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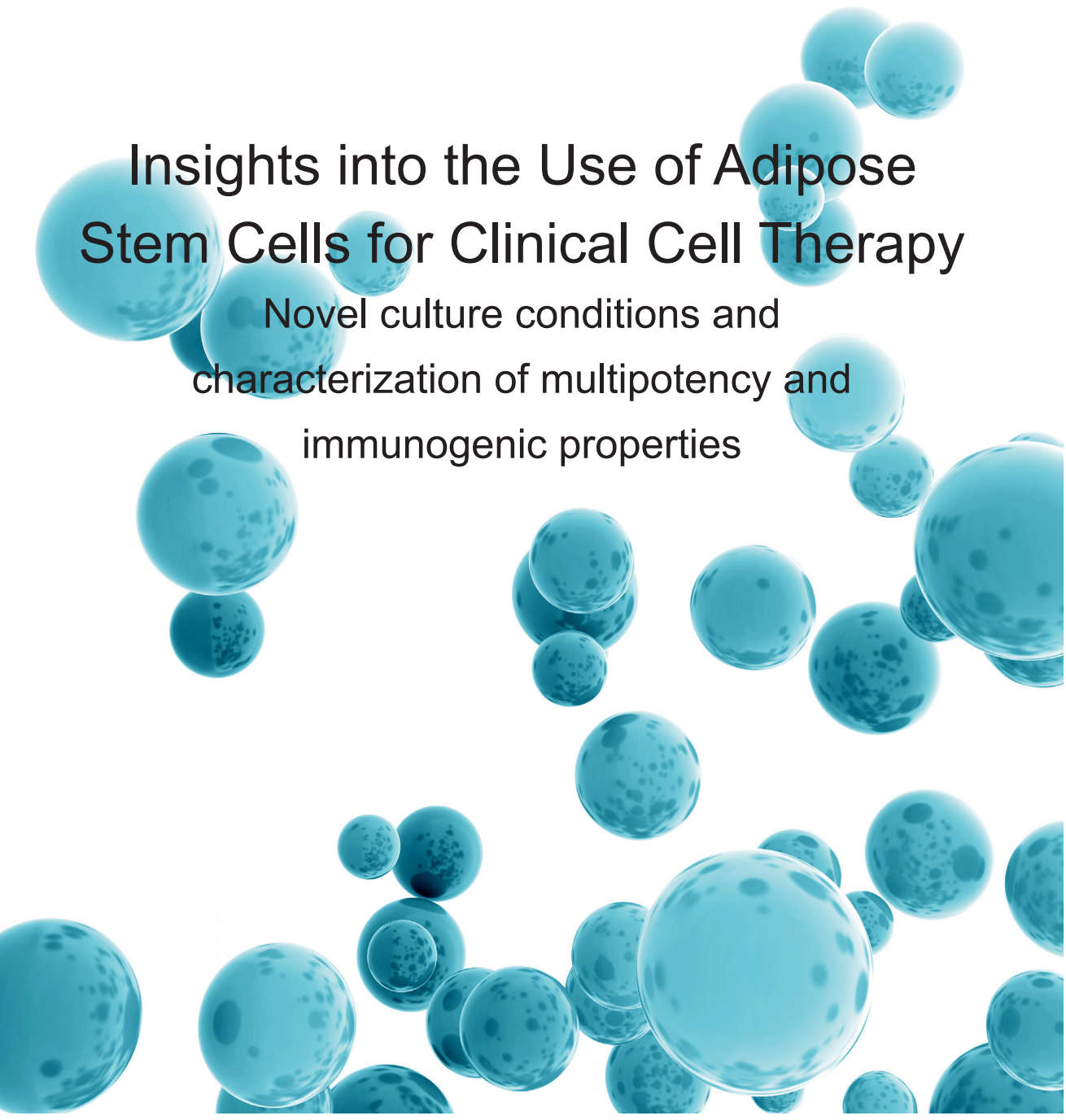
MIMMI PATRIKOSKI

Insights into the Use of Adipose Stem Cells for Clinical Cell Therapy

Novel culture conditions and characterization of multipotency and immunogenic properties



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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Finland

Supervised by
Docent Susanna Miettinen
University of Tampere
Finland
PhD Bettina Mannerström
University of Tampere
Finland

Reviewed by
Professor Jeffrey Gimble
Tulane University
USA
Docent Johanna Nystedt
Åbo Akademi
Finland

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To my dearest

Henri and Pihla

Abstract

Adipose tissue is an abundant source of multipotent stem cells, known as adipose stromal/stem cells (ASCs). The excellent proliferation capacity, multilineage differentiation potential, low immunogenicity and ability for immunomodulation make ASCs promising candidates for diverse clinical applications in regenerative medicine and immunomodulation therapies. Nevertheless, expansion of ASCs is often necessary to obtain a clinically sufficient cell number for effective cell treatments. Standard cell-culture techniques use animal-derived reagents that are not recommended in clinical use because of safety concerns with respect to allergic reactions, rejection and zoonoses. Therefore, xeno- and serum-free (XF/SF) reagents are highly desirable for enhanced safety and quality of transplanted ASCs.

In this thesis, animal-component-free isolation and cell-expansion protocols were developed for ASCs. Basic stem cell characteristics such as immunophenotype, proliferation, and differentiation potential were assessed in XF/SF conditions and compared with those in allogeneic human serum (HS) or the traditionally used fetal bovine serum (FBS) cultures. Additionally, macromolecular crowding (MMC) was used to support cells in re-creating their own microenvironments in vitro. The effects of MMC on ASC characteristics were analyzed under three culture conditions, i.e., in FBS, HS and XF/SF. The immunogenicity and immunosuppression of ASCs as well as the secretion of signaling proteins were also determined through mixed lymphocyte reactions after cell isolation and expansion in FBS- and HS-supplemented medium and in XF/SF conditions. Finally, the effects of bioactive glass (BAG) S53P4 or β- tricalcium phosphate (β-TCP) and bone morphogenetic protein (BMP) -2 and -7 on the osteogenic differentiation of ASCs were investigated in HS-supplemented medium.

The results showed that ASCs cultured in XF/SF conditions had significantly higher proliferation rates compared with those in HS and FBS cultures. The characteristic immunophenotype and multilineage differentiation potential of ASCs were maintained in all conditions. Although MMC did not support ASC proliferation under any of the studied conditions, adipogenic and osteogenic differentiation was efficient under MMC in FBS and HS media. However, the MMC method was not suitable for the XF/SF cultures studied because the ASCs did not remain viable in

long-term exposure to MMC. Furthermore, our immunology studies showed that ASCs were weakly immunogenic when expanded under the three conditions. The significantly strongest suppression was observed with cells expanded in FBS conditions, whereas higher ASC numbers were required to display suppression in HS or XF/SF conditions. The results of the biomaterial studies showed that BMP supplementation decreased the cell number and osteogenic differentiation of ASCs with both the BAG and β-TCP materials. The most efficient osteogenic differentiation of ASCs was observed with BAG cultured without osteogenic supplements.

Our findings demonstrated that the novel XF and XF/SF conditions maintained the basic stem cell features of ASCs, and thus, these animal-free workflows may have potential in clinical cell therapies. Careful characterization of ASCs with respect to in vitro culture conditions, immunological properties and cell behavior with clinically approved biomaterials is highly important for positive development of cell-based therapies. Thus, these novel findings of ASC characteristics will provide valuable tools for regulatory authorities. Nevertheless, additional preclinical safety and efficacy studies are required prior to clinical translation.

Tiivistelmä

Rasvakudos on monikykyisten kantasolujen ehtymätön lähde. Rasvakudoksen kantasoluilla on erinomainen kyky jakautua ja erilaistua usean eri solutyypin suuntaan. Ne eivät myöskään aiheuta voimakasta immunologista vastetta, vaan niillä on kyky säädellä elimistön immunologisia reaktioita. Edellä mainitut ominaisuudet tekevät rasvakudoksen kantasoluista lupaavan vaihtoehdon erilaisiin kliinisiin sovelluksiin uudistavan lääketieteen alalla sekä immuunipuolustuksen säätelyä vaativiin hoitoihin. Kantasoluhoitoihin tarvittavat solumäärät ovat kuitenkin usein suuria, joten solujen määrää tulee lisätä laboratorio-olosuhteissa tehokkaan hoitovasteen aikaansaamiseksi. Perinteiset laboratoriossa käytettävät soluviljelytekniikat hyväksikäyttävät eläinperäisiä ainesosia, joita ei suositella kliiniseen käyttöön mahdollisten turvallisuusriskien, kuten allergisten reaktioiden tai hyljinnän takia. Tästä syystä täysin seerumittomat ja eläinperäisistä aineista vapaat reagenssit ovat toivottuja ja niillä voidaan parantaa rasvakudoksen kantasolujen turvallisuutta ja laatua kliinisiä soluhoitoja ajatellen.

Tässä väitöskirjatyössä kehitettiin seerumittomat ja eläinperäisistä aineista vapaat soluviljelymenetelmät rasvakudoksen kantasolujen laboratoriossa tapahtuvaan eristykseen ja kasvatukseen. Solujen perusominaisuudet, kuten immunofenotyyppi, jakautumiskyky ja erilaistuspotentiaali arvioitiin seerumittomissa ja eläinperäisistä aineista vapaissa kasvatusolosuhteissa ja niitä verrattiin ihmisen seerumia sekä naudan seerumia sisältäviin olosuhteisiin. Lisäksi tutkittiin viljelymediumin makromolekulaarisen täytön eli Ficollin lisäyksen vaikutusta soluihin ja kykyä tukea solujen luonnollista mikrotason elinympäristön tuottoa laboratorio-olosuhteissa. Ficollin lisäyksen vaikutusta rasvakudoksen kantasolujen ominaisuuksiin tutkittiin kolmessa yllämainitussa kasvatusolosuhteessa. Lisäksi rasvakudoksen kantasolujen immunogeenisyys, kyky estää immuunivasteen muodostusta sekä signaaliproteiini-eritys määritettiin käyttäen lymfosyyttien yhteisviljelmiä kolmessa yllämainitussa laboratorio-olosuhteessa. Lopuksi tutkittiin bioaktiivisen lasin sekä β-trikalsiumfosfaatin sekä luun morfogeneettisten proteiinien (BMP) vaikutusta rasvakudoksen kantasolujen luuerilaistuskykyyn ihmisen seerumia sisältävässä kasvatusliuoksessa.

Tulokset osoittivat, että seerumittomissa ja eläinperäisitä aineista vapaissa viljelyolosuhteissa rasvakudoksen kantasolut jakautuivat merkittävästi nopeammin verrattuna viljelyliuoksiin, jotka sisälsivät ihmisen tai naudan seerumia. Soluille ominainen immunofenotyyppi sekä solujen monikykyinen erilaistumispotentiaali säilyivät kaikissa kolmessa viljelyolosuhteessa. Ficollin lisääminen kasvatusliuokseen ei tukenut solujen jakautumista, mutta solujen erilaistuskyky rasva- ja luusolujen suuntaan oli tehokasta seerumia sisältävissä viljelyolosuhteissa Ficollin lisäyksen jälkeen. Menetelmä ei kuitenkaan soveltunut seerumittomiin olosuhteisiin, sillä rasvakudoksen kantasolujen elinkyky heikentyi merkittävästi Ficollin vaikutuksesta. Rasvakudoksen kantasolut eivät aiheuttaneet voimakasta immunologista vastetta missään tutkitussa olosuhteessa. Solujen kyky estää immuunivasteen muodostumista oli voimakkain naudan seerumia sisältävässä olosuhteessa, mutta suuremmilla kantasolumäärillä estävä vaikutus voitiin nähdä myös muissa tutkituissa olosuhteissa. Luun morfogeneettiset proteiinit vähensivät rasvakudoksen kantasolujen määrää ja luuerilaistuskykyä molempien biomateriaalien kanssa. Rasvakudoksen kantasolujen luuerilaistuskyky oli tehokkainta, kun niitä viljeltiin bioaktiivisella lasilla ilman osteogeenisiä suplementteja.

Tämä väitöstutkimus osoitti, että uudet seerumittomat ja eläinperäisistä aineista vapaat viljelyolosuhteet pitävät yllä rasvakudoksen kantasolujen perusominaisuuksia ja kyseisillä olosuhteissa voi olla edellytyksiä myös kliiniseen käyttöön. Rasvakudoksen kantasolujen huolellinen karakterisointi erilaisissa viljelyolosuhteissa, immunologisten omaisuuksien arviointi sekä solujen käyttäytymisen tutkiminen kliinisessä käytössä olevien biomateriaalien kanssa ovat ensiarvoisen tärkeitä soluterapioiden positiivisen kehityksen kannalta. Uudet tutkimustulokset rasvakudoksen kantasolujen ominaisuuksista tarjoavat työkaluja myös kantasoluhoitojen regulaatioon. Lisää solujen turvallisuutta ja tehokkuutta arvioivia prekliinisiä tutkimuksia kuitenkin tarvitaan ennen siirtymää kliinisiin soluhoitoihin.

Contents

Abbreviations

3D	three-dimensional
ALP	alkaline phosphatase activity
ANOVA	analysis of variance
aP2	adipocyte Protein 2
APC	allophycocyanin
ASC	adipose stromal/stem cell
ATMP	advanced therapy medicinal product
BAG	bioactive glass
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BM-MSC	bone marrow-derived mesenchymal stem cell
BrdU	bromodeoxyuridine
CAT	Committee for Advanced Therapies
CBA	cytometric bead array
CCK-8	Cell Counting Kit -8
CCL	C-C chemokine ligand
CD	cluster of differentiation
CFU-F	fibroblastic colony-forming unit
cDNA	complementary DNA
CM	control medium
Col-1	collagen type-1
CXCL	CXC chemokine ligand
D-MMC	differentiation under standard condition
D+MMC	differentiation under macromolecular crowding
DC	dendritic cell
Dlx5	distal-less homeobox transcription factor 5
DMEM	Dulbecco's modified Eagle's medium
E-MMC	expansion under standard condition
E+MMC	expansion under macromolecular crowding
ECM	extracellular matrix

ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESC	embryonic stem cells
EVE	excluded volume effect
Fabp4	adipocyte fatty-acid binding protein
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
Frw	forward
FVO	fractional volume occupancy
GAG	glycosaminoglycan
GMP	good manufacturing practice
GTP	good tissue practice
GVHD	graft-versus-host-disease
HA	hydroxyapatite
HCA	carbonated hydroxyapatite
HLA	human leucocyte antigen
HP	horseradish peroxidase
HS	human serum
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
IDO	indoleamine 2,3-deoxygenase
IFATS	International Federation of Adipose Therapeutics and Science
IFN-γ	interferon γ
IGF	insulin-like growth factor
IL	interleukin
iPSC	induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
MBCP	macro porous biphasic calcium phosphate
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMC	macromolecular crowding
MRI	magnetic resonance imaging
MSC	mesenchymal stromal/stem cell
NK	natural killer

OC	osteocalcin
OM	osteogenic medium
OPN	osteopontin
p/s	penicillin/ streptomycin
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PE	phycoerythrin
PECy7	phycoerythrin-cyanine
PFA	paraformaldehyde
PHA	phytohemagglutinin
PL	platelet lysate
PPARγ	peroxisome proliferator activated receptor γ
qALP	quantitative alkaline phosphatase activity
qRT-PCR	quantitative real-time polymerase chain reaction
Rev	reverse
RNA	ribonucleic acid
Runx2	runt-related transcription factor 2
SD	standard deviation
SF	serum-free
SFM	serum-free media
SOP	standard operating procedure
SVF	stromal vascular fraction
TGF-β1	transforming growth factor β1
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
XF	xeno-free
XF/SF	xeno-free/serum-free
XF/SF CM	xeno-free/serum-free medium with Coating Matrix Kit
XF/SF CS	xeno-free/serum-free medium with CELLstart™ coating
α-MEM	alpha modified Eagle's medium
β-TCP	β-tricalsium phosphate

Original publications

The present study is based on the following articles, which are referred to in the text by their Roman numerals (**I-IV**):

I. **Patrikoski M**, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerström B, Miettinen S. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy compliant human adipose stem cells. *Stem Cell Res Ther. 2013 Mar 7;4(2):27.*

II. **Patrikoski M**, Lee MHC, Ang XM, Mäkinen L, Mannerström B, Raghunath M, Miettinen S. Effect of macromolecular crowding on human adipose stem cell differentiation and proliferation under FBS, HS and defined XF/SF cultures. *Submitted.*

III. **Patrikoski M**, Sivula J, Huhtala H, Helminen M, Salo F, Mannerström B, Miettinen S. Different culture conditions modulate the immunological properties of adipose stem cells. *Stem Cells Transl Med. 2014 Oct;3(10):1220-30.*

IV. Waselau M, **Patrikoski M**, Juntunen M, Kujala K, Kääriäinen M, Kuokkanen H, Sándor GK, Vapaavuori O, Suuronen R, Mannerström B, von Rechenberg B, Miettinen S. Effects of bioactive glass S53P4 or betatricalcium phosphate and bone morphogenetic protein-2 and bone morphogenetic protein-7 on osteogenic differentiation of human adipose stem cells. *J Tissue Eng 2012 ;3(1)2041731412467789.*

1 Introduction

Tissue engineering and regenerative medicine are interdisciplinary fields that focus on the development of alternative therapies for the repair or replacement of damaged tissues and organs. To achieve an optimal clinical outcome, scaffolds and cell populations from different sources are combined in tissue engineering applications (Salgado *et al.*, 2013).

Human adipose tissue was previously described as worthless and unwanted; however, over the last 15 years it has emerged as a premiere source of cells for regenerative therapies (Kapur *et al.*, 2015). Human adipose stromal/stem cells (ASCs) are multipotent cells that have the ability to differentiate into adipogenic, chondrogenic, and osteogenic cells (Zuk *et al.*, 2001; Zuk *et al.*, 2002), and moreover, certain studies have demonstrated a much wider potential for differentiation, e.g., into neuronal cells or hepatocytes and beta islet cells (Tsuji *et al.*, 2014). In addition to wide differentiation potential, ASCs have a low immunogenic profile (McIntosh *et al.*, 2006; Niemeyer *et al.*, 2007) and a capacity for immunomodulation (Cui *et al.*, 2007; Kronsteiner *et al.*, 2011; Lee *et al.*, 2012; McIntosh *et al.*, 2006; Puissant *et al.*, 2005; Yoo *et al.*, 2009). Due to the mentioned characteristics, ASCs have potential for diverse clinical applications in regenerative medicine and immunomodulation therapies and have been the subject of preclinical and clinical studies directed toward numerous applications, as previously reviewed (Casteilla *et al.*, 2011; Lim *et al.*, 2014).

In delivery of effective stem cell treatments, cell expansion is often necessary to obtain sufficient cell numbers. In vitro cell expansion protocols traditionally use cultures based on fetal bovine serum (FBS) (Bunnell *et al.*, 2008; Gimble and Guilak, 2003; Haimi *et al.*, 2009b; Zuk *et al.*, 2001), despite the safety concerns associated with its clinical use. Alternatives to FBS have been studied, including autologous or allogeneic human serum (HS) (Josh *et al.*, 2012; Koellensperger *et al.*, 2014; Lindroos *et al.*, 2009), platelet lysate (PL) based cultures (Naaijkens *et al.*, 2012) as well as completely xeno-free/serum-free (XF/SF) cultures (Al-Saqi *et al.*, 2014a; Dromard *et al.*, 2011; Konno *et al.*, 2010; Lindroos *et al.*, 2009; Yang *et al.*, 2012); however, the search for optimal conditions for in vitro cell expansion remain ongoing. Currently, performed cell therapies have relied primarily on the use of autologous cells, whereas the use of allogeneic cells would be more straightforward because allogeneic stem

cells could theoretically be stored and offered to the patient as off-the-shelf products as necessary.

In stem cell therapies, cells are often combined with a suitable biomaterial, followed by transplantation into a patient. Thus, the scaffold structure serves as an initial foundation for cell attachment and also provide signals for cell differentiation. In bone tissue engineering, bioactive glass (BAG) and β-tricalcium phosphate (β-TCP) are commonly used as scaffold materials due to their osteoinductive abilities and superior biocompatibility with bone tissue (Baino and Vitale-Brovarone, 2011; Q. Z. Chen *et al.*, 2006; Haimi *et al.*, 2009a; Mesimaki *et al.*, 2009; Sandor *et al.*, 2013; Thesleff *et al.*, 2011; Vitale-Brovarone *et al.*, 2007; Yuan *et al.*, 2001). Tissue engineered constructs serve as an attractive alternative to bone autografts and allografts that have previously been considered as the golden standard for bone reconstruction.

This thesis work demonstrates the significance of culture conditions on ASC behavior with respect to clinical use. In this work, novel XF/SF culture conditions were developed and tested for their ability to maintain the basic stem cell characteristics of ASCs and compared with those of HS-based and traditional FBS-based cultures. The potential of ASCs for allogeneic treatments or immunomodulation therapies was also demonstrated by determining the immunogenic properties of ASC under the above-mentioned clinically relevant conditions. The osteogenic differentiation potential of ASCs was investigated in combination with clinically used BAG and β-TCP materials as well as the bone morphogenetic proteins -2 and -7 relative to their ability to support osteogenic commitment of ASCs.

2 Literature review

2.1 Stem cells

Stem cells are defined by two basic properties: the ability to self-renew for long periods of time and the ability to differentiate into one or more specialized cell types (Kolios and Moodley, 2013; Lerou and Daley, 2005; Thomson *et al.*, 1998). These basic properties make stem cells valuable tools for regenerative medicine because they possess the capacity to replace differentiated cells that are lost after disease or trauma or after natural cell apoptosis, and thus, the ability to prevent or repair tissue damage. However, these basic stem cell properties differ among various sources of stem cells, and they can be classified based on their origin and/or differentiation potential (Kolios and Moodley, 2013).

Embryonic stem cells (ESCs) have the ability to grow indefinitely and to differentiate into any type of cell in an organism (Thomson *et al.*, 1998). Totipotent cells are the least differentiated cells and exist only for a short period during development of a pre-implantation embryo (Alvarez *et al.*, 2012; Kolios and Moodley, 2013; Thomson *et al.*, 1998). A fertilized oocyte and the cells of the first two divisions, up to the 8-cell stage of morula, are totipotent cells capable of differentiating into both embryonic and extra-embryonic tissues, thereby forming the embryo and the placenta (Kolios and Moodley, 2013). After further cell divisions, ESCs can be derived from the inner cell mass of the blastocyst, and because they are pluripotent cells, they can differentiate into tissues of the three primary germ layers: ectoderm, endoderm and mesoderm (Kolios and Moodley, 2013).

Moreover, generation of pluripotent cells directly from a patient's own cells is possible today (Takahashi and Yamanaka, 2006). Induced pluripotent stem cells (iPSCs) share characteristics similar to those of ESCs but are derived from differentiated somatic cells and reprogrammed into a pluripotent state. Takahashi and Yamanaka demonstrated that four factors, i.e., Oct3/4, Sox2, c-Myc, and Klf4, are required to reprogram somatic cells back into an embryonic state (Takahashi and Yamanaka, 2006). This finding was a groundbreaking discovery that enabled researchers to create autologous human iPSC lines from somatic cells. These cells are useful tools for personal medicine, e.g., for study of difficult genetic diseases and

their pathophysiology and drug sensitivity (Lahti *et al.*, 2012). Although iPSCs and ESCs have numerous advantages, such as great self-renewal and differentiation potential, certain ethical and regulatory challenges remain connected to their use in regenerative medicine (Alvarez *et al.*, 2012; Csete, 2010). Additionally, the fact that ESCs and iPSCs possess teratoma-producing properties poses critical safety issues that should be carefully evaluated before further clinical use (Bongso *et al.*, 2008). A risk of immunorejection problems could also occur if stem cells are not a close tissue match to the patient (Bongso *et al.*, 2008).

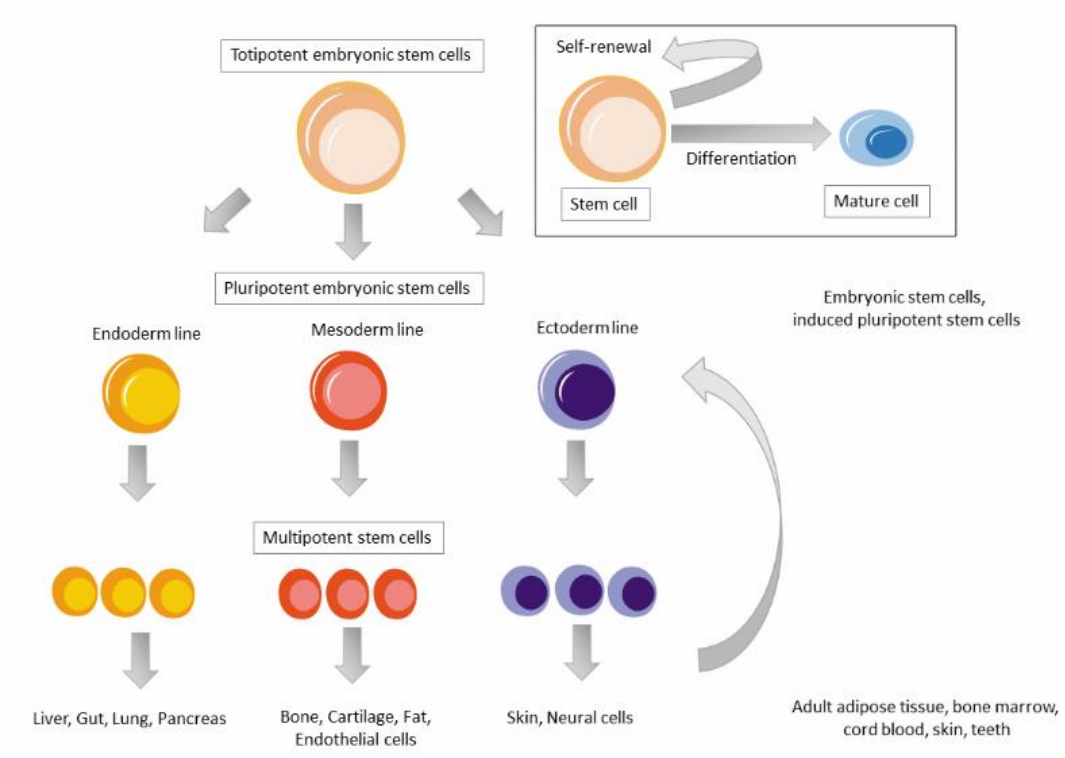

Figure 1. Hierarchy of stem cells and their basic properties: The ability to self-renew and to differentiate into specialized cell types. Totipotent stem cells exist for a short period of time during development of a pre-implantation embryo. Pluripotent embryonic stem cells (ESCs) can be derived from the inner cell mass of a blastocyst, whereas induced pluripotent stem cells (iPSCs) are obtained from somatic cells via genetic reprogramming. Multipotent adult stem cells are more restricted stem cells that can be obtained from several tissues of an adult individual.

Ethical problems also exist with respect to the use of human embryos as well as the problem of potential tissue rejection following transplantation in patients. Certain problems can be overcome with adult stem cells, which are multipotent cells capable of differentiating into one or several cell types; these cells are responsible for the maintenance and repair of the host tissue and have a limited self-renewal and differentiation capacity restricted by the particular tissue or organ (Choumerianou *et al.*, 2008; Kolios and Moodley, 2013). Adult stem cells can be derived from practically any tissue of an adult individual and can be divided into three categories according to the germ layer from which they originate: endodermal stem cells (such as pulmonary epithelial stem cells or gastrointestinal tract stem cells), mesodermal stem cells (such as bone marrow or adipose tissue derived stem cells), and ectodermal stem cells (such as neural and skin stem cells) (Choumerianou *et al.*, 2008).

2.2 Mesenchymal stem cells

Mesenchymal stromal/stem cells (MSCs) were first isolated and characterized from bone marrow in 1974 by Friedenstein *et al.* (Friedenstein *et al.*, 1974). The International Society for Cellular Therapy (ISCT) has recently recommended use of the term mesenchymal *stromal* cells; however, the term mesenchymal *stem* cells is also commonly used and approved among the scientific community. Since 1974, MSCs have been isolated from various tissues such as blood, umbilical cord blood, amniotic membrane, adult connective tissues, and adipose and dental tissues, as previously reviewed (Tatullo *et al.*, 2015). The MSCs are heterogeneous cell populations that are plastic-adherent under standard culture conditions and express the mesenchymal stem cell markers CD73, CD90 and CD105; lack hematopoietic markers CD14, CD19, CD34, CD45, and human leukocyte antigen (HLA)-DR; and have the capacity to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (Dominici *et al.*, 2006) (see Section 2.5.1). Apart from their differentiation capacity, MSCs have immunomodulatory functions that are exerted by direct cell-cell contacts, secretion of cytokines or by combination of both mechanisms (Fernandez Vallone *et al.*, 2013; Kyurkchiev *et al.*, 2014) (see Section 2.6).

It is also hypothesized that MSCs are a subgroup of vessel-lining pericytes that can be found in multiple organ systems (Crisan *et al.*, 2008) and are involved in vessel homeostasis by reacting to tissue damage with regenerative processes (Mosna *et al.*, 2010). Crisan *et al.* showed that these cells express markers typical of MSCs and may be present in virtually any vascularized tissue throughout the human body and have

the potential to differentiate towards myocytes, osteocytes, chondrocytes, and adipocytes. Consequently, these researchers suggested that pericytes may be potential progenitor cells to non-bone marrow-derived MSCs, but the true identity of MSCs is still under debate, and MSC-like cells are also found in avascular tissues (Lv *et al.*, 2014).

Although MSCs isolated from different sources have similar characteristics, a certain amount of variation in abundance, phenotype, differentiation potential and immunomodulatory capabilities has been reported (Hanley, 2014). Bone marrow-derived mesenchymal stem cells (BM-MSCs) are likely the most frequently studied MSCs, and most clinical trials to date have used BM-MSCs. Still, multiple passages, each accompanied by multiple cell doublings, are usually required to reach clinically relevant cell doses due to relatively low MSC numbers in bone marrow. A bone marrow transplant contains approximately 6×10^6 nucleated cells per mL, of which only 0.001–0.01% are stem cells (Baer and Geiger, 2012). By comparison, the number of stromal vascular fraction (SVF) cells per gram of adipose tissue is approximately $0.5\text{-}2.0\times10^6$, whereas the percentages of stem cells range from 1% to 10%. Thus, a more than 2,500-fold frequency of stromal/stem cells can be found from the uncultured SVF compared with that of bone marrow (Baer and Geiger, 2012). In addition, superior immunosuppressive capabilities of ASCs have been reported compared with BM-MSCs (Najar *et al.*, 2010). However, certain studies have suggested that the osteogenic capacity of adipose tissue-derived MSCs is lower than that of BM-MSCs (G. I. Im *et al.*, 2005; Liao and Chen, 2014; Lotfy *et al.*, 2014). Indeed, it is suggested that the tissue of origin regulates the epigenetic memory of MSCs, and thus, their subsequent differentiation potential is somewhat determined (Eilertsen *et al.*, 2008). Another potential harvest site of MSCs is cord blood, but this source has the lowest frequency of stem cells at approximately 0.00003 % of all cells in the cord blood unit (Peters *et al.*, 2010); however, the extensive proliferation potential of cord blood-derived MSCs has been reported (Bieback *et al.*, 2004). Additionally, low oxygen conditions have been used for efficient isolation and expansion of cord blood-derived MSCs (Laitinen *et al.*, 2011).

2.3 Adipose stem cells

Katz, Zuk and co-workers were first to show that the SVF isolated from human lipoaspirates contained cells with multilineage potential, and they referred to theses cells as processed lipoaspirate cells (Katz *et al.*, 1999; Zuk *et al.*, 2001). Since then

adipose tissue has been extensively studied as a cell source for tissue engineering and regenerative medicine. As opposed to bone marrow-derived MSCs, adipose-derived MSCs can be obtained from adipose tissue using a minimally invasive procedure that results in a high number of cells (Tsuji *et al.*, 2014); one milliliter of lipoaspirate can yield as much as 1 to 6 million ASCs (Aust *et al.*, 2004; Suga *et al.*, 2010). Zuk *et al.* introduced a widely used method for ASC isolation in 2001 (Zuk *et al.*, 2001) in which the adipose tissue is first mechanically minced followed by enzymatic digestion. After centrifugation, the resulting pellet is referred to as the SVF from which ASCs are selected based on their plastic adherence property. Because ASCs are of mesodermal origin, they have the potential to differentiate into adipogenic, chondrogenic, and osteogenic cells (Zuk *et al.*, 2001; Zuk *et al.*, 2002). In addition, certain studies suggest that ASCs have the potential to differentiate into cells of ectodermal and endodermal origin such as neuronal cells or hepatocytes and beta islet cells, as previously reviewed (Tsuji *et al.*, 2014).

2.3.1 Adipose stem cells differentiation

Lineage-specific induction media is used in vitro to direct ASC differentiation toward a desired direction. This section describes the typical media compositions for ASC multilineage differentiation; however, certain variation in protocols exist among different laboratories. To induce ASCs into adipogenesis, cells are cultured in the presence of isobutylmethylxanthine (IBMX), insulin, indomethacin, pantothenate and biotin (Brayfield *et al.*, 2010; Lindroos *et al.*, 2011). Following induction, ASCs develop multiple lipid droplets and express several types of extracellular matrix (ECM) proteins, including fibronectin, laminin, and various types of collagen. As the cells differentiate into mature adipocytes in 2 to 3 weeks, the lipid vacuoles accumulate inside cells and the cells express agipogenesis-specific genes, such as *lipoprotein lipase (LPL)*, *proliferator-activated receptor γ (PPARγ)*, *adipocyte fatty-acid binding protein (FABP4/aP2)* and *leptin* (Lindroos *et al.*, 2011). Lipid droplets containing triglycerides can be detected histologically using Oil red O or Nile Red staining.

For osteogenic differentiation, ASCs are cultured in the presence of ascorbate-2-phosphate, β-glycerophosphate, dexamethasone and/or 1,25 vitamin D_3 (Gimble and Guilak, 2003; Halvorsen *et al.*, 2001). After 2 to 4 weeks of induction, the differentiated cells begin to produce calcium phosphate within the ECM which can be assessed with Alizarin Red or von Kossa staining. During osteogenesis, several genes are typically upregulated, such as *alkaline phosphatase*, *type I collagen*, *osteopontin*

(OPN), *osteocalcin (OC)*, *bone sialoprotein*, *runt-related transcription factor 1 (Runx-1)*, *BMP-2*, *BMP-4*, *parathyroid hormone receptor*, *BMP receptor 1* and *2 phosphatase* (Tsuji *et al.*, 2014).

To undergo chondrogenic differentiation, ASCs require 3D micro-mass or pellet culture systems. The micro-mass pellet culture model mimics pre-cartilage condensation during embryonic development, which increases cell-to-cell interactions and leads to the production of a cartilage-like matrix (Wei *et al.*, 2007). For chondrogenic inductions, ASCs are cultured in defined SF media in the presences of TGF-β1, insulin, dexamethasone, ascorbate-2-phosphate, and BMP-6 (Tsuji *et al.*, 2014). After differentiation, chondrocytes begin to secrete the extracellular matrix proteins of cartilage, such as type II and IV collagen, aggrecan, prolyl endopeptidase, and sulfate-proteoglycan (Lindroos *et al.*, 2011; Tsuji *et al.*, 2014). Chondrogenic differentiation of micro-mass cultures can be detected by Alcian blue, collagen type II, Safranin O and Toluidine blue stainings.

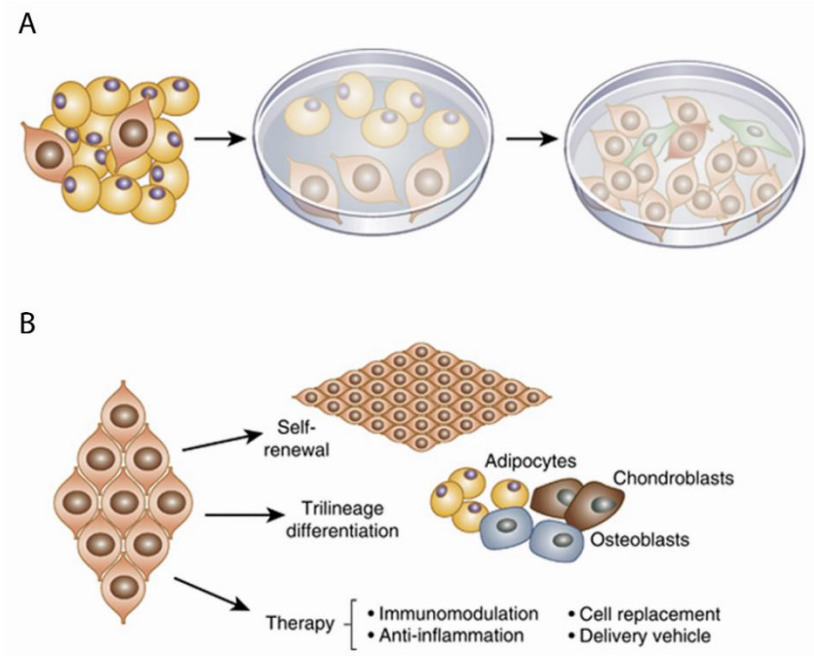

Figure 2. Isolation, characterization, and therapeutic use of adipose stem cells. (A) ASCs can be isolated based on their plastic-adherence capacity. After initial cell isolation, the cell population remains heterogeneous and is composed of ASCs, stromal cells, and endothelial cells, at minimum. (B) ASCs are characterized by their properties of self-renewal and differentiation into adipocytes, chondroblasts and osteoblasts. These cells could potentially be used to treat diseases by providing immunomodulation, anti-inflammatory actions, and cell replacement as well as delivery of therapeutic agents. Modified from Lin (F. Lin, 2012).

2.3.2 Adipose stem cell heterogeneity

One important characteristic of ASCs is their rather heterogenic cell population (H. Li *et al.*, 2011; Zuk *et al.*, 2002). Accordingly, there is no single unique surface marker for ASCs, but these cells express typical mesenchymal markers such as CD13, CD29, CD44, CD63, CD73, CD90, and CD105 and are negative for hematopoietic markers such as CD14, CD31, CD45, and CD144 (Section 2.5.1). During the first passages, ASCs show positive expression for CD34, which decreases after further passaging (Mitchell *et al.*, 2006), as well as other hematopoietic markers such as CD11a/b and CD14. In contrast, the expression of MSC markers CD73, CD90 and CD166 may increase from SVF to later passages (McIntosh *et al.*, 2006; Mitchell *et al.*, 2006). Passaging is considered to select for a cell population with a more homogenous cell surface marker profile as compared with that of SVF.

In addition to passaging, the basic characteristics of ASCs may be affected by several factors, e.g., age and sex of the adipose tissue donor as well as the anatomic harvest location. It has been demonstrated that ASCs isolated from aged donors (>60 years) display senescent features (reduced viability, proliferation and differentiation potential) compared with cells isolated from younger (<30 years) donors (Alt *et al.*, 2012; Choudhery *et al.*, 2014). The growth kinetics and the osteogenic and chondrogenic differentiation potentials of ASCs were negatively affected by increased donor age; however, the adipogenic potential appeared to be maintained during aging. A similar negative correlation between donor age and proliferation efficiency was observed by Van Harmelen *et al.* (Van Harmelen *et al.*, 2004), who also showed that stromal cells from the subcutaneous adipose tissue region proliferated more efficiently than those from the omental region, but no regional difference in differentiation potential was observed. In fact, the depth of adipose tissue harvest appears to be critical to function (Baglioni *et al.*, 2012). Baglioni *et al.* demonstrated that subcutaneous ASCs have significantly higher proliferation rate and adipogenic potential compared with ASCs derived from visceral omental fat pads. Further, Schipper *et al.* observed variability in apoptotic susceptibility of ASCs that were harvested from different subcutaneous fat tissues (Schipper *et al.*, 2008). Cells from superficial abdominal fat were significantly more resistant to apoptosis compared with 4 other tested subcutaneous fat tissue locations, and the greatest number of cells were recovered from the arm fat compared with the thigh, abdomen, and breast. Additionally, it was demonstrated that male gender has an effect on ASC differentiation with more rapid and effective osteogenic potential of ASCs from male donors compared with female donors (Aksu *et al.*, 2008).

2.4 Culture of ASCs

2.4.1 Good manufacturing practice regulations

As cell-based therapies become more common, GMP facilities that perform advanced cell manipulation must be well controlled to ensure safe and efficient treatment for patients. In Europe, the Committee for Advanced Therapies (CAT) at the European Medicines Agency (EMA) regulates the use of ASC-based tissue engineering products that are defined as advanced therapy medicinal products (ATMPs) (Schneider *et al.*, 2010). The ATMPs are medicines for human use that are based on gene therapy, somatic-cell therapy or tissue engineering (Ilic *et al.*, 2012) and are often at the forefront of innovation, offering potential treatment opportunities for diseases that currently have limited or no effective therapeutic options (Schneider *et al.*, 2010). The regulations on ATMPs provide a consistent legal system covering the collection, testing, processing, storage and distribution of human tissues, cells and blood as well as the manufacturing of ATMPs made from human materials. The European regulations are similar to the regulatory framework set up in the United States (US), although it is much more standardized, systematic and comprehensive (Rehmann and Morgan, 2009). In the US, stem cell -based products are regulated under the authority of the Food and Drug Administration (FDA) (Fink, 2009), which implements a risk-based approach to the regulation of human cells. This regulation focuses on three general areas: limiting the risk of transmission of disease from donors to recipients, establishing manufacturing practices (GMP) that minimize the risk of contamination, and requiring an appropriate demonstration of safety and effectiveness (European Medicines Agency, 2011). Similar principles apply to European standards, and both the FDA and EMA classify procedures according to the degree of manipulation involved and the risk of adverse processing-related events (Fink, 2009; Schneider *et al.*, 2010). Minimal manipulation, such as cryopreservation of autologous cells, can be performed using good tissue practices (GTPs), which is a less-defined standard used mostly in industries. However, processes beyond minimal manipulation or substantial manipulation, e.g., ex vivo expansion, activation, combination with non-tissue components, or use for other than the tissue's normal function, requires a higher degree of process control designated as good manufacturing practices (GMPs) (Ancans, 2012; Burger, 2000; Schneider *et al.*, 2010). The term, substantial

manipulation, denotes that the biological characteristics, functions, or properties relevant for the therapeutic effect have been altered (Ancans, 2012).

Good manufacturing processes are a component of quality assurance that ensures products are consistently produced and controlled by the quality standards appropriate to their intended use. The unifying concept that underlies all aspects of GMP is control, and GMP can be understood as unified group of processes that are highly controlled (Burger, 2000). The GMP regulations can be divided into ten elements that concentrate on different aspects of the cell-based product manufacture: production and process, personnel management, record keeping, calibration, validation, error management, standard operating procedures (SOPs), labeling, quality control and auditing, and facilities and equipment. To ensure control over each aspect of GMP and to document that control, GMP facilities that perform advanced cell therapies are required for well-controlled protocols. In stem cell therapies, ex vivo cell expansion should be reproducible, robust, and efficient according to GMP. This process requires fully defined culture conditions that enable efficient cell proliferation without risk of genomic instability, as well as maintenance of basic stem cell characteristics such as immunophenotype and differentiation capacity (Sensebe *et al.*, 2013). In the following chapters, the effects of culture media on quality, safety and efficacy of ASCs are discussed.

2.4.2 Standard ASC cultures

In vitro culture of ASCs requires optimal conditions that support the maintenance of cells in their undifferentiated state as well as their differentiation after induction. Traditionally, cell culture media has consisted of basal media such as alpha modified Eagle's medium (α-MEM) or Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F-12) supplemented with 10% serum, 1% antibiotics (usually penicillin or streptomycin), and 1% L-glutamine (Sotiropoulou *et al.*, 2006a; Zuk *et al.*, 2001). The amount of glucose in cell culture media can range from 1 g/L (5.5 mM) to 4.5 g/L (25 mM); however, a commonly used basal media for ASC expansion such as DMEM/F-12 contains 17.5 mM D-glucose which represents a diabetic level of glucose supplementation (Saki *et al.*, 2013). Furthermore, FBS is typically used in ASC cultures (Bunnell *et al.*, 2008; Gimble and Guilak, 2003; Haimi *et al.*, 2009b; Zuk *et al.*, 2001) because it offers a cocktail of growth factors, cytokines, adhesion proteins and other nutrients for the cells (Wang *et al.*, 2000). However, FBS is of animal origin and thus would be recommended for replacement with XF alternatives

for cell therapy purposes. In fact, allogeneic HS is a XF alternative for FBS with similar properties. Supplementation with HS has an enhanced or equivalent effect on ASC doubling times compared with FBS (Josh *et al.*, 2012; Koellensperger *et al.*, 2014; Lindroos *et al.*, 2009; Tateishi *et al.*, 2008), and no substantial differences in cell morphology or immunophenotype have been observed between cells cultured in HS- versus FBS-supplemented medium (de Paula *et al.*, 2013; Josh *et al.*, 2012). In contrast, Lindroos *et al.* have reported that early osteogenic differentiation was significantly enhanced in FBS medium compared with HS medium, and at least 15% HS was required to reach cell proliferation levels comparable with those of 10% FBS (Lindroos *et al.*, 2010). These observations were contradictory to previous published studies (Josh *et al.*, 2012; Lindroos *et al.*, 2009; Tateishi *et al.*, 2008) in which enhanced proliferation rates in HS medium were demonstrated. A similar favorable effect of HS medium on ASC proliferation and osteogenic differentiation was recently observed by Kyllönen *et al.* (Kyllonen *et al.*, 2013). These variable results may be due to lot-to-lot variation within serum-supplemented media performance, which can affect the proliferation rate and differentiation potential (Parker *et al.*, 2007). Therefore, the safety and quality of transplanted ASCs could be enhanced by replacing undefined and/ or animal-derived components with fully defined GMP-compliant XF and SF reagents that are more reproducible, robust, and efficient (Al-Saqi *et al.*, 2014a; Chase *et al.*, 2012; Lindroos *et al.*, 2009).

2.4.3 XF and/or SF cultures

During clinical cell therapies, ASCs are often expanded in vitro to obtain clinically sufficient cell number for effective treatments. However, if ASCs are cultured in the presence of FBS, zoonoses are potentially transferred into the patient, which could cause severe sequelae related to xenogeneic infections (Kadri *et al.*, 2007; Selvaggi *et al.*, 1997). Furthermore, antibodies towards bovine antigens may be produced in successive administration of cells, which can affect the efficacy of cell-based treatments. Due to these concerns associated with the use of animal-derived components (Mackensen *et al.*, 2000; Selvaggi *et al.*, 1997) and the variability of media performance (Parker *et al.*, 2007), there is growing interest in development of novel cultivation media for ASCs. Various XF and/or SF alternatives have been developed and studied for ASC cultures but relatively few formulations are commercially available. The XF/SF conditions should be safe and efficient in terms of cell proliferation and differentiation if designated for clinical use.

One approach to replace FBS from cell culture is the use of human platelet-derived supplements. Schallmoser *et al.* (Schallmoser and Strunk, 2009) introduced a standard protocol for the preparation of pooled human PL. Trojahn Kølle *et al.* cultured ASCs in PL-based media and reported high proliferation rates, retained multipotency and chromosomal stability (Trojahn Kolle *et al.*, 2013). Furthermore, results of increased proliferation rates and retained differentiation capacity in PL cultures were reported by Naajikens *et al.* (Naaijkens *et al.*, 2012). Additionally, the suitability of PL for BM-MSC cultures was recently demonstrated by Laitinen *et al.*, who reported a robust and reproducible PL-based culture method in which the cells maintained their normal karyotype, typical immunophenotype and differentiation potential (Laitinen *et al.*, 2015). However, contradictory results are also available in which the ASC population doubling time in PL cultures was significantly lower compared with that in FBS media, but similar immunophenotype and differentiation potential was maintained (Blande *et al.*, 2009). In addition to the above-mentioned studies, several promising results have been reported that demonstrate the suitability of PL for ASC cultures (Hildner *et al.*, 2013; Kocaoemer *et al.*, 2007; Schallmoser and Strunk, 2013; Shih *et al.*, 2011; Torensma *et al.*, 2012).

Additionally, completely serum-component-free culture conditions for ASCs have been investigated, but few studies exist in which both cell isolation and expansion as well as cell differentiation were performed using only SF/XF reagents. However, encouraging results have been observed by several researchers who cultured adipose stem cells in chemically defined SF media (Al-Saqi *et al.*, 2014a; Dromard *et al.*, 2011; Konno *et al.*, 2010; Lindroos *et al.*, 2009; Yang *et al.*, 2012). Al-Saqi *et al.* tested defined SF and XF Mesencult-XF media (Stem Cell Technologies) and observed a shorter population doubling time, stable morphology and immunophenotype, and a higher differentiation potential compared with FBS cultures (Al-Saqi *et al.*, 2014a). Dromard *et al.* demonstrated successful SF-culture systems in which ASCs were expanded as floating spheres (Dromard *et al.*, 2011), and Santos *et al.* investigated a microcarrier-based bioreactor system for XF/SF culture of ASCs (Santos *et al.*, 2011). Lindroos *et al.* and Yang *et al.* both tested XF/SF Stem Pro media (Life Technologies) and observed significantly higher proliferation rates while maintaining differentiation potential and surface marker expressions (Lindroos *et al.*, 2009; Yang *et al.*, 2012). It was also demonstrated that XF/SF cryopreservation of ASCs is feasible and does not affect basic stem cell characteristics such as multipotency, proliferation or immunophenotype (Al-Saqi *et al.*, 2014b).

Table 1. Commercially available SF media, coatings and manufacturers.

Product	*Coating*	*Manufacturer*	*Reference*	*Webpage*
STEMPRO® MSC SFM	CELLstart™	Life Technologies	(Agata *et al.*, 2009)	www.lifetechnologies.com
MesenCult™-XF	MesenCult™-SF Attachment Substrate	Stem Cell™ Technologies	(Al-Saqi *et al.*, 2014a)	www.stemcell.com
StemXVivo Serum-Free Human MSC Expansion Media	Fibronectin	R&D systems	(Gottipamula *et al.*, 2013)	www.rndsystems.com
MSC NutriStem® XF	MSC Attachment Solution	Biological Industries	(Genser-Nir *et al.*, 2013)	www.bioind.com
TheraPEAK™ MSCGM-CD™	Fibronectin	Lonza Walkersville	(Trubiani *et al.*, 2015)	www.lonza.com
StemMACS MSC Expansion Media Kit XF	None	Miltenyi Biotec		www.miltenyibiotec.com
Mesenchymal Stem Cell Growth Medium DXF	Fibronectin	PromoCell		www.promocell.com

Furthermore, a few commercially available SF media have been introduced for MSCs expansion, and the media and coatings used are presented in Table 1. Several bioscience companies such as Life Technologies, Stem Cell™ Technologies, R&D systems, Lonza Walkersville and Miltenyi Biotec provide their own XF and/or SF culture media that are typically offered together with a coating supplement to support XF/SF cell attachment. Two of the most frequently used SF media are the STEMPRO® MSC SFM (Agata *et al.*, 2009; Chase *et al.*, 2012; Lindroos *et al.*, 2009) from Life Technologies and MesenCult™-XF media (Al-Saqi *et al.*, 2014a; Swamynathan *et al.*, 2014) from Stem Cell™ Technologies. However, it is likely that additional XF and/or SF culture protocols for ASCs will be developed in the near future because the need for safe and efficient culture conditions is increasing as clinical use of ASCs becomes more common.

In conclusion, ASCs in XF/SF culture media show higher proliferation rates compared with traditional serum-containing medium, which is a promising result for considering the use of ASCs in clinical applications in which efficient cell expansion

protocols are essential. However, the proliferation capacity of ASCs may be diminished sooner in XF/SF conditions compared with cells expanded in serum-containing medium (Lindroos *et al.*, 2009) and thus, population doubling studies at high passages would be justified in XF/SF media. Moreover, XF/SF cultures typically require coating of cell culture plastics to ensure proper cell attachment, which is highly important for cell functions and changes of matrix architecture during ASC differentiation (J. M. Kang *et al.*, 2012; I. S. Park *et al.*, 2009). Additional supplementation of growth factors such as basic fibroblast growth factor (bFGF) or transforming growth factor beta (TGF)-β also may be required in defined SF cultures (Jung *et al.*, 2010; van der Valk *et al.*, 2010). Consequently, cell attachment and differentiation under XF/SF cultures may be insufficient without additional growth factors or coatings; however, the coating may also select for a different type of cell populations compared with that of standard serum-based cultures. Further investigation should be conducted on the possible selective effect of coating prior to clinical use of XF/SF expanded cells. However, several studies have demonstrated that ASCs maintain their immunophenotype and differentiation potential under XF/SF conditions (Al-Saqi *et al.*, 2014a; Dromard *et al.*, 2011; Lindroos *et al.*, 2009; Sato *et al.*, 2015) and thus, the cell characteristics should be similar to those of cells expanded in serum-containing medium. A defined GMP-grade cell product of good quality and efficient performance is critical for positive development of cellular therapies, and these fully defined, regulatory-friendly XF/SF conditions represent one step closer to that goal.

2.4.4 Macromolecular crowding

Traditionally, the composition of culture medium has been maintained plain and simple and only reagents that are vital for the cell growth and proliferation have been provided, although this approach is opposite to any biological system. Inside the human body, cells face a microenvironment that is physiologically crowded with soluble factors, other cells and extracellular matrix. The typical biological fluid concentrations are, e.g., 30-70 g/L in interstitial fluid, 80 g/L in blood plasma and even 200-350 g/L in cell cytoplasm, which are significantly higher compared with typical in vitro cell cultures with concentrations of 1-10 g/L (Zeiger *et al.*, 2012). The in vitro conditions differ greatly from the original tissue microenvironment from which the cells are derived; however, the cell microenvironment plays a central role in stem cell maintenance in the undifferentiated state as well as in cell differentiation.

Macromolecular crowding (MMC) is a method that can be used to support cells in re-creating their own microenvironment in vitro. When macromolecules are added to cell culture media, they function via the excluded volume effect (EVE) (Figure 3) (C. Z. Chen *et al.*, 2011). The volume surrounding a given molecule becomes unavailable for the other molecules, and thus, the effective concentrations of different macromolecules in the solution are higher. The amount of EVE is dependent on the fraction volume occupancy (FVO), which is defined as the fraction of the total volume occupied by macromolecules. Macromolecular crowders can generate a high level of FVO, which greatly influences the thermodynamic states of equilibria and rates of biochemical reactions. The free energy of the system is reduced by non-covalent associations or conformational changes, e.g., protein and nucleic acid synthesis, intermediary metabolism, cell signaling, gene expression, and fibril formation (C. Z. Chen *et al.*, 2009; C. Z. Chen *et al.*, 2011; Zeiger *et al.*, 2012; H. X. Zhou *et al.*, 2008).

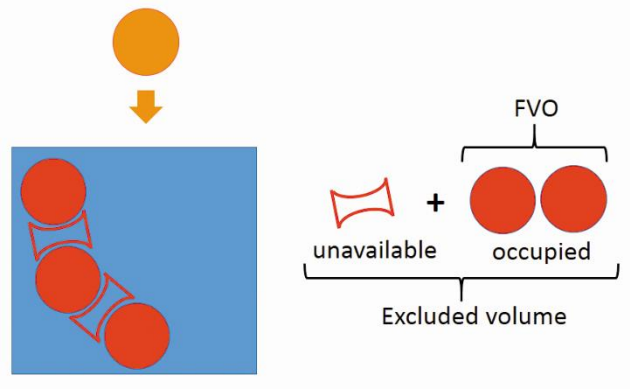

Figure 3. Schematic representation of the generation of excluded volume effect (EVE) through the presence of macromolecules. The volume surrounding a given molecule (red) becomes unavailable for the other molecules (orange).

Several macromolecules have been tested for suitability as MMC agents. Polyethylene glycol is widely used for crowding purposes but it may have undesirable interactions with other proteins present in the in vitro cultures (H. X. Zhou *et al.*, 2008). Better results have been shown with Ficoll (trademark of GE Healthcare), which is an uncharged, small and non-cytotoxic polymer of sucrose that dissolves readily in aqueous solutions without notable alterations in viscosity. Because of its neutrality, this polymer does not interact with or alter ordinary charge-dependent cellular reactions. Zeiger *et al.* reported the effects of MMC on mesenchymal stem cells using a mixture of 70 kDa and 400 kDa Ficoll at physiological levels as a crowding agent (Zeiger *et al.*, 2012). This study compared intra- and extracellular protein organization between cultures in the presence (+MMC) and absence (-MMC) of MMC showing that crowding aligned the ECM organization even in the

absence of cellular interaction. Furthermore, MMC affected cell-matrix interactions and promoted adhesion by also exerting an influence on the formation and structure of the cytoskeleton. As a consequence, organized cells secreted additional matrix proteins to enhance its formation in a feedback-like manner. By promoting cell-matrix interaction and adhesion, the crowding also increased cell proliferation and decreased the cells' ability to migrate (Zeiger *et al.*, 2012) (Figure 4).

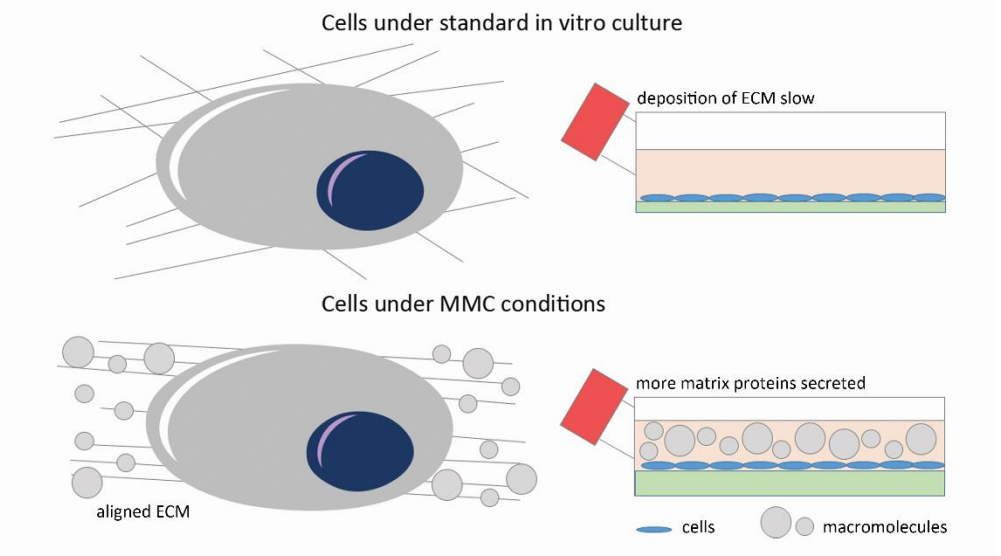

Figure 4. Schematic illustration of cell-matrix interactions and ECM secretions under standard in vitro culture compared with MMC conditions.

2.5 Characterization of ASCs

The International Federation of Adipose Therapeutics (IFATS) and ISCT have established phenotypic and functional criteria for characterization of SVF as well as ASCs. As the IFATS and ISCT have stated, adipose-derived stem cell research takes place in a dynamic field that requires further standardization, and thus, guidance in support of safety and biologic clarifications for clinical practices was provided by IFATS and ISCT (Bourin *et al.*, 2013). The final aim of those instructions is to develop efficacious adipose tissue-derived cell therapies that benefit society in an optimal manner.

The initial cell fraction that is gained after tissue processing is known as the stromal vascular fraction, which is separated from mature adipocytes by differential centrifugation. The SFV contains a heterogeneous mesenchymal cell population, e.g., cells of endothelial, hematopoietic and pericytic origin and many others (Table 2). The adipose tissue collection site and the digestion protocol may affect the heterogeneity of SVF cell types. When SVF cells are seeded into cell culture, a subset of elongated cells begins to adhere to the tissue culture plastic ware that select the ASCs.

Table 2. Cell populations in stromal vascular fraction. Table modified from Bourin *et al.* (Bourin *et al.*, 2013).

Hematopoietic-lineage cell	Stem and progenitor cells	<0.1%
	Granulocytes	10–15%
	Monocytes	5–15%
	Lymphocytes	10–15%
Endothelial cells		10–20%
Pericytes		3–5%
Stem cells		15–30%

2.5.1 Surface marker expression of ASCs

No single markers are available for recognition of ASCs, but instead, the use of a multi-color identification panel of several cell surface markers is recommended. Additionally, a viability marker is also suggested to eliminate dead or apoptotic cells induced by the isolation procedures. According to recommendations by the IFATS and ISCT, ASCs should be negative (<2%) for hematopoietic markers such as CD14 or CD11b, CD45, CD86, and HLA-DR and positive (>90%) for stromal markers such as CD13, CD73, CD90 and CD105. Similar to ASCs, BM-MSCs should be negative for hemopoietic markers CD11b and CD45, and express stromal markers CD13, CD73, CD90 and CD105. To distinguish ASCs from BM-MSC, use of two other markers is proposed, i.e., CD36 (fatty acid translocase) and CD106 (VCAM-1). In contrast to BM-MSCs it has been reported that ASCs do not express CD106 but are positive for CD34 (Katz *et al.*, 2005; Maumus *et al.*, 2011; Pachon-Pena *et al.*, 2011). However, the expression of CD34 is greatly dependent on the in vitro culture period. It is generally expressed during the early phase of culture, but its expression decreases with continued cell division (Maumus *et al.*, 2011; Mitchell *et al.*, 2006). In contrast to ASCs, BM-MSCs do not express CD34 (Liao and Chen, 2014). Of note,

CD34 is a marker that is generally used for hematopoietic stem and progenitor cells (Bensinger *et al.*, 1993; Trischmann *et al.*, 1993) but is also highly expressed in vascular endothelial cells and their precursors (Asahara *et al.*, 1997; Rafii *et al.*, 1994). Multiple classes of CD34 antibodies exist that recognize unique immunogens. Consequently, the choice of CD34 antibody can substantially influence the signal intensity detected on a given cell population, and therefore the use of class III CD34 antibodies is highly recommended. Moreover, histological analysis of adipose tissue has revealed that CD34 positive cells are widely distributed among adipocytes and are primarily associated with vascular structures (Traktuev *et al.*, 2008). Although small numbers of these cells are probably CD31 positive capillary endothelial cells, a CD34+/CD31- cell population of pericytic origin may be derived from adipose tissue (Johal *et al.*, 2015).

Furthermore, additional markers will still strengthen the characterization. Bourin *et al.* suggested that CD10, CD26 (DPPIV), CD49d (VLA4), CD49e (VLA5) and CD146 (MUC18) can be added as additional positive markers but with variable expression depending on the donor or culture passage. In contrast, minor expression (<2%) levels can be observed with additional negative markers CD3, CD11b (Mac-1), CD49f (VLA6) and Podocalyxin-like protein. However, when ASCs are defined using basic surface antigens, it is likely that ASC populations will display heterogeneity for additional surface antigens (Pachon-Pena *et al.*, 2011). In contrast, flow cytometry analysis can be considered as a definition of the relative homogeneity or alternatively, the relative heterogeneity of the ASCs. Guidelines for characterization of ASCs and SVF are collected in Table 3.

Of note, these characterization criteria were originally determined for ASCs cultured in traditional FBS culture medium, but the IFATS and ISCT do not take a stand on the effect of serum conditions on cell surface marker expression. For instance, Bourin *et al.* stated that one main difference between SVF cell and ASC populations is the high level of CD45+ cells in the SVF cells and a notably low or undetectable level in ASCs. This definition applies to ASC cultures in FBS or HS conditions, but somewhat higher expression of CD45 has been observed in defined XF/SF conditions (Rajala *et al.*, 2010). Overall, cell phenotypes still remain highly similar when cultured in different serum conditions or defined XF/SF conditions (Al-Saqi *et al.*, 2014a; Rajala *et al.*, 2010).

In addition to cell surface markers, the fibroblastoid colony-forming unit (CFU-F) assay is recommended for use in defining the number of progenitor cells. The number of colonies allows for an estimation of the rate of doubling of the population during the primary phase of culture. The information gained from CFU-F could be

particularly useful in enhancing the quality control of any resulting cell therapy product (Bourin *et al.*, 2013).

Table 3. Guidelines for characterization of adipose tissue-derived cells. Modified from Bourin *et al.* (Bourin *et al.*, 2013).

Feature	Assay	Cells of SVF	ASCs
Immunophenotype	Flow cytometry	Primary stable positive markers for stromal cells: CD13, CD29, CD44, CD73, CD90 (>40%), CD34 (<20%) Primary negative markers for stromal cells: CD31 (<20%), CD45 (<50%).	Primary stable positive markers: CD13, CD29, CD44, CD73, CD90, CD105 (>80% in ASC)
			Primary unstable positive marker: CD34 (present at variable levels)
			Primary negative markers: CD31, CD45, CD235a (<2%)
			Secondary other positive markers: CD10, CD26, CD36, CD49d, CD49e
			Secondary other low or negative markers: CD3, CD11b, CD49f, CD106, PODXL
Adipogenic differentiation	Histochemistry, RT-PCR, Western blot immunoblot, ELISA		Histology: oil red O, Nile red or stain specific for lipid inclusions Biomarkers: adiponectin, C/EBPα, FABP4, leptin, PPARγ
Osteogenic differentiation			Histology: alizarin red or von Kossa Biomarkers: alkaline phosphatase, bone sialoprotein, osteocalcin, osterix, runx2
Chondrogenic differentiation			Histology: alcian blue or safranin O Biomarkers: aggrecan, collagen type II, Sox 9
Proliferation and frequency	CFU-F	Anticipated frequency: >1%	Anticipated frequency: >5%

2.5.2 Differentiation potential of ASCs

One of the characterization criteria of ASCs is their multipotency and ability to give rise to osteoblastic, chondrocytic and adipocytic lineages (Gimble and Guilak, 2003; Zuk *et al.*, 2001; Zuk *et al.*, 2002). However, as demonstrated with BM-MSCs, long-term ex vivo culture of MSCs may lead to a loss of osteogenic differentiation capacity (Banfi *et al.*, 2000; Noer *et al.*, 2009). A recent study compared the impact of aging on the regenerative properties of MSCs from different tissue sources and showed that BM-MSCs may display impaired proliferation and chondrogenic response, whereas ASCs exhibited no negative effects on cell differentiation (Beane *et al.*, 2014). This same study also demonstrated that the osteogenic differentiation potential should be retained after long-term ex vivo culture with MSCs from different tissue sources. Nevertheless, protocols that induce cell differentiation have been published extensively. Although a qualitative evaluation of cell differentiation based on histochemistry is helpful, the IFATS and ISCT have stated that it may not be sufficient for comprehensive analysis. A better approach for characterization of differentiation is the use of quantitative methods such as RT-PCR, western blot immunoblot, and ELISA assay. A selection of lineage-specific gene or protein biomarkers can be analyzed based on published data: for adipogenesis adiponectin (GBP-28), aP2, leptin, PPAR-γ, glycerol 3 phosphate dehydrogenase (GPDH); for chondrogenesis, aggrecan (CSPCP), collagen type II, Sox9; for osteogenesis, alkaline phosphatase (ALP), bone sialoprotein (BSP), OC, OPN, osterix (OSX), RUNX-2, and DLX5 (Erickson *et al.*, 2002; Seda Tigli *et al.*, 2009; G. Yu *et al.*, 2010; Zuk *et al.*, 2001; Zuk *et al.*, 2002). These biomarkers analyzed by quantitative methods provide reliable indications of cell differentiation.

2.6 Immunological properties of ASCs

2.6.1 ASC immunogenicity and immune regulatory properties

Apart from the demonstrated differentiation potential of ASCs, these cells also possess immunomodulatory functions that have gained much interest recently due to their potential for future clinical treatments. The ASCs have a low immunogenic profile, as shown by low expression of major histocompatibility complex (MHC) class II molecules and T- and B-cell co-stimulatory molecules CD80, CD86, and

CD40 that are required for complete T-cell activation (McIntosh *et al.*, 2006; Niemeyer *et al.*, 2007). When used as stimulator cells in a one-way mixed lymphocyte reaction (MLR) assay, ASCs do not stimulate a proliferative response of allogeneic T-cells (McIntosh *et al.*, 2006; Niemeyer *et al.*, 2007; Puissant *et al.*, 2005; M. L. Ren *et al.*, 2012). McIntosh *et al.* also demonstrated that the immunogenicity of ASCs may decrease with passaging of the cells, and SVF may remain more immunogenic compared with cells at higher passages (McIntosh *et al.*, 2006). Furthermore, the differentiation status of the cells may change the immunogenic profile of ASCs; as shown by in vivo study, the osteogenically induced ASCs appeared to be eliminated by the host's immune system. However undifferentiated ASCs were proven as potential candidates for allogeneic cell transplantation (Niemeyer *et al.*, 2008). Still, contradictory in vitro results are available that demonstrate the low immunogenicity and immunomodulatory functions of ASCs after osteogenic differentiation (Montespan *et al.*, 2014; Niemeyer *et al.*, 2007), and thus, additional investigation is required to fully analyze the effect of differentiation on cell immunogenic properties.

Previous published studies support the use of allogenic ASCs in cell therapies that would significantly increase their therapeutic interest, especially if they maintain a non-immunogenic profile after implantation in the patient. If allogeneic ASCs were used in cell therapies the problem of extensive donor variation would be overcome, e.g., the ASCs with optimal response to osteogenic induction could be chosen for cellular therapies. Certain in vivo studies on allogeneic transplantation of ASCs have been performed with promising results (Beggs *et al.*, 2006; Jeong *et al.*, 2014; Kuo *et al.*, 2011; McIntosh *et al.*, 2009). Kuo *et al.* demonstrated that ASCs modulate the immune system and significantly prolong allotransplant survival by changing the anti-inflammatory cytokine expression pattern and affecting T-cell functions (Kuo *et al.*, 2011; Niemeyer *et al.*, 2008). A recent study by Jeong *et al.* showed similar results of prolonged allotransplant survival time and reduced inflammatory cell infiltration in a rat hind limb allotransplantation model (Jeong *et al.*, 2014); this study also suggested that ASCs act through the induction of regulatory T-cells. These studies support the use of allogenic ASCs in clinical cell therapy, but further investigation is still required to confirm the low immunogenic profile of ASCs in vivo. Of note, certain contradictory results exist that suggest MSCs may not be intrinsically immunoprivileged and cannot serve as a universal donor in immunocompetent MHC-mismatched recipients (Eliopoulos *et al.*, 2005). Based on these results, the immunosuppressive ability of MSCs is not sufficient to prevent their own rejection if complete MHC class I and II mismatch donors are used. Although this study was performed in a murine model with BM-MSCs and may not be fully comparable to

human ASCs, the possibility of immune rejection should be considered in early-phase clinical trials.

In addition to the low immunogenicity of ASCs and their potential use in allogeneic cell therapies, ASCs possess the ability to suppress the proliferation of lymphocytes, as demonstrated in an MLR assay in vitro (Cui *et al.*, 2007; Kronsteiner *et al.*, 2011; Lee *et al.*, 2012; McIntosh *et al.*, 2006; Puissant *et al.*, 2005; Yoo *et al.*, 2009). Several published studies have shown that ASCs suppress the immunogenic responses by modulating the cytokine milieu and finally by affecting the lymphocyte functions (Crop *et al.*, 2010a; Cui *et al.*, 2007; Yoo *et al.*, 2009). The ability of ASCs to modulate immunity and inflammation has increased the number of both pre-clinical and clinical in vivo studies that attempt to elucidate the therapeutic potential of the cells for immunomodulation purposes; several clinical trials to evaluate the immunomodulation potential of ASCs are ongoing and already completed, as previously reviewed (Casteilla *et al.*, 2011) (Section 2.9.).

The immunomodulatory effect of ASCs has been demonstrated in several in vivo disease models, e.g., autoimmune disease, Alzheimer's disease and graft-versus-host disease (GVHD) models. For instance, the immunosuppressive potential of ASCs was studied in an autoimmune hearing loss murine model in which the immunosuppressive effects of ASCs on autoreactive T-cells were investigated (Y. Zhou *et al.*, 2011). The results showed that systemic infusion of ASCs significantly improved hearing function and protected hair cells in vivo; the proliferation of antigen-specific Th1/Th17 cells was reduced whereas production of anti-inflammatory cytokine interleukin-10 was increased (Y. Zhou *et al.*, 2011). These results showed that ASCs act as important regulators of immune tolerance with the ability to suppress effector T-cells and to induce the generation of antigen-specific Treg cells. In addition, the potential of ASCs to treat Alzheimer's disease was evaluated in a mouse model in which intravenously transplanted ASCs were shown to improve the memory deficit and neuropathology of the disease by up-regulating interleukin (IL) -10 and vascular endothelial growth factor (VEGF) secretion (S. Kim *et al.*, 2012). Furthermore, allogeneic ASCs have been shown to prevent acute GVHD in a mouse model (Yanez *et al.*, 2006), and the improved outcome is likely a result of changing the cytokine milieu from a pro-inflammatory to an anti-inflammatory direction (C. S. Lin *et al.*, 2012). The feasibility of ASCs in treating GVHD was also evaluated in clinical case studies (Fang *et al.*, 2007a) and currently a phase I/II clinical trial (NCT01222039; www.clinicaltrials.gov) is ongoing to evaluate the use of allogeneic ASCs to treat GVHD (Section 2.9.). Furthermore, several

clinical studies using BM-MSCs for treatment of GVHD have been reported (N. Kim *et al.*, 2013; Le Blanc *et al.*, 2008; Resnick *et al.*, 2013; Ringden *et al.*, 2006).

The mechanism behind the immunosuppression has been studied primarily with BM-MSCs; however, it is suggested that no significant differences exist among the immunomodulatory properties of MSCs derived from different adult human tissues (Yoo *et al.*, 2009). The MSC-mediated immunosuppression includes soluble factors as well as direct cell-cell contacts, and the surrounding inflammatory environment also affects the immune plasticity of MSCs by enhancing the immunosuppressive capacity of the cells (Krampera *et al.*, 2006; G. Ren *et al.*, 2008; G. Ren *et al.*, 2010; Waterman, 2010). The importance of cell-cell contact in MSC-mediated immunosuppression was first reported by Di Nicola *et al.* (Di Nicola *et al.*, 2002); this study demonstrated that the suppressive capacity of BM-MSCs was stronger in direct co-culture than in trans-well cultures. The BM-MSCs have been shown to express integrins, intercellular adhesion molecule 1 (ICAM-1, CD54) and vascular cell adhesion protein 1 (VCAM-1, CD106), among other adhesion molecules, by which they bind to T-lymphocytes with high affinity (Haddad and Saldanha-Araujo, 2014). The importance of adhesion molecules ICAM-1 and VCAM-1 in BM-MSC-mediated immunosuppression was also later verified by Ren *et al.* (G. Ren *et al.*, 2010). However, ASCs do not express VCAM-1 on the cell surface and thus, ASC-mediated immunosuppression should be ICAM-1- but not VCAM-1-mediated. Puissant *et al.* have demonstrated that direct cell-cell contact is required for the strong suppressive effect mediated by ASCs because supernatants from mixed lymphocyte cultures did not suppress T-cell proliferation without cell-cell contact (Puissant *et al.*, 2005). Moreover, Quaedackers *et al.* studied the cell-cell interactions between ASCs and lymphocytes and showed that activated PBMCs are bound to ASCs; they also suggested that subpopulation of lymphocytes may be selectively captured by ASC (Quaedackers *et al.*, 2009). An increase in the secretion of the T-cell attractants CXCL9, CXCL10 and CXCL11 was also observed under inflammatory conditions, which recruit T-lymphocytes to the vicinity of ASCs. Recent evidence has suggested that ASC/T-cell interactions and local microenvironment factors such as IFN-γ produce induction signals for ASCs to obtain the phenotypic and functional properties of antigen-presenting cells. This transformation of ASCs leads to increased production of anti-inflammatory molecules that will further induce or target immunoregulatory effector cells such as Tregs (Davis *et al.*, 2014; Ma *et al.*, 2014). The immunosuppressive effects of MSCs will remain for a longer period, although the cells may disappear relatively quickly after in vivo administration (Ma *et*

al., 2014). Thus, it is hypothesized that MSCs produce factors that modify the tissue microenvironment and lead to intrinsic recovery.

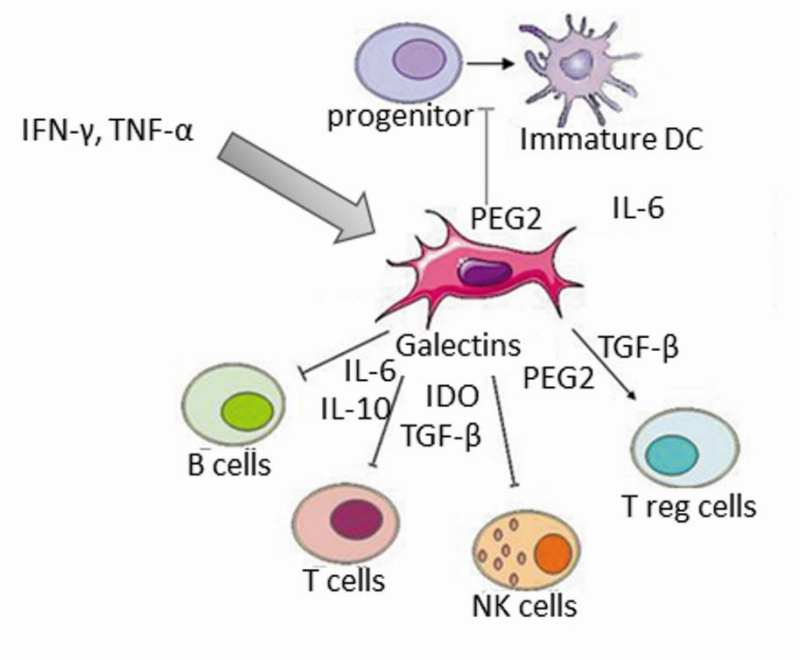

Figure 5. Schematic representation of the interactions between MSCs and immune cells. After INF-γ and/or TNF-α activation, MSCs secrete soluble mediators such as prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), interleukin (IL) -6, and galectins. Production of these mediators regulates the proliferation and function of a variety of immune cells as well as the induction of regulatory T (reg) cells. NK, natural killer; DC, dendritic cell. Figure modified from Ghannam *et al.* (Ghannam *et al.*, 2010).

For instance, the secretion of IFN-γ, tumor necrosis factor α (TNF-α) and IL-6 is increased under inflammatory conditions that further induce the immunosuppressive potential of MSCs (Crop *et al.*, 2010a). However, IL-6 has several functions because it is involved in inflammation and infection responses and also in the regulation of metabolic, regenerative, and neural processes (Scheller *et al.*, 2011); thus, it can either support or suppress inflammation depending on the context. Several other anti-inflammatory factors have been shown to be involved in immunomodulation, such as transforming growth factor β (TGF-β) (Melief *et al.*, 2013a) and prostaglandin E2 (Cui *et al.*, 2007), which both suppress T-cell activation and IL-2 synthesis. In addition, the role of indoleamine 2,3-deoxygenase (IDO) (Crop *et al.*, 2010a) and galectin-1 and -3 (Sioud *et al.*, 2011a; Sioud, 2011b) have been

demonstrated in MSC-mediated suppression. To conclude, the immune response is always a result of several factors because the secretion of many chemokines and cytokines is increased under inflammatory conditions. These signaling proteins may also play distinct roles depending on the current cellular environment. A set of signaling proteins and certain functions during MSC-mediated immunomodulation is listed in Table 4. The interactions between MSCs and immune cells are illustrated in Figure 5.

Table 4. Signaling proteins and certain of their functions during MSC-mediated immunomodulation.

