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Influence of culture conditions on the *ex-vivo* expansion and hematopoietic supportive capacity of human Mesenchymal Stromal Cells

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Declaration

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Stem Cell Bioengineering Group, Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period March-September 2022, under the supervision of Dr. Ana Fernandes-Platzgummer and Prof. Cláudia Lobato da Silva.

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It's done! Please do not fall asleep while reading the rest of the thesis, I promise it is interesting!

Resumo

Células Mesenquimais Estromais (MSC) são uma população bastante heterogénica que tem sido bastante estudada devido ao seu potencial terapêutico. No entanto, diferentes fontes, protocolos de isolamento ou expansão, ou condições durante todo o processo de produção, podem originar populações de MSC com propriedades diferentes, levando a resultados contraditórios. A expansão destas células em condições que garantam a possibilidade de serem usadas clinicamente é essencial. Neste trabalho, foi testada uma solução de transporte para MSC (Nutristor), e a influência do uso de laminina-521 e do meio de cultura utilizado, tanto na expansão das MSC como na sua funcionalidade. Nutristor manteve a capacidade das MSC aderirem ao plástico e a viabilidade celular praticamente constante durante 5 dias de armazenamento. Porém, a percentagem de células perdidas durante o processo foi considerável. A utilização de laminina-521 permitiu a obtenção de 2.2 ± 0.4 mais MSC, e teve um impacto mínimo na sua capacidade de suportar a expansão de células hematopoiéticas estaminais e progenitoras (HSPC). Finalmente, expandir as MSC com meio suplementado com lizado plaquetário humano (DMEM/hPL), em substituição de soro fetal bovino (DMEM/FBS), não só aumentou a sua capacidade proliferativa, como alterou a sua morfologia, e expressão de CD271. Adicionalmente, quando as HSPC foram co-cultivadas com MSC cuja última passagem tinha sido com DMEM/hPL, a sua capacidade proliferativa diminuiu e a diferenciação aumentou, obtendo-se 2.4 vezes menos células $CD34^{+}$. Em última análise, este trabalho comprova a necessidade de analisar o impacto que diferentes condições durante a produção de terapias celulares podem ter na função das células.

Palavras-chave: Células Mesenquimais Estromais, Solução de transporte, Condições de cultura, Substrato de revestimento, Meios de cultura, Suporte hematopoiético.

Abstract

Ex-vivo expanded Mesenchymal Stromal Cells (MSC) have been intensively studied due to their therapeutic potential. Different sources, isolation methods, or culture conditions can originate MSC populations with different properties leading to conflicting results. There is a need to establish GMP-compliant protocols throughout the entire process of cell-based therapies development. In this work, the impact of Nutristor, a cold cell storage solution in beta testing phase, on MSC viability and proliferation was assessed. Throughout 5 days of storage with Nutristor solution, MSC viability was roughly constant, and in the end of storage time, cells maintained the ability to adhere to plastic and proliferate. Nevertheless, a significant cell loss could be observed. In the second part of this work, the influence of culture conditions, namely coating substrate (laminin-521 (BL) vs no coating) and culture media supplementation (fetal bovine serum (FBS) vs human platelet lysate (HPL)), on MSC expansion and hematopoietic supportive capacity was also evaluated. MSC were expanded on tissue culture plates with (BL-MSC) and without (MSC) laminin-521 coating, and at the end of the expansion BL-MSC were 2.2 ± 0.4 more than MSC. Hematopoietic Stem/Progenitor Cells (HSPC) clonogenic capacity seemed to be enhanced after expansion with BL-MSC feeder layers, although almost no differences could be found. Finally, MSC were cultured with basal culture medium supplemented with hPL (DMEM/hPL) or FBS (DMEM/FBS) and it was found that the culture medium affected MSC's properties. When grown with DMEM/hPL, MSC were smaller, had a higher proliferation capacity and seemed to have a higher expression of CD271. However, when MSC cultured with DMEM/hPL were used as feeder layers, their hematopoietic supportive capacity was diminished (originating 2.4 times less $CD34^+$ cells after expansion). Ultimately, this work highlights the necessity to be aware of the impact of the culture conditions during the entire process of the development of MSC-based therapies.

Keywords: Mesenchymal Stromal Cells, Short Storage Solution, Culture Conditions, Coating, Culture Media, Hematopoietic Support

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Nomenclature

α-MEM Minimum Essential Medium Eagle - Alpha Modification

BFU-E Erythroid burst-forming unit

BL-MSC MSC cultured with Biolaminina-521 coating

CFU-GEMM Multilineage colony-forming unit

CFU-GM Granulocyte-macrophage colony-forming unit

CFU Colony-forming unit

CV Coefficient of variation

DMEM/FBS DMEM supplemented with 10% FBS-MSC qualified

DMEM/hPL DMEM supplemented with 5% hPL

DMEM Dulbecco's modified Eagle's medium

DMSO Dimethyl Sulfoxide

ECM Extra-cellular matrix

EMA European Medicines Agency

FBS Fetal Bovine Serum

FI Fold Increase

FL Feeder Layers

FSC Forward Scattering

GMP Good Manufacturing Practices

GvHD Graft-versus-host disease

HLA Human Leucocyte Antigen

hPL Human Platelet Lysate

HSPC Hematopoietic Stem/Progenitor Cells

ISCT International Society for Cell and Gene Therapy

L/D LIVE/DEAD Dye

LN Laminin

mmC Mitomycin-c

MNC Mononuclear Cells

MSC(AT) Mesenchymal Stromal Cells isolated from Adipose Tissue

MSC(DP) Mesenchymal Stromal Cells isolated from Dental Pulp

MSC(M) Mesenchymal Stromal Cells isolated from Bone Marrow

MSC(WJ) Mesenchymal Stromal Cells isolated from Wharton's jelly

MSC-EV MSC-derived extracellular vesicles

MSC Mesenchymal Stromal Cells

PBS Phosphate Buffered Saline

SEM Standard Error of the Mean

SSC Side Scattering

TNC Total Nucleated Cells

UCB Umbilical Cord Blood

WHO World Health Organization

Chapter 1

Introduction

1.1 Mesenchymal Stromal Cells

Back in the 1970s a plastic-adherent, fibroblast-like, clonogenic stromal population was isolated from the bone marrow by Friedenstein and his colleagues [1]. The same authors later found out that those stromal cells were important to regulate the hematopoietic niche [2].

In the end of the 20^{th} century, this population was proved to be multipotent - cells could differentiate into osteoblasts, chondrocytes, and adipocytes - and they were able to maintain the multilineage potential after being expanded *in vitro* [3, 4].

Since the aforementioned cells were able to differentiate into different cell types and self renew, they were named ”Mesenchymal Stem Cells” [4, 5]. However, due to the heterogeneity of the population the International Society for Cell and Gene Therapy (ISCT) recommended the use of Mesenchymal Stromal Cells (MSC) instead, since probably only a small percentage of the population could be truly considered stem cells [6]. Following the ISCT indications, in this work we will refer to this population as ”Mesenchymal Stromal Cells”.

Furthermore, to better define the so called MSC population, ISCT has issued a series of guidelines that go from proposing minimal criteria to define MSC to nomenclature suggestions [6–8]. To be considered MSC, cells should (1) be plastic adherent, (2) express CD73, CD90 and CD105, and lack expression of CD11b or CD14, CD19 or CD79α, CD34, CD45, and HLA-DR (hematopoietic and endothelial markers), (3) and lastly, they should be capable to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro* [7].

Besides the intrinsic heterogeneity of MSC, these cells can be retrieved from several sources, and their properties are reported to be affected by their source [9, 10]. The main source of MSC in adults is bone marrow (MSC(M)) [11], however, they can be isolated from other adult tissues, like adipose tissue (MSC(AT)) [12], or dental pulp (MSC(DP)) [13], and also from whartson’s jelly from the umbilical cord (MSC(WJ)) [14].

ISCT guidelines are not enough to guarantee that the population that is considered MSC have the same cellular composition. It is important to standardize procedures between laboratories, understand

the implications of different culture conditions, and implement reproducible, cost-effective, and scalable protocols for cell culture, compliant with Good Manufacturing Practices (GMP) [15]. Furthermore, it is needed to define clear characterization methods and robust potency assays to access quality and predict MSC's therapeutic effectiveness [15, 16].

1.1.1 Clinical Interest

Beyond their ability to differentiate into given cell lines, MSC have important roles regarding tissue maintenance and homeostasis, which holds promise for the treatment of a variety of disorders [17]. MSC can home to injured sites, inducing tissue repair and reducing inflammation by cell-to-cell contact or resorting to their paracrine action, since they are known for the production of a variety of cytokines, growth factors, and exosomes that act in different conditions [18–20]. Overall, MSC show, between others, tissue regenerative [21], immunoregulatory [20, 22–24], angiogenic [25] and neural-protective [26] properties, and have the ability to support and regulate hematopoiesis [2]. All these properties make MSC-based therapies a focus of research interest, and they are serious candidates to be used in clinical trials for several diseases.

Furthermore, MSC have a low expression of Human Leucocyte Antigen (HLA)-I, and they do not express HLA-II nor co-stimulatory molecules, hence being considered hypoimmunogenic [27, 28]. MSC can then avoid immune recognition, and allogeneic cells should not create immune responses, regardless the HLA match between patient and donor, which makes MSC prone to constitute a off-the-shelf therapy.

A search at ClinicalTrials.gov with the study type "Interventional (Clinical Trial)" and key words "Mesenchymal Stem Cells" or "Mesenchymal Stromal Cells" finds a total of 1948 studies [29]. From these studies immune diseases, heart conditions, neurological diseases, and graft-versus-host disease (GvHD) are amongst the most common illnesses from what MSC have been studied.

In order to develop MSC-based therapies, these cells need to be expanded *in vitro* to achieve therapeutically relevant doses. Therefore, it is needed to guarantee MSC's biosafety after the expansion [30]. Research shows that even after extensive expansion, MSC can keep a constant immunophenotype and show no major alterations on their chromosomal structure [31]. Besides the results of *in vitro* studies, both animal models and results of clinical trials, show MSC as a safe therapeutical option [32, 33]. Nevertheless, MSC products for therapies must be developed in GMP compliant conditions, and thoroughly tested for safety before administration in patients.

Globaly, there are 10 MSC products approved to be used therapeutically (see Table 1.1). Nevertheless, only in 2018, Europe had its first (and for now only) therapeutic product that comprised the use of allogeneic MSC approved. Alofisel is composed of expanded human allogeneic MSC extracted from adipose tissue and it was approved by the European Medicines Agency (EMA) to treat complex perianal fistulas in adults with non-active/mildly active luminal Crohn's disease [34].

Table 1.1: Approved MSC Therapies over the world

Product Name	Product Composition	Therapeutic Target	Countries of Approval	Ref.
Queencell®	*Autologous MSC(AT)* mixed with preadipocytes, endothelial progenitor cells, pericytes, mast cells, and fibroblast.	Subcutaneous tissue defect	South Korea (2010)	[35]
Cellgram®	*Autologous MSC(M)*	Acute Myocardial Infarction (Improves ejection fraction)	South Korea (2011)	[36]
Cartistem®	*Allogeneic MSC(WJ)*	Repetitive and/or traumatic cartilage degeneration including Degenerative Osteoarthritis	South Korea (2012)	[37]
Cupistem®	*Autologous MSC(AT)*	Complex Crohn's fistula	South Korea (2012)	[35]
Prochymal®	*Allogeneic MSC(M)*	Acute Graft vs. Host Disease in pediatric patients	New Zealand (2012) Canada (2014)	[38, 39]
NeuroNata-R®	*Autologous MSC(M)*	Amyotrophic lateral sclerosis (it has a neuroprotective effect and relieves its progression)	South Korea (2014)	[40]
TEMCELL®	*Allogeneic MSC(M)*	Acute Graft vs. Host Disease in pediatric patients	Japan (2015)	[41]
Stempeucel®	*Allogeneic MSC(M)*	Critical Limb Ischemia and Knee Osteoarthritis	India (2016)	[42]
Alofisel	*Allogeneic MSC(AT)*	Complex perianal fistulas in adult patients with non-active/mildly active luminal Crohn's disease	Europe (2018) Japan (2021)	[34, 43]
Stemirac	*Autologous MSC(M)*	Spinal cord injury (Improvement of neurological symptoms and functional disorders)	Japan (2018)	[43]

MSC for immunological diseases

At the moment, the majority of diseases for which MSC are being clinically examined are immune-mediated disorders. Between autoimmune diseases like, Crohn's disease, Alzheimer's, multiple sclerosis, or rheumatoid arthritis, and acquired disorders like GvHD, the application of MSC-based therapies has a lot of potential.

MSC can modulate not only the adaptive immune system [20], but also the innate response [23]. Suppression of immune reaction is achieved by inhibiting maturation and proliferation of a range of immune cells like T-cells [20], natural killer cells [24], B cells [22], dendritic cells [23], or macrophages[44] Nevertheless, MSC environment can affect the cocktail of cytokines, growth factors, and chemokines released by them, having the potential to change MSC's immunomodulatory activities [45]. A technique that is widely used to enhance MSC therapeutic effect is to precondition them to obtain the desired secretome [46]. Oxidative stress, hypoxia, heat shok, or priming with biological factors are all examples of techniques that are used to precondition MSC [46].

Note that specific MSC subpopulations seem to be more prone to be immunomodulatory. Sun *et al.* investigated transcriptomic variations between MSC populations from different sources, creating a panel of genes, that when expressed, make MSC more prone to immunomodulation [47]. They identified that the expression of immune-response related genes (CCL2, CCL7, VCAM1, ICAM1, etc) was highly

variable between sources and samples, suggesting that the assessment of gene expression prior to the realization of other assays could be used to create an enriched subpopulation of immunomodulatory MSC [47]. In an easier way, MSC immunimudolatory could be assessed by MSC surface markers, more about it will be discussed at section 1.2.

MSC for neurologic diseases and heart conditions

Neurologic diseases are a growing problem in the developed world, were the expectancy of life is increasing. Most of such disorders do not have an effective therapy, and an interesting approach is the use of stem cell-based therapies, namely MSC-based therapies [26].

Neurodegenerative diseases usually have an autoimmune cause, Alzheimer's disease or multiple sclerosis are examples of such cases. Unfortunately, nowadays, standard therapies for neurodegenerative diseases only slow their progress. The use of stem cell-based therapies can possibly change this paradigm [26]. Due to their immune-mediated component MSC appeared as a logical choice the develop therapies for neurodegenerative diseases. Based mostly on pre-clinical trials and animal models, it seems like MSC can regulate microglia and astrocytes, hence controling neuroinflamation [48]. Furthermore, besides the immunomodulatory capacity of MSC, their presence seems to be able to increase neuronal survival and it even seems to enhance neurogenesis and stabilize synapses [49, 50]. Traumatic brain disorders, like stroke or traumatic brain injury may also benefit of MSC's neuroprotection, neurogenic potential, and immunomodulatory ability, but also from MSC's angiogenic potential, since usually parts of the brain become ischemic [26]. Furthermore, MSC interaction with neurons, pericytes, and astrocytes seems to help the maintenance of the blood brain barrier integrity [51]. The combination of the different MSC properties previously mentioned, makes them serious candidates to reverse the progression of neurodegenerative diseases and repair brain damage (therapeutic effects that, at the moment, cannot be achieved with regular medicines).

According to the World Health Organization (WHO), heart conditions are also a major problem, being the main cause of death worldwide [52]. However, as in the case of neurological disorders, current treatments for chronic heart disease can only delay its progress [53]. Such diseases are usually characterized by the migration of immune cells to the heart, creating an inflammatory response, and by myocyte death followed by further replacement with fibrous scar tissue [54]. Once more, the combination of MSC's properties makes them an interesting candidate for heart conditions therapies. MSC can protect the myocardium since they can reduce the inflammation [55], but also by promoting angiogenesis [25] and regeneration of myocardial cells MSC can avoid fibrosis, reducing the probability of further heart failure [53, 56].

MSC to support Hematopoietic Stem/Progenitor Cells (HSPC)-based therapies

MSC are therapeutically interesting, not only to be the focus of the therapy themselves, but also to help to develop Hematopoietic Stem/Progenitor Cells (HSPC)-based therapies. As mentioned previously, MSC were found out in the bone marrow adult hematopoietic niche, but its localization near

hematopoietic zones since embryonic developmental stages, further supports the importance of MSC to regulate proliferation, differentiation, and self renewal of HSPC [57, 58].

HSPC were the first adult stem cells to be identified, and can be found in the adult bone marrow and harvested by bone marrow aspiration or peripheral stem cell mobilization, but can also be found in neonatal tissues, namely the cord blood [59–61]. These cells are capable to replace an entire hematopoietic system. Hence, HSPC are the main components of bone marrow transplantations which have been an established therapy for hematologic disorders, namely for blood cancers [62]. In fact, according to the European Cancer Information System (ECIS), in 2019, 48 512 bone marrow transplantations were performed only in europe. Furthermore, cases of blood malignancies are increasing, ECIS also predicts that by 2040 there will be a 25% increase of leukemia and lymphoma cases [63].

Unfortunately, HSPC are a rare population, they constitute only around 1% of the mononucleated cells of the cord blood and bone marrow aspirates [64]. So, to increase the number of cells available and the clinical outcome of hematopoietic stem cell transplantation, *ex vivo* expansion of HSPC have been a focus of research.

Despite the importance of HSPC *ex vivo* expansion, these cells tend to differentiate when cultured *in vitro*. Hematopoietic niche *in vivo* is composed of several cell types which help regulating HSPC fate, namely their hability to self-renew (and maintain the stemness), the lack of ablity to do it *in vitro* has been associated with the lack of factors to mimic their natural microenvironment [65]. Logically, MSC capacity to support the expansion of HSPC *in vitro* was tested, and it is now widely accepted that co-culturing HSPC with MSC feeder layers can help the maintenance of hematopoietic stemness [66].

There are still a lot of unanswered questions about the mechanisms behind MSC capability to regulate HSPC, but it is known that cell-to-cell contact has a big impact on their interacion, despite the existance of paracrine regulatory mechanisms as well [67, 68]. One of the mechanisms that is known to be associated with regulation of HSPC growth and differentiation is the Notch signaling pathway. In reality, MSC express Notch ligands which can then activate Notch signals in HSPC, decreasing the tendency of HSPC to differentiate [69, 70]. It was already observed, that alteration of Notch ligand expression in MSC decreases their capability to avoid HSPC's premature differentiation [70].

Interaction between MSC and HSPC is important, not only to allow the expansion of the latest, but they can also be combined in mixed therapies. As aforementioned, MSC are known for being immunomodulatory, and have been reported to enhance the engraftment of HSPC *in vivo*. Therefore, co-transplantation of both cell types have been investigated, since it should reduce the problematics associated with the immune response (namely GvHD) and should also fasten the hematopoietic system regeneration [71, 72]. Nonetheless, clinical trials that make use of this strategy show contradictory results, with some trials showing promising results [71, 72] and others showing no significant differences between performing a co-transplant with MSC or transplant only HSPC [73].

While disease-to-disease or patient-to-patient may affect the clinical trials' results, the heterogeneity of MSC populations probably plays a big role in it. It is reported that MSC's tendency to support hematopoiesis is highly variable, depending on several factors like the donor, source, culture media, or cells senescence [10, 74, 75]. While these factors can be controlled, the same cannot be said of

the intrinsic heterogeneity of an MSC population [74, 75]. Furthermore, it is known that different MSC populations seem to have different functions in the hematopoietic niche, indicating a biologic variability between different MSC populations.

