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THE ESTABLISHMENT OF EFFICIENT METHODS TO CULTURE IMMUNOSUPPRESSIVE MESENCHYMAL STROMAL CELLS FROM CORD BLOOD AND BONE MARROW

Anita Laitinen

ACADEMIC DISSERTATION

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III. Laitinen A, Oja S, Kilpinen L, Kaartinen T, Möller J, Laitinen S, Korhonen M, Nystedt J. A robust and reproducible animal serum-free culture method for clinical-grade bone marrow-derived mesenchymal stromal cells. Cytotechnology 2015 Mar 17. (Epub ahead of print)

IV. Kerkelä E, Laitinen A, Räbinä J, Valkonen S, Takatalo M, Larjo A, Veijola J, Lampinen M, Siljander P, Lehenkari P, Alfthan K, Laitinen S. Adenosinergic immunosuppression by human mesenchymal stromal cells requires co-operation with T cells. Stem Cells. 2016 Mar;34(3):781-90

The publications are referred to in the text by their roman numerals.

Author contribution
Anita Laitinen (AL) is fully responsible for the summary of this doctoral thesis. AL was the main author in publications I, II and III. In publication **I,** AL designed the work and established the culture method. In publication **II,** AL established the cell culture method, designed, performed and analyzed the comparative long term cell culture studies. AL performed and analyzed the immunophenotypic characterization and differentiation assays. AL also participated in the design of the angiogenesis study and designed and participated in the performance and analysis of the T cell proliferation assays. In publication **III,** AL participated in the study design and performed and analyzed the comparative short term cell culture studies presented. AL also designed, performed and analyzed most of the T cell proliferation assays and participated in the phenotypic characterizations and differentiation assays. In publication

IV, AL participated in the conception and design of the study. AL participated in the design, performance and analysis of the phenotypic characterization of the cells. AL participated in the design of nucleotide measurements and in the design, performance and analysis of T cell proliferation assays. AL also participated in the manuscript writing.

ABSTRACT

Advanced cell therapies are evolving at an enormous pace. Mesenchymal stromal cells (MSCs) have become the focus of cell based therapies due to their capacity to home to the site of infection or tissue damage, to suppress immune reactions, and to support the regeneration of tissues. Bone marrow (BM) is a rich source of MSCs but other sources, such as cord blood (CB), have been presented as an attractive alternative because of the ease of access and abundance of this material. A good culture method constitutes the basis for clinical production of MSCs. The traditional cell culture method of BM-derived MSCs (BM-MSCs) is not an efficient technique for obtaining MSCs from CB. Thus, the development of cell culture methods is necessary for this material to be utilized in MSC research and therapy. Even small changes in the environment of MSCs may alter the phenotype or functional capacity of these cells. The knowledge of the functional capacities of MSCs and conditions that impact them will help in production of specific MSCs for various indications.

The aim of this thesis was to establish a culture condition for efficient production of MSCs from human CB and to compare different good manufacturing practice (GMP)-compliant methods to establish a robust method to culture human BM-MSCs in platelet lysate (PL)-containing culture medium. The impact of culture conditions, both culture media and oxygen (O_2) concentration, on the characteristics and functionality of MSCs was studied. Another aim of this thesis was to gain knowledge about the immunosuppressive mechanisms of MSCs.

The culture method to obtain MSCs from human CB established in this thesis was efficient in obtaining MSCs from almost 90% of processed CB-units. This figure is much higher than presented previously in literature. As a result of the comparison of two different GMP-compliant culture methods for obtaining adequate numbers of BM-MSCs, we identified a robust method to culture MSCs for clinical purposes. The results from culturing MSCs in different O_2 concentrations indicated that low O_2 concentrations are beneficial for the MSC proliferation at late passages but this supportive impact is minimal at early passages. Culturing the cells in different media was demonstrated to impact the phenotype and functional capacities of MSCs. The expression of cell surface markers CD90 and HLA-DR was altered by different culture media. The functional capacities of MSCs were also influenced by different culture media. CB-MSCs cultured in medium without dexamethasone (DX) and containing only small amounts of growth factors showed a higher capacity to support angiogenesis than MSCs cultured in the presence of DX and in growth factor-rich medium. On the other hand, MSCs cultured in the presence of DX and

growth factor-rich medium were more efficient in suppressing T cell proliferation.

In this thesis, a novel mechanism for human MSCs and MSC-derived extracellular vesicles (MSC-EVs) to suppress T cell proliferation via adenosine (Ado) production was demonstrated. Ado was efficiently produced from ATP by the action of CD73 molecule on MSCs in concert with CD39 expressing T cells.

In conclusion, this thesis presents an efficient method for obtaining MSCs from CB and a GMP-compliant method for culturing BM-MSCs for clinical use. The culture medium and O_2 concentration have an effect on the proliferative potential, surface characteristics and functionality of MSCs, and they should be carefully studied when altering the culture protocols. This thesis also presents a novel immunomodulatory mechanism of MSCs, mediated by the activity of cell surface molecule CD73.

ABBREVIATIONS

Ado	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AT	Adipose tissue
AT-MSCs	Adipose tissue-derived mesenchymal stromal cells
ATMP	Advanced therapy medicinal products
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSCs	Bone marrow-derived mesenchymal stromal cells
CAT	Committee for Advanced Therapies, at EMA
CB	Cord blood
CB-MSCs	Cord blood-derived mesenchymal stromal cells
CD	Cluster of differentiation
CFU-F	Colony-forming unit –fibroblasts
CM	Conditioned medium
CO_2	Carbon dioxide
DC	Dendritic cell
DX	Dexamethasone
EGF	Epidermal growth factor
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine, adenosine deaminase inhibitor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESC	Embryonic stem cell
EV	Extracellular vesicle
FBS	Fetal bovine serum
FN	Fibronectin
GMP	Good manufacturing practice
GVHD	Graft versus host disease
HLA	Human leucocyte antigen
HOX	Homeobox
HPLC	High-performance liquid chromatography
HSC	Hematopoietic stem cell
IDO	Idoleamine-2,3-oxygenase
IFNγ	Interferon gamma
IL	Interleukin
iPS cell	Induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
M1	Medium 1
M2	Medium 2

M3	Medium 3
MAPC	Multi potent adult progenitor cell
MC	Mononuclear cell
MIAMI cells	Marrow-isolated adult multi-lineage inducible cells
MSC	Mesenchymal stromal/stem cell
MSC-EV	Mesenchymal stromal cell-derived extracellular vesicles
MV	Microvesicle
NK cell	Natural killer cell
O_2	Oxygen
PBMC	Peripheral blood mononuclear cell
PD	Population doubling
PDGF-BB	Platelet derived growth factor-BB
PL	Platelet lysate
PRP	Platelet rich plasma
qRT-PCR	Quantitative real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
sCTMP	Somatic cell therapy medicinal product
StdM	Standard growth medium
TEP	Tissue-engineered product
TGF	Transforming growth factor
TLR	Toll like receptor
Treg	Regulatory T cell
UC	Umbilical cord
UC-MSCs	Umbilical cord-derived MSCs
USSC	Unrestricted somatic stem cells
VEGF	Vascular endothelial growth factor

1 REVIEW OF THE LITERATURE

1.1 CELL THERAPIES

Cell therapy is defined as the administration of live cells into a patient with the intention to replace, augment, or modify the function of patients' cells that are diseased, dysfunctional, or missing. Cell therapy is another therapeutic application to complement the traditional pharmaceuticals, biopharmaceuticals, and medical devices in treatment of patients (Mason et al. 2011). Cell therapies can be conducted using either autologous or allogenic cell products. In autologous cell therapy, the patient is treated with his or her own cells in contrast to allogenic cell therapy which requires the transplantation of cells from a donor to a patient (Buckler et al. 2016).

The field of cell therapies has grown enormously during last decades and it is still steadily expanding (see Figure 1). The worldwide stem cell therapy market is estimated to grow at a rate of 39.5% from 2015 to 2020 (Buckler et al. 2016). Once limited to blood and bone marrow (BM) transplantation and reproductive *in vitro* fertilization, the field of cell therapies has moved towards advanced cell therapies. Cell therapies cover a broad range of specialties and applications, divided in permanent and transient cell therapy categories. The permanent cell therapies include the replacement of damaged tissue with functional cells such as damaged cornea replaced with corneal epithelial stem cells. The transient cell therapies include therapies such as the immunomodulatory therapy provided by adult stem cells (Mason et al. 2011).

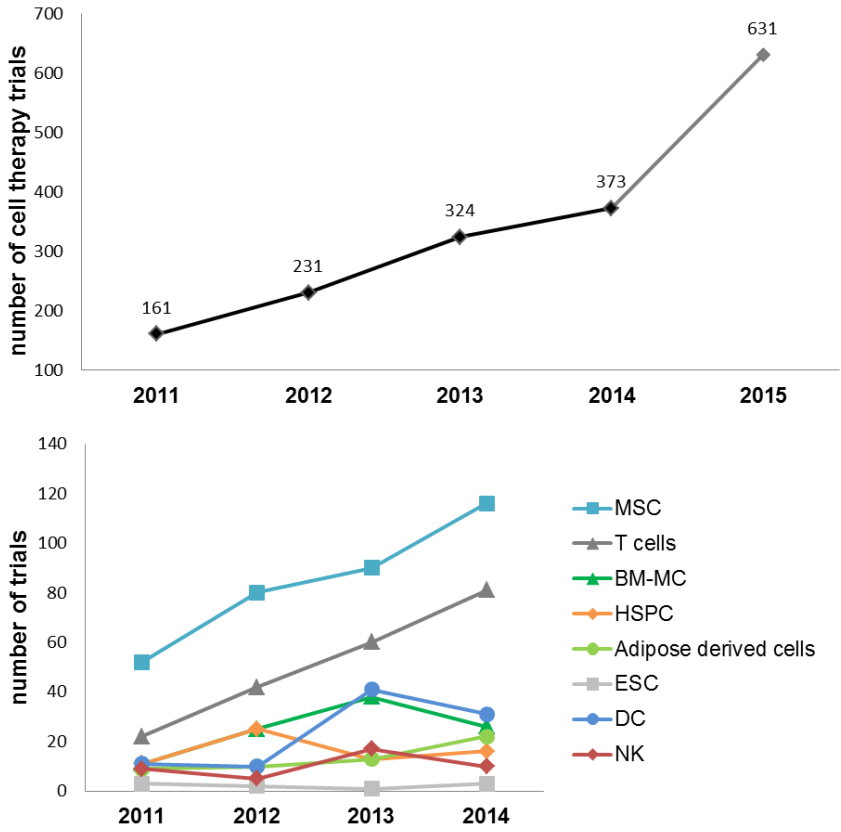

Figure 1 Number of cell therapy trials. **(A)** The total number of cell therapy trials from 2011 to 2015. The total number of cell therapy trials was 631 in 2015 according to Alliance for Regenerative Medicine. **(B)** The number of cell therapy trials by cell type from 2011 to 2014 modified from Bersenev 2015. Data from Bersenev 2015 (Bersenev 2015) and from Annual Data Report by Alliance for Regenerative Medicine (http://alliancerm.org/sites/default/files/ARM_Annual_Report_2015_Web_Version_FINAL.pd). Abbreviations: MSC, mesenchymal stromal cells; BM-MC, bone marrow mononuclear cells; HSPC, hematopoietic stem/progenitor cells; ESC, embryonic stem cells; DC, dendritic cells; NK, natural killer cells.

1.1.1 CELL TYPES IN CELL THERAPIES

Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst stage of an embryo (Evans and Kaufman 1981; Thomson et al. 1998). These rapidly proliferating pluripotent cells can be propagated *in vitro* indefinitely in particular culture conditions. The characteristics of cultured ESCs include the expression of some pluripotent markers (NANOG, SOX2, OCT4, and KLF4) and the cells have active telomerase and alkaline

phosphatase enzymes. Undifferentiated ESCs form teratomas (tumors with differentiated structures of ectoderm, mesoderm, and endoderm origin) when injected into an immune-deficient mouse, indicating their capacity to differentiate towards all three embryonic germ layers. ESCs can also be induced to differentiate into a range of committed somatic cells. Finding appropriate culture conditions to achieve efficient cell differentiation is one of the active areas within the ESC field (Alvarez et al. 2012).

Although ESCs have properties that sound promising for regenerative medicine and tissue replacement after an injury or a disease, these cells have several disadvantages in cell therapies. These problems include ethical issues, teratoma formation, and immune reactions after transplantation (Jung 2009). However, some clinical trials have been conducted, the first being a treatment of patients with acute spinal cord injury by Geron corporation. Geron Corporation started a phase I trial in 2010, using ESC-derived oligodendrocytes and treated 4 of the 10 planned patients before stopping the trial for economic reasons (Scott and Magnus 2014). In addition, some other phase I trials with ESC-derived cells are ongoing for treating macular degeneration (Mead et al. 2015).

Induced pluripotent stem cells

In 2006, Takahashi and Yamanaka (Takahashi and Yamanaka 2006) described the creation of induced pluripotent stem (iPS) cells. These cells share similar characteristics with ESCs including self-renewal capacity and the capacity to differentiate into various cell types. These cells were initially generated from adult somatic cells by retroviral delivery of four transcription factors: Oct4, Sox2, Klf4, and cMyc (Takahashi and Yamanaka 2006). Since then, it has been demonstrated that most of these four factors can be substituted with different factors, though some key factors such as OCT4 cannot be omitted (Yamanaka and Blau 2010).

The use of virus vectors to create iPS cells may produce insertional mutations, thus resulting even in tumor genesis (Hyun et al. 2007). To overcome this, Yu et al. presented a vector and transgene free system to induce iPS (Yu et al. 2009). However, despite the recent progress in designing protocols that reduce the risk of viral integrations and oncogene expression in generation of iPS cells, there are still technical challenges in the production of these cells and safety concerns regarding their use in clinical applications which require solving before clinical use (Ji et al. 2016).

While iPS cells possess similar pluripotent potential as ESCs, they do not have the same ethical problems as ESCs as they are derived from adult cells. They hold great potential for regenerative medicine as patient specific iPS cells can be generated to overcome the immune rejection typical of ESCs. iPS cells can also be used to model human diseases and screen drug candidates *in vitro*. In 2013, a clinical trial for macular degeneration with autologous iPS was started by Japanese RIKEN Center for Developmental Biology. The trial

was halted one year later due to mutations in second product and regulatory changes. The company also changed their strategy, deciding to move forward with allogenic iPS cells (http://stemcellassays.com/2015/09/first-ips-cell-clinical-trial-insights/).

