutic potential, as illustrated by transplantation studies in EAE and humans. Recently, Liang and colleagues reported the first successful treatment of MS with umbilical cord derived MSC transplantation (43).

3.1 The human umbilical cord as a source of stem cells

The umbilical cord represents the link between mother and fetus and is composed of a specialized mucous connective tissue, the Wharton's Jelly. This jelly-like material functions to prevent torsion, compression and bending of the vessels within the cord, to maintain the bidirectional flow of blood between fetus and placenta (44). Within various compartment of the cord (see Figure 3), cells with a mesenchymal morphology are present, which in recent years have gained much attention in the search for alternative stem cell sources. The umbilical cord is an easy accessible, non-controversial source of multipotent stem cells (45). Unlike BM-MSC, no painful harvesting procedures are involved and relatively large amounts of tissue for stem cell extraction can be obtained straight away. In addition, stem cells derived from the Wharton's Jelly, also termed umbilical cord matrix derived mesenchymal stem cells (UCMS) are easily isolated and expanded in culture (46). UCMS have been reported to differentiate neurons and glia (15, 46, 47) and show immunomodulatory properties in vitro (48). Taken together, the fact that umbilical cord derived mesenchymal stem cells (a) have a favorable immunological profile, (b) have multilineage differentiation capacity and (c) are easily accessed and expanded, makes these cells promising candidates for transplantation therapy in MS.

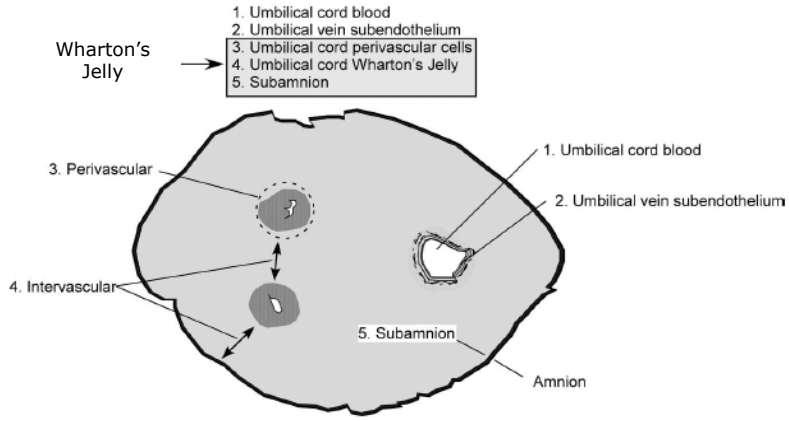

Figure 3: Schematic overview of the different compartments within the umbilical cord. 5 separate regions have been shown to contain MSC. (Adapted from Troyer et al. 2008 (45)).

4. Research goals & experimental setup

In the present study, the neural differentiation potential and immunomodulatory capacity of easy accessible umbilical cord matrix derived mesenchymal stem cells (UCMS) will be investigated. UCMS are isolated according to the explant culturing procedure (46). At first the mesenchymal stem cell properties of the isolated cells will be determined. Secondly, the neural differentiation potential of the stem cells will be addressed. Differentiation into neural cell types is performed using basic fibroblast growth factor (bFGF), as was already described for BM-MSC (19). bFGF has been described as controlling NSC renewal and differentiation. In parallel to the bFGF treatment, a commercially available neural differentiation medium will be tested as well. The morphology of the cells during differentiation will be observed using phase contrast microscopy. After bFGF treatment the expression of specific neural and glial markers will be assessed using immunocytochemical staining. True differentiation is confirmed at the ultrastructural level with transmission electron microscopy. Furthermore, the immunomodulatory potential of UCMS will be explored in co-culture assays with peripheral blood mononuclear cells (PBMC). Their capacity to escape or modulate allogeneic responses will be evaluated as well as their potential to suppress activated T-cells. In order to identify the mechanisms implicated in the observed responses, the involvement of soluble factors and/or the need for cellular contact will be determined. In this setup, the co-cultures are repeated without cell-cell contact in transwell cultures. When soluble molecules are involved, their expression will be analyzed using standard sandwich enzyme-linked immunosorbent assay (ELISA). In addition, co-cultures of PBMC and irradiated UCMS are performed to confirm whether stem cell inactivation affects the responses seen in the other assays.

Materials and methods

1. Isolation and culture of UCMS

Umbilical cords were obtained from local hospitals after vaginal delivery or cesarean section with informed consent of the mother. The cords were transported to the laboratory in sterile conical tubes containing 0,01M phosphate buffered saline (PBS; pH 7,2) supplemented with 1% penicillin/streptomycin (P/S; Gibco, Paisley, UK) and 0,2% amphotericin B (fungizone®; Gibco). Tissue processing for stem cell isolation occurred within 48 hrs according to the explant culture method (46, 49). First, the cords were washed in PBS to remove residual blood cells. Next, the tissue was transferred to a Petri dish containing PBS and cut into small pieces (1-2 cm). The arteries and vein were stripped off to avoid endothelial cell contamination. The remaining tissue (Wharton's Jelly) was further diced into pieces (2-3 mm^3) and transferred for culture expansion into 6 well plates (Nunc, Roskilde, Denmark) containing culture medium consisting of Dulbecco's modified Eagle's medium with GlutaMAX™-I and low glucose (DMEM-GL; Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 1% P/S and 0,2% fungizone®. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and observed regularly with a phase contrast microscope. When explants attached and outgrowth of cells was seen, the medium was changed every 3 days. At 80% confluence, the cultures were harvested with 0,05% trypsin-EDTA (Gibco, Paisley, UK), the obtained cell suspension was pelleted at 300g for 5 min and resuspended in culture medium. A small sample was taken to determine the total cell number by means of trypan blue exclusion (Fuchs-Rosenthal counting chamber). Afterwards cells were seeded for further expansion (25 & 75cm^2 culture flasks; Nunc). Morphology and growth of UCMS cells in culture were monitored using a Nikon Eclipse TS100 inverted phase contrast microscope provided with a digital camera (Nikon, Japan). Digital images were acquired and processed using LNET software.

2. Immunocytochemistry

Protein expression was assessed using immunocytochemistry (ICC) to determine mesenchymal phenotype or differentiation-related protein expression. UCMS grown on glass coverslips (Menzel Gläser, Thermo Scientific) were fixed in 4% formaldehyde (Unifix, Duiven, The Netherlands) for 20 min. Immunocytochemical stainings were performed using the peroxidase-based EnVision System® (Dakocytomation, Glostrup, Denmark). Fixed cells were washed with PBS, permeabilized with 0,05% Triton X-100 (Boehringer, Mannheim, Germany) in PBS for 30 minutes at 4°C and washed again. Prior to labeling, non-specific binding sites were blocked with 3% normal goat serum (Dakocytomation) or normal donkey serum (Jackson ImmunoResearch, Suffolk, UK) in PBS for 20 minutes depending on the secondary antibody. After washing, cells were incubated for 1 hr with the primary antibody (see table 1), washed, and incubated for another 30 min with the appropriate secondary antibody. Either the horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit secondary antibody from the EnVision kit or the donkey anti-goat secondary antibody (Peroxidase-conjugated AffiniPure $F(ab')_2$ Fragment IgG, 1/1000; Jackson ImmunoResearch) was used. The 3,3'-diaminobenzidine (DAB) chromogen solution was used to visualize the antibodies and Mayer's hematoxylin nuclear counterstain was performed. After mounting on a glass slide with Aquatex

(Merck, Darmstadt, Germany), coverslips were examined with a Nikon Eclipse 80i microscope provided with a digital camera or scanned with a MIRAX Desk microscope (Carl Zeiss MicroImaging, New York, USA). Digital images were processed using LNET software and MIRAX Scan software respectively.

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

Primary antibody	Species	Dilution	Source (clone)
CD29	MM IgG1	1/50	Abcam (4B7R)
CD44	MM IgG1	1/250	Abcam (NKI-P2)
CD105	MM IgG1	1/1000	Abcam (105 C02)
CD146	RP IgG	RTU	Abcam
osteocalcin	MM	1/10	R&D Systems stem cell kit
fatty acid binding protein 4 (FABP-4)	GP	1/10	R&D Systems stem cell kit
aggrecan	GP	1/10	R&D Systems stem cell kit
STRO-1	MM IgM	1/50	R&D Systems (stro-1)
nestine	MM IgG1	1/500	Chemicon (10C2)
glial fibrillary acidic protein (GFAP)	MM IgG1	1/200	NovoCastra (GA5)
Neurofilament (NF) 70kD	MM IgG1	RTU	Dako (2F11)
neural cell adhesion molecule (NCAM)	MM IgG1	1/250	Santa Cruz Biotech (123C3)
Neuron specific nuclear protein (NeuN)	MM IgG1	1/100	Chemicon (A60)
β tubulin III	MM IgG2a	1/4000	Sigma Aldrich (2G10)

MM = mouse monoclonal, RP = rabbit polyclonal, GP = goat polyclonal, RTU = ready to use.

3. Transmission electron microscopy (TEM)

The ultrastructure of the UCMS cells was examined using TEM. Cells were prepared according to standard procedures. The methodological details are provided in the supplemental information.

4. Assessment of UCMS differentiation potential

4.1 Mesenchymal differentiation

To evaluate the mesenchymal stem cell properties of the isolated cells, their ability to functionally differentiate into 3 mesodermal cell types, the adipocyte, osteoblast and chondroblast, was investigated. To this end the Human mesenchymal stem cell functional identification kit (SC006, R&D Systems, Minneapolis, USA) was used. Differentiation was confirmed with ICC, histological stainings and TEM.