Signaling protein	Abbreviation	Function
Interferon γ	IFN-γ	• stimulates MSCs to elicit immunosuppressive factors (Ghannam *et al.*, 2010) • immunomodulatory functions (Montespan *et al.*, 2014; Roemeling-van Rhijn *et al.*, 2013) • immunosuppression (Crop *et al.*, 2010a; G. Ren *et al.*, 2008) • induces adhesion molecule expression (G. Ren *et al.*, 2008; G. Ren *et al.*, 2010) • regulates the expression of chemokines (Croitoru-Lamoury *et al.*, 2007)
Tumor necrosis factor α	TNF-α	• immunomodulatory functions (Montespan *et al.*, 2014; Roemeling-van Rhijn *et al.*, 2013) • immunosuppression (Crop *et al.*, 2010a; G. Ren *et al.*, 2008) • regulates the expression of chemokines (Croitoru-Lamoury *et al.*, 2007)
Indolamine 2,3 - deoxygenase	IDO	• immunosuppression (Crop *et al.*, 2010a; DelaRosa *et al.*, 2009; J. W. Kang *et al.*, 2008; Meisel *et al.*, 2004) • inhibits T-cell proliferation (G. Ren *et al.*, 2009) • promotes type II macrophage differentiation (Francois *et al.*, 2012) • impairs NK cell activity (Pittenger *et al.*, 1999)
Prostaglandin E2	PGE2	• immunosuppression (Cui *et al.*, 2007; J. W. Kang *et al.*, 2008; Najar *et al.*, 2010; Sotiropoulou *et al.*, 2006b) • induces Foxp3+ T regs (English *et al.*, 2009) • inhibits NK cell function (Sotiropoulou *et al.*, 2006b; Spaggiari *et al.*, 2008) • induces type II macrophages (Ylostalo *et al.*, 2012) • inhibits dendritic cell maturation (Spaggiari *et al.*, 2009)
Galectin-1	Gal-1	• immunosuppression (Gieseke *et al.*, 2010; Sioud *et al.*, 2011a; Sioud, 2011b) • inhibits T-cell proliferation (Lepelletier *et al.*, 2010) • modulates the release of cytokines such as TNF-α, IFN-γ, IL-2, IL-10 (Gieseke *et al.*, 2010)

Galectin-3	Gal-3	• immunosuppression (Hernandez and Baum, 2002; Sioud *et al.*, 2011a; Sioud, 2011b) • induction T-cell proliferation (Hernandez and Baum, 2002)
Transforming growth factor β1	TGF-β1	• multiple actions in innate and adaptive immunity, important factor in maintaining immune tolerance (Wahl *et al.*, 2006) • immunosuppression, suppress T cells and several cytokines such as TNF-α and IFN-γ (J. W. Kang *et al.*, 2008; Letterio and Roberts, 1998; Melief *et al.*, 2013a; Tiemessen *et al.*, 2003) • induces T regs (Tasso *et al.*, 2012) • inhibits NK cell activation and function (Sotiropoulou *et al.*, 2006b)
Interleukin 6	IL-6	• supports or suppresses the inflammation depending on the context (Scheller *et al.*, 2011) • prevents monocyte differentiation toward antigen-presenting cells (Melief *et al.*, 2013b) • inhibits T-cell proliferation (Najar *et al.*, 2009) • inhibits the differentiation of dendritic cells (Djouad *et al.*, 2007) • anti-inflammatory effects mediated through inhibition of TNF-α (Ulich *et al.*, 1991)
Interleukin 10	IL-10	• inhibits T-cell responses, decreases Th17 cell differentiation (Beyth *et al.*, 2005; Qu *et al.*, 2012)
Interleukin 8	CXCL8/IL-8	• induces extracellular matrix degradation (Anton *et al.*, 2012) • promotes angiogenesis (Strieter *et al.*, 2005) • regulates neutrophil and mast cell functions (Kouji *et al.*, 2011)
C-C chemokine ligands 2 and 5	CCL2/MCP-1 CCL5/RANTES	• promote T-cell chemotaxis, attracts immune cell or MSC migration to the sites of injury or inflammation (Anton *et al.*, 2012) • induce extracellular matrix degradation (Anton *et al.*, 2012) • regulate monocyte and effector and memory T cell functions (Kouji *et al.*, 2011) • CCL2 regulates monocyte mobilization and macrophage infiltration (Tsou *et al.*, 2007) • CCL5 has T cells co-stimulatory functions (Wong and Fish, 2003)
CXC chemokine ligand 10	CXCL10/IP-10	• induces MSC migration to inflammation sites (Croitoru-Lamoury *et al.*, 2007; Rice and Scolding, 2010) • regulates dentritic cells and effector, memory, and regulatory T cell functions (Kouji *et al.*, 2011)

2.6.2 Immunological characterization

As demonstrated by several research groups (described above), ASCs possess immune regulatory properties that can be utilized clinically for treatment of different types of immunological disorders (Casteilla *et al.*, 2011; Krampera *et al.*, 2013). A large number of experimental approaches with qualitative and quantitative methods as well as in vitro and in vivo models have been used to assess immune regulatory properties of MSCs (Crop *et al.*, 2010b; Eliopoulos *et al.*, 2005; Kronsteiner *et al.*, 2011; Kuo *et al.*, 2011; Kuo *et al.*, 2011; Nauta and Fibbe, 2007; Niemeyer *et al.*, 2007; Niemeyer *et al.*, 2008; Niemeyer *et al.*, 2008; Puissant *et al.*, 2005; Puissant *et al.*, 2005; Waterman, 2010). For instance, the immunosuppressive potential of ASCs has been compared with that of well-characterized BM-MSCs using an MLR in vitro model, with or without osteogenic differentiation (Niemeyer *et al.*, 2007; Puissant *et al.*, 2005). These investigations were followed by in vivo studies in which BM-MSCs and ASCs were transplanted subcutaneously into immunocompetent mice (Niemeyer *et al.*, 2008) or use of intravenous administrations into orthotopic hind-limb in vivo model (Kuo *et al.*, 2011). Moreover, murine BM-MSCs were engineered to release erythropoietin and tested for their feasibility in supporting transplantations into MHC-mismatched allogeneic mice without immunosuppressive medication (Eliopoulos *et al.*, 2005). The results suggested that MSCs are not intrinsically immunoprivileged and may not be used for MHC-mismatched donors. Consequently, the amount of data in the published literature is relatively large; however, this information may be inconsistent and not fully comparable. Naturally, this observation is due to several factors, such as different species and tissue sources, different culture conditions, number of passages, activation status of both MSCs and responsive immune effector cells, and the analytical methods and animal models used. To combine the scientific research in this field, the MSC Committee of the ISCT has published a working proposal paper on the need for shared guidelines for immunological characterization of MSCs for clinical use (Krampera *et al.*, 2013).

According to the ISCT Committee guidelines, the immunological characterization of MSCs should include the following: (Krampera *et al.*, 2013) 1) A standard immune plasticity assay with IFN-γ and TNF-α used as in vitro priming agent. Previous studies have demonstrated that the local inflammatory environment, especially the expression of cytokines IFN-γ and TNF-α, is highly important in the regulation of MSC plasticity (Crop *et al.*, 2010a). Regardless, IFN-γ remains the first key licensing agent for MSC suppressor functions (Krampera *et al.*, 2013); 2) Functional analyses of cells using standardized immune assays that provide a

mechanistic understanding on clinical responses. The immunological properties of MSCs, both phenotypic and functional, are dependent on the activation status of cells after interaction with lymphocytes or other soluble mediators (Menard *et al.*, 2013). Thus, comparison of the results of resting and primed MSCs would be the most informative; 3) Use of purified responders is recommended to provide a more generalized image of the functional potency of MSCs (Krampera *et al.*, 2006). Both unselected PBMCs and purified T-cells can be used, but the latter usually provide more reproducible results because third-party cells are lacking; 4) The IDO response should be included as a component of an in vitro assay. Activation of IDO (in humans) (Meisel *et al.*, 2004) and inducible nitric oxide synthase (in animal species) (G. Ren *et al.*, 2008) is a central mechanism in MSC-mediated immunosuppression; 5) Careful conclusions should be drawn with respect to transplantation of human MSCs into xeno-recipient animal models in conducting clinical trials. Most in vivo models that assess MSC immunomodulation potential are based on rodent species (Gimble *et al.*, 2010), and thus critical evaluation should be performed on data obtained from these studies. Additionally, important for clinical applicability is a clarification of the mechanisms underlying the therapeutic functions; 6) Analysis of lymphocyte populations in patients to be treated with MSC should be encouraged. Immune cell populations should be prospectively analyzed using standardized immune assays in patients who receive MSC therapy. In conclusion, the aim of the described methodological standardization is to obtain shared, reproducible and consistent data for validation of MSC-based clinical approaches as potentially useful treatments for immunological diseases (Krampera *et al.*, 2013).

2.7 Bone tissue engineering

Interestingly, human bone tissue has a relatively high potential for regeneration (Bessa *et al.*, 2008a; Reddi, 2005); however, the natural healing capacity of bone tissue may fail if the bone defect is large, also referred to as critical-sized. Bone autografts are considered a gold standard for bone reconstruction due to their histocompatibility and non-immunogenicity and possess the essential factors for bone healing: osteoinductive factors such as bone morphogenetic proteins, osteoprogenitor cells required for osteogenesis, and functional material for osteoconduction such as three-dimensional and porous matrix structure (Amini *et al.*, 2012). Nevertheless, the use of autografts requires an additional surgical operation for bone harvesting, and several studies have reported significant

limitations and complications such as donor site injury and morbidity, deformity, scarring and surgical risks (Amini *et al.*, 2012). Bone tissue engineering is a novel multidisciplinary approach for bone regeneration and requires the collaborative work of biologists, engineers, and surgeons (O'Keefe and Mao, 2011). Several critical factors exist in bone tissue engineering that affect the final outcome of regenerated bone. A biocompatible scaffold is a key component that should provide a supportive structure for bone regeneration. The design of biocompatible scaffolds requires a balance of an osteoinductive cellular microenvironment, diffusion of soluble factors, sufficient flexibility, and mechanical loading appropriate for the anatomical site (Mravic *et al.*, 2014). In certain cases, osteogenic cells may be seeded on a biomaterial scaffold in combination with growth signals to direct osteogenic differentiation. Moreover, sufficient vascularization is essential for the survival of newly formed bone to provide nutrients for the growing tissue (Weigand *et al.*, 2015).

2.7.1 Biomaterials in clinical bone applications

A biomaterial is defined as a nonviable material used in a medical device that is intended to interact with biological systems (Williams, 1999). Biocompatibility is essential for biomaterials and is defined as the ability of a material to perform with an appropriate host response in a specific situation (Williams, 1999). The first materials referred as biomaterials were invented almost 50 years ago for the purpose of simply replacement of diseased, damaged or aged tissues (Hench *et al.*, 2004). Thus, these materials were selected to match the physical properties of the replaced tissues with a minimal toxic response in the host and thus were as bioinert as possible (Hench *et al.*, 2004). In the 1970s, Hench *et al.* discovered that certain bioglasses containing CaO and SiO_2 were able to form a strong adhesive bond with bone and soft connective tissues (Hench, 1998). These biomaterials became known as bioactive materials due to a controlled reaction in the physiological environment.

Subsequently, bioactive glass (BAG) and beta-tricalcium phosphate (β-TCP) ceramic have been widely studied and used for bone tissue engineering purposes due to their biocompatibility and ability to support osteoblastic growth and maturation (Baino and Vitale-Brovarone, 2011; Q. Z. Chen *et al.*, 2006; Haimi *et al.*, 2009a; Mesimaki *et al.*, 2009; Sandor *et al.*, 2013; Thesleff *et al.*, 2011; Vitale-Brovarone *et al.*, 2007; Yuan *et al.*, 2001). The BAG develops a carbonated phosphate surface layer that allows chemical bonding to the host bone. This bone-bonding behavior is referred to as bioactivity and has been associated with the formation of a carbonated

hydroxyapatite (HCA) layer on the glass surface after implantation (Hench, 1998). A biologically active HCA layer has been shown to be essential for the bone bonding. In addition to BAGs, hydroxyapatite (HA) and related calcium phosphates have shown excellent bioactive properties and abilities in bone bonding. Approximately 60 wt% of bone consists of HA $Ca_{10}(PO_4)_6(OH)_2$, and therefore, HA and related calcium phosphates such as α-TCP and β-TCP have been investigated for use as scaffold materials in bone tissue engineering, as previously reviewed (Rezwan *et al.*, 2006). Due to the high similarity of the chemical and crystal structure of calcium phosphates to bone minerals, these materials exhibit a superior biocompatibility with bone tissue. Although they may not be osteoinductive, calcium phosphates have osteoconductive abilities and bind directly to bone under certain conditions (Rezwan *et al.*, 2006). Osteoinduction is defined as the stimulation and activation of host stem cells from the surrounding tissue or, alternatively, activation of transplanted stem cells for differentiation into bone-forming osteoblasts, also in ectopic tissues. In contrast, osteoconduction is defined as the ability of a material to serve as a scaffold to which bone cells attach, migrate and divide, but osteogenesis is not directly induced (Albrektsson and Johansson, 2001). With advanced material processing techniques, optimal porous and interconnected 3D structures can be manufactured to further support osteoblastic growth and maturation (Rezwan *et al.*, 2006).

2.7.2 Bone morphogenetic proteins

To identify more efficient and reliable methods for osteogenic differentiation of MSCs, several growth factors and cytokines have been investigated for their potential to direct MSC osteogenesis. When osteoinductive biomaterials are combined with optimal osteoinductive factors, effective osteogenic differentiation can be achieved. The BMPs were discovered in 1965 and are probably the most important growth factors in bone formation and healing (Bessa *et al.*, 2008a; Reddi, 2005). In 1972, it was suggested by Reddi and Huggins that BMPs are responsible for the initiation of a cascade of developmental events in which progenitor cells are induced to produce bone cells, thus leading to bone regeneration (Bessa *et al.*, 2008a). The BMPs belong to the transforming growth factor-β (TGF-β) superfamily that includes several other growth factors, such as activins, inhibins and TGF-βs. These growth factors have functions in several tissues and organs, but BMPs play significant roles, especially in bone and cartilage formation (Bessa *et al.*, 2008a). BMP superfamily includes a large number of growth factors from BMP-2 to BMP-18 of which only BMPs -2, -4, -6, -

7, and -9 are known to induce complete bone morphogenesis. In contrast, BMP-3 and -3b have been shown to have negative effect on osteogenesis by downregulating the expression of ALP in bone cells (Hino *et al.*, 2004). Currently, human BMPs can be produced in large amounts using recombinant technology, and in 2002, the FDA approved two models of collagen sponges (Infuse™, Medtronik, US/Wyeth, UK; Osigraft™, Stryker Biotech) that deliver recombinant human BMP-2 or BMP-7 for human use as an alternative to bone grafts for spinal fusion and long bone fractures (Bessa *et al.*, 2008b).

Signaling of BMP is mediated to the cell nucleus through serine-threonine kinase receptors on the cell surface, where a specific intracellular pathway is activated, leading to gene transcription and finally affecting the cell proliferation and differentiation (Shi and Massague, 2003). Smads are the main signal transducers of these complex pathways that are strictly regulated (Bessa *et al.*, 2008a; Derynck and Zhang, 2003). Upon BMP binding, type I receptors phosphorylate receptor-regulated Smads (R-Smads) that form a heterotrimeric complex with one Co-Smad, which translocates the signal into the nucleus and modulates gene transcription in co-operation with other transcription factors. Smads regulate the transcription of several genes such as *RUNX-1*, *-2* and *-3*, *OSX, HOXC-8* and *MyoD* (Bessa *et al.*, 2008a). One of the most studied early markers of osteogenic differentiation is *RUNX-2,* the expression of which is low in mesenchymal cells and is further induced after BMP signaling (Ito and Miyazono, 2003). *RUNX-2* regulates processes such as bone formation and hematopoiesis and upregulates several osteogenic markers such as ALP, osteocalcin and osteopontin (Bessa *et al.*, 2008a).

2.7.3 Towards allogeneic bone treatments with ASCs

For practical purposes, a cell product should be available as an off-the-shelf product, immediately upon demand at the point of care (Liu *et al.*, 2013). The current use of autologous ASCs in a clinical setting does not meet this criterion. Isolation and expansion of ASCs is time-consuming and importantly, cells derived from aged donors or cancer patients should be replaced, possibly by the allogeneic cells of young healthy donors (Gu *et al.*, 2014). Therefore, the ability to use allogeneic ASCs for bone repair is more suitable for clinical demands. Gu *et al.* have observed that similar to undifferentiated ASCs, osteogenically differentiated ASCs show low expression for MHC I antigens and are negative for MHC II (Gu *et al.*, 2014) and concluded that the allogeneic differentiated ASCs maintain their low

immunogenicity and can be used in allogeneic settings, as demonstrated in an ulnar bone rabbit model without any immunosuppressive therapies. The use of allogeneic osteogenically differentiated ASCs for the treatment of critical-sized bone defects in vivo was also investigated by Liu *et al.* (Liu *et al.*, 2013), who demonstrated the potential of allogeneic ASCs in the treatment of bone diseases using an immunocompetent canine cranial model without immunosuppressive therapies. As previous studies have demonstrated, ASCs show low immunogenicity and possess immunomodulatory functions both in vitro and in vivo (Section 2.6.1), but translation of these findings to the clinical settings is still challenging. Limitations remain, such as differences in immune systems between human and animal species associated with many of the used models, which may confuse interpretation of the results if the aim is to provide safe, effective, and reproducible treatments for the patient. Well-designed and standardized clinical trials are necessary to verify the safety and efficacy of ASCs for allogeneic stem cell treatments and for immune modulating therapies. Clinical trials for ASCs are described in more detail in Section 2.9.

2.8 Safety aspects of ASC treatments

Adipose stem cell -based therapies have shown potential for repair, replacement or regeneration of damaged cells and tissues. However, a major challenge in cell therapies is ensuring efficacy and safety. During clinical therapies, cells are often expanded in vitro outside their natural environment, which may increase risk for genomic instability or altered differentiation potential; furthermore, the cells may induce significant adverse effects such as tumors, cell growth in ectopic tissues or severe immune reactions.

Cancer treatments generally rely on tumor destruction techniques that may lead to major functional defects in the surrounding tissues (Semont *et al.*, 2006). This post-therapy damage requires the development of safe regenerative therapies for cancer remission. For breast cancer patients, an autologous fat graft comprising SVF cells is often used as a filler for breast reconstruction to correct possible irregularities after mastectomy (Eterno *et al.*, 2014). In addition to formal breast reconstruction, ASCs and BM-MSCs favor tissue-healing processes and promote local tissue repair by modulation of the tissue microenvironment (Caplan and Dennis, 2006). However, interactions between MSCs and cancer cells in modulating tumor microenvironment are critical for safety matters. Many components that are required for successful

regenerative therapy, e.g., revascularization, immunosuppression, cellular homing, are also critical for tumor progression and metastasis (Djouad *et al.*, 2003; Zimmerlin *et al.*, 2013). Mesenchymal stem cells are well known to secrete cytokines, chemokines and growth factors that are essential for development and maintenance of an inflammatory state, thus inducing tissue regeneration after injury (Caplan and Dennis, 2006; Eterno *et al.*, 2014; Eterno *et al.*, 2014; Fritz and Jorgensen, 2008). However, inflammatory responses may also create an optimal microenvironment that stimulates cancer cells for continuous proliferation with anti-apoptotic and pro-proliferative paracrine signals and promotes tumor neoangiogenesis (Karnoub and Weinberg, 2006; Karnoub *et al.*, 2007; J. L. Yu and Rak, 2003; Zimmerlin *et al.*, 2013).

Eterno *et al.* reported that ASCs are not internally tumorigenic, but they reveal a tumorigenic potential in the presence of c-Met-expressing breast cancer cells (Eterno *et al.*, 2014). This study suggests that c-Met could be used as a marker to predict risk of cancer recurrence if ASCs are used in cancer patients for regenerative and reconstructive purposes. A similar conclusion is drawn by Bielli *et al.*, who suggested that only active cancer cells are affected by the tumor microenvironment, thus promoting neoangiogenesis or matrix remodeling and further supporting tumor growth and invasiveness (Bielli *et al.*, 2014). Rowan *et al.* demonstrated that human ASCs increased migration and metastasis of human MDA-MB-231 triple-negative cancer cells in a xenograft in vivo model that was likely due to facilitation of the early steps of the metastatic process (Rowan *et al.*, 2014). This study showed that co-cultures of ASCs with MDA-MB-231 cells had no effect on MDA-MB-231 growth; however, the migration of MDA-MB-231 cancer cells was increased after co-culture with ASCs or with use of ASC-conditioned medium. Thus, these results suggested that ASCs stimulated metastasis of MDA-MB-231 breast tumors to multiple mouse organs (Rowan *et al.*, 2014). As a conclusion, the results of different research groups suggest that the effects of MSCs on tumor cells are various and may depend on the state of the tumor cell, the properties of specific MSC populations, and interactions with other cell types, such as tumor infiltrating immune cells (Zimmerlin *et al.*, 2013). Although several published clinical studies suggest that ASCs do not increase the risk of new cancer compared with a control group (Delay *et al.*, 2009; Kolle *et al.*, 2013; Rigotti *et al.*, 2010), additional studies are still needed to better clarify the crosstalk between aggressive cancer cells and MSCs. Additionally, it would be helpful to maintain a registry for patients treated with ASC in the context of breast cancer to monitor long-term outcomes.

Another important safety aspect in clinical translation of ASC is the possibility for genomic instability when cells are expanded ex vivo. Importantly, cultured ASCs

have appeared to be genomically stable in long-term cultures after multiple cell doublings, thus supporting their suitability for regenerative applications (Grimes *et al.*, 2009). However, certain clonal chromosomal aberrations may arise transiently in early passage of ASC cultures that may disappear or become negligible later on (Meza-Zepeda *et al.*, 2008). Moreover, Rubio *et al.* demonstrated similarities between stem cell and cancer stem cell genetic programs and showed spontaneous transformation of ASCs after long-term in vitro culture (Rubio *et al.*, 2008). This study characterized the molecular mechanisms behind transformation and suggested a two-stage model by which ASCs may become tumor cells, up-regulation of c-myc and suppression of p16 levels and modulation of mitochondrial metabolism. In contrast, Rubio *et al.* concluded that ASCs can be safely expanded during the early passages, and only long-term in vitro expansion may occasionally shift ASCs into a tumorigenic state. Furthermore, the influence of a novel XF medium on the genomic stability was analyzed with BM-MSCs after cell expansion for 25 (P5) and 45 (P9) days under XF conditions (Chase *et al.*, 2012). These results showed no gross chromosomal aberrations, thus suggesting that the cells preserved their normal genotype even when cultured under XF conditions.

Nevertheless, these results of ASC transformation should be cautiously interpreted because subsequent reports have suggested that cells used in the transformation studies were cross-contaminated by malignant cells that initially grew slowly in the presence of human MSCs (Torsvik *et al.*, 2010). Similar conclusions were drawn by Carcia *et al.*, who showed that the most plausible explanation for the spontaneous transformation of MSC is an artifact mediated by an unnoticed minimal cross-contamination of some of the original samples with HT1080 cancer cells (Garcia *et al.*, 2010). This discrepancy in the transformation studies may be explained by minimal contamination, and thus, more rigorous cell culture procedures are required for use of primary cells (Torsvik *et al.*, 2010). In theory, even a small percentage of chromosomally abnormal cells within a population may increase the risk for uncontrolled cell divisions or tumor formation upon transplantation; however, strong conclusions should not be drawn based on these inconsistent results. Nevertheless, clinical safety and efficacy studies should be performed to carefully evaluate the stem cell product before further clinical use to avoid possible adverse effects connected to cell-based therapies.

2.9 Clinical studies for evaluation of the potential of ASCs

In May 2015, a total of 164 clinical trials were found on the www.ClinicalTrials.gov database evaluating the potential of ASCs for treating different types of medical disorders (Figure 6). However, all 164 trials did not use expanded ASCs, but instead, SVF was used in at least 37 trials. Some of the 37 trials actually used the term ASCs, and thus, the terminology may be misleading in certain cases. Only 10 out of 164 trials progressed into phase III or IV, whereas 120 trials were still in phase I or II. The remainder of the trials did not specify the current status. A commercial sponsor was involved in 65 trials, whereas the remainder of the trials were conducted in academic or hospital settings. The most common application of ASCs was the treatment of complex fistulas that are often associated with Crohn's disease. Equally common were clinical studies evaluating the applicability of ASCs for treatment of joint disorders such as osteoarthritis and ASCs were also widely tested for the treatment of neurological disorders such as Parkinson's disease, spinal cord injury and Multiple Sclerosis as well as for the treatment of cardiovascular diseases such as myocardial infarction.

Garcia-Olmo *et al.* reported promising results of a randomized controlled phase II trial of ASCs to treat complex perianal fistulas associated with Crohn's disease (Garcia-Olmo *et al.*, 2009a). The efficacy of ASC-based treatment was demonstrated over conventional treatment and 20 to 60 million expanded ASCs were administered in combination with fibrin glue. One half of the cells were injected at 2 mm under the epithelium along the fistula, and the other half was combined with fibrin glue and used to fill in the fistula directly. The ASCs proved to be an effective and safe treatment for complex perianal fistula and appeared to achieve higher rates of healing than fibrin glue alone (Garcia-Olmo *et al.*, 2005; Garcia-Olmo *et al.*, 2009a; Garcia-Olmo *et al.*, 2009b). Garcia-Olmo *et al.* performed various trials with both SVF cells and expanded ASCs and reported healing of 75% of chronic fistulas using expanded ASCs. Although ASCs display immunosuppressive features and low expression of immunoreactive surface antigens in vitro, SVF cells lack any immunosuppressive function and stimulate T-cell proliferation in parallel assays (Gimble *et al.*, 2010). In light of the autoimmune etiology of Crohn's disease, the immunomodulatory features of ASCs may be responsible for the relative success or failure of the treatment.

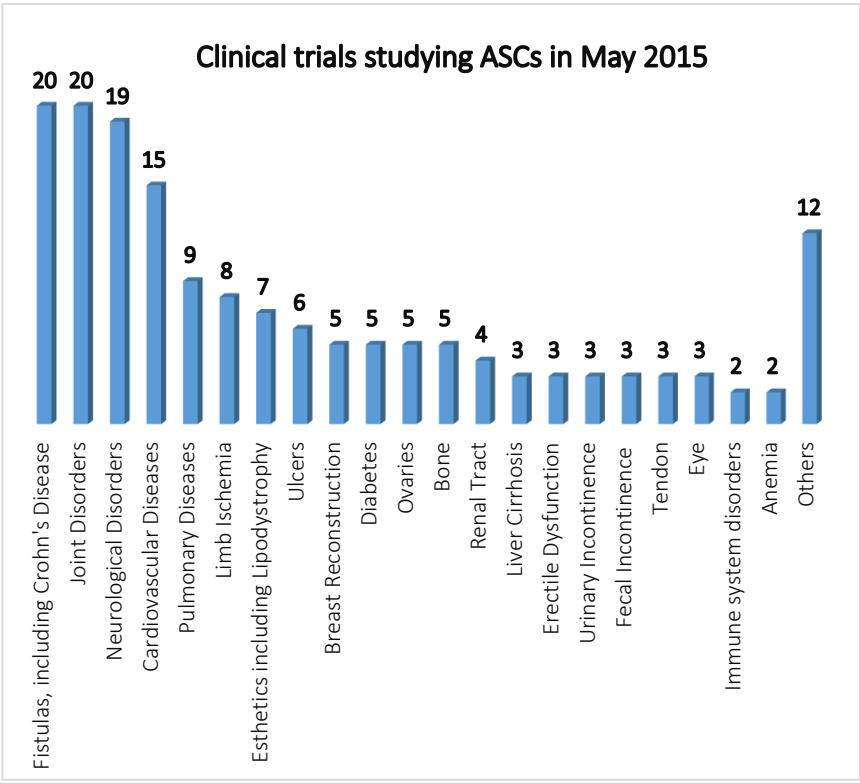

Figure 6. A total number of 164 clinical trials using ASCs were ongoing on 19 May 2015, as searched from clinicaltrials.gov. Clinical studies are categorized based on the disease or target tissue of the treatment. The "Others" group includes clinical trials that could not be categorized into defined groups, e.g., trials for treatment of HIV, burn wounds, leukemia, amputation stumps, obesity, depressed scars, and urethral strictures in males. Only 10 out of 164 trials progressed into phase III or IV, whereas 120 trials were still in phase I or II. The remainder of the trials did not specify the current status.

Riordan *et al.* reported a clinical study of three Multiple Sclerosis patients who were treated with intravenous infusions of 25 to 75 million autologous adipose-derived SVF cells combined with intravenous infusions of allogeneic CD34+ cells and ASCs (Riordan *et al.*, 2009). Their study demonstrated significant improvements in patient conditions, and the need for regular medication was clearly reduced after ASC treatment. However, MRI images obtained 6-7 months after the stem cell treatment showed lesions that were highly similar to the lesions observed prior to the stem cell treatment. Nevertheless, this study demonstrated that intravenous injections of SVF cells are well tolerated and may yield symptomatic improvements.

The results of randomized clinical trials in a larger patient cohort are still needed before any conclusions can be drawn.

A clinical study using ASC injections for the treatment of critical limb ischemia was also published with encouraging results (Bura *et al.*, 2014). The feasibility and safety of autologous ASCs was demonstrated in 10^8 cells that were intramuscularly injected into the ischemic leg of patients followed by improved wound healing that supported a functional efficiency of the treatment (Bura *et al.*, 2014). Pre-clinical in vivo studies have suggested that ASCs exposed to ischemia or hypoxia secrete cytokines that improve cell proliferation and vasculogenesis directly without the presence of the ASCs themselves (Eto *et al.*, 2011). Similar conclusions were drawn by Bhang *et al.*, who demonstrated in vivo that the therapeutic efficacy of the ischemia treatment may be duo to paracrine effects (Bhang *et al.*, 2011). These results suggest that ASC-conditioned medium alone may be sufficient for treatment of ischemic injuries, without the need for direct cell transplantation (Gimble *et al.*, 2012).

The immunomodulation capacity of ASCs is likely involved in the therapeutic effects of many treatments described in this work; however, the overall number of clinical trials of ASCs that study severe immunological diseases such as sepsis or GVHD is still relatively small. The advanced biopharmaceutical company TiGenix NV has focused on developing novel therapeutics with allogeneic ASCs for treatment of inflammatory and autoimmune diseases (http://www.TiGenix.com). This company has recently announced a phase I trial designed to demonstrate the safety and efficacy of allogeneic ASCs for treatment of sepsis using intravenous infusions of 0.25 - 4 $x10^6$ ASCs/kg (NCT02328612; www.clinicaltrials.gov). This trial uses healthy volunteers challenged with a bacterial endotoxin that elicits an inflammatory response that induce sepsis-like clinical symptoms. TiGenix expects to complete the phase I trial by the third quarter of 2015 and to follow with a Phase II trial. Apart from studies performed by TiGenix, another phase I/II clinical trial evaluating the use of allogeneic ASCs for the treatment of chronic, extensive GVHD is currently ongoing (NCT01222039; www.clinicaltrials.gov). This clinical study investigates the safety and efficacy of intravenously injected allogenic ASCs (1 - 3 $x10^6$ cells/kg) used in combination with a gradually descending dosage of the conventional treatment.

Moreover, Fang and co-workers have reported clinical case studies on the promising use of allogeneic ASCs for preventing steroid-resistant acute GVHD. Transplantation of allogeneic ASCs was reported with successful results in 7 of 9 patients when patients received intravenous infusions of 1.0-1.5$x10^6$ ASCs/kg (Fang

et al., 2007a; Fang *et al.*, 2007b; Fang *et al.*, 2007c; Fang *et al.*, 2009). The mechanism responsible for the improved outcomes of these patients is likely connected to a shift from a pro-inflammatory to anti-inflammatory cytokine milieu (C. S. Lin *et al.*, 2012). Similar to the work of Fang *et al.*, the use of BM-MSC has been demonstrated in a phase II study as an effective therapy for patients with steroid-resistant, acute GVHD (Le Blanc *et al.*, 2008). Although these clinical studies show encouraging results for treatment of steroid-resistant acute GVHD patients, preliminary trials using ASCs must be carefully followed to ensure both safety and efficacy for future treatments.

An important aspect in clinical translation is the ex vivo cell expansion method often required for ASC-based therapies to obtain clinically sufficient cell numbers for effective treatments. One possibility is use of a non-expanded adipose SFV, as has been performed by Riordan *et al.* (Riordan *et al.*, 2009). In this way, the cell expansion step can be avoided, but relatively little is known about the potential clinical effects of whole lipoaspirate that contains numerous cell populations in addition to ASCs. For larger cell numbers, safe and efficient in vitro cell isolation and expansion techniques are required for clinical use of cells. The majority of the protocols use reagents derived from animal sources, although the risks and benefits of using of animal-derived reagents should be carefully assessed because of safety concerns (Mackensen *et al.*, 2000; Selvaggi *et al.*, 1997). In the clinical trials described above, Garcia-Olmo *et al.* used FBS supplemented medium for cell expansion (Garcia-Olmo *et al.*, 2009a), whereas Bura *et al.* used human-platelet-growth-factor-enriched plasma for cell expansion (Bura *et al.*, 2014). Fang *et al.* used 2% fetal calf serum in combination with growth factor bFGF, insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) (Fang *et al.*, 2007a).

However, the identification of XF alternatives for standard FBS-based media is important for considering clinical applicability of ASCs. An ideal culture media should be cost effective, display minimal batch variability and low immunogenicity and be free of all animal products and likely allogenic derivatives as well (Johal *et al.*, 2015). Commercially available XF and SF media exist that meet these criteria, such as the MesenCult™-SF Culture Kit from Stem Cell Technologies and STEMPRO® MSC SFM from Life Technologies, which both support the multilineage capacity of ASCs with significantly improved ASC proliferation rates (Al-Saqi *et al.*, 2014a; Lindroos *et al.*, 2009) (Section 2.4.3.). If the cost effectiveness of these XF/SF media is reasonable, and adequate experimental data on ASC performance exist, these defined culture conditions will become clinically favored. Nevertheless, the culture media manufacturers should guarantee that a given media is available in the long-

term; if the pre-clinical and clinical data are demonstrated with a given media, it should be available for patient treatments. Otherwise, many additional studies should be performed to demonstrate the cell performance under distinct media, which would be substantially expensive for the producer of cell therapies.

Clinical trials are a required step forward in translation to cell-based therapies and for commercial use of cell-based products as well. However, licensed products and those approaching marketing authorization are still few. A major challenge in commercialization of cell-based products is the manufacturing and quality assurance of these complex products because cells are much more complex entities than small molecules and therapeutic proteins (Salmikangas *et al.*, 2015). Demonstration of quality, safety and efficacy may be demanding because it is difficult to ensure the comparability between production processes and batches for cell-based medicinal products (Salmikangas *et al.*, 2015). However, a flexible case-by-case and risk-based approach has been applied in the legislation for ATMPs (Schneider *et al.*, 2010). During clinical transition, ATMPs provide treatments for diseases with limited or no effective therapeutics for which safety and efficacy over conventional treatments is still not shown by clinical trials. The risk-based approach of ATMPs is based on the identification of specific risks (or lack thereof) associated with the clinical use such as risks linked to quality, manufacturing and administration of the product. These risks may include unwanted immunogenicity, tumor formation, and ectopic tissue formation as well as contaminations from the production process and toxicity due to toxic degradation products of biomaterial components (Salmikangas *et al.*, 2015).

Currently, four cell-based medicinal products have received marketing authorization in the European Union (Salmikangas *et al.*, 2015). Holoclar was the first cell-based product (ATMP) containing stem cells that was recommended for approval in the European Union (EMA, European Public Assessment Report: Holoclar, 2015). This product was developed to replace damaged cells on cornea epithelium and can be used in adult patients with moderate to severe limbal stem cell deficiency due to physical or chemical burns to the eye. The other three products include two for cartilage repair, i.e., ChondroCelect® (EMA, European Public Assessment Report: ChondroCelect, 2009) and MACI® (EMA, European Public Assessment Report: MACI, 2013), and one for treatment of metastatic castrate-resistant prostate cancer, i.e., Provenge® (EMA, European Public Assessment Report: Provenge, 2013). At the moment, clinical translation and commercialization of cell-based medicinal products are experiencing strong progress worldwide, although most developers in European Union originate from hospitals/academia or spin-off companies of those institutions (Salmikangas *et al.*, 2015).

TiGenix NV is one of the leading European cell therapy companies with a commercialized cell-based product ChondroCelect® that uses expanded autologous chondrocytes for cartilage repair in the knee (www.TiGenix.com). Additionally, this company has a clinical stage pipeline of three ASC programs in which the applicability of allogeneic ASCs under investigation in clinical trials for the treatment of 1) complex perianal fistulas in Crohn's disease (NCT01541579; www.clinicaltrials.gov), 2) rheumatoid arthritis (NCT01663116; www.clinicaltrials.gov) and 3) autoimmune diseases via intralymphatic administration (NCT01743222; www.clinicaltrials.gov). For treatment of complex fistulas, phase III clinical trials are currently ongoing. Based on the report from the phase II trial, a high efficacy of the product was demonstrated compared with other products in the closing of fistulas in Crohn's patients. The trial also confirmed the strong safety profile of the product. TiGenix has reported that the cell product acts by controlled reduction of inflammation in the fistula and through the release of anti-inflammatory factors, which in turn promote natural fistula closure (www.TiGenix.xom). Moreover, a phase IIa clinical trial of refractory rheumatoid arthritis was completed in 2013, and the safety and tolerability of the product was confirmed with encouraging signs of efficacy. A phase I study has been conducted for autoimmune diseases. The results confirmed the safety, tolerance and the feasibility of intra-lymphatic cell administration, which enables the possibility of achieving efficacy at lower dosage. According to TiGenix, the intra-lymphatic administration system would further increase the safety and feasibility of expanded ASCs and it would significantly reduce the cost of goods (www.TiGenix.com). These and other novel cell products offer promise for future medicine, but time will reveal the final outcome of these complex medicinal products.

2.10 Patent landscape in the field of ASC research

Until recently, stem cell research conducted within academic settings has paid no or little attention to patent questions. However, academic researchers are increasingly beginning to focus on patenting matters, especially when universities are involved in commercially sponsored research projects or are searching for opportunities to license university inventions (Bergman and Graff, 2007). In the field of stem cell research, patents may cover different methods, including optimized culture conditions with defined profiles of growth factors, proteins, hormones or small molecules for proliferation and differentiation or de-differentiation of cells. Many of

these methods are separately patented technologies that do not have many alternatives and therefore may be protected by intellectual property rights.

To provide an overview of the latest inventions in the field, investigation of patent applications is a considerably fast and useful method to gain access to the latest data. In this review, a patent search was performed using the Espacenet Worldwide database (http://www.epo.org/) that covers patents and patent applications from over 90 countries worldwide. When a patent search was conducted with key words "mesenchymal stem cell" in the patent title or abstract, 2,238 hits were found, whereas the key words "mesenchymal stromal cell" produced 120 hits (searches conducted on 23rd June, 2015). With more detailed key words of "adipose stem cell" and "adipose stromal cell" in the patent title or abstract 464 and 83 hits were found, respectively. Still more specific key words "adipose stem cell culture medium" and "adipose stromal cell culture medium" garnered 51 and 5 search results, respectively. These patented culture media formulations included protocols for isolation, expansions and cryopreservation as well as differentiation of ASCs. Additionally, patents for ASC reprogramming into iPSCs were found. The search results described are collected in Table 5.

Table 5. Patents related to adipose stem cells research. The search was conducted on 23rd June, 2015.

Key words in title or abstract	**Number of hits**	**Patent**
Adipose stem cell culture medium	14	Isolation, expansion or cryopreservation of ASCs
	13	Differentiation induction media for ASCs
	5	Media for reprogramming ASCs into iPSCs
	15	Media compositions of ASCs for cellular therapies
	4	Cell therapy -compliant SF media for ASC culture
Adipose stromal cell culture medium	1	Isolation of ASCs
	2	Differentiation induction media for ASCs
	2	Media compositions of ASCs for cellular therapies
Mesenchymal stem cell	2,238	
Mesenchymal stromal cell	120	
Adipose stem cell	464	
Adipose stromal cell	83	

The technical content of the patent landscape is highly complex. As the number of patent applications increase in the field, the potential risk of bottlenecks might emerge (Bergman and Graff, 2007), although access to data and materials is critical for the positive progress of stem cell science (Mathews *et al.*, 2011). A number of

factors currently limit the sharing of data in the field, including the potential competitive spirit of individual scientists, ethical regulations in use of human cell lines and a complex landscape of intellectual property rights (Mathews *et al.*, 2011). Although these potential obstacles should be kept in mind for patenting, at the same time, it is justifiable to offer protection for researchers who invest in the development of products and processes that have significant medical benefits.

The original patent that covers ASCs was published in 2004 by Katz *et al.* (Katz *et al.*, 2004). This particular patent was notable broad and covered the cells alone and within biologically compatible materials as well as the methods used to generate differentiated tissues and structures both in vivo and in vitro. Additionally, Katz's patent included the secreted hormones and conditioned media of ASC cultures as well as production of an extracellular matrix lattice from adipose tissue. However, due to prior art in the academic literature, Katz's patent claims were challenged and overturned in the US patent court. Because this particular overturned patent is prior art for all subsequent patents on the use of ASCs, the current patent holders may not be able to withhold licensing of ASC-derived methods or restrain freedom to operate in future. The above-mentioned detail may change the patent landscape for SVF and ASC in the coming years.

3 Aims of the current study

The aim of this thesis was to characterize ASCs with respect to clinical cell therapy and to evaluate ASC characteristics such as multipotency and immunogenic properties under clinically relevant culture conditions. In the first study, defined XF/SF culture conditions were developed for ASCs. In the second study, the MMC method was tested under defined XF/SF conditions versus different serum conditions. In the third study, the immunological properties of ASCs were evaluated in XF/SF conditions versus serum conditions, and finally in the fourth study, the osteogenic differentiation efficiency was tested with growth factors and clinically used biomaterials. The long-term goal of this study is establishment of high-quality ASCs potentially available as off-the-shelf products for cell-based therapies. The specific aims of each study are listed below:

I: To test novel XF/SF culture conditions for ASCs for maintenance of basic MSC characteristics, including multilineage differentiation potential, immunophenotype, and proliferation capacity.

II: To enhance the proliferation and differentiation capacity of ASCs using a MMC method in defined XF/SF conditions versus different serum conditions.

III: To evaluate the effect of cell culture medium on ASC immunologic properties and characterize the cytokine secretion profile of ASCs in defined XF/SF conditions versus different serum conditions.

IV: To evaluate and compare the effects of 1) BAG and β-TCP and 2) BMP-2 and BMP-7 on the osteogenic differentiation and angiogenic potential of ASCs.

4 Matherials and methods

4.1 Ethical considerations and tissue procurement

The collection of adipose tissue and peripheral blood was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere, Finland (ethical approval R03058) and the study was conducted in accordance with the Declaration of Helsinki 1975, revised in Hong Kong 1989. Adipose tissue samples were obtained from Tampere University Hospital and the buffy coat samples were from the Finnish Red Cross Blood Service. ASCs were isolated from adipose tissue samples acquired from elective surgical procedures in the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland, with the patients' written consent. The adipose tissue samples in study **I** were obtained from 4 female donors (mean age 36 ± 9 years), in study **II** from 4 female donors (mean age 52 ± 12), in study **III** from 9 female donors (mean age 41 ± 10), and in study **IV** form 6 donors (5 female and 1 male, mean age 39 ± 18).

4.2 Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Allogeneic human PBMCs were isolated from buffy coat samples (n = 7) by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077 g/mL; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com) according to manufacturer's instructions. Subsequently, the cells were aliquoted and cryopreserved in the nitrogen gas phase until co-cultures in MLRs.

4.3 Isolation and expansion of ASCs

ASCs were isolated from adipose tissue samples under three different culture conditions in medium containing FBS, HS, or under defined XF/SF culture conditions. Isolation of ASCs from adipose tissue samples was performed using a mechanical and enzymatic method as described previously with minor modifications (Gimble and Guilak, 2003; Haimi *et al.*, 2009a; Lindroos *et al.*, 2009; Zuk *et al.*, 2002). Briefly, the adipose tissue was minced manually into small fragments and digested with collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany, http://www.serva.de) in a water bath at 37°C under shaking conditions. The digested tissue was centrifuged and filtered in sequential steps through a 100-mm pore size filter to separate the ASCs from the surrounding tissue. For FBS and HS conditions, Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 (Life Technologies, Rockville, MD, http://www.lifetech.com) was supplemented with 1% L-analyl-L-glutamine (GlutaMAX I; Life Technologies), 1% antibiotics (p/s; 100 U/mL penicillin, 100 U/mL streptomycin; Lonza, Walkersville, MD, http://www.lonza.com) and either 10% FBS (Life Technologies) or 10% HS (human serum type AB; Lonza). ASCs isolated and expanded in FBS medium were detached using 1% trypsin (Lonza), and ASCs isolated in HS medium were detached using TrypLE Select (Life Technologies). Prior to use, the FBS and HS serum lots were screened based on ASC proliferation and differentiation capacity under serum supplemented media. All culture-media formulations are presented in Table 6.

Table 6. Culture-media formulation overview. Two different manufacturers were used for HS, which are separated by superscripts [1] and [2]: HS[1] (Lonza, Walkersville, MD) was used in studies **I, II** and **III** whereas HS[2] (PAA Laboratories, Pasching, Austria) in studies **III** and **IV**. The used HS is specified later with respect to each study. MSC, mesenchymal stem cell; SFM, serum free medium; p/s, penicillin/streptomycin.

Acronym	Manufacturer	Basal media	Serum	Coating/ coating-free supplements	Supplements
HS[1] studies **I**,**II**,**III**	Lonza, Walkersville, MD	DMEM/ F-12	Human serum	none	1% GlutaMAX, 1% p/s (100 U/mL penicillin, 100 U/mL streptomycin)
HS[2] studies **III**,**IV**	PAA Laboratories, Pasching, Austria	DMEM/ F-12	Human serum	none	1% GlutaMAX, 1% p/s
FBS studies **I**,**II**,**III**	Life Technologies, Gibco, Rockville, MD	DMEM/ F-12	Fetal bovine serum	none	1% GlutaMAX, 1% p/s
XF/SF CS studies **I**,**II**,**III**	Life Technologies, Gibco, Rockville, MD	StemPro® MSC SFM	none	CELLstart™ coating	StemPro® MSC SFM Xeno-free supplement, 1% GlutaMAX, 0,3% p/s (30 U/mL penicillin, 30 U/mL streptomycin)
XF/SF CM study **I**	Life Technologies, Gibco, Rockville, MD	StemPro® MSC SFM	none	Coating Matrix Kit	StemPro® MSC SFM Xeno-free supplement, 1% GlutaMAX, 0,3% p/s

4.3.1 Optimization of XF/SF isolation and expansion protocol

For XF/SF culture, the cells were isolated under XF/SF conditions and seeded in carboxyl-coated flasks (PureCoat Carboxyl T75; BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) and expanded in STEMPRO MSC SFM (Life Technologies) supplemented with 1% GlutaMAX I, 0,3% antibiotics (p/s; 30 U/mL penicillin, 30 U/mL streptomycin), and 10% StemPro MSC SFM Xeno-Free supplement. From passage 1 onwards, XF/SF cells were expanded in STEMPRO MSC medium supplemented with CELLstart CTS coating (Life Technologies) according to the manufacturer's instructions. ASCs isolated and expanded in SF/XF medium were detached using TrypLE Select. Flow chart of the isolation as well as performed analyses in different culture conditions in studies **I** and **II** are illustrated in Figure 7.

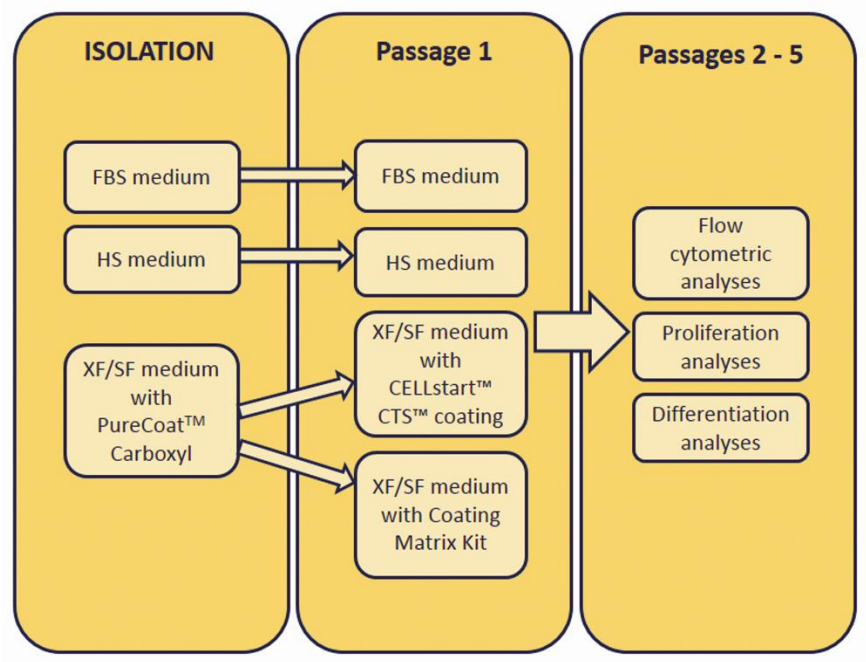

Figure 7. Work flow of the isolation and performed analyses of ASCs in study I and II under different culture conditions. XF/SF isolation of ASCs was carried out by using carboxyl-coated flasks, and onward from passage 1, two different XF/SF conditions were tested in basic Nunclon flasks; Coating Matrix Kit, and CELLstart™ coating. Cell-proliferation rate, differentiation potential, and immunophenotype were analyzed in four (I) or three (II) different culture conditions at passages 2 – 5. MSC, mesenchymal stem cell; SFM, serum free medium; p/s, penicillin/streptomycin. Modified from Study **I**.

4.3.2 Macromolecular crowding in ASC cultures

The macromolecular crowding method was tested for its ability to support ASC proliferation and differentiation capacity. A cocktail of macromolecules containing Ficoll™400 (PM400, 17-0300-50; GE Healthcare, Bio-sciences AB) and Ficoll™70 (PM70, 17-0310-50; GE Healthcare, Bio-Sciences AB) was dissolved into culture media at room temperature with gentle agitation. Fractional volume occupancy of 17% was achieved with concentrations 37.5mg/mL of Ficoll™70 and 25mg/mL of Ficoll™400 as described previously (C. Z. Chen *et al.*, 2011). Media was sterile filtered after the addition of Ficoll particles.

From passage 1 onwards, the cells isolated in FBS- and HS-containing medium were divided into two populations, and the cells were expanded under +/- MMC conditions until the analyses in passage four. Due to technical reasons, the XF/SF cells were divided into two populations from passage 2 onwards, and expanded under +/-MMC conditions until the analyses in passage 4.

4.4 Analyses of ASC characteristics

4.4.1 Immunophenotype of ASCs

ASCs expanded in FBS, HS and SF/XF media were analyzed with flow cytometry (FACSAria; BD Biosciences, Erembodegem, Belgium) to determine whether different culture conditions have an effect on the immunophenotype of the cells. The list of analyzed cell surface markers is presented in Table 7. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

Table 7. Panel of cell surface markers used in all studies **I-IV**.

Antigen	Fluorophores	Surface protein	Manufacturer
CD3	phycoerythrin (PE)	T-cell co-receptor	BD Biosciences
CD11a	allophycocyanin (APC)	Integrin alpha L (lymphocyte function-associated antigen 1)	R&D Systems Inc., Minneapolis, MN, USA
CD14	phycoerythrin-cyanine (PECy7)	Lipopolysaccharide receptor	BD Biosciences, Franklin Lakes, NJ, USA
CD19	PECy7	B lymphocyte-lineage differentiation antigen	BD Biosciences
CD34	APC	Hematopoietic progenitor cell antigen 1	Immunotools GmbH, Friesoythe, Germany
CD45-RO	APC	RO isoform of leucocyte common antigen	BD Biosciences
CD54	fluorescein isothiocyanate (FITC)	Intercellular adhesion molecule 1 (ICAM-1)	BD Biosciences
CD73	PE	Ecto 5' nucleotidase	BD Biosciences
CD80	PE	B7-1	R&D Systems Inc.
CD86	PE	B7-2	R&D Systems Inc.
CD90	APC	Thy-1 (T cell surface glycoproteins)	BD Biosciences
CD105	PE	SH-2, endoglin	R&D Systems Inc.
HLA-DR	PE	Major histocompatibility class II antigen (MHC-II)	Immunotools GmbH

4.4.2 Morphology of ASCs

Morphological characteristics of ASCs were observed by light microscopy to support the results of proliferation analyses. Additionally, the morphological changes during cell expansion under different culture conditions (FBS/HS versus XF/SF; +/- MMC), as well as changes during adipogenic, osteogenic or chondrogenic inductions were monitored by light microscopy.

4.4.3 Viability of ASCs

In study **IV**, the cell attachment and viability were evaluated using quantitative Live/Dead staining method (Molecular Probes, Eugene, OR, USA). Briefly, samples were incubated for 45 min with a mixture of 5μM CellTracker™ green (5-chloromethylfluorescein diacetate [CMFDA]; Molecular Probes) and 2.5μM Ethidium Homodimer-1 (EH-1; Molecular Probes). Fluorescence microscope was used to obtain cell images in which the viable cells stained green and red fluorescence indicated dead cells.

4.4.4 Proliferation assays

In studies **I** and **II**, the cell viability and metabolic activity in the different culture conditions (FBS, HS, and SF/XF) and in MMC condition were assessed with the PreMix WST-1 Cell Proliferation Assay System (study **I**) or Cell Counting Kit -8 (CCK-8) (study **II**) (Takara Bio Inc., Shiga, Japan). Both assays are based on the cleavage of tetrazolium salts (WST-1/WST-8) by mitochondrial dehydrogenase in viable cells, which enable to measure the cell proliferation and viability with colorimetric assay. Tetrazolium salts are cleaved to formazan dye by the succinate-tetrazolium reductase which exists in mitochondrial respiratory chain and is active only in viable and metabolically active cells. The amount of the formazan dye that is generated by the activities of dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity of CCK-8 was higher than WST-1 tetrazolium salt. ASCs were seeded on 48-well plates at a density of 2,500 cells/cm^2, and the proliferation was assessed at 1, 4, 7, and 11 days. In brief, at each time point, the cell-culture medium was removed, and DPBS (Dulbecco Phosphate-Buffered Saline, Lonza, BioWhittaker, Verviers, Belgium) and PreMix WST-1/CCK-8 reagent were added 10:1. The 48-well plate was incubated for 4 hours (**I**) or 3 hours (**II**) at 37°C, and the relative cell proliferation activity was measured in a microplate reader (Victor 1429 Multilabel Counter; Wallac; Turku, Finland) at 450 nm.

In study **I**, the population doubling was determined by using the formula x = log2(NH)/(N1), where N1 is the absorbance value at day 1, and NH is the absorbance value at observed time point 4, 7, or 11, as described previously (Cristofalo *et al.*, 1998). To calculate the cumulative population doubling, the population doubling was determined in each passage and compared with the population doubling of earlier passages. In study **II**, the metabolic activity was

detected by CCK-8 Cell Proliferation Assay and was normalized to the number of cells in culture by quantifying the total DNA from of the cultures with CyQUANT® cell proliferation assay.

In studies **II** and **IV**, the cell number was quantitatively analyzed by determining the amount of total DNA with a cell proliferation assay kit (CyQUANT®, Molecular Probes, Invitrogen, Paisley, UK). CyQUANT dye expresses fluorescence when bound to cellular nucleic acids. Briefly, cells were lysed using 0.1% Triton-X 100 buffer (Sigma-Aldrich), and the supernatant was collected and stored at -80°C until final analyses. Twenty micro liters of each sample were mixed with CyQUANT GR dye and lysis buffer in a 96-well plate (Nunc). Fluorescence signals were measured with a microplate reader at 480/520nm.

4.5 ASC multilineage differentiation

The trilineage differentiation potential of ASCs towards osteogenic, adipogenic and chondrogenic cells was evaluated in XF/SF conditions versus HS and traditionally used FBS-supplemented medium in study **I** and **II**. In addition to different serum or XF/SF conditions, the effect of MMC on ASC differentiation was evaluated in study **II**. In study **IV**, the osteogenic differentiation of ASCs together with osteoinductive biomaterials and growth factors was evaluated. Differentiation capacity of ASCs was observed after 14 days (**I**) of induction in either adipogenic, osteogenic or chondrogenic medium versus cells cultured in control medium. In study **II**, 28 days of osteogenic induction, 14 days of adipogenic induction and 21 days of chondrogenic induction periods were used to evaluate the ASC multipotency. The osteogenic and adipogenic differentiation was analyzed in four different treatment groups in each culture condition in study **II**: 1) expansion (E) -MMC, differentiation (D) -MMC; 2) E-MMC, D+MMC; 3) E+MMC, D-MMC; 4) E+MMC, D+MMC and the chondrogenic differentiation was performed in two different treatment groups: 1) E+MMC, D-MMC; 2) E-MMC; D-MMC. In study **IV**, osteogenic differentiation was observed after 1, 7, and 14 days of induction. The culture media formulations used for differentiation assays are shown in Table 9.

4.5.1 Osteogenic differentiation

The osteogenic differentiation ASCs was determined qualitatively by alkaline phosphatase (ALP) -staining (**I** and **IV**) and quantitatively by colorimetric ALP measurements (**IV**). Cells were seeded on 12-well plates at a density of 2.5 X 10^3 cells/cm^2 (**I**) and 2.0 X 10^3 cells/cm^2 (**II**) in control media and the induction was initiated 24 h (**I**) or 48 h (**II**) after cell seeding. The differentiation degree after osteogenic induction was determined by the level of ALP activity by using a leukocyte ALP kit (Sigma-Aldrich, #86R-1KT) as described previously (Haimi *et al.*, 2009a). The ALP staining solution is a mixture of naphthol AS-BI alkaline solution and fast red violet LB, which forms insoluble purple salts in the sample indicating alkaline phosphatase activity. In brief, cells were fixed with 4% paraformaldehyde (PFA) and incubated with ALP staining solution for 15 minutes. After rinsing the cells with deionized water, color formation was analyzed microscopically. Osteogenic differentiation was identified as purple staining of cells, as the alkaline phosphatase activity is shown by red dye deposits.

For quantitative ALP measurements, cells were lysed with 0.1% Triton-X 100 and cell lysates were frozen and stored to -70°C until analysis. After a freeze – thaw cycle, cell lysates were incubated with an alkaline buffer solution (2-amino-2-methyl propanol; 1.5 M; pH 10,3; Sigma Aldrich) and *p*-nitrophenol phosphate (Sigma Aldrich) for 15 min at 37 °C. The reaction was stopped by adding 50 µl of NaOH (1M Sigma Aldrich) and color intensity was measured with as microplate reader at 405 nm. The method is based on the hydrolysis of *p*-nitrophenol phosphate by ALP, yielding a yellow complex of *p*-nitrophenol and phosphate. The intensity of color formation is proportional to the concentration of ALP.

In study **II,** the quantitative alizarin Red S method was used to detect calcium compounds deposited in the ECM during osteogenic differentiation. Briefly, cells were fixed with 4% PFA and stained with 2% Alizarin Red solution (Sigma-Aldrich; pH 4.2), followed by several washes with H_2O. Light microscope was used to obtain cell images for qualitative analysis, and for quantitative analysis the Alizarin Red dye was extracted with 100 mM cetylpyridinium chloride (Sigma-Aldrich) at a shaking for 3 h. The dye intensity was determined at 540 nm with a microplate reader.

In study **IV**, the osteogenic differentiation capacity of ASCs was evaluated in combination with osteoinductive biomaterials. Commercially available materials BAG (BoneAlive granules, 1.0-2.0mm; BoneAlive Biomaterials Ltd., Turku, Finland) and β-TCP granules (ChronOS granules, 1.4-2.8mm, porosity 60%; Synthes, Oberndorf, Switzerland) were selected for the study. In addition to material testing,

BMP-2 and BMP-7 (Sigma–Aldrich) were chosen as additives for the osteogenic media. Used concentrations and combinations of BMPs are presented in Table 8. 400 µl of sterile BAG or β-TCP granules were transferred onto 24-well plate and incubated in corresponding medium for 48 h prior to cell seeding. After the incubation, 5.0 x 10^4 cells were suspended with individual treatment medium and seeded on both biomaterials to initiate osteogenic differentiation.

Table 8. Different treatment groups and medium components used in study **IV**. All treatments groups were studied separately with both biomaterials BAG and β-TCP. BAG, bioactive glass; β-TCP, beta-tricalcium phosphate; OM, osteogenic medium; BMP-2, bone morphogenetic protein 2; BMP-7, Bone morphogenic protein 7.

Medium	Supplements
1) Control medium (CM)	10% HS, 1% GlutaMAX, 1% p/s
2) Osteogenic medium (OM)	10% HS, 1% GlutaMAX, 1% p/s, 50 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 10mM beta-glycerophosphate (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich)
3) OM + BMP-2	OM + 100 ng/mL BMP-2 (Sigma-Aldrich)
4) OM + BMP-7	OM + 100 ng/mL BMP-7 (Sigma-Aldrich)
5) OM + BMP-2/-7	OM + 100 ng/mL BMP-2 + 100 ng/mL BMP-7

4.5.2 Adipogenic differentiation

For adipogenic differentiation in study **I**, ASCs were seeded on 12-well plates at a density of 2.0 × 10^4 cells/cm^2. After 14 days of adipogenic induction culture, differentiation was confirmed by qualitative Oil Red-O staining, indicating the formation of intracellular lipid accumulation, as described earlier (Zuk *et al.*, 2002). In brief, the cells were washed with DPBS and fixed with 4% PFA followed by several washes with deionized water. Cells were pre-treated with 60% isopropanol and stained with 0.5% Oil Red-O staining solution in 60% isopropanol (Sigma-Aldrich) for 15 min. Adipocytes were identified with light microscopy as cells with red-stained lipid vesicles.

For adipogenic differentiation in study **II**, ASCs were seeded on 24-well plates at an initial density of 1.05x10^4 cells/cm^2. Adipogenic differentiation was initiated when the cells reached confluence as described previously (Pittenger *et al.*, 1999) with

sequential method of 4 days of induction, followed by 3 days of maintenance. After 21 days of differentiation, Nile Red staining and quantitative adherent cytometry was used to assess area of cytoplasmic lipid accumulation. In brief, cell cultures were rinsed with phosphate-buffered saline (PBS), fixed in 4% PFA and co-stained for 30min with 5mg/mL Nile Red (N3013; Sigma), for cytoplasmic lipid droplets and 0.5 mg/mL of DAPI (D3571, Molecular Probes®; Life Techonologies) for nuclear DNA as previously described (Greenspan *et al.*, 1985). Adherent cytometry was performed according to previously described protocol (C. Z. Chen *et al.*, 2009). Extent of adipogenic differentiation was quantified by area of Nile Red fluorescence and normalized to nuclei count. End data corresponded to total area of lipid droplets present per well normalized to cell number (mm^2/ nuclei). Cell images were acquired using 2x magnification with a cool-SNAP HQ camera attached to a Nikon TE2000 microscope (Nikon Instruments), and analyzed using the Metamorph Imaging System Software 6.3v3 (Molecular Devices). More detailed descriptions of cell count procedures and the analysis of raw data is presented by Ang *et al.* (Ang *et al.*, 2014).

4.5.3 Chondrogenic differentiation

The chondrogenic differentiation potential was assessed by a micro-mass culture method as described previously (Denker *et al.*, 1995; Lindroos *et al.*, 2009; Maenpaa *et al.*, 2010; Zuk *et al.*, 2001). In brief, 8 x 10^4 cells were seeded on a 24-well culture plate in a 10 µl volume and were allowed to adhere for 3 hour prior the addition of chondrogenic induction medium. After 14 days (**I**) or 21 days (**II**) of chondrogenic induction, differentiation was confirmed using Alcian blue staining method as described earlier (Maenpaa *et al.*, 2010). In brief, the micro-mass cultures were fixed with 4% PFA and stored in 70% ethanol. Pellets were dehydrated, embedded in paraffin, and sectioned at 5-mm thickness. The sections were stained with Alcian blue (pH 1.0) to verify the presence of sulfated glycosaminoglycans (GAGs) by using Nuclear Fast Red solution (Biocare Medical, Concord, MA, USA) as a counterstain.

Table 9. Culture media formulations used for differentiation assays. Different manufacturers for HS are separated by superscripts [1] and [2]: HS[1] (Lonza, Walkersville, MD) was used in studies **I, II** and **III** whereas HS[2] (PAA Laboratories, Pasching, Austria) in studies **III** and **IV**.

Medium	Basal media	Serum	Coating/ coating-free supplements	Supplementation
Osteogenic FBS/HS[1] **I**,**II**	DMEM/F-12	10% Fetal bovine serum/ 10% Human serum	none	1% GlutaMAX, 1% p/s, 150 µM L-ascorbic acid 2-phosphate (Sigma), 10mM beta-glycerophosphate (Sigma), 10 nM dexamethasone (Sigma)
Osteogenic XF/SF CS **I**,**II** and XF/SF CM **I**	StemPro® MSC SFM	none	CELLstart™ coating/ Coating Matrix Kit	StemPro® MSC SFM Xeno-free supplement, 1% GlutaMAX, 0,3% p/s, same osteogenic supplements than in FBS/HS cultures in study **I** and **II**
Osteogenic HS[2] **IV**	DMEM/F-12	10% Human serum	none	1% GlutaMAX, 1% p/s, 50 µM L-ascorbic acid 2-phosphate (Sigma), 10mM beta-glycerophosphate (Sigma), 10 nM dexamethasone (Sigma)
Adipogenic FBS/HS[1] **I**	DMEM/F-12	10% Fetal bovine serum/ 10% Human serum	none	1% GlutaMAX, 1% p/s, 33 µM biotin (Sigma), 1 µM dexamethasone (Sigma), 100nM insulin (Life Technologies), 17 µM pantothenate (Fluka, Buchs, Switzerland), 250 µM isobutylmethylxanthine (IBMX; Sigma) for 48 hour induction after cell seeding
Adipogenic FBS/HS[1] **II**	DMEM/F-12	10% Fetal bovine serum/ 10% Human serum	none	For 4 days induction during cyclic differentiation: 1% GlutaMAX, 1% p/s (Gibco, Life Technologies), 0.5mM isobutylmethylxanthine (IBMX; Sigma), 0.2mM indomethacin (Sigma), 1 µM dexamethasone (Sigma), and 10 mg/mL insulin (Sigma).

Adipogenic XF/SF CS **I** and XF/SF CM **I**	StemPro® MSC SFM	none	CELLstart™ coating/ Coating Matrix Kit	StemPro® MSC SFM Xeno-free supplement, 1% GlutaMAX, 0,3% p/s, same adipogenic supplements than in FBS/HS cultures in study **I**
Adipogenic XF/SF CS **II**	StemPro® MSC SFM	none	CELLstart™ coating	For 4 days induction during cyclic differentiation: 1% GlutaMAX, 0.3% p/s, same adipogenic supplements than in FBS/HS cultures in study **II**
Chondrogenic FBS/HS[1] **I**	DMEM/F-12	none	none	1% GlutaMAX, 0,3% p/s, 10 mg/mL human serum albumin (Sigma), 8 µg/mL holo-transferrin human (Sigma), 5 ng/mL sodium selenite (Sigma), 10 µg/mL insulin (Life Technologies), 1 µg/mL linoleic acid (Sigma), 50 µM L-ascorbic acid 2-phosphate (Sigma), 55 µM sodium pyruvate (Life Technologies), 23 µM L-proline (Sigma), 10 ng/mL TGF-β1 (Sigma)
Chondrogenic XF/SF CS **I** and XF/SF CM **I**	StemPro® MSC SFM	none	none	1% GlutaMAX, 0,3% p/s, same chondrogenic supplements than in FBS/HS cultures in study **I**
Chondrogenic FBS/HS[1] **II**	DMEM/F-12	none	none	1% GlutaMAX, 0,2% p/s, 1x ITS+1 (Sigma), 50 µM L-ascorbic acid 2-phosphate (Sigma), 55 µM sodium pyruvate (Life Technologies), 23 µM L-proline (Sigma), 10 ng/mL TGF-β1 (Sigma)
Chondrogenic XF/SF CS **II**	StemPro® MSC SFM	none	none	1% GlutaMAX, 0,2% p/s, Xeno-free supplement, same chondrogenic supplements than in FBS/HS cultures in study **II**

4.5.4 Real-time quantitative PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to analyze the adipogenesis and osteogenesis associated gene expressions in studies **I** and **IV** as described previously (Maenpaa *et al.*, 2010). Total RNA was isolated by using the NucleoSpin RNA® II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The RNA samples were reverse transcribed to first strand cDNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). In brief, the real-time detection of PCR product was monitored by using the SYBR Green dye (Applied Biosystems, Warrington, UK). The housekeeping gene, the ribosomal phosphoprotein P0 (RPLP0), was used as an internal control, and the relative expression level for each gene was calculated according to a previously described mathematical model (Pfaffl, 2001). In study **I**, the expression of adipogenesis-associated genes *PPARγ*, and *aP2* was analyzed as well as osteogenesis-associated genes such as *DLX5*, *ALP*, and *RUNX-2*. In study **IV**, expression of early markers in osteogenesis including *OPN*, *RUNX-2*, *COL-1* and *OC* were measured. Sequences and accession numbers of all primers (Oligomer Oy, Helsinki, Finland) are displayed in Table 10. The reactions were conducted and monitored with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

4.6 Quantitating VEGF protein secretion

In study **IV**, the VEGF secretion was measured using a quantitative human VEGF immunoassay (R&D Systems, UK) according to the manufacturer's instructions. Briefly, 50 µl of assay diluent and 200 µL of cell culture supernatants were mixed and incubated for 2 h in microplate, pre-coated with specific antibody for VEGF. Followed by several washes, the samples were incubated additional 2 h with VEGF conjugate, horseradish peroxidase (HP) labeled detection antibody. After washes, tetramethylbenzidine (TMB) substrate solution was added and incubated for 20 min. Finally, the reaction was terminated with stop solution, and color intensity was determined at 450 nm with a microplate reader. The blue color development was proportional to the amount of VEGF present in the sample.

Table 10. Primer sequences of marker genes. hRPLP0, Ribosomal protein, large P0; haP2, fatty acid-binding protein 4; hPPARG, peroxisome proliferator-activated receptor gamma; hRUNX-2, runt related transcription factor 2; hDLX5, distal-less homeobox transcription factor 5; hALP, alkaline phosphatase; hCOL-1, Collagen, type I, alpha I; hOC, Osteocalcin; hOPN, Osteopontin.

Name	Acc. No.	Primer direction	Sequences	Product size (bp)
hRPLP0	NM_001002	Frw	5'-AAT CTC CAG GGG CAC CAT T-3'	70
		Rev	5'-CGC TGG CTC CCA CTT TGT-3'	
haP2	NM_001442	Frw	5'-GGTGGTGGAATGCGTCATG-3'	71
		Rev	5'-CAACGTCCCTTGGCTTATGC-3'	
hPPARG	NM_015869	Frw	5'-CAGTGTGAATTACAGCAAACC -3'	100
		Rev	5'-ACAGTGTATCAGTGAAGGAAT-3'	
hRUNX-2	NM_004348	Frw	5'-CCCGTGGCCTTCAAGGT-3'	76
		Rev	5'-CGTTACCCGCCATGACAGTA-3'	
hDLX5	NM_005221	Frw	5'-ACCATCCGTCTCAGGAATCG-3'	75
		Rev	5'-CCCCCGTAGGGCTGTAGTAGT-3'	
hALP	NM_000478	Frw	5'-ATGTCATCATGTTCCTGGGAGAT-3'	79
		Rev	5'-TGGTGGAGCTGACCCTTGAG-3'	
hCOL-1	NM_00088	Frw	5'-CCAGAAGAACTGGTACATCAGCAA-3'	94
		Rev	5'-CGCCATACTCGAACTGGAATC-3'	
hOC	NM_000711	Frw	5'-AGCAAAGGTGCAGCCTTTGT-3'	94
		Rev	5'-GCGCCTGGGTCTCTTCACT-3'	
hOPN	J04765	Frw	5'-GCCGACCAAGGAAAACTCACT-3'	71
		Rev	5'-GGCACAGGTGATGCCTAGGA-3'	

4.7 Analyses of ASC immunology

One-way and two-way MLR assays were used to determine the immunogenic properties of ASCs after cell isolation and expansion in different culture conditions: FBS, HS and chemically defined XF/SF conditions. The MLR assay was used to obtain information with respect to the immunogenic and suppressive properties of ASCs. Also the signaling protein secretion of ASCs was characterized through MLR assay. HS (PAA Laboratories) medium (10%) was chosen to serve as a constant environment for MLR cultures because of significantly decreased proliferation and metabolic activity of PBMCs when cultured in XF/SF condition (Figure 8). Therefore, prior to MLR assays, ASCs isolated and expanded in three different culture conditions, FBS, HS (Lonza), and XF/SF conditions, received the same treatment of medium change and were allowed to adjust in HS medium (PAA Laboratories) for 24 hours prior to co-culture. The same five patient ASC lines were used for MLR assays and for flow cytometric analyses, whereas the protein secretion studies were performed with four different ASC lines.

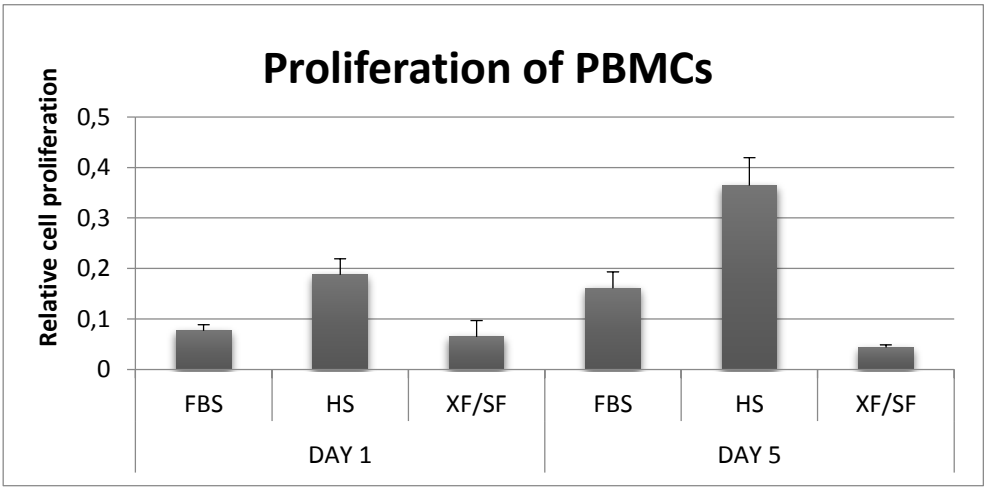

Figure 8. The proliferation of PBMCs under different culture media (FBS, HS and XF/SF conditions) at 1 and 5 day time points, assessed by the PreMix WST-1 Cell Proliferation Assay. Significantly lower proliferation of PBMCs was observed under XF/SF conditions as compared to FBS- and HS-supplemented media at day 5.

4.7.1 One-way mixed lymphocyte reaction (MLR) immunogenicity assay

One-way MLRs were performed as described previously by McIntosh *et al.* (McIntosh, 2011). PBMCs, acting as responder cells, were seeded on 96-well plates at cell density of $2.5x10^5$ cells/cm^2. Three different donor PBMCs were used and co-cultured with three different stimulator populations: 1) autologous PBMCs (baseline control response), 2) allogeneic BPMCs (positive control response), both plated at $1.0x10^4$ cells per well and 3) the ASC test population, plated at $0.5x10^4$, $1.0x10^4$ and $2.0x10^4$ cells per well (Figure 9). Stimulator PBMCs and test ASCs were irradiated with γ-rays (40 Gy) prior to the co-culture to inhibit the proliferation of the stimulator cells. ASCs in medium alone were plated as control cultures. In addition, control cultures of PBMCs alone were added as well as PBMCs supplemented with mitogen phytohemagglutinin (PHA, 1 mg/mL) to activate the responder PBMC lines to serve as a maximal positive control response. Quadruplicate reactions were performed from each treatment, and the cultures were incubated at 37° C in 5% CO2 for 5 days in HS medium (PAA Laboratories).

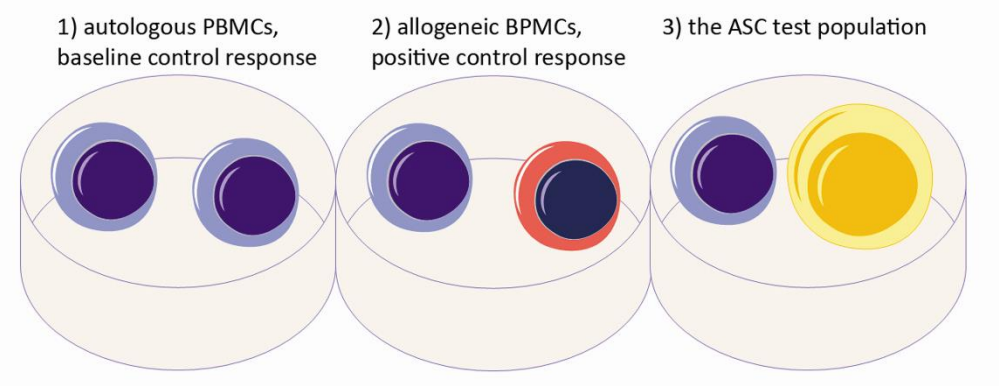

Figure 9. Schematic illustration of one-way MLR assay. Autologous PBMCs (blue) represent a baseline control response, allogeneic BPMCs (red) a positive control response and ASCs (yellow) a test population in one-way MLR assay. PBMCs, peripheral blood mononuclear cells; ASCs, adipose stem cells.

4.7.2 Two-way MLR immunosuppression assay

Two-way MLRs were also performed as described previously by McIntosh *et al.* (McIntosh, 2011). Two different MLR combinations were formed from three different responder PBMCs based on their cross-reactivity and HLA dissimilarities that were observed during pre-tests (data not shown). For each MLR combination, cells from two different donors were mixed in equal amounts to activate the proliferative response of each PBMC line. A total number of $2.5x10^5$ PBMCs were seeded per well on 96-well plates. After initiating the MLRs, test ASCs were added to the reactions at cell densities of $0.5x10^4$, $1.0x10^4$ or $2.0x10^4$ cells per well (Figure 10). Control wells containing only MLR combinations without ASCs, and ASCs alone were also seeded. Quadruplicate reactions were performed from each treatment group, and the cultures were incubated at 37°C in 5% CO_2 for 5 days in HS (PAA Laboratories) medium.

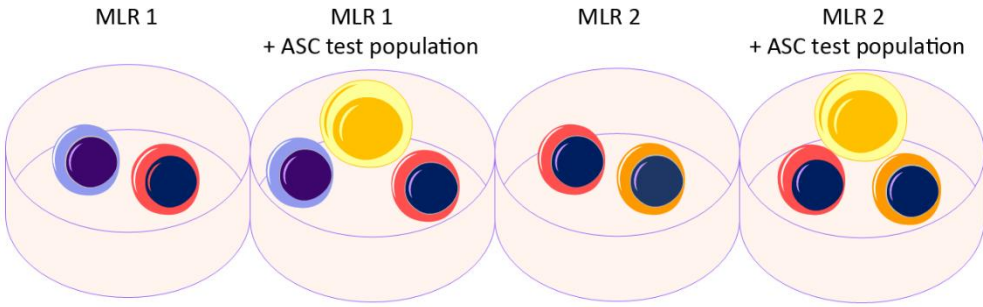

Figure 10. Schematic illustration of two-way MLR assay. Two different MLR combinations were formed from allogeneic PBMCs (blue and red; red and orange). MLRs 1 and 2 were co-cultured with allogeneic ASCs (yellow) to evaluate the suppressive potential of ASCs on MLR proliferative response. PBMCs, peripheral blood mononuclear cells; ASCs, adipose stem cells.

4.7.3 Bromodeoxyuridine (BrdU) ELISA

On day 4 of the MLRs, 10 mM bromodeoxyuridine (BrdU) was added to mono- and co-cultures, and the cells were incubated for additional 16 hours at 37°C. BrdU is a pyrimidine analogue that instead of thymine is incorporated into the DNA of dividing cells during the incubation. On day 5, PBMC proliferation was assessed by BrdU enzyme-linked immunosorbent assay (ELISA) (Roche Applied Science, Penzberg, Germany, https://www.roche-applied-science.com) according to the

manufacturer's instructions. Briefly, cells were fixed, permeabilizated and the DNA was denatured, followed by antibody binding to the incorporated BrdU. Detector anti-BrdU monoclonal antibody was incubated for one hour. After washes, HP-conjugated goat anti-mouse antibody was added and bound the detector antibody. The HP catalyzes the conversion of the substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution. Finally, the reaction was terminated with sulfuric acid (H_2SO_4) and color intensity was determined at 450 nm with a microplate reader. The intensity of the color is proportional to the amount of dividing cells in the sample.

4.7.4 Quantitative protein secretion in mono- and co-cultures

Signaling protein secretion was analyzed using a two-way MLR assay. Four different donor PBMC lines were used as responder cells, in two different MLR combinations. PBMCs from two donors were mixed in equal amounts to activate the MLR, and a total cell number of $8.0x10^5$ PBMCs was seeded per well on 24-well plate. After initiating the MLRs, ASCs were added at densities of $3.0x10^4$ cells per well either in direct co-culture or using a semipermeable membrane inserts to prevent direct cell-cell contacts between ASCs and PBMCs. When using the inserts, PBMCs were pipetted into the inserts (pore size, 0.4 mm; ThinCert, Greiner Bio-One, Frickenhausen, Germany) and ASCs were seeded on the bottom of the wells. Cell culture supernatants from mono- and co-cultures were collected on day 5 and stored in -20°C until analysis. Cytokines and chemokines secreted by the cells were analyzed using Cytometric Bead Arrays (CBA): Human Chemokine Kit, Human Th1/Th2/Th17 cytokine kit and Human TGF-β1 Single Plex Flex Set (BD Biosciences). IDO, Galectin-1 and Galectin-3 were analyzed using colorimetric ELISA assays: ELISA kit for IDO (Cloud-Clone Corporation, USCN Life Science Inc.), and Human Galectin-1 and-3 Quantikine ELISA Kits (R&D Systems). A list of the analyzed signaling proteins is presented in Table 11. Each colorimetric ELISA reaction was done in triplicate, and averages of the parallel reactions were then taken into account in statistical analysis. CBA output data were analyzed using FCAP Array software version 3.0 (BD Biosciences) according to the manufacturer's instructions.