Mechanisms behind the effect of MSC on both *ex* and *in vivo* HSPC are still being studied, as the hematopoietic niche itself is not fully defined. Information on the niche organization, components, and mechanisms will help not only to create more efficient protocols to allow the expansion of HSPC, but also to select the best cells for therapies.

Together with the selection of the source or culture protocol that leads to higher hematopoietic support, the identification of surrogate markers that allow the evaluation of MSC's potency to support hematopoiesis - and potentially allow the enrichment of a suitable sub-population - may reduce the variability between clinical trials [47, 75]. More about surface markers and MSC subpopulations and their characteristics can be found on the section 1.2.

Use of MSC-derived products as therapies

Lately, MSC-derived products have been investigated as alternatives to the administration of MSC themselves for therapies, since there is evidence that most of the therapeutic potential of MSC is related with their paracrine effectors [15, 76]. MSC secretome is mainly composed of a cocktail of cytokines, growth factors, chemokynes, and MSC-derived extracellular vesicles (MSC-EVs). Recently, it has been suggested that MSC-EVs can preserve MSC therapeutic action, hence, MSC-EVs are a potential candidate to ease the translation of cell-derived therapies to clinical practice [16, 77].

MSC-EVs are nanovesicles that are loaded with biomolecules (proteins, lipids, or nucleic acids) and are responsible for transporting them between cells [78]. It is widely accepted that certain types of MSC-EVs are essential for several physiological processes.

Besides the evidence that MSC-EVs maintain therapeutic roles, they have several advantages over the use of MSC themselves: they are easier to store, have a high safety profile, present low immunogenicity, cross biological barriers, have a transient presence in the body, and avoid certain complications connected to MSC - for example, entrapment in lung microvasculature or induced ectopic tumor formation [76, 78].

Furthermore, it is possible to use a selected immortalized cell line to produce MSC-EVs continually. While it is not possible to use immortalized cell lines directly as therapeutic products since there are several risks involved, if it can be assured that the immortalization process does not affect the produced EVs, the variability for the MSC-EVs for a given application would significantly decrease, and it would be much easier to obtain a significant number of vesicles for a off-the-shelf therapeutic product [16, 76]. In reality, there are some proof-of-concept papers stating that the immortalization of MSC by overexpressing c-Myc seems to not affect EV preparations [79].

The use of MSC-EVs holds great potential as a cell-free substitute to MSC administration. However, there are some obstacles that still need to be considered. MSC secretome varies a lot with their sources and culture conditions, which makes MSC-EVs composition very heterogeneous. Furthermore, isolotion protocols are still not optimal and a scale-up production of MSC-EVs would need to be implemented.

Lastly, there are some therapeutical outcomes that may be related with cell-to-cell interactions, so it should also be considered whether MSC-EVs are therapeutically appropriate for a given application or not.

1.1.2 MSC Manufacturing

The low ammount of MSC that can be harvested from the donor tissues requires scalable, cost-effective, and reproducible expansion protocols to make their use in a clinical context a viable option. Despite the lack of knowledge on the ideal doses for therapeutics, Olsen *et. al* predicted that by 2040 around 300 Trillion MSC would be needed per year for therapeutic products considering only 10 FDA approved products [80]. Parameters like culture systems, culture conditions, or storage and transport conditions can be relevant to allow one to reach the necessary ammount of cells for a therapy in a suitable timeframe and with controlled costs.

Culture Systems

One of the first things that is considered when one wants to scale-up MSC production is the choice of a culture system. MSC can be cultured using plannar tecnhonologies or bioreactors, depending on the clinical strategy and necessary dose.

For small scales, usually at a laboratorial scale, static 2D planar cultures where MSC adhere as a monolayer to the plastic surface of the culture system are usually used (for example, T-flasks or culture plates), however to generate the large number of cells needed for therapy other strategies need to be considered [81].

Total surface area and cell density at harvest are the main parameters that control harvest size in 2D cultures [82]. Multiflasks or cells stackers are options that increase the surface area by creating multiple layers where MSC can adhere in a single flask, increasing the number of cells obtained. However, multiflasks still present several limitations to be considered a scalable production system for MSC-based therapies [81, 82]. Namely, each flask is susceptible to culture variability, the use of these systems is very labor intensive which limits the number of cells that can realistically be harvested [81].

3D dynamic culture systems, such as spinner flasks, vertical wheel, wave bag, stirred tank, hollow fiber bioreactors and bed perfusion systems, represent reliable alternatives to planar culture systems for a scalable production of MSC. These dynamic culture systems usually make use of microcarries for MSC propagation, which have a high surface area and can be chemically modified with adhesion motifs. Consequently, bioreactors allow the harvest of the same ammount of cells in less time than plannar systems and avoid the passage cycles which makes it less likely to occur phenotypic changes and decreases the risk of senescence [83–85]. Furthermore, such systems usually provide simpler operation protocols, can function as closed systems which decreases the probability of contamination, frequently permit the incorporation of online monitoring and control, allow a better control of environmental conditions, can provide a controlled delivery of biobimetic stimuli, have simpler harvesting processes, and are more cost-effective at larger scales [81].

Overall, the use of these systems can improve standardization and reproducibility of MSC expansion, and allow the expansion of those cells on a clinical relevant level. However, there is still a need to further optimize culture of MSC in bioreactors, namely to allow more efficient cell recovery [85].

Coating Strategies

A simple way of accelerating MSC production is by culture them in surfaces that can enhance their adhesion and proliferation, instead of simply expanding MSC on polystyrene surfaces. To achieve better proliferation rates, surfaces can be modified,for example, a increase on the surface's hydrophilicity will allow a better adsorption of adhesive proteins [86]. Alternatively, the addition of immobilized cell-binding motifs to the surface can also be done [87]. However, one of the most common techniques to enhance MSC proliferation is by using coating strategies.

In vivo, MSC are anchored to the extracellular matrix (ECM), which is composed by a range of proteins and glycosaminoglycans (GAGs). Key cellular processes like cell adhesion, migration, proliferation, and differentiation are regulated by specific interactions between MSC surface receptors and ligands on ECM, so the presence of different ECM molecules on the culture system can activate those pathways [88].

When MSC are cultured with FBS- or hPL-supplemented media, adhesive proteins (like fibronectin or vitronectin) adsorb to the plastic surface of culture flasks, allowing the attachment of MSC, even before the presence of their own produced ECM [86].

The use of coating strategies become specially relevant to culture MSC with xeno-free and human-free culture media, since such culture media are not as rich in proteins. A common strategy to enhance adhesion and proliferation of MSC on their expansion *in vitro* is to pre-coat cultureware surfaces with ECM adhesion components, for example fibronection, laminin, collagen typo I and IV, and gelatin are commonly used as coating solutions for MSC expansion. Coating with different ECM molecules can affect MSC expansion in different ways, since these can activate different pathways [89].

Fibronectin is an adhesive glycoprotein that is present in the ECM of MSC and it is commonly used as coating for MSC *ex vivo* expansion. It has been shown that fibronectin coatings enhance MSC migration, adhesion, and proliferation [89, 90]. However, it can also be responsible for the modulation of MSC differentiation. By instance, it has been shown that fibronectin has a pivotal role in osteoblast differentiation, moreover this molecule seem to inhibit adipogenic differentiation [87]. Nevertheless, other authors reported no effect of fibronectin on adipogenic differentiation [89, 91].

Collagen molecules are the most common component of the ECM. Collagen type I, II, III, and IV have been reported to also enhance migration, adhesion and proliferation of MSC [89]. Coating of cultureware surfaces with collagen type I (and IV, but at a lower extent), have proved to not only enhance MSC proliferation, but also to increase their tendency for osteogenic differentiation [91–93]). Furthermore, it has been shown that combined coating of collagen type 1 with heparin, besides of what was previously mentioned, can also modulate MSC secretome and their immunosuppressive potency [94].

Laminins are another key element of the ECM. There are 15 different laminin (LN) isomers composed by different combinations of α, β, and γ chains (table 1.2). Laminin chains differ structurally depending on

the number, size, and arrangement of constitutive domains, giving the diverse members of the laminin family both similar and distinctively significant activities [95]. In fact, it has been shown that different laminin isoforms have different binding affinities to integrins. By instance, LN-511/521 seem to have a higher affinity to $\alpha 3\beta 1$, $\alpha 6\beta 4$, and $\alpha 6\beta 1$ integrins followed by LN-332, other laminins could show affinity to specific integrins, while LN-411 seemed to be the one with overall lower affinity [96].

Coating with LN-332 have shown to be more efficient increasing MSC adhesion and proliferation than collagen IV or fibronectin and than other laminin isomeres [92, 97, 98]. LN-521 and LN-511 also have shown to increase MSC proliferation but at a lower extent than LN-332 [98]. However, for example, coating with LN-211 and LN-221 did not show major improves on cell adhesion [98], and LN-111 coating shows contradictory results, it has been shown that it was as efficient as LN-332 regarding MSC proliferation but it has also been shown that coating with this isomer decreased MSC adhesion [97, 98]. Interestingly, LN-332 seems to promote osteogenesis [92], however there are some reports that it has no effect on osteogenic differentiation, simply promotes the formation of bone due to the enhancement of MSC proliferation and the inhibition of chondrogenic differentiation [97, 98].

Table 1.2: Laminin isomers and their composition.

Chain Composition	Former Designation	Current Designation
$\alpha 1\ \beta 1\ \gamma 1$	LN-1	LN-111
$\alpha 2\ \beta 1\ \gamma 1$	LN-2	LN-211
$\alpha 1\ \beta 2\ \gamma 1$	LN-3	LN-121
$\alpha 2\ \beta 2\ \gamma 1$	LN-4	LN-221
$\alpha 3\ \beta 3\ \gamma 2$	LN-5	LN-332
$\alpha 3\ \beta 1\ \gamma 1$	LN-6	LN-311
$\alpha 3\ \beta 2\ \gamma 1$	LN-7	LN-321
$\alpha 4\ \beta 1\ \gamma 1$	LN-8	LN-411
$\alpha 4\ \beta 2\ \gamma 1$	LN-9	LN-421
$\alpha 5\ \beta 1\ \gamma 1$	LN-10	LN-511
$\alpha 5\ \beta 2\ \gamma 1$	LN-11	LN-521
$\alpha 2\ \beta 1\ \gamma 3$	LN-12	LN-213
$\alpha 4\ \beta 2\ \gamma 3$	LN-14	LN-423
$\alpha 5\ \beta 2\ \gamma 3$	LN-15	LN-523

Overall, it seems to be consensual that coating of cultureware with ECM proteins enhances MSC adhesion, proliferation and can influence their differentiation capacity. Moreover, it seems like when a combination of ECM proteins is used as coating there is a higher enhancement of MSC adhesion and proliferation than it is simply used a single protein coating [92, 97].

Culture Media

Selecting an appropriate culture medium is essential for the successful isolation and growth of MSC from various sources. Usually, culture media formulations include a basal medium with nutrients like glucose and glutamine, like Dulbecco's modified Eagle's medium (DMEM) or Minimum Essential Medium Eagle alpha (αMEM), as well as a protein-rich supplement with growth and adhesion factors necessary for the adhesion and expansion of MSC [99].

Fetal Bovine Serum (FBS) is one of the most common culture media supplements for *in vitro* cell

expansion. FBS is well established for its cost-effectiveness and richness of growth factors, adhesion molecules, micronutrients, and hormones that stimulate attachment, growth, and proliferation of MSC [100, 101]. However, there are some disadvantages to its use: FBS' composition is undefined and varies from batch to batch. Furthermore, it is a xenogenic product, which not only rises ethical problems regarding the use of animal-derived products for scientific purposes, but also it can induce immunologic reactions and transmit prions or zoonic viruses [101, 102]. Keeping in mind the objective of using MSC as therapeutic products, the aforementioned problems make FBS not compliant with GMP, since it may affect the safety of the final cell product. Therefore, alternatives to FBS have been intensively studied.

Human Platelet Lysate (hPL) appeared as an attractive cell culture supplement to replace FBS. hPL is produced from human outdated platelets which contain a wide range of growth hormones, cytokines, and proteins [100, 101]. Due to the lyse of platelets, immunoglobulins, albumin, folate, vitamin B12, glucose, triglycerides, and a variety of growth factors, including transforming growth factor beta (TGF-ß), platelet-derived growth factors (PDGF), insulin-like growth factor 1 (IGF-1), brain-derived growth factor (BDNF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) are released [103]. In fact, hPL shows a higher concentration of growth factors than FBS which can be the explanation for why most current research concur that hPL enhances cell growth to a greater extent than FBS [103–105].

Despite the problem of batch to batch variation not being solved with this supplement, it addresses the ethical concerns about the use of xenogeneic products, and, as long as it is treated previously, it can allow a GMP compliant manufacture [101, 106]. Furthermore, most studies show that MSC cultured with hPL (MSC-hPL), compared to FBS (MSC-FBS), can maintain the cluster of differentiation markers (positive for CD73, CD90, CD105, and negative for CD34 and CD45), and do not present major differences in their differentiation capacity (in osteocyte, adipocyte, and chondrocyte) [104]. Nevertheless, MSC-hPL showed a spindle-shaped elongated morphology and have higher proliferation capacity. Overall, GMP-compliant hPL can be a good candidate to substitute FBS in translational regenerative medicine. However, while some claim that using hPL-supplemented culture media do not have effect on MSC function [104, 105], others found that hPL-MSC had less immunosuppressive capacity [107, 108].

Despite the overall advantages of hPL over FBS, the lack of definition of its components and the batch variation remains a problem that can affect MSC phenotype and function. Therefore, in order to guarantee standardized culture protocols, the ideal substitute would be a fully chemically defined xeno-free human-free medium [101].

The development of a chemically defined culture medium is however a problem. Not only it would be necessary to identify from the high number of components of FBS and hPL which ones are important for attachment, growth and proliferation of MSC, but also one would need to understand the combined action of the factors and the right concentrations. In the end, all necessary components (growth factors, attachment factors, nutrients, vitamins and transport proteins) must be present in precisely the right amounts. Furthermore, for clinical applications, it is essential that such culture media do not affect cellular characteristics and they should maintain MSC's clinically relevant properties [109, 110].

Chemically defined media are not only hard to develop and to be approved, but also, they usually

have a poor cost-effectiveness relationship, being to expensive for the development of cell therapies, that are already costly by themselves. Nevertheless, there are options in the market for xeno-free, human-free options (despite not being chemically defined yet). For example, StemPro SFM MSC is one of the commercial options that have been widely used and that showed the capacity to maintain MSC minimal definition criteria [107, 111, 112]. In fact, a study performed by Oikonomopoulos *et. al*, showed that MSC cultured with StemPro did not have their immunomodulatory function impaired [107], and more recently Kim and collegues even showed an enhanced regenerative capacity of MSC exosomes after expansion with StemPro [112].

In the end, the choice of culture media can always affect MSC phenotype and function. Table 1.3 displays a collection of studies that examined the effects of different culture media on MSC, mainly differences between FBS- and hPL-supplemented media.

Storage & Transport

High quality storage and transportation systems are required to deliver MSC safely and efficiently for therapies [117]. It is important that such systems consider not only the number of MSC that will be recovered at the end, but also their quality. When considering autologous therapies, transport solutions (or short storage systems) should be enough. However, if one aims to develop of-the-shelve therapies, long storage systems also need to be considered [117].

Cryopersevation is one of the main techniques to store MSC in long term. In order to cryopreserve cells it is needed to supplement the culture media with cryoprotetancts - substances that protect cells from freezing damage, otherwise water crystallization would damage cells' internal structure compromising their viability [118, 119]. Despite its cytotoxic effects, Dimethyl sulfoxide (DMSO) is still one of the most used cryoprotectants. Nevertheless, for cells to be used therapeutically DMSO should be thoroughly washed out which can be expensive and time consuming, but also can lead to a reduction of the cell yield [118]. Less toxic (or ideally non-toxic) alternatives to DMSO are being investigated - sugars and sugar alcohols arrive as a natural alternative to DMSO since those molecules can stabilize cell membranes and influence hydrogen bonding of the water molecules, reducing the cellular damage from ice, nevertheless the results are still bellow the expected [119]. NutriFreez® D5 (DMSO-reduced freezing solution) and BIOFREEZE (DMSO-free freezing solution) are examples of commercially available serum-free and xeno-free freezing media alternatives that are closer to be GMP compliant and therefore more prone to be used in clinical context.

Regarding short storage options, there are some cold storage solutions that rise as an alternative to cryopreservation. Such solutions act by hypothermic preservation and allow cells to be transported between 2 and 8ºC without compromising them, which is a big advantage upon cryopreservation, since in that case cells should be transported at -80ºC [120, 121].

Since hypothermia causes ion imbalances - affecting essential ions like Na^+, K^+, Ca^{2+} and Cl^- - it causes osmotic and oxidative stress to the cells. Therefore, a good storage medium would need to have an ionic balance similar to the intracellular milieu [121]. Despite MSC being relatively resistant to stress, without a solution to to tackle the perturbations of ionic balance caused by the low temperatures,

Table 1.3: Collection of articles that compare effect of different culture media on MSC

MSC Source	Culture Media	Main Conclusions	Ref.
MSC(M)	FBS vs hPL	hPL-MSC: - Express lower levels of adipogenic and osteogenic markers - Have the ability to fully differentiate into osteoblastic, adipogenic, chondrogenic and vascular smooth muscle lineages - Have increased secretion of interleukin (IL)-6 and IL-8 - Have increased growth rate (shorter culture time)	[113]
MSC(M)	FBS vs hPL	hPL-MSC: - Have increased proliferation and are smaller - Show enhanced osteogenic differentiation and suppressed chondrogenic differentiation - Present transcriptomic changes in comparison to FBS-MSC - Have impaired immunomodulatory and angiogenic functions	[108]
MSC(M)	FBS vs hPL	hPL-MSC: - Showed the same immunomodulatory properties of FBS-MSC	[114]
MSC(M), MSC(AT), and MSC(WJ)	FBS vs hPL	hPL-MSC: - Show impaired hematopoietic support ability (for the 3 sources)	[10]
MSC(AT)	FBS vs hPL	hPL-MSC: - Have increased proliferation capacity- Show significantly enhanced neurotrophic properties	[115]
Periosteum derived MSC	FBS vs hPL	hPL-MSC: - Have increased proliferation capacity - Maintain phenotype and differentiation capacity - Show suppressed adipogenic differentiation - Present transcriptomic changes in comparison to FBS-MSC - After implantation, create mineralized tissue, while FBS-MSC generate fibrous tissue.	[116]
MSC(M) and MSC(AT)	FBS vs hPL vs StemPro	hPL-MSC: - Have increased proliferation capacity, alongside StemPro-MSC: - Exhibited diminished immunosuppressive properties (in comparison with both FBS-MSC and StemPro-MSC)	[107]
MSC(WJ)	FBS vs StemPro	StemPro-MSC: - Produced exosomes with higher expression of cytokines related to regenerative bioactivities which resulted in enhanced wound healing and angiogenesis.	[112]

irreversible changes in MSC cellular structure would happen, leading to death and decrease in cell viability over time [120].

In a perfect scenario, cells would be infused together with such solutions to the patient. Hence, solutions that were previously used for infusion, have been studied for transportation and hypothermic storage of cell-based therapies. Solutions like Ringer's solution or Plasma-Lyte mimic electrolyte concentration, pH, and osmolality of physiological plasma, nevertheless, it looks like they are not able to maintain viable MSC in cold storage for periods of time longer than 24h [120, 122]. There are GMP compliant approved solutions that allow MSC cold storage for a few more days (for example HypoThermosol® FRS can maintain MSC(M)'s viability for up to 3 days [120], and Cellsius 2-8 could maintain viability of MSC(AT) for up to five days in storage [123]). However, these storage media are not injectable, requiring a washing step prior application of MSC-based therapeutic products.