Adult stem / progenitor cells

Adult stem and progenitor cells, including tissue specific stem cells, are multipotent cells found in differentiated tissues. These cells have a self-renewal capacity and the capacity to differentiate into the specialized cell types of the tissue. In contrast to ESCs, the differentiation capacity of adult stem cells is often restricted to certain lineages. Progenitor cells are described to have a more limited differentiation and self-renewal capacity than adult stem cells. The primary role of adult stem and progenitor cells is to maintain and repair the tissue where they reside.

The best known adult stem cell type is the hematopoietic stem cells (HSCs), which reside in the BM and form all types of blood cells. HSCs are routinely used for therapeutic purposes as BM transplantations have been conducted for several decades (Storb 2003). Another adult stem cell or progenitor cell type, initially found in BM (Friedenstein et al. 1968), is mesenchymal stromal/stem cells (MSCs). They are multipotent adult cells found in virtually all tissues of the body (Crisan et al. 2008). These multipotent cells have been called by several names due to their unique characteristics noticed *in vitro*. Besides MSCs, these multipotent cells include unrestricted somatic stem cells (USSC), marrow-isolated adult multi-lineage inducible (MIAMI) cells, multi potent adult progenitor cells (MAPCs), and very small embryonic-like (VSEL) cells (D'Ippolito et al. 2004; Jiang et al. 2002; Kogler et al. 2004; Kucia et al. 2006). These cells have been demonstrated to have several unique features but it is still not clear if they are hierarchically related to each other and constitute overlapping populations of early-development stem cells or whether they are just a consequence of differential culture procedures (Suszynska et al. 2014).

Several adult cell based products are already on the market. Holoclar® (Holostem Advanced Therapies), a stem cell therapy product, was the first advanced therapy medicinal product (ATMP) containing adult stem cells approved in the European Union (EU). This product consists of patient derived limbal stem cells and is used for the treatment of limbal stem cell deficiency due to physical or chemical burns of eyes (http://www.eurostemcell.org/story/europe-approves-holoclar-first-stem-cell-based-medicinal-product). Another tissue specific product with market approval in Europe is ChondroCelect® (TiGenix), which contains autologous *in vitro* expanded chondrocytes for repair of cartilage defects of the knee (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000878/WC500026031.pdf.).

Immune cells

Several immune cell types have potential for use in cell therapies. Widely studied cell types in this field include T cells, natural killer (NK) cells, and dendritic cells, with the main focus on malignant and infectious disease therapies.

In T cell therapies, T cells are cultured and/or engineered *ex vivo* and adoptively transferred into a patient, or T cells can be directly targeted by vaccination or biological compounds (Guo et al. 2015). T cell therapies include the use of pathogen specific T cells, tumor infiltrating leucocytes (TIL) (Rosenberg et al. 1988), cytotoxic T lymphocytes (CTL) against virus associated malignancies (Schuessler et al. 2014), and genetically engineered T cells such as chimeric antigen receptor (CAR) T cells (Gill et al. 2016). Pathogen-specific T cells include virus-specific T cells against viruses such as cytomegalovirus (CMV) (Einsele et al. 2002) and fungus-specific T cells (Deo and Gottlieb 2015). Donor lymphocyte infusion (DLI) is used to tackle a hematopoietic malignancy relapse after HSC transplantation (Chang and Huang 2013). Regulatory T cells (Treg) have also been used in clinic to reduce the incidence of graft versus host disease (GVHD) (Trzonkowski et al. 2009).

Besides T cells, also other immune cells have been found to be applicable in cell therapies. Early clinical and preclinical studies have indicated that NK cells have great potential for targeting tumor cells. Autologous and allogenic NK cells have been demonstrated to be safe and without toxic effects in several clinical studies aimed at targeting tumors. The major risk involved in the use of allogenic NK cells is the development of GVHD. Although NK cell therapies represent a promising therapy against cancer, clinical trials have not clearly demonstrated their benefits in patients with malignancies (Dahlberg et al. 2015).

Dendritic cells (DCs) have also been used as immunotherapeutic agents against tumors. DC vaccines have been used in several clinical trials in cancer patients and sipuleucel-T (Provenge®, Dendreon Corporation), a product approved by authorities has shown significant survival advantage in metastatic castration-resistant prostate cancer (Mantia-Smaldone and Chu 2013).

1.2 MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs) have been studied since 1960s, pioneered by Friedenstein (Friedenstein et al. 1968). He managed to culture a plastic adherent fibroblastic cell type from BM, which he named colony-forming unit -fibroblasts (CFU-F) (Friedenstein et al. 1976). Since then, these cells have been studied worldwide, with several names given to them (Charbord 2010; Horwitz et al. 2005). In 1991, these cells were named mesenchymal stem cells (Caplan 1991). Later on, International Society for Cellular Therapy

(ISCT) recommended that they be named multipotent mesenchymal stromal cells, using the same acronym MSC (Horwitz et al. 2005). This name was recommended as these cells do not literally fulfill the criteria for a true stem cell. MSCs have not been demonstrated to be capable of producing a tissue *in vivo*. A true stem cell should have self-renewal capacity, the capacity to produce differentiated progeny, and, when transplanted, they should be capable of regenerating a tissue.

Since the initial finding of MSCs from BM, these cells have been derived from various sources (Erices et al. 2000; Gronthos et al. 2000; in 't Anker et al. 2004; Wang et al. 2004; Zuk et al. 2002). They can be found in nearly all adult tissues where they are mostly located in the perivascular areas (Crisan et al. 2008). Actually, these cells share similarities with pericytes and thus there is a theory that the *in vivo* niche of MSCs is the perivascular area and the cultured MSCs are descendants of periendothelial cells (Caplan 2008; da Silva Meirelles et al. 2008).

MSCs are a heterogeneous population of cells containing just a small proportion of cells with stem cell characteristics. MSCs are defined as having certain characteristics. The minimal criteria defined by the ISCT (Dominici et al. 2006) are that these cell should be positive for CD90, CD73, and CD105 and negative for hematopoietic lineage cell surface markers such as CD34, CD14, CD45, CD19, and HLA-DR (see Table 1). They ought to have the capacity to differentiate into adipocytes, chondrocytes, and osteoblasts and they must be plastic adherent. These criteria were established as markers for human BM-derived and fetal bovine serum (FBS) cultured MSCs and were set as a basis for additional characterization. Other markers commonly expressed on MSCs derived from different tissues are the molecules CD13, CD29, CD44, CD166, and HLA I (Hass et al. 2011; Lv et al. 2014), see Table 1.

Table 1. *Cell surface markers commonly identified in MSCs and their expression on other cell types.*

Cell surface protein	activity/function	expression on other cells	expression on MSCs
CD13	zinc metalloproteinase	fibroblasts, endothelial cells, granulocytes, monocytes and mast cells	+
CD14	receptor for endotoxin (LPS)	macrophages, monocytes, granulocytes, dendritic cells and B-cells	- *
CD19	signal transduction molecule	B cells and follicular dendritic cells	-*
CD29	cell adehesion molecule (integrin β-1)	fibroblasts, platelets, T cells, monocytes, granulocytes, mast cells, endothelial cells and myoepithelial cells	+
CD34	cell-cell adhesion factor	hematopoietic stem and progenitor cells	-*
CD44	involved in cell-cell interactions, cell adhesion and cell migration	on most mammalian cell types	+
CD45	tyrosine phosphatase	hematopoietic cells	-*
CD49e	integrin alpha 5 subunit (join with β-1 unit)	T cells, thymocytes, B cells, platelets, monocytes and neutrophils	+
CD73	5'-nucleotidase	T cells, B cells, follicular dendritic cells, epithelial cells and endothelial cells	+*
CD90	cell-cell and cell-matrix interactions	hematopoietic stem cells, neurons, fibroblasts, thymocytes, follicular dendritic cells, lymph node HEV endothelium	+*
CD105	regulatory component of TGF-β receptor	endothelial cells, activated monocytes and tissue macrophages, pre B cells	+*
CD166	adhesion molecule	neurons, activated T cells, activated monocytes, epithelium, fibroblasts	+
HLA-ABC	major histocompatibility complex class I	all nucleated cells and platelets	+
HLA-DR	major histocompatibility complex class II	B cells, T cells, monocytes, macrophages and NK cells	-*

* criteria according to ISCT

1.2.1 FUNCTIONAL MECHANISMS OF MSCs

Beside the tri-lineage differentiation capacity of the MSCs described above, these cells have been demonstrated to have several other capabilities. These capabilities include the diverse plasticity to differentiate, the capacity to support the growth and differentiation of stem and progenitor cells (especially HSCs), the immunosuppressive and the angiogenic supporting capacity of the cells and the anti-apoptotic effects on other cells (Bronckaers et al. 2014; da Silva Meirelles et al. 2009). The mechanisms behind MSCs' functioning in cell therapies can be divided into two categories: permanent cell replacement and soluble factor mediated mechanisms (see Figure 2).

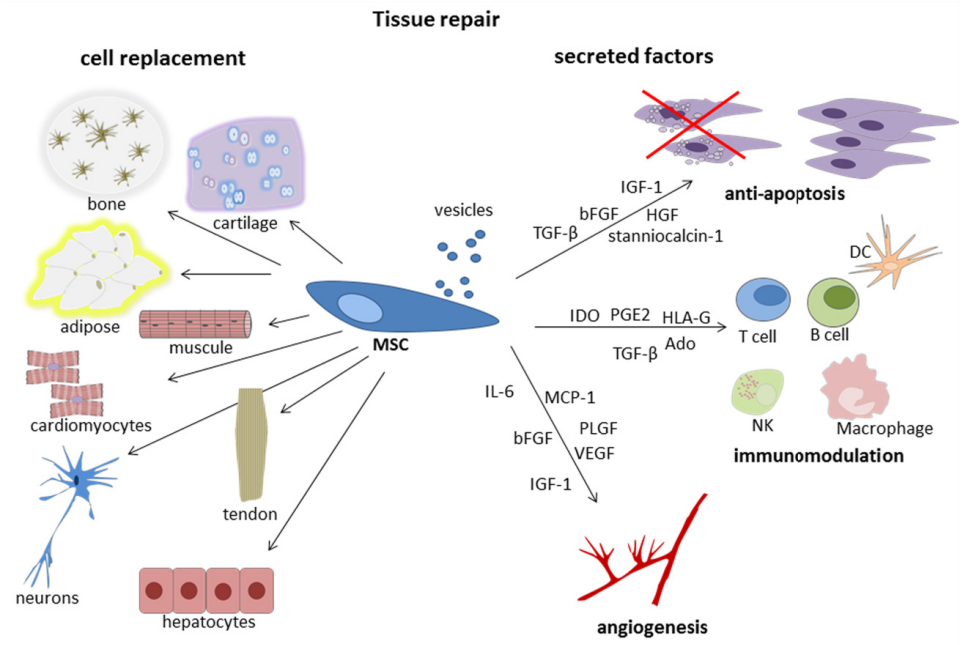

Figure 2 Functional mechanisms of MSCs. MSCs have the capacity to differentiate into different cell types at least *in vitro*. This capacity has been employed in permanent cell replacement in tissue repair. The regenerative benefits of MSCs have been demonstrated to be mainly consequences of the secreted factors.

1.2.1.1 Tissue repair by cell replacement and differentiation

MSCs have been demonstrated to have the capacity to differentiate, at least *in vitro*, into mesodermal, endodermal and ectodermal lineages. Besides the osteogenic, chondrogenic, and adipogenic differentiation, mesodermal differentiation into skeletal muscle, tendon, myocardium, smooth muscle, and endothelium has been demonstrated (Dezawa et al. 2005; Kuo and Tuan 2008; Makino et al. 1999; Oswald et al. 2004; Ross et al. 2006). The ectodermal and endodermal differentiation into epithelial cells, neurons, and

hepatocytes has been shown to occur in certain conditions (Lee et al. 2004a; Spees et al. 2003; Woodbury et al. 2000). However, the non-mesodermal differentiation of the cells is controversial and it has not been demonstrated convincingly *in vivo* (Strioga et al. 2012). Some of the *in vivo* trans-differentiation observations on MSCs have been shown to be due to the fusion of the MSCs with the contacted cells, and not via differentiation of MSCs (Alvarez-Dolado et al. 2003). Thus, the *in vitro* differentiation potential of MSCs does not necessarily correlate with the *in vivo* differentiation potential of the cells.

Due to the *in vitro* differentiation capacity of MSCs, it was initially hypothesized that the differentiation capacity of MSCs would be the curative mechanism of these cells in tissue regeneration/engineering. It was envisioned that the transplanted cells would directly replace the damaged tissue. Later on, the regenerative benefits of MSCs have been demonstrated to be mainly a consequence of the paracrine mechanisms of MSCs, rather than a result of their differentiation (Bronckaers et al. 2014; Noiseux et al. 2006; Togel et al. 2005). In tissue engineering, such as in repair of extensive bone defects, MSCs have been successfully used when seeded into scaffolds yielding a 3D bioimplant (Quarto et al. 2001; Sandor et al. 2014). The soluble factors secreted by MSCs together with osteoconductive biomaterials have shown to synergistically favor the production of good constructs (Wolff et al. 2013).

1.2.1.2 Secreted soluble factors

Immunomodulation

The discovery of the immunosuppressive capacity of MSCs has provided a potential tool for down-regulating the unwanted immune reactions. As MSCs do not express cell surface MHC II molecules without stimulation and they do not express co-stimulatory molecules CD80, CD86, or CD40 (Tse et al. 2003), they are often said to be immune privileged. Nowadays it is, however, known that also MSCs are recognized by the host immune system and they may stimulate the immune cells (Ankrum et al. 2014). MSCs have been demonstrated to induce the proliferation of allogenic unstimulated lymphocytes (Karlsson et al. 2012; Le Blanc et al. 2003b) and the production of antibodies by B cells (Rasmusson et al. 2007; Traggiai et al. 2008). Allogenic MSCs have also been shown to cause a rejection of BM transplant if co-transplanted to sub-lethally irradiated mice. They can also induce a memory T cell response (Nauta et al. 2006). Thus, MSCs should be called “immune evasive”, rather than immune privileged (Ankrum et al. 2014).

MSCs are able to suppress many types of immune cells of the innate and adaptive immune system. The overall picture of how MSCs influence the immune system is not yet clear, but an enormous number of different possible factors and mechanisms functioning via both soluble and cell

contact-dependent interactions have been demonstrated to be responsible for the immune modulatory effects. Several of the factors are listed in table 2. Contact dependent mechanisms such as PD-1 – PD-L1/2, Jagged1-Notch1, and Fas-Fas-L interactions in T and B cells have also been implicated (Akiyama et al. 2012; Augello et al. 2005; Liotta et al. 2008)

MSCs may influence the activation, differentiation, proliferation, and the cytokine production of T cells, B cells, NK cells, DCs, and macrophages via diverse mechanisms. MSCs have been shown to influence the expression of co-stimulatory molecules on antigen presenting cells (Beyth et al. 2005). They have also been demonstrated to inhibit the degranulation of mast cells (Brown et al. 2011) and there are indications that they influence the apoptosis of immune cells (Glenn and Whartenby 2014). As MSCs promote the macrophage polarization into the immunosuppressive M2 subset (Cho et al. 2014; Francois et al. 2012; Kim and Hematti 2009), they influence the creation of anti-inflammatory milieu and thus also reduce the migration of inflammatory cells to the site of tissue damage.

