Platelet lysates manufactured from fresh and expired platelet concentrates as a culture supplement for human, bone marrow-derived mesenchymal stem cells

Effects on morphology, expansion, osteogenic differentiation and immunomodulation

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Blóðflögulýsöt framleidd úr ferskum og útrunnum blóðflögueiningum sem íbæti fyrir vaxtaræti mesenchymal stofnfruma úr beinmerg manna

Áhrif á útlit, vöxt, beinsérhæfingu og hæfni til ónæmismótunar

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Ágrip

Mesenchymal stofnfrumur (MSC) eru fjölhæfar frumur sem hægt er að einangra úr beinmerg manna. Þær hafa hæfni til ónæmismótunar og búa yfir þríþátta sérhæfingarhæfni yfir í bein, brjósk og fitu. Frumurnar hafa verið rannsakaðar með tilliti til þessa, og þá sérstaklega hæfni til ónæmisbælingar og beinsérhæfingar skoðuð til notkunar í læknisfræði. Kálfasermi er oftast notað til ræktunar á MSC frumum en nauðynlegt er að finna staðgengil í stað kálfasermis ef nota á MSC frumur í læknisfræði, vegna óæskilegra eiginleika, hugsanlegs ónæmissvars þega og smithættu.

Blóðflögulýsöt úr blóðflögum manna eru hugsanlegur staðgengill kálfasermis. Árlega rennur stór hluti þeirra blóðflögueininga sem framleiddar eru í blóðbönkum út og er þeim þá fargað. Hugsanlega er hægt að nýta þessar útrunnu einingar til að útbúa blóðflögulýsöt til ræktunar á MSC frumum. Blóðflögulýsöt eru oftast unnin úr ferskum blóðflögum og því vantar upplýsingar um áhrif útrunnina eininga á ræktun og eiginleika MSC frumna.

Einangraðar MSC frumur úr beinmerg manna voru ræktaðar í ræktunaræti, bættu með kálfasermi fyrir MSC frumur og ræktunaræti bættu með blóðflögulýsötum unnum úr ferskum eða útrunnum blóðflögueiningum. Skoðuð voru áhrif ætisins á útlit, vöxt og hæfni til ónæmismótunar og beinsérhæfingar.

Tegund ræktunaræt is hafði ekki áhrif á hæfni til ónæmismótunar eða beinsérhæfingar. Vöxtur var hraðari hjá frumum ræktuðum í blóðflögulýsati úr ferskum eða útrunnum blóðflögueiningum samanborið við frumur sem ræktaðar voru í kálfasermi. Smávægilegur munur var einnig á útliti frumnanna í ræktun.

Hér kemur fram að blóðflögulýsat úr útrunnum blóðflögueiningum getur verið notað sem staðgengill kálfasermis til *in vitro* ræktunar á MSC frumum, til jafns við blóðflögulýsat úr ferskum einingum, þegar vöxtur og hæfni til ónæmismótunar og beinsérhæfingar er haft í huga.

Abstract

Mesenchymal stem cells (MSC) are multipotential cells that can be isolated from the human bone marrow. They have been studied based on their differentiation potential, especially towards osteoblasts, chondrocytes and adipocytes, and for their immunomodulatory abilities. With this is mind they are thought to hold a great therapeutic potential for future use in regenerative medicine. MSCs are frequently expanded in fetal bovine serum (FBS) but, due to undesirable features and possible pathogen contamination, FBS needs to be substituted before the clinical use of MSCs is possible. Human platelet lysates (HPL) have been suggested as a substitute to FBS. A significant proportion of platelet units expire and are discarded from blood banks every year. These expired products could serve as a resource for HPL production. Fresh platelet units have frequently been used for HPL production (HPLF) but information about the effects of HPL from expired platelet units (HPLO) on MSCs is lacking.

Human bone-marrow MSCs acquired from three donors were expanded in mesenchymal stem cell screened FBS (MSC-FBS), HPLF and HPLO supplemented media and effects on morphology, expansion, immunomodulation and osteogenic differentiation were analyzed.

Expanding MSCs in each of the three different media had comparative effects on immunomodulation and osteogenic differentiation. Proliferation of MSCs expanded in HPL containing media was faster compared to MSCs from MSC-FBS containing media. Slight morphological differences were also observed.

In this thesis, our results demonstrate that HPL from expired platelet rich concentrates can be used as a substitute of FBS for *in vitro* culture of MSCs to the same extend as HPL from fresh platelet rich concentrates with regard to MSC proliferation, immunomodulation and osteogenic differentiation.

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Sandra Mjöll Jónsdóttir Buch

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Formulas

Abbreviations

ALP	Alkaline phosphatase
BC	Buffy coat
CFU-Fs	Colony forming unit fibroblasts
CPD	Cumulative population doublings
D1	Donor 1
ECM	Extra-cellular matrix
FBS	Fetal bovine serum
GT	Generation time
GvHD	Graft versus host disease
HPL	Human platelet lysate
HPLF	Human platelet lysate – fresh
HPLO	Human platelet lysate – outdated (expired)
HSC	Hematopoietic stem cell
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
INF-γ	Interferon gamma
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cells
MSC	Mesenchymal stem cell
NO	Nitric oxide
P1	Passage one
PBS	Phosphate buffered saline
PD	Population doublings
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
PHA	Phytohemaglutinin
PPARgamma	Peroxisome proliferator-activated receptor
PRP	Platelet rich plasma
RT-qPCR	Real time quantitative polymerase chain reaction
RUNX2	Runt related transcription factor 2
SDF	Stromal derived factor
SEM	Standard error of the mean
SPP1	Secreted phosphoprotein 1 (osteopontin)
β-TG	Beta thromboglobulin
PF4	Platelet factor 4
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
EGF	Epidermal growth factor
HGF	Hepatocyte growth factor
IGF	Insulin like growth factor
MEM	Minimal essential medium
DMEM	Dulbecco’s minimal essential medium
F12	Ham’s F12 supplement

RPMI	Roswell Park Memorial Institute medium
BCA	Bicinchoninic acid
pNPP	p-Nitrophenyl phosphate
ISCT	The International Society for Cellular Therapy
TNF-α	Tumor necrosis factor alpa
PECAM	Platelet/endothelial cell adhesion molecule
VLA	Very late antigen
VCAM	Vascular cell adhesion molecule
MMP	Matrix metalloproteinase

1 Introduction

1.1 Mesenchymal Stem Cells

1.1.1 Basic characteristics

1.1.1.1 Historical perspective and nomenclature

Bone marrow has been extensively studied in relationship with hematopoietic stem cells (HSC) and hematopoiesis. During the second half of the 20th century scientists were experimenting with *ex vivo* culturing of HSCs but discovered that cell growth survival depended on a supportive layer of stromal cells. The stromal cells were isolated from the bone marrow, similar to the HSCs, and proved to originate from the mesodermal germ layer (Charbord, 2010). Alexander Friedenstein first described and identified mesenchymal stromal cells as a part of the hematopoietic-inductive microenvironment when culturing bone marrow. He identified the formation of fibroblast-like colonies and referred to the cells as colony forming unit fibroblasts (CFU-Fs) (Bianco, Riminucci et al., 2001; Wuchter, 2011). Further research showed that CFU-Fs had stem cell-like capabilities, were distinct from endothelial and hematopoietic cells and could differentiate into osteoblasts, chondrocytes or adipocytes (Charbord, 2010; Delorme, Ringe et al., 2009; Prockop, 1997). Today, these CFU-Fs are regarded as non-hematopoietic multipotent cells that can be isolated from various tissues of the body and differentiated into other cells of mesodermal origin such as osteoblasts, adipocytes and chondrocytes (Charbord, 2010; Keating, 2006; Phinney & Prockop, 2007). Some confusion regarding the nomenclature of these cells has arisen. As previously mentioned, Friedenstein referred to the cells as CFU-Fs and later adopted the term osteogenic stem cells. The same cells have also been termed marrow stromal stem cells, mesenchymal progenitor cells and most recently multipotent mesenchymal stromal cells (Charbord, 2010; Horwitz, Le Blanc et al., 2005). Arnold Caplan first used the term mesenchymal stem cells which is the term used in this thesis (Caplan, 1991, 2004). Conveniently, all these terms have the acronym MSC which is widely used in the literature. Here, the acronym MSC will refer to mesenchymal cells isolated from bone marrow, unless otherwise stated.

1.1.1.2 Definition

The MSC population is highly heterogeneous, which is evident when individual colonies are examined. Broad range of colony sizes can be observed as well as varying cellular morphology. Some cells appear long and spindle shaped while others are more round and flat. Culturing for a prolonged time can affect the phenotype and the colonies will vary in their differentiation potential (Bianco et al., 2001; Charbord, 2010). Based on the expression of different classes of regulatory proteins, MSC populations can be divided into distinct subpopulations including progenitors, precursor cells and lineage-committed cells with limited differentiation potential (Phinney, 2007; Phinney & Prockop, 2007). Due to above described heterogeneity, it has proven difficult to define the MSC signature and hence development of better isolation procedures is affected. No unique markers are known for MSCs and they are traditionally isolated based on their ability to adhere to plastic (da Silva Meirelles, Caplan et al., 2008; Harichandan & Buhring, 2011). The cell surface proteome of MSCs was recently defined and numerous markers identified that might enhance the isolation of MSCs in the future (Niehage, Steenblock et al., 2011).

The International Society for Cellular Therapy (ISCT) proposed the following minimal criteria for defining MSCs, now commonly accepted in the field for *in vitro* culture (Dominici, Le Blanc et al., 2006; Salem & Thiemermann, 2010; Sensebe, Krampera et al., 2010). Per definition MSCs :

1. Are Plastic-adherent under standard culture conditions
2. Express CD105, CD73 and CD90, and don't express CD45, CD34, CD14, CD11b, CD79alpha, CD19 and HLA-DR surface molecules.
3. Must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.

Unlimited self-renewal and multilineage differentiation potential define a stem cell (Horwitz et al., 2005; Salem & Thiemermann, 2010). Very few, if any, of the known types of tissue specific stem cells, actually fulfill these criteria. It is a matter of debate whether mesenchymal cells should be categorized as stem cells or not. The ISCT recognizes that within MSC populations there are cells which are bona fide stem cells, but emphasizes that not all MSCs isolated based on plastic adherence should be considered stem cells due to the heterogeneity of the population (Horwitz et al., 2005). The self-renewing capacity has been shown in a mouse-model but whether the term pluripotent or multipotent should be used to describe these cells is currently under debate (Charbord, 2010; Kuroda, Kitada et al., 2011; Sacchetti, Funari et al., 2007).

1.1.1.3 The MSC microenvironment

Mesenchymal stem cells are thought to originate from mesodermal progenitors but the *in vivo* origins and native role is in fact poorly understood, since the cells are difficult to identify (Augello, Kurth et al., 2010; Sensebe et al., 2010). This is true for other types of tissue-specific stem cells as well, based on the lack of stem cell specific markers and histological methods for identification (Morrison & Spradling, 2008). Stem cells are thought to reside in a microenvironment called a niche, which consists of cells, extra-cellular matrix (ECM) and signaling molecules that support stem cell renewal and regulate their quiescent state (Augello et al., 2010; Schraufstatter, Discipio et al., 2012). Within the niche, stem cells remain quiescent and differentiate upon departure from it, thus the niche plays a role in maintaining the stem cell pool (Kiefer, 2011; Morrison & Spradling, 2008). Stem cell niches have been identified at various anatomical locations in the body, the HSC niche in the bone marrow represents the classic example (Morrison & Spradling, 2008).

The MSC niche remains an enigma. MSCs have been isolated from most tissues of the body, from bone marrow, adipose tissue and umbilical cord tissue (Malgieri, Kantzari et al., 2010; Sensebe et al., 2010). There are some indications that MSCs from different tissue sources exhibit phenotypic heterogeneity and biological variation, but they all share the same differentiation potential and expression of common surface markers (Augello et al., 2010; Charbord, Livne et al., 2011).

The location of MSCs in tissues has been a source of vast speculations. One suggestion is that MSCs are all located in one organ or tissue type and migrate from there to other sites of the body. Another idea is that tissue-specific stem cells reside in their niches in various tissues and MSC characteristics

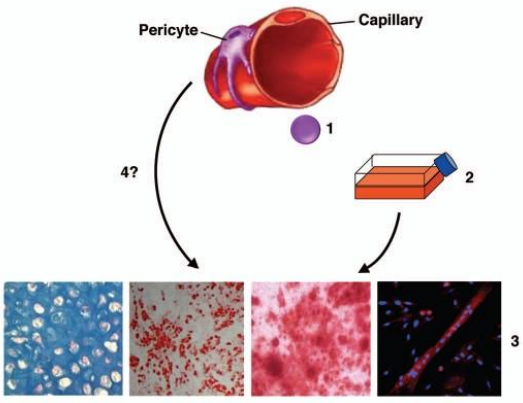

Figure 1 MSC like abilities of pericytes

Pericytes line the walls of the blood vessels but can be dissociated from the wall (1) and differentiated in vitro into multilineage progeny (2-3, cartilage, fat, bone, myocytes). Whether this represents a natural model remains unknown (4)(Crisan, Corselli et al., 2011).

can only be seen when stem cells are cultured *in vitro*. However, both of these theories have limited evidence and so the search for the precise niche remains (da Silva Meirelles et al., 2008).

The hypothesis that MSCs are actually progenies of perivascular nature has been gaining ground in recent years (Corselli, Chen et al., 2010; Crisan et al., 2011; da Silva Meirelles et al., 2008; Nombela-Arrieta et al., 2011). Pericytes are perivascular cells associated with blood vessels and show MSC like behavior in *in vitro* culture, including differentiation potential into osteoblasts, chondrocytes and adipocytes (Figure 1), fibroblastic morphology, and expression of defined surface markers (Nombela-Arrieta et al., 2011). *In vivo,* pericytes of the bone marrow are able to maintain the HSC pool, as is the case of MSCs (da Silva Meirelles et al., 2008). This data supports the observation that MSCs are widely distributed in the body and from a perivascular niche, MCSs could quickly migrate into the circulation and home (navigate) to local sites of injury (da Silva Meirelles et al., 2008).

In the bone marrow, MSCs have a fundamental role in supporting hematopoiesis and are a part of the HSC microenvironment. During embryonic development, MSCs have been shown to colonize hematopoietic sites before HSCs. They are therefore thought to participate in HSC regulation both through direct interaction and through organization of vascular networks (Nombela-Arrieta et al., 2011). This notion further supports the idea of a perivascular niche.

1.1.1.4 Multi-lineage differentiation potential

The tri-lineage differentiation potential is the hallmark of MSCs and essential to understanding their biology (Figure 2). Their differentiation was first examined and defined by observing *in vitro* cultures. However, in recent years it has been shown that differentiation pathways between lineages interact and regulate each other both *in vitro* and *in vivo* (Muruganandan, Roman et al., 2009). Differentiation is dependent on local factors and regulatory pathways (Marie & Fromigue, 2006; Muruganandan et al., 2009). Osteogenic differentiation is characterized by different stages, including proliferation, ECM production and mineralization. Osteoblast formation can be induced *in vitro* by supplying dexamethasone, ascorbic acid and β-glycerophosphate (Kassem, Abdallah et al., 2008; Marie & Fromigue, 2006).

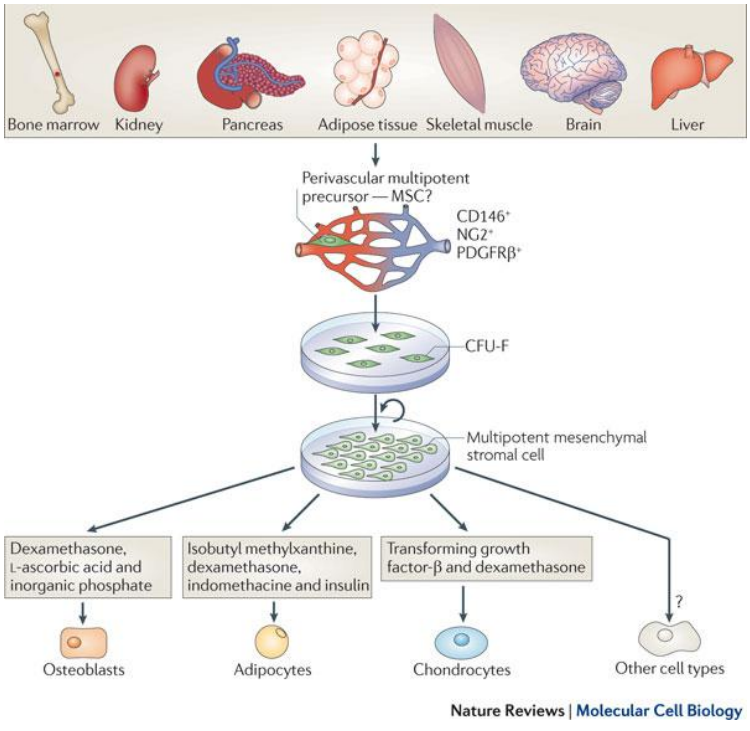

Figure 2 Isolation and differentiation of MSCs

MSCs can be isolated from various types of tissue and after expansion in culture induced to differentiate into osteoblasts, adipocytes and chondrocytes. Differentiation into other cell types remains to be confirmed (Nombela-Arrieta, Ritz et al., 2011)

Adipogenesis can be divided into two stages, determination and terminal differentiation. During determination, adipogenic precursors are lineage-primed and become pre-adipocytes. During terminal differentiation, cells start to show features of mature adipocytes (Rosen & MacDougald, 2006). Adipogenic differentiation can be induced *in vitro* using dexamethasone, isobutyl methyl xanthine, insulin and a peroxisome proliferator-activated receptor gamma 2 agonist (Kassem et al., 2008).

Both osteogenic and adipogenic differentiation pathways are under the regulation of the canonical Wnt pathway. Wnt proteins bind to Frizzled receptors and signals are mediated through β-catenin. In the absence of Wnt stimulation, β-catenin is bound to a cytoplasmic complex that leads to its phosphorylation and degradation. Wnt signaling stimulates a factor called disheveled (Dvl), which inhibits the cytoplasmic complex and degradation of β-catenin, enabling its translocation to the nucleus (Galli, Passeri et al., 2010). In the nucleus, β-catenin up-regulates the expression of runt-relater transcription factor 2 (*RUNX2)*, the master regulator of osteogenesis, while peroxisome proliferator-activated receptor gamma (*PPARgamma)*, the master regulator of adipogenesis, is down-regulated. This results in a vice versa inhibition after initiation of the respective differentiation pathway.

In absence of Wnt signaling, adipogenesis of mesenchymal progenitors can be spontaneously initiated (Rosen & MacDougald, 2006). The relationship between these two pathways is medically important, since it could shed light on diseases such as osteoporosis and diabetes (Muruganandan et al., 2009).