Despite the efforts that are being done to find proper storage solutions and conditions, there is still no ideal solution. Furthermore, there is evidence that cell storage can induce changes in the therapeutic properties of cells, compromising the therapeutic outcome [121]. It is important to establish strong and reproducible assays to control MSC's quality before treatment.

1.1.3 Quality Control

Despite the high interest in MSC-based therapies, the results of studies and clinical trials are often contradictory and present mixed outcomes. One of the main reasons to justify such results is the use of cell products with uncertain quality [15, 124]. As it has been mention throughout this work, besides the different sources from where MSC can be isolated, different isolation and culture conditions (expansion protocols, coating strategies, culture media, etc) can alter MSC characteristics and select different subpopulations. Different laboratories use different expansion conditions and different assays to evaluate MSC quality.

In order to guarantee the production of high quality cell-based therapeutic products some preventive measures should be taken. First of all, since donor characteristics (like age, gender, or health conditions) can affect MSC [125], a rigorous donor selection should be made. Moreover, once cell-based therapies have the capacity to spread infectious diseases, screening for transmittable diseases should be done before the donation [126].

During the isolation and expansion proccess, it is important to guarantee that all materials and reagents that are used during the production are GMP compliant and from qualified manufacturers. Furthermore, tests to guarantee that cell-products are free of any contaminants (like bacteria, fungi, endotoxins, or mycoplasma) should be performed [126]. Moreover, it is necessary to confirm if MSC expanded *ex vivo* comply with the ISCT minimal criteria to define them, and if they are viable [126].

Lastly, since tumorogenicity is one of the most critical safety concerns when using stem cells, karyotype assay, to guarantee that there are no chromosomal abnormality after the culture *in vitro*, and *in vivo* tumorigenicity assays should be performed prior infusion to the patient [126].

Nevertheless, even if all the safety measures and assays are performed, it is also important to make

sure that the MSC that will be infused kept their function after the *ex vivo* expansion. Therefore, it is important to apply potency and functional assays.

Potency and Functional Assays

According to EMA, ”Potency is the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties” and ”an appropriately validated potency assay should be based on a defined biological effect as close as possible to the mechanism(s) of action/clinical response” [127]. Overall, a robust potency assay should reflect a relevant mechanism of action (MoA), for a specific disease, preferably, in a quantitative way [15, 16].

Ideally, product attributes that predict clinical efficacy should be measured, however, it does not exist a test that can do it adequately [128–130]. Alternatively, substantial evidence of clinical efficacy is collected by designing well-controlled investigations, which includes designing well-defined and robust potency assays [129].

Potency tests, together with several others, are used to assure the quality of a cell product. These tests will access the identity, purity, strength, and stability of the cell product. For a product to have market approval, it needs to have well-designed, reproducible, and robust potency assays for every stage of clinical investigation [128, 129].

Defining potency assays for MSC-like structures is particularly challenging, mainly due to (1) inherent variability for starting materials, (2) limited lot size and material for testing, (3) limited stability, (4) lack of appropriate reference standards, (5) complex and not fully understood MoA, (vi) *in vivo* fate of the product (that can not be fully reproduced *in vitro*) [15, 128]. What has been suggested by the FDA to solve this issue is the adoption of an assay matrix composed of several complementary potency tests that can measure product quality, consistency, and stability [128, 129].

In order to measure potency, one can use biological or non-biological analytical assays [15, 128, 129]. Traditionally, quantitative biological assays (bioassays) are used by evaluating the activity of the product in a living system. Nevertheless, the design of a suitable bioassay for MSC is not always possible, in that case, non-biological analytical assays may complement the results [128]. Analytical assays measure properties of the product outside of a living system and can be used to demonstrate potency if a correlation between them and the product-specific biological activity can be proved [128, 129]. Quantitative flow cytometry and enzyme-linked immunosorbent assay; reverse transcription polymerase chain reaction, quantitative polymerase chain reaction and microarray; or protein binding and enzymatic reactions, are examples of analytical assays approved by the FDA that measure immunochemical, biological, or molecular attributes (respectively) [128]. Bioassays and analytical assays can complement each other, and can be used together in a potency assay matrix to better indicate MSC potency [128].

Due to the lack of consensual surrogate markers to evaluate MSC quality and functionality, co-culture systems between MSC and different cell types are common assays. By instance, PBMC (or specific immune cells) can be used to confirm MSC’s immunomodulatory capacity [128], and HSPC can be used to test their ability to support hematopoiesis.

1.2 MSC's Subpopulations

As it has been mentioned throughout this review, MSC are a population that is very heterogeneous regarding both phenotype and function, which may diminish their therapeutic capacity and lead to contraditory results between studies. Recently, different MSC's subpopulations, with different characteristics and therapeutic potentials have been proposed. The characterization of MSC's subpopulations regarding their biological functions, and selection of the proper subpopulations with enhanced potential for a given condition, its an interesting path to create more effective MSC-based therapies and allow a better comparison between different clinical studies.

An easy way to identify and sort cells is by their surface markers, and in fact, expression of different surface markers on MSC have been reported to influence their main characteristics, allowing the identification of subpopulations with different biological activities and therefore, different therapeutic applications.

1.2.1 MSC Subpopulations for immunoregulation

There are several reports that state the existance of a correlation between expression level of different MSC surface markers and their immunosuppressive potential.

For starters, it has been shown that CD106^{+} MSC have augmented immunosupressive properties. MSC's surface protein CD106 (also known as VCAM-1 (vascular cell adhesion molecule 1)) seems to be essential for mediating cell-cell contact with immune cells. In a work performed by Ren *et. al*, not only was concluded that CD106 expression in MSC was induced by T cells, but also that higher the expression of this surface marker, higher MSC's immunosupressive capacity. Lastly these results were confirmed by a significant decrease on MSC's immunosupressive ability after genetic deletion of CD106 or after its functional blockage ([131]). In another work, Yang *et. al* compared the immunomodulatory potential of CD106^{+} and CD106^{-} MSC, and concluded that CD106^{+} produced more cytokines associated with the immune response (COX-2, IL-1a, IL-1b, IL-6 and IL-8) and were more effective modulating T-helper cells [132].

Another population of MSC that proved to have enhanced immunomodulatory capacity are CD200^{+} MSC. CD200 (or OX-2 membrane glycoprotein) interacts with a receptor that exists on the surface of myeloid cells (CD200R) causing the dowregulation of immune cells (mainly macrophages). It has been shown that contrarily to CD200$^{-/low}$, CD200^{+} MSC can suppress the secretion of TNF-α from macrophages, moreover when this glycoprotein is blocked with anti-CD200 antibody its ability to downregulate macrophages activity decreases [133]. It has also been shown that MSC genetically manipulated to express CD200 on their surface have increased immunomodulatory capacities [134]. Finally, it looks like CD200/CD200R pathway is responsible for the innibition of dendritic cells maturation [135]. Overall, CD200^{+} MSC are well established as having an important role in the regulation of immune cells.

CD271^{+} MSC and CD146^{+} MSC are examples of populations for which the enhancement of immunomodulatory proprerties have been reported, however it seems like it is not directly related with the surface markers themselves. By instance, MSC with high expression of CD271 (also known as

low-affinity nerve growth factor receptor (LNGFR)) have shown to produce more cytokines, significantly reduce the proliferation of allogeneic T cells, and suppress the proliferation of mononuclear cells during mixed-lymphocyte reaction than unsorted MSC [136, 137]. CD146 (also known as melanoma cell adhesion molecule (MCAM)) seems to identify a population of cells with enhanced secretory capacity [138]. Immunopotency tests with stimulated PBMC and T cells showed CD146$^+$ MSC to be a potent immunosuppressant [138, 139]. Furthermore, CD146$^+$ MSC promoted macrophage polarization from M1 to M2, reducing their phagocytic capacity and promoting tissue repair [138, 139].

1.2.2 MSC Subpopulations for hematopoietic support

It is widely known that MSC are central members of the hematopoietic niche. Despite the limited knowledge about the hematopoietic niche, it is known that through paracrine production of soluble molecules and direct interaction, MSC regulate HSPC homeostasis [140]. As some light is shedded in the structure of this niche, *in vivo* research has revealed various MSC subpopulations involved on HSPC homeostasis, identified by the expression of different surface markers, namely CD146 and CD271 [57, 136].

CD146$^+$ MSC are located in the sinusoidal wall and seem to have an enhanced clonogenic capacity. Furthermore, when transplanted into immunocompromised mice , they supported hematopoietic activity by re-establishing the hematopoietic environment. Lastly, regulatory genes related with maintenance of HSPC homeostasis, like Angiogenin-1 and C-X-C motif chemokine 12 (CXCL12), have shown to be overexpressed by CD146$^+$ MSC [141].

CD271$^+$ MSC are located in the trabecular region of the bone marrow, and as CD146$^+$ MSC this subpopulation also has an enhanced clonogenic capacity. Compared to CD271$^-$ MSC, they have greater proliferation capacity, and propensity to differentiate into mesodermal tissues [136]. Transcriptional analysis of both CD271$^+$ and CD271$^-$/CD140a$^{-/low}$ MSC has shown increased expression levels of hematopoiesis-related genes [142, 143]. For CD271$^+$ MSC, CXCL12, FLT3L, IL-3, TPO, and KITL are examples of genes that had an enhanced expression [142]. Furthermore, *in vivo* testing has shwon that CD271$^+$ MSCs are more effective at promoting HSPC engraftment [136].

It was demonstrated that CD271$^+$ and CD271$^+$CD146$^{-/low}$ MSC seem to be present in bone-lining low oxygenated areas and are associated with long-term HSPC, while CD146$^+$ and CD271$^+$CD146$^+$ seem to be located in the sinusoids of the bone marrow and to be associated with proliferating HSPC. Nevertheless, in both regions HSPC location is nearby MSC's, allowing them to interact directly [144]. Nevertheless, it also has been shown that CD146$^+$ MSC can support long-term *ex vivo* expansion of HSPC through activation of the Notch signaling pathway, while CD146$^-$ MSC induced HSPC differentiation, compromising their expansion. [70]

Appart from populations that have been identified in the hematopoietic niche, PDGFRα^+CD51$^+$ cells represent a small subset of CD146$^+$ cells and have been suggested to have enhanced hematopoietic supportive functions, since PDGFRα^+CD51$^+$ cells seem to be Nestin$^+$ [145]. Nestin$^+$ cells can create non-adherent mesenpheres that can self-renew and expand, and upon adherent culture these cells

can meet the requirements to be classified as MSC (nevertheless, expression of nestin is lost during the expansion). It has been shown that Nestin$^+$ cells are located closely to HSPC in the hematopoietic niche, and expression of HSPC maintenance genes is enhanced. Furthermore, *in vivo* tests with mice showed that depletion of Nestin$^+$ cells reduced the presence of HSPC in the bone marrow [146]. In reality, Pinho *et. al* demonstrated that PDGFRα^+CD51$^+$ cells were able to support the *ex vivo* expansion of HSPC [145].

1.2.3 Other functions and characteristics

Other functions have been attributed to the previously mentioned MSC populations and to others. By instance, CD200, CD271, and CD146, but also CD73, and CD130 have been reported to identify MSC that have enhanced clonogenic capacity [136, 147]. Different subpopulations can have different proliferation potentials. CD200$^+$ and CD271$^+$ MSC tend to differentiate into osteogenic and chodrogenic lineage [134, 148]. On the other hand, CD146$^+$ MSC are able to differentiate into the myogenic lineage, namely can differentiate into cardiomyocites and be used for heart repair [149, 150]. As another example, PDGFRα^+ cells have can differentiate into dermal fibroblasts and ectodermal keratinocytes, which can be very useful for wound healing [151].

Table 1.4: Examples of MSC subpopulations and their specific characteristics

Subpopulation	Characteristics	References
CD106	Increased immunomodulatory properties	[131, 132]
	Decreased colony-forming capacity	[132]
CD200	Increased immunomodulatory properties	[133–135]
	Increased colony-forming capacity	[134, 147]
	Increased osteogenic and chodrogenic differentiation capacity	[134]
CD271	Increased immunomodulatory properties	[136, 137]
	Enhancement of engraftement capacity *in vivo*	[136]
	Increased colony-forming capacity	[136]
	Increased osteogenic and chondrogenic differentiation capacity	[148]
CD146	Increased immunomodulatory properties	[138, 139]
	Enhanced secretory capacity	[138]
	Enhanced hematopoietic supportive properites	[70]
	Increased colony-forming capacity	[147]
	Myogenic differentiation / high potential for heart repair	[149, 150]
PDGFRα	Enhanced hematopoietic supportive properites	[145]
	Dermal fibroblasts and ectodermal keratinocytes differentiation / high potential for wound healing	[151]
Nestin	Enhanced hematopoietic supportive properites	[145, 146]
	Neuroregenerative properties (astrogliogenesis promoters)	[152]

In table 1.4 it is possible to observe a summary of different functions for different MSC subpopulations. Note that, it is just a sample, since there are other populations and functions that could be part of this summary. Due to the heterogeneity of MSC, it is important to perform thorough identification of specific subpopulations, and understand their respective strenghts and weaknesses, and mechanisms behind their specific properties.

Despite MSC being one of the more promising cell types for therapeutics, its heterogeneity can be a drawback for the translation from bench to clinic. Overall, it is important to understand how different

procedures during cell-based therapeutic products can affect MSC function and what are the mechanisms that cause alterations in the populations. In the end of the day, main objective should be to take advantage of the different MSC properties and learn how to enhance or diminish them, depending on the wanted application. This specificity of MSC production towards a specific application as the potential to, not only enhance therapeutic outcomes, but also decrease the variability on clinical trials outcome.

Chapter 2

Materials and Methods

2.1 MSC Samples

In this study, human MSC available in the Stem Cell Engineering Research Group (SCERG) cell bank, at iBB-IST, Lisboa, Portugal, previously isolated from bone marrow aspirate and characterized to guarantee compliance with ISCT minimal MSC identity and characterization criteria were used.

Bone marrow aspirates for MSC isolation were previously donated from Instituto Português de Oncologia Francisco Gentil, Lisboa, under collaboration agreements with Institute for Bioengineering and Biosciences (iBB) at Instituto Superior Técnico (IST). Samples were collected from healthy donors, after written and informed consent and according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 regarding standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of the respective clinical institution.

Throughout this work three different MSC(M) donors with different ages were used, from which one was a male donor and the other two were females (table 2.1).

Table 2.1: Donor age and sex distribution (M - male; F - female)

	Donor 1	Donor 2	Donor 3
Age	29	57	19
Sex	M	F	F

2.2 Cell Thawing

Cryopreserved MSC(M) were withdrawn from the cellbank and partially thawed in a water bath at 37ºC. DMEM (31600-091 Gibco) supplemented either with 5% (v/v) hPL ultraGRO™-PURE GI (HPCHX-CGLI50 Avantacell) or 10% (v/v) FBS (10270-106 Gibco) and 1% (v/v) Antibiotic-Antimycotic (15240-062 Gibco) was pre-heated. Cells were then fully thawed by slowly suspending them in 5mL of pre-heated culture media.

Cell suspensions were centrifuged at 350G for 7 minutes and the supernatant was discarded while the pellet was re-suspended in 1.5 to 4 mL of their respective culture media, depending on the expected cell number. Cells thawed in DMEM supplemented with 10% FBS were resuspended in DMEM supplemented with 10% FBS-MSC qualified (12662-029 Gibco).

From here on, DMEM supplemented with 5% hPL will be referenced as DMEM/hPL and supplemented with 10% FBS-MSC qualified as DMEM/FBS.

2.3 Cell Passage

MSC exhausted medium was removed and centrifuged at 350G for 10 minutes. Cells were then washed with the same volume of Phosphate buffered saline (21600-044 Gibco) (PBS). To detach MSC from the plastic surface of T-flasks, cells were incubated with an enzimatic agent for 7 minutes at 37ºC. Depending if the cells were previously cultured with FBS-MSC qualified or hPL supplements, either a solution of 0.05% (v/v) Trypsin (15090-046 Gibco) and 0.1 mM EDTA (J15694-AE Thermo Scientific) in PBS or TrypLE™ Select 10x concentrated solution (A12177-02 Gibco) diluted with PBS were used.

Once cells were detached, enzymatic agents were inactivated by diluting them with the supernatant of the respective centrifuged exhausted medium. If a significant amount of cells were still adherent to the surface of the T-flask, a second cycle of detachment could be performed.

After collecting the cell solutions, they were centrifuged for 7 minutes at 350G and the pellet was once again resuspended in the appropriate volume of the desired culture medium.

2.4 Cell Expansion

After thawing or detachment of MSC, cell counts and viability were assessed resorting to Trypan Blue (15250-061 Gibco) exclusion test. Cell suspensions were resuspended in the appropriate volume of the respective culture medium for seeding. Cells were seeded in T-flasks at a cell density from 2 300 to 4 100 cells/cm^2.

Cells were incubated at 37ºC in a 5% CO_2 humidified atmosphere until a confluence of 80-90% was reached or up to 10 days in culture. If a high number of cells in suspension was observed in the first day of culture, the culture medium was changed. Otherwise, it was changed every 3 to 4 days until cells were passaged. In this study, MSC were expanded with DMEM/FBS or DMEM/hPL, depending on the desired condition.

2.5 Nutristor Testing

MSC(M) from one donor (Donor 1) were centrifuged after passaging for 7 minutes at 350G. The obtained pellet was then suspended in Nutristor (05F3F30011B Sartorius) or DMEM/hPL at a concentration of 1 million cells/mL and kept at 4ºC. This test was performed 3 times, with cells from different conditions. Furthermore, for each test, cells were counted after different duration in storage:

Test 1. MSC(M) in P4 adapted from DMEM/FBS to DMEM/hPL, measurements were performed after 0h and 48h (2 days) in storage.

Test 2. MSC(M) in P4 cultured with DMEM/hPL, measurements were performed after 0h, 24h, 48h, 72h, 96h, and 120h (5 days) in storage.

Test 3. MSC(M) in P5 thawed in DMEM/hPL, adapted to DMEM/FBS, and readapted to DMEM/hPL, measurements were performed after 0h, 2h, 18h, 24h, 48h, and 72h (3 days) in storage.

Viability and recovery were calculated from the results of cell countings for each timepoint. Viability depicts the ratio of live cells over the total, and recovery represents the percentage of cells that are recovered from the ones that were placed in suspension. Cell viability lower than 70%, cell recovery lower than 50%, or insufficient cell number were used as conclusion criteria.

For test 2 and 3, cells were plated at each time point to test their capacity to re-adhere to plastic and proliferate. For test 3 flow cytometry (see section 2.7.1) with only LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (L34974, Invitrogen) (L/D) dye was performed to cells after 30', 2h, 18h, and 72h stored, to confirm viability results.

2.6 Influence of Biolaminin Coating on MSC

To understand the influence of biolaminin coating in MSC function, MSC(M) from one donor at P4 (Donor 1) previously isolated and expanded with DMEM/FBS were seeded at 3 000 cells/cm^2 and cultured with DMEM/FBS for two passages with Biolaminina LN 521 coating (LN521-05, Biolamina) and without coating. At each passage cell number and morphology were analyzed. MSC fold increase was calculated as the ratio between the recovered cells after each passage and the plated cells.

MSC from both conditions were then plated in a 12-well plate and kept in culture until they reach 100% confluency, creating feeder layers to test them for hematopoietic support capacity. The presence or absence of the coating was maintained also in the 12-well plate.