The immunosuppressive effects of MSCs have mostly been studied with T cells (van den Akker et al. 2013). Studies have shown that the suppressive effect of MSCs on T cell proliferation is enhanced when MSCs are pre-exposed to effector cytokines such as interferon gamma (IFNγ), TNFα, and interleukin (IL)-1β (Chang et al. 2006a; Le Blanc et al. 2003a; Ren et al. 2008). Besides their suppressive effect on the proliferation of T cells, MSCs also suppress the IFNγ production of T_{h1} cells and promote the secretion of IL-4 by T_{h2} cells, while also increasing the amount of regulatory Tregs (Aggarwal and Pittenger 2005; English et al. 2009).

MSCs have been reported to express several toll-like receptors (TLRs) (Delarosa et al. 2012; van den Akker et al. 2013) and the activation of these innate immune system associated receptors may contribute to the polarization of MSCs into an anti- or pro-inflammatory phenotype (Waterman et al. 2010). Although, as there are many conflicting results regarding the effects of TLRs (Munir and McGettrick 2015), more research will be necessary to clarify their role in MSC immunomodulation properties.

Extracellular nucleotides (such as ATP, ADP, UTP, and UDP) are mediators of intercellular communications in virtually all tissues and they are important factors in cell stress (Burnstock 2006). These nucleotides are degraded into nucleosides, such as adenosine (Ado). Ado is found in every tissue and organ and it regulates their function. It has an important role in a wide variety of biochemical processes such as energy transfer and it also acts as an inhibitor in presynaptic excitatory neurotransmitter release (Borea et al. 2016). Ado mediates cardioprotective, neuroprotective, vasodilatatory, and angiogenic responses and it counteracts the inflammatory/stress signal triggered by adenosine triphosphate (ATP) (Scarfi 2014). Ado is either released directly by cells or generated by de-phosphorylation of adenine nucleotides. This de-phosphorylation is performed by two ectonucleotidases, CD39 and CD73 (Scarfi 2014). The CD39 CD73 mediated adenosinergic

pathway has been demonstrated to be an important machinery used by Tregs to inhibit T cell proliferation and cytokine secretion (Deaglio et al. 2007). Recently this pathway has also been shown to have a relevant function in MSCs, as demonstrated with mice MSCs (Sattler et al. 2011). Chen et al. demonstrated that human gingiva-derived MSCs suppress the proliferation of murine CD4+CD25- T cell proliferation via the CD39 CD73 pathway (Chen et al. 2013). Recently, Amarnath et al. demonstrated the Ado signalling to be responsible for the therapeutic effect in a xenogeneic mouse GVHD model (Amarnath et al. 2015). The role of MSCs' Ado pathway has also been indicated in autoimmune responses in experimental autoimmune uveitis model in mice (Chen et al. 2016). Also, *in vitro* studies showing that human MSCs impact the suppression of human T cell functions via the Ado pathway have been published (Chen et al. 2016; Lee et al. 2014; Saldanha-Araujo et al. 2011).

As there are many different mechanisms behind the immunomodulatory effects of MSCs, it is most probable that a combination of different pathways will provide the optimal effect in each certain situation.

Angiogenesis supporting effects

Angiogenesis means the formation of new blood vessels from the pre-existing vasculature. Angiogenesis is a fundamental physiological event in the development of an individual and in wound and fracture healing (Bronckaers et al. 2014). Angiogenesis is a tightly regulated process initiated only in response to specific stimuli such as inflammation and hypoxia. The induction of endothelial cells to start a new branch of blood vessel requires the liberation of pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and stromal derived factor-1 (SDF-1). Angiogenic factors are naturally released during the proteolytic breakdown of extracellular matrix (ECM) of the capillaries (Bronckaers et al. 2014). MSCs secrete numerous angiogenic factors (Table 2) including VEGF, bFGF, placental growth factor (PLGF), monocyte chemoattractant protein-1 (MCP-1), IL-6, and insulin like growth factor-1 (IGF-1) (da Silva Meirelles et al. 2009).

Studies have shown that various conditions and factors have an impact on the capacity of MSCs to secrete angiogenic factors. It has been demonstrated that the induction of MSCs with chemokines and growth factors, such as transforming growth factor (TGF)-α and angiotensin II, induce the MSCs to secrete several angiogenic factors. Additionally, the conditioned medium (CM) of the induced cells is more efficient in supporting angiogenesis than the CM of non-treated MSCs (De Luca et al. 2011; Liu et al. 2015). According to studies, the angiogenic supporting capacity of MSCs is also enhanced by hypoxic environment (Aranha et al. 2010; Chen et al. 2008). In addition, serum-deprivation has been shown to increase the capacity of MSCs to be angiogenic (Oskowitz et al. 2011).

Studies have shown MSCs to be involved in all steps of angionenesis *in vitro*. They induce endothelial cell proliferation, migration, invasion, and tube formation, and they have also been indicated to protect endothelial cells from apoptosis (Bronckaers et al. 2014). The capacity of MSCs to stabilize the newly formed vessels in a manner similar to pericytes has supported the pericyte theory (Caplan and Correa 2011). Besides the secretion of angiogenic factors, MSCs have also been demonstrated to differentiate into endothelial cell-like cells at least *in vitro* (Oswald et al. 2004), and this differentiation can be induced by several treatments of MSCs (Bekhite et al. 2014).

Table 2. *Soluble factors secreted by MSCs (non-comprehensive)*

Factors in	Targets	References
immunomodulation		
Idoleamine-2,3-oxygenase (IDO)	T cells, macrophages, NK cells	(Francois et al. 2012; Meisel et al. 2004; Spaggiari et al. 2008)
prostaglandin E2 (PGE2)	macrophages, DC, T cells, Mast cells, NK cells	(Aggarwal and Pittenger 2005; Brown et al. 2011; Spaggiari et al. 2008)
IL-6	neutrophils, DC, macrophages	(Djouad et al. 2007; Raffaghello et al. 2008; Zhang et al. 2010)
GM-CSF	macrophages	(Zhang et al. 2010)
IL-10	T cells	(Qu et al. 2012)
TGF- β1	Treg	(Patel et al. 2010)
Nitric oxide (especially in mice)	T cells	(Shi et al. 2011)
heme oxygenase-1	T cells	(Chabannes et al. 2007)
HLA-G5	T cells	(Selmani et al. 2009)
Galectin-3	T cells	(Sioud et al. 2010)
Adenosine (Ado)	T cells	(Saldanha-Araujo et al. 2011)
angiogenesis and growth support		
VEGF	endothelial cells	(Kinnaird et al. 2004; Rehman et al. 2004)
IGF-1	endothelial cells	(Togel et al. 2007)
bFGF	endothelial cells	(Kinnaird et al. 2004)
PLGF	induction of vessel formation	(Kinnaird et al. 2004)
IL-6	endothelial cells	(Zhang et al. 2013)
MCP-1	endothelial cells	(Boomsma and Geenen 2012)
SDF-1	hematopoietic stem/progenitor cells	(Van Overstraeten-Schlogel et al. 2006)
angiopoietin 1	endothelial cells	(Wu et al. 2007)
anti-apoptosis		
VEGF	endothelial cells	(Rehman et al. 2004; Togel et al. 2007)
HGF	endothelial cells	(Rehman et al. 2004; Togel et al. 2007)
IGF-1	endothelial cells	(Togel et al. 2007)
stanniocalcin 1	epithelial cells, fibroblasts	(Block et al. 2009)
TGF- β	endothelial cells, cardiomyocytes	(Rehman et al. 2004; Wang et al. 2009)
bFGF	endothelial cells	(Rehman et al. 2004)

Extracellular vesicles

Besides the secretion of soluble molecules such as immunomodulatory and angiogenic factors, MSC have been described to secrete extracellular vesicles (EVs), subtyped as microvesicles (MVs) and exosomes. MVs are generated by budding of the cell membrane and they are 50 nm - 1,000 nm in size. Exosomes, which were originally thought a by-product of cell turnover, are slightly smaller (40-100 nm) or of the same size. Exosomes are thought to be of endosomal origin and to have homogenous characteristics with defined biophysical and biochemical properties, e.g. the constitution of their lipid membrane is well characterized (Madrigal et al. 2014; Rani et al. 2015). The types of different vesicles are not clearly defined and thus the nomenclature of these vesicles is confusing and the same term can mean different things to different investigators. It has been suggested that while there is no consensus about the nomenclature, the preferred term for these vesicles would be EVs (Gould and Raposo 2013).

EVs can be collected from all types of body fluids and also from cell culture supernatants. EVs facilitate the communication between cells by transferring bioactive molecules and they are involved in normal physiological processes. Liberated EVs may be internalized by other cells via endocytosis or cell type specific phagosytosis. Extracellular signals, such as hypoxia and inflammation, have been demonstrated to regulate the protein packing into EVs and their release from MSCs (Rani et al. 2015). EVs may also have a role in the development and progress of diseases. EVs have been linked to tumorigenesis, spread of viruses and pathogenic agents. In addition, factors involved in neurodegenerative diseases have been associated with EVs (Iraci et al. 2016).

The advantageous effect of MSC-derived EVs (MSC-EV) on several diseases has been demonstrated in several animal models. There is also evidence from animal models that points to their beneficial role in cutaneous wound healing and in the healing of myocardial infraction, acute kidney injury (AKI), liver diseases, and lung diseases (Arslan et al. 2013; Bruno et al. 2009; Kilpinen et al. 2013; Li et al. 2013; Timmers et al. 2007; Zhang et al. 2015; Zhu et al. 2014). MSC-EVs have also been demonstrated to have immunomodulatory capacities (Mokarizadeh et al. 2012) and anti-tumor activities, and their role in drug delivery has also been studied (Pascucci et al. 2014; Wu et al. 2013).

In animal models, MSC-EVs have been shown to specifically accumulate in the site of injury (Grange et al. 2014). This has also been seen with MSCs, although they are, at least first, trapped in the lungs when administered intravenously (Gao et al. 2001; Kerkela et al. 2013; Mahmood et al. 2003; Nystedt et al. 2013; Schrepfer et al. 2007). Amarnath et al. have shown that MSCs trapped in the lungs deliver their systemic immune suppression via MSC-EVs (Amarnath et al. 2015). There has also been a case study with MSC-EVs administrated to a human to treat GVHD and the results indicated

that MSC-EVs may have potential for the treatment of GVHD (Kordelas et al. 2014).

One of the mechanisms behind the beneficial effects of MSC-EVs has been suggested to be the delivery of mRNA and miRNA (Chen et al. 2010; Tomasoni et al. 2013). Other forms of biological information transmitted by EVs include proteins, glycoproteins, and lipids. MSC-EVs are hypothesized to have advantages over MSCs in clinical use in the future (Rani et al. 2015).

1.2.2 TISSUE SOURCES

MSCs have been derived from many different tissues. The most well characterized MSCs are BM-derived MSCs (BM-MSCs). Other, well characterized MSC sources include adipose tissue (AT)[1], cord blood (CB), umbilical cord (UC), and placenta (Mattar and Bieback 2015). The afterbirth (such as placenta and umbilical cord) is considered a good source of MSCs as these tissues are abundantly available and would otherwise be biological waste. Also, excess AT removed by liposuction is a medical waste and is thus available for other uses (Strioga et al. 2012). The collection of BM is an invasive procedure which always contains a risk of infection (Hass et al. 2011), making alternative sources to bone marrow very attractive.

All MSCs are not equal and MSCs from different tissues vary. The cells are in principal similar, but some variance is seen in their differential proliferation potential, surface marker expression, and functional capacities. Also, the gene expression profiles of MSCs from different tissues have been shown to have significant differences between different tissues (Wagner et al. 2005). Along with AT-MSCs, the initial CFU-F numbers are described as the highest, as compared to BM- and CB-derived MSCs (CB-MSCs) (Kern et al. 2006). Although the initial number of MSC in CB is very low, the proliferative capacity of CB-MSCs and other afterbirth tissue-derived MSCs is reported to be higher than that of MSCs from adult tissues (Barlow et al. 2008; Kern et al. 2006). An example of the variance in the immunophenotype of MSCs from different tissues is the CD34 positivity of AT-MSCs, reported at least at early stages *in vitro* (Maumus et al. 2011; Traktuev et al. 2008), whereas MSCs from other sources are negative for this marker (see Table 1). Variance in the differentiation capacities has been observed among MSCs from different sources. CB-MSCs are reported to differentiate poorly into adipocytes *in vitro* (Bieback et al. 2004; Chang et al. 2006b; Manca et al. 2008; Montesinos et al. 2009; Yoshioka et al. 2015; Zhang et al. 2011), while *in vivo* it has been shown that dental pulp derived MSCs differentiate into dentin and not into bone as do similarly handled BM-

[1] AT-derived MSCs (AT-MSCs) are named with a variety of names and the name adipose derived stem/stromal cells (ASC) is currently the most accepted term for these cells (Lindroos et al. 2011). These cells are however named AT-MSCs in this thesis to indicate their similarity with MSCs from other sources.

MSCs (Gronthos et al. 2000). The capacity of MSCs to support the construction of functional hematopoietic niche has recently been shown to be restricted to BM-derived MSCs, as opposed to AT-MSCs and umbilical cord-derived MSCs (UC-MSCs) (Reinisch 2015).

The comparison of immunomodulatory capacities of MSCs from different tissues has been reviewed by Mattar and Bieback (2015). Although it has been reported that MSCs from all tested sources are capable of suppressing immune reactions *in vitro* there is some variability in their capacities (Mattar and Bieback 2015). In many studies, AT-MSCs, for instance, have been demonstrated to have enhanced immunosuppressive capabilities compared to BM-MSCs, though conflicting results also exists. The heterogeneity in the experimental protocols makes it difficult to compare the immunosuppressive capabilities of MSCs from different sources (Mattar and Bieback 2015). The angiogenic supporting potential of MSCs has also been described to vary between different sources. AT-MSCs have been demonstrated to have significantly greater angiogenic potential than BM-MSCs (Kim et al. 2007). As these results are to some extent conflicting and most of the comparative studies have been conducted *in vitro*, the real difference in the capacities of MSCs from different sources still needs to be studied further to establish whether one source is more beneficial than another in therapy.

