Expired human platelets for mesenchymal stromal cell propagation

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Thesis for the degree of Philosophiae Doctor

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2017

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE

Notkun útrunnina blóðflaga til fjölgunar á mesenkímal stofnfrumum

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Ritgerð til doktorsgráðu

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ISBN 978-9935-9365-2-3

Printing by Háskólaprent ehf.

Reykjavik, Iceland 2017

Vits er þörf

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Dælt er heima hvað.

Að augabragði verður

sá er ekki kann

og með snotrum situr

~ Úr Hávamálum

Fyrir Þór og Birtu

Ágrip

Mesenkímal stofnfrumur úr beinmerg (MSC) lofa góðu fyrir notkun í vefjalækningum sökum hæfni þeirra til að mynda vefi stoðkerfisins og til að stýra ónæmissvari. Notkun þeirra eru þó vandasöm vegna þess að kálfasermi þarf til að rækta þær utan líkamans. Kálfasermi er óæskilegt því það getur haft skaðleg áhrif í för með sér, svo sem hættu á dýrbornusmiti og ónæmis- og bólgusvari í frumuþega. Nauðsynlegt er að finna aðra lausn sem er ekki upprunnin úr dýrum, leysir kálfasermi af hólmi og styður við MSC frumur í rækt. Blóðflögulausnir úr blóðflögum manna hafa verið ræddar í þessu samhengi sökum þess hve ríkar þær eru af vaxtarþáttum og frumuboðum sem finnast í seytiögnum þeirra.

Blóðflögur sem má breyta í blóðflögulausnir fást hjá blóðbönkum. Blóðbankar eiga hinsvegar ekki umframmagn af blóðflögum til að láta af hendi þar sem þeir búa nú þegar við skort á blóðgjöfum. Engu að síður þarf að farga töluverðu magni af blóðflögum árlega vegna þess að þær renna út. Útrunnar blóðflögur væri hægt að nota til að útbúa ræktunarlausnir á hagkvæman, ódýran og skilvirkan hátt sem staðgengil kálfasermis fyrir frumuræktir. MSC frumur sérhæfðar frá stofnfrumum úr fósturvísum (hES-MP) hafa líka verið skoðaðar sem möguleiki fyrir vefjalækningar og því einnig mikilvægt að finna ræktunarlausnir fyrir þær sem eru ekki upprunar frá dýrum.

Í þessari rannsókn skoðuðum við hæfni útrunnina blóðflagna og útrunnina smithreinsaðra blóðflagna frá Blóðbankanum til að styðja við MSC og hES-MP frumur í rækt. Frumuvöxtur, tjáning á yfirborðssameindum, þátttaka í ónæmissvari og hæfni þeirra til að mynda vefi stoðkerfisins var skoðuð sérstaklega.

Blóðflögulausnir úr útrunnum blóðflögum voru jafngildar eða betri en kálfasermi og blóðflögulausnir úr ferskum blóðflögum þegar frumuvöxtur, tjáning á yfirborðssameindum og vefjamyndun var skoðuð hjá MSC og hES-MP frumum. Blóðflögulausnir hentuðu sérstaklega vel til að styðja við beinmyndun sem sást með aukinni virkni alkalísks fosfatasa, útfellingu steinefna í vef og aukinni genatjáningu fyrir beinmyndun. Sambærilegar niðurstöður komu fram við notkun á blóðflögulausnum úr útrunnum smithreinsuðum blóðflögum. Þegar slíkum blóðflögulausnum var bætt út í æti fyrir vefjasérhæfingu kom fram aukin bein- og brjóskmyndun umfram það sem sást við notkun á kálfasermi. hES-MP frumur, bæði í blóðflögulausnum

og kálfasermi, fjölguðu sér hraðar heldur en MSC frumur. hES-MP frumur gátu hinsvegar ekki dregið úr fjölgun ónæmisfruma líkt og MSC frumur gera. Báðar frumutegundir mynduðu þó bein, brjósk og fituvef.

Blóðflögulausnir úr útrunnum blóðflögum, smithreinsuðum eður ei, henta sem ræktunarlausnir fyrir MSC og hES-MP frumur án þess að draga úr hæfni þeirra til frumufjölgunar, þátttöku í ónæmissvari eða vefjamyndun. Útrunnar blóðflögur eru því ákjósanlegur efniviður fyrir blóðflögulausnir sem nota má í stað kálfasermis.

Lykilorð:

Stofnfrumur, Blóðflögur, Stofnfrumur frá fósturvísum, Bein, Ónæmisfræði.

Abstract

Mesenchymal stromal cells (MSC) are promising candidates for cellular therapy due to their ability to regenerate bone and cartilage and modulate immune responses. The use of MSC in cellular therapy is problematic due to the need for fetal bovine serum (FBS) during ex vivo expansion of the cells. The use of FBS may lead to detrimental side effects for patients such as cross-species viral infections and severe immune responses. Alternative cell culture supplements are therefore needed. Lysates derived from platelets have been suggested, mainly due to the abundance of various growth factors and cytokines that are found in platelet granules.

Platelets to make lysates can be obtained from blood banks. Blood banks, however, do not possess a large surplus of platelets since they face a shortage of platelet donors. Still, a significant number of platelet concentrates expire annually in blood banks and are discarded. These expired platelets could be used as an alternative to FBS to make lysates in an economical and effective way. Furthermore, the resources of blood banks would be utilized in a more efficient manner. Human embryonic-derived mesenchymal progenitor (hES-MP) cells have been envisioned as a future source of MSC which, for the same reason as MSC, also require serum-free culture supplements.

In this thesis, we evaluated the suitability of expired platelets and expired pathogen-inactivated platelets to support the growth, phenotype, immune function, and tissue formation of MSC and hES-MP cells.

Platelet lysates from expired platelets supported MSC and hES-MP growth, phenotype, and tissue formation to an equal or greater extent than lysates from fresh platelets or FBS. Platelet lysates supported osteogenic differentiation particularly well, causing upregulation of alkaline phosphatase activity, tissue mineralization, and osteogenic gene expression. Similar observations were obtained for platelet lysates from expired pathogen-inactivated platelets which supported MSC in all aspects evaluated. Furthermore, adding platelet lysates from expired pathogen-inactivated platelets to the tissue differentiation media enhanced both osteogenic and chondrogenic differentiation of MSC and hES-MP compared to FBS.

In both platelet lysate and FBS, hES-MP cells proliferated faster than MSC and reached higher cell numbers in shorter time. However, hES-MP

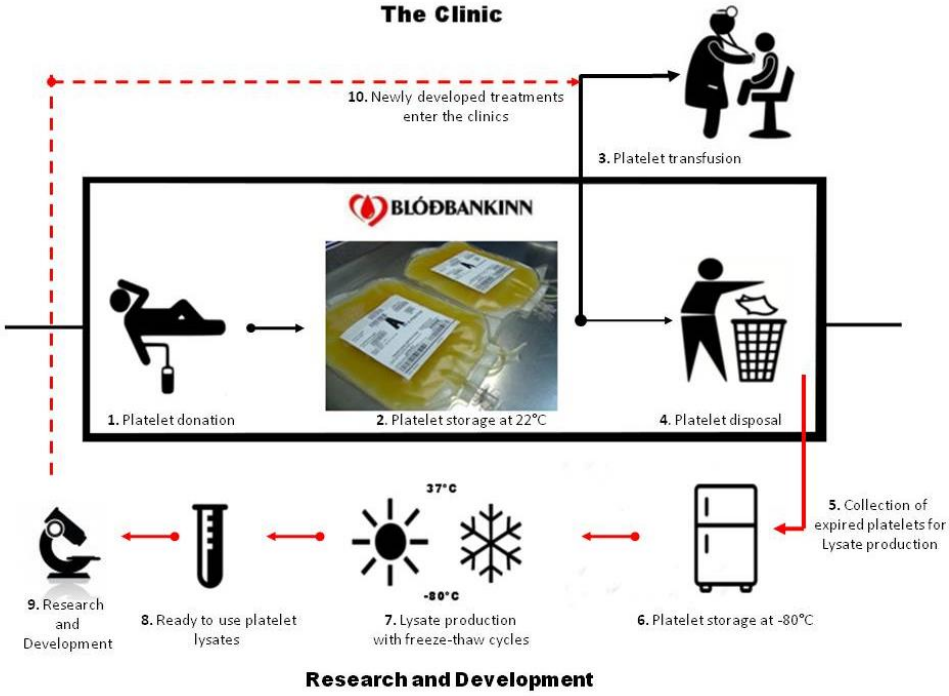
failed to suppress immune cell proliferation, while MSC did so effectively. Both cell types, nevertheless, differentiated successfully toward osteogenic, chondrogenic, and adipogenic lineages independent of the culture supplement used.

We conclude that lysates from expired platelets, pathogen-inactivated or not, successfully support the expansion of MSC and hES-MP while also allowing the cells to maintain their capacity to modulate immune responses and differentiate. Expired platelets therefore represent a feasible and attractive source material to make platelet lysates for FBS replacement.

Keywords:

Mesenchymal stromal cells, Platelets, Embryonic stem cells, Bone, Immunology

Graphical Abstract



The aim of this thesis was to evaluate whether expired platelets are suitable as source material for platelet lysate production. The graphical abstract illustrates the traditional lifetime of blood bank produced platelet concentrates (black process) and how this thesis aims to extend their lifetime and use post-expiration (red process). Platelet concentrates are made from donated blood in blood banks (1) and stored at 22°C for five to seven days (2). The majority of the produced platelets are transfused (3), but the platelets that expire are discarded (4). In this thesis, we collected the expired platelets (5) and stored them at -80°C until platelet lysate production (6). The platelets were lysed in freeze-thaw cycles (7) and platelet lysate solutions made (8). The platelet lysates were then used to grow and study bone marrow-derived mesenchymal stromal cells (MSC) and embryonic-derived MSC (9) to contribute to the increasing knowledge in regenerative medicine that may one day lead to novel cellular therapies (10).

Acknowledgements

The entire work presented in this thesis was carried out in the Icelandic Blood Bank. I owe the staff of the Blood Bank, past and present, my deepest gratitude for their willingness to aid me in any way possible whenever needed, for constant encouragement and moral support. I am especially thankful for the assistance of Ragna Landrö, Björn Harðarson and the Blood Processing Unit for providing study material as well as Steinunn Jóna Matthíasdóttir for making sure I received all expired units. Without your help this work would never have been possible.

I will be forever grateful to my supervisor and mentor, Dr. Ólafur Eysteinn Sigurjónsson, who has not only guided my scientific work but also taught me lessons about life. Thank you, Óli, for giving me this opportunity years ago and for having constant faith in me, even at times when I doubt myself. Thank you for always knowing when to allow me independence and when to give me guidance. Thank you for your trust, respect and selflessness. A better man is hard to find. I will pay it forward.

To all the colleagues I have been so fortunate to work with; I would like to express my appreciation. Especially to Dr. Ramona Lieder whose outstanding scientific work and efforts inspired me to pursue a Ph.D. to begin with. My greatest friend, Hildur Sigurgrímsdóttir, is also entitled to my gratitude but we have had many insightful conversations on science, life and the universe. I hope we will continue to do so into old age, both inside and outside of the lab. Kristbjörg Gunnarsdóttir was an instrumental part of the research team behind the work of this thesis and contributed greatly with her own work. I would also like to thank Kristján Torfi Örnólfsson for his contribution as well as the people at the Department of Pathology – University Hospital of Iceland. Dr. Martha Ásdís Hjálmarsdóttir from the Department of Biomedical Sciences at the University of Iceland gets my thanks for inspiring me and other biomedical scientists to seek higher education and supporting me on the way. It is an honor to work with you. Kristine Wichuk deserves appreciation for all the time she spent proofreading abstracts and manuscripts prior to publishing. Lastly, my thanks go to Helena Montazeri and Christian Christensen for moral support and understanding during the writing of this thesis.

Nothing would have been accomplished without the constant motivation, understanding and love showed by my better half, Þór Friðriksson, whose keen interest in science and mathematics inspired me to change my chosen path in life more than ten years ago. He has been with me every step of the way, read everything written on this project and on numerous occasions shared insightful comments leading to the betterment of the whole study. Thank you, Þór, for the your support, endless love and for being the best father Birta could have.

At last I would like to thank my doctoral committee for their work and helpful comments: Dr. Martina Seifert, Dr. Guðrún Valdimarsdóttir, Dr. Hilmar Viðarsson and Dr. Þorbjörn Jónsson.

This project was funded by The Icelandic Research Council (RANNÍS) and Landspítali – University Hospital of Iceland research fund (Vísindasjóður).

Ungur var ég forðum,

fór ég einn saman:

þá varð ég villur vega.

Auðigur þóttumst

er ég annan fann:

Maður er manns gaman.

~Úr Hávamálum

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List of abbreviations

ADIPOQ	Adiponectin
ALK	Activin receptor-like kinase
ALP	Alkaline phosphatase
BC	Buffy coat
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic proteins
CD	Cluster of differentiation
CFU	Colony forming units
COL	Collagen
CPD	Cumulative population doublings
DC	Dendritic cells
DTS	Dense tubular system
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ESC	Embryonic stem cell
ESC-MSC	Embryonic stem cell-derived MSC
EV	Extracellular vesicle
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GP	Glycoprotein
GMP	Good manufacturing practice
GVHD	Graft versus host disease
hES-MP	Human embryonic cell-derived mesenchymal progenitor cells
HGF	Hepatocyte growth factor

HLA	Human leukocyte antigen
hPL	Human platelet lysate
hPL-EX	Platelet lysate made from expired platelets
hPLF	Platelet lysate made from fresh platelets
hPLO	Platelet lysate made from expired platelets
hPL-PI	Platelet lysate made from expired pathogen-inactivated platelets
HSC	Hematopoietic stem cells
IDO	Indolamin-2,3-dioxygenase
IFNγ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
iPSC	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
LRP	Lipoprotein receptor-related protein
M1	Macrophages type 1 (pro-inflammatory)
M2	Macrophages type 2 (anti-inflammatory)
MHC	Major histocompatibility complex
MSC	Mesenchymal stromal cells
MSC1	MSC type 1 (pro-inflammatory)
MSC2	MSC type 2 (anti-inflammatory)
NK cells	Natural killer cells
NO	Nitric oxide
OCS	Open canalicular system
P	Passage
PBMC	Peripheral blood mononuclear cells
PC	Platelet concentrate
PD	Population doubling

PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI	Pathogen inactivation
PPARG	Peroxisome proliferator-activated receptor gamma
PRP	Platelet rich plasma
RUNX2	Runt-related transcription factor 2
SOX9	SRY-Box 9
SP7	Osterix
SPP1	Secreted Phosphoprotein 1
TF	Tissue factor
TGFβ	Transforming growth factor beta
Th1	T helper cell type 1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tregs	T regulatory cells
TSG6	Tumor necrosis factor-inducible gene 6
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
β-TG	β-Thromboglobulin

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I - IV:

I. **Jonsdottir-Buch, S.M**., Lieder, R. & Sigurjonsson, O.E. (2013). Platelet lysates produced from expired platelet concentrates support growth and osteogenic differentiation of mesenchymal stem cells. *PloS One, 8*(7), e68984

II. **Jonsdottir-Buch, S.M.**, Sigurgrimsdottir, H. Lieder, R. & Sigurjonsson, O.E. (2015). Expired and pathogen-inactivated platelet concentrates support differentiation and immunomodulation of mesenchymal stromal cells in culture. *Cell Transplantation, 24,* 1545-1554

III. **Jonsdottir-Buch, S.M.**, Gunnarsdottir, K. & Sigurjonsson, O.E. Embryonic-derived mesenchymal progenitor cells (hES-MP cells) are fully supported in culture with human platelet lysates. Manuscript submitted.

IV. **Jonsdottir-Buch, S.M.** & Sigurjonssons, O.E. Embryonic-derived mesenchymal cells (hES-MP cells) participate in immunomodulation without being immunosuppressive. Manuscript submitted.

In addition, some unpublished data may be presented:

V. **Jonsdottir-Buch, S.M.**, Gunnarsdottir, K., Ornolfsson, K.T. & Sigurjonsson, O.E. Platelet lysates from expired and pathogen-inactivated platelets enhance tissue differentiation of BM-MSC and hES-MP cells. Unpublished data.

Declaration of contribution

The doctoral candidate, Sandra Mjöll Jónsdóttir-Buch, is responsible for experimental design, experimentation, data analysis, interpretation, and writing of all papers and manuscripts presented in this thesis.

Dr. Ólafur E. Sigurjónsson mentored and guided the doctoral candidate as needed and carefully examined and approved all experimental setups, manuscripts, and papers.

Dr. Ramona Lieder aided with experimental design, data analysis, and manuscript writing in Paper I. In Paper II, Dr. Lieder critically reviewed the manuscript before submission.

Hildur Sigurgrímsdóttir participated in experimentation, data analysis, and manuscript writing in Paper II.

Kristbjörg Gunnarsdóttir carried out all evaluations regarding immunophenotyping in Paper III along with data analysis. Kristbjörg also carried out the majority of the chondrogenesis presented as unpublished findings in chapter 4.5.

Kristján Torfi Örnólfsson evaluated tissue mineralization during osteogenic differentiation presented here as unpublished findings in chapter 4.5.

Histology stainings on cartilage pellets presented in Papers I and III and Chapter 4.5 were performed in collaboration with Sigrún Kristjánsdóttir at the Department of Pathology, Landspitali University Hospital.

The Blood Bank provided study material for all experiments. The wonderful staff of the Blood Bank further assisted the doctoral candidate in any way possible when needed and thus contributed to all work presented in the thesis.

1 Introduction

1.1 Somatic and embryonic mesenchymal stromal cells

Stem cells play a fundamental role in normal growth and tissue regeneration from the very first cell of an individual throughout life. Stem cells are found in all tissues of the body and have the unique ability to self-renew through asymmetrical cell division and thus maintain the stem cell pool while also forming more differentiated progeny (de Peppo & Marolt, 2012; Fernández-Vallone et al., 2013). Stem cells vary in their potential. Totipotent cells such as the zygote can give rise to all tissues of the body (Fernández-Vallone et al., 2013; Power & Rasko, 2011). Pluripotent cells such as embryonic stem cells are more restricted but can still form cells from all three germ layers (Kolios & Moodley, 2013). Cells that are limited to a single germ layer are termed multipotent, and cells that can form only a few or single cell types are oligopotent or unipotent (Kolios & Moodley, 2013; Power & Rasko, 2011). Exploiting the potentials of pluri- and multipotent cells for tissue regeneration as a part of cellular therapy has raised considerable interest, both within the scientific community and among the public (Ilic & Ogilvie, 2016). Especially the use of multipotent mesenchymal stromal cells (Fernández-Vallone et al., 2013). Hence, this thesis will focus on multipotent mesenchymal stromal cells from the bone marrow and similar cells derived from embryonic stem cells.

1.1.1 Defining mesenchymal stromal cells

Researchers first identified mesenchymal stromal cells in the post-war era while studying the relationship between the bone marrow and hematopoiesis (Bianco, 2015). These studies initially revealed the presence of hematopoietic stem cells (HSC) in the bone marrow (Charbord, 2010). Further experiments showed that their ex vivo survival depended on a supportive layer of stromal cells. This layer consisted of cells that exhibited distinct morphology and characteristics different from the HSC (Charbord, 2010). The work of Alexender Friedenstein and, later, Maureen Owen demonstrated that these cells were osteogenic mesenchymal stromal cells (MSC), an instrumental part of the bone marrow microenvironment (Bianco, 2015; Bianco et al., 2008). Today, the existence of MSC has been demonstrated for various tissues of the body (Dominici et al., 2006; Uccelli et al., 2008).

Originally, MSC were termed colony-forming unit-fibroblasts and osteogenic stem cells (Horwitz et al., 2006). Other terms such as marrow stromal stem cells, mesenchymal progenitor cells, multipotent mesenchymal stromal cells, and the widely used term mesenchymal stem cells were later adopted (Caplan, 1991; Charbord, 2010). Disputes have arisen regarding the accuracy of calling them stem cells. True stem cells demonstrate self-renewing abilities, clonality, and potency (Kolios & Moodley, 2013). MSC are most often isolated as a heterogenic group, which is a combination of individual cell populations that demonstrate self-renewal and potency to various extents. Therefore, the term mesenchymal stromal cells has been adopted in this thesis.

1.1.2 MSC characterization

The International Society for Cellular Therapy (ISCT) formed the minimum criteria for defining MSC in 2006. The cells should be plastic-adherent, express certain surface markers (CD105, CD73, and CD90) but not common hematopoietic markers, and the cells should be capable of trilineage differentiation into osteoblasts, chondrocytes, and adipocytes as demonstrated with histological staining (Dominici et al., 2006). These criteria still form the guidelines along which MSC characterization is performed, a decade after its publication (Kfoury & Scadden, 2015).

Defining the MSC signature has proven problematic due to lack of MSC-specific biomarkers. MSC are commonly isolated as a heterogeneous population of cells as is evident during colony formation. Colonies often vary greatly in size, differentiation potential, and cell morphology (Rosu-Myles et al., 2012). It is recognized that within the population true stem cells can be found. However, they cannot be distinguished from less potent cells (Rennerfeldt & Van Vliet, 2016). This has hampered improvement in isolation procedures that still commonly rely on the plastic adherence of the cells (Sivasubramaniyan et al., 2012).

Both the MSC gene expression and surface marker expression have been demonstrated to change as the cells are grown in vitro (Kfoury & Scadden, 2015). This complicates the identification of potential MSC markers as findings on cultured cells do not reflect the state of freshly isolated cells. Expression of markers can also vary between tissue sources and depending on culture conditions (Al-Nbaheen et al., 2013; Kfoury & Scadden, 2015; Mafi, 2011). Several markers have been suggested (Table 1); however, the combination of both positive and negative markers is likely to give the best results for effective isolation of potent MSC (Mafi, 2011; Niehage et al., 2011; Pérez-Silos et al., 2016; Wong et al., 2015).

Table 1. Markers for the isolation of mesenchymal stromal cells. Adapted from Salem & Thiemermann (2010).

Suggested markers for MSC isolation	
Positive Markers	**Negative Markers**
CD9, CD10, CD13, CD29, CD44, CD49a, CD49b, CD49c, CD49e, CD51, CD54, CD58, CD61, CD62L, CD71, CD73, CD90, CD102, CD104, CD105, CD106, CD119, CD120a, CD120b, CD121. CD123, CD124, CD126, CD127, CD140a, CD166, CD340, CD349, CCR1, CCR4, CCR7, CXCR5, CCR10, VCAM-1, AL-CAM, ICAM-1, STRO-1 (CD140b), W8B2, W3D5, W4A5, W5C4, W5C5, W7C6, 9A3, 58B1, F9-3C2F1, HEK-3D6	CD11a, CD14, CD15, CD18, CD19, CD25, CD31, CD34, CD45, CD49d, CD50, CD62E, CD62P, CD80/40, CD86, CD117

1.1.3 The MSC microenvironment

Tissue-resident adult stem cells dwell in a specific microenvironment that maintains and regulates the balance between a quiescent and active state of the cells within the tissue. This microenvironment is also known as the stem cell niche (Ferraro et al., 2010). The niche is composed of cells and extracellular matrix (ECM), both of which provide structure as well as physical and chemical signals that influence the occupying stem cells. Within the niche, stem cells remain quiescent but differentiate upon departure from it. Thus, the niche is important to support self-renewal and to maintain the stem cell pool (Ferraro et al., 2010).

Various contributing factors participate in niche regulation. The cells modulate the surrounding ECM that in turn impacts the state of the cells. The dynamic interaction between the cells and the ECM in the niche is mediated directly through the binding of molecules such as integrins and indirectly through signaling mediated by soluble factors (Ahmed & Ffrench-Constant, 2016). Mechanical forces also participate since the stiffness of the ECM plays

a role in determining the stem cell fate (Ahmed & Ffrench-Constant, 2016; Li, Y. Y. et al., 2015). Stem cell niches have been identified at various anatomical locations in the body such as in the brain and skin (Ahmed & Ffrench-Constant, 2016; Ferraro et al., 2010). The hematopoietic niche in the bone marrow, however, was the first niche described and remains the best-understood example as of today.

It has been known for a long time that MSC are an instrumental part of the hematopoietic niche (Nombela-Arrieta et al., 2011; Reinisch et al., 2015). MSC can form both osteoblasts and adipocytes. The two cell types exert opposing effects on the HSC in the niche and can regulate their state. Adipocytes act as antagonists while osteoblasts provide the needed endosteal environment (Nombela-Arrieta et al., 2011). Hence, the differentiation potential of MSC and the balance between cell types is crucial for regulation of the HSC niche. The MSC secretions also play a role, and their age and stage of senescence have been shown to contribute to HSC dysfunction (O'Hagan-Wong et al., 2016). The regulation is thus most likely more complex than previously thought.

1.1.4 Different tissue sources

MSC have traditionally been isolated from the bone marrow. However, isolation from various sources has been widely described (Figure 1). Common sources include adipose tissue, umbilical cord blood, Wharton's jelly, periodontal tissue, dental pulp tissue, and amniotic membrane (Augello et al., 2010; Marquez-Curtis et al., 2015; Pievani et al., 2014).

The location and biological role of MSC between different tissues have been a source of extensive speculation. One hypothesis is that MSC belong to a single specific niche in the body, such as the bone marrow, and migrate from there to other sites of the body (da Silva Meirelles et al., 2008). A different opinion suggests that MSC belong to niches in various tissues and have different properties between anatomical locations (da Silva Meirelles et al., 2008). A third possibility is that MSC are in fact of perivascular nature, line blood vessels, and reside in a niche from which they can quickly reach the circulation and home in on sites of injury (Nombela-Arrieta et al., 2011). It has been demonstrated that pericytes can support the HSC niche and demonstrate trilineage differentiation. Consequently, there has been a substantial focus on the pericyte hypothesis since it offers an explanation of why MSC can be found in most vascularized tissue (de Souza et al., 2016).