Adipogenic differentiation: A monolayer of cells was grown for 3 weeks in a 24 well plate (Nunc) at 37°C in a humidified atmosphere with 5% CO_2. Differentiation was induced in α-MEM (Gibco) containing 10% FBS, 1% P/S, 2mM L-glutamine and adipogenic supplement from the kit. The medium was changed every 3 days. To assess intracellular lipid accumulation, cells were fixed and stained for 30 minutes with 0,3% Oil Red O in distilled water. Subsequently, the cells were rinsed with 60% isopropanol in distilled water for 3 seconds, washed again, and counterstained with Mayer's haematoxylin for 8 minutes. The coverslips were rinsed with tap water for colorization and mounted on a glass slide with Aquatex. In addition, differentiation was confirmed with TEM and immunocytochemical staining for FABP-4.

Osteogenic differentiation: A monolayer of cells was grown for 3 weeks in a 24 well plate on fibronectin coated coverslips, at 37°C in a humidified atmosphere with 5% CO_2. To promote

osteogenic differentiation UCMS cells were cultured in α-MEM containing 10% FBS, 1% P/S, 2mM L-glutamine and osteogenic supplement. The medium was changed every 3 days. For evaluation of mineralized (calcified) matrix formation, cells were fixed and stained with 2% Alizarin Red S solution for 20 minutes. After washing with distilled water cells were inspected on the phase contrast microscope. Also, differentiation was confirmed with TEM and immunocytochemical staining for osteocalcin.

Chondrogenic differentiation: UCMS were pelleted and cultured for 3 weeks in conical tubes, at 37°C in a humidified atmosphere with 5% CO_2. Differentiation was induced in DMEM/F12 (Gibco) containing 1% P/S and ITS-chondrogenic supplement from the kit. The medium was changed every 3 days. The globular cell pellets were assessed for acidic mucopolysaccharides which are present in cartilage tissue. To this end the globules were fixed and transferred for cryosectioning to a vial containing Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands). Cryosections of differentiated chondrogenic pellets and control pellets were mounted on adhesive glass slides (Superfrost Menzel Gläser, Thermo Scientific) and kept overnight at -20°C. Following thawing, the sections were hydrated with distilled water and stained with Alcian Blue for 30 min. After washing with distilled water, the slides were counterstained for 5 min with nuclear fast red, dehydrated in a graded alcohol series, and cleared in xylene. Finally a glass coverslip was mounted over the pellets with DPX (VWR, Leuven, Belgium). In addition, immunocytochemical staining was performed to determine aggrecan production and TEM images were taken.

4.2 Neural differentiation

UCMS were seeded in a 24 well plate at a density of 10^4 cells per well. Differentiation was induced by treatment with basic fibroblast growth factor (bFGF; 25ng/ml or 50ng/ml; Peprotech, New Jersey, USA) in culture medium containing 1% FBS (19). In parallel, cells were treated with neural differentiation medium from the Hyclone AdvanceSTEM™ Neural differentiation kit (Thermo Scientific, SC protocol sheet 00014). Cells cultured in normal culture medium (10% & 1% FBS) served as controls. Morphological changes were monitored with phase contrast microscopy. After 1 week the cells were fixed and ICC staining for specific neural and glial markers was performed (see Table 1). If the UCMS show morphological changes and/or specific marker expression, true differentiation will be assessed at the ultrastructural level with TEM.

5. Assessment of immunological properties of UCMS

5.1 Immunophenotypical analysis of UCMS using FACS

Immunophenotyping of UCMS was performed by staining with cell-surface antigen-specific monoclonal antibodies and subsequent fluorescent activated cell sorting (FACS) analysis. UCMS cells were cultured for 3 days in the presence or absence of 100 U/ml human recombinant interferon (IFN)-γ (Immunotools, Friesoythe, Germany). Cells were harvested by treatment with 0,05% trypsin-EDTA, washed with FACS-buffer (PBS supplemented with 2% FBS and sodium azide) and incubated in the dark for 30 minutes at 4°C with the following phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies: HLA-ABC-PE and HLA-DR-PE (Immunotools), CD14-PE and CD31-FITC (BD Biosciences) and CD80-PE (2D10.4), CD86-PE (IT2.2), CD45-PE (HI30) (eBioscience, California, USA). The samples were washed and the specific

fluorescence was analyzed on a FACSCalibur instrument using CellQuest software (Becton Dickinson, Temse, Belgium). At least 25.000 events were counted. Cells were all single-labeled and percentage of positive cells was determined.

5.2 Isolation of PBMC and labeling with CFSE

Human peripheral blood mononuclear cells (PBMC) were prepared from heparinized whole blood samples from healthy donors, by density gradient centrifugation (Ficoll Histopaque 1077, Sigma-Aldrich, Missouri, USA) according to the manufacturer's instructions. The isolated PBMC were resuspended at a concentration of $20x10^6$ cells/ml in PBS containing 0,1% bovine serum albumine (BSA; Immunosource, Halle-Zoersel, Belgium), and then labeled with 2µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Vybrant CFDA SE cell tracer kit, Molecular Probes, Oregon, USA) for 7 min at 37°C. Subsequently the cells were washed and incubated for another 30 min at 37°C in co-culture medium (RPMI 1640 Glutamax^{TM}-I + 1% sodium-pyruvate + 1% non-essential amino acids (all from Gibco) + 0,5% P/S + 10%FBS) to stabilize the staining. After a final wash step, cells were resuspended in co-culture medium at the desired cell concentration, to be used in the different assays.

5.3 Co-culture of UCMS and PBMC

To investigate whether UCMS could provoke an in vitro allogeneic response, UCMS were co-cultured with allogeneic PBMC (*alloresponse assay*). In addition their potential to suppress CD3 induced PBMC proliferation was assessed (*suppression assay*). UCMS were harvested as described before, plated in a 48-well plate and left to adhere overnight. The next day, $5x10^4$ CFSE-labeled PBMC were added with or without addition of anti-CD3 monoclonal antibodies (aCD3, clone 2G3, final concentration: 2µg/ml; BIOMED, Diepenbeek, Belgium). Experiments were performed with a constant number of PBMC in the presence of different numbers of UCMS to obtain UCMS:PBMC ratios of 1:100; 1:10; 1:5; 1:2; 1:1; 2:1; 3:1 and 5:1. Co-cultures were carried out in duplicate, in a final volume of 500µl of co-culture medium. CFSE-labeled PBMC (± aCD3) without UCMS were used as control. After 5 days, the co-cultures were processed for FACS analysis.

5.4 Transwell culture of UCMS and PBMC

For co-culture assays with separated cell populations, 24-well transwell chambers with 0,4 µm pore size membranes (Corning Costar, Massachusetts, USA) were used to physically separate the UCMS from the PBMC. Stem cells were seeded into the lower chamber in a volume of 500 µl of culture medium and left to adhere overnight. The next day, the medium was replaced with 500 µl of co-culture medium. Then, $5x10^4$ CFSE-labeled PBMC were added to the upper chamber in a volume of 250 µl of co-culture medium, with or without aCD3 stimulation (final concentration: 2µg/ml). Experiments were performed in duplicate with ratio's of UCMS:PBMC of 1:10; 1:2; 1:1; 2:1 and 5:1. CFSE-labeled PBMC (± aCD3) without UCMS were used as control. After 5 days, the co-cultures were processed for FACS analysis.

5.5 Co-culture of irradiated UCMS and PBMC

Prior to the co-culture, the UCMS were seeded in a 24-well plate and left to adhere overnight. The next day the cells were γ-irradiated (dose = 30 Gy) (IBL 437 C irratiator, CIS Bio International,

France). Afterwards, $5x10^4$ CFSE-labeled PBMC were added to the plate in a volume of 500µl of co-culture medium, with or without aCD3 stimulation (final concentration: 2µg/ml). Experiments were performed in duplicate using the same PBMC:UCMS ratio's as in the transwell cultures (see 5.4). CFSE-labeled PBMC (± aCD3) without UCMS were used as control. After 5 days, the co-cultures were processed for FACS analysis.

5.6 Measuring proliferation of $CD4^+$ & $CD8^+$ T-cells using FACS analysis

In the co-cultures, T-cell proliferation was assessed after 5 days. The medium in each co-culture condition was carefully resuspended to ensure aspiration of the PBMC population and transferred to eppendorf tubes. The tubes were centrifuged for 1 minute at 5000 rpm. The supernatant was kept for further analysis and the cell pellets were resuspended in FACS-buffer. The PBMC suspension was transferred to a V-bottomed 96 well-plate and simultaneously labeled with peridinin-chlorophyll-protein complex (PerCP)-conjugated anti-CD8 (Becton Dickinson) and PE-conjugated anti-CD4 (Immunotools, Friesoythe, Germany) antibodies for 30 min at 4°C in the dark. Cells were analyzed for proliferation (CFSE dye dilution; 10,000 gated events) using a FACSCalibur cytometer and processed using CellQuest software (Becton Dickinson, Temse, Belgium).

5.7 Viability test

Viability of PBMC in co-culture was assessed with 7-amino-actinomycin D (7-AAD; BD Bioscience) staining (for protocol see supplemental material and methods S1.2).

5.8 Cytokine analysis

Supernatant harvested from the co-culture assays were analyzed for IFN-γ, TGF-β1 and IL-10 content using the ELISA Ready-SET-Go reagent kit (eBioscience) according to the manufacturer's instructions. The supernatants were also assessed for NO content. The nitrite (NO_2^-) concentration was determined using the Griess reagent system (Promega, Wisconsin, USA) according to the manufacturer's instructions. Samples were analyzed using a Bio-Rad 680 Microplate Reader equipped with Microplate Manager software (Bio-Rad, Nazareth Eke, Belgium).