1.2.3 CLINICAL USE OF MSCs AND CLINICAL TRIALS

The basis for using MSCs for clinical purposes relies on their immune regulation capacity, hematopoiesis, and tissue regeneration supporting functions, as well as on their migratory capacity. Numerous promising *in vitro* studies with animal models have rapidly made MSCs a major focus of cell-based therapies (see Figure 1).

In the first trials, autologous MSCs were utilized in the treatment of patients suffering from hematological malignancies, without any indications of adverse effects (Lazarus et al. 1995). Osteogenesis imperfect was the first disease treated with allogenic MSCs in a clinical study (Horwitz et al. 2002). The effects of allogenic MSCs have also been studied in a clinical trial with patients with inborn metabolic errors (Koc et al. 2002). Although the clinical results of the first trials did not clearly demonstrate the efficacy of MSCs they were important as they provided preliminary evidence that MSCs can be administered without adverse effects, indicating the safety of MSC therapy (Singer and Caplan 2011).

Since then, the number of clinical trials has increased enormously, with the number of completed or ongoing clinical trials being nearly 350 in 2013 (Ankrum et al. 2014), and rising close to 500 in 2015 (Squillaro et al. 2016). The current number of clinical trials is 652 including completed, terminated, and ongoing studies (data from clinicaltrials.gov, March 2016, with search terms “Mesenchymal Stem cells”, “Mesenchymal Stromal Cells”, “Multipotent stromal cells”, and “bone marrow stromal cells”). Most of the

clinical trials occur, at an early phase, which demonstrates that the therapeutic effectiveness still needs to be proven. Based on the first systematic review and meta-analysis that comprehensively summarizes the safety of systemic MSC administration, this therapy appears to be safe (Lalu et al. 2012). The long term safety of MSC based cell therapies is still poorly investigated, which hinders the translation of these therapies into clinical practice (Squillaro et al. 2016).

GVHD is an adverse condition where the transplanted cells attacks host tissues and organs and it occurs in 30-80% of recipients after allogeneic hematopoietic stem cell transplantation (Squillaro et al. 2016). The capacity of MSCs to treat GVHD has been extensively studied since the first case study reported by Le Blanc et al. in 2004 (Le Blanc et al. 2004). Data from most studies suggest that MSCs are effective for GVHD (Ankrum et al. 2014; Sharma et al. 2014; Squillaro et al. 2016), although also conflicting data exists (Parekkadan and Milwid 2010; von Bonin et al. 2009). Besides GVHD, MSCs have been used to treat several other diseases and conditions including cardiovascular diseases, neurologic diseases, autoimmune diseases, organ transplantation, wound healing, and defects in bone and cartilage (Table 3).

Table 3. *Examples of reported clinical trials and pilot studies with MSCs*

target of therapy	MSC type	Reference
supporting function HSC transplantation	autologous BM-MSC, HLA-identical/haploidentical-related BM-MSC	(Macmillan et al. 2009; Meuleman et al. 2009)
GVHD	allogenic BM-MSCs, identical/haploidentical-related BM-MSC, allogenic AT-MSCs	(Arima et al. 2010; Fang et al. 2007; Kebriaei et al. 2009; Le Blanc et al. 2004; Le Blanc et al. 2008; Ringden et al. 2006; Zhao et al. 2015)
stroke	autologous BM-MSC	(Bang et al. 2005; Honmou et al. 2011; Lee et al. 2010)
myocardial infarction	allogenic/autologous BM-MSC	(Chen et al. 2004; Hare et al. 2009; Mohyeddin-Bonab et al. 2007; Yang et al. 2010)
kidney transplantation	allogenic AT-MSCs, autologous BM-MSC	(Perico et al. 2011; Vanikar et al. 2011)
chronic obstructive pulmonary disease (COPD),	allogenic BM-MSCs	(Weiss et al. 2013)
osteogenesis imperfecta	allogenic BM-MSCs	(Horwitz et al. 2002)
cartilage lesions	autologous BM-MSC	(Wakitani et al. 2011)
wound healing	autologous BM-MSC	(Falanga et al. 2007)
liver diseases	autologous BM-MSC, allogenic UC-MSC	(Kharaziha et al. 2009; Mohamadnejad et al. 2007; Zhang et al. 2012)
Crohn's disease	allogenic BM-MSCs autologous AT-MSCs, autologous BM-MSC	(de la Portilla et al. 2013; Duijvestein et al. 2010; Garcia-Olmo et al. 2005; Liang et al. 2012)
inborn metabolic disorders	allogenic BM-MSCs	(Koc et al. 2002)
systemic lupus erythematosus (SLE)	allogenic UC-MSC, allogenic BM-MSC	(Liang et al. 2010; Sun et al. 2010)
multiple sclerosis	autologous BM-MSC	(Karussis et al. 2010; Llufriu et al. 2014)
Parkinson's disease	autologous BM-MSC	(Venkataramana et al. 2010)
amyotrophic lateral sclerosis (ALS)	autologous BM-MSC	(Karussis et al. 2010; Kim et al. 2014; Mazzini et al. 2003)
spinal cord injury	autologous AT-MSCs	(Ra et al. 2011)
cerebral palsy (CP)	autologous BM-MSC	(Wang et al. 2013)

It has also been suggested that because of their capacity to migrate to tumour environment, MSCs may be useful as cellular vehicles for targeted delivery of chemotherapeutics into tumours (Adjei and Blanka 2015). Virus

transduced MSCs have already been used in clinical studies to deliver tumour destroying virus into tumours such as neuroblastoma (Garcia-Castro et al. 2010). Although MSCs themselves are not shown to be tumorigenic, (Tarte et al. 2010) their tumour migrating capacity and immunosuppressive effects may also be a threat in a case that the recipient has an un-known tumour when treated with MSCs. In animals with a pre-existing tumour, MSCs have been shown to promote tumour growth (Djouad et al. 2006). Thus, there are still several issues to resolve before all the potential held by MSCs and their possible adverse effects in clinical settings are known.

1.3 CULTURE OF MSCs

There are several methods for culturing MSCs. The beginning of the culture usually starts with the isolation of mononuclear cells (MCs) when using tissues such tissues as BM, CB, or peripheral blood as starting material. These sources do not need to be mechanically or enzymatically treated, as do such sources as adipose tissue, placenta, and umbilical cord (Dehkordi et al. 2015; Gimble and Guilak 2003; Parolini et al. 2008). MSC cultures have also been established by enriching first certain cell populations using magnetic selection (Tondreau et al. 2005; Zhang and Chan 2010). Although these selections may enrich MSCs, the resulting cell populations are still heterogeneous and the majority of the isolated cells do not give rise to CFU-F (Ho et al. 2008).

Cell isolation is followed with plating of the cells. Cells are most often plated on uncoated culture flasks made of plastic or on flasks coated with a protein, usually extracellular matrix protein (Ho et al. 2008). MSCs are assumed to adhere within a few days after initial plating, and the following media changes remove the contaminating non-adherent hematopoietic cells (Bara et al. 2014). It has been suggested that the variation between groups in these initial culture procedures may result in differences in the outcome of MSC cultures and the following cell-based therapies conducted with these cells (Bara et al. 2014).

Also seeding density and confluency of cultures have been shown to impact the properties of MSCs. MSCs seeded at low density have been shown to have the highest capacity to proliferate (Both et al. 2007; Colter et al. 2000). It has also been demonstrated that if MSC cultures are grown to confluence, the proliferative capacity of the cells is diminished (Lennon et al. 2012). In contrast, the effect of culturing MSCs to their confluence does not affect their capacity to differentiate (Lennon et al. 2012). Ylöstalo et al. have shown that MSCs in the middle of a dense cell colony are partially differentiated, compared to cells in the outer part of the colony. This difference can be reversed when the cells are re-plated. When re-plated at clonal densities, the cells from the outer and inner part of the initial colony become indistinguishable (Ylostalo et al. 2008). Thus the sub-culturing of

MSCs on a regular basis seems to be important when propagating these cells (Lennon et al. 2012).

Culture conditions have been shown to impact the properties of MSCs. Many of the cell preparations given different names may just be creations of the conditions they have been cultured in. Roobrouck et al. demonstrated that MSCs and MAPCs have different properties that are at least partially mediated by culture conditions (Roobrouck et al. 2011a). Roobrouck et al. specifically showed that changing the MSC culture condition to MAPC culture condition shifted the MSC gene expression profile toward the MAPC gene expression profile and vice versa (Roobrouck et al. 2011a). Others have also demonstrated that different culture conditions change the gene expression profile of MSCs (Wagner et al. 2005) and it has been noted that slight experimental modifications can lead to the production of completely different cell population (Ho et al. 2008).

1.3.1 CULTURE MEDIA

MSCs have been cultured in a variety of different culture media. The basic media is most commonly DMEM/DMEM F12 or alpha MEM (Bara et al. 2014), supplemented with various supplements. The glucose concentration used in MSC culture media is 1,000 mg/ml (5.5 mM) which approximates normal blood sugar levels *in vivo*. This glucose concentration has been demonstrated to have better capacity to support MSC growth compared to the higher glucose concentration of 4,500 mg/ml (Sotiropoulou et al. 2006). Glutamine is an essential nutrient needed in cell culture. It is unstable, leading to formation of ammonia in cell cultures and finally to cell growth inhibition. The use of Glutamax™ (dipeptide L-alanine-L-glutamine) provides L-glutamine in a more stable form and it has proved to have better capacity to support the growth of MSCs (Sotiropoulou et al. 2006) than the use of L-glutamine. For successful cell culture, a mixture of factors for cell attachment, growth, and proliferation is also needed. FBS is the most common supplement containing these essential factors in MSC cultures. MSCs cultured from different sources seem to have different needs regarding the medium. In their studies, Wagner et al. noticed that MSCs from CB could not be obtained using the same low serum conditions with few additives as for BM-MSCs (Wagner et al. 2005). CB-MSCs seem to need more FBS or other supporting components in the medium (Kogler et al. 2004). Different supplements have been used to enhance the growth of MSCs; some of these are listed in table 4.

Table 4. *Growth supporting factors used in MSC culture media*

growth factor	cell type	reference
bFGF	CB-MSC, BM-MSC	(Gharibi and Hughes 2012; Jung et al. 2010; Lee et al. 2004b; Ng et al. 2008; Zhang et al. 2011)
platelet derived growth factor-BB (PDGF-BB)	BM-MSC	(Gharibi and Hughes 2012; Ng et al. 2008)
epidermal growth factor (EGF)	BM-MSC	(Gharibi and Hughes 2012)
dexamethasone (DX)	CB-MSC, BM-MSC	(Xiao et al. 2010; Zhang et al. 2011)
TGF-β	BM-MSC	(Jung et al. 2010; Ng et al. 2008)
stem cell factor (SCF)	CB-MSC	(Zhang et al. 2011)
ascorbic acid	BM-MSC	(Gharibi and Hughes 2012; Jung et al. 2010)
Wnt3	BM-MSC	(Gharibi and Hughes 2012)
IL-3	CB-MSC	(Zhang et al. 2011)

1.3.1.1 FBS and its substitutes

FBS has been the most commonly used supplement in MSC culture media. The concentration of FBS varies from 2% up to 30% (Kogler et al. 2004; Pittenger et al. 1999; Reyes et al. 2002), 10% being the most common concentration. Although FBS supports the MSC growth well, there are several disadvantages associated with it, one being the huge lot-to-lot variation (Tekkatte et al. 2011). The major disadvantages pertain to the safety of using FBS for culturing cells for clinical purposes. Animal-derived components hold the risk of possible contamination with viruses, bacteria, mycoplasma, yeast, fungi, and endotoxins (Tekkatte et al. 2011). It has also been demonstrated that FBS cultured MSCs may induce antibody production against FBS when administered to patients (Horwitz et al. 2002; Sundin et al. 2007).

For clinical purposes the use of xeno-free culture conditions are encouraged by regulators. The term xeno-free means a product that does not contain any animal-derived components. Several different substitutes for FBS have been tested (listed in Table 5). These medium supplements include human serum, plasma, CB serum, and platelet derivatives (Tekkatte et al.

2011). Although chemically defined medium would be the optimal solution, the design of the composition of the medium is extremely challenging since the effect of individual growth factors on MSC growth is poorly understood (Tekkatte et al. 2011). Platelet lysate (PL), which is derived from platelet rich plasma (PRP), has been the most studied FBS substitute and it is an acceptable medium supplement according to regulators (Wuchter et al. 2015). The use of PL instead of FBS does not necessarily make the culture medium xeno-free as porcine-derived heparin is usually needed to prevent the clotting of coagulation factors-containing PL. The use of heparin has been accepted as it is an approved pharmaceutical. Heparin-free medium using serum converted PL instead PL containing coagulation factors has also been demonstrated to support MSC growth (Mojica-Henshaw et al. 2013). In addition, a commercially available PL-product with reduced coagulation factors as a supplement in culture medium without heparin has already been tested in MSC cultures. The capacity of the coagulation factor reduced PL to support MSC growth might, however, be diminished (Juhl et al. 2016).

Table 5. *Pros and cons of FBS substitutes tested in MSC cultures.*

FBS substitute	+	-	reference
autologous serum	high proliferation rate	diminished differentiation, limited availability	(Shahdadfar et al. 2005)
allogenic serum	expansion kinetics comparable with FBS at early passages	inhibitory effects on growth and survival of MSCs and decelerated proliferation from p4 on	(Bieback et al. 2009; Shahdadfar et al. 2005)
thrombin activated platelet lysate	expansion kinetics comparable with FBS at early passages	decelerated proliferation from p4 on, requires fresh preparation, complicate preparation	(Bieback et al. 2009)
PL (plasma-containing)	high expansion rate, no lot-to-lot variability	need of porcine derived heparin	(Bieback et al. 2009)
serum converted PL	heparin-free culture, proliferative capacity comparable to plasma-containing PL	complicate preparation	(Mojica-Henshaw et al. 2013)
coagulation factor reduced PL (Stemulate ™)	heparin-free culture	diminished proliferation compared to PL	(Juhl et al. 2016)
CB/placental serum	High expansion capacity, availability	no attachment to plates after p3	(Julavijitphong et al. 2014; Shafaei et al. 2011; Shetty et al. 2007)

1.3.2 **OXYGEN CONCENTRATION**

In vitro culture of cells is usually performed in incubators in normal atmospheric oxygen (O_2) concentration in the presence of 5% carbon dioxide (CO_2). 5% CO_2 is optimal for the maintenance of appropriate pH conditions and it resembles the CO_2 concentration *in vivo* (Csete 2005; D'Ippolito et al. 2006). The O_2 concentration is not usually controlled due to the more complicated controlling procedures as, besides the surrounding atmospheric O_2 conditions, the O_2 tension depends on the depth of medium, density of cells, and cellular respiration (Csete 2005). Thus the standard O_2

concentration in incubators supplied by room air is 20% (Haque et al. 2013). The physiological O_2 level is significantly lower. The O_2 concentration varies from tissue to tissue, being 1% in cartilage and bone, 1-6% in BM and 4-14% in circulation, and 3-8% in placenta (Haque et al. 2013; Sullivan et al. 2006).