Coating was performed according with the manufacturer instructions. Shortly, Biolaminina LN 521 was diluted with DPBS(Ca^{++}/Mg^{++}) to a final concentration of 10 μg/mL. To coat T-12.5 flasks, used for MSC expansion, the volume of coating solution was 2mL, while for the 12-well plate 500 μL/well were used. Cultureware were then incubated for 2h at 37ºC. Afterwards, the coating solution was discarded and cells were plated as described previously.

2.7 Influence of culture media on MSC

To study the influence of culture media on MSC, MSC(M) previously isolated with DMEM/hPL or DMEM/FBS at passage 1 or 2 from three donors (Donor 1, 2 , and 3), were seeded in T-flasks at a cell density from 2 300 to 4 100 cells/cm^2. MSC(M) were then cultured for 4 passages with DMEM/hPL or DMEM/FBS, in three scenarios as present in Figure 2.1, note that the first culture medium with which these cells were expanded was the one with which they were previously isolated:

(1) continuously in the same culture medium

(2) adapted to a different culture medium halfway

(3) adapted to a different culture medium and then re-adapted to the original one.

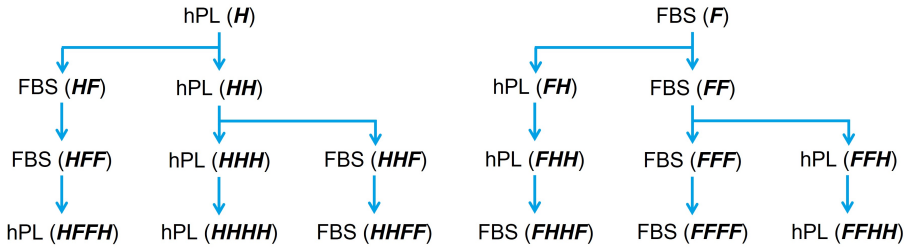


Figure 2.1: Scheme of culture media conditions. DMEM/FBS is represented as ”FBS” and DMEM/hPL as ”hPL”. ”H” or ”F” represent the culture medium that was used at each passage, for example ”FH” means that in the first passage of the study cells were cultured with FBS supplement and in the second one with hPL.

At each passage, MSC were observed, counted with the trypan blue exclusion test, and the expression of CD146 and CD271 surface markers was assessed by flow cytometry.

Average velocity of growth was calculated as the ratio between the number of cells that grew during the culture (difference between recovered and plated cells) and the number of days in culture.

After the last passage, MSC from the three scenarios aforementioned were co-cultured with HSPC to evaluate the influence of MSC culture medium on their function, namely hematopoietic support capacity (see section 2.8).

2.7.1 MSC Flow Cytometry

After detaching the cells, between 50 and 150 thousand cells were placed in 5 mL round bottom polystyrene tubes, for each condition three tubes were prepared. Samples were washed with PBS by adding around 2 mL of PBS and centrifuging them at 500G for 5 minutes. After centrifugation the supernatant was discarded and cells were resuspended in the remaining supernatant.

In order to test cell viability, L/D was diluted with PBS in 1:100 proportion and 10 μL of the diluted L/D solution were placed at two of the three tubes. Cells were then incubated for 15 minutes at room temperature in the dark, and washed with PBS as described before.

One of the tubes was then stained with CD146 PE (342004 Biolegend) mouse anti-human monoclonal antibody and the other one with CD271 PE (345106 Biolegend) antibody to assess the presence of those antigens. Once more, cells were incubated for 15 minutes at room temperature in the dark and washed with PBS.

In the end, for each condition, there was one unstained tube as negative control, one tube stained with L/D and CD146 PE, and one tube with L/D and CD271 PE.

After sample preparation, data acquisition was performed using a FACSCalibur Cytometer. Data analysis was then performed resorting to FlowJo V.10 software.

2.8 Hematopoietic Support Assay

Umbilical cord blood (UCB) units samples used in this study were originally obtained from Hospital São Francisco Xavier, Centro Hospitalar de Lisboa, under collaboration agreements with iBB at IST. Samples were collected from healthy donors, after written and informed consent with the same legal framework as explained to MSC donation.

2.8.1 CD34$^+$ Pool

Two different pools of CD34$^+$-enriched cells were created, one to test the influence of the culture media and another one to test the influence of the biolaminin coating. Enrichment of CD34$^+$ cells was done with Magnetic Activated Cell Sorting (MACS) using the CD34 MicroBead Kit human (130-046-702 Miltenyi Biotec)

To create CD34$^+$-enriched pools, firstly mononuclear cells (MNC) previously isolated from UCB were thawed in pre-heated DMEM supplemented with 10% (v/v) FBS. Up to 5 mL of thawed cell suspension were added into a 50 mL tube and topped with DMEM supplemented with 10% FBS.

As done before, cells were centrifuged at 350G for 7 minutes, and the supernatant was discarded. The pellet was resuspended in around 20 mL of MACS Buffer and counted. If visible cumps were present after resuspension, cells would be filtered through a 70μm filter. Afterwards, the kit instructions were followed.

Once the sorting process was complete, cells in the CD34$^+$-enriched fraction were counted. Flow cytometry of the same fraction was also done to characterize the sample and guarantee the success of the sorting proccess. CD34$^+$-enriched cells were then cryopreserved with culture medium (DMEM supplemented with 10% FBS) supplemented with 10% (v/v) Dimethyl Sulfoxide (DMSO) (D850-100ML, Sigma). After addition of DMSO to the cell suspension, cells were distributed by 1.8 mL cryovials which were placed inside a CoolCell and left at 80ºC overnight. Afterwards, cryovials were transfered and maintained in the cell bank (in liquid nitrogen).

MNC for the CD34$^+$-enriched pool that were used to test the influence of the culture media on MSC hematopoietic support capacity were from 3 different donors. In the case of the cells used to test the influence of the biolaminin, CD34$^+$-enrichment was done from MNC from one donor of umbilical cord blood.

2.8.2 HSPC Expansion

As mentioned before, to test influence of culture media and biolaminin coating on MSC function, hematopoietic support assays were performed.

For each condition MSC were plated in 12-well plates and kept in culture until they formed 100% confluent feeder layers. One of the feeder layers for each condition was kept non-inactivated, while the other was inactivated resorting to mitomycin-c (M4287-2MG Sigma Life Sciences) (mmC).

To inactivate MSC, a stock solution of mitomicin-c in PBS at a concentration of 0.5 mg/mL was

prepared. Such solution could be used up to 1 month, requiring a fresh solution to be prepared after that. MSC were incubated for 3 hours at 37ºC with 1 mL of the stock solution diluted 1:1000 with the respective culture medium (reaching a final concentration of mmC of 0.5μg/mL). Afterwards, feeder layers (FL) were carefully washed with the respective culture medium two times and kept in culture medium inside the incubators until the start of the hematopoietic support assay. FL could be used until 1 day after the inactivation.

Previously frozen vials of a UCB CD34$^+$-enriched cells pool for each experiment were withdrawn from the cellbank. Vials were first partially thawed in a water bath at 37ºC and then fully thawed by slowly suspending them in 5mL of pre-heated DMEM supplemented with 10% (v/v) FBS and 1% (v/v) Antibiotic-Antimycotic. Cell suspensions were centrifuged at 350G for 7 minutes, supernatants were discarded while pellets were resuspended in up to 1mL of StemSpan Serum-Free Expansion Medium (SFEM) II (09655 Stemcell Technologies) supplemented with 1% (v/v) Antibiotic-Antimycotic. As explained previously for MSC, cell counts and viability were assessed resorting to Trypan Blue exclusion test.

UCB CD34$^+$-enriched cells were expanded in StemSpan SFEM II supplemented with 1% (v/v) Antibiotic-Antimycotic and with a previously optimized cytokine cocktail: Stem Cell Factor (SCF - 90 ng/mL) (AF-300-07-100UG PeproTech), FMS-like tyrosine kinase 3 ligand (Flt-3L - 82 ng/mL) (AF-300-19-100UG PeproTech), Thrombopoietin (TPO - 77 ng/mL) (AF-300-18-100UG PeproTech), and Basic Fibroblast Growth Factor (bFGF - 5 ng/mL) (AF-100-18B PeproTech). A 7-day expansion at 37ºC was performed at a density of 60 000 cells/well (30 000 cells/mL) in co-culture with MSC FL (inactivated and non-inactivated) and without the presence of MSC.

2.8.3 HSPC characterization

In the end of the expansion, total nucleated cells (TNC) were counted once more with the trypan blue exclusion test. Furthermore HSPC used for the hematopoietic support assay were characterized prior (D0) and after the expansion (D7) by their immunophenotype and clonogenical potential.

Fold Increase (FI) was calculated by dividing the number of cells obtained after each 7-day expansion, by the number of HSPC plated at D0 (60 000). Normalized Fold Increase was calculated by dividing FI for each FL condition, by FI of the the control condition (no FL) for each hematopoietic cell expansion.

Immunophenotype was analyzed by flow cytometry, as explained before for MSC. An Unstained tube was used as negative control for each condition, and in the sample tube cells were marked with the following mouse anti-human monoclonal antibodies: CD45RA FITC (555488 BdBiosciences), CD90 PE (328110 Biolegend) and CD34 PerCP-Cy5.5 (34722 BdBiosciences).

To analyze the clonogenic potential a colony forming assay (CFU) was performed. Between 1 000 and 2 500 HSPC in 100 μL were placed on top of 2 mL of MethoCult™ H4434 Classic medium (04434 Stemcell Technologies). Cells were resuspended resorting to a 18G seringe and the medium with the cells was divided by 3 wells on a 24-well plate, while empty wells were filled with PBS. Cells were kept in culture at 37ºC for 14 days. At day 14, colonies were counted and classified as multilineage

colony-forming unit (CFU-GEMM), granulocyte-macrophage colony-forming unit (CFU-GM), and erythroid burst-forming unit (BFU-E).

Colony number was divided by the number of seeded cells and multiplied by 10^4 to obtain the number of colonies generated per 10^4 HSPC. Percentage of each colony type were also calculated. Finally, colony FI was calculated by dividing the total number of colonies at D7 by the total number of colonies at D0.

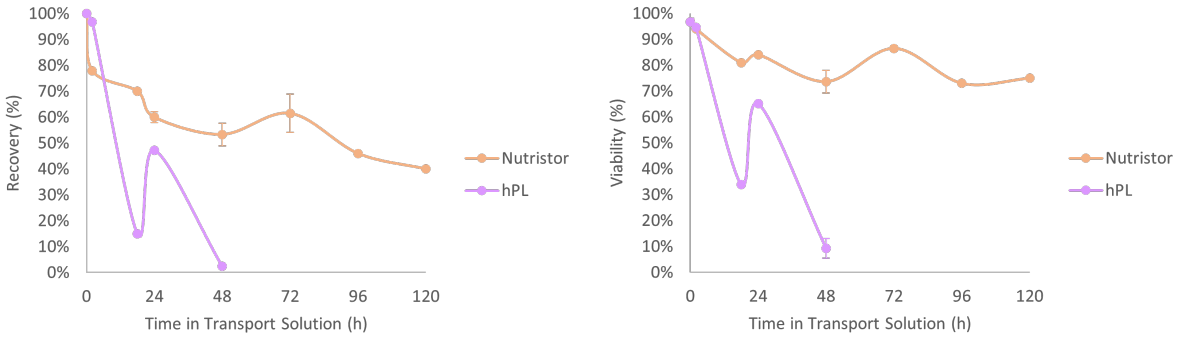
Chapter 3

Results and Discussion

3.1 Nutristor - a cold storage medium for MSC

The use of MSC as therapeutic products requires transportation processes. Therefore, short storage solutions for MSC-based products should be able to guarantee high viability (ideally, higher than 70%), good recovery rates, and maintain MSC characteristics. Furthermore, it should be possible to maintain cell-products in storage for as long as possible, since it is important to allow flexibility upon transport process and administration date.

Nutristor is a cold short storage media for MSC. To test its performance MSC(M) that were being expanded with DMEM/hPL were harvested and stored in suspension at 4ºC in both Nutristor and DMEM/hPL (used as control condition), cell viability and recovery were calculated after different periods in storage. In figure 3.1 both cell recovery (figure 3.1(a)) and viability (figure 3.1(b)) over a maximum of 5 days (120h) in cold storage can be observed.



(a) Cell Recovery

(b) Cell Viability

Figure 3.1: Cell recovery (a) and viability (b) after a maximum of 120h in cold storage. Data is represented by the mean value for each timepoint 2h, 18h, 96h and 120h (n=1); 24h and 72h (n=2); 48h (n=3). Orange represents cells stored in Nutristor, while purple represents cells stored in DMEM/hPL. Error bars represent the SEM.

For cells stored in DMEM/hPL, it was not possible to recover viable cells after 48h of storage, and even after only 18h only 39% of the cells were alive. Regarding cells stored in Nutristor, within the first

2 hours, it was observed a cell loss of 22%, while the average loss per hour, considering the entire storage time, was 0.5%/h. After the initial cell loss, between 1 and 3 days (24h to 72h) in Nutristor, the recovery rate was roughly constant (approximately 60%). After 3 days in storage, the recovery rate started decreasing until the end of the storage (after 5 days in Nutristor), when it was possible to recover 40% of the cells. Regarding cell viability, at the moment of harvest, MSC had a viability of 97$\pm$1%. During the entire storage in Nutristor (from 1 to 5 days) viability was roughly constant, being always higher than 70% (on average, 78$\pm$5%) (figure 3.1(b)). Such results were confirmed for MSC that were 30', 2h, 18h, and 72h cold storage resorting to L/D staining (figure 3.2). Overall, for a storage time shorter than 2h the use of Nutristor is not justifiable. However, when one wants to maintain cells viable for a few days, the use of culture medium would not be an appropriate option.

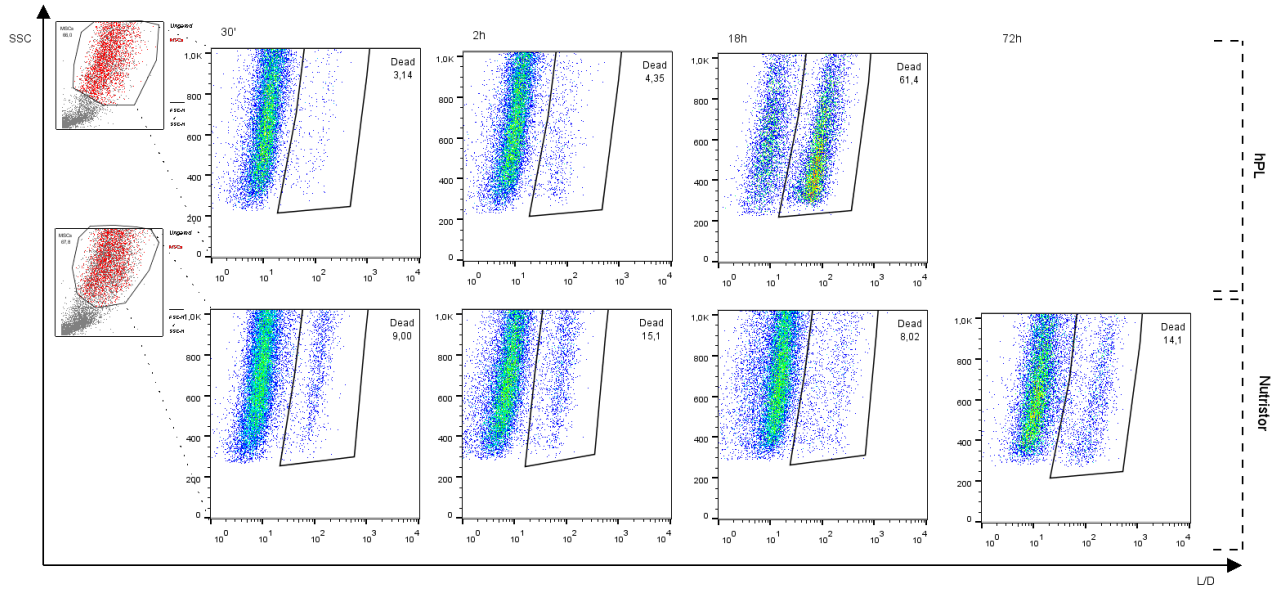


Figure 3.2: Pseudocolor plots of flow cytometry results of L/D dye, with gating and percentage of the population of dead MSC for the following durations in cold storage: 30', 2h, 18h (for hPL and Nutristor), and 72h (for Nutristor).

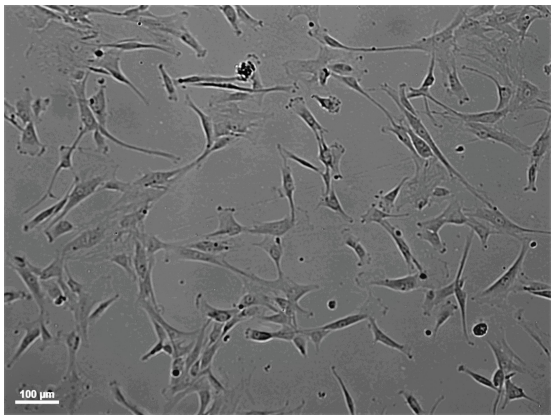
Studies focusing the efficacy of other commercially available storage solutions have been reported. By instance, Petrenko *et. al* compared the efficacy of Ringer's solution, Plasma-Lyte® 148, HypoThermosol® FRS, and a buffered trehalose solution (BTS) with MSC(M) being kept up to 72h (3 days) in cold storage [120]. It was concluded that Ringer's solution and Plasma-Lyte 148 could not maintain MSC viability after 24h in storage, but both HypoThermosol and BTS had viabilities around 75% at the end of the storage time (after 3 days) [120]. Such results are comparable with the ones obtained in the present study. Nevertheless, while Petrenko *et. al* only guaranteed the viability of MSC over 3 days, here, it was shown the potential of Nutristor to store cells until up to 5 days, maintaining their viability higher than 70%. Furthermore, the same authors concluded that the use of HypoThermosol and BTS as short storage solutions had recovery rates around 70% after 3 days in storage and maintained the ability of cells to attach and proliferate, immunophenotype, differentiation capacity, and immunomodulatory properties

also did not seem to be affected by storage with the aforementioned solutions [120]. It is important to note that recovery results were not possible to compare between Nutristor and the solutions tested by Petrenko *et. al*, since they were calculated differently. While here we calculated the percentage of cells that could be recovered from the initial number of cells stored, in this other study, authors replated the stored cells and calculated the percentage ratio between the number of cells that could be found in the culture flask after some time of culture and the number of cells in the same condition prior storage.

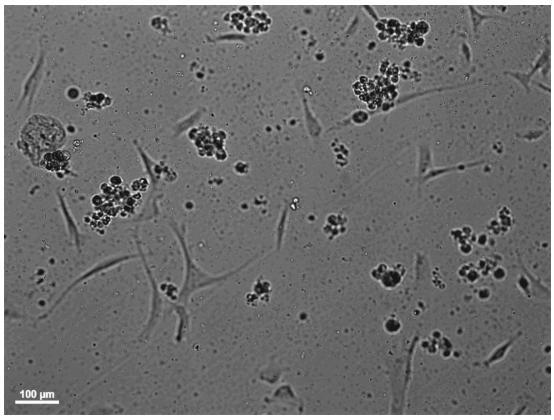
In a previous study performed at our group, the use of fresh media, conditioned media, or 2-8 Cellsius (Protide Pharmaceuticals) solution to store MSC(AT) and MSC(WJ) were compared [123]. It was concluded that 2-8 Cellsius was able to maintain MSC viability for 5 (MSC(AT)) to 7 (MSC(WJ)) days. For the adult MSC, it was possible to recover around 60% of the cells after 5 days 2-8 Cellsius at 4ºC, which was considerably higher than the recovery rate observed in the present study after 5 days in storage with Nutristor (40%). Nevertheless, in this past study, MSC(AT) stored in DMEM/hPL at 4ºC could maintain MSC viability for 4 days, with a recovery of around 70%, which was significantly higher than the results obtained for the same condition in the current study (from 18h onward, the viability of MSC stored in DMEM/hPL was lower than 70% and after 48h (2 days) it was only possible to recover 3% of the cells). To better understand the differences between the performance of Nutristor and 2-8 Cellsius, or other storage solutions, they should be compared for cells in the same conditions, i.e. from the same source, donor, with the same expansion conditions, etc.