Chondrogenesis and osteogenesis act together during skeletogensis (Kronenberg, 2003). Some parts of the skeleton, like the skull, develop through direct differentiation of MSCs into osteoblasts. Other parts, like the long bones of the limbs, are formed through endochondral ossification, where chondrocytes lay down a matrix that can be infiltrated by blood vessels and used as a scaffold for osteoblasts (Freyria & Mallein-Gerin, 2012; Kronenberg, 2003; Pelttari, Steck et al., 2008). Chondrogenesis in vitro can be induced by various compounds including dexamethasone and ascorbic acid but the presence of Transforming growth factor beta seems to be imperative (Freyria & Mallein-Gerin, 2012; Pelttari et al., 2008).

Differentiation into other tissue types such as neuronal cells, cardiac cells, hepatocytes, pancreatic-like cells and myocytes has been reported (Kuroda et al., 2011). However, differentiation across germ layers is highly unorthodox and has conventionally been assumed to be under strict developmental control, even in the embryo (Bianco et al., 2001). Therefore, these reports need to be read critically, verified and confirmed. To do so, isolation and culture of MSCs need to be standardized and improved, and differentiation pathways thoroughly defined (Charbord, 2010). Currently, the standardized and defined culture procedures used within 30 European research units (The Genostem Project), have shown that MSC differentiation potential is restricted to the previously defined three mesodermal differentiation pathways (Charbord, 2010; Charbord et al., 2011). Thus, further research is required before accepting the differentiation of MSC across germ layers.

1.1.1.5 Proposed biological function

In the body, MSCs aid at sites of tissue damage where they enhance tissue repair either directly, by secreting soluble factors, producing ECM and differentiating, or indirectly through their anti-inflammatory abilities (Augello et al., 2010; da Silva Meirelles et al., 2008; Schraufstatter et al., 2012).

Mesenchymal stem cells appear to be relatively resistant to harm by external factors such as hypoxia, radiation and chemotherapy as would be expected from cells participating in tissue repair (Schraufstatter et al., 2012). They also seem to be tolerant against oxidative stress (Fehrer & Lepperdinger, 2005). Under normal conditions, MSCs are not found circulating in peripheral blood. It is hypothesized that under inflammatory state cells are released into the vasculature and home to sites of injury (Kode et al., 2009; Schraufstatter et al., 2012).

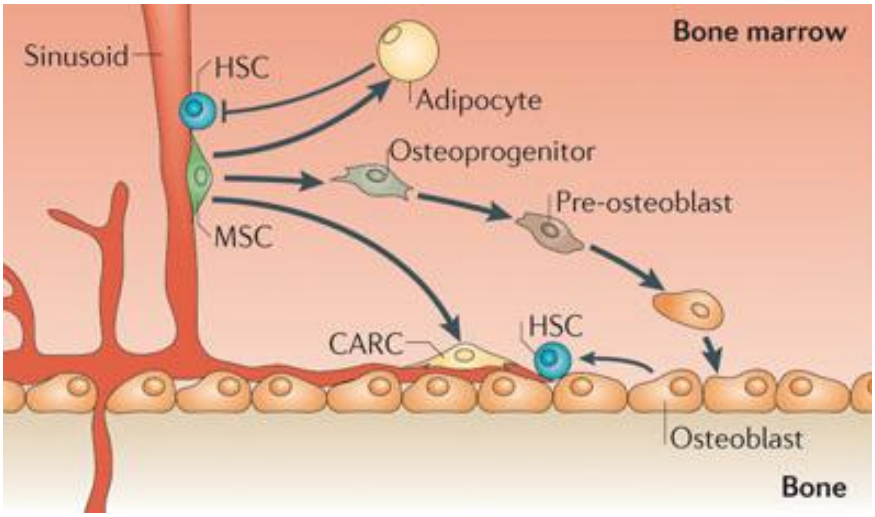

Figure 3 Proposed mechanism of HSC regulation by MSCs

MSCs regulate HSCs in the bone marrow niche. The progeny of MSCs, osteoblasts and adipocytes, regulate HSC quiescent state and activity, respectively. MSCs also secrete factors that provide a hemosupportive environment (Nombela-Arrieta et al., 2011)

As previously mentioned, MSCs have a critical role in maintaining the HSC niche and regulating hematopoiesis (Hao, Sun et al., 2012). The exact mechanism of regulation is currently unclear but is probably based on the same mechanisms as tissue repair, including the secretion of soluble factors, ECM production and differentiation (Figure 3) (Hao et al., 2012). Because of the importance of MSCs for the HSC niche and their diverse locations, there might be a role of MSC in the maintenance of other tissue-specific stem cell niches in the body

In healthy young donors, MSCs can be expected to be about 0.001-0.01% of bone marrow cells, but the numbers decrease with age (Caplan, 2007; Fehrer & Lepperdinger, 2005; Malgieri et al., 2010). Telomerase activity is low to non-existant and the cells lose their multi-lineage differentiation potential with time. Hence, the MSC pool can be expected to be almost exhausted by the end of the lifespan (Fehrer & Lepperdinger, 2005). Limited renewal capacity has also been demonstrated *in vitro* and MSCs usually discontinue proliferation after 25-50 cell population doublings (Charbord et al., 2011; Fehrer & Lepperdinger, 2005). This is consistent with increased healing time with age and their proposed role in tissue repair.

1.1.2 Immunomodulatory effects

1.1.2.1 Antigen expression

A unique ability of the MSCs is to avoid immunodetection. By expressing major histocompatibility complex (MHC) molecules, the body can distinguish between self and non-self (Le Blanc & Ringden, 2007). MSCs are an exception, since they only express MHC class I and until recently were believed to completely lack MHC class II expression (Keating, 2006). It is now clear that they express MHC class II intra-cellularly and translocation to the outer surface of the cell membrane only occurs under certain conditions. Translocation is regulated by interferon gamma (IFN-γ) levels, with low levels up-regulating the expression of MHC class II and high levels suppressing its expression (Shi et al., 2012; Yagi, Soto-Gutierrez et al., 2010). Additionally, MSCs lack the expression of important co-stimulatory

molecules for immune detection, such as CD80, CD86 or CD40 and even fail to elicit an immune response when induced to express them (Keating, 2006; Kode et al., 2009; Le Blanc & Ringden, 2007). Using this mechanism, MSCs avoid detection and immune reactions result in anergy (Salem & Thiemermann, 2010).

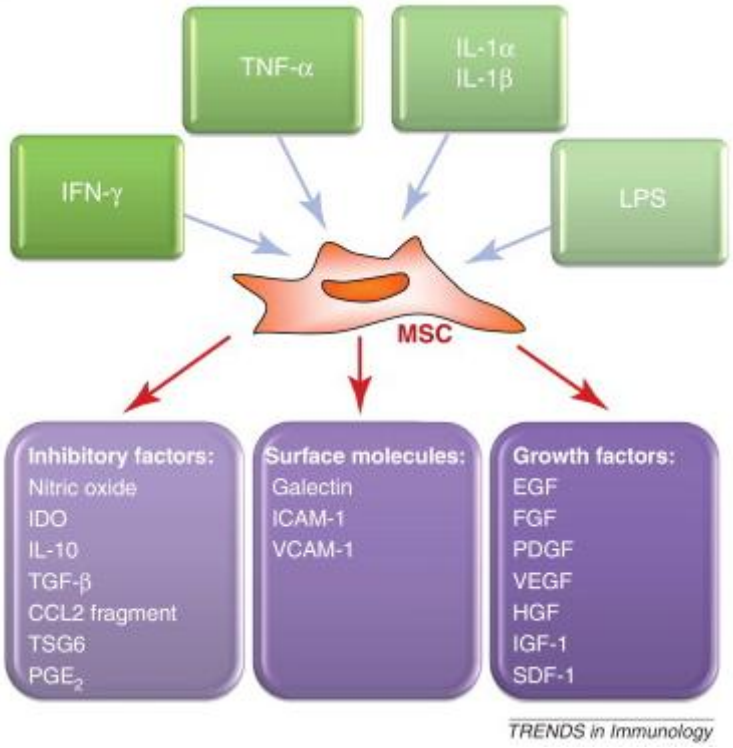

Figure 4 Immunoactivation of MSCs by inflammatory cytokines

When MSCs are stimulated with pro-inflammatory molecules, they secrete soluble factors that aid in immunosuppression. Surface receptors that facilitate homing and binding to the ECM are also expressed by MSCs (Shi, Su et al., 2012)

1.1.2.2 Mechanism of immunosuppression

In the proper environment, MSCs can be stimulated to show immunosuppressive abilities (Figure 4). At sites of tissue damage, pro-inflammatory molecules are released from necrotic cells. INF-γ, tumor necrosis factor alpha (TNF-α), interleukin – 1 (IL-1) and lipopolysaccharide especially stimulate MSC mediated immune suppression (Shi et al., 2012). Various cytokine and ECM receptors are expressed by MSCs (Table 1) but the presence of at least some of the above mentioned factors is considered necessary for immunosuppression (Kode et al., 2009; Shi et al., 2012).

When stimulated, MSCs secrete soluble factors with anti-inflammatory properties such as nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), TNF-stimulated gene 6 protein, chemokine ligand 2 and IL-10. NO is known to inhibit immune response and induce T-cell apoptosis. IDO catalyses the degradation of tryptophan into kynurenine. Kynurenine accumulation suppresses T-cell proliferation and induces apoptosis. Tryptophan is also important for lymphocyte metabolism and so the decrease in tryptophan subsequently also suppresses their proliferation (Keating, 2006; Shi et al., 2012). Other molecules such as PGE2 act in combination with NO and IDO.

In addition to secreting immunosuppressive mediators, MSCs also secrete various growth factors (Figure 4). These growth factors attract lymphocytes to zones rich in soluble factors and

Table 1 MSC Cell surface receptors

MSCs express various surface molecules that aid in signaling and binding ECM. Only examples are given in this table (Kode, Mukherjee et al., 2009)

Marker type	Examples
Surface markers	CD13, CD29, CD44, CD73, CD90, CD105, CD106, Stro-1, Sca-1
Cytokine receptors	IL-1R, IL-3R, IL-4R, IL-6R, IL-7R
Extracellular matrix receptors	ICAM-1, ICAM-2, VCAM-1, ALCAM, endoglin, hyaluronate receptor integrins α1, α2, α3, αA, αV, β1, β2, β3, β4
Growth factor receptors	BFGF-R, PDGF-R
Other receptors	Thy-1, IFN-γR, TGF-βR, TNF-R

messengers, such as IDO and NO, which eventually suppress their proliferation or induce apoptosis

Suppression of T-cell proliferation has been shown effectively *in vitro* by evaluating the effect of MSCs on mixed lymphocyte reactions (MLR). The inhibition of lymphocyte proliferation is dose dependent and more prominent depending on the number of MSCs present, usually most noticeable at the MSC:lymphocyte ratio of 1:10 (Le Blanc & Ringden, 2007). The presence of MSCs and the consequent down-regulation of IFN-γ secretion introduces a shift from T-helper cells type 1 towards T-helper cells 2, resulting in increased IL-4 production and a switch from pro-inflammatory to anti-inflammatory effects (Kode et al., 2009; Le Blanc & Ringden, 2007; Uccelli, Moretta et al., 2008). It has been shown *in vitro* that MSCs are able to inhibit antibody production by arresting B-cells in the G0/G1 phase of the cell cycle through secretion and cell-cell contact (Uccelli et al., 2008). At the same time, proliferation of T-regulatory cells is up-regulated (Le Blanc & Ringden, 2007).

MSCs also participate in innate immune responses by inhibiting the maturation of monocytes into antigen presenting cells and decreasing the cytotoxic activity of resting natural killer cells (Uccelli et al., 2008). Thus, MSCs are able to inhibit both innate and adaptive immune responses.

1.1.2.3 Homing to sites of tissue damage

After intravenous administration, MSCs have been known to specifically home to sites of tissue injury (Fox, Chamberlain et al., 2007). It has proven difficult to track MSCs *in vivo*. Donated MSCs from donors of the opposite sex can be administered and chromosomal investigation used to identify them at sites of tissue damage (Fox et al., 2007; Le Blanc, Rasmusson et al., 2004).

It has been proposed that MSCs use a homing mechanism similar to leukocytes (Figure 5) although they differ in at least two ways i) MSCs don't express L-selectins and only express E-

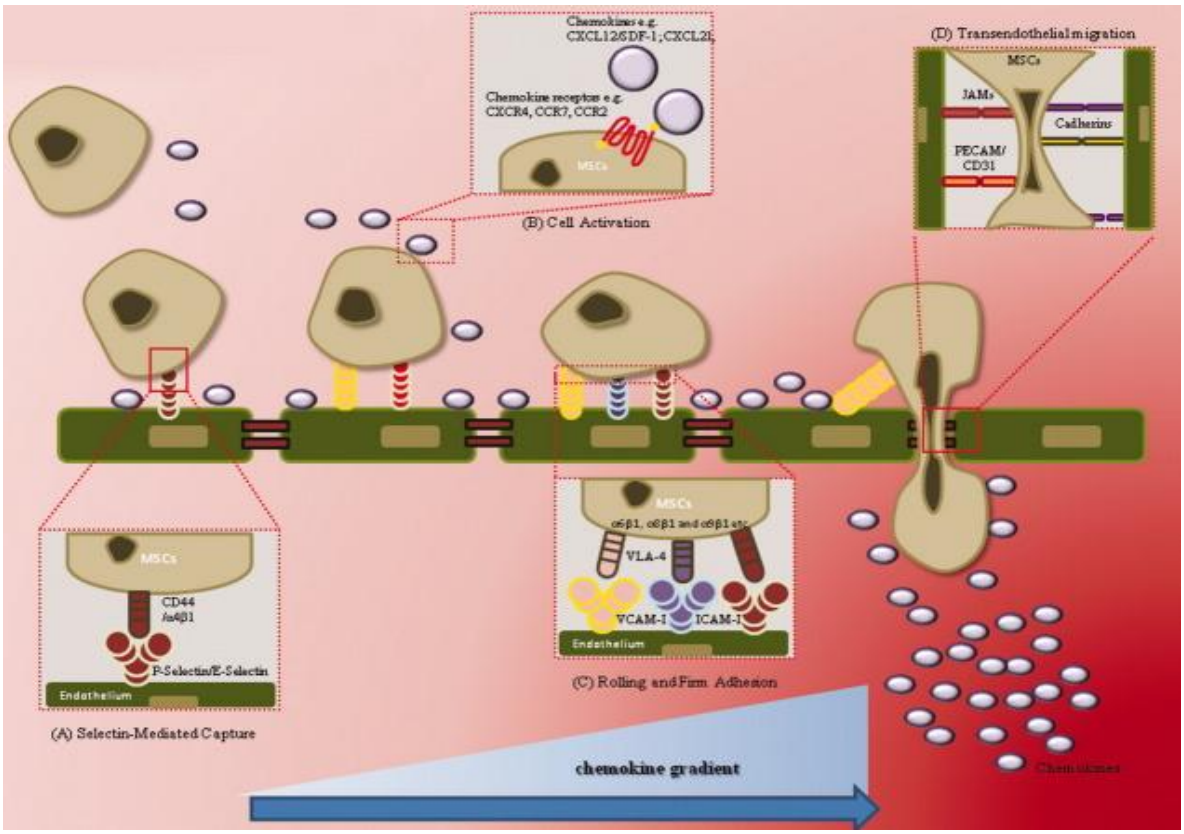

Figure 5 Mechanism of MSC homing to sites of tissue damage

MSCs are attracted to sites of damage via chemotaxis. Binding of SDF-1 with CXCR4 commits the cells to rolling and migration. Rolling is mediated through P-selectins and VCAM-1. Other adhesion molecules enhance the binding and facilitate migration through the endothelium (Salem & Thiemermann, 2010)

selectins in low quantity ii) platelet/endothelial cell adhesion molecule 1 (PECAM-1) expression is absent or low (Fox et al., 2007; Salem & Thiemermann, 2010). Stromal cell derived factor 1 (SDF-1) is expressed in various tissues and is up-regulated after tissue damage and in the presence of TNF-α (Deak, Seifried et al., 2010; Fox et al., 2007; Yagi et al., 2010). SDF-1 binds to CXCR4 on the MSC surface and attracts other MSCs (Pourrajab, Forouzannia et al., 2011; Yagi et al., 2010). In the vasculature, MSC rolling depends on binding to P-selectins through MSC specific ligand and through the binding of very late antigen (VLA) 4 to vascular cell adhesion molecule (VCAM) 1 (Salem & Thiemermann, 2010; Uccelli et al., 2008). Strong binding and migration through the endothelium is facilitated through the binding of junctional adhesion molecule, cadherins and PECAM-1 expressed on the endothelium. Matrix metalloproteinase (MMP) 2 is secreted by the MSCs to degrade the basal membrane and from there they migrate further into the tissue (Salem & Thiemermann, 2010; Uccelli et al., 2008).

1.1.3 Clinical approach and the potential malignancy

1.1.3.1 Regenerative medicine and tissue engineering of MSCs

Regenerative medicine approaches use either the transplantation of *ex vivo* expanded stem cells, such as MSCs, or try to activate and mobilize endogenous cells in the patient for tissue repair (Andersson & Lendahl, 2009). MSCs constitute only a small proportion of the bone marrow and recruitment of sufficient numbers to sites of damage or disease could prove difficult.

Therapeutically-relevant cell counts can be obtained using *ex vivo* expansion after 4-10 population doublings and have received greater focus in research (Kuraitis, Ruel et al., 2011). Administration of MSCs is safe and has been used in order to treat immunological disorders, such as graft versus host disease (GvHD) and arthritis (Hao et al., 2012; Le Blanc, Samuelsson et al., 2007; Lin & Hogan, 2011). Since the first report of therapeutic MSC use in treating severe GvHD in 2004, increasing numbers of clinical studies have been applying MSCs to either treat or prevent GvHD using different infusion approaches (Le Blanc et al., 2004). Results have varied between complete healing and no benefit at all depending on the patient and the approach. (Lin & Hogan, 2011). There is some evidence that MSC infusion may enhance engraftment of HSCs following HSC transplantation (Le Blanc et al., 2007). Disease models for collagen-induced arthritis, inflammatory bowel disease, diabetes and systemic lupus erythematosus have been studied in animals but results are conflicting (Shi et al., 2012). It has been pointed out that immunomodulatory effects differ between animals and humans, so animal models may not constitute a predictive model for clinical situations in humans (Le Blanc & Ringden, 2007).