6. Statistical analysis

All statistical analysis were performed using Graphpad Prism 5 (Graphpad, California, USA). Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. Significant differences between the means of two groups was tested using the two-tailed unpaired t test with Welch's correction. To compare 3 ore more groups, the one-way analysis of variance test (ANOVA) was used. Data are represented as mean ± standard error of the mean (SEM). P-values $< 0,05$ were considered statistically significant.

Results

1. Characterization of UCMS

Stem cells were isolated from the Wharton's Jelly of the umbilical cord using explant culturing. Approximately 7 days after isolation the explants attached to the culture plate and UCMS started to grow out (Figure 4A). These cells were plastic adherent, showed a fibroblast-like morphology and had a large nucleus containing multiple nucleoli (Figure 4A, arrowhead). When cultures reached confluence, UCMS became spindle-shaped and were able to form colonies. At the ultrastructural level the cells displayed large euchromatic nuclei, a perinuclear region rich in organelles, and stress fibers located near the cell membrane (Figure 4B). No deposits of extracellular matrix were seen.

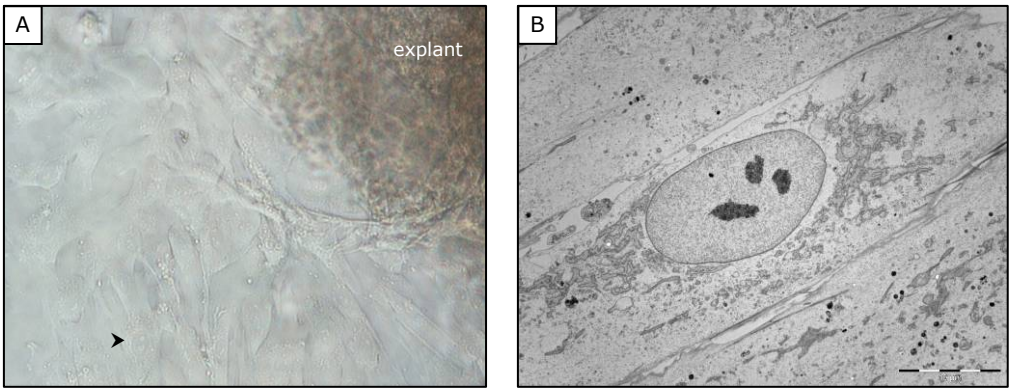

Figure 4: Morphological characterization of isolated UCMS. (A) Phase contrast image showing cellular outgrowth from an attached explant. Cells had a fibroblast-like morphology with large nuclei, which often contained multiple nucleoli (arrowhead). (B) Transmission electron microscopy image of UCMS in vitro. UCMS show a large euchromatic nucleus surrounded by cell organelles. Stress fibers are near the cell membrane and the extracellular space contains no deposits. (A: 10x magnification; B: scale bar = 10 µm).

Culture-expanded cells were identified as MSC based on their phenotype and multilineage differentiation potential. Since no single marker exists to identify mesenchymal stem cells, UCMS were screened for a panel of different surface molecules. Immunocytochemical staining showed expression of CD29, CD44, CD105, CD146 and STRO-1 (Figure 5). FACS analysis showed that UCMS did not express CD31 and the haematopoietic markers CD14 and CD45, as shown in supplemental figure 1 (Figure S1).

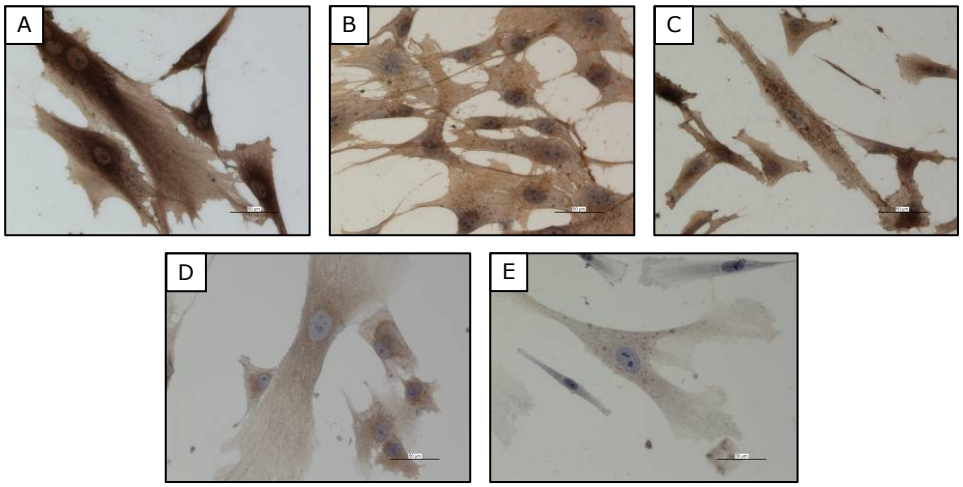

Figure 5: Mesenchymal marker expression. Stem cells stained positive for CD29 (A), CD44 (B), CD105 (C), CD146 (D) and STRO-1 (E). (A-E: Scale bar = 50 µm).

The isolated UCMS demonstrated multilineage differentiation potential into adipocytes, osteoblasts and chondroblasts, after culturing in defined differentiation media. Adipogenic, chondrogenic and osteogenic differentiation was confirmed by histological staining (Oil Red O, Alcian Blue and Alizarine Red respectively (Figure 6)), immunocytochemical staining (FABP-4, aggrecan and osteocalcin respectively) and TEM (Figure S2).

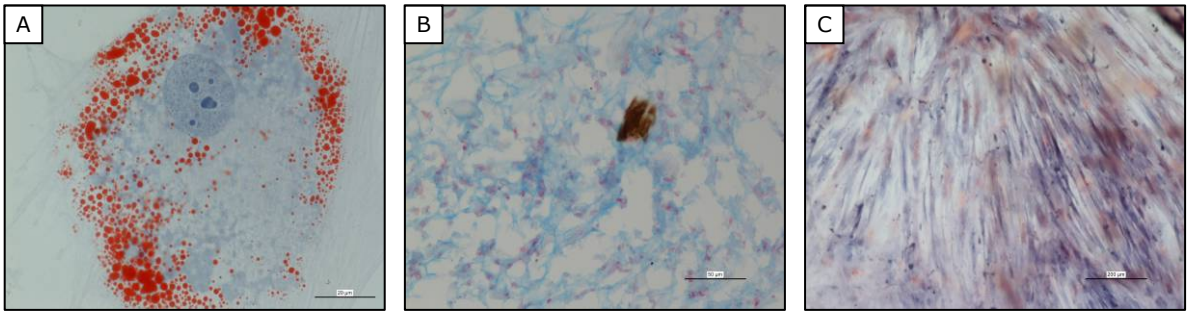

Figure 6: Histological stainings confirming mesenchymal differentiation capacity. Adipogenic: Oil Red O (A), chondrogenic: Alcian Blue (B) and osteogenic: Alizarin Red (C). (A: bar = 20 µm; B: bar = 50 µm; C: bar = 200 µm).

2. Neural differentiation

The isolated UCMS were cultured in monolayer and neural induction was performed with bFGF-conditioned medium. Cells were cultured for 1 week with medium changed every 3 days. During the culture period the cellular morphology was monitored regularly with phase contrast microscopy. However, no morphological differences were detected between control cells and bFGF-reated cells (data not shown). After 1 week of treatment, the cells were fixed and stained for specific neural markers (see Table 3). In the control condition (1% and 10% FBS), UCMS expressed β-tubulin III and nestine. No differences between both control conditions were observed (Figure S3 and S4). After bFGF treatment, UCMS continued to express both nestin and β-tubulin III (Figure 7) The number of nestin positive cells appeared to be slightly increased compared to control, however the amount remained very low (<5%). The stem cells did not express NeuN, NF or GFAP after differentiation.

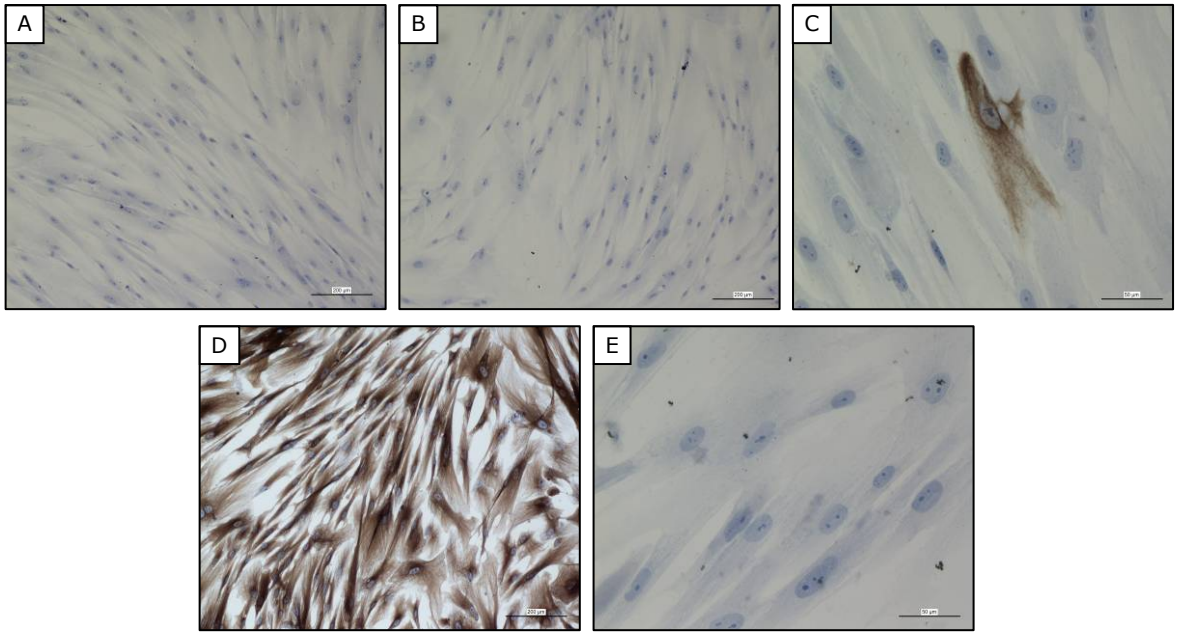

Figure 7: Neural marker expression after bFGF treatment. UCMS were treated for 1 week with 25 ng/ml bFGF and subsequently stained for NeuN (A), GFAP (B), nestin (C), β-tubulin III (D) & NF (E). (Scale bar: A, B&D: 200µm; C&E: 50µm).