To understand if MSC kept their proliferative capacity after the cold storage, cells were re-plated and expanded with DMEM/hPL (figure 3.3). After 18h in cold storage, cells stored in DMEM/hPL were not able to attach to the plastic surface and proliferate (figure 3.3(b)), while the ones that were stored in Nutristor could do so, and appeared to have normal morphology (figure 3.3(a)). In fact, even after 5 days in Nutristor, MSC were able to adhere to the plastic and proliferate, presenting a normal morphology (figure 3.3(c)).

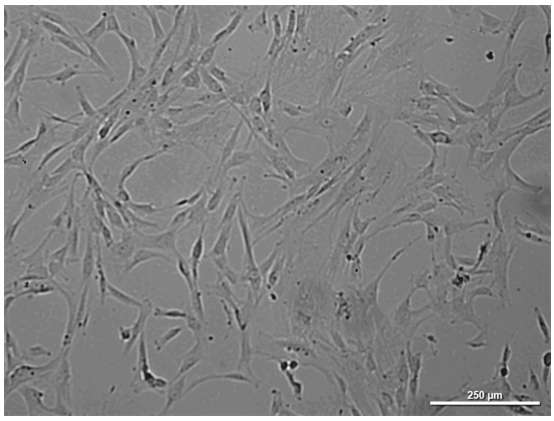
Since the nutristor solution is in a beta testing phase, there are no studies using it as a storage solution for hypothermic preservation to allow a comparison of results. Overall, Nutristor seems to be able to maintain MSC for a longer period than other options. However, a significant percentage of the cells was lost during the process, which is a problem when therapeutic doses require a high number of cells at a time. Further studies would need to be performed to guarantee MSC quality after storage in Nutristor. Namely, characterization studies, as the analysis of surface markers expression and differentiation assays, would be necessary to guarantee that after storage MSC keep compliant with the minimal criteria to define them. Moreover, the three tests that were performed were done from the same donor MSC, and most of the timepoints only have one measurement which is not representative. It would be necessary to test for more donors, each one with more replicates.



(a) MSC(M) at day 2 of culture after 18h in Nutristor



(b) MSC(M) at day 2 of culture after being 18h in hPL



(c) MSC(M) at day 4 of culture after 120h (5 days) in Nutristor

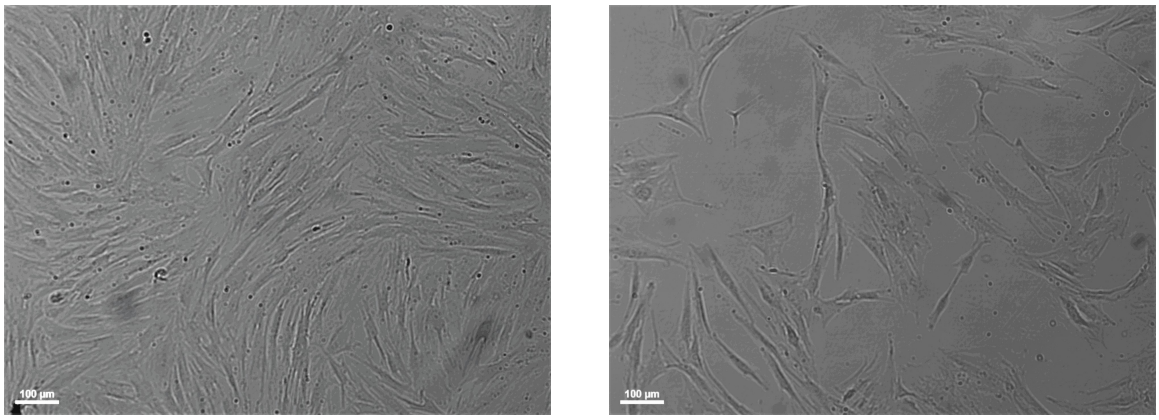
Figure 3.3: Representative microscopic images (100x) of MSC(M) at day 2 of culture after being stored for 18h in Nutristor (a) and in DMEM/hPL (b), and at day 4 of culture after being stored for 120h (5 days) in Nutristor at 4ºC (c). Scale bar represents 100 μm for (a) and (b), and 250 μm for (c).

3.2 Biolaminina-521 Coating to Enhance MSC Properties

3.2.1 Effect on MSC Expansion

MSC(M) from one donor (N=1) were expanded for two passages with DMEM/FBS in the presence of Biolaminina-521 coating (BL-MSC). As control, the same cells were also expanded for two passages in a regular T-flask, with no coating (MSC). Passages were performed at the same day for both conditions and were determined by the condition with higher confluence. In figure 3.4, it is possible to observe that at the same day, BL-MSC were much more confluent than the control condition.

It was observed that BL-MSC in the second passage had a higher proliferation capacity than in the first one. Not only did they reach confluence in less time (table 3.1), but also its fold increase was higher (figure 3.5). Nevertheless, on both passages BL-MSC had a higher fold increase (FI = 4 and 6, respectively) than the control condition (FI = 2, on both passages), which was expected since upon passaging, MSC expanded without coating were not confluent.



(a) BL-MSC(M) (b) MSC(M)

Figure 3.4: Bright field microscopic images (with 100x ampliation) of (a) BL-MSC and (b) MSC at day 7 of culture. Scale bar represents 100μm.

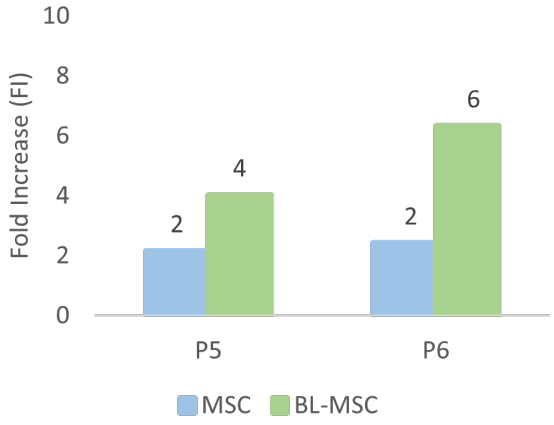


Figure 3.5: Fold increase of MSC (blue) and BL-MSC (green) on the first and second passage after coating (N=1).

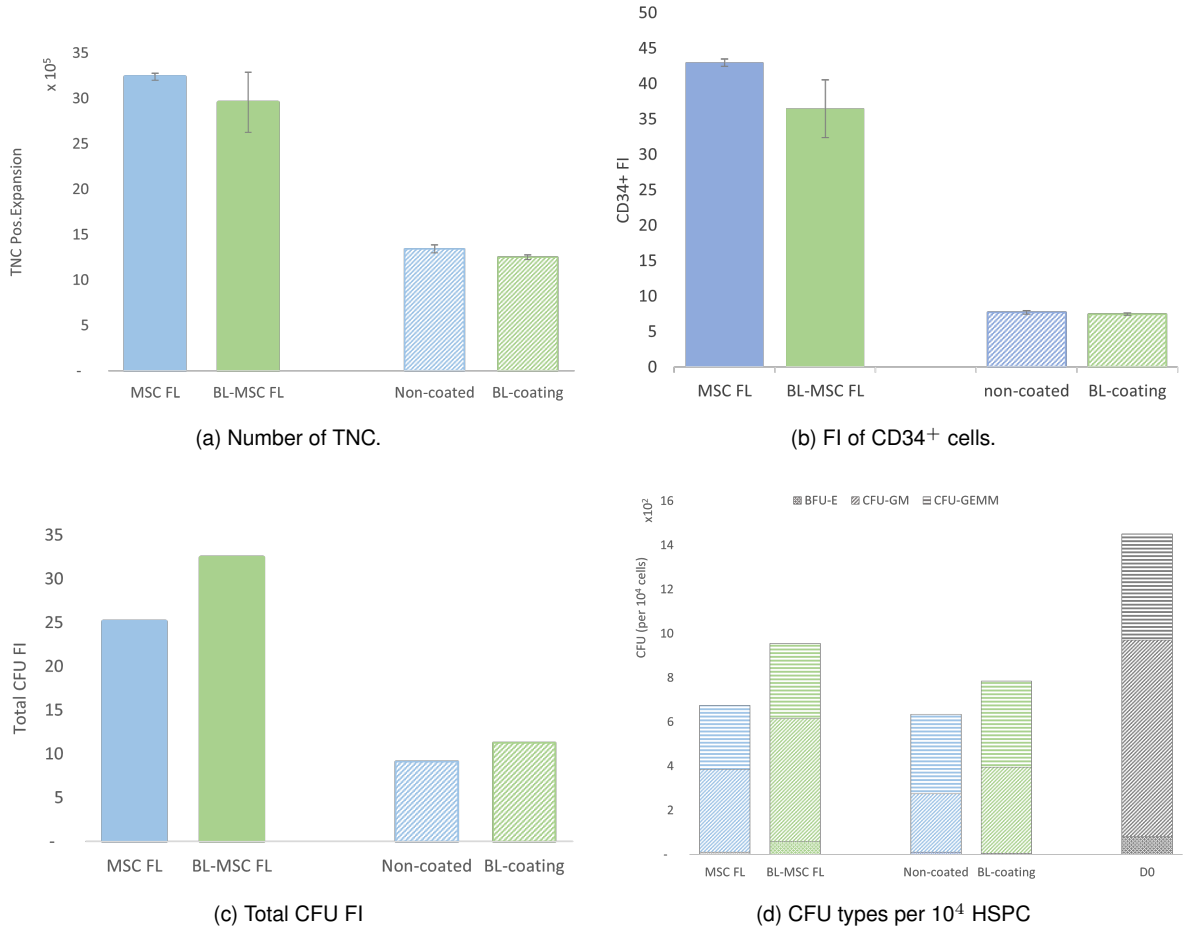
Table 3.1: Number of days in culture on the first and second passage with and without coating. Cells were passaged once BL-MSC reach confluence (N=1)

Passage	Days of culture
P5	7
P6	5

The higher proliferation capacity of BL-MSC was expected, since laminins are a major component of the ECM and other studies also reported a higher proliferation rate of MSC in the presence of different laminin isomers. While studying the effect of laminin-332 on MSC proliferation and osteogenic differentiation, Hashimoto *et. al* also studied the influence of other laminins present in the bone marrow. Despite the highest capacity to enhance proliferation being observed with laminin-332, the laminin-521 coating also showed an increase in MSC proliferation and attachment [98]. In the same study, it was observed that MSC enhanced proliferation was dose-dependent with the laminin-332 coating concentration. Interestingly, it was observed that the addition of laminin-332 to the culture medium as a soluble factor had the same effect as using it as coating, also enhancing MSC proliferation [98]. Lindner *et. al* also found out that the presence of ECM proteins improve MSC(M) proliferation, from the tested proteins (fibronectin, collagen IV, laminin-111 and 332) laminins were the ones who better promoted MSC adhesion and proliferation [97], in another study Mittag *et. al* reached the same conclusion about laminin-111 and 332 [92]. Overall, the effect of other laminin isomers on MSC adhesion and proliferation is well reported, and specifically for laminin-521 it also was observed before.

3.2.2 Hematopoietic Support Assay

Despite the effect on MSC adhesion and proliferation, it was necessary to understand if MSC's function was maintained. Hence, a hematopoietic support assay to confirm if there was any alteration on the hematopoietic cell supportive function of MSC was performed with BL-MSC feeder layers (FL) and MSC FL. Since laminins exist in the hematopoietic niche and are known to interact with HSPC [153–155], a condition only with laminin-521 coating but without FL (BL-coating) was also tested as well as a condition without any feeder layer nor coating (non-coated).



(a) Number of TNC.

(b) FI of CD34$^+$ cells.

(c) Total CFU FI

(d) CFU types per 10^4 HSPC

Figure 3.6: Characterization of HSPC after expansion with MSC FL (solid blue), BL-MSC FL (solid green), without FL nor coating (stripped blue), without FL but with laminin-521 coating (stripped green). (a) total nucleated cells (TNC), (b) CD34$^+$-cells FI , (c) Total colony forming unit (CFU) FI, and (d) CFU types per 10^4 HSPC. For (A) and (B) bars represent the average result of two technical replicates (n=2) of one MSC donor (N=1). Error bars display the SEM between replicates.

Nevertheless, the presence of laminin-521 coating during MSC expansion seemed to have a minimal impact on MSC's capacity to support the expansion of HSPC (figure 3.6). As can be seen in figure 3.6(a), proliferation capacity of HSPC was not affected whether cells were co-cultured with BL-MSC or MSC FL (TNC at D7 = 3.2x10^6±3.9x10^4 vs 3.0x10^6±3.3x10^5, respectively), the coefficient of variation (CV) between both conditions was 5.1%. Also in the conditions where the hematopoietic cell expansion

Table 3.2: Percentage of $CD34^+$, $CD34^+CD45RA^-$, and $CD34^+CD45RA^-CD90^+$ cells before hematopoietic cell expansion (D0) and after 7-day expansion in the previously mentioned conditions: with MSC FL (MSC FL), with BL-MSC FL (BL-MSC FL), wihout coating nor FL (non-coated), without FL but with laminin-521 coating (BL-coating). Percentages were assessed resorting to FlowJo V.10 software.

	$CD34^+$	$CD34^+CD45RA^-$	$CD34^+CD45RA^-CD90^+$
D0	77.10%	51.33%	5.00%
MSC FL	61.40%	17.27%	2.54%
BL-MSC FL	57.10%	16.91%	2.46%
non-coated	26.50%	8.03%	1.54%
BL-coating	27.50%	9.29%	1.78%

occurred without FL (non-coated and BL-coated), HSPC proliferation capacity was not affected by the coating (TNC at D7 = $1.3x10^6 \pm 4.4x10^4$ vs $1.3x10^6 \pm 2.5x10^4$, respectively. CV=2.6%).

Results of immunophenotypic characterization were also very similar between conditions with and without laminin-521 coating, as can be seen in table 3.2. The percentage of $CD34^+$ cells had a CV of 3.6% between the both conditions of HSPC expanded with FL, and of 1.9% between both conditions without FL (non-coated and BL-coated). Overall, FI of $CD34^+$ (figure 3.6(b)), was slightly decreased after expansion with BL-MSC FL (FI= 36.5 ± 4.1) in comparison with MSC FL (FI=43.0 ± 0.5). The percentage of $CD34^+CD45RA^-$ and $CD34^+CD45RA^-CD90^+$ were also assessed to investigate the influence of the coating on more primitive HSPC populations, and, for both HSPC expanded with and without FL, no difference could be found when laminin-521 coating was used.

Lastly, clonogenic potential of HSPC after expansion was also studied by colony-forming unit assay (CFU) (figure 3.6(c) and 3.6(d)). HSPC clonogenic potential seemed to be slightly enhanced after hematopoietic cell expansion with BL-MSC FL and BL-coating (in comparison to expansion with MSC FL and non-coated wells). During the hematopoietic cell expansion, HSPC's total capacity to create colonies had a 25-fold increase when co-cultured with MSC FL and a 32-fold increase when with BL-MSC FL. Also for conditions without FL, the clonogenic capacity was enhanced with the laminin-521 coating (FI=11 and 9 for BL-coated and non-coated, respectively). Focusing on the CFU types, CFU-GM were the ones whose production was more enhanced by the presence of laminin-521. HSPC expanded with BL-MSC FL originated 555 CFU-GM colonies per 10^4 cells (in comparison with 375 with MSC FL), and the ones expanded in BL-coated condition created 390 CFU-GM colonies per 10^4 cells (in comparison to 265 for non-coated). This indicates that not only does laminin-521 coating enhance HSPC capacity to differentiate into granulocyte-myeloid progenitors, but also that MSC expanded in the presence of this coating (BL-MSC) also enhanced it.

Despite the relevance of laminin-521 on the bone marrow extracellular matrix and its strong interaction with HSPC that have been reported previously [153, 154], these results showed that laminin-521 influence on hematopoietic cell expansion and on MSC ability to support it was minimal.In a recent study, Godvarthy *et. al*, observed that not only laminin-521 and 511 strongly adhered to receptors on HSPC, but also these substracts induced a decrease of HSPC proliferation in a dose-dependent manner [155]. However, in the study performed by Godvarthy and colleagues, laminin isoforms were given to the culture as growth factors, while in this study they were used for coating, which can greatly reduce

the amount of HSPC that can interact with them.

Regarding the influence of laminin isoforms on MSC function, namely the hematopoietic supportive capacity, no other studies were found. The only characteristic that has been reported repeatedly is the enhanced tendency of MSC cultured with laminins towards osteogenic differentiation [92, 97, 156], which as been suggested by Hashimoto *et. al* to be observed due to a decreased ability to differentiate into chondroblasts [98].

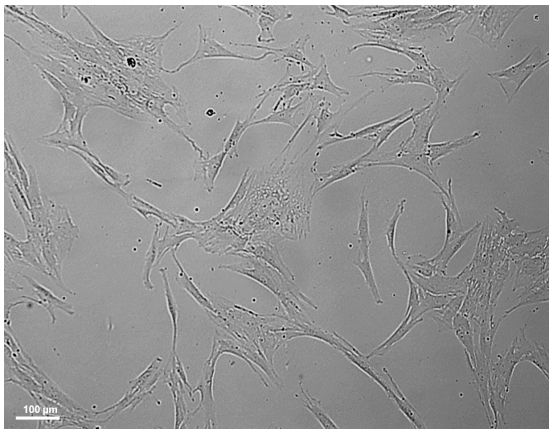
Ultimately, the use of laminin-521 as a coating for MSC culture appears as a candidate to accelerate their expansion, without the concern of affecting MSC's hematopoietic supportive function. However, an economic analysis of the impact of reducing the culture time would need to be conducted. If the use of laminin coating proves to be advantageous, further tests to characterize BL-MSC would need to be performed to confirm the obtained results for more MSC donors and to further evaluate its impact on other MSC functions.

3.3 Effect of Culture Media on MSC Properties

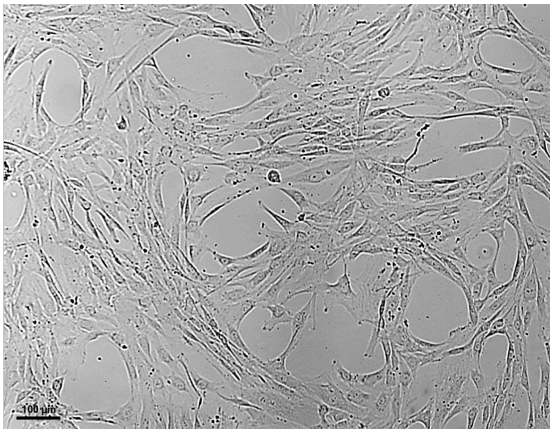
3.3.1 MSC Expansion

Proliferation

MSC(M) were expanded in DMEM/FBS and DMEM/hPL as explained in figure 2.1 for 3 different MSC donors, with ages between 19 and 57 years old (table 2.1). For the different donors and conditions, it was observed that MSC proliferated faster in the presence of DMEM/hPL (table 3.3), in figure 3.7, it is possible to observe that, at the same day, MSC cultured with DMEM/FBS (figure 3.7(a)) are significantly less confluent than MSC cultured with DMEM/hPL (figure 3.7(b)).