The possible clinical use of MSCs in the treatment of immune mediated diseases is of great interest, especially when considering increasing life expectancy and the burden of disease. MSCs retain their immunosuppressive effects with age. Elderly patients in need of transplantation often display low tolerance for the side effects of immunosuppressive treatments, and for them, treatment with MSCs could be beneficial (Landgraf, Brunauer et al., 2011).

Improved cardiac function has been reported following MSC injection into the myocardium, reduction in scar formation and possible activation of endogenous stem cells in cardiac tissue (Kuraitis et al., 2011). These effects are most likely mediated in a paracrine manner (Brignier & Gewirtz, 2010). MSCs have also been used to improve the healing of skin and bone defects, in auto-immune disorders of the nervous system and renal disease (Kode et al., 2009; Salem & Thiemermann, 2010; Sensebe et al., 2010).

Tissue engineering is a concept originally defined in 1993 as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes (Langer & Vacanti, 1993). Common approaches include using scaffolds for implantation that are either

seeded with cells or made to attract cells from the body. Bio-fabrication has been an emerging field in recent years and includes the formation of 3D tissue by cell assembly and cell printing (Gojo, Toyoda et al., 2011; Mironov, Trusk et al., 2009). Recently, new techniques have been developed, including the use of cell sheets and whole-organ tissue engineering by employing ECM components as a structural platform after decellularization (Gojo et al., 2011; Ott, Matthiesen et al., 2008).

In applied tissue engineering, MSCs have mostly been studied for their differentiation potential. Therefore, MSCs have been seeded on scaffolds and differentiated into osteoblasts to treat bone defects but they have also been suggested for the use in cartilage engineering. This field of research is currently very active and MSCs are generally regarded to hold a great potential for bone tissue engineering (Costa-Pinto, Reis et al., 2011; Freyria & Mallein-Gerin, 2012; Polini, Pisignano et al., 2011).

1.1.3.2 Potential malignancies

Malignant disease is in part characterized by the expression of inflammatory mediators and growth factors and thus MSCs can be attracted towards tumors. It has been suggested to use MSCs as delivery vehicles for highly toxic therapeutic drugs to tumors (Schraufstatter et al., 2012). In this approach, a delicate balance has to be kept, since the characteristics of MSCs making them an attractive tool in battling cancer, can also support tumor growth. The role of MSCs in cancer can be dual, either through involvement of modulatory effects or directly via malignant transformation (Wong, 2011). Tumor protection is mediated through MSCs anti-apoptotic effects and are thereby able to promote metastasis. MSCs have also been associated with drug-resistant cancer cells, which is thought to be mediated through paracrine factor secretion and immunosuppression (Wong, 2011).

Before administration of MSC to patients, the cells need to be expanded *ex vivo* to obtain therapeutically significant cell numbers. This leaves the cells susceptible to malignant transformation and aberrant karyotypic changes, as has sometimes been observed during long-term culture (Kode et al., 2009). Exposure to a local tumor in the stromal environment has also been shown to lead to differentiation of MSCs into cancer-associated fibroblasts, leading to tumors that resemble sarcoma (Wong, 2011). The potential for further disease progression has to be considered before employing MSCs as therapeutic agents in cancer treatment.

1.2 The skeletal system

1.2.1 Osteogenesis

1.2.1.1 Bone development

The skeleton provides mechanical support, muscle and tendon attachment and stores minerals. It is maintained through the delicate balance between osteoblasts (bone-forming cells) and osteoclasts (bone absorbing cells) (Long, 2012). The skeleton is derived from two different parts of the embryonic tissue, the mesoderm and the neural crest. The paraxial and lateral plate mesoderm form tissue units on each side of the neural tube. By the fourth week of gestation, the mesodermal units form a loose tissue called the mesenchyme. The mesenchyme is able to migrate and differentiate along different lineages and gives rise to structural components of the skeleton, *e.g.* cartilage, bone and fibroblasts. Cells derived from the neural crest also form mesenchyme which later on develops into bones of the cranium (Sadler, 2006).

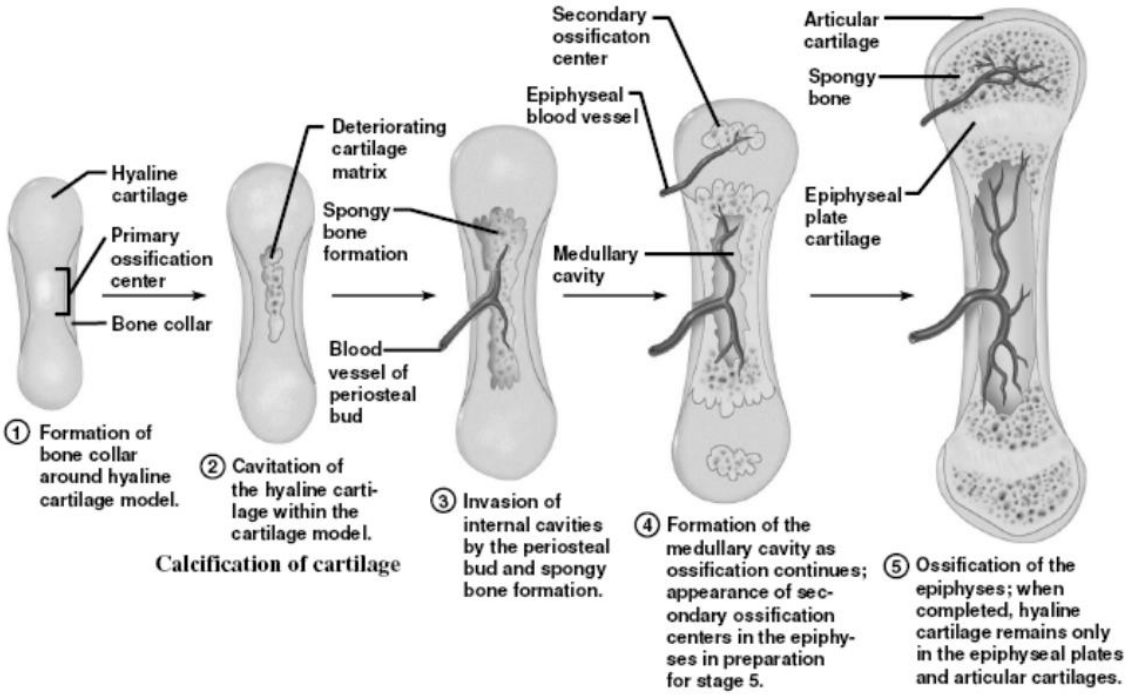

Figure 6 Bone formation through endochondral ossification in a stepwise manner

Primary ossification is initiated in the cartilage scaffold when chondrocytes reach a hypertrophic state (1-2). Blood vessels penetrate the developing bone, delivering chondroclasts which degrade the cartilage matrix and form the marrow cavity (3-4). Secondary ossification centers form and contribute to bone formation (4-5) (Cummings, 2006).

Bone development can occur in two ways; intramembranous ossification and endochondral ossification. During intramembranous ossification, osteoblasts will form directly from the mesenchyme, but this process is limited to the cranium. The remaining bones of the skeleton form through endochondral ossification. In endochondral ossification, the mesenchyme first forms cartilage scaffolds that later are used for the deposition of skeletal components. A part of these cartilage-scaffold chondrocytes proliferate and mature until they reach a hypertrophic state which in turn activates osteogenic differentiation (Long, 2012). Limb formation begins by week 6 of embryonic development by which time the limbs mainly consist of cartilage. It is not until the end of embryonic week 12 that ossification is initiated (Sadler, 2006).

After the onset of osteogenic differentiation. blood vessels penetrate the hypertrophic matrix and a marrow cavity forms (Figure 6). When the developing bone has been vascularized, chondroclasts enter the marrow cavity and degrade the cartilage matrix, thereby increasing the size of the cavity. Perichondrial cells and progenitor cells, such as MSCs, then arrive and differentiate into osteoblasts, contributing to bone formation.

In the third trimester, skeletal mineralization occurs inducing the deposition of calcium, magnesium and phosphorus. In the femur, secondary ossification takes place but most epiphyses will remain cartilagenous until after birth to allow bone development and growth (Kovacs, 2011).

1.2.1.2 Osteogenic regulation

Bone development and turn-over is a tightly regulated process depending on the interplay of various lineage specific transcription factors. Runt-related transcription factor 2 (*RUNX2*) is the master regulator of osteogenesis and plays a central role in the commitment of progenitor cells to the osteochondro-progenitor lineage (Franceschi et al., 2007). *RUNX2* is essential for bone formation and

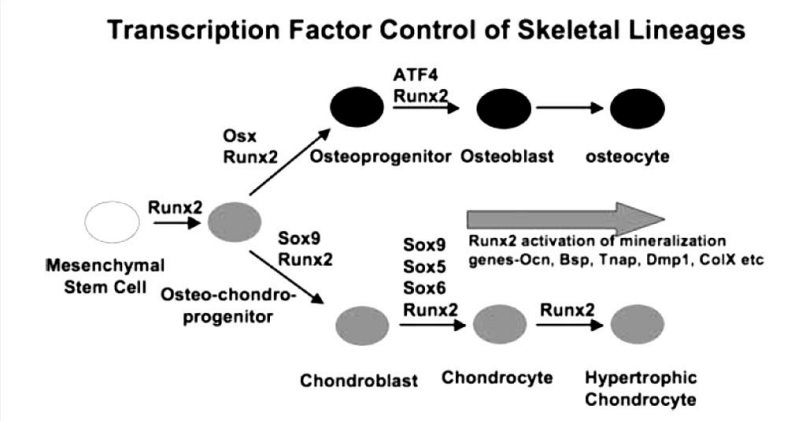

Figure 7 Transcriptional regulation of skeletogensis

The master regulator RUNX2 is expressed early and co-expression with SOX9 initiates chondrogenesis. Expression of SP7 (OSX) in RUNX2 expressing cells commits progenitor cells to the osteoblast linage (Franceschi, Ge et al., 2007)

the homozygous lack of this regulator will result in the complete absence of skeletogensis, whereas haplo-expression leads to severe bone defects (Long, 2012).

During mesenchymal condensation, expression of *SOX9* is up-regulated. *SOX9* is one of the regulators of chondrogenesis and is required for the expression of cartilage specific matrix proteins, i.e. collagen type I and type II, and aggrecan (Dancer, Henry et al., 2010). Initially, co-expression of *SOX9* and *RUNX2* initiates cartilage formation, but gradually, *RUNX2* expression becomes more restricted to perichondrial cells and osteoblasts (Figure 7)

Expression of another regulator, SP7 transcription factor (*SP7,* previously known as osterix), commits progenitor cells to the osteoblast linage (Franceschi et al., 2007). *SP7* is a downstream target of *RUNX2* but lack of this transcription factor will also results in complete lack of osteoblasts (Long, 2012).

During *in vitro* differentiation of MSCs towards the osteoblastic lineage, the same markers are expressed. Osteogenic differentiation *in vitro* can be divided into three stages. The first stage includes mainly cell proliferation followed by a stage of early differentiation, which is characterized by the expression of alkaline phosphatase (*ALP*) and collagen type I (*COL1)*. The last stage is characterized by the high expression of bone gamma-carboxyglutamate protein (*BGLAP*, previously known as osteocalin),secreted phosphoprotein 1 (*SPP1*, previously known as osteopontin) and by mineral deposition (Birmingham, Niebur et al., 2012; Marie & Fromigue, 2006).

1.2.1.3 Bone remodeling and repair

Bone is constantly absorbed and degraded by osteoclasts of hematopoietic origin, and then reconstructed by osteoblasts under hormonal control (Confavreux, 2011). Osteogenesis is active through adulthood but at a slower pace due to a decreased number of progenitor cells and increased mechanical force on adult bones (Deschaseaux, Sensebe et al., 2009)

The bone healing process has a remarkable resemblance to osteogenesis during development, such as high ALP activity (Deschaseaux et al., 2009; Pagani, Francucci et al., 2005). Healing of bone injuries is not mediated through the formation of fibrous scar tissue but rather through the initiation of callus formation by ossification. Therefore, newly formed bone cannot be distinguished from un-injured bone based on morphology (Deschaseaux et al., 2009). Bone formation during healing depends on several factors, such as MSC access to the site of injury, scaffold attachment, soluble factors and mechanical stimuli. The process relies on adequate vascularisation and transport of soluble growth factors ensuring homing of MSCs and survival of osteoblasts. Osteo-progenitors and MSCs adhere to the ends of the fracture and can use the ECM as a scaffold for depositing the callus. After serious injury, disease and severe fractures, bones can fail to repair. This can be due to disruption of vasculature and lack of a supportive matrix. Non-union of bones is difficult to treat and can usually

only be repaired by using invasive techniques, such as metal or bone implants (Kanczler & Oreffo, 2008)

1.2.2 Bone defects and current strategies

With increasing life expectancy, the number of severe fractures requiring invasive repair, *e.g*. hip fractures and spinal fusion surgery, has increased (Kanczler & Oreffo, 2008). Diseases such as osteoporosis, osteogenesis imperfecta and tumor growth can also result in bone defects. Skeletal disorders often affect the patient's quality of life dramatically by inhibiting locomotion and causing pain and deformity (Costa-Pinto et al., 2011).

The use of autologous bone grafts is considered the gold standard for treating bone defects (Costa-Pinto et al., 2011; Kraus & Kirker-Head, 2006). Autologous grafts are obtained from donor sites using the patient's own body, for example the iliac crest. In severe cases, autologous bone is not sufficient to treat the defect and additionally, obtaining the grafts can lead to side effects at the site of collection such as blood loss, non-union or infection (Kanczler & Oreffo, 2008; Kraus & Kirker-Head, 2006). Millions of surgical treatments are performed every year with the number of operations and expenses steadily increasing, raising the demand for alternative therapies and better solutions (Kanczler & Oreffo, 2008; Panetta, Gupta et al., 2009).

Various tissue engineering strategies have been developed over the years to meet the demand for bone repair (Kanczler & Oreffo, 2008). The most successful strategy so far includes the use of scaffolds to provide a platform for osteogenesis and guided regeneration. Scaffolds can be produced from various materials including hydroxyapatite, tricalcium phosphate, calcium sulfates, titanium, bioactive glass and chitosan (Kraus & Kirker-Head, 2006; Polini et al., 2011). Nevertheless, the problem remains that scaffolds in tissue engineering lack osteogenic properties and rely on the adherence and proliferation of progenitor cells.

In this field, MSCs have been studied for their osteogenic differentiation potential and the results are promising (Marie & Fromigue, 2006). Methods to direct MSCs to engraft at sites of bone fractures using scaffolds or cell modifications have been developed (Centeno, Schultz et al., 2010; Guan, Yao et al., 2012). Most often MSCs are expanded *in vitro* to obtain sufficient numbers of cells and then induced to differentiate into osteoblasts (Marie & Fromigue, 2006). Undifferentiated MSCs can be used, but osteogenic differentiation potential and colonization of the scaffolds strongly depends on the appropriate environment. Nevertheless, a recent long-term analysis has shown better sustainability of primed osteoblasts compared to undifferentiated MSCs in tissue engineering approaches (Gomide, Zonari et al., 2012).

This development of tissue engineering is of great interest to clinicians and might soon be introduced into the clinical practice. Therefore, the development and standardization of good isolation practices, culture and differentiation methods for MSCs need to be solved before approval by regulatory authorities.

1.3 Platelet lysates

1.3.1 Platelet biology

1.3.1.1 Biological role

Plat**e**lets, or thrombocytes, are a-nucleated products of large multi-nucleated cells in the bone marrow called megakaryocytes. Each megakaryocyte can produce thousands of platelets during thrompopoieses by the formation of long pseudopods (Italiano & Hartwig, 2007).

Platelets play a key role in hemostasis, participating both in primary and secondary stages of the process. In primary hemostasis, platelets adhere to the vascular endothelium upon injury and release platelet derived growth factor (PDGF), which aids in endothelial repair. Adhesion to the vascular wall also triggers platelet activation resulting in shape changes, from smooth discs to structures with pseudopods that facilitate platelet aggregation. Fibrinogen binds to platelets already adhering to the injured vascular wall, forming a platelet plug to reduce blood loss from the injured vessel. Secretion of factors, such as adenosine diphosphate, serotonin, calcium and platelet specific molecules like β-thromboglobulin, platelet factor 4 and thrombospondin, induces aggregation and cause vasoconstriction. In secondary hemostasis the platelet plug formed during the primary stage is strengthened through activation of coagulation factors present in the plasma, to further reduce bleeding (Harmening, Escobar et al., 2009).

A reduction in the number of platelets in the circulation is called thrombocytopenia and can result in dispensed bleeding into skin and mucosa and easy bruising. This can be caused by a number of reasons and can sometimes be treated with platelet transfusions (Liles & Knupp, 2009). Cancer patients with hematological disorders frequently need platelet transfusions since thrombocytopenia can arise from intense cytotoxic therapy and cause life-threatening hemorrhages (Perrotta & Snyder, 2007).

Platelets are sensitive to activation by blood vessels, blood flow and the composition of the blood. The platelet surface, when activated, is catalytic and able to initiate coagulation. Changes in levels of coagulation factors in the blood can result in a hypercoagulable state, leading to the formation of thromboembolism with up to 30% mortality rate (Liles & Knupp, 2009). Hence, platelet activity and quantity need to be under strict homeostatic control in order to function properly, to provide protection against injury and prevent disease.

1.3.1.2 Platelet structure

Platelets, released from the bone marrow, are on average 2 – 5 µm in diameter and circulate in the blood stream for 7 - 10 days before being replaced. The interior can be divided into three zones, the peripheral zone, the sol-gel zone and the organelle zone, each with distinct functions (Harmening et al., 2009).

The peripheral zone includes the cell membrane, glycolcalyx and the open canalicular system. The glycolcalyx is a sticky coat on the outside of the membrane and participates in adhesion and aggregation. The open canalicular system is a path for delivery of secretory products of platelet granules (White, 2007).