Table 11. Cell signaling proteins analyzed in study **III**.

Signaling protein	Used method	Manufacturer
CXCL8/IL-8 **CCL5/RANTES** **CXCL9/MIG** **CCL2/MCP1** **CXCL10/IP-10**	CBA, Human Chemokine Kit	BD Biosciences, Franklin Lakes, NJ, USA
IL-2 **IL-4** **IL-6** **IL-10** **TNF-α** **IFN-γ** **IL-17A**	CBA,Human Th1/Th2/Th17 Cytokine Kit	BD Biosciences
TGF-β1	Single Plex Flex Set	BD Biosciences
IDO	ELISA kit	Cloud-Clone Corporation, Uscn Life Science Inc., Wuhan, China
Galectin-1	ELISA kit	R&D Systems Inc., Minneapolis, MN, USA
Galectin-3	ELISA kit	R&D Systems

4.8 Statistical analyses

Statistical analyses were performed with IBM SPSS software version 19 - 21 (IBM Corp., Armonk, NY, www.ibm.com) in studies **I**-**III** and with GraphPad Prism 5.01 software (GraphPad Software, CA, USA, www.graphpad.com) in study **IV**. To analyze the effect of different culture conditions on cell-proliferation rate, cell surface-marker expression, and differentiation potential, a one-way ANOVA with Bonferroni post hoc test was used in study **I**, and a nonparametric Mann-Whitney U test in study **II**. A nonparametric Kruskal-Wallis test with Mann-Whitney U post hoc test was used in study **III** to compare different culture conditions and their effect on immunogenic proliferative response in one-way and two-way MLR, as well as for

determining the effect of different culture conditions on cell surface protein expression of cytometric data. The cytokine secretion data in study **III** was analyzed using a regression analysis with ranked values. In study **IV**, a one-way analysis of variance with the Bonferroni post hoc test for multiple comparisons was used to study statistically significant differences between study groups. The nonparametric Spearman correlation test was used to study correlation between DNA amounts and metabolic activity in study **II**, and correlation between DNA amounts, expression of VEGF, and ALP activity in study **IV**. When multiple comparisons were performed, the p values were multiplied by the number of comparisons before the interpretation of the data. The results were considered statistically significant when the p value was under 0.05. All data were presented as mean ± standard deviation (SD).

5 Results

5.1 Isolation of ASC under different culture conditions

ASCs were successfully isolated in FBS- and HS-containing medium and efficient cell proliferation was observed following the initial cell adhesion. Thus, the ASC isolation success rate under FBS and HS medium remained close to 100%. The XF/SF isolation of ASCs was also successful but more donor dependent. In studies **I**-**III**, the initial XF/SF isolation step was carried out using carboxyl-coated flasks that provided support for the cell adhesion. Correspondingly, the surface of standard Nunclon™ cell-culture flasks was not supportive enough during XF/SF isolation although a CELLstart™ coating was tested. In study **I**, the isolation efficiency of XF/SF cells was four out of six isolations, and in subsequent XF/SF isolations the success rate remained around 75% (three out of four isolations). Nevertheless, if the initial cell adhesion was successful the cell proliferation in XF/SF medium was efficient in further passages, and the cell-population doubling was notably faster than that in standard FBS- and HS-containing medium (Figure 13). After the first passaging, standard Nunclon™ flasks were used because ASCs were able to grow in standard flasks under XF/SF medium in the presence of Coating Matrix kit (study **I**) or CELLstart™ coating (studies **I**, **II**, **III**). Thus, the isolation of ASCs in defined XF/SF conditions was the most critical step of the cell culture, however from that step onwards XF/SF expansion was notably more efficient as compared to traditionally used FBS and HS cultures (Figure 13).

5.2 Cell surface profile of ASCs

Cell-surface marker expression of ASCs was performed in all studies **I**-**IV** to confirm the mesenchymal origin of the cells. In studies **I**, **II** and **III**, the cell surface profiles were determined for ASCs cultured in different serum conditions FBS, HS and XF/SF. In general, the characteristic immunophenotype of ASCs was maintained in every culture condition, with minor differences observed between XF/SF conditions and serum-containing media. The results of flow cytometric analyses from all studies

I-**IV** are combined and average values are presented in Figure 11. The mean of SD was calculated based on mean values of studies **I**-**IV**.

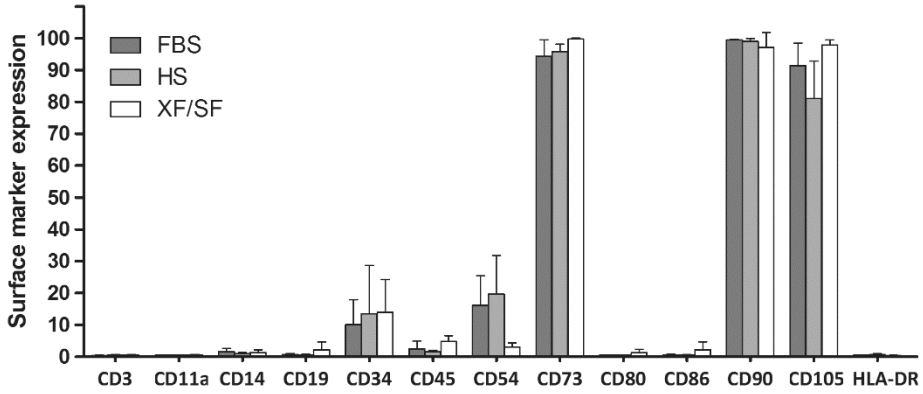

Figure 3. Surface marker expression of undifferentiated ASCs at passages 1-5 cultured in different serum conditions (FBS, HS and XF/SF) as analyzed by flow cytometry (studies I-IV) (n = 12 for FBS, XF/SF conditions; n = 18 for HS condition). Data are presented as mean ± SD.

ASCs expressed markers CD73, CD90 and CD105 in all of the studied culture conditions (Figure 11). Importantly, in studies **I**-**III** the expressions of stromal cell markers CD73, CD90 and CD105 were clearly positive (>90%) in defined XF/SF conditions. ASCs lacked the expression of CD3, CD11a, CD14, CD19, CD80, CD86, and HLA-DR in every culture condition with minor variations. ASCs showed average moderate expression for the hematopoietic progenitor and endothelial cell marker CD34 in all studied culture conditions (FBS 10.1±7.9; HS 13.6±15.1; XF/SF 12.7±10.3), although a variation between donors cell lines was observed.

The largest variation between different culture conditions was seen in the expressions of lymphocyte common antigen CD45 and the immune-related marker CD54 in studies **I**-**III**. On average, ASCs lacked the expression (<2%) of CD45 in FSB and HS media, and showed on average low expression (2% to ≤10%) in XF/SF conditions. Furthermore, moderate expression on average (5% to ≤40%) of CD54 was observed in FBS and HS conditions, whereas in XF/SF conditions the cells lacked the expression or showed very low expressions. The effect of passaging on the homogeneity of cell population was observed in study **I**, where the expression of CD34 and CD54 was higher in passage 2, but decreased in later passages indicating a more homogeneous population (Study I). However, this influence was not shown as clearly in subsequent studies **II**-**III**.

5.2.1 The effect of MMC on cell surface markers (II)

Cell surface marker expression of ASCs was analyzed after MMC treatment to understand the influence of crowding on cell surface protein expressions. ASCs cultured in FBS- and HS-supplemented medium were exposed to MMC during the cell expansion from P1 to P4. Nevertheless, the MMC treatment was not beneficial for XF/SF cultured ASCs and, as a consequence, the cell proliferation capacity was significantly decreased under MMC culture. For this reason, the flow cytometric analysis of XF/SF cultured cells was performed after seven days culture in MMC medium to ensure sufficient cell number for the analysis.

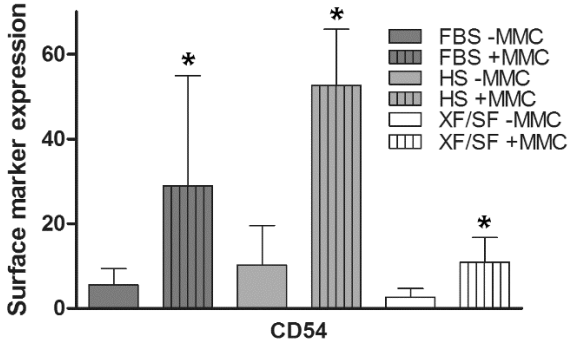

Figure 12. The expression of marker CD54 that was significantly increased ($p<0.05$) under MMC cultures in every culture condition (FBS, HS, and XF/SF) (n = 4). Data are presented as mean ± SD. * = $p<0.05$.

In general, the characteristic immunophenotype of ASCs was maintained after MMC exposure. ASCs showed expression for stromal markers CD73, CD90 and CD105 in both -/+MMC conditions in all of the studied culture conditions FBS, HS and in XF/SF (study **II**). Additionally, ASCs lacked the expression of CD3, CD11a, CD14, CD19, CD80, CD86, and HLA-DR in in both -/+MMC conditions. The most notable effect of MMC was observed in the expression of CD54, wherein the expression was significantly higher ($p<0.05$) in MMC cultures in all of the studied culture conditions (Figure 12).

5.3 Adipose stem cell morphology, viability and proliferation capacity

The cell morphology was examined by light microscopy to observe the differences between culture conditions, such as serum versus XF/SF conditions, as well as the effect of macromolecular crowding under different conditions (**I**, **II**). Cells grown in

the presence of serum adopted a wide spindle shaped morphology, whereas XF/SF cells were smaller, more spindle-shaped, and more fibroblast like (Figure 13). The adhesion of the cells grown in XF/SF medium was relatively weak, which was also reflected in the morphology of ASCs in XF/SF conditions.

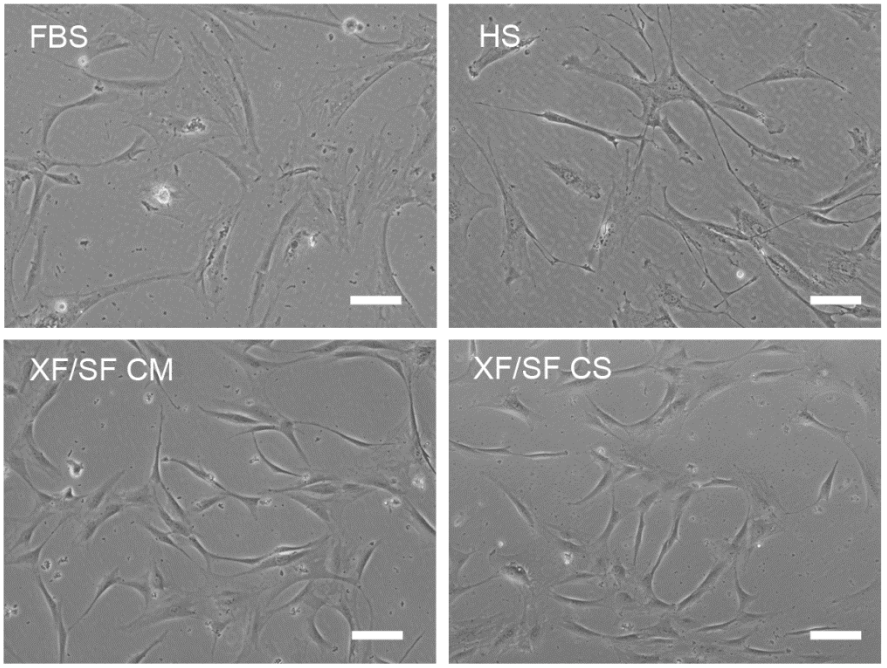

Figure 4. Morphologic images of cells cultured in different conditions: FBS, HS, XF/SF CM, and XF/SF CS at timepoint 4 days in P2. The morphology pictures of one donor cell line are presented, but cell proliferation was studied separately by four donor cell lines. The morphology of ASCs grown in XF/SF medium is more spindle-shaped and smaller than in cells grown under serum-containing medium (FBS, HS). Scale bar, 100 µm. XF/SF CM, xeno-free/serum-free media with coating matrix kit; XF/SF CS, xeno-free/serum-free media with CELLstart™ coating. (Study **I**)

The effect on MMC on cell morphology was visible in every condition and especially the morphology of XF/SF cultured cells was dramatically changed under MMC culture (Figure 14). During the culture in MMC medium, cells adopted rounded cell morphology and became larger in size compared to standard cultures. Many cell extensions were observed in XF/SF cultured cells under MMC medium. In XF/SF conditions, the cell proliferation rate was significantly altered under MMC culture as compared to standard cultures which could also be seen from

morphological images. Cell number decreased dramatically compared to standard cultures and cells under MMC were substantially larger with many nuclei in some cases (Figure 14).

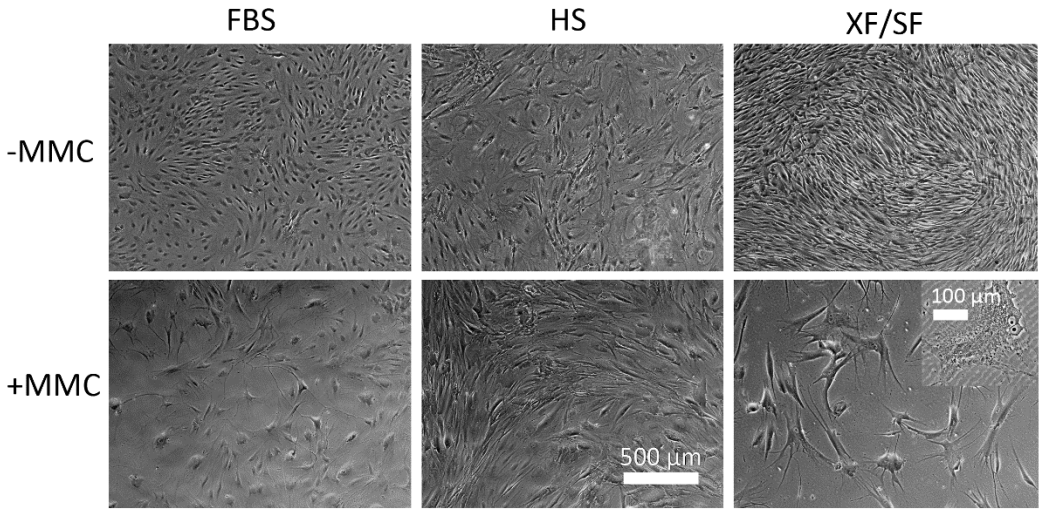

Figure 5. ASC morphology under different culture conditions: FBS, HS and XF/SF media. ASCs cultured under MMC adopted rounded morphology and became larger in size. The morphology pictures of one donor cell line are presented, although a cell proliferation was studied separately by four donor cell lines. The cell viability was poor under MMC in XF/SF cultures and thus, cell number decreased dramatically compared to standard cultures. Many nuclei were observed in some XF/SF cultured cells. MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free

5.3.1 Cell viability and proliferation

Cell viability and proliferation in different culture conditions was analyzed with WST-1 assay in study **I**. A statistically significant increase in population doubling was observed in XF/SF cultured cells when compared to FBS and HS conditions. Figure 15 demonstrates the efficient cell proliferation in XF/SF conditions in passages 2 (A) and 5 (B). Significantly higher cumulative population doubling was observed in XF/SF conditions compared with HS/FBS cultures in both passages 2 and 5 in study **I**.

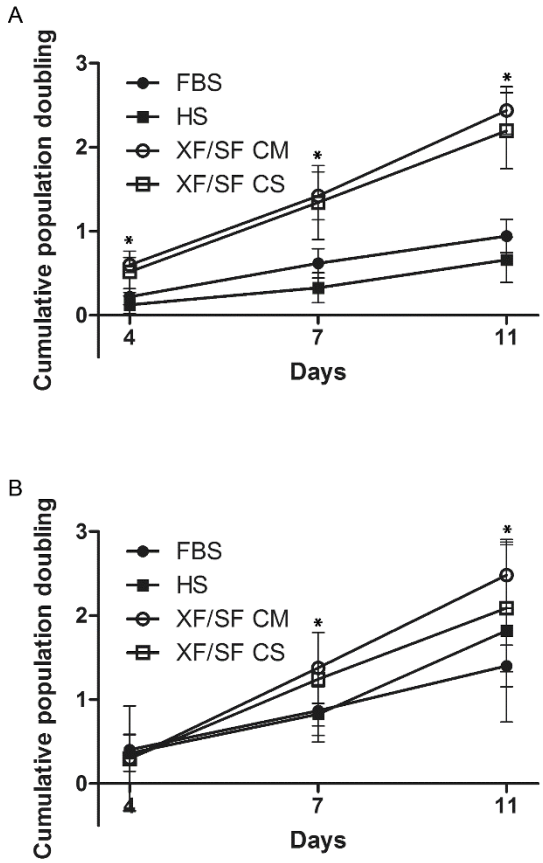

Figure 15. Cumulative population doubling was analyzed in different culture conditions (n = 4), in FBS- and HS-containing medium, as well as in two XF/SF media at time points 1, 4, 7, and 11 day in two passages 2 (A) and 5 (B). The data in the diagrams are presented as mean ± SD. Significantly higher cumulative population doubling was observed in XF/SF conditions compared with HS/FBS cultures in passage 2 at 4-, 7-, and 11-day time points. Furthermore, statistically significant difference in population doubling were seen in passage 5 at 7- and 11-day time points between cells grown in XF/SF CM medium and FBS-containing medium. * = $p<0.05$. XF/SF CM, xeno-free/serum-free media with coating matrix kit; XF/SF CS, xeno-free/serum-free media with CELLstart™ coating. (Study **I**)

In study **IV**, the DNA amount indicating the cell numbers in HS-supplemented cultures with BAG and β-TCP was analyzed with CyQUANT® cell proliferation assay because the WST-1/CCK-8 Cell Proliferation assays were not suitable for biomaterial studies. The DNA amount was significantly greater in the BAG cultures as compared to the β-TCP cultures, suggesting a greater cell proliferation rate in response to BAG. The DNA amount in CM, OM and OM with BMPs were analyzed with both biomaterials independently. Overall, OM increased the cell number significantly as compared to CM and interestingly, BMP supplementation resulted in reduced cell amounts as compared to OM with both biomaterials. Cell number in different treatment groups over time is presented in Figure 16.

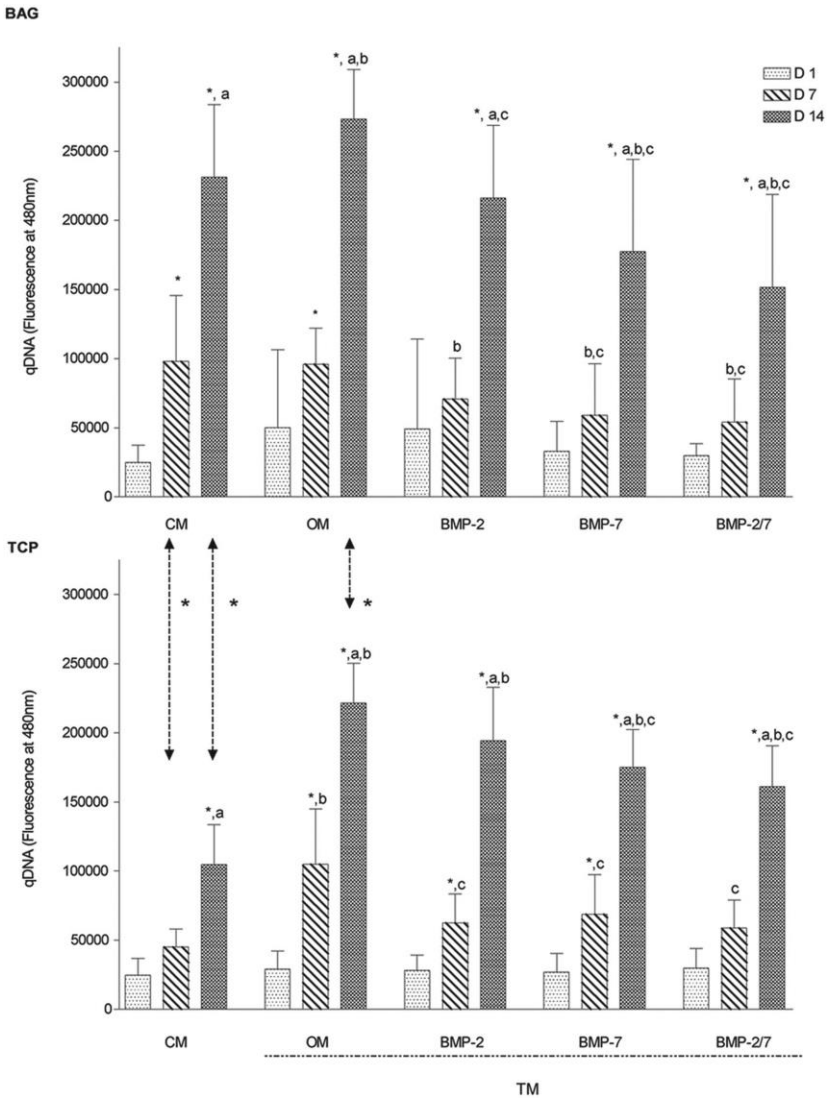

Figure 16. Comparative overview on cell amount over time analyzed by CyQUANT® cell proliferation assay (n = 6). Overall, cell amount increased continuously over time independent on biomaterial evaluated. Cell amount was greatest after exposure to BAG in OM. After 7 days, BAG increased cell amount significantly more in CM as compared to β-TCP. BMP supplementation decreased cell amount independent on biomaterial. BAG: bioactive glass; β-TCP: beta-tricalcium phosphate; CM: control medium; OM: osteogenic medium; BMP: bone morphogenetic protein; TM: treatment media. The * indicates significant difference between days 1–7 and days 7–14; a indicates significant difference between days 1 and 14; b indicates significant difference as compared to CM; c indicates significant difference as compared to OM; arrow with * represents significant differences between biomaterials. (Study **IV**)

In study **IV**, the cell viability was assessed by Live/Dead staining at day 14 (Figure 17). Live/Dead staining revealed a good cytocompatibility of both biomaterials, and the greater cell number that was confirmed by CyQUANT® analysis could also be seen by qualitative Live/Dead method. Nonetheless, Live/Dead staining showed good cell viability and successful cell attachment on both materials BAG and β-TCP which both efficiently supported the cells spreading and migration.

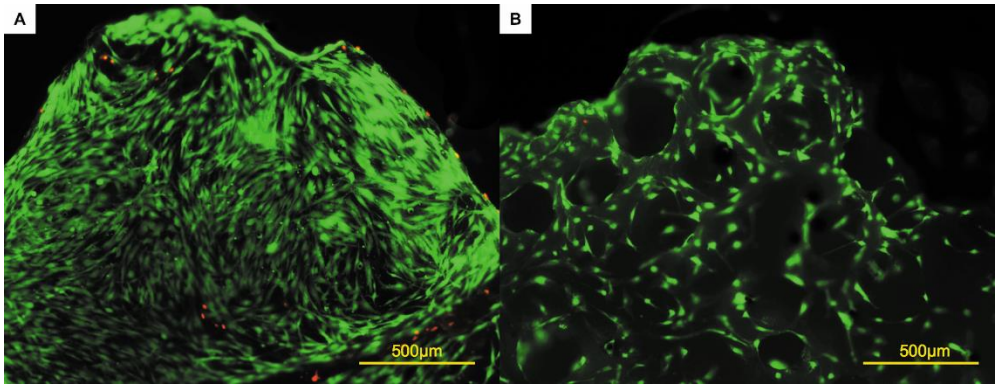

Figure 6. Live/Dead staining at day 14. Live-dead staining confirmed successful attachment and viability of cells on both biomaterials. A: BAG-granules; B: β-TCP-granules.

In study **II**, the DNA amount demonstrating the cell number was analyzed with CyQUANT® cell proliferation assay (Figure 18). The cell number was significantly higher under standard cultures in XF/SF conditions compared to FBS ($p<0.001$) and HS ($p<0.05$) media at each time point 1, 4, 7, and 11 days. Compared to MMC cultures, the cell number was significantly higher under standard culture conditions in HS media at day 1 ($p<0.05$), and in FBS media at days 4 ($p<0.05$), 7 and 11 ($p<0.001$) days. Under MMC condition at times points 7 and 11 days, the cell number was significantly higher in HS media ($p<0.05$) compared to FBS media. Although the metabolic activity was decreased in XF/SF cultures after MMC, no statistical differences could be established due to the low number of repeats, due to significantly decreased proliferation capacity of ASCs under MMC in XF/SF conditions. A statistically significant increase in cell number from day 1 to day 11 was observed in each culture media in both +/- MMC conditions, except for the cells in XF/SF media under MMC.

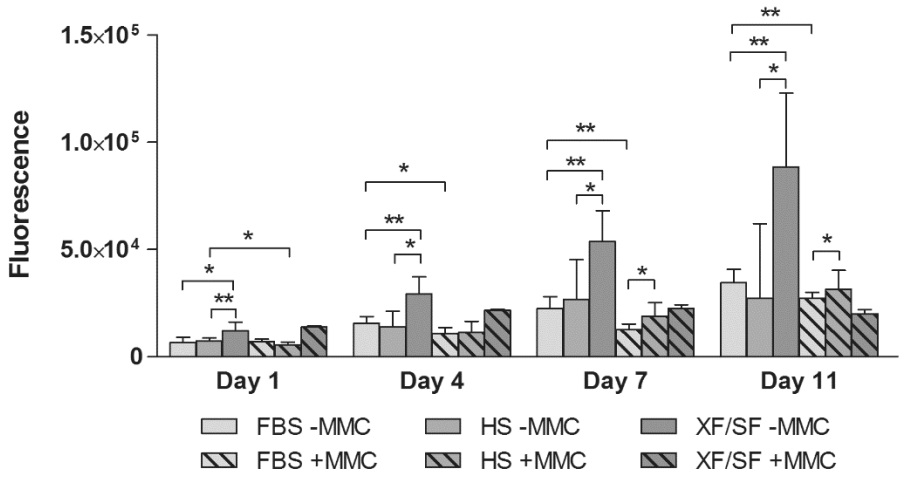

Figure 18. Cell number under different culture conditions was analyzed with CyQUANT® cell proliferation assay (n = 4). Significantly higher cell number was observed under standard cultures in XF/SF conditions compared to FBS ($p<0.001$) and HS ($p<0.05$) media at each time point 1, 4, 7, and 11 days. Cell number was also significantly decreased after MMC crowding in FBS, HS and XF/SF media. * = $p<0.05$; ** = $p<0.001$. Due to significantly decreased proliferation capacity of ASCs under MMC in XF/SF conditions, the CyQUANT® assay coud only be performed with one donor cell line under MMC in XF/SF media.

5.3.2 Metabolic activity under MMC conditions

The cell metabolic activity under -/+MMC cultures was analyzed with CCK-8 assay, and normalized to total DNA that was quantified by CyQUANT® cell proliferation assay (Section 5.3.1) (Figure 19). The MMC efficiently increased the metabolic activity in FBS media compared with standard conditions (-MMC) and a significant increase in metabolic activity in FBS conditions at 1 day time point ($p<0.001$) was observed. Furthermore, cells cultured under MMC conditions in FBS media had significantly higher metabolic activity compared to the ASCs cultured HS media at time points 4 ($p<0.001$), 7 and 11 ($p<0.05$) days. Although the metabolic activity was decreased in XF/SF cultures after MMC, no statistical differences were observed due to low number of repeats. The cell metabolic activity increased significantly ($p <0.001$) over time (from day 1 to day 11) in each culture conditions FBS, HS and XF/SF under standard cultures, and in FBS media under MMC conditions. The metabolic activity also correlated with the ASC number, that was studied with the Spearman correlation test (coefficient 0.78; $p <0.001$).

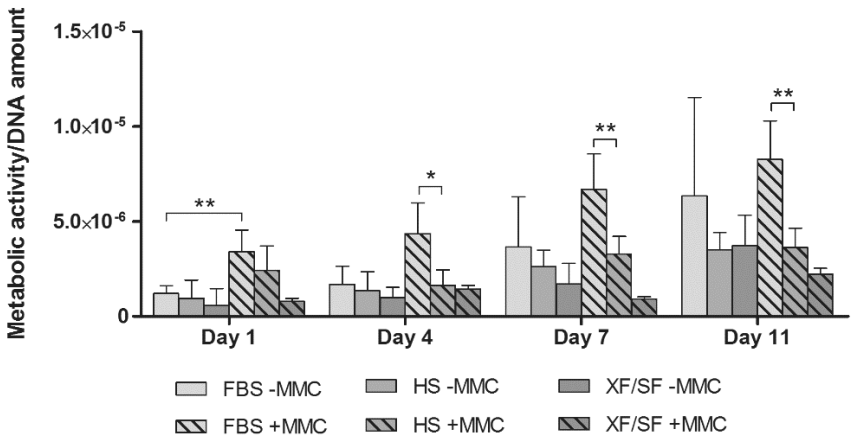

Figure 19. Metabolic activity was analyzed with CCK-8 assay and normalized to DNA amount (CyQUANT proliferation assay) (n = 4). Metabolic activity was increased in FBS media after MMC conditions compared with standard FBS conditions at day 1 and compared with HS conditions under MMC at days 4, 7 and 11. Metabolic activity also correlated with the cell number. * = $p<0.05$; ** = $p<0.001$.

5.4 Differentiation

The multilineage differentiation potential is defined as one of the characterization criteria for ASCs. In our studies **I** and **II,** the differentiation potential towards osteogenic, adipogenic and chondrogenic cells was evaluated in different serum supplementation conditions FBS, HS and XF/SF, as well as using MMC method. In study **IV**, osteogenic differentiation potential of ASCs combined with biomaterials BAG and β-TCP, and growth factors BMP-2 and-7 was evaluated.

5.4.1 Multipotent adipose stem cells in XF/SF conditions (I)

In the osteogenic induction cultures of study **I**, cells expanded in HS- or FBS-containing medium showed slightly enhanced capacity to undergo osteogenic differentiation compared with cells expanded under XF/SF conditions, based on the ALP staining (Figure 20). However, to confirm the osteogenic potential of XF/SF cultured ASCs, XF/SF expanded cells were primed with FBS- or HS-containing media. As a consequence, the differentiation of XF/SF cells was efficient after FBS or HS priming, which demonstrated that the multilineage capacity of ASCs was maintained also in XF/SF conditions (study **I**).

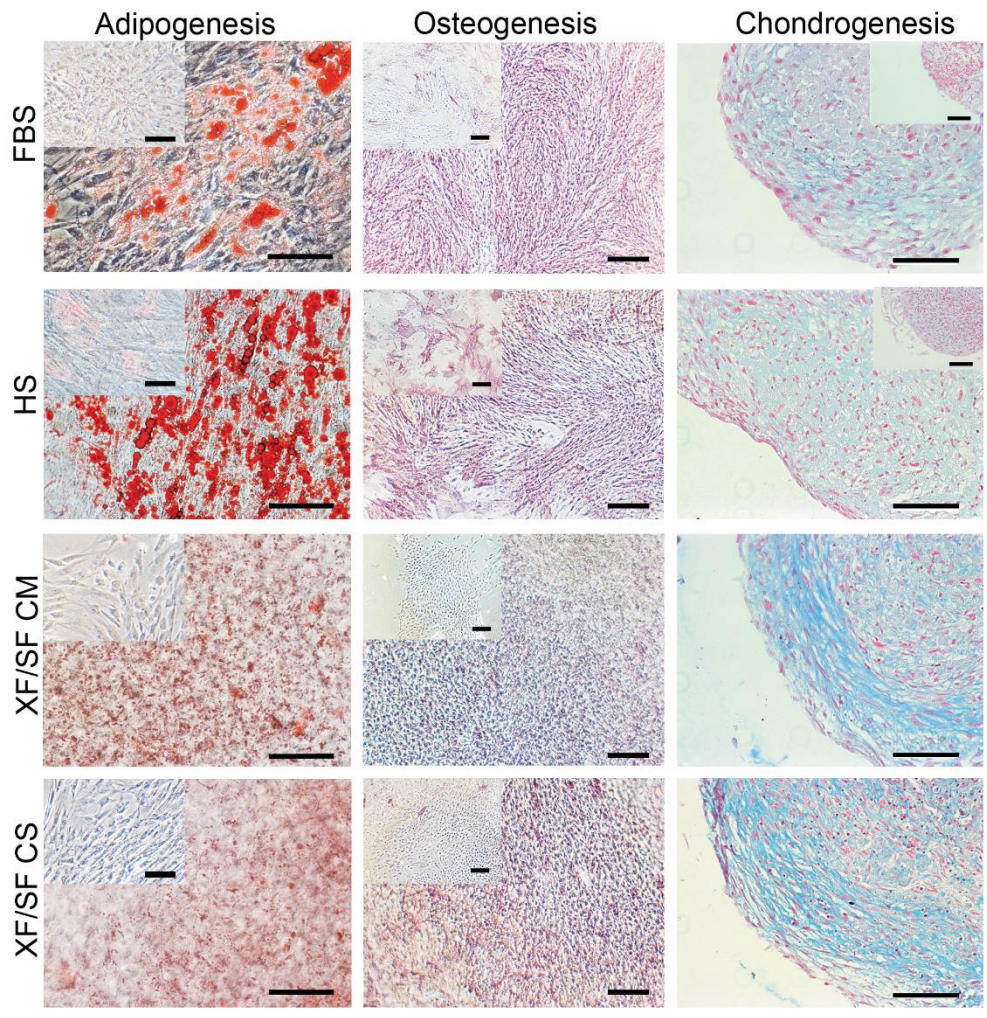

Figure 20. Multilineage differentiation potential of ASCs cultured in four different conditions FBS, HS and two XF/SF conditions. Differentiation results of one donor cell line are presented, although the analyses were performed separately by four donor cell lines. Oil Red-O staining indicates the formation of intracellular lipid in cells going through adipogenic differentiation (scale bar, 100 µm); ALP staining reveals the alkaline phosphatase activity in osteogenic-differentiation cultures (scale bar, 300 µm), and Alcian blue staining recognizes the glycosaminoglycans of the cells going through chondrogenic differentiation (scale bar, 100 µm). Adipogenesis and osteogenesis was more effective in serum-containing media, whereas the most intense chondrogenesis was seen in XF/SF cultures. XF/SF CM, xeno-free/serum-free media with coating matrix kit; XF/SF CS, xeno-free/serum-free media with CELLstart™ coating. (Study **I**)

In addition to ALP staining, the osteogenic differentiation was evaluated by the expression of osteogenesis specific genes. In contrast to ALP staining results, the expression of *RUNX2* was increased in XF/SF conditions compared with serum-containing medium, and a statistically significant increase was seen between XF/SF CM and serum conditions in passage 5 (Study **I**; Figure 6C). The response of gene *DLX5* (Study **I**; Figure 6E) to the osteogenic induction was stronger in XF/SF cultured cells compared with serum conditions, and a statistically significant increase in *DLX5* expression was seen in cells cultured in XF/SF CM induction when compared with serum induction, as well as XF/SF CM control. The ALP staining result was verified at gene-expression level, where a trend of increased expression was observed in all differentiation cultures compared with control samples (Study **I**; Figure 6D). Albeit the expression of ALP was increased after induction, no significant differences were seen because of the high standard deviation between different donor cell lines.

In the adipogenic induction cultures of study **I**, oil droplets were visible by light microscopy in cells expanded in HS- or FBS-containing medium. In XF/SF cultures, differentiation was clearly initiated but not progressed efficiently towards adipogenic cells, which was shown by smaller oil droplets in Oil Red-O staining (Figure 20). To confirm the adipogenic potential of XF/SF cells, FBS- or HS-containing induction media were used for XF/SF expanded ASCs and as a consequence, effective adipogenic differentiation was observed, compared with earlier XF/SF differentiation without serum priming. As predicted, differentiation was more efficient when HS or FBS priming was used and HS media clearly displayed increased differentiation compared with FBS media (study **I**).

The results acquired by Oil Red-O staining were still confirmed by the analysis of the adipogenesis associated gene expressions. A trend of a higher expression of the PPARγ gene was noted in XF/SF conditions compared with serum-containing medium, but no significant differences were observed because of the high standard deviation between different donor cell lines (Study **I**; Figure 6A). Further, the expression of a gene aP2 (Study **I**; Figure 6B) was significantly increased in HS-supplemented induction medium compared with HS control medium and the cells in all the other induction media, which was in line with the results of Oil Red-O staining.

Chondrogenic differentiation was more intense in XF/SF conditions compared with serum-containing medium based on the qualitative Alcian blue staining of proteoglycans after micro-mass culture in chondrogenic induction medium (Figure 20). The pellet size was also larger in XF/SF conditions versus HS or FBS cultures.

5.4.2 The effect of MMC on cell differentiation (II)

Based on the results of quantitative Alizarin Red staining (Figure 21), ASCs expanded in HS-containing medium showed significantly the most efficient osteogenic differentiation capacity compared with FBS cultures in three treatment groups 1) E-MMC; D-MMC, 3) E+MMC; D-MMC, 4) E+MMC; D+MMC ($p<0.05$) and compared with XF/SF conditions in two treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC ($p<0.05$). Moreover, significantly stronger differentiation potential was observed in FBS conditions in treatment group 2) E-MMC; D+MMC compared with XF/SF conditions in the same treatment group ($p<0.05$).

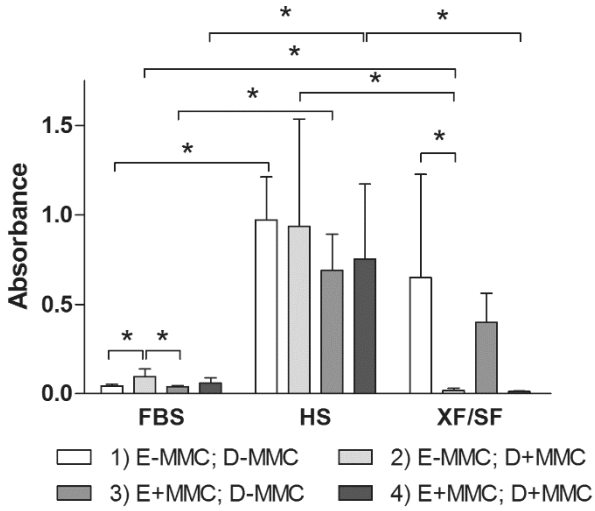

Figure 21. Quantitative Alizarin Red staining results showing the osteogenic differentiation potential of ASCs under different treatment groups (n = 4). The most efficient osteogenic differentiation was observed in HS media. Numbering 1) - 4) corresponds to treatment groups described in text. * = $p<0.05$. (Study **II**)

Cells cultured in FBS media in treatment group 2) E-MMC; D+MMC showed significantly stronger osteogenic differentiation potential compared with treatment groups 1) E-MMC; D-MMC and 3) E+MMC; D-MMC ($p<0.05$) in FBS media. The viability of ASCs was poor in XF/SF conditions under MMC and thus, the most efficient osteogenic differentiation was observed in treatment group 1) E-MMC; D-MMC, in which a significantly stronger differentiation capacity was observed compared with group 2) E-MMC; D+MMC ($p<0.05$). Interestingly, one of the four donor cell lines that survived during expansion under MMC in XF/SF conditions showed relatively efficient differentiation capacity after induction in standard XF/SF media.

Compared with control cultures of the same treatment group, significantly stronger differentiation potential was observed in all four induction cultures in HS media ($p<0.05$). In FBS media a significant difference between induction and control cultures was only observed in two treatment groups 1) E-MMC; D-MMC and 2) E-MMC; D+MMC ($p<0.05$). The AR staining results in different treatment groups under FBS, HS and XF/SF conditions are presented in Figure 22. The altered morphology of XF/SF cells as well as increased cell size after exposure to MMC is also visible in Figure 22: XF/SF cells in treatment group 4) E+MMC; D+MMC.

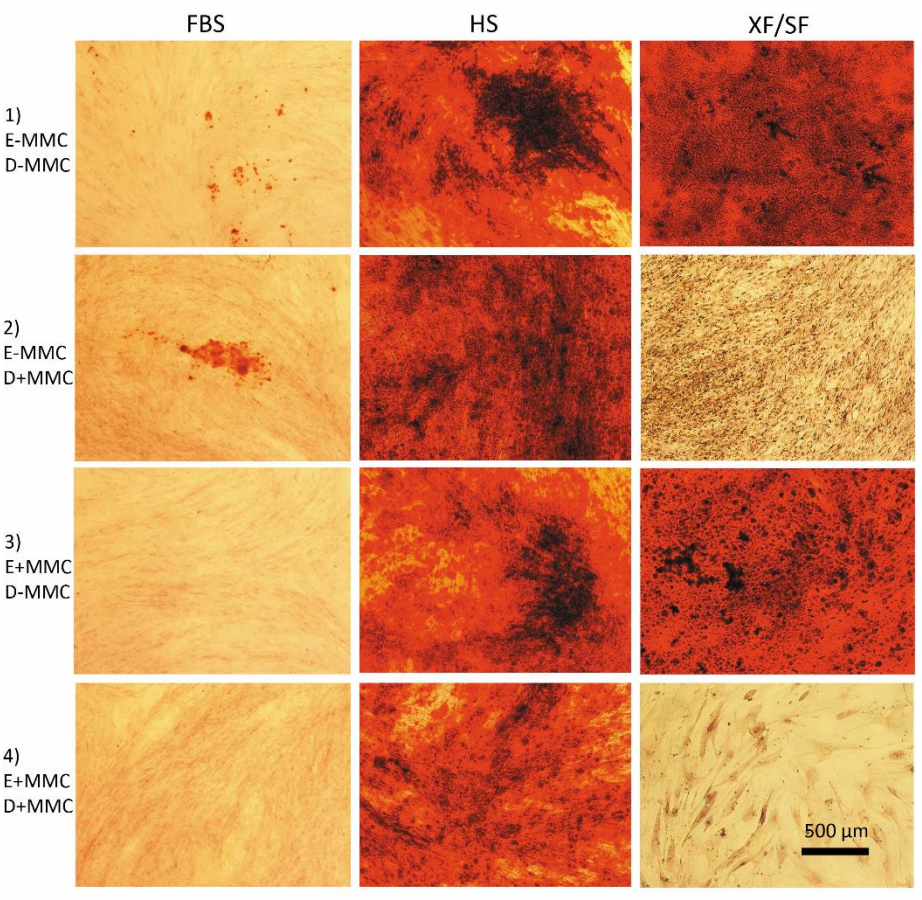

Figure 22. Alizarin Red staining in different culture condition FBS, HS and XF/SF conditions under four MMC treatment groups in study **II**. Osteogenic differentiation results of one donor cell line are presented, although the differentiation was analysed separately by four donor cell lines. The most efficient osteogenic differentiation was observed in HS-supplemented culture media. (Study **II**)

The most efficient adipogenic differentiation was observed in HS-containing media, based on the results of Nile Red staining that was normalized to cell number (Figure 23). MMC clearly supported adipogenic commitment of ASCs cultured in FBS and HS conditions, and cells accumulated more lipid content under MMC induction in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC compared with induction in standard media in treatment groups 1) E-MMC; D-MMC and 3) E+MMC; D-MMC. Compared with control cultures of the same treatment group, clearly stronger differentiation potential was observed in all four induction cultures in FBS and HS media. The cell viability was poor under MMC in XF/SF cultures and thus, the adipogenic induction could only be performed after expansion in standard XF/SF media, however, MMC did not support adipogenic differentiation of XF/SF cells. The Nile Red staining of lipid droplets in different treatment groups under FBS and HS conditions is presented in Figure 24.

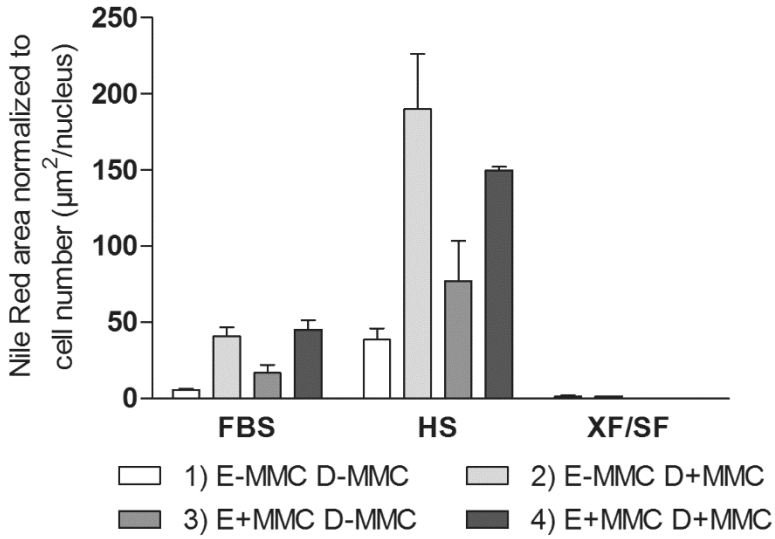

Figure 23. Quantitative Nile Red staining normalized to cell number (n=1). Adipogenic differentiation was enhanced under MMC induction in FBS and HS conditions in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC compared with induction in standard media, as quantitated by the area of Nile Red staining of lipid droplets. The most efficient adipogenic differentiation was observed in HS-containing media. Numbering 1) - 4) corresponds to treatment groups described in the text. (Study **II**)

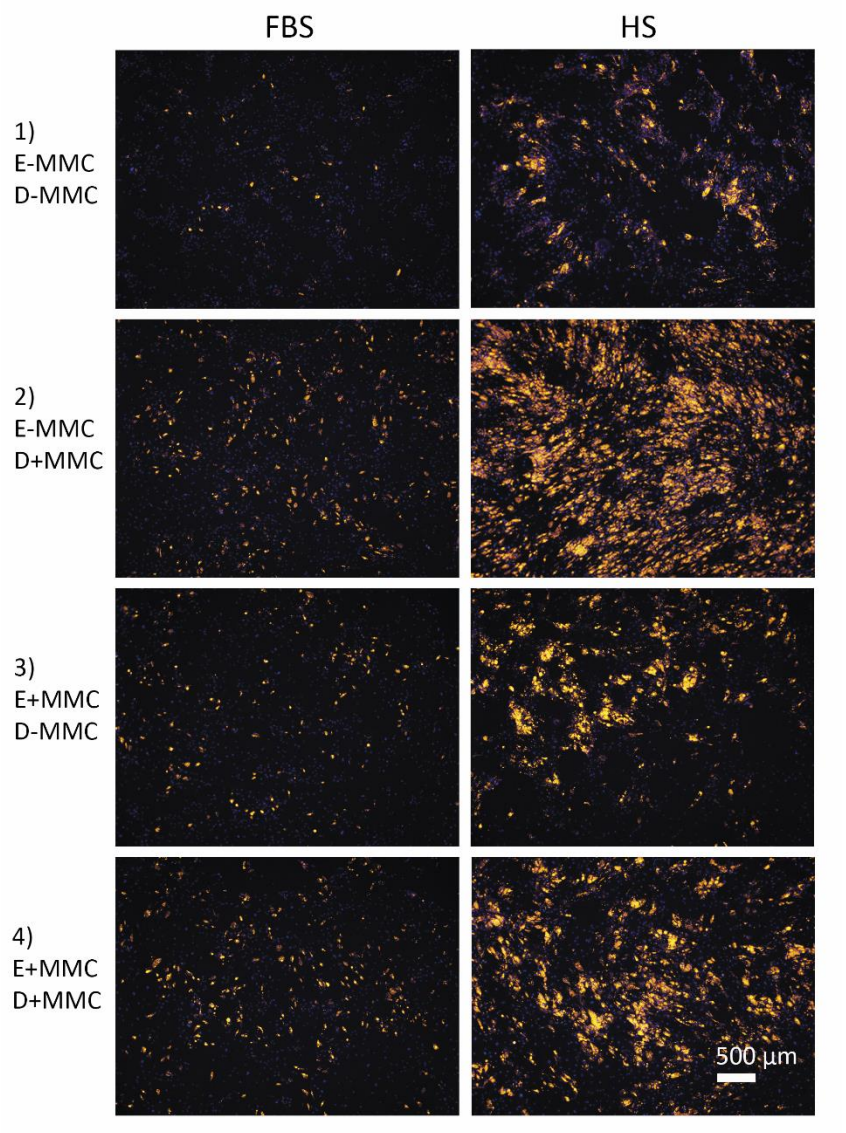

Figure 24. Nile Red staining indicating the adipogenic differentiation in different treatment groups. The osteogenic differentiation results of one donor cell line are presented. The accumulation of lipid droplets was increased under MMC induction in FBS and HS conditions in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC compared with induction in standard media. Numbering 1) - 4) corresponds to treatment groups described in the text. (Modified from study **II**)

Chondrogenic differentiation of ASCs was efficient in all studied culture conditions, however more ECM was deposited after expansion in standard conditions based on qualitative Alcian blue staining. Interestingly, the cells expanded under MMC conditions prior to chondrogenic induction formed a less dense histological architecture of the micro-mass pellet. Alcian blue staining results of two donor cell lines are presented in Figure 25.

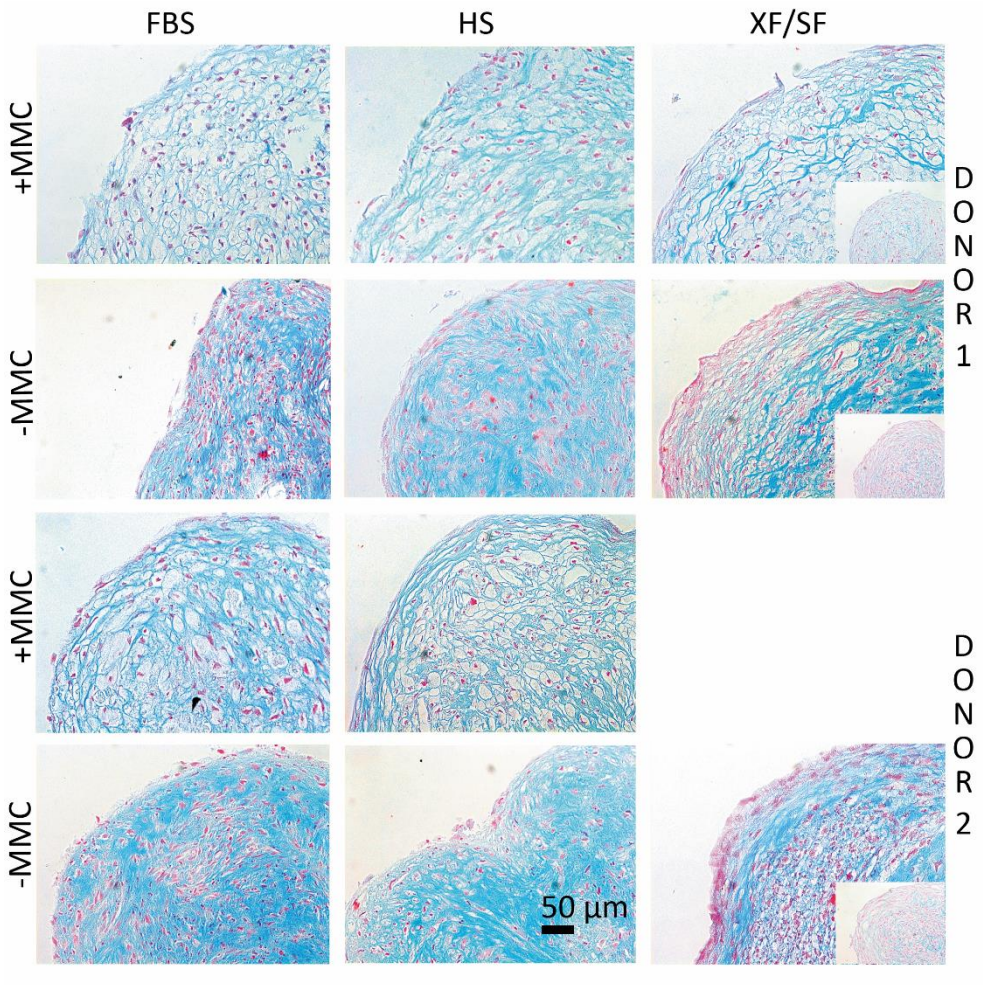

Figure 25. Alcian blue staining indicating the chondogenic differentiation in different conditions after cell expansion +/-MMC. The chondrogenic differentiation results of two donor cell line are presented, although the differentiation was analysed by four donor cell lines. More ECM was deposited after expansion in standard conditions and the histological structure of the micro-mass pellets was more compacted, compared with expansion under MMC conditions. (Modified from study **II**)

5.4.3 BMP-2- and BMP-7-supplemented media did not enhance osteogenic differentiation with BAG and β-TCP in vitro (IV)

Osteogenic differentiation was determined by ALP staining and by quantitative ALP (qALP) analyses, and the results of both methods were consistent. Osteogenic differentiation of ASCs on BAG was the most efficient in CM without osteogenic supplements (Figure 26), whereas decreased ALP activities were observed when osteogenic induction media or BMP supplementation was used. On the contrary, ASCs cultured on β-TCP required OM to undergo osteogenic differentiation. The BMP supplementation did not enhance the osteogenic differentiation independent of biomaterial, but a significantly lower ALP activity was quantitatively detected from BAG group with BMP-2 and -7 when compared with β-TCP group. In general, BAG in CM showed superior osteogenic potential confirmed by significantly greater qALP activities at days 1, 7, and 14 (Figure 26).

The qALP activity was decreased in both biomaterials groups from day 1 to 7 which did not correlate to DNA amount, however an increase of qALP was observed from days 7 to 14 (Figure 26). In BAG group, significantly lower qALP activities were measured in all treatment media at days 7 and 14 compared with CM, suggesting an overall stimulatory effect of BAG alone on early osteogenesis. BMP supplementation did not enhance the osteogenic differentiation in BAG group but instead, significantly lower qALP activities were measured in all BMP media at day 14 compared with OM. In general, significantly lower ALP expressions were measured in BMP-7 and BMP-2/7 medium at day 14 ($p<0.05$) compared with OM in both biomaterial groups, suggesting an inhibitory effect of BMPs in early osteogenic differentiation.

To confirm the effect of biomaterials and growth factors on osteogenic differentiation of ASCs, the expression of osteogenesis related genes *OPN*, *RUNX-2*, *Col-1*, and *OC* were analyzed at day 14 (study **IV;** Figures 4 and 5). In the BAG group, the cell response to the BMP supplementation was shown in the expression of OPN that was significantly higher in the BMP-2/7 medium compared with CM, OM, and BMP-2 medium. Interestingly, this trend was not seen in the β-TCP group. In alignment with the results of ALP activity, the *Col-1* expressions were significantly lower in any treatment media compared with control media in the BAG group. In the β-TCP group, there were no differences between treatment groups in expressions of *Col-1*, which was also confirmed by ALP activity measurements. The expression levels of genes *RUNX-2* and *OC* were also analyzed but no significant differences were observed between different treatment media.

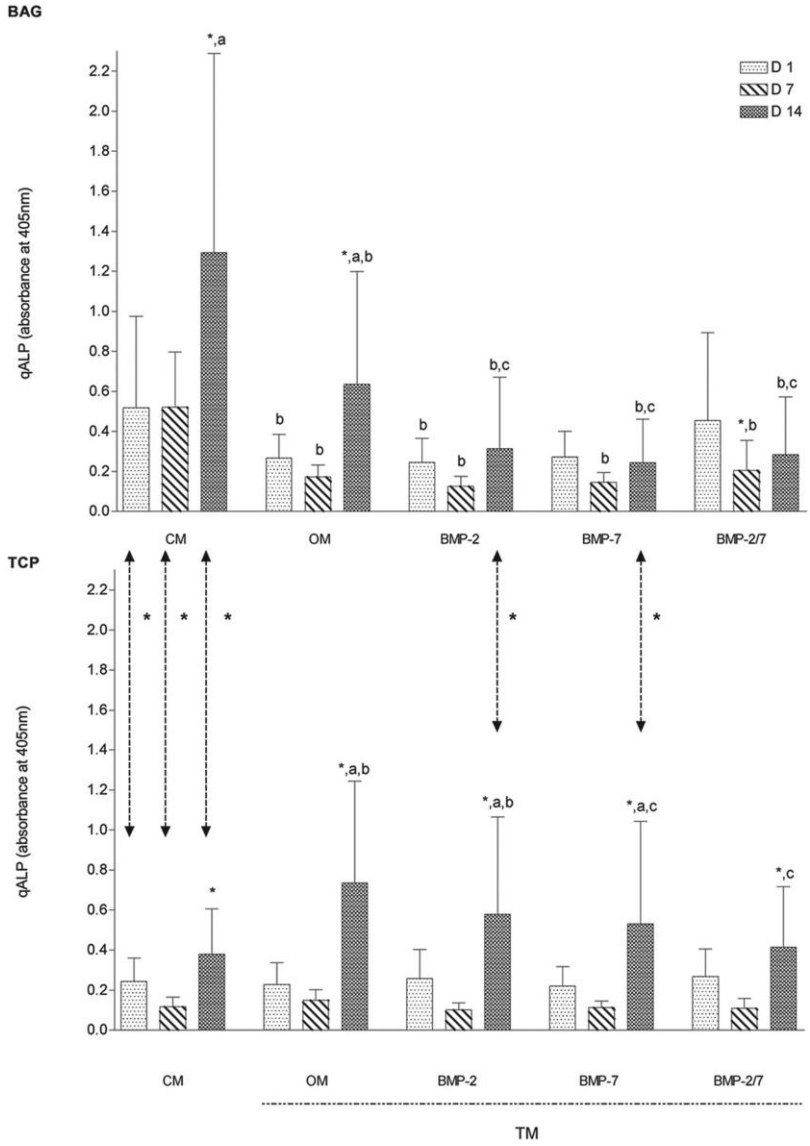

Figure 26. Comparative overview on quantitative ALP measurements over time (n=6). Osteogenic differentiation of ASC on BAG was the most efficient in CM without osteogenic supplements, whereas decreased ALP activities were observed when osteogenic induction media or BMP supplementation was used. ASCs cultured on β-TCP required OM to undergo osteogenic differentiation. The BMP supplementation did not enhance the osteogenic differentiation independent of biomaterial. * indicates significant difference between days 1–7 and days 7–14; a indicates significant difference between days 1 and 14; b indicates significant difference when compared with CM; c significant different when compared with OM; arrows with * indicates significant differences between biomaterials. (Study **IV**)

5.5 Angiogenic potential of ASCs (IV)

VEGF analyses were used to study the effect of the biomaterials on the angiogenic potential of ASCs at 1, 7 and 14 day time points (Figure 27). In both biomaterial groups, the expression of VEGF correlated with cell amounts, and the VEGF expression levels rose continuously over time and reached highest levels after 14 days in CM and all treatment media. The Spearman correlation coefficient was 0.75 for BAG ($p = 0.0012$) and 0.56 for β-TCP ($p = 0.031$). In BAG group, VEGF levels in all treatment media were significantly lower ($p<0.05$) when compared with CM in each time points. Furthermore, after BMP supplementation the expression levels were significantly decreased at days 7 and 14 ($p<0.05$) compared with OM. In β-TCP group, significantly lower VEGF levels were measured in all treatment media at days 1 and 7 ($p<0.05$) compared with CM. When compared with OM, significantly decreased VEGF levels were measured in BMP-2 medium at day 7 and in BMP-7 and BMP-2/7 at days 1 and 7 ($p<0.05$). In overall, the BMP supplementation did not enhance the VEGF expression in either material group. However, β-TCP in combination with BMPs was more beneficial for inducing VEGF expression compared with BAG.

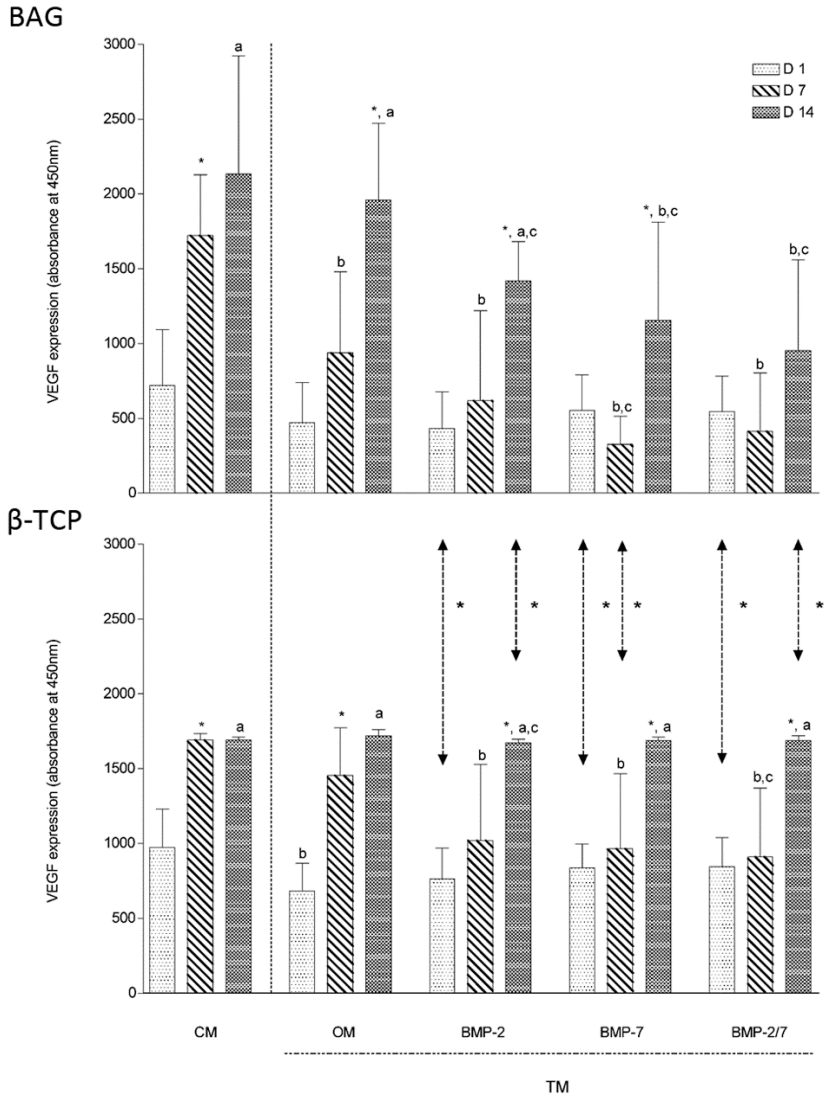

Figure 27. Comparative overview on VEGF measurements over time (n=6). Supplementation with BMPs did not enhance the angiogenic potential of ASCs in either material BAG or β-TCP. Although a higher expression of VEGF was measured in β-TCP group after BMP-supplementation compared with BAG, an increased angiogenic potential was observed with BAG in CM compared with β-TCP. * indicates significant difference between 1–7 and 7–14 days; a indicates significant difference between days 1 and 14; b indicates significant difference when compared with CM; c indicates significant difference when compared with OM; arrow with * represents significant differences between biomaterials. (Study **IV**)

5.6 Immunological properties of adipose stem cells (III)

5.6.1 ASCs elicited a weak immunogenic response

One-way MLR assays were performed to assess the immunogenicity of ASCs (Figure 28 A, B). Although the different culture conditions of the ASCs had a significant effect on the immunogenicity of the cells, the ASCs expanded in all of the studied conditions elicited only a weak immunogenic response compared with the maximal positive control response of PBMCs activated with PHA mitogen (Figure 28 A, B). In Figure 28A and B, a value of 1 indicates the baseline response of PBMCs without ASCs, and values above 1 indicate activation. ASCs expanded in FBS-containing medium induced the lowest immunogenic response on PBMCs in both passages 2 and 5. In passage 2, a significantly lower immunogenic response on PBMCs was observed in FBS medium ($p<0.05$) when compared with ASCs expanded in HS or XF/SF conditions, with $1.0x10^4$ and $2.0x10^4$ stimulator ASCs (Figure 28 A, a). In passage 5, a significantly lower PBMC immunogenic response was observed in FBS medium ($p<0.05$) compared with HS medium, with $1.0x10^4$ stimulator ASCs (Figure 28B, *). Furthermore, the immunogenic response in passage 5 was significantly stronger in HS medium ($p<0.05$), with $2.0x10^4$ stimulator ASCs, compared with both FBS and XF/SF conditions (Figure 28B, b).

5.6.2 ASCs showed a suppressive effect on PBMC proliferation

Differences between culture conditions were observed with respect to suppression potential of ASCs (Figure 28C and D). The suppression potential of ASCs was dependent on the ASC number, as well as the culture condition. Co-culture results in two-way MLRs were standardized using values from MLRs without ASCs, and therefore a value of 1 represents the baseline and values below 1 indicate suppression in Figure 28 C and D. ASCs expanded in FBS medium efficiently suppressed proliferation of PBMCs stimulated in MLR in both passages 2 and 5, whereas ASCs expanded in HS medium showed suppression in passage 2 with $1.0x10^4$ and $2.0x10^4$ cells and in passage 5 with $2.0x10^4$ cells. By contrast, ASCs expanded in XF/SF conditions were not able to suppress PBMC proliferation in passage 2 and suppressed the proliferation in passage 5 only with $1.0x10^4$ or $2.0x10^4$ cells.

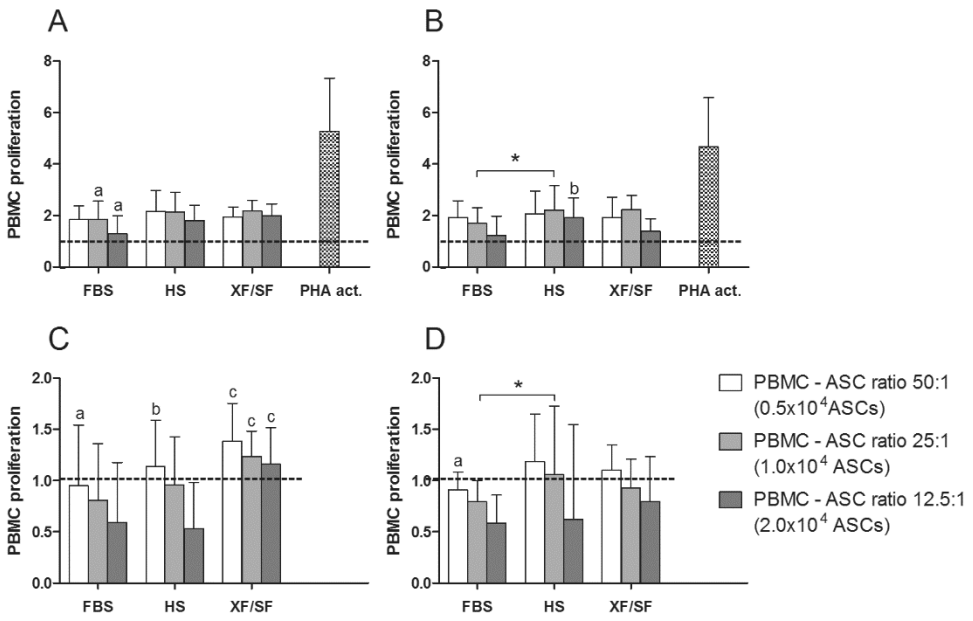

Figure 28. The immunogenicity (A, B) and suppression potential (C, D) of ASCs are presented in passages 2 (A, C) and 5 (B, D) (n=5). ASCs expanded in FBS, HS, and XF/SF conditions elicited only a weak immunogenic response compared with the maximal positive control response activated with PHA. Suppression potential was dependent on the ASC number, as well as the culture condition. The dashed line represents a baseline response; values above 1 indicate activation (A, B), and values below 1 indicate suppression (C, D). The results are presented as means ± SD. a = $p<0.05$ when FBS is compared with HS and XF/SF conditions; b = $p<0.05$ when HS is compared with FBS and XF/SF conditions; c = $p<0.05$ when XF/SF is compared with FBS and HS conditions; * = $p<0.05$ when FBS in compared with HS but not XF/SF condition. (Modified from study **III**)

Some of the differences in suppressive potential between different serum conditions were statistically significant. In both passages 2 and 5, FBS showed significantly stronger suppression ($p>0.05$), with the lowest ASC number ($0.5x10^4$) (Figure 28C and D, a) compared with HS and XF/SF conditions that were not capable of inducing suppression. In addition, XF/SF-expanded ASCs were significantly less suppressive ($p<0.05$) compared with HS and FBS conditions in passage 2 (Figure 28C, c). Furthermore, a significantly stronger suppression was observed with $1.0x10^4$ cells in FBS medium ($p<0.05$) when compared with HS medium in passage 5.

5.6.3 Signaling protein secretion of ASCs

A panel of secreted proteins was analyzed from cell culture supernatants either in direct or indirect co-cultures of ASCs and PBMCs after the expansion of ASCs in FBS, HS, and XF/SF conditions (study **III**). Altered secretion profiles were observed between the different serum conditions of the ASCs, but the effect of direct versus indirect contact between ASCs and PBMCs was also evident (Figure 29). Figure 29 demonstrates the statistically significant differences between different culture conditions with respect to the protein secretion data. The results of all studied protein secretions are presented in original publications (study **III**).

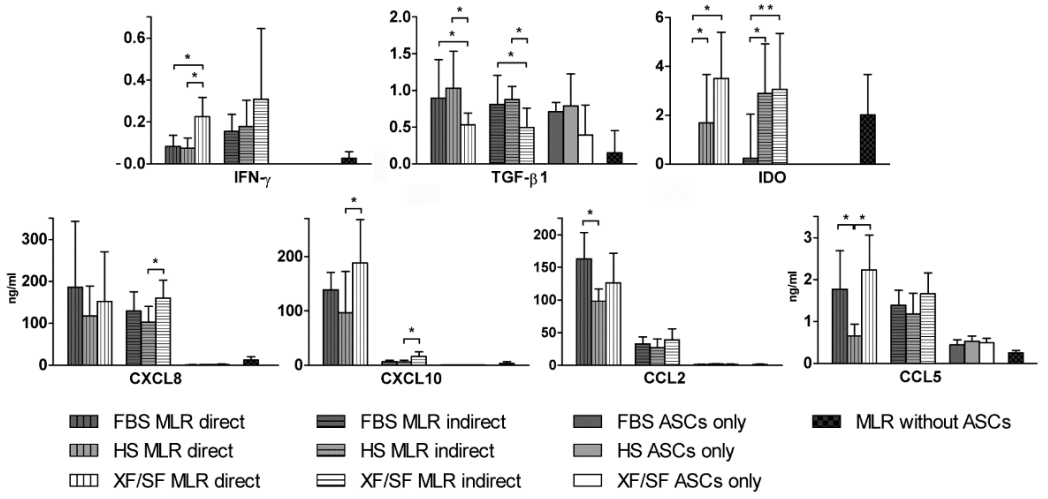

Figure 29. Secreted proteins analyzed in direct co-cultures and using separating membranes between ASCs and peripheral blood mononuclear cells (n = 4). Prior to the co-cultures, ASCs were expanded in different serum conditions: FBS, HS, and XF/SF. Altered secretion profiles were observed between the different conditions of the ASCs. TGF-β1 and IDO amounts of medium alone were subtracted from quantitated concentrations of TGF-β1 and IDO in mono- and cocultures. The results are presented as means ± SD. * = p<0.05; ** = p<0.001. Abbreviations: CCL2 and -5, C-C chemokine ligands 2 and 5; CXCL8 and CXCL10, CXC chemokine ligands 8 and 10; IDO, indoleamine 2,3-deoxygenase; IFN-γ, interferon γ; MLR, mixed lymphocyte reaction; TGF-β1, transforming growth factor β1. (Modified from study **III**)

Direct co-cultures

A significantly higher secretion of IFN-γ was detected in XF/SF conditions ($p<0.05$) compared with co-cultures with FBS- and HS expanded ASCs. Similarly, the lowest TGF-β1 concentrations were measured in direct co-cultures containing XF/SF expanded ASCs ($p<0.05$) compared with either FBS or HS conditions. Moreover, secretion of CCL2 was significantly higher in co-cultures with FBS-expanded ASC ($p<0.05$) compared with HS-expanded ASCs and a significantly lower secretion of chemokine CCL5 was measured in HS conditions ($p<0.05$) compared with XF/SF and FBS conditions. The secretion of chemokine CXCL10 was significantly higher in co-cultures with XF/SF-expanded ASCs ($p>0.05$) compared with HS conditions. Furthermore, the lowest IDO concentrations were measured in direct co-cultures containing ASCs expanded in FBS conditions compared with either HS ($p<0.05$) or XF/SF ($p<0.001$) conditions.

Table 12. Statistically significant differences in secretions of signaling proteins (n = 4).

	Indirect vs. direct co-cultures	Direct co-cultures			Indirect co-cultures			ASCs only	PBMCs only
		FBS vs. HS	FBS vs. XF/SF	HS vs. XF/SF	FBS vs. HS	FBS vs. XF/SF	HS vs. XF/SF		
CXCL8/IL-8							*↓	**↓	**↓
CCL5/RANTES		*↑		*↓				**↓	**↓
CXCL9/MIG	**↓							**↓	**↓
CCL2/MCP-1	**↓	*↑						**↓	**↓
CXCL10/IP-10	**↓			*↓			*↓	**↓	**↓
IL-6	**↓							**↓	**↓
TNF-α	**↑							**↓	
IFN-γ	*↑		*↓	*↓				**↓	*↓
TGF-β1			*↑	*↑		*↑	*↑		*↓
IDO		*↓	**↓		*↓	*↓		**↓	
Galectin-1	**↓							**↓	**↓
Galectin-3	**↑							**↓	

*, $p<0.05$, **, $p<0.001$. An arrow indicates the increase or decrease in the amount of secreted protein.

Reactions containing only ASCs or PBMCs showed a significantly lower secretion of cytokines and chemokines IL-6, CCL2, CCL5, CXCL8, CXCL9, and CXCL10 ($p<0.001$) compared with direct co-cultures. IFN-γ and TNF-α secretions were not detected in reactions containing only ASCs, and a significantly lower secretion of IFN-γ and TGF-β1 ($p<0.05$) was measured in reactions containing only PBMCs compared with direct co-cultures. Concentrations of IDO, galectin-1, and galectin-3 were significantly lower ($p<0.001$) in reactions containing ASCs alone compared with direct co-cultures, and the concentration of galectin-1 ($p<0.001$) was also significantly lower in reactions containing only PBMCs when compared with direct co-cultures. Statistically significant differences in secretions of signaling proteins are presented in Table 12.

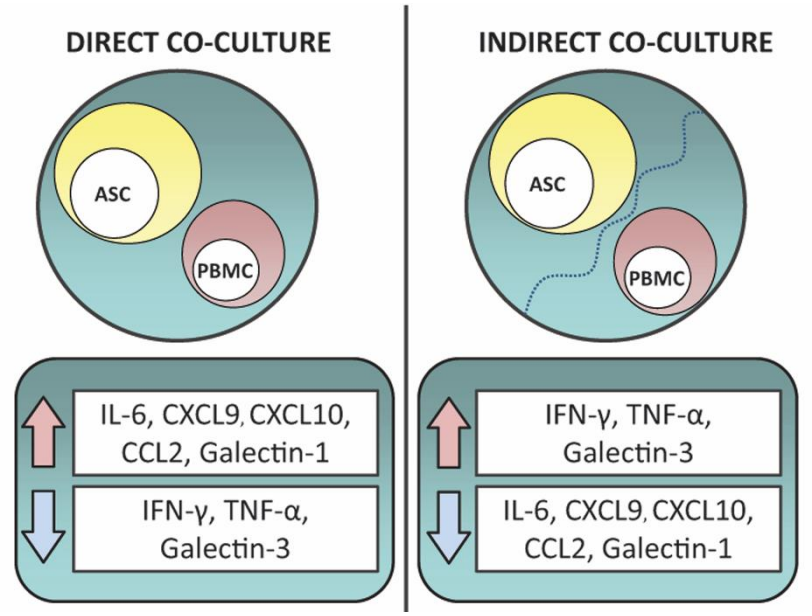

Figure 30. Schematic overview highlighting the key signaling proteins in direct versus indirect co-cultures. The increase or decrease in protein secretion between direct versus indirect co-cultures were statistically significant. (Study **III**)

Indirect Co-cultures

Compared with direct cultures, the secretions of IL-6 ($p<0.001$), CCL2 ($p<0.001$), CXCLX9 and -10 ($p<0.001$) and galectin-1 ($p<0.001$) were significantly decreased in indirect co-cultures when culture conditions were not assessed separately in the statistical regression analysis with ranked values. By contrast, the secretion of IFN-γ ($p<0.05$), TNF-α ($p<0.001$) and galectin-3 ($p<0.001$) was significantly increased in the indirect co-culture, when compared with the direct cultures (Figure 30).

Similarly to direct co-cultures, the lowest TGF-β1 concentrations were measured from cultures containing ASCs expanded in XF/SF conditions ($p<0.05$) compared with either FBS or HS conditions (Figure 29). Additionally, a significantly higher secretion of chemokine CXCL8 ($p<0.05$) and CXCL10 ($p<0.05$) was measured in XF/SF conditions compared with the HS medium using the indirect co-culture. Moreover, significantly lowest IDO concentrations were measured from indirect cultures containing ASCs expanded in FBS conditions compared with either HS or XF/SF ($p<0.05$) conditions.

6 Discussion

6.1 Effect of culture conditions on adipose stem cell characteristics

6.1.1 Cell surface markers under different culture conditions

The IFATS and ISCT have established minimal criteria for phenotypic identification of adherent stromal/stem cell population, which were first published in 2006 by Dominici *et al.* (Dominici *et al.*, 2006) and recently updated by Bourin *et al.* (Bourin *et al.*, 2013). Although this guidance is a valuable tool for cell characterization, the criteria are defined for cells cultured under standard conditions in FBS-supplemented medium, and certain differences may arise if cells are cultured in different serum conditions. Our flow cytometry results demonstrate that the cell surface-marker profile defined by Dominici *et al.* primarily applies to ASCs cultured under FBS, HS and XF/SF conditions because cell surface-marker expression of ASCs was highly similar between cells grown under different serum conditions. However, small variations were observed in the expression of CD11a (Integrin alpha), CD14 (lipolysaccharide receptor), CD19 (leukotriene B4 receptor), and CD86 (co-stimulatory molecule for T-cell activation) between cells grown in XF/SF conditions versus serum-containing medium (**I**). All of these markers are known to interact with immune-related cells, and thus the culture conditions indeed have a potential effect on the immunogenicity of ASCs, as demonstrated in study **III**.

Of note, the expression of CD54 was altered depending on the serum conditions, and statistically significant higher expression was observed in serum-containing medium versus SF/XF conditions (**I, III**). The CD54 binds to integrins of type CD11a or CD11b and is reported to be present in endothelial cells, antigen-presenting cells, and certain stromal cells. Furthermore, it was demonstrated that CD54 and CD106 play a key role in mesenchymal stem cell-mediated immunosuppression (Kronsteiner *et al.*, 2011; G. Ren *et al.*, 2010; G. Ren *et al.*, 2011). These previous published studies are in line with the results of our immunological studies (**III**) in which FBS expanded ASCs showed high expression of CD54 and

had the strongest immunosuppressive potential. In addition, the low expression of the adhesion molecule CD54 as observed under XF/SF conditions may be reflected by a notably weaker cell adhesion of ASCs. The lower CD54 expression also suggest that a different cell populations could be selected through XF/SF isolation and expansion protocols compared with cells grown in the presence of serum.

Additionally, statistically significant differences were observed for the expression of CD45 with higher expression in XF/SF conditions (**III**). The CD45 is a receptor-linked protein-tyrosine phosphatase that is shown to be essential for leukocyte differentiation and antigen receptor-mediated signal transduction (Altin and Sloan, 1997; Matsuda *et al.*, 1998). The XF/SF expanded ASCs were less immunosuppressive compared with those in serum-containing medium, which was in line with higher expression of CD45 in XF/SF conditions. Nevertheless, due to a lack of stronger evidence, the immunosuppressive capacity of ASCs should not be evaluated based on the expression of CD45. Further, as stated in the updated guidance by IFATS and ISCT, one of the main differences between SVF cells and ASCs should be the high expression levels of CD45 in SVF cells and a notably low or undetectable level in ASCs (Bourin *et al.*, 2013). This definition applies to ASC cultures in FBS or HS conditions, but slightly higher expression of CD45 appears to be present in defined XF/SF conditions (**I, III)**. Compared with BM-MSCs, CD45 is more readily expressed in ASCs because BM-MSCs do not express CD45 even at low levels (Liao and Chen, 2014; Pachon-Pena *et al.*, 2011). Although CD45 is the classic marker used to identify cells of hematopoietic origin, various isoforms of CD45 exist, and a chosen isoform of CD45 marker may also affect the intensity of the expression (Tchilian and Beverley, 2006). In humans, a high-molecular-weight isoform (CD45RA) is expressed in naive T-lymphocytes, whereas the low-molecular-weight isoform (CD45RO) is expressed after T-cell activation (Tchilian and Beverley, 2006).

Furthermore, the hematopoietic stem cell marker CD34 (Bensinger *et al.*, 1993; Trischmann *et al.*, 1993) was moderately expressed in both XF/SF and serum-supplemented conditions (**I**) in contrast to the originally defined criteria (Dominici *et al.*, 2006). Since 2006, similar variable expression for CD34 has been reported by others (Mirabet *et al.*, 2008; Rebelatto *et al.*, 2008), and the expression of CD34 appears to be greatly dependent on the length of the in vitro culture period; it is typically expressed during the early phase of culture, but its expression subsequently decreases after continued cell divisions (Maumus *et al.*, 2011; Mitchell *et al.*, 2006). We observed a similar phenomenon in our studies (**I, III**) in which the expressions of both CD34 and CD54 were higher in passage 2 but decreased in later passages,

thus indicating a more homogeneous population. Compared with BM-MSCs, CD34 is more readily expressed on ASCs because BM-MSCs do not express CD34 even during the early phase of culture (Liao and Chen, 2014; Pachon-Pena *et al.*, 2011). Additionally, CD34 is a marker of endothelial progenitor cells, and moderate expression of CD34 has been reported in endothelial cells (43±19), as analyzed from human umbilical vein endothelial cells (HUVECs) (Huttala *et al.*, 2015).