(a) DMEM/FBS (FF) - Day 4



(b) DMEM/hPL (FH) - Day 4

Figure 3.7: Representative bright field microscopic images (with 100x ampliation) of MSC proliferation when cultured with (a) DMEM/FBS (FF) and (b) DMEM/hPL (FH), after 4 days in culture. Scale bars represent 100μm.

MSC cultured with DMEM/hPL not only took fewer days to become confluent, but upon passage, the number of MSC retrieved was also higher. In figure 3.8 it is possible to observe that the fold increase

Table 3.3: Number of days in culture until MSC become confluent. Note that a maximum of 10 days in culture was previously decided, even if MSC did not become confluent. Results represented as the interval of days of culture between the 3 donors.

F	FF	FFF 7 to 10	FFFF 7 to 9
	6 to 10	FFH 5 to 7	FFHH 5 to 7
7 to 9	FH 4 to 5	FHH 4 to 6	FHHF 9 to 10

H	HH	HHH 5 to 8	HHHH 7 to 8
	4 to 6	HHF 10	HHFF 7 to 9
6 to 9	HF 6 to 10	HFF 7 to 9	HFFH 6 to 7

per cell passage was always higher in conditions with DMEM/hPL (purple bars) reaching its maximum when cells were adapted from DMEM/FBS to DMEM/hPL (FFH, FI=16.1$\pm$4.4) and minimum when cells were re-adapted from DMEM/hPL to DMEM/FBS (FHHF, FI=1.8$\pm$0.6).

It is important to note that proliferation capacity was different from donor to donor (table 3.4). Nevertheless, it was always higher for MSC cultured with DMEM/hPL. Variability of proliferation capacity between donors from different ages and genders had already been reported [157, 158].

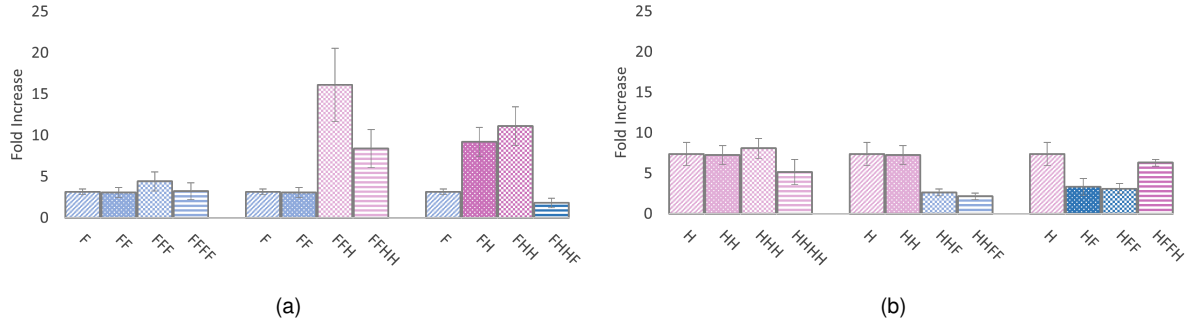


Figure 3.8: Fold increase of MSC on the 3 branchs of conditions previously described for cells thawed in DMEM/FBS (a) and in DMEM/hPL (b), from right to left, (1) continuously in the same culture medium, (2) adapted to a different culture medium halfway, (3) adapted to a different culture medium and then re-adapted to the original one. Note that there are data points that are repeated, using figure (a) as an example, the condition 'F' is repeated in the 3 branches, and 'FF' repeated in the 2 first ones. Blue bars represent timepoints were MSC were cultured with DMEM/FBS and purple bars were they were cultured with DMEM/hPL. Each bar represents the average fold increase of 3 MSC donors (N=3), and error bars depict the SEM.

Table 3.4: Average MSC's velocity of growth (#cells/day) per donor. Values depict the average of the average velocity of growth on the 4 passages of the branch without any change of culture medium (av(F,FF,FFF,FFFF) and av(H,HH,HHH,HHHH) $\pm$ SEM).

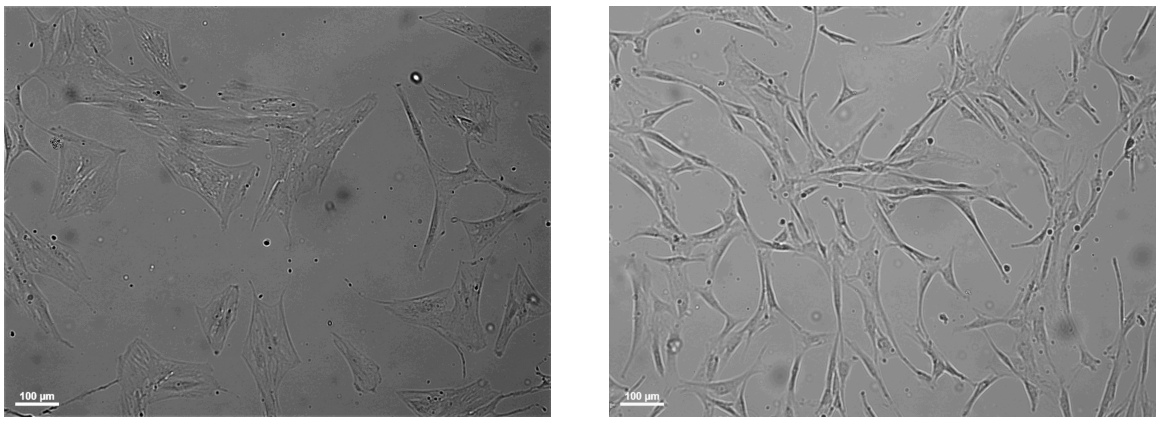
	Donor 1 (M, 29)	Donor 2 (F, 57)	Donor 3 (F, 19)
FBS	(1.7±0.2)x10^5	(8.6±3.8)x10^4	(3.7±0.9)x10^5
hPL	(4.2±0.6)x10^5	(4.3±1.0)x10^5	(2.5±0.7)x10^5

Overall, the higher proliferation capacity of MSC when cultured with DMEM/hPL was expected since these results are in line with which have been reported until now [108, 115, 116]. Different authors, using different techniques, also concluded that hPL-supplemented media promoted MSC growth in comparison with FBS-supplemented media. By instance, Palombella *et. al*, observed increased proliferation of MSC(AT) by measuring their metabolic activity when cultured with DMEM/hPL instead of DMEM/FBS [115]. In a different study, Gupta *et. al* also observed higher numbers of MSC(M) cultured in spinner

cultures with DMEM supplemented with 10% hPL by both cell count and DNA quantification [116], in comparison to FBS-supplemented medium.

Morphology

In figure 3.9 it is possible to see that MSC's morphology is different when cells are cultured with DMEM/hPL and DMEM/FBS. Visually, MSC cultured with DMEM/hPL (figure 3.9(a)) seem to have a more spindle shape and also seem to be smaller whereas the ones under influence of DMEM/FBS (figure 3.9(b)) seem to be bigger and with a more flattened shape.



(a) DMEM/FBS (HHF) - Day 3

(b) DMEM/hPL (HHH) - Day 3

Figure 3.9: Representative bright field microscopic images (with 100x ampliation) of MSC's morphology when cultured with (a) DMEM/FBS and (b) DMEM/hPL after 3 days in culture. Scale bar represents 100μm.

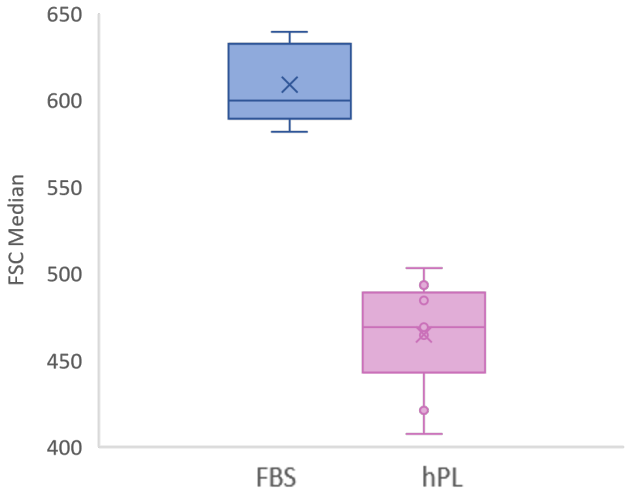


Figure 3.10: Boxplot of the forward scattering (FSC) median of every conditions where MSC were expanded with DMEM/FBS (blue) and DMEM/hPL (purple). Results for 3 MSC donors (N=3).

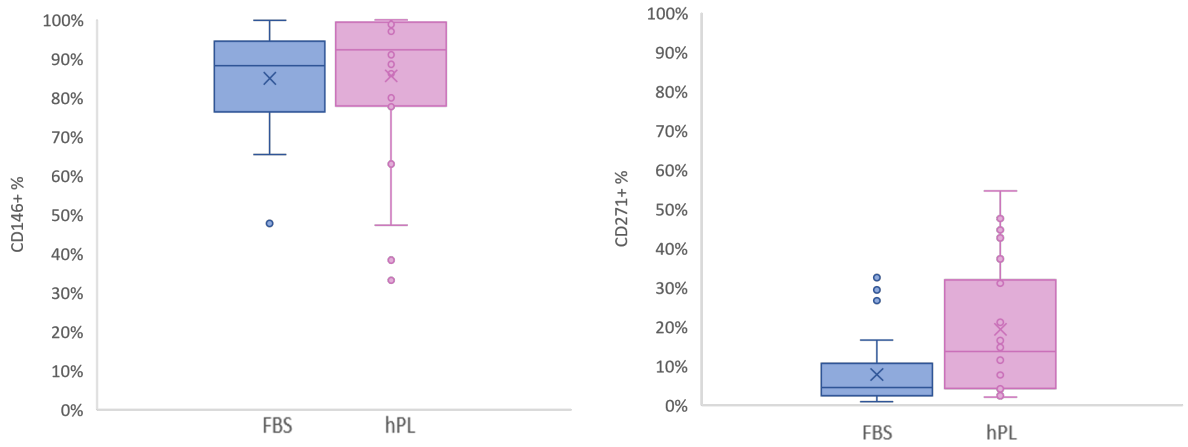
To confirm the relationship between the size of the cells and the culture medium, the forward scattering (FSC) obtained through flow cytometry was analyzed as can be seen in 3.10. This parameter is known to be proportional to cell size and it is used to select given populations [159]. The average FSC

median for conditions in DMEM/hPL was 465 $\pm$ 10, while for conditions in DMEM/FBS was 609 $\pm$ 7, which is significantly higher. Hence, in this study MSC size proved to be smaller, in a quantitative way, when DMEM/hPL was used in oposition to DMEM/FBS. Accordingly, a recent study by Du *et. al*, also demonstrated the reduced size of MSC(M) cultured with DMEM/hPL in comparison with DMEM/FBS but by using an automated cell counter [108].

CD146 and CD271 surface markers

Finally, expression of CD146 (figure 3.13) and CD271 (figure 3.14) surface markers were analyzed at each passage for the 3 MSC donors, since CD146$^+$ and CD271$^+$ MSC were described as having an important role in the hematopoietic niche and regulation of hematopoiesis [57, 70, 160].

While the expression of CD146 did not seem to be influenced by the culture medium used to expand MSC, nor present a lot of donor variability, its expression was higher than 70% in most conditions (figure 3.11(a), table 3.5). On the other hand, CD271 expression was almost always lower than 50% and had great variability from donor to donor, but it seemed to be over-expressed when MSC were cultured with DMEM/hPL (figure 3.11(b), table 3.5).



(a) CD146 expression

(b) CD271 expression

Figure 3.11: Boxplot of (a) CD146 and (b) CD271 expression of every conditions where MSC were expanded with DMEM/FBS (blue) and DMEM/hPL (purple).

Riis *et. al*, verified that the expression of CD146 on MSC(AT) when expanded with hPL-supplemented media was higher than when cultured with fetal calf serum [161]. However, Ma *et. al* compared, between others, cell morphology, proliferation capacity, and surface phenotypic profiles of MSC from dental pulp (MSC(DP)) cultured with culture medium supplemented with serum (SE) and with serum-free supplement (SF). In agreement with the current study, they concluded that the use of SF culture conditions did not affect the expression of CD146 surface marker [48].

In the same study, the authors concluded that CD146$^+$ populations tend to have an enhanced proliferation capacity [48], which was also concluded by Siegel *et. al* with MSC(M) cultured with α-MEM supplemented with pooled human blood group AB serum [125]. Nevertheless, there are also works where CD146$^{-/low}$ populations proliferated faster than CD146$^+$ populations. For example, Paduano *et.*

al verified it for human periapical cyst (dental tissue) MSC cultured with α-MEM supplemented with FBS [162], and Scioli *et. al* observed a decreased proliferation of CD146$^+$ MSC(AT) [163]. With the current work, no relationship was found between proliferation capacity and expression of CD146.

Table 3.5: Average expression of CD146 and CD271 when MSC are cultured with DMEM/FBS (FBS) and DMEM/hPL (hPL) by donor. Results presented as average $\pm$ SEM.

		Donor 1 (M, 29)	Donor 2 (F, 57)	Donor 3 (F, 19)
CD146 (%)	FBS	90.9±3.5%	81.9±4.8%	82.1±3.9%
	hPL	73.3±9.0%	88.7±4.4%	95.2±2.2%
CD271 (%)	FBS	3.1±0.4%	16.6±3.4%	4.0±1.1%
	hPL	6.2±1.6%	38.2±3.9%	15.7±3.9%

Despite the tendency to have higher percentages of CD271$^+$ cells when MSC were cultured with DMEM/hPL being consistent between the 3 donors, relative expression of the surface marker was highly variable (table 3.5). Barilani *et. al* also identified a high variability of CD271 expression between MSC donors for both MSC(M) and MSC(AT), in line with the results obtained in the current work, percentage of CD271$^+$ was always lower than 40%. Nevertheless, contrarily to the obtained results, they visualized a higher CD271 expression when MSC were cultured with FBS-supplemented medium (in comparison with hPL-supplemented and chemically defined media) [164].

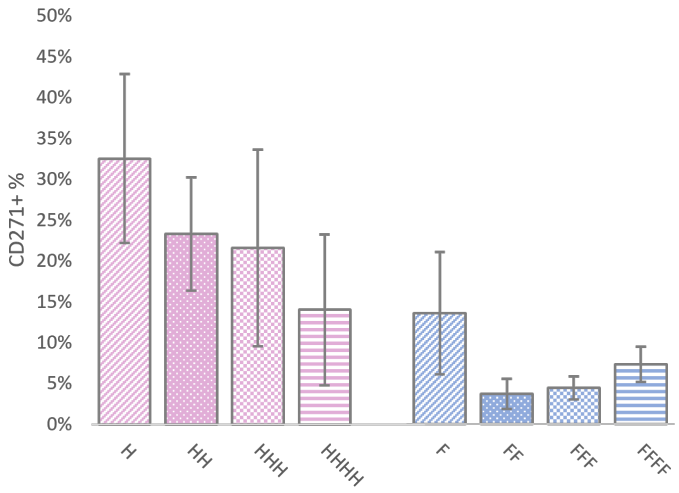


Figure 3.12: CD271 expression by passage for the branches that did not suffer alterations on the culture media (H/F-P1; HH/FF-P2; HHH/FFF-P3; HHHH/FFFF-P4). Purple bars represent MSC expanded with DMEM/hPL and blue bars expanded with DMEM/FBS. Bars represent the average results for 3 MSC donors (N=3), and the error bars display the SEM.

When no change of culture medium was performed expression of CD271 decreased over passages (figure 3.12). When cells were cultured with DMEM/hPL from the first (H) to the last (HHHH) passage there was a decrease of almost 19% in the expression of the surface marker. For DMEM/FBS this result was not so clear, since the decrease was only 6% and there was a slight increase in the intermediate passages. Note that a decrease in the expression of CD271 over passages when MSC are cultured *in vitro* has been described before [164, 165].

All in all, different results regarding the relationship between surface markers and MSC characteris-

tics may be justified by the use of MSC from different sources and expanded with different conditions, which makes it difficult to compare results between studies.

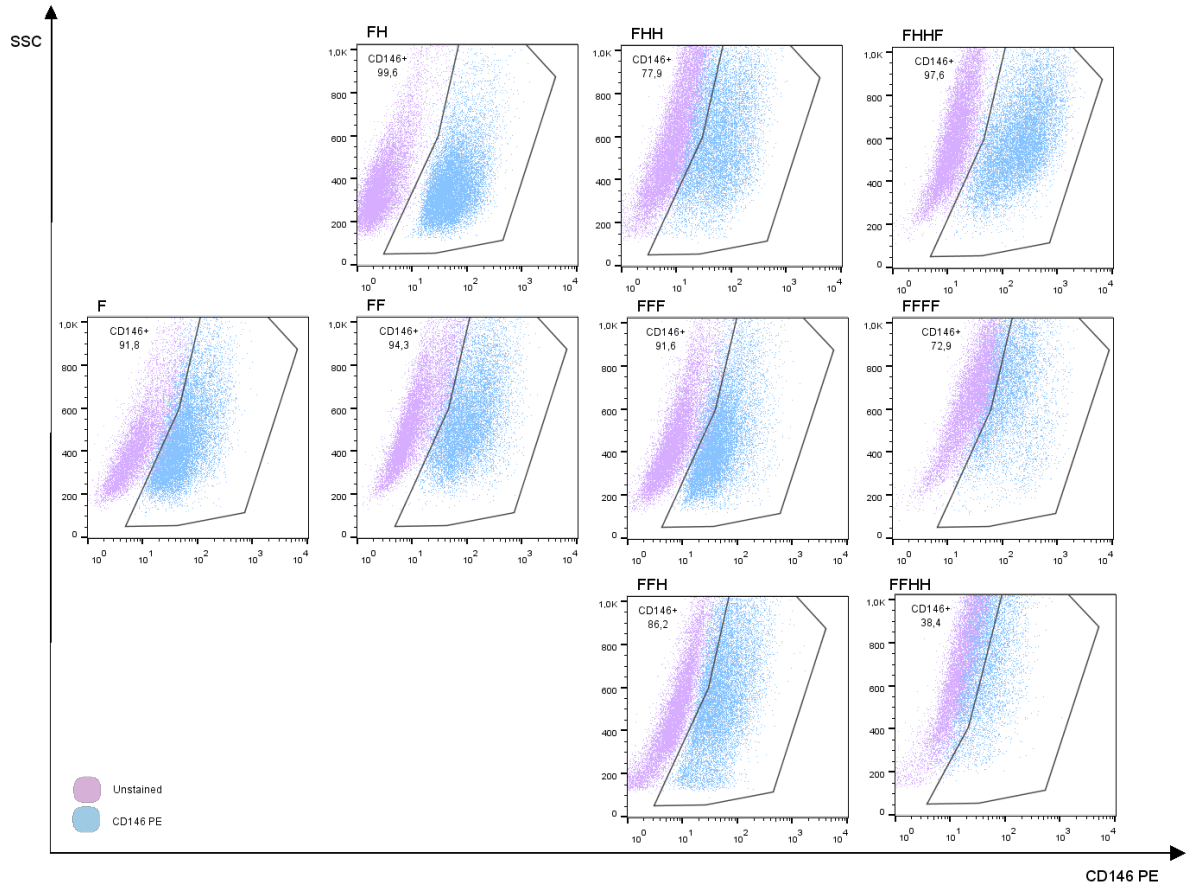


Figure 3.13: Representative image of flow cytometry results, vertical axis represents side scattering (SSC) and horizontal axis CD146 PE expression. Purple population represents the unsatined sample, blue represents the stained one. Images obtained with FlowJo V.10.