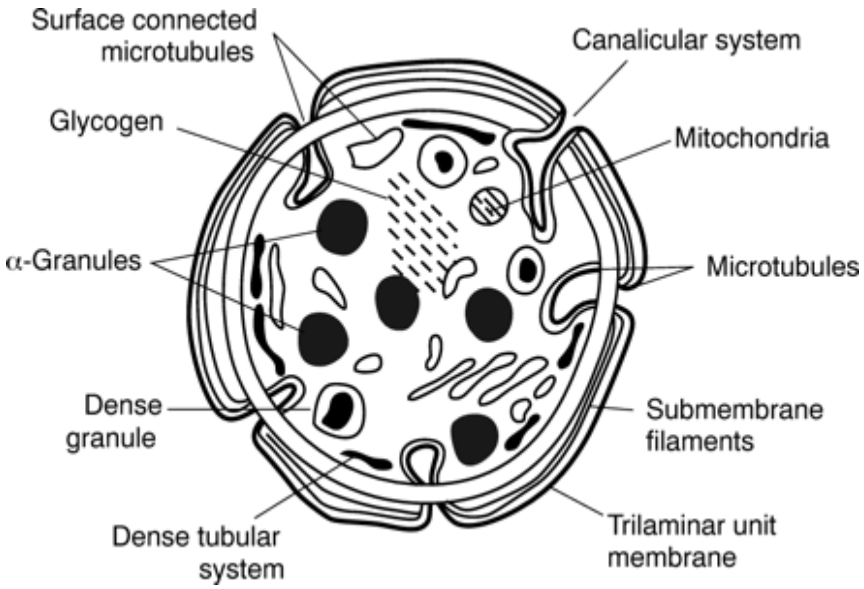

Figure 8 Platelet structure

Platelets contain various cellular organelles and their structure can be divided into three zones; the peripheral zone consisting of the outer surface of the platelet membrane , sol-gel zone consisting of microtubules and the organelle zone, which contains the α-granules (Saif & Hamilton, 2001).

The sol-gel zone, also known as the structural zone, is made up of microtubules and microfilaments. It is responsible for maintaining the platelet structure and participates in shape changes during activation (White, 2007).

The organelle zone is centrally located in the platelet and includes granules, lysosomes, mitochondria and glycogen (White, 2007).

Platelets have both α-granules and dense granules with the former being more abundant. The α-granules are rich in various soluble factors such as coagulation factors, thrombolytic molecules, growth factors, chemokines and more (

Table 2). Platelet specific molecules, such as PF4 and β-TG, are located in the α-granules. The dense granules are less abundant than α-granules and contain mainly small molecules such as ions, nucleotides and serotonin (Reed, 2007).

Table 2 Contents of α-granules

The various molecules of α-granules can be divided into groups based on their biological role and function. Each group contains a number of molecules and only examples are given in this table (Reed, 2007).

Molecule type	*Examples*
Adhesion molecules	P-selectin, van Willebrand factor, thrombospondin, fibrinogen, integrin αIIbβ3, integrin αvβ3, fibronectin
Chemokines	PF 4, β-TG, CCL3, CCL5, CCL7, CCL17, CSCL1, CXCL5, CXCL8
Coagulation pathway	Factor V, multimerin, factor VIII
Fibrinolytic pathway	α_2- macroglobulin, plasminogen, plasminogen activator inhibitor 1
Growth and angiogenesis	bFGF, EGF, HGF, IGF, TGF-β, VEGF-A, VEGF-C, PDGF
Immunological molecules	β1H globulin, factor D, c1 inhibitor, IgG
Other proteins	Albumin, α_1-antitrypsin, Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectin protease nexin-II (amyloid beta-protein precursor)

Platelets contain numerous growth factors that are known to aid in tissue repair and angiogenesis (Blair & Flaumenhaft, 2009; Reed, 2007). Among other factors, α-granules contain PDGF, vascular endothelium growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocytes growth factor (HGF) and insulin-like growth factor (IGF) in enough quantity to promote wound healing (Blair & Flaumenhaft, 2009). Products enriched in platelet secretory molecules from the α-granules also support cellular proliferation of osteogenic and tendon cells among others (Blair & Flaumenhaft, 2009). These factors initiated the use of platelet rich plasma in the treatment of orthopedic defects and further research is focused on the potential clinical role of platelets apart from hemostasis.(Feltsan, Mracna et al., 2011).

1.3.2 Platelets and cell-culture medium

1.3.2.1 Serum supplemented medium

Successful cell culturing depends on the quality of the growth medium used, proper incubation environment and handling of cells. *In vitro* culturing of cells requires an environment that mimics the natural habitat of the cells, taking into account pH, temperature, gas concentration, osmolarity, nutrients and other vital factors. The choice of growth medium is especially important with different types of media being available for different cell lines (Freshney, 2005).

Complete growth medium, by definition, contains all necessary factors and supplements for its intended use. Different types of media are available, ranging from straightforward solutions such as Eagle's Minimal Essential Medium (MEM) to more complex types such as Dulbecco's modified minimal essential medium (DMEM), Ham's F12 supplement (F12) and Roswell Park Memorial Institute medium 1640 (RPMI 1640). The combination of two complex media to obtain a more complete solution is also known and commercially available. Most media are enriched with amino acids and contain vitamins, salts and glucose. Under the appropriate incubation conditions the medium also provides the cells with stable pH from 7,0-7,4, accurate osmolarity and allows flow of gases such as oxygen and CO_2 (Freshney, 2005).

Growth medium is often supplemented with serum which adds minerals, lipids, hormones and growth factors to the media, increasing its suitability for cell culture. Adding serum also increases the viscosity of the medium, protecting the cells from damage and rupture during handling and adhesion molecules within the serum can furthermore improve cell attachment. Serum can be obtained from different sources including horses, humans and bovine animals, with fetal bovine serum being most widely used (Freshney, 2005; Rauch, Feifel et al., 2011).

Fetal bovine serum (FBS) is harvested from bovine fetuses older than three months via cardiac puncture when the mother is brought in for slaughter. A needle is inserted through the chest straight into the heart and the blood is then drained from the fetus through a tube using vacuum, gravity or massage to facilitate the process. The collected blood is then allowed to clot and serum is obtained after centrifugation. The estimated annual production of FBS is about 500.000 L from over 1.000.000 bovine fetuses (Jochems, van der Valk et al., 2002; Tekkatte, Gunasingh et al., 2011).

Fetal bovine serum will last for one year before expiration, after which time it cannot be used anymore. Based on the process of FBS production, two batches will never be identical (batch to batch variation) and has to be considered in before the set-up of experiments. (Freshney, 2005; Tekkatte et al., 2011). The choice of a suitable FBS batch for a specified cell culture system is time consuming and expensive. Furthermore, the production of FBS is dependent on the availability of bovine fetuses that can vary annually due to environmental and financial factors. Finally, FBS can contain xenogenic pathogens, including viruses, bacteria and mycoplasma (Freshney, 2005; Tekkatte et al., 2011).

With the advancement of cellular biology and the increasing interest in the clinical use of cellular therapies, the demand for serum alternatives are raised. For clinical treatments, the risk of pathogen contamination during culture and the varying quality of serum between batches is not acceptable. Antibodies against bovine proteins have been detected in patients after receiving MSCs previously expanded in media containing FBS , leading to anaphylactic shock in the most severe cases (Sundin, Ringden et al., 2007; Tekkatte et al., 2011).

Animal welfare is also an issue that needs to be considered during the harvesting and production of FBS. The fetal heart is often still beating during the blood collection and the fetus is developed enough to sense pain and distress (Jochems et al., 2002). Russell and Burch came up with the concept of 3Rs (reduce, refine and replace) in animal research 1957. Modern legislations, like the Animal Scientific Procedure Act in Europe, are based on this concept which aims to reduce the number of experimental animals used, refine the experimental procedures to minimize suffering as much as possible and replace experiments with animals with non-animal experiments (Wells, 2011).

1.3.2.2 Platelet-derived serum substitutes

Several types of serum-free culture media are already commercially available, but often they have been formulated only by trial and error to suit specific cell lines (Tekkatte et al., 2011). Serum free media solutions lack important constituents and need to be supplemented with growth factors, adhesion molecules, hormones, lipids, minerals and other factors. In most cases of serum free media, nutrient rich complex media, such as the DMEM/F12 blend, is used as basal media and supplemented with serum-free solutions. (Freshney, 2005).

Serum-free blends offer the possibility to eliminate most of the problems concerning serum supplementation. Due to high specificity, they can also be used to select for subpopulations in cultures or regulate proliferation and differentiation. When culturing heterogeneous populations of cells, the need for several types of media to accommodate the requirements of all subpopulations, might arise. Furthermore, specialized blends are not suitable for primary or continuous cultures since they could select for subpopulations not representative for the whole cell population (Freshney, 2005). This has led to the development of a great variety of cell culture media, making the choice for the correct blend for the cells of interest a difficult one. In fact, so many different kinds of media are available nowadays, that it has even been discussed to organize a growth media data bank to aid scientists in the selection of the most suitable media type. The great selection of speciliazed blends is on its own justified, as each of them serves a special purpose, but a single growth solution, fitting the culture of most cell types, has not been identified to date (Gstraunthaler, 2003).

Growth factors and other substances supplied to serum-free media need to be thoroughly purified and preferably not be produced in prokaryotic or animal models. Another disadvantage of serum free media is reduced cell proliferation and increased costs as compared to conventional serum supplemented media. Furthermore, cells cultured in serum free medium might be more susceptible to proteolytic activity of trypsin since protective abilities of the serum are missing. Hence, care must be taken during trypsination of the cells and proteolytic inhibitors added (Freshney, 2005).

Ideally, for a growth medium intended for MSC culture in the clinical arena, serum should be substituted with human-derived supplements including human growth factors, hormones and proteins (Mannello & Tonti, 2007). In the search for a versatile solution for serum replacement. human blood - derived elements have been suggested, such as human plasma, serum, platelet rich plasma (PRP) and platelet lysates (HPL) (Mannello & Tonti, 2007).

The use of autologous and allogenic human plasma and serum has been proposed. Serum is the remaining solution after whole blood has clotted and is generally thought to contain growth factors in more abundance than plasma, due to the release of growth factors during clotting. The use of autologous plasma or serum is a viable choice since it would both prevent cellular contact to FBS and

other allogeneic products limiting immunoreactions (Perez-Ilzarbe, Diez-Campelo et al., 2009). Nevertheless, the substitution of FBS with serum or plasma is an unlikely scenario for the clinics, since the amount needed to obtain clinically relevant numbers of cells in culture are beyond the donor's capabilities, especially during a state of disease. (Mannello & Tonti, 2007; Perez-Ilzarbe et al., 2009). Pooling plasma or serum to obtain a heterogeneous solution has been attempted but the results are contradicting, with some studies claiming successful isolation and expansion of MSCs while others describe poor proliferation rates and growth arrest (Mannello & Tonti, 2007; Perez-Ilzarbe et al., 2009). Even though serum and plasma don't fully support the growth of MSCs, adding platelets or platelet secretory products has been shown to greatly improve the expansion while maintaining basic characteristics (Mannello & Tonti, 2007; Sankaranarayanan, Tekkatte et al., 2011; Schallmoser, Bartmann et al., 2010; Tekkatte et al., 2011).

Platelet rich plasma (PRP) is high in the concentration of platelets within a relatively low volume of plasma. Due to high growth factor concentration and potential benefits in tissue healing, PRP has been successfully used to treat orthopedic disorders such as rotator cuff syndrome, achilles tendon rupture and patellar tendinopathy (Redler, Thompson et al., 2011). Reportedly, PRP also displays antimicrobial activity against *E. coli* and *S. aureus,* evidently beneficial in the clinical environment (Bielecki, Gazdzik et al., 2007; Redler et al., 2011). As a supplement for MSCs growth media, PRP has proven to enhance cellular proliferation and retain and even improve chondrogenic and osteogenic differentiation (Drengk, Zapf et al., 2009; Duan, Kuang et al., 2011; Mishra, Tummala et al., 2009). For platelets to secrete growth factors into the PRP solution, an activation step is need. Activation is typically achieved by adding calcium or thrombin to PRP but addition of external factors is undesirable, especially if zoonotic thrombin is used. Recombinant thrombin is an alternative but comes at higher cost (Mishra et al., 2009). Lysing the platelets in PRP, and thereby releasing the growth factors into the plasma, is one possibility that is gradually gaining more and more attention (Tekkatte et al., 2011).

Platelet lysates can be prepared from PRP by adding chemical factors, but mechanically lysing of platelets is preferred. In the mechanical approach, PRP units are frozen and subsequently thawed again at 37°C, resulting in platelet rupture and growth factor release (Schallmoser & Strunk, 2009; Tekkatte et al., 2011). Human platelet lysates (HPL) are a promising supplement for MSCs growth medium allowing MSCs to retain their basic characteristics and immunomodulatory abilities to the same extent as MSCs cultured in FBS (Bieback, Hecker et al., 2009; Flemming, Schallmoser et al., 2011; Schallmoser, Bartmann et al., 2007). MSCs have been reported to proliferate at a higher rate when cultured in HPL compared to FBS, without showing chromosomal abnormalities (Bieback et al., 2009; Crespo-Diaz, Behfar et al., 2011; Sankaranarayanan et al., 2011). Similar results have been obtained when culturing cells other than MSCs, such as renal epithelial cells and myocardial fibroblasts (Rauch et al., 2011; Riem Vis, Bouten et al., 2010). Many factors found in HPL are known to participate in osteogenesis and bone turn-over and there are some indications that HPL might enhance osteogenic differentiation of MSCs on osteogenic inducing environment (Chevallier, Anagnostou et al., 2010; Verrier, Meury et al., 2010; Wahlstrom, Linder et al., 2011).

The discovery of the potential use of platelets as growth medium supplement is of major interest and an important step towards the development of serum-free growth medium for MSCs. Since HPL supplemented growth media seems to have positive effects on other cell types as well, HPL could be a step towards a zoonotic, serum-free, multi-purpose solution that is not restricted to a special cell line (Tekkatte et al., 2011).

1.4 Blood banks as platelet resources

Blood banks store and process blood components for transfusion. The advancement in blood storage was accompanied by major advances in surgical procedures, like coronary artery bypass grafting

(Despotis, Joist et al., 1997). With advances in the field of organ and tissue transplantation the role of the hospital blood banks is gradually shifting towards bio-banking to store biospecimens other than blood components as well. The emerging demand for stem cell availability has led to the International Stem Cell Banking Initiative that aims to form a worldwide system of stem cell banks (Crook, Hei et al., 2010; Diaferia, Cardano et al., 2012). Since the knowledge and resources of tissue caretaking is already located in blood banks, they have been suggested to include banking of stem cells as well (Rebulla, Lecchi et al., 2007).

Blood banks are the main producers of platelet concentrates for transfusion. Platelet concentrates are produced either from donated whole blood or from platelet apheresis, where platelets are removed in a cell separator and the remaining blood is returned to the donor. Two preparation methods from whole blood are prominent, the PRP method and the buffy coat method. The PRP method is favored in the United States of America, where whole blood is subjected to slow centrifugation followed by hard centrifugation. The buffy coat method (Figure 10, page 33) is favored more in Europe with hard centrifugation followed by slow centrifugation (Harmening & Moroff, 2005; Perrotta & Snyder, 2007).

Platelets have short shelf-life of five to seven days after which they are discarded due to the increased risk of pathogen contamination and platelet storage lesion causing impaired platelet function (Harmening & Moroff, 2005; Ohto & Nollet, 2011). This short shelf-life leads to a significant proportion of platelet concentrates being discarded yearly due to expiration (Fuller, Uglik et al., 2011). These expired products could be a valuable source of platelet lysates for use in cell culture media.

Most studies exploring the use of HPL in culture media, use lysates prepared from fresh platelet concentrates. In this thesis, we show that expired platelet concentrates (HPLO) can be used as FBS substitute to the same extent as platelet lysates from fresh concentrates (HPLF). Proliferation, immune-modulatory functions and osteogenic differentiation are maintained.

2 Objectives

The aim of this study was to analyze the effect of expanding mesenchymal stem cells (MSC) in media supplemented either with MSC-screened fetal bovine serum versus the use of lysates produced from fresh and expired platelets on:

1. MSC morphology and proliferation
2. Immunomodulatory properties of MSCs
3. Osteogenic differentiation

3 Materials and methods

3.1 Experimental set-up

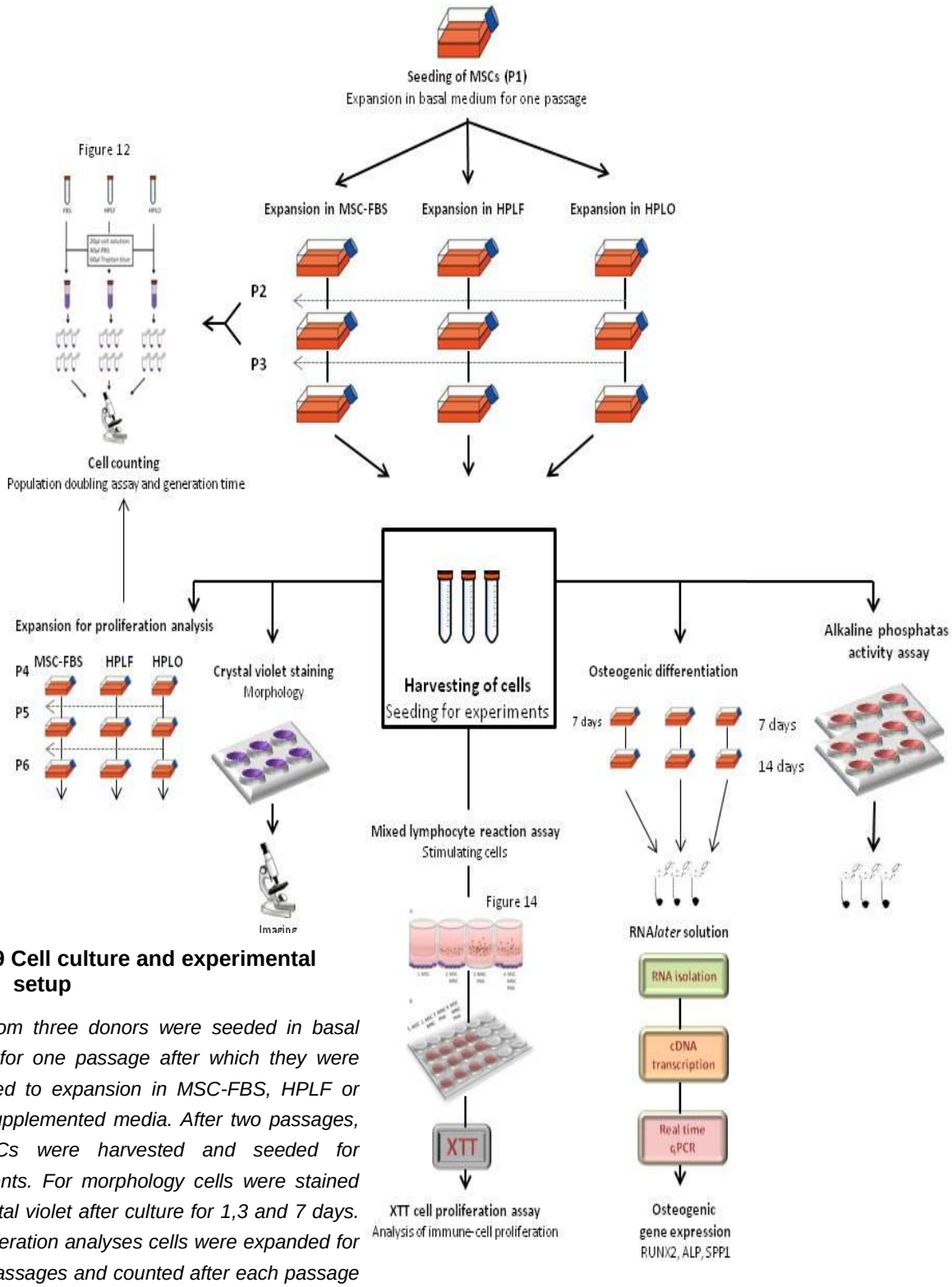

Figure 9 Cell culture and experimental setup

MSCs from three donors were seeded in basal medium for one passage after which they were transferred to expansion in MSC-FBS, HPLF or HPLO supplemented media. After two passages, the MSCs were harvested and seeded for experiments. For morphology cells were stained with crystal violet after culture for 1,3 and 7 days. For proliferation analyses cells were expanded for further passages and counted after each passage (Figure 12 in text)

Immunomodulation was analyzed using an MLR assay (Figure 14 in text) and evaluated with XTT cell proliferation assay. In osteogenic differentiation cells were seeded for alkaline phosphatase activity assay or for osteogenic gene expression. After expansion in osteogenic media for evaluation of osteogenic gene expression, RNA was isolated, cDNA synthesized and gene expression analyzed with a real-time qPCR

The experimental setup can be seen in Figure 9. Cells from three different MSC donors were seeded in basal growth medium for one passage. After one passage the MSCs were harvested and transferred to expansion in mesenchymal stem cell screened FBS (MSC-FBS), HPL from fresh platelet rich concentrates (HPLF) or HPL from expired (outdate) platelet rich concentrates (HPLO). After two passages in expansion the MSCs were harvested and seeded for experiments.