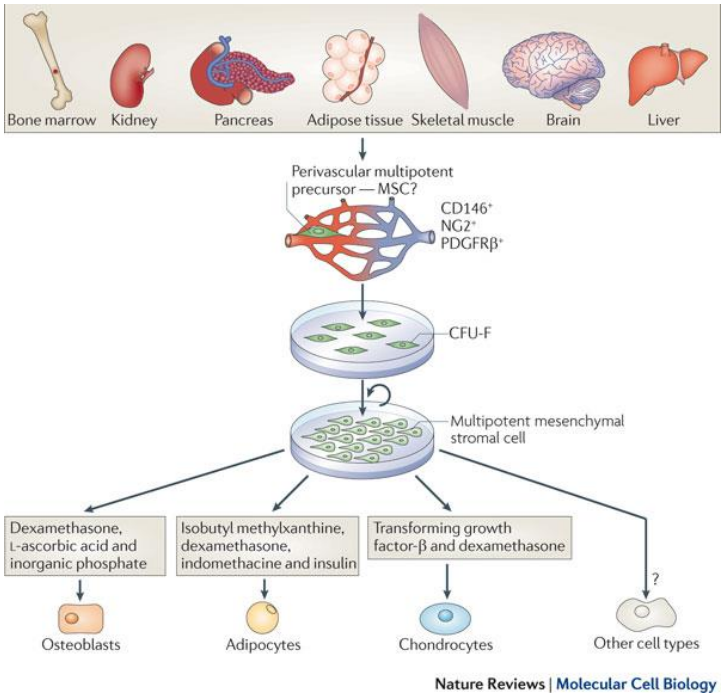

Figure 1. Mesenchymal Stromal Cells. MSC can be isolated from the vascular fraction of several different tissues based on adherence to plastic surfaces. MSC can then be induced to form osteoblasts, adipocytes, chondrocytes and potentially other cell types in vitro under the right stimulus. (Nombela-Arrieta et al., 2011)**.**

MSC from different sources contribute to homeostasis by cell replacement, modulation of tissue microenvironments, and by secretion of soluble factors and mediators (Bernardo & Fibbe, 2013; Keating, 2012; Nombela-Arrieta et al., 2011). Significant differences in the extent of their abilities are nonetheless observed between sources (Augello & De Bari, 2010). Recently, Reinisch et al. demonstrated that when comparing MSC from bone marrow, adipose tissue, umbilical cord blood, and skin, only MSC from the bone marrow were able to contribute to a formation of an HSC niche through endochondral ossification (Reinisch et al., 2015). Marinkovic et al. further showed that when mimicking the MSC niche in adipose tissue and the niche in bone marrow in vitro, MSC demonstrated significantly different characteristics based on in which niche they were grown (Marinkovic et al., 2016). Best results were obtained when MSC were grown in an ECM specific for the tissue from which they were isolated. Together, these findings underscore the differences seen between cell sources and the importance of further defining subpopulations of MSC (Marinkovic et al., 2016). To date, it

has been challenging to arrive at a narrow definition of what comprises an MSC. This partly stems from the uncertainty about whether findings, markers, and characteristics of MSC seen in vitro actually represent their in vivo state (Kfoury & Scadden, 2015).

1.1.5 Multilineage differentiation potential

MSC are derived from the mesodermal germ layer of the embryo. The mesoderm gives rise to the tissues of the skeletal system through development (Eriksen, 2010). The trilineage differentiation potential to form osteoblasts, chondrocytes, and adipocytes is thus the hallmark of MSC (Dominici et al., 2006). Bone and cartilage formation are closely related developmental processes while the formation of adipocytes is more distinct (Li, J. et al., 2016).

The bones of the skeleton form either through intramembranous or endochondral ossification (Long, 2011). Intramembranous ossification is restricted to limited parts of the cranium and entails a direct bone formation through osteoblast formation from progenitor cells. The rest of the skeleton is formed through endochondral ossification where chondrogenesis precedes osteogenesis (Long, 2011). Endochondral ossification starts with condensation of mesenchymal progenitor cells and proceeds with the early formation of hyaline cartilage (Liu, C.-F. et al., 2016). The cartilage constructs are made up of proliferating chondrocytes and are used as scaffolds to guide subsequent osteogenesis (Long, 2011). As the chondrocytes advance through differentiation, they become hypertrophic, a signal that triggers the activation of osteogenesis and the already formed cartilage is slowly replaced by bone (Liu, C.-F. et al., 2016).

The in vitro differentiation of MSC is dependent on factors and signaling pathways that interact and regulate each other. Early osteogenesis is characterized by cell proliferation and upregulation of alkaline phosphatase (*ALP*) and collagen type I (*COL1*) (Birmingham et al., 2012). As the osteogenesis advances, the cell proliferation is reduced, but ECM production and mineralization increase (Birmingham et al., 2012). This occurs as the cells go from a population of preosteoblasts toward matrix-producing osteoblasts and then mature osteocytes (Eriksen, 2010). The cells also go from a fibroblast appearance toward a more cuboidal shape typical for osteoblasts (Mechiche Alami et al., 2016). Osteoblast formation from MSC can be induced by the addition of dexamethasone, ascorbic acid, and β-glycerophosphate to their environment (Langenbach & Handschel, 2013).

Chondrogenesis can also be seen to take place in a stepwise manner. During early chondrogenesis, cellular condensation occurs as microspheres or aggregates form. This is followed by morphological changes, chondrocyte formation, upregulation of *SOX9* (SRY-Box 9), and fibril formation that strengthens the chondrocytic structure (Yamashita et al., 2010). ECM production is subsequently upregulated as collagen II (COL2), collagen X (COL10*)*, aggrecan and glycosaminoglycans (GAG) are secreted (Chen, W.-H. et al., 2009). Cell proliferation is then downregulated and the chondrocytes become hypertrophic and induce a shift toward ossification including matrix degradation (Yamashita et al., 2010). In vitro chondrogenesis is frequently induced using dexamethasone, ascorbic acid, and TGFβ, which is vital for successful differentiation (Freyria & Mallein-Gerin, 2012).

Adipogenesis is not fully understood, but when MSC are treated with a combination of factors such as dexamethasone, isobutyl methylxanthine, insulin, and a peroxisome proliferator-activated receptor gamma 2 (PPARG) agonists, they acquire a round shape, and accumulation of lipid droplets in the cytosol are observed (Tencerova & Kassem, 2016).

1.1.6 Transcriptional control of differentiation

Osteoblasts, chondrocytes, and adipocytes are all derived from the same common progenitor, and their regulatory processes are intertwined (Li, J. et al., 2016; Liu, C.-F. et al., 2016). Each lineage is under a strict transcriptional control, and the master transcription factors runt-related transcription factor 2 (*RUNX2*), *SOX9* and *PPARG* play a significant role (Franceschi et al., 2007; Liu, C.-F. et al., 2016; Tencerova & Kassem, 2016).

RUNX2 is central to committing MSC to the osteochondroprogenitor lineage (Franceschi et al., 2007). It was first described to be a specific activator of osteoblast differentiation, but it is now known that *RUNX2* is expressed in chondrocytes as well (Ducy et al., 1997; Franceschi et al., 2007). *RUNX2* is upregulated early in the differentiation process and its expression often precedes osteogenesis (Freeman et al., 2016). Its expression is, however, essential for osteogenesis as its downstream targets are required for osteoblast formation, including regulators such as *SP7* (osterix) and *SPP1* (secreted Phosphoprotein 1) (Franceschi et al., 2007; Long, 2011). Absence of *RUNX2* expression results in severe bone defects and abnormal skeletogenesis (Long, 2011). This is also true for endochondral ossification indicating the importance of *RUNX2* not only for osteoblasts but also for chondrocytes (Freeman et al., 2016; Liu, C.-F. et al., 2016).

Chondrogenic transcriptional control involves several SOX factors with *SOX9* being especially important for the lineage commitment and upregulation of ECM components such as collagen and aggrecan (Pritchett et al., 2011). *SOX5*/*SOX6* work as enhancers for *SOX9* (Akiyama & Lefebvre, 2011). Insufficient *SOX9* expression is known to cause a severe chondrodysplasia (Nishimura et al., 2012). Mesenchymal condensation upregulates *SOX9* expression early in endochondral ossification. At that the time the osteochondroprogenitor cells are bipotent, expressing both *RUNX2* and *SOX9* (Akiyama & Lefebvre, 2011). Lineage commitment toward chondrocytes is then marked by reduced expression of *RUNX2* and upregulated expression of *SOX9* (Dancer et al., 2010; Nishimura et al., 2012). That is opposite to what is seen for osteoblast differentiation where *RUNX2* expression is upregulated along with other osteogenic regulators (Franceschi et al., 2007). For chondrocytes, *SOX9* expression stimulates proliferation and ECM secretion but inhibits maturation (Nishimura et al., 2012). *RUNX2* expression stimulates chondrocytic hypertrophy and a shift toward ossification (Nishimura et al., 2012). Hence, the upregulation of *RUNX2* during chondrogenesis is indicative for chondrocyte maturation, hypertrophy, and late differentiation stage (Dancer et al., 2010). The right balance between chondrocyte proliferation and maturation as determined by the expression of these two lineage-committing factors is a key factor for healthy bone formation. Any imbalances can severely affect skeletogenesis (Pritchett et al., 2011).

Adipogenesis is under the transcriptional regulation of *PPARG*, a transcription factor that is both necessary and sufficient to drive adipocyte differentiation. Inhibition of *PPARG* enhances osteogenesis, suggesting a negative regulation between these two lineages (Kawai et al., 2012; Tencerova & Kassem, 2016)

1.1.7 Signaling pathways of differentiation

Various signaling pathways participate in fate determination of MSC. Bone morphogenetic proteins (BMP) and TGFβ signaling have been observed to exert antagonistic effects on differentiation. Members of the BMP family stimulate osteogenesis while TGFβ inhibits it (Dexheimer et al., 2016; Keller et al., 2011; Spinella-Jaegle et al., 2001). BMP-2 and BMP-4 seem to act together after the upregulation of *RUNX2* expression to stimulate other osteogenic regulators downstream of *RUNX2* (Long, 2011; Nishimura et al., 2012). TGFβ, on the other hand, is vital for chondrogenesis (Dexheimer et al., 2016).

β-catenin-dependent Wnt signaling also plays a crucial role (Long, 2011). Wnt signaling is mediated by the binding of Wnt to Frizzled receptors and co-receptors such as lipoprotein receptor-related protein 5 (LRP5) and LRP6. The binding of Wnt sets in place an intracellular signaling cascade where cytosolic β-catenin is stabilized and translocated to the nucleus (Karner & Long, 2016). There, it interacts with transcription factors to stimulate expression of target genes. In the absence of Wnt signaling, cytosolic β-catenin is degraded (Karner & Long, 2016). *RUNX2* is a direct target of β-catenin and is upregulated through their interaction (Karner & Long, 2016). Hence, Wnt signaling stimulates osteogenesis.

The absence of Wnt signaling in MSC results in adipogenesis. Both when Wnt is blocked from binding its Frizzled receptors and also when co-receptors are impaired, especially LPR5 (Tencerova & Kassem, 2016). This results in degradation of β-catenin that is then unable to stimulate *RUNX2* expression. *PPARG* expression is, however, active (Kawai et al., 2012). Thus adipogenesis seems to be spontaneous in the absence of osteogenic stimulators (Li, J. et al., 2016). Understanding the regulation of Wnt signaling during osteogenesis and adipogenesis is of clinical interest since it can shed further light on the pathology of osteoporosis where bone formation is reduced and fat formation increased (Kawai et al., 2012).

1.1.8 MSC role in immunomodulation

A large body of literature centers on the unique ability of MSC to modulate immune responses (Bernardo & Fibbe, 2013). They have been shown to home to sites of injury and inflammation and participate in wound healing (English, 2012). Their role as regulators of inflammation is becoming more evident and potential use in immunotherapies more attractive. It is now clear that MSC participate not only in cell replacement through differentiation but also by modulating the microenvironment during inflammation (Keating, 2012; Wang et al., 2014; Wong et al., 2015). MSC are sensitive to changes in their surroundings and respond to inflammation by secreting soluble factors, with cell-cell signaling and by contributing to a more tolerogenic microenvironment (Bernardo & Fibbe, 2013; Najar et al., 2016).

1.1.8.1 MSC and cells of immunity

Mesenchymal stromal cells interact both with cells of the innate and the adaptive immune system (Bernardo & Fibbe, 2013; Le Blanc & Davies, 2015). The nature of the inflammatory microenvironment determines how MSC respond (Figure 2). A pro-inflammatory environment with high levels of

interferon (IFN)γ and tumor necrosis factor (TNF)α encourages MSC to adopt an anti-inflammatory phenotype (MSC2). Anti-inflammatory microenvironment conversely drives MSC toward pro-inflammatory phenotype (MSC1) (Bernardo & Fibbe, 2013; Waterman et al., 2010).

Activation through toll-like receptors (TLR) is central to this polarization and enhances the immunomodulatory properties of MSC (Krampera et al., 2013). TLR4 stimulation is chiefly associated with MSC1 phenotype while TLR3 stimulation is associated with MSC2 (Waterman et al., 2010). Following TLR activation, MSC abilities to suppress and avoid natural killer cells (NK cells), induce regulatory T cells (Tregs) and recruit neutrophils are enhanced (Giuliani et al., 2014; Lu et al., 2015; Rashedi et al., 2016). This is achieved by upregulating cytokine secretion of various cytokines such as interleukin (IL)-6 and IL-8 (Le Blanc & Davies, 2015).

Depending on their phenotype after activation, MSC can polarize monocytes to mature into either type 1 pro-inflammatory macrophages (M1) or type 2 anti-inflammatory (M2) (Molina et al., 2015; Spaggiari & Moretta, 2013). IL-6 secretion by MSC2 stimulates the formation of IL-10 secreting M2 macrophages. Conversely, MSC1 secretes minor levels of IL-6 and thus monocytes mature into M1 macrophages that secrete high levels of IFNγ and TNFα (Bernardo & Fibbe, 2013). TLR activation, similar to what is seen for MSC, plays an important role in macrophage polarization (Keating, 2012; Molina et al., 2015; Spaggiari & Moretta, 2013).

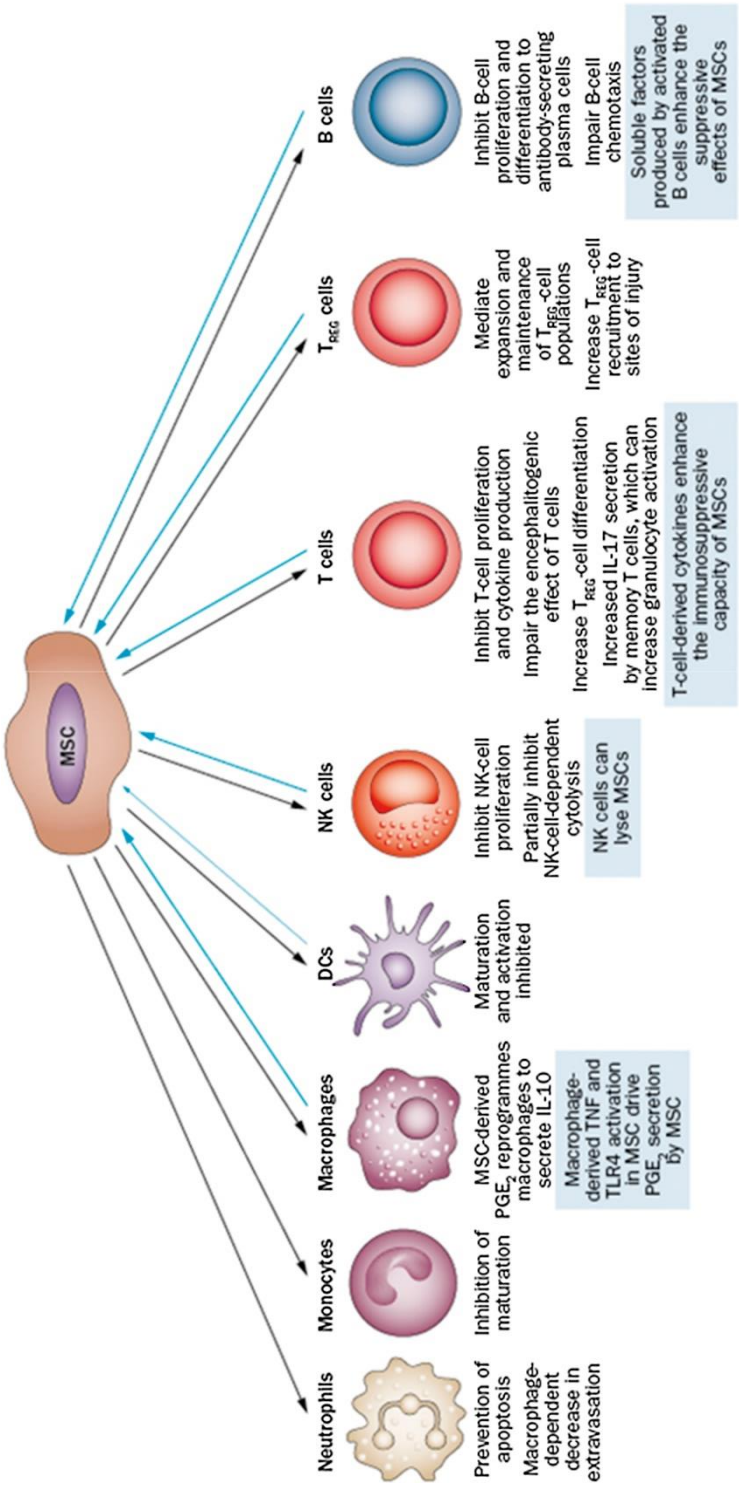

Figure 2. MSC interaction with cells of the immune system. MSC interact with the cells of the immune system and can inhibit their function and proliferation through various mechanisms. Immune cells are likewise able to influence MSC activity. The crosstalk between these cell types is a crucial part of the immune response and resolution. (Tyndall, 2015a)

MSC express low levels of molecules such as HLA-II, CD40, CD80, and CD86, surface-antigens necessary for the maturation of dendritic cells (Le Blanc & Davies, 2015). Dendritic cells (DCs) are an important link between the innate and the adaptive immune system since they present antigens to T-cells and modulate their activity (Le Blanc & Davies, 2015; Molina et al., 2015). By failing to stimulate DC maturation, MSC contribute to a tolerogenic DC phenotype that in turn does not activate the adaptive immune system (English, 2012; Najar et al., 2016). For this reason, they have sometimes been described as immunoprivileged (Waterman et al., 2010). MSC are nonetheless susceptible to the cytotoxic effects of activated NK cells which specifically target cells with low HLA-I expression (Liao et al., 2013; Spaggiari et al., 2006). TLR activation and licensing with IFNγ enables MSC to prevent the activation of NK cells by IL-2 and avoid detection by upregulating MHC-I expression (Giuliani et al., 2014; Le Blanc & Davies, 2015; Lu et al., 2015; Molina et al., 2015). Licensing thus allows MSC to avoid lysis by NK cells.

The ability to suppress cells of the adaptive immune system, especially T cells, was among the first MSC immunomodulatory properties to be reported (Wang et al., 2014). MSC are, however, not only able to suppress T and B cells but are also able to induce the formation of T regulatory cells (Tregs) and B regulatory cells (Bregs). Formation of regulatory cells allows for the generation of a more tolerogenic environment that enables immunomodulation (Najar et al., 2016). $CD4^+$-mediated Th2 response is favored over Th1 and Th17 response, and cytotoxic $CD8^+$ T cells are arrested (Bernardo & Fibbe, 2013; Glenn & Whartenby, 2014). Several studies have described that MSC reduce B cell activation; this, however, seems to vary between tissue sources for the MSC (Glenn & Whartenby, 2014; Ribeiro et al., 2013).

1.1.8.2 The inflammatory microenvironment

Licensing of MSC with IFNγ or other inflammatory molecules has been described as enhancing their properties and resulting in greater immunosuppression (Le Blanc & Davies, 2015). Some will even argue that MSC are not spontaneously immunomodulatory but require licensing (English, 2012). IFNγ seems to be especially potent in activating MSC (Vigo et al., 2016). Other inflammatory molecules such as TNFα, IL1β, prostaglandin E2 (PGE2), indolamin-2,3-dioxygenase (IDO), TGFβ, tumor necrosis factor-inducible gene 6 (TSG6) and nitric oxide (NO) that are normally found during inflammation can act as activators as well, especially through TLR stimulation (English, 2012). Evidence suggests that MSC are

not only activated by IFNγ but by the general inflammatory microenvironment (Ma, S. et al., 2014; Szabó et al., 2015).

A complex combination of cytokines and cells is found at the inflammatory site. MSC influence the cytokine secretion of surrounding cells and secrete several factors themselves. The main molecules secreted by MSC that partake in immunomodulation are IDO, PGE2, NO, TSG-6, hepatocyte growth factor (HGF), TGFβ, and human leukocyte antigen G (HLA-G) (Bruno et al., 2015; English, 2012). IDO is a potent regulator for T cell proliferation since the enzyme catabolizes tryptophan into kynurenine that is required for T cell regulation (de Lara Janz et al., 2015). IDO expression by MSC is stimulated by IFNγ licensing or TLR activation, and the factor has been shown to participate in modulating both the innate and adaptive immune response by polarizing macrophages, stimulating regulatory DC maturation as well as by encouraging Th2 responses over Th1 (English, 2012). PGE2 expression is upregulated in activated MSC and participates in immunomodulation at several levels (de Lara Janz et al., 2015). A regulatory connection has been identified between IL-6 and PGE2 since PGE2 can upregulate IL-6 production which in turn can stimulate PGE2, generating a feedback loop (Liu, X. H. et al., 2006). This produces an environment that stimulates M2 macrophages and the resolution of inflammation (Bouffi et al., 2010; Ylöstalo et al., 2012). TSG-6 secretion by MSC is stimulated by TNFα and can act as an anti-inflammatory agent systemically as well as locally (English, 2012; Lee et al., 2009).

Recent evidence suggests that MSC can secrete immunomodulatory factors in the form of extracellular vesicles (EV) (Bruno et al., 2015). MSC-derived EVs have been demonstrated to exert immunomodulation similar to MSC, indicating that the role of MSC-derived factors in immunomodulation might be more significant than previously assumed (Bruno et al., 2015). This has been observed in culture with peripheral blood mononuclear cells (PBMC), in rat models of Parkinson's disease, models of acute lung injury, lung inflammatory conditions, and in mouse model of myocardial infarction (Chen, W. et al., 2016; Lee et al., 2009; Monsel et al., 2016; Teixeira et al., 2016). The notion that MSC can regulate inflammation at a distance opens up new possibilities for immunotherapy and underlines the importance of addressing the immune function of MSC further (Bruno et al., 2015).

1.1.9 Embryonic derived mesenchymal stromal cells

Mesenchymal stromal cells have already been the subject of numerous clinical trials (Lalu et al., 2012; Tyndall, 2015b). MSC have been administered successfully to treat conditions such as acute graft-versus-host-disease (aGVHD) and Crohn's disease and are generally considered safe (Lalu et al., 2012). Both allogenic and autologous cells have been used, but due to low MSC numbers in most tissues, they have to be expanded ex vivo before being administered to a patient (Kitagawa & Era, 2010; Sharma et al., 2014). This and several other challenges regarding MSC, such as their heterogeneity and senescence after few passages in culture, have prompted the development of protocols to derive MSC from pluripotent cells (Ikebe & Suzuki, 2014). The pluripotent cells commonly used are embryonic stem cells (ESC) and, more recently, induced pluripotent stem cells (iPSC) (Brown et al., 2014; Sheyn et al., 2016). By deriving MSC from ESC or iPSC, it is hoped to combine the advantages of both cell types in one. That is, combining the differentiation potential and immunomodulation of MSC with the immortality of ESC (Brown et al., 2014).

1.1.9.1 Embryonic stem cells

Embryonic stem cells (ESC) are isolated from the inner layer of the blastocyst and can give rise to cells from all the three embryonic germ layers. They have an unlimited proliferative capacity and can be expanded and manipulated ex vivo (Mountford, 2008). Outside of the body, ESC need to be maintained on a layer of feeder cells to ensure their survival and undifferentiated state. The feeder cells most commonly used are mouse embryonic fibroblasts (de Peppo & Marolt, 2012). ESC were first derived by Thomson et al. and immediately gained a lot of attention (Thomson et al., 1998), not only by the scientific community for their unrestricted plasticity and potential for medical research but also by the public where they became the center of an ethical debate due to their biological origins. ESC are still considered controversial by some groups (Ilic & Ogilvie, 2016).

ESC have been studied intensely and increased our understanding of development and tissue formation (de Peppo & Marolt, 2012). They have been implanted into experimental animals, but upon doing so, they generally form tumors called teratoma (Bulic-Jakus et al., 2016). During teratoma formation, the cells undergo uncontrolled differentiation and give rise to various tissue types within the same tumor. As of today, no practical methods exist to control the behavior of undifferentiated ESC after implantation in order to prevent teratoma formation (Bulic-Jakus et al., 2016).

1.1.9.2 ESC-derived MSC

MSC do not form teratomas after implantation since their differentiation potential is more restricted than for ESC (Fernández-Vallone et al., 2013). MSC are also able to modulate immune responses and generate a more tolerogenic environment, reducing the probability of rejection by the host (Najar et al., 2016). Several protocols have been published that result in the successful derivation of MSC from ESC (ESC-MSC). The cells demonstrate long-term proliferation, trilineage differentiation potential, and no tumor formation after implantation (Brown et al., 2014; Luzzani & Miriuka, 2016). The published protocols do, however, vary significantly from each other (Luzzani & Miriuka, 2016). Some protocols rely on the use of feeder cells such as OP9 cells and the spontaneous formation of ESC-MSC through epithelial-to-mesenchymal transition (EMT) (Barberi et al., 2005). The ESC-MSC are removed from the ESC culture according to surface markers or scraped off as they migrate from the ESC colonies (the raclure method) and are then cultured separately (Olivier et al., 2006). Some protocols try to induce EMT by depriving the cells of pluripotency signals with starvation (Trivedi & Hematti, 2008). Other methods try to encourage the ESC-MSC formation by adding external factors such as insulin-transferrin-selenite (ITS), basic fibroblast growth factor (bFGF) or by blocking TGFβ signaling (Karlsson et al., 2009; Sánchez et al., 2011; Stavropoulos et al., 2009).