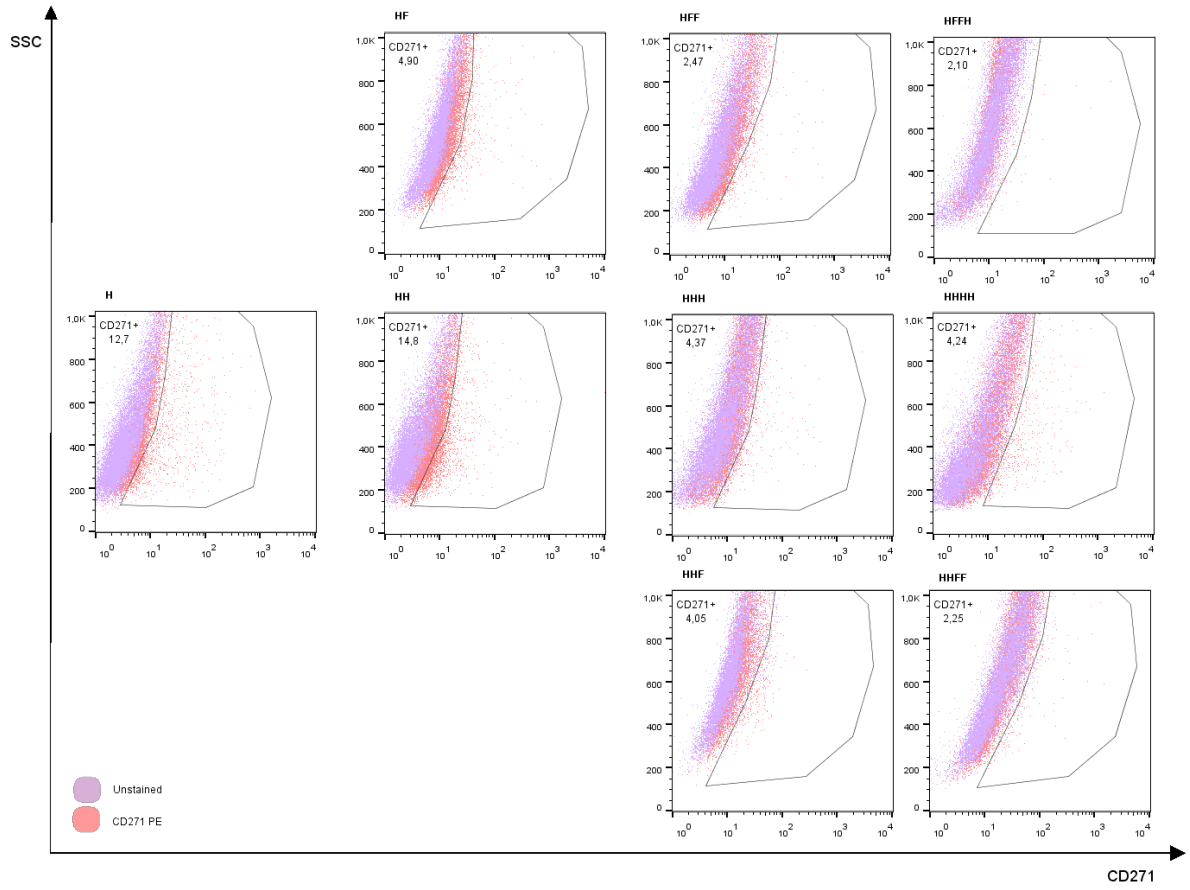


Figure 3.14: Representative image of flow cytometry results, vertical axis represents SSC and horizontal axis CD271 PE expression. Purple population represents the unstained sample, pink represents the stained one. Images obtained with FlowJo V.10.

3.3.2 Hematopoietic Support Assay

To evaluate the influence of culture media on MSC hematopoietic supportive capacity, FL were established for all the final conditions (FFFF, FHHF, HHFF, FFHH, HFFH, and HHHH). At the end of the hematopoietic cell expansion, not only the number of total nucleated cells (TNC) was assessed, but also the percentage of $CD34^{+}$, $CD34^{+}CD45RA^{-}$, and $CD34^{+}CD45RA^{-}CD90^{+}$ cells, and the number of colony-forming units (CFU) formed per 10^{4} HSPC were assessed.

Such assays were performed before the hematopoietic cell expansion (D0) and after the 7-day expansion for the following conditions:

(1) In the absence of FL (No-FL) (n=8)

(2) In the presence of MSC FL inactivated for each one of the conditions previously mentioned (N=3, except for the FFHH FL (N=2))

(3) In the presence of MSC FL non-inactivated for each one of the conditions previously mentioned (N=3, except for the FFHH FL (N=2))

Inactivated vs non-inactivated FL

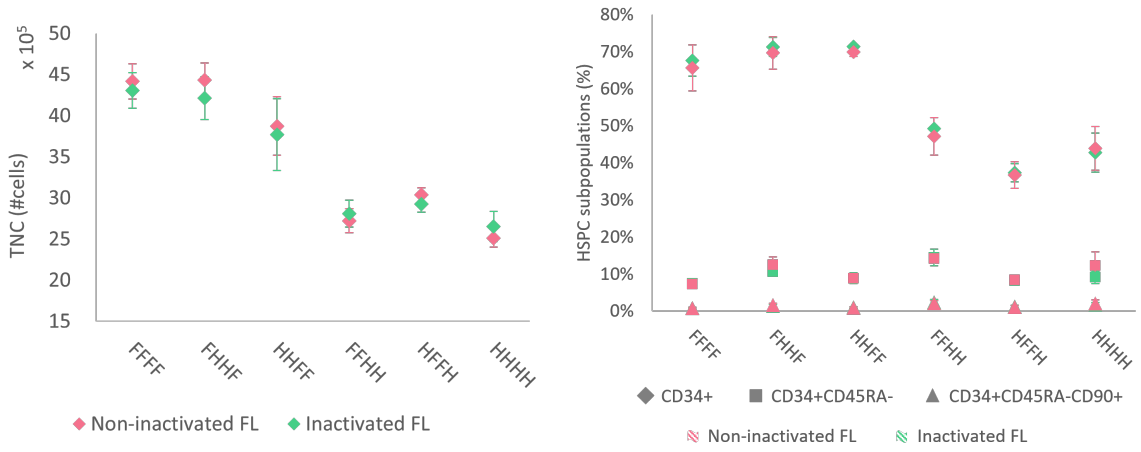
HSPC were co-cultured with both MSC feeder layers that were inactivated with mitomycin C (mmC) and feeder layers that did not go through the inactivation process (non-inactivated). Feeder layers are usually inactivated to arrest MSC proliferation, mmC inhibits DNA synthesis leading to inhibition of cell proliferation, however, this process should not alter cells function [90].

In this study, it was confirmed that the inactivation of MSC feeder layers with mmC did not improve nor prejudice MSC's ability to support the *in vitro* expansion of HSPC (figure 3.15). In fact, the coefficient of variation (CV) between the TNC obtained after hematopoietic cell expansion with inactivated and non-inactivated FL was 1.9$\pm$0.5% (note that the CV between replicates from the same condition was 6.5$\pm$1.8% and 5.2%$\pm$1.4%, respectively). Similarly, CV between both conditions for the percentage of $CD34^+$, $CD34^+CD45RA^-$, and $CD34^+CD45RA^-CD90^+$ subpopulations were lower than 5%, and the average of CV between CFU types of HSPC expanded with inactivated and non-inactivated FL is of 5.3$\pm$3.0% (the lowest CV within the same condition is of 12.2$\pm$5.1%). The CV being for the different parameters being lower between conditions than within the same condition confirms the lack of variation between HSPC co-cultured with inactivated and non-inactivated MSC FL.

Such result was expected since it has been suggested that MSC(M) themselves suffer from contact inhibition. Aziza *et. al* concluded that, for MSC(M), proliferation rates and population doublings were significantly reduced when MSC had a confluence of 100% [166]. Furthermore, their glucose consumption rate was comparable with the one for MSC with a confluence of 20% [166]. Such results are in accordance with the inhibition of MSC(M) due to contact with surrounding cells. A natural inhibition of MSC(M) when they form a FL may be the reason why the inactivation with mmC or not of MSC(M) FL does not seem to affect the hematopoietic cell expansion. No other studies with this comparison were found, so it was not possible to compare results. Note that other authors have established protocols for HSPC expansion where FL inactivation is not performed (namely since the expansion protocol is considered short, only 7 days in culture) [167–169].

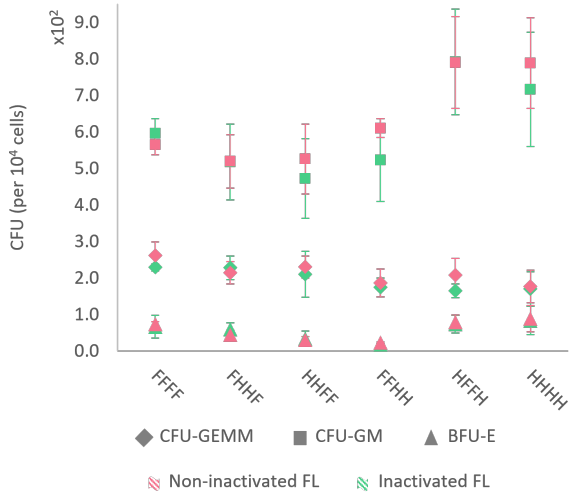
Effect of MSC FL from different conditions

Regarding the total number of nucleated cells, performance of MSC as FL for the expansion of HSPC seem to be enhanced when MSC had DMEM/FBS as their last culture medium before the hematopoietic cell expansion, regardless the adaptations that they have suffered before (TNC ranged from (4.4$\pm$0.2)x10^6 to (3.9$\pm$0.4)x10^6 after co-culture with DMEM/FBS FL, and from (3.0$\pm$0.1)x10^6 to (2.5$\pm$0.1)x10^6 after co-culture with DMEM/hPL FL) (figure 3.16(a)). However, when the control condition for each expansion (No FL) was taken into consideration, normalized FI of HSPC cultured with FL that were always in DMEM/FBS (FFFF) was the highest (2.4$\pm$0.3), followed by the other two conditions with DMEM/FBS as the last culture medium (FHHF: 1.8$\pm$0.3; HHFF: 1.7$\pm$0.0) (figure 3.16(b)). In a previous work, Bucar *et. al* reported that HSPC proliferation was not affected by the culture media where MSC were expanded, since the FI TNC was similar for both DMEM/FBS and DMEM/hPL expanded FL, for MSC(M), MSC(AT), and MSC(WJ) [10]. However, the results obtained in the current study were very



(a) TNC after expansion

(b) HSPC Subpopulations



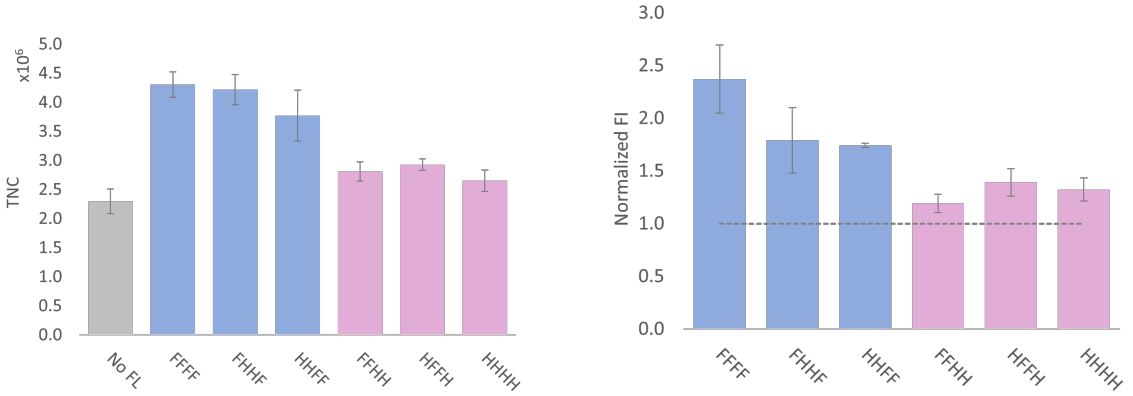
(c) CFU

Figure 3.15: HSPC characterization when expanded with inactivated (pink) and non-inactivated (green) FL. Values for total nucleated cells (a), expression of $CD34^+$, $CD34^+CD45RA^-$, and $CD34^+CD45RA^-CD90^+$ (b), and number of CFU formed per 10^4 HSPC (c) are represented as the average value between 3 MSC donors (N=3) and error bars depict SEM within the same condition.

coherent between the 3 MSC donors.

Immunophenotypic characterization was performed right after the isolation process (pre-cryopreservation) to guarantee that the isolation had been successful, and after thaw immediately before the hematopoietic cell expansion (D0). In table 3.6, it is possible to observe that there were no relevant differences between the percentage of $CD34^+$, $CD34^+CD45RA^-$, and $CD34^+CD45RA^-CD90^+$ populations on both conditions. The maintenance of the immunophenotype of UCB HSPC had already been described [170]. Results for D0 were used to compare with the profile of cells after expansion.

In order to characterize the cells obtained after the hematopoietic cell expansion (at day 7), the expression of surface markers to identify stem and progenitor cells was assessed through flow cytometry analysis (figure 3.18 and 3.19). For HSPC expanded with DMEM/FBS FL, between 60 to 76% of TNC were $CD34^+$ cells (FFFF: 67.6$\pm$4.2%; FHHF: 71.3$\pm$2.6%; HHFF: 71.4$\pm$0.6%), while when DMEM/hPL FL were used, only from 34 to 53% of cells were $CD34^+$ (HHHH: 49.3$\pm$0.3%; HFFH: 37.4$\pm$2.4%; FFHH:



(a) TNC after expansion

(b) Normalized FI

Figure 3.16: Number of total nucleated cells (a) and normalized FI (b) of expanded umbilical cord HSPC after 7 days of co-culture with mesenchymal stromal cells previously expanded in different culture media. Blue bars represent conditions where the last culture medium before the hematopoietic cell expansion was DMEM/FBS and purple bars where it was DMEM/hPL. Normalized FI represents the ratio between TNC of conditions with FL and TNC of the condition without FL on the same expansion. Values are presented as mean ± SEM. n=8 for 'No FL' and N=3 for all the other conditions with the exception of FFHH: N=2.

Table 3.6: Immunophenotypical expression of CD34, CD45RA, and CD90 surface markers prior hematopoietic cell expansion. Comparison between cells after isolation (pre-thawing) (n=1) and post-thawing (D0) (n=2). Results for D0 presented as mean±SEM.

	$CD34^+$ %	$CD34^+CD45RA^-$ %	$CD34^+CD45RA^-CD90^+$ %
Pre-thawing	79%	34%	7%
Post-thawing (D0)	81±1%	33±0%	7±2%

42.8±5.3%). Furthermore, for the control condition (no FL) the $CD34^+$ population comprised 28.7±1.0% of the total amount of cells.

Overall, when the total number of $CD34^+$ cells was calculated for each condition, when HSPC were co-cultured with DMEM/FBS FL it was possible to obtain (2.9±0.1)x10^7 cells (FI of 59±2). However, when DMEM/hPL FL were used only (1.2±0.1)x10^7 $CD34^+$ cells were counted (FI of 22±2). Ultimately, the number of $CD34^+$ cells after 7-day expansion of HSPC co-cultured with DMEM/FBS FL was approximately 2.4 times higher than when using DMEM/hPL FL (table 3.7).

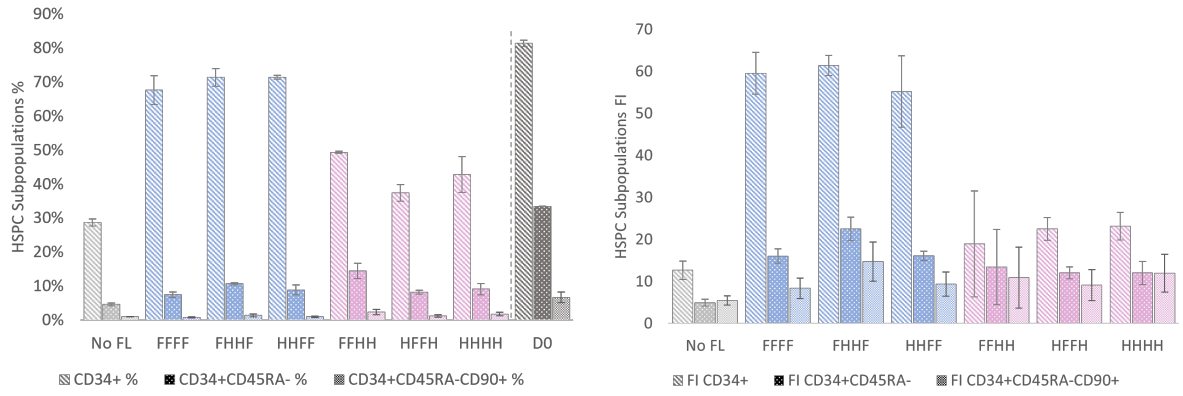
Table 3.7: Total number of $CD34^+$ cells after 7-day expansion of HSPC. Calculated by multiplying TNC with the percentage of $CD34^+$ cells. Results in the table are depicted as mean±SEM.

	Condition	#$CD34^+$ cells (x10^5)	Average (x10^5)
	No FL	6.2±0.8	
DMEM/FBS	FFFF	29.1±1.9	28.7±1.1
	FHHF	30.0±1.0	
	HHFF	26.9±3.1	
DMEM/hPL	FFHH	13.9±0.9	12.0±1.2
	HFFH	11.0±1.0	
	HHHH	11.3±1.2	

To analyze the presence of more primitive HSPC, the expression of CD45RA and CD90 surface markers was also assessed, since $CD34^+CD45RA^-$ and $CD34^+CD45RA^-CD90^+$ are known to be more primitive cells (being the $CD34^+CD45RA^-CD90^+$ the most primitive population in analysis [171])

(figure 3.19).

For HSPC expanded in the control condition (no FL), after the expansion 4.7±0.4% of the cells were $CD34^+CD45RA^-$, and only 1.0±0.1% were $CD34^+CD45RA^-CD90^+$. When the expansion was done in the presence of MSC FL percentages of $CD34^+CD45RA^-$ cells were comprised between 7.5±0.8% (FFFF) and 14.5±2.3% (FFHH), in average conditions with DMEM/FBS FL preserved 9.0±1.1% of $CD34^+CD45RA^-$ cells and DMEM/hPL FL 10.6±2.6%. Despite the percentage of $CD34^+CD45RA^-$ seeming to be slightly increased after the expansion with DMEM/hPL FL, CV between expansion DMEM/FBS and DMEM/hPL is 8.1%, while CV within DMEM/FBS conditions is of 12.2% and within DMEM/hPL of 24.4%, so no significant difference could be found between both conditions. Percentage of $CD34^+CD45RA^-CD90^+$ was also very similar between HSPC co-cultured with DMEM/FBS and DMEM/hPL FL, it ranged from 0.8±0.2% (FFFF) to 2.4±0.8% (FFHH). In average 1.1±0.2% and 1.8±0.4% of the TNC was $CD34^+CD45RA^-CD90^+$ after expansion of HSPC with DMEM/FBS FL and DMEM/hPL FL, respectively (figure 3.17(a)).