The typical immunophenotype of ASCs was also maintained in MMC cultures (**II**) in all of the studied serum conditions of FBS, HS and XF/SF. However, in XF/SF conditions the immunophenotypic analysis was performed after 7 days of exposure to MMC because longer-term culture with MMC was not feasible. The XF/SF cultured ASCs showed poor viability and proliferation capacity under MMC, and as a result the typical immunophenotype of ASC was lost under longer-term exposure to MMC, which was confirmed with one ASC donor cell line. Interestingly, the expression of CD54 was significantly higher under MMC in all of the studied serum conditions. Several explanations might exist for these results. The proliferative capacity of ASCs was significantly decreased under MMC culture, especially in XF/SF conditions, and it could be speculated that the cell viability under MMC media was not 100%. Although use of a cell viability marker during a flow cytometry analysis would have strengthened this result, a uniform cell population was still observed in the forward and side scatter data, which suggested that no substantial amount of dead cells was present in the analysis.

As shown in previous studies, ECM is extensively deposited under MMC conditions (Ang *et al.*, 2014; C. Z. Chen *et al.*, 2011) leading to more mature ECM, which might reflected stronger cell adhesion and higher expression of adhesion molecule CD54. In previous studies, the intra- and extracellular protein organization between +/- MMC cultures were compared, and these studies showed that it is possible to further align the ECM even in the absence of cellular interaction (Zeiger *et al.*, 2012). The ECM in turn affects cell-matrix interactions and promotes cell adhesion, thus exerting an influence on the formation and structure of the cytoskeleton (Zeiger *et al.*, 2012). As shown by previous studies, the matrix deposition is increased under MMC conditions (Ang *et al.*, 2014; C. Z. Chen *et al.*, 2011), and when ECM is mature and extensively deposited, the cell adherence is also strong (C. Z. Chen *et al.*, 2011), as can be observed in the higher expression of adhesion molecule CD54. Thus, higher CD54 expression may be associated with stronger cell adherence because higher CD54 expression levels were observed for MMC exposed cells, as opposed to the lower CD54 levels of weakly attached XF/SF cells.

6.1.2 Cell proliferative behavior under different culture conditions

Today, clinical cell therapies using ASCs are in progress, and several clinical trials are ongoing, which require reliable, reproducible and safe methods for rapid in vitro expansion of cells. Traditionally, ASC culture medium has been supplemented with FBS, which may not be optimal for clinical cell therapies because of possible complications as a result of interspecies interactions (Kadri *et al.*, 2007; Mackensen *et al.*, 2000; Selvaggi *et al.*, 1997). As shown in previous studies, therapeutic use of ASCs cultured in FBS containing medium may expose the patient to bovine antibodies with possible clinical sequelae triggered by allergic reactions or rejection. Thus, different substitutes for FBS are recommended for therapeutic applications, and alternatives such as human albumin (Trivedi *et al.*, 2008), PL (Hildner *et al.*, 2013; Naaijkens *et al.*, 2012; Schallmoser and Strunk, 2013; Trojahn Kolle *et al.*, 2013), autologous or allogenic HS (W. Im *et al.*, 2011; Josh *et al.*, 2012; Koellensperger *et al.*, 2014; Kyllonen *et al.*, 2013; Thesleff *et al.*, 2011) or completely defined XF/SF conditions (Al-Saqi *et al.*, 2014a; Blande *et al.*, 2009; Chase *et al.*, 2010; Dromard *et al.*, 2011; Konno *et al.*, 2010) have been extensively studied for their suitability in clinical use (Section 2.4.3). The use of HS or serum derivatives instead of FBS may be preferable for ASC cultures; however, limitations exist, such as lot-to-lot variability, limited availability, and undefined composition that may hinder the use of HS (Parker *et al.*, 2007; Schallmoser *et al.*, 2007).

However, FBS supplementation of culture medium was recently accepted by EMA for in vitro expansion of cartilage cells for use in patients for repair of knee cartilage damages (EMA, European Public Assessment Report: ChondroCelect, 2009). Additionally, FBS is currently used for cell expansion in ongoing clinical trials designed to study the potential of ASCs for the treatment of complex fistulas (Garcia-Olmo *et al.*, 2009a), but its use remains controversial. The use of FBS in clinical cell therapies may be justified by the fact that pathogens of animal origin may not be directly transmitted to humans (Antia *et al.*, 2003; Wolfe *et al.*, 2012) with FBS cultured cells, as may be the case with human pathogens. Nevertheless, in the public assessment report on the ChondroCelect product (EMA, European Public Assessment Report: ChondroCelect, 2009), the EMA stated that the ChondroCelect must not be used in people who are hypersensitive to any of the ingredients of bovine serum, and consequently concerns about the risks connected FBS are still present.

In addition to safety aspects, in vitro expansion of cells should be rapid and efficient during clinical cell therapies, which can be ensured with novel XF or SF

culture conditions. In our study **I**, the cell cumulative population doubling in XF/SF medium was superior compared with that of both the FBS and HS conditions. The results of our studies were consistent with earlier studies of mesenchymal stem cell cultures under XF or SF conditions (Al-Saqi *et al.*, 2014a; Chase *et al.*, 2010; Yang *et al.*, 2012) in which a higher proliferation rate of XF- or SF-cultured cells was demonstrated compared with FBS cultures. These results support the use of XF/SF conditions in cell expansion for clinical application in which a large number of cells is required on a minimum time scale.

The effect of MMC on cell proliferation capacity was also evaluated in different culture conditions of FBS, HS and in XF/SF. In opposition to earlier results in which increased proliferation rates were observed after MMC treatment (C. Z. Chen *et al.*, 2011; Rashid *et al.*, 2014a), the cell proliferation capacity was decreased under MMC in all studied culture conditions (study **II)**. In addition, the metabolic activity of the cells was determined based on the mitochondrial dehydrogenase enzyme, which revealed that cells expanded in FBS conditions under MMC showed increased metabolic activities compared with standard FBS cultures. A similar effect was not observed in HS or XF/SF conditions, but instead, both cell proliferation and metabolic activity were reduced under MMC. It was previously shown that mitochondrial properties and thus cell metabolic activity are altered during osteogenic differentiation of BM-MSCs; increased mitochondrial volume and maturation have been observed after osteogenic differentiation (Pietila *et al.*, 2012). In our study, MMC supported osteogenic differentiation of ASCs in FBS media and metabolic activity was increased in FBS conditions under MMC compared with that of standard FBS cultures. However, a similar connection between strong osteogenic differentiation and increased mitochondrial activities could not be observed in HS conditions under MMC.

Of note, these earlier results of increased proliferation rates under MMC were performed with BM-MSCs in FBS containing media, which could explain the different outcome. The effect of MMC is based on the EVE, which is dependent on the FVO (C. Z. Chen *et al.*, 2011). Furthermore, the FVO is defined as the fraction of the total volume occupied by macromolecules. The FVO is calculated as optimal for BM-MSCs, and calculations are performed based on the blood serum and its dominant component albumin concentrations as a baseline. Thus, 17% FVO is optimal for BM-MSC crowding but should be further optimized for ASC cultures. In addition, the effect of different serum conditions may change the equilibrium of the optimized 17% MMC occupancy. The basis for MMC functions is its ability to support cells in re-creating their own microenvironment in vitro. As discussed by

Chen *et al.*, cells that do not produce much ECM, cannot be induced to build the microenvironment even under MMC (C. Z. Chen *et al.*, 2011). It can be speculated that, at least in chemically defined basic XF/SF cultures, the ECM production by ASCs may be reduced, which hinder the suitability of MMC methods for XF/SF cells. Additionally, the weak cell adherence that was observed with XF/SF cells may interfere with the effects of MMC under XF/SF culture.

During clinical cell therapies, ASCs are often combined with biomaterial scaffolds (G. I. Im, 2013; Sandor *et al.*, 2014; Thesleff *et al.*, 2011) that exert an additional effect on stem cell behavior. Two biomaterials, i.e., BAG and β-TCP, were evaluated in study **IV** for their ability to support ASC proliferation and osteogenic differentiation. The proliferation of ASCs in CM was significantly increased in the BAG cultures compared with that of the β-TCP cultures, thus suggesting a greater cell proliferative response to BAG. The surface roughness and porosity differ between these materials, which may affect the proliferation capacity; however, the cell number at day one was similar on both materials, suggesting an efficient attachments of ASCs on both material surfaces. Our results were in line with previous studies in which the positive effect of BAG and β-TCP on cell proliferation was demonstrated (Haimi *et al.*, 2009a; Hench, 1998; Rahaman *et al.*, 2011; Zhao *et al.*, 2010). In our study, OM increased the cell proliferation significantly with both biomaterials compared with CM, and interestingly, BMP supplementation reduced the cell number with both biomaterials compared with OM. Similar effects of OM and BMP supplementation on cell proliferation activities were demonstrated by Tirkkonen *et al.* (Tirkkonen *et al.*, 2013). In line with our results, decreasing proliferation activities were observed after BMP supplementation, whereas OM induced increased proliferation.

6.1.3 Morphological features of ASCs under different culture conditions

Typical MSC morphology was retained in all FBS, HS and XF/SF cultures. However, ASCs grown under XF/SF conditions displayed smaller and more spindle-shaped morphology (I) that may be explained by the weaker cell adherence of XF/SF cells. The weaker cell attachment to the cell culture vessel was also shown by the decreased enzymatic digestion time during cell detachment. When ASCs were cultured under MMC, changes in cell morphology were noticed in all serum conditions. As MMC induced efficient ECM production, the cell adherence also became stronger, which may be visible in the larger and more cubical cell morphology. Interestingly, the major effect of MMC was evident in XF/SF cells whose morphology was fully

transformed by MMC culture. As the cell number significantly decreased in XF/SF conditions after longer-term MMC exposure, the cells became large and round with many extensions. Of note, several nuclei were observed in certain XF/SF cells after a longer MMC culture period. Similar observations were not reported earlier; however, our study was the first report to use MMC with XF/SF cultured ASCs. Our results clearly showed that the MMC method is not suitable for the studied XF/SF cultures because the cells are not capable of processing Ficoll particles advantageously for their microenvironment. It could be speculated that Ficoll also has toxic effects on ASCs, which are well demonstrated under XF/SF conditions. Ficoll may be transported inside XF/SF cells, but they lack methods to process these particles. However, the changes observed in morphology may be the result of attachment difficulties caused by Ficoll molecules. Nevertheless, additional studies should be performed to analyze the molecular mechanisms behind these observations.

6.1.4 Differentiation potential affected by different culture conditions, biomaterials and growth factors

As defined by the criteria from the IFATS and ISCT, a hallmark of the ASCs is their multipotency and ability to give rise to osteoblastic, chondrocytic and adipocytic lineages (Bourin *et al.*, 2013). Hence, the differentiation potential toward osteogenic, adipogenic and chondrogenic cells was investigated in novel XF/SF conditions in compared with that in HS and FBS conditions in study **I**. Unlike in previous studies (Blande *et al.*, 2009; Chase *et al.*, 2010; Dromard *et al.*, 2011), osteogenic and adipogenic inductions were performed out in completely XF/SF differentiation media in which the serum was substituted with XF/SF supplement of the STEMPRO MSC SFM kit. The differentiation potential of XF/SF-expanded cells was retained, although the differentiation was not as efficient as that observed in serum-containing medium. However, efficient differentiation was demonstrated in a follow-up study in which FBS- or HS-based induction media were used for XF/SF expanded cells, thus suggesting that nutrient-rich media is required to promote robust differentiation. Additionally, the weaker cell adherence that was observed in XF/SF cultures may affect the differentiation efficiency of the cells. In accordance with our observations, previous studies have demonstrated that cell adhesion and nutrients are important during the cell differentiation (J. M. Kang *et al.*, 2012; I. S. Park *et al.*, 2009). Moreover, ASCs expanded in XF/SF conditions showed strong

chondrogenic differentiation potential compared with those in serum-containing media based on Alcian blue staining results, although we did not use quantitative analysis of study chondrogenic differentiation. Similar findings were reported by Chase *et al.* (Chase *et al.*, 2010), who observed robust chondrogenesis in SF culture conditions. However, our results suggested that the structure of the micro-mass pellet differs to a certain extent depending on the culture conditions because it is typical for XF/SF cells to form an intensely stained strip of ECM at the rim of the pellet with more apoptotic-like cells in the middle. In contrast, after cell expansion in standard serum-containing medium, the micro-mass pellet structure was more consistent throughout the pellet. Although these results of effective chondrogenic differentiation in XF/SF conditions are promising for the use of ASCs in cartilage applications, additional research is still required to confirm the efficient chondrogenic potential of XF/SF cultured ASCs.

The aim of study **I** was to remove all animal-derived and undefined components from the cell-culture workflow and demonstrate that ASCs maintain their basic stem cell characteristics in these defined XF/SF conditions. Although serum or other supporting attachment factors may be required for more efficient differentiation, study **I** demonstrated that cells cultured under XF/SF conditions retain their capacity for trilineage differentiation. The differentiation efficacy could easily be improved by serum induction if required during clinical treatments, e.g., use of autologous HS supplementation. However, cell expansion should still be performed under defined XF/SF conditions that are superior for effective cell expansion. Currently, XF/SF cultures of ASCs have been reported in several papers, but few studies are available in which the isolation and differentiation is also performed in completely XF/SF media. Our study **I** was the first published report of fully defined XF/SF isolation, expansion and differentiation protocols. However, Al-Saqi *et al.* have recently published their results of XF/SF isolation and expansion of ASCs using serum-containing differentiation protocols (Al-Saqi *et al.*, 2014a). In addition, Laitinen *et al.* have established a clinically compliant XF/SF culture protocol for BM-MSCs based on an optimized platelet-derived supplement that can be used for osteogenic differentiation of BM-MSCs (Laitinen *et al.*, 2015). Furthermore, Skog *et al.* have recently published a clinically compliant defined XF/SF differentiation protocol for human BM-MSCs for chondrogenic differentiation (Skog *et al.*, 2014).

Furthermore, the effect of MMC on ASC differentiation was studied in FBS- and HS-containing medium and in XF/SF conditions. As shown in the previous study with BM-MSCs, crowding facilitates microenvironment formation and stabilizes or drives differentiation (Ang *et al.*, 2014). Ang *et al.* showed that under adipogenic

induction media in MMC conditions, the ECM was more extensively remodeled toward a pro-adipogenic microenvironment, which further promoted the adipogenic differentiation of cells. Thus, the MMC method could be considered as an alternative method to direct ASC differentiation in vitro.

Although MMC exposure did not enhance ASC proliferation in the current study, the cells displayed efficient osteogenic and adipogenic differentiation, as observed with FBS- and HS-expanded cells. In FBS conditions, significantly stronger osteogenic differentiation was observed in the MMC condition compared with induction in standard conditions when the cells were expanded under standard culture conditions. The ASCs accumulated plenty of lipid droplets under MMC induction compared with induction under standard conditions after expansion in both -/+MMC conditions, which suggests the supportive influence of MMC on adipogenic commitment of cells in serum-containing medium. In contrast, cells cultured in XF/SF conditions did not respond to MMC during differentiation. This result demonstrates the compatibility of the MMC method with XF/SF cultures; however, it may not indicate the osteogenic or adipogenic potential of XF/SF expanded ASCs. As shown in the serum priming experiments in study **I**, XF/SF cells still retain the capacity for multipotential differentiation; however, the induction media should be optimized for XF/SF conditions. Significant differences in the intensity of osteogenic differentiation were also observed between HS and FBS cultures. Compared with HS cultures, FBS medium did not support efficient osteogenic differentiation. However, lot-to-lot variation in serum performance may affect the differentiation potential of ASCs (Parker *et al.*, 2007). In line with this result, a similar diminished osteogenic differentiation potential for ASCs in FBS medium compared with that of HS medium was reported previously by our group (Kyllonen *et al.*, 2013).

Moreover, chondrogenic differentiation was studied in standard induction media (-MMC) after cell expansion in both -/+MMC culture conditions. Our results showed more efficient differentiation and more extensive ECM deposition after expansion in standard conditions compared with the +MMC condition based on qualitative Alcian blue staining. The micro-mass pellet structure was altered depending on the MMC treatment, and consequently, the cells differentiated under MMC conditions were less dense histologically. However, one explanation for dissimilar structure after MMC expansion would be that Ficoll is transported inside ASCs and observed as gaps inside the histological pellet structure. In line with our hypothesis, Rashid et a. have recently shown that Ficoll is pinocytosed by BM-MSCs and subsequently transported into mitochondria (Rashid *et al.*, 2014b); however, they

also demonstrated that the intracellular level of Ficoll was decreased over time, suggesting that it does not persist within cells.

In the final study **IV**, the effect of BAG and β-TCP on osteogenic differentiation of ASC was investigated. Similar to previous studies performed with BM-MSCs and ASCs (Haimi *et al.*, 2009a; Haimi *et al.*, 2009b; Liu *et al.*, 2007; Tirkkonen *et al.*, 2013), both biomaterials supported osteogenic differentiation based on the results of ALP activity. Interestingly, with BAG, the osteogenic differentiation of ASCs was the most efficient in CM without osteogenic induction or BMP supplementation. Unlike with BAG, chemical induction (OM) was required with β-TCP for cells to undergo osteogenic differentiation. These results indicate that BAG granules may be more osteoconductive compared with β-TCP in the absence of osteogenic supplements, which is likely due to BAG ion release that strongly induces ASCs toward osteogenesis (Tsigkou *et al.*, 2009).

Additionally, the potential osteopromoting effect of growth factors BMP-2 and BMP-7 was evaluated in study **IV**. According to several published studies, BMP-2 (Knippenberg *et al.*, 2006; Song *et al.*, 2011; Zhao *et al.*, 2010) and BMP-7 (Al-Salleeh *et al.*, 2008; Giannoudis and Tzioupis, 2005) supplementation efficiently induces ASCs toward osteogenic differentiation. However, the use of BMPs in regenerative bone applications is still under intense debate because contradictory results of the osteogenic effects of BMPs exist (Tirkkonen *et al.*, 2013; Zuk *et al.*, 2011). Our results from study **IV** suggested a negative impact from both growth factors on osteogenic differentiation of ASCs. In addition, a decrease in ALP activities was observed over time after BMP supplementation with both biomaterials. Interestingly, OPN was the only marker for which expression was increased by BMPs, but this effect was only detected in BAG and thus requires additional investigation. In line with our results, Zuk *et al.* demonstrated that in vitro treatment of ASCs with BMP-2 had no consistent or significant effect on matrix mineralization or the expression of osteogenic markers, and thus, they concluded that the osteogenic differentiation of ASCs may not be influenced by BMP-2 (Zuk *et al.*, 2011). Zuk *et al.* also suggested that the canonical BMP-2 signaling pathway may not be functional in human ASCs. This conclusion was supported by Lindroos *et al.*, who previously showed that the BMP receptor mediated signaling pathway was underexpressed in ASCs cultured in HS medium compared with those cultured in FBS medium (Lindroos *et al.*, 2010). The negative effect of BMPs was also demonstrated by Tirkkonen *et al.* (Tirkkonen *et al.*, 2013), who showed that OM significantly enhanced ASC differentiation towards bone-forming cells compared with growth factors BMP-2 and BMP-7.

The inconsistency between our results and previous observations may be explained by the exogenous delivery method of BMPs. In our study, BMPs were introduced to the cells by frequent medium changes; however; gene therapy would be a more efficient method for supporting osteogenic differentiation. In addition, several other factors may also explain the great variation in the outcomes of BMP-2 studies, as concisely discussed by Tirkkonen *et al.* (Tirkkonen *et al.*, 2013). These factors include the use of animal versus human cells (Knippenberg *et al.*, 2006), the origin of recombinant BMP in mammalian or bacterial cells (Bessa *et al.*, 2008a), ASC donor variability, different biomaterials, varied cell densities and different BMP concentrations. In addition, the timing of cell signaling and the complex interplay among different effectors and inhibitors will have an effect on the final outcome.

Moreover, concern exists that the use of BMP-2 for patient treatments is associated with cancer risk; a high dose of BMP-2 for treatment of lumbar spinal arthrodesis was reported to be associated with an increased risk of cancer recurrence as well as secondary cancer (Carragee *et al.*, 2013). Previous studies reported multiple tumor types, and certain of these tumors occurred distant to the site of BMP-2 implantation. Additionally, it was shown that patient treatment with BMP-2 triggered not only bone healing but also several undesired effects, such as frequent soft tissue inflammation, bone resorption or excessive bone formation and heterotopic ossification (Kisiel, 2013). Although the cancer risk may be dose dependent (Devine *et al.*, 2012), it is still a safety concern, and thus alternatives to BMP-2 should be studied for regenerative bone treatments.

Further, adipose tissue is an active endocrine organ that is known to secrete several angiogenic cytokines and such as growth factors VEGF, bFGF and IGF-I (Gimeno and Klaman, 2005; Sarkanen *et al.*, 2012). The formation of vasculature is essential for bone formation, and therefore, the secretion of VEGF was evaluated in study **IV**. Both biomaterials BAG and β-TCP consistently supported VEGF expression. The expression levels rose continuously over time and were also correlated with the cell amount. Our results were in agreement with previous studies that reported beneficial effects of BAG and β-TCP on VEGF secretion (Ghanaati *et al.*, 2010; Gorustovich *et al.*, 2010). Interestingly, with BMP supplementation, ASCs cultured on β-TCP showed significantly higher VEGF secretion compared with cells cultured on BAG. Potentially, β-TCP granules in co-operation with BMP-2 or BMP-7 may support the angiogenic potential of ASCs. However, ASCs cultured in CM on BAG showed the highest secretion of VEGF compared with all treatment groups of both biomaterials. Thus, the choice of biomaterial or other additional factors may

play a more important role in the vasculature formation of ASC in 3D cultures than the use of growth factors BMP-2 or BMP-7.

Overall, several factors may affect the outcome of ASC differentiation; these factors include the effect of serum conditions, the choice of biomaterials, growth factors, composition of induction media and duration of induction. Additionally, cell responses are definitely donor dependent, and certain donor cell lines respond better to the osteogenic, whereas others to adipogenic induction. Variation always occurs in differentiation efficiency, and the cells cultured under different serum conditions may exist at different stages of the differentiation process as well. With respect to gene expression results, the mRNA expression and enzymatic activities may not be in line because regulation occurs on a posttranscriptional and translational level, and thus, the expression of certain osteogenic or adipogenic markers may fluctuate between different serum conditions. All of these factors can be observed in the differentiation results, as shown the high standard deviations that are typical for biological data. For clinical trials, the quality or commitment into an osteogenic versus adipogenic pathway of ASCs derived from different donors could be evaluated using potency assays (Bravery *et al.*, 2013). Potency can be defined as the ability of a treatment to elicit a particular response at a certain dose, and thus, it is a quantitative measure of relevant biologic function based on the attributes linked to relevant biologic properties. To achieve adequate assessment of cellular therapy product, appropriate in vitro or in vivo laboratory assays (referred to as potency assays) must be created (Bravery *et al.*, 2013).

6.2 Immunological characterization of ASCs in different serum conditions

6.2.1 ASCs show low immunogenicity in all studied culture conditions

Our results from study III demonstrate that ASCs have a low immunogenicity whether cultured in FBS, HS, or XF/SF conditions. This article was the first published study on the immunological properties of ASCs after expansion in different culture conditions; however, our results were consistent with earlier studies performed with ASCs expanded in FBS medium (McIntosh *et al.*, 2006; McIntosh, 2011; Niemeyer *et al.*, 2007; Puissant *et al.*, 2005). Interestingly, based on our studies, ASCs cultured in FBS medium proved to be the least immunogenic, as shown by the

MLR assay. It may be speculated that the low immunogenicity in the FBS medium is explained by the origin of bovine serum, which is harvested from the blood of bovine fetuses with immature immune systems and is unable to trigger full immune responses (Sharma *et al.*, 2012). Still, more detailed studies are needed to fully determine the mechanisms behind the low immunogenicity observed with FBS cultured cells. Nevertheless, the results of study **III** suggest that ASCs elicit only a low immunogenic response toward allogeneic PBMCs in all studied culture conditions. Due to the low immunological profile, allogeneic ASC treatments may be conceivable in the future, although in vivo studies are still required to systemically confirm the in vitro results.

6.2.2 ASCs possess a promising immunosuppressive capacity that is strongest in FBS conditions

In addition to low immunogenicity, our study **III** confirmed that ASCs have potential for immunosuppression, as previously demonstrated (Kronsteiner *et al.*, 2011; Lee *et al.*, 2012; McIntosh *et al.*, 2006; Puissant *et al.*, 2005; Yoo *et al.*, 2009). Our results revealed that the suppressive potential was affected by the culture conditions and functioned in a dose-dependent manner. Interestingly, the strongest suppression was observed with FBS-cultured ASCs, whereas ASCs cultured in the HS condition suppressed PBMC proliferation only with higher cell numbers. The ASCs cultured in the XF/SF condition exhibited reduced capacity for immunosuppression and even stimulation of PBMC proliferation was observed with lower ASC number. Le Blanc *et al.* have previously demonstrated that in lower concentrations MSCs may have less inhibitory and occasionally stimulating effects (Le Blanc *et al.*, 2003a). Their findings suggested that the surface structures of MSCs may occasionally act as antigens, especially if MSCs are added in low concentrations. However, our results of one-way MLR still demonstrated that XF/SF-expanded ASCs have low immunogenicity, and thus the stimulatory effect is probably MLR assay-related.

One explanation for the strong suppressive capacity in FBS medium may be found in the expression levels of CD54 in FBS medium. As discussed earlier in Section 6.1.1, a significantly higher expression of CD54 was observed on ASCs grown in FBS-containing medium compared with cells cultured in XF/SF conditions. In line with the CD54 expression levels, the strongest suppression was observed in FBS medium, whereas XF/SF cells could barely suppress PBMC

proliferation. It was previously reported that CD54 and CD106 are required for lymphocyte-BM-MSC adhesion, and with the aid of chemokines, they are responsible for inducing immunosuppression mediated by BM-MSCs (Kronsteiner *et al.*, 2011; G. Ren *et al.*, 2010; G. Ren *et al.*, 2011). In contrast to BM-MSCs, ASCs have been shown to be negative for CD106 (De Ugarte *et al.*, 2003; Lindroos *et al.*, 2009), and thus, it is likely that immunosuppression of ASC is CD54- but not CD106-mediated. Nevertheless, ASCs expanded in serum-containing media have potential for immunomodulation therapies, and currently, several clinical trials are ongoing for study of the suitability of ASCs for treatment of autoimmune diseases or inflammatory conditions (Section 2.9.).

6.2.3 Characterization of signaling proteins secreted during ASC and PBMC co-cultures

Signaling proteins such as cytokines and chemokines are strongly involved in processes mediating the immunogenicity and immunosuppression of ASCs. Thus, a set of signaling proteins was analyzed from the co-cultures of ASCs and PBMCs and mono-cultures of ASCs and PBMCs alone. It has been demonstrated in previous studies, that the local inflammatory environment is highly important in the regulation of MSC plasticity, and especially the expression of cytokines IFN-γ, TNF-α, and IL-6 are important in regulating the immunosuppressive phenotype of ASCs (Crop *et al.*, 2010a). IFN-γ has been shown to induce MHC-I and MHC-II expressions that further increase the antigen-presenting capacity and immunogenicity of MSCs (Chan *et al.*, 2008; Le Blanc *et al.*, 2003b).

In our study **III**, the secretion of IFN-γ, TNF-α, and IL-6 was increased after co-culture of ASCs and PBMCs compared with mono-cultures. Furthermore, IFN-γ and TNF-α levels were higher in indirect co-culture compared with direct co-cultures, which suggest the inhibitory effect of direct contact between ASCs and lymphocytes with respect to the secretion of IFN-γ and TNF-α. A similar phenomenon of reduced IFN-γ and TNF-α levels after direct co-culture of CD4+T-cells and human umbilical cord MSCs was reported earlier (Wu *et al.*, 2014). In this previous study, T-cells were first activated for high IFN-γ and TNF-α secretion via PHA/IL-2 exposure, followed by co-culture with MSCs that reduced these secretion levels. It may be speculated that, similar to PHA/IL-2, the indirect contact between ASCs and lymphocyte changes the local inflammatory environment and activates PBMCs for higher IFN-γ and TNF-α secretion, as demonstrated by Crop *et al.* (Crop

et al., 2010a); however, direct contact between ASCs and lymphocytes will reduce the activation.

In contrast to IFN-γ and TNF-α secretion, direct contact between ASCs and PBMCs induced cells toward higher IL-6 production in our studies. Additionally, IL-6 has several functions because it is involved in inflammation and infection responses and also in the regulation of metabolic, regenerative, and neural processes (Scheller *et al.*, 2011). Thus, IL-6 can either support or suppress inflammation depending on the context, and the anti-inflammatory effect is mediated in co-operation with IFN-γ by inhibiting the secretion of TNF-α (Ulich *et al.*, 1991). Through production of IL-6, MSCs prevent monocyte differentiation toward antigen-presenting immunogenic cells and drive differentiation toward an anti-inflammatory IL-10-producing cell type (Melief *et al.*, 2013b). In our study, IL-6 secretion was higher in FBS conditions compared with XF/SF conditions, which is in agreement with the strong suppression potential observed in FBS media. In addition, high IL-6 levels are correlated with low TNF-α levels and vice versa. Based on our studies, IL-6 is more heavily involved in ASC-mediated suppression functions.

The secretion levels of IFN-γ and TNF-α may be decreased by the cytokine TGF-β1, which is an important factor in maintaining immune tolerance, in addition to several other functions in control of cell growth, proliferation, differentiation, and apoptosis (Wahl *et al.*, 2006). In addition, proliferation and activation of T-cells can be diminished by the effect of TGF-β1 (Gilbert *et al.*, 1997; Tiemessen *et al.*, 2003). In our study, significantly lower levels of TGF-β1 were secreted in co-cultures with XF/SF-expanded ASCs, which was in line with the immunosuppression results in which significantly less suppressive potential was observed with ASCs expanded in XF/SF conditions. These results were in accordance with the increased IFN-γ and TNF-α and decreased IL-6 secretion levels that were measured in co-cultures containing XF/SF-expanded ASCs.

Additionally, our study demonstrates the secretion profile of chemokines CXCL8, -9, -10 and CCL2 and -5 in co- and mono-cultures of ASCs and lymphocytes. It was demonstrated that the family of chemotactic cytokines known as chemokines regulate cell adhesion and chemotaxis of all types of leucocytes, including hematopoietic precursors, mature leucocytes of the innate and naive immune system and memory and effector lymphocytes (Moser and Willimann, 2004). Thus, breakdown in the control of leukocyte mobilization may lead to chronic inflammatory diseases. However, by controlling the secretion of chemokines, ASCs may have the capacity of immunomodulation. The secretion of several chemokines

has been shown to increase when ASCs are co-cultured with lymphocytes (Crop *et al.*, 2010a). Our results support these previous studies and further demonstrate that the direct contact between ASCs and lymphocytes is particularly required for stronger chemokine secretion and T-cell recruitment. Overall, chemokine secretion was strongly increased in direct co-cultures compared with indirect co-cultures, and interestingly, greater secretion of CXCL-8 and -10 was significantly more secreted in cultures with XF/SF expanded ASCs. It is likely that high IFN-γ levels that were detected in XF/SF media have induced XF/SF cells for higher CXCL-8 and -10 secretion, which would be typical example of IFN-γ induced chemokine secretion. As described earlier, our study demonstrates that XF/SF cells were more immunogenic compared with FBS-expanded cells and less immunosuppressive compared with those in both FBS and HS conditions. Thus, it may be speculated that higher IFN-γ and CXCL-8 and -10 levels detected in XF/SF conditions are associated with weaker suppressive potential and higher immunogenicity.

The secretion of CCL2 and CCL5 was lower in HS conditions compared with FBS and XF/SF conditions. These chemokines are involved in MSC chemotaxis and cell migration to the sites of injury or inflammation (Anton *et al.*, 2012). The chemokines CXCL8, CCL2 and CCL5 may be produced by immune cells, which induce extracellular matrix degradation and further facilitate the increased migration of MSCs through the extracellular matrix. However, the effect of CCL2 and CCL5 on cell homing to inflammation sites could probably be better demonstrated in vivo. Based on our results, it is difficult to conclude how the lower levels of CCL2 and CCL5 that we observed in HS conditions affect the immunogenicity of ASCs.

The role of IDO (Crop *et al.*, 2010a) has been demonstrated in MSC-mediated immunosuppression and is often secreted in response to IFN-γ. However, in our studies, IDO was secreted at significantly higher levels in XF/SF and HS media compared with FBS conditions, which contradicted the result of the strong immunosuppressive potential observed in FBS media. It may be speculated that the high initial concentration of IDO that was detected in basal culture media could have interfered with the result or that other factors in the co-culture reaction assay had a stronger effect on IDO secretion. Furthermore, a greater amount of IDO was clearly measured in co-cultures of ASC and lymphocytes compared with mono-cultures of ASCs only, suggesting that ASCs do not readily express IDO without activation, which is in line with previous results demonstrated with MSCs (Meisel *et al.*, 2004). According to previous observations, IFN-γ levels and IDO secretion were correlated, as also observed in our study. Furthermore, Meisel *et al.* concluded that IDO-mediated T-cell inhibition depends on MSC activation (Meisel *et al.*, 2004), and

consequently, modulation of IDO activity may alter the immunosuppressive properties of MSCs.

The secretion of galectin-1 and -3 was included in the characterization panel because their expression was clearly linked to the immunosuppressive potential of ASCs (Sioud *et al.*, 2011a; Sioud, 2011b). However, it has been shown that in addition to suppressive functions, galectin-3 also has T-cell proliferative functions (Hernandez and Baum, 2002). It was therefore rational that the secretion patterns of galectin-1 and -3 were somewhat different in our studies, and galectin-3 was less secreted overall. However, the secretion of both galectin-1 and -3 was higher in direct and indirect co-cultures compared with mono-cultures of ASCs and PBMCs alone. Galectin-1 suppresses immune responses and induces T-cell apoptosis, and thus, increased galectin-1 concentrations and the detected suppression potential in the co-cultures were expected. According to our results, galectin-3 expression is not as good an indicator of immune suppression as galectin-1, but galectin-1 concentrations were better correlated with the suppression assay results.

Taking these cytokine secretion results together, the final determination of inflammatory responses may be elicited through a combined action of direct cell-cell contacts and secretion of soluble factors following modulation of the local inflammatory environment. Moreover, the suppressive capacity of ASCs is likely a result of several factors that can act in a synergistic manner. Our study clearly demonstrated that direct versus indirect contact between ASCs and lymphocytes has an effect on the secretion of suppressive soluble factors. Although this result was expected, the difference was substantially strong, especially for the chemokine secretion. Puissant *et al.* have previously shown that ASCs do not spontaneously release suppressive factors, but instead, interaction between ASCs and lymphocytes is required for the secretion (Puissant *et al.*, 2005). Moreover, Puissant *et al.* showed that the MLR-conditioned media was not able to suppress the lymphocyte proliferation; however, the suppressive capacity of ASCs was retained in indirect MLR. In our study, increased secretion levels of IFN-γ and TNF-α were observed in indirect culture compared with direct culture, which was an interesting result. It may be speculated that the local inflammatory environment in indirect MLR is more beneficial for inducing the immunosuppressive phenotype of ASCs compared with direct MLR. In line with our result, Crop *et al.* reported that secretion of suppressive factors such as IDO was increased in indirect co-culture compared with direct MLR (Crop *et al.*, 2010a).

Nevertheless, additional studies on the cell signaling mechanisms associated with ASC-mediated immunosuppression should be performed to draw a conclusion on

the importance of direct versus indirect contact. To underline a few key factors, the low secretion of TGF-β1 as well as high secretion of INF-γ and chemokines CXCL-8 and -10 that were measured in XF/SF conditions may be associated with increased immunogenicity under XF/SF conditions. Furthermore, it may be speculated that the low IFN-γ and high IL-6 secretion that was observed in FBS conditions may be linked to stronger suppressive potential of ASCs. Still, relatively small differences were observed between different serum conditions, and the effect of direct versus indirect contact between ASCs and lymphocytes on signaling protein secretion was more evident. The results of ASC immunogenic properties and cytokine secretion under different culture condition are illustrated in Figure 31.

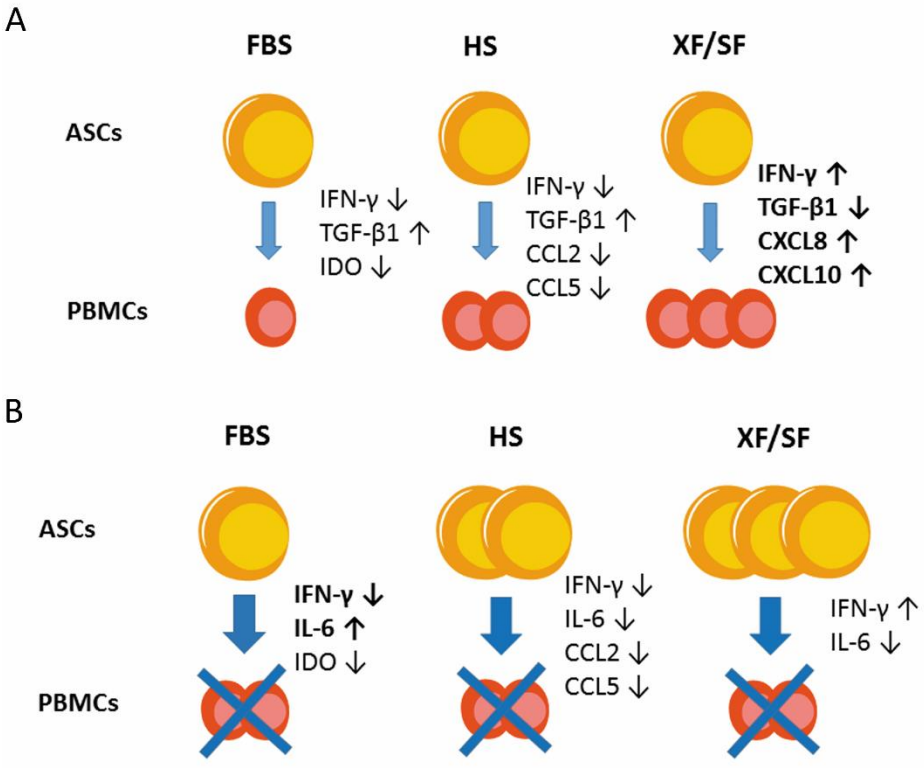

Figure 31. Schematic illustration of the immunogenic properties of ASCs and signaling protein secretion under different culture conditions. (A) Despite the low immunogenicity in all studied conditions, increased proliferation of PBMCs was observed in HS and XF/SF conditions. The high secretion of IFN-γ and chemokines CXCL-8 and -10, as well as low secretion of TGF-β1 in XF/SF conditions may be associated with increased immunogenicity. (B) A higher ASC number was required for suppressive effects in HS and XF/SF conditions compared with FBS medium. The low IFN-γ and high IL-6 secretion that was measured in FBS conditions may be associated with stronger suppressive potential.

6.3 Methodological considerations and limitations of study

The study protocols chosen for the in vitro studies in this thesis are standardized methods that have been widely used for the investigation of MSC behavior by our laboratory and others. Naturally, limitations are associated with each method because in vitro culture is rarely completely comparable with biological environment, and thus, histological, PCR-based, flow cytometry and other additional methods give only a narrow perspective of the biological truth. Critical assessment of selected methods is presented below.

The ASCs were isolated from adipose tissue samples obtained from 22 female donors and 1 male donor, and thus, the gender ratio was not balanced. It has been previously shown that donor gender may influence the osteogenic differentiation potential of ASCs, and more rapid and efficient osteogenic differentiation has been observed with ASCs derived from male donors (Aksu *et al.*, 2008). Hence, the osteogenic differentiation results may have been biased because the off-balanced ratio of gender and perhaps stronger osteogenic commitment of ASCs could have been visible with higher number of male ASCs.

The isolation of ASCs was performed using a mechanical and enzymatic method, as described previously (Gimble and Guilak, 2003). These standardized isolation protocols have been used in our laboratory for a long time, and therefore, the standard serum-based isolation procedure should not cause any bias in the results. At the same time, it is generally recognized that ASCs are a heterogeneous cell population that is selected through their plastic-adherent property and cell passaging (Bourin *et al.*, 2013). In the current thesis work, we developed a new XF/SF isolation method for ASCs. The isolation protocol is partially dependent on ASC adherence, and thus, the new XF/SF isolation protocol may have an effect on the heterogeneity of the ASC population, as discussed above. These XF/SF cultures require coating of the cell culture vessel, which may select for somewhat different cell populations during the isolation step, although the immunophenotype of ASCs isolated and expanded under XF/SF conditions was primarily retained compared with cells expanded in HS and traditionally used FBS media.

In studies **I** and **IV**, the differentiation capacity of ASCs was analyzed using specific histological staining methods and analysis of gene expression. For detection of osteogenic differentiation, ALP staining was used in studies **I** and **IV**. The ALP is a membrane-bound enzyme that regulates phosphate metabolism and is abundant in early osteogenic differentiation (Desai *et al.*, 2013); however, ALP activity may not be specific only for osteogenesis. Thus, additional staining (e.g., Alizarin Red or von

Kossa staining) of the calcified matrixes would have been recommended to confirm the result of ALP staining. Based on the experience with ALP staining, we decided to analyze the osteogenic differentiation using Alizarin Red staining in study **III**. Additionally, in studies **I** and **IV,** the osteogenic differentiation potential was confirmed by analyses of osteogenesis specific gene expressions, and thus, the results should be reliable. Nevertheless, limitations might be connected to each method, and the PCR-based methods may not be fully comparable to protein expression levels. The mRNA expression and enzymatic activities may might not be in agreement because regulation occurs on posttranscriptional and translational levels. Nevertheless, the aim of study **I** was to demonstrate the multipotency of the cells after expansion in different culture conditions and not the optimization of differentiation protocols for each condition. For these purposes, the selected methods should be fully adequate.

In study **III,** which investigated the immunological properties of ASCs, we used multiple PBMC responder cell donors in MLRs to minimize the possibility of HLA similarity between responder and stimulator cell populations. Although unlikely, it is possible that the lack of response to the ASC population may be due to HLA similarity between the ASC and PBMC populations and not to the non-immunogenic profile of the ASC population (McIntosh, 2011). With use of multiple PBMC donors, this possibility was diminished.

For testing the effect of different culture condition on the immunogenic properties of ASCs, we expanded the cells in three different culture conditions of FBS, HS and in XF/SF prior to co-culture. Still, the MLRs were all performed in HS-containing medium due to the low viability of PBMCs in the XF/SF condition, and thus, the medium was changed to match each condition 24 hours prior to co-culture. Consequently, the possibility exists that incubation of ASCs in HS-supplemented medium for 24 hours may have caused an effect on the phenotype of the cells or that certain cell characteristics may have been normalized by the medium change. However, as mentioned in the methods section, the PBMCs were not viable in XF/SF conditions for more than a few days, and the results of XF/SF cultures would have been biased without the medium change. Thus, we concluded that the chosen experimental set up was the most optimal approach to performing the assay. Of note, cells cultured in different conditions all received the same treatment of medium change prior to MLR analysis, and the possible normalization step was similar in all conditions; also the HS medium (Lonza, BioWhittaker) was replaced by a different type of HS medium manufactured by PAA Laboratories.

In study **II**, the MMC method was studied in combination with XF/SF conditions to find novel methods that support XF/SF differentiation of ASCs. Unfortunately, the MMC method could not be combined with XF/SF cultures because ASC survival was low under MMC conditions in XF/SF media. Low proliferation capacity and altered morphology were observed in each culture condition under MMC, which may also suggest decreased cell viability. Therefore, cautious conclusions should be drawn based on our results of ASCs cultured under MMC. Because a cell viability marker was not used during flow cytometry analysis, the increased CD54 expression levels under MMC may be partially due to unspecific intracellular staining in the case in which the cell membranes were not intact. However, a uniform cell population was observed in the forward and side scatter data, which suggests that a few dead cells were included in the analysis. Nevertheless, some of the intended experiments could not be completed after long-term culture under MMC conditions, but this experiment still produced important evidence of the applicability of the MMC method and demonstrated the unpredictable characteristics of in vitro stem cell research.

6.4 Not lost in translation: Challenges in translation of vitro to in vivo and finally to clinical practice

The literature that evaluates the potential of ASCs for regenerative medicine applications has expanded significantly during the past decade. The current literature includes a rather comprehensive overview of ASCs and their immunophenotype, differentiation capacity, expansion protocols, and immunomodulatory properties in vitro, as previously reviewed (Gimble *et al.*, 2007; Gimble *et al.*, 2010; Johal *et al.*, 2015; Lindroos *et al.*, 2011; McIntosh *et al.*, 2013). However, as discussed in Section 2.9, challenges connected to cell-based products and their clinical use remain, and probably the most important issue is ensuring the safety and quality of these complex entities (Salmikangas *et al.*, 2015). The heterogeneous nature of ASCs adds to the challenge because variation occurs between donor cell lines, and it may be difficult to find reliable and consistent methods to characterize and validate the cells. Despite the widespread acceptance of characterization by CD markers CD70, CD90 and CD105, it is recognized that these and additional markers might not represent a definitive phenotype of ASCs (Johal *et al.*, 2015). Furthermore, additional attention should be paid to the terminology used (Bourin *et al.*, 2013) and clearly distinguish

SVF cells from ASCs that are relatively homogeneous compared with SVF containing several cell populations.

Still, the transition from in vitro and in vivo studies to clinical practice is in progress. Unfortunately, ASC features that are observed on cell culture plastic may correlate poorly with those observed in the patient, and thus, optimization of in vivo models is critical to bridging the link from culture to clinic (Johal *et al.*, 2015). However, it may be challenging to find an appropriate in vivo model that could reliably correlate to patients because limitations are associated with many of the models used. Multiple factors must be considered when designing in vivo studies and interpreting the data, which include: 1) the species and strain used (such as mouse, rat, dog or pig); 2) the status of immune system (immunocompetent versus immunocompromised); 3) the immunological aspects of the donor cells (autologous/syngeneic, allogeneic, xenogeneic); 4) the method, site, and timing of cell delivery (intramuscular injection, subcutaneous transplantation); and 5) the imaging and quantitative methods applied (MRI, nuclear imaging, histology) (Gimble *et al.*, 2007). Nevertheless, several published studies in animal models are available that show evidence of the safety and efficacy of ASCs, as previously reviewed (Gimble *et al.*, 2010). Most of these studies have been performed using rodents, but studies in ovine, porcine, and other large animal models also have been reported. Time will show whether these studies are sufficient for prove safety and efficacy to regulatory authorities. It is likely that additional studies will be required, especially if considering cell migration into major organs and possible tumor formation as well as long-term safety and efficacy studies in larger animal models. However, preclinical animal studies suggest that ASCs will have the potential for soft and hard tissue repair (Levi *et al.*, 2010; K. C. Li *et al.*, 2015; Shah *et al.*, 2014) and for treatment of disorders related to immune response, such as autoimmune disorders (Gonzalez *et al.*, 2009; S. A. Park *et al.*, 2013; Yousefi *et al.*, 2013). Although further studies that address the regulatory concerns still must be completed prior to clinical translation, ongoing and upcoming clinical trials with a wide array of patient materials will finally determine the outcome of cell therapies that use ASCs. Figure 32 summarizes the different stages required for clinical transition of ASCs.

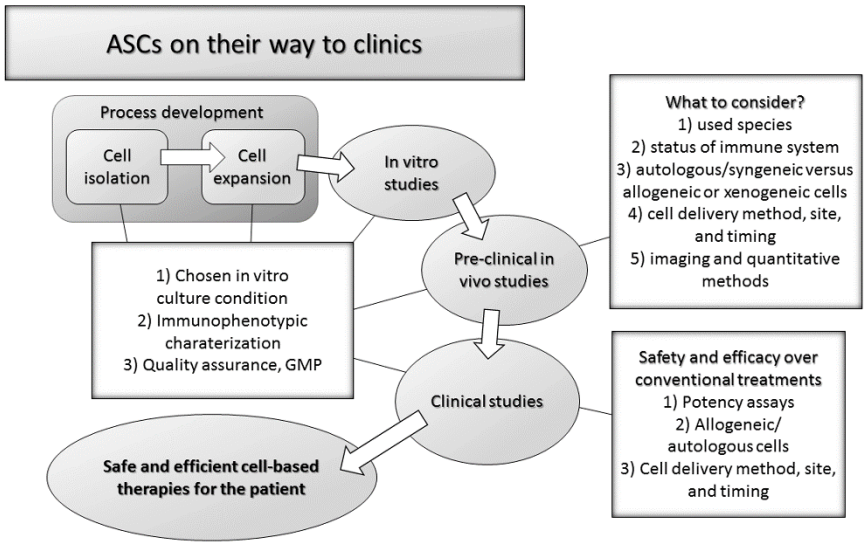

Figure 32. Required steps during clinical transition of ASCs.

One critical aspect of future cell-based therapies is the wide donor variation in ASCs, which will influence the cell response to differentiation induction as well as other properties, such as immunomodulation capacity. Several factors may affect cell characteristics, which include donor age and sex as well as the anatomic harvest location and depth (Section 2.3.2.). Unfortunately, no valid method exists for selecting patients for ASC-based therapies, e.g., for finding patients whose ASCs will efficiently respond to osteogenic induction and therefore be suitable candidates for bone regeneration therapies. Nevertheless, if allogeneic ASCs were used in cell therapies, the problem of extensive donor variation would be overcome. A few clinical studies were recently completed to evaluate the safety and efficacy of allogeneic ASCs for treatment of acute respiratory distress syndrome (ARDS) (Zheng *et al.*, 2014), refractory rheumatoid arthritis (NCT01663116; www.clinicaltrials.gov) and complex perianal fistulas (NCT01372969; www.clinicaltrials.gov). These studies reported that intravenous administration of allogeneic ASCs is a safe and feasible method for treating medical conditions, but further clinical safety and efficacy studies should still be performed. Safety testing after cell expansion in different culture conditions is especially important because regulatory authorities will require in vivo evidence of cell behavior under the culture condition chosen for patient treatments. Currently, the safety and efficacy of allogeneic ASCs have been demonstrated in several preclinical in vivo studies that investigate bone regeneration, spinal fusion, joint diseases and the systemic immunomodulatory effects of ASCs (Gu *et al.*, 2014; Liu *et al.*, 2013; Lopez *et al.*,

2009; Marx *et al.*, 2014; S. A. Park *et al.*, 2013; M. L. Ren *et al.*, 2012; Webb and Webb, 2014). The potential use of allogeneic ASCs in the future could enable manufacture of "off-the-shelf" products in which cells from donors with the best potential characteristics could be used for treatment of various diseases.

6.5 Future perspectives

Overall, ASC research is at a crucial turning point from basic science to clinical cell therapy. However, many important scientific questions still remain only partially answered. This thesis was conducted to further support the applicability of ASCs for clinical use by focusing on the safety and efficacy aspects of in vitro cultured cells as well to determine the immunogenic properties of ASCs. The development of a complete XF/SF workflow is an important step forward to avoid issues related to xenogeneic infections and lot-to-lot variation. The novel XF/SF conditions also proved superior in terms of effective cell expansion with respect to clinical cell therapies in which a large number of cells is required on a minimum time-scale.

In the future, it would be interesting to combine XF/SF-expanded ASCs with clinically used biomaterials, e.g., BAG or β-TCP. Only a few studies have been performed to investigate osteopromoting materials under XF/SF conditions; however, in one paper, a polypeptide polymer conjugate was investigated as a coating material for bone implants due to its ability to improve the cell adhesion under SF conditions (Tatrai *et al.*, 2013). In fact, loose cell adhesion under XF/SF conditions was one of the drawbacks that emerged during this thesis. Thus, in future studies, biomaterials with appropriate coatings should be tested for their ability to support cell adhesion under XF/SF conditions and thus to enhance the differentiation of ASCs under XF/SF conditions.

At the same time, the immunological properties of ASC should be determined when cells are combined with biomaterials. In addition, the low immunogenicity and immunosuppressive potential of ASCs should be confirmed after differentiation. If ASCs could combine the abilities of bone regeneration and immunomodulation, they could be considered as allogenic sources for treatment of bone defects. However, these novel approaches must be critically examined before entering the clinical stage. A recently published study investigated the effect of macroporous biphasic calcium phosphate (MBCP) granules on ASC immune properties (Montespan *et al.*, 2014). The results were promising because they demonstrated that the MBCP material did not modify the immunomodulatory functions of ASCs. Furthermore, only a few

studies have been published on the immunological properties of ASC after osteogenic differentiation, but in these studies, the low immunogenicity and immunosuppressive capacity of differentiated ASCs was successfully demonstrated (Montespan *et al.*, 2014; Niemeyer *et al.*, 2007). Nevertheless, additional studies are still required to fully determine the immunogenic properties of ASCs when cultured on biomaterials or differentiated into osteogenic cells.

In conclusion, careful characterization of ASCs with respect to in vitro culture conditions, immunological properties and cell behavior with clinically approved biomaterials is highly important during clinical transition and will also provide further evidence for regulatory authorities. The appropriate quality assurance in agreement with current GMP guidelines is critical during clinical transition, but these requirements might be demanding for cell-based products. Therefore, development should be based on well-controlled manufacturing processes (Salmikangas *et al.*, 2015) facilitated by clinically relevant culture conditions that were developed and tested in this thesis work. Further characterization and safety assessment of ASCs should be performed before ASCs can be considered as a standard clinical treatment; however, every study brings us closer to that goal.

7 Conclusions

In this thesis, a fully defined XF/SF culture system for ASCs was developed and evaluated in parallel with serum-containing media. Furthermore, the osteogenic differentiation of ASCs in response to biomaterials BAG and β-TCP and growth factors BMP- 2 and -7 was investigated. The main conclusions and findings from the studies are described as follows:

I The novel XF/SF culture conditions maintain the mesenchymal stem cell characteristics of ASCs with a few exceptions. The immunophenotype of ASCs was retained in every condition with minor differences; cells expanded in XF/SF conditions showed significantly lower expression of CD54 (intercellular adhesion molecule 1, ICAM-1) at low passage numbers. Importantly, the proliferation rate of ASCs was significantly increased in XF/SF conditions compared with HS- and FBS-containing medium. Furthermore, the chondrogenic differentiation potential was intense in XF/SF conditions, whereas adipogenic and osteogenic differentiation were comparable to the FBS condition after serum priming.

II The characteristic immunophenotype of ASCs was maintained under MMC conditions compared with standard conditions, except for the expression of CD54, which was significantly increased in all of the studied serum conditions under MMC. Although the MMC method did not support ASC proliferation in any of the studied conditions, metabolic activity was increased under MMC in FBS cultures. Importantly, MMC had a supportive influence on adipogenic and osteogenic differentiation of ASCs in FBS- and HS-containing media. Based on Alcian blue staining, chondrogenic differentiation was stronger after expansion in the MMC condition, and moreover, MMC altered the histological structure of micro-mass pellets into a less dense architecture. Nevertheless, the MMC method was not suitable for ASCs cultured in the studied XF/SF conditions because the cells do not remain viable after long-term exposure to MMC, which was observed by reduced cell number and dramatically changed morphology of XF/SF cultured ASCs.

III The isolation and expansion conditions of ASCs have an effect on immunogenicity, suppressive potential, and protein secretion profile of the cells. The strongest immunosuppression and lowest immunogenicity was observed with ASCs expanded in

the FBS-supplemented medium. By contrast, ASCs expanded in XF/SF conditions induced the strongest proliferative response in PBMCs and showed less immunosuppressive potential. However, the differences between culture conditions were minor; ASCs elicit only a low immunogenic response whether cultured in FBS, HS, or in XF/SF conditions, and suppressive potential is detectable in FBS and HS containing media high ASC numbers. ASCs expanded in XF/SF conditions had reduced capacity for immunosuppression as compared with FBS and HS media. Relatively small differences were observed in cytokine secretion between different serum conditions, and even more evident was the effect of direct versus indirect contact between ASCs and lymphocytes on signaling protein secretion. However, to underline few factors, the low IFN-γ and high IL-6 and secretions that were observed in FBS conditions may be associated with a stronger suppressive potential of ASCs. In conclusion, ASCs have potential for immunomodulation therapies, and allogeneic stem cell treatments may be conceivable in the future.

IV Both biomaterials BAG S53P4 and β-TCP granules supported successful cell attachment, viability and proliferation. The BAG S53P4 stimulated osteogenic differentiation of ASCs without osteogenic induction, whereas β-TCP required OM to induce osteogenic commitment. Interestingly, supplementation of OM with BMP-2 and BMP-7 decreased early osteogenic differentiation with both biomaterials. Both materials BAG and β-TCP consistently supported VEGF expression, thus indicating early signs of vasculature formation; however, significantly higher VEGF secretion was observed with BMP supplementation in cultures with β-TCP compared with cultures with BAG. Overall, culture of ASCs with BAG without osteogenic supplements was a beneficial approach to enhancing proliferation and osteogenic differentiation of ASCs while minimizing safety and regulatory concerns for clinical purposes.

In summary, the novel XF/SF conditions could be applied for effective ASC expansion during clinical cell therapies. Due to low immunogenicity in all studied culture conditions and promising immunosuppressive potential in serum-containing media, ASCs are attractive candidates for allogenic clinical use and immunomodulation therapies. The ASCs cultured on BAG showed an efficient osteogenic differentiation and thus, BAG could be used to induce successful osteogenic commitment of ASCs instead of BMPs or other osteogenic factors. Additional preclinical safety and efficacy studies will still be necessary and must be standardized prior to clinical transition.

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"It's nothing a few stem cells and another 20 years of research can't fix."

Figure modified from Ron Morgan. www.cartoonstock.com.

8 References

Agata H, Watanabe N, Ishii Y, Kubo N, Ohshima S, Yamazaki M, Tojo A, Kagami H (2009). Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions. Biochemical and Biophysical Research Communications 382: 353-358.

Aksu AE, Rubin JP, Dudas JR, Marra KG (2008). Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. Annals of Plastic Surgery 60: 306-322.

Albrektsson T, Johansson C (2001). Osteoinduction, osteoconduction and osseointegration. European Spine Journal : Official Publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society 10 Suppl 2: S96-101.

Al-Salleeh F, Beatty MW, Reinhardt RA, Petro TM, Crouch L (2008). Human osteogenic protein-1 induces osteogenic differentiation of adipose-derived stem cells harvested from mice. Archives of Oral Biology 53: 928-936.

Al-Saqi SH, Saliem M, Asikainen S, Quezada HC, Ekblad A, Hovatta O, Le Blanc K, Jonasson AF, Gotherstrom C (2014a). Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells. Cytotherapy 16: 915-926.

Al-Saqi SH, Saliem M, Quezada HC, Ekblad A, Jonasson AF, Hovatta O, Gotherstrom C (2014b). Defined serum- and xeno-free cryopreservation of mesenchymal stem cells. Cell and Tissue Banking

Alt EU, Senst C, Murthy SN, Slakey DP, Dupin CL, Chaffin AE, Kadowitz PJ, Izadpanah R (2012). Aging alters tissue resident mesenchymal stem cell properties. Stem Cell Research 8: 215-225.

Altin JG, Sloan EK (1997). The role of CD45 and CD45-associated molecules in T cell activation. Immunology and Cell Biology 75: 430-445.

Alvarez CV, Garcia-Lavandeira M, Garcia-Rendueles ME, Diaz-Rodriguez E, Garcia-Rendueles AR, Perez-Romero S, Vila TV, Rodrigues JS, Lear PV, Bravo SB (2012). Defining stem cell types: Understanding the therapeutic potential of ESCs, ASCs, and iPS cells. Journal of Molecular Endocrinology 49: R89-111.

Amini AR, Laurencin CT, Nukavarapu SP (2012). Bone tissue engineering: Recent advances and challenges. Critical Reviews in Biomedical Engineering 40: 363-408.

Ancans J (2012). Cell therapy medicinal product regulatory framework in europe and its application for MSC-based therapy development. Frontiers in Immunology 3: 253.

Ang XM, Lee MH, Blocki A, Chen C, Ong LL, Asada HH, Sheppard A, Raghunath M (2014). Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Engineering.Part A 20: 966-981.

Antia R, Regoes RR, Koella JC, Bergstrom CT (2003). The role of evolution in the emergence of infectious diseases. Nature 426: 658-661.

Anton K, Banerjee D, Glod J (2012). Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. PloS One 7: e35036.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science 275: 964-967.

Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, Sen A, Willingmyre GD, Gimble JM (2004). Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 6: 7-14.

Baer PC, Geiger H (2012). Adipose-derived mesenchymal stromal/stem cells: Tissue localization, characterization, and heterogeneity. Stem Cells International 2012: 812693.

Baglioni S, Cantini G, Poli G, Francalanci M, Squecco R, Di Franco A, Borgogni E, Frontera S, Nesi G, Liotta F, Lucchese M, Perigli G, Francini F, Forti G, Serio M, Luconi M (2012). Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. PloS One 7: e36569.

Baino F, Vitale-Brovarone C (2011). Three-dimensional glass-derived scaffolds for bone tissue engineering: Current trends and forecasts for the future. Journal of Biomedical Materials Research.Part A 97: 514-535.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. Experimental Hematology 28: 707-715.

Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM (2014). Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. PloS One 9: e115963.

Beggs KJ, Lyubimov A, Borneman JN, Bartholomew A, Moseley A, Dodds R, Archambault MP, Smith AK, McIntosh KR (2006). Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. Cell Transplantation 15: 711-721.

Bensinger W, Singer J, Appelbaum F, Lilleby K, Longin K, Rowley S, Clarke E, Clift R, Hansen J, Shields T (1993). Autologous transplantation with peripheral blood mononuclear cells collected after administration of recombinant granulocyte stimulating factor. Blood 81: 3158-3163.

Bergman K, Graff GD (2007). The global stem cell patent landscape: Implications for efficient technology transfer and commercial development. Nature Biotechnology 25: 419-424.

Bessa PC, Casal M, Reis RL (2008a). Bone morphogenetic proteins in tissue engineering: The road from the laboratory to the clinic, part I (basic concepts). Journal of Tissue Engineering and Regenerative Medicine 2: 1-13.

Bessa PC, Casal M, Reis RL (2008b). Bone morphogenetic proteins in tissue engineering: The road from laboratory to clinic, part II (BMP delivery). Journal of Tissue Engineering and Regenerative Medicine 2: 81-96.

Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J (2005). Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105: 2214-2219.

Bhang SH, Cho SW, La WG, Lee TJ, Yang HS, Sun AY, Baek SH, Rhie JW, Kim BS (2011). Angiogenesis in ischemic tissue produced by spheroid grafting of human adipose-derived stromal cells. Biomaterials 32: 2734-2747.

Bieback K, Kern S, Kluter H, Eichler H (2004). Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22: 625-634.

Bielli A, Scioli MG, Gentile P, Agostinelli S, Tarquini C, Cervelli V, Orlandi A (2014). Adult adipose-derived stem cells and breast cancer: A controversial relationship. SpringerPlus 3: 345-1801-3-345. eCollection 2014.

Blande IS, Bassaneze V, Lavini-Ramos C, Fae KC, Kalil J, Miyakawa AA, Schettert IT, Krieger JE (2009). Adipose tissue mesenchymal stem cell expansion in animal serum-free medium supplemented with autologous human platelet lysate. Transfusion 49: 2680-2685.

Bongso A, Fong CY, Gauthaman K (2008). Taking stem cells to the clinic: Major challenges. Journal of Cellular Biochemistry 105: 1352-1360.

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM (2013). Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: A joint statement of the international federation for adipose therapeutics and science (IFATS) and the international society for cellular therapy (ISCT). Cytotherapy 15: 641-648.

Bravery CA, Carmen J, Fong T, Oprea W, Hoogendoorn KH, Woda J, Burger SR, Rowley JA, Bonyhadi ML, Van't Hof W (2013). Potency assay development for cellular therapy products: An ISCT review of the requirements and experiences in the industry. Cytotherapy 15: 9-19.

Brayfield C, Marra K, Rubin JP (2010). Adipose stem cells for soft tissue regeneration. Organ Der Vereinigun 42: 124-128.

Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C (2008). Adipose-derived stem cells: Isolation, expansion and differentiation. Methods (San Diego, Calif.) 45: 115-120.

Bura A, Planat-Benard V, Bourin P, Silvestre JS, Gross F, Grolleau JL, Saint-Lebese B, Peyrafitte JA, Fleury S, Gadelorge M, Taurand M, Dupuis-Coronas S, Leobon B, Casteilla L (2014). Phase I trial: The use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. Cytotherapy 16: 245-257.

Burger SR (2000). Design and operation of a current good manufacturing practices cell-engineering laboratory. Cytotherapy 2: 111-122.

Caplan AI, Dennis JE (2006). Mesenchymal stem cells as trophic mediators. Journal of Cellular Biochemistry 98: 1076-1084.

Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, Yoon ST, Comer G, Kopjar B (2013). Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. The Journal of Bone and Joint Surgery.American Volume 95: 1537-1545.

Casteilla L, Planat-Benard V, Laharrague P, Cousin B (2011). Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. World Journal of Stem Cells 3: 25-33.

Chan WK, Lau AS, Li JC, Law HK, Lau YL, Chan GC (2008). MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. Experimental Hematology 36: 1545-1555.

Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC (2010). A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Research & Therapy 1: 8.

Chase LG, Yang S, Zachar V, Yang Z, Lakshmipathy U, Bradford J, Boucher SE, Vemuri MC (2012). Development and characterization of a clinically compliant xeno-free culture medium in good manufacturing practice for human multipotent mesenchymal stem cells. Stem Cells Translational Medicine 1: 750-758.

Chen CZ, Loe F, Blocki A, Peng Y, Raghunath M (2011). Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Advanced Drug Delivery Reviews 63: 277-290.

Chen CZ, Peng YX, Wang ZB, Fish PV, Kaar JL, Koepsel RR, Russell AJ, Lareu RR, Raghunath M (2009). The scar-in-a-jar: Studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well. British Journal of Pharmacology 158: 1196-1209.

Chen QZ, Thompson ID, Boccaccini AR (2006). 45S5 bioglass-derived glass-ceramic scaffolds for bone tissue engineering. Biomaterials 27: 2414-2425.

Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT (2014). Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. Journal of Translational Medicine 12: 8-5876-12-8.

Choumerianou DM, Dimitriou H, Kalmanti M (2008). Stem cells: Promises versus limitations. Tissue Engineering.Part B, Reviews 14: 53-60.

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3: 301-313.

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC (1998). Relationship between donor age and the replicative lifespan of human cells in culture: A reevaluation. Proceedings of the National Academy of Sciences of the United States of America 95: 10614-10619.

Croitoru-Lamoury J, Lamoury FM, Zaunders JJ, Veas LA, Brew BJ (2007). Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and copaxone. Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon and Cytokine Research 27: 53-64.

Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, van Ijcken WF, Dahlke MH, Eggenhofer E, Weimar W, Hoogduijn MJ (2010a). Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clinical and Experimental Immunology 162: 474-486.

Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Weimar W, Hoogduijn MJ (2010b). Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. Stem Cells and Development 19: 1843-1853.

Csete M (2010). Translational prospects for human induced pluripotent stem cells. Regenerative Medicine 5: 509-519.

Cui L, Yin S, Liu W, Li N, Zhang W, Cao Y (2007). Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2. Tissue Engineering 13: 1185-1195.

Davis TA, Anam K, Lazdun Y, Gimble JM, Elster EA (2014). Adipose-derived stromal cells promote allograft tolerance induction. Stem Cells Translational Medicine 3: 1444-1450.

de Paula AC, Zonari AA, Martins TM, Novikoff S, da Silva AR, Correlo VM, Reis RL, Gomes DA, Goes AM (2013). Human serum is a suitable supplement for the osteogenic differentiation of human adipose-derived stem cells seeded on poly-3-hydroxibutyrate-co-3-hydroxyvalerate scaffolds. Tissue Engineering.Part A 19: 277-289.

De Ugarte DA, Alfonso Z, Zuk PA, Elbarbary A, Zhu M, Ashjian P, Benhaim P, Hedrick MH, Fraser JK (2003). Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunology Letters 89: 267-270.

DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, Rico L, Camarillo E, Garcia L, Abad JL, Trigueros C, Delgado M, Buscher D (2009). Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Engineering.Part A 15: 2795-2806.

Delay E, Garson S, Tousson G, Sinna R (2009). Fat injection to the breast: Technique, results, and indications based on 880 procedures over 10 years. Aesthetic Surgery Journal / the American Society for Aesthetic Plastic Surgery 29: 360-376.

Denker AE, Nicoll SB, Tuan RS (1995). Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1. Differentiation; Research in Biological Diversity 59: 25-34.

Derynck R, Zhang YE (2003). Smad-dependent and smad-independent pathways in TGF-beta family signalling. Nature 425: 577-584.

Desai HV, Voruganti IS, Jayasuriya C, Chen Q, Darling EM (2013). Live-cell, temporal gene expression analysis of osteogenic differentiation in adipose-derived stem cells. Tissue Engineering.Part A 19: 40-48.

Devine JG, Dettori JR, France JC, Brodt E, McGuire RA (2012). The use of rhBMP in spine surgery: Is there a cancer risk? Evidence-Based Spine-Care Journal 3: 35-41.

Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99: 3838-3843.

Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, Cantos C, Jorgensen C, Noel D (2007). Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem Cells 25: 2025-2032.

Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noel D, Jorgensen C (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102: 3837-3844.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. Cytotherapy 8: 315-317.

Dromard C, Bourin P, Andre M, De Barros S, Casteilla L, Planat-Benard V (2011). Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres. Experimental Cell Research 317: 770-780.

Eilertsen KJ, Floyd Z, Gimble JM (2008). The epigenetics of adult (somatic) stem cells. Critical Reviews in Eukaryotic Gene Expression 18: 189-206.

Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J (2005). Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 106: 4057-4065.

EMA, European Public Assessment Report: ChondroCelect (2009). ChondroCelect: Characterised viable autologous cartilage cells expanded ex vivo expressing specific marker proteins.http://www.ema.europa.eu

EMA, European Public Assessment Report: Holoclar (2015). Holoclar: Ex vivo expanded autologous human corneal epithelial cells containing stem cells. www.ema.europa.eu/ema

EMA, European Public Assessment Report: MACI (2013). Maci: Matrix-applied characterised autologous cultured chondrocytes. www.ema.europa.eu/ema

EMA, European Public Assessment Report: Provenge (2013). Provenge: Autologous peripheral-blood mononuclear cells activated with prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor. www.ema.europa.eu/ema

English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP (2009). Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(high) forkhead box P3+ regulatory T cells. Clinical and Experimental Immunology 156: 149-160.

Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F (2002). Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochemical and Biophysical Research Communications 290: 763-769.

Eterno V, Zambelli A, Pavesi L, Villani L, Zanini V, Petrolo G, Manera S, Tuscano A, Amato A (2014). Adipose-derived mesenchymal stem cells (ASCs) may favour breast cancer recurrence via HGF/c-met signaling. Oncotarget 5: 613-633.

Eto H, Suga H, Inoue K, Aoi N, Kato H, Araki J, Doi K, Higashino T, Yoshimura K (2011). Adipose injury-associated factors mitigate hypoxia in ischemic tissues through activation of adipose-derived stem/progenitor/stromal cells and induction of angiogenesis. The American Journal of Pathology 178: 2322-2332.

European Medicines Agency (2011). European medicines agency, CAT secretariat & US food and drug administration. Regenerative Medicine 6: 90-96.

Fang B, Song Y, Li N, Li J, Han Q, Zhao RC (2009). Mesenchymal stem cells for the treatment of refractory pure red cell aplasia after major ABO-incompatible hematopoietic stem cell transplantation. Annals of Hematology 88: 261-266.

Fang B, Song Y, Liao L, Zhang Y, Zhao RC (2007a). Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. Transplantation Proceedings 39: 3358-3362.

Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao RC, Ma Y (2007b). Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. Pediatric Transplantation 11: 814-817.

Fang B, Song Y, Zhao RC, Han Q, Lin Q (2007c). Using human adipose tissue-derived mesenchymal stem cells as salvage therapy for hepatic graft-versus-host disease resembling acute hepatitis. Transplantation Proceedings 39: 1710-1713.

Fernandez Vallone VB, Romaniuk MA, Choi H, Labovsky V, Otaegui J, Chasseing NA (2013). Mesenchymal stem cells and their use in therapy: What has been achieved? Differentiation; Research in Biological Diversity 85: 1-10.

Fink DW,Jr (2009). FDA regulation of stem cell-based products. Science 324: 1662-1663.

Francois M, Romieu-Mourez R, Li M, Galipeau J (2012). Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2

macrophage differentiation. Molecular Therapy: The Journal of the American Society of Gene Therapy 20: 187-195.

Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV (1974). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. cloning in vitro and retransplantation in vivo. Transplantation 17: 331-340.

Fritz V, Jorgensen C (2008). Mesenchymal stem cells: An emerging tool for cancer targeting and therapy. Current Stem Cell Research & Therapy 3: 32-42.

Garcia S, Bernad A, Martin MC, Cigudosa JC, Garcia-Castro J, de la Fuente R (2010). Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. Experimental Cell Research 316: 1648-1650.

Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA (2005). A phase I clinical trial of the treatment of crohn's fistula by adipose mesenchymal stem cell transplantation. Diseases of the Colon and Rectum 48: 1416-1423.

Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M (2009a). Expanded adipose-derived stem cells for the treatment of complex perianal fistula: A phase II clinical trial. Diseases of the Colon and Rectum 52: 79-86.

Garcia-Olmo D, Herreros D, Pascual M, Pascual I, De-La-Quintana P, Trebol J, Garcia-Arranz M (2009b). Treatment of enterocutaneous fistula in crohn's disease with adipose-derived stem cells: A comparison of protocols with and without cell expansion. International Journal of Colorectal Disease 24: 27-30.

Genser-Nir, M., Daniliuc, S., Tevrovsky, M., & Fiorentini, D (2013). Toward a serum-free, xeno-free culture system for optimal growth and expansion of hMSC suited to therapeutic applications . 23rd European Society for Animal Cell Technology (ESACT) Meeting, France. , 23rd European Society for Animal Cell Technology (ESACT) Meeting

Ghanaati S, Barbeck M, Orth C, Willershausen I, Thimm BW, Hoffmann C, Rasic A, Sader RA, Unger RE, Peters F, Kirkpatrick CJ (2010). Influence of beta-tricalcium phosphate granule size and morphology on tissue reaction in vivo. Acta Biomaterialia 6: 4476-4487.

Ghannam S, Bouffi C, Djouad F, Jorgensen C, Noel D (2010). Immunosuppression by mesenchymal stem cells: Mechanisms and clinical applications. Stem Cell Research & Therapy 1: 2.

Giannoudis PV, Tzioupis C (2005). Clinical applications of BMP-7: The UK perspective. Injury 36 Suppl 3: S47-50.

Gieseke F, Bohringer J, Bussolari R, Dominici M, Handgretinger R, Muller I (2010). Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. Blood 116: 3770-3779.

Gilbert KM, Thoman M, Bauche K, Pham T, Weigle WO (1997). Transforming growth factor-beta 1 induces antigen-specific unresponsiveness in naive T cells. Immunological Investigations 26: 459-472.

Gimble JM, Bunnell BA, Guilak F (2012). Human adipose-derived cells: An update on the transition to clinical translation. Regenerative Medicine 7: 225-235.

Gimble JM, Guilak F (2003). Adipose-derived adult stem cells: Isolation, characterization, and differentiation potential. Cytotherapy 5: 362-369.

Gimble JM, Guilak F, Bunnell BA (2010). Clinical and preclinical translation of cell-based therapies using adipose tissue-derived cells. Stem Cell Research & Therapy 1: 19.

Gimble JM, Katz AJ, Bunnell BA (2007). Adipose-derived stem cells for regenerative medicine. Circulation Research 100: 1249-1260.

Gimeno RE, Klaman LD (2005). Adipose tissue as an active endocrine organ: Recent advances. Current Opinion in Pharmacology 5: 122-128.

Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M (2009). Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 136: 978-989.

Gorustovich AA, Roether JA, Boccaccini AR (2010). Effect of bioactive glasses on angiogenesis: A review of in vitro and in vivo evidences. Tissue Engineering.Part B, Reviews 16: 199-207.

Gottipamula S, Muttigi MS, Kolkundkar U, Seetharam RN (2013). Serum-free media for the production of human mesenchymal stromal cells: A review. Cell Proliferation 46: 608-627.

Greenspan P, Mayer EP, Fowler SD (1985). Nile red: A selective fluorescent stain for intracellular lipid droplets. The Journal of Cell Biology 100: 965-973.

Grimes BR, Steiner CM, Merfeld-Clauss S, Traktuev DO, Smith D, Reese A, Breman AM, Thurston VC, Vance GH, Johnstone BH, Slee RB, March KL (2009). Interphase FISH demonstrates that human adipose stromal cells maintain a high level of genomic stability in long-term culture. Stem Cells and Development 18: 717-724.

Gu H, Xiong Z, Yin X, Li B, Mei N, Li G, Wang C (2014). Bone regeneration in a rabbit ulna defect model: Use of allogeneic adipose-derivedstem cells with low immunogenicity. Cell and Tissue Research 358: 453-464.

Haddad R, Saldanha-Araujo F (2014). Mechanisms of T-cell immunosuppression by mesenchymal stromal cells: What do we know so far? BioMed Research International 2014: 216806.

Haimi S, Moimas L, Pirhonen E, Lindroos B, Huhtala H, Raty S, Kuokkanen H, Sandor GK, Miettinen S, Suuronen R (2009b). Calcium phosphate surface treatment of bioactive glass causes a delay in early osteogenic differentiation of adipose stem cells. Journal of Biomedical Materials Research.Part A 91: 540-547.

Haimi S, Suuriniemi N, Haaparanta AM, Ella V, Lindroos B, Huhtala H, Raty S, Kuokkanen H, Sandor GK, Kellomaki M, Miettinen S, Suuronen R (2009a). Growth and osteogenic differentiation of adipose stem cells on PLA/bioactive glass and PLA/beta-TCP scaffolds. Tissue Engineering.Part A 15: 1473-1480.

Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM (2001). Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Engineering 7: 729-741.

Hanley PJ (2014). Therapeutic mesenchymal stromal cells: Where we are headed. Methods in Molecular Biology

Hench LL (1998). Bioactive materials: The potential for tissue regeneration. Journal of Biomedical Materials Research 41: 511-518.

Hench LL, Xynos ID, Polak JM (2004). Bioactive glasses for in situ tissue regeneration. Journal of Biomaterials Science.Polymer Edition 15: 543-562.

Hernandez JD, Baum LG (2002). Ah, sweet mystery of death! galectins and control of cell fate. Glycobiology 12: 127R-36R.

Hildner F, Eder MJ, Hofer K, Aberl J, Redl H, van Griensven M, Gabriel C, Peterbauer-Scherb A (2013). Human platelet lysate successfully promotes proliferation and subsequent chondrogenic differentiation of adipose-derived stem cells: A comparison with articular chondrocytes. Journal of Tissue Engineering and Regenerative Medicine

Hino J, Kangawa K, Matsuo H, Nohno T, Nishimatsu S (2004). Bone morphogenetic protein-3 family members and their biological functions. Frontiers in Bioscience: A Journal and Virtual Library 9: 1520-1529.

Huttala O, Vuorenpaa H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, Sarkanen JR, Heinonen T (2015). Human vascular model with defined stimulation medium - a characterization study. Altex 32: 125-136.

Ilic N, Savic S, Siegel E, Atkinson K, Tasic L (2012). Examination of the regulatory frameworks applicable to biologic drugs (including stem cells and their progeny) in europe,

the U.S., and australia: Part I--a method of manual documentary analysis. Stem Cells Translational Medicine 1: 898-908.

Im GI (2013). Adipose stem cells and skeletal repair. Histology and Histopathology 28: 557-564.

Im GI, Shin YW, Lee KB (2005). Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis and Cartilage / OARS, Osteoarthritis Research Society 13: 845-853.

Im W, Chung JY, Kim SH, Kim M (2011). Efficacy of autologous serum in human adipose-derived stem cells; cell markers, growth factors and differentiation. Cellular & Molecular Biology 57: O1470-5.

Ito Y, Miyazono K (2003). RUNX transcription factors as key targets of TGF-beta superfamily signaling. Current Opinion in Genetics & Development 13: 43-47.

Jeong SH, Ji YH, Yoon ES (2014). Immunosuppressive activity of adipose tissue-derived mesenchymal stem cells in a rat model of hind limb allotransplantation. Transplantation Proceedings 46: 1606-1614.

Johal KS, Lees VC, Reid AJ (2015). Adipose-derived stem cells: Selecting for translational success. Regenerative Medicine 10: 79-96.