Morphology was evaluated after staining the MSCs with crystal violet and imaging them. Proliferation analysis was done by continuing the expansion in each media type and counting the cells after each passage.

Immunomodulation was evaluated by seeding the cells in a mixed lymphocyte reaction assay (MLR) and analyze the proliferation of immune-cells with a XTT cell proliferation analyses.

Osteogenic differentiation was initated both for osteogenic gene expression of *RUNX2, ALP* and *SPP1* with realt-time qPCR and also for alkaline phosphatase activity assay. Methods are described in detail in the following sections.

3.2 Preparation of human platelet lysates

3.2.1 Preparation of platelet rich concentrates

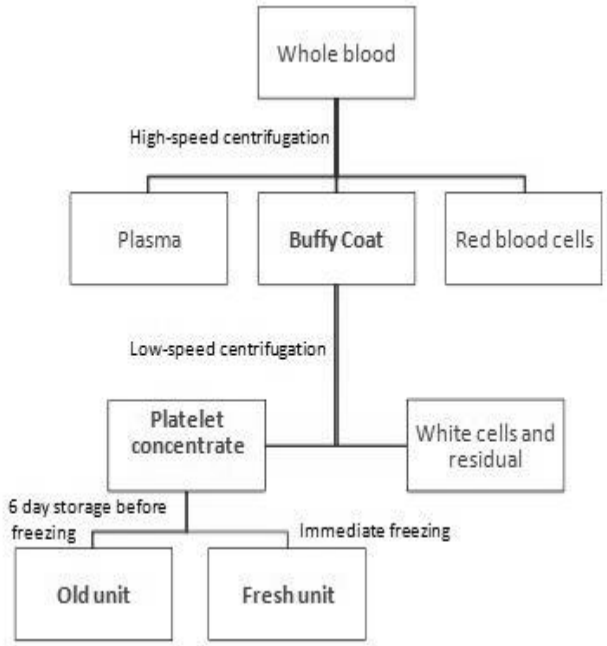

Figure 10. Preparation process of platelet rich units from buffy coats.

Units of platelet rich concentrate were prepared from BC after low speed centrifugation. The units were divided into two parts. One part was placed in -80°C immediately while the other part was stored for six days until expired before being placed in -80°C (Perrotta & Snyder, 2007)

Units of platelet concentrates using the buffy coat (BC) method were prepared in the blood processing department at the Blood bank, Iceland (Figure 10). Buffy coats, rich in platelets and leukocytes, were derived from 10 donor units of whole blood by high-speed centrifugation, which separated whole blood into plasma, erythrocytes and buffy coats. Each of separated cell populations were stored in separate units.

Five BCs were combined in a plasma supplemented bag (Fenwal, Lake Zhurich, IL, USA) and centrifuged at low-speed to obtain a supernatant, which is rich in platelets. The supernatant was transferred to a separate bag using Optipress® II (Fenwal, Baxter S.A., Maurepas, France).

The press automatically detects erythrocytes and stops; the resulting product is a platelet rich concentrate low in leukocytes.

The platelet rich concentrate was separated into two bags and allowed to rest at room temperature, because the process could have lead to platelet activation.

After the resting period, platelets rich concentrates were separated into two groups, one being frozen at -80°C immediately (fresh unit) and the other stored until expiration after six days before freezing at -80°C (expired unit). This part of the preparation was performed by trained clinical staff at the blood processing department of the Blood bank.

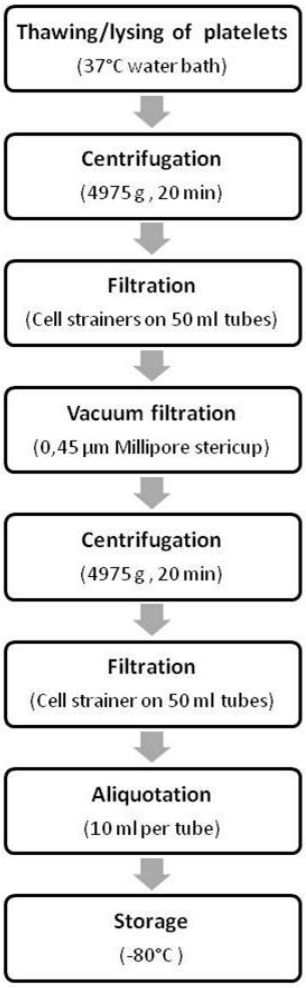

Figure 11. Lysis and filtration process of platelet rich concentrates

Frozen platelets at -80°C were lysed by thawing at 37°C. The resulting lysate was purified by repeated filtration and centrifugation. The final product was aliquoted and stored at -80°C.

3.2.2 Lysing and filtration of platelet rich concentrates

Human platelet lysates (HPL) were prepared by lysing platelets from the platelet rich concentrates in a single freeze-thaw cycle (Figure 11). Expired units were treated separately from the fresh units to prevent cross-contamination Stored units were thawed in a 37°C water bath and wiped with ethanol before transfer to a sterile hood, where contents were divided equally into 50 ml plastic tubes. After centrifugation at 4975 g for 20 min, the supernatant was filtered through 40 µm cell strainer (BD Falcon, Franklin lakes, NJ, USA) into empty 50 ml plastic tubes and the pellet discarded. The filtrate was then transferred into 0.45 µm Stericup (Millipore, Billerica, MA, USA) and filtered under -0.9 bar pressure resulting in a pool of filtered lysate. The filtered lysate was then centrifuged for the second time at 4975 g for 20 min and filtered into empty 50 ml plastic tubes. The resulting lysate was then divided into 10 ml aliquots (to reduce repeated freeze-thaw cycles during studies), labeled and placed in -80°C for storage.

3.3 Cell culture

Mesenchymal stem cells (MSC) were seeded and cultured for one passage before being subjected to different expansion media (10% HPLF, 10% HPLO or 10% MSC-FBS). After two passages in expansion media, cells were harvested.

3.3.1 Seeding of cells

Human, bone marrow-derived mesenchymal stem cells, negative for HIV-I, hepatitis B and C viruses (Lonza, Walkersville, MD, USA), at first passage (P1) from two different donors (D1 and D3) and at passage zero (P0) from one donor (D4) were seeded in culture flasks. The cells were taken from storage in liquid nitrogen and carefully thawed in a 37°C water bath. When thawed, the cells were carefully transferred into pre-warmed basal growth medium (MSCBM, Lonza) and centrifuged at 509 g for five min to minimize the effects of DMSO (IDT Biologika Gmbh, Steinbach, Germany) preservation. DMSO is used during the storage of MSC in liquid nitrogen to prevent cell rupture. The supernatant was discarded and cells resuspended in pre-warmed medium before being seeded into culture flasks at a density of 6000 cells/cm^2. Flasks were immediately placed in a 5% CO_2 incubator at 95% humidity and 37°C (standard culture conditions).

3.3.2 Subculturing and harvesting of cells

When seeded cells had reached 80%-90% confluency, they were subcultured and cells from each donor seeded for expansion in the three different expansion media (10% MSC-FBS, 10% HPLF or 10% HPLO). Basal medium was prepared by adding 4 IU/ml of heparin (LEO Pharma A/S, Ballerup, Denmark) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) into DMEM/F12 growth medium (Gibco), The three different expansion media were prepared from basal medium by adding HPLF, HPLO or MSC-FBS (Gibco) at 10% concentration. HPLF and HPLO were centrifuged for five min at 4975 g to separate precipitates from the solution. The supernatant was then used in the medium

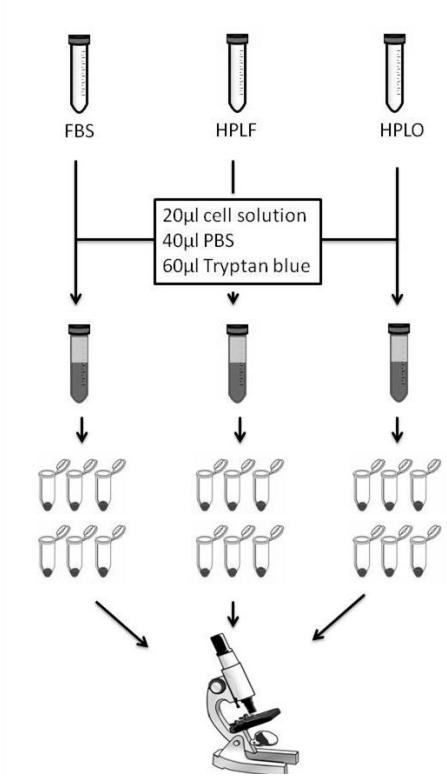

Figure 12 Staining and counting of MSCs

MSCs expanded in MSC-FBS, HPLF or HPLO supplemented media were harvested and a sample from each stained with trypan blue. Cell counts in six samples from the stained solution were determined in an inverted microscope and the concentration of cells/ml determined using Formula 1

Cells were washed with phosphate buffered saline (Giboc, PBS) before being trypsinized with the proteolytic enzyme trypsin to detach them form the surface of the culture vessels. When cells had detached from the plastic surface of the culture flasks, trypsin (Gibco) was neutralized with basal growth medium (Lonza) and the resulting solution transferred into 15 ml plastic tubes. The tubes were centrifuged at 509 g, after which the supernatant was discarded and the pellet resupsended in basal growth medium. This was repeated twice. A 20 µl sample was stained with Trypan blue (Gibco) for viability and counted with an improved Neubauer hemocytometer (Assistent, Munich, Germany). Cells were then seeded at a density of 5000-6000 cells/cm^2 in the three different media and incubated at standard culture conditions (5% CO2, 95% humidity and 37°C) until 80%-90% confluent. Medium was fully changed every 2-3 days. Cells were subcultured two times in the treatment medium before harvesting.

3.4 Morphological analysis

MSC morphology was observed in an inverted microscope and imaged frequently for five passages (P2-P6) and during osteogenic differentiation. For better visibility of cellular morphology, MSCs in expansion were stained with crystal violet.

3.4.1 Crystal violet staining

After one passage in basal growth medium (Lonza) and two passages in expansion media, cells were harvested and seeded in duplicate in 6- well plates for one, three and seven days of culture, respectively.

At the indicated time points, cultures were stained with crystal violet as following: Media was removed and cells washed with PBS before fixation in 4% para-formaldehyde for 15 minutes. After fixation, cells were incubated at room temperature with crystal violet solution for 30 minutes. After treatment with the staining solution, cells were washed four times in PBS and once in water before drying over night. Images were taken in an inverted microscope using Infinity Capture 2.0 Software (Lumenerea, Capella court, Ottawa, ON, Canada)

3.5 Population doubling assay

Expansion of MSCs from D1 and D4 was continued for six passages and quantified at the end of each passage in an improved Neubauer hemocytometer (Assistent). Population doublings (PD) and generation time (GT, time between two PDs) was determined from cell counts.

3.5.1 Cell counting

During subculturing, samples were obtained from each cell culture expanded in MSC-FBS, HPLF or HPLO supplemented media. The samples (20 µl) were stained with trypan blue (60 µl), after dilution in PBS (40 µl), and incubated at room temperature for 20 min. Each sample was counted six times in an improved Neubauer hemocytometer in an inverted microscope at 10x magnification (Figure 12). All viable cells in 4 large squares of the hemocytometer were counted but non-viable cells, that stained blue, were excluded (Figure 13). Cell count per ml was determined using Formula 1.

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Formula 1 Cell concentration per ml

This formula was used to determine the cell concentration per ml of cell solution obtained after each passage. $Cells_{mean}$ refers to the mean count of cells in the four large squares, five is the dilution factor and 10^4 is the conversion factor for converting to ml.

$$Cells/_{ml} = Cells_{Mean} \cdot 5 \cdot 10^4$$

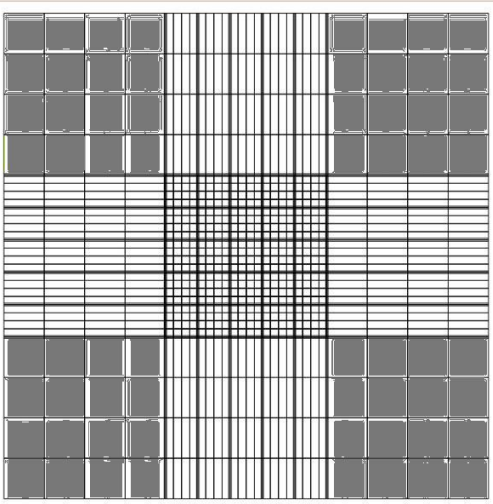

Figure 13 Layout of an improved Neubauer counting chamber

MSCs cultured in MSC-FBS, HPLF or HPLO were stained with trypan blue and counted six times at the end of each passage. Cells were counted in all squares of the four shaded areas.

3.5.2 Calculations for proliferation analysis

Population doublings (PD) at each passage from P1-P6 were determined using Formula 2a and added to the PD of former passages to obtain the number of cumulative PD of all passages (CPD). Generation time was calculated as well, using Formula 2b (Bieback et al., 2009).

Formula 2 Population doublings and generation time

The following formula was used to determine population doublings (a) and generation time (b) of MSCs expanded in MSC-FBS, HPLF or HPLO supplemented media from cell counts. N_H is the number of harvested cells at the end of each passage, N_1 is the number of cells seeded at the beginning of each passage and Δt is the time between passages.

a)	b)
$PD = \frac{[\log_{10}(N_H) - \log_{10}(N_1)]}{\log_{10}(2)}$	$GT = \frac{[\log_{10}(2) - \Delta t]}{[\log_{10}(N_H) - \log_{10} N_1]}$

3.6 Osteogenic differentiation

After two passages in the three different expansion cells were harvested by trypsination (described above). Cells were seeded in pre-warmed osteogenic culture medium containing dexamethasone

(1 ml), ascorbate (1 ml), mesenchymal stem cell growth supplement (20 ml), L-glutamin (4 ml), penicillin/streptomycin (2 ml) and β – glycerophosphate (2 ml). Osteogenic culture media along with necessary supplements was purchased from Lonza poietics™. Seeding density was 3000 cells per cm^2. Medium was fully changed every 2-3 days for 7 and 21 days after which MSCs were harvested. Cells were harvested with trypsination after osteogenic differentiation. The differentiated MSCs adhered tightly to the flask wall and were further detached after trypsination using a cell scraper (BD Falcon). Cells were washed in PBS with 10% MSC-FBS to neutralize the trypsin and reduce harm from handling and scraping. Cells were centrifuged at 509 g, the supernatant was discarded and the remaining pellet suspended in 1 ml of RNA*later*® solution (Ambion, Stocholm Sweden). After suspension it split into two 0,5 ml samples and stored at -20°C.

3.7 Mixed lymphocyte reaction assay

Immunosuppressive abilities were verified by mixed lymphocyte reaction assay. After two passages in the three different expansion media (10% MSC-FBS, 10% HPLF or 10% HPLO) MSCs were harvested by trypsination (described above). Cells from each donor and each expansion medium were transferred into three 24-well plates and 50.000 MSCs were seeded into each well with one ml of RPMI 1640 medium (Lonza) supplemented with 10% MSC-FBS. Cells were incubated for 24 hours at standard conditions to allow adherence to the surface.

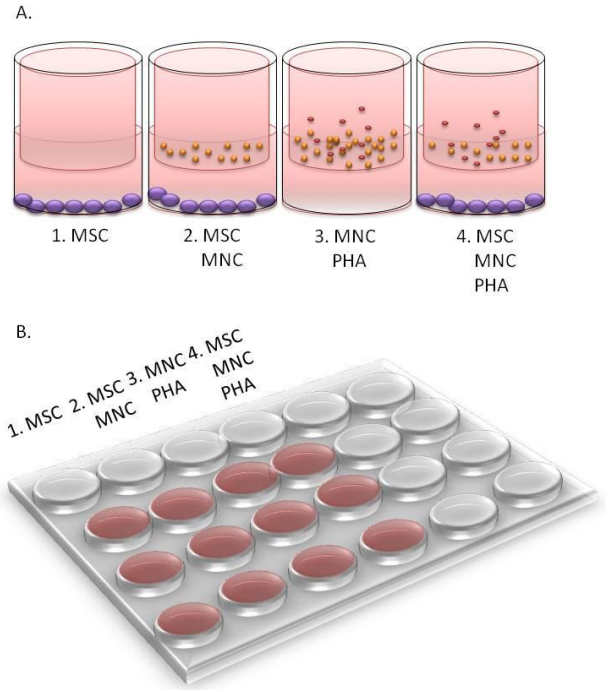

Figure 14 MLR assay setup

A. MSCs (50.000 cells) were seeded in the bottom of wells of a 24-well plate (A1, A2 and A4). Inserts were placed in the wells and 500.000 MNCs seeded in the inserts (A2-A4). MNC proliferation was stimulated by adding PHA to the inserts (A3-A4). Well A1 served as a blank, wells A2-A3 were used as controls and well A4 was the test subject. B. The layout of the plates can be seen, each type was set up in triplicate.