(a) Percentage of HSPC subpopulations

(b) FI of HSPC subpopulations

Figure 3.17: Immunophenotypic characterization of expanded umbilical cord HSPC after 7 days of co-culture with MSC previously expanded in different culture media. Percentage of $CD34^+$, $CD34^+CD45RA^-$, and $CD34^+CD45RA^-CD90^+$ after expansion and at D0 (a) and FI of the same populations after expansion (b) were represented as mean ± SEM. n=8 for 'No FL' and N=3 for all the other conditions with the exception of FFHH: N=2.

In figure 3.17(b), it is possible to observe the FI of $CD34^+$, $CD34^+CD45RA^-$ and $CD34^+CD45RA^-CD90^+$ populations after the hematopoietic cell expansion. A slight increase in the FI of $CD34^+CD45RA^-$ population could be observed when HSPC were co-cultured with DMEM/FBS FL (FI=18±3) in opposition to DMEM/hPL FL (FI=12±1). FI of $CD34^+CD45RA^-CD90^+$ seemed to not be affected by the culture medium on which MSC FL were established, HSPC expanded with DMEM/hPL FL showed a FI of 11±1 and with DMEM/FBS FL of 11±3.

Despite the different conclusions between the current study and the one mentioned before by Bucar *et. al* about the proliferation potential of HSPC, Bucar *et. al* also concluded that the percentage of $CD34^+$ cell was reduced after co-culture with DMEM/hPL FL (adapted from MSC isolated with DMEM/FBS) in comparison with co-culture with DMEM/FBS FL [10]. From the current analysis, it was concluded that regardless of the culture medium used for isolation, when MSC are cultured with DMEM/hPL in their last passage before the hematopoietic cell expansion, the presence of $CD34^+$ populations is greatly affected, overall DMEM/FBS FL have a remarkably better performance. These results

suggest that less primitive progenitors tend to differentiate faster when cultured with DMEM/hPL FL. However, the use of DMEM/FBS or DMEM/hPL FL seems to have minimal or no effect on the maintenance of more primitive populations ($CD34^{+}CD45RA^{-}$, and $CD34^{+}CD45RA^{-}CD90^{+}$).

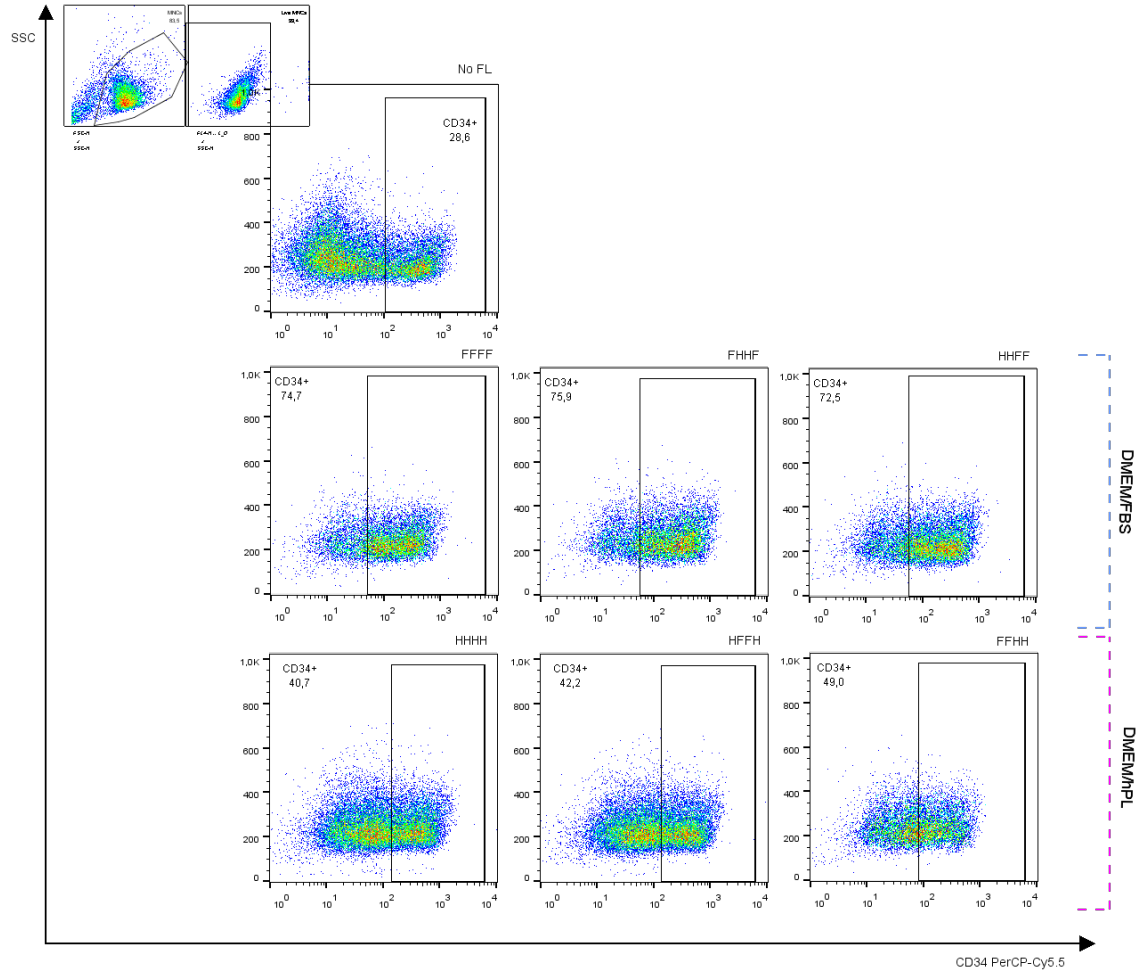


Figure 3.18: Representative image of immunophenotypic analysis by flow cytometry of HSPC after 7-day expansion without FL and with FFFF, FHHF, HHFF, HHHH, HFFH, and FFHH FL. Vertical axis represents SSC and horizontal axis CD34 PerCP-Cy5.5 expression.

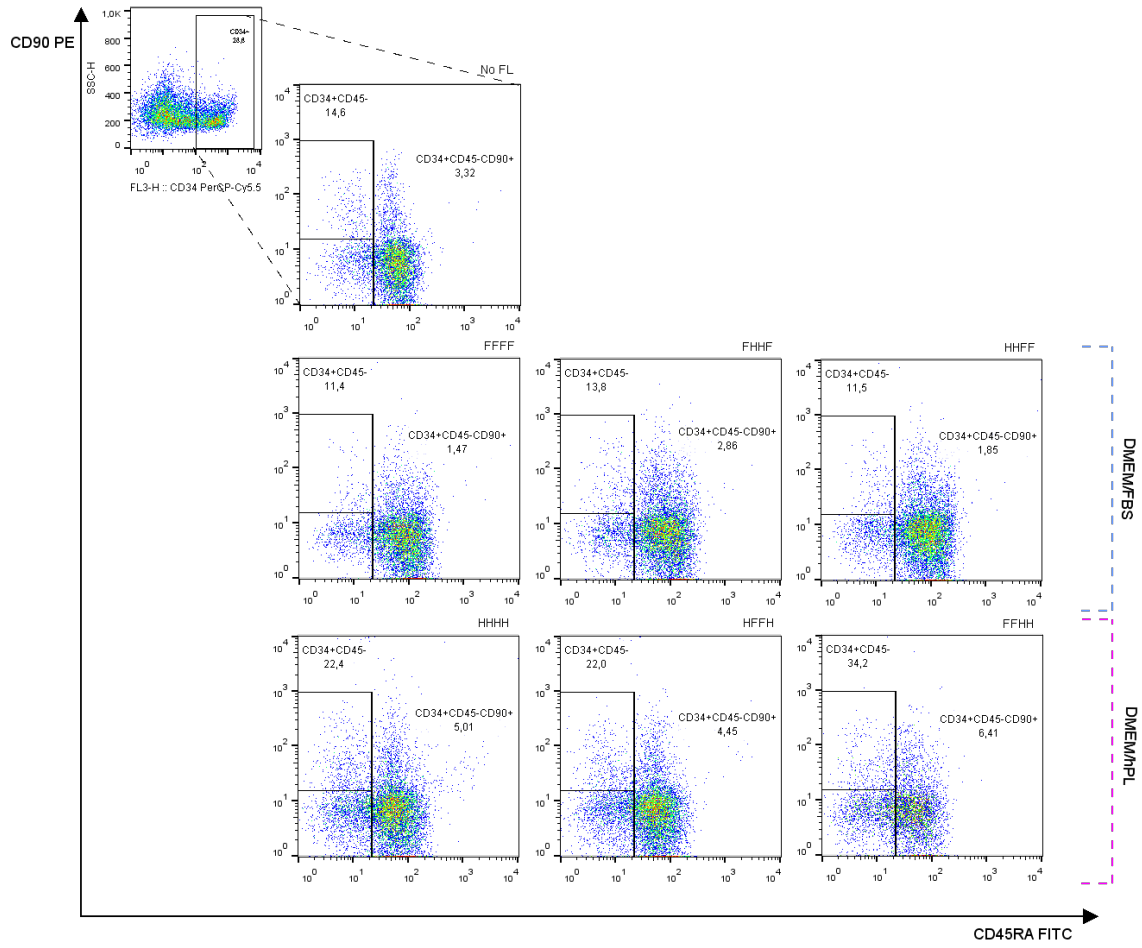
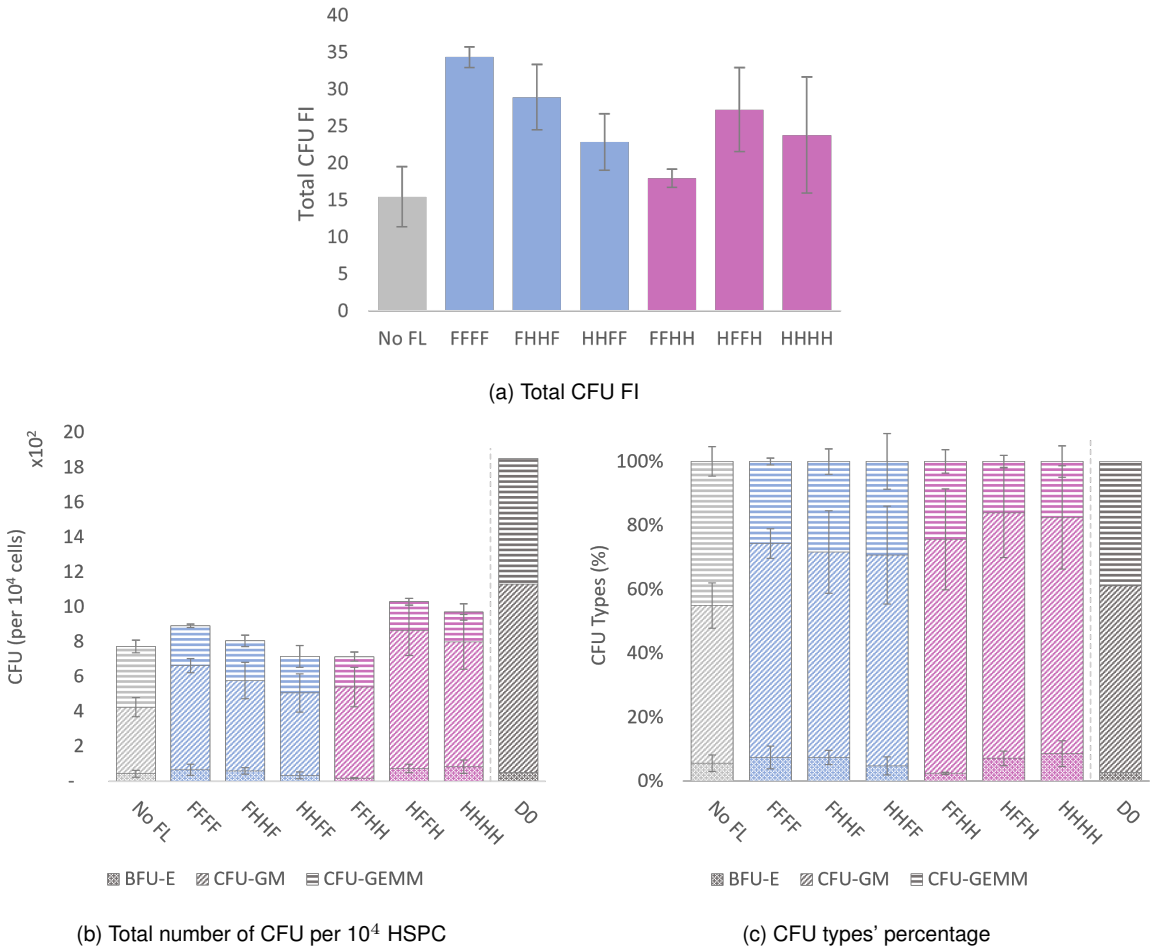


Figure 3.19: Representative image of immunophenotypic analysis by flow cytometry of HSPC after 7-day expansion without FL and with FFFF, FHHF, HHFF, HHHH, HFFH, and FFHH FL. Vertical axis represents CD90 PE and horizontal axis CD45RA FITC expression. Expression of the markers was analysed for previously identified $CD34^+$ cells.

In order to identify the clonogenic potential of the progenitor cells, the colony-forming unit (CFU) assay was performed for every condition. In figure 3.20(a) is possible to observe that considering the TNC obtained after expansion, HSPC expanded with FFFF FL would be the ones that would be able to create a higher number of colonies (total CFU FI=15.4$\pm$4.1), and cells expanded with FFHH FL would generate the lower amount of colonies (total CFU FI=18.0$\pm$1.2). This means, that overall, the condition with a higher capability of repopulating a hematopoietic system would be the expansion with FL of MSC that were always cultured with DMEM/FBS.

Nevertheless, when considering the number of CFU that 10^4 cells could create, for the different conditions, it was concluded that, as it was expected, the total number of CFU obtained per 10^4 cells was considerably higher before the expansion (D0) than for the different conditions after expansion. At D0 a total of 1.9x10^3 CFU were obtained per 10^4 HSPC. After the expansion no major difference was found regarding to the total number of CFU per 10^4 for the different conditions, values were comprised between (7.1$\pm$1.4)x10^2, for HSPC expanded with FFHH FL, and (1.0$\pm$0.2)x10^3, for HSPC expanded with HFFH FL. Note that the total number of CFU that HSPC expanded without the presence of FL

(No FL) could originate after the expansion is included in the previously mentioned range of colonies, (7.7±1.1)x10^2 colonies could be obtained from 10^4 HSPC (figure 3.20(b)).



(a) Total CFU FI

(b) Total number of CFU per 10^4 HSPC

(c) CFU types' percentage

Figure 3.20: CFU produced by HSPC before and after a 7-day expansion in a co-culture system MSC(M) FL previously expanded in different culture media. (a) Total CFU FI considering TNC obtained from the hematopoietic cell expansion for each condition. (b) Total number of CFU (by type) that can be originated per 10^4 HSPC. (c) Percentage of Percentage of each CFU type (BFU-E, CFU-GM, CFU-GEMM. Values are presented as mean ± SEM. n=8 for 'No FL' and N=3 for all the other conditions with the exception of FFHH: N=2.

Despite the similarities between the total number of CFU originated by HSPC after expansion with and without FL, it is possible to observe differences in the distribution of CFU types, erythroid burst-forming unit (BFU-E), colony-forming unit granulocyte-monocyte (CFU-GM) or multilineage colony-forming unit (CFU-GEMM) (figure 3.20(c)). Before the comparison between the different conditions after the expansion, it is important to understand the clonogenic potential of HSPC before being expanded (D0). At D0, 58.4% of the colonies were classified as CFU-GM, which means that they were derived from specific progenitors for granulocyte/monocyte cells. HSPC able to differentiate into granulocyte, monocyte, erythrocyte, and megakaryocyte cells formed 38.9% of the colonies (CFU-GEMM), while erythroid-specific progenitors were only able to generate 2.7% of the colonies (BFU-E). After a 7-day expansion without a FL (No FL), the percentage of CFU-GM was 49.3±7.1%, slightly lower than at D0. However, interestingly, both CFU-GEMM and BFU-E were present in higher percentages (45.1±4.6% and 5.6±2.3%,

respectively). Regarding the clonogenic potential of HSPC after expansion in contact with FL, all conditions had lower percentages of CFU-GEMM (between 16.0±1.8%, HFFH, and 29.3±8.7%, FHHF), and higher percentages of CFU-GM (from 64.3±13.0%, FHHF to 76.9±14.0%, HFFH) than at D0 and after the expansion without FL (between 16.0±1.8% and 29.3±8.7%), percentages of BFU-E were comprised between 2.4±0.4% (FFHH) and 8.6±4.0% (HHHH). Moreover, it seems that HSPC expanded in the presence of DMEM/FBS FL can originate a slightly higher percentage of CFU-GEMM and a slightly lower percentage of CFU-GM, both with differences around 10%.

All in all, the culture medium with which MSC FL were established, did not seem to have a major effect on HSPC clonogenic potential. This conclusion is also in accordance with the results obtained by Bucar *et. al* regarding the clonogenic potential of HSPC [10].

Besides the previous study of Bucar *et. al*, no other studies focusing the influence of MSC culture medium on MSC hematopoietic supportive capacity were found. However, several studies show that the use of hPL-supplemented media, instead of FBS-supplemented ones, affects other MSC's functions. Du *et. al*, demonstrated that MSC cultured with hPL-supplemented medium tend to differentiate towards osteocytes or adipocytes (over chondrocytes) since they have a tendency towards aerobic metabolism. Furthermore, they also verified that hPL-supplemented medium impaired MSC's paracrine function, since the secreted factor could not induce angiogenesis nor stimulate polarization of M2 macrophages [108]. In another work, Oikonomopoulos *et. al* showed that for MSC(M) and MSC(AT) as co-culture systems for the expansion of peripheral blood mononuclear cells (PBMC), MSC expanded with hPL-supplemented medium did not affect the proliferation of PBMC, exhibiting diminished immunosuppressive properties when compared with MSC expanded with FBS-supplemented medium [107]. Overall, there are reports that the use of hPL-supplemented media can affect different MSC functions.

CD146 and CD271 expression on MSC and their ability to support hematopoietic cell expansion

In contrary to what was expected, no major relationship was found between the expression of CD146 and CD271 on MSC and the outcome of the hematopoietic cell expansion with the correspondent FL (figure 3.21). To better understand if there could be any relationship between the expression of CD146 and CD271 surface markers and the tendency of MSC to support hematopoietic cell expansion, the correlation coefficient (r) between the expression of the surface markers on the last passage before the establishment of the FL and the parameters used to evaluate the hematopoietic cell expansion (TNC, $CD34^+$, $CD34^+CD45^-$, and $CD34^+CD45^-CD90^+$ populations) were calculated (table 3.8). r higher than 0.7 (positive or negative) were considered strong correlations.

Tormin *et. al* studied the phenotypical and anatomical distribution of MSC populations in the bone marrow. They concluded that both $CD271^+/CD146^+$ and $CD271^+/CD146^{-/low}$ were able to form hematopoietic stroma *in vivo*. Furthermore, they reported $CD271^+$ and $CD271^+/CD146^{-/low}$ MSC as being bone-lining cells associated with the expansion of long-term (LT)-HSPC in low oxygen areas, on the other hand, $CD146^+$ and $CD271^+/CD146^+$ were reported to be located around BM sinusoids in association with proliferating HSPCs [144].

Nevertheless, regarding the expression of CD271, across all donors and parameters, only the per-