It has been reported that the proliferation of stem cells is enhanced and their apoptosis is reduced in low O_2 concentrations (Csete 2005). Also, the proliferation of MSCs has been demonstrated to be enhanced when cultured in low O_2 concentrations (Carrancio et al. 2008; Dos Santos et al. 2010; Estrada et al. 2012; Tsai et al. 2011). Although low O_2 concentrations enhance the proliferation of MSCs, this effect is only seen clearly at late passages (Fehrer et al. 2007; Tsai et al. 2011). Besides the supportive effect on the proliferation of MSCs, low O_2 concentrations are also known to reduce the genetic instability and oxidative stress in MSCs (Estrada et al. 2012).

A few reports propose that different cell populations are generated under low O_2 concentrations. MIAMI cells have been cultured from BM in low O_2 concentrations on fibronectin (FN)-coated culture plates in the medium supplemented with 5% FBS. These cells have been reported to have extensive proliferative capacity and a wide *in vitro* differentiation repertoire (D'Ippolito et al. 2004). The same group also cultured MIAMI cells in different O_2 concentrations (1%, 3%, 5%, 10%, and 21%) and noticed that the cells cultured in 3% O_2 concentration have the highest capacity to proliferate (D'Ippolito et al. 2006).

Buchheiser *et al.* reported that cells with different properties can be derived from CB in different O_2 concentrations (5% and 20%) (Buchheiser et al. 2012). In 20% O_2, they were able to generate both CB-MSCs and so called USSCs. USSCs are defined as differing from CB-MSCs by their *Homeobox* (*HOX)* gene expression pattern, *Delta-like 1 homolog* (*DLK-1)* expression, and by their adipogenic differentiation capacity (Kluth et al. 2010). *HOX* genes are known to be involved in tissue specification and cell identity during embryonic development and the *HOX* expression pattern of murine MSCs points to the topographic origin of the cells (Ackema and Charite 2008). The cells generated in 5% O_2 concentrations differed from both CB-MSCs and USSCs (Buchheiser et al. 2012). They also showed that growth kinetics of the cells generated in 20% O_2 concentration did not change when converted to 5% O_2 concentration. On the other hand, the cells generated in 5% O_2 concentration showed decreased proliferation capacity and they seemed to be less adaptive to higher O_2 concentrations. Buchheiser et al. hypothesized that culture conditions during the generation of cells is more critical than temporary changes in O_2 concentrations. Thus different cell populations may be generated at different frequencies depending on the O_2 concentration (Buchheiser et al. 2012).

An example of the influence of environmental conditions on the functionality of MSCs is the effect of hypoxia on these cells. With hypoxic (1%) O_2 concentration, MSCs have been shown to increase the production of trophic factors to enhance angiogenesis and immune modulatory activity

(Madrigal et al. 2014). These examples indicate the responsiveness of MSCs to the surrounding environment.

1.3.3 CULTURE OF MSCs FOR CLINICAL USE

MSCs have been cultured from different sources in a variety of different conditions. Clinical trials are also conducted with MSCs produced with different culture protocols and consequently the data obtained from these trials may be difficult to interpret. A position paper written by researchers, clinicians, and regulators (Wuchter et al. 2015) gives recommendations and relevant information that promote the goal of having a common standard for the production of MSCs for clinical use. The paper presents recommendations that pertain to the source of MSCs, media composition, and duration of cell expansion, as well as recommendations for storage and measures of quality controls. As BM is the most commonly used source of MSCs, the position paper concentrates on this tissue. Xenogeneic-free medium with defined and certified ingredients is recommended, with PL recommended as the supplement for the medium. The duration of culture should be as limited as possible, counted by means of the population doubling (PD) numbers (Wuchter et al. 2015).

For quality control testing, it is recommended that the MSCs should be positive for CD73, CD90, and CD105 and negative for CD45. Measuring other markers is recommended depending on the source of the cells and their therapeutic use (see Table 1). No recommendations can be given about measuring the immunomodulatory capacity of MSCs as the existing *in vitro* assays do not necessarily reflect their *in vivo* capacity. Although there is no indication of adult MSCs having tumorigenic potential, the occurrence of chromosomal abnormalities should be checked by karyotyping. The monitoring of PD time is recommended to help to detect highly proliferating, possibly transformed cell populations (Wuchter et al. 2015).

1.3.3.1 Regulation and good manufacturing practice (GMP)

Culture expanded MSCs are classified as advanced therapy medicinal products (ATMPs) according to EU legislation (Regulation (EC) 1394/2007 on advanced therapy medicinal products). Subclasses of ATMPs include gene therapy medicinal products (GTMPs), somatic cell therapy medicinal products (sCTMPs), and tissue engineered medicinal products (TEPs) (Celis et al. 2015). CTMPs and TEPs, also referred to as cell-based medicinal products (CBMPs), differ from cell and tissue transplants as they have undergone additional manipulation and they can be used for a function in the recipient that differs from the original function in the donor (Celis et al. 2015). MSCs belong to the group of sCTMPs.

As ATMPs are complex pharmaceuticals, a risk-based approach has been developed to enhance the regulatory flexibility needed for the regulation of these products (Celis et al. 2015). The Committee for Advanced Therapies (CAT) within the European Medicines Agency (EMA) is responsible for all regulatory procedures concerning ATMPs in the EU (Ancans 2012). It offers classification, certification, and evaluation procedures concerning ATMPs, and thus also all regulatory procedures for MSCs. CAT reviews the quality, safety, and efficacy of ATMPs prior to marketing authorization. After authorization, ATMPs are subject to a strict post-authorization surveillance program to ensure the patient safety (Celis et al. 2015). Alternatively, an ATMP therapy product may be considered for a national authorized procedure under the hospital exemption. For hospital exemption authorization, the product has to be prepared on a non-routine basis according to specific quality standards, and it has to be used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner (Ancans 2012). The national authorities (in Finland Finnish Medicines Agency) have a crucial role in the supervision of clinical trials of ATMPs as well as the supervision of hospital exemptions.

Prior to clinical studies, non-clinical studies are mandatory for the assessment of risks related to a new ATMP. Non-clinical studies should be performed in relevant animal models to demonstrate the proof of principle for the product and to define the pharmacological and toxicological effects. Also, the safe doses for subsequent clinical studies and the route of administration should be studied (Celis et al. 2015; Salmikangas et al. 2010). The beneficial effects of MSCs have been demonstrated in a wide range of pre-clinical development disease models and the results of clinical studies have led to the conclusion that MSC applications are safe and feasible (Ancans 2012).

One of the central elements in the ATMP regulation is the GMP requirements that are mandatory to ensure safety and reproducibility of the product. For the culture of MSCs for clinical studies, a formal approval from competent regulators is needed. This approval is only granted if the cells are cultured according to GMP. Thus, the cells need to be cultured under the highest standards of sterility, quality control, and documentation, in accordance with a standard operating procedure. The basic GMP requirements mean that all manufacturing processes are clearly defined and systematically reviewed. The critical steps (including processes, equipment, and facilities) of the manufacture need to be validated, the personnel must be appropriately qualified and trained, traceability of all materials must be arranged and instructions must be clearly written, with records of all the steps during manufacture being mandatory (on-time recording). Also all significant deviations need to be fully recorded and investigated, and preventative actions must be implemented. To assure the quality of the cell product, quality controls (process and final product) must include bacteriological, phenotypic, viability, safety, and efficacy tests. The release

criteria are product dependent and must be defined separately for each therapeutic product.

2 AIMS OF THE STUDY

The aims of this study were:

1. To establish an efficient culture method to obtain MSCs from CB.

2. To establish a robust culture method to culture BM-MSCs for clinical use.

3. To gain knowledge about the impact of different culture conditions on MSC characteristics and functionality.

4. To gain basic knowledge about the immunomodulatory mechanisms of MSCs.

3 MATERIALS AND METHODS

3.1 ETHICS

The study was approved by the ethical review board of the Finnish Red Cross Blood Service and the Ethical Committee of the Hospital District of Helsinki and Uusimaa. Human CB and BM were collected from voluntary healthy donors after written informed consent. Only CB-units unsuitable for clinical banking due to a too low blood volume or cell amount were used in the studies.

3.2 METHODS

Materials and methods used in the studies are described in detail in the original publications (I-IV) and are listed in Table 6.

Table 6. *Materials and Methods used in publications I-IV in the thesis.*

Method	**used in study**
Cell and EV isolation methods	
- density-gradient centrifugation	
• isolation of MCs from CB	I, II
• isolation of MCs from BM	III
• peripheral blood MC (PBMC) isolation	II, III, IV
- magnetic bead cell separation	
• isolation of $CD3^+$ T cells	IV
- ultracentrifugation	
• EV collection from cell culture supernatant	IV
Preparation of platelet-derived cell culture supplements	III
Cell culture	
- culture of CB-MSCs	I, II, IV
- culture of BM-MSCs	III, IV
- MSC differentiation assays	II, III
• bone, adipose and cartilage	
Cell functionality tests	
- T cell suppression assay	
• co-culture of PBMCs and MSCs	II, III
• co-culture of T cells and MSCs	IV
- angiogenesis assay	II
• tube formation assay of endothelial cells	
Fluorescence based characterization of cells	
- Flow cytometry	
• immunophenotype of MSCs	II, III, IV
• immunophenotype of T cells	IV
- Immunofluorescence microscopy	IV
• expression of CD73 and CD39 in MSCs	
Karyotyping	III
HOX expression	II
- Reverse transcription polymerase chain reaction (RT-PCR)	
VEGF expression	II
- Quantitative real-time polymerase chain reaction (qRT-PCR)	
- Enzyme-linked immunosorbent assay (ELISA)	
EV analysis	IV
- Nanoparticle tracking analysis (NTA)	
Nucleotide measurements	IV
- High-performance liquid chromatography (HPLC)	

3.2.1 DIFFERENT CULTURE MEDIA

Different culture media used in the CB-MSC studies are listed in table 7 and in table 8 for the BM-MSC studies.

Table 7. *The compositions of culture media used in the CB-MSC studies. StdM was used as standard growth medium of CB-MSCs (publications I, II, IV) and in the functional studies of CB-MSCs (publication II). M1 medium was used in the long term culture studies of CB-MSCs and in the functional studies of CB-MSCs (publication II). M2 medium was used in the functional studies of CB-MSCs (publication II). M3 medium was used as an assay medium in the functional studies of CB-MSCs (publication II).*

medium component	StdM	M1	M2	M3
alpha-MEM Glutamax™	x	x	x	x
penicillin	100U/ml	100U/ml	100U/ml	100U/ml
streptomycin	100µg/ml	100µg/ml	100µg/ml	100µg/ml
FBS	10%	5 %	5 %	5 %
DX	50 nM	-	-	50 nM
EGF	10 ng/ml	-	10 ng/ml	10 ng/ml
PDGF-BB	10 ng/ml	10 ng/ml	10 ng/ml	-

StdM = Standard growth medium, M1= Medium 1 and M2 = Medium 2, M3 = Medium 3,
X=included in medium

Table 8. *The composition of growth media used for the BM-MSC studies (publication III).*

medium component	FBS-medium	PL1-medium	PL2-medium
DMEM-LG	x	x	x
penicillin	100U/ml	100U/ml	100U/ml
streptomycin	100µg/ml	100µg/ml	100µg/ml
FBS	10%	-	-
Heparin	-	40 IU/ml	40 IU/ml
PL1*	-	10%	-
PL2*	-	-	0.5%
AB-plasma	-	-	2.5%

FBS = fetal bovine serum, PL1 = platelet lysate 1 supplement, PL2 = platelet lysate 2 supplement
*Different platelet concentration in lysate
X=included in medium

4 RESULTS

4.1 AN EFFICIENT METHOD TO CULTURE MSCs FROM CB

4.1.1 THE SUCCESS RATE OF OBTAINING MSCs FROM CB

CB has proven to be a challenging material to obtain MSCs from. In our laboratory, in the early stages of the development of the method to culture MSCs from CB, several trials were made using either low serum (2% FBS) containing medium with several growth factors or high serum (15% FBS) containing medium with fewer growth factors (Laitinen and Laine 2007). These methods were carried out in normal atmospheric O_2 concentration. As the success rate of obtaining MSCs with these methods was low (data not shown), the method was further improved. The standard growth medium (StdM, Table 7) was developed based on information on MSC supporting factors in literature.

With the improved method, MSCs were successfully generated from 14 out of 16 CB-units, which corresponds to a success rate of 87.5%, when cells from CB were cultured in StdM on FN-coated plates in 3 % O_2 concentration. The mean volume of the CB units was 58.6 ± 19.2 ml. MSC could even be obtained from small CB-units of 42 ml in volume. The mean MC count after density gradient centrifugation was 382.6 ± 124.2. There was no correlation between the CB unit volume or MC number and MSC amount in passage 1 (p1) (R^2 = 0.0374 and R^2 = 0.0023, respectively). The storage time of CB-units before processing was over 19 h (mean 23.97 ± 5.88 h, publication II Table 2). The success rate of obtaining MSCs from almost 90% of CB-units that were stored for over 19 hours (and included even small units) is much higher than the success rate reported earlier in literature for human CB-MSCs.

4.1.2 THE IMPACT OF CULTURE CONDITIONS ON THE PROLIFERATION OF CB-MSCs

Several growth factors are thought to be beneficial for MSC growth. In our laboratory, the importance of different components in the StdM on cell proliferation was tested by excluding growth factors from medium. These tests indicated PDGF-BB to be a critical factor for efficient CB-MSC proliferation since the proliferation was radically diminished without PDGF-BB (data not shown) and therefore it was included in all growth media used for CB-MSCs in the studies of this thesis. Also O_2 concentration in cell culture has been demonstrated to influence cell proliferation.

In this thesis, the impact of culture conditions on the proliferation of CB-MSCs was studied. To see the effects of different culture media on long term proliferation of CB-MSCs, the cells were cultured in parallel in two different media, StdM and M1 (Table 7), from passage 2 on. For the purpose of investigating the impact of O_2 on the long term cultures of CB-MSCs, the cells were also cultured in two different O_2 concentrations (3% and 20%).

The results showed that CB-MSCs from the studied cell batches were capable of proliferating for up to 17 passages before senescence. Although the capacity of CB-MSCs to proliferate differed between CB-MSC batches, MSCs cultured in StdM, which contained more growth factors compared to M1 medium, proliferated better and reached higher PD numbers in shorter time than CB-MSCs cultured in M1 medium, irrespective of the O_2 concentration. The combination of an optimal culture medium and 3 % O_2 concentration gave the best results in terms of the CB-MSC growth. CB-MSCs showed the greatest capacity to proliferate when cultured in StdM in 3% O_2 concentration. The supportive effect of 3% O_2 concentration on cell proliferation was seen more clearly at late passages than at early passages (Table 9, Figure 3 and publication II Figure 3).