Josh F, Kobe K, Tobita M, Tanaka R, Suzuki K, Ono K, Hyakusoku H, Mizuno H (2012). Accelerated and safe proliferation of human adipose-derived stem cells in medium supplemented with human serum. Journal of Nippon Medical School = Nippon Ika Daigaku Zasshi 79: 444-452.

Jung S, Sen A, Rosenberg L, Behie LA (2010). Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. Cytotherapy 12: 637-657.

Kadri N, Potiron N, Ouary M, Jegou D, Gouin E, Bach JM, Lieubeau B (2007). Fetal calf serum-primed dendritic cells induce a strong anti-fetal calf serum immune response and diabetes protection in the non-obese diabetic mouse. Immunology Letters 108: 129-136.

Kang JM, Han M, Park IS, Jung Y, Kim SH, Kim SH (2012). Adhesion and differentiation of adipose-derived stem cells on a substrate with immobilized fibroblast growth factor. Acta Biomaterialia 8: 1759-1767.

Kang JW, Kang K, Koo HC, Park JR, Choi EW, Park YH (2008). Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. Stem Cells and Development 17: 681-693.

Kapur SK, Dos-Anjos Vilaboa S, Llull R, Katz AJ (2015). Adipose tissue and Stem/Progenitor cells: Discovery and development. Clinics in Plastic Surgery 42: 155-167.

Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449: 557-563.

Karnoub AE, Weinberg RA (2006). Chemokine networks and breast cancer metastasis. Breast Disease 26: 75-85.

Katz AJ, Llull R., Futrell WJ, Hedrick MH, Benhaim, P, Lorenz, HP, Less, MZ (2004). Patent. Adipose-derived stem cells and lattices. US20010936665 20010910; US20010936665 20010910; National class code: C12N5/0068; C12N5/06; C12N5/0647; C12N5/0654; C12N5/0655; C12N5/0658; C12N5/0667; A61K35/12; A61K48/00; C12N2500/25; C12N2500/38; C12N2500/42; C12N2501/01; C12N2501/33; C12N2501/39; C12N2502/1305; C12N2506/1384; C12N2510/00; C12N2533/90; International class code: C12N5/00; C12N5/077; C12N5/0775; C12N5/0789; A61K35/12; A61K48/00; (IPC1-7): C12N5/00; C12N5/08, University of California.

Katz AJ, Llull R, Hedrick MH, Futrell JW (1999). Emerging approaches to the tissue engineering of fat. Clinics in Plastic Surgery 26: 587-603, viii.

Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC (2005). Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells 23: 412-423.

Kim N, Im KI, Lim JY, Jeon EJ, Nam YS, Kim EJ, Cho SG (2013). Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: Experiments and practice. Annals of Hematology 92: 1295-1308.

Kim S, Chang KA, Kim J, Park HG, Ra JC, Kim HS, Suh YH (2012). The preventive and therapeutic effects of intravenous human adipose-derived stem cells in alzheimer's disease mice. PloS One 7: e45757.

Kisiel, M (2013). Bone enhancement with BMP-2 for safe clinical translation. ACTA UNIVERSITATIS UPSALIENSIS UPPSALA).

Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PI, Klein-Nulend J (2006). Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochemical and Biophysical Research Communications 342: 902-908.

Kocaoemer A, Kern S, Kluter H, Bieback K (2007). Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25: 1270-1278.

Koellensperger E, Bollinger N, Dexheimer V, Gramley F, Germann G, Leimer U (2014). Choosing the right type of serum for different applications of human adipose tissue-derived stem cells: Influence on proliferation and differentiation abilities. Cytotherapy 16: 789-799.

Kolios G, Moodley Y (2013). Introduction to stem cells and regenerative medicine. Respiration; International Review of Thoracic Diseases 85: 3-10.

Kolle SF, Fischer-Nielsen A, Mathiasen AB, Elberg JJ, Oliveri RS, Glovinski PV, Kastrup J, Kirchhoff M, Rasmussen BS, Talman ML, Thomsen C, Dickmeiss E, Drzewiecki KT (2013). Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: A randomised placebo-controlled trial. Lancet 382: 1113-1120.

Konno M, Hamazaki TS, Fukuda S, Tokuhara M, Uchiyama H, Okazawa H, Okochi H, Asashima M (2010). Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture. Biochemical and Biophysical Research Communications 400: 461-465.

Kouji M, Yuya T, Etsuko T, Francis S, Satoshi U (2011). Chemokines in inflammatory and immune diseases. Inflammation and Regeneration Vol.31 No.1 January

Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F (2006). Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 24: 386-398.

Krampera M, Galipeau J, Shi Y, Tarte K, Sensebe L, MSC Committee of the International Society for Cellular Therapy (ISCT) (2013). Immunological characterization of multipotent mesenchymal stromal cells--the international society for cellular therapy (ISCT) working proposal. Cytotherapy 15: 1054-1061.

Kronsteiner B, Wolbank S, Peterbauer A, Hackl C, Redl H, van Griensven M, Gabriel C (2011). Human mesenchymal stem cells from adipose tissue and amnion influence T-cells depending on stimulation method and presence of other immune cells. Stem Cells and Development 20: 2115-2126.

Kuo YR, Chen CC, Goto S, Lee IT, Huang CW, Tsai CC, Wang CT, Chen CL (2011). Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. Plastic and Reconstructive Surgery 128: 661e-72e.

Kyllonen L, Haimi S, Mannerstrom B, Huhtala H, Rajala KM, Skottman H, Sandor GK, Miettinen S (2013). Effects of different serum conditions on osteogenic differentiation of human adipose stem cells in vitro. Stem Cell Research & Therapy 4: 17.

Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, Kyurkchiev S (2014). Secretion of immunoregulatory cytokines by mesenchymal stem cells. World Journal of Stem Cells 6: 552-570.

Lahti AL, Kujala VJ, Chapman H, Koivisto AP, Pekkanen-Mattila M, Kerkela E, Hyttinen J, Kontula K, Swan H, Conklin BR, Yamanaka S, Silvennoinen O, Aalto-Setala K (2012). Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. Disease Models & Mechanisms 5: 220-230.

Laitinen A, Nystedt J, Laitinen S (2011). The isolation and culture of human cord blood-derived mesenchymal stem cells under low oxygen conditions. Methods in Molecular Biology 698: 63-73.

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

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O, Developmental Committee of the European Group for Blood and Marrow Transplantation (2008). Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: A phase II study. Lancet 371: 1579-1586.

Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O (2003a). HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Experimental Hematology 31: 890-896.

Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O (2003b). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scandinavian Journal of Immunology 57: 11-20.

Lee JM, Jung J, Lee HJ, Jeong SJ, Cho KJ, Hwang SG, Kim GJ (2012). Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. International Immunopharmacology 13: 219-224.

Lepelletier Y, Lecourt S, Renand A, Arnulf B, Vanneaux V, Fermand JP, Menasche P, Domet T, Marolleau JP, Hermine O, Larghero J (2010). Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. Stem Cells and Development 19: 1075-1079.

Lerou PH, Daley GQ (2005). Therapeutic potential of embryonic stem cells. Blood Reviews 19: 321-331.

Letterio JJ, Roberts AB (1998). Regulation of immune responses by TGF-beta. Annual Review of Immunology 16: 137-161.

Levi B, James AW, Nelson ER, Vistnes D, Wu B, Lee M, Gupta A, Longaker MT (2010). Human adipose derived stromal cells heal critical size mouse calvarial defects. PloS One 5: e11177.

Li H, Zimmerlin L, Marra KG, Donnenberg VS, Donnenberg AD, Rubin JP (2011). Adipogenic potential of adipose stem cell subpopulations. Plastic and Reconstructive Surgery 128: 663-672.

Li KC, Chang YH, Lin CY, Hwang SM, Wang TH, Hu YC (2015). Preclinical safety evaluation of ASCs engineered by FLPo/Frt-based hybrid baculovirus: In vitro and large animal studies. Tissue Engineering.Part A

Liao HT, Chen CT (2014). Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells. World Journal of Stem Cells 6: 288-295.

Lim MH, Ong WK, Sugii S (2014). The current landscape of adipose-derived stem cells in clinical applications. Expert Reviews in Molecular Medicine 16: e8.

Lin CS, Lin G, Lue TF (2012). Allogeneic and xenogeneic transplantation of adipose-derived stem cells in immunocompetent recipients without immunosuppressants. Stem Cells and Development 21: 2770-2778.

Lin F (2012). Adipose tissue-derived mesenchymal stem cells: A fat chance of curing kidney disease? Kidney International 82: 731-733.

Lindroos B, Aho KL, Kuokkanen H, Raty S, Huhtala H, Lemponen R, Yli-Harja O, Suuronen R, Miettinen S (2010). Differential gene expression in adipose stem cells cultured in allogeneic human serum versus fetal bovine serum. Tissue Engineering.Part A 16: 2281-2294.

Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, Haataja R, Vemuri M, Suuronen R, Miettinen S (2009). Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy 11: 958-972.

Lindroos B, Suuronen R, Miettinen S (2011). The potential of adipose stem cells in regenerative medicine. Stem Cell Reviews 7: 269-291.

Liu G, Zhang Y, Liu B, Sun J, Li W, Cui L (2013). Bone regeneration in a canine cranial model using allogeneic adipose derived stem cells and coral scaffold. Biomaterials 34: 2655-2664.

Liu G, Zhao L, Cui L, Liu W, Cao Y (2007). Tissue-engineered bone formation using human bone marrow stromal cells and novel beta-tricalcium phosphate. Biomedical Materials 2: 78-86.

Lopez MJ, McIntosh KR, Spencer ND, Borneman JN, Horswell R, Anderson P, Yu G, Gaschen L, Gimble JM (2009). Acceleration of spinal fusion using syngeneic and allogeneic adult adipose derived stem cells in a rat model. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society 27: 366-373.

Lotfy A, Salama M, Zahran F, Jones E, Badawy A, Sobh M (2014). Characterization of mesenchymal stem cells derived from rat bone marrow and adipose tissue: A comparative study. International Journal of Stem Cells 7: 135-142.

Lv FJ, Tuan RS, Cheung KM, Leung VY (2014). Concise review: The surface markers and identity of human mesenchymal stem cells. Stem Cells 32: 1408-1419.

Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y (2014). Immunobiology of mesenchymal stem cells. Cell Death and Differentiation 21: 216-225.

Mackensen A, Drager R, Schlesier M, Mertelsmann R, Lindemann A (2000). Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. Cancer Immunology, Immunotherapy: CII 49: 152-156.

Maenpaa K, Ella V, Mauno J, Kellomaki M, Suuronen R, Ylikomi T, Miettinen S (2010). Use of adipose stem cells and polylactide discs for tissue engineering of the temporomandibular joint disc. Journal of the Royal Society, Interface / the Royal Society 7: 177-188.

Marx C, Silveira MD, Selbach I, da Silva AS, Braga LM, Camassola M, Nardi NB (2014). Acupoint injection of autologous stromal vascular fraction and allogeneic adipose-derived stem cells to treat hip dysplasia in dogs. Stem Cells International 2014: 391274.

Mathews DJ, Graff GD, Saha K, Winickoff DE (2011). Access to stem cells and data: Persons, property rights, and scientific progress. Science 331: 725-727.

Matsuda A, Motoya S, Kimura S, McInnis R, Maizel AL, Takeda A (1998). Disruption of lymphocyte function and signaling in CD45-associated protein-null mice. The Journal of Experimental Medicine 187: 1863-1870.

Maumus M, Peyrafitte JA, D'Angelo R, Fournier-Wirth C, Bouloumie A, Casteilla L, Sengenes C, Bourin P (2011). Native human adipose stromal cells: Localization, morphology and phenotype. International Journal of Obesity (2005) 35: 1141-1153.

McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Di Halvorsen Y, Ting JP, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006). The

immunogenicity of human adipose-derived cells: Temporal changes in vitro. Stem Cells 24: 1246-1253.

McIntosh KR (2011). Evaluation of cellular and humoral immune responses to allogeneic adipose-derived stem/stromal cells. Methods in Molecular Biology 702: 133-150.

McIntosh KR, Frazier T, Rowan BG, Gimble JM (2013). Evolution and future prospects of adipose-derived immunomodulatory cell therapeutics. Expert Review of Clinical Immunology 9: 175-184.

McIntosh KR, Lopez MJ, Borneman JN, Spencer ND, Anderson PA, Gimble JM (2009). Immunogenicity of allogeneic adipose-derived stem cells in a rat spinal fusion model. Tissue Engineering.Part A 15: 2677-2686.

Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D (2004). Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 103: 4619-4621.

Melief SM, Geutskens SB, Fibbe WE, Roelofs H (2013b). Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. Haematologica 98: 888-895.

Melief SM, Zwaginga JJ, Fibbe WE, Roelofs H (2013a). Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. Stem Cells Translational Medicine 2: 455-463.

Menard C, Pacelli L, Bassi G, Dulong J, Bifari F, Bezier I, Zanoncello J, Ricciardi M, Latour M, Bourin P, Schrezenmeier H, Sensebe L, Tarte K, Krampera M (2013). Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: Standardization of immune quality controls. Stem Cells and Development 22: 1789-1801.

Mesimaki K, Lindroos B, Tornwall J, Mauno J, Lindqvist C, Kontio R, Miettinen S, Suuronen R (2009). Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. International Journal of Oral and Maxillofacial Surgery 38: 201-209.

Meza-Zepeda LA, Noer A, Dahl JA, Micci F, Myklebost O, Collas P (2008). High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. Journal of Cellular and Molecular Medicine 12: 553-563.

Mirabet V, Solves P, Minana MD, Encabo A, Carbonell-Uberos F, Blanquer A, Roig R (2008). Human platelet lysate enhances the proliferative activity of cultured human fibroblast-like cells from different tissues. Cell and Tissue Banking 9: 1-10.

Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM (2006). Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24: 376-385.

Montespan F, Deschaseaux F, Sensebe L, Carosella ED, Rouas-Freiss N (2014). Osteodifferentiated mesenchymal stem cells from bone marrow and adipose tissue express HLA-G and display immunomodulatory properties in HLA-mismatched settings: Implications in bone repair therapy. Journal of Immunology Research 2014: 230346.

Moser B, Willimann K (2004). Chemokines: Role in inflammation and immune surveillance. Annals of the Rheumatic Diseases 63 Suppl 2: ii84-ii89.

Mosna F, Sensebe L, Krampera M (2010). Human bone marrow and adipose tissue mesenchymal stem cells: A user's guide. Stem Cells and Development 19: 1449-1470.

Mravic M, Peault B, James AW (2014). Current trends in bone tissue engineering. BioMed Research International 2014: 865270.

Naaijkens BA, Niessen HW, Prins HJ, Krijnen PA, Kokhuis TJ, de Jong N, van Hinsbergh VW, Kamp O, Helder MN, Musters RJ, van Dijk A, Juffermans LJ (2012). Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. Cell and Tissue Research 348: 119-130.

Najar M, Raicevic G, Boufker HI, Fayyad Kazan H, De Bruyn C, Meuleman N, Bron D, Toungouz M, Lagneaux L (2010). Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: Combined comparison of adipose tissue, wharton's jelly and bone marrow sources. Cellular Immunology 264: 171-179.

Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, Bron D, Toungouz M, Martiat P, Lagneaux L (2009). Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: The importance of low cell ratio and role of interleukin-6. Cytotherapy 11: 570-583.

Nauta AJ, Fibbe WE (2007). Immunomodulatory properties of mesenchymal stromal cells. Blood 110: 3499-3506.

Niemeyer P, Kornacker M, Mehlhorn A, Seckinger A, Vohrer J, Schmal H, Kasten P, Eckstein V, Sudkamp NP, Krause U (2007). Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. Tissue Engineering 13: 111-121.

Niemeyer P, Vohrer J, Schmal H, Kasten P, Fellenberg J, Suedkamp NP, Mehlhorn AT (2008). Survival of human mesenchymal stromal cells from bone marrow and adipose tissue after xenogenic transplantation in immunocompetent mice. Cytotherapy 10: 784-795.

Noer A, Lindeman LC, Collas P (2009). Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. Stem Cells and Development 18: 725-736.

O'Keefe RJ, Mao J (2011). Bone tissue engineering and regeneration: From discovery to the clinic--an overview. Tissue Engineering.Part B, Reviews 17: 389-392.

Pachon-Pena G, Yu G, Tucker A, Wu X, Vendrell J, Bunnell BA, Gimble JM (2011). Stromal stem cells from adipose tissue and bone marrow of age-matched female donors display distinct immunophenotypic profiles. Journal of Cellular Physiology 226: 843-851.

Park IS, Han M, Rhie JW, Kim SH, Jung Y, Kim IH, Kim SH (2009). The correlation between human adipose-derived stem cells differentiation and cell adhesion mechanism. Biomaterials 30: 6835-6843.

Park SA, Reilly CM, Wood JA, Chung DJ, Carrade DD, Deremer SL, Seraphin RL, Clark KC, Zwingenberger AL, Borjesson DL, Hayashi K, Russell P, Murphy CJ (2013). Safety and immunomodulatory effects of allogeneic canine adipose-derived mesenchymal stromal cells transplanted into the region of the lacrimal gland, the gland of the third eyelid and the knee joint. Cytotherapy 15: 1498-1510.

Parker AM, Shang H, Khurgel M, Katz AJ (2007). Low serum and serum-free culture of multipotential human adipose stem cells. Cytotherapy 9: 637-646.

Patrikoski M, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerstrom B, Miettinen S (2013). Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. Stem Cell Research & Therapy 4: 27.

Peters R, Wolf MJ, van den Broek M, Nuvolone M, Dannenmann S, Stieger B, Rapold R, Konrad D, Rubin A, Bertino JR, Aguzzi A, Heikenwalder M, Knuth AK (2010). Efficient generation of multipotent mesenchymal stem cells from umbilical cord blood in stroma-free liquid culture. PloS One 5: e15689.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29: e45.

Pietila M, Palomaki S, Lehtonen S, Ritamo I, Valmu L, Nystedt J, Laitinen S, Leskela HV, Sormunen R, Pesala J, Nordstrom K, Vepsalainen A, Lehenkari P (2012). Mitochondrial function and energy metabolism in umbilical cord blood- and bone marrow-derived mesenchymal stem cells. Stem Cells and Development 21: 575-588.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147.

Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A (2005). Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. British Journal of Haematology 129: 118-129.

Qu X, Liu X, Cheng K, Yang R, Zhao RC (2012). Mesenchymal stem cells inhibit Th17 cell differentiation by IL-10 secretion. Experimental Hematology 40: 761-770.

Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ (2009). Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. European Journal of Immunology 39: 3436-3446.

Rafii S, Shapiro F, Rimarachin J, Nachman RL, Ferris B, Weksler B, Moore MA, Asch AS (1994). Isolation and characterization of human bone marrow microvascular endothelial cells: Hematopoietic progenitor cell adhesion. Blood 84: 10-19.

Rahaman MN, Day DE, Bal BS, Fu Q, Jung SB, Bonewald LF, Tomsia AP (2011). Bioactive glass in tissue engineering. Acta Biomaterialia 7: 2355-2373.

Rajala K, Lindroos B, Hussein SM, Lappalainen RS, Pekkanen-Mattila M, Inzunza J, Rozell B, Miettinen S, Narkilahti S, Kerkela E, Aalto-Setala K, Otonkoski T, Suuronen R, Hovatta O, Skottman H (2010). A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PloS One 5: e10246.

Rashid R, Beyer S, Blocki A, Le Visage C, Trau D, Wohland T, Raghunath M (2014b). Mitochondrial routing of glucose and sucrose polymers after pinocytotic uptake: Avenues for drug delivery. Biomacromolecules 15: 2119-2127.

Rashid R, Lim NS, Chee SM, Png SN, Wohland T, Raghunath M (2014a). Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. Tissue Engineering.Part C, Methods 20: 994-1002.

Rebelatto CK, Aguiar AM, Moretao MP, Senegaglia AC, Hansen P, Barchiki F, Oliveira J, Martins J, Kuligovski C, Mansur F, Christofis A, Amaral VF, Brofman PS, Goldenberg S, Nakao LS, Correa A (2008). Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Experimental Biology and Medicine 233: 901-913.

Reddi AH (2005). BMPs: From bone morphogenetic proteins to body morphogenetic proteins. Cytokine & Growth Factor Reviews 16: 249-250.

Rehmann W, Morgan G (2009). The regulation of advanced therapies: Perspectives from the EU. RAJ Pharma, Informa UK Ltd February

Ren G, Roberts AI, Shi Y (2011). Adhesion molecules: Key players in mesenchymal stem cell-mediated immunosuppression. Cell Adhesion & Migration 5: 20-22.

Ren G, Su J, Zhang L, Zhao X, Ling W, L'huillie A, Zhang J, Lu Y, Roberts AI, Ji W, Zhang H, Rabson AB, Shi Y (2009). Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells 27: 1954-1962.

Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2: 141-150.

Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, Roberts AI, Le AD, Shi S, Shao C, Shi Y (2010). Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. Journal of Immunology 184: 2321-2328.

Ren ML, Peng W, Yang ZL, Sun XJ, Zhang SC, Wang ZG, Zhang B (2012). Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs. Cell Transplantation 21: 2711-2721.

Resnick IB, Barkats C, Shapira MY, Stepensky P, Bloom AI, Shimoni A, Mankuta D, Varda-Bloom N, Rheingold L, Yeshurun M, Bielorai B, Toren A, Zuckerman T, Nagler A, Or R (2013). Treatment of severe steroid resistant acute GVHD with mesenchymal stromal cells (MSC). American Journal of Blood Research 3: 225-238.

Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR (2006). Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 27: 3413-3431.

Rice CM, Scolding NJ (2010). Adult human mesenchymal cells proliferate and migrate in response to chemokines expressed in demyelination. Cell Adhesion & Migration 4: 235-240.

Rigotti G, Marchi A, Stringhini P, Baroni G, Galie M, Molino AM, Mercanti A, Micciolo R, Sbarbati A (2010). Determining the oncological risk of autologous lipoaspirate grafting for post-mastectomy breast reconstruction. Aesthetic Plastic Surgery 34: 475-480.

Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, Marschall HU, Dlugosz A, Szakos A, Hassan Z, Omazic B, Aschan J, Barkholt L, Le Blanc K (2006). Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 81: 1390-1397.

Riordan NH, Ichim TE, Min WP, Wang H, Solano F, Lara F, Alfaro M, Rodriguez JP, Harman RJ, Patel AN, Murphy MP, Lee RR, Minev B (2009). Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. Journal of Translational Medicine 7: 29-5876-7-29.

Roemeling-van Rhijn M, Mensah FK, Korevaar SS, Leijs MJ, van Osch GJ, Ijzermans JN, Betjes MG, Baan CC, Weimar W, Hoogduijn MJ (2013). Effects of hypoxia on the immunomodulatory properties of adipose tissue-derived mesenchymal stem cells. Frontiers in Immunology 4: 203.

Rowan BG, Gimble JM, Sheng M, Anbalagan M, Jones RK, Frazier TP, Asher M, Lacayo EA, Friedlander PL, Kutner R, Chiu ES (2014). Human adipose tissue-derived stromal/stem cells promote migration and early metastasis of triple negative breast cancer xenografts. PloS One 9: e89595.

Rubio D, Garcia S, Paz MF, De la Cueva T, Lopez-Fernandez LA, Lloyd AC, Garcia-Castro J, Bernad A (2008). Molecular characterization of spontaneous mesenchymal stem cell transformation. PloS One 3: e1398.

Saki N, Jalalifar MA, Soleimani M, Hajizamani S, Rahim F (2013). Adverse effect of high glucose concentration on stem cell therapy. International Journal of Hematology-Oncology and Stem Cell Research 7: 34-40.

Salgado AJ, Oliveira JM, Martins A, Teixeira FG, Silva NA, Neves NM, Sousa N, Reis RL (2013). Tissue engineering and regenerative medicine: Past, present, and future. International Review of Neurobiology 108: 1-33.

Salmikangas P, Menezes-Ferreira M, Reischl I, Tsiftsoglou A, Kyselovic J, Borg JJ, Ruiz S, Flory E, Trouvin JH, Celis P, Ancans J, Timon M, Pante G, Sladowski D, Lipnik-Stangelj M, Schneider CK (2015). Manufacturing, characterization and control of cell-based medicinal products: Challenging paradigms toward commercial use. Regenerative Medicine 10: 65-78.

Sandor GK, Numminen J, Wolff J, Thesleff T, Miettinen A, Tuovinen VJ, Mannerstrom B, Patrikoski M, Seppanen R, Miettinen S, Rautiainen M, Ohman J (2014). Adipose stem cells used to reconstruct 13 cases with cranio-maxillofacial hard-tissue defects. Stem Cells Translational Medicine 3: 530-540.

Sandor GK, Tuovinen VJ, Wolff J, Patrikoski M, Jokinen J, Nieminen E, Mannerstrom B, Lappalainen OP, Seppanen R, Miettinen S (2013). Adipose stem cell tissue-engineered construct used to treat large anterior mandibular defect: A case report and review of the clinical application of good manufacturing practice-level adipose stem cells for bone regeneration. Journal of Oral and Maxillofacial Surgery : Official Journal of the American Association of Oral and Maxillofacial Surgeons 71: 938-950.

Santos F, Andrade PZ, Abecasis MM, Gimble JM, Chase LG, Campbell AM, Boucher S, Vemuri MC, Silva CL, Cabral JM (2011). Toward a clinical-grade expansion of mesenchymal stem cells from human sources: A microcarrier-based culture system under xeno-free conditions. Tissue Engineering.Part C, Methods 17: 1201-1210.

Sarkanen JR, Vuorenpaa H, Huttala O, Mannerstrom B, Kuokkanen H, Miettinen S, Heinonen T, Ylikomi T (2012). Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering. Cells, Tissues, Organs 196: 385-397.

Sato K, Itoh T, Kato T, Kitamura Y, Kaul SC, Wadhwa R, Sato F, Ohneda O (2015). Serum-free isolation and culture system to enhance the proliferation and bone regeneration of adipose tissue-derived mesenchymal stem cells. In Vitro Cellular & Developmental Biology.Animal

Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D (2007). Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47: 1436-1446.

Schallmoser K, Strunk D (2009). Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. Journal of Visualized Experiments : JoVE 32: 10.3791/1523.

Schallmoser K, Strunk D (2013). Generation of a pool of human platelet lysate and efficient use in cell culture. Methods in Molecular Biology 946: 349-362.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica Et Biophysica Acta 1813: 878-888.

Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP (2008). Regional anatomic and age effects on cell function of human adipose-derived stem cells. Annals of Plastic Surgery 60: 538-544.

Schneider CK, Salmikangas P, Jilma B, Flamion B, Todorova LR, Paphitou A, Haunerova I, Maimets T, Trouvin JH, Flory E, Tsiftsoglou A, Sarkadi B, Gudmundsson K, O'Donovan M, Migliaccio G, Ancans J, Maciulaitis R, Robert JL, Samuel A, Ovelgonne JH, Hystad M, Fal AM, Lima BS, Moraru AS, Turcani P, Zorec R, Ruiz S, Akerblom L, Narayanan G, Kent A, Bignami F, Dickson JG, Niederwieser D, Figuerola-Santos MA, Reischl IG, Beuneu C, Georgiev R, Vassiliou M, Pychova A, Clausen M, Methuen T, Lucas S, Schussler-Lenz M, Kokkas V, Buzas Z, MacAleenan N, Galli MC, Line A, Gulbinovic J, Berchem G, Fraczek M, Menezes-Ferreira M, Vilceanu N, Hrubisko M, Marinko P, Timon M, Cheng W, Crosbie GA, Meade N, di Paola ML, VandenDriessche T, Ljungman P, D'Apote L, Oliver-Diaz O, Buttel I, Celis P (2010). Challenges with advanced therapy medicinal products and how to meet them. Nature Reviews. Drug Discovery 9: 195-201.

Seda Tigli R, Ghosh S, Laha MM, Shevde NK, Daheron L, Gimble JM, Gumusderelioglu M, Kaplan DL (2009). Comparative chondrogenesis of human cell sources in 3D scaffolds. Journal of Tissue Engineering and Regenerative Medicine 3: 348-360.

Selvaggi TA, Walker RE, Fleisher TA (1997). Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood 89: 776-779.

Semont A, Francois S, Mouiseddine M, Francois A, Sache A, Frick J, Thierry D, Chapel A (2006). Mesenchymal stem cells increase self-renewal of small intestinal epithelium and accelerate structural recovery after radiation injury. Advances in Experimental Medicine and Biology 585: 19-30.

Sensebe L, Gadelorge M, Fleury-Cappellesso S (2013). Production of mesenchymal stromal/stem cells according to good manufacturing practices: A review. Stem Cell Research & Therapy 4: 66.

Shah AR, Cornejo A, Guda T, Sahar DE, Stephenson SM, Chang S, Krishnegowda NK, Sharma R, Wang HT (2014). Differentiated adipose-derived stem cell cocultures for bone regeneration in polymer scaffolds in vivo. The Journal of Craniofacial Surgery 25: 1504-1509.

Sharma AA, Jen R, Butler A, Lavoie PM (2012). The developing human preterm neonatal immune system: A case for more research in this area. Clinical Immunology 145: 61-68.

Shi Y, Massague J (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113: 685-700.

Shih DT, Chen JC, Chen WY, Kuo YP, Su CY, Burnouf T (2011). Expansion of adipose tissue mesenchymal stromal progenitors in serum-free medium supplemented with virally inactivated allogeneic human platelet lysate. Transfusion 51: 770-778.

Sioud M (2011b). New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. Scandinavian Journal of Immunology 73: 79-84.

Sioud M, Mobergslien A, Boudabous A, Floisand Y (2011a). Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. International Journal of Oncology 38: 385-390.

Skog M, Muhonen V, Nystedt J, Narcisi R, Kontturi LS, Urtti A, Korhonen M, van Osch GJ, Kiviranta I (2014). Xeno-free chondrogenesis of bone marrow mesenchymal stromal cells: Towards clinical-grade chondrocyte production. Cytotechnology

Song I, Kim BS, Kim CS, Im GI (2011). Effects of BMP-2 and vitamin D3 on the osteogenic differentiation of adipose stem cells. Biochemical and Biophysical Research Communications 408: 126-131.

Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M (2006b). Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 24: 74-85.

Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M (2006a). Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 24: 462-471.

Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L (2009). MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: Central role of MSC-derived prostaglandin E2. Blood 113: 6576-6583.

Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L (2008). Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: Role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood 111: 1327-1333.

Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP (2005). CXC chemokines in angiogenesis. Cytokine & Growth Factor Reviews 16: 593-609.

Suga H, Eto H, Aoi N, Kato H, Araki J, Doi K, Higashino T, Yoshimura K (2010). Adipose tissue remodeling under ischemia: Death of adipocytes and activation of stem/progenitor cells. Plastic and Reconstructive Surgery 126: 1911-1923.

Swamynathan P, Venugopal P, Kannan S, Thej C, Kolkundar U, Bhagwat S, Ta M, Majumdar AS, Balasubramanian S (2014). Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of wharton's jelly derived mesenchymal stem cells? A comparative study. Stem Cell Research & Therapy 5: 88.

Takahashi K, Yamanaka S (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676.

Tasso R, Ilengo C, Quarto R, Cancedda R, Caspi RR, Pennesi G (2012). Mesenchymal stem cells induce functionally active T-regulatory lymphocytes in a paracrine fashion and ameliorate experimental autoimmune uveitis. Investigative Ophthalmology & Visual Science 53: 786-793.

Tateishi K, Ando W, Higuchi C, Hart DA, Hashimoto J, Nakata K, Yoshikawa H, Nakamura N (2008). Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: Potential feasibility for clinical applications. Cell Transplantation 17: 549-557.

Tatrai P, Sagi B, Szigeti A, Szepesi A, Szabo I, Bosze S, Kristof Z, Marko K, Szakacs G, Urban I, Mezo G, Uher F, Nemet K (2013). A novel cyclic RGD-containing peptide polymer improves serum-free adhesion of adipose tissue-derived mesenchymal stem cells to bone implant surfaces. Journal of Materials Science.Materials in Medicine 24: 479-488.

Tatullo M, Marrelli M, Paduano F (2015). The regenerative medicine in oral and maxillofacial surgery: The most important innovations in the clinical application of mesenchymal stem cells. International Journal of Medical Sciences 12: 72-77.

Tchilian EZ, Beverley PC (2006). Altered CD45 expression and disease. Trends in Immunology 27: 146-153.

Thesleff T, Lehtimaki K, Niskakangas T, Mannerstrom B, Miettinen S, Suuronen R, Ohman J (2011). Cranioplasty with adipose-derived stem cells and biomaterial: A novel method for cranial reconstruction. Neurosurgery 68: 1535-1540.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147.

Tiemessen MM, Kunzmann S, Schmidt-Weber CB, Garssen J, Bruijnzeel-Koomen CA, Knol EF, van Hoffen E (2003). Transforming growth factor-beta inhibits human antigen-specific CD4+ T cell proliferation without modulating the cytokine response. International Immunology 15: 1495-1504.

Tirkkonen L, Haimi S, Huttunen S, Wolff J, Pirhonen E, Sandor GK, Miettinen S (2013). Osteogenic medium is superior to growth factors in differentiation of human adipose stem cells towards bone-forming cells in 3D culture. European Cells & Materials 25: 144-158.

Torensma R, Prins HJ, Schrama E, Verwiel ET, Martens AC, Roelofs H, Jansen BJ (2012). The impact of cell source, culture methodology, culture location, and individual donors on gene expression profiles of bone marrow-derived and adipose-derived stromal cells. Stem Cells and Development

Torsvik A, Rosland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lonning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R (2010). Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: Putting the research field on track - letter. Cancer Research 70: 6393-6396.

Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R, Johnstone BH, March KL (2008). A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circulation Research 102: 77-85.

Trischmann TM, Schepers KG, Civin CI (1993). Measurement of CD34+ cells in bone marrow by flow cytometry. Journal of Hematotherapy 2: 305-313.

Trivedi HL, Vanikar AV, Thakker U, Firoze A, Dave SD, Patel CN, Patel JV, Bhargava AB, Shankar V (2008). Human adipose tissue-derived mesenchymal stem cells combined with

hematopoietic stem cell transplantation synthesize insulin. Transplantation Proceedings 40: 1135-1139.

Trojahn Kolle SF, Oliveri RS, Glovinski PV, Kirchhoff M, Mathiasen AB, Elberg JJ, Andersen PS, Drzewiecki KT, Fischer-Nielsen A (2013). Pooled human platelet lysate versus fetal bovine serum-investigating the proliferation rate, chromosome stability and angiogenic potential of human adipose tissue-derived stem cells intended for clinical use. Cytotherapy 15: 1086-1097.

Trubiani O, Piattelli A, Gatta V, Marchisio M, Diomede F, D'Aurora M, Merciaro I, Pierdomenico L, Maraldi NM, Zini N (2015). Assessment of an efficient xeno-free culture system of human periodontal ligament stem cells. Tissue Engineering.Part C, Methods 21: 52-64.

Tsigkou O, Jones JR, Polak JM, Stevens MM (2009). Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 bioglass conditioned medium in the absence of osteogenic supplements. Biomaterials 30: 3542-3550.

Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. The Journal of Clinical Investigation 117: 902-909.

Tsuji W, Rubin JP, Marra KG (2014). Adipose-derived stem cells: Implications in tissue regeneration. World Journal of Stem Cells 6: 312-321.

Ulich TR, Yin S, Guo K, Yi ES, Remick D, del Castillo J (1991). Intratracheal injection of endotoxin and cytokines. II. interleukin-6 and transforming growth factor beta inhibit acute inflammation. The American Journal of Pathology 138: 1097-1101.

van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino ML, Gstraunthaler G (2010). Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. Toxicology in Vitro : An International Journal Published in Association with BIBRA 24: 1053-1063.

Van Harmelen V, Rohrig K, Hauner H (2004). Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. Metabolism: Clinical and Experimental 53: 632-637.

Vitale-Brovarone C, Verne E, Robiglio L, Appendino P, Bassi F, Martinasso G, Muzio G, Canuto R (2007). Development of glass-ceramic scaffolds for bone tissue engineering: Characterisation, proliferation of human osteoblasts and nodule formation. Acta Biomaterialia 3: 199-208.

Wahl SM, Wen J, Moutsopoulos N (2006). TGF-beta: A mobile purveyor of immune privilege. Immunological Reviews 213: 213-227.

Wang P, Hao J, Shi J (2000). Actions of bovine plasma fibronectin on cultured human dental pulp cells. The Chinese Journal of Dental Research: The Official Journal of the Scientific Section of the Chinese Stomatological Association (CSA) 3: 55-62.

Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM (2010a). A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. PloS One 5: e10088.

Webb TL, Webb CB (2014). Stem cell therapy in cats with chronic enteropathy: A proof-of-concept study. Journal of Feline Medicine and Surgery

Wei Y, Sun X, Wang W, Hu Y (2007). Adipose-derived stem cells and chondrogenesis. Cytotherapy 9: 712-716.

Weigand A, Beier JP, Hess A, Gerber T, Arkudas A, Horch RE, Boos AM (2015). Acceleration of vascularized bone tissue engineered constructs in a large animal model combining intrinsic and extrinsic vascularization. Tissue Engineering.Part A

Williams, DF (1999). Williams dictionary of biomaterials. Liverpool University Press.

Wolfe, N., Dunavan, C., & Diamond, J (2012). Origins of major human infectious diseases. Improving food safety through a one health approach: Workshop summary. Washington (DC): National Academies Press (US).

Wong MM, Fish EN (2003). Chemokines: Attractive mediators of the immune response. Seminars in Immunology 15: 5-14.

Wu M, Han ZB, Liu JF, Wang YW, Zhang JZ, Li CT, Xin PL, Han ZC, Zhu XP (2014). Serum-free media and the immunoregulatory properties of mesenchymal stem cells in vivo and in vitro. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 33: 569-580.

Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA (2006). Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells 24: 2582-2591.

Yang S, Pilgaard L, Chase L, Boucher S, Vemuri M, Fink TD, Zachar V (2012). Defined xenogeneic-free and hypoxic environment provides superior conditions for long-term expansion of human adipose-derived stem cells. Tissue Engineering.Part C, Methods 18: 593-602.

Ylostalo JH, Bartosh TJ, Coble K, Prockop DJ (2012). Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. Stem Cells 30: 2283-2296.

Yoo KH, Jang IK, Lee MW, Kim HE, Yang MS, Eom Y, Lee JE, Kim YJ, Yang SK, Jung HL, Sung KW, Kim CW, Koo HH (2009). Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cellular Immunology 259: 150-156.

Yousefi F, Ebtekar M, Soleimani M, Soudi S, Hashemi SM (2013). Comparison of in vivo immunomodulatory effects of intravenous and intraperitoneal administration of adipose-tissue mesenchymal stem cells in experimental autoimmune encephalomyelitis (EAE). International Immunopharmacology 17: 608-616.

Yu G, Wu X, Dietrich MA, Polk P, Scott LK, Ptitsyn AA, Gimble JM (2010). Yield and characterization of subcutaneous human adipose-derived stem cells by flow cytometric and adipogenic mRNA analyzes. Cytotherapy 12: 538-546.

Yu JL, Rak JW (2003). Host microenvironment in breast cancer development: Inflammatory and immune cells in tumour angiogenesis and arteriogenesis. Breast Cancer Research: BCR 5: 83-88.

Yuan H, de Bruijn JD, Zhang X, van Blitterswijk CA, de Groot K (2001). Bone induction by porous glass ceramic made from bioglass (45S5). Journal of Biomedical Materials Research 58: 270-276.

Zeiger AS, Loe FC, Li R, Raghunath M, Van Vliet KJ (2012). Macromolecular crowding directs extracellular matrix organization and mesenchymal stem cell behavior. PloS One 7: e37904.

Zhao J, Hu J, Wang S, Sun X, Xia L, Zhang X, Zhang Z, Jiang X. (2010). Combination of beta-TCP and BMP-2 gene-modified bMSCs to heal critical size mandibular defects in rats. Oral Diseases 16: 46-54.

Zheng G, Huang L, Tong H, Shu Q, Hu Y, Ge M, Deng K, Zhang L, Zou B, Cheng B, Xu J (2014). Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: A randomized, placebo-controlled pilot study. Respiratory Research 15: 39-9921-15-39.

Zhou HX, Rivas G, Minton AP (2008). Macromolecular crowding and confinement: Biochemical, biophysical, and potential physiological consequences. Annual Review of Biophysics 37: 375-397.

Zhou Y, Yuan J, Zhou B, Lee AJ, Lee AJ, Ghawji M,Jr, Yoo TJ (2011). The therapeutic efficacy of human adipose tissue-derived mesenchymal stem cells on experimental autoimmune hearing loss in mice. Immunology 133: 133-140.

Zimmerlin L, Park TS, Zambidis ET, Donnenberg VS, Donnenberg AD (2013). Mesenchymal stem cell secretome and regenerative therapy after cancer. Biochimie 95: 2235-2245.

Zuk PA, Chou YF, Mussano F, Benhaim P, Wu BM (2011). Adipose-derived stem cells and BMP2: Part 2. BMP2 may not influence the osteogenic fate of human adipose-derived stem cells. Connective Tissue Research 52: 119-132.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002). Human adipose tissue is a source of multipotent stem cells. Molecular Biology of the Cell 13: 4279-4295.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001). Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Engineering 7: 211-228.

9 Original publications

CORRIGENDA

There is one error in the original publications:

Study IV:

Page 3: Seeding and osteogenic differentiation of hASCs on biomaterial combinations.

"..was supplemented with l-ascorbic acid-2-phosphate (50 mM), β-glycerophosphate (500 μM), and dexamethasone (10 μM).." should read "..was supplemented with l-ascorbic acid-2-phosphate (50 μM), β-glycerophosphate (10mM) and dexamethasone (10 nM).."

stem cell research&therapy

RESEARCH Open Access

Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells

Mimmi Patrikoski[1,2,3*], Miia Juntunen[1,2,3], Shayne Boucher[4], Andrew Campbell[5], Mohan C Vemuri[4], Bettina Mannerström[1,2,3] and Susanna Miettinen[1,2,3]

Abstract

Introduction: Adipose tissue is an attractive and abundant source of multipotent stem cells. Human adipose stem cells (ASCs) have shown to have therapeutic relevancy in diverse clinical applications. Nevertheless, expansion of ASCs is often necessary before performing clinical studies. Standard *in vitro* cell-culture techniques use animal-derived reagents that should be avoided in clinical use because of safety issues. Therefore, xeno- and serum-free (XF/SF) reagents are highly desirable for enhancing the safety and quality of the transplanted ASCs.

Methods: In the current study, animal component-free isolation and cell-expansion protocols were developed for ASCs. StemPro MSC SFM XF medium with either CELLstart™ CTS™ coating or Coating Matrix Kit were tested for their ability to support XF/SF growth. Basic stem-cell characteristics such as immunophenotype (CD3, CD11a, CD14, CD19, CD34, CD45RO, CD54, CD73, CD80, CD86, CD90, CD105, HLA-DR), proliferation, and differentiation potential were assessed in XF/SF conditions and compared with human serum (HS) or traditionally used fetal bovine serum (FBS) cultures.

Results: ASCs cultured in XF/SF conditions had significantly higher proliferation rates compared with HS/FBS cultures. Characteristic immunophenotypes of ASCs were maintained in every condition; however, cells expanded in XF/SF conditions showed significantly lower expression of CD54 (intercellular adhesion molecule 1, ICAM-1) at low passage number. Further, multilineage differentiation potential of ASCs was maintained in every culture condition.

Conclusions: Our findings demonstrated that the novel XF/SF conditions maintained the basic stem cell features of ASCs and the animal-free workflow followed in this study has great potential in clinical cell therapies.

Keywords: Adipose stem cells, Xeno-free, Serum-free, Human serum, Fetal bovine serum, Multipotentiality, Proliferation rate, Immunophenotype, Flow cytometry, Cell therapy

Introduction

Human adipose tissue is an abundant source of multipotent stem cells known as adipose stem cells (ASCs), and they have the ability to differentiate toward various mesenchymal cell types, including bone, cartilage, and fat cells [1,2]. Since Zuk *et al.* [3] described this unlimited source of multipotent cells, growing interest exists toward the clinical applicability of ASCs. The therapeutic relevance of the cells has been noticed and, in fact, the number of clinical cell therapies using ASCs has been steadily increasing [4-6]. Therefore, increased focus occurs on the safety, efficacy, reproducibility, and quality of the cells used in clinical treatments.

ASCs and bone marrow-derived stem cells (BMSCs) alike are mesenchymal stem cells (MSCs) that are defined as plastic-adherent cells with the potential to differentiate toward bone-, fat-, and cartilage-like cells. Furthermore, for their characterization, it is required that the cells express (≥95%) certain markers on the cell surface (CD105, CD73, and CD90) and lack the expression of hematopoietic antigens (≤2%) [7].

* Correspondence: mimmi.patrikoski@uta.fi
[1]Adult Stem Cell Group, Institute of Biomedical Technology, University of Tampere, Tampere, Finland
[2]BioMediTech, Tampere, Finland
Full list of author information is available at the end of the article

Of particular interest is that ASCs have immunomodulatory properties such as regulation of T-cell functions, antiinflammatory cytokine expression, and prolongation of allotransplant survival [8,9]. Thus, ASCs have been shown to suppress allogeneic lymphocytes both *in vitro* and *in vivo* [9,10]. In addition, ASCs lack the expression of MHC class II molecules as well as T- and B-cell costimulatory molecules CD80, CD86, and CD40 on their cell surfaces [11]. Because of these characteristics, ASCs are strong candidates for the treatment of immunologic disorders such as severe graft-versus-host disease or Crohn disease. Furthermore, because of their low immunogenicity, they may be used in allogeneic stem cell therapies, such as in the treatment of bone defects. High proliferation rate and good differentiation potential are important from the clinical point of view, and therefore, off-the-shelf cell products could be used to achieve effective treatments by using allogeneic cells with functional stem cell characteristics.

Cell-based therapies typically require large numbers of cells, and expansion of ASCs is often necessary before clinical use. Traditionally, fetal bovine serum (FBS) has been used in ASC cultures because of its ability to support cell growth and attachment by providing nutrients and attachment factors for the cells. However, in clinical cell therapies, the use of animal-derived reagents should be avoided, and the risks and benefits carefully assessed because of safety concerns [12,13]. Alternatives for FBS have been studied, such as allogeneic human serum (alloHS) or autologous HS (autoHS) [14], as well as platelet-derived supplements [15,16]. Nevertheless, limitations connected to the use of serum or its components exist, such as lot-to-lot variation with serum-supplemented media performance, affecting differentiation potential and proliferation rate [17]. In addition, limited availability of autoHS can make long-term cultures of ASCs impractical. It is noteworthy that no standardized, fully defined xeno- and serum-free (XF/SF) cultivation protocols are available. However, the safety and the quality of transplanted ASCs would be significantly enhanced by replacing undefined and animal-derived components with defined XF/SF reagents.

The aim of the current study was to develop safe and efficient XF/SF culture conditions for ASCs and to show that ASCs cultured under these novel XF/SF conditions maintained their stem-cell characteristics, including the multilineage differentiation potential, immunophenotype, and proliferation capacity. The cell isolation and expansion was carried out in parallel in three different culture conditions, under fully defined completely XF/SF conditions, as well as in medium containing HS or FBS to compare the cell characteristics between these conditions. This study demonstrates the development of a fully defined animal origin-free culture system for the propagation and expansion of clinically relevant human adipose stem cells for the purpose of cell therapy.

Methods

Isolation and culture of ASCs

The study was conducted in accordance with the ethics committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058). ASCs were isolated from adipose tissue samples obtained with written informed consent from four female donors (age, 36 ± 9 years) undergoing elective surgical procedures in the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland. To assess how serum supplementation of the culture media affects the cell characteristics, ASCs were isolated under three different culturing conditions: in medium containing FBS, HS, or in XF/SF culture conditions. FBS- and HS-containing media were used as reference media for testing two different XF/SF culture conditions: (a) XF/SF media with CELLStart coating, and (b) XF/SF media with novel coating-free supplement, referred to hereafter as Coating Matrix Kit.

Isolation of adipose stem cells (ASCs) from adipose tissue samples was carried out by using a mechanical and enzymatic method, as described previously [1,18,19]. In brief, the adipose tissue was minced manually into small fragments and digested with collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany) in a water bath at 37°C under shaking conditions. The digested tissue was centrifuged and filtered in sequential steps through a 100-μm pore-size filter to separate the ASCs from the surrounding tissue. The first passage after the seeding of cells on cell-culture plastics, after dissociation of fat tissue, was designated passage 0. Cells were expanded in T75 flasks and passaged after reaching 80% confluency.

For HS and FBS conditions, Dulbecco modified Eagle medium (DMEM)/F-12 1:1 (Life Technologies, Gibco, Carlsbad, CA, USA) was supplemented with 1% l-analyl-l-glutamine (GlutaMAX I; Life Technologies, Gibco), 1% antibiotics (p/s; 100 U/ml penicillin, 0.1 mg/ml streptomycin; Lonza, BioWittaker, Verviers, Belgium) and serum from either 10% FBS (Life Technologies, Gibco) or 10% alloHS (Human Serum Type AB; Lonza, BioWhittaker, Walkersville, MD, USA) was used. ASCs isolated and expanded in FBS medium were detached by using 1% trypsin (Lonza, Biowhittaker, Verviers, Belgium), and ASCs isolated in HS medium were detached by using TrypLE Select (Life Technologies, Gibco) for XF detachment of cells.

For SF/XF conditions, one third of the cells were isolated under SF/XF conditions, and seeded on carboxyl-coated flasks (PureCoatCarboxyl T75; BD Biosciences, Franklin Lakes, NJ, USA) and expanded in STEMPRO® MSC SFM (Life Technologies, Gibco) supplemented with 1% l-analyl-l-glutamine, 0.3%

antibiotics, and 10% StemPro MSC SFM XenoFree supplement. Amine-coated flasks (PureCoat™ Amine T75; BD Biosciences) were initially tested for their suitability for XF/SF, but the coating was not supportive enough for cell attachment, and instead of amine flasks, the carboxyl-coated flasks were selected for further studies.

From passage 1 onward, additional supplements were used in XF/SF conditions to support cell attachment and growth in normal Nunclon flasks. Thus, XF/SF cells were expanded in STEMPRO MSC medium supplemented with either Coating Matrix Kit (XF/SF CM) (Life Technologies, Gibco) or CELLstart™ CTS™ coating (XF/SF CS) (Life Technologies, Gibco), according to manufacturer's instructions. ASCs isolated and expanded in SF/XF medium were detached by using TrypLE Select for XF detachment of cells.

All culture-media formulations are presented in Table 1, and a flow chart of the isolation as well as performed analyses in different culture conditions are illustrated in Figure 1. All the analyses were performed separately with four donor cell lines isolated in FBS, HS, and XF/SF conditions.

Proliferation assay

The cell viability and proliferation activity were assessed in the different culture conditions (FBS, HS, and SF/XF) by using the PreMix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan). ASCs (n = four donor cell samples/analysis, passages 2 and 5) were seeded on 48-well plates at a density of 2,500 cells/cm^2, and the proliferation was assessed at 1, 4, 7, and 11 days. In brief, at each time point, the cell-culture medium was removed, and DPBS (Dulbecco Phosphate-Buffered Saline, Lonza, BioWhittaker, Verviers, Belgium) and PreMix WST-1 were added 10:1. The 48-well plate was incubated for 4 hours at 37°C, and the relative cell-proliferation activity was measured in a microplate reader (Victor 1429 Multilabel Counter) at 450 nm.

The population doubling was determined by using the formula x = log2(NH)/(N1), where N1 is the absorbance value at day 1, and NH is the absorbance value at observed time point 4, 7, or 11, as described previously [20]. To calculate the cumulative population doubling, the population doubling was determined in each passage and compared with the population doubling of earlier passages.

Flow-cytometric analysis of immunophenotype

ASCs expanded in SF/XF, HS, and FBS (n = 4, passages 2 and 5) media were analyzed with flow cytometry (FACSAria; BD Biosciences, Erembodegem, Belgium) to determine whether different culturing conditions have an effect on the immunophenotype of the cells. Monoclonal antibodies (MAbs) against CD11a–allophycocyanin (APC), CD80–phycoerythrin (PE), CD86–PE, CD105–PE (R&D Systems Inc., Minneapolis, MN, USA), CD-3 (PE), CD14–phycoerythrin-cyanine (PECy7), CD19-PECy7, CD45RO-APC, CD54-fluorescein isothiocyanate (FITC), CD73-PE, CD90-APC (BD Biosciences), and CD34-APC, HLADR-PE (Immunotools GmbH, Friesoythe, Germany) were used. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

Differentiation analyses

The trilineage differentiation potential of ASCs (n = 4, passages 2 to 5) toward osteogenic, adipogenic and chondrogenic cells was evaluated in XF/SF conditions versus HS and traditionally used FBS-supplemented medium. Differentiation capacity of ASCs was evaluated after 14 days of differentiation in either adipogenic, osteogenic, or chondrogenic medium versus cells cultured in control medium. Media for differentiation and control cultures were changed 3 times per week during the differentiation studies. The culture-media formulations used for differentiation assays are shown in Table 2. In a subsequent smaller-scale study, ASCs were primed for 3 days under FBS- or HS-supplemented media before differentiating under osteogenic or adipogenic condition. For this, commercial serum-based StemPro Adipogenesis and Osteogenesis differentiation kits (Life Technologies, Gibco) were used during the 14-day induction for XF/SF cells.

Table 1 Culture-media formulation overview

Acronym	Basal media	Serum	Coating/coating-free supplements	Supplementation
HS	DMEM/F-12	Human serum	None	1% GlutaMAX, 1% p/s
FBS	DMEM/F-12	Fetal bovine serum	None	1% GlutaMAX, 1% p/s
XF/SF CM	StemPro MSC SFM	None	Coating Matrix Kit	StemPro® MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s
XF/SF CS	StemPro MSC SFM	None	CELLstart™ coating	StemPro MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s

MSC, mesenchymal stem cell; p/s, penicillin/streptomycin; SFM, serum-free medium.

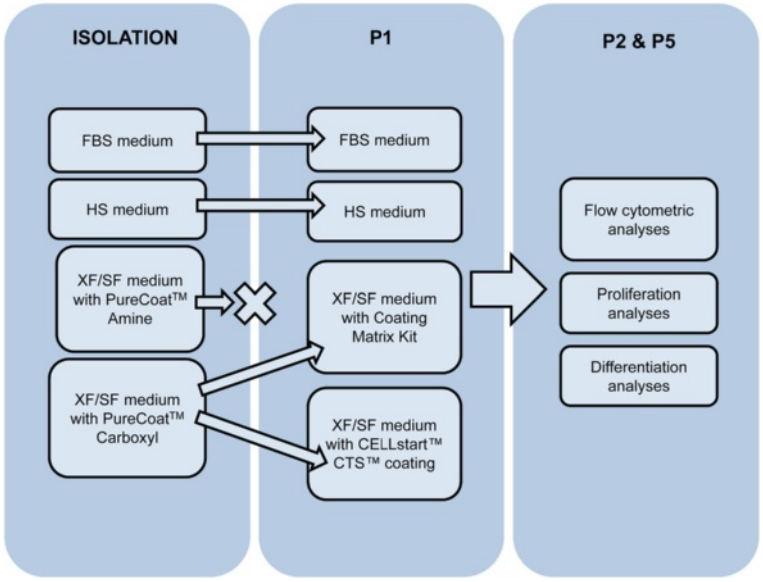

Figure 1 Work flow of the isolation and performed analyses of ASCs under different culture conditions. XF/SF isolation of ASCs was carried out by using carboxyl-coated flasks, and onward from passage 1, two different XF/SF conditions were tested in basic Nunclon flasks; Coating Matrix Kit, and CELLstart™ coating. Cell-proliferation rate, differentiation potential, and immunophenotype were analyzed in four different culture conditions at passages 2 and 5.

ALP staining

For alkaline phosphatase (ALP) staining, cells were seeded on 12-well plates at a density of 2.5×10^3 cells/cm^2. The differentiation degree after 14 days of osteogenic induction was determined by the level of ALP activity by using a leukocyte ALP kit (Sigma-Aldrich, St. Louis, MO, USA), as described previously [21]. In brief, cell cultures were washed twice with DPBS and fixed with 4% paraformaldehyde (PFA) or citrate-buffered formaldehyde-acetone solution. Subsequently, cells were rinsed with deionized water, and ALP staining solution was added and incubated for 15 minutes. After rinsing the cells with deionized water, color formation was analyzed microscopically.

Oil Red-O staining

For adipogenic differentiation, ASCs were seeded on 12-well plates at a density of 2.0×10^4 cells/cm^2. After 14 days of adipogenic induction culture, differentiation was confirmed by Oil Red-O staining, indicating the formation of intracellular lipid accumulation, as described earlier [2]. In brief, the cells were washed 3 times in DPBS and fixed with 4% PFA. Subsequently, cells were rinsed with deionized water and pretreated with 60% isopropanol after the additions of the 0.5% Oil Red-O staining solution in 60% isopropanol (Sigma-Aldrich). After 15-minute incubation in RT, the cells were rinsed with deionized water, and adipocytes were identified with microscopy as cells with red-stained lipid vesicles. In a later study, the cells were directly fixed and stained with 0.5% Oil Red-O staining solution with 60% isopropanol and then rinsed with distilled water before conducting microscopic assessment of adipocyte generation.

Alcian blue staining

The chondrogenic differentiation potential was assessed with a micromass culture method, as described previously [3,19,22]. In brief, 8×10^4 cells were seeded on a 24-well culture plate in a 10-μl volume and were allowed to adhere for 3 hours before the addition of chondrogenic induction medium. After 14 days of chondrogenic induction, differentiation was confirmed by using the Alcian blue staining method, as described earlier [23]. In brief, ASC pellets were rinsed with DPBS and fixed with 4% PFA. Subsequently, cells were rinsed twice with deionized water and stored in 70% ethanol. Pellets were dehydrated, embedded in paraffin, and sectioned at 5-mm thickness. The sections were rehydrated and stained with Alcian blue (pH 1.0) to detect sulfated glycosaminoglycans (GAGs) by using Nuclear Fast Red solution (Biocare Medical, Concord, MA, USA) as a counterstain.

Real-time quantitative PCR

Total RNA was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The RNA samples were reverse transcribed to first-strand cDNA by using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). The mRNA levels of

Table 2 Culture media formulations used for differentiation assays

Medium	Basal media	Serum	Coating/coating-free supplements	Supplementation
Adipogenic (FBS)	DMEM/F-12	10% Fetal bovine serum	None	1% GlutaMAX, 1% p/s, 33 μ*M* biotin (Sigma), 1 μ*M* dexamethasone (Sigma), 100 n*M* insulin (Life Technologies), 17 μM pantothenate (Fluka, Buchs, Switzerland), 250 μ*M* isobutylmethylxanthine (IBMX; Sigma) for 48-hour induction after cell seeding
Osteogenic (FBS)	DMEM/F-12	10% Fetal bovine serum	None	1% GlutaMAX, 1% p/s, 150 μ*M* L-ascorbic acid 2-phosphate (Sigma), 10 m*M* β-glycerophosphate (Sigma), 10 n*M* dexamethasone (Sigma)
Chondrogenic (FBS/HS)	DMEM/F-12	None	None	1% GlutaMAX, 0.3% p/s, 10 mg/ml human serum albumin (Sigma), 8 μg/ml holo-transferrin human (Sigma), 5 ng/ml sodium selenite (Sigma), 10 μg/ml insulin (Life Technologies), 1 μg/ml linoleic acid (Sigma), 50 μ*M* L-ascorbic acid 2-phosphate (Sigma), 55 μ*M* sodium pyruvate (Life Technologies), 23 μ*M* L-proline (Sigma), 10 ng/ml TGF-β (Sigma)
Adipogenic (HS)	DMEM/F-12	10% Human serum	None	1% GlutaMAX, 1% p/s, 33 μ*M* biotin, 1 μ*M* dexamethasone, 100 n*M* insulin, 17 μ*M* pantothenate, 250 μ*M* IBMX for 48-hour induction after cell seeding
Osteogenic (HS)	DMEM/F-12	10% Human serum	None	1% GlutaMAX, 1% p/s, 150 μ*M* L-ascorbic acid 2-phosphate, 10 m*M* β-glycerophosphate, 10 n*M* dexamethasone
Adipogenic (XF/SF CS)	StemPro MSC SFM	none	CELLstart™ coating	StemPro MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s, 33 μ*M* biotin, 1 μ*M* dexamethasone, 100 n*M* insulin, 17 μ*M* pantothenate, 250 μ*M* IBMX for 48-hour induction after cell seeding
Osteogenic (XF/SF CS)	StemPro MSC SFM	none	CELLstart™ coating	StemPro MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s, 150 μ*M* L-ascorbic acid 2-phosphate, 10 m*M* β-glycerophosphate, 10 n*M* dexamethasone
Chondrogenic (XF/SF CS and XF/SF CM)	StemPro MSC SFM	None	None	1% GlutaMAX, 0.3% p/s, 10 mg/ml human serum albumin, 8 μg/ml holo-transferrin human, 5 ng/ml sodium selenite, 10 μg/ml insulin, 1 μg/ml linoleic acid, 50 μ*M* L-ascorbic acid 2-phosphate, 55 μ*M* sodium pyruvate, 23 μ*M* L-proline, 10 ng/ml TGF-β
Adipogenic (XF/SF CM)	StemPro MSC SFM	None	Coating Matrix Kit	StemPro MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s, 33 μ*M* biotin, 1 μ*M* dexamethasone, 100 n*M* insulin, 17 μ*M* pantothenate, 250 μ*M* IBMX for 48-hour induction after cell seeding
Osteogenic (XF/SF CM)	StemPro MSC SFM	None	Coating Matrix Kit	StemPro MSC SFM XenoFree supplement, 1% GlutaMAX, 0.3% p/s, 150 μM L-ascorbic acid 2-phosphate, 10 mM beta-glycerophosphate, 10 nM dexamethasone

adipogenesis/osteogenesis-associated genes were analyzed by the qRT–PCR method as described previously [23]. In brief, the real-time detection of PCR product was monitored by using the SYBR Green dye (Applied Biosystems, Warrington, UK). The housekeeping gene, the ribosomal phosphoprotein P0 (*RPLP0*), was used as an internal control, and the relative expression level for each gene was calculated according to a previously described mathematical model [24]. The expression of adipogenesis-associated genes, peroxisome proliferator-activated receptor γ (*PPARγ*), and adipocyte Protein 2 (*aP2*) was analyzed as well as osteogenesis-associated genes such as distal-less homeobox transcription factor 5 (*DLX5*), *ALP*, and runt-related transcription factor 2 (*RUNX2*). Sequences and accession numbers of all primers (Oligomer Oy, Helsinki, Finland) are displayed in Table 3. The reactions were conducted and monitored with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

Statistical analyses

One-way ANOVA with Bonferroni *post hoc* test was used to analyze the effect of different culture conditions on cell-proliferation rate, cell surface-marker expression, and differentiation potential by using IBM SPSS software version 19 (IBM SPSS Statistics 19, USA). Differences in proliferation rate between different culture conditions were analyzed separately at each time point. The statistical analyses were performed at the significance level $P < 0.05$, and data are presented as mean ± SD.

Table 3 Primer sequences of marker genes determined

Name	Primer direction	Sequences	Product size (bp)
hRPLP0[1]	Frw	5′-AAT CTC CAG GGG CAC CAT T-3′	70
	Rev	5′-CGC TGG CTC CCA CTT TGT-3′	
haP2[2]	Frw	5′-GGTGGTGGAATGCGTCATG-3′	71
	Rev	5′-CAACGTCCCTTGGCTTATGC-3′	
hPPARG[3]	Frw	5′-CAGTGTGAATTACAGCAAACC –3′	100
	Rev	5′-ACAGTGTATCAGTGAAGGAAT-3′	
hRUNX2[4]	Frw	5′-CCCGTGGCCTTCAAGGT-3′	76
	Rev	5′-CGTTACCCGCCATGACAGTA-3′	
hDLX5[5]	Frw	5′-ACCATCCGTCTCAGGAATCG-3′	75
	Rev	5′-CCCCCGTAGGGCTGTAGTAGT-3′	
hALP[6]	Frw	5′-ATGTCATCATGTTCCTGGGAGAT-3′	79
	Rev	5′-TGGTGGAGCTGACCCTTGAG-3′	

[1]Ribosomal protein, large, P0, (Acc. No: NM_001002); [2]fatty acid-binding protein 4, (Acc. No: NM_001442); [3]peroxisome proliferator-activated receptor gamma (Acc. No: NM_015869), [4]runt-related transcription factor 2 (Acc. No: NM_004348); [4]distal-less homeobox transcription factor 5 (Acc. No: NM_005221); [5]alkaline phosphatase (Acc. No: NM_000478).

Results

XF/SF isolation of ASCs was the most critical step of the cell culture

The isolation of ASCs was conducted in three different culture conditions, in completely XF/SF conditions by using carboxyl-coated flasks (PureCoat™, BD), as well as in HS- and FBS-supplemented medium by using normal Nunclon™ cell-culture flasks. Carboxyl coating was used during the passage 0 after isolation in XF/SF conditions because Nunclon™ cell-culture flasks were unable to provide sufficient initial cell adhesion for ASCs in XF/SF medium. Still, after the first passaging, ASCs were able to grow in normal Nunclon™ flasks in XF/SF medium in the presence of Coating Matrix kit or CELLstart™ coating. The adhesion of ASCs after isolation in XF/SF medium was a critical step of XF/SF culture, and the XF/SF isolation was not successful with all the cell lines; cells from six donors were isolated, but only four donor cell lines were able to adhere and stay viable under XF/SF conditions. Thus, the isolation efficiency in XF/SF conditions was donor dependent. Nevertheless, if the cells were initially able to adhere, cell proliferation in XF/SF medium was efficient in further passages, and the cell-population doubling was notably faster than that in FBS/HS-containing medium (Figure 2). Subsequently, all experiments were carried out with four donor cell lines isolated in HS, FBS, and XF/SF conditions.

Cumulative population doubling of ASCs expanded under XF/SF conditions versus medium containing HS or FBS

The cumulative population doubling of ASCs in XF/SF medium versus serum-containing medium was analyzed with WST-1 assay at time points 1, 4, 7, and 11 days in two passages, 2 and 5. A statistically significant increase in population doubling was seen in cells grown in XF/SF conditions compared with serum containing medium in passage 2 at 4-, 7-, and 11-day time points (Figure 2). Furthermore, a statistically significant difference was seen in passage 5 at 7- and 11-day time points between cells grown in XF/SF CM medium and FBS-containing medium (Figure 2).

Differences in population doublings between passages 2 and 5 were also statistically significant. In HS-supplemented medium; population doubling in passage 5 was significantly increased as compared with passage 2 at 4-, 7-, and 11-day time points, and in FBS medium, a statistically significant increase was seen in passage 5 at days 4 and 11 time points (Figure 2). Of note in passage 5, the population doubling in HS medium was higher than in FBS-containing medium at 11 days, whereas in passage 2, it was vice versa.

Morphology of ASCs expanded under XF/SF conditions versus HS- or FBS-containing medium

The morphologic differences between cells cultured in different conditions were consistent with the cell characteristics seen during the proliferation experiments. The adhesion of the cells grown in XF/SF medium was relatively weak during the isolation, which was also reflected in the morphology of ASCs in XF/SF conditions. Cells grown in the presence of serum adopted wide spindle-shaped and almost cuboidal morphology, whereas XF/SF cells were smaller, more spindle-shaped, and more fibroblast like (Figure 3). The morphologic differences suggest that spindle-shaped cells may not be as strongly attached as cells grown in serum-containing medium.

In addition to light microscopy, cell populations were examined by flow cytometry, especially the uniformity of

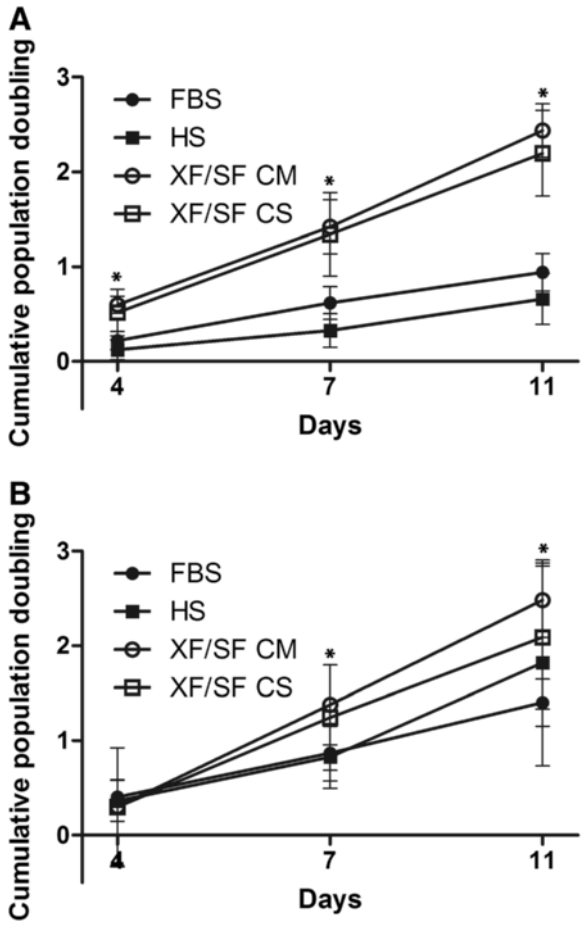

Figure 2 WST-1 proliferation assay. Cumulative population doubling was analyzed in different culture conditions, in FBS- and HS-containing medium, as well as in XF/SF medium with Coating Matrix Kit and with CELLstart™ coating at time points 1, 4, 7, and 11 day in two passages 2 (**A**) and 5 (**B**). The data in the diagrams are presented as mean ± SD. Significantly higher cumulative population doubling was observed in XF/SF conditions compared with HS/FBS cultures in passage 2 at 4-, 7-, and 11-day time points. Furthermore, statistically significant difference in population doubling were seen in passage 5 at 7- and 11-day time points between cells grown in XF/SF CM medium and FBS-containing medium.

the cells in the forward and side scatter. The cells expanded in XF/SF medium displayed a more homogeneous population, seen as a uniform cluster with less debris when compared with cells expanded in serum-containing medium (Figure 3).

Immunophenotype of ASCs expanded under XF/SF conditions versus HS- or FBS-containing medium

Cell-surface marker expression of ASCs was analyzed with flow cytometry to compare the expression profile of cells expanded in XF/SF conditions against cells expanded in HS- or FBS-containing medium at passages 2 and 5 (Figure 4). In general, the characteristic immunophenotype of ASCs was maintained in every culture condition, with some minor differences observed between XF/SF conditions and serum-containing media, as well as in the expression of specific markers between passages 2 and 5.

ASCs showed positive expression (>90%) for the markers CD73 (Ecto 5' nucleotidase), CD90 (Thy-1) and CD105 (Endoglin) in all of the studied culture conditions in both passages (Figure 4), except the slightly lower expression of CD73 in FBS medium in P2 and CD105 in HS medium in P5. In contrast, ASCs lacked the expression (<2%) of CD11a (Integrin α-L), CD14 (LPS-Receptor), CD19 (B4), CD80 (B7-1), CD86 (B7-2), and HLA-DR (major histocompatibility class II receptor) in every culture condition with a few exceptions. Low expression (>2% to <7%) was observed for cells grown in XF/SF CM condition at P2 (CD19, CD86), and in XF/SF CS condition at P2 (CD19, CD80, CD86).

Moderate expression (>7% to <41%) was observed for the hematopoietic progenitor and endothelial cell marker CD34, except for the low expression in FBS and HS cultures at P5. ASCs lacked the expression of leukocyte common antigen CD45 in FBS and HS cultures, and low expression was observed in XF/SF conditions. The largest variation between different culture conditions was seen in the expression of CD54, which showed significantly lower expression in cells expanded under XF/SF conditions compared with cells expanded in serum-containing medium at P2. Furthermore, statistically significant differences were seen between HS medium and XF/SF conditions at passage 5. Generally, ASCs cultured in FBS or HS medium showed moderate expression of CD54 (intercellular adhesion molecule 1, ICAM1), whereas low expression was observed for cells cultured in XF/SF conditions. In addition, whereas the expression of CD34 and CD54 was decreased from passage 2 to passage 5, no statistical differences were observed between passages.

Multipotentiality of ASCs expanded under XF/SF conditions versus HS- or FBS-containing medium

To test the multilineage differentiation potential of ASCs expanded under XF/SF conditions versus HS or FBS medium, the differentiation capacity toward the adipogenic, osteogenic, and chondrogenic lineages was analyzed. After the 14 days of differentiation induction, the differentiation degree was examined by specific staining methods and by the analysis of gene expression.

Adipogenic differentiation

In the adipogenic-induction cultures, oil droplets were visible by light microscopy in cells expanded in HS- or FBScontaining medium. In XF/SF induction culture, differentiation was clearly initiated but did not progress

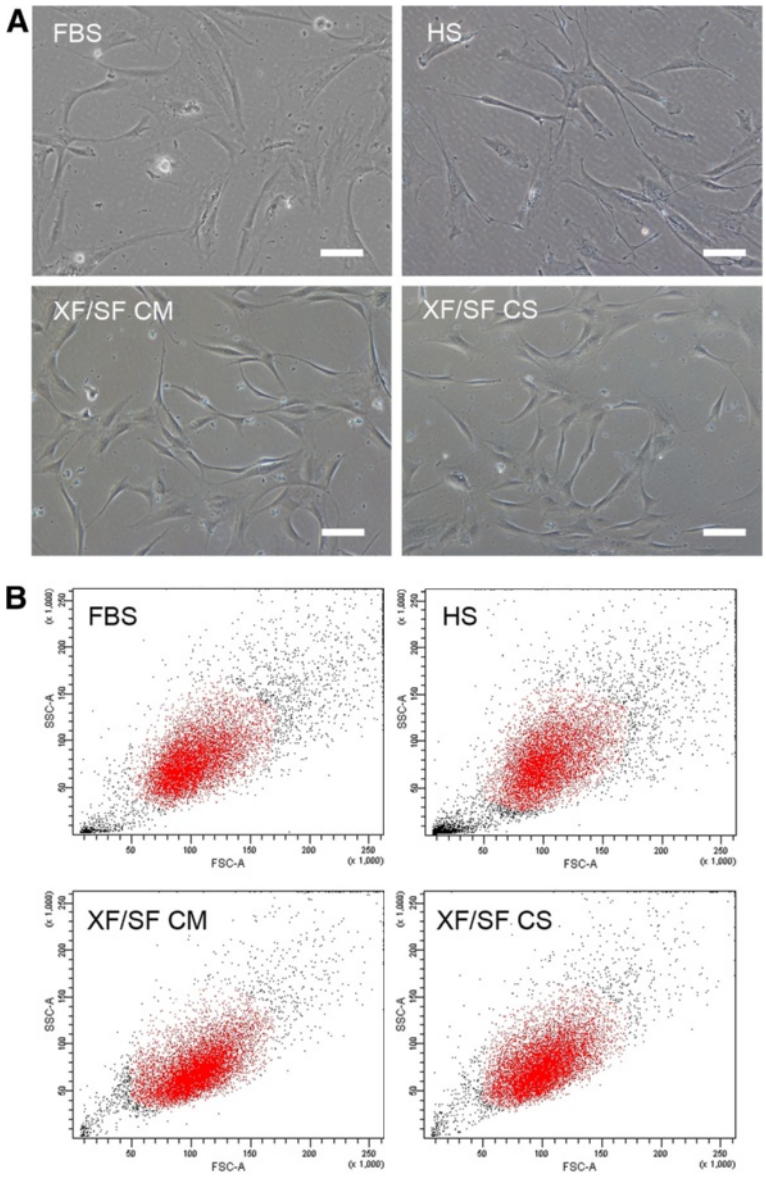

Figure 3 Cell morphology. (**A**) Morphologic images of cells cultured in different conditions: FBS, HS, XF/SF CM, and XF/SF CS at time point 4 days in passage 2. The morphology of ASCs grown in XF/SF medium is more spindle-shaped and smaller than in cells grown under serum-containing medium (FBS, HS). Scale bar, 100 μm. (**B**) Flow-cytometric analysis confirms the morphologic characteristics observed with light microscopy. In XF/SF culture conditions (CM and CS), the cell cloud in the forward and side scatter is more uniform and contains less debris than does a cloud of cells grown in FBS or HS medium, suggesting a more homogeneous population.

very efficiently, which was shown by smaller oil droplets in Oil Red-O staining (Figure 5).

Nevertheless, a trend of higher expression of the gene *PPARγ*, the central transcriptional regulator of adipogenesis, was noted in XF/SF conditions as compared with serum-containing medium, but no significant differences were seen because of high standard deviation (Figure 6A). Further, the expression of *aP2* (fatty acid-binding protein) (Figure 6B) was consistent with the results of Oil Red-O staining (Figure 5). The most intense differentiation was seen in HS medium, which was demonstrated by large oil droplets in Oil Red-O staining and by a significant increase in the expression of *aP2* gene (Figure 6B) in ASCs cultured in HS-supplemented induction medium compared with HS control medium and the cells in all the other induction media in passage 2. Although the serum-containing medium and especially HS medium appeared to be the best condition for adipogenic differentiation, cells cultured under XF/SF conditions showed signs of early differentiation.

Because the induction response of XF/SF cells to adipogenic differentiation appeared to be attenuated under

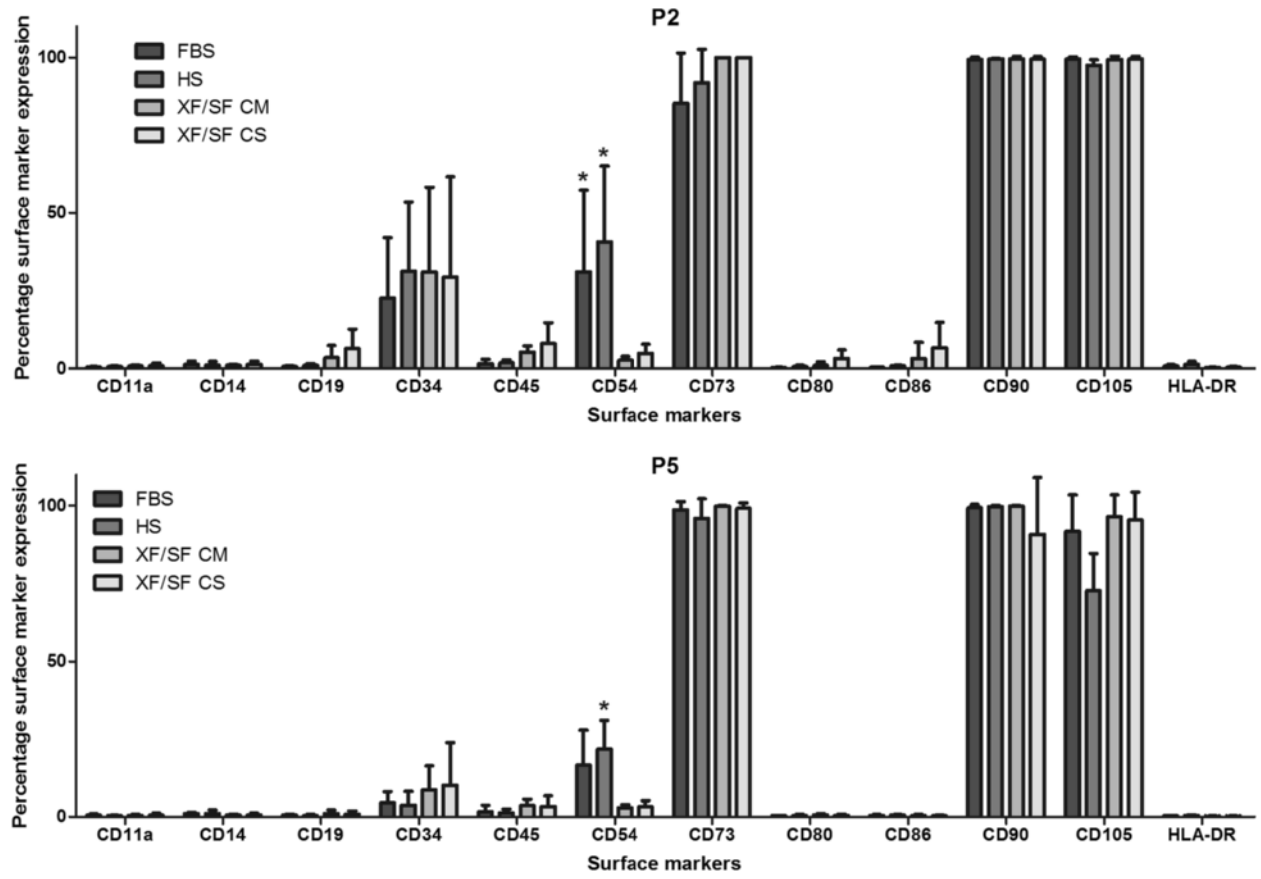

Figure 4 Surface-marker expression of undifferentiated ASCs. Immunophenotype of cells expanded in four different culture conditions; FBS, HS, XF/SF CM, and XF/SF CS was investigated in passages 2 and 5. The data in the diagrams are presented as mean ± SD. Cells expanded under XF/SF conditions showed significantly lower expression of CD54 (ICAM-1) compared with cells expanded in serum containing medium in passage 2. Furthermore, statistically significant differences in the expression of CD54 were seen between HS medium and XF/SF conditions in passage 5. Characteristic immunophenotypes of ASCs were maintained in every culture conditions with minor differences.

serum-free condition, we hypothesized that the cells needed more nutrient-rich media to promote efficient adipogenesis. Cryopreserved ASCs cultured in FBS-containing media (passage 1), XF/SF CS (passage 2), and XF/SF CM (passage 2) were thawed, recovered, and grown in their own media. After reaching near confluency, the ASCs were harvested and plated into both FBS-containing or HS-containing media and let grow for 3 days. Then the medium was replaced with adipogenic induction media in either FBS- or HS-containing condition and cultured for 14 days. As predicted, differentiation was more efficient when primed with HS- or FBS-containing medium, but HS media clearly displayed increased differentiation than did FBS media (Figure 7). No noticeable differences were noted between XF/SF CS and XF/SF CM cells when induced with either FBS- or HS-based adipogenic media.