3.7.1 Isolation of Buffy coat-Mononuclear cells

Mononuclear cells (MNC) were isolated from buffy coat blood using the ficoll method. Buffy coat blood was obtained from the blood processing department at the Blood Bank and diluted at a ratio of 1:2 in PBS. Histopaque-1077 (Sigma, St. Louis, MO, USA) solution was placed in the bottom of a plastic tube and the diluted blood-solution carefully layered on top at ratio of 3:5, respectively. After centrifugation for 20 min at 796 g (no acceleration or deceleration), blood components in the tube had separated into visible layers. A layer of MNCs had formed between the plasma at the top and erythrocytes in the bottom. The MNC layer was carefully removed and transferred to a separate tube. After centrifugation for 10 min at 575 g (acceleration and deceleration active), the resulting supernatant was discarded. The pellet was resuspended in 2 ml of erythrocyte lysis buffer, BD Pharma Lyse™ Lysing Buffer (BD Bioscience, Franklin Lakes, NJ, USA), for three min at room temperature. Then, cells were washed with 10% MSC-FBS containing RPMI 1640 medium (Gibco) and centrifuged for 10 min at 575 g. The resulting supernatant was discarded and the pellet resuspended in 10% MSC-FBS containing RPMI 1640 medium before cell counting was performed (described above).

3.7.2 Seeding and stimulation of mononuclear cells with PHA

In the previously seeded plates containing MSC, media was changed and semi-permeable inserts with 8 µm pores (BD Falcon, Franklin Lakes, NJ, USA) were placed in the wells to serve as a physical barrier preventing direct MSC and MNC contact. The assay setup can be seen in Figure 14. Mononuclear cells were seeded in 10% MSC-FBS supplemented RPMI 1640 media into the inserts at the MSC:MNC ratio of 1:10, respectively. Phytohemaglutinin (PHA, 25µl, Sigma), at the concentration of 1,5 µg/ml was used to stimulate MNC proliferation. As a blank, wells containing only MSCs were used. Control wells constituted of MSCs and MNCs without PHA stimulation and MNCs without MSCs with PHA stimulation. Plates were then incubated in standard conditions for 48 hours.

3.7.3 XTT proliferation assay

An XTT cell proliferation assay (ATCC, Munassas, VA, USA) was performed, to test the proliferation of MNCs, following manufacturer's instructions. An active XTT-solution was prepared by adding 0.1 ml of activation reagent to 5 ml of XTT reagent at 37°C. Samples (100µl) of each well from the MLR plates were transferred to a 96-well plate in duplicate

Activated XTT solution (50 µl) was added to each sample and the plate covered with adhesive aluminum foil. The plate was incubated in the dark at standard conditions for four hours after which it was swirled gently and bubbles removed. Optical density of the sample was measured at 475 nm but optical noise present in the sample was measured at 660 nm and deducted from the sample value. Multiskan® spectrum spectrometer was used for measuring and specific absorbance was determined using Formula 3. (Thermo Scientific, Vantaa, Finland).

Formula 3 Specific absorbance for XTT cell proliferation assay

The following formula was used to determine the specific absorbance of XTT cell proliferation assay performed on the MLR assay. A stands for measured absorbance.

$$Specific\ absorbance = A_{475nm}(sample) - A_{475nm}(Blank) - A_{660nm}(sample)$$

3.8 Alkaline phosphatase (ALP) activity assay

3.8.1 ALP assay

The enzymatic activity of alkaline phosphatase (ALP) during osteogenic differentiation was evaluated by ALP activity assay. After two passages in the three expansion media, MSCs were harvested by trypsination (described above). Cells were seeded in complete osteogenic medium (Lonza) at a density of 3000 cells per cm^2 in quadruplicate. Cells were cultured at standard culture conditions and osteogenic media completely changed every two to three days. After 7 and 14 days in culture, media was removed and cells washed three times with PBS before being lysed and detached from the plastic surface using 0,02% Triton-100 (Sigma) in PBS. Each well was scraped with a cell scraper (BD Falcon) and thoroughly washed by pipetting to detach adhering cells. The resulting cell solution in each well was then transferred to an eppendorf tube and each tube then vortexed vigorously for 30

sec to further lyse the cells and release the ALP. Tubes were then centrifuged at 15.700 g at 4°C for 15 min.

A p-nitrophenyl phosphate solution was prepared by dissolving a pNPP tablet (Sigma) in Tris buffer (Sigma) and stored at 37°C in the dark until needed.

After centrifugation, half of the tubes were separated from the rest and the supernatant transferred to empty labeled eppindorf tubes. These tubes were stored at -80°C for protein quantification in a BCA assay. The supernatant for each of the remaining tubes was divided into two other eppendorf tubes and pNPP solution added at a 1:1 ratio. Tubes were then incubated at 37°C in the dark for 30 min. A sample of 300 µl from each tube was transferred in duplicate into a 96-well plate and measured in Multiskan® spectrum spectrometer (Thermo Scientific, Vantaa, Finland) at a wavelength from 400-415 nm. ALP conversion of p-nitrophenol phosphate in nMol/min was calculated using Formula 4.

Formula 4 ALP activity

The following formula was used to calculate the conversion of p-nitrophenyl phosphate into p-Nitrophenol as nMol/min. The absorbance (A) was divided by the extinction coefficient 18,8 and divided by the incubation time (30 min). The value was then multiplied by 1000 to obtain the concentration in nMol/min.

$$nMol/_{min} = \frac{A/_{18,8}}{30} \cdot 1000$$

3.8.2 Bicinchoninic acid assay

To determine the protein concentration of samples used during ALP activity assays, Bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockfor, IL, USA) was used.

A serial dilution of one Albumin standard (BSA) ampule, ranging from 0 – 2000 µg/ml BSA, was prepared by dilution in distilled water. BCA working reagent was prepared according to manufacturer's instructions (Pierce). Serial dilutions for the standard curve and protein samples were transferred into a 96-well microplate (25 µl) and 200 µl of working reagent added to each well. Contents of each well were mixed thoroughly and then incubated at 37°C for 30 min in the dark and subsequently allowed to cool to room temperature. Absorbance was measured at 562 nm in a Multiskan® spectrum spectrometer (Thermo Scientific).

A standard curve was prepared from the values of the serial dilutions and the protein content of the samples derived from a linear trendline equation in µg/ml. ALP activity was then normalized according to the protein concentration.

3.9 Gene expression

To analyze the expression of the osteogenic marker genes *RUNX2, ALP* and *SPP1* a quantitative real time polymerase chain reaction (RT-qPCR) was performed on samples obtained after 7 and 21 days in osteogenic medium and on samples previously expanded in MSC-FBS, HPLF or HPLO supplemented expansion media. Before RT-qPCR was performed, RNA had to be isolated and cDNA

synthesized. RNA extraction, cDNA synthesis and Real-Time PCR was performed at TATAA Biocenter Sweden. Analysis of RT-qPCR gene expression levels was performed using GenEx 5.3.2.13 software (MultiD Analyses AB, Guthenborg, Sweden).

3.9.1 RNA isolation

Samples were stored in RNA*later*® solution (Ambion) after 0, 7 and 21 days of osteogenic differentiation and kept frozen at -20°C. For RNA isolation, samples were thawed and centrifuged for 10 min at 16.000 g. RNA*later*® was removed and the cell pellet resuspended in RLT buffer and RNA isolated using RNeasy® Mini Kit #74104 (Qiagen, Hamburg, Germany) in a Quiagen BioRobot Workstation (Quiagen, Germany). During extraction all samples were treated with RNase free DNase Set #1023460 (Qiagen) to reduce gDNA contamination. All steps were performed according to manufacturer's instructions.

Extracted RNA was analyzed for RNA quantity and quality by spectrophotometer NanoDrop ND-1000 (Thermo Scientific) according to manufacturer's instruction.

3.9.2 cDNA reverse transcription

RNA samples were normalized to 100 ng/µl where possible. Samples with a concentration less than 100 ng/µl were used undiluted. Reverse transcription was performed with TATAA GrandScripTTM kit #A103b (TATAA Biocenter AB, Gothenburg, Sweden) according to manufacturer's instructions in single 20 µl reactions, 5 µl Reverse Transcription Mix, 5µl RNase free H_2O and 10 µl sample. Reverse transcription controls (no enzymes added) were included to monitor the presence of gDNA. Controls were analyzed in pools of five, containing 2 µl of each sample (total volume 10 µl). After reverse transcription, cDNA was diluted ten fold and stored at -20°C.

3.9.3 Quantitative real-time polymerase chain reaction

RT-qPCR analysis of the generated cDNA was performed on all samples with *RUNX2*, *ALP* and *SPP1* as genes of interest. All qPCR assays were performed with 10 µl reaction volume in duplicates on the LightCycler 480 instrument (Roche Applied Science Inc., Indianapolis, IN, USA) with TATAA SYBR® GrandMaster™ Mix #TA01 (TATAA Biocenter AB). Detection was performed using FAM as fluoresence label and allowed to cycle for 45 amplification cycles (Table 3). All samples were analyzed with TATAA Human Reference Gene panel. Suitable reference genes were identified by analyzing qPCR data with GenEx 5.3.2.13 software (MultiD Analyses) using functions for geNorm and NormFinder. Primers for genes of interest were designed by TATAA biocenter AB. All experiments were performd according to manufacturer‘s instructions.

Table 3 RT-qPCR temperature protocol

Genes of interest were amplified using RT-qPCR in LightCycler 480 instrument. The following three step temperature protocol, ranging from 60°C-95°C, was used for amplification.

Step	*Time*	*Temperature (°C)*	*Cycles*	*Function*
1	1 min	95°C	1	Activation
2	5 sec	95°C	45	Amplification
	15 sec	60°C		
	20 sec	72°C		Fluorescence detection
3	15 sec	95°C	1	Melt curve
		60°C-95°C		Fluorescence detection

3.10 Statistical analysis

GraphPad® version 5.0 software was used for all analysis except gene expression. GenEx 5.2.3.13 software was used for analysis of gene expression. Statistical tests used for each assay are summarized in Table 4. Two-way ANOVA was used where possible and student's t-test used to confirm statistical significance. Using ANOVA to evaluate XTT proliferation results could not be performed due to the nature of the data set. All statistical analysis was performed at the Blood bank, Iceland.

Table 4 Summary of statistical analysis

Two-way ANOVA was used where possible and statistical significance confirmed with student's t-test to account for multiple-testing errors.

Assay	*ANOVA*	*Student's t-test*
Population doubling assay	X	X
Generation time	X	X
XTT proliferation assay		X
ALP activity assay	X	X
Gene expression	X	X

4 Results

4.1 Morphology and population doublings

4.1.1 Morphology

Morphology was examined visually and imaged in an inverted microscope at 5x magnification during expansion in growth medium supplemented with MSC-FBS, HPLO or HPLF at 10% concentration. After culture for 1, 3 and 7 days crystal violet staining was performed for better visibility of cellular morphology.

Crystal violet staining of MSCs at first passage in treatment media revealed spindle-shaped and elongated cells after 1 day in culture. After 3 days in culture, cells grown in HPLF and HPLO supplemented media showed spherical growth patterns and denser growth than cells grown in MSC-FBS supplemented medium. After 7 days in culture, MSCs had reached complete confluence, with cells grown in HPLF and HPLO supplemented media exhibiting denser growth and indistinguishable cell boundaries (Figure 15).

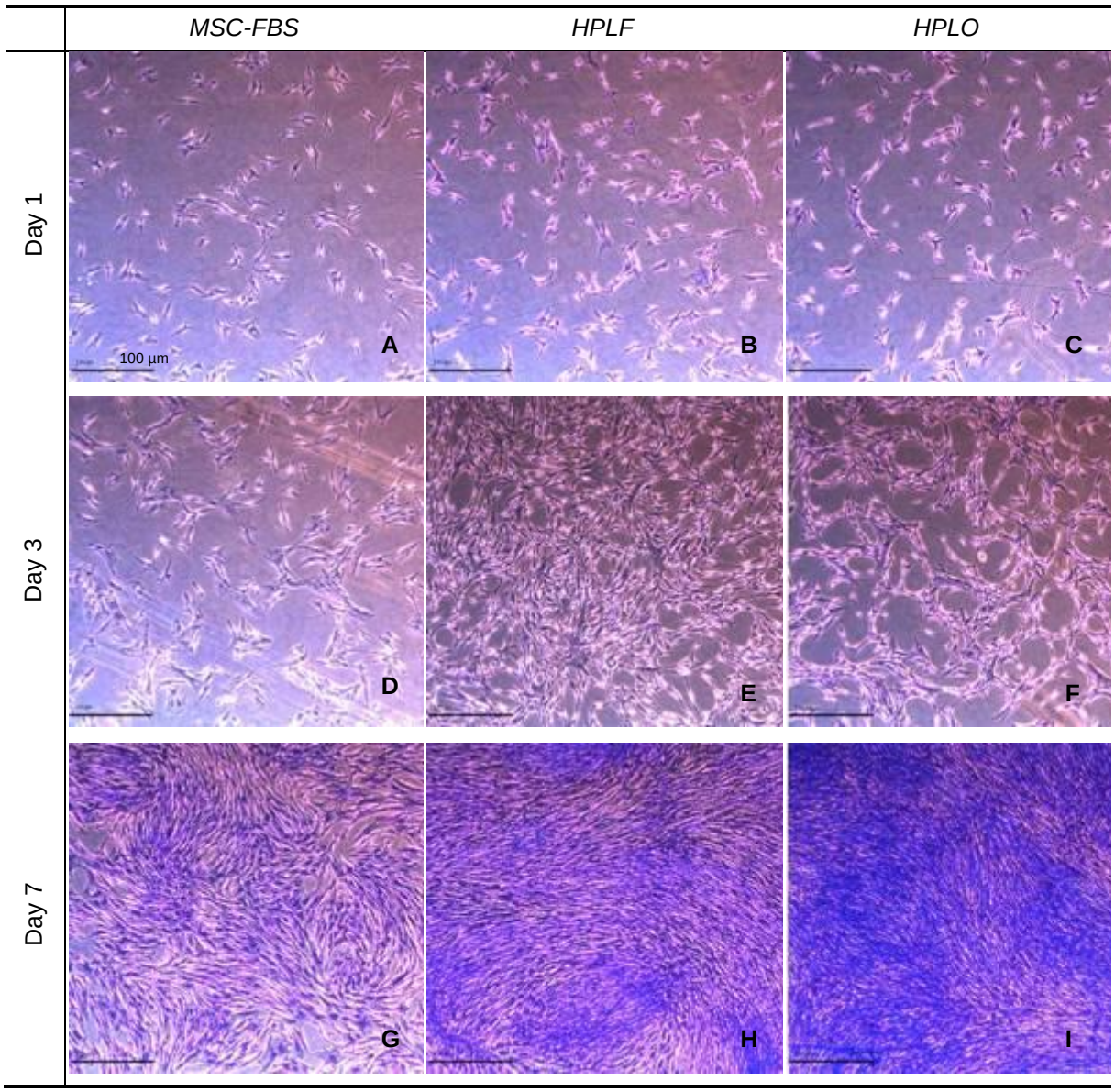

Figure 15 Crystal violet staining of MSCs

Images of MSCs stained with crystal violet after 1 (A-C), 3 (D-F) and 7 days (G-I) in culture with MSC-FBS, HPLF or HPLO supplemented media. Cells grown in HPLF or HPLO exhibit spherical growth patterns (E-F) and faster growth compared to MSC-FBS grown cells (G-I). Images were taken in an inverted microscope at 5x magnification. Images are representative of thee experiments.

4.1.2 Population doubling assay

Mesenchymal stem cells, expanded from two donors (D1 and D4), were quantified at the end of every passage from P1 to P6 for population doubling and generation time analysis.

MSCs cultured in either HPLF or HPLO supplemented media exhibited a higher expansion rate than MSCs cultured in MSC-FBS (n=2 for each expansion media at each passage). It was observed that soon after the introduction of either MSC-FBS, HPLF or HPLO supplemented media into the culture conditions (P2), the PD rate between cells grown in HPL containing media and cells grown in MSC-FBS began to differ, with HPL grown cells expanding faster. The maximum difference was observed at P4-P5 with mean difference of 4.233 ± 0.7313 CPD ($p<0.05$, Figure 16) between cells expanded in MSC-FBS or HPL containing media.

Cells grown in either HPLF or HPLO showed similar proliferation rates

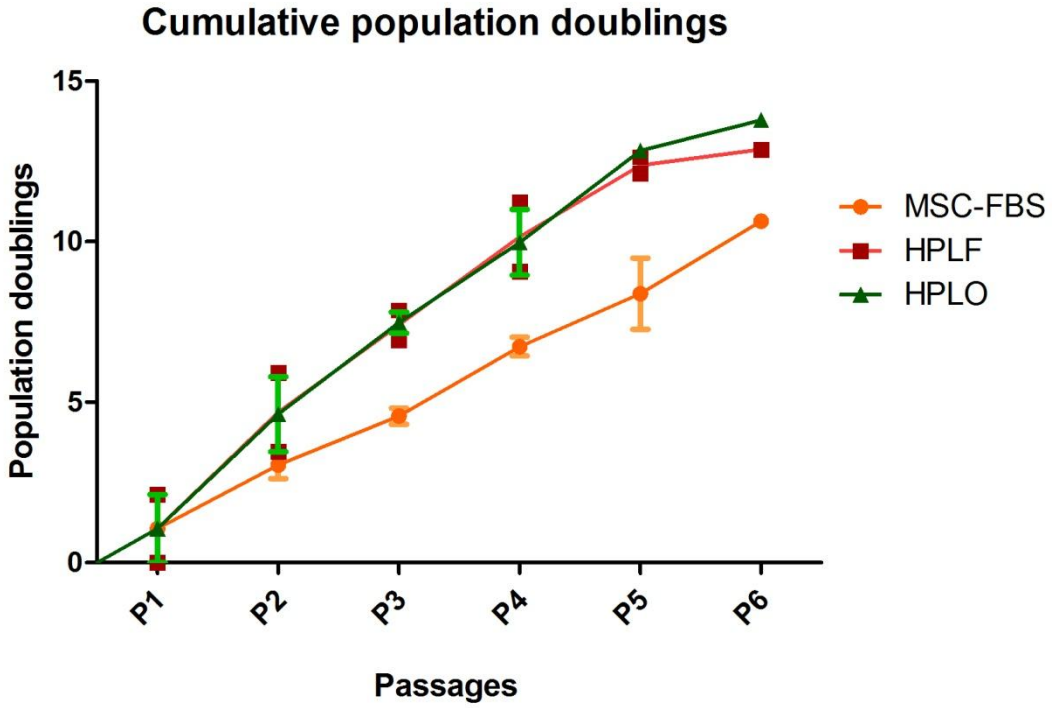

Figure 16 Cumulative population doublings of MSCs after six passages

The average CPDs for two experiments are shown with the standard error of mean (SEM) represented as error bars. MSCs cultured in HPLF or HPLO supplemented media expanded faster than MSCs cultured in MSC-FBS (n=2 at each time point, $p<0.05$). MSCs exhibit a similar growth curve at early passages but at later passages the difference between MSCs cultured in HPLF or HPLO compared to MSC-FBS increased reaching the maximum at P4-P5 with mean difference of 4.233 ± 0.7313 CPD ($p<0.05$) between cells grown in MSC-FBS or HPL supplemented media. Data is representative for two experiments.