Table 9. *The proliferative capacity of the MSCs in different culture conditions in the long term cultures of CB-MSCs. The numbers of cumulative PDs, the culture length in days (d) and passage numbers (p) reached by CB-MSCs from two different CB-units. (Modified from Laitinen et al 2016, publication II Figure 3).*

CB-unit name	**391P**						**454T(7)**					
medium	**StdM**			**M1**			**StdM**			**M1**		
	PD	d	p	PD	d	p	PD	d	p	PD	d	p
3% O_2	51	57	16	42	85	17	48	59	17	32	71	14
20% O_2	47	63	17	35	66	14	40	56	15	26	66	13

StdM = Standard growth medium, M1= Medium 1

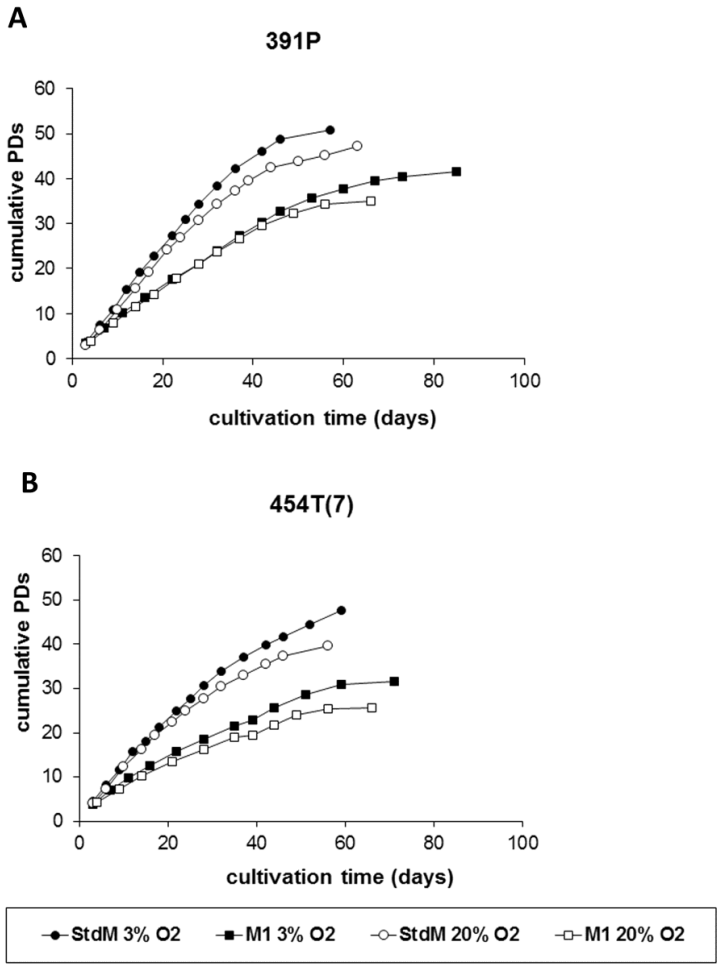

Figure 3 Long term culture of CB-MSCs in two different media and in two different oxygen concentrations. Cells from CB-units **(A)** 391P and **(B)** 454T(7) cultured from passage 2 until senescence. The supportive effect of 3 % O_2 on cell proliferation was more obvious at late passages. (Modified from Laitinen et al 2016, publication II Figure 3).

4.2 DEVELOPMENT OF CULTURE METHOD FOR CLINICAL-GRADE BM-MSCs

4.2.1 DEVELOPMENT OF PL SUPPLEMENTS

Efficient xeno-free expansion methods to culture MSCs for clinical use are strongly encouraged by regulators. Human platelet-derived supplements have been widely studied to find substitutes for bovine derived FBS. In this

thesis, two different platelet-derived PLs, PL1 and PL2, were produced for use as supplements in BM-MSC culture media (Table 8). PL1 was produced by pooling several PRP units that had been frozen once before pooling. PL2 was produced from platelet concentrates by efficiently lysing the platelets with five freeze-thaw cycles before pooling several of these units. The efficiency of these supplement-pools to support MSC growth were tested in a 5-7 day BM-MSC proliferation assay.

PL1 pools of different sizes (produced from different number of PRP units) were tested using 2–4 individual MSC batches as responder cells. The tested pools supported consistently the proliferation of BM-MSCs, with the cells reaching 3-6.2 PDs during the proliferation assay (publication III Table 2). The pools produced from two PRP units were noted to be as efficient in supporting MSC proliferation as those produced from 13 units. For PL2, all units used for the pool were individually tested and shown to support MSC growth at least as well as FBS-containing medium.

4.2.2 THE IMPACT OF CULTURE CONDITIONS ON BM-MSC PROLIFERATION

Efficient GMP-compliant methods are necessary for the expansion of a sufficient number of BM-MSCs for clinical needs from a limited amount of BM. In this thesis, two different GMP-compliant culture media, PL1 and PL2, were compared against FBS-medium (Table 8) in regard to their ability to support the proliferation of BM-MSCs at early passages of the cells (up to passage 2). We also tested the capacity of these different media to support the long term proliferation of BM-MSCs. The impact of O_2 concentration on the cell proliferation at early passages was also studied.

The short term cultures (up to passage 2) indicated that the PL1-medium had better capacity to support the growth of BM-MSCs regardless of O_2 concentration compared to PL2-medium as the PD time was significantly shorter when BM-MSCs were cultured in PL1-medium (p=0.015). There was no statistical difference observed in the PD times between FBS- and PL1-medium cultured cells. Also, there was no statistical difference in total cell yield or cumulative PDs between different culture conditions (p = 0.42 and 0.99, respectively) (Figure 4). Though the difference was not statistically significant, there was a trend indicating that 3% O_2 concentration might support a shorter PD time (Figure 4c). The most obvious difference in PD times was observed between cultures of PL1-medium in 3% O_2 and PL2-medium in 20% O_2 (p=0.04), with the PD time at passage 2 being 1.7 ± 0.3 and 4.2 ± 2.0 days, respectively. The long term culture of BM-MSCs in different media also indicated the effectiveness of PL1-medium as PL1-medium cultured cells reached higher PD numbers (46) before senescence than PL2- and FBS-media cultured cells (27 and 38 PDs, respectively, publication III Figure 4).

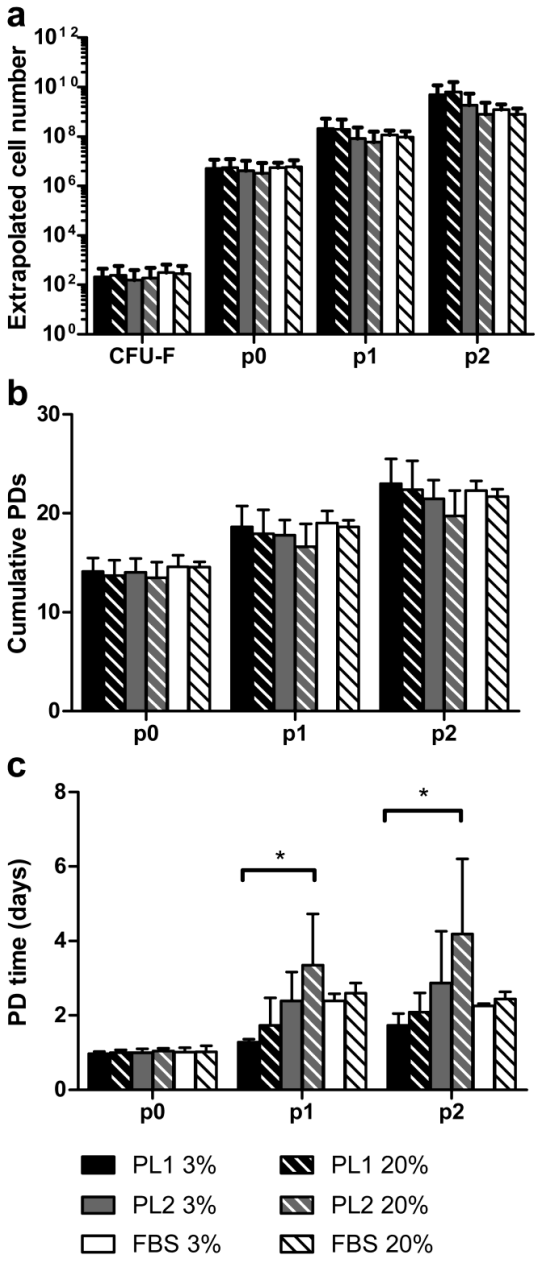

Figure 4 Results from BM-MSC cultures in three different media and in two different oxygen concentrations. **(a)** Extrapolated cell yields from 20 ml of BM up to passage two. **(b)** Cumulative PDs and **(c)** PD time at different passages from BM-MC up to passage 2. Data represented as mean ± SD. (From Laitinen et al. 2015, publication III Figure1)

As the PL1-medium proved to be the most potent proliferation medium of the media tested, it was chosen for further studies. The further studies were conducted in 20% O_2 concentration as the 3% O_2 concentration did not significantly enhance cell proliferation. The effectiveness in obtaining clinically relevant numbers of BM-MSCs in PL1-medium within two cell passages was studied in detail. The aim was to reach clinically relevant cell numbers (10^9) starting from 20 ml of BM within two cell passages. This aim was reached with 73 % of the cultured BM batches within 21-35 days (publication III Figure 2). The main reason for not reaching this limit set for cell numbers was the initial quality of the BM sample (clots in BM sample). Thus with good quality BM samples, the success percentage was even higher.

4.3 CHARACTERISTICS OF MSCs AND THE IMPACT OF CULTURE CONDITIONS

4.3.1 PHENOTYPE

MSCs from both BM and CB expressed the typical MSC markers, as shown by immunophenotyping with flow cytometry. The cells were positive for CD13, CD29, CD44, CD49e, CD73, CD90, CD105, and HLA-ABC, and they were negative for CD14, CD19, CD34, and CD45. Differences in expression of HLA-DR and CD90 were seen when culturing the cells in different conditions.

BM-MSCs cultured in PL1-medium were constantly positive for HLA-DR but the level of positivity varied between cell batches, with 7.5%-66.1% of the cells expressing this molecule on their surface. The expression of HLA-DR was also seen on PL2-medium cultured cells. On FBS-medium cultured cells, no HLA-DR positivity was detected (publication III Figure 6).

With CB-MSCs, the flow cytometric analysis of the cells revealed the cell surface expression of CD90 to be lower on cells cultured in growth factor rich and DX-containing medium, StdM, compared to cells cultured in medium with less growth factors, M1. The expression of CD90 was sustained through the passages on the cell surface of the CB-MSCs cultured in M1 but it was clearly diminished on StdM cultured cells (publication II, Figure 4).

4.3.2 *HOX* FINGERPRINT

The *HOX* code has been introduced as a marker to distinguish CB-derived cell types, such as *HOX*-positive CB-MSC and *HOX*-negative USSC (Liedtke et al. 2010). In this thesis, to characterize the *HOX* fingerprint of CB-MSCs the expression of 39 *HOX* genes distributed among four clusters (ABCD) were tested by RT-PCR. The expression profile of 39 *HOX* genes showed varied distribution within the A-D *HOX* clusters in MSCs from different CB-units, indicating heterogeneity in CB-MSC populations of different cell batches. There were two batches of CB-MSCs expressing the genes from all

four clusters, two CB-MSC batches expressing the genes from ACD clusters, and one cell batch expressing the genes from BCD clusters. In two CB-MSC batches, there was almost no expression of the *HOX* genes in any of the clusters (publication II, Figure 2A). The *HOX* fingerprint indicated that there is heterogeneity in MSC populations obtained from CB.

Changes seen in the expression profile during passaging were minimal as only some minor differences of the expression profile within the same *HOX* gene group were detected (publication II, Figure 2B).

4.3.3 DIFFERENTIATION POTENTIAL

According to the minimal criteria set for MSCs, these cells should have tri-lineage differentiation capacity, with the ability to differentiate into osteoblasts, adipocytes, and chondrocytes (Dominici et al. 2006). In this thesis, the differentiation capacity of CB- and BM-MSCs was tested. While BM-MSCs were found to have all these differentiation capacities, the differentiation capacity of CB-MSCs was heterogenic as not all batches showed true tri-lineage differentiation.

Only two of the tested CB-MSC batches retained the tri-lineage differentiation capacity with the others showing the capacity to differentiate into only two of the three lineages (publication II Table3 and publication II Figure 1). 10 of the tested 12 CB-MSC batches were capable of differentiating into osteoblasts and 5 of 10 tested batches differentiated into chondrocytes. Adipogenic differentiation was tested with two different methods, the traditional and the enhanced method (with PPARγ ligand), with positive differentiation seen in 1 of 9 CB-MSC batches when tested with traditional method and in 11 of 13 when using the enhanced method. Chondrogenic differentiation was detected with 5 of the 10 tested CB-MSC batches (publication II Table3 and publication II Figure 1).

BM-MSCs were showed to have the capacity to differentiate into all three lineages (publication III Figure 5). Osteogenic differentiation was conducted using two different osteogenic differentiation media: FBS-containing and PL1-containing differentiation media. When the differentiation was conducted in FBS-containing osteogenic differentiation medium, BM-MSCs cultured in PL1-supplemented growth medium did not show strong osteogenic differentiation potential as the accumulation of calcium deposits was modest. In contrast, conducting the differentiation of BM-MSCs in PL1-supplemented differentiation medium resulted in notably stronger differentiation (publication III Figure 5c and 5d).

4.4 THE IMPACT OF CULTURE CONDITIONS ON MSC FUNCTIONALITY

4.4.1 THE CAPACITY OF MSCs TO SUPPORT ANGIOGENESIS

To study the angiogenic supporting capacity of CB-MSCs cultured in different media, their ability to produce angiogenic factor, VEGF, was investigated with qRT-PCR and ELISA tests and their capacity to support tube formation of endothelial cells was verified in an *in vitro* angiogenesis assay.

CB-MSCs expressed VEGF on mRNA and on protein levels. The VEGF mRNA expression was up-regulated in cells primed for five days in M1 and M2 media, the media without DX (fold change difference values 3.7 and 4.3, respectively), as compared to StdM primed cells. A similar difference was detected on the protein level with CB-MSCs first primed in different media (StdM and M1) and then transferred into identical culture medium (M3) for 21h. The capacity of the CB-MSCs to produce VEGF varied between tested CB-MSC batches but the production was higher with cells primed in M1 medium (publication II Figure 5A and B).