In addition, prolonged culturing in bFGF conditioned medium (2 weeks) or exposure to a higher concentration of bFGF (50 ng/ml) did not induce any morphological changes or difference in neural marker expression (data not shown).

In parallel with the bFGF experiments, a commercially available neural differentiation protocol, described to induce neural differentiation of a variety of human somatic stem cells, was tested. UCMS were cultured in monolayer and neural induction with Hyclone AdvanceSTEM^{TM} neural differentiation medium was performed during 1 week with medium changes every 2 days. Similar results were obtained when compared to the bFGF experiment. The Hyclone medium did not induce any morphological changes. As shown in Figure 8, no expression of GFAP, NCAM and NF was seen after differentiation. β-tubulin III was ubiquitously expressed. Nestin was expressed in a minority of treated cells (<1%) but this was also seen in control cells (shown in Figure S4).

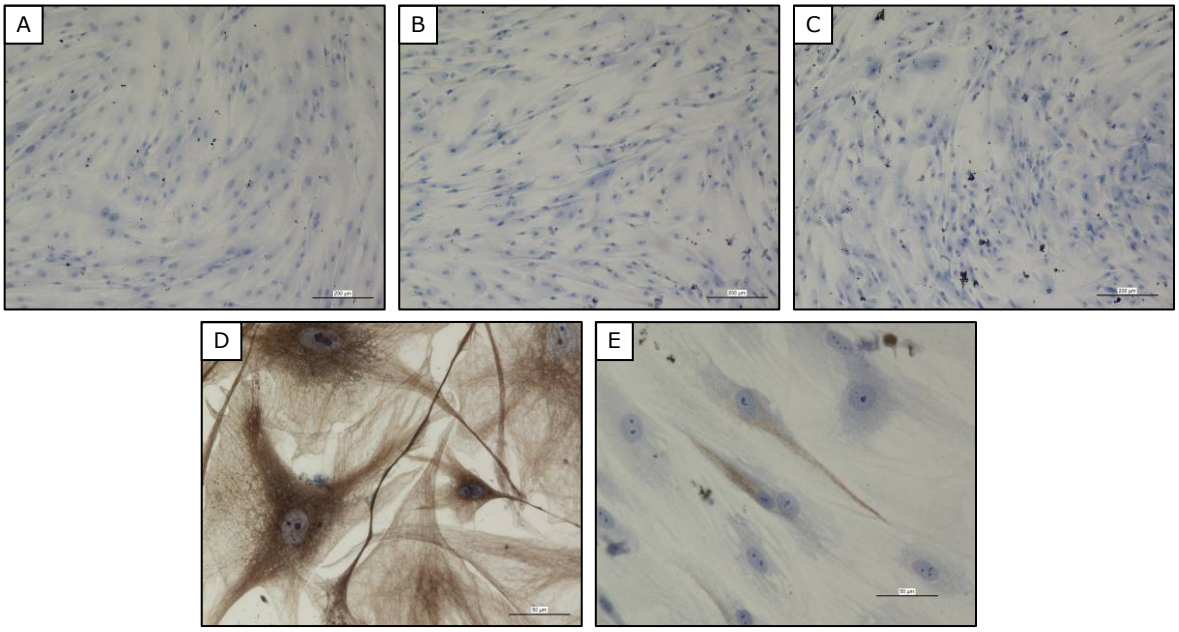

Figure 8: Neural marker expression after Hyclone AdvanceSTEM^{TM} treatment. UCMS were treated for 1 week and subsequently stained for GFAP (A), NCAM (B), NF (C), β-tubulin III (D) & nestin (E). (Scale bar: A-C: 200µm; D&E: 50µm).

3. Immunological properties

In order to gain more knowledge about possible immune responses after stem cell transplantation, a series of immunological assays were performed. First, the expression of immunologically relevant surface molecules on UCMS was analyzed. Next, the alloreactivity of HLA-mismatched PBMC (T-cell response) towards the UCMS was evaluated in co-culture assays. In addition, we examined the immunomodulatory potential of the UCMS in similar co-culture assays with PBMC containing activated T-cells. Further, the mechanisms involved in alloreactivity and immunomodulation were addressed. To reveal whether soluble factors are involved in the suppression, co-cultures were performed without cell-cell contact (transwell cultures). Co-cultures of PBMC with irradiated UCMS (30 Gy) were performed to confirm whether stem cell inactivation would affect the responses seen in the other assays.

3.1 Immunological marker expression

The immunological phenotype of UCMS was determined by flow cytometry. Cultured UCMS showed expression of HLA-ABC (66,7±14%) but they did not express HLA-DR (0,7±0,3%) nor the co-stimulatory molecules CD80 and CD86. Exposure to IFN-γ (100 U/ml) for 72 hours, significantly increased expression of HLA-ABC (97,8±0,8%) and HLA-DR (38,5±4,8%). In contrast, IFN-γ did not induce expression of the co-stimulatory molecules (Figure 9).

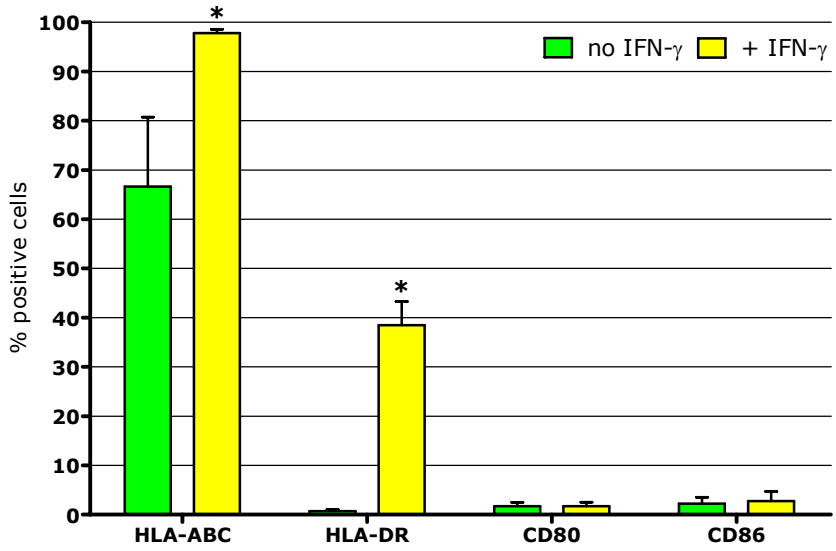

Figure 9: Expression of immunological surface molecules. FACS analysis showed expression of HLA class I (ABC) in cultured UCMS. Exposure to IFN-γ significantly increased HLA class I and induced HLA class II (DR) expression. In contrast, UCMS did not express the co-stimulatory markers both in control and treated conditions. Data were obtained from 6 independent experiments and are represented as mean ±SEM. Significant differences in marker expression between normal cultured stem cells and IFN-γ treated cells was determined using the unpaired t-test (two-tailed with Welch's correction). * p-value < 0,05.

3.2 Assessment of alloreactivity and immunosuppressive capacity of UCMS

A potential alloreactive immune response against the stem cells was assessed in co-culture with PBMC (alloresponse assay). After 5 days of co-culturing, the proliferative response of T-cells was examined using FACS analysis. As shown in Figure 10A, the conditions with a low number of stem cells (1:100 → 1:2) showed an increased (but not significant) proliferation (±10%) as compared to background proliferation (PBMC). However, when the number of UCMS increased (1:1 → 5:1), T-cell proliferation decreased to background level.

The suppressive capacity of the UCMS was further demonstrated in co-cultures with aCD3-stimulated PBMC (suppression assay, Figure 10B). At low stem cell numbers a similar proliferative response of T-cells was found (compare Figure 10A and 10B). Low numbers of stem cells increased T cell proliferation with approximately 10% compared to control (PBMC+aCD3). At higher cell numbers (1:1 → 5:1), a significant reduction in aCD3 stimulated T-cell proliferation was observed.

The data presented in Figure 10 are representative for all experiments in which both UCMS and PBMC were derived from multiple donors (UCMS: n=6, PBMC: n=3) (data not shown). These observations indicate a low alloreactivity of T cells towards these stem cells and suggest that UCMS can actively suppress this response when present in sufficient amounts.