Generation time (y-axis) between passages (x-axis) is shown in Figure 17. Data for P5 and P2 was excluded from calculations due to outlaws present in the data-set for those time points. The number of days between two PDs increased with every passage for all expansion media (MSC-FBS, HPLF or HPLO supplemented media). The GT ranged from 0,94 days (n=3, P1) to 9,27 days (n=1, HPLF P6).

The difference between GT at P4 and P6 is prominent for every expansion media, with difference ranging from 3,15-7,44 days in GT for cells grown in MSC-FBS and HPLF supplemented media, respectively. At passage six, cells grown in HPLF supplemented media exhibit a noticeably longer time between population doublings indicating a slower growth compared to cells grown in HPLO or MSC-FBS, which exhibit a similar GT at P6. Overall, the same trend for a slower growth with increased passage number was noticed for all cells irrespective of treatment.

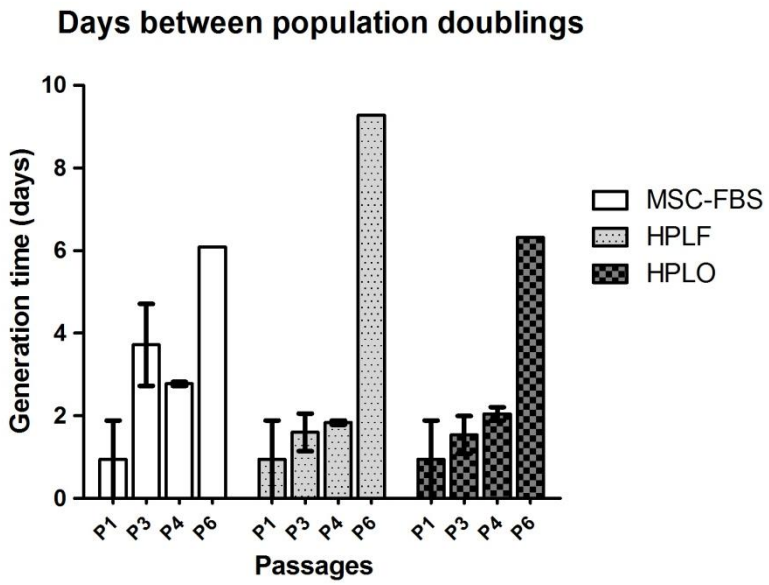

Figure 17 Changes in generation time between passages

The average generation time for MSCs obtained from two donors (D1 and D4) is shown on y-axes with SEM presented as error bars. Number of passages is shown on x-axis. The higher the GT value the slower the cell proliferation. Generation time increased with higher passage number for all expansion media. At P6, GT for HPLF grown cells stands out with longer time between population doublings indicating a slower growth compared to cells grown in HPLO or FBS which exhibit a similar GT at P6. Data and is representative for two experiments.

4.2 Immunomodulation

4.2.1 MLR assay

Sustained immunomodulatory capacity of MSCs after culture in MSC-FBS, HPLF or HPLO supplemented media was assessed in a MLR assay.

Results are presented as fold change in proliferation in Figure 18. Proliferation of mononuclear cells, stimulated with PHA, in the absence of MSCs served as a control and was assigned the fold change level 1 (n=18, MNC column Figure 18). Proliferation of MNCs stimulated with PHA in the presence of MSCs cultured in MSC-FBS, HPLF or HPLO (n=6 for each) was compared to that of the control using the ratio between. Mononuclear cell proliferation was significantly reduced in the presence of MSCs regardless of the type of media they had previously been cultured in ($p < 0.05$). No difference in abilities to immunosuppression were noticed between different expansion media.

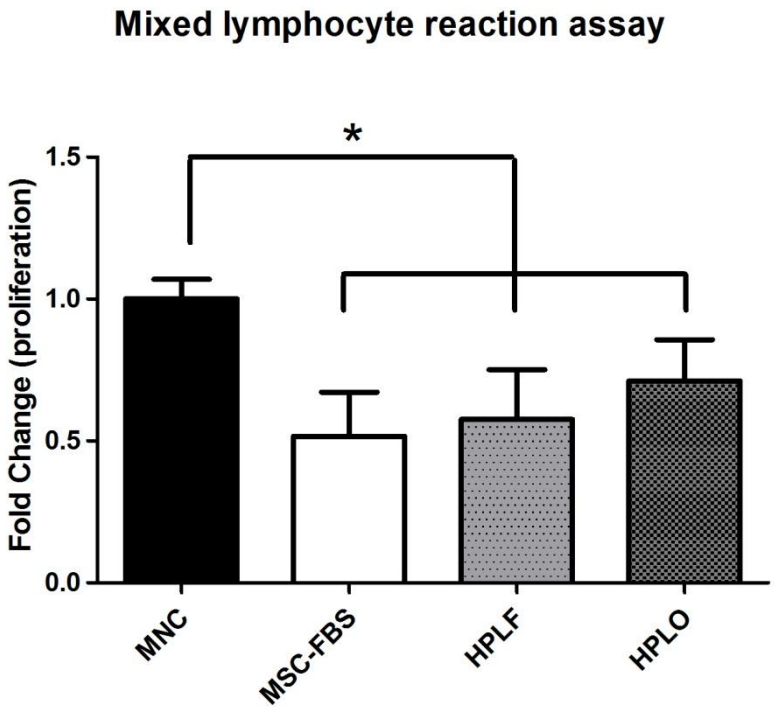

Figure 18 Relative MNC proliferation in an MLR assay

*The average proliferation of control (n=18) and of MNCs in presence of MSCs from MSC-FBS, HPLF or HPLO supplemented media (n=6) is presented as fold change with SEM as error bars. The control (MNC) has been assigned level 1 fold change and test subjects are shown relative to it (MSC-FBS, HPLF and HPLO). The proliferation was 0,29-0,48 fold lower in the presence of MSCs, regardless of the expansion media ($p < 0.05$, n=6 for each media type). No difference in abilities to immunosuppression were noticed between different treatments. Data is representative for two experiments. * = $p < 0.05$.*

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4.3 Osteogenic differentiation

4.3.1 Osteogenic morphology

Cells were imaged after 7 and 21 days in osteogenic culture. After 7 days in culture, MSCs previously grown in HPL containing media showed denser growth with osteoblast-like cells in more abundance than MSC-FBS (Figure 19). Cells appeared more cuboidal and less fibroblast like. After 21 days in osteogenic media all MSCs were completely confluent and no morphologic differences could be observed between cells from different media.

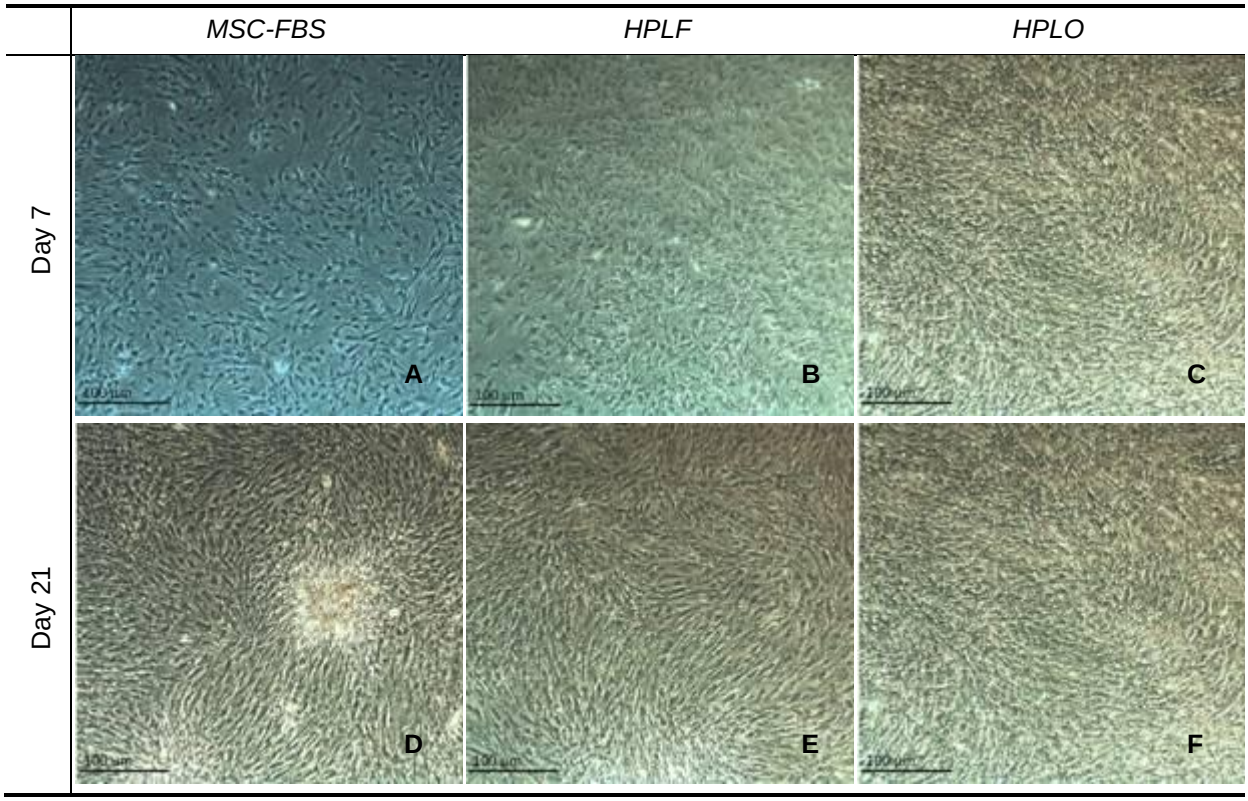

Figure 19 Morphology of MSCs during osteogenic differentiation

During osteogenic differentiation cells were constantly monitored and imaged at time of harvest after 7 and 21 days in culture. MSCs previously cultured in MSC-FBS (A,D), HPLF (B,E) or HPLO (C,F) are presented here. After 7 days in culture, cells from HPL containing media (B,C) showed denser growth and more osteoblast-like morphology than cells from FBS. After 21 days in culture all MSCs were completely confluent and no morphological differences evident between MSCs from different expansion media.

4.3.2 Alkaline Phosphatase (ALP) activity assay

Mesenchymal stem cells were cultured for 7 and 14 days in osteogenic media and then ALP activity was evaluated. The activity of ALP in nMol(p-nitrophenol)/min/mg protein can be seen in Figure 20. Activity of ALP was significantly higher after 14 days in culture than after 7 days ($p<0.05$, $n=6$ for each type of media).

Cells grown in HPLF supplemented media showed a statistically significant decrease in ALP activity after 7 days in culture as compared to cells grown in FBS ($p<0.05$, $n=6$). Influence of treatment was not observed after 14 days in culture with ALP activity being similar for all treatments. Data was normalized to total protein amount in samples.

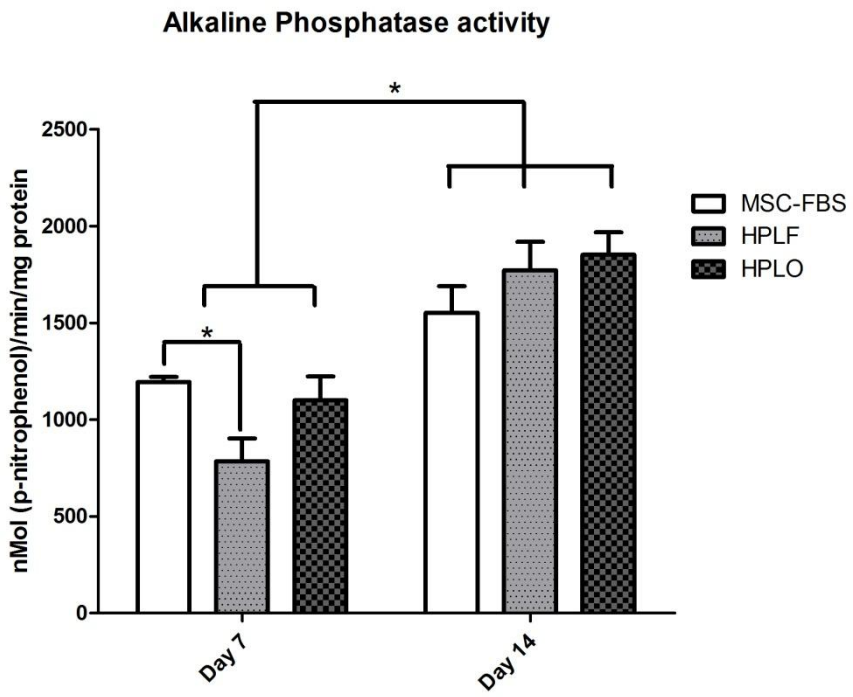

Figure 20 ALP activity after 7 and 14 days in osteogenic culture

The average ALP activity (y-axis) for MSCs from two donors (D1 and D4) after 7 and 14 days (x-axis) is presented with SEM as error bars. MSCs cultured for 14 days in osteogenic media had significantly higher ALP activity than MSCs cultured for 7 days regardless of treatment ($p<0.05$, $n= 6$ for each media type). Treatment influenced ALP activity after 7 days of culture with HPLF grown cells showing less activity than FBS grown cells ($p< 0.05$). Influence of treatment was not observed after 14 days of osteogenic culture. Data is representative for two experiments. $*$ = $p< 0.05$.

4.3.3 Gene expression

Samples from MSCs grown in MSC-FBS, HPLF or HPLO supplemented media from three donors (D1, D3 and D4) were obtained before initiation of osteogenesis (0 days). Samples were also obtained after osteogenic differentiation for 7 and 21 days in osteogenic medium. All samples were analyzed for gene expression of *RUNX2, SPP1* and *ALP.*

At 0 days of osteogenic culture, samples were analyzed for gene expression and it was observed that expansion in those three different expansion media used before initiation of osteogenic differentiation did not affect osteogenic gene expression. Gene expression was not affected by different donors.

Overall, the treatment did not significantly influence osteogenic gene expression. *RUNX2* expression was found to be significantly higher in samples from HPLO grown cells compared to MSC-FBS grown cells using one-way ANOVA but this could not be proven with multiple-testing (t-tests) when combining time points, only when analyzing individual time-points. Expression of *RUNX2* and *SPP1* was significantly different depending on time ($p<0,01$, $n=18$) but *ALP* expression was not significantly different depending on time in culture (Figure 21). *ALP* expression was dependant on the different donor origins of the cells with D4 being significantly different from the other donors ($p<0,02$).

At day 7 of osteogenic culture the expression of *RUNX2* was significantly higher in samples from HPLO compared to samples from MSC-FBS ($p<0,05$, $n=6$, validated with a t-test). No difference was found between HPLF and MSC-FBS samples in *RUNX2* expression after 7 days. Expression of *SPP1* and *ALP* did not depend on different treatments after 7 days in osteogenic culture. Gene expression did not depend on different donors.

After 21 days in osteogenic culture the expression of *RUNX2* was significantly higher in samples from both HPLO and HPLF samples compared to MSC-FBS samples ($p<0,01$, $n=6$, validated with a t-test). Expression of *SPP1* and *ALP* did not depend on different treatments after 21 days in osteogenic culture. Gene expression did not depend on different donors.

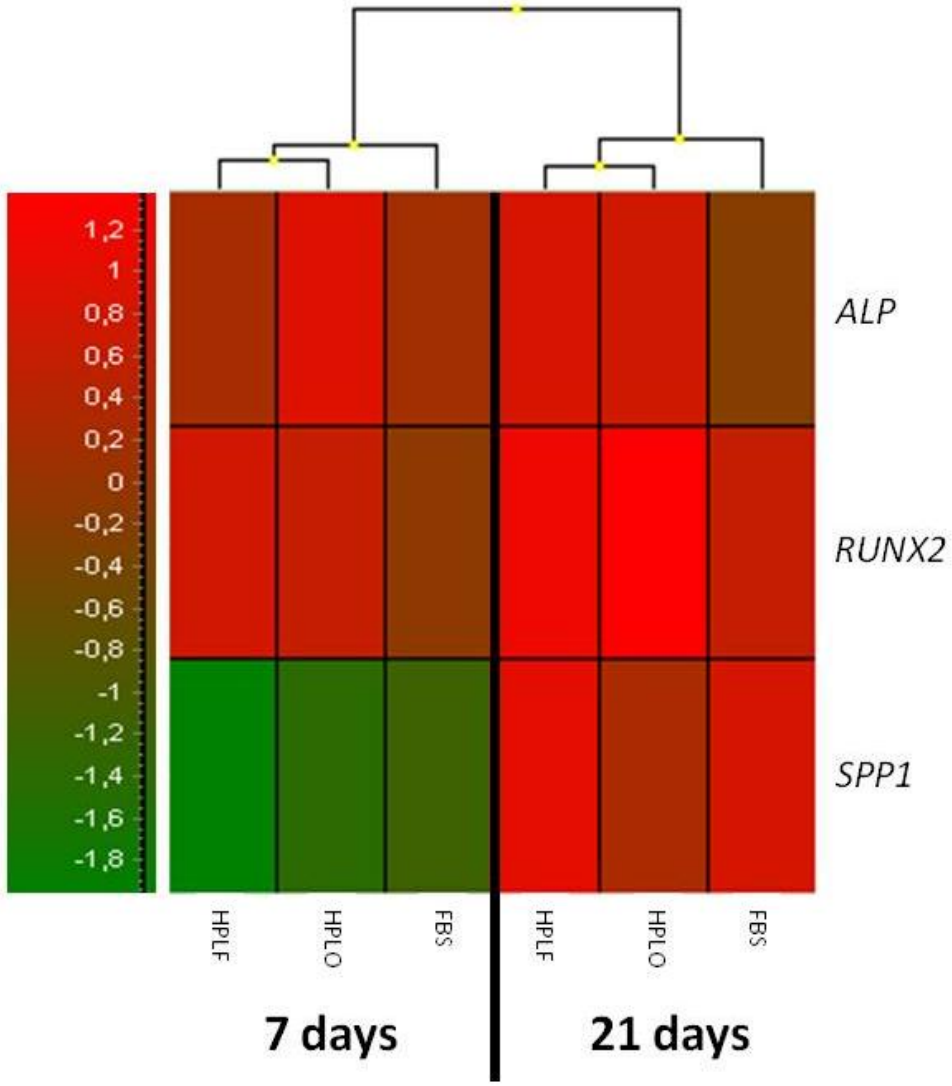

Figure 21 Heat-map of osteogenic gene expression after 7 and 21 days

Osteogenic gene expression of ALP, RUNX2 and SPP1 presented as a heat-map. High expression is indicated with shades of red but low expression is indicated with shades of green, as shown on a color scale to the left. The central bold line separates the two different time-points with 7 days presented on the left side of the line and 21 days presented to the right. Expression of ALP was not different between the time-points. SPP1 was expressed significantly higher after 21 days than 7 day (p<0,01). RUNX2 expression was significantly higher at 7 days in ostgenic culture than at 21 days (p<0,01).