The concept of ESC-MSC is to generate cells that can be produced in significant quantity for cell therapies but are not tumorigenic or rejected by the recipients body (Luzzani & Miriuka, 2016). Animal or xenogeneic components should be limited as much as possible. Especially now with the passing of stricter regulations for advanced tissue engineering products (Ancans, 2012; Ram-Liebig et al., 2015). Hence, clinically feasible protocols for derivation of ESC-MSC need to be examined and standardized.

Karlsson et al. described one such protocol to obtain human embryonic cell derived mesenchymal progenitor cells (hES-MP) (Karlsson et al., 2009). Shortly after, the first commercial ESC-MSC cell line (hES-MP002.5) was generated. Our group has explored the properties of the hES-MP002.5 cell line in the papers presented here. The hES-MP002.5 cell line is thus especially important for this thesis.

1.1.9.3 hES-MP002.5

Unlike previous methods to derive ESC-MSC, the Karlsson protocol does not require transfection steps, coculture with other cells, assisted cell sorting, or

manual selection and handling of the cells (Karlsson et al., 2009; Luzzani & Miriuka, 2016). Undifferentiated ESC are plated onto 0.1% gelatin-coated tissue culture plates at a high density and grown with media containing 10% FBS or 10% human serum and 10ng/ml human recombinant bFGF (Karlsson et al., 2009). After seven days, an outgrowth of various cell types is observed. No specific selection of cells is performed. All the cells are moved to uncoated plates and passaged weekly until only cells that exhibit an hES-MP morphology are left in the culture (Karlsson et al., 2009). Karlsson et al. demonstrated the repeatability of this protocol for 10 different ESC lines with consistent results. They also derived a completely xeno-free hES-MP line (hES-MP611) from the xeno-free ESC cell line SA611 using human serum instead of FBS and human recombinant gelatin instead of porcine (Karlsson et al., 2009). The cell line used for this thesis, hES-MP002.5 is derived from the ESC line SA002.5. They will be referred to as hES-MP cells from here on.

The hES-MP cell lines do not express typical markers for undifferentiated ESC, endodermal markers, or epithelial markers. They do, however, express mesodermal markers and demonstrate robust growth up to 20 passages. The cells successfully differentiate toward osteoblastic, chondrocytic, and adipocytic lineages and do not form teratomas when transplanted in SCID mice (Karlsson et al., 2009). Further characterization of the transcriptome has revealed that the hES-MP cells have significantly lower expression of genes associated with pluripotency (the OCT family and *NANOG*) than ESC (de Peppo, Svensson, et al., 2010). The hES-MP cells have higher expression of genes associated with proliferation than MSC but a similar expression of genes encoding for important membrane receptors associated with MSC differentiation (*TGFBR2* and *BMPR2*) (de Peppo, Svensson, et al., 2010). Subsequently, it has been stated that the hES-MP cells demonstrate osteogenic potential superior to MSC in vitro, both in a monolayer culture and in a packed bed/column bioreactor system on coral scaffolds (de Peppo, Sjovall, et al., 2010; de Peppo et al., 2013). Li et al. demonstrated the capacity of hES-MP to engraft in bone marrow and support hematopoiesis after intrafemoral transplantation in NSG mice (Li, O. et al., 2013). They also demonstrated the inability of hES-MP to suppress in vitro lymphocyte proliferation (Li, O. et al., 2013).

Our group has continued this work, as demonstrated in this thesis, and further characterized the immunomodulation by hES-MP and their growth when cultured with human platelet lysates (Papers III and IV).

1.2 Platelets

Platelets are small, anucleated structures with a discoid shape and a lifespan of about seven to 10 days. Approximately 10^{11} platelets are released into the circulation daily (Harmening et al., 2009). They are central to hemostasis and are equipped with the necessary organelles, such as mitochondria and granules, to fulfill their role in the circulation (Harmening et al., 2009). Apart from their role in hemostasis, they also contain an abundance of growth factors and cytokines that participate in wound healing, inflammation and vasoconstriction (Thomas & Storey, 2015; Thon & Italiano, 2012).

1.2.1 Megakaryocytes and platelet formation

Platelets are the progeny of megakaryocytes, and their formation is the result of a series of steps that begins with the formation and maturation of megakaryocytes (Italiano & Hartwig, 2013). Megakaryocytes are of hematopoietic origin and are derived from a common bipotential myeloid progenitor (Klimchenko et al., 2009; Sigurjónsson et al., 2002). They are formed within the osteoblastic niche in the bone marrow, and as they mature, they gradually migrate toward vascular spaces where they release platelets into the circulation (Bluteau et al., 2009; Italiano & Hartwig, 2013; Machlus & Italiano, 2013).

Megakaryocytes are polyploid cells with multiple karyotypes (N). Cells up to 128N have been reported (Tomer et al., 1988). The formation of a polyploid nucleus is achieved through a process called endomitosis where the genetic material is replicated, but cell division does not happen (Italiano & Hartwig, 2013). The megakaryocytes begin the mitotic cycle as in normal mitosis and proceed from prophase to anaphase but do not undergo cytokinesis (Geddis & Kaushansky, 2006; Lordier et al., 2008). Polyploidization is essential for megakaryocyte maturation and efficient platelet formation (Deutsch & Tomer, 2006). When endomitosis is completed, the megakaryocytes enter final maturation where the cytoplasm is prepared for platelet formation with synthesis of platelet-specific proteins, organelles, granules, and membrane systems (Italiano & Hartwig, 2013).

The mechanism by which the megakaryocytes release platelets remains controversial (Bluteau et al., 2009). One of the most prevalent hypotheses focuses on the formation of a kind of pseudopodia, termed proplatelets, that protrude from the megakaryocytes and release platelets from their distal ends (Avanzi et al., 2015; Bender et al., 2015; Bluteau et al., 2009; Richardson, 2005; Thon et al., 2010). The proplatelet formation is regulated by various

factors such as the dephosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) and the stiffness of the microenvironment (Aguilar et al., 2016; Machlus et al., 2016). An alternative hypothesis for platelet formation focuses on explosive-fragmentation of megakaryocytes giving rise to platelets. According to this hypothesis, platelets are formed within megakaryocytes and then released following a global fragmentation event of the cytoplasm (Deutsch & Tomer, 2006; Reems et al., 2010; Zucker-Franklin, D., 1984; Zucker-Franklin, Dorothea & Philipp, 2000). The scientific community has yet to reach a consensus on the subject; however, the proplatelet hypothesis appears to be the more accepted hypothesis for platelet formation (Italiano & Hartwig, 2013; Reems et al., 2010).

Platelet formation or platelet release has been observed to happen at various locations in the body such as the bone marrow, the blood stream, and the lungs (Italiano & Hartwig, 2013). Apparently, shear stress plays a significant role in facilitating platelet release within the circulation. In vitro, it has been shown that submitting human megakaryocytes to high shear stress results in considerably more platelet release than when no shear stress is applied (Dunois-Larde et al., 2009; Thon et al., 2010). In vivo, platelets are found in high concentrations in the pulmonary vein (Thon et al., 2010). Pre- and proplatelets may become trapped in the capillary bed due to their size and be driven toward terminal platelet formation under shear forces (Dunois-Larde et al., 2009). This might also apply to the network of microcapillaries in the spleen and bone marrow (Machlus & Italiano, 2013). Platelet formation, therefore, seems to be dependent on the interplay between megakaryocytes, proplatelets, and shear forces.

1.2.2 Platelet structure

The anatomy of the platelet (Figure 3) is classically divided into four systems or zones; the peripheral zone, the sol-gel zone, the organelle zone, and the platelet membrane system (White, 2013,).

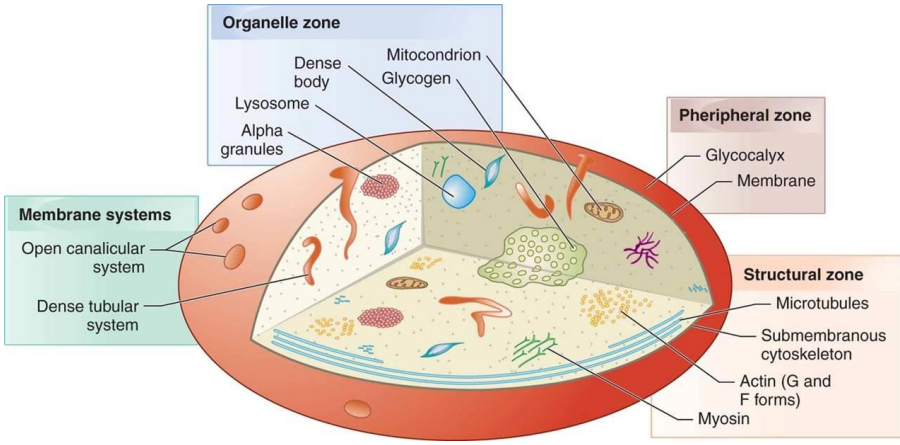

Figure 3. Platelet structure. Platelets are composed of four structural zones. The peripheral zone is the platelet membrane, the sol-gel zone or structural zone is equivalent to cytoplasm, the membrane system participates in uptake, storage, and secretion of molecules, and the organelle zone is composed of all the platelet organelles such as mitochondria and secretory granules (McKenzie & Williams, 2016).

1.2.2.1 The peripheral zone

The peripheral zone is the platelet plasma membrane and consists of three layers. The layers act together to facilitate both primary and secondary hemostasis during vascular injury by expressing molecules important for the initiation of blood clotting (White, 2013).

The top layer is the glycocalyx in which key glycoproteins and receptors are embedded (White, 2013). The glycocalyx makes up the exterior surface and plays an important role in sensing changes in the vascular environment and mediating signals regarding hemostasis (Harmening et al., 2009; White, 2013). It contains numerous glycoprotein (GP) receptors to fulfill its role, but the GPIb-IX complex and GPIIb-IIIa (integrin αIIbβ3) are the primary receptors (Bennett, 2005; Harmening et al., 2009; Li, R. & Emsley, 2013). Each is expressed abundantly on the platelet exterior with approximately 25,000 GPIb-IX and 80,000 GPIIb-IIIa receptors covering each platelet (Bennett, 2005; White, 2013). The absence of either of these two receptors is the cause of the two most common platelet membrane defects: Bernard-Souler Syndrome (GPIb-IX) and Glanzmann's thrombasthenia (GPIIb-IIIa). These defects vary in severity, and symptoms range from minor bruising,

mucosal bleeding and prolonged bleeding time, to severe hemorrhages (Li, R. & Emsley, 2013; Liles & Knupp, 2009).

The glycocalyx rests on top of the unit membrane which is a lipid bilayer resembling the plasma membrane of other cells. The unit membrane plays a vital role in accelerating blood coagulation during secondary hemostasis by exposing tissue factor molecules (TF) on the surface when the platelet becomes activated (del Conde, 2005; Vignoli et al., 2013). The third layer, the submembrane area of the peripheral zone, is found below the unit membrane and plays a role in allowing shape changes, translocation of receptors and anchoring of filaments (White, 2013).

1.2.2.2 The sol-gel zone

The sol-gel zone is the equivalent to the cytoplasm of other cells. It is, however, more fibrous and viscous due to a dense network of cytoskeletal microtubules, microfilaments, and glycogen (White, 2013).

The main cytoskeletal components of the sol-gel zone are the circumferential microtubule coil and actinomyosin filaments that connect to the submembrane area of the peripheral zone (White, 2013). The microtubule coil helps resting platelets to maintain a discoid shape and integrity under high shear forces in the circulation while actin forms a dense mesh in which organelles and other structural components are suspended (Hartwig, 2013). Following platelet activation the microtubule coil is degraded and the cytoskeleton reorganized (Qiu et al., 2014). The process is triggered by the influx of calcium and activation of the remodeling protein gelsolin that fragments the microtubule coil. As a result, the platelet loses its discoid shape and becomes spherical (Sorrentino et al., 2015).

Actin filaments assemble in the sol-gel zone in parallel to the fragmentation of the microtubule coil. The actin filaments are associated with myosin heads that aid in the contraction of the platelet plug during coagulation and shape changes (Hartwig, 2013; Qiu et al., 2014). The amount of actin in the platelet doubles, causing fingerlike projections to protrude from the platelet surface. The platelet is then able to spread over the surface it adheres to and participate in coagulation and platelet plug formation (Sorrentino et al., 2015). The filaments found in the sol-gel zone are thus crucial for the proper function of platelets.

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1.2.2.3 The platelet membrane system

Two distinctive membrane systems are found within platelets: the surface connected open canalicular system (OCS) and the dense tubular system (DTS) (White, 2013).

The OCS connects to the peripheral zone and opens to the exterior of the platelet. Within the platelet, it tunnels through the cytoplasm and serves as a route for uptake and secretion of chemicals to and from the platelet granules (Thon & Italiano, 2012). The OCS composition resembles the layers of the peripheral zone, and during platelet spreading, it serves as an important source of additional platelet membrane (Thon & Italiano, 2012; White, 2013).

The DTS, on the other hand, is not connected to the surface membrane but remains separate from other structures of the platelet. Its origins are thought to be from the endoplasmic reticulum of the megakaryocyte, and it is abundant in ionized calcium, storing up to 30% of the total calcium molecules of the platelet (Rendu & Brohard-Bohn, 2001). Calcium is important for platelet activation and is released from the DTS following a decrease in the concentration of cytosolic cAMP (Rendu & Brohard-Bohn, 2001). The release of calcium from the DTS causes platelet activation, cytoskeletal reorganization, and secretion of granular content (Thon & Italiano, 2012). The balance between cytosolic calcium and cytosolic cAMP needs to be regulated, in part by the DTS (Rendu & Brohard-Bohn, 2001).

1.2.2.4 The Organelle zone

The organelle zone of the platelet contains its secretory granules, mitochondria, and other structures such as glycosomes (White, 2013).

The α-granules are the most abundant of all the platelet granules with 40 to 80 α-granules present in each platelet (Thon & Italiano, 2012). The α-granules contain numerous proteins and molecules that influence the platelet bioactivity and allow it to fulfill its function. These proteins participate in platelet adhesion, hemostasis, wound healing, and inflammation (Jurk & Kehrel, 2005; Rendu & Brohard-Bohn, 2001). They originate from the endoplasmic reticulum of the megakaryocytes and contain both proteins that were endogenously synthesized and proteins that have been incorporated through endocytosis (Koseoglu & Flaumenhaft, 2013). The α-granules remain separate from each other in resting platelets, but upon activation, the granules fuse with each other and with the OCS as their contents are secreted from the platelets (Thon & Italiano, 2012). Proteomic studies have revealed the presence of 827 different granule-related proteins that

participate in at least 32 different processes and pathways (Zufferey et al., 2014). Some of the proteins are platelet-specific, some are taken up through the OCS, and others associate with the cytoskeleton and facilitate secretion (Rendu & Brohard-Bohn, 2001; Zufferey et al., 2014).

The platelet-specific proteins, platelet factor 4 (PF4; CXCL4) and β-Thromboglobulin (β-TG; CXCL7), are located in the α-granules along with their proteoglycan precursor proteins (Rendu & Brohard-Bohn, 2001). PF4 is released in large quantities following platelet activation and binds to glycosaminoglycans and heparin-like molecules, influencing their activity and promoting coagulation (Amelot et al., 2007). PF4 has also been shown to suppress lymphocytes and attract neutrophils, suggesting its role in modulating inflammation at the site of vascular injury (Fleischer et al., 2002; Martí et al., 2002). β-TG is a chemoattractant and a mitogen for fibroblasts and can attract them to sites of tissue damage to promote healing (Kalwitz et al., 2009; Ravindran & Krishnan, 2007). Adhesive proteins and coagulation factors can also be found within the α-granules (Jurk & Kehrel, 2005).

The α-granules harbor important growth factors and cytokines with the most noticeable growth factors being platelet-derived growth factor (PDGF), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), and basic fibroblast growth factor (bFGF) (Rendu & Brohard-Bohn, 2001; Rožman & Bolta, 2007). Together, these growth factors act as regulators of wound healing and new vessel growth (Thon & Italiano, 2012). Cytokines secreted from the α-granules include RANTES and CD40L (Rondina et al., 2013).

Among the α-granule membrane-bound proteins, P-selectin plays a major role and is expressed on the platelet surface after activation (Rendu & Brohard-Bohn, 2001). P-selectin mediates platelet-leukocyte interaction and allows platelet rolling along the endothelium (Jurk & Kehrel, 2005; Rondina et al., 2013). Other membrane-bound proteins include GTP receptors, osteonectin, and glycoprotein receptors (Rendu & Brohard-Bohn, 2001).

Dense granules are another major type of granule found in platelets. They contain a high concentration of ADP and ATP as well as bioactive amines such as serotonin and histamine (Flaumenhaft, 2013). Dense granules are also rich in calcium (Ambrosio et al., 2015). This calcium pool, unlike the calcium of the DTS, is not in an ionized form and does not contribute to platelet activation. The calcium found in dense granules contributes to stability and provides a concentration gradient to attract serotonin (Rendu & Brohard-Bohn, 2001). Other molecules in the dense granules have

hemostatic effects like ADP which is a platelet agonist that triggers shape change, granule release, and aggregation (Thon & Italiano, 2012).

Platelets also contain lysosomes which contain degrading enzymes and the recently described T granules that were demonstrated by Thon et al. (2012) to store TLR 9 (Flaumenhaft, 2013).

1.2.3 Role in hemostasis

Vascular damage triggers a tightly regulated chain of events that aims to stop bleeding and initiate wound healing. This chain of events consists of primary hemostasis, secondary hemostasis, and fibrinolysis. Platelets are central to the process by forming a loose platelet plug to seal the injury and provide the negatively charged phospholipid surface needed to activate secondary hemostasis (Harmening et al., 2009).

Platelet adhesion to the endothelium and platelet aggregation are important steps in primary hemostasis. The platelet membrane plays a key role in mediating the signals from extracellular agonists to intracellular processes through the expression of specific receptors (Harmening et al., 2009). Platelet adhesion relies on the interaction of endothelium-bound von Willebrand factor (vWF) with the GPIb/V/IX complex on the platelet membrane (Clemetson, 2012). The contact between vWF and GPIb/V/IX is the first step in platelet-mediated hemostasis, and its main role is to slow down platelets at the site of injury, facilitating both platelet adhesion and binding of signaling receptors that mediate platelet activation (Cimmino & Golino, 2013; Clemetson, 2012; Gale, 2011).

Activated platelets lose their discoid shape as a result of cytoskeletal reorganization mediated by increased concentration of cytosolic calcium (Qiu et al., 2014). They form pseudopodia, adhere to the site of injury, and gradually spread over the surface like a blanket (Clemetson, 2012). The shape changes bring the platelet granules near the OCS, which opens to the surface (Thon & Italiano, 2012). The granules fuse with the OCS and their contents are released (Clemetson, 2012). The α granules release coagulation factors (factors V, XIII), receptors (GPVI, GPIIb/IIIA), vWF, fibrinogen, prothrombin, plasminogen, and mitogenic factors such as PDGF (Flaumenhaft, 2013; Jurk & Kehrel, 2005). The dense granules, on the other hand, release secondary agonists such as ADP, Ca^{2+}, and serotonin (Cimmino & Golino, 2013; Flaumenhaft, 2013). These agonists interact with the platelet surface membrane and cause further shape change and aggregation.

The GPIIb/IIIa complex is indispensable for successful platelet aggregation and is solely expressed on platelets and megakaryocytes (Bennett, 2005). In its active form, it binds soluble fibrinogen and contributes to the formation of a loose platelet plug. The platelet plug is subsequently strengthened through an enzymatic cascade which turns fibrinogen into fibrin mesh with the aid of thrombin (Bennett, 2005; Harmening et al., 2009). Plasmin later cleaves the fibrin back into fibrinogen during fibrinolysis to dissolve blood clots, aid in wound healing and prevent thrombus formation. The blood clot is slowly replaced by new tissue (Mutch, 2013).

1.2.4 Role in inflammation and wound healing

Apart from their important role in hemostasis, platelets also participate actively in inflammation and wound healing through secretion of mediators (Herter et al., 2014). The entire proteome of the platelet granules has not been deciphered yet, but they are known to contain various chemokines and factors such as CXCL1, CXCL5, CXCL7, Interleukin-8 (IL-8), PF4, RANTES, P-selectin and CD40L (Golebiewska & Poole, 2015; Thomas & Storey, 2015). These molecules interact with leukocyte receptors and contribute to a pro-inflammatory response (Morrell et al., 2014).

Activated platelets express P-selectin. It binds its ligand (PSGL-1) on the surface of innate immune cells causing them to slow down in the circulation. Slowing down allows them to bind to the endothelium and the platelet plug and form aggregates (Thomas & Storey, 2015). Platelets also express CD40L and secrete it in its soluble form. Interaction of CD40L with CD40 on monocytes encourages them to express tissue factor and initiate the coagulation cascade (Morrell et al., 2014). Binding of CD40L to endothelial cells further upregulates the expression of adhesion molecules and enables the migration of immune cells to the site of injury (Herter et al., 2014; Thomas & Storey, 2015).

While recruiting immune cells, platelets also secrete an abundance of mitogens such as PDGF, TGFβ, VEGF, EGF, IGF, and bFGF (Rendu & Brohard-Bohn, 2001; Rožman & Bolta, 2007). Together, these factors stimulate the proliferation and migration of smooth muscle cells, fibroblasts, and endothelial cells to close the wound, repair the vessel wall, and encourage de novo angiogenesis (Golebiewska & Poole, 2015). Platelets in the circulation are equipped with the necessary factors for wound healing which makes them well suited for their important role as guardians of the circulation (Harper et al., 2014).

1.3 Platelet transfusion

Platelets are used both prophylactically to treat patients at a risk of bleeding and as a measure against active bleeding (Perrotta et al., 2013). Platelet transfusion became available in the 1960s and early 1970s when methods to prepare platelet concentrates were developed (Djerassi et al., 1963; Harmening & Moroff, 2005; Klein et al., 1956). Before the introduction of platelet transfusion therapy, leukemia linked mortality in children was approximately 90% at one year post diagnosis, often due to fatal hemorrhages. After platelet transfusions became available the frequency of fatal hemorrhages dropped by half in a decade (Freireich, 2011; Hersh et al., 1965). Since then platelet transfusion sciences have changed the clinical landscape with regard to treating patients at risk of bleeding (Brand et al., 2006).

1.3.1 Platelets in clinical use

Approximately three million platelet units are transfused annually in Europe and two million in North-America (Brecher et al., 2013; Stroncek & Rebulla, 2007). A considerable increase in demand has been observed over the past decade with an increase of 20-30% seen in many countries (Estcourt, 2014). Most of the transfused units are administered prophylactically to prevent bleeding rather than to stop active bleeding, for example during surgery and trauma (Heal & Blumberg, 2004).

Platelet transfusions are generally associated with a low occurrence of adverse effects, even in the event of transfusion across ABO barriers (Cid et al., 2013; Dunbar et al., 2012; Shehata et al., 2009). Both acute and delayed adverse effects are nonetheless well known (Cap et al., 2016). Bacterial and viral contamination remains one of the major concerns, but allergic reactions, hemolytic transfusion reactions, post-transfusion purpura, and transfusion-associated graft-versus-host disease (GvHD) are also commonly reported (Heal & Blumberg, 2004; Holbro et al., 2013; Stroncek & Rebulla, 2007). To minimize adverse effects, blood banks, transfusion centers, and other manufacturers of blood components need to fulfill strict quality criteria and regulations regarding collection, manufacturing, preparation, and distribution of blood components (Ciaraldi & Williams, 2005). Standard guidelines require the pH, volume, and platelet counts to be monitored (Hughes & Wright, 2005). All blood and platelet donors have to pass a screening program consisting of a medical history questionnaire, physical examination, and blood screening for transmitted diseases (Hughes & Wright, 2005). Donors

are most commonly screened for hepatitis B and C, HIV, HTLV, syphilis, and West Nile virus. Additional screening tests may be performed based on location (Williams et al., 2005).

1.3.2 Preparing platelet concentrates for transfusion

Platelet concentrates for transfusion are prepared according to three different protocols. The platelet-rich plasma (PRP) method is favored in North America, and the buffy coat (BC) method is favored in Europe (Figure 4). Both regions commonly use preparation by apheresis as well (Holbro et al., 2013; Perrotta et al., 2013). Platelet preparation procedures in South-East Asia and countries classified as “developing” are poorly documented (Marwaha, 2010; Verma & Agarwal, 2009). For those reasons, the focus here is on platelet utilization in North America and Europe.

Platelet-rich plasma (PRP) is prepared from anticoagulated whole blood that is centrifuged at a low speed, leukoreduced, and then centrifuged again at higher speed. The first step eliminates the red blood cells and the second produces platelet-poor plasma as a supernatant and a pellet rich in platelets. The platelet pellet is removed and resuspended in plasma to produce a unit of PRP from a single donor. The PRP is then stored at 20°C to 24°C with agitation (Hughes & Wright, 2005; Perrotta et al., 2013). Several PRP units are pooled before transfusion.

When preparing platelet concentrate with the buffy coat method, a unit of anticoagulated whole blood is first centrifuged at high speed. This step separates the whole blood into components where the red blood cells reside at the bottom while plasma and white blood cells rest on top. Platelets are located on the interface between the two layers. The platelet layer or buffy coat and small portions of the plasma and red cells are removed (Perrotta et al., 2013; Stroncek & Rebulla, 2007). Several buffy coat units are then pooled together, leukoreduced, and centrifuged at a low speed allowing a platelet-rich supernatant to form. The supernatant is transferred to a storage bag and stored at 20°C to 24°C with agitation (Hughes & Wright, 2005).

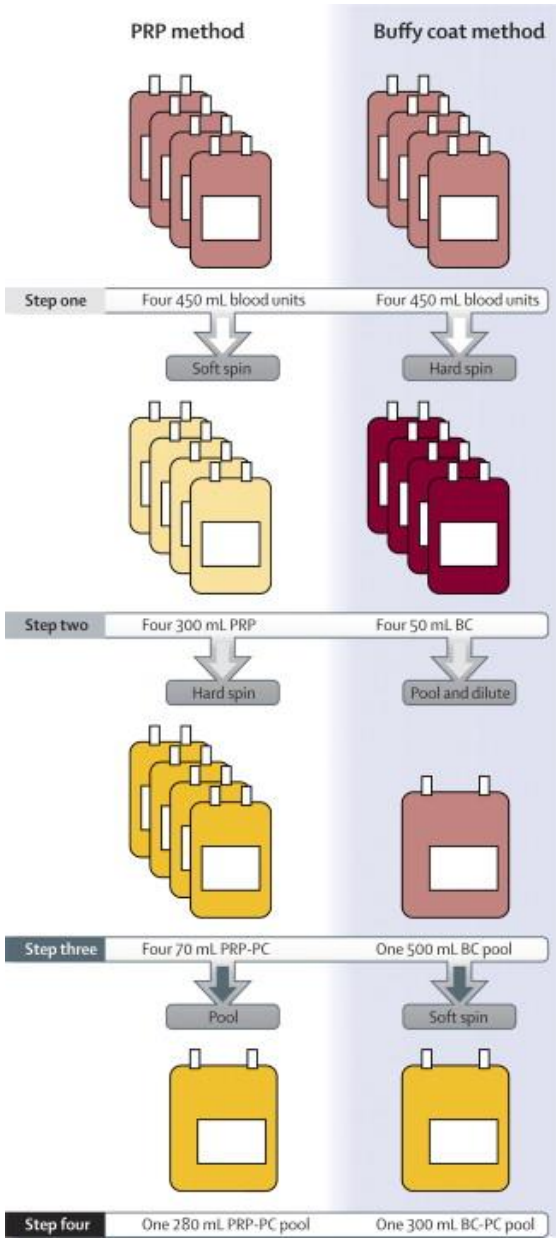

Figure 4. Manufacturing process of platelet concentrates. Platelet concentrates from whole blood are commonly made using either the platelet-rich plasma (PRP) method or the buffy coat method. The PRP method starts with a soft spin, and the supernatant then goes through a hard spin to produce a platelet concentrate. Conversely, the buffy coat starts with a hard spin, the platelets are then isolated and submitted to a soft spin to produce a platelet concentrate for transfusion (Stroncek & Rebulla, 2007).

Platelet transfusion units can also be generated through apheresis with a cell separator. Blood from a single donor is collected through a catheter and the platelets separated from other blood components using differential centrifugation before the blood is returned to the donor (Perrotta et al., 2013). Preparation by apheresis is a common practice in both Europe and North-America (Cap et al., 2016; Heal & Blumberg, 2004).

There does not appear to be any significant difference in the performance of platelets prepared with these different methods (Marwaha & Sharma, 2009). The BC method is favored in Europe due to evidence that there is a lower cytokine concentration in those concentrates and the platelets are not activated to the same extent during preparation. These difference are, however, observed only within the first 48 hours (Flegel et al., 1995; Marwaha & Sharma, 2009).

1.3.3 Platelet storage lesion

To maintain platelet count and function during storage, platelets need to be stored at 20°C to 24°C with agitation. The shelf life is five to seven days (Gulliksson, 2003). This is different from red blood cells that can be stored at 4°C for over 30 days and plasma that can be stored frozen at -18°C for at least a year (Paglia et al., 2012; Prowse et al., 2014). The specific storage conditions for platelets aim at maximizing the storage time by overcoming two major obstacles: the platelet storage lesion and the risk of bacterial contamination.

Platelet storage lesion is characterized by morphological changes, reduction in the ability to aggregate, increase in activation markers, oxidative stress, glucose depletion, build-up of metabolites, granular secretion, and a drop in pH over time (Cap et al., 2016; Manasa & Vani, 2016; Milford & Reade, 2016). The overall quality of the platelet is thus reduced as indicated by poor post-transfusion survival in the circulation (Perrotta et al., 2013). Several factors contribute to platelet storage lesion such as the method of blood collection, manufacturing, storing and post-production handling (Cap et al., 2016; Paglia et al., 2014; Perrotta et al., 2013). The detailed mechanism behind platelet storage lesion is not fully understood; however, the platelets seem to go through few distinct metabolic stages during storage that affect the regulation of energy metabolism (Paglia et al., 2014). To limit the effects of storage, specialized conditions are employed, including certain temperature values, gas-permeable storage bags, agitation, and platelet additive solutions (Gulliksson, 2003).

1.3.4 Bacterial contamination

Apart from the storage lesion, the risk of bacterial contamination remains a major concern that limits the storage time of platelet concentrates. Bacteria represent a significant threat. It is estimated that 0.1% of all platelet concentrates may be contaminated and bacteria are associated with up to 20% of all transfusion-related deaths (Walther-Wenke, 2008). The incidence of transfusion-related bacterial infections far exceed transfusion-related viral infections (Palavecino et al., 2006).

The storage conditions for platelets provide bacteria, if present, with ideal conditions for growth. Bacterial contamination can arise from transient skin flora of the blood donor if not properly disinfected before venipuncture. Bacteria can also originate from the donated blood if the blood donor is bacteremic at the time of donation (Vasconcelos & Seghatchian, 2004). Measures to limit or detect bacterial contamination have not been efficient enough (Brecher et al., 2013). Standards from regulatory bodies state that blood banks should practice both preventive measures and have a systematic bacterial detection program (Palavecino et al., 2006). This is done by evaluating the pH levels after the expiration of the platelet units and by sampling for bacterial detection (Brecher et al., 2013). Bacterial detection can be performed with various methods such as flow cytometry, genetic studies, and automated detection systems (Vasconcelos & Seghatchian, 2004). The ideal detection method would require a small sample, be both sensitive and specific, have short turnaround time, and be cost efficient (Palavecino et al., 2006).

Currently, most detection methods require 24 to 48 hours, by which time the platelets might already have been transfused (Palavecino et al., 2006). Novel approaches that offer rapid detection are emerging such as the BactiFlow system that uses flow cytometry and the use of PCR to detect bacterial 16S rDNA (Mathai, 2009; Müller et al., 2015; Walther-Wenke, 2008). The time of detection is nonetheless an important factor, since early testing may not be sensitive enough to detect a small number of bacteria as was shown in a study by the Irish Blood Transfusion Service. The study demonstrated less than 40% sensitivity of early screening using the most common method for bacterial detection in blood components, the BacTAlert system (Brecher et al., 2013; Murphy, W. G. et al., 2008). Hence, more focus has been on preventive measures, such as pathogen inactivation, rather than detection methods in recent years.

1.3.5 Pathogen inactivation

Considerable advancements in the development of pathogen inactivation (PI) technology have taken place in the past decade. The role of PI is to reduce and inactivate bacteria, pathogens, and other cellular entities present in blood components, thereby reducing the incidence of transfusion-related infections. Inactivating currently unrecognized and emerging pathogens such as dengue virus, Plasmodium spp., and the Zika virus is also within the scope of this technology (Marano et al., 2015; Schmidt et al., 2014). Methods to inactivate plasma have been in place for some time and rely on either a solvent-detergent approach or the addition of methylene blue to the plasma pool. Those methods result in excessive reduction in factor VIII activity and fibrinogen concentration and cannot be applied to cellular blood components due to cytotoxic effects (Schlenke, 2014; Seltsam & Müller, 2013).

Three commercially available platforms have now been developed that can pathogen inactivate cellular components: INTERCEPT™ (Cerus Corporation, Concord, CA, USA), Mirasol® (Terumo BCT, Lakewood, CO, USA), and THERAFLEX® UV (Macopharma, Mouvaux, France), all of which are based on the use of UV illumination to damage nucleic acids (Schlenke, 2014).

The Mirasol® system uses riboflavin (vitamin B2) and broad-spectrum UV light to cause irreversible damages in DNA and RNA through oxidation. Riboflavin is converted to lumichrome and photoproducts that cause damage without binding nucleic acids and proteins. The damages both block replication and inhibit the repair mechanism. Secondary effects on factor VIII and fibrinogen have been described following Mirasol® inactivation, and further toxicological studies are needed (Schlenke, 2014).

THERAFLEX® relies on UV illumination only. Narrow-bandwidth UV-C light is applied causing the formation of pyrimidine dimers. This method has been tested on dogs and demonstrated tolerability and low immunogenicity (Schlenke, 2014).

The INTERCEPT™ system has been adopted by the Icelandic Blood Bank and is especially relevant to this thesis. The Intercept™ system will hence be discussed in detail. This method uses a synthetic substance called amotosalen (S-59). Amotosalen is a photoactive psoralen similar to other naturally occurring psoralens found in plants such as lime and celery (Irsch & Lin, 2011; Schlenke, 2014). It is a tricyclic molecule with an added amine side chain that gives high water solubility and allows it to pass easily through lipid cellular membranes without any alterations to its structure (Irsch & Lin, 2011).

Access to non-lipid enveloped viruses and bacterial spores is, however, not as high due to its chemical nature (Prowse, 2013). Amotosalen binds nucleic acids (DNA and RNA) with high affinity in a sequence-unspecific manner. Upon UV-A illumination, cross-links form between the reactive groups of amotosalen and the pyrimidine bases of the nucleic acids. It has been demonstrated to happen at a high frequency and can happen both between strands and within a single strand (Irsch & Lin, 2011). The crosslinking does not happen in the absence of UV-A and is not dependent on the formation of oxygen reactive species known to be harmful to cells.

Following the PI process, residual amotosalen is filtered from the final platelet product, so the final concentration levels are approximately 0.5 µmol/l. For a transfused patient, it is an estimated exposure of 1µg amotosalen per transfusion (Irsch & Lin, 2011; Schlenke, 2014). Amotosalen is cleared from the circulation within 24 hours after transfusion (Lozano & Cid, 2013). Satisfying safety margins have been demonstrated for amotosalen and safety has been demonstrated in four randomized clinical trials (Janetzko et al., 2005; Lozano et al., 2011; McCullough et al., 2004; van Rhenen et al., 2003). It is not considered carcinogenic, mutagenic, or genotoxic (Seltsam & Müller, 2013). Toxicological studies examining effects of high amotosalen concentrations (1µg/kg) also failed to demonstrate any toxic effects (Irsch & Lin, 2011). This system has demonstrated good inactivation capacity and is also able to inactivate white blood cells, thus reducing transfusion-related alloimmunization and GVHD (Schlenke, 2014; Seltsam & Müller, 2013). The system has, however, not been demonstrated to be effective against bacterial spores (Seltsam & Müller, 2013)

Photochemical treatment with the INTERCEPT™ system can be performed immediately after production of the platelet concentrate without affecting metabolism or activation markers (Janetzko et al., 2004). The same inactivation performance is observed for BC-derived platelets and apheresis platelets (Chavarin et al., 2011). Applying the inactivation treatment early is important since the load of infectious particles (e.g. viruses and bacteria) is generally considered lowest soon after blood collection and before storage (Goodrich et al., 2010). For bacteria, this is often 10 to 100 colony-forming units (CFU) per product. As the number of bacteria grows with time, so does the concentration of bacteria-derived endotoxins, often reaching sufficiently high levels to cause severe reactions in the patient (Goodrich et al., 2010). Inactivating the bacteria as early as possible is therefore of major importance and most likely outweighs the disadvantages of the bacteria detection systems.

1.3.6 Managing the platelet inventory

The next decade will see an estimated 10% increase in demand for blood available for transfusion (Williamson & Devine, 2013). For a country to be self-sufficient, 3-4% of its inhabitants need to donate blood. In Iceland, approximately 5% of the nation donates blood (Jóhannsdóttir et al., 2016). The annual decrease in donors is however approximately 3.4% (Jóhannsdóttir et al., 2016). Constant marketing campaigns are needed to recruit new donors and retain previous ones (Riley et al., 2007). Keeping a balanced blood inventory is a challenging task, especially since running out of blood is not considered an acceptable option in any developed health-care system (Williamson & Devine, 2013).

The increase in demand for platelets seems to be especially prominent with an increase of over 7% seen in just three years (2008-2011) in the United States (Dunbar, 2015). Still, in the same period between, 12% and 17% of all platelet units were wasted due to expiration (Dunbar, 2015). The reason is the short-shelf life of platelets, retention period due to bacterial screening, and fluctuations in demand that can be hard to predict. The recommended storage time for platelets has traditionally been only five days. The limited storage time complicates platelet inventory management since a fine balance between supply and demand needs to be kept under conditions of high uncertainty. The storage time was determined based on the increased likelihood of bacterial infections with time as well as the platelet storage lesion where platelets lose their function over time (Gulliksson, 2003). The introduction of pathogen inactivation now allows the platelets to be stored for up to seven days (Irsch & Lin, 2011; Schlenke, 2014). This increase by two days greatly facilitates inventory management.

1.4 Platelet lysates

Platelets are considered promising candidates for the formulation of cell culture supplements. Their richness in growth factors makes them suitable for the task (Rožman & Bolta, 2007). The platelets are stimulated or lysed, causing them to release growth factors from their granules (Schallmoser & Strunk, 2013; Shih & Burnouf, 2015). The obtained solution is then used for the preparation of different materials such as platelet lysate, platelet glue, platelet gel, or platelet-rich plasma (PRP), all of which are used to stimulate tissue regeneration, wound healing, and cellular growth (Burnouf, T. et al., 2013; Piccin et al., 2016).

Platelet activation is achieved by using different kinds of agents that stimulate the granular release of platelets. The most commonly used factors are either thrombin or $CaCl_2$, but collagen, epinephrine, Triton-X, and ADP have all been used, as well as sonication above 20 kHz (Astori et al., 2016; Herrmann et al., 2014). The solution obtained after activation is termed platelet releasate and is commonly used for its intended application shortly after production (Astori et al., 2016).

Growth factor and granular release can also be attained by lysing the platelets. The solution obtained is termed human platelet lysate (hPL) and can be stored frozen for later use (Shih & Burnouf, 2015). The main difference between a platelet releasate and an hPL is that no platelet activating agents are added during the formation of a platelet lysate (Hemeda et al., 2014). The platelets are instead subjected to repeated freeze-thaw cycles, a process that causes the platelets to rupture and release their contents into the surrounding solution (Bieback, 2013; Schallmoser & Strunk, 2013). The cellular debris, platelet membranes, and fibrin clots are then separated from the solution by centrifugation and filtration (Astori et al., 2016). The resulting lysate is used as a source of growth factors for various applications, mainly wound healing and in cell culture as an animal serum replacement (Shih & Burnouf, 2015). Platelet lysates are the subject of this thesis and will be discussed further in the chapters below.

1.4.1 Platelet lysates in tissue regeneration

Human platelet lysates (hPL) are rich in various growth factors and other molecules that originate from the platelet granules. These factors include PDGF, TGFβ, EGF, bFGF, and VEGF that are found in high quantities in hPL and demonstrate low batch-to-batch variability (Altaie et al., 2016). The exact contribution of those factors to cell expansion or wound healing is not fully understood. Both PDGF-BB and bFGF seem to be especially important since a lack of those factors significantly reduces cellular proliferation (Fekete, Gadelorge, et al., 2012; Hao et al., 2011). The exact quantity needed to support cells is, however, unknown (Astori et al., 2016)

Solutions based on hPL have nonetheless been used successfully for wound healing (Chiara Barsotti et al., 2013). Corneal lesions have been treated with hPL-containing eye drops and contact lenses (Fea et al., 2016; Sandri, G. et al., 2016, 2012). Promising results have also been obtained when using hPL to treat ocular GvHD (Pezzotta et al., 2017, 2012; Zallio et al., 2016). Other applications include treatment of skin ulcers, epicondylitis,

and tendon lesions, stimulation of hair growth and various other orthopedic uses (Al-Ajlouni et al., 2014; Dastan et al., 2016; Pirvu et al., 2014; Rizzo et al., 2014; Rožman & Bolta, 2007; Tan, X. et al., 2016). The hPL is applied either directly for topical use or injected as a PRP product (Al-Ajlouni et al., 2014; Fabi & Sundaram, 2014). Incorporation into various biomaterials and wound dressings for controlled release of growth factors is done as well (Lima et al., 2015; Mori et al., 2016; Sandri, Giuseppina et al., 2013, 2015; Tenci et al., 2016). As indicated, the applications vary a great deal but nonetheless share the common goal of improving tissue regeneration and stimulating cellular growth.

The use of hPL and other PRP products for wound healing has been found to give inconsistent results (Fabi & Sundaram, 2014). Some studies have demonstrated no beneficial effects beyond placebo (Stacey et al., 2000). Others have highlighted the suitability of hPL for wound healing (Chiara Barsotti et al., 2013). The differences observed between studies are partly due to the lack of standardization in hPL production (Fabi & Sundaram, 2014). Large randomized controlled trials with adequate power are needed to clear this matter (Fabi & Sundaram, 2014).

1.4.2 Platelet lysates as serum replacement in cell culture

1.4.2.1 Fetal bovine serum

A considerable focus is being placed on the improvement of current cell culture techniques, standardization of media, and the elimination of animal serum (Astori et al., 2016). Such efforts go hand in hand with progress in regenerative medicine and the clinical use of advanced tissue engineering products (Burnouf, T. et al., 2016). For over half a century, fetal bovine serum (FBS) has been the main cell culture supplement in use (Gstraunthaler et al., 2013). It effectively supports most types of human, animal, and insect cells and is rich in fetal growth factors and hormones that stimulate cellular proliferation and maintenance. It is also known to contain transport proteins, adhesion factors, vitamins and minerals, fatty acids, and protease inhibitors making it a versatile culture supplement (Hemeda et al., 2014; Kinzebach & Bieback, 2012; Mannello & Tonti, 2007). However, detailed composition remains unknown (Hemeda et al., 2014).

FBS use is linked to several disadvantages and drawbacks (Shih & Burnouf, 2015). Especially for the culture of human cells meant for regenerative medicine (Hemeda et al., 2014). These drawbacks concern unethical production methods, questions about biosafety, and undesirable

variations in quality between batches (Mannello & Tonti, 2007). FBS is a side product from the beef industry. If a cow is pregnant at the time of slaughter, the pregnant uterus is removed and the blood drained from the fetus (Van Der Valk et al., 2004). Methods of blood collection vary, but applying cardiac puncture is a common procedure (Hemeda et al., 2014; Van Der Valk et al., 2004). The collected blood is chilled, and after clotting, the serum is obtained as supernatant (Hemeda et al., 2014). Annual production of FBS has been estimated to be around 500,000 L, a quantity requiring approximately 1,000,000 fetuses (Gstraunthaler et al., 2013; Hemeda et al., 2014; Van Der Valk et al., 2004). Procedures to reduce pain and discomfort for the fetuses have been outlined and are recommended. However, no regulations are in place to ensure ethical treatment of the animals at the time of blood collection (Van Der Valk et al., 2004).

Variations in quality are also a great concern. FBS demonstrates significant lot-to-lot variations, a problem that has been recognized since the 1970s (Honn et al., 1975; Zheng et al., 2008a). Laboratories have been forced to perform FBS screening of their own to find lots that are suitable for their particular research (Phelan & May, 2015). When the preselected lot has been found, the laboratory in question needs to reserve large enough quantities to last the whole study since switching between lots could adversely affect the research. This process is both time-consuming and costly (Mannello & Tonti, 2007; Phelan & May, 2015). Lot-to-lot variations also make it hard to compare data received by different groups since the FBS lots used may not be comparable (Mannello & Tonti, 2007).

The supply of FBS fluctuates and is dependent on external factors such as geographical location, droughts, and conditions in cattle (Gstraunthaler et al., 2013). Demand for FBS is highest in the United States and Europe, but the raw serum producers are located far away in Central- and South America, South Africa, Australia, and New Zealand (Hawkes, 2015a). The production of FBS has been loosely regulated in the past, and serious cases of fraud and false labeling have occurred (Gstraunthaler et al., 2013; Zheng et al., 2008b). For example, it was revealed in 2011 that PAA Laboratories (acquired be GE Healthcare Life Sciences) had for nine years, from 2003 to 2011, practiced false labeling of their FBS. The serum contained, in some cases, added bovine serum albumin (BSA), water, or added growth factors (Astori et al., 2016; Gstraunthaler et al., 2013; U.S. Food and Drug Administration, 2013). The stated country of origin was in many cases intentionally incorrect as well (Gstraunthaler et al., 2013). Such cases of

fraud are a serious offense. They can, at best, damage research and results and, at worst, be hazardous for humans.

Lastly, FBS is not considered to meet the requirements for use in cellular therapy (Mannello & Tonti, 2007). FBS contains factors of animal origin that can be immunogenic when used as a part of treatment, (Hemeda et al., 2014; Shih & Burnouf, 2015). It is estimated that between 7 to 30 mg of FBS derived proteins can be transferred with each cell dose (Hao et al., 2011; Hemeda et al., 2014). Anaphylaxis, Arthus-like reactions, and the development of antibodies to bovine proteins have been observed following administration of FBS treated cells into patients (Mackensen et al., 2000; Selvaggi et al., 1997; Tuschong et al., 2002). This was seen although the FBS was used only during the cell expansion phase and the cells were washed prior to injection (Shih & Burnouf, 2015).

Apart from immunogenicity, the risk of animal-borne disease being transmitted with the FBS in the form of bacteria, viruses, prions, or other pathogens is also a cause for concern (Hawkes, 2015b). In recent years, the world has seen how zoonotic pathogens can adapt and become virulent to humans. Such pathogens include HIV, the severe acute respiratory disease (SARS) and H7N9 flu virus (Shih & Burnouf, 2015). It is therefore sobering that between 20% and 50% of all commercially available FBS is believed to be virally contaminated with bovine viruses such as bovine viral diarrhea virus (BVDV) (Bolin & Ridpath, 1998; Even et al., 2006; Hemeda et al., 2014; Wessman & Levings, 1999). An outbreak of bovine spongiform encephalopathy (mad cow disease) in the UK in the 1990s sparked a needed discussion of the matter (Chou et al., 2015; Hawkes, 2015b).

1.4.2.2 Suitability of platelet lysates

It has been demonstrated that hPL supports the proliferation of numerous cell types even better than FBS (Burnouf, T. et al., 2016; Hemeda et al., 2014). Cells from different sources that are supported by hPL include mesenchymal stromal cells (Doucet et al., 2005), dental pulp stem cells (Marrazzo et al., 2016), tenon fibroblasts (Carducci et al., 2016), endothelial cells (Hofbauer et al., 2014), renal epithelial cells (Rauch et al., 2011a), CHO cells (Kao et al., 2016), HUVECs (Ma, J. et al., 2014), keratinocytes (Baik et al., 2014), and various other human cell lines including leukemia cell lines and HeLa (Fazzina, Iudicone, Mariotti, et al., 2016). This broad applicability is directly related to the high growth factor concentration found in hPL (Kinzebach & Bieback, 2012).

hPL made from platelet concentrates (PC) obtained from accredited blood banks is more standardized than the production of FBS and demonstrates lower lot-to-lot variations (Altaie et al., 2016; Warnke et al., 2013). This is because the source material has to pass strict quality control for transfusion before being used for hPL production (Kinzebach & Bieback, 2012; Shih & Burnouf, 2015). Using a standardized source material facilitates standardization of the final hPL product (Shih & Burnouf, 2015). Still, hPL is not a fully defined solution and does demonstrate lot-to-lot variations (Hemeda et al., 2014; Jung et al., 2012). Its function is dependent on factors such as donor age and the heparin concentration used as well (Hemeda et al., 2013; Lohmann et al., 2012). The variations in pooled hPL are, however, less than what is seen for FBS and, in general, hPL outperforms FBS (Horn et al., 2010; Schallmoser & Strunk, 2013; Wuchter et al., 2014).

Since hPL is of human origin, concerns regarding transmission of zoonotic pathogens or immune reactions to bovine protein do not apply and hPL has been used to grow cells for clinical application without any side effects (Hemeda et al., 2014). There is, however, the same risk for transmission of infectious disease as there is for platelet transfusions (Burnouf, T. et al., 2016; Hemeda et al., 2014). Using pathogen-inactivated PC for hPL production may significantly reduce this risk, but the effects of pathogen inactivation on hPL function must then also be evaluated (Burnouf, T. et al., 2016; Kinzebach & Bieback, 2012; Shih & Burnouf, 2015). Also, immune reactions to plasma proteins and ABO antigens in pooled allogenic hPL products cannot be excluded (Moll et al., 2014; Shih & Burnouf, 2015).

The use of hPL to support mesenchymal stromal cells (MSC) in culture has gained special attention (Burnouf, T. et al., 2016). Expectations are placed on the potential use of MSC in regenerative medicine and cell therapy due to their nature (Hao et al., 2011). MSC have already been applied for treatment in numerous clinical trials (Glovinski et al., 2017; Hao et al., 2011). Defining how cells are to be handled before their use in cellular therapies is a critical task (Fekete et al., 2014; Jung et al., 2012; Kinzebach & Bieback, 2012). FBS has been commonly used in the past for MSC propagation in basic research but, as discussed above, is not suitable for use in clinical regenerative medicine (Jung et al., 2012). Doucet et al. (2005) published a seminal work in which hPL was deemed suitable as a safety substitute for FBS for MSC culture (Doucet et al., 2005). A series of publications that further explored this concept followed in the next decade (Burnouf, T. et al., 2016; Schallmoser et al., 2007, 2008). Now, hPL is generally considered the most promising alternative to FBS for MSC propagation (Burnouf, T. et al., 2016).

1.4.3 Preparation of platelet lysates

1.4.3.1 Production procedures

For hPL production, platelet lysis is generally achieved through a freeze-thaw step that is followed by centrifugation to remove debris (Figure 5).

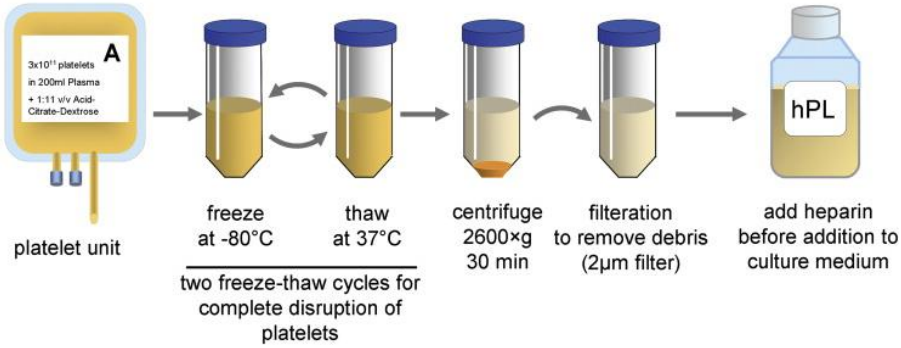

Figure 5. Platelet lysate production. Platelet lysates (hPL) are made by submitting platelet concentrates to a few freeze-thaw cycles to cause lysis. Centrifugation and filtration are subsequently applied for debris removal. Several different protocols have been published. The protocol of Hemeda et al. is illustrated here (Hemeda et al., 2014).

Still, published production procedures vary a great deal with regard to the number of freeze-thaw cycles, temperature, filtration steps, centrifugation, and heparin concentration (Astori et al., 2016; Shih & Burnouf, 2015). Repeating the freeze-thaw step two to five times seems to be a common practice, and freezing temperatures range from -196°C to -20°C. The most commonly reported freezing temperature is -80°C, while the thawing temperature is 37°C (Burnouf, T. et al., 2013, 2016; Schallmoser & Strunk, 2013). Centrifugation is performed one or two times at 600 x g to 16,000 x g at either room temperature or 4°C (Burnouf, T. et al., 2016; Schallmoser & Strunk, 2013). Most protocols centrifuge at 4,000 x g to 5,000 x g and filtration is not always applied (Altaie et al., 2016; Burnouf, T. et al., 2016). Since hPL contains fibrinogen and coagulation factors, the addition of heparin as an anticoagulant is necessary (Hemeda et al., 2014). However, heparin concentration should not exceed 0.61 IU/ml for unfractionated heparin or 0.024 mg/ml for low-molecular-weight heparin as it has been demonstrated to have negative effects on hPL performance (Altaie et al., 2016; Hemeda et al., 2013)

1.4.3.2 Autologous or allogenic

Human platelet lysate can be made both as autologous and allogenic (Gottipamula, S et al., 2013). The preparation is similar in both cases, except that autologous hPL is made with platelets from a single donor while allogenic hPL is frequently a pool from many donors (Burnouf, T. et al., 2016). The use of autologous hPL is ideal for the growth of a small number of cells meant for transplantation or for the direct application of hPL for wound healing, such as eye drops (Burnouf, T. et al., 2016; Pezzotta et al., 2017; van der Meer et al., 2016). Using autologous hPL circumvents the risk of transmitted disease or immune reaction since both the donor and the patient are the same individual (Burnouf, T. et al., 2016). Still, the hPL volume needed is a concern since the capability of the patient to provide enough for his/her own treatment cannot always be guaranteed (Bieback, 2013). Hence, it is unlikely that autologous hPL will suffice and using allogenic hPL will also be necessary (Bieback, 2013).

Using allogenic hPL requires the source material to be of high quality. Platelet concentrates (PC) prepared either with the PRP, BC, or apheresis method (see chapter 1.3.2.) have been used as a source material for pooled allogenic hPL. Apheresis PC are most commonly used followed by BC derived PC (Burnouf, T. et al., 2016). No significant differences in hPL performance based on whether it is made from apheresis-PC or BC-PC has been reported, and hPL from both types are used equally (Fekete, Gadelorge, et al., 2012). Generating a large pool of hPL from many PC increases the risk of contamination by blood-borne pathogens. The risk can be minimized by using PC, especially pathogen-inactivated PC, from accredited blood banks that meet international requirements for testing and processing of blood components (Shih & Burnouf, 2015). The quality control should include donor screening before blood collection, screening for infectious disease, leucofiltration, proper storage of components to reduce storage lesion, and a bacterial detection system as discussed above (Bieback et al., 2009; Shih & Burnouf, 2015).

1.4.3.3 Standardization and supply

Even though protocols for hPL preparation vary from each other, they generally result in hPL that effectively support cells in culture (Burnouf, T. et al., 2016). It is nonetheless important to reach international agreement on the production methods, quality testing, and safety criteria for GMP-compliant (good manufacturing practices) hPL. The guidelines should include quality

criteria for the PC used as source material and performance specifications for the hPL including parameters such as key growth factor concentrations, sterility, and endotoxins (Burnouf, T. et al., 2016; Schallmoser & Strunk, 2013).

Establishing such guidelines has proven challenging, especially since the optimal growth factor concentration has not yet been defined and the effects of blood group antigens, if any, still need to be evaluated in detail (Astori et al., 2016; Burnouf, T. et al., 2016). It is particularly relevant for clinical use (Moll et al., 2014). Platelets are either suspended in plasma, an additive solution, or both when preparing a PC (Gulliksson, 2003). Residual plasma may contaminate the PC with ABO antigens (Moll et al., 2014). Thus, some protocols use only platelets from blood group O donors suspended in blood group AB plasma (Altaie et al., 2016). However, most studies do not report on the blood type of the PC used to make hPL. Fortunately, no negative or adverse effects related to blood group antigens in hPL have been reported to date, so the risk is considered low (Burnouf, T. et al., 2016; Moll et al., 2014).

As has been discussed, PC manufactured according to blood bank standards would be the best source material for hPL. However, obtaining freshly donated platelets for such production at a large scale might be difficult. Blood banks are already struggling to recruit blood donors (Riley et al., 2007). Competition between blood banks and hPL providers for platelet donors might, therefore, generate a tension that would burden blood banks even further and act against the public interest. Nonetheless, as outlined in chapter 1.3, a significant number of PC expire annually (Dunbar, 2015). The expired PC may represent an ideal source material for hPL production, chiefly because they have fulfilled strict regulations, quality control, and screening that apply to the production of blood components for clinical use (Bieback, 2013). Their use also prevents rivalry over platelet donors between blood banks and hPL providers. Using such source material may, therefore, facilitate standardization of hPL production (Bieback, 2013). It is estimated that between 100,000 and 250,000 L can be generated annually from outdated PC, suggesting that hPL can be made at an industrial scale to supply cell research and clinical applications (Burnouf, T. et al., 2016). It is, therefore, important to demonstrate the suitability of hPL from expired PC in order to validate their use for cell culture and clinical use (Shih & Burnouf, 2015).

2 Aims

The overall aim of this thesis is to evaluate the suitability of expired platelets and expired pathogen-inactivated platelets as a source material for the formulation of human platelet lysate for expansion and differentiation of MSC and hES-MP.

Specific aims are:

I. To compare platelet lysates made from expired platelets and fresh platelets to fetal bovine serum for expansion and differentiation of MSC.

II. To compare platelet lysates from expired pathogen-inactivated platels with amotosalen treatment to platelet lysates from untreated expired platelets. Their effects on growth, immune function and differentitation of MSC was evaluated.

III. To evaluate if platelet lysate from expired platelets can be applied as a growth supplement for hES-MP cells to the same extent as fetal bovine serum.

IV. To compare the immune function of MSC and hES-MP cells when grown in platelet lysate or fetal bovine serum.

V. To evaluate the effects of differentiation medium containing platelet lysate from expired pathogen-inactivated platelets on osteogenic and chondrogenic differentiation of MSC and hES-MP.

3 Materials and methods

The main methods are briefly summarized here. For more detailed descriptions refer to Papers I-IV.

3.1 Preparation of platelet lysates

3.1.1 Platelet lysates

For all papers, platelet lysates (hPL) were prepared according to a commonly described methodology (Figure 5, Astori et al., 2016; Bieback, 2013; Schallmoser et al., 2007; Schallmoser & Strunk, 2013; Shih & Burnouf, 2015). Platelet concentrates (PC) were submitted to freeze-thaw cycles. Platelet depletion was then performed with a two-step centrifugation and microfiltration. All PC starting material was obtained from the Blood Bank (Landspitali University Hospital, Reykjavík, Iceland) and passed standardized quality control for platelet transfusion. The quality control includes donor screening with health questionnaire, screening for infectious disease, and testing for parameters such as the platelet count, pH, and PC weight. Two PC were used for preparation of each hPL lot, representing between two and 16 healthy blood donors behind each batch. Both PC obtained from buffy coats (BC-PC) and with apheresis were applied. Only PC that had exceeded the expiration date were used, except for Paper I where fresh PC were used for comparison.

In Paper I, hPL was made from both freshly obtained BC-PC and expired BC-PC. The starting material was stored frozen at -80°C and thawed at 37°C once before being centrifuged at 4975 x g for 20 min. The supernatant was then filtered through a cell strainer and a 0.45 µm vacuum filter (Millipore, Billerica, MA, USA). After a second centrifugation step the hPL was used for cell supplementation or stored frozen at -20°C.

Paper II examined what effects pathogen inactivation with the INTERCEPT™ system (Cerus Corporation, Concord, CA, USA) would have on hPL if it were applied to the PC starting material. BC-PC and pathogen-inactivated BC-PC, past the expiration date, were obtained for hPL production. Unlike Paper I, three freeze-thaw cycles were performed to achieve better platelet rupture and hence greater release of platelet factors into the solution. The lysate was centrifuged at 4975 x g for 20 min two times.

After the first step, the resulting platelet pellet was discarded, but the supernatant was submitted to a second centrifugation step. Filtration with 0.45 µm vacuum filter was performed after the second centrifugation, unlike in Paper I where it was performed between centrifugation steps. The hPL used in Papers III and IV was produced using the same protocol as for Paper II; however, the starting material was different. Both BC-PC and Apheresis PC were used, but no pathogen inactivation was applied. The reason for this is that the INTERCEPT™ system had not been implemented in the Blood Bank at the time of hPL preparation for Papers III and IV.

3.1.2 Characterization

In Paper III, five different batches of hPL were prepared and characterized for albumin and selected growth factor concentrations. Albumin is the most abundant protein within the circulation and acts as a carrier for other proteins. It is constantly and abundantly expressed, is stable, and has a long half-life (Sleep, 2015). PC contain up to 20% plasma and contain comparable levels of albumin as well. Here, albumin was adopted as a marker to assess variability between hPL batches. The concentrations of BMP-2, bFGF, VEGF, IGF, PDGF-BB, and TGFβ growth factors were also measured in the hPL batches (n=5). Their concentration was compared to concentrations found in FBS (n=3). These factors are known to be present in hPL and play a significant role in cell growth, especially bFGF and PDGF-BB (Altaie et al., 2016; Fekete, Gadelorge, et al., 2012). Evaluation of their concentration was performed to characterize further hPL prepared using the protocol described in Papers II-IV. Albumin was evaluated using the Human Albumin ELISA Quantitation Kit from Bethyl Laboratories (Montgomery, TX, USA), and TGFβ was measured using the Human TGF-beta1 Quantikine ELISA Kit (RnD Systems, Minneapolis, MN USA). Other factors were measured with appropriate Standard ELISA Development Kits from PeproTech (Rocky Hill, NJ, USA).

3.2 Cell culture

3.2.1 BM-MSC and hES-MP002.5

Mesenchymal stromal cells (MSC) isolated from bone marrow were used for experimentation in all papers (I-IV) and the hES-MP002.5 cell line (hES-MP) was used in Papers III and IV. Bone marrow-derived MSC have been studied intensely and remain the best characterized of MSC (Bianco, 2015). MSC from the bone marrow were thus used in the papers accompanying this

thesis. The hES-MP002.5 cell line was developed at Cellartis in Gothenburg, Sweden, (now Takara) and kindly donated for research purposes. The cells are termed mesenchymal progenitor cells and are derived from the hESC cell line SA002.5 (Karlsson et al., 2009). The BM-MSC were purchased from Lonza (Walkersville, MD, USA).

Cells were grown in tissue culture vessels with DMEM/F12+glutamax media (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (Gibco), and 4 IU/ml heparin (Leo Pharma A/S, Ballerup, Denmark). The cell culture media was supplemented with either hPL or FBS to the concentration of 10%. The culture surface was coated with 0.1% gelatin (Sigma, St. Louis, MO, USA) for hES-MP002.5 to facilitate adherence. Coating was not necessary for MSC. MSC from three human donors were used in all papers, except for Paper II where cells from two donors were used. The cells were used for experimentation before reaching passage 8. Only for long-term analysis of proliferation and immunophenotype were cells allowed to reach passage 10 (Paper III). Media were changed every two to three days and passaging was performed when the cultures reached 80% to 90% confluency.

3.2.2 Proliferation and immunophenotyping

Long-term proliferation was assessed with a population doubling (PD) assay over several passages (Papers I and III). The population-doubling assay estimates the number of times the cell population in the culture doubled over a single passage and is indicative for the proliferation rate. The population-doubling number is found using the logarithms of the number of cells seeded at the beginning of a passage and the number of cells retrieved at the end of a passage (Bieback et al., 2009). Adding the PD number of each passage to the PD of previous passages gives a cumulative number that can be used to observe the continuous proliferation rate over several passages and notice when the cells slow in proliferation or reach senescence (Greenwood et al., 2004). An XTT assay (ATCC, Manassas, VA, USA) was used to evaluate short-term proliferation over a few days (Paper II).

For immunophenotyping, the expression of CD29, CD45, CD73, CD90, CD105, and HLA-DR surface antigens was evaluated according to the ISCT minimal criteria for MSC (Dominici et al., 2006). In Paper III, CD10, CD13, CD44 and CD184 were analyzed in addition to previously stated markers. The presence of those markers on MSC surface has been described, but they are not part of the minimal criteria (Niehage et al., 2011). To estimate

fluctuations in surface antigen expression with time and between different supplements, the immunophenotype was analyzed repeatedly after 4, 6, and 10 passages in continuous culture (Paper III). All experiments were performed on FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using the CellQuestPro Software 4.0.2.

3.3 In vitro differentiation

Trilineage differentiation is the hallmark of MSC (Dominici et al., 2006). Osteogenic and chondrogenic differentiation were induced in Papers I-III. Adipogenic differentiation was performed in Papers I and II. The cells were first expanded in media containing different supplements for two to three passages. At the end of the expansion phase, in vitro differentiation was initiated. All cell cultures were treated with the same differentiation media to allow the evaluation of whether the different media supplements used for expansion affected the differentiation capacity of the cells.

3.3.1 Osteogenic differentiation

Cells were seeded at the density of 3000 cells/cm^2 and differentiated using the hMSC Differentiation BulletKit® Osteogenic Media (Lonza). The differentiation process was evaluated after seven, 14, and 21 days (Papers I and II) and after seven, 14, 21, and 28 days (Paper III).

Increased activity of alkaline phosphatase (ALP) is suggestive for active formation of osteoblasts in MSC cultures (Birmingham et al., 2012). The ALP activity was thus evaluated at different time points throughout the osteogenic phase by measuring the color change as p-nitrophenylphosphate is converted to p-nitrophenyl by ALP (Papers I-III). Mineralization is a vital part of bone formation. Depositions of minerals during osteogenesis were thus examined with von Kossa staining (Paper I), alizarin red staining (Paper III), and semi-quantified by dissolving the alizarin red staining with 10% cetylpyridinium chloride and measuring optical density at 562 nm (Paper III). Gene expression of the transcription factor *RUNX2* and its downstream targets *SPP1* (Paper I-III) and *ALP* (Paper I) was analyzed after seven and 21 days in Papers I and II and after seven, 14, 21, and 28 days in Paper III.

3.3.2 Chondrogenic differentiation

During early chondrogenesis, cellular condensation occurs as microspheres or aggregates form. This is followed by morphological changes, chondrocyte formation, upregulation of *SOX9* and fibril formation that strengthens the

chondrocytic structure (Yamashita et al., 2010). Attempting to imitate this process, chondrogenic differentiation was allowed to take place in a pellet culture. After initial expansion, 250,000 cells were seeded in sterile 1.5 ml microtubes and centrifuged at 150 x g for five min to generate a pellet. The tube lids were punctured with a needle to allow gas exchange and the pellets were placed in a cell culture incubator. The induction media used was hMSC Differentiation BulletKit® Chondrogenic media that was changed every two to three days. Care was taken to ensure that the pellets were loosely placed in the tubes and did not adhere to the walls. The chondrogenic formation was assessed after 14 and 28 days in Papers I and II and after 7, 14, 28, and 35 days in Paper III.

The tissue morphology was visualized with histological stainings. The pellets were fixed in paraformaldehyde, embedded in paraffin, sectioned and stained with toluidine blue (Paper I), hematoxylin and eosin (Paper III), and Masson's trichrome stain (Paper III). Concentration of the ECM components glycosaminoglycans (GAG) was measured with the Blyscan assay (Biocolor, Carrickfergus, UK) after digesting the pellets in papain extraction reagent (Papers II and III). Gene expression of the transcription factor *SOX9* was evaluated after 14 and 28 days in Paper II and after 7, 14, 28, and 35 days in Paper III.

3.3.3 Adipogenic differentiation

The StemPro® Adipogenesis Differentiation media was used for adipogenic differentiation. Cells were seeded at the density of 10,000 cells/cm^2 and differentiated for 14 days. To demonstrate the accumulation of lipid droplets within the cells, an Oil Red O staining was used (Paper I) and the gene expression of *ADIPOQ*, a downstream target of *PPARG,* was evaluated at the end of the differentiation phase (Paper II).

3.4 Immunomodulatory activity

The immune function of the MSC and hES-MP cells was evaluated in co-cultures with peripheral blood mononuclear cells (PBMC, Papers I, II, and IV). PBMC were isolated from buffy coat blood using density gradient centrifugation, stimulated with the mitogen phytohemagglutinin (PHA) and grown in the presence of stromal cells (only MSC in Papers I and II but both MSC and hES-MP were used in Paper IV). The cells remained in culture for 48 hours (Paper I) or 72 hours (Papers II and IV), after which the PBMC proliferation was analyzed using an XTT assay (ATCC). PHA-stimulated PBMCs were grown both with and without stromal cells and their proliferation

compared to see whether the stromal cells could suppress the PBMC proliferation. Transwell culture system was applied in Paper I using a semi-permeable membrane to separate PBMCs from MSC. However, a direct system was applied in Paper II where all cell types were allowed to remain in direct contact. Paper IV assessed both direct and Transwell co-culture systems.

The immune function of MSC and hES-MP when expanded using different supplements to enrich the culture media was further assessed in Paper IV. At the end of the 72-hour co-cultures, the PBMCs were carefully removed and stained with CD3, CD8, CD4, CD14, and CD19 antibodies. The cells were then analyzed using a flow cytometer to examine individual PBMC populations. The results were compared to similar findings for the original PBMC population before co-culture. Spent media were also collected at the end of the 72-hour co-cultures and the concentration of inflammatory cytokines evaluated with a Standard ELISA Development Kit (PeproTech). Together, these experiments were used to shed light on the immunomodulatory effects MSC and hES-MP have on PMBC proliferation, PBMC sub-populations, and cytokine secretion.

3.5 Molecular biology

Expression of selected genetic markers was evaluated to monitor in vitro differentiation in Papers I-III. Depending on the tissue being studied, genes of interest were *RUNX2*, *SPP1* (Papers II and III) and *ALP* (Paper I) for bone, *SOX9* for cartilage (Papers II and III) and *ADIPOQ* for fat (Paper II).

For Paper I, RNA isolation, reverse transcription, and real-time quantitative PCR (qPCR) were performed by TATAA Biocenter, Gothenburg, Sweden. RNA isolation for Paper II was done using the RNeasy® Mini Kit (Qiagen, Hamburg, Germany) after homogenizing the samples in RLT buffer with 1% β-mercaptoethanol. In Paper III, samples were homogenized and RNA isolated using TRIzol® Reagent (Thermo Scientific). RNA clean-up was subsequently performed using RNeasy® Plus Mini Kit (Qiagen). The RNA quantity and quality was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). GeneAmp® RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA) was used to achieve reverse transcription (Papers II and III), and real-time qPCR performed in StepOne™ Real-Time PCR system (Thermo Scientific). Solely TaqMan® Gene Expression Assays (primers) were used (Papers II and III). *TBP* and *YWHAZ* served as reference , and all experiments were normalized to two reference genes. The reference genes were selected based on a screening of several genes to find the most stable ones for the cell types being tested.

3.6 Statistics

Data were analyzed using the GraphPad® version 5.0 (GraphPad Software, La Jolla, CA, USA) and Microsoft Office Excel 2013. Student's T-test was used for the direct comparison between treatment and control groups. Furthermore, One-way ANOVA and Two-way ANOVA were performed where appropriate, followed by Bonferroni correction. Relative gene expression was analyzed in REST-384© version 2 using a pair-wise fixed reallocation randomization test (Pfaffl et al., 2002). $P < 0.05$ was considered statistically significant.

4 Results and Discussion

This thesis is composed of two published papers (Papers I and II), two submitted manuscripts (Papers III and IV), and unpublished findings (Chapter 4.5). In all the papers, hPL was used to expand MSC or hES-MP and the effects on cellular growth, immunobiology, and tissue differentiation evaluated. In this chapter, the main results of each paper are summarized and discussed as well as the unpublished findings that further add to the results of the individual papers.

The first paper evaluates whether outdated platelet concentrates are a suitable starting material for hPL when compared to freshly obtained hPL and FBS. The second paper compares hPL made from either outdated PC or outdated pathogen-inactivated PC. In the third paper, the applicability of using hPL to support the expansion of hES-MP is assessed, and the fourth paper compares the immune function of MSC and hES-MP when grown with hPL. Unpublished findings on the use of hPL made from pathogen-inactivated platelets to support both expansion and differentiation of MSC and hES-MP are included here as well.

4.1 Paper I — Suitability of outdated platelets to make hPL

The use of hPL for expansion of cells in vitro is promising but remains to be standardized (Shih & Burnouf, 2015). As has been discussed, the starting material needs to be of high quality and obtainable in sufficient quantity to allow large-scale hPL production (Bieback, 2013; Schallmoser & Strunk, 2013). Platelets can be obtained from blood banks, which produce and store platelet concentrates for transfusion. Blood banks are, however, faced with an imminent donor shortage, and ensuring adequate platelet production is a growing struggle (Riley et al., 2007). There is no surplus available to others so relying only on freshly donated platelets for hPL production would lead to competition for platelet donors between blood banks and hPL providers. Recruiting donors would thus become harder for blood banks and ensuring the availability of platelets for transfusion more challenging. Such tension would act against the public interest. Other factors are also important in this context, namely the short-shelf life of platelets and unpredictable demand (Gulliksson, 2003; Williamson & Devine, 2013). Keeping a platelet inventory balance is hard, and as a result, a considerable amount of platelets expire annually (Dunbar, 2015).

Expired platelets are not transfused and are generally discarded. However, at the time of expiration, the platelets may still contain the desired mitogens needed to support cellular growth in the form of hPL (Dzieciatkowska et al., 2015; Slichter et al., 2014). Whether storing platelets until their expiration date affects their cell-promoting abilities had not been explored before the publication of Paper I of this thesis (Burnouf, T. et al., 2014). In this paper, we examined the suitability of expired platelets for hPL production and compared them to hPL made from freshly donated platelets and FBS. Others at the time suggested the potential use of expired platelets, but a direct comparison between fresh and expired platelets was still lacking (Bieback et al., 2009; Crespo-Diaz et al., 2011; Griffiths et al., 2013; Rauch et al., 2011b). Since Paper I was published, the findings have been recognized by other groups and cited more than 40 times ("Google Scholar," 2017). Transition toward using expired material has also taken place with an increasing number of studies and hPL providers turning toward expired platelets to make lysates (Burnouf, T. et al., 2014; Glovinski et al., 2017; Luzzani et al., 2015; Shih & Burnouf, 2015).

In our study, bone marrow-derived MSC from three human donors were grown using hPL made from outdated platelets (hPLO), hPL made from freshly donated platelets (hPLF) or FBS. The expression of surface markers, cell proliferation, morphology, trilineage differentiation, and immunomodulation was then evaluated and compared.

No difference in the immunophenotype of MSC or their capacity for trilineage differentiation was observed in relation to whether the MSC had been grown using FBS, hPLF, or hPLO. The obtained results were in compliance with the ISCT minimal criteria for MSC (Dominici et al., 2006). MSC from all supplements also successfully suppressed PBMC proliferation, as has been shown by others (Bernardo et al., 2007; Flemming et al., 2011; Najar et al., 2016). The PBMC proliferation was reduced by 29%-48% on average ($p \leq 0.05$) with no differences found between culture supplements. Using hPL, therefore, neither enhanced nor reduced the immune suppression exerted by MSC, compared to FBS. Furthermore, hPL from fresh and expired platelets were comparable, indicating that one is not superior to the other in this regard.

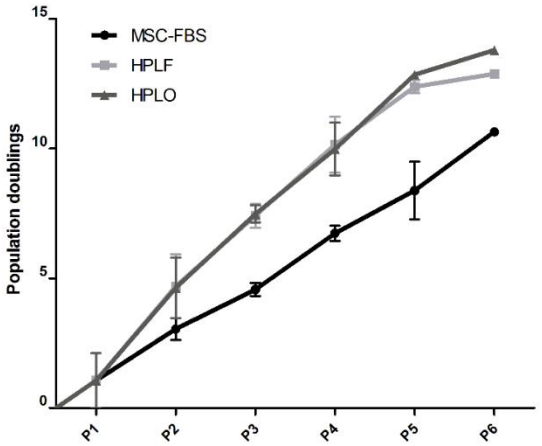

Figure 6. MSC proliferation in hPL and FBS. MSC proliferation over six passages when grown using either hPLO, hPLF or FBS (Jonsdottir-Buch et al., 2013)

Regarding MSC proliferation, hPL was found to be superior to FBS resulting in a significantly higher cell number obtained from hPL supplemented cultures over a period of six passages (Figure 6). MSC from hPL doubled in number four times as often as MSC from FBS (4.2 ± 0.7 CPD, $p \leq 0.05$) and a faster proliferation was evident from passage 2. This is in agreement with previous findings where accelerated MSC growth in hPL has been widely documented (Bieback et al., 2009; Burnouf, T. et al., 2014; Doucet et al., 2005; Griffiths et al., 2013). This held true for MSC from both hPLF and hPLO, which exhibited very similar growth kinetics throughout the study period, where MSC from hPLO doubled in number 13.77 ± 0.77 times and MSC from hPLF 12.77 ± 0.12 times. Similar observations have recently been reported by others and confirm our findings (Glovinski et al., 2017).

The accelerated proliferation may reflect a difference in growth factor concentration between hPL and FBS for factors such as PDGF-BB and bFGF that have known importance for cellular growth (Fekete, Gadelorge, et al., 2012; Fekete et al., 2014). It must also not be ignored that the growth factors from hPL and FBS do not originate from the same species and might therefore not interact with cellular receptors in an identical manner. Although fast MSC proliferation is desirable for large-scale and rapid MSC production, the cells may enter senescence sooner than their counterparts in FBS and care needs to be taken to ensure that the speed does not induce chromosomal aberrations (Crespo-Diaz et al., 2011; Hemeda et al., 2014; Schallmoser et al., 2010). Using a lower percentage of hPL for

supplementation is one way to control the proliferation, and numerous studies have adopted the use of 5% hPL instead of 10% which is the concentration used in this thesis and is commonly used for FBS (Burnouf, T. et al., 2014; Fekete et al., 2014). The results here do, however, signify that both expired platelets and fresh platelets can be applied for hPL production while still ensuring adequate growth support.

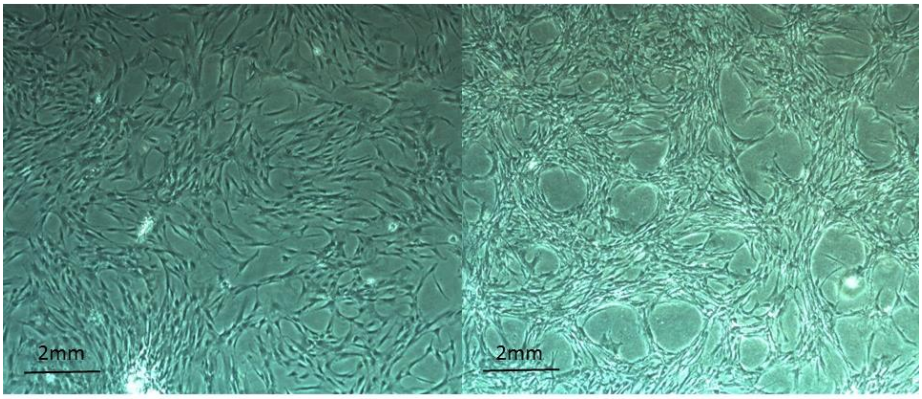

Figure 7. MSC morphology in FBS and hPL. Different MSC morphology was observed between cells grown in FBS (left) and hPLO (right). Cells growing in hPLO appear elongated and dense compared to cells in FBS and grow in a manner where circular cell-free areas appear during growth. The cells here are at passage 4, four days post seeding.

In parallel with the rapid growth of MSC in hPLF and hPLO, morphological changes were seen. While MSC grown in hPL still had characteristic fibroblast morphology similar to MSC grown with FBS, the cells seemed more elongated and dense. Their growth behavior differed as well since they grew in a circular fashion leaving circular spaces between them rather than covering the culture surface in an even manner as they became confluent (Figure 7). This morphology was observed consistently in all papers of the thesis but has not been noticed to markedly affect the MSC behavior, neither in the presented work nor the work of others (Gottipamula, Sanjay et al., 2012; Griffiths et al., 2013). Why the cells adopt this morphology remains speculative, but it may be due to factors present in hPL but not in FBS, such as serotonin. Platelets store serotonin in their dense granules (Zufferey et al., 2014). It is a known vasoconstrictor and has been linked to morphological changes of cells such as endothelial cells and fibroblasts during cellular growth (Boswell et al., 1992). It is known to interact directly with the cytoskeleton and influence actin polymerization and microtubule formation

(Azmitia, 2001). Whether the morphology in either FBS or hPL reflects how MSC appear within the body is impossible to know, since MSC most surely adopt a different shape when removed from their natural surroundings (Kfoury & Scadden, 2015). The difference between MSC in FBS and hPL is nonetheless noticeable and the cause unknown.

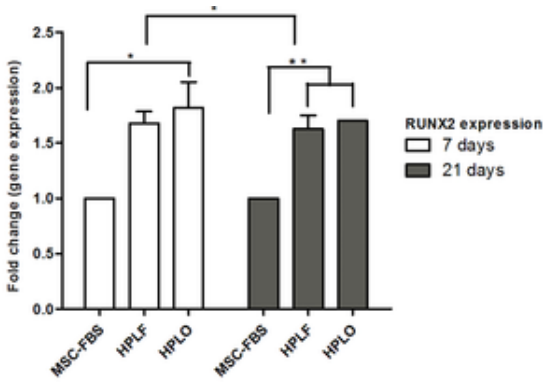

Figure 8. *RUNX2* expression in MSC from FBS, hPLF, and hPLO. MSC grown in hPLO expressed *RUNX2* significantly more than cells in FBS at both time points (p≤0.05 and p≤0.01 after 7 and 21 days). MSC grown in hPLF had higher *RUNX2* expression than cells in FBS after 21 days (p≤0.01). No difference in *RUNX2* expression was found between cells in hPLO and hPLF. *= p≤0.05, **=p≤0.01. (Jonsdottir-Buch et al., 2013)**.**

Following expansion for three passages in either FBS, hPLF, or hPLO, trilineage differentiation was induced. Osteogenic differentiation was evaluated in terms of alkaline phosphatase (ALP) activity after seven and 14 days and by evaluating expression of *RUNX2, SPP1,* and *ALP* after seven and 21 days. The ALP activity increased with time for MSC from all treatments as expected since an increase in ALP activity during MSC differentiation correlates with the formation of osteoblasts and hence adequate differentiation (Birmingham et al., 2012). Still, the genetic expression of *ALP* remained constant with time and comparable between treatments. An increase in the activity of the ALP protein, despite constant genetic expression, has been described for other cell types and linked to post-transcriptional regulation (Mikami et al., 2009). Kiledjian and Kadesch (1991) compared the *ALP* expression of Saos-2 osteoblast-derived cells and HepG2 hepatoblastoma cells and found that the cells had similar *ALP* expression despite an evident difference in ALP activity. Based on their

findings they concluded that ALP is under post-transcriptional control most likely due to the presence of destabilizing sequences in the *ALP* mRNA (Kiledjian & Kadesch, 1991). *ALP* expression is therefore not an accurate indicator for the activity of its protein product and osteoblast formation, since the enzymatic activity does not always correlate with the genetic expression.

Expression of *RUNX2* and *SPP1,* on the other hand, followed previously described expression patterns associated with osteogenesis. *RUNX2* was upregulated early and then decreased while the opposite was observed for *SPP1* ($p \leq 0.01$). *SPP1* was expressed in a similar manner between treatment groups, unlike what was seen for *RUNX2. RUNX2* expression was markedly higher in MSC from hPLO when compared to hPLF and FBS at day 7 ($p \leq 0.05$) and from both types of hPL compared to FBS at day 21 ($p \leq 0.01$, Figure 8). *RUNX2* expression is vital for successful osteogenesis, and strong early upregulation is associated with a stringent commitment toward the osteoblast lineage (Franceschi et al., 2007). Under osteogenic stimulation, the expression is upregulated and triggers processes that upregulate other osteogenic genes downstream of *RUNX2,* such as *SPP1* (Freeman et al., 2016; Long, 2011). The results here indicate enhanced lineage commitment toward osteoblasts after expansion in hPL as compared to FBS. Still, during expansion, osteogenic marker genes were not upregulated or differently expressed between treatment groups. That suggests that the MSC were not primed toward a single lineage before differentiation but when under osteogenic stimuli can upregulate *RUNX2* to a further extent than MSC from FBS. This difference in *RUNX2* expression between MSC from FBS and hPL was consistently observed throughout this thesis.

However, no difference in *RUNX2* expression was seen between MSC expanded in hPLO and hPLF. In fact, no significant differences between hPLO and hPLF were found in this study when evaluating surface markers, cell proliferation, morphology, trilineage differentiation, and immunomodulation. MSC grown in both hPLO and hPLF demonstrated enhanced proliferation and stronger lineage commitment compared to MSC from FBS. The reasons for the differences seen are not clear but are in all likelihood due to the differences in composition (Hemeda et al., 2014). Taken together, the results confirm that expired platelets are suitable as a source material for hPL production intended for in vitro expansion of MSC and that hPLO, like hPLF, supports rapid MSC growth and commitment toward osteogenesis more than FBS. Therefore, expired platelet concentrates should be collected from blood banks for hPL production instead of competing with blood banks for fresh platelets for the same purpose.

4.2 Paper II — Effects of pathogen inactivation on hPL quality

Storage time for platelet concentrates ranges from five to seven days (Gulliksson, 2003). The main reason for this brief shelf-life is the increased risk of bacterial contamination that far outweighs the threat of viral infections (Palavecino et al., 2006). Transfusion-associated sepsis due to bacterial contamination is the cause of death in up to 20% of all transfusion-related deaths; therefore special care is taken to limit the risk of infection (Müller et al., 2015; Walther-Wenke, 2008). The storage conditions for platelets proves problematic in this context since platelets are stored at 22°C in additive solutions which contain glucose (Gulliksson, 2014). The storage conditions are tailored to reduce the platelet storage lesion, but a small number of transient bacteria can reach a life-threatening concentration in five to seven days under these same conditions (Vasconcelos & Seghatchian, 2004). Bacterial screening programs are a requirement for blood banks (Palavecino et al., 2006). They have, however, been associated with low sensitivity for early screening and long turnaround time for routine screening (Brecher et al., 2013; Murphy, W. G. et al., 2008). Preventive measures should, therefore, also be practiced and pathogen inactivation technologies have been emerging to limit this risk and increase the storage time (Schlenke, 2014).

The Blood Bank of Iceland has implemented and validated the INTERCEPT™ Blood System for platelet concentrates to pathogen-inactivate different types of pathogens, including bacteria, viruses, and parasites. The system is based on the chemical crosslinking between amotosalen and nucleic acids when illuminated with UV-A (Irsch & Lin, 2011; Schlenke, 2014). The potential benefits of using virally or pathogen-inactivated platelets for hPL preparation has been reflected on in the past (Bieback, 2013; Burnouf, T. et al., 2014). However, only a few publications exist that evaluate their suitability for that purpose (Burnouf, P. et al., 2010; Shih et al., 2011).

To our knowledge, only one other group has studied the use of amotosalen treated platelets for hPL production. Their first paper was published during the review process of Paper II of this thesis (Iudicone et al., 2014). The study reported in Paper II was thus carried out without any knowledge of the study by Iudicone et al. Both studies compared hPL from pathogen-inactivated platelets to hPL from untreated platelets. The findings of Iudicone et al. (2014) are consistent with our results. Still, the starting material was different since Iudicone et al. (2014) collected fresh platelets from volunteers while our group focused on the use of expired pathogen-inactivated platelets. Paper II thus remains the only paper that discusses the

potential of using pathogen-inactivated platelets for hPL preparation after their expiration.

Here, we examined the effects of pathogen inactivation (PI) on hPL (hPL-PI) and compared them to hPL made from expired untreated platelets (hPL-EX). Bone marrow-derived MSC from two separate donors were grown in both types of hPL, and the expression of surface markers, cell proliferation, trilineage differentiation, and immunomodulation was then evaluated and compared.

MSC grown in hPL-EX and hPL-PI exhibited comparable proliferation, expression of surface antigens and significantly inhibited the proliferation of mixed PHA-stimulated PBMCs compared to the control ($p \leq 0.001$,Figure 9). MSC grown in hPL-PI were, however, significantly more suppressive than MSC grown in hPL-EX ($p \leq 0.01$). The difference between the treatments was 6.16% ± 1.16%, where MSC in hPL-PI demonstrated 20.25% ± 0.98% suppression, compared to the 14.09 ± 1.31% suppression by MSC in hPL-EX (Figure 9). These results are consistent with Iudicone et al. (2014) where the PBMC suppression ranged from 12% to 25%, but the difference between hPL-PI and hPLF did not meet statistical significance as was shown here. Fazzina et al. (2016) further demonstrated adequate PBMC suppression by MSC in hPL-PI but no comparison to hPLF was made (Fazzina, Iudicone, Fioravanti, et al., 2016).

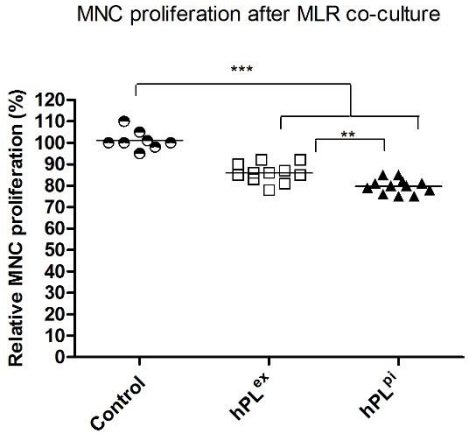

Figure 9. PBMC proliferation after MSC co-culture. MSC grown in both hPL-EX and hPL-PI successfully reduced PBMC (MNC) proliferation after PHA stimulation compared to the control. MSC from hPL-PI demonstrated greater immune suppression than MSC from hPL-EX (Jonsdottir-Buch et al., 2015). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Observing greater immunosuppression in hPL-PI is not surprising since the PI process inactivates residual leukocytes resulting in a lower concentration of leukocyte-derived cytokines. Despite leukofiltration, all platelet concentrates can be expected to contain residual leukocytes to a small extent (Fast et al., 2011). During storage, these cells are metabolically active and secrete inflammatory factors, cytokines, and chemokines that build up in the concentrate over time (Jackman et al., 2013; Vetlesen et al., 2013).

Pathogen inactivation with amotosalen does not discriminate between eukaryotic or prokaryotic cells, viruses, or other structures containing nucleic acids (Irsch & Lin, 2011). Leukocytes are thus inactivated as well. Since activation with amotosalen causes DNA and RNA crosslinking to occur every 84 base pairs, it is likely not only to inhibit replication but may also hinder transcription (Irsch & Lin, 2011). In that case, pathogen-inactivated leukocytes would secrete lower levels of inflammatory molecules than untreated leukocytes resulting in lower concentrations in the treated platelet concentrate. Amotosalen treated leukocytes have already been demonstrated to have lower secretion of inflammatory cytokines and do not contribute to immune responses to the same extent as untreated leukocytes (Fast et al., 2011; Vetlesen et al., 2013). Their failure to cause post-transfusion alloimmunization has also been demonstrated in mice (Jackman et al., 2013). Lower levels of inflammatory molecules in hPL-PI could explain the difference observed in PBMC suppression compared to hPL-EX since hPL-PI would be expected to be less immunogenic than hPL-EX and hence better support MSC immune suppression.

The level of endotoxins is another possible explanation for better hPL-PI immune suppression. Since pathogen inactivation greatly reduces bacterial proliferation, less buildup of endotoxins can be expected compared to untreated platelet concentrates. Although bacteria should not be present at all in platelet concentrates, the presence of bacterially derived endotoxins can nonetheless not be excluded (Blajchman et al., 2008). Endotoxins are notoriously inflammatory and also affect the MSC immune response (Lieder, Gaware, et al., 2013; Lieder, Petersen, et al., 2013). It is crucial to keep endotoxins at bay which can at least partially be achieved with pathogen inactivation before platelet storage. (Blajchman et al., 2008).

Osteogenic differentiation was monitored over 21 days. The ALP activity increased progressively throughout differentiation, with no differences observed between MSC in hPL-EX and hPL-PI. Furthermore, no difference in *RUNX2* expression between groups was observed (Figure 10a). The expression did not vary with time. *SPP1* expression, on the other hand, increased with time and was significantly upregulated at 21 days compared to day seven ($p \leq 0.001$, Figure 10b). A 1.69 ± 0.33-fold difference in *SPP1* expression was seen after 21 days where cells from hPL-PI had higher expression ($p \geq 0.05$, not significant). The opposite had been seen after seven days where cells from hPL-EX had significantly higher *SPP1* expression (0.47 ± 0.30-fold difference, $p \leq 0.001$). The *SPP1* expression in MSC from hPL-PI thus increased to greater extent than in MSC from hPL-EX (3.19 ± 0.32-fold increase). *SPP1* is a downstream target of *RUNX2* and upregulated at later stages of osteogenesis, the increase seen here with time is thus in agreement with previous findings (Franceschi et al., 2007).

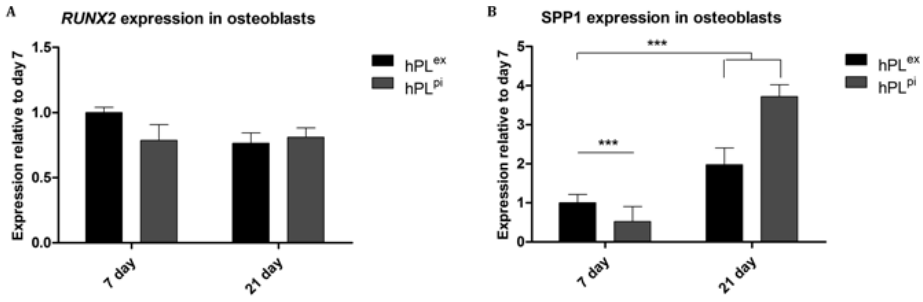

Figure 10. Osteogenic gene expression of MSC-derived osteoblasts

A) *RUNX2* expression did not increase with time during osteogenic differentiation and no difference was observed between MSC from hPL-EX and hPL-PI B) Expression of *SPP1* increased with time for MSC from both hPL-EX and hPL-PI. Difference in *SPP1* between MSC from hPL-EX and hPL-PI was detected after seven days but this difference had disappeared by day 21 of osteogenic differentiation. *=p≤0.05, **=p≤0.01, ***=p≤0.001 (Jonsdottir-Buch et al., 2015).

To further asses the commitment of MSC toward osteogenesis, genetic markers for other lineages were also evaluated. *SOX9* was chosen to represent chondrogenesis and *ADIPOQ* for adipogenesis. No differences in *RUNX2, SOX9,* or *ADIPOQ* expression between hPL-EX and hPL-PI were observed (Figure 11). MSC submitted to osteogenesis expressed *RUNX2* markedly more than *SOX9* and *ADIPOQ* at day seven ($p \leq 0.001$). This early

upregulation of *RUNX2* under osteogenic conditions is beyond what was seen for markers of other lineages and points toward true commitment toward osteogenesis and hence successful in vitro differentiation.

The differences in *RUNX2* and *SOX9* expression were obscure after 21 days (Figure 11b). The *SOX9* expression was examined relative to the *RUNX2* expression and the results observed here are most likely due to the downregulation of *RUNX2* with time rather than upregulation of *SOX9.* As genes involved in osteoblast formation, such as *SPP1* and *SP7*, are upregulated, *RUNX2* is commonly downregulated (Franceschi et al., 2007; Long, 2011). Hence, the relative difference between *RUNX2* and *SOX9* would appear to be less than at earlier stages of differentiation. The minimal expression of *ADIPOQ* relative to *RUNX2* further supports this and points toward active Wnt signaling necessary to support osteogenesis (Li, J. et al., 2016).

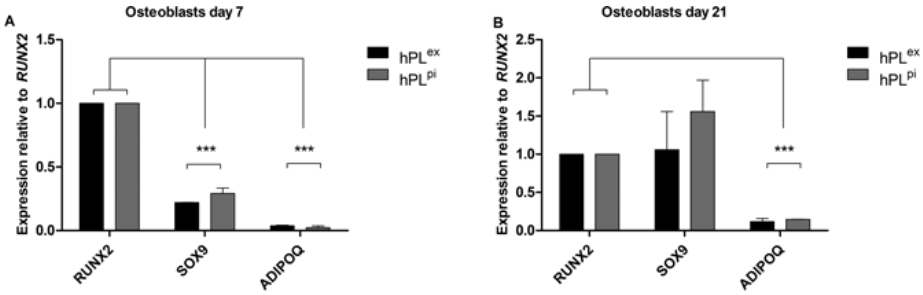

Figure 11. Lineage-specific gene expression in MSC-derived osteoblasts. A) *RUNX2* expression was significantly stronger in osteoblasts at day seven when compared to genetic markers of other lineages (*SOX9* for chondrocytes and *ADIPOQ* for adipocytes). This was seen for both hPL-EX and hPL-PI. B) After 28 days of differentiation, *RUNX2* was still more strongly expressed than *ADIPOQ,* but no difference between *RUNX2* and *SOX9* was detected. The same observations were found in hPL-EX and hPL-PI (Jonsdottir-Buch et al., 2015). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Chondrogenesis was evaluated in terms of the ECM components glycosaminoglycans (GAG) and by assessing the expression of *SOX9.* In the first 14 days of chondrogenic differentiation, the GAG concentration increased significantly (p≤0.001) but then decreased (p≤0.05, Figure 12). Significantly higher levels of GAG were observed for MSC from hPL-PI than from hPL-EX throughout the process (p≤0.05). The accumulation of ECM components, first GAG and then increasingly collagen, is commonly

observed during early chondrogenesis, but at later stages, before ossification, the cells reach hypertrophy and the matrix is degraded (Chen, W.-H. et al., 2009; Yamashita et al., 2010). The initial increase and then decrease in GAG seen here correlates with previous evidence and suggests that later stages of chondrogenic differentiation were reached (Figure 12). The time-dependent increase in *SOX9* expression further supports this (6.83 ± 0.22-fold and 6.12 ± 0.20-fold increase for hPL-EX and hPL-PI respectively, p≤0.001).

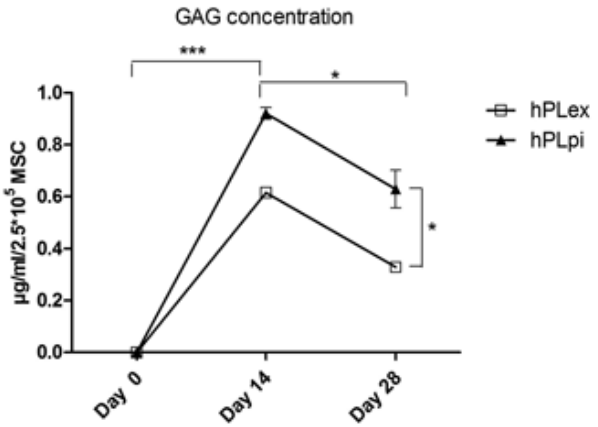

Figure 12. GAG concentration. Concentration of glycosaminoglycans (GAG) increased until day 14 after which a decrease was seen. MSC from hPL-PI had significantly higher GAG concentration than MSC from hPL-EX at all time points (Jonsdottir-Buch et al., 2015). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

As for the osteogenesis, genetic markers for the three lineages of MSC were examined for MSC undergoing chondrogenesis (Figure 13). At day 14, *RUNX2* expression in MSC from hPL-EX under chondrogenic stimulus was significantly lower relative to the *SOX9* expression as expected (p≤0.05). The *RUNX2* expression in MSC from hPL-PI was, however, higher than the *SOX9* expression (p≤0.01). A significant difference between hPL-EX and hPL-PI regarding *RUNX2* expression was furthermore detected (p≤0.05). It is known that osteochondroprogenitor cells are bipotent and express both *RUNX2* and *SOX9* (Akiyama & Lefebvre, 2011; Ducy et al., 1997; Freeman et al., 2016). For osteogenesis, *SOX9* is then downregulated while *RUNX2* and other osteogenic genes are expressed (Akiyama & Lefebvre, 2011). For chondrogenesis, *SOX9* upregulation happens as lineage commitment takes place (Dancer et al., 2010). Later upregulation of *RUNX2* during

chondrogenesis induces a shift toward ossification (Liu, C.-F. et al., 2016; Nishimura et al., 2012). By day 28, that same expression pattern was observed for both hPL-EX and hPL-PI as *RUNX2* was expressed to a significantly lower extent than *SOX9* (p≤0.001), as would be expected during chondrogenic lineage commitment.

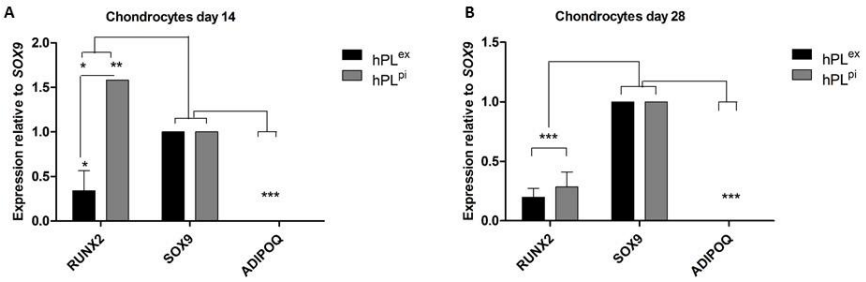

Figure 13. Lineage-specific gene expression in MSC-derived chondrocytes. C) After 14 days of chondrogenic differentiation, MSC from hPL-EX expressed significantly lower levels of *RUNX2* relative to *SOX9.* The opposite was observed for hPL-PI where *RUNX2* levels were significantly higher than *SOX9*. A difference in *RUNX2* expression between hPL-EX and hPL-PI was also observed D) After 28 days of chondrogenic differentiation, the *SOX9* expression was significantly stronger than the expression of genetic markers for osteogenesis (*RUNX2*) and adipogenesis (*ADIPOQ*) (Jonsdottir-Buch et al., 2015). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The results observed at day 14 are nonetheless hard to interpret (Figure 13). For hPL-EX, the low expression of *RUNX2* relative to *SOX9* demonstrates upregulation in compliance with chondrogenic lineage commitment. The high *RUNX2* expression relative to *SOX9* seen for hPL-PI does, however, stand out and implies that osteochondroprogenitor cells are more numerous in the hPL-PI-treated MSC cultures than the hPL-EX-treated cultures. More chondroblasts would correspondingly be expected to be found in the hPL-EX group. These differences had disappeared by day 28, and *SOX9* expression was significantly stronger than expression of markers for other lineages. So, if chondrogenesis proceeded at a slower pace in MSC from hPL-PI early in the process, a balance had been reached by day 28, and cells from both hPL types were committed toward chondrogenesis.

Adipogenic differentiation took place over 14 days, after which the expression of *ADIPOQ* was evaluated. No difference in *ADIPOQ* expression

was seen between MSC from hPL-EX and hPL-PI. The *ADIPOQ* expression was furthermore significantly higher in the developing adipocytes than *RUNX2* or *SOX9,* supporting the occurrence of adequate adipogenesis.

Trilineage differentiation for MSC expanded in hPL-PI was demonstrated by Iudicone et al. (2014) and Fazzina et al. (2016) using histological stains. Lineage-specific gene expression was not assessed. The results presented here are the first on MSC gene expression after being treated with hPL-PI. All other aspects evaluated—antigen expression, immunosuppression, and proliferation—were in agreement with previous reports (Fazzina, Iudicone, Fioravanti, et al., 2016; Iudicone et al., 2014). MSC characteristics were either comparable or enhanced after expansion in hPL-PI compared to hPL-EX. The evidence presented here, therefore, suggests that pathogen inactivation of platelets with amotosalen does not negatively affect their potential to be used as raw material for hPL production, even after their expiration.

4.3 Paper III — Using hPL to support hES-MP expansion

A growing body of literature is focused on how the advantages of MSC can be efficiently exploited and protocols standardized for large-scale expansion (Escacena et al., 2015; Sharma et al., 2014). To be feasible for use in practice, the cells need to be easily obtainable and expandable to create therapeutic doses efficiently (Fekete, Rojewski, et al., 2012; Warnke et al., 2013). Their potency and safety need to be guaranteed, whichever they are, allogenic or autologous (Murphy, M. B. et al., 2013). The substantial variability in MSC observed between donors, and even tissue sources, complicates their use and standardization (Escacena et al., 2015; Sharma et al., 2014).

Development of cell lines that resemble primary MSC has been pursued by several groups (Barberi et al., 2005; Brown et al., 2014; Luzzani & Miriuka, 2016; Olivier et al., 2006; Trivedi & Hematti, 2008). The cell lines are often derived from embryonic stem cells (ESC) and are intended as standardized and reproducible alternatives to primary MSC (Luzzani & Miriuka, 2016; Olivier et al., 2006). Using ESC, however, requires the use of animal-derived feeder cells in the derivation process (de Peppo & Marolt, 2012). As previously discussed, the use of animal-derived components is undesirable for regenerative medicine.

Human embryonic-derived mesenchymal progenitor cells (hES-MP) can be derived from ESC lines using completely xenofree methods (Karlsson et

al., 2009). These cells have been shown to possess similar abilities as MSC with regard to proliferation, immunophenotype, and trilineage differentiation. Furthermore, they lack expression of ESC markers and fail to form teratomas after in vivo transplantation (de Peppo et al., 2013). Several studies have evaluated their resemblance to MSC, but the majority of the studies use FBS as a culture supplement (de Peppo, Sjovall, et al., 2010; de Peppo et al., 2013; de Peppo, Svensson, et al., 2010; Karlsson et al., 2009; Li, O. et al., 2013). The suitability of using hPL for hES-MP propagation has not previously been evaluated. Using hPL to support hES-MP, derived under animal-free settings, could be a step toward establishing an MSC-like cell line that does not require any animal components and generates more reproducible results than primary MSC. In Paper III, we therefore evaluated for the first time whether hPL is as suitable for hES-MP expansion as FBS.

The proliferation of hES-MP was evaluated over the course of 10 passages and compared to the proliferation of bone marrow-derived MSC. The cells were grown in either FBS or hPL.

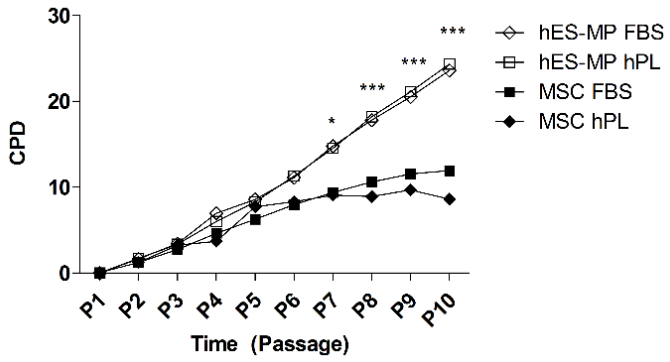

Figure 14. hES-MP and MSC proliferation. hES-MP from both hPL and FBS exhibited more rapid growth over 10 passages than MSC. hES-MP and MSC proliferated at a similar rate until passage 6 after which hES-MP continued at the same rate but MSC proliferation gradually slowed down. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Until passage 6, hES-MP and MSC exhibited similar proliferation rates; after passage 6, hES-MP demonstrated a significantly higher proliferation rate (Figure 14). MSC growth also gradually slowed down while hES-MP exhibited continuous proliferation throughout the experiment without showing any signs of cellular senescence. Seeding density-dependent CPD has been reported for MSC. Colter et al. compared high and low seeding density of

MSC and showed significantly different CPD reached based on the density (Colter et al., 2000). When a low seeding density (1.5 cells/cm^2) was used, MSC reached up to 50 CPD, but only about 15 CPD when a high seeding density was used (5000 cells/cm^2). A high seeding density was used in this study, and the results were comparable to Colter et al. The same seeding density does not seem to affect the hES-MP growth since significantly higher CPD were reached here and a steady proliferative rate observed. This is similar to what has been previously reported for hES-MP in FBS, where about 3 PD were consistently seen for every passage for up to 30 passages, resulting in over 60 CPD (de Peppo, Svensson, et al., 2010). Equivalent results have also been obtained for other ESC-MSC (Brown et al., 2014; Sánchez et al., 2011). This is not entirely unexpected since the hES-MP precursors, ESC, are well known for their robust growth (Luzzani & Miriuka, 2016). The cells seem to retain some of that robustness through the hES-MP derivation process and hence are likely to yield higher cell numbers in a shorter time than MSC.

MSC and hES-MP both exhibited similar growth kinetics, independent of the supplement used (Figure 15). The long-term proliferation of hES-MP in hPL has not been explored before. Luzzani et al. (2015) did, however, evaluate the proliferation of ESC-MSC and iPSC-MSC when grown in hPL or FBS. They examined the growth over 14 days and found a significant difference in proliferation after nine days of culture, where cells in hPL demonstrated higher growth (Luzzani et al., 2015). Such difference for hES-MP was never detected throughout our study.

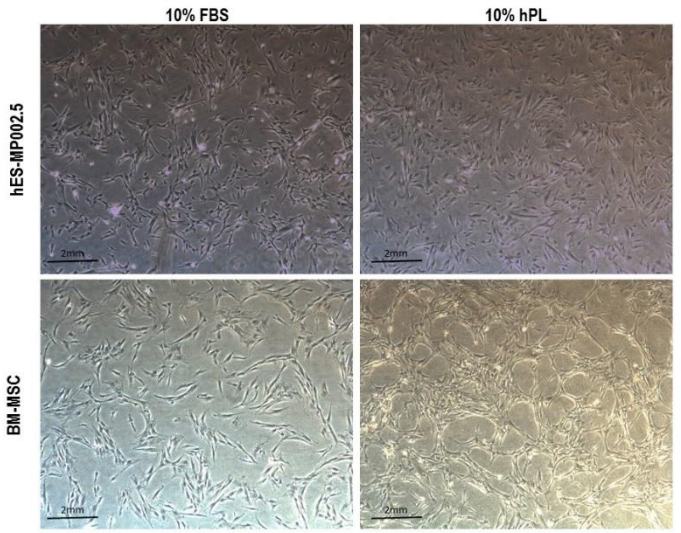

Figure 15. hES-MP and MSC morphology. hES-MP showed fibroblast-like morphology similar to MSC in both FBS and hPL. MSC and hES-MP here are at passage 1, 72 hours post seeding.

The lack of difference between FBS and hPL for MSC growth is surprising since we previously demonstrated a significant difference between the supplements (Figure 6). MSC have been reported by many to grow faster in hPL than FBS, but reports demonstrating no difference also exist (Bieback et al., 2009; Burnouf, T. et al., 2014; Schallmoser et al., 2010, 2007). The discrepancies between Papers I and III may be because the MSC used were not from the same donors, and that different lots of FBS and hPL were used. These different results underline how the variability between MSC donors and supplement lots can impact results and complicate study comparisons.

The surface antigen expression of hES-MP was evaluated periodically over 10 passages to determine the expression stability over time and to study differences between hPL and FBS. The antigen expression was determined after four, six, and 10 passages. The hES-MP from both FBS and hPL expressed CD29, CD44, and CD73 at all time points, but expression of CD45, CD184, and HLA-DR was not detected. This expression pattern resembles the expression previously seen for MSC (Dominici et al., 2006). Furthermore, no differences dependent on the culture supplement were seen.

The expression of three surface antigens, CD105, CD13, and CD10, stood out and fluctuated based on supplement type and time (Figure 16). CD105, an antigen that is expressed on MSC per ISCT minimal criteria,

varied in expression on hES-MP based on whether the cells were expanded in FBS or hPL. Significantly lower expression was repeatedly detected after expansion in hPL compared to FBS ($p \leq 0.01$ at passage 4 and $p \leq 0.001$ at passage 10, Figure 16). CD105 (endoglin) is a co-receptor for members of the TGFβ family and participates in Smad signaling (Nassiri et al., 2011; Pérez-Gómez et al., 2010). hPL was found to contain significantly higher levels of TGFβ-1 than FBS (59,628.2 ± 1517.2 pg/ml versus 10.1 ± 0.1 pg/ml, $p \leq 0.001$). The reason for these differences in CD105 expression remain hypothetical, but the downregulation of CD105 as a response to high TGFβ-1 concentration in hPL is a possibility. This downregulation would inhibit TGFβ signaling through activin receptor-like kinase (ALK) 1 but promote signaling through ALK5 and help maintain an undifferentiated state (Dexheimer et al., 2016; Mishra et al., 2005; ten Dijke et al., 2008).

The high TGFβ-1 concentration could also explain the time-dependent increase seen in the CD13 expression for hES-MP in hPL. The hES-MP cultured in either FBS or hPL expressed CD13 (aminopeptidase N) on their surface. At passage 4, no difference was seen between FBS and hPL in CD13 expression, but at both passages 6 and 10, hES-MP in hPL had significantly higher expression than cells in FBS ($p \leq 0.001$). The expression was highest at passage 10 and lowest at passage 4 ($p \leq 0.001$). TGFβ-1 has been shown to increase CD13 expression in a concentration and time-dependent manner for other cell types (Gabrilovac et al., 2008). The role of CD13 on MSC (and hence hES-MP) remains to be determined, but it might participate in the survival of the stem cell pool as has been shown for liver cancer and muscle satellite stem cells (Kim, H. M. et al., 2012; Rahman et al., 2014)

CD10 has also been demonstrated to participate in stem cell pool maintenance, especially for early progenitors (Bachelard-Cascales et al., 2010). Here, CD10 was expressed by hES-MP grown with hPL at all passages but not by hES-MP grown in FBS ($p \leq 0.001$). For hES-MP from hPL, the highest expression was noted after passage 6 and was significantly higher than at other time points ($p \leq 0.05$ for passage 4 and $p \leq 0.01$ for passage 10). Whether CD10 is expressed on MSC or not has been debated (Javazon et al., 2004; Mafi, 2011). Since CD10 is associated with early progenitor cells, the different expression in CD10 on MSC might be related to the heterogeneity of the cell population being studied each time. MSC populations with a high number of true stem cells and progenitor cells would then be expected to have higher CD10 expression than MSC populations consisting mainly of lineage-committed cells. Karlsson et al. (2009)

demonstrated CD10 expression on hES-MP when grown with human serum, but other reports focusing on the hES-MP surface proteome have all used FBS as supplement, and none has reported on the CD10 expression (Chen, W. et al., 2013; de Peppo, Svensson, et al., 2010; Lai et al., 2010; Li, O. et al., 2013). Here, we demonstrate for the first time the effects of hPL on CD10 expression for hES-MP. It is notable that the use of human serum and hPL seems to support CD10 expression while FBS does not. This raises questions about the ability of different culture supplements not only to support the cell population as a whole but specifically to support the stem cells and the progenitor cells within the population. Further studies regarding the CD10 expression on MSC are relevant since it is important in a heterogeneous MSC population to support the early progenitor cells to an even further extent than more committed cells.

Following initial expansion, osteogenesis was induced and evaluated over a period of 28 days. The ALP activity was examined after seven, 14, and 28 days of differentiation, while the osteogenic gene expression and mineralization were analyzed at four time points (seven, 14, 21, and 28 days). ALP activity increased in a time-dependent manner throughout the study period ($p \leq 0.001$) with no differences seen between hPL and FBS (Figure 17). Our findings are contradictory to others, where the failure of hES-MP to increase ALP protein expression when under osteogenic stimuli has been found (Bigdeli et al., 2010; de Peppo et al., 2013). These observations have, furthermore, been shown to depend on the age of the cells (passage number) for both hES-MP and MSC (de Peppo et al., 2013). The hES-MP at passage 3 were used in this study, and the ALP activity was consistently seen to increase during osteogenic differentiation as would be expected in the case of MSC.

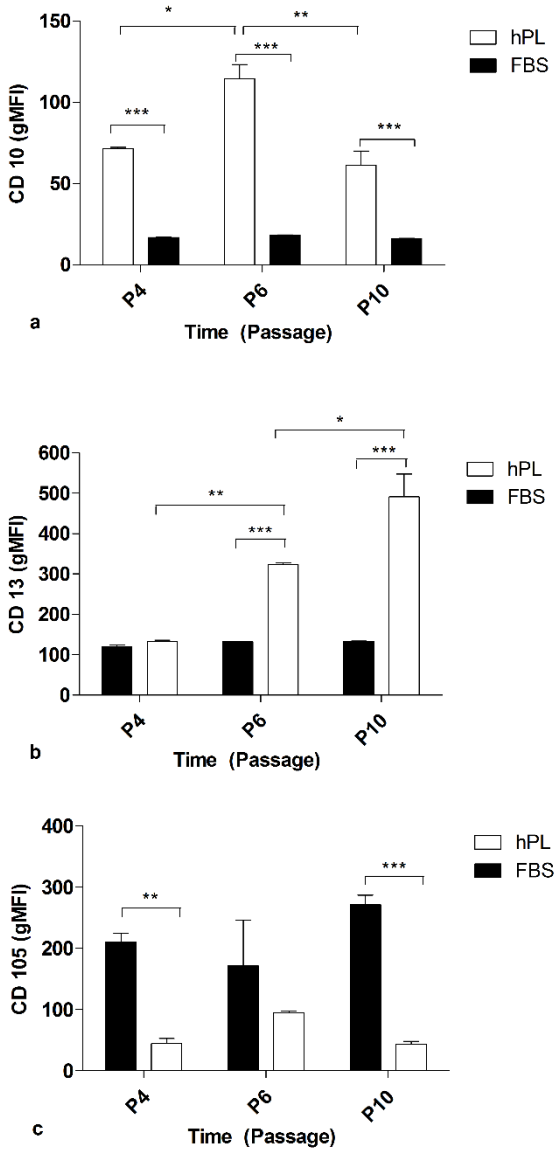

Figure 16. Surface expression of CD10, CD13, and CD105 on hES-MP. A. hES-MP from hPL expressed CD10 at all time points, but hES-MP from FBS did not. B. The expression of CD13 increased in a time-dependent manner on hES-MP from hPL, but the CD13 expression remained constant for hES-MP from FBS C. hES-MP from hPL were found to express significantly lower levels of CD105 as compared to hES-MP from FBS at all time points. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

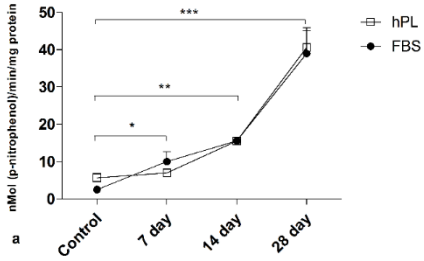

Figure 17. ALP activity in hES-MP in osteogenic differentiation. During osteogenic differentiation, the ALP activity significantly increased with time for hES-MP in both FBS and hPL. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The hES-MP from hPL demonstrated significantly stronger osteogenic gene expression at all time points compared to hES-MP from FBS (Figure 18). *RUNX2* and *SPP1* expression in both hPL and FBS followed the same expression pattern, but significantly stronger expression was nonetheless observed at all time points for hPL (p≤0.05 for day 14 and 21, p≤0.01 for day 7 and 28). *RUNX2* expression in hES-MP compared to MSC has been evaluated by others, and hES-MP are generally seen to express *RUNX2* to a lesser extent than MSC (Bigdeli et al., 2010; de Peppo et al., 2013). The *RUNX2* expression is also commonly stable over time as is shown in this study. As has been discussed for MSC, *RUNX2* expression is important for osteogenesis. The differences observed here, between hPL and FBS, suggest a stronger lineage commitment toward osteogenesis for hES-MP in hPL compared to FBS. These results agree with previous results for MSC in Papers I and II (Figure 8). How the hES-MP compare to MSC was, however, not assessed here.

Chondrogenic differentiation occurred over 35 days. Concentration of GAG and expression of *SOX9* were evaluated after seven, 14, 28, and 35 days. The GAG concentration increased for the first 14 days after which a decrease was seen for days 28 and 35. No difference was found between FBS and hPL. The pattern observed here for hES-MP is comparable to the results obtained for MSC in Paper II and is in agreement with the step-wise chondrogenic differentiation previously discussed and hES-MP findings from other groups (Brown et al., 2014). This was further supported with histological staining of hES-MP chondrocyte pellets that demonstrated an accumulation of ECM and hypertrophic cells at later stages of differentiation (Figure 19).

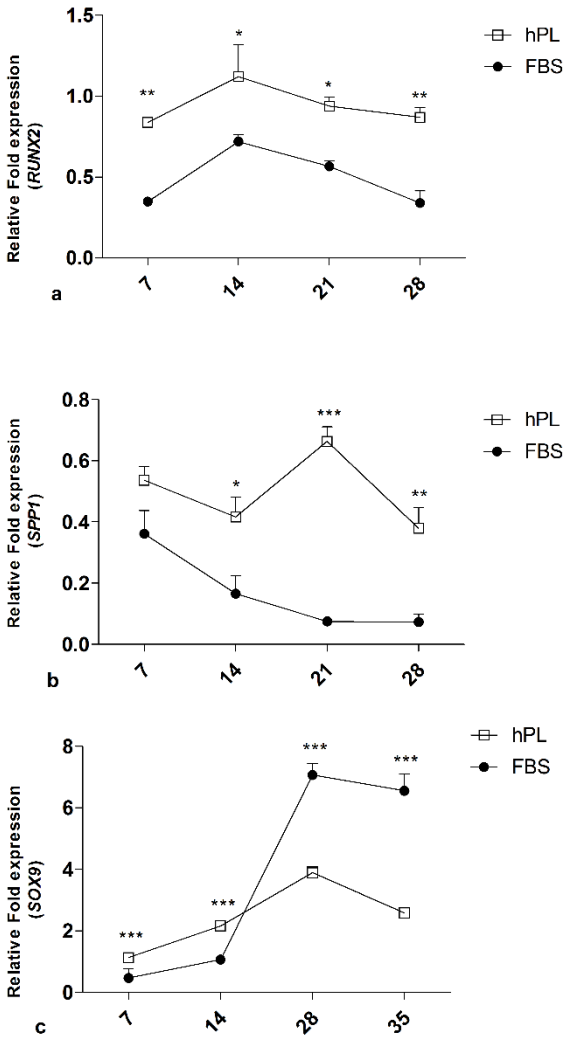

Figure 18. Gene expression during hES-MP differentiation. The expression of *RUNX2* and *SPP1* (A and B) was evaluated over four weeks of osteogenic differentiation. Both genes were expressed significantly more in hES-MP from hPL as compared to hES-MP from FBS at all time points. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

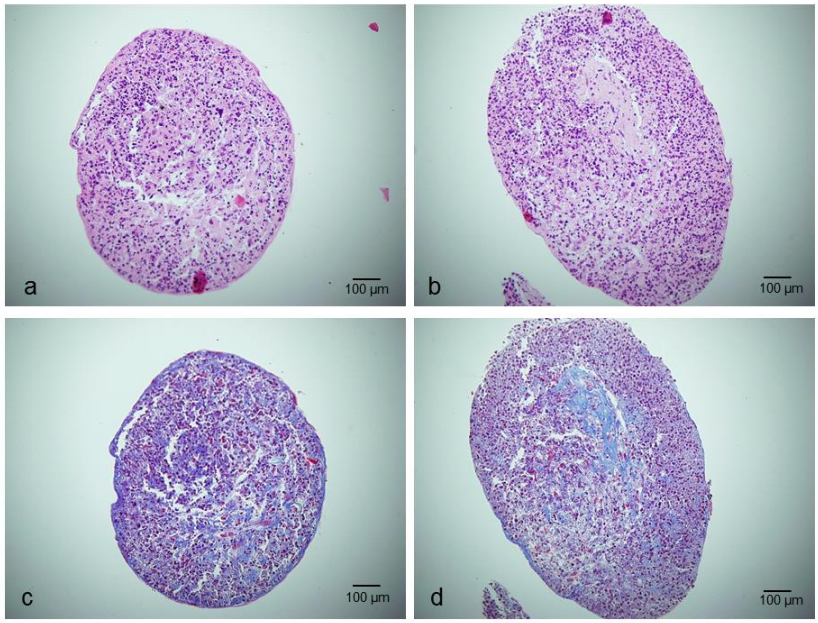

Figure 19. hES-MP cartilage pellets. hES-MP from FBS (A and C) and hPL (B and D) underwent chondrogenic differentiation. Cartilage pellets after 35 days of differentiation are illustrated here stained with hematoxylin and eosin stain (A and B) and Masson trichrome stain (C and D).

The expression of *SOX9* was significantly higher in hES-MP from hPL compared to FBS at days 7 and 14 ($p \leq 0.001$). At days 28 and 35, the opposite was observed where *SOX9* expression was higher in FBS than hPL ($p \leq 0.001$). As was seen in Paper II, the highest *SOX9* expression was noted at day 28, for both FBS and hPL.

Results for the osteogenic gene expression suggest that hES-MP grown in hPL are more committed toward osteogenesis than hES-MP from FBS. Arriving at a similar conclusion for the chondrogenesis is more challenging. *SOX9* is commonly described to be upregulated during chondrogenesis, but as the cells become mature, *SOX9* is downregulated, and the cells reach hypertrophy. For both hPL and FBS, the *SOX9* expression increased until day 28 and then downregulation was observed as would be expected so late in the differentiation. Still, the expression was significantly higher in FBS compared to hPL. The importance of this difference is hard to determine

since no differences could be seen in GAG concentrations or morphology where both FBS and hPL consisted of ECM and hypertrophic cells.

The hES-MP exhibit robust growth beyond what is seen for MSC and do so both in hPL and FBS. They have similar surface antigen expression and are capable of osteogenic and chondrogenic differentiation like MSC. Osteogenic marker genes are expressed more strongly in hES-MP from hPL during osteogenic differentiation like we have seen for MSC in Papers I and II. The evidence presented here thus demonstrate that hPL is a suitable supplement for hES-MP growth and supports their osteogenic differentiation. The findings are in agreement with previous findings from others (Luzzani et al., 2015).

4.4 Paper IV — Immunological properties of hES-MP and MSC in hPL

Despite their tissue-forming potential, MSC have unique immune properties. They actively influence immune responses and can adopt both an anti-inflammatory and pro-inflammatory phenotype depending on circumstances (Bernardo & Fibbe, 2013). They secrete various mediators that participate in inflammation, and MSC-derived extracellular vesicles have been demonstrated to modify immune responses in the absence of MSC (Bruno et al., 2015; Monsel et al., 2016; Teixeira et al., 2016). These immunological characteristics may be of significance to regenerative medicine as the importance of immunomodulation in facilitating tissue regeneration becomes more recognized (Keating, 2012; Lee et al., 2009; Molina et al., 2015).

The hES-MP were derived from ESC in order to resemble MSC (Karlsson et al., 2009). They have already been demonstrated to possess MSC-like characteristics in terms of phenotype and differentiation potential (Bigdeli et al., 2010). However, their abilities to participate in immune suppression and modulation have not been evaluated to the same extent. A single study has reported on the interaction of hES-MP and PBMC in co-culture. A failure of hES-MP to suppress PBMC was reported, which is in line with our results (Li, O. et al., 2013). Still, active participation in the immune response has been reported for other types of ESC-MSC (Lotfinia et al., 2016; Sánchez et al., 2011; Tan, Z. et al., 2011; Trivedi & Hematti, 2008; Yen et al., 2009). Since the immunological properties of MSC are a distinctive feature, hES-MP should ideally exhibit the same properties. In Paper IV, we evaluate for the first time the immune suppression, immunomodulation, and cytokine secretion of hES-MP in hPL and compare to MSC.

The hES-MP were placed in co-culture with PHA-stimulated PBMC, both in a Transwell system and allowing direct contact. hES-MP from both hPL and FBS were tested. Surprisingly, hES-MP consistently failed to suppress PBMC proliferation as compared to the control (Figure 20). When bone marrow-derived MSC were tested, they significantly suppressed PBMC proliferation in both Transwell and direct contact settings, from both hPL and FBS (Figure 20), as previously described in Papers I and II. This failure of hES-MP was previously described by Li et al. (2012). There, hES-MP failed to suppress PBMC, even after IFNγ stimulation. The capability of hES-MP to suppress immune cells has not been specifically assessed in other studies. However, they have been claimed to have favorable immunological properties and to have greater immune privilege than MSC (de Peppo, Svensson, et al., 2010). These claims are mainly based on lower HLA-DR expression by hES-MP than MSC in a study by de Peppo et al. (2010) who concluded that hES-MP represent a suitable MSC alternative for in vivo applications. The results of de Peppo et al. (2010) indicate that hES-MP may not be immunogenic but are not sufficient to determine whether the cells participate in immunomodulation. Failure to participate in immune suppression greatly reduces their potential as an MSC replacement in regenerative medicine.

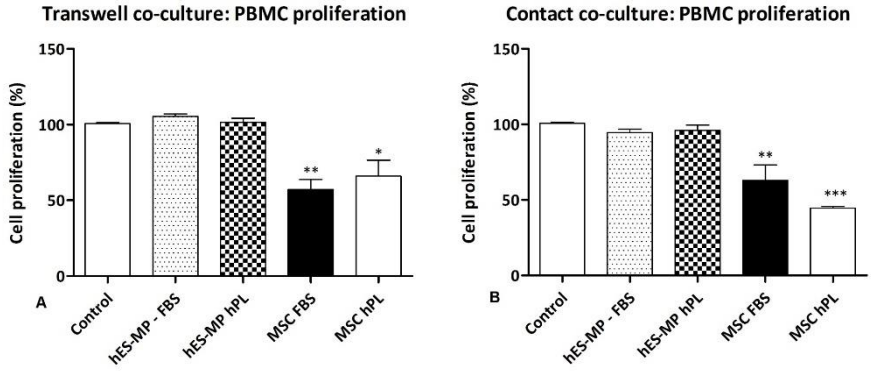

Figure 20. Immune suppression by hES-MP and MSC. The hES-MP in both FBS and hPL failed to suppress PBMC proliferation after PHA stimulation. Both in Transwell culture (A) and in direct contact (B). MSC, unlike hES-MP, suppressed PBMC proliferation consistently. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Bone marrow-derived MSC dwell in the hematopoietic niche within the body. In their microenvironment, they interact with immune cells as they are being formed from the HSC (Hoggatt et al., 2016). This environment most likely gives rise to all the necessary signals to educate the MSC and allow them to acquire immune suppressive characteristics. The hES-MP are derived in experimental settings where such signals are missing. The hES-MP may thus not develop the same characteristics. Where favorable immunological properties have been reported for other ESC-MSC, feeder cells have been used in the derivation process or for ESC maintenance (Lotfinia et al., 2016; Sánchez et al., 2011; Tan, Z. et al., 2011; Trivedi & Hematti, 2008; Yen et al., 2009). The interaction of fibroblasts or stromal cells with ESC-MSC might, therefore, play a role in providing the signals needed for the development of certain immunological properties.

The composition of the PBMC population used was further characterized in a flow cytometer before co-culture, and again after the co-culture, to examine if the hES-MP or MSC affected the proportions of individual cell populations differently. Following co-culture with both hES-MP and MSC, proportions of $CD8^+$, $CD14^+$, and $CD19^+$ cells were generally reduced but not the proportions of $CD4^+$ cells. No difference was seen based on whether FBS or hPL was used as a supplement.

Under pro-inflammatory conditions, such as created in this experiment, MSC adopt an anti-inflammatory phenotype that aids in suppressing immune cell proliferation and shifting the immune response toward Th2 response, while inhibiting $CD8^+$ mediated response, B cells and monocyte maturation (Waterman et al., 2010). Changes in line with our observations. Therefore, it seems like the hES-MP were contributing to a more tolerogenic inflammatory environment without suppressing the general PBMC proliferation. MSC, on the other hand, demonstrated both, as has been so widely described in the literature (Le Blanc & Davies, 2015).

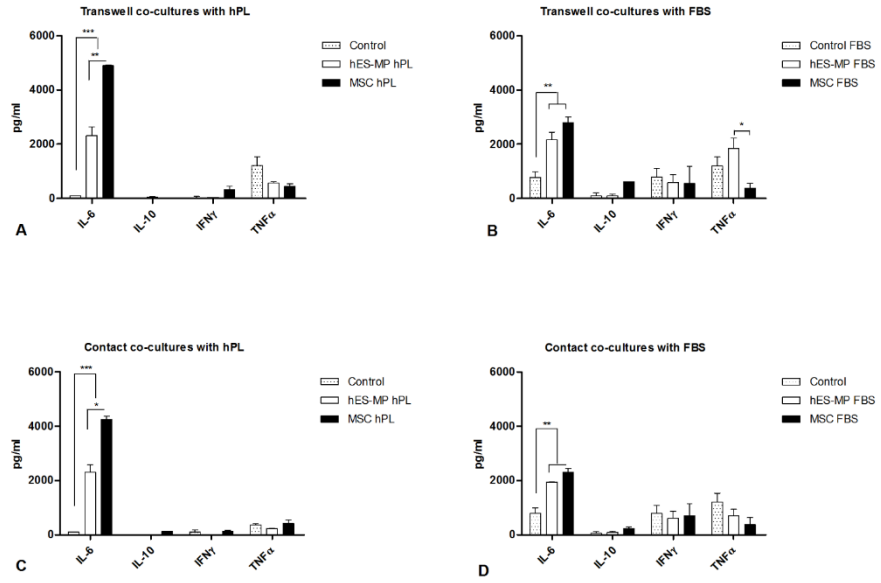

Figure 21. Cytokine secretion after PBMC co-culture with hES-MP or MSC. Secretion of IL-10, INFγ and TNFα was comparable to the control in both hPL and FBS cultures. IL-6 levels were significantly higher compared to the control, especially in hPL cultures with MSC (A and C). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The cytokine secretion in PBMC co-cultures with hES-MP and MSC was comparable apart from the IL-6 secretion. IL-6 was consistently higher in co-cultures compared to PBMC alone (p≤0.01 for FBS and p≤0.001 for hPL, Figure 21), especially for MSC co-cultures supplemented with hPL, where significant differences were seen in IL-6 secretion between hES-MP and MSC co-cultures (p≤0.01). When PBMC were grown by themselves, different results were obtained, where a significant difference in cytokine secretion between hPL and FBS PBMC was seen.

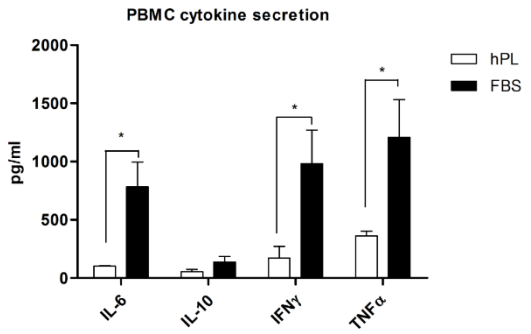

Figure 22. Cytokine secretion by PBMC in hPL and FBS. PBMC secreted significantly higher levels of IL-6, IFNγ, and TNFα when grown in FBS compared to hPL. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The secretion of IL-6, IFNγ, and TNFα was significantly higher if PBMC were grown with FBS (p≤0.05) suggesting a priming toward pro-inflammatory state taking place in FBS. Low levels were, however, seen for PBMC in hPL. When hES-MP or MSC were added to the culture, IL-6 concentration was nevertheless significantly higher in hPL than FBS, and the difference in IFNγ and TNFα disappeared. IL-6 has been portrayed as a potent inflammatory agent in the past, but it is now known that IL-6 participates in the polarization of MSC toward an anti-inflammatory phenotype through COX2 upregulation (Bouffi et al., 2010; Liu, X. H. et al., 2006). MSC then further secrete IL-6 themselves to stimulate the formation of M2 anti-inflammatory macrophages (Kim, J. & Hematti, 2009). IL-6 secretion by MSC is hence associated with an anti-inflammatory response (Bouffi et al., 2010). An IL-6 increase was observed in all co-cultures. An IL-6 increase to a further extent was observed in hPL compared to FBS pointing toward a stronger anti-inflammatory response in hPL.

Here, hES-MP failed to suppress PBMC proliferation but had an immunomodulatory effect on cell populations like MSC. The hPL did not affect the immune modulation of hES-MP when compared to FBS, although MSC in hPL resulted in higher IL-6 levels in co-culture than MSC in FBS. Clearly, the immunological properties of hES-MP are not comparable to MSC in all aspects and need to be evaluated further before considering the cells as suitable MSC alternatives.

4.5 Unpublished findings — hPL-PI in MSC and hES-MP differentiation

In addition to evaluating the suitability of hPL from expired platelets for the expansion of MSC and hES-MP, we examined their differentiation potential when using hPL from expired pathogen-inactivated platelets (hPL-PI), both during their expansion and during differentiation. We previously assessed the suitability of hPL-PI for MSC expansion in Paper II. However, hPL-PI was not present throughout the differentiation. We had also not tested hPL-PI for hES-MP expansion and differentiation. In an additional study for this thesis, we therefore evaluated the suitability of hPL-PI for hES-MP and MSC differentiation compared to FBS by applying differentiation media containing hPL-PI or FBS.

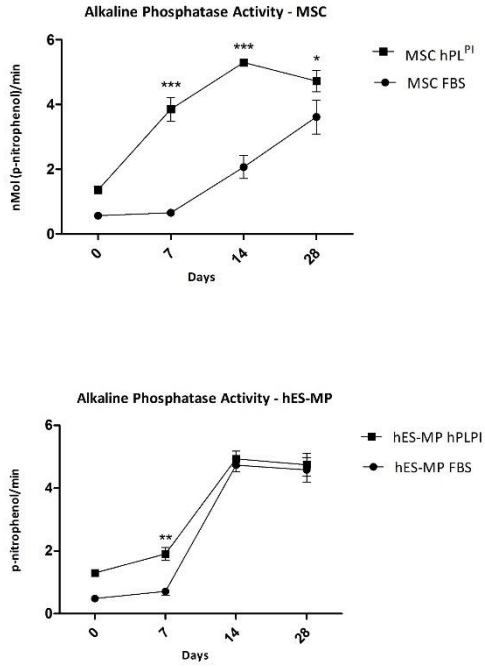

Figure 23. ALP activity of hES-MP and MSC in hPL-PI and FBS Top. The ALP activity increased with time for MSC during osteogenesis, significantly more so in hPL-PI compared to FBS (top). The ALP activity also increased with time for hES-MP during osteogenesis with significant differences seen between hPL-PI and FBS at day 7 (bottom). *=p≤0.05, **=p≤0.01, ***=p≤0.001.

The hES-MP and MSC underwent osteogenic differentiation for 28 days. ALP activity increased significantly with time for both MSC and hES-MP in both FBS and hPL-PI ($p \leq 0.01$, Figure 23). For MSC, however, a significant difference was seen between hPL-PI and FBS from day 7 ($p \leq 0.01$). The ALP activity increased rapidly early in the differentiation period for MSC in hPL-PI, an increase greater than what had been observed before in Papers I and II. The increase in ALP activity was moderate early in differentiation but increased from day 7 following a similar pattern as was observed for MSC in Papers I and II. These findings were further supported by observations for *RUNX2* expression which was significantly stronger in MSC from hPL-PI compared to FBS at days 7 and 28 ($p \leq 0.001$, Figure 24). Mineralization was also significantly stronger for MSC in hPL-PI compared to FBS by day 28 ($p \leq 0.001$). Adding hPL-PI to the osteogenic differentiation media for MSC, therefore, seems to improve osteogenic lineage commitment and improve osteogenesis.

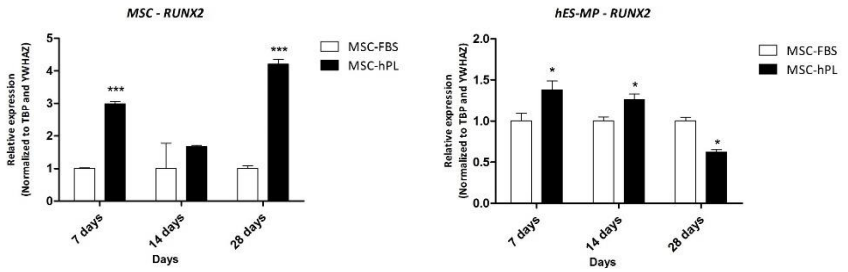

Figure 24. RUNX2 expression in MSC and hES-MP. *RUNX2* expression was significantly higher at seven and 28 days for MSC in hPL-PI compared to MSC in FBS (left). In hES-MP, the *RUNX2* expression was significantly higher in hPL-PI compared to FBS at days 7 and 14 but significantly lower at 28 days (right). *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$.

Results for hES-MP were not as conclusive. No differences between hPL-PI and FBS were seen for hES-MP ALP activity, except at day 7 ($p \leq 0.01$). *RUNX2* was upregulated in hPL-PI at days 7 and 14 ($p \leq 0.05$) but downregulated at day 28 compared to FBS ($p \leq 0.05$). The hES-MP from hPL-PI were, furthermore, observed to mineralize less than hES-MP in FBS ($p \leq 0.05$). MSC and hES-MP, therefore, do not respond to the same osteogenic stimuli in an identical manner.

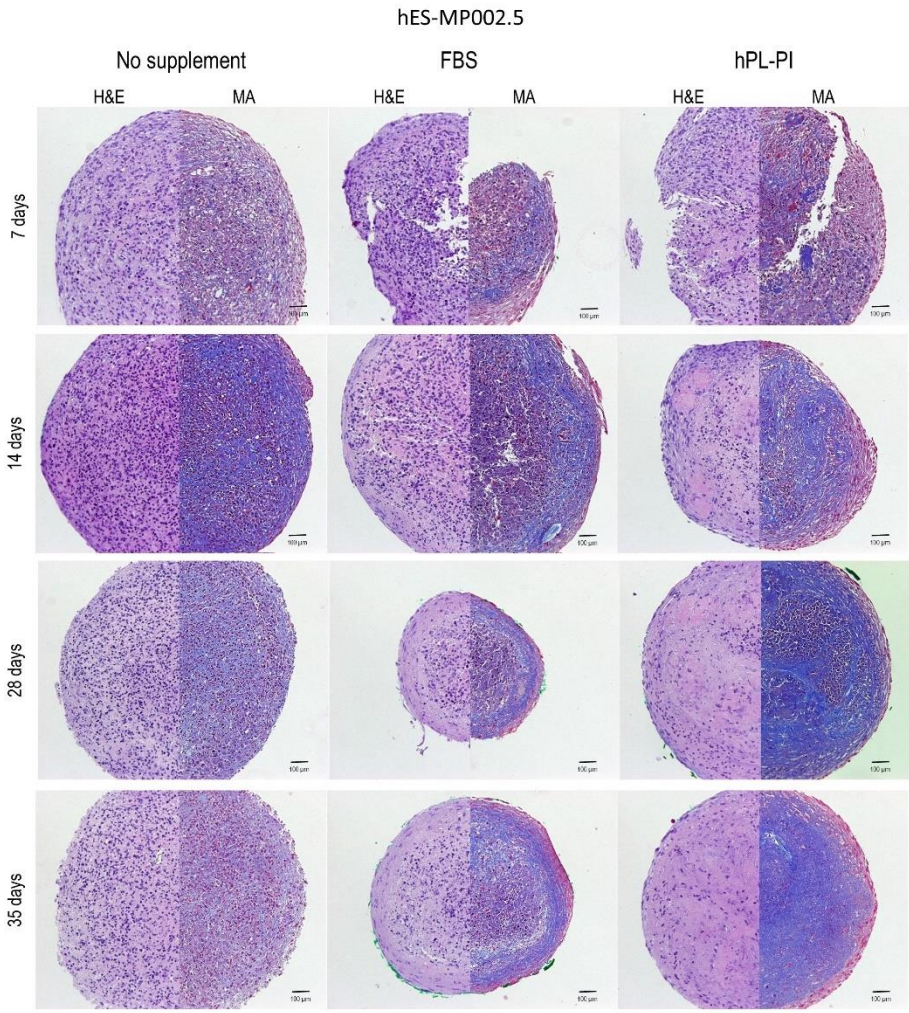

Figure 25. hES-MP-derived cartilage pellets. The hES-MP underwent chondrogenesis for 35 days in the form of a cartilage pellets. Pellets were stained with hematoxylin and eosin staining (H&E) and Masson trichrome staining (MA) to examine tissue morphology.

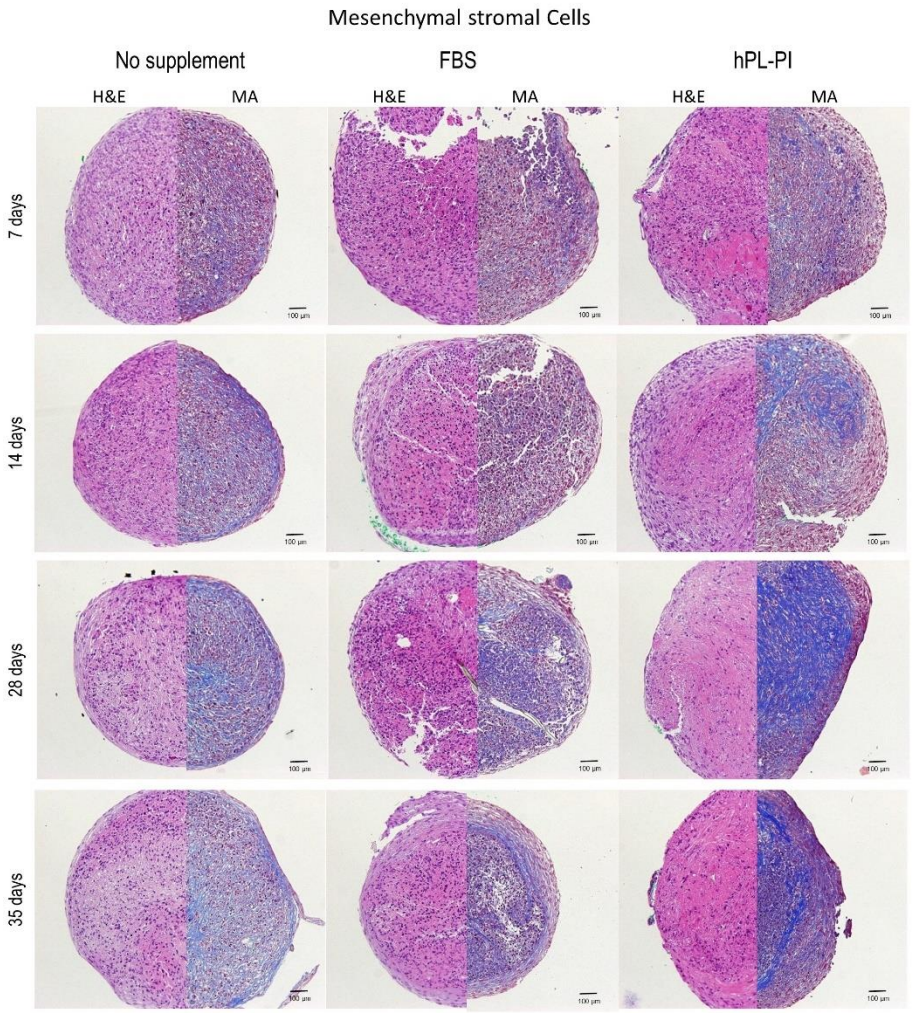

Figure 26. MSC-derived cartilage pellets. MSC underwent chondrogenesis for 35 days in the form of a cartilage pellets. Pellets were stained with hematoxylin and eosin staining (H&E) and Masson trichrome staining (MA) to examine tissue morphology.

Chondrogenesis was induced for MSC and hES-PM in FBS and hPL-PI. The process was monitored for 35 days by evaluating the histology of the cartilage pellets and analyzing the *SOX9* expression. The hES-MP and MSC in both FBS and hPL-PI successfully formed cartilage pellets (Figure 25 and Figure 26). The pellets from hPL-PI bore a higher resemblance to articular cartilage than pellets from FBS since hPL-PI-grown pellets demonstrated a higher amount of collagen containing ECM, shown with Masson trichrome staining, and a greater decrease in cell numbers with time. Articular cartilage is rich in ECM and contains few cells (Fox et al., 2009). Cartilage differentiation is characterized by an early increase in ECM followed by a decrease in cell proliferation and number at later stages of differentiation as was seen here (Yamashita et al., 2010). The results are further supported by SOX9 expression (Figure 27). For MSC in hPL-PI, there was a significant upregulation of SOX9 at day 7 ($p \leq 0.05$) and days 28 and 35 ($p \leq 0.001$) compared to FBS. For hES-MP in hPL-PI, *SOX9* upregulation was noted at all timepoints except day 35 ($p \leq 0.001$).

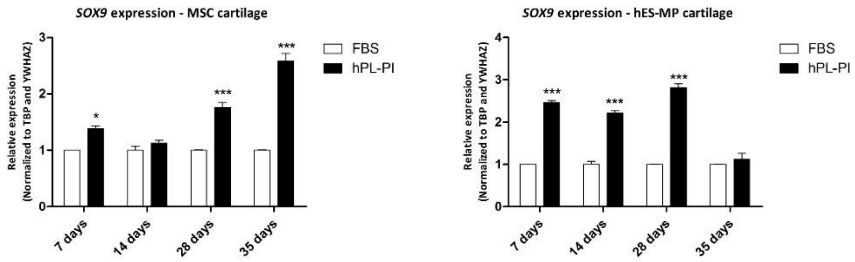

Figure 27. SOX9 expression in MSC and hES-MP. *SOX9* expression was significantly higher in MSC from hPL-PI during chondrogenesis at days 7, 28, and 35 compared to MSC in FBS (left). In hES-MP, the *SOX9* expression was significantly higher in hPL-PI compared to FBS at days 7, 14, and 28 during chondrogenesis (right). *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$.

When evaluating the cartilage histology and *SOX9* expression, it can be observed that chondrogenesis appears to have proceeded faster in hES-MP than MSC. By the end of the experiment, hES-MP cartilage pellets were further along in the differentiation process, based on their morphology and downregulation of *SOX9* expression between the last time points. These results indicate that successful differentiation occurred and reached the last stage of the differentiation process, where cells prepare for ossification by decreasing *SOX9* expression. The results thus strongly point to a more

favorable chondrogenesis for MSC and hES-MP when expanded and differentiated using hPL-PI as a culture supplement. The hPL-PI also supported MSC osteogenic differentiation more than FBS, although the same conclusions could not be made for hES-MP. The hPL-PI is, therefore, a favorable alternative to FBS to support the growth and differentiation of both MSC and hES-MP.

5 Concluding remarks

The field of tissue engineering and regenerative medicine (TERM) has expanded rapidly in recent years (Wobma & Vunjak-Novakovic, 2016). The field is focused on generating advanced therapies that can restore the function of damaged tissue when healing is beyond the body's capacity (Dlaska et al., 2015). The cells are often expanded, manipulated, or differentiated ex vivo before injection or transplantation (Escacena et al., 2015). Hence, all TERM strategies need to adhere to strict regulations about advanced therapy medicinal products number 1394/2007 in Europe and 21st code of federal regulation 1271 for human cells, tissues, and cellular and tissue-based products in the United States (Escacena et al., 2015; Kinzebach & Bieback, 2012; Murphy, M. B. et al., 2013).

The use of MSC is one of the greatest hopes of TERM (Keating, 2012). They have been used in numerous clinical trials to treat different types of illnesses in various organs (Lalu et al., 2012; Sharma et al., 2014). Still, several challenges associated with MSC need to be overcome before their clinical use can be fully established. MSC are not fully defined cells and are isolated according to various protocols (da Silva Meirelles et al., 2008). Their properties can differ between tissue sources and their potency is affected by the age of the MSC donor (Fehrer & Lepperdinger, 2005; Raggi & Berardi, 2012). MSC can grow only for a limited time outside the body and reach senescence after several passages (Schallmoser et al., 2010). The best mode of delivery, optimal dose, and frequency remain to be determined (Sharma et al., 2014).

In this thesis, we have demonstrated the suitability of hPL from expired platelets, pathogen-inactivated or not, as an FBS alternative for the growth and differentiation of MSC and their embryonic-derived counterparts, hES-MP. The frequent use of FBS for MSC culture is a concern, and the use of suitable serum alternatives such as hPL must be put into practice. As has been shown by us and others, hPL supports rapid growth of MSC and hES-MP while allowing them to retain their differentiation and tissue-forming potential (Doucet et al., 2005; Luzzani et al., 2015; Schallmoser et al., 2007, 2008). We showed that hPL use supports osteogenesis more than FBS for both MSC and hES-MP, especially if added to the differentiation media. Furthermore, supplementation with hPL and hPL-PI does not affect the

abilities of MSC to suppress proliferation of immune cells or the ability of hES-MP to participate in immunomodulation.

When all is said and done, FBS is not an ideal supplement to grow human cells. Strict regulations are being passed on its use, and regulatory bodies and institutions such as The World Health Organization (WHO) have considered banning its use entirely for cell therapy protocols (Shih & Burnouf, 2015; van der Valk et al., 2010; Van Der Valk et al., 2004). Nonetheless, such a ban cannot take effect until a suitable replacement for FBS has been found (Astori et al., 2016). The replacements should preferably be chemically defined or of human origin, be applicable for various cell types, easily available, and standardized (Hemeda et al., 2014; van der Valk et al., 2010; Van Der Valk et al., 2004). In this context, hPL made from expired pathogen-inactivated platelets can be regarded as a feasible and practical alternative.

Using expired platelets for hPL production has a distinct advantage since high-quality starting material can be obtained without increasing the demand for blood donors. Also, biological waste coming from blood banks is reduced and both research grade and GMP grade hPL can be easily made. Eliminating FBS from cellular growth settings and applying hPL instead will shorten the research and development phase of new cell therapies since the process from basic research to clinical translation will be faster. This will allow regenerative medicine to take a step forward and meet the growing demand for new cell-based solutions.

Several clinical trials have already been conducted using MSC grown in hPL under standardized conditions (Bieback, 2013; Capelli et al., 2015). Capelli et al. (2015) described the setup of a cell factory with defined cell culture systems using GMP hPL. At the time of publication, seven clinical trials with MSC grown in hPL had been conducted with cells from their cell factory. A total of 103 MSC batches had been produced and used to treat 57 patients (Capelli et al., 2015). Fazzina et al. (2016) tested eight different hPL lots for MSC expansion and demonstrated less than 4% variation in growth and potency (Fazzina, Iudicone, Fioravanti, et al., 2016). Such promising results raise hopes that the development of cellular therapies is moving toward more standardized and serum-free processes. Clinical-scale production of cells intended for cellular therapies should result in cells with consistent and predictable qualities. It is critical to limit treatment failures (Jung et al., 2012). Moving toward the use of GMP hPL rather than FBS will be an important step in the right direction.

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Original publications

Paper I

Paper II

Paper III

Paper IV

Appendix I

Paper III – Supplementary material

Appendix II

Paper IV – Supplementary material