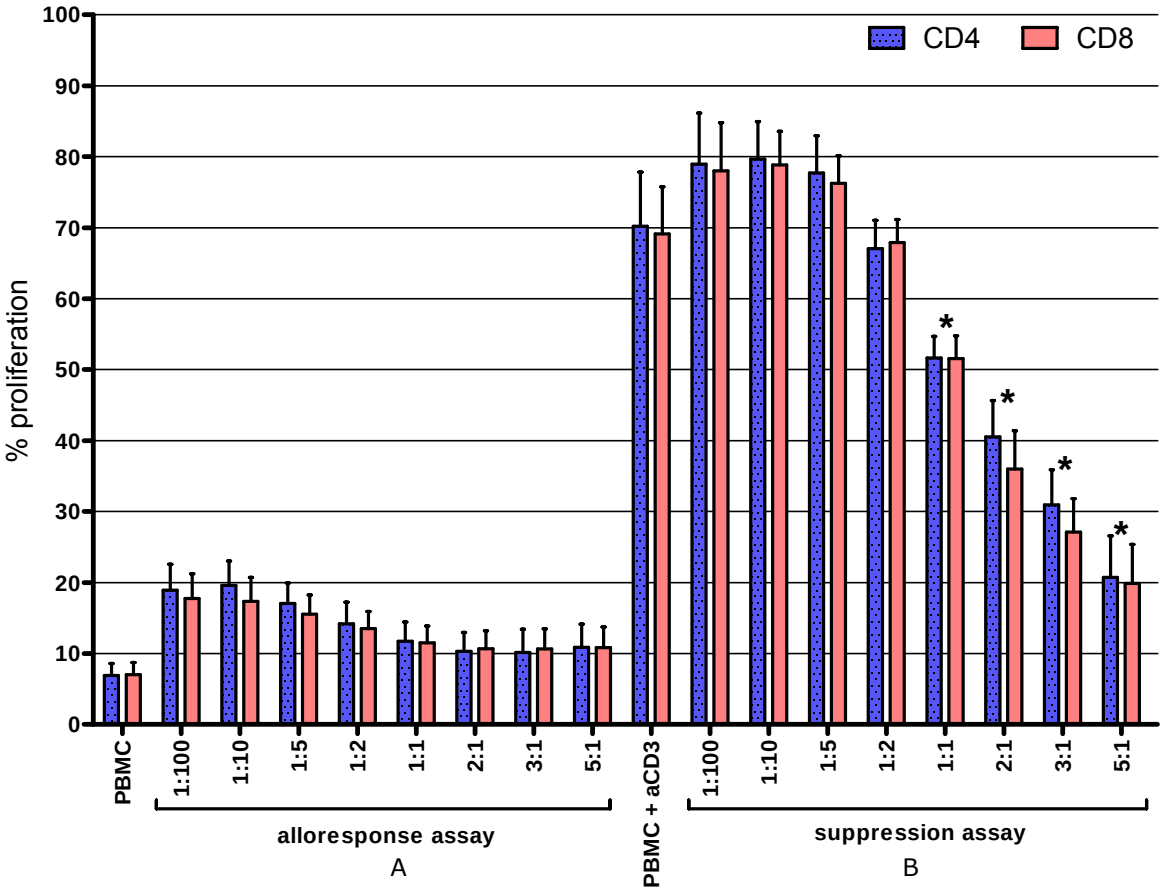

Figure 10: Proliferative response of T-cells in co-culture with UCMS. (A) Alloresponse assay: $5x10^4$ CFSE-labelled PBMC were co-cultured with increasing numbers of UCMS and the T-cell proliferation was measured after 5 days. At low cell numbers (1:100 – 1:2), stem cells show low immunogenicity. (B) Suppression assay: $5x10^4$ CFSE-labelled PBMC were stimulated with aCD3 and co-cultured with increasing numbers of UCMS. The T-cell proliferation was measured after 5 days. At high cell numbers (1:1 - 5:1) UCMS were able to significantly suppress activated T-cell proliferation in a dose dependent manner. Data were derived from 6 independent experiments and are represented as mean ±SEM. Significant differences in proliferation were determined by one way ANOVA with Tukey post test. * p-value < 0,05.

3.3 Assessment of potential mechanisms involved in T-cell suppression

The observed decrease in T cell proliferation could be a consequence of cell death. Due to the fact that more stem cells are present, the culture medium could be depleted of growth factors more rapidly. Viability of PBMC (T-cells) in co-culture was assessed with 7-AAD staining. No differences in viability were observed between PBMC in co-culture with stem cells and PBMC cultured alone (data not shown).

To determine whether cell-cell contact between PBMC and UCMS was required for T cell suppression, PBMC (+/- aCD3) were cultured in the upper chamber of a transwell, separated by a semi-permeable membrane from the UCMS which were cultured in the lower chamber. No allogeneic response against the stem cells could be observed (Figure 11A). However, UCMS were able to suppressed activated T-cell proliferation (Figure 11B). Similar to the response depicted in Figure 10, proliferation of activated T-cells was suppressed by high numbers of stem cells. Yet, the only significant decrease in proliferation was seen for the $CD4^+$ T-cells in condition 5:1 (compared to 1:10).

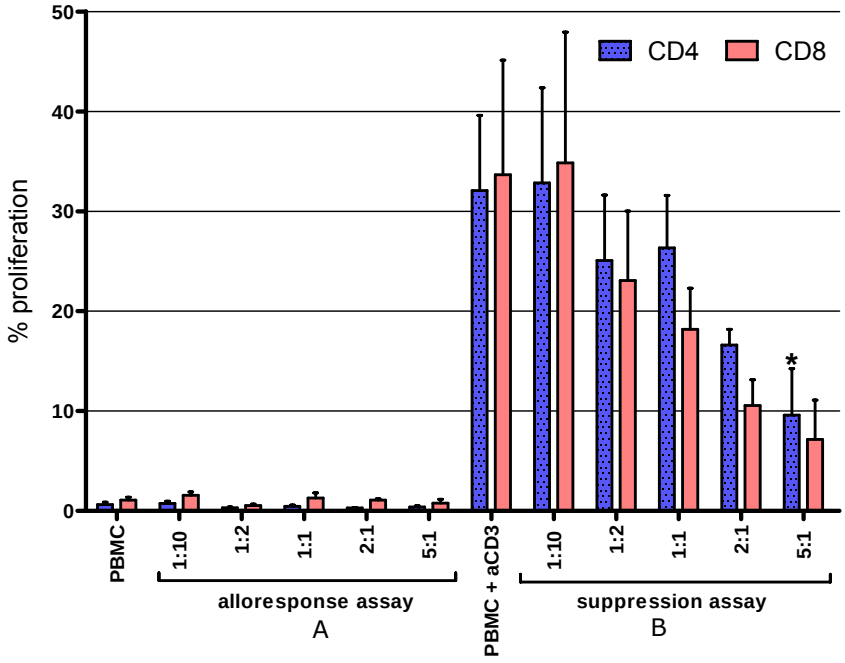

Figure 11: Proliferative response of T-cells cultured in a transwell system with UCMS. (A) Alloresponse assay: $5x10^4$ CFSE-labelled PBMC were co-cultured with increasing numbers of UCMS and both cell populations were physically seperated using a semipermeable membrane. T-cell proliferation was measured after 5 days. No allogeneic response was detected. (B) Suppression assay: $5x10^4$ CFSE-labelled PBMC, stimulated with aCD3, were co-cultured with increasing numbers of UCMS and both cell populations were physically seperated using a semipermeable membrane. T-cell proliferation was measured after 5 days. At high cell numbers (1:1 - 5:1) UCMS were able to suppress activated T-cell proliferation. Data were derived from 6 independent experiments and are represented as mean ±SEM. Significant differences in proliferation were determined by one way ANOVA with Tukey post test. * p-value < 0,05.

Because transwell experiments indicated the possible involvement of soluble factors, the cytokine profile of the supernatants collected from the co-cultures was examined. The production of IFN-γ, IL-10 and TGF-β1 using standard sandwich ELISA was analyzed. In addition the nitrite content of the supernatants was analyzed to determine NO production.

IFN-γ production was induced by aCD3 activation which correlates with aCD3 induced PBMC proliferation (Figure 12). However much more IFN-γ production was seen in the conditions of the

suppression assays compared to aCD3 activated control PBMC, whereas no IFN-γ was produced in the allogeneic setting. Addition of a low number of stem cells significantly increased the production of IFN-γ compared to both control conditions. Nevertheless, conditions with increasing stem cell numbers contained significantly less IFN-γ compared to 1:100.

No TGF-β1 or NO was detected in the supernatants of 6 independent co-culture experiments (transwell & cell-cell contact: n=3, data not shown). On the other hand, IL-10 production was found in all stem cell conditions (Figure 13). Surprisingly, IL-10 production reached its maximum level of 136 pg/ml at low stem cell numbers (1:5), and shows on average, a production between 60-100 pg/ml throughout all different stem cell amounts (conditions 1:5 → 5:1). No increased production was seen with increasing stem cell numbers.