In the *in vitro* angiogenesis assay, the CM of CB-MSCs was able to support tubule formation of endothelial cells. To compare this capacity in CB-MSCs primed in different media, the cells were first cultured in either StdM or M1 media and then transferred into the same medium (M3) for 21h before the collection of CM. The CM produced by M1 primed cells showed significantly better capacity ($p<0.001$) to support tube formation than the CM of StdM primed cells (publication II, Figure 5C).

4.4.2 THE CAPACITY OF MSCs TO SUPPRESS T CELL PROLIFERATION

The capacity of both BM- and CB-MSCs to suppress T cell proliferation was tested with an *in vitro* co-culture assay of MSCs and peripheral blood MCs (PBMCs) in which T cells were activated with anti-CD3 antibody. Both BM- and CB-MSCs had the capacity to suppress T cell proliferation.

The capacity of CB-MSCs to suppress T cell proliferation differed significantly depending on whether the cells were primed in media with or without DX supplementation (StdM vs. M1 and M2). The suppression capacity was significantly diminished if cells were primed in M1 and M2 media, compared to StdM primed cells ($p<0.01$ and $p<0.05$, respectively, publication II Figure 6).

With BM-MSCs, there was no statistically significant difference detected in the MSCs' capacity to suppress T cell proliferation when they were cultured in different culture conditions (different media PL1, PL2 and FBS and different O_2 concentrations, 3% and 20%, $p=0.14$) (publication III Figure 7).

Table 10. *Summary of the results from characterization and functionality studies of CB- and BM-MSCs cultured in different media.*

character/ functionality	general criteria*	BM-MSCs			CB-MSCs		
		FBS	PL1	PL2	StdM	M1	M2
CD90	+ (≥95%)	+	+	+	↓	↑	nd
HLA-DR	- (≤2%)	-	↑	↑	-	-	nd
adipogenesis	+	+	+	+	↓	nd	nd
osteogenesis	+	+	+	+	↓	nd	nd
chondrogenesis	+	+	+	+	↓	nd	nd
immunosuppression	+	+	+	+	↑	↓	↓
angiogenic support	+	nd	nd	nd	↓	↑	↑

FBS=FBS containing medium, PL1= PL1 containing medium, PL2=PL2 containing medium
StdM = Standard growth medium, M1= Medium 1, M2=Medium 2
* ISCT criteria for human BM-MSCs cultured in FBS-medium (Dominici et al. 2006).
↑ = increased surface expression/ differentiation/functional capacity
↓ = decreased surface expression/differentiation/functional capacity
nd = no data

4.5 ADENOSINE PRODUCTION, ONE OF THE IMMUNOSUPPRESSIVE MECHANISMS OF MSCs

CD73 is one of the positive markers for MSCs included in the minimal criteria for MSCs (Dominici et al. 2006). As it is an ectonucleotidase that converts AMP into immunosuppressive Ado, its role as an immunosuppressive factor of MSCs was studied in this thesis. In the extracellular space, AMP is produced from ATP by another ectonucleotidase, CD39. Thus the expression of this molecule, along with CD73, was studied on MSCs and MSC-EVs, as well as on T cells, the targets of immunosuppressive action of MSCs. To connect the expression of CD73 to the immunosuppressive function of MSCs, the production of Ado was measured from MSC cultures and from the co-cultures of MSCs and T cells, and the impact of Ado on the proliferation of T cells was studied.

4.5.1 EXPRESSION OF CD73 AND CD39

The expression of CD73 and CD39 was studied by flow cytometric analysis and immunofluorescence microscopy. The results showed that CB- and BM-MSCs expressed CD73 on their cell surface (publication IV Figure 1H). CD39 expression could be detected on the surface of BM-MSCs, but not on CB-MSCs. In both cell types, intracellular expression of CD39 was detected and it co-localized with CD73 in filopodia and the bulge areas of cell membrane

(publication IV Figure 1). The positive labeling of CD73 was also seen on CB-MSC-EVs (publication IV Figure 2B).

The expression of CD73 and CD39 on T cells was studied by flow cytometry after *in vitro* proliferation assay of these cells with or without the presence of MSCs in the assay. The expression of CD73 decreased significantly on T cells upon stimulation with CD3 and CD28 antibodies ($p<0.05$ for $CD4^+$ T cells and $p<0.001$ for $CD8^+$ T cells). On $CD4^+$ T cells, the expression level of CD73 was low even without the stimulation, with about 10% of the cells being positive. The unstimulated $CD8^+$ T cells expressed CD73 at a higher level, with about 72% of the cells being positive. The co-culture of stimulated T cells with MSCs significantly increased the expression of CD73 on both $CD4^+$ and $CD8^+$ T cells, compared to culture of stimulated T cells alone ($p<0.001$ and $p<0.01$, respectively). In $CD4^+$ T cells, the expression of CD73 increased to a higher level than with unstimulated cells (publication IV Figure 6A).

The expression of CD39 increased significantly on both $CD4^+$ and $CD8^+$ T cells upon stimulation ($p<0.05$ and $p<0.01$, respectively). The co-culture with MSCs did not have an impact on CD39 expression of the stimulated T cells (publication IV Figure 6B).

4.5.2 THE PRODUCTION OF ADENOSINE

To study if the enzyme activity of CD73 molecule is seen on BM- and CB-MSCs, the production of Ado was studied by nucleotide measurements with HPLC from CM of MSC cultures supplemented with AMP or ATP. MSCs were capable of efficiently producing Ado from AMP but not from ATP. This production from AMP was inhibited dose dependently with CD73 inhibitor. The nucleotide measurements from CD3 enriched T cell cultures indicated that T cells produced AMP from ATP but could not produce any significant amounts of Ado (publication IV Figure 3A). Similarly to MSCs, CB-MSC-EVs had the capacity to produce Ado from AMP but not to any great extent from ATP (publication IV Figure 3B), and this capacity was also blocked with CD73 inhibitor.

When stimulated T cells were combined with MSCs in the culture, Ado could be produced from ATP (publication IV Figure 3C). The stimulation of MSCs with cytokines did not significantly impact their capacity to produce Ado from ATP (publication IV Figure 4).

4.5.3 THE EFFECT OF ADENOSINE IN AN IMMUNOSUPPRESSION ASSAY

To assess if Ado has the capacity to suppress T cell proliferation in an *in vitro* assay, pure Ado was added into the culture of stimulated T cells. The results showed that the addition of Ado could dose dependently suppress T cell proliferation if the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-

nonyl)adenine (EHNA), was also present in the assay (publication IV Figure 5A and B). A similar effect could not be detected without EHNA, indicating the presence of adenosine deaminase, which rapidly degrades Ado, in the assay.

To study if Ado plays a role in a four day co-culture assay of CB-MSCs and stimulated T cells, ATP was added to the assay with EHNA. In this assay, IDO is the predominant mechanism used by MSCs to suppress T cell proliferation. The addition of ATP to the co-culture in the presence of EHNA resulted in enhanced suppression of T cell proliferation, as compared to the suppressive effect of co-cultures without these additions. The effect was seen more clearly with $CD8^+$ cells and in assays where the original suppressive capacity of MSCs was not very high (publication IV Figure 5C and D). Thus, it is likely that Ado production is one mechanism used by MSCs to suppress T cell proliferation.

5 DISCUSSION

5.1 THE ESTABLISMENT OF EFFICIENT AND ROBUST CULTURE CONDITIONS FOR CB- AND BM-MSCs

MSCs are isolated and cultured from many different tissues (Caplan 1991; Erices et al. 2000; Friedenstein et al. 1976; Gronthos et al. 2000; in 't Anker et al. 2004; Wang et al. 2004; Zuk et al. 2002). As MSCs are only found in small numbers in the body they cannot just be collected and given directly to a patient. The *in vitro* culture of MSCs is an unavoidable step required to gain an adequate number of cells for research and clinical use. Thus, an efficient method to culture these cells is crucial when establishing the basis for clinical cell production.

For years, BM has been the standard source of MSCs, which have traditionally been thought to be part of the same general population, regardless of the tissue source. Comparative studies have, however, indicated clear differences between cells from different tissues (Chang et al. 2006b; Jin et al. 2013; Kern et al. 2006; Wagner et al. 2005) and thus it has been suggested that different MSC sources may generate MSC products suited to specific clinical applications (Horwitz and Dominici 2008).

In this thesis the aim was to investigate if there was a possibility to establish a method to increase the gain of MSCs from CB. The aim was also to establish a method to produce MSCs for clinical purposes. As BM is an easier material to produce MSCs and BM-MSCs have already been used in clinical studies, we compared two different GMP-compliant methods to produce clinically relevant numbers of BM-MSCs. The GMP-compliant medium efficient for BM-MSC culture was also tested for CB-MSC establishment, but it seemed not to be sufficient to obtain MSC from this source (data no shown). Thus, the GMP-compliant method to produce MSCs from CB still needs a lot of effort in further studies.

CB-MSCs

CB has been an attractive source due to its abundance and ease of access and the high proliferative capacity of the cells. But this source has turned out to be a difficult material to obtain MSCs from, with the success rate ranging from non-existing to 10%-63% (Bieback et al. 2004; Mareschi et al. 2001; Montesinos et al. 2009). Higher success rates have been gained only if the processing time has been kept to a minimum or units of a certain size have been used (Zhang et al. 2011). In this thesis, an efficient method to obtain MSCs from almost 90% of CB units is presented (publications I, II). This figure is considerably higher than the rates reported previously.

MSCs from CB seem to need more support for their *in vitro* growth than MSCs from BM. In our method for CB-MSCs, the supporting factors for these cells come in the form of growth factor-rich medium (StdM) containing EGF, PDGF, and DX in addition to FBS. The other important constituents contributing to the support of CB-MSC growth are FN-coated culture plates and an atmosphere with an O_2 concentration close to the physiological O_2 concentration, which is on average 3% on the tissue level (Haque et al. 2013).

Although we have established an efficient method for receiving MSCs from CB, plenty of further development is required before the method is GMP-and clinically-compliant. As using animal-derived components in cell culture always carries a risk of transmission of xenogeneic infectious agents and immunization(Cervenakova et al. 2011; Horwitz et al. 2002; Liu et al. 2008), the regulators urge the use of alternative supplements (EMEA/CHMP/410869/2006 and EMA/410/01 rev.3). Thus, the first critical step is to replace FBS in the culture medium. As also the quality of biologically active additives, such as growth factors, needs to be tested and documented with respect to identity, purity, sterility, and biological activity of the components as well as for the absence of adventitious agents, the amount of work is tremendous if several components are included in the culture medium, as in StdM. Also, the practicality and financial aspects need to be considered when establishing culture methods.

BM-MSCs

BM seems to be an easier source in terms of obtaining and culturing MSCs than CB as MSC cultures can be established from every BM sample of good quality (Kern et al. 2006). BM-MSCs have also been the first MSCs used for clinical purposes even though the production of the MSCs has been based on FBS-containing medium (Horwitz et al. 2002; Lazarus et al. 1995; Le Blanc et al. 2004). In our studies, we compared two different GMP-compliant culture media (PL1 and PL2) based on platelet lysate against FBS-containing culture medium in regard to their capacity to support the growth of BM-MSCs (publication III). Based on these studies, we found the PL1-medium to be better suited to clinical production of MSCs as its production was more practical and it was superior in its MSC proliferation supporting ability. Thus we established our clinical BM-MSC manufacturing protocol based on this method.

Crucial factors in MSC culture

As a good cell yield is important when producing MSCs for clinical use, we analyzed the capacity of different cell culture conditions to support MSC proliferation. Another critical factor in *in vitro* cell culture, besides the composition of the medium, is the surrounding environment. Cells are

usually cultured in 5% CO_2 concentration, which reflects the CO_2 conditions *in vivo* (Csete 2005). The buffering capacity of the traditional culture media is optimized for use in this CO_2 concentration. The O_2 conditions are often ignored in tissue culture as the O_2 concentration is more complicated to regulate. However, the concentration of O_2 is important for virtually all cell processes (Csete 2005). In our studies, we produced evidence showing that low O_2 (3%) enhances the capacity of the MSCs to proliferate but this effect is more evident at late cell passages than in the early stages of culture (publications II, III). The combined effect of optimized culture medium and low O_2 gave the best results as the BM-MSCs cultured in PL1-supplemented medium in 3% O_2 concentration showed the shortest PD (publication III) time. Correspondingly, the CB-MSCs cultured in medium with the richest growth factor content (StdM) in 3% O_2 concentration reached the highest cumulative PD numbers in the shortest time (publication II). Although a positive influence of low O_2 concentration was seen in our studies, the impact of low O_2 was only minimal at the early passages of cell culture (publication III). For safety reasons, the method of choice for MSCs to be used for clinical purposes is to culture the cells *in vitro* for as short a time as possible (Wuchter et al. 2015) and therefore the O_2 concentration is not a critical factor when improving the cell yield in the early passages. In our studies, more impressive differences in the proliferative capacity of MSCs were seen between cells cultured in different media, with StdM being better than M1 for CB-MSCs and the PL1-medium being better compared to PL2 for BM-MSCs (publications II, III).

As expected, we found the growth factor-rich medium, StdM, to be more efficient in supporting the growth of CB-MSCs (publication II). The culture supplements EGF, PDGF, and DX have also been used by others to support cell growth (Reyes et al. 2001). The combination of PDGF and FN-coating of plastic-ware has been demonstrated to have a positive influence on the MSC proliferation (Veevers-Lowe et al. 2011). Veevers-Lowe et al. have shown that MSC adhesion to FN induces the α5β1-integrin (CD49e and CD29, both strongly expressed on MSCs) dependent phosphorylation of PDGF-receptor β (PDGFR-β). The adhesion to FN also strongly potentiates the PDGF-BB-induced phosphorylation of the receptor. This phosphorylation of PDGFR-β leads to downstream signal transduction, finally resulting in the stimulation of cell growth and motility as well as cell proliferation (Veevers-Lowe et al. 2011). EGF has been shown to maintain the colony forming capacity of MSCs, thus EGF helps to preserve early progenitors in MSC populations (Tamama et al. 2010). It has been indicated that DX increases proliferation, and the positive effect of DX at the beginning of CB-MSC culture may be a result of its inhibitory effect on monocytes adherence on culture plates (Roberts et al. 1998), thus allowing more space for MSCs to proliferate.

It could be speculated that the excess of growth factors may negatively affect MSC growth, for example shortening the life span of MSCs. However, in our studies we did not see any negative effect of growth factor rich

medium on CB-MSC growth, as MSCs in StdM reached higher population doubling numbers than MSCs in M1 medium. We also did not notice any abnormalities in the karyotype of CB-MSCs that were tested in early passages (passage 4) and at late passages (passage 14-16, data not shown). Nevertheless, to resolve if growth factor rich medium affects the stability of MSCs in other respect still needs further studies.