Osteogenic differentiation

In the osteogenic induction cultures, cells expanded in HS- or FBS-containing medium showed slightly enhanced capacity to undergo osteogenic differentiation than did the cells expanded under XF/SF conditions, based on the ALP staining (Figure 5). However, the proliferation rate of ASCs grown in XF/SF medium was increased compared with serum-containing medium and, as a result, the wells became confluent, and cells started to detach. Therefore, the weak ALP staining in XF/SF conditions may indicate attachment difficulties of the cells in confluent wells, although the osteogenic differentiation is ongoing (Figure 5).

In addition to ALP staining, the osteogenic differentiation was evaluated by the expression of osteogenesis-specific genes. In contrast to ALP staining results, the expression of *Runx2* was increased in XF/SF conditions as compared with serum-containing medium, and a statistically significant increase was seen between XF/SF CM and FBS/HS induction media in passage 5 (Figure 6C). Further, the response of *DLX5* (Figure 6E) to the osteogenic induction was stronger in XF/SF cultured cells when compared with FBS/HS conditions, and a statistically

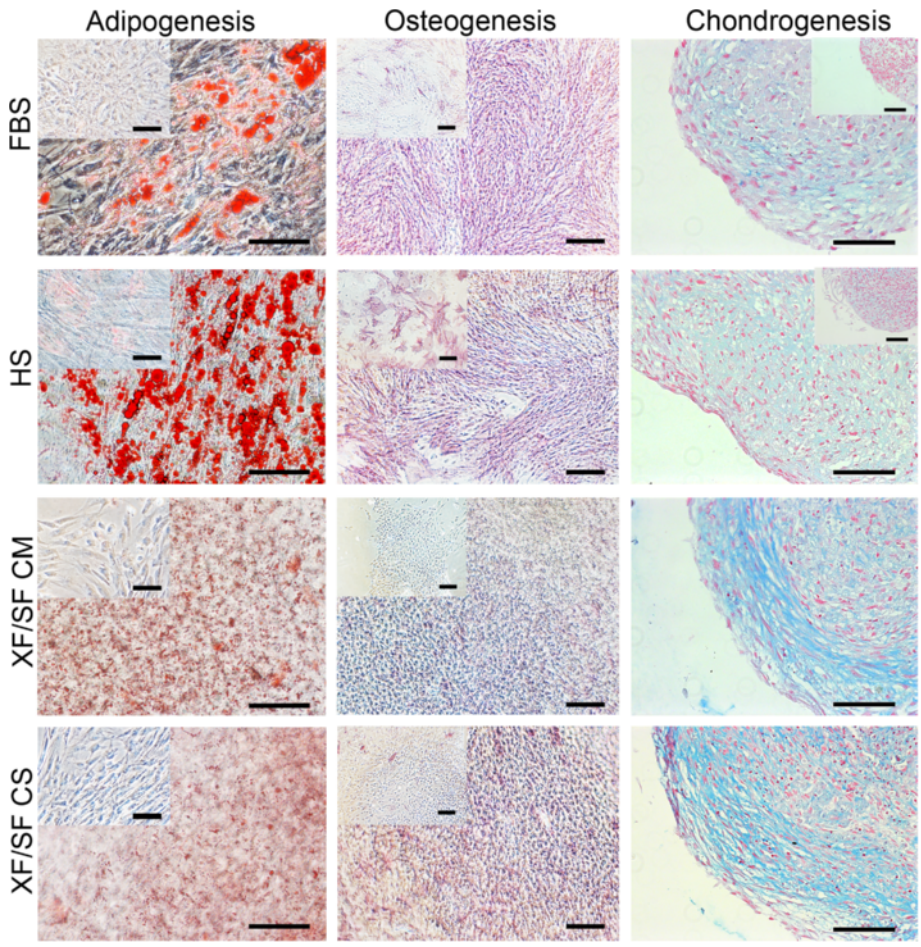

Figure 5 Multilineage differentiation potential of ASCs. Differentiation potential of ASCs cultured in four different conditions; FBS, HS, XF/SF medium with Coating Matrix Kit, or CELLstart coating was investigated toward adipogenic, osteogenic, and chondrogenic cells. Oil Red-O staining indicates the formation of intracellular lipid in cells going through adipogenic differentiation (scale bar, 100 μm); ALP staining reveals the alkaline phosphatase activity in osteogenic-differentiation cultures (scale bar, 300 μm), and Alcian blue staining recognizes the glycosaminoglycans of the cells going through chondrogenic differentiation (scale bar, 100 μm). Adipogenesis and osteogenesis was more effective in serum-containing media, whereas clearly the most intense chondrogenesis was seen in XF/SF cultures.

significant increase in *DLX5* expression was seen in cells cultured in XF/SF CM induction media when compared with FBS/HS induction conditions, as well as XF/SF CM control. Also, the alkaline phosphatase staining result was verified at gene-expression level, where a trend of increased expression was observed in every differentiation culture condition when compared with control samples (Figure 6D). Although the expression of alkaline phosphatase (*ALP*) was increased after induction, no significant differences were seen because of high standard deviation.

To determine whether ALP staining could be enhanced, XF/SF cells were also tested with a serum-based medium to see whether more-efficient osteogenesis could be induced. Cryopreserved ASCs cultured under FBS-containing media (passage 1), XF/SF CS (passage 2), and XF/SF CM (passage 2) were thawed, recovered, and grown in their own media. After reaching near confluency, the ASCs were harvested and plated into both FBS- and HS-containing media and let grown for 3 days. After 3 days, the media were replaced with osteogenic induction medium in either FBS- or HS-containing condition and cultured for 14 days. Differentiation was more efficient when primed with HS- or FBS-containing medium (Figure 7). No noticeable differences were noted between XF/SF CS and XF/SF CM cells when induced with either FBS- or HS-based osteogenic media.

Chondrogenic differentiation

Chondrogenic differentiation was more intense in XF/SF conditions compared with serum containing medium, shown by the Alcian blue staining of proteoglycans after the micromass culture in chondrogenic induction medium (Figure 5). The size of the pellet was also larger in XF/SF conditions versus HS or FBS cultures. These

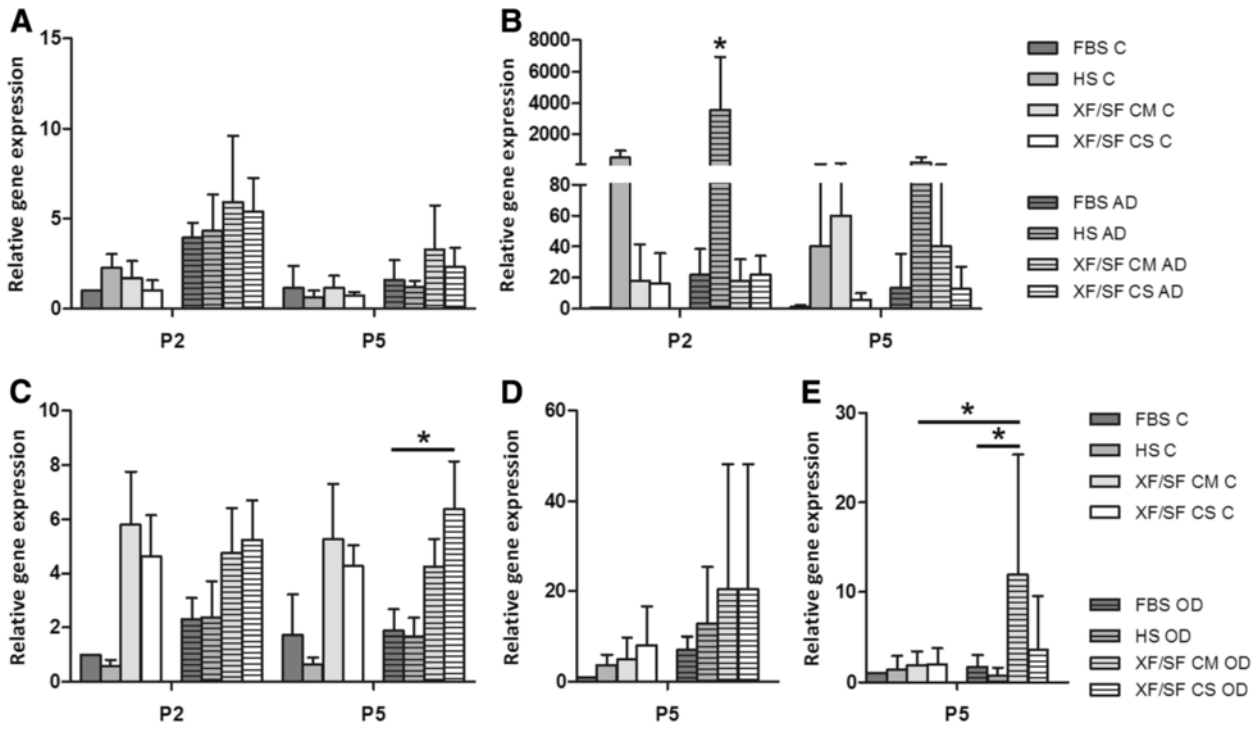

Figure 6 Gene-expression analysis. Differentiation-specific gene-expression analysis was performed after 14 days of differentiation induction, versus control medium, in different culture conditions: FBS, HS, XF/SF medium with Coating Matrix Kit, and with CELLstart coating in passages 2 and 5. The expression of *PPARγ* gene (**A**) and *aP2* gene (**B**) demonstrate adipogenesis after 14 days of adipogenic induction. Respectively, the expression of *Runx2* (**C**) *ALP* (**D**), and *DLX5* (**E**) genes indicate osteogenic differentiation after 14 days of osteogenic induction. The expression of *ALP* and *DLX5* are presented in passage 5. A significant increase in the expression of aP2 was observed in HS-supplemented induction medium compared with HS control medium, and the cells in all the other induction media in passage 2. Further, the expression of *Runx2* was significantly increased in XF/SF CM induction medium as compared with FBS/HS inductions in passage 5. Moreover, the response of *DLX5* to the osteogenic induction was significantly stronger in XF/SF CM-cultured cells when compared with FBS/HS conditions, as well as XF/SF CM control in passage 5. AD, adipogenic differentiation; OD, osteogenic differentiation. The data in the diagrams are presented as mean ± SD.

results suggest the XF/SF conditions promote the cartilage differentiation since the formation of proteoglycans, central components of cartilage tissue, is enhanced in XF/SF cultures.

Discussion

Today, clinical cell therapies using ASCs are in progress, and several clinical trials are ongoing [5] and require more-reliable, reproducible, and safe methods for *in vitro* expansion of the cells. Therefore, the transition from FBS- or HS-medium supplementation to defined XF/SF culture conditions would be one of the most important steps forward in considering the suitability of ASCs for clinical use. By removing all the animal-derived components as well as the undefined serum from the cell-culture workflow, the safety of the patient receiving cell transplant can be improved.

Traditionally, ASC culture medium has been supplemented with FBS, which is not a preferred option in clinical therapies because of xenogeneic components with critical safety issues [12,25]. Consequently, different kinds of alternatives for FBS have been studied considering the clinical use of ASCs. Trivedi and colleagues [26] replaced FBS with 20% human albumin during the ASC expansion for clinical use to treat diabetes, whereas Tzouvelekis and colleagues [27] used autologous platelet-rich plasma for the cell expansion to treat patients with pulmonary fibrosis. AutoHS is currently used for the expansion of ASC by our group for the reconstruction of bone defects in the craniomaxillofacial area [4,28]. However, as mentioned earlier, limitations are associated with the use of autoHS or serum derivatives, such as lot-to-lot variability [17], limited availability, and undefined composition, and therefore, the use of XF/SF medium would be the preferred option.

Studies have been performed on ASCs/BMSCs studying the defined XF- or SF-culture conditions; Dromard and colleagues [29] demonstrated that ASCs can be expanded as floating spheres in defined SF-culture systems supplemented with 2% human plasma and specific growth factors. Further, Santos *et al*. [30] investigated a microcarrier-based bioreactor system for the XF/SF

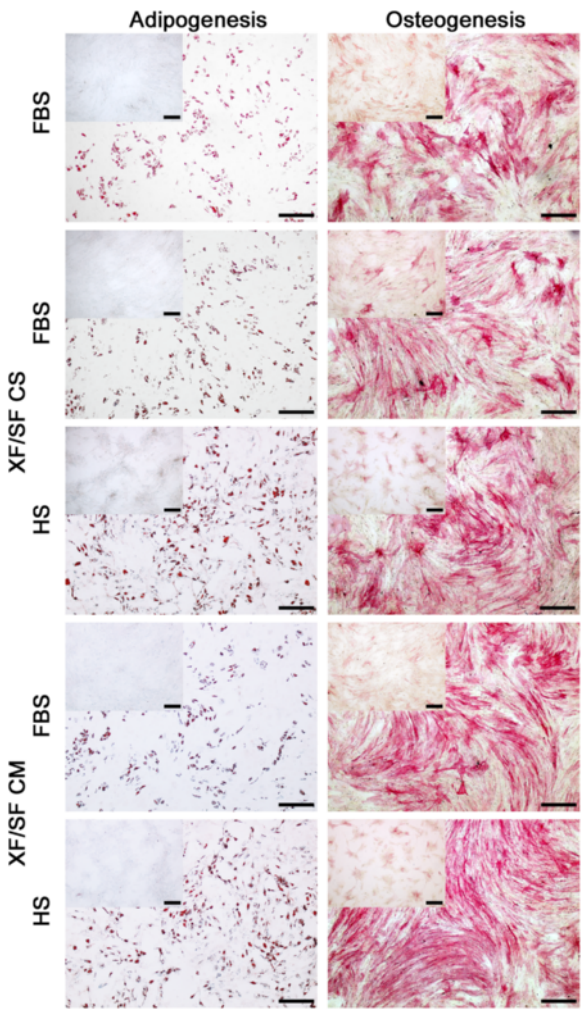

Figure 7 Adipogenic and osteogenic differentiation potential of ASCs after serum priming. Follow-up study on the differentiation of ASCs performed in five different conditions: (1) FBS, (2) XF/SF CS primed initially in FBS, (3) XF/SF CS primed initially in HS, (4) XF/SF CM primed initially in FBS, and (5) XF/SF CM primed initially in HS. Oil Red-O staining indicates the formation of intracellular lipid in cells going through adipogenic differentiation (scale bar, 100 μm); ALP staining reveals the alkaline phosphatase activity in osteogenic differentiation cultures (scale bar, 100 μm). Inset images are the paired undifferentiated negative controls. Differentiation toward adipogenic and osteogenic cells was more efficient when primed with HS- or FBS-containing medium.

expansion of ASCs and BMSCs. Moreover, the suitability of human platelet lysate (PL) for FBS substitution has been investigated by several groups. Schallmoser and colleagues [15] introduced a standard protocol for platelet preparation for animal protein-free cultures of ASCs, and Naajikens *et al.* [31] showed that PL-cultured ASCs had a similar differentiation capacity and increased proliferation rate when compared with FBS cultures. Blande *et al.* [32], in contrast, showed that ASC population doubling time in PL cultures was significantly lower than that in FBS cultures, but the immunophenotype was similar, and both cultures retained the differentiation potential of the cells.

Still, a better-defined culture environment is needed, and to our knowledge, this study is the first report describing ASC isolation and expansion in completely XF/SF conditions maintaining the basic stem cell characteristics of ASCs. In the past, XF or SF expansion of mesenchymal stem cells has been reported, but the cell isolation and early expansion and differentiation studies were carried out in serum-containing medium [19,30,33-35]. In this study, we isolated the cells without serum exposure in XF/SF conditions by using BD PureCoat carboxyl flasks. Onward from passage 1, the cells were able to grow on basic Nunclon Δ surface vessels in XF/SF conditions when either CELLstart CTS coating or XF/SF CM was used. Considering the future clinical applications, it is an advance that the cells are isolated and expanded in fully XF/SF culture conditions instead of using serum supplementation at any point of the culture. If the patient is exposed to undefined components under *in vitro* expansion, an increased risk occurs for cross-contaminations and immune reactions in a patient receiving the cell transplant. Nevertheless, patient safety is still the most important aspect considering clinical use of ASCs.

In addition to safety, it is advantageous for *in vitro* expansion of cells to be performed in a shorter time scale. Our studies on the cell-proliferation rate were consistent with the results of earlier studies of mesenchymal stem cell cultures under XF or SF conditions [19,33,34] in which the higher proliferation rate of XF- or SF-cultured cells compared with FBS cultures was demonstrated. In our study, the cumulative population doubling in XF/SF medium was superior when compared with both FBS and HS conditions. Efficient expansion of ASCs in XF/SF conditions is crucial for clinical sustainability where a large cell number is required in a minimum time scale.

Cell surface-marker expression profile of ASCs was largely similar between cells grown under different culture conditions, except the significant difference in the expression of CD54 (ICAM-1), which is a marker of endothelial cells and cells of the immune system. To our knowledge, CD54 expression of ASCs has not been studied earlier in XF/SF conditions. The lower expression of CD54 may suggest that a more homogeneous cell population is achieved through more-selective isolation and expansion protocols compared with cells isolated in the presence of serum. In addition, weaker cell adhesion under XF/SF conditions was observed, which may reflect on lower expression of the adhesion molecule ICAM1 (CD54). This aspect of XF/SF cultures and the possible selective effect on cell population has to be investigated

in subsequent XF/SF studies, especially when a coating is used.

Some variations were also seen in the expression of CD11a (integrand am), CD14 (lip polysaccharide receptor), CD19 (leukotriene B4 receptor), and CD86 (costimulatory molecule for T-cell activation) on cells grown in XF/SF conditions versus serum-containing medium. All of these markers are known to interact with immune-related cells, and therefore, the culture conditions may affect the immunogenicity of ASCs.

The minimal criterion for the immunophenotype of MSCs described by Dominici *et al*. [7] was defined for cells cultured under standard condition in a medium with FBS supplementation. However, our current results with XF/SF cells demonstrate that the cell surface-marker profile applies to ASCs cultured under XF/SF conditions as well. The hematopoietic progenitor cell marker CD34 was moderately expressed in both XF/SF and serum-supplemented conditions in contrast to earlier described criteria. However, similar results for CD34 expression have been reported by others [36,37], and the variable interpretations could be explained by differences cell-culturing and -passaging protocols. In the current study, the expression of CD34 and CD54 was higher in passage 2, but the expression level was decreased in later passages, indicating a more homogeneous population.

The chondrogenic differentiation capacity of ASCs cultured under XF/SF conditions was strong compared with cells cultured in serum-containing medium based on the Alcian blue staining. Similar findings have been shown by Chase *et al*. [31], in which a robust chondrogenesis in SF-culture conditions was seen when compared with serum-containing medium. In our study, the Alcian blue staining of proteoglycans was intense in cells expanded under XF/SF conditions, and also the pellet size after micromass-culture was larger compared with serum-containing medium, which was in agreement with Chase's results. This result promises potential use of ASCs in chondrogenic applications, but further research is needed to investigate the chondrogenic-differentiation potential of ASCs in XF/SF conditions.

Moreover, the differentiation potential toward osteogenic and adipogenic cells was investigated in XF/SF conditions. Unlike in previous studies [28,31,32], osteogenesis and adipogenesis was induced in totally XF/SF differentiation media, and serum was substituted by XF/SF supplement of the STEMPRO MSC SFM kit. When ASCs were cultured under XF/SF conditions, they showed moderate differentiation potential toward osteogenic and adipogenic cells, as demonstrated by ALP and Oil Red-O staining, as the differentiation was not as efficient as seen in serum-containing medium. By optimizing the differentiation protocols for each condition, the efficiency of osteogenic and adipogenic differentiation can be enhanced. Furthermore, the reduced differentiation potential may be due to decreased cell adhesion for cells undergoing differentiation under XF/SF conditions. The weak cell-attachment hypothesis was supported by altered morphology and cell detachment during the proliferation studies, as well as decreased enzymatic digestion time. Another explanation is that ASCs need a more nutrient-rich media to promote robust differentiation. The follow-up differentiation studies with FBS- or HS-based media strongly indicate that nutrients play a key role in efficient differentiation. These findings are consistent with previous studies in which the importance of cell adhesion and nutrients during the cell differentiation has been shown [38,39].

In addition, the responses seem to be donor specific, and possibly, some cell lines respond better to the osteogenic induction, whereas others respond better to adipogenic induction. Thus, variation in the responses exists, and different stages of differentiation are evident, as shown by the high standard deviations of gene expressions. Also, the mRNA expression and enzymatic activity may not be in line because the regulation occur on posttranscriptional and translational levels, and finally, on the level of formation of an active enzyme. The cells cultured in different conditions may also be at different stages of their differentiation process, thus not expressing the same markers simultaneously. Still, the activity increase in gene-expression level shows commitment to osteogenic or adipogenic pathway, depending on donor cell line and culture condition.

Nevertheless, although at an early stage, differentiation occurred in XF/SF medium, showing that the cells have the capacity for trilineage differentiation, as shown by Oil Red-O, ALP, and Alcian blue staining. Chase *et al.* [33] demonstrated that BMSCs expanded in SF conditions and differentiated in serum containing induction medium retained their ability to differentiate into adipocytes, chondrocytes, and osteoblasts. In addition, Yang *et al.* [34] published similar results on the differentiation potential of ASCs expanded in a hypoxic XF environment. The cells expanded in XF medium had equal multilineage differentiation capacity, as compared with cells expanded in traditional serum-containing medium when serum induction was used during the differentiation. Taking these previous results into account, the efficiency of differentiation could easily be improved by serum induction during the culture in differentiation medium.

However, the aim of this study was to remove all the undefined components from the cell-culture workflow, and therefore, serum induction is not a preferred option for use during differentiation. Conversely, autoHS supplementation may be used in clinical treatments for differentiation induction, and the cells would still be expanded in defined XF/SF conditions before differentiation.

Also, ASCs can be implanted to the defect site in their undifferentiated state, and the cell differentiation then occurs *in vivo,* as has been performed by our group [4,28].

Additionally, growth factors and biomaterials can be used to support cell differentiation in XF/SF conditions. Cordonnier and co-workers [40] showed that growth-factor induction is especially effective for cells cultured in low serum. In their study, the effect of bone morphogenetic proteins (BMPs) -2, -4, and –7 on osteogenic differentiation of BMSCs was evaluated in low (2%) and higher (10%) FBS-supplemented cultures, leading to a conclusion that BMP-4 induction in low-serum cultured cells was the most effective osteogenic inducer. Similarly, the osteogenic differentiation potential of XF/SF cultured ASCs could be enhanced by growth-factor induction. Furthermore, the differentiation capacity of XF/SF-cultured ASCs can be enhanced by inductive biomaterials, such as osteopromoting bioactive glass [41] or β-tricalcium phosphate [42], or alternatively, chondrogenesis-inductive materials such as 3-D woven polycaprolactone scaffolds [43].

Conclusions

Effective and safe *in vitro* methods to isolate and expand ASCs are critical for the positive development of cell-therapy applications. Our current results demonstrate that the novel XF/SF culture conditions maintains the stem cell characteristics of ASCs. The cells grown in different culture conditions displayed the characteristic immunophenotype of ASCs with minor differences. Importantly, the proliferation rate of ASCs was significantly increased in XF/SF conditions, compared with HS- and FBS-containing medium. Furthermore, the chondrogenic differentiation potential was intense in XF/SF conditions, whereas adipogenic and osteogenic differentiation were comparable to the FBS condition after serum priming.

These novel XF/SF culture conditions have great potential for clinical use, but additional preclinical safety and efficacy studies will be needed and standardized before using in clinical treatments. Off-the-shelf cell products will require effective XF/SF conditions in which the basic stem-cell characteristics of ASCs are maintained, the proliferation rate is high, and the cells retain their functionality. Naturally, a substantial number of safety-assessment studies would have to be done before allogeneic ASCs can be used in clinical cell treatments. The development of efficient and safe XF/SF-culture conditions is one step closer to that goal.

Abbreviations

AD: Adipogenic differentiation; alloHS: Allogeneic human serum; ALP: Alkaline phosphatase; aP2: Adipocyte protein 2; ASC: adipose stem cells; autoHS: Autologous human serum; BMPs: Bone morphogenic proteins; BMSC: Bone marrow-derived stem cell; CD: Chondrogenic differentiation; CM: Coating matrix; CS: CELLStart; DLX5: Distal-less homeobox transcription factor 5; FBS: Fetal bovine serum; HS: human serum; ICAM1: Intercellular adhesion molecule 1; MSC: mesenchymal stem cell; OD: Osteogenic differentiation; PPARγ: peroxisome proliferator-activated receptor γ; RUNX2: runt-related transcription factor 2; XF/SF: Xeno- and serum-free.

Competing interests

SB, AC, and MV are regular employees of Life Technologies and have not received any financial gains. They hold some stocks of Life Tech as employees of Life Technologies. MP, MJ, BM, and SM declare that they have no competing interests. The authors alone are responsible for the content and writing of the manuscript.

Authors' contributions

MP performed the laboratory work, the isolation and expansion of adipose stem cells, proliferation, immunophenotypic, and differentiation studies in cooperation with MJ. MP performed statistical analyses and wrote the manuscript, and MJ participated in producing the figure panels and reviewing the manuscript. BM designed and supervised the study and participated in reviewing the manuscript. SB participated in designing the study, performed the follow-up differentiation studies, and reviewed the manuscript. SM supervised the study and participated in reviewing the manuscript. AC participated in the development of XF/SF coating-free supplements and reviewed the manuscript. MV participated in planning and reviewing of the results and discussion. All authors read and approved the final manuscript.

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Author details

[1]Adult Stem Cell Group, Institute of Biomedical Technology, University of Tampere, Tampere, Finland. [2]BioMediTech, Tampere, Finland. [3]Science Center, Tampere University Hospital, Tampere, Finland. [4]Life Technologies, Cell Therapy Systems, Frederick, MD, USA. [5]Life Technologies, BioProduction, Grand Island, MD, USA.

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References

1. Gimble J, Guilak F: **Adipose-derived adult stem cells: isolation, characterization, and differentiation potential.** *Cytotherapy* 2003, **5:**362–369.
2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH: **Human adipose tissue is a source of multipotent stem cells.** *Mol Biol Cell* 2002, **13:**4279–4295.
3. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH: **Multilineage cells from human adipose tissue: implications for cell-based therapies.** *Tissue Eng* 2001, **7:**211–228.
4. Thesleff T, Lehtimaki K, Niskakangas T, Mannerstrom B, Miettinen S, Suuronen R, Ohman J: **Cranioplasty with adipose-derived stem cells and biomaterial: a novel method for cranial reconstruction.** *Neurosurgery* 2011, **68:**1535–1540.
5. Casteilla L, Planat-Benard V, Laharrague P, Cousin B: **Adipose-derived stromal cells: their identity and uses in clinical trials: an update.** *World J Stem Cells* 2011, **3:**25–33.
6. Mizuno H: **Adipose-derived stem and stromal cells for cell-based therapy: current status of preclinical studies and clinical trials.** *Curr Opin Mol Ther* 2010, **12:**442–449.
7. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: **Minimal criteria for defining multipotent mesenchymal stromal cells: The International Society for Cellular Therapy position statement.** *Cytotherapy* 2006, **8:**315–317.
8. Niemeyer P, Vohrer J, Schmal H, Kasten P, Fellenberg J, Suedkamp NP, Mehlhorn AT: **Survival of human mesenchymal stromal cells from bone marrow and adipose tissue after xenogenic transplantation in immunocompetent mice.** *Cytotherapy* 2008, **10:**784–795.

9. Kuo YR, Chen CC, Goto S, Lee IT, Huang CW, Tsai CC, Wang CT, Chen CL: **Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model.** *Plast Reconstr Surg* 2011, **128:**661e–672e.
10. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A: **Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells.** *Br J Haematol* 2005, **129:**118–129.
11. Niemeyer P, Kornacker M, Mehlhorn A, Seckinger A, Vohrer J, Schmal H, Kasten P, Eckstein V, Sudkamp NP, Krause U: **Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro.** *Tissue Eng* 2007, **13:**111–121.
12. Mackensen A, Drager R, Schlesier M, Mertelsmann R, Lindemann A: **Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells.** *Cancer Immunol Immunother* 2000, **49:**152–156.
13. Selvaggi TA, Walker RE, Fleisher TA: **Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions.** *Blood* 1997, **89:**776–779.
14. Tateishi K, Ando W, Higuchi C, Hart DA, Hashimoto J, Nakata K, Yoshikawa H, Nakamura N: **Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications.** *Cell Transplant* 2008, **17:**549–557.
15. Schallmoser K, Strunk D: **Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures.** *J Vis Exp* 2009. doi:10.3791/1523.
16. Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, Kluter H: **Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow.** *Stem Cells* 2009, **27:**2331–2341.
17. Parker AM, Shang H, Khurgel M, Katz AJ: **Low serum and serum-free culture of multipotential human adipose stem cells.** *Cytotherapy* 2007, **9:**637–646.
18. Haimi S, Moimas L, Pirhonen E, Lindroos B, Huhtala H, Raty S, Kuokkanen H, Sandor GK, Miettinen S, Suuronen R: **Calcium phosphate surface treatment of bioactive glass causes a delay in early osteogenic differentiation of adipose stem cells.** *J Biomed Mater Res A* 2009, **91:**540–547.
19. Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, Haataja R, Vemuri M, Suuronen R, Miettinen S: **Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro.** *Cytotherapy* 2009, **11:**958–972.
20. Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC: **Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation.** *Proc Natl Acad Sci U S A* 1998, **95:**10614–10619.
21. Haimi S, Suuriniemi N, Haaparanta AM, Ella V, Lindroos B, Huhtala H, Raty S, Kuokkanen H, Sandor GK, Kellomaki M, Miettinen S, Suuronen R: **Growth and osteogenic differentiation of adipose stem cells on PLA/bioactive glass and PLA/beta-TCP scaffolds.** *Tissue Eng Part A* 2009, **15:**1473–1480.
22. Denker AE, Nicoll SB, Tuan RS: **Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1.** *Differentiation* 1995, **59:**25–34.
23. Maenpaa K, Ella V, Mauno J, Kellomaki M, Suuronen R, Ylikomi T, Miettinen S: **Use of adipose stem cells and polylactide discs for tissue engineering of the temporomandibular joint disc.** *J R Soc Interface* 2010, **7:**177–188.
24. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR.** *Nucleic Acids Res* 2001, **29:**e45.
25. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, Hsu SC, Smith J, Prockop DJ: **Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy.** *Mol Ther* 2004, **9:**747–756.
26. Trivedi HL, Vanikar AV, Thakker U, Firoze A, Dave SD, Patel CN, Patel JV, Bhargava AB, Shankar V: **Human adipose tissue-derived mesenchymal stem cells combined with hematopoietic stem cell transplantation synthesize insulin.** *Transplant Proc* 2008, **40:**1135–1139.
27. Tzouvelekis A, Koliakos G, Ntolios P, Baira I, Bouros E, Oikonomou A, Zissimopoulos A, Kolios G, Kakagia D, Paspaliaris V, Kotsianidis I, Froudarakis M, Bouros D: **Stem cell therapy for idiopathic pulmonary fibrosis: a protocol proposal.** *J Transl Med* 2011, **9:**182.
28. Mesimaki K, Lindroos B, Tornwall J, Mauno J, Lindqvist C, Kontio R, Miettinen S, Suuronen R: **Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells.** *Int J Oral Maxillofac Surg* 2009, **38:**201–209.
29. Dromard C, Bourin P, Andre M, De Barros S, Casteilla L, Planat-Benard V: **Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres.** *Exp Cell Res* 2011, **317:**770–780.
30. Santos F, Andrade PZ, Abecasis MM, Gimble JM, Chase LG, Campbell AM, Boucher S, Vemuri MC, Silva CL, Cabral JM: **Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions.** *Tissue Eng Part C Methods* 2011, **17:**1201–1210.
31. Naaijkens BA, Niessen HW, Prins HJ, Krijnen PA, Kokhuis TJ, de Jong N, van Hinsbergh VW, Kamp O, Helder MN, Musters RJ, van Dijk A, Juffermans LJ: **Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications.** *Cell Tissue Res* 2012, **348:**119–130.
32. Blande IS, Bassaneze V, Lavini-Ramos C, Fae KC, Kalil J, Miyakawa AA, Schettert IT, Krieger JE: **Adipose tissue mesenchymal stem cell expansion in animal serum-free medium supplemented with autologous human platelet lysate.** *Transfusion* 2009, **49:**2680–2685.
33. Chase LG, Lakshmipathy U, Solchaga LA, Rao MS, Vemuri MC: **A novel serum-free medium for the expansion of human mesenchymal stem cells.** *Stem Cell Res Ther* 2010, **1:**8.
34. Yang S, Pilgaard L, Chase L, Boucher S, Vemuri M, Fink TD, Zachar V: **Defined xenogeneic-free and hypoxic environment provides superior conditions for long-term expansion of human adipose-derived stem cells.** *Tissue Eng Part C Methods* 2012, **18:**593–602.
35. Konno M, Hamazaki TS, Fukuda S, Tokuhara M, Uchiyama H, Okazawa H, Okochi H, Asashima M: **Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture.** *Biochem Biophys Res Commun* 2010, **400:**461–465.
36. Mirabet V, Solves P, Minana MD, Encabo A, Carbonell-Uberos F, Blanquer A, Roig R: **Human platelet lysate enhances the proliferative activity of cultured human fibroblast-like cells from different tissues.** *Cell Tissue Bank* 2008, **9:**1–10.
37. Rebelatto CK, Aguiar AM, Moretao MP, Senegaglia AC, Hansen P, Barchiki F, Oliveira J, Martins J, Kuligovski C, Mansur F, Christofis A, Amaral VF, Brofman PS, Goldenberg S, Nakao LS, Correa A: **Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue.** *Exp Biol Med (Maywood)* 2008, **233:**901–913.
38. Park IS, Han M, Rhie JW, Kim SH, Jung Y, Kim IH, Kim SH: **The correlation between human adipose-derived stem cells differentiation and cell adhesion mechanism.** *Biomaterials* 2009, **30:**6835–6843.
39. Kang JM, Han M, Park IS, Jung Y, Kim SH, Kim SH: **Adhesion and differentiation of adipose-derived stem cells on a substrate with immobilized fibroblast growth factor.** *Acta Biomater* 2012, **8:**1759–1767.
40. Cordonnier T, Langonne A, Sohier J, Layrolle P, Rosset P, Sensebe L, Deschaseaux F: **Consistent osteoblastic differentiation of human mesenchymal stem cells with bone morphogenetic protein 4 and low serum.** *Tissue Eng Part C Methods* 2011, **17:**249–259.
41. Bosetti M, Cannas M: **The effect of bioactive glasses on bone marrow stromal cells differentiation.** *Biomaterials* 2005, **26:**3873–3879.
42. Marino G, Rosso F, Cafiero G, Tortora C, Moraci M, Barbarisi M, Barbarisi A: **Beta-tricalcium phosphate 3D scaffold promote alone osteogenic differentiation of human adipose stem cells: in vitro study.** *J Mater Sci Mater Med* 2010, **21:**353–363.
43. Moutos FT, Estes BT, Guilak F: **Multifunctional hybrid three-dimensionally woven scaffolds for cartilage tissue engineering.** *Macromol Biosci* 2010, **10:**1355–1364.

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Title page

Effect of macromolecular crowding on human adipose stem cell proliferation and differentiation under FBS, HS and defined XF/SF cultures

*Mimmi Patrikoski[1,2], Michelle Hui Ching Lee[3], Laura Mäkinen[1,2], Xiu Min Ang[3,4], Bettina Mannerström[1,2], Michael Raghunath[3,4], Susanna Miettinen[1,2].

[1] Adult Stem Cell Group, BioMediTech, Institute of Biosciences and Medical Technology, University of Tampere, Tampere, Finland

[2] Science Center, Tampere University Hospital, Tampere, Finland

[3] NUS Tissue Engineering Program, Life Science Institute, National University of Singapore, 117510, Singapore

[4] Department of Biomedical Engineering, National University of Singapore, 117575, Singapore

*corresponding author

Mimmi Patrikoski (mimmi.patrikoski@uta.fi)

Lee Hui Ching Michelle (lsilhcm@nus.edu.sg)

Mäkinen Laura (makinen.laura.M@student.uta.fi)

Ang Xiu Min (a0082391@u.nus.edu)

Mannerström Bettina (bettina.mannerstrom@helsinki.fi)

Raghunath Michael (bierm@nus.edu.sg)

Miettinen Susanna (susanna.miettinen@uta.fi)

Abstract

Introduction: Culture microenvironment plays an important role in the expansion and differentiation of stem cells. The macromolecular crowding (MMC) method is used to promote cells to produce their microenvironment in vitro.

Methods: In this study, the applicability of MMC was evaluated for its ability to support ASC proliferation, metabolic activity and multilineage differentiation under different culture conditions: fetal bovine serum (FBS), human serum (HS) and xeno- and serum- free (XF/SF) conditions. Furthermore, the effect of MMC on the immunophenotype of ASCs was evaluated under different culture conditions.

Results: The proliferation capacity of ASCs was reduced under MMC in each culture condition compared to standard conditions, although ASCs cultured under MMC in FBS media showed higher metabolic activity compared to cells cultured in standard FBS media. The osteogenic and adipogenic differentiation was more efficient under MMC induction in FBS and HS cultures. The XF/SF cultured ASCs did not show a positive response to MMC, but poor viability under MMC was observed. Based on Alcian blue staining, the chondrogenic differentiation of ASCs was more efficient after cell expansion in standard (-MMC) culture conditions as compared to the MMC expanded ASCs that showed a histologically less dense micromass structure. The characteristic immunophenotype was maintained under MMC conditions, however the expression of CD54 was significantly higher under MMC in all studied culture conditions.

Conclusions: ASCs cultured in serum containing media under MMC maintained the characteristic immunophenotype and multilineage differentiation potential, although the proliferation capacity was reduced under MMC. Although being suboptimal method for the studied XF/SF cultures, the adipogenic and osteogenic differentiation of ASCs was enhanced under MMC in serum containing media.

Keywords

adipose stem cells, culture condition, macromolecular crowding, fetal bovine serum, human serum, xeno- and serum-free, multipotency

Introduction

Human adipose tissue is an abundant source of multipotent stem cells, known as adipose stem cells (ASCs). They have the ability to differentiate toward several cell types of mesodermal origin [1,2], show low immunogenicity [3-5] and ability for immunomodulation [6-9]. Due to these characteristics, ASCs are promising candidates for clinical applications and several clinical trials are currently ongoing as reviewed [10,11].

Stem cell therapies often require large numbers of cells, and usually in vitro cell expansion precedes in vivo implantation. In vitro culture media typically provide only the essential components for the cells in order to support cell attachment and growth while maintaining basic in vitro conditions. Unfortunately, this poorly corresponds to the original tissue microenvironments, especially in regard to the overall concentration of biological macromolecules in the medium [12]. The macromolecular crowding (MMC) method addresses this question by modifying the micro-environment and facilitating the formation and remodeling of the extracellular matrix (ECM). Macromolecular crowders function by way of the excluded volume effect (EVE) [13]; the volume surrounding a given molecule becomes unavailable for other molecules and thus, effective concentrations of different macromolecules in the solution are higher. The amount of EVE is dependent on the fractional volume occupancy (FVO), which is defined as the fraction of the total volume occupied by macromolecules. Consequently, MMC influences many fundamentals such as rates of enzymatic reactions, formation of cytoskeleton, cell adhesion and migration [12-16].

The effect of MMC on cell behavior has mainly been studied in traditional fetal bovine serum (FBS) supplemented cultures [17-19]. However, in clinical use reagents of animal origin may elicit safety concerns due to risk of allergic reactions and rejection [20,21]. Therefore, xeno-free (XF) alternatives such as human serum (HS) [22-24] or platelet (PL) derived supplements [25-27] as well as fully defined XF and serum-free (SF) conditions [28,29] have been developed for ASC cultures. Although of human origin, the use of HS or its derivatives also has disadvantages such as limited availability and lot-to-lot variation affecting proliferation rate and differentiation potential of ASCs [30] that can be avoided by replacing undefined and/ or animal-derived components with fully defined XF/SF reagents that are more reproducible, robust, and efficient [28,29].

The aim of this study was to investigate if MMC supports the proliferation and multilineage differentiation capacity of ASCs and to analyze the effect of MMC on ASC immunophenotype and

morphology. In the current study, the cell isolation and expansion was carried out in parallel in FBS and HS containing media and compared to fully defined XF/SF conditions. To our knowledge, the MMC method has not been previously reported in ASC cultures, and especially the use of different culturing conditions (FBS, HS and XF/SF) under MMC is a novel approach. Our current study demonstrate the applicability of the MMC method on ASC cultures in different serum conditions, and show promising results of osteogenic and adipogenic differentiation of ASCs under MMC in FBS and HS cultures.

Methods

Ethical considerations and tissue procurement

The collection of adipose tissue was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere, Finland (ethical approval R03058). Adipose tissue samples were acquired from elective surgical procedures performed in the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland, with the patients' written consent. ASCs were isolated from adipose tissue samples that were obtained from four female donors (mean age 52 ± 12).

Isolation and expansion of ASCs

ASCs were isolated in parallel from adipose tissue samples into three different culture conditions: medium containing FBS, HS, or defined XF/SF culture conditions. Isolation of ASCs was carried out using a mechanical and enzymatic method as described previously [1,2,29,31]. Briefly, the adipose tissue was minced manually into small fragments and digested with collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany, http://www.serva.de) in a water bath at 37°C under shaking conditions. The digested tissue was centrifuged and filtered in sequential steps through a 100-mm pore size filter to separate the ASCs from the surrounding tissue. For FBS and HS conditions, Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 (Life Technologies, Rockville, MD, http://www.lifetech.com) was supplemented with 1% L-analyl-L-glutamine (GlutaMAX I; Life Technologies), 1% antibiotics (p/s; 100 U/mL penicillin, 0.1 mg/mL streptomycin; Lonza, Walkersville, MD, http://www.lonza.com) and either 10% FBS (Life Technologies) or 10% HS (human serum type AB; Lonza). For XF/SF conditions, the cells were isolated using a previously described method [29]. Briefly, adipose tissue was minced, digested, centrifuged and filtered similarly to FBS

and HS cultures, and finally seeded in carboxyl-coated flasks (PureCoat Carboxyl T75; BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) and expanded in STEMPRO MSC SFM (Life Technologies) supplemented with 1% GlutaMAX I, 0,3% antibiotics, and 10% StemPro MSC SFM Xeno-Free supplement. From passage 1 onwards, XF/SF cells were expanded in STEMPRO MSC medium supplemented with CELLstart CTS coating (Life Technologies) according to the manufacturer's instructions. ASCs were detached in all conditions using TrypLE Select (Life Technologies). The culture media formulations used for FBS, HS and XF/SF cultures are presented in Table 1. The analyses of immunophenotype, proliferation, metabolic activity and differentiation towards osteogenic and chondrogenic direction were performed separately with four donor cell lines isolated in FBS, HS, and XF/SF conditions, and the adipogenic differentiation was analyzed with one donor cell lines in FBS, HS, and XF/SF conditions.

Macromolecular crowding in ASC culture

A cocktail of macromolecules containing FicollTM400 (PM400, 17-0300-50; GE Healthcare, Bio-sciences AB) and FicollTM70 (PM70, 17-0310-50; GE Healthcare, Bio-Sciences AB) was dissolved into culture media at room temperature with gentle agitation. Fractional volume occupancy of 17% was achieved with concentrations 37.5mg/mL of FicollTM70 and 25mg/mL of FicollTM400 as described previously [13]. The MMC culture media was sterile filtered before use.

From passage 1 onwards, the cells isolated in FBS and HS containing medium were divided into two populations, and the cells were expanded under -/+MMC conditions until the analyses. For technical reasons, the XF/SF cells were divided into two populations from passage 2 onwards, and expanded under -/+MMC conditions until the analyses.

Adipose stem cell immunophenotype

ASCs expanded under -/+MMC in FBS, HS and SF/XF were analyzed with flow cytometry in passage 4 (FACSAria; BD Biosciences, Erembodegem, Belgium) to determine whether different culturing conditions have an effect on the immunophenotype of the cells. Monoclonal antibodies (MAbs) against CD11a–allophycocyanin (APC), CD80–phycoerythrin (PE), CD86–PE, CD105–PE (R&D Systems Inc., Minneapolis, MN, USA), CD-3 (PE), CD14–phycoerythrin-cyanine (PECy7), CD19-PECy7, CD45RO-APC, CD54-fluorescein isothiocyanate (FITC), CD73-PE, CD90-APC (BD Biosciences), and CD34-APC, HLADR-PE (Immunotools GmbH, Friesoythe, Germany) were used. Analysis was

performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels [31].

ASC morphology, metabolic activity and proliferation

ASCs were observed by light microscopy to detect morphological changes during cell expansion in FBS, HS and XF/SF conditions -/+MMC. The metabolic activity of ASCs in the aforementioned culture conditions were assessed with Cell Counting Kit -8 (CCK-8) (Takara Bio Inc., Shiga, Japan) in passage 4. The assay is based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase enzyme, which enables measuring of the cell proliferation and viability with a colorimetric assay. Tetrazolium salts are cleaved to formazan dye by the succinate-tetrazolium reductase which exists in mitochondrial respiratory chain and is active only in viable and metabolically active cells. ASCs were seeded on 48-well plates at a density of 2,500 cells/cm^2, and the metabolic/mitochondrial activity was assessed at 1, 4, 7, and 11 days. In brief, at each time point, the cell-culture medium was removed, and DPBS (Dulbecco Phosphate-Buffered Saline, Lonza, BioWhittaker, Verviers, Belgium) and CCK-8 reagent was added 10:1. The 48-well plate was incubated for 3 hours at 37°C, and the relative mitochondrial activity was measured in a microplate reader (Victor 1429 Multilabel Counter; Wallac; Turku, Finland) at 450 nm.

The cell number was quantitatively analyzed with CyQUANT® cell proliferation assay kit (Molecular Probes, Invitrogen, Paisley, UK) in passage 4, as described previously [32]. Briefly, cells were lysed using 0.1% Triton-X 100 buffer (Sigma-Aldrich), and the supernatant was collected and stored at -80°C until final analyses. Twenty micro liters of each sample were mixed with CyQUANT GR dye and lysis buffer in a 96-well plate (Nunc). Fluorescence signals were measured with a microplate reader at 480/520nm. The metabolic activity detected by CCK-8 Cell Proliferation Assay was normalized to the cell number that was quantified by CyQUANT® cell proliferation assay kit.

Trilineage differentiation potential

The osteogenic, adipogenic and chondrogenic differentiation potential was evaluated in XF/SF conditions versus HS and traditionally used FBS supplemented medium under -/+MMC conditions in passage 4. The osteogenic and adipogenic differentiation capacity of ASCs was analyzed in four different treatment groups in each culture condition: 1) expansion (E) -MMC; differentiation (D) -MMC 2) E-MMC; D+MMC 3) E+MMC; D-MMC 4) E+MMC; D+MMC, whereas the chondrogenic

differentiation was performed in two different treatment groups: 1) E+MMC; D-MMC 2) E-MMC; D-MMC. The culture media formulations used for differentiation assays are shown in Table 1.

For osteogenic differentiation, cells were seeded on 12-well plates at a density of 2.0 X 10^3 cells/cm^2 in control media and the induction was initiated 48 h after cell seeding. The osteogenic differentiation was determined by the quantitative Alizarin Red S method to detect calcium compounds deposited in the ECM during osteogenic differentiation, as described previously [24]. Briefly, cells were fixed with 4% paraformaldehyde (PFA) and stained with 2% Alizarin Red solution (Sigma-Aldrich; pH 4.2), followed by several washes with distilled water. Light microscopic cell imaging was used for qualitative analysis, and for quantitative analysis the Alizarin Red dye was extracted with 100 mM cetylpyridinium chloride (Sigma-Aldrich) under shaking for 3 h. The dye intensity was determined at 540 nm with a microplate reader.

For adipogenic differentiation, ASCs were seeded on 24-well plates at an initial density of 1.05 x 10^4 cells/cm^2. Adipogenic differentiation was initiated when the cells reached confluence as described previously [17] via three cycles of 4 days of induction, followed by 3 days of maintenance. After 21 days of differentiation, Nile Red staining and quantitative adherent cytometry was used to assess area of cytoplasmic lipid accumulation. In brief, cell cultures were rinsed with PBS, fixed in 4% PFA and co-stained for 30min with 5mg/mL Nile Red (N3013; Sigma), for cytoplasmic lipid droplets and 0.5 mg/mL of DAPI (D3571, Molecular Probes®; Life Techonologies) for nuclear DNA as previously described [33]. Adherent cytometry was performed according to a previously described protocol [17]. Briefly, cell images were acquired using 2x magnification with a cool-SNAP HQ camera attached to a Nikon TE2000 microscope (Nikon Instruments), and analyzed using the Metamorph Imaging System Software 6.3v3 (Molecular Devices). The extent of adipogenic differentiation was quantified by area of Nile Red fluorescence and normalized to nuclei count. End data corresponded to total area of lipid droplets present per well normalized to cell number (µm^2/ nuclei).

The chondrogenic differentiation potential was assessed by a micro mass culture method as described previously [31,34-36]. In brief, 8 x 10^4 cells were seeded on a 24-well culture plate in a 10 µl volume and were allowed to adhere for 3 hour prior the addition of chondrogenic induction medium. After 21 days of chondrogenic induction, differentiation was confirmed using Alcian blue staining method as described earlier [36]. In brief, the micro mass cultures were fixed with 4% PFA and stored in 70% ethanol. Pellets were dehydrated, embedded in paraffin, and sectioned at 5-mm thickness. The sections were stained with Alcian blue (pH 1.0) to verify the presence of sulfated

glycosaminoglycans (GAGs) with Nuclear Fast Red solution (Biocare Medical, Concord, MA, USA) as a counterstain.

Statistical analyses

Statistical analyses were performed with IBM SPSS software version 21 (IBM Corp., Armonk, NY, www.ibm.com). Since the data was not normally distributed, a nonparametric Mann-Whitney U test was used to analyze the effect of different culture conditions on cell proliferation rate, cell surface marker expression, and differentiation potential. When multiple comparisons were performed, the p values were multiplied by the number of comparisons before the interpretation of the data. Thus, for proliferation (CyQUANT), metabolic activity (CCK-8) and quantitative AR staining data, the p value was multiplied by 42 that was a total number of performed comparisons. The nonparametric Spearman correlation test was used to study correlation between DNA amounts and metabolic activity of the cells. The results were considered statistically significant when the p value was under 0.05. The data were presented as mean ± standard deviation (SD).

Results

The effect of MMC on cell surface markers

Cell surface marker expression of ASCs was analyzed after MMC treatment to determine the effect of crowding on cell surface protein expressions. ASCs cultured in FBS and HS supplemented medium were exposed to MMC during the cell expansion from passage 1 to 3. The MMC conditions did not support the expansion of ASCs in XF/SF media, and the cells showed poor viability after culture with MMC agents. Thus, the flow cytometric analysis of XF/SF cells was performed after one week of culture under MMC conditions. At large, the characteristic immunophenotype of ASCs was maintained after MMC exposure. ASCs expressed stem markers CD73, CD90 and CD105 in both -/+MMC conditions in all of the studied culture conditions (Figure 1A). ASCs also lacked the expression of CD3, CD11a, CD14, CD19, CD80, CD86, and HLA-DR in both -/+MMC conditions (Figure 1B). The effect of MMC on the expression of CD54 was statistically significant and a higher expression of CD54 was observed under MMC cultures ($p<0.05$) in all studied culture conditions. On average, ASCs showed low to moderate expression for hematopoietic progenitor stem cell marker CD34 in all of the studied culture conditions. Although some variation were observed between

culture conditions for CD34, no statistical differences were observed due to large standard deviations between donor cell lines.

Cell morphology, number and metabolic activity

The effect of MMC on cell morphology was evident in all studied culture conditions, especially the morphology of XF/SF cultured cells which was changed dramatically after exposure to MMC (Figure 2). During the culture under MMC, cells adopted rounded morphology and became larger in size. Many cell extensions were observed in XF/SF cells after culture in MMC medium. In XF/SF conditions, the cell viability was poor in MMC cultures which could also be seen from the morphological images. The cell number dramatically decreased compared to standard cultures and cells cultured under MMC were larger with many nuclei in some cases (Figure 2, right column).

The DNA amount demonstrating cell number in different culture conditions was analyzed with CyQUANT® cell proliferation assay (Figure 3). A significantly higher cell number was observed under standard cultures in XF/SF conditions compared to FBS ($p<0.001$) and HS ($p<0.05$) media at each time points day 1, 4, 7, and 11. Compared to MMC cultures, the cell number was significantly higher under standard culture conditions in HS media at day 1 ($p<0.05$), and in FBS media at days 4 ($p<0.05$), 7 and 11 ($p<0.001$). Under MMC condition at time points day 7 and 11, the cell number was significantly higher in HS media ($p<0.05$) compared to FBS media. Nevertheless, a statistically significant increase in cell number from day 1 to day 11 was observed in each culture media in both -/+MMC conditions, except for the cells cultured in XF/SF under MMC. The cell proliferation assay could only be performed with one donor cell line after MMC expansion in XF/SF conditions due to low viability. Although a decrease in XF/SF cell number was notable under MMC, no statistical differences could be established due to the low number of repeats.

The cell metabolic activity under -/+MMC cultures was analyzed with CCK-8 assay, and normalized to total DNA quantified by CyQUANT® cell proliferation assay (Figure 4). A statistically significant increase in metabolic activity was observed in FBS media ($p<0.001$) in MMC conditions at 1 day time point compared standard FBS conditions. Furthermore, cells cultured under MMC condition in FBS media had a significantly higher metabolic activity compared to ASCs cultured in HS media at time points 4 ($p<0.001$), 7 and 11 ($p<0.05$) days. Although the metabolic activity was decreased in XF/SF cultures after MMC, no statistical differences could be established due to the low number of repeats. The metabolic activity increased significantly ($p <0.001$) over time (from day 1 to day 11) in

each culture condition in standard cultures, and in FBS media under MMC conditions. The metabolic activity also correlated with the ASC number, that was analyzed with the Spearman correlation test (coefficient 0.78; $p < 0.001$).

Multilineage differentiation potential of ASCs under MMC

Based on the results of quantitative Alizarin Red staining (Figure 5), ASCs expanded in HS containing medium showed the most efficient osteogenic differentiation capacity compared to FBS cultures in three treatment groups 1) E-MMC; D-MMC, 3) E+MMC; D-MMC, 4) E+MMC; D+MMC ($p<0.05$), and compared to XF/SF conditions in two treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC ($p<0.05$). Moreover, stronger differentiation potential was observed in FBS conditions in treatment group 2) E-MMC; D+MMC compared to XF/SF conditions in the same treatment group ($p<0.05$).

Cells cultured in FBS media in treatment group 2) E-MMC; D+MMC showed stronger osteogenic differentiation potential compared to treatment groups 1) E-MMC; D-MMC and 3) E+MMC; D-MMC ($p<0.05$) in FBS media. The viability of ASCs was poor under MMC in XF/SF conditions and thus, the most efficient osteogenic differentiation was observed in treatment group 1) E-MMC; D-MMC, in which a stronger differentiation capacity was observed compared to group 2) E-MMC; D+MMC ($p<0.05$). Interestingly, one of the four donors cell lines survived during expansion under MMC in XF/SF conditions, and showed strong differentiation capacity after induction in standard XF/SF media in treatment group 3) E+MMC; D-MMC. Furthermore, ASCs cultured in HS conditions showed strong osteogenic differentiation potential in all studied treatment groups, however, no statistical differences were observed between groups in HS media.

Compared to control cultures of the same treatment group, superior differentiation potential was observed in all four induction cultures in HS media ($p<0.05$), whereas in FBS media a significant difference between induction and control cultures was only observed in two treatment groups 1) E-MMC; D-MMC and 2) E-MMC; D+MMC ($p<0.05$) (supplementary Figure 1). The AR staining results in different treatment groups under FBS, HS and XF/SF conditions are presented in Figure 6. The altered cell morphology as well as large cell size in XF/SF conditions under MMC exposure is also visible in Figure 6 (XF/SF cells in treatment group 4) E+MMC; D+MMC).

The most efficient adipogenic differentiation was observed in HS containing media based on the results of Nile Red staining that was normalized to cell number (Figure 7). MMC clearly supported

adipogenic commitment of ASCs cultured in FBS and HS conditions, and cells accumulated more lipid content under MMC induction in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC as compared to induction in standard media in treatment groups 1) E-MMC; D-MMC and 3) E+MMC; D-MMC. Compared to control cultures of the same treatment group, clearly stronger differentiation potential was observed in all four induction cultures in FBS and HS media. The cell viability was poor in XF/SF cultures under MMC and thus, MMC did not support adipogenic differentiation of XF/SF cells. The Nile Red staining of lipid droplets in different treatment groups under FBS, HS and XF/SF conditions is presented in Figure 8.

Chondrogenic differentiation of ASCs was efficient in all studied culture conditions, however more ECM was deposited after expansion in -MMC conditions based on qualitative Alcian blue staining. Interestingly, the cells expanded under MMC conditions prior to chondrogenic induction formed a less dense histological architecture of the micro mass pellet (Figure 9). However, apart from the dissimilar structure, blue color intensity of the stained proteoglycans was strong in both -/+MMC treatment groups based on Alcian blue staining.

Discussion

As demonstrated in previous studies, MMC of culture medium will increase the thermodynamic activities and biological processes by several orders of magnitude, and promote cells to re-create more robust microenvironment in vitro [15,37]. As cell-based treatments are becoming more common, efficient in vitro cell expansion methods are required to obtain clinically sufficient cell number for effective cell treatments. In the current study, we demonstrate the effect of MMC on ASCs proliferation and differentiation under clinically relevant culture conditions. To our knowledge, this is a novel approach, and the influence of the MMC method on ASCs characteristics has not been previously reported. However, we and others have shown that culture conditions influence the proliferation rate and the differentiation potential of the ASCs [28,29]. Therefore, in the current study, MMC was combined with different culture conditions in order to determine the potential of MMC for supporting ASC proliferation and differentiation under XF/SF conditions as compared FBS and HS media. In previous studies, the benefits of MMC cultures have been shown to increase proliferation rates as well as efficient adipogenic differentiation of human bone marrow mesenchymal stem cells (BMSCs) [13,17,38].

Contrary to previous results on BMSCs, the proliferation capacity of ASCs was diminished under MMC in all studied culture conditions. MMC especially affected the proliferation capacity of ASCs under XF/SF conditions. Cells expanded in standard XF/SF conditions had superior proliferation rates compared to cells cultured in standard FBS and HS cultures, however proliferation of XF/SF cells was clearly reduced under MMC. Interestingly, ASCs expanded in FBS conditions under MMC showed increased metabolic activity compared to standard FBS cultures, whereas the metabolic activity was reduced under MMC in HS and XF/SF cultures.

In addition, changes in cell morphology were noticed in all serum conditions in ASCs expanded under MMC. As MMC induce efficient ECM production [13,17], cell adherence became stronger, which was possibly reflected by larger and more cubical cell morphology. Interestingly, the major effect of MMC was evident in XF/SF cells whose morphology was fully transformed by the MMC culture. As the cell number significantly decreased in XF/SF conditions after longer-term MMC exposure, the cells became large and round with many extensions. Of note, several nuclei were observed in some XF/SF cells after longer MMC culture period. To our knowledge, similar observations have not been reported earlier, however our study was the first report to use MMC with XF/SF cultured ASCs. The results clearly showed that MMC method is not suitable for studied XF/SF cultures.

Of note, previous reports of increased proliferation rates under MMC were performed with BMSCs in FBS containing media, which may explain the different outcome. The MMC functions by way of the EVE that is dependent on the FVO that denotes the fraction of the total volume occupied by macromolecules [13]. A mixture of 70 and 400 kDa Ficoll as a crowding agent was first reported by Chen et al. who calculated the final concentration of the culture medium to a level corresponding to the protein concentration of blood plasma that is approximately 80 mg/mL [13]. The 17% FVO is calculated as being optimal for BMSCs, however, it should be further optimized for ASC cultures. Also the effect of different serum conditions may change the equilibrium of the optimized 17% MMC occupancy. The basis for MMC function is its ability to support cells to re-create their own microenvironment in vitro. As discussed by Chen et al., cells that do not produce much ECM, cannot be induced to build the microenvironment even under MMC [13]. It can be speculated that, at least in chemically defined basic XF/SF cultures, the ECM production by ASCs may be reduced hindering the suitability of MMC methods for XF/SF cells. Also, the weak cell adherence that has been observed with XF/SF cells [29] may interfere with the effects of MMC under XF/SF cultures.

The typical immunophenotype of ASCs was maintained in MMC cultures in all of the studied culture conditions. Low to moderate expression was observed for CD34 which is typical for ASCs, and similar expression levels has been reported previously [39,40]. CD34 is typically expressed during the early phase of culture and the expression is greatly dependent on the in vitro culture period of cells [41,42]. In XF/SF conditions, however, the flow cytometric analysis was performed after 7 days exposure to MMC, since longer-term culture with MMC was not feasible. The immunophenotype of HS and FBS cultured cells was analyzed after MMC culture until passage 3. Interestingly, the expression of CD54 was significantly higher under MMC conditions in all of the studied serum conditions. As shown in previous studies, ECM is extensively deposited under MMC conditions [13,17] leading to more mature ECM, which may be reflected by stronger cell adhesion and higher expression of the adhesion molecule CD54. In previous studies, the intra- and extracellular protein organization between -/+MMC cultures have been compared, and it has been shown that crowding itself is able to further align ECM even in the absence of cellular interaction [12]. The ECM in turn affects cell-matrix interactions and promotes cell adhesion, thus having an influence on the formation and structure of the cytoskeleton [12]. Thus, the increased CD54 expression may be associated with stronger cell adhesion, as higher CD54 expression was observed for MMC exposed cells, as oppose to lower CD54 levels of weakly attached XF/SF cells.

The effect of MMC on ASC differentiation towards adipogenic, osteogenic and chondrogenic directions was studied in different culture conditions. As shown in a previous study using BMSCs, crowding facilitates microenvironment formation and stabilizes or drives differentiation [17]. Ang et al. have demonstrated that in adipogenic induction media in MMC conditions the ECM was extensively remodeled toward a pro-adipogenic microenvironment, which further promoted the adipogenic differentiation of cells. Thus, MMC method could be considered to direct ASC MSC differentiation in vitro.

While MMC exposure did not enhance ASC proliferation in the present study, the cells displayed efficient osteogenic and adipogenic differentiation, as observed with FBS and HS expanded cells. In FBS conditions, significantly stronger osteogenic differentiation was observed in MMC condition as compared to induction in standard condition, when the cells were expanded under standard culture conditions. ASCs accumulated plenty of lipid droplets under MMC induction compared to induction under standard conditions, after expansion in both -/+MMC conditions, which suggest supportive influence of MMC on adipogenic commitment of cells in serum containing medium. In contrast, cells

cultured in XF/SF conditions did not respond to MMC during differentiation induction. Significant differences in the intensity of osteogenic differentiation were also observed between HS and FBS cultures. As compared to HS cultures, FBS medium did not support efficient osteogenic differentiation. However, lot-to-lot variation in serum performance may affect the differentiation potential of ASCs [30]. In line with this result, similar diminished osteogenic differentiation potential in FBS medium compared HS medium has been reported previously for ASCs by our group [24].

Moreover, chondrogenic differentiation was studied in standard induction media (-MMC) after cell expansion in both -/+MMC culture conditions. Based on qualitative Alcian blue staining, more efficient chondrogenic differentiation and more ECM deposition was observed for cells that were expanded in standard condition compared to +MMC condition. The micro mass pellet structure was altered depending on the MMC treatment and consequently, the cells that were differentiated under MMC conditions were less dense histologically. It may be speculated that Ficoll particles are transported into ASCs, and observed as gaps inside the histological pellet structure. Thus, Ficoll would have also other than MMC effects on ASCs. In line with our hypothesis, Rashid et a. have recently shown that Ficoll is pinocytosed by BMSCs and subsequently transported into mitochondria [43]. However, they also demonstrated that the intracellular level of Ficoll was decreased over time, suggesting that it does not persist within cells.

Conclusions

Careful characterization of ASC behavior with regards to different in vitro culture conditions is highly important for the development of cell-based therapies. In the current study, MMC was studied as an alternative method to facilitate ASC differentiation and proliferation. Characteristic immunophenotype of ASCs was maintained after MMC cultures, except for the expression of CD54 that was significantly increased in all of the studied serum conditions under MMC. The higher CD54 expression may be associated with a more mature ECM and stronger cell adhesion under MMC. MMC method did not support ASC proliferation in any of the studied conditions, however metabolic activity was increased under MMC in FBS cultures. Importantly, MMC had supportive influence on adipogenic and osteogenic differentiation of ASCs in FBS and HS containing media. Osteogenic differentiation was stronger when cells were expanded in standard culture medium prior to differentiation under MMC, whereas adipogenic differentiation under MMC was efficient after expansion in both -/+MMC conditions. Based on Alcian blue staining, chondogenic differentiation

was stronger after expansion in -MMC condition and moreover, MMC altered the histological structure of micro mass pellets into less dense architecture. MMC method was not suitable for ASC cultures in studied XF/SF conditions since the cells were not viable after long-term exposure to MMC, which was observed by reduced cell number and dramatically changed morphology of XF/SF cultured ASCs.

List of abbreviations

APC, allophycocyanin; ASCs, adipose stem cell; CCK-8, Cell Counting Kit -8; CD, cluster of differentiation; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; E-MMC; D-MMC; expansion-MMC; differentiation-MMC; EVE, excluded volume effect; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FVO, fractional volume occupancy; HS, human serum; ICAM-1, intercellular adhesion molecule 1; MMC, macromolecular crowding; MSC SFM, mesenchymal stem cell serum-free media; PE, phycoerythrin; PECy7, phycoerythrin-cyanine; PFA, paraformaldehyde; SD, standard deviation; XF/SF, xeno-free and serum-free

Competing interests

The author(s) declare that they have no competing interests. The authors alone are responsible for the content and writing of the manuscript.

Authors' contributions

MP and LM performed the proliferation, metabolic activity, immunophenotypic, and osteogenic and chondrogenic differentiation experiments, whereas LHCM performed the adipogenic differentiation studies in co-operation with AXM. MP performed statistical analyses and wrote the manuscript. LM and LHCM also participated in drafting the manuscript. SM, BM and MR participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

[1] Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy. 2003;5:362-369.

[2] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002;13:4279-4295.

[3] McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, et al. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells. 2006;24:1246-1253.

[4] Niemeyer P, Kornacker M, Mehlhorn A, Seckinger A, Vohrer J, Schmal H, et al. Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. Tissue Eng. 2007;13:111-121.

[5] Ren ML, Peng W, Yang ZL, Sun XJ, Zhang SC, Wang ZG, et al. Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs. Cell Transplant. 2012;21:2711-2721.

[6] Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol. 2005;129:118-129.

[7] Kronsteiner B, Wolbank S, Peterbauer A, Hackl C, Redl H, van Griensven M, et al. Human Mesenchymal Stem Cells from Adipose Tissue and Amnion Influence T-Cells Depending on Stimulation Method and Presence of Other Immune Cells. Stem Cells Dev. 2011;20:2115-2126.

[8] Cui L, Yin S, Liu W, Li N, Zhang W, Cao Y. Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2. Tissue Eng. 2007;13:1185-1195.

[9] Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol. 2010;162:474-486.

[10] Casteilla L, Planat-Benard V, Laharrague P, Cousin B. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. World J Stem Cells. 2011;3:25-33.

[11] Lim MH, Ong WK, Sugii S. The current landscape of adipose-derived stem cells in clinical applications. Expert Rev Mol Med. 2014;16:e8.

[12] Zeiger AS, Loe FC, Li R, Raghunath M, Van Vliet KJ. Macromolecular crowding directs extracellular matrix organization and mesenchymal stem cell behavior. PLoS One. 2012;7:e37904.

[13] Chen C, Loe F, Blocki A, Peng Y, Raghunath M. Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Adv Drug Deliv Rev. 2011;63:277-290.

[14] Chen CZ, Peng YX, Wang ZB, Fish PV, Kaar JL, Koepsel RR, et al. The Scar-in-a-Jar: studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well. Br J Pharmacol. 2009;158:1196-1209.

[15] Zhou HX, Rivas G, Minton AP. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. Annu Rev Biophys. 2008;37:375-397.

[16] Ellis RJ. Macromolecular crowding: an important but neglected aspect of the intracellular environment. Curr Opin Struct Biol. 2001;11:114-119.

[17] Ang XM, Lee MH, Blocki A, Chen C, Ong LL, Asada HH, et al. Macromolecular crowding amplifies adipogenesis of human bone marrow-derived mesenchymal stem cells by enhancing the pro-adipogenic microenvironment. Tissue Eng Part A. 2014;20:966-981.

[18] Dewavrin JY, Hamzavi N, Shim VP, Raghunath M. Tuning the architecture of three-dimensional collagen hydrogels by physiological macromolecular crowding. Acta Biomater. 2014;10:4351-4359.

[19] Satyam A, Kumar P, Fan X, Gorelov A, Rochev Y, Joshi L, et al. Macromolecular crowding meets tissue engineering by self-assembly: a paradigm shift in regenerative medicine. Adv Mater. 2014;26:3024-3034.

[20] Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood. 1997;89:776-779.

[21] Kadri N, Potiron N, Ouary M, Jegou D, Gouin E, Bach JM, et al. Fetal calf serum-primed dendritic cells induce a strong anti-fetal calf serum immune response and diabetes protection in the non-obese diabetic mouse. Immunol Lett. 2007;108:129-136.

[22] Josh F, Kobe K, Tobita M, Tanaka R, Suzuki K, Ono K, et al. Accelerated and Safe Proliferation of Human Adipose-derived Stem Cells in Medium Supplemented with Human Serum. J Nippon Med Sch. 2012;79:444-452.

[23] Tateishi K, Ando W, Higuchi C, Hart DA, Hashimoto J, Nakata K, et al. Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications. Cell Transplant. 2008;17:549-557.

[24] Kyllonen L, Haimi S, Mannerstrom B, Huhtala H, Rajala KM, Skottman H, et al. Effects of different serum conditions on osteogenic differentiation of human adipose stem cells in vitro. Stem Cell Res Ther. 2013;4:17.

[25] Trojahn Kolle SF, Oliveri RS, Glovinski PV, Kirchhoff M, Mathiasen AB, Elberg JJ, et al. Pooled human platelet lysate versus fetal bovine serum-investigating the proliferation rate, chromosome stability and angiogenic potential of human adipose tissue-derived stem cells intended for clinical use. Cytotherapy. 2013;15:1086-1097.

[26] Blande IS, Bassaneze V, Lavini-Ramos C, Fae KC, Kalil J, Miyakawa AA, et al. Adipose tissue mesenchymal stem cell expansion in animal serum-free medium supplemented with autologous human platelet lysate. Transfusion. 2009;49:2680-2685.

[27] Naaijkens BA, Niessen HW, Prins HJ, Krijnen PA, Kokhuis TJ, de Jong N, et al. Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. Cell Tissue Res. 2012;348:119-130.

[28] Al-Saqi SH, Saliem M, Asikainen S, Quezada HC, Ekblad A, Hovatta O, et al. Defined serum-free media for in vitro expansion of adipose-derived mesenchymal stem cells. Cytotherapy. 2014;16:915-926.

[29] Patrikoski M, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerstrom B, et al. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. Stem Cell Res Ther. 2013;4:27.

[30] Parker AM, Shang H, Khurgel M, Katz AJ. Low serum and serum-free culture of multipotential human adipose stem cells. Cytotherapy. 2007;9:637-646.

[31] Lindroos B, Boucher S, Chase L, Kuokkanen H, Huhtala H, Haataja R, et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy. 2009;11:958-972.

[32] Lindroos B, Maenpaa K, Ylikomi T, Oja H, Suuronen R, Miettinen S. Characterisation of human dental stem cells and buccal mucosa fibroblasts. Biochem Biophys Res Commun. 2008;368:329-335.

[33] Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. J Cell Biol. 1985;100:965-973.

[34] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211-228.

[35] Denker AE, Nicoll SB, Tuan RS. Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1. Differentiation. 1995;59:25-34.

[36] Maenpaa K, Ella V, Mauno J, Kellomaki M, Suuronen R, Ylikomi T, et al. Use of adipose stem cells and polylactide discs for tissue engineering of the temporomandibular joint disc. J R Soc Interface. 2010;7:177-188.

[37] Kumar P, Satyam A, Fan X, Rochev Y, Rodriguez BJ, Gorelov A, et al. Accelerated Development of Supramolecular Corneal Stromal-Like Assemblies from Corneal Fibroblasts in the Presence of Macromolecular Crowders. Tissue Eng Part C Methods. 2015;.

[38] Rashid R, Lim NS, Chee SM, Png SN, Wohland T, Raghunath M. Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. Tissue Eng Part C Methods. 2014;20:994-1002.

[39] Mirabet V, Solves P, Minana MD, Encabo A, Carbonell-Uberos F, Blanquer A, et al. Human platelet lysate enhances the proliferative activity of cultured human fibroblast-like cells from different tissues. Cell Tissue Bank. 2008;9:1-10.

[40] Rebelatto CK, Aguiar AM, Moretao MP, Senegaglia AC, Hansen P, Barchiki F, et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Exp Biol Med (Maywood). 2008;233:901-913.

[41] Maumus M, Peyrafitte JA, D'Angelo R, Fournier-Wirth C, Bouloumie A, Casteilla L, et al. Native human adipose stromal cells: localization, morphology and phenotype. Int J Obes (Lond). 2011;35:1141-1153.

[42] Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells. 2006;24:376-385.

[43] Rashid R, Beyer S, Blocki A, Le Visage C, Trau D, Wohland T, et al. Mitochondrial routing of glucose and sucrose polymers after pinocytotic uptake: avenues for drug delivery. Biomacromolecules. 2014;15:2119-2127.

Figure legends

Figure 1. Surface marker expression of undifferentiated ASCs in different culture conditions FBS, HS and XF/SF -/+MMC.

The characteristic immunophenotype of ASCs was maintained under MMC, except the expression of marker CD54 that was significantly increased under MMC culture in every culture condition. Immumophenotype of ASCs was analyzed by flow cytometry at passages 3. Data are presented as mean ± SD. MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 2. Morphology of ASCs in standard cultures and in MMC conditions.

ASCs cultured under MMC adopted rounded morphology and became larger in size. The cell viability was poor under MMC in XF/SF cultures and thus, cell number decreased dramatically compared to standard cultures. MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 3. Cell number under different culture conditions analyzed with CyQUANT® cell proliferation assay.

Significantly higher cell number was observed under standard cultures in XF/SF conditions compared to FBS ($p<0.001$) and HS ($p<0.05$) media at each time point 1, 4, 7, and 11 days. Cell number was also significantly decreased after MMC crowding in FBS and HS media. * indicates $p<0.05$; ** indicates $p<0.001$; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free

Figure 4. Metabolic activity of ASCs in different culture conditions -/+MMC.

Metabolic activity was analyzed with CCK-8 assay and normalized to DNA amount (CyQUANT proliferation assay). Metabolic activity was increased in FBS media under MMC conditions compared standard FBS conditions at day 1, as well as compared to HS conditions under MMC at days 4, 7 and 11. Metabolic activity also correlated with the cell number. * indicates $p<0.05$; ** indicates $p<0.001$; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 5. Quantitative Alizarin Red staining indicating the osteogenic differentiation potential of ASCs under different treatment groups.

The most efficient osteogenic differentiation was observed in HS media. Numbering 1) - 4) corresponds to treatment groups described in the text. * indicates $p<0.05$; E, expansion; D, differentiation; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 6. Alizarin Red staining in FBS, HS and XF/SF conditions under four different MMC treatment groups.

The most efficient osteogenic differentiation was observed in HS supplemented culture media. XF/SF cells only differentiated under osteogenic induction in standard XF/SF cultures (-MMC). E, expansion; D, differentiation; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 7. Quantitative Nile Red staining normalized to cell number.

Adipogenic differentiation was enhanced under MMC induction in FBS and HS conditions in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC as compared to induction in standard media, as quantitated by the area of Nile Red staining of lipid droplets. The most efficient adipogenic differentiation was observed in HS containing media. Numbering 1) - 4) corresponds to treatment groups described in the text. E, expansion; D, differentiation; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 8. Nile Red staining indicating the adipogenic differentiation in different treatment groups.

The accumulation of lipid droplets was increased under MMC induction in FBS and HS conditions in treatment groups 2) E-MMC; D+MMC and 4) E+MMC; D+MMC as compared to induction in standard media. However, the cell viability was poor under MMC in XF/SF conditions and thus, MMC did not support the adipogenic differentiation of XF/SF cells. Numbering 1) - 4) corresponds to treatment groups described in the text. E, expansion; D, differentiation; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Figure 9. Alcian blue staining indicating the chondogenic differentiation in different conditions after cell expansion +/-MMC.

More ECM was deposited after expansion in -MMC conditions and the histological structure of the micro mass pellets was more compacted, as compared to expansion under MMC conditions. MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Supplementary Figure 1. Quantitative Alizarin Red staining of ASCs under different treatment groups in FBS, HS and XF/SF conditions.