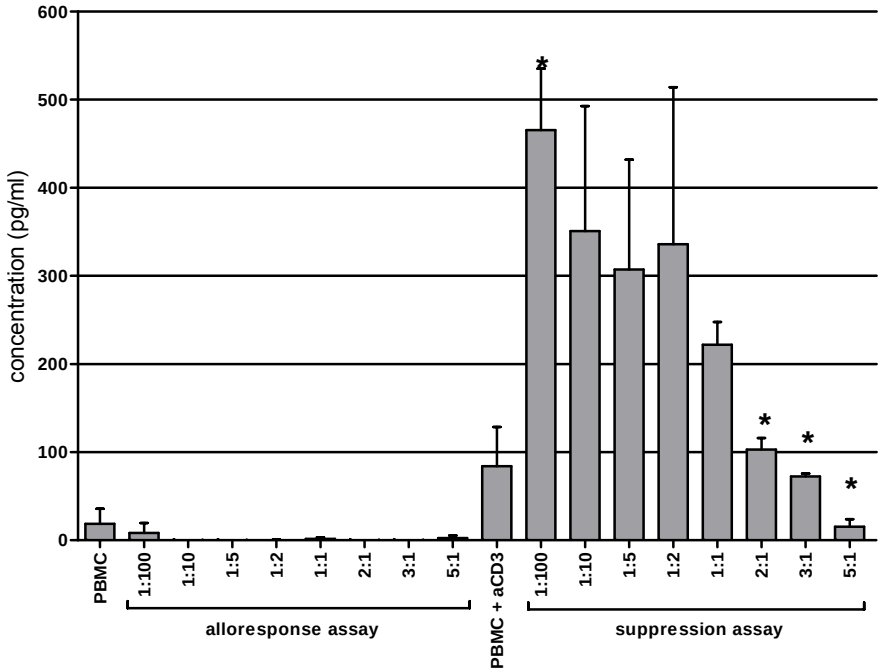

Figure 12: Production of IFN-γ during co-culture. The concentration (pg/ml) of IFN-γ for each co-culture condition is given. Cytokine content is significantly increased in the activated T-cell conditions. The addition of UCMS 1:100 induced significantly higher amounts of IFN compared to control (PBMC & PBMC+aCD3). When the stem cell number was increased, IFN-gamma content significantly decreased. Data were derived from 6 independent experiments and are represented as mean ±SEM. Significant differences in cytokine concentration were determined by one way ANOVA with Tukey post test. * p-value < 0,05.

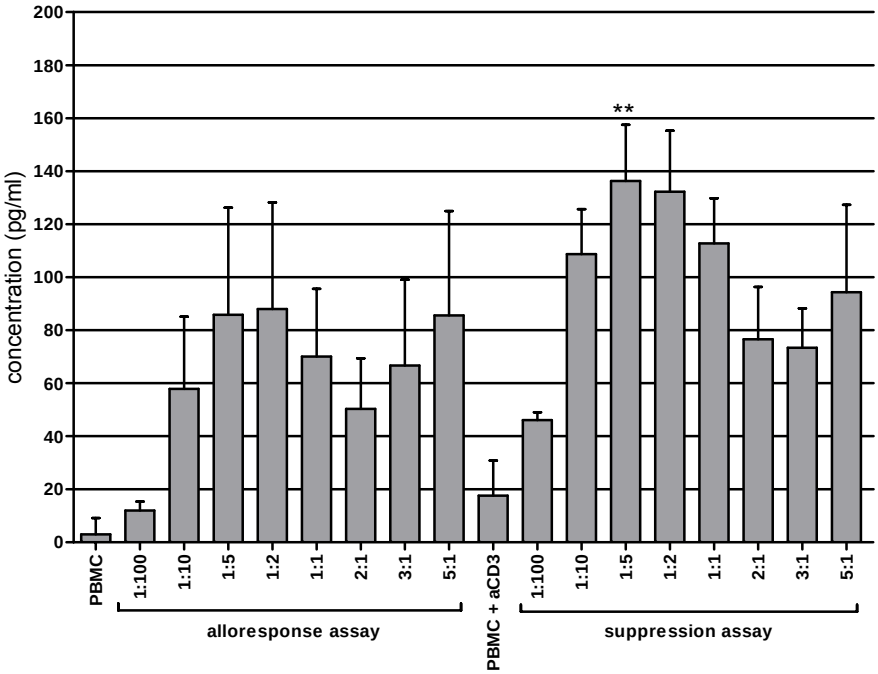

Figure 13: Production of IL-10 during co-culture. The concentration (pg/ml) of IL-10 for each co-culture condition is given. IL-10 production reached a maximum level of 136 pg/ml at UCMS 1:5, and shows on average, a production between 60-100 pg/ml throughout all different stem cell amounts (conditions UCMS 1:5 → 5:1). Data were derived from 6 independent experiments and are represented as mean ±SEM. Significant differences in cytokine concentration were determined by one way ANOVA with Tukey post test. * p-value < 0,05.

To investigate whether the immunosuppressive effect is abrogated when the stem cells are inactivated, a co-culture experiment with irradiated stem cells was performed. As shown in Figure 14A, no allogeneic immune response was observed. T-cell proliferation decreased at higher stem cell numbers (Figure 14B). However, as the stem cells are inactivated, a contact dependent mechanism that suppresses T-cells is likely to be involved as well.

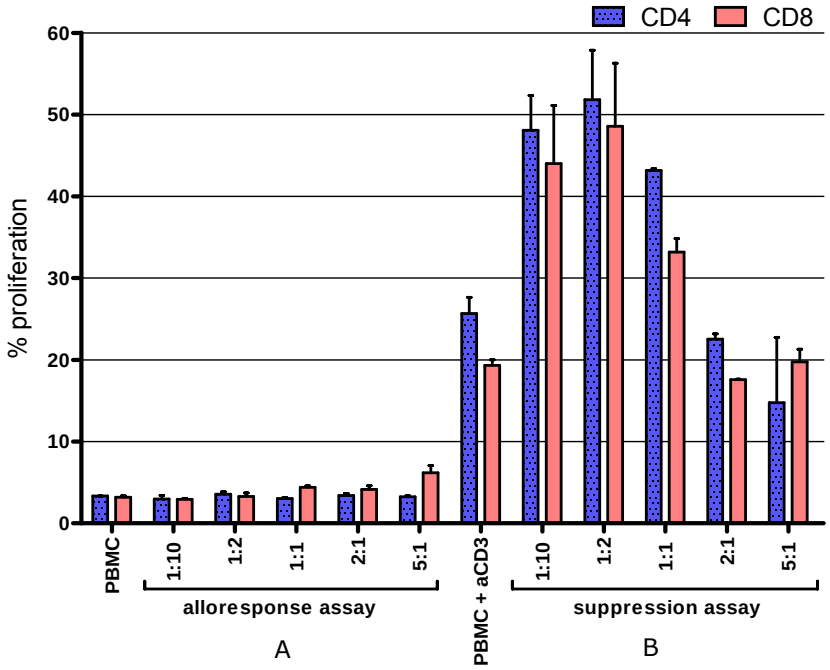

Figure 14: Proliferative response of T-cells in co-culture with irradiated UCMS. $5x10^4$ CFSE-labelled PBMC were co-cultured with irradiated stem cells (30Gy) and the T-cell proliferation was measured after 5 days. No allogeneic immune response was observed (PBMC+UCMS accolade) and activated T-cell proliferation decreased at higher stem cell numbers. Data were derived from 1 experiment and are represented as mean ±SEM.

Discussion

MSC show great potential to be used as a stem-cell based therapies in degenerative disorders. However, before therapeutic use can be considered it is important to evaluate the characteristics of the candidate stem cells in vitro first. Findings in vitro may provide new insights in our knowledge regarding the stem cell's behaviour. Unexpected cellular responses could have severe clinical implications e.g. tumour formation. In this setting, the present study aims to investigate the neural differentiation capacity and immunological properties of stem cells derived from the umbilical cord matrix (Wharton's jelly). The ultimate goal would be to use these cells as a stem-cell based transplantation therapy for MS.

The stem cells that were isolated from the umbilical cord matrix were characterized as mesenchymal stem cells as they expressed several mesenchymal surface markers and showed multilineage differentiation potential into bone, fat and cartilage. Cells were found positive for CD29, CD44, CD146, CD105 and HLA-class I and did not express CD14, CD31, CD45 and HLA class II. This expression pattern was found to be similar to that of BM-MSC (49). The analysis's that were performed in the present study met in part the minimum criteria for defining MSC, set by the International Society for Cellular Therapy (13). These are as follows: the cells (a) are plastic adherent under standard culture conditions, (b) express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR, (c) differentiate into osteoblasts, adipocytes and chondroblasts in vitro. From the findings in the present study and other reports (48, 50) it was concluded that the isolated cells showed a mesenchymal phenotype.

After the initial characterization, a neural differentiation was performed using either bFGF. bFGF is an important regulator in (neural) stem cell renewal and differentiation and was reported to induce neuronal differentiation in BM-MSC (19). Differentiation was initially assessed in culture by observing the morphology of the cells. No obvious morphological differences could be seen during differentiation. Afterwards the cells were analyzed for the expression of neuronal markers (NeuN, NF, β-tubulin III, nestin) and GFAP (glial marker). Solely, nestin and β-tubulin III expressed was observed in both treated and control conditions. The expression of nestin appeared to be slightly increased after bFGF treatment however the amount remained very low (<5%). Tropel and coworkers found that bFGF increased the number of nestin positive cells and induced neuronal cell types that could respond to glutamate and veratridine (classical neuronal activators) within 1 week. They treated BM-MSC in poly-lysine coated dishes, since neuronal cells are classically cultured as such, and reported that fibronectin-coating yielded poor morphological differences after treatment. As no coating was applied to the culture system in the present study, perhaps no morphological differences could be seen. This was also reported by Lian et al., who used bFGF in a pre-induction step before inducing differentiation with Salvia Milthiorrizha. No obvious morphological changes were observed and the UCMS were only weakly positive for nestin, β tubulin III , GFAP and neurofilament. In the current study, differentiation was induced in normal culture conditions as well as under low-serum conditions, as this was reported to enhance neuronal differentiation capacity (15). However, no differences were found regarding marker expression or morphology. It is likely

that differentiation did not work because the UCMS did not express the bFGF receptor. In the future bFGF receptor expression could be addressed with Western blot prior to differentiation.
Another possibility that could explain the unsuccessful differentiation is the fact that the umbilical cord harbors subpopulations of MSC-like cells. It has been reported that 2 types of cells are present in umbilical cord derived primary cultures of which the type II cells show a greater neurogenic potential (45). It is also possible that cells with less replicative potential (senescent cells) were present in the cultures at time of differentiation (51). In parallel with the bFGF experiments the Hyclone AdvanceSTEM^{TM} neural differentiation medium was tested. We did not find any difference in morphology or marker expression after treating the stem cells for 1 week. Because of these poor results is was decided that no TEM images were taken, as it was very likely we would not see any differences at the ultrastructural level.
In conclusion, neural differentiation of UCMS using bFGF or a commercially available differentiation medium failed. Nevertheless, this does not indicate that the UCMS hold no potential to differentiate into neural cells. To this end, further characterization of their neural differentiation capacity is essential.