The two different PL supplements, PL1 and PL2, used in BM-MSC culture media, differed in their composition (Table 11) as PL2 was produced from PRP units and PL2 from platelet concentrates. The PL2 supplement was more concentrated and thus only a PL content of 0.5% was used in the final PL2-medium, compared to 10% PL1 supplement in PL1-medium, to have comparable numbers of lysed platelets between these supplements in final media. The PL1-supplement contained more AB-plasma, thus it was not necessary to add plasma to the PL1-medium. The plasma concentration of PL2-medium was lower although some AB-plasma was added to it. Another difference between these supplements was the number of freeze-thaw cycles applied, with PL2 being efficiently lysed with five cycles. Although the initial platelet counts of the supplements used to prepare the final media were comparable, the PL1 supplemented medium had better capacity to support MSC growth. It has been demonstrated that the freezing and thawing of PL has an effect on the growth factor composition of the supplement (Mojica-Henshaw et al. 2013) and consequently the high number of freeze–thaw cycles during the manufacture of PL2 may have inactivated some critical components in this supplement. Also the plasma content of medium has proved to be an important factor for cell proliferation (Horn et al. 2010).

Table 11. *Characteristics of the PL1 and PL2 supplements and their use in BM-MSC growth media (Modified from Laitinen et al. 2015, publication III Table 1)*

	COMPOSITION OF PLATELET UNITS/BAGS		PLATELET LYSATE				MSC GROWTH MEDIA		
Supp-lement	**Description**	**Platelets x10^9***	**Lysate additive**	**Lysed platelets x10^9/ml***	**Freeze-thaw cycles**	**Pool size (nr of platelet units)**	**Concentration of platelet lysate (%)**	**Lysed platelets x10^8/ml***	**Concentration of plasma (%)**
PL1	Platelets in plasma, 4 donors BC	300	AB plasma	0.1	2	2-13	10	1.0	10
PL2	Platelets in 30% SSP/70% plasma, 4 donors BC	300	Octaplas AB (pooled virus-inactivated fresh frozen plasma)	15	5	15	0.5 (+ 2.5 % Octaplas AB)	0.8	3

*mean numbers
BC= buffy coat

5.2 IMPACT OF CULTURE CONDITONS ON MSC CHARACTERISTICS AND FUNCTIONALITY

Although a good cell yield is the basis for producing cells for clinical use, it cannot override the quality and the properties of the cells. Culture conditions have been suggested to influence the properties of MSCs (Bieback et al. 2010; Roobrouck et al. 2011b; Wagner et al. 2005) and thus the impact of different culture conditions on cell properties should always be carefully examined. The possible alterations in the properties of MSCs can be observed by studying the phenotype or expression of certain molecules of the cells. Also, conducting several functional tests may help to elucidate the influence of culture conditions on cell properties. Any possible alterations in cellular phenotype are easily observed but the link between the expression of a particular molecule and a relevant function of MSCs is not usually known.

HLA-DR is one of the molecules that should not be expressed on the MSC cell surface according to the original minimal criteria to define MSCs (Dominici et al. 2006). This molecule is an example of how culture conditions may affect the phenotype. Certain inducing factors, such as IFN-γ and bFGF, have been shown to induce the expression of HLA-DR on MSCs (Le Blanc et al. 2003a; Romieu-Mourez et al. 2007; Sotiropoulou et al. 2006). Lately, the classification of this molecule as a negative marker for MSCs has been criticized as its expression is dependent on the culture conditions (Tarte et al. 2010). In our studies, HLA-DR expression levels differed depending on whether the BM-MSCs were cultured in PL1, PL2, or FBS-containing media (publication III). Although HLA-DR was expressed at different levels on these cells, the capacity of the cells to suppress T cell proliferation did not change in our *in vitro* assay. Others have also indicated that the expression of HLA-DR is not connected with the capacity of MSCs to inhibit T cell proliferation (Sotiropoulou et al. 2006; Tarte et al. 2010).

One MSC marker, for which variable expression was observed in our CB-MSC culture experiments when culturing the cells in different media, was CD90 (publication II). Its expression was decreased when culturing the cells in StdM, the growth factor-rich medium. Others have not reported the effects of growth factors on the expression of CD90 but the impact on other molecules, such as CD105 and CD146, has been reported (Gharibi and Hughes 2012). The connection between this marker and the functionality of MSCs is not been clearly demonstrated. A decrease in CD90 expression has been linked to the differentiation processes and diminished immunosuppressive capacities of MSCs (Sibov et al. 2012). In our experiments, functional consequences were not studied. There are also arguments against CD90 as a "specific" MSC marker as several groups have noticed only low-level expression of CD90 on CB-MSCs (Goodwin et al. 2001; Lee et al. 2004b; Montesinos et al. 2009; Nagano et al. 2010). Until a clear association between a particular phenotypic character and the functionality of MSCs is demonstrated, it cannot be concluded that a specific

phenotype leads to certain functional capacities of the cells and thus the functionality of MSCs should be somehow tested when altering culture conditions.

In our studies, the MSC batches obtained differed in their *HOX* gene expression pattern, which did not considerably change during passaging. This indicates heterogeneity in the cell populations obtained from individual CB units. Therefore, although the characteristics of MSCs can be influenced by culture conditions, there may be limitations as the initial culture conditions may eliminate some subtypes of cells from the MSC population. This was also proposed by Buchheiser et al. when they noted that culture conditions during cell generation may be more critical than temporary changes in O_2 concentrations and different cell populations may be generated at different frequencies depending on the O_2 concentration (Buchheiser et al. 2012).

It has been suggested that cell culture conditions could be modified to receive MSCs optimized for a specific clinical indication (Horwitz and Dominici 2008). In our studies, we show that culture conditions have an effect on the functionality of MSCs *in vitro*. With CB-MSCs, we demonstrated that although StdM, the growth factor-rich medium, had the greater ability to support the growth of MSCs, its ability to promote the angiogenic supporting capacity of MSCs was diminished, as MSCs cultured in this medium expressed VEGF mRNA at a lower level than cells cultured in medium containing less growth factors and without DX, M1, and M2 media. MSCs induced in different media and then transferred into similar conditions still showed divergent potential to produce VEGF and to support angiogenesis in an *in vitro* tubule formation assay (publication II). This demonstrates the inductive impact of culture conditions to influence the angiogenic capacity of MSCs. On the other hand, our experiments showed that MSCs cultured in the presence of DX had a greater capacity to suppress T cell proliferation in an *in vitro* T cell proliferation assay (publication II). Thus when optimizing the culture conditions, for instance, to achieve higher cell yields, one should bear in mind that the changes in culture conditions may have an effect on the functionality of the cells, which is not always seen simply as alterations in the phenotype of the cells.

Another indication of culture conditions having an impact on the functionality of MSCs was seen in our experiment aimed at differentiating BM-MSCs into osteoblasts (publication III). When MSCs were initially cultured in PL1-containing medium their capacity to differentiate into bone forming osteoblasts was diminished if the differentiation was performed in traditional FBS-containing differentiation medium. When the differentiation was performed in PL1-containing medium the differentiation was much more pronounced. The adipogenic differentiation of the CB-MSCs also suggested that by altering the adipogenic differentiation medium, the CB-MSCs could be differentiated into adipocytes with increased frequency (publication II). The adipogenic differentiation of CB-MSCs has been demonstrated to be

weak (Bieback et al. 2004; Chang et al. 2006b; Manca et al. 2008; Yoshioka et al. 2015; Zhang et al. 2011). In our studies, the adipogenic differentiation was also shown to be very weak if the traditional differentiation medium was used. When the differentiation medium was optimized for CB-MSCs by using the PPARγ ligand, ciglitazone, the adipogenic differentiation was enhanced.

5.3 ADENOSINE PRODUCTION AS AN IMMUNOSUPPRESSIVE MECHANISM OF MSCs

MSCs have demonstrated to have many mechanisms to suppress immune reactions (Castro-Manrreza and Montesinos 2015). In this thesis, a relatively unknown immunosuppressive mechanism of MSCs via adenosinergic signalling is presented.

We became interested in the CD73 molecule, an ectonucleotidase that converts AMP into a highly immunosuppressive purine nucleoside Ado, as the anti-CD73 antibody was one of the few antibodies produced in our experiments of whole cell immunizations of the mice (unpublished data in collaboration with Medix Biochemica). This CD73 molecule is constantly expressed on MSCs at high intensity. The immunosuppressive effects of CD73 have been demonstrated with Tregs, tumor cells, and recently with MSCs (Amarnath et al. 2015; Chen et al. 2013; Chen et al. 2016; Deaglio et al. 2007; Ghiringhelli et al. 2012; Sattler et al. 2011). In our studies, we explored the capacity of MSCs to suppress T cell proliferation in an *in vitro* assay and our aim was to demonstrate the role of Ado in the MSC mediated T cell suppression (publication IV).

The expression of CD73 was noted to differ between individual cell batches, but we did not notice any effect of the different culture conditions on the capacity of MSCs to express CD73. Interestingly the co-expression of CD73 and CD39 was observed in filopodia and the bulge areas of cell membrane, indicating the possibility that MSCs may have the capacity to transfer CD39 to their cell surface under certain stimuli, as demonstrated by others in a co-culture assay of MSCs and T cells (Saldanha-Araujo et al. 2011).

Using HPLC analysis, we saw that BM- and CB-MSCs and CB-MSC-EVs could efficiently produce Ado only from AMP. From ATP this was possible only if stimulated T cells were present together with MSCs in the assay, indicating that MSCs act synergistically with immune cells to produce immunosuppressive Ado in conditions containing ATP. As the Ado production was inhibited by CD73 inhibitor we could conclude that CD73 has a critical role in the production of Ado by MSCs. Though we saw the impact of CD73 inhibitor in the short time co-culture assays with HPLC analysis, we could not demonstrate its impact in our four day *in vitro* co-culture assay.

The immuosuppressive impact of MSCs in the *in vitro* T cell suppression assay comes mainly from the effects of MSC-produced IDO as its inhibition

restores the proliferative capacity of T cells. This has also been demonstrated by others (Francois et al. 2012; Jones et al. 2007; Menard et al. 2013). Although the suppressive capacity of MSCs in the assay was mainly due to the action of IDO we could, however, see improvement in the suppressive impact of MSCs when the cultures were supplemented with ATP and adenosine deaminase inhibitor, EHNA, to stabilize the produced Ado. Even though the promoting effect of the adenosinergic pathway on the suppression of T cells was not outstanding in this assay, it indicated the Ado route as one of the mechanisms used by MSCs to suppress T cell proliferation.

As *in vitro* assays are artificial with several additives that need to be added to co-cultures the conditions never truly resemble the situation *in vivo*. Therefore it is difficult to demonstrate the immunosuppressive mechanisms of MSCs in a co-culture assay and to predict their true *in vivo* capacities. The suppressive effect of Ado has been demonstrated with an *in vivo* mouse GVHD-model and autoimmune uveitis model, as blocking Ado-receptor with a receptor antagonist or blocking CD73 with CD73 inhibitor abrogated the ability of MSCs to suppress the inflammatory effects of T cells (Amarnath et al. 2015; Chen et al. 2016). Thus, adenosinergic pathway seems to be one relevant mechanism of MSCs to suppress immune reactions.

In our studies, we also explored the expression of CD73 and CD39 on T cells in the *in vitro* T cell suppression assay. The capacity of MSCs to increase the expression of CD73 on stimulated T cells was significant. Similar impacts by MSCs on the CD73 expression on T cells have been demonstrated by others (Chen et al. 2016; Saldanha-Araujo et al. 2011). This indicates the role of MSCs to prime T cells to express CD39 and CD73, which both are needed for Ado production. TGF-β has been introduced to act as an agent by which MSCs up-regulate the expression of CD73 on T cells (Chen et al. 2016). In our studies, we showed that also MSC-EVs are CD73 positive and thus it is intriguing to hypothesize that the CD73 positivity of T cells might be mediated by CD73 positive EVs. Whether EVs mediate the expression of CD73 on T cells or not, they still offer an active enzyme to enable the production of Ado via CD39 and CD73 molecules.

As natural Tregs are described as being positive for CD39 but negative for CD73, they would need assistance from neighboring CD73 positive cells or EVs to produce Ado. *In vitro* induced Tregs, on the other hand, are described as being positive for CD73, and therefore capable of producing Ado on their own (Whiteside et al. 2011). Our results indicated that the expression of CD73 increased on T cells in the presence of MSCs, but we did not specifically determine whether the number of Tregs had increased. $CD8^+$ T cells expressed higher levels of both CD39 and CD73, thus pointing to the adenosinergic pathway being important also for other cell populations and not just for CD4+CD25+ Tregs. In any case, as our results showed, the overall capacity of stimulated T cell populations to produce Ado from ATP increases in the presence of MSCs as both adenosinergic pathway enzymes are present. This adenosinergic pathway might be a relevant mechanism for

MSCs to suppress immune cell reactions especially in tissue damage, where ATP is abundantly available and since MSCs have the capacity to home into areas of injury and inflammation.

5.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this thesis, it was demonstrated that MSCs can be efficiently cultured from CB though this material is not as reliable as a source of MSCs as BM. Succeeding in obtaining MSCs from CB requires more from the culture conditions than to obtaining MSCs from BM. In this thesis, it was shown that many different growth factors, along with low oxygen concentration, were needed to efficiently obtain MSCs from CB. And as each additive in the culture medium should be of GMP-grade, the development of a GMP-compliant CB-MSC production method still needs a considerable amount of effort. For BM-MSCs, one robust GMP-compliant culture method is presented in this thesis.

The culture conditions of MSCs have impact on both the proliferative capacity of MSCs and the functional capacities of these cells. In this thesis, the impact of different culture media on the functional capacity of CB-MSCs to support angiogenesis and their capacity to suppress T cell proliferation was demonstrated. Similarly, the culture conditions certainly impact the functionality of MSCs from other sources.

Many mechanisms for MSCs to produce their therapeutic effects have been presented. In this thesis, the Ado producing capacity of MSCs is demonstrated to be a novel mechanisms used by these cells to suppress T cell proliferation. As MSCs are capable of producing Ado from ATP in cooperation with T cells and as the level of ATP is usually high in situations such as tissue damage, this mechanism may be a crucial factor influencing the therapeutic effects of these cells in tissue damage indications. Extensive research is still required to elucidate, how the Ado producing mechanism, as well as other functional mechanisms of MSCs, operate *in vitro* and whether a particular mechanism is more important in a certain indication. If the importance of these mechanisms could be linked to the therapeutic outcomes of different MSC treatments, it might be possible to tailor specific MSCs for each indication, possibly by merely optimizing the culture conditions for the MSCs. Also, *in vitro* functional tests to determine the true *in vivo* potential of MSCs must be developed to ensure that the cells given to a patient are functional for that particular purpose.

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