Compared to control cultures of the same treatment group, superior differentiation potential was observed in all four induction cultures in HS media, whereas in FBS media a significant difference between induction and control cultures was observed in two treatment groups 1) E-MMC; D-MMC and 2) E-MMC; D+MMC. Numbering 1) - 4) corresponds to treatment groups described in the text. E, expansion; D, differentiation; MMC, macromolecular crowding; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Table 1. Culture media formulations used for cell expansion and differentiation assays. MSC, mesenchymal stem cell; SFM, serum free medium; p/s, penicillin/streptomycin; FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Table

Medium	Basal media	Serum	Coating/coating-free supplements	Supplementation
Expansion FBS	DMEM/F-12 (Life Technologies, Gibco, Rockville, MD)	10% FBS (Life Technologies)	none	1% GlutaMAX (GlutaMAX I; Life Technologies), 1% p/s (p/s; 100 U/mL penicillin, 0.1 mg/mL streptomycin; Lonza)
Expansion HS	DMEM/F-12	10% HS (Lonza, Walkersville, MD)	none	1% GlutaMAX, 1% p/s
Expansion XF/SF	StemPro® MSC SFM (Life Technologies)	none	CELLstart™ coating (Life Technologies)	1% GlutaMAX, 0,3% p/s, StemPro® MSC SFM Xeno-free supplement (Life Technologies)
Adipogenic FBS/HS	DMEM/F-12	10% FBS/HS	none	For 4 days induction during cyclic differentiation: 1% GlutaMAX, 1% p/s, 0.5mM isobutylmethylxanthine (IBMX; Sigma), 0.2mM indomethacin (Sigma), 1µM dexamethasone (Sigma), and 10µg/mL insulin (Sigma)
Adipogenic XF/SF	StemPro® MSC SFM	none	CELLstart™ coating	For 4 days induction during cyclic differentiation: 1% GlutaMAX, 0,3% p/s, StemPro® MSC SFM Xeno-free supplement, same adipogenic supplements than in FBS/HS cultures
Osteogenic FBS/HS	DMEM/F-12	10% FBS/HS	none	1% GlutaMAX, 1% p/s, 150 µM L-ascorbic acid 2-phosphate (Sigma), 10mM beta-glycerophosphate (Sigma), 10 nM dexamethasone (Sigma)
Ostegenic XF/SF	StemPro® MSC SFM	none	CELLstart™ coating	1% GlutaMAX, 0,3% p/s, StemPro® MSC SFM Xeno-free supplement, same osteogenic supplements than in FBS/HS cultures
Chondrogenic FBS/HS	DMEM/F-12	none	none	1% GlutaMAX, 0,2% p/s, 1x ITS+1 (Sigma), 50 µM L-ascorbic acid 2-phosphate, 55 µM sodium pyruvate (Life Technologies), 23 µM L-proline (Sigma), 10 ng/mL TGF-β (Sigma)
Chondrogenic XF/SF	StemPro® MSC SFM	none	none	1% GlutaMAX, 0,2% p/s, StemPro® MSC SFM Xeno-free supplement, same chondrogenic supplements than in FBS/HS cultures

Figure 1

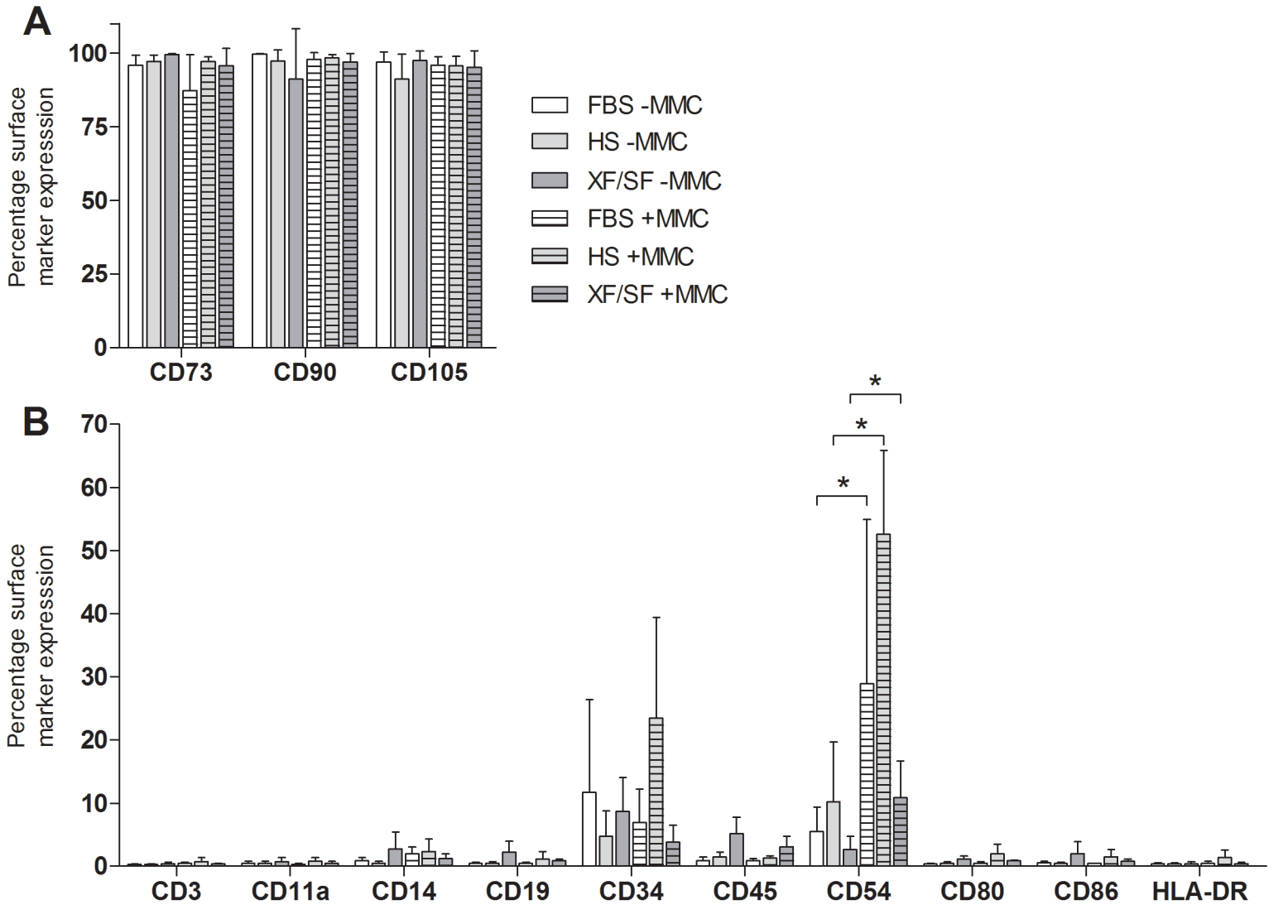

A
Percentage surface marker expresssion
100
75
50
25
0
CD73
CD90
CD105
FBS -MMC
HS -MMC
XF/SF -MMC
FBS +MMC
HS +MMC
XF/SF +MMC
B
Percentage surface marker expresssion
70
60
50
40
30
20
10
0
*
*
*
CD3
CD11a
CD14
CD19
CD34
CD45
CD54
CD80
CD86
HLA-DR

Figure 2

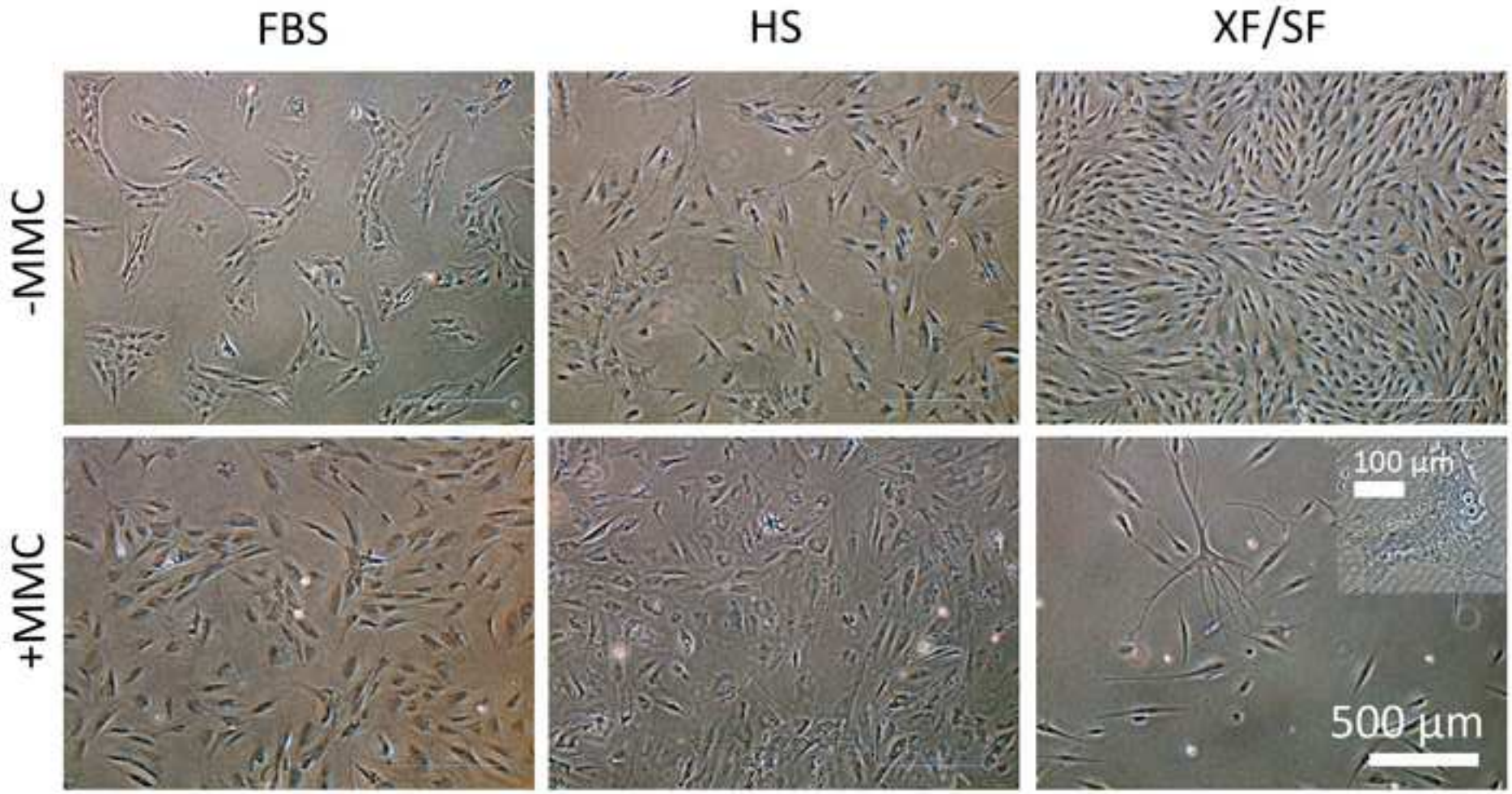


Figure 3

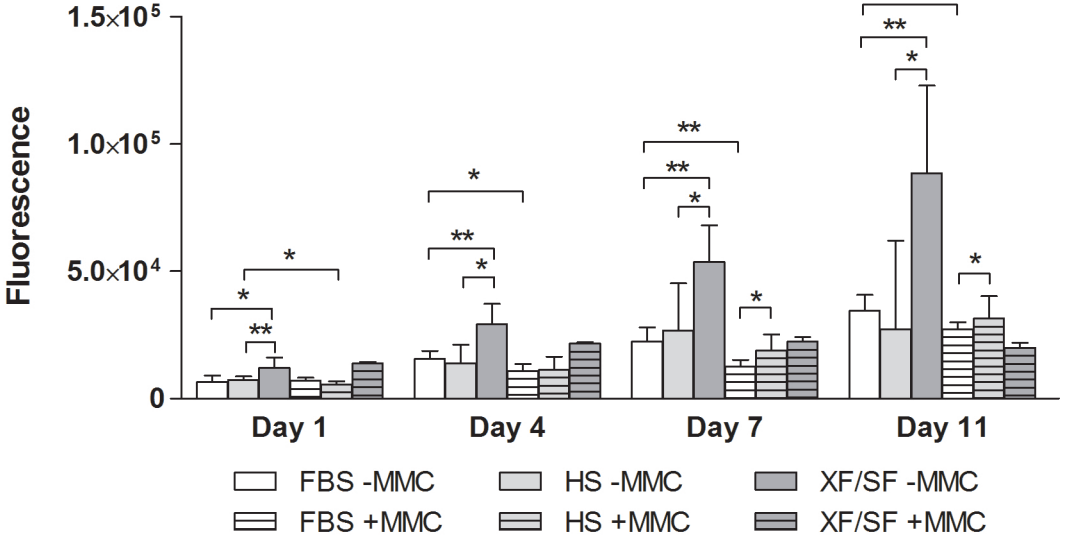

Fluorescence
1.5×10^5
1.0×10^5
5.0×10^4
0
Day 1
Day 4
Day 7
Day 11
FBS -MMC
FBS +MMC
HS -MMC
HS +MMC
XF/SF -MMC
XF/SF +MMC

Figure 4

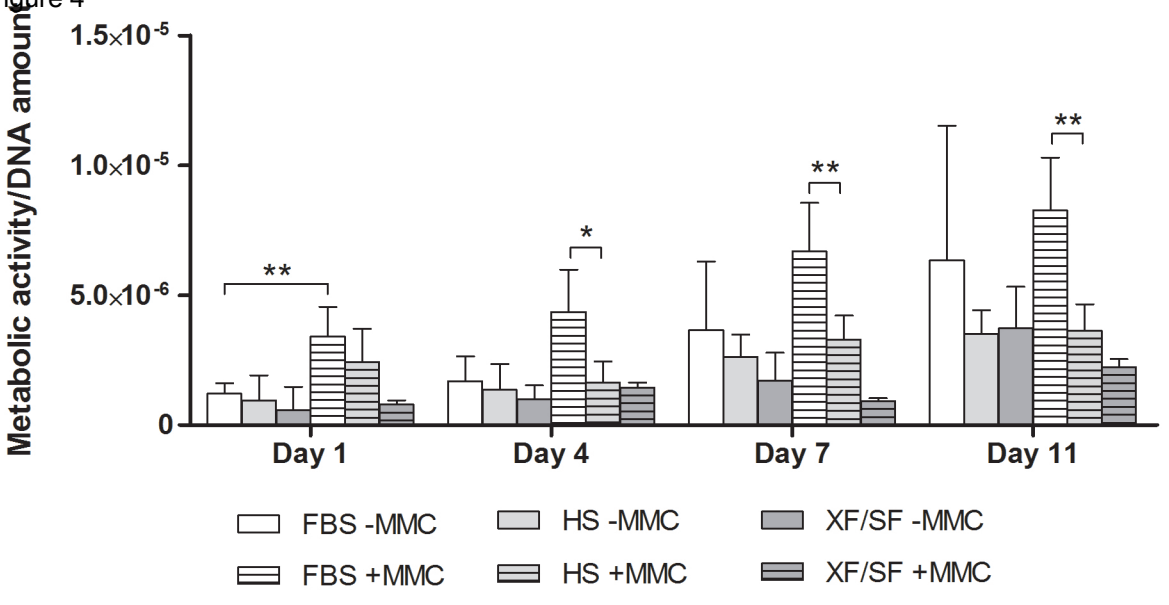

Metabolic activity/DNA amount
1.5×10-5
1.0×10-5
5.0×10-6
0
**
*
**
**
Day 1
Day 4
Day 7
Day 11
FBS -MMC
FBS +MMC
HS -MMC
HS +MMC
XF/SF -MMC
XF/SF +MMC

Figure 5

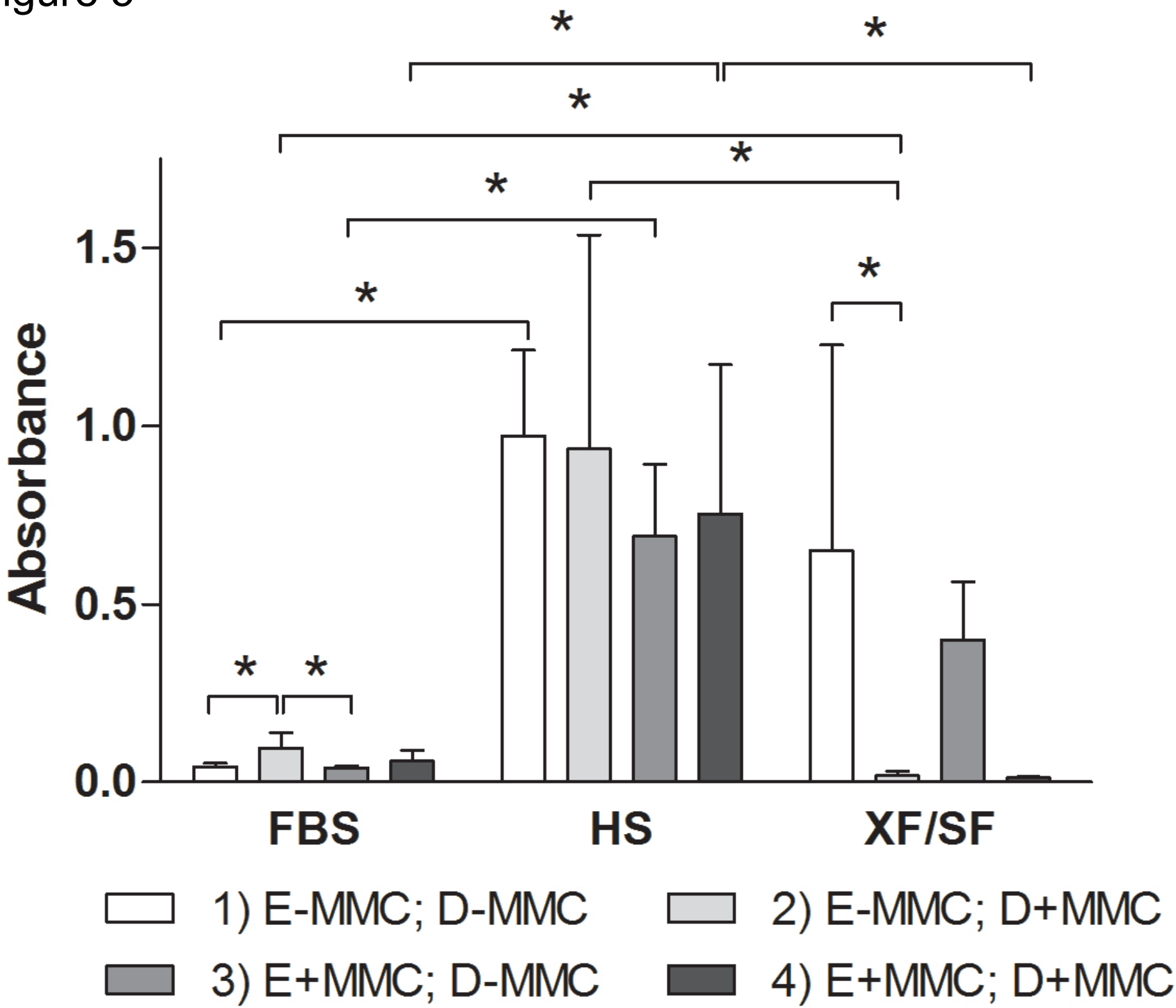

Absorbance
1.5
1.0
0.5
0.0
FBS
HS
XF/SF
1) E-MMC; D-MMC
2) E-MMC; D+MMC
3) E+MMC; D-MMC
4) E+MMC; D+MMC

Figure 6

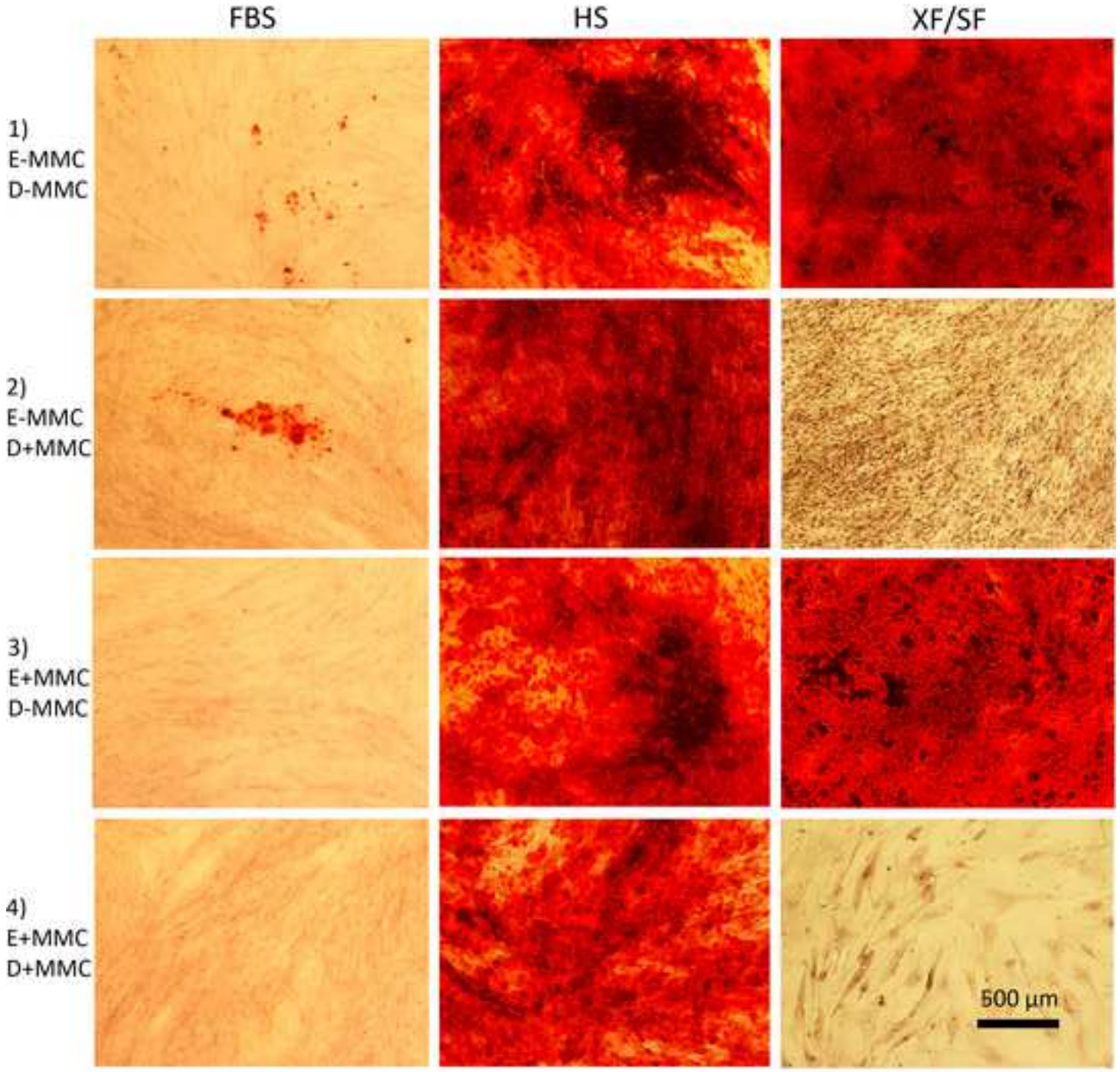

FBS
HS
XF/SF
1)
E-MMC
D-MMC
2)
E-MMC
D+MMC
3)
E+MMC
D-MMC
4)
E+MMC
D+MMC
500 µm

Figure 7

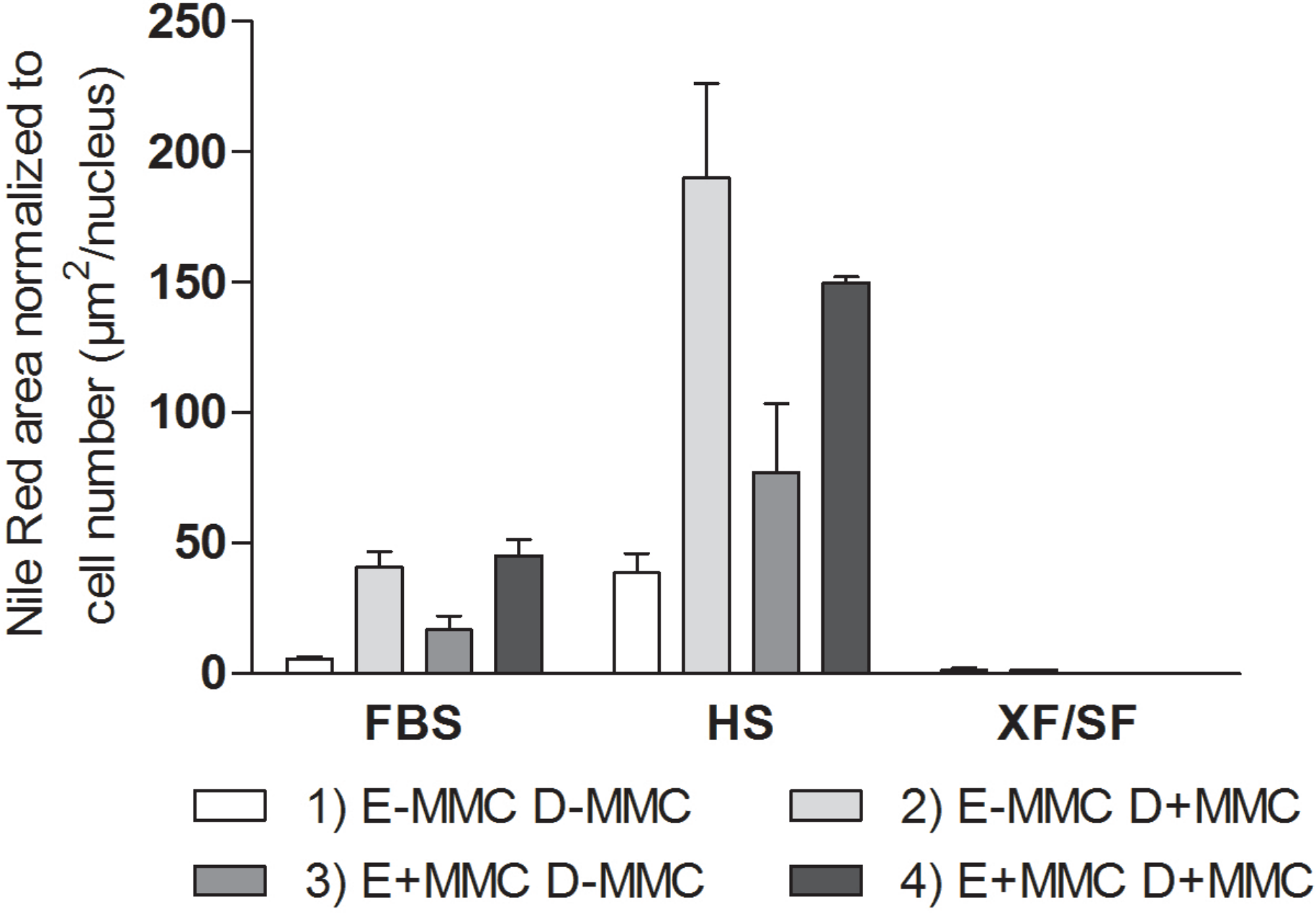

Nile Red area normalized to cell number (µm²/nucleus)
250
200
150
100
50
0
FBS
HS
XF/SF
1) E-MMC D-MMC
2) E-MMC D+MMC
3) E+MMC D-MMC
4) E+MMC D+MMC

Figure 8

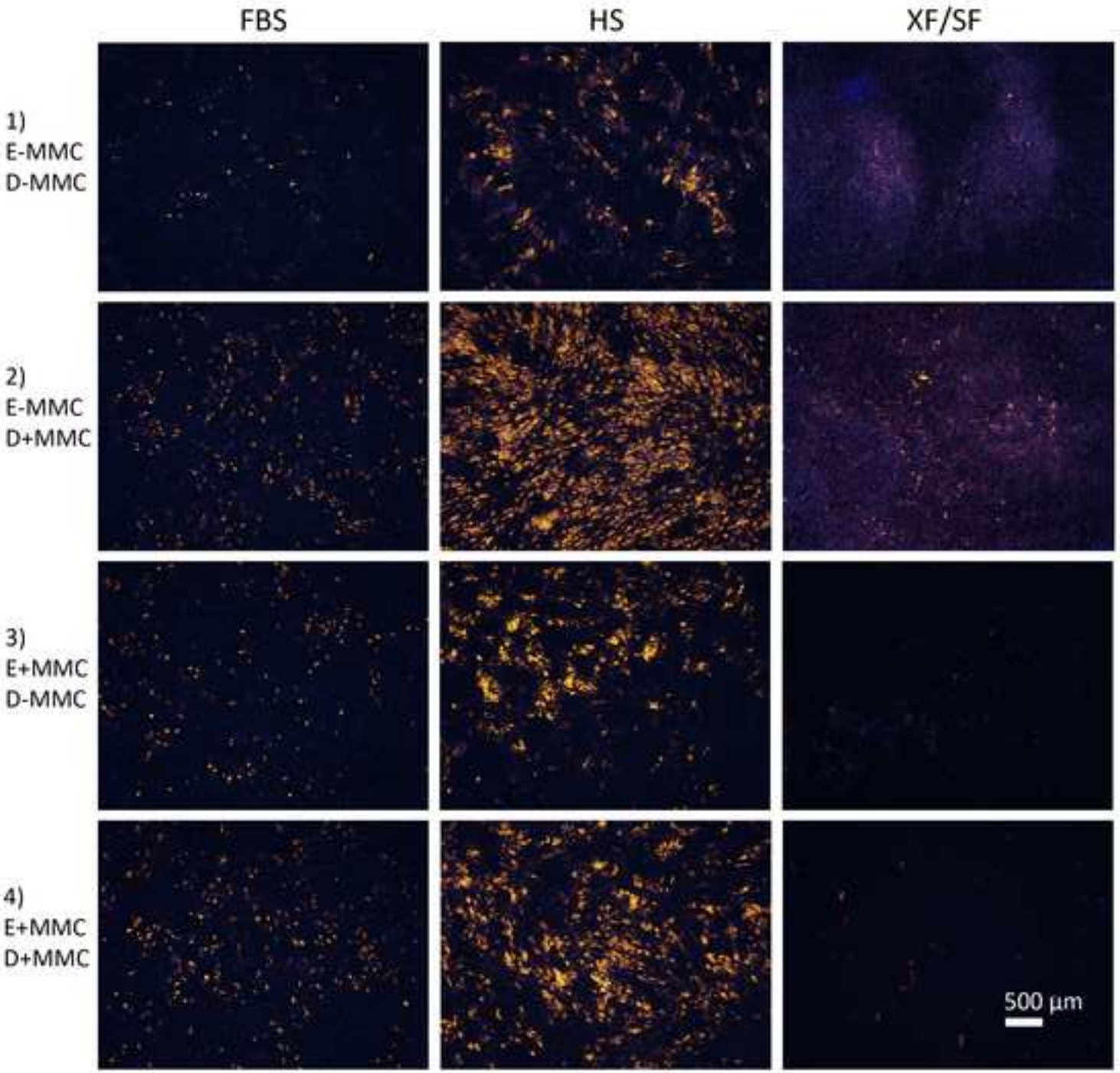
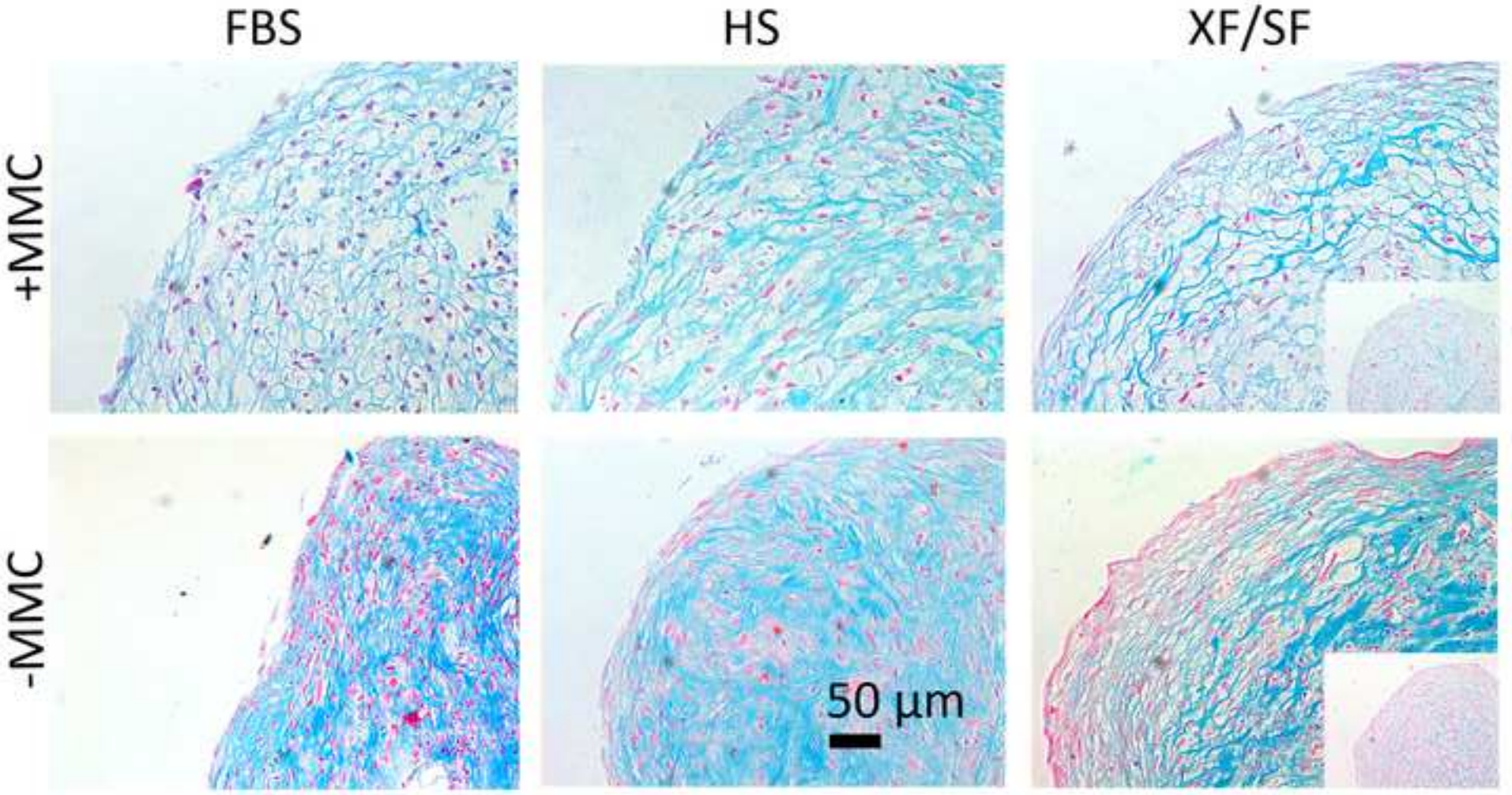

FBS
HS
XF/SF
1)
E-MMC
D-MMC
2)
E-MMC
D+MMC
3)
E+MMC
D-MMC
4)
E+MMC
D+MMC
500 µm

Figure 9



STEM CELLS TRANSLATIONAL MEDICINE®

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Different Culture Conditions Modulate the Immunological Properties of Adipose Stem Cells

MIMMI PATRIKOSKI,[a,b] JYRKI SIVULA,[a,b] HEINI HUHTALA,[c] MIKA HELMINEN,[b,c] FANNY SALO,[a,b] BETTINA MANNERSTRÖM,[a,b,*] SUSANNA MIETTINEN[a,b,*]

Key Words. Adipose stem cells • Immunogenicity • Immunosuppression • Cytokines • Serum-free • Cellular therapy

[a]Adult Stem Cell Group, Institute of Biosciences and Medical Technology (BioMediTech), and [c]School of Health Sciences, University of Tampere, Tampere, Finland; [b]Science Center, Tampere University Hospital, Tampere, Finland

[*]Contributed equally.

Correspondence: Mimmi Patrikoski, M.Sc., Adult Stem Cell Group, Institute of Biosciences and Medical Technology (BioMediTech), Biokatu 12, University of Tampere, 33520 Tampere, Finland. Telephone: 358-40-190-1789; E-Mail: mimmi.patrikoski@uta.fi

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ABSTRACT

The potential of human adipose stem cells (ASCs) for regenerative medicine has received recognition owing to their ease of isolation and their multilineage differentiation capacity. Additionally, low immunogenicity and immunosuppressive properties make them a relevant cell source when considering immunomodulation therapies and allogeneic stem cell treatments. In the current study, immunogenicity and immunosuppression of ASCs were determined through mixed lymphocyte reactions. The immunogenic response was analyzed after cell isolation and expansion in fetal bovine serum (FBS), human serum (HS)-supplemented medium, and xeno-free and serum-free (XF/SF) conditions. Additionally, the immunophenotype and the secretion of CXC chemokine ligand 8 (CXCL8), CXCL9, CXCL10, C-C chemokine ligand 2 (CCL2), CCL5, interleukin 2 (IL-2), IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor-α, interferon-γ, transforming growth factor-β1, indoleamine 2,3-deoxygenase, Galectin-1, and Galectin-3 were analyzed. The results showed that ASCs were weakly immunogenic when expanded in any of the three conditions. The significantly strongest suppression was observed with cells expanded in FBS conditions, whereas higher ASC numbers were required to display suppression in HS or XF/SF conditions. In addition, statistically significant differences in protein secretion were observed between direct versus indirect cocultures and between different culture conditions. The characteristic immunophenotype of ASCs was maintained in all conditions. However, in XF/SF conditions, a significantly lower expression of CD54 (intercellular adhesion molecule 1) and a higher expression of CD45 (lymphocyte common antigen) was observed at a low passage number. Although culture conditions have an effect on the immunogenicity, immunosuppression, and protein secretion profile of ASCs, our findings demonstrated that ASCs have low immunogenicity and promising immunosuppressive potential whether cultured in FBS, HS, or XF/SF conditions. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1–11*

INTRODUCTION

Human adipose tissue possesses an abundant source of multipotent mesenchymal stem cells (MSCs) known as adipose stem cells (ASCs). They are promising candidates for clinical applications because of their ability to differentiate toward several cell types of mesodermal origin [1, 2]. ASCs also have low immunogenicity [3], and they are capable of immunomodulation [4], which makes them an even more relevant cell source when considering clinical applications.

Several ongoing clinical trials are studying the suitability of ASCs in tissue repair and in treatment of autoimmune diseases or immunological disorders. For instance, the potential of ASCs is being evaluated in clinical trials for the treatment of craniofacial injuries of bone and soft tissue (NCT01633892), articular cartilage defects (NCT02090140), and urinary incontinence (NCT01799694; NCT01804153) (http://clinicaltrials.gov/). There is also a growing interest in clinical applications using the potential of ASCs to modulate immunity and inflammation, as reviewed by Casteilla et al. [5]. Specifically, the potential of ASCs is currently being evaluated in clinical trials for the treatment of Crohn's disease (NCT01157650; NCT01011244), osteoarthritis (NCT01739504; NCT01585857), graft versus host disease (GVHD) (NCT01222039), and multiple sclerosis (NCT01453764) (http://clinicaltrials.gov/).

The immunomodulatory capacity of ASCs has been studied by many research groups [4, 6–9], and interestingly, ASCs are considered immunologically privileged, lacking the expression of major histocompatibility complex (MHC) class II molecules, as well as T- and B-cell costimulatory molecules CD80, CD86, and CD40 [3, 9], required for complete T-cell activation. Nevertheless, immunological privilege of MSCs may not be absolute [10], and consequently the ability of MSCs to escape the immune system remains under debate. Still, ASCs have been shown to have

immunomodulatory properties, such as regulation of T-cell functions and modulation of cytokine secretion [3, 4, 7, 11, 12]. As shown by McIntosh et al. [3], the immunogenic properties of ASCs may also change as the passage increases. They showed that the stromal vascular fraction is more immunogenic when compared with higher passages that are also capable of immunosuppression. The immunomodulatory effect has also been demonstrated in vivo [8, 13, 14], where ASCs have been shown to suppress allogeneic lymphocytes and prolong allotransplant survival [8, 14]. In addition, the potential of MSCs to treat patients with GVHD has been reported in several clinical studies [15–18].

The mechanisms behind the immunosuppressive capacity are being investigated, so far mainly using bone marrow-derived mesenchymal stem cells (BMSCs). MSCs have been shown to actively inhibit the functions of several immune cells through cytokine secretion [19–21], and several soluble mediators have been suggested to be involved in the suppression process. These mediators include transforming growth factor β (TGF-β) [22] and prostaglandin E2 [23], both of which modulate interleukin 2 (IL-2) synthesis and T-cell activation, indoleamine 2,3-deoxygenase (IDO) [24, 25], and galectin-1 and -3 [26, 27]. In addition to soluble factors, direct cell-cell contact is an essential part of the interaction, but the mechanism has not been completely defined. Ren et al. [28] demonstrated that cell-cell adhesion mediated by intercellular adhesion molecule 1 (ICAM-1, CD54) and vascular cell adhesion molecule 1 (VCAM-1, CD106) is critical for MSC-mediated immunosuppression. In addition, the inflammatory environment has been shown to affect the suppression process, enhancing the immunosuppressive capacity of ASCs [4, 19, 24, 29]. Consequently, soluble factors, direct cell-cell contacts, and the inflammatory environment are all involved in the process, but more studies are required to fully determine the suppression mechanisms and to receive a comprehensive picture of the immunomodulatory potential of ASCs and other MSCs.

As the number of clinical therapies using ASCs increases, further requirements regarding the safety and efficiency of the cells arise. In fact, several reports have been published to fully characterize ASCs and to develop safe and efficient in vitro culture protocols for clinical cell therapies [30–34]. Completely defined xeno-free and serum-free (XF/SF) isolation and expansion protocols were recently developed for ASCs by our team [30] to replace traditional fetal bovine serum (FBS)-based culturing protocols, which should be avoided in clinical treatments because of safety concerns [35, 36]. Cells cultured in the presence of animal-derived components may transfer xenogeneic antibodies into the human body and trigger severe immune responses [37, 38]. By contrast, novel XF/SF culture conditions meet the current good manufacturing practice (GMP) requirements, and cells are cultured in fully defined media using only GMP grade reagents.

In this study, we evaluated the effect of the cell culture medium on the immunologic properties of ASCs. To our knowledge, this is a novel approach, and the aspect of the culture medium on cell immunologic properties has not been reported previously. However, in our previously published reports, we demonstrated that the culture conditions have an effect on the proliferation rate, as well as on the differentiation potential of the cells [30], and we hypothesized that the immunomodulatory properties also differ between cells isolated and expanded in different serum conditions and in XF/SF conditions. The isolation and expansion of ASCs was carried out in parallel in three different culture conditions: in an FBS-containing medium, in a human serum (HS)-containing medium, and in XF/SF conditions. After cell expansion in these conditions, ASCs were cocultured with peripheral blood mononuclear cells (PBMCs) in mixed lymphocyte reactions (MLRs) to evaluate the immunogenic properties of the cells in low (passage 2) and high (passage 5) passage numbers.

Materials and Methods

The collection of adipose tissue and peripheral blood was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere, Finland (R03058). Adipose tissue samples were obtained from Tampere University Hospital, and the buffy coat samples were from the Finnish Red Cross Blood Service. All analyses were performed separately with four or five donor cell lines isolated in FBS, HS, and XF/SF conditions. The same five patient cell lines were used for MLR assays and for flow cytometric analyses (donors 1–5), whereas the protein secretion studies were performed with four different ASC lines (donors 6–9).

Isolation and Culture of ASCs

ASCs were isolated from adipose tissue samples (n = 9) collected from female donors (age 41 $\pm$ 10 years) undergoing elective surgical procedures in the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland. ASCs were isolated under three different culturing conditions: medium containing FBS, HS, or XF/SF culture conditions.

Isolation of ASCs from adipose tissue samples was carried out using a mechanical and enzymatic method as described previously [2, 31, 39]. Briefly, the adipose tissue was minced manually into small fragments and digested with collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany, http://www.serva.de) in a water bath at 37°C under shaking conditions. The digested tissue was centrifuged and filtered in sequential steps through a 100-μm pore size filter to separate the ASCs from the surrounding tissue.

For FBS and HS conditions, Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 (Life Technologies, Rockville, MD, http://www.lifetech.com) was supplemented with 1% L-analyl-L-glutamine (GlutaMAX I; Life Technologies), 1% antibiotics (p/s; 100 U/ml penicillin, 0.1 mg/ml streptomycin; Lonza, Walkersville, MD, http://www.lonza.com) and either 10% FBS (Life Technologies) or 10% HS (human serum type AB; Lonza). ASCs isolated and expanded in FBS medium were detached using 1% trypsin (Lonza), and ASCs isolated in HS medium were detached using TrypLE Select (Life Technologies).

For XF/SF conditions, the cells were isolated under XF/SF conditions and seeded in carboxyl-coated flasks (PureCoat Carboxyl T75; BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) and expanded in STEMPRO MSC SFM (Life Technologies) supplemented with 1% GlutaMAX I, 0,3% antibiotics, and 10% StemPro MSC SFM Xeno-Free supplement as described previously [30]. From passage 1 onwards, XF/SF cells were expanded in STEMPRO MSC medium supplemented with CELLstart CTS coating (Life Technologies) according to the manufacturer's instructions. ASCs isolated and expanded in SF/XF medium were detached using TrypLE Select.

Isolation of PBMCs

Allogeneic human PBMCs were isolated from buffy coat samples (n = 7) by density gradient centrifugation using Ficoll-Paque PLUS

(density 1.077 g/ml; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com) according to manufacturer's instructions, aliquoted, and cryopreserved in the nitrogen gas phase until cocultures.

Immunogenicity and Immunosuppression Analyses

The one-way and two-way MLR assays were used to determine the immunogenic properties of ASCs after cell isolation and expansion in different culture conditions, in FBS, HS-containing medium, or XF/SF conditions. MLRs were performed separately with four to five ASC donor cell lines (donors 1–5) in passages 2 and 5. The MLRs were seeded on 96-well plates using DMEM/F-12 1:1 supplemented with 1% GlutaMAX I (Life Technologies), 1% antibiotics (p/s; 100 U/ml penicillin, 0.1 mg/ml streptomycin; Life Technologies), and 10% HS (PAA Laboratories, Pasching, Austria, http://www.paa.at). 10% HS (PAA Laboratories) medium was chosen to serve as a constant environment for MLR cultures because of low viability of PBMCs when cultured in XF/SF condition (data not shown). Therefore, prior to MLR assays, ASCs isolated and expanded in three different culture conditions, FBS, HS, (Lonza), and XF/SF conditions received the same treatment of medium change and were allowed to adjust in HS medium (PAA Laboratories) for 24 hours prior to coculture.

One-Way MLR Measuring the Immunogenicity of ASCs

PBMCs derived from three different donors were seeded at 2.5×10^5 cells per well and acted as responder cells. In addition to PBMC responders, various stimulator cells were added to the reactions: autologous PBMCs (baseline response) and allogeneic PBMCs (positive-control response), both plated at 1.0×10^4 cells per well, and the test ASCs, plated at 0.5×10^4, 1.0×10^4 and 2.0×10^4 cells per well. Stimulator PBMCs and ASCs were irradiated with γ-rays (40 Gy) prior to the coculture to inhibit the proliferation of the stimulator cells. ASCs in medium alone were plated as control cultures. In addition, control cultures of PBMCs alone were added as well as PBMCs supplemented with mitogen phytohemagglutinin (PHA, 1 μg/ml) to activate the responder PBMC lines to serve as a maximal positive control response in the one-way reaction. Quadruplicate reactions were performed from each treatment, and the cultures were incubated at 37°C in 5% CO_2 for 5 days in HS medium (PAA Laboratories). One-way MLRs were performed as described previously by McIntosh et al. [40].

Two-Way MLR Measuring the Immunosuppression of ASCs

Two different MLR combinations were formed from three different PBMC lines. For each MLR combination, cells from two donors were mixed at a time in equal amounts to activate the proliferative response of each PBMC line, and a total 2.5×10^5 cells were seeded per well. After seeding the MLRs, ASCs were added to the reactions at cell densities of 0.5×10^4, 1.0×10^4 or 2.0×10^4 cells per well. In addition, control wells containing only MLR combinations without ASCs, and ASCs alone were also seeded. Quadruplicate reactions were performed from each treatment group, and the cultures were incubated at 37°C in 5% CO_2 for 5 days in HS (PAA Laboratories) medium. Two-way MLRs were performed as described previously by McIntosh et al. [40].

Proliferation Assay

On day 4 of the MLRs, 10 μM bromodeoxyuridine (BrdU) was added to mono- and cocultures, and the cells were incubated for additional 16 hours at 37°C. On day 5, PBMC proliferation was assessed by BrdU enzyme-linked immunosorbent assay (ELISA) (Roche Applied Science, Penzberg, Germany, https://www.roche-applied-science.com) according to the manufacturer's instructions using a microplate absorbance reader (Victor 1429 Multilabel Counter; Wallac, Turku, Finland, http://www.perkinelmer.com).

Performed Calculations From Proliferation Assay's Absorbance Values

Prior to statistical analysis, the following calculations were performed for the raw absorbance data of BrdU ELISA. The average absorbance values of ASC control wells were calculated. Medium background was subtracted from the average ASC control values. These modified ASC control values were then subtracted from MLR absorbance values taking into account the used ASC numbers. Finally, these corrected MLR values were divided by the average autologous PBMCs value that was considered as baseline response in one-way MLR or by the average control value of wells containing only MLR cultures without ASCs, considered as baseline response in two-way MLR. According to the performed calculations, in one-way MLRs, the value 1 indicates the baseline response, and values above 1 indicate activation. Respectively, in two-way MLRs, a value of 1 represents a baseline response, and reaction values below 1 indicate suppression. Performed calculations are modified from the protocols described by McIntosh et al. [40].

Flow Cytometric Analyses

ASCs expanded in FBS, HS, and XF/SF media (n = 4–5, donors 1–5, passages 2 and 5) were analyzed by flow cytometry (FACSAria; BD Biosciences) to determine whether different culturing conditions affect the immunophenotype of the cells. Monoclonal antibodies against CD11a-allophycocyanin (APC), CD80-phycoerythrin (PE), CD86-PE, CD105-PE (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), CD-3 (PE), CD14-phycoerythrin-cyanine (PECy7), CD19-PECy7, CD45RO-APC, CD54-fluorescein isothiocyanate, CD73-PE, CD90-APC (BD Biosciences), and CD34-APC, HLADR-PE (Immunotools GmbH, Friesoythe, Germany, http://www.immunotools.de) were used. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

Quantitative Protein Measurements

Two-way MLR assay was used to evaluate the secreted protein expression in 24-well plate format. Four different donor PBMC lines were used as responder cells, in two different MLR combinations. PBMCs from two donors were mixed at a time in equal amounts to activate the proliferative response of each PBMC line, and a total cell number of 8.0×10^5 PBMCs was seeded per well. After seeding the MLRs, ASCs were added at densities of 3.0×10^4 cells per well (donors 6–9) either in direct coculture or using a semipermeable membrane inserts to prevent direct cell-cell contacts between ASCs and PBMCs. When using the inserts, PBMCs were pipetted into the inserts (pore size, 0.4 μm;

ThinCert, Greiner Bio-One, Frickenhausen, Germany, http://www.gbo.com/en), whereas ASCs remained on the bottom of the wells. In addition, control wells containing MLR cultures with no ASCs or ASCs alone were seeded. Similarly to immunogenicity assays, the reactions were performed with ASCs isolated and expanded in three different culture conditions (FBS, HS, and XF/SF conditions), and were subsequently let to adjust to the 5% HS (PAA Laboratories) medium for 24 hours prior to coculture. HS lot (PAA laboratories) was changed prior to cytokine studies, and 5% HS was chosen as a standard culturing condition to control excess cell growth during cytokine measurements. Quadruplicate reactions were performed from each treatment. Cell culture supernatants from mono- and cocultures were collected on day 5 and stored in −20°C until analysis. Cytokines and chemokines secreted by the cells were analyzed using Cytometric Bead Arrays (CBAs; BD Biosciences); human chemokine kit (CXC chemokine ligand 8 [CXCL8/IL-8], -9 [CXCL9/MIG], -10 [CXCL10/IP-10], C-C chemokine ligand 2 [CCL2/MCP-1], and -5 [CCL5/RANTES]), human Th1/Th2/Th17 cytokine kit (interleukin 2 [IL-2], -4 [IL-4], -6 [IL-6], -10 [IL-10], and -17A [IL-17A]; tumor necrosis factor α [TNF-α]; and interferon γ [IFN-γ]) and human TGF-β1 Single Plex Flex Set (TGF-β1). IDO and Galectin-1 and -3 were analyzed using colorimetric ELISA assays; ELISA kit for IDO (Cloud-Clone Corporation, Uscn Life Science Inc., Wuhan, People's Republic of China, http://www.uscnk.com), Human Galectin-1 Quantikine ELISA Kit (R&D Systems), and Human Galectin-3 Quantikine ELISA Kit (R&D Systems). Each colorimetric ELISA reaction was done in triplicate, and averages of the parallel reactions were then taken into account in statistical analysis. Substantially high concentrations of IL-6, CXCL8, CXCL9, CCL2, and CXCL10 were detected from the culture media, exceeding the detection limits of the human chemokine and human Th1/Th2/Th17 cytokine kits. As a consequence, the cell culture supernatants were diluted 1:150, and these cytokines were reanalyzed using multiplexed Flex Set array (BD Biosciences). CBA output data were analyzed using FCAP Array software version 3.0 (BD Biosciences) according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed with SPSS version 19 (IBM Corp., Armonk, NY, http://www.ibm.com). A nonparametric Kruskal-Wallis test with Mann-Whitney U post hoc test was used to compare different culture conditions and their effect on immunogenic proliferative response in one-way and two-way MLR, as well as for determining the effect of different culture conditions on cell surface protein expression of cytometric data. The cytokine secretion data were analyzed using a regression analysis with ranked values. The effect of culture condition on cytokine secretion was analyzed using a model in which the FBS or HS conditions were defined as reference. Secretion values of other two conditions were then compared with reference condition separately in direct and indirect cocultures. Differences in cytokine secretion between direct versus indirect cultures, as well as between cocultures and monocultures, were analyzed using a different model. In this model, monocultures (ASCs alone or MLR without ASCs) and indirect cocultures were compared with direct cocultures that were defined as reference. The results of MLRs, cytokine secretion studies, and flow cytometric analyses were reported as means and SD. When three different culturing conditions were compared, a Bonferroni correction was performed to avoid the problem of multiple comparisons. As a consequence, the p values were multiplied by 3 before the interpretation of statistical data. The results were considered statistically significant when the p value was under 0.05.

Results

ASCs Elicited a Weak Immunogenic Response on PBMCs in All Culture Conditions

One-way MLR assays were performed to assess the immunogenicity of ASCs (Fig. 1A, 1B). In Figure 1A and 1B, a value of 1 indicates the baseline response of PBMCs without ASCs, and values above 1 indicate activation.

ASCs expanded in the FBS-containing medium induced the lowest immunogenic response on PBMCs in both passages 2 and 5 (Fig. 1A, 1B). In passage 2, a significantly lower immunogenic response on PBMCs was observed in the FBS medium ($p < .05$) when compared with ASCs expanded in HS or XF/SF conditions, with 1.0×10^4 and 2.0×10^4 stimulator ASCs (Fig. 1A, a). In passage 5, a significantly lower PBMC immunogenic response was observed in the FBS medium ($p < .05$) compared with the HS medium, with 1.0×10^4 stimulator ASCs (Fig. 1B). Furthermore, the immunogenic response in passage 5 was significantly stronger in the HS medium ($p < .05$), with 2.0×10^4 stimulator ASCs, compared with both FBS and XF/SF conditions (Fig. 1B, b). However, although the different culture conditions of the ASCs had a significant effect to the immunogenicity of the cells, the ASCs expanded in all of the studied conditions elicited only a weak immunogenic response compared with the maximal positive control response of PBMCs activated with PHA mitogen (Fig. 1A, 1B).

ASCs Showed a Suppressive Effect on PBMC Proliferation

Differences between culture conditions were also evident regarding the suppression potential of ASCs (Fig. 1C, 1D). Coculture results in two-way MLRs were standardized using values from MLRs without ASCs. Therefore, in Figure 1C and 1D, a value of 1 represents the baseline, and values below 1 indicate suppression.

ASCs expanded in the FBS medium efficiently suppressed the proliferation of PBMCs stimulated in MLR in both passages 2 and 5 (Fig. 1C, 1D). ASCs expanded in the HS medium showed suppression of PBMCs in passage 2 (Fig. 1C) with 1.0×10^4 and 2.0×10^4 cells per well and in passage 5 (Fig. 1D) with 2.0×10^4 cells per well. By contrast, ASCs expanded in XF/SF conditions were not able to suppress the PBMC proliferation in passage 2 and suppressed the proliferation in passage 5 with only 1.0×10^4 or 2.0×10^4 cells per well (Fig. 1C, 1D). Some of these differences proved to be statistically significant. In passage 2, FBS showed significantly stronger suppression ($p < .05$), with 0.5×10^4 stimulator ASCs (Fig. 1C, a–c) compared with HS and XF/SF conditions that were not capable of inducing suppression. In addition, XF/SF-expanded ASCs were significantly less suppressive ($p < .05$) compared with HS and FBS conditions in passage 2 (Fig. 1C, c). In passage 5 (Fig. 1D), ASCs expanded in the FBS medium showed significantly stronger suppression ($p < .05$), with a cell number of 0.5×10^4 (Fig. 1D, a), compared with HS and XF/SF conditions. With a cell number of 1.0×10^4, a significantly stronger suppression was also observed in the FBS medium ($p < .05$) when compared with the HS medium. Therefore, the

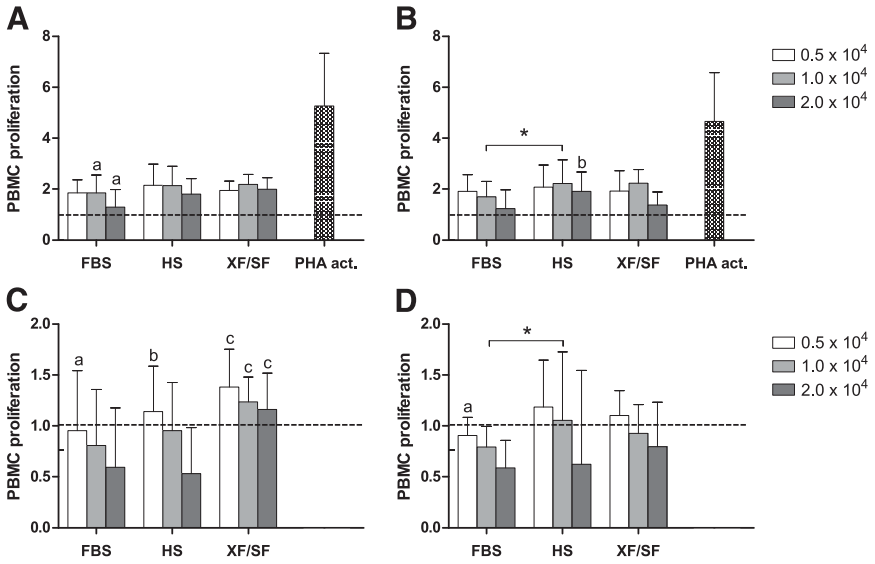

Figure 1. Immunogenicity and immunosuppression of adipose stem cells (ASCs). The immunogenicity **(A, B)** and suppression potential **(C, D)** of ASCs are presented in passages 2 **(A, C)** and 5 **(B, D)**. ASCs expanded in FBS, HS, and XF/SF conditions elicited only a weak immunogenic response compared with the maximal positive control response activated with PHA. Suppression potential was dependent on the ASC number, as well as the culture condition. The dashed line represents a baseline response; values above 1 indicate activation **(A, B)**, and values below 1 indicate suppression **(C, D)**. The results are presented as means ± SD. a, $p < .05$ when FBS is compared with HS and XF/SF conditions; b, $p < .05$ when HS is compared with FBS and XF/SF conditions; c, $p < .05$ when XF/SF is compared with FBS and HS conditions; ∗, $p < .05$ when FBS in compared with HS but not XF/SF conditions. Abbreviations: act., activated; FBS, fetal bovine serum; HS, human serum; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; XF/SF, xeno-free/serum-free.

suppression potential of ASCs was dependent on the ASC number, as well as the culture condition.

Protein Secretion Profile Was Altered Depending on Direct Versus Indirect Cocultures and Expansion Conditions of ASCs

A panel of secreted proteins was analyzed from cell culture supernatants either in direct or indirect cocultures after the expansion of ASCs in different serum conditions: FBS, HS, and XF/SF conditions. Altered secretion profiles were observed between the different expansion conditions of the ASCs, but more evident was the effect of direct versus indirect contact between ASCs and PBMCs (Fig. 2).

Direct Cocultures

The secretion of IL-6 was increased in cocultures with ASCs expanded in FBS conditions compared with ASCs expanded in HS and XF/SF conditions, but no statistical significances were observed because of the large standard deviation. A significantly higher secretion of IFN-γ was detected in XF/SF conditions ($p < .05$) compared with cocultures with FBS- and HS-expanded ASCs. TNF secretion was higher in XF/SF conditions compared with the FBS and HS media, but no statistical significances were observed. However, statistically significant differences were seen between different culture conditions for the secretion of TGF-β1. Direct cocultures containing ASCs expanded in XF/SF conditions had the lowest TGF-β1 concentrations ($p < .05$) compared with either FBS or HS conditions. There was no significant difference between FBS and HS conditions.

The chemokine CCL2 secretion was significantly higher in cocultures with FBS-expanded ASC ($p < .05$) compared with HS-expanded ASCs. A significantly lower secretion of chemokine CCL5 was measured in HS conditions ($p < .05$) compared with XF/SF and FBS conditions. The secretion of chemokines CXCLX9 and -10 was strong in each culture condition, and the secretion of chemokine CXCL10 was significantly higher in cocultures with XF/SF-expanded ASCs ($p < .05$) compared with HS conditions. The chemokine CXCL8 was strongly secreted in cocultures with ASCs expanded in all of the studied conditions.

Statistically significant differences were also seen between different culture conditions for the secretion of IDO. Direct cocultures containing ASCs expanded in FBS conditions had the lowest IDO concentrations compared with either HS ($p < .05$) or XF/SF ($p < .001$) conditions. There was no significant difference between HS and XF/SF conditions. The concentration of galectin-1 was highest in the direct coculture containing ASCs expanded in HS conditions, but no statistically significant differences were seen between different culture conditions for galectin-1 or galectin-3.

Reactions containing only ASCs or PBMCs showed a significantly lower secretion of cytokines and chemokines IL-6, CCL2, CCL5, CXCL8, CXCL9, and CXCL10 ($p < .001$) compared with direct cocultures. IFN-γ and TNF-α secretion was not detected in reactions containing only ASCs. Therefore, a significantly lower secretion of IFN-γ and TNF-α ($p < .001$) was measured in reactions containing only ASCs, and a significantly lower secretion of IFN-γ ($p < .05$) was measured in reactions containing only PBMCs. A significantly lower secretion of TGF-β1 was detected in reactions containing PBMCs only compared with direct cocultures ($p < .05$). The secretion of TGF-β1 was also lower in reactions containing ASCs alone, but there was no significant difference compared with direct cocultures. Concentrations of IDO, galectin-1, and galectin-3 were significantly lower ($p < .001$) in reactions containing ASCs alone compared with direct cocultures. In addition, the concentration of galectin-1 ($p < .001$) was significantly

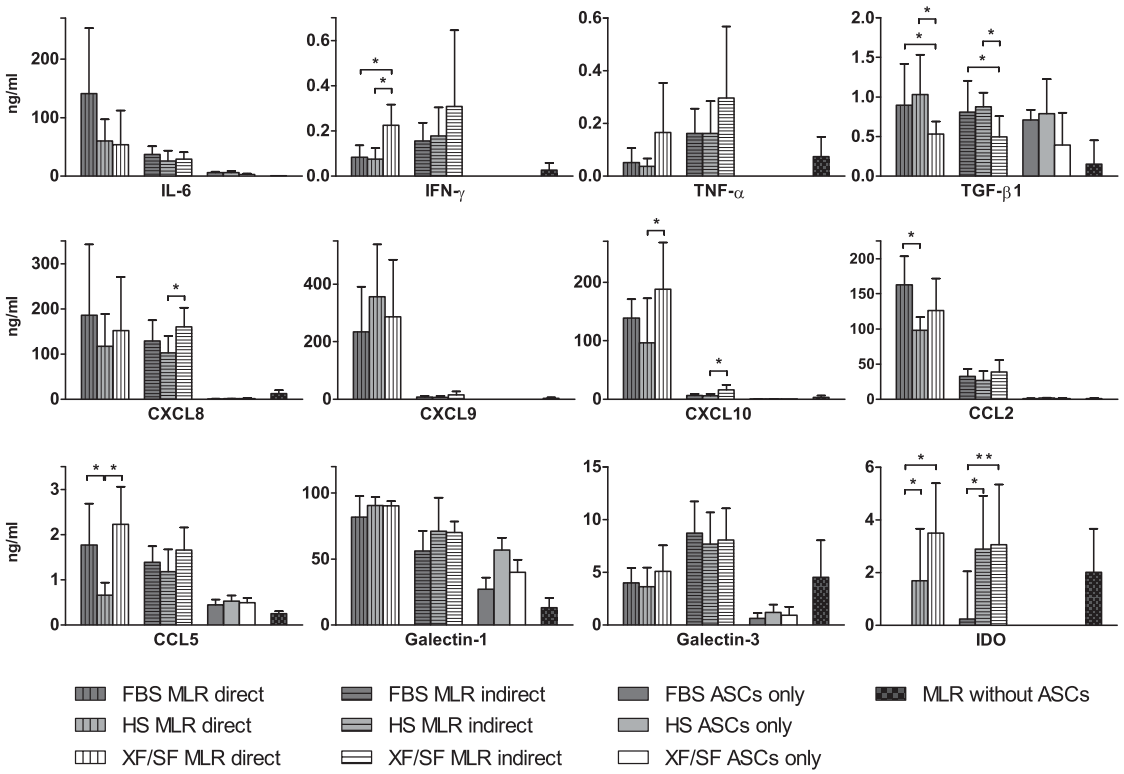

Figure 2. Protein secretion. Secreted proteins analyzed in direct cocultures and using separating membranes between ASCs and peripheral blood mononuclear cells. Prior to the cocultures, ASCs were expanded in different culture conditions: FBS, HS, and XF/SF. Altered secretion profiles were observed between the different expansion conditions of the ASCs. TGF-β1 and IDO amounts of medium alone were subtracted from quantitated concentrations of TGF-β1 and IDO in mono- and cocultures. The results are presented as means $\pm$ SD. $*$, $p < .05$; $**$, $p < .001$. Abbreviations: ASC, adipose stem cell; CCL2 and -5, C-C chemokine ligands 2 and 5; CXCL8,CXCL9, and CXCL10, CXC chemokine ligands 8, 9, and 10; FBS, fetal bovine serum; HS, human serum; IDO, indoleamine 2,3-deoxygenase; IFN-γ, interferon γ; IL-6, interleukin 6; MLR, mixed lymphocyte reaction; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; XF/SF, xeno-free/serum-free.

lower in reactions containing only PBMCs when compared with direct cocultures. However, there was no significant difference between direct cocultures and reactions containing PBMCs alone for the secretion of IDO and galectin-3. The secretion of cytokines IL-2, IL-4, IL-10, or IL-17A was not detected in cocultures or in reactions containing only ASCs or PBMCS in any of the studied culture conditions.

Indirect Cocultures

Compared with direct cultures, the secretion of IL-6 was significantly decreased ($p < .001$) in indirect cocultures when culture conditions were not assessed separately in the regression model. By contrast, the secretion of IFN-γ ($p < .05$) and TNF-α ($p < .001$) was significantly increased in the indirect coculture, when compared with the direct cultures. There was no significant difference in TGF-β1 secretion between direct and indirect cocultures. However, statistically significant differences in TGF-β1 secretion were also seen between different culture conditions in indirect cocultures. Similarly to direct cocultures, cultures containing ASCs expanded in XF/SF conditions had the lowest TGF-β1 concentrations ($p < .05$) compared with either FBS or HS conditions. There was no significant difference between FBS and HS conditions.

The chemokine CCL2 secretion was significantly lower ($p < .001$) in each culture condition when separating membranes were used. Chemokine CCL5 secretion was altered depending on the culture conditions. The secretion was decreased in indirect cultures in XF/SF and FBS conditions, whereas higher concentrations were measured in the HS medium in the indirect culture. Significantly lower secretion ($p < .001$) of chemokines CXCLX9 and -10 was measured in indirect cultures compared with the strong secretion that was measured in direct cocultures. Similarly to direct cultures, the secretion of chemokine CXCL10 was significantly higher in indirect cocultures with XF/SF-expanded ASCs ($p < .05$) compared with HS conditions. The chemokine CXCL8 was also strongly secreted in indirect cocultures with ASCs expanded in all of the studied conditions. Significantly higher secretion of CXCL8 was measured in XF/SF conditions ($p < .05$) compared with the HS medium using the indirect coculture.

Statistically significant differences in the secretion of IDO were also observed between different culture conditions in indirect cocultures. Cultures containing ASCs expanded in FBS conditions had the lowest IDO concentrations compared with either HS or XF/SF ($p < .05$) conditions. There was no significant difference between HS and XF/SF conditions.

Compared with direct cultures, the concentration of galectin-3 was significantly higher ($p < .001$) in indirect cocultures when culture conditions were not assessed separately in the regression model. By contrast, galectin-1 concentration was significantly lower ($p < .001$) in the indirect coculture compared with direct cultures. The concentration of galectin-1 was highest in

cocultures containing ASCs expanded in HS conditions, but no statistically significant differences were observed between different culture conditions for galectin-1 or for galectin-3.

Flow Cytometric Analyses

The cell surface marker expression of ASCs was analyzed by flow cytometry to compare the expression profile of cells expanded in different culture conditions, in FBS, HS, and XF/SF media. In general, the characteristic immunophenotype of ASCs was maintained in every condition, with some minor differences observed between XF/SF conditions and the serum-containing media, and in the expression of specific markers between passages 2 and 5. Flow cytometric results were very similar to our previously published results [30].

The largest variation between different culture conditions was seen in the expression of lymphocyte common antigen CD45 and the immune-related marker CD54 (Fig. 3). On average, ASCs lacked the expression (<2%) of CD45 in all conditions, except the average low expression (2% to ≤10%) in XF/SF conditions in both passages and in FBS conditions in passage 5. A significantly higher expression of CD45 was observed in XF/SF conditions ($p < .05$) compared with HS and FBS conditions in passage 2. Furthermore, the average moderate expression (10% to ≤25%) of CD54 was observed in FBS and HS conditions, whereas in XF/SF conditions, the cells lacked the expression in passage 2 and showed low expression in passage 5. A statistically significant difference between FBS and XF/SF conditions ($p < .05$) was observed in passage 2 for the expression of CD54.

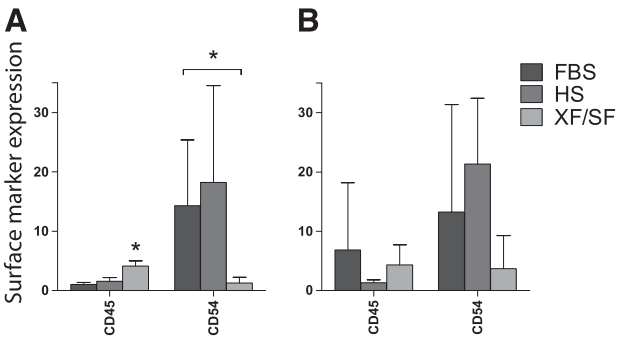

Figure 3. Surface-marker expressions of adipose stem cells. The expression of lymphocyte common antigen CD45 and immune-related marker CD54 on cells expanded in three different culture conditions (FBS, HS, and XF/SF) was investigated in passages 2 **(A)** and 5 **(B)**. A significantly higher expression of CD45 was observed in XF/SF conditions ($p < .05$) compared with HS and FBS conditions in passage 2. By contrast, significantly lower expression of CD54 was observed in XF/SF conditions ($p < .05$) compared with FBS conditions in passage 2. The data in the diagrams are presented as means ± SD; ∗, $p < .05$. Abbreviations: FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Discussion

Previous studies have demonstrated that MSCs are not strongly immunogenic and that they have an immunosuppressive capacity against T-cell functions and cytokine secretion [4, 7, 12]. The immunomodulatory properties of MSCs have been described both in vitro and in vivo; however, markedly more studies have been performed with BMSCs than with ASCs [17, 41–44]. In our previous study, we showed that culture conditions affect the proliferation rate and the differentiation potential of the cells [30]. In the current study, we demonstrate that different cell expansion conditions also affect the immunogenicity, immunosuppressive capacity, and immunogenicity-related protein secretion of ASCs. To our knowledge, this is a novel approach, and the effect of culture media on cell immunogenicity has not previously been reported.

Traditionally, the ASC culture medium has been supplemented with FBS, which is not a preferred option for clinical cell therapies because of safety concerns [35, 36]. Different alternatives to FBS have therefore been studied, such as human albumin [45], platelet-rich plasma [46], autologous HS [47], and a completely defined XF/SF culture medium [30, 31, 33, 48].

Our results show that ASCs elicit only a low immunogenic response to PBMCs in a one-way MLR, whether cultured in FBS, HS, or XF/SF conditions. This result is consistent with earlier studies performed with cells expanded in FBS medium [3, 9, 49]. However, statistically significant differences were observed between different culture conditions, and cells cultured in FBS medium proved the least immunogenic. It can be speculated that the reason for the low immunogenicity in the FBS medium is that FBS is harvested from the blood of bovine fetuses whose immune system is not fully mature at the point of harvest [50], and therefore immunomodulatory factors in the FBS medium may differ from the HS-supplemented medium. Nevertheless, a significantly lower activation of PBMCs was measured in MLR cultures with ASCs expanded in any of the studied conditions, when compared with a positive control response measured in PBMCs activated with PHA mitogen. This result suggests that ASCs elicit only a very low immunogenic response toward allogeneic PBMCs, and the different culture conditions do not affect the result substantially. Because of the low immunogenicity of ASCs, allogeneic stem cell treatments may be conceivable in the future, although in vivo studies are still required to systemically confirm the in vitro results shown in this study.

In addition to low immunogenicity, our present study confirms that ASCs have the potential for immunosuppression, as has been demonstrated previously [3, 4, 7, 8]. Our results revealed that the suppressive potential was affected by the culture condition of ASCs and that the suppression functioned in a dose-dependent manner. Two different MLR combinations were used in a two-way MLR assay, and these combinations were not identically reactive. This biological variation in the intensity of each MLR can be seen as the larger standard deviation after the coculture with ASCs. However, the strongest suppression was seen with ASCs cultured in FBS conditions, whereas ASCs cultured in HS or XF/SF conditions suppressed PBMC proliferation only with higher cell numbers. It was somewhat surprising that FBS-expanded ASCs elicited the strongest suppression on PBMC proliferation, but there are previous published studies that may explain this result. Certain adhesion molecules, such as ICAM-1 (CD54) and VCAM-1 (CD106), have been shown to play a key role in mesenchymal stem cell-mediated immunosuppression [4, 28, 51]. CD54 is reported to be present in endothelial cells, antigen-presenting cells, and some stromal cells. In our present study, a significantly higher expression of CD54 was observed on FBS-expanded ASCs, compared with cells cultured in XF/SF conditions, as detected by the flow cytometric analyses. In our previous published studies [24, 30] we have seen similar results when ASCs cultured in FBS- or HS-containing media have shown a significantly

higher expression of CD54 compared with XF/SF conditions. These findings are consistent with previous studies recognizing the importance of CD54 and CD106 in MSC-mediated immunosuppression. As demonstrated by Ren et al. [28, 51], CD54 and CD106 are required for lymphocyte-BMSC adhesion, and with the help of chemokines they induce immunosuppression mediated by BMSCs. Moreover, when the functions of these adhesion molecules were inhibited by gene knockout, BMSC-mediated suppression was significantly reversed, as shown by an in vivo study [52]. In contrast to BMSCs, ASCs have been shown to be negative for CD106 [31, 53], and thus it is likely that immunosuppression of ASC is CD54- but not CD106-mediated.

Previous studies have suggested that MSC-mediated immunosuppression would not be stable but rather induced by the inflammatory environment and cytokines, such as IFN-γ, TNF-α, and IL-6 [29]. Production of these cytokines is greatly increased under inflammatory conditions [24], and MSCs may respond to this increase by changing their immunomodulatory functions. IFN-γ has been shown to induce expression of MHC-I and to a lesser extent also MHC-II, increasing the antigen-presenting capacity and hence immunogenicity of MSCs [54]. Low levels of IFN-γ may induce MSCs to express MHC-II as antigen-presenting cells, whereas high levels can mediate a decreased expression of MHC-II [42]. These studies indicate the importance of local inflammatory conditions in the regulation of MSC plasticity. In our present study, we observed that the production of IFN-γ, TNF-α, and IL-6 was increased after coculture in a two-way MLR, as compared with monocultures of PBMCs and ASCs. IFN-γ and TNF-α levels were higher in the indirect culture compared with the direct coculture of ASCs and PBMCs. The result suggests that direct contact between ASCs and PBMCs inhibits the secretion of IFN-γ and TNF-α. Wu et al. [55] reported a similar phenomenon of reduced IFN-γ and TNF-α secretion of $CD4^+$T cells after coculture with MSCs. In their study, T cells were activated for higher IFN-γ and TNF-α secretion by PHA/IL-2 exposure, and reduced secretion of these factors was observed after coculture with MSCs. It can be speculated that similarly to PHA/IL-2, the indirect contact between PBMCs and ASCs changes the local inflammatory environment and activates PBMCs for higher secretion, as demonstrated by Crop et al. [24]. However, direct contact will reduce that activation.

In contrast to IFN-γ and TNF-α, direct contact between ASCs and PBMCs was required for higher production of IL-6, whereas lower levels of IL-6 were detected when the cells were separated by a membrane. Melief et al. [56] have shown that through production of IL-6, MSCs prevent monocyte differentiation toward antigen-presenting immunogenic cells and drive differentiation toward an anti-inflammatory IL-10-producing cell type. IL-6 can act to either support or suppress the inflammation depending on the context [57], and the anti-inflammatory effect is mediated through inhibition of TNF-α [58]. In our study, IL-6 secretion was significantly higher in FBS-expanded ASCs compared with XF/SF-expanded cells, which is in line with the stronger suppression potential observed in FBS conditions, as compared with XF/SF conditions. In addition, the secretion of TNF-α was low in FBS conditions compared with the higher secretion observed in XF/SF conditions. These results support the hypothesis proposed by Melief et al. [56], which suggests a connection between IL-6 and the anti-inflammatory environment.

Furthermore, signaling protein TGF-β1 has multiple functions in controlling cell growth, proliferation, differentiation, and apoptosis, and in addition, it is an important factor in maintaining immune tolerance [59]. Proliferation and activation of T cells can be diminished by the effect of TGF-β1 [60, 61]; similarly, it can downregulate many cytokines, including IFN-γ and TNFα [62]. The medium used in the MLRs contained a relatively large amount of TGF-β1, which may have obscured some of our results. Although there was a difference between reactions containing FBS- and HS-expanded ASCs, this was not significant. However, the significantly lower expression of TGF-β1 in cocultures containing XF/SF-expanded ASCs is in line with the proliferation assay results, and higher IFN-γ and TNFα and lower IL-6 concentrations that were measured in cocultures containing XF/SF-expanded ASCs.

Inflammatory mediators also induce the expression of CXCLX chemokines, and, for example, chemokine CXCL10 is secreted in response to higher IFN-γ levels. Accordingly, the significantly higher secretion of IFN-γ that we observed in XF/SF conditions induced significantly higher CXCL10 secretion in XF/SF-expanded ASCs compared with FBS and HS conditions. As we showed in this study, ASCs expanded in XF/SF conditions elicited a stronger immunogenic response in a one-way MLR compared with FBS conditions and proved to be less immunosuppressive in a two-way MLR compared with FBS and HS conditions. Thus, there may be a link between higher IFN-γ and CXCL10 levels in XF/SF conditions and the weaker suppression potential that was measured by the two-way MLR in XF/SF conditions, as compared with the HS and FBS media.

On the other hand, chemokines are needed to promote T-cell chemotaxis, and once T cells have made contact with MSCs, the function of T cells may be blocked by the combined action of soluble factors and direct cell-cell contact [20]. The expression of several chemokines has been shown to increase when ASCs are cultured in MLRs. Crop et al. [24] have demonstrated a strong increase in the expression of T-cell attractants; chemokine ligands CXCL9 and -10, as well as ligands with CC-motives CCL2 and -5, increased after MLR culture when compared with the control cultures of ASCs. In our studies, the secretion of chemokine ligands CXCL9 and -10, as well as CCL2, was increased after coculture in MLR, and significantly stronger secretion was observed in direct cocultures. This result suggests that direct contact between ASCs and PBMCs stimulates cells for stronger chemokine secretion to strengthen T-cell recruitment. Thus, the final determination of inflammatory responses may be elicited through a combined action of cell-cell contacts and soluble factors.

The secretion of CCL2 and CCL5 was significantly lower in HS conditions compared with FBS conditions (CCL2) and when compared with FBS and XF/SF conditions (CCL5). In addition, significantly lower secretion of CXCL8 was observed in the HS medium when compared with XF/SF conditions. Chemokines CCL2 and CCL5 have been shown to be involved in MSC chemotaxis and cell migration to the sites of injury or inflammation [41]. The increase in the production of chemokines such as CXCL8, CCL2, and CCL5 may also induce extracellular matrix degradation. These chemotactic factors are produced by immune cells, and the phenomenon is facilitated by the increased migration of human MSCs through the extracellular matrix. Based on our results, it is still difficult to conclude how the lower levels of CCL2 and CCL5 that we observed in HS conditions may affect the immunogenicity of ASCs. The effect of CCL2 and CCL5 on cell homing to inflammation sites could probably be better demonstrated in vivo.

Similarly to TGF-β1, the reaction medium contained a relatively large amount of IDO. Our results still show a significantly higher secretion of IDO in cocultures compared with reactions containing ASCs only. In reactions in which ASCs were cultured alone,

the measured IDO was lower than in the medium controls, which suggests that ASCs do not readily express IDO without activation [63]. When considering the potent immunosuppressive effects of IDO, our results are somewhat opposed to the two-way MLR assay results. The lowest levels of IDO were in the cocultures containing ASCs expanded in the FBS medium, although the stronger suppression potential was also observed in FBS conditions. This might be due to the high initial concentration of IDO or due to the fact that complete secretion of IDO would have required a longer coculture, and thus other factors have a stronger effect than IDO in the reactions performed here [64].

Galectin-1 and -3 were included in the secreted protein panel because of their reported importance in regulating immune reactions and reported function as mediators of immune suppression caused by MSCs [26, 65]. Galectin-1 is clearly linked to immunosuppression, and galectin-3 also has T-cell proliferative functions in addition to suppressive functions [66]. Thus, it is not surprising that our results on galectin-1 and -3 are partially opposing. Galectin-1 was low in the reactions containing only PBMCs, and although ASCs alone expressed some galectin-1, the expression was higher in direct and indirect cocultures. Galectin-1 induces apoptosis in T cells and suppresses immune responses, which is in line with the suppression of proliferation and elevated galectin-1 concentrations in the cocultures. Similar to galectin-1, galectin-3 was upregulated in cocultures and compared with reactions with ASCs alone; there were no significant differences between the cocultures containing ASCs expanded in different conditions. Unlike with galectin-1, the galectin-3 concentration was relatively high in the reactions with PBMCs only, and it was also higher in indirect cocultures than in direct cocultures. The higher concentrations of galectin-3 that were measured in cocultures are the opposite of the proliferation assay results, but might well be due to the relatively high expression seen already in reactions with PBMCs only and the very low expression in reactions containing only ASCs. These results might also be partially explained by the differences in overall expression patterns and functions between the two galectins [67], but according to our results, galectin-3 expression is not as good an indicator of immune suppression as galectin-1: galectin-1 concentrations correlated well with the proliferation assay results. Figure 4 summarizes the key signaling proteins investigated in the present study. The increase or decrease in protein secretion is presented in the direct coculture compared with the indirect culture and vice versa.