In a second aim of this study was to assess the immunomodulatory properties of UCMS.
Immunophenotypical analysis of UCMS showed that the stem cells expressed HLA class I and lacked class II and costimulatory molecules. Yet, exposure to interferon gamma increased class I and *induced* class II expression. These observations are consistent with current findings by others and suggest that UCMS have an immunoprivileged status (50, 52). It has been reported that HLA class II antigens are present intracellularly and can be induced by interferon gamma to be expressed on the cell surface (13). In the context of allogeneic transplantation, the expression of class II molecules could prone the UCMS to immune recognition. However, experiments performed by other research groups showed that IFN-γ treated MSC did not induce an immune response (50). Considering these results and because of the lack of HLA class II in normal cultured stem cells and the absence of co-stimulatory molecules, a allogeneic T-cell response would not be expected upon challenging PBMC with unactivated UCMS (not exposed to IFN-γ).

In co-cultures with HLA-mismatched PBMC it was tested whether UCMS would initiate an allogeneic response. At low stem cell numbers they induced more proliferation of $CD4^+$ and $CD8^+$ T-cells (10% increase). When PBMC and UCMS were co-cultured in a transwell setup, in which both cell populations were physically separated by a semi-permeable membrane, this response was not observed. In addition, it was found that with increasing stem cell number the T-cell proliferation reduced to background levels. These data suggest that the UCMS are capable of providing an MHC-restricted first signal to T-cells and that they can actively suppress the alloantigen-induced T-cell proliferation. The group of Magatti et al. reported similar findings with amniotic mesenchymal tissue cells. Furthermore they implicated the expression of HLA-DR in a sub fraction of the stem cells as a potential cause for the observed proliferation with low stem cell numbers (53). It is reasonable to believe that this could also be the case for UCMS, which already have been reported to be a heterogeneous stromal cell population (51).

To further characterize the observed suppression, the UCMS were co-cultured with aCD3 stimulated PBMC (inducing activated T-cells). At low cell numbers a similar allogeneic response could be observed (10% increased proliferation). However when more stem cells were present in the co-culture, the proliferation of the activated T-cells was suppressed in a dose dependent manner. This observation could also be seen in the transwell setup, suggesting the possible involvement of soluble T-cell inhibitory factors. Indeed, cytokine analysis of the co-culture supernatants showed production of IL-10 in the stem cells conditions. IL-10, TGF-β1 and NO are well-known inhibitors of T-cell activation and proliferation (24, 54). Yet, both TGF-β1 and NO were not detected in the co-culture supernatant.

These assays revealed that UCMS are capable of suppressing activated T-cell responses in vitro. Whether this suppressive function acts directly on the T-cells or is mediated via other immune cells present in the PBMC population (e.g. antigen presenting cells) is not yet known. MSC have been reported to escape from the immune system via limited expression of allo-antigens (e.g. HLA class II), modulation of host dendritic cell function or induction of regulatory T cells (see table 2).
Sato et al. put forward the suppressive action of NO, which required initial activation of the MSC by cytokines. Whether the suppressive capacity of UCMS was induced by exposure to inflammatory cytokines, needs to be determined (24, 55). In the present experiment, activation of the stem cells with IFN-γ did not appear to be necessary to induce the observed T-cell suppression. In addition, IFN-γ production gradually decreased, as more stem cells were used in co-culture. The observations correlated with a reduced T-cell activity rather than with stem cell activation. In addition the supernatants of the co-cultures did not contain NO, indicating that this pathway of immune suppression was not expressed.

In addition to the above mentioned immunological assays, a pilot experiment which involved a co-culture of PBMC with irradiated UCMS, showed a similar decline in T-cell proliferation as was observed in the transwell assays. Yet in this setup, the stem cells were inactivated by a dose of 30 Gy of gamma irradiation. This could implicate that the factor affecting T-cell proliferation is both expressed as a soluble molecule and membrane bound protein. A recent report of Weiss and colleagues showed expression of an HLA-G isoform in UCMS. HLA-G, which is a non classic MHC class I molecule, is both expressed at the cellular membrane and secreted as a soluble molecule, and has been reported to be involved in inhibiting various immune cells (26). It is possible that HLA-G accounts for the observed suppression in both the co-culture/transwell assays and the co-culture containing irradiated UCMS.

In conclusion, the results of the immunological experiments indicate that UCMS have only limited alloreactivity and can suppress the proliferative response of (activated) T-cells at high cell numbers. This suggests that UCMS have immunomodulatory functions and thus may be useful in allogeneic cell therapy.

Future research could further address the mechanism(s) involved in immune suppression (e.g. HLA-G) and investigate whether the UCMS show immunomodulatory properties in vivo (EAE). Also, in an animal model it would be possible to test for the migration capacity of UCMS, which could

shed a light on whether or not the cells are able to home to the inflamed lesions. A site-directed delivery (intracisternal injection) or systemic delivery (intravenous injection) strategy could be addressed. However, due to the multifocality of MS it is rather impossible and certainly dangerous to inject stem cells directly into lesions of human patients. In this setting, it is certainly interesting to investigate whether the stem cells can be genetically engineered to express specific receptors (e.g. adhesion molecules, chemokine receptors). Additionally it would be appealing to asses the interaction between the stem cells and different types of immune cells. e.g. interaction between UCMS and dendritic cells or regulatory T-cells, as those two cell types play a central role in the induction of immunity and tolerance (56). Moreover the response of NK cells when exposed to UCMS could be determined. Besides T-cells, NK cells play an important role in transplantation immunology. Depending on the level of HLA class I expression, interactions of NK and UCMS might affect the survival of the stem cells and their persistence after allotransplantation.

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Supplemental information

S1. Supplemental materials and methods

S1.1 TEM

UCMS grown on plastic coverslips (Thermanox®; Nunc, New York, USA) were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, Pennsylvania, USA) in 0,05M cacodylate buffer (pH 7,3). After two washes with cacodylate buffer, cells were post fixed with 2% osmium tetroxide for 1 h, washed again and stained with 2% uranyl acetate in 10% acetone during 20 min. Next, cells were dehydrated in a graded acetone series and imbedded in epoxy resin (Araldite) according to the pop-off method (57). Ultra-thin sections (0,06 µm) were mounted on 0,7% formvar-coated grids, contrasted with uranyl acetate followed by lead citrate. The grids were examined with a Philips EM 208 transmission electron microscope. Images were acquired with a Morada CCD camera and processed with iTEM-FEI software (Olympus SIS, Münster, Germany).

S1.2 Viability of PBMC

Viability of PBMC in co-culture was assessed with 7-amino-actinomycin D (7-AAD; BD Bioscience) staining (according to manufacturer's instructions). Cells were processed for FACS analysis as described in Materials and methods section 5.6. with the difference that a PE-conjugated anti-CD8 antibody (Immunotools) was used. Cells were double labeled with 7-AAD and PE-conjugated anti-CD4 or anti-CD8 monoclonal antibody. Viable lymphocytes were gated based on forward/side scatter and negative 7-AAD staining.

2. Supplemental data

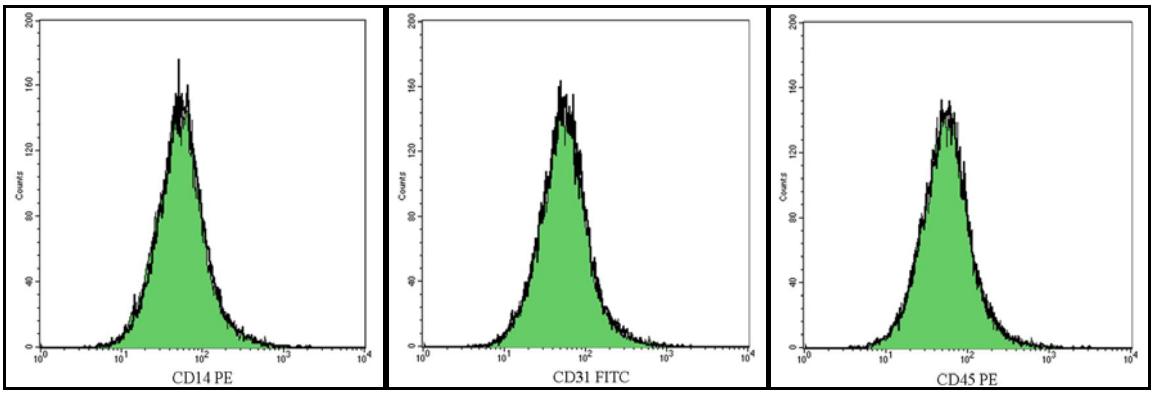

Figure S1: Flow cytometric phenotyping of UCMS. The isolated cells did not express CD14 (left), CD31 (middle) nor CD45 (right). Filled curve represents unstained control (green). Black hollow curves represent stained cells.