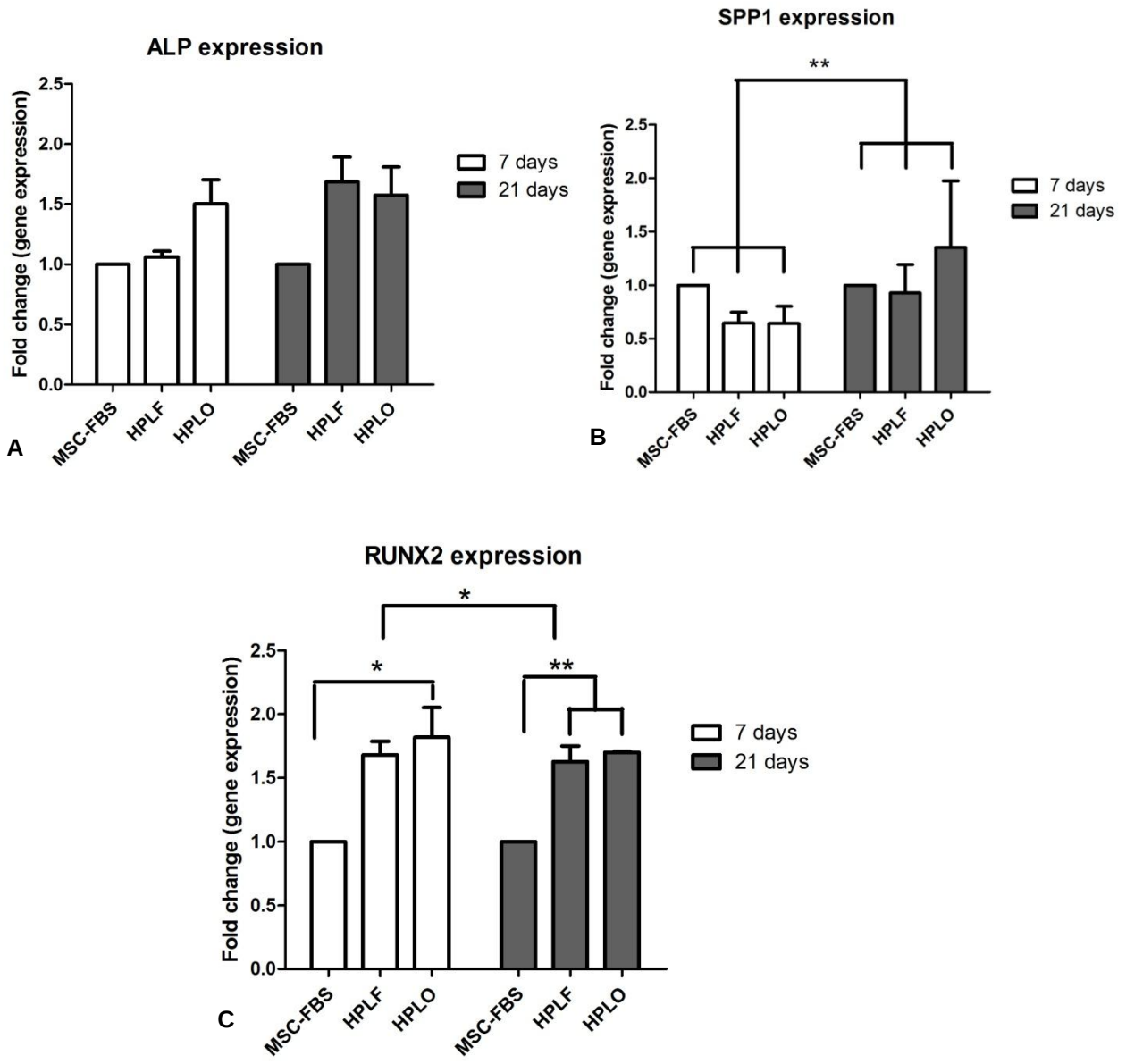

Figure 22 Osteogenic gene expression of individual genes after 7 and 21 days

Gene expression is presented as fold change with SEM as error bars. The control (MSC-FBS) has been assigned level 1 fold change and test subjects are shown relative to it (HPLF and HPLO). A) Expression of ALP did not differ significantly between 7 and 21 days of osteogenic culture. Expansion in treatment media prior to osteogenic differentiation did not affect ALP expression during osteogenic differentiation. B) SPP1 was higher expressed after 21 days in osteogenic culture than after 7 days (p<0,01). SPP1 expression did not depend on which kind of media the MSCs had previously been expanded in. C) RUNX2 expression was significantly higher after 7 days in osteogenic culture compared to 21 days. After 7 days RUNX2 expression was higher in samples from HPLO supplemented media compared to samples from MSC-FBS supplemented media (p<0,05). After 21 days RUNX2 expression was significantly higher in samples from both HPLO and HPLF supplemented media compared to samples from MSC-FBS supplemented media (p<0,01). $*$ *= p< 0.05,* $**$ *= p< 0.01.*

5 Discussion

In this thesis, the effect of three different expansion media (10% MSC-FBS, 10% HPLF and 10% HPLO) on MSC basic characteristics were compared. The characteristics analyzed were proliferation, immunomodulation and osteogenic differentiation. Mesenchymal stem cells from three independent donors were expanded for two passages in each media and then used in experiments evaluating the basic characteristics. Expansion in each of the three media did not affect basic properties such as immunosuppression and osteogenic differentiation. However, expansion in both preparations of platelet lysates induced morphological changes and increased proliferation compared to MSC-FBS supplemented media.

5.1 Morphology and population doublings

Following expansion in platelet lysates and FBS supplemented media, all cells showed the typical morphology of MSC, long, spindle-shaped cells resembling fibroblasts.

Cells grown in HPL supplemented media exhibited a slightly different morphology from cells grown in MSC-FBS with longer and denser cells, able to grow in higher numbers per area. The cells grown in HPL supplemented media grew in a spherical growth pattern leaving circular areas free of growth between them when 60-70% confluent (Figure 15). This growth tendency was not seen in cells grown in MSC-FBS. The reasons for this are still unknown but could be due to factors present in HPL and absent in MSC-FBS, most notably serotonin. Serotonin is stored in platelets and is known to affect cellular morphology of neural cells and cause vasoconstriction of endothelial cells (Daubert & Condron, 2010; Reed, 2007). There are some indications that MSCs are of a perivascular nature and could therefore be affected by serotonin levels, causing cellular contraction. The impact of changes in morphology on MSC biology if any, remain to be determined.

Proliferation of MSCs cultured in HPLF or HPLO tended to be faster than of MSCs cultured in MSC-FBS, reaching more cumulative population doublings in fewer passages (Figure 16, page 44). The difference in the rate of proliferation was significant but due to the limited amount of data points in this study, the term “significant” is avoided and we prefer to describe the results as a “trend in proliferation”. Furthermore, this same trend has been described in larger studies and the same is true for other growth supplements produced from or containing platelets (Bieback et al., 2009; Duan et al., 2011).

The generation time was short at earlier passages but increased with every passage. The same as was seen for the analysis of population doublings, short at earlier passages but increased with every further passage (Figure 17, page 45). Again, the power of the study is limited but these results are nevertheless able to show a trend of decreased proliferation with longer time in culture and increasing number of passages, as has been described in other studies (Bieback et al., 2009; Schallmoser et al., 2007). There was no difference in proliferation between expired and fresh platelet lysates.

Platelet lysates are extremely rich in various growth factors necessary for MSC growth, with even higher concentrations than in FBS (Tekkatte et al., 2011). The life time of MSCs *in vitro* is limited to 25-50 population doublings (Charbord et al., 2011). Since cells grown in HPL supplemented media proliferate faster and reach the limit of population doublings earlier, there is the possibility that these cells will cease growth sooner than cells grown in media supplemented with FBS. Furthermore, the effective proliferation rate after long term culture in FBS media compared to HPL supplemented media can be expected to be very similar since a plateau phase is observed after a few passages in cultures with platelet lysates.

All the same, there still is some evidence that MSCs grown in HPL have the potential to reach even higher population doublings than cells grown in FBS (Cholewa, Stiehl et al., 2011; Horn, Bokermann et al., 2010). Therefore, HPL media might be a more attractive choice for fast cellular expansion compared to FBS. Ultimately, there was no difference observed between expired and fresh platelet

lysates, suggesting that the expired units at blood banks, instead of being discarded, can be used for MSC culture.

During the handling and passing of cells, no difference in cell adherence could be observed. Trypsin, an enzyme used to detach adherent cells from cell culture plastic, was neutralized with HPL containing media to the same extent as can be observed for FBS. No negative effects on cellular proliferation or cell adherence were found following the use of HPL, which indicates that platelet lysates might display some of the same protective abilities attributed to MSC-FBS without the external addition of proteolytic inhibitors.

5.2 Immunomodulation

The immunosuppressive abilities of MSC after expansion in the three different kinds of expansion media was analyzed in a mixed lymphocyte reaction assay (MLR). After stimulation with PHA, an initiator of immune-responses in mononuclear cells, MNC proliferation in the presence of MSC was markedly decreased as compared to MNC alone. This effect was not influenced by the expansion in expired or fresh platelet lysates.

Isolated MNC cell population used in this study is a combination of several cell populations, such as lymphocytes, natural killer cells and monocytes. Furthermore, lymphocytes can be divided into different subpopulations, most notably T-cells (Regulatory T-Cells, T-helper cells 1 and T-helper cells 2) and B-cells (Kode et al., 2009). Depending on the type of immune cell, MSC affect proliferation differently, stimulating proliferation in cell populations such as T-regulatory cells and T-helper cells 2 and suppressing others like T-cells, T-helper cells 1, B-cells and natural killer cells (Le Blanc & Ringden, 2007; Uccelli et al., 2008). MLR assays are widely used throughout the literature but might not be the best model to study immunosuppression of MSC. To most effectively evaluate immunosuppression in an MLR assay, a ratio of 1 MSC to 10 lymphocytes is recommended (Le Blanc & Ringden, 2007).

However, when mononuclear cells are used during this kind of assay, the number of actual lymphocytes in the sample is underestimated based on the presence of other “contaminating” cell populations. Furthermore, the distribution of cell populations other than lymphocytes might not be homogeneous, introducing an additional level of variation between individual wells in the experiment, independent of immunosuppressive abilities. Therefore, this method can be used to determine a general suppression of MNC proliferation, but the precise mechanisms and the effects on each individual immune cell type is impossible to conclusively determine. This issue can be accounted for in a more sophisticated set-up of the same assay, isolating each subpopulation from the original MNC fraction and evaluating the MSC immunomodulatory effect. These cell populations could be isolated from MNC samples and then each cell population seeded for the MLR assay, separate from other cell types. Interactions between each cell population might affect the proliferation and must be accounted for and thus the cellular combination of MNC samples could also be determined before and after the MLR assay to see the shift in individual cell populations.

For the purpose of this study, however, the general effect of immunodulatory abilities rather than the individual effect on each immune cell type was important. It can be ruled out that the immunosuppression observed was mediated through cell to cell contact due to the presence of a physical barrier created by the semi-permeable membrane. Hence, the suppressive effect on MNC proliferation was mediated through diffusible soluble factors that could be exchanged between both cell populations by a semi-permeable membrane. The analysis of which soluble factors played a role in this process was not included in this thesis but time-dependent factor release assay to better model the time line of immunosuppression by MSCs constitutes an interesting project for further research.

Nevertheless, even when the issues related to the MLR assay mentioned above are considered, the reduction in immune cell proliferation was significant and indicates that platelet lysates do not affect immunomodulatory abilities during the expansion of MSC.

5.3 Osteogenic differentiation and gene expression

The quality of osteogenic differentiation in this study was evaluated by analysing Alkaline Phosphatase activity and osteogenic marker gene expression, i.e. Alkaline Phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX2*) and secreted phosphoprotein 1 (*SPP1*).

During osteogenic differentiation MSCs lost their fibroblast-like morphology and became more cuboidal, indicating successful osteogenesis (Figure 19, page 47). A significant increase in ALP enzymatic-activity was observed after 14 days of osteogenic differentiation compared to 7 days, indicative of increased osteoblast activity over time ($p<0.05$). At early time points (7 days), a significantly less ALP activity in HPLF supplemented compared to MSC-FBS supplemented cultures was seen, but since the secretion of active ALP enzyme reached similar levels after 14 days, the effect on osteogenic differentiation quality is thought to be minimal. No difference in ALP acitivity was detected between expired platelet lysates and MSC-FBS.

To evaluate whether platelet lysates induced spontaneous osteogenic differentiation in the absence of *in vitro* osteogenic inducers, gene expression levels of osteogenic marker genes were evaluated after 7 day expansion. No induction of osteogenic marker gene expression could be observed in any of the samples irrespective of the growth media used, indicating that MSC expanded in platelet lysate are not primer for osteogenic differentiation and can be used for the differentiation towards any mesenchymal lineage.

After induction of osteogenic differentiation, a continuous expression of *ALP* was found, without no difference between early (7 days) and late (21 days) osteogenic differentiation. *RUNX2* expression decreased over time, as expected, since this transcription factor is indicated in the lineage commitment to osteochondroprogenitor cells (Birmingham et al., 2012). On the other hand, the expression of *SPP1*, a late marker of osteogenic differentiation, increased over the time course of the experiment. Altogether, the results for gene expression and *ALP* activity indicate successful osteogenic differentiation in all samples.

Concerning the effect of platelet lysates on gene expression, no difference was observed in the expression of *ALP* and *SPP1* at either time point. However, *RUNX2* expression was significantly higher in HPLF and HPLO supplemented cultures as compared to MSC-FBS at 21 days for both and also at 7 days for HPLO. Expression of *RUNX2* plays a central role in committing MSCs towards the osteoprogenitor lineage and subsequently upregulates expression of other factors necessary for osteogenesis (Franceschi et al., 2007). High *RUNX2* expression is indicative of good quality osteogenic differentiation and limited differentiation towards other lineages

The effect on *RUNX2* gene expression is not entirely surprising, since platelet lysates can contain factors that participate in osteogenesis and normal bone turn-over and might therefore be able to stimulate the expression of osteogenic marker genes. Therefore, expansion of MSC in platelet lysates might induce better and faster osteogenic differentiation, but as described above, does not prime cells to commit to the osteogenic lineage. The indications presented here are in agreement with previous publications (Chevallier et al., 2010; Verrier et al., 2010).

One important factor to consider when working with MSCs is donor variation, which makes it difficult to compare results between studies. Per definition, MSCs are a heterogeneous cell population and differences in differentiation potential, proliferation and cytokine secretion can be observed between individual donors but also within the MSC population itself. The analysis of three independent donors, as performed in this study, showed significant differences between cells obtained from each of the donors. Many factors can play a role in affecting MSC cell behavior, including donor's age, lifestyle and health status. Therefore, to prove the suitability of platelet lysates as replacement for MSC-FBS, the study of several donors was important. However, samples from healthy young donors are often difficult to obtain based on the collection procedure for this cell type. MSC are derived from the bone-marrow, requiring bone marrow aspiration from healthy donors, which is both agonizing and risky. In other cases, bone marrow biopsies can be obtained at the same time as samples for clinical analysis,

but these donors do not represent the normal healthy population and samples obtained from these donors might not be suitable for analyzing MSC biology. Therefore, it is of great importance to further improve the characterization of MSC and enhance isolation techniques to increase comparability between studies. Until then, donor variability will be part of MSC research.

5.4 Error evaluation and improvement suggestions

As with every research, there is always the possibility for improvement and doing further experiments. Several additional experiments and variations of methods have been suggested in the discussion above, to be implemented in further research. However, based on the scope of the study, the selection of experimental methods and the sample number is considered appropriate.

The lack of power could still have been improved by increasing the number of experiments behind each data point, but was sufficient to prove the general trend already described in the literature.

Additionally, several other interesting genes with functions in osteogenesis could be analyzed, including Collagen type 1 (*COL1A2*) and SP7 (previously known as osterix) as well as marker genes for additional lineages, i.e. *SOX9* (chondrogenic differentiation) and *PPARgamma* (adipogenic differentiation).

Finally, the production of platelet lysates could be improved by increasing the number of freeze/thaw cycles during the lysis process. Commonly, only a single freeze/thaw cycle is described in the literature, but increasing this number might result in increased lysing of the platelets and higher quality lysates.

5.5 Future directions

The results presented in this thesis suggest that HPL from expired platelet rich concentrates can be used for the culture of MSC *in vitro*. Ongoing research will further evaluate the effects of platelet lysates on basic cell characteristics, including phenotype and marker expressions. Effects of long term culture up to 10-15 population doublings remains to be evaluated and the effect of platelet lysates on the differentiation along other mesenchymal lineages awaits evaluation. Based on the indications for enhanced osteogenic differentiation with platelet lysates, cells could be cultured on a 3-dimensional support matrix to better model the *in vivo* bone environment. This scaffold could then also be used for implantation and evaluation in mice models *in vivo*.

Finally, before platelet lysates can replace the use of MSC-FBS in the expansion of MSC, further analysis needs to be directed at its content, the concentration of growth factors and possible contaminants.

6 Conclusion

This study shows that human platelet lysates derived from expired and fresh platelet rich concentrates can be used for the expansion of MSC to the same extent as traditionally used fetal bovine serum (FBS). Cell culture in growth media containing platelet lysates increases proliferation but does not affect immunosuppressive abilities or osteogenic differentiation potential of these cells. Expired platelet lysate, currently a waste product in blood processing departments, can be used as a potential substitute for fetal bovine serum. By using expired platelet lysates many concerns relating to the use of FBS can be solved such as pathogen contamination, immune-reactions to xenogenic proteins and batch-to-batch variations can be limited. No ethical concerns are related to the use of expired platelets and it comes at a lower price than the FBS. Therefore, platelet lysates from expired platelet rich concentrates are a potential substitute for FBS in *in vitro* culturing of bone-marrow derived mesenchymal stem cells.

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