Furthermore, flow cytometric analyses revealed that the cell surface marker expression profile of ASCs was largely similar in cells grown under different culture conditions. However, statistically significant differences were still found for the expression of CD45, which was more expressed in XF/SF conditions in passage 2. CD45 is a receptor-linked protein-tyrosine phosphatase that is expressed on all leukocytes playing a crucial role in the function of these cells, and the expression has been shown to be essential for differentiation and antigen receptor-mediated signal transduction in leukocytes [68, 69]. XF/SF-expanded ASCs showed a higher expression of CD45, and in line with higher expression, they also proved to be less immunosuppressive in XF/SF conditions in passage 2. However, because of large patient variation, no significant differences were seen in passage 5, and more studies are needed to draw a conclusion and show a link between immunosuppression capacity and the expression of CD45 in ASCs.

The suppression capacity of MSCs holds promise for the future treatments of severe immunological disorders, such as GVHD. Several recognized clinical studies have thus been performed to investigate the potential of BMSCs for the treatment of GVHD [15, 70–73]. In addition, both preclinical and clinical studies have been performed to evaluate the use of ASCs to prevent acute GVHD, as reviewed by Leto Barone et al. [74]. Nonetheless, if ASCs are used for patients for immunomodulatory purposes, it is important to carefully assess the risk for the possibility of tumor formation connected with ASC-mediated suppression. There is evidence that tumors are potential sites for inflammatory cytokine and chemokine production, which may enable MSCs to home in on tumor sites and as a result immunomodulate the tumor environment [75, 76]. Furthermore, if MSCs may play a role in tumor growth and metastasis as has been stated [77, 78], there is a potential risk in the use of MSCs in cell-based therapies. Compte et al. have shown that tumor angiogenesis in particular may be enhanced by MSCs, which is critical considering tumor development [79]. Taking these previous results into account, the possible supportive effect on the pathogenesis and progression of tumors should be clarified carefully before further clinical applications of immunomodulation.

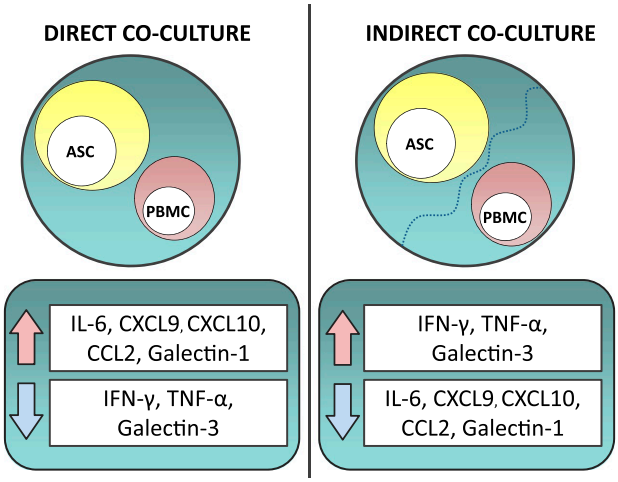

Figure 4. Schematic overview highlighting the key signaling proteins of our study. The increase or decrease in protein secretion is presented in direct coculture compared with indirect culture. All changes in secretion between direct versus indirect cocultures were statistically significant ($p < .05$). Abbreviations: ASC, adipose stem cell; CCL2, C-C chemokine ligand 2; CXCL9, and -10, CXC chemokine ligands 9 and 10; IFN-γ, interferon γ; IL-6, interleukin 6; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor α.

Conclusion

Our findings demonstrated that the isolation and expansion conditions of ASCs have an effect on immunogenicity, suppressive potential, and protein secretion profile of the cells. Nevertheless, ASCs elicit only a low immunogenic response whether cultured in FBS, HS, or in XF/SF conditions, and suppressive potential is detectable in every condition with high ASC numbers.

The strongest immunosuppression and lowest immunogenicity was observed with ASCs expanded in the FBS-supplemented medium. High IL-6 and low IFN-γ secretion was observed in FBS conditions, which may have a link to stronger suppressive potential. By contrast, ASCs expanded in XF/SF conditions induced the strongest proliferative response in PBMCs and showed less immunosuppressive potential, that was in line with low IL-6

and TGF-β1 and high IFN-γ and TNF-α secretion in XF/SF conditions. However, the differences between culture conditions were minor, especially for immunogenicity. In addition to low immunogenicity, the safety and efficacy of allogeneic ASC treatments should be evaluated based on the proliferation and differentiation potential of the cells in these different conditions. Therefore, considering allogeneic stem cell treatments, FBS may not be the optimal culture condition for clinical use. For immunosuppressive purposes, FBS-expanded cells may have potential, but there are several factors affecting the immunosuppression process. The mechanism behind the suppressive capacity involves direct cell-cell contact, secretion of soluble factors, and the modulation inflammatory environment. Moreover, by increasing the ASC number, suppression can also be achieved with HS- or XF/SF-expanded cells. In conclusion, ASCs have potential for immunomodulation therapies and allogeneic stem cell treatments in the future. Still, the effect of the culture medium on cell characteristics should not be disregarded when clinical applications are considered.

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Author Contributions

M.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; H.H. and M.H.: data analysis and interpretation, final approval of manuscript; F.S.: collection and/or assembly of data, final approval of manuscript; B.M. and S.M.: conception and design, administrative support, data analysis and interpretation, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279–4295.
2 Gimble J, Guilak F. Adipose-derived adult stem cells: Isolation, characterization, and differentiation potential. Cytotherapy 2003;5:362–369.
3 McIntosh K, Zvonic S, Garrett S et al. The immunogenicity of human adipose-derived cells: Temporal changes in vitro. Stem Cells 2006;24:1246–1253.
4 Kronsteiner B, Wolbank S, Peterbauer A et al. Human mesenchymal stem cells from adipose tissue and amnion influence T-cells depending on stimulation method and presence of other immune cells. Stem Cells Dev 2011;20:2115–2126.
5 Casteilla L, Planat-Benard V, Laharrague P et al. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. World J Stem Cells 2011;3:25–33.
6 Crop MJ, Baan CC, Korevaar SS et al. Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. Stem Cells Dev 2010;19:1843–1853.
7 Lee JM, Jung J, Lee HJ et al. Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. Int Immunopharmacol 2012;13:219–224.
8 Kuo YR, Chen CC, Goto S et al. Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. Plast Reconstr Surg 2011;128:661e–672e.
9 Niemeyer P, Kornacker M, Mehlhorn A et al. Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. Tissue Eng 2007;13:111–121.
10 Eliopoulos N, Stagg J, Lejeune L et al. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 2005;106:4057–4065.
11 Puissant B, Barreau C, Bourin P et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. Br J Haematol 2005;129:118–129.
12 Yoo KH, Jang IK, Lee MW et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol 2009;259:150–156.
13 McIntosh KR, Lopez MJ, Borneman JN et al. Immunogenicity of allogeneic adipose-derived stem cells in a rat spinal fusion model. Tissue Eng Part A 2009;15:2677–2686.
14 Niemeyer P, Vohrer J, Schmal H et al. Survival of human mesenchymal stromal cells from bone marrow and adipose tissue after xenogenic transplantation in immunocompetent mice. Cytotherapy 2008;10:784–795.
15 Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: A phase II study. Lancet 2008;371:1579–1586.
16 Ringdén O, Uzunel M, Rasmusson I et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 2006;81:1390–1397.
17 Resnick IB, Barkats C, Shapira MY et al. Treatment of severe steroid resistant acute GVHD with mesenchymal stromal cells (MSC). Am J Blood Res 2013;3:225–238.
18 Kim N, Im KI, Lim JY et al. Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: Experiments and practice. Ann Hematol 2013;92:1295–1308.
19 Waterman RS, Tomchuck SL, Henkle SL et al. A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS One 2010;5:e10088.
20 Ren G, Zhang L, Zhao X et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141–150.
21 Krampera M, Cosmi L, Angeli R et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386–398.
22 Melief SM, Zwaginga JJ, Fibbe WE et al. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. Stem Cells Translational Medicine 2013;2:455–463.
23 Cui L, Yin S, Liu W et al. Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2. Tissue Eng 2007;13:1185–1195.
24 Crop MJ, Baan CC, Korevaar SS et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol 2010;162:474–486.
25 Burr SP, Dazzi F, Garden OA. Mesenchymal stromal cells and regulatory T cells: The Yin and Yang of peripheral tolerance? Immunol Cell Biol 2013;91:12–18.
26 Sioud M. New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. Scand J Immunol 2011;73:79–84.
27 Sioud M, Mobergslien A, Boudabous A et al. Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. Int J Oncol 2011;38:385–390.
28 Ren G, Zhao X, Zhang L et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol 2010;184:2321–2328.
29 Shi Y, Hu G, Su J et al. Mesenchymal stem cells: A new strategy for immunosuppression and tissue repair. Cell Res 2010;20:510–518.
30 Patrikoski M, Juntunen M, Boucher S et al. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. Stem Cell Res Ther 2013;4:27.

31 Lindroos B, Boucher S, Chase L et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy 2009; 11:958–972.

32 Chase LG, Yang S, Zachar V et al. Development and characterization of a clinically compliant xeno-free culture medium in good manufacturing practice for human multipotent mesenchymal stem cells. STEM CELLS TRANSLATIONAL MEDICINE 2012;1:750–758.

33 Santos F, Andrade PZ, Abecasis MM et al. Toward a clinical-grade expansion of mesenchymal stem cells from human sources: A microcarrier-based culture system under xeno-free conditions. Tissue Eng Part C Methods 2011;17:1201–1210.

34 Rajala K, Lindroos B, Hussein SM et al. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One 2010;5:e10246.

35 Mackensen A, Dräger R, Schlesier M et al. Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. Cancer Immunol Immunother 2000; 49:152–156.

36 Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood 1997;89:776–779.

37 Spees JL, Gregory CA, Singh H et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9:747–756.

38 Heiskanen A, Satomaa T, Tiitinen S et al. *N*-Glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. STEM CELLS 2007;25:197–202.

39 Haimi S, Moimas L, Pirhonen E et al. Calcium phosphate surface treatment of bioactive glass causes a delay in early osteogenic differentiation of adipose stem cells. J Biomed Mater Res A 2009;91:540–547.

40 McIntosh KR. Evaluation of cellular and humoral immune responses to allogeneic adipose-derived stem/stromal cells. Methods Mol Biol 2011;702:133–150.

41 Anton K, Banerjee D, Glod J. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. PLoS One 2012;7:e35036.

42 Chan JL, Tang KC, Patel AP et al. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma. Blood 2006;107:4817–4824.

43 Chen PM, Yen ML, Liu KJ et al. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J Biomed Sci 2011;18:49.

44 Jacobs SA, Roobrouck VD, Verfaillie CM et al. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. Immunol Cell Biol 2013;91:32–39.

45 Trivedi HL, Vanikar AV, Thakker U et al. Human adipose tissue-derived mesenchymal stem cells combined with hematopoietic stem cell transplantation synthesize insulin. Transplant Proc 2008;40:1135–1139.

46 Tzouvelekis A, Koliakos G, Ntolios P et al. Stem cell therapy for idiopathic pulmonary fibrosis: A protocol proposal. J Transl Med 2011;9:182.

47 Thesleff T, Lehtimäki K, Niskakangas T et al. Cranioplasty with adipose-derived stem cells and biomaterial: A novel method for cranial reconstruction. Neurosurgery 2011;68:1535–1540.

48 Dromard C, Bourin P, André M et al. Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres. Exp Cell Res 2011;317:770–780.

49 Ren ML, Peng W, Yang ZL et al. Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs. Cell Transplant 2012;21:2711–2721.

50 Sharma AA, Jen R, Butler A et al. The developing human preterm neonatal immune system: A case for more research in this area. Clin Immunol 2012;145:61–68.

51 Ren G, Roberts AI, Shi Y. Adhesion molecules: Key players in mesenchymal stem cell-mediated immunosuppression. Cell Adhes Migr 2011;5:20–22.

52 Shimada Y, Hasegawa M, Kaburagi Y et al. L-selectin or ICAM-1 deficiency reduces an immediate-type hypersensitivity response by preventing mast cell recruitment in repeated elicitation of contact hypersensitivity. J Immunol 2003;170:4325–4334.

53 De Ugarte DA, Alfonso Z, Zuk PA et al. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunol Lett 2003;89:267–270.

54 Chan WK, Lau AS, Li JC et al. MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. Exp Hematol 2008;36:1545–1555.

55 Wu M, Han ZB, Liu JF et al. Serum-free media and the immunoregulatory properties of mesenchymal stem cells in vivo and in vitro. Cell Physiol Biochem 2014;33:569–580.

56 Melief SM, Geutskens SB, Fibbe WE et al. Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. Haematologica 2013;98:888–895.

57 Scheller J, Chalaris A, Schmidt-Arras D et al. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813:878–888.

58 Ulich TR, Yin S, Guo K et al. Intratracheal injection of endotoxin and cytokines: II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. Am J Pathol 1991;138:1097–1101.

59 Wahl SM, Wen J, Moutsopoulos N. TGF-beta: A mobile purveyor of immune privilege. Immunol Rev 2006;213:213–227.

60 Tiemessen MM, Kunzmann S, Schmidt-Weber CB et al. Transforming growth factor-beta inhibits human antigen-specific CD4+ T cell proliferation without modulating the cytokine response. Int Immunol 2003;15:1495–1504.

61 Gilbert KM, Thoman M, Bauche K et al. Transforming growth factor-beta 1 induces antigen-specific unresponsiveness in naive T cells. Immunol Invest 1997;26:459–472.

62 Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. Annu Rev Immunol 1998;16:137–161.

63 Meisel R, Zibert A, Laryea M et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004; 103:4619–4621.

64 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–1822.

65 Gieseke F, Böhringer J, Bussolari R et al. Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. Blood 2010;116:3770–3779.

66 Hernandez JD, Baum LG. Ah, sweet mystery of death! Galectins and control of cell fate. Glycobiology 2002;12:127R–136R.

67 Perillo NL, Marcus ME, Baum LG. Galectins: Versatile modulators of cell adhesion, cell proliferation, and cell death. J Mol Med (Berl) 1998 76:402–412 [BERL].

68 Altin JG, Sloan EK. The role of CD45 and CD45-associated molecules in T cell activation. Immunol Cell Biol 1997;75:430–445.

69 Matsuda A, Motoya S, Kimura S et al. Disruption of lymphocyte function and signaling in CD45-associated protein-null mice. J Exp Med 1998;187:1863–1870.

70 Lazarus HM, Koc ON, Devine SM et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005;11:389–398.

71 Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439–1441.

72 Papewalis C, Topolar D, Gotz B et al. Mesenchymal stem cells as cellular immunotherapeutics in allogeneic hematopoietic stem cell transplantation. Adv Biochem Eng Biotechnol 2013;130:131–162.

73 Vaes B, Van't Hof W, Deans R et al. Application of MultiStem(®) allogeneic cells for immunomodulatory therapy: Clinical progress and pre-clinical challenges in prophylaxis for graft versus host disease. Front Immunol 2012;3:345.

74 Leto Barone AA, Khalifian S, Lee WP et al. Immunomodulatory effects of adipose-derived stem cells: Fact or fiction? Biomed Res Int 2013; 2013:383685.

75 Zimmerlin L, Park TS, Zambidis ET et al. Mesenchymal stem cell secretome and regenerative therapy after cancer. Biochimie 2013; 95:2235–2245.

76 Hall B, Dembinski J, Sasser AK et al. Mesenchymal stem cells in cancer: Tumor-associated fibroblasts and cell-based delivery vehicles. Int J Hematol 2007;86:8–16.

77 Karnoub AE, Dash AB, Vo AP et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007; 449:557–563.

78 Djouad F, Plence P, Bony C et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003;102:3837–3844.

79 Compte M, Cuesta AM, Sánchez-Martín D et al. Tumor immunotherapy using gene-modified human mesenchymal stem cells loaded into synthetic extracellular matrix scaffolds. STEM CELLS 2009;27:753–760.

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Effects of bioactive glass S53P4 or beta-tricalcium phosphate and bone morphogenetic protein-2 and bone morphogenetic protein-7 on osteogenic differentiation of human adipose stem cells

Martin Waselau[1,2], Mimmi Patrikoski[2,3,4], Miia Juntunen[2,3,4], Kasperi Kujala[2,3,4,5], Minna Kääriäinen[6], Hannu Kuokkanen[6], George K Sándor[2,3,4,5], Outi Vapaavuori[1], Riitta Suuronen[2,3,4], Bettina Mannerström[2,3,4], Brigitte von Rechenberg[7] and Susanna Miettinen[2,3,4]

Abstract

The effects of bioactive glass S53P4 or beta-tricalcium phosphate; and bone morphogenetic proteins bone morphogenetic protein-2, bone morphogenetic protein-7, or bone morphogenetic protein-2 + 7 on osteogenic differentiation of human adipose stem cells were compared in control medium, osteogenic medium, and bone morphogenetic protein-supplemented osteogenic medium to assess suitability for bone tissue engineering. Cell amount was evaluated with qDNA measurements; osteogenic differentiation using marker gene expression, alkaline phosphate activity, and angiogenic potential was measured by vascular endothelial growth factor expression. As compared to beta-tricalcium phosphate, cell amount was significantly greater for bioactive glass in control medium after 7 days and in osteogenic medium after 14 days, and alkaline phosphate activity was always significantly greater for bioactive glass in control medium. However, alkaline phosphate activity increased for beta-tricalcium phosphate and decreased for bioactive glass granules in osteogenic medium. For both biomaterials, bone morphogenetic protein supplementation decreased cell amount and osteogenic differentiation of human adipose stem cells, and vascular endothelial growth factor expressions correlated with cell amounts. Effects of culture medium on human adipose stem cells are biomaterial dependent; bioactive glass in control medium enhanced osteogenic differentiation most effectively.

Keywords

bioactive glass, beta-tricalcium phosphate, bone morphogenetic protein-2, bone morphogenetic protein-7, human adipose stem cells, osteogenic differentiation, in vitro

Introduction

The regeneration potential of normal bone is excellent due to extensive vascular supply. However, healing of large bone defects after severe trauma, reconstructive or ablative surgery is still challenging despite recent advances in technical and surgical methods.[1] Over the past decades, bone grafts and different biomaterials have been employed with variable results.[1] Therefore, there is still a need to enhance fracture or bone defect repair. Autologous bone grafts are still considered the gold standard for reconstructive bone surgery due to low immunogenicity, simultaneous presence of stem cells, and growth factors as well as their osteoinductive/osteoconductive properties. However, donor site morbidity and limited availability are of concern.[1]

[1]Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland
[2]Adult Stem Cell Group, Institute of Biomedical Technology, University of Tampere, Tampere, Finland
[3]BioMediTech, Tampere, Finland
[4]Science Center, Tampere University Hospital, Tampere, Finland
[5]Faculty of Medicine, Institute of Dentistry, University of Oulu, Oulu, Finland
[6]Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland
[7]Musculoskeletal Research Unit, Competence Center for Applied Biotechnology and Molecular Medicine, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

Corresponding author:
Martin Waselau, Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 57 (Viikintie 49), FIN-00014 Helsinki, Finland.
Email: martin.waselau@uta.fi

Therefore, suitability of bone tissue engineering and cell-based therapies utilizing stem cells, co-incubated with biological factors, and seeded on biomaterials have recently been explored to regenerate bone.[2] We and others have treated successfully patients with bone defects in cranio-maxillofacial area using autologous human adipose stem cells (hASCs) in combination with biomaterials.[3,4] Among the stem cells used, hASCs have gained popularity as alternative source of mesenchymal stem cells (MSCs) to human bone marrow stromal cells (hBMSCs) due to abundant availability, ease of harvesting, simple processing, and proven ability to differentiate into multiple lineages including osteoblasts.[5]

Human ASCs are traditionally cultured and expanded in medium supplemented with animal-derived fetal bovine serum (FBS) posing the risk for zoonotic diseases and allergic reactions. Hence, the use of FBS is one concern for its direct clinical application in humans.[6] Therefore, standardized human serum (HS) has recently being investigated, and research is directed toward the development of even xeno-free culture media, produced according to good manufacturing practice.[7,8] It is also possible to use autologous HS for cell expansion.[3,4] Isolated hASCs have also co-incubated with osteogenic nutrients to promote osteogenic differentiation in vitro and, finally to stimulate new bone formation in vivo.[3] Among the additives, the osteogenic potential of bone morphogenetic protein-2 and -7 (BMP-2 and BMP-7) on hASCs has been investigated over the last years.[9] Currently, the osteoinductive growth factors BMP-2 and BMP-7 are in clinical use.[3,10,11] However, controversial results were recently reported regarding their beneficial role in osteogenesis requiring further clarification.[11]

Implanted biomaterials serve as initial scaffolds, cell attachment bases and may induce signals for cell differentiation, and in the past, several bioactive materials were thoroughly investigated.[12] Among those, bioactive glass (BAG) and beta-tricalcium phosphate (β-TCP) have been used widely in oral–maxillofacial and orthopedic surgery due to good biocompatibility and ability to support osteoblastic growth and maturation.[13–15] Customized implants that contain osteogenic cells (e.g. hASCs), osteoinductive factors (e.g. BMPs) along with a synthetic osteoconductive matrix (e.g. BAG or β-TCP) represent an attractive alternative to autografts and allografts while uniting all three bone-forming properties in a more controlled and effective combination.

To our knowledge, the response of hASCs to BMP-2/7 and BAG or β-TCP after expansion in HS has not been reported yet. Therefore, the aim of the current study was to evaluate and compare the effect of (1) BAG and β-TCP and (2) BMP-2 and BMP-7 or both on osteogenic differentiation of hASCs when maintained in medium containing HS.

Materials and methods

Ethics statement, hASCs isolation, and culture

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058), and the Declaration of Helsinki 1975, revised Hong Kong 1989. Adipose tissue samples were harvested as by-products of open surgical procedures from six patients (age = 39 ± 18 years, one male and five female patients) at the Tampere University Hospital, Finland. A written consent form was obtained from each patient before the procedure. Subsequently, hASCs were isolated and cultured as described elsewhere.[5] Briefly, samples were washed with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, UK), minced manually into smaller pieces, digested with 1.5 mg/mL collagenase type I (Life Technologies, UK) and were incubated in a water bath at 37°C for 90 min. Subsequently, the digested tissue was centrifuged (600g, 10 min) in consecutive steps achieving sufficient segregation of hASCs from connective tissue. The supernatant was discarded, the cell pellet resuspended in 10% HS and, finally, filtered through a 100 μm strainer. Subsequently, isolated cells were maintained and expanded in polystyrene flasks (Nunc, Denmark) in control medium (CM) containing Dulbecco's modified Eagle medium (DMEM)/Ham's nutrient mixture F-12 (F-12 1:1; Invitrogen) that was supplemented with 1% L-glutamine (GlutaMAX; Invitrogen), 1% antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin; Invitrogen), and 10% HS (PAA Laboratories GmbH, Austria) at 37°C and 5% CO_2. The cells were passaged when the flasks approached about 80% confluency and were detached enzymatically using trypsin (TrypLE Select™, Invitrogen). Finally, expanded hASCs were cryopreserved using liquid nitrogen in a freezing solution containing 10% dimethyl sulfoxide (Hybri-Max, Sigma–Aldrich, USA) and 10% HS. Before experiments, hASCs were thawed and expanded in CM, and cell passages 1–4 were used.

Flow cytometric analysis of hASC surface marker expression

After primary culture for cell passages 1–2, hASCs were harvested and characterized by flow cytometry (FACSAria™; BD Biosciences, Belgium) as described previously.[16] Monoclonal antibodies against CD14–phycoerythrin–cyanine (PECy7), CD19–PECy7, CD90–allophycocyanin (APC) (BD Biosciences), CD34–APC, HLA-DR–phycoerythrin (PE) (ImmunoTools GmbH, Germany), and CD105–PE (R&D Systems Inc., USA) were employed. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

Table 1. Overview on experimental design—group assignments

BAG[a] + CM[b]	BAG + OM[c]	BAG + BMP-2[d]	BAG + BMP-7[e]	BAG + BMP-2/7[f]
TCP[g] + CM	TCP + OM	TCP + BMP-2	TCP + BMP-7	TCP + BMP-2/7

BAG: bioactive glass; CM: control medium; OM: osteogenic medium; BMP: bone morphogenetic protein; TCP: tricalcium phosphate; β-TCP: beta-tricalcium phosphate.
[a]Bioactive glass granules (BAG group).
[b]Control medium.
[c]Osteogenic medium.
[d]Bone morphogenic protein 2 (BMP-2 medium).
[e]Bone morphogenic protein 7 (BMP-7 medium).
[f]Combination of bone morphogenic protein 2 and 7 (BMP-2/7 medium).
[g]Beta-tricalcium phosphate granules (β-TCP group).

Biomaterials and growth factors

Commercially available biomaterials accepted for clinical use were selected for the study. BAG granules (S53P4, 23% Na_2O, 20% CaO, 53% SiO_2, 4% P_2O_5, BoneAlive granules, 1.0–2.0 mm; BoneAlive Biomaterials Ltd, Finland) and β-TCP granules (ChronOS granules, 1.4–2.8 mm, porosity 60%; Synthes, Switzerland) were used in the study. Also, clinically used BMP-2 and BMP-7 (Sigma–Aldrich) were chosen as additive for the osteogenic media evaluated.

Seeding and osteogenic differentiation of hASCs on biomaterial combinations

Sterile BAG and β-TCP granules (400 µL biomaterials/well) were incubated with CM in a 24-well plate (Nunc) for 48 h before cell seeding for equilibration purposes. For osteogenic medium (OM), CM was supplemented with L-ascorbic acid-2-phosphate (50 mM), β-glycerophosphate (500 µM), and dexamethasone (10 µM) (all Sigma–Aldrich). Subsequently, individual treatment media (TM) were produced containing OM only, OM + BMP-2 (Sigma–Aldrich, dose 100 ng/mL), OM + BMP-7 (Sigma–Aldrich, dose 100 ng/mL), and OM + BMP-2 + BMP-7 (both 100 ng/mL) resulting in the following combinations: (1) BAG + CM (BAG + CM), (2) BAG + OM (BAG + OM), (3) BAG + OM + BMP-2 (BAG + BMP-2), (4) BAG + OM + BMP-7 (BAG + OM + BMP-7), (5) BAG + OM + BMP-2/7 (BAG + BMP-2/7), (6) β-TCP + CM (TCP + CM), (7) β-TCP + OM (TCP + OM), (8) β-TCP + OM + BMP-2 (TCP + BMP-2), (9) β-TCP + OM + BMP-7 (TCP + BMP-7), and (10) β-TCP + OM + BMP-2/7 (TCP + BMP-2/7) (Table 1). Dosages for BMPs were chosen based on earlier studies.[17] Previously isolated hASCs were suspended with each individual treatment medium to initiate osteogenic differentiation and, finally, were seeded on both biomaterials (50,000 cells/well) as described earlier.[17] Seeded grafts were maintained in culture at 37.5°C and 5% CO_2 changing individual media every 48 h until final analyses.

Cell amount

After 1, 7, and 14 days in culture, the amount of hASCs was evaluated quantitatively using CyQUANT®, Cell Proliferation Assay Kit (Molecular Probes, Invitrogen) that measured DNA amounts in samples as described elsewhere.[8] Briefly, all cells were lysed using 0.1% Triton-X 100 buffer (Sigma–Aldrich), and the supernatant was collected and stored at −80°C until final analyses. A volume of 20 µL of each sample were mixed with CyQUANT GR dye and lysis buffer in a 96-well plate (Nunc) after a freeze–thaw cycle. Fluorescence signals were measured with a multiple plate reader (Victor 1420 Multilabel Counter; Wallac, Finland) at 480 or 520 nm.

Alkaline phosphatase staining and quantitative alkaline phosphatase analyses

The osteogenic differentiation of isolated hASCs was determined qualitatively by alkaline phosphatase (ALP) staining and quantitatively by ALP measurements. Subsequently, after 1, 7, and 14 days in culture, all samples were stained using a leukocyte ALP kit (Sigma–Aldrich, #86R-1KT). Therefore, cell cultures were washed twice using DPBS and fixed in 4% paraformaldehyde. ALP staining solution was pipetted into each well, incubated for 15 min, washed in deionized water, and finally evaluated macroscopically.

After 1, 7, and 14 days in culture, quantitative alkaline phosphatase (qALP) activities were measured photometrically according to Sigma ALP (Sigma–Aldrich) at 405 nm (Victor 1420). The ALP activities were measured from the same Triton-X 100 lysates as used for the cell numbers.

Quantitative real-time reverse transcription polymerase chain reaction to measure early markers of osteogenic differentiation

The mRNA expression levels of early markers in osteogenesis including osteopontin (OPN), runt-related

Table 2. Overview on sequences of osteogenic marker genes determined

Name	Primer direction	Sequences	Product size (bp)
hRPLP0[a]	Forward	5′-AAT CTC CAG GGG CAC CAT T-3′	70
	Reverse	5′-CGC TGG CTC CCA CTT TGT-3′	
hCOL1[b]	Forward	5′-CCA GAA GAA CTG GTA CAT CAG CAA-3′	94
	Reverse	5′-CGC CAT ACT CGA ACT GGA ATC-3′	
hOC[c]	Forward	5′-AGC AAA GGT GCA GCC TTT GT-3′	94
	Reverse	5′-GCG CCT GGG TCT CTT CAC T-3′	
hOPN[d]	Forward	5′-GCC GAC CAA GGA AAA CTC ACT-3′	71
	Reverse	5′-GGC ACA GGT GAT GCC TAG GA-3′	
hRUNX2[e]	Forward	CCCGTGGCCTTCAAGGT	76
	Reverse	CGTTACCCGCCATGACAGTA	

[a]Ribosomal protein, large, P0 (Acc.No.: NM_001002).
[b]Collagen, type I, alpha 1 (Acc.No.: NM_00088).
[c]Osteocalcin (Acc.No.: NM_000711).
[d]Osteopontin (Acc.No.: J04765).
[e]Runt-related transcription factor 2 (Acc.No.: NM_004348).

transcription factor 2 (RUNX-2), collagen type-1 (Col-1), and osteocalcin (OC) were measured 14 days after cell seeding and incubation using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The expression of RPLP0 was used to normalize expression levels between samples. Therefore, total RNA was isolated from hASCs using TRIzol reagent (Invitrogen) following the manufacturer's guidelines. Sequences and accession numbers of all primers (Oligomer Oy, Finland) are displayed in Table 2. All reactions were performed using ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, UK), and the relative gene expression for each individual marker was calculated according to a mathematical model.

Expression of vascular endothelial growth factor

At days 1, 7, and 14, vascular endothelial growth factor (VEGF) was measured using a human VEGF immunoassay (R&D Systems, UK) according to the manufacturer's instructions. Briefly, 50 µL of assay diluent and 200 µL cell culture sample supernate were added into each well and incubated for 2 h at room temperature (RT). Subsequently, all samples were aspirated and washed for three times before 200 µL of VEGF conjugate was pipetted into each well and incubated for 2 h at RT. The previous aspiration-washing cycle was repeated followed by addition of 200 µL substrate solution and incubated for 20 min at RT. Finally, the reaction was terminated with 50 µL of stop solution, and optical density of each well was determined using a microplate reader set at a wave length of 450 nm.

Statistical analyses

Quantitative analyses of cell amounts and qALP were run in duplicates per experiment, and experiments were repeated five times. Quantitative measurements of OPN, RUNX-2, Col-1, OC, and VEGF were run in duplicates per experiment, and experiments were repeated three times. All data were presented as mean ± standard deviation (SD). A one-way analysis of variance with the Bonferroni post hoc test for multiple comparisons was used to study statistically significant differences between study groups. The nonparametric Spearman correlation test was used to study correlation between DNA amounts, expression of VEGF, and ALP activity. Values of $p < 0.05$ were regarded as significant. All graphs and statistics were done using GraphPad Prism 5.01 software.

Results

Cell surface maker profile of hASC in medium containing 10% HS

Human ASCs were isolated and expanded using cell culture medium containing 10% HS. After expansion, cell surface marker expression profile of hASCs was analyzed by flow cytometry. Human ASCs showed positive expression (>70%) for the surface markers CD73 (Ecto-5′-nucleotidase), CD90 (Thy-1), and CD105 (Endoglin) and lacked (<2%) the expression of CD14, CD19, and HLA-DR. Furthermore, moderate expression was recorded for the hematopoietic progenitor marker CD34. Cytometric analyses confirmed the characteristic immunophenotype of hASCs, which was similar to earlier reports.[16]

Cell amounts in BAG were greater than in β-TCP in OM and CM

Quantitatively, the DNA amount was analyzed as an indicator of cell amounts at days 1, 7, and 14. Results of both

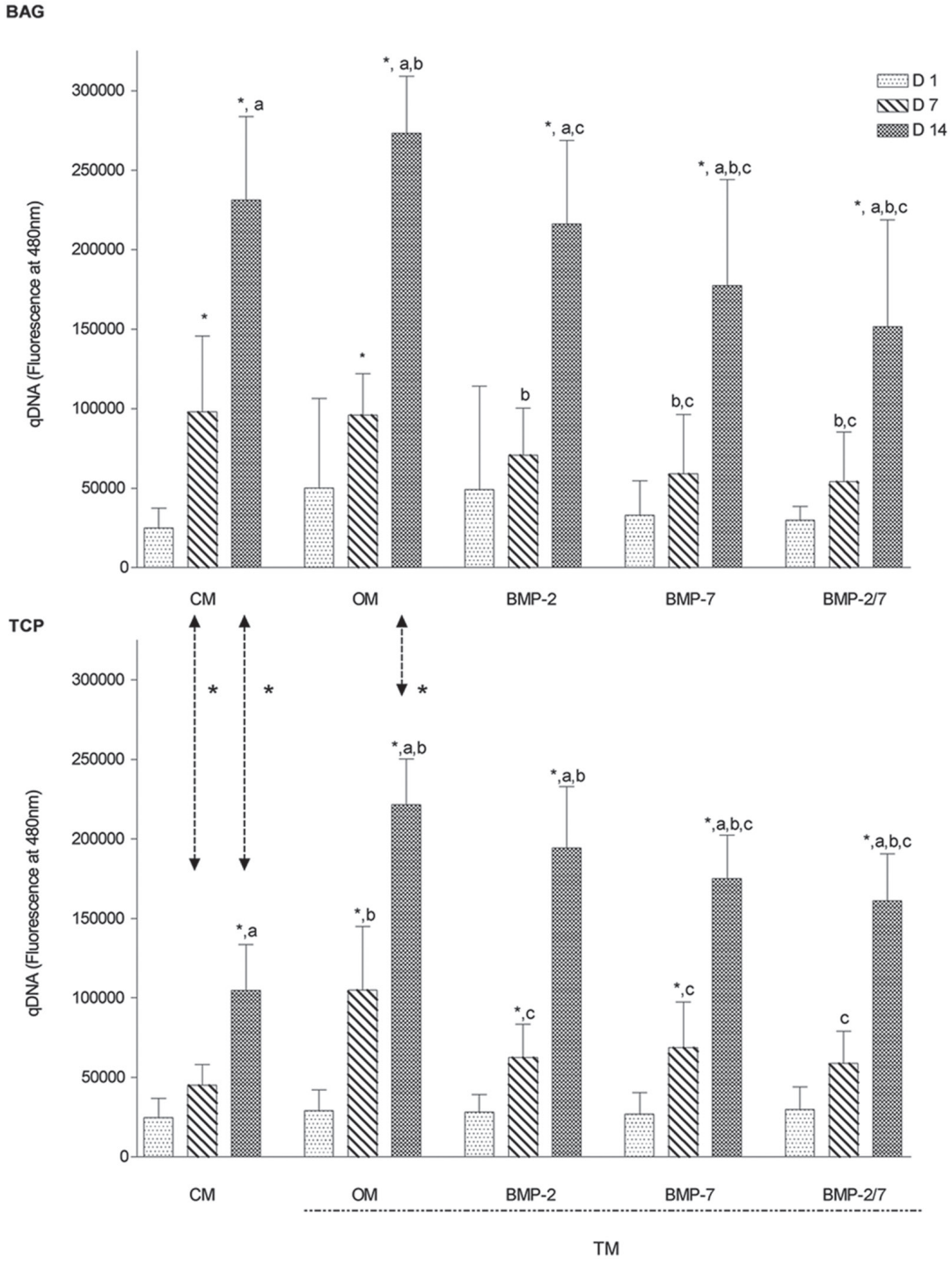

Figure 1. Comparative overview on cell amount over time (N = 10 replicates per time point). Overall, cell amount increased continuously over time independent on biomaterial evaluated. Cell amount was greatest after exposure to BAG in OM. After 7 days, BAG increased cell amount significantly more in CM as compared to β-TCP. Generally, BMP supplementation decreased cell amount independent on biomaterial.

BAG: bioactive glass; β-TCP: beta-tricalcium phosphate; CM: control medium; OM: osteogenic medium; BMP: bone morphogenetic protein; TM: treatment media.

The symbol "*" indicates significant difference between days 1–7 and days 7–14; "a" indicates significant difference between days 1 and 14; "b" indicates significant difference as compared to CM; "c" indicates significant difference as compared to OM; arrows represent significant differences between biomaterials.

biomaterials, when maintained under the same culture conditions, were compared to each other. In the BAG group, cell number increased continuously and significantly in CM and TM ($p < 0.0001$). In the TCP group, however, cell amount increased similarly in OM, BMP-2, and BMP-7 medium (Figure 1). DNA amount was significantly greater in the BAG group in CM at days 7 and 14 ($p < 0.001$) and in OM at day 14 ($p < 0.0001$) as compared to the β-TCP group, suggesting a greater cell proliferation rate in response to BAG (Figure 1).

BMP-2- and BMP-7-supplemented media decrease cell amount regardless of biomaterial

For the BAG and β-TCP group individually, results of CM and TM were compared among each other to evaluate the effects of different media on cell amount. In the BAG group, when compared to the CM, significantly lower qDNA amounts were measured in BMP-2 medium at day 7 as well as in BMP-7 and BMP-2/7 medium at days 7 and 14 ($p < 0.03$), but significantly higher qDNA levels were measured in OM at day 14 ($p < 0.01$) (Figure 1). When compared to OM, cell amount was significantly lower in all BMP media at days 7 and 14 ($p < 0.03$) except for BMP-2 at day 7, suggesting a negative impact of BMP supplementation on cell amount in BAG (Figure 1). In the β-TCP group, when compared to CM, overall qDNA amounts were significantly higher in OM at days 7 and 14 and in BMP-supplemented media at day 14 ($p < 0.05$) suggesting enhanced cell proliferation. When compared to OM, cell amount was significantly lower in BMP-2 media at day 7 as well as in BMP-7 and BMP-2/7 at days 7 and 14 ($p < 0.03$). Overall, BMP supplementation of TM resulted in reduced cell amounts as compared to OM independent of biomaterial (Figure 1).

ALP activity in response to CM and OM is differently regulated in hASCs cultured with BAG and β-TCP

Qualitatively, osteogenic differentiation of hASCs was determined by ALP staining and quantitatively by ALP analyses. Results of BAG and β-TCP groups, when maintained in the same media, were compared to evaluate the effects of each biomaterial on osteogenic differentiation. Overall, our qALP measurements were consistent with the ALP staining results.

ALP staining indicated osteogenic differentiation of hASCs in BAG and β-TCP groups, but both biomaterials influenced this process differently. BAG exerted the most beneficial effect on differentiation when maintained in CM only (Figure 2), and ALP activity was decreased when OM was used. On the contrary, hASCs cultured with β-TCP required OM for osteogenic differentiation (Figure 2). Although BMP supplementation demonstrated no qualitative enhancing effect on osteogenic differentiation independent of biomaterial, a significantly lower ALP activity was quantitatively measured in BAG group in BMP-2 and BMP-7 medium at day 14 ($p < 0.05$) when compared to β-TCP group (Figure 3). The superior osteogenic potential of BAG when maintained in plain CM was confirmed by significantly greater qALP activities at days 1, 7, and 14 ($p < 0.001$) (Figure 3).

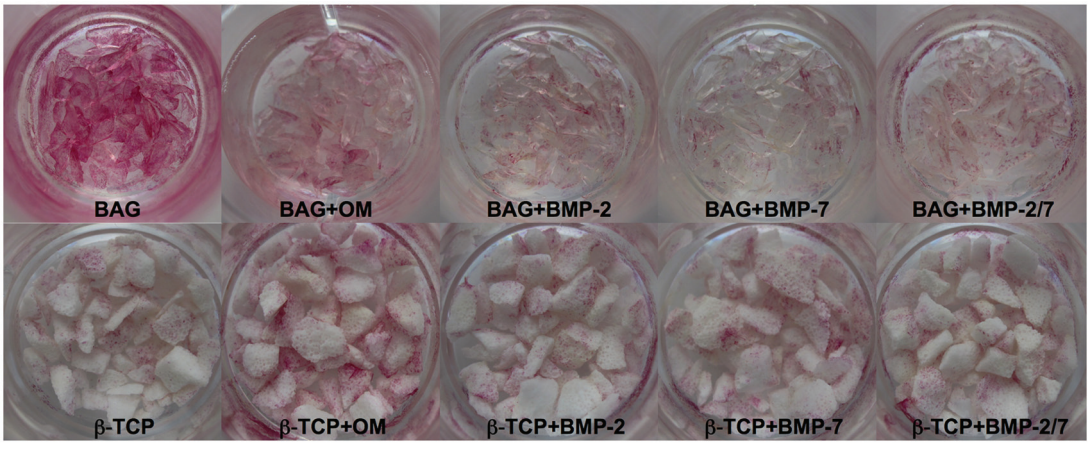

Figure 2. Alkaline phosphatase (ALP) staining at day 14 (N = 5 replicates per group). BAG stimulated osteogenic differentiation in CM most, whereas β-TCP exerted the greatest effects in OM. An increase in color intensity correlates with increased ALP synthesis.
BAG: bioactive glass; CM: control medium; β-TCP: beta-tricalcium phosphate; OM: osteogenic medium; BMP: bone morphogenetic protein.

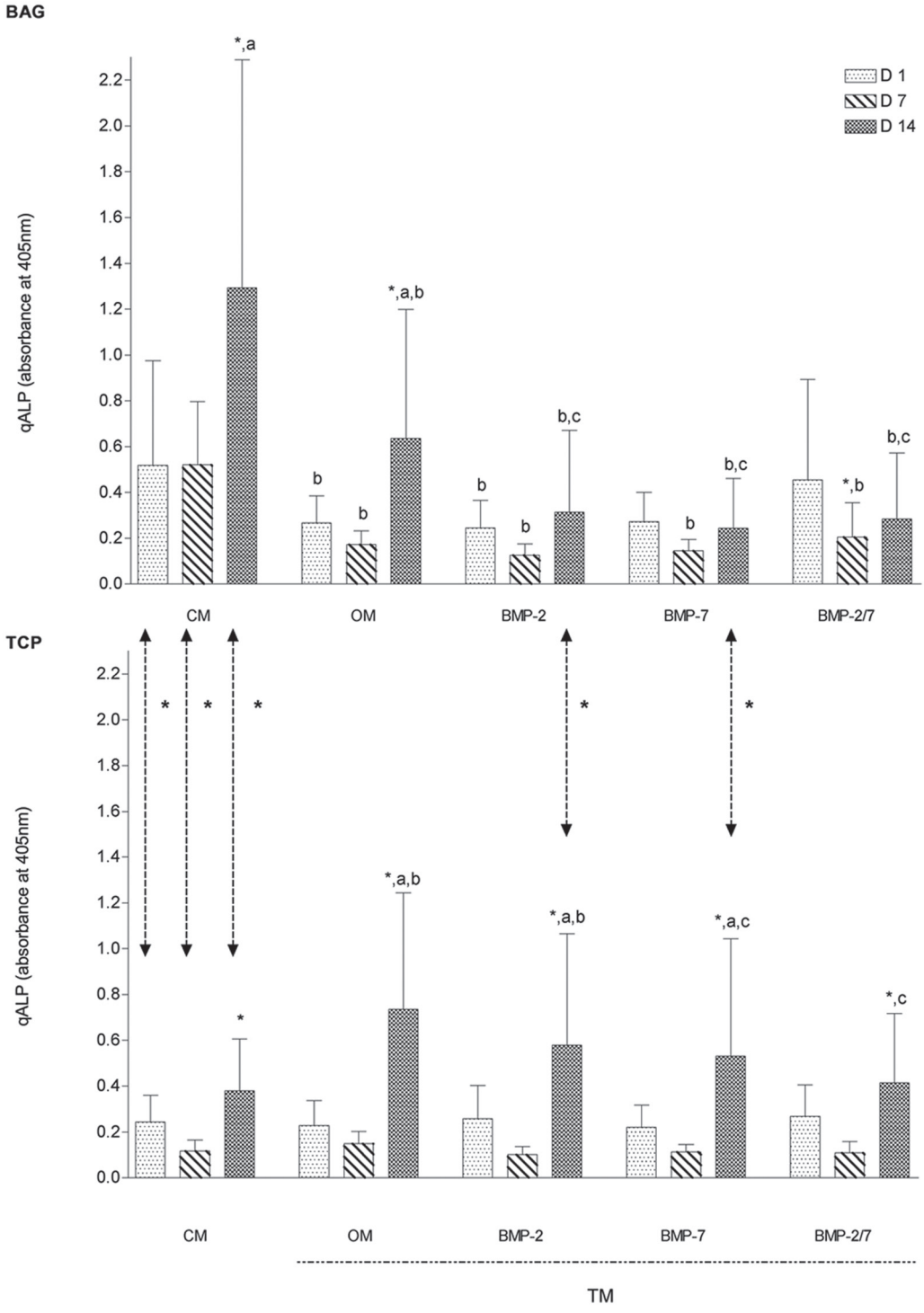

Figure 3. Comparative overview on quantitative ALP measurements over time (N = 10 replicates per time point). Human ASCs responded to both biomaterials differently. Bioactive glass promoted greatest ALP gene expression in CM after 14 days whereas β-TCP provoked highest ALP expressions in OM. BMPs decreased ALP activities independent on biomaterial evaluated. Overall, the results were supported by ALP staining.

ALP: alkaline phosphatase; ASC: adipose stem cell; CM: control medium; OM: osteogenic medium; β-TCP: beta-tricalcium phosphate; BMP: bone morphogenetic protein; TM: treatment media.

The symbol "*" indicates significant difference between days 1–7 and days 7–14; "a" indicates significant difference between days 1 and 14; "b" indicates significant difference when compared to CM; "c" significant different when compared to OM; arrows represent significant differences between biomaterials.

BMP-2- and BMP-7-supplemented media decrease ALP activity regardless of biomaterial

Both biomaterials showed an initial reduction in qALP activity from days 1 to 7 that did not correspond with qDNA measurements, followed by an increase of qALP from days 7 to 14 (Figures 1 and 3). We did not find statistically significant correlation between cell amounts and qALP for either biomaterial. Specifically, in the BAG group, an initial decrease in qALP activity was measured on day 7 followed by an increase on day 14 in all TMs. This initial activity decline was significant for OM, BMP-2/7 medium only ($p < 0.03$), whereas the subsequent rise from days 7 to 14 was significant for CM and OM ($p < 0.02$). The overall increase from days 1 to 14 was significantly greater in CM and OM only ($p < 0.04$). When compared to CM, significantly lower activities were measured at day 1 in OM and BMP-2 medium only, but for all TMs at days 7 and 14 ($p < 0.05$), also suggesting an overall stimulatory effect of plain CM on early osteogenic differentiation in BAG group. When compared to OM, significantly lower qALP activities were measured in all BMP media at day 14 ($p < 0.03$), which was consistent with ALP staining and was suggestive of an inhibitory effect of BMPs on osteogenic differentiation (Figures 2 and 3). Similarly in the β-TCP group, an initial nonsignificant drop in qALP activity was measured on day 7 followed by a significant rise from days 7 to 14 in CM and all TMs ($p < 0.01$). The overall increase from days 1 to 14 was significantly greater in OM, BMP-2, and BMP-7 medium ($p < 0.05$). When compared to the CM, expressions were significantly higher in OM and BMP-2 medium at day 14 ($p < 0.01$), which was in alignment with our ALP staining for OM (Figures 2 and 3). When compared to OM, significantly lower ALP expressions were measured in BMP-7 and BMP-2/7 medium at day 14 ($p < 0.02$), suggesting an inhibitory effect of both BMPs in early osteogenic differentiation (Figure 3). Qualitatively, ALP staining was consistent with our qDNA measurements regarding the inhibitory effect of BMP supplementation to TM in both biomaterials.

BMP-2/7-supplemented media induces expression of OPN in hASCs cultured on BAG but not on β-TCP

To further analyze the influence of biomaterials and growth factors on osteogenic differentiation of hASCs, the expression of osteogenic marker genes OPN, RUNX-2, Col-1, and OC was measured at day 14.

In the BAG group, mRNA expression of OPN was significantly higher in the BMP-2/7 medium as compared to CM, OM, and BMP-2 medium ($p < 0.03$) (Figure 4(a)), which was also in alignment with ALP activities recorded for BAG in CM and OM at day 14. Surprisingly, this effect was not seen in the β-TCP group, and mRNA expressions of OPN among TMs were not different. However, OPN gene expression in the BAG group was significantly stronger in BMP-2/7 medium ($p < 0.01$) as compared to the β-TCP group (Figure 4(a)).

In the BAG group and in alignment with the ALP activity, Col-1 expressions were significantly lower in TM as compared to CM ($p < 0.01$) (Figure 5(a)).

In the β-TCP group, mRNA expressions for Col-1 were not different among TMs, which was also confirmed by ALP activity measurements. However, Col-1 expressions were significantly lower in the BMP-7 and BMP-2/7 medium when compared to CM ($p < 0.009$) (Figure 5(a)).

The mRNA expression for RUNX-2 was significantly lower in OM in the BAG group ($p < 0.01$) than in the β-TCP group (Figure 4(b)), which was in alignment with ALP staining (Figure 2). However, the expression was not regulated by OM or growth factors when compared in the same biomaterial group. Similar expressions of OC were measured in BAG and β-TCP groups in CM and TM. When compared within biomaterial groups, the expression was neither different between CM and TM nor among TMs (Figure 5(b)).

VEGF expression correlated the cell amounts with both biomaterials

The effect of the biomaterial on the angiogenic potential of hASCs was determined by VEGF analyses. The expression of VEGF correlated with the cell amounts in both biomaterials. The Spearman correlation coefficient was 0.75 ($p = 0.0012$) and 0.56 ($p = 0.031$) for BAG and β-TCP, respectively.

The results of BAG and β-TCP group were compared to evaluate the effects of each biomaterial when maintained in the same media. In the BAG group, VEGF expression was significantly lower in CM and TM as compared to β-TCP group except for CM at days 7 and 14 as well as in OM at day 14 ($p < 0.04$). This observation is suggestive for an overall positive effect of β-TCP in combination with BMPs on angiogenic potential when compared to BAG granules (Figure 6).

For each biomaterial individually, the results of CM and TM were compared to determine the influence of culture media on angiogenic potential of hASCs. In both biomaterial groups, VEGF levels rose continuously over time and reached highest levels after 14 days in CM and TM (Figure 6). In the BAG group, a continuously significant increase was observed from days 1 to 14 in CM and all TMs ($p < 0.002$). When compared to CM, VEGF levels were significantly lower in all TMs at all time points ($p < 0.04$). When compared to OM, significantly reduced VEGF levels were measured in all BMP media at days 7

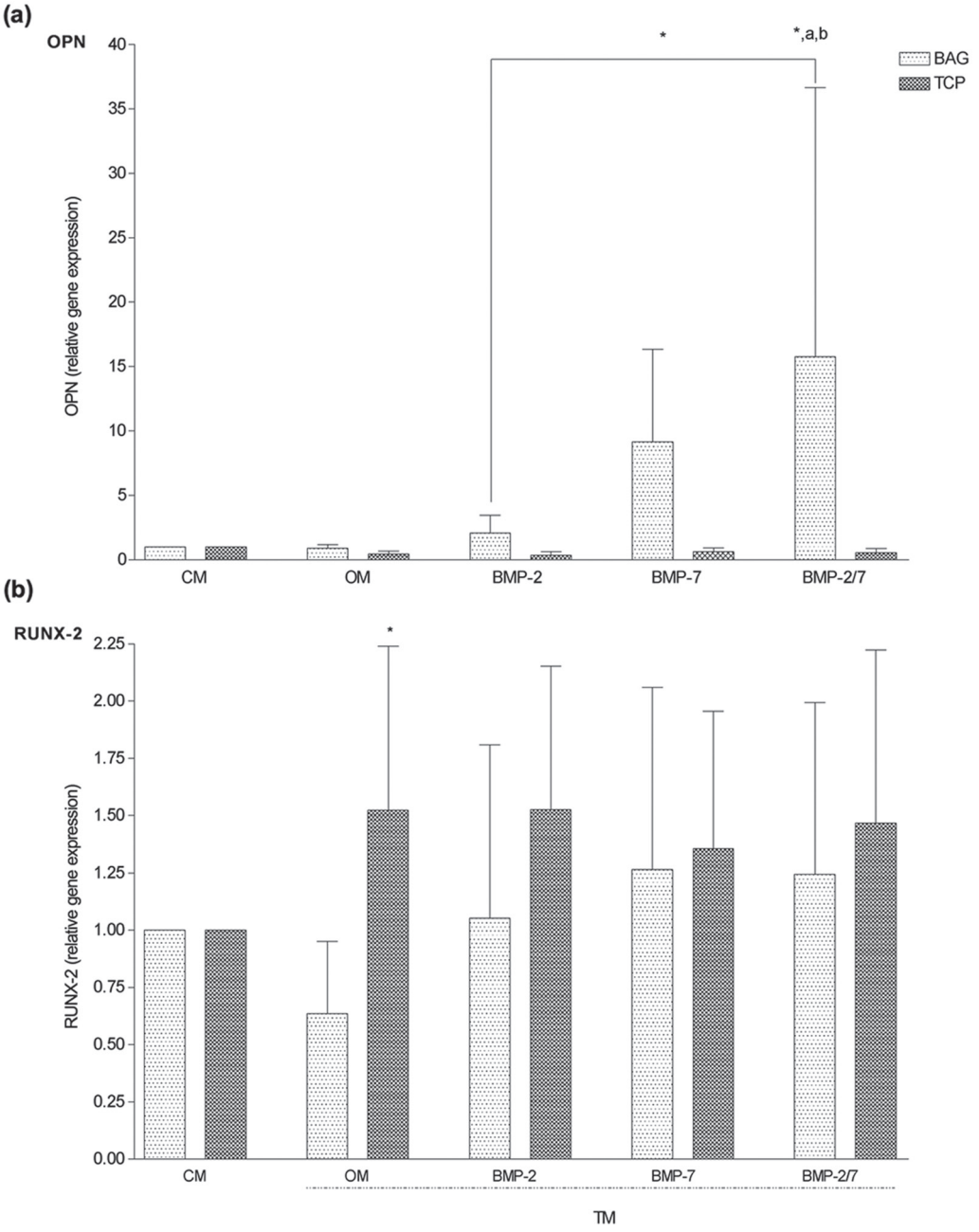

Figure 4. Comparative overview on gene expression of OPN and RUNX-2 (N = 6 replicates per time point). (a) OPN gene expression was significantly upregulated by BAG in BMP-2/7 medium as compared to β-TCP. Furthermore, BMP-2/7 induced significantly higher OPN expression as compared to BMP-2 in BAG group, suggesting a permissive effect on early osteogenesis. (b) RUNX-2 gene expression was significantly upregulated by β-TCP in OM as compared to BAG. However, additional BMP supplementation failed to enhance the expression. These findings were in alignment with ALP staining.
OPN: osteopontin; RUNX-2: runt-related transcription factor 2; BAG: bioactive glass; CM: control medium; OM: osteogenic medium; BMP: bone morphogenetic protein; β-TCP: beta-tricalcium phosphate; ALP: alkaline phosphatase; TM: treatment media.
The symbol "*" indicates significant difference between biomaterials; "a" indicates significant different as compared to CM; "b" indicates significant different as compared to OM.

and 14 ($p < 0.04$) (Figure 6). In the β-TCP group, the gradual increase of VEGF expression was significant in CM, OM, BMP-2, and BMP-7 media from days 1 to 7 ($p < 0.0007$), in all TMs from days 7 to 14 ($p < 0.01$) and in CM and all TMs from days 1 to 14 ($p < 0.0005$). When compared to CM, VEGF levels were significantly lower in all TMs at days 1 and 7 ($p < 0.04$). When compared to OM, significantly decreased VEGF levels were measured in BMP-2 medium at day 7 and in BMP-7 and BMP-2/7 at days 1 and 7 ($p < 0.03$).

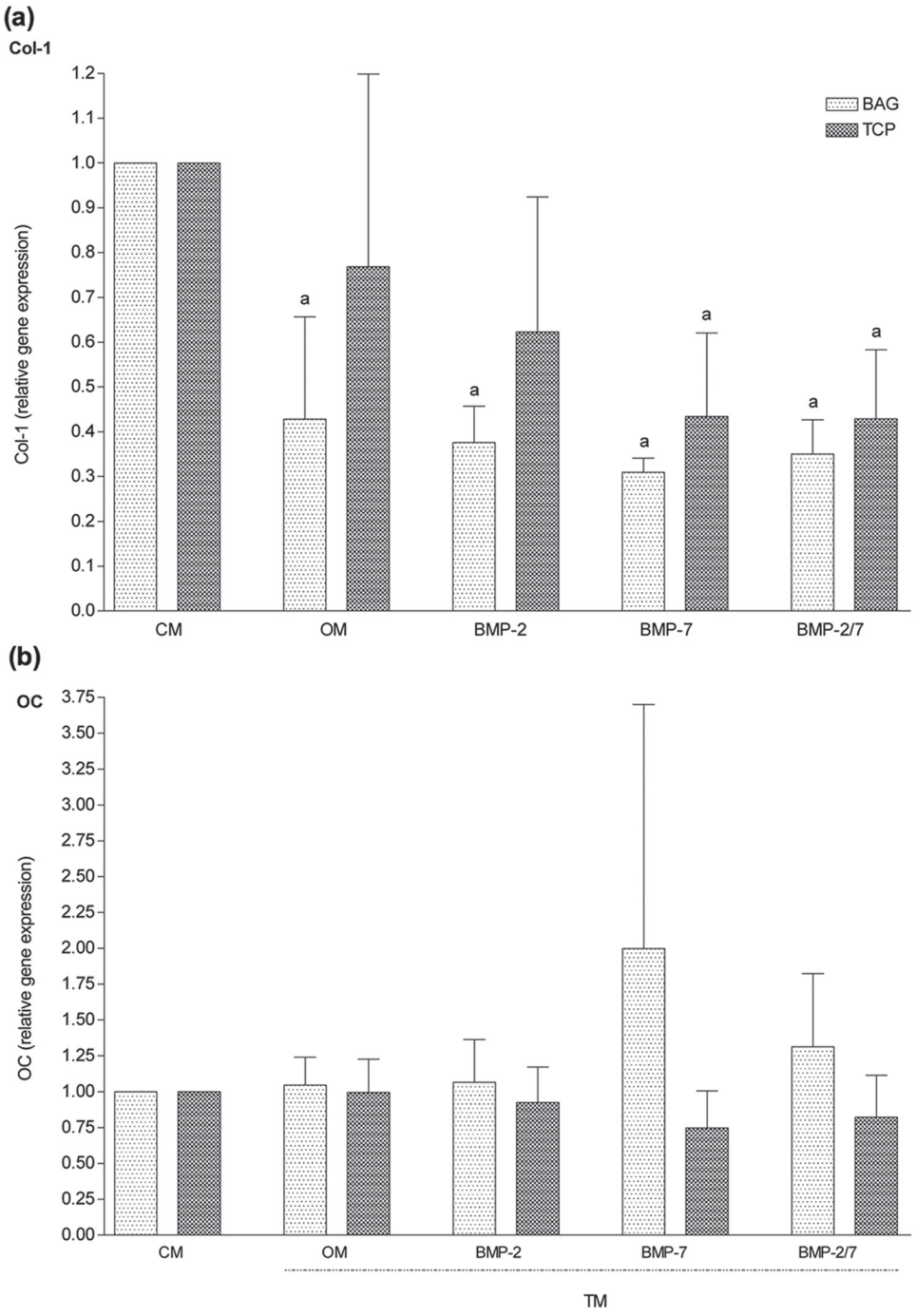

Figure 5. Comparative overview on gene expression of Col-1 and OC (N = 6 replicates per time point). (a) Our data revealed significantly lower marker expression for Col-1 in all TMs in the BAG group as compared to CM and was in accordance with ALP stainings. However, β-TCP downregulated Col-1 expression in BMP-7 and BMP-2/7 media only as compared to CM. (b) The gene expression for OC was neither different between biomaterials nor among media evaluated.
Col-1: collagen type-1; OC: osteocalcin; CM: control medium; ALP: alkaline phosphatase; β-TCP: beta-tricalcium phosphate; OM: osteogenic medium; BMP: bone morphogenetic protein; TM: treatment media.
"a" indicates significant different as compared to CM.

Discussion

To our knowledge, our study is the first report evaluating the effect of BAG and β-TCP on osteogenic differentiation of hASCs in HS-supplemented medium. The effects of CM, OM, and growth factor–supplemented (BMP-2, BMP-7, or BMP-2/7) OM on osteogenic differentiation of hASCs on BAG, and β-TCP granules were investigated concurrently.

In the current study, hASCs were used as a stem cell source for bone tissue engineering since they are readily

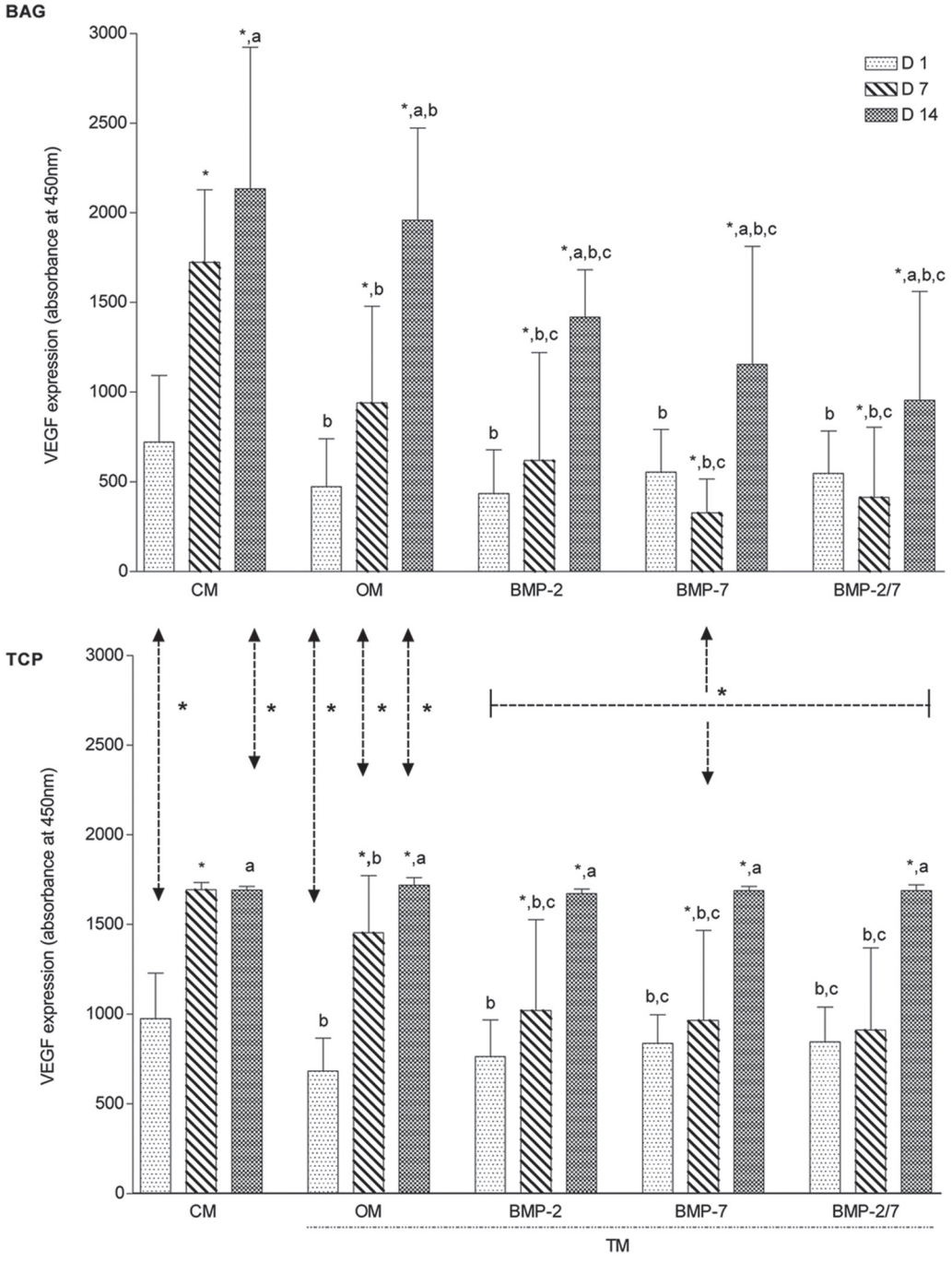

Figure 6. Comparative overview on VEGF measurements over time (N = 6 replicates per time point). Overall, a superior effect of β-TCP on early angiogenic potential was detected in BMP-supplemented media as compared to BAG. Supplementation with BMPs decreased early angiogenic potential of BAG. However, BAG had an increased potential in CM as compared to β-TCP.
VEGF: vascular endothelial growth factor; β-TCP: beta-tricalcium phosphate; CM: control medium; OM: osteogenic medium; BMP: bone morphogenetic protein; TM: treatment media.
The symbol "*" indicates significant difference between days 1–7 and days 7–14; "a" indicates significant difference between days 1 and 14; "b" indicates significant difference when compared to CM; "c" indicates significant difference when compared to OM; arrows represent significant differences between biomaterials.

available and show similar osteogenic differentiation potential as compared to hBMSCs.[18]

In the past, FBS has been used for expansion and differentiation of stem cells but recently, safety concerns have arisen regarding the use of this animal-derived product for clinical application in human medicine. Therefore, alternatives including HS have been investigated.[7,16] In theory, substitution of FBS by HS would overcome safety risks and, therefore, peaked interest in orthopedic research.[19] Recently, the supportive effect of pooled HS on

proliferation of hASCs was demonstrated,[7] but the impact of HS on osteogenic differentiation when combined with biomaterials remained to be determined. Therefore, in the current study, hASCs cultured in HS-supplemented medium, and clinically used biomaterials and growth factors were investigated to meet current safety standards while mimicking a feasible clinical application.

The cell amounts were increasing over time with both biomaterials suggesting that HS-supplemented medium in combinations with BAG and β-TCP can provide sufficient amounts of attachment factors for hASCs. The positive effect of BAG and β-TCP on MSC proliferation has been reported previously.[13,20–22] Our results were in accordance with these studies; however, BAG appeared to have a superior effect on hASC mount as compared to β-TCP when maintained in CM and OM. The surface roughness and porosity differ between BAG and β-TCP materials used in our study. However, the obtained DNA amount was similar with both biomaterials on day 1 in all groups, suggesting similar amounts of attached cells at the beginning of the experiments.

The effect of BAG and β-TCP on osteogenic differentiation of hBMSCs and hASCs has been previously studied.[20,23,24] Similarly to these reports, both biomaterials supported osteogenic differentiation in the current study as indicated by ALP activity. The most enhancing effect of BAG on differentiation was measured in CM, whereas the use of OM decreased ALP activity. In contrast to BAG, osteogenic induction of hASCs was greatest in OM when cultured with β-TCP.

Interestingly but consistent for both biomaterials independent on culture medium, a discrepancy between cell proliferation and osteogenic differentiation was noticed at day 7. Although cells steadily proliferated, concurrent osteogenic differentiation was not observed within the first seven days as indicated by decreasing qALP activities. This was in contrast with a previous report, in which ALP activity increased consistently over 14 days followed by a decrease after 21 days and requires further investigation.[20]

Currently, BMP-2 and BMP-7 are in clinical use since their osteoinductive effect has been demonstrated in various studies.[10,11] Due to their clinical approval, their potential use for other applications in human medicine is appealing and has resulted in further research.

In our current study, BMP supplementation decreased cell amounts regardless of the used biomaterial. In contrast to our findings, the beneficial effects of BMP-2 and BMP-7 on proliferation of hBMSCs have been demonstrated.[25] However, consistent with our findings, the negative impact of BMP-2 on cell growth rates was recently demonstrated in primary immortalized human fetal and primary human osteoblasts.[26] Additionally, higher apoptosis rates were recorded after exposure to BMP-2 and BMP-7.[26] These observations may suggest decreased cell growth via apoptosis after BMP supplementation but remains to be determined.

Although the supportive effect of BMP-2 and BMP-7 on the osteogenic differentiation of MSC has been well documented in vivo,[27,28] conflicting results have been obtained in vitro. Our in vitro findings were suggestive for a negative impact of both growth factors on osteogenic differentiation of hASCs. Over time, decrease in ALP activities was observed in BMP-supplemented OM independent on biomaterial evaluated. Recently,[17] it was suggested that the canonical BMP-2 signaling pathway may not be functional in hASCs, and thus, their osteogenic differentiation may not be influenced by BMP-2 even in vivo.[29] However, those results were in contrast with a recent study, in which rat bone marrow stromal cells (BMSCs) differentiated in the presence of BMP-2 and BAG into more rounded osteoblast-like cells resulting in greater ALP expression.[28] Similarly, when β-TCP containing collagen sponges were used as carrier for rat BMSCs and BMP-2, an enhanced osteogenic differentiation was confirmed by increased ALP and OC gene expression and histology.[27]

The negative effect on differentiation may also originate in the autoregulatory negative feedback loop of BMP preventing excessive stimulation. BMPs exert their effects via receptors that activate Smad-dependent and Smad-independent mechanisms resulting in linear expression of target genes including early markers of osteogenesis such as OC, ALP, or Col-1.[30] Several factors may be involved in this negative feedback loop including negative nonsignaling pseudoreceptors (BAMBI), inhibitory Smads (Smad 6 or 7), Smad-binding proteins (Ski and Tob), or degradation of Smads and extracellular BMP antagonists.[31] Among those, continuous exposure to BMP may have resulted in Smad 6 or 7 and/or Ski accumulation operating the negative feedback loop. This assumption is supported by a recent study in which no BMP-induced nuclear translocation of Smad 1/Smad 4 complexes was observed, suggesting a nonfunctional BMP-2 signaling pathway in hASCs.[17] However, a consistent downregulation of all osteogenic markers should have been observed. The exact timing after BMP stimulation of each individual marker remains to be determined.

The discrepancy between our results and previous observations may also originate in the BMP delivery method. In general, growth factors can be delivered exogenously via frequent media changes or endogenously via gene therapy. The effect of BMP-2 and BMP-7 on hASCs was investigated using frequent medium changes in the current study. The osteogenic differentiation of BMP-2 transfected rat BMSCs cultured with β-TCP scaffolds were recently investigated in vivo. Contrary to our findings, mRNA expression of OPN and OC was significantly increased, and ALP staining was more intensive.[21] In another study, MSCs were co-transfected with BMP genes, resulting in endogenous BMP synthesis. A prolonged effect for weeks was achieved after transfection.[32] These data may suggest that gene therapy is a more efficient method in supporting osteogenic differentiation of MSCs than regular

media changes. Those contradictory results highlight the importance of delivery systems for BMPs and call for additional investigations. Interestingly, in our study, OPN was the only marker of which expression was increased by BMPs but this was only detected in BAG and requires further investigations.

Formation of vasculature is essential for bone formation. In the current study, VEGF was measured since its validity as early marker for angiogenic potential is well documented and the VEGF secretion is considered as a permissive factor for bone formation.[33] In our study, both biomaterials consistently supported VEGF expression in all samples. This is in accordance with recent studies, in which the beneficial effects of BAG and β-TCP on VEGF secretion were reported.[34,35] Our results suggest a significantly better effect of β-TCP on the VEGF secretion of hASCs in BMP-supplemented OM when compared to BAG at all times. These findings are supported by a previous study, in which the efficacy of MSCs seeded on β-TCP scaffolds was investigated in rats.[36] An upregulation of VEGF mRNA and protein levels was measured over time, which also resulted in greater bone formation in vivo. Potentially, β-TCP granules in BMP-2- and BMP-7-supplemented OM may support the angiogenic potential of hASCs, whereas BAG under similar culture conditions may even decrease the VEGF expression but this requires further clarification.

Although the positive effects of both BMPs on angiogenesis have been well reported using different cell lines, media, and biomaterials,[37] this is the first in vitro study investigating the VEGF secretion of hASCs when exposed to BAG or β-TCP and supplemented with BMPs. Contrary to those well-known supportive effects, BMP-2 and BMP-7 supplementation of BAG decreased the angiogenic potential of hASCs in our study.

The VEGF secretion solely followed the pattern of cell amount. Interestingly, a recent study demonstrated an inhibitory effect of BMPs on cell proliferation and angiogenesis. Recently, the negative effect of BMP-2 on oral squamous cell carcinoma proliferation and angiogenesis was demonstrated.[38] Also, the interaction of VEGF and BMP-2 was investigated in BMSC in vitro and in vivo. Those coculture experiments revealed VEGF as a potent inhibitor of BMP-2 expression and, thus, may inhibit osteogenesis.[39] Similarly, in our study, inhibition of early osteogenesis may have been indicated by reduced qALP activity secondary to VEGF expression. These findings call for studies elucidating the exact role of BMP-2, BMP-7, and VEGF on the angiogenic and osteogenic potentials of hASCs.

In conclusion, hASCs can be successfully isolated and expanded in HS but the response differs on BAG S53P4 and β-TCP granules. In the current study, BAG S53P4 stimulated osteogenic differentiation of hASCs in CM, whereas β-TCP required OM for osteogenic induction. Overall, supplementation of OM with BMP-2 and BMP-7 decreased early osteogenic differentiation independent on biomaterial evaluated. Based on our measurements, a combination of BAG S53P4 and CM may be an applicable way in enhancing proliferation and osteogenic differentiation of hASCs while minimizing safety and regulatory concerns in bone tissue engineering. Further studies elucidating the individual role of growth factors and biomaterials are required to design an effective bone tissue engineered implant suitable for clinical use. This study is a preliminary step forward in achieving this goal.

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Conflict of interest

The authors declare that there are no conflicting interests.

References

1. De Long WG, Einhorn TA, Koval K, et al. Bone grafts and bone graft substitutes in orthopaedic trauma surgery. A critical analysis. *J Bone Joint Surg Am* 2007; 89(3): 649–658.
2. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* 2000; 21(24): 2529–2543.
3. Mesimäki K, Lindroos B, Tornwall J, et al. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int J Oral Maxillofac Surg* 2009; 38(3): 201–209.
4. Thesleff T, Lehtimaki K, Niskakangas T, et al. Cranioplasty with adipose-derived stem cells and biomaterial. A novel method for cranial reconstruction. *Neurosurgery* 2011; 68(6): 1535–1540.
5. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001; 7(2): 211–228.
6. Tekkatte C, Gunasingh GP, Cherian KM, et al. "Humanized" stem cell culture techniques: the animal serum controversy. *Stem Cells Int* 2011; 504723.
7. Kocaoemer A, Kern S, Kluter H, et al. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. *Stem Cells* 25(5): 1270–1278.
8. Lindroos B, Boucher S, Chase L, et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. *Cytotherapy* 2009; 11(7): 958–972.
9. Kim M and Choe S. BMPs and their clinical potentials. *BMB Rep* 2011; 44(10): 619–634.
10. Haid RW, Branch CL, Alexander JT, et al. Posterior lumbar interbody fusion using recombinant human bone

morphogenetic protein type 2 with cylindrical interbody cages. *Spine J* 2004; 4(5): 527–538; discussion 538–539.
11. Giannoudis PV and Tzioupis C. Clinical applications of BMP-7: the UK perspective. *Injury* 2005; 36(Suppl. 3): S47–S50.
12. Jones JR and Hench LL. Regeneration of trabecular bone using porous ceramics. *Curr Opin Solid State Mater Sci* 2003; 7(4–5): 301–307.
13. Hench LL.. Bioactive materials: the potential for tissue regeneration. *J Biomed Mater Res* 1998; 41(4): 511–518.
14. Hench LL, Xynos ID and Polak JM. Bioactive glasses for in situ tissue regeneration. *J Biomater Sci Polym Ed* 2004; 15(4): 543–562.
15. Knop C, Sitte I, Canto F, et al. Successful posterior interlaminar fusion at the thoracic spine by sole use of beta-tricalcium phosphate. *Arch Orthop Trauma Surg* 2006; 126(3): 204–210.
16. Lindroos B, Aho KL, Kuokkanen H, et al. Differential gene expression in adipose stem cells cultured in allogeneic human serum versus fetal bovine serum. *Tissue Eng Part A* 2010; 16(7): 2281–2294.
17. Zuk P, Chou YF, Mussano F, et al. Adipose-derived stem cells and BMP2: part 2. BMP2 may not influence the osteogenic fate of human adipose-derived stem cells. *Connect Tissue Res* 2011; 52(2): 119–132.
18. Lindroos B, Suuronen R and Miettinen S. The potential of adipose stem cells in regenerative medicine. *Stem Cell Rev* 2011; 7(2): 269–291.
19. Kobayashi T, Watanabe H, Yanagawa T, et al. Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. *J Bone Joint Surg Br* 2005; 87(10): 1426–1433.
20. Gomide VS, Zonari AAC, Breyner NM, et al. Attachment and proliferation of human-adipose-tissue-derived stem cells on bioactive glass/PVA hybrid scaffolds. *ISRN Mater Sci* 2011; 240864.
21. Zhao J, Hu J, Wang S, et al. Combination of beta-TCP and BMP-2 gene-modified bMSCs to heal critical size mandibular defects in rats. *Oral Dis* 2010; 16(1): 46–54.
22. Rahaman MN, Day DE, Bal BS, et al. Bioactive glass in tissue engineering. *Acta Biomater* 2011; 7(6): 2355–2373.
23. Haimi S, Gorianc G, Moimas L, et al. Characterization of zinc-releasing three-dimensional bioactive glass scaffolds and their effect on human adipose stem cell proliferation and osteogenic differentiation. *Acta Biomater* 2009; 5(8): 3122–3131.
24. Liu G, Zhao L, Cui L, et al. Tissue-engineered bone formation using human bone marrow stromal cells and novel beta-tricalcium phosphate. *Biomed Mater* 2007; 2(2): 78–86.
25. Pountos I, Georgouli T, Henshaw K, et al. The effect of bone morphogenetic protein-2, bone morphogenetic protein-7, parathyroid hormone, and platelet-derived growth factor on the proliferation and osteogenic differentiation of mesenchymal stem cells derived from osteoporotic bone. *J Orthop Trauma* 2010; 24(9): 552–556.
26. Gautschi OP, Cadosch D, Zellweger R, et al. Apoptosis induction and reduced proliferation in human osteoblasts by rhBMP-2, -4 and -7. *J Musculoskelet Neuronal Interact* 2009; 9(1): 53–60.
27. Tadokoro M, Matsushima A, Kotobuki N, et al. Bone morphogenetic protein-2 in biodegradable gelatin and beta-tricalcium phosphate sponges enhances the in vivo bone-forming capability of bone marrow mesenchymal stem cells. *J Tissue Eng Regen Med* 2011. DOI: 10.1002/term.427.
28. Radin S, Reilly G, Bhargave G, et al. Osteogenic effects of bioactive glass on bone marrow stromal cells. *J Biomed Mater Res A* 2005; 73(1): 21–29.
29. Chou YF, Zuk PA, Chang TL, et al. Adipose-derived stem cells and BMP2: part 1. BMP2-treated adipose-derived stem cells do not improve repair of segmental femoral defects. *Connect Tissue Res* 2011; 52(2): 109–118.
30. Miyazono K, ten Dijke P and Heldin CH. TGF-beta signaling by Smad proteins. *Adv Immunol* 2000; 75: 115–157.
31. Canalis E, Economides AN and Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev* 2003; 24(2): 218–235.
32. Liu SB, Hu PZ, Hou Y, et al. Recombinant human bone morphogenetic protein-2 promotes the proliferation of mesenchymal stem cells in vivo and in vitro. *Chin Med J (Engl)* 2009; 122(7): 839–843.
33. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004; 109(10): 1292–1298.
34. Ghanaati S, Barbeck M, Orth C, et al. Influence of beta-tricalcium phosphate granule size and morphology on tissue reaction in vivo. *Acta Biomater* 2010; 6(12): 4476–4487.
35. Gorustovich AA, Roether JA and Boccaccini AR. Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. *Tissue Eng Part B Rev* 2010; 16(2): 199–207.
36. Deckers MM, Van Bezooijen RL, Van der Horst G, et al. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* 2002; 143(4): 1545–1553.
37. Shoji T, Mifune Y, Matsumoto T, et al. Local transplantation of human multipotent adipose-derived stem cells accelerates fracture healing via enhanced osteogenesis and angiogenesis. *Lab Invest* 2010; 90(4): 637–649.
38. Gao Q, Tong W, Luria JS, et al. Effects of bone morphogenetic protein-2 on proliferation and angiogenesis in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* 2010; 39(3): 266–271.
39. Schonmeyr BH, Soares M, Avraham T, et al. Vascular endothelial growth factor inhibits bone morphogenetic protein 2 expression in rat mesenchymal stem cells. *Tissue Eng Part A* 2010; 16(2): 653–662.