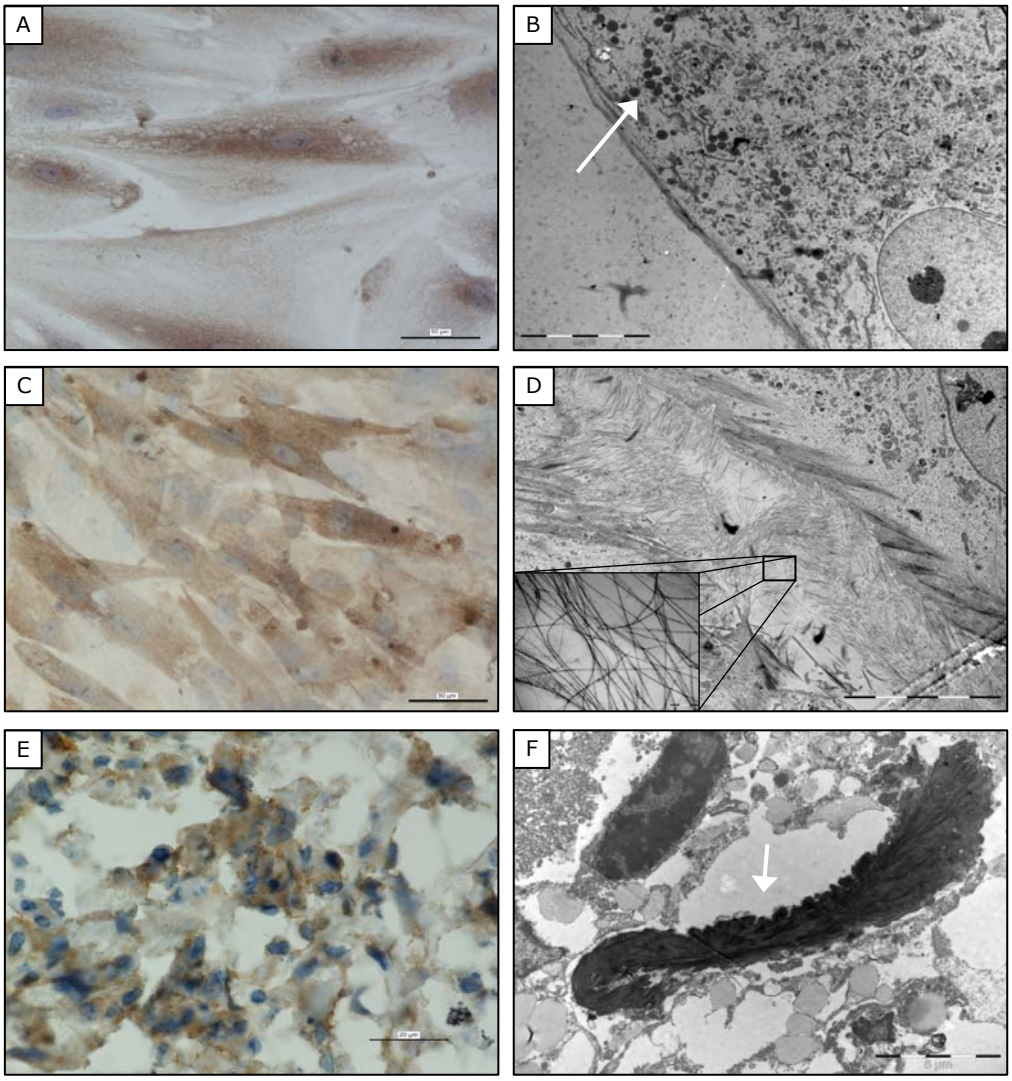

Figure S2: Confirmation of mesenchymal differentiation by ICC and TEM. A&B: Adipogenic differentiation: UCMS express FABP-4 (A) and show intracellular lipid accumulation (B, white arrow). C&D: Osteogenic differentiation: UCMS express osteocalcin (C) and secrete collagen (see insert) into the ECM (D). E&F: Chondrogenic differentiation: UCMS express aggrecan (E) and produce cartilage-like structures (F, white arrow).

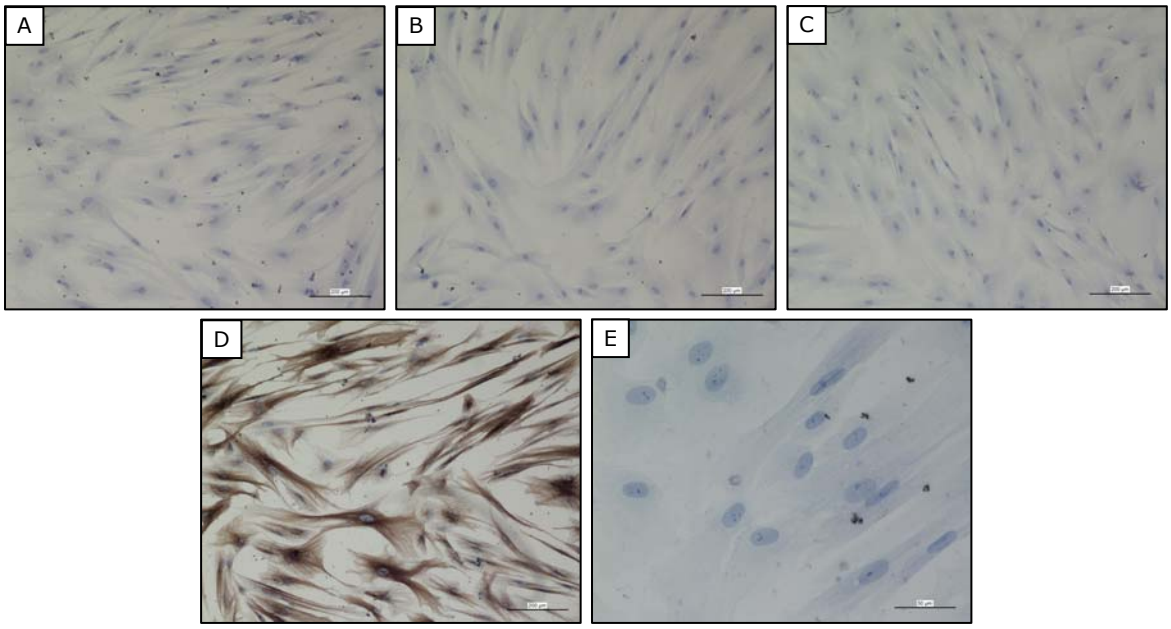

Figure S3: Neural marker staining of bFGF control cells (1%FBS). Control UCMS were grown in culture medium containing 1% FBS and subsequently stained for NeuN (A), GFAP (B), nestin (C), β-tubulin III (D), NCAM (E) & NF (F). (Scale bars A-F: 200µm).

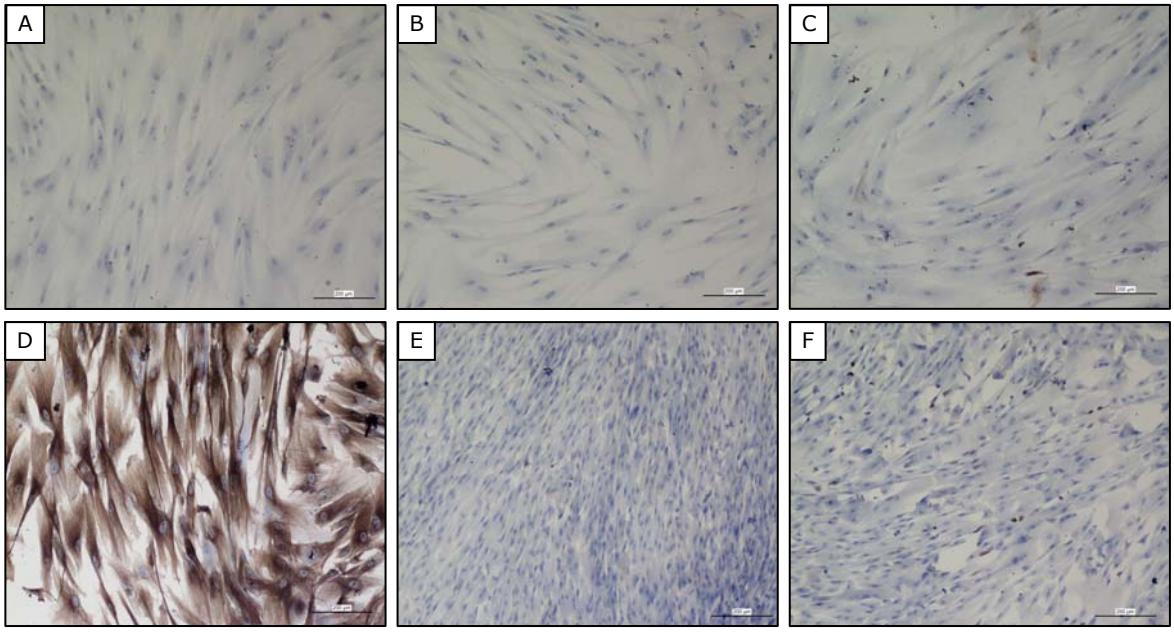

Figure S4: Neural marker staining of bFGF and Hyclone AdvanceSTEM™ control cells (10%FBS). Control UCMS were grown in culture medium containing 10% FBS and subsequently stained for NeuN (A), GFAP (B), nestin (C), β-tubulin III (D), NCAM (E) & NF (F). (Scale bars A-F: 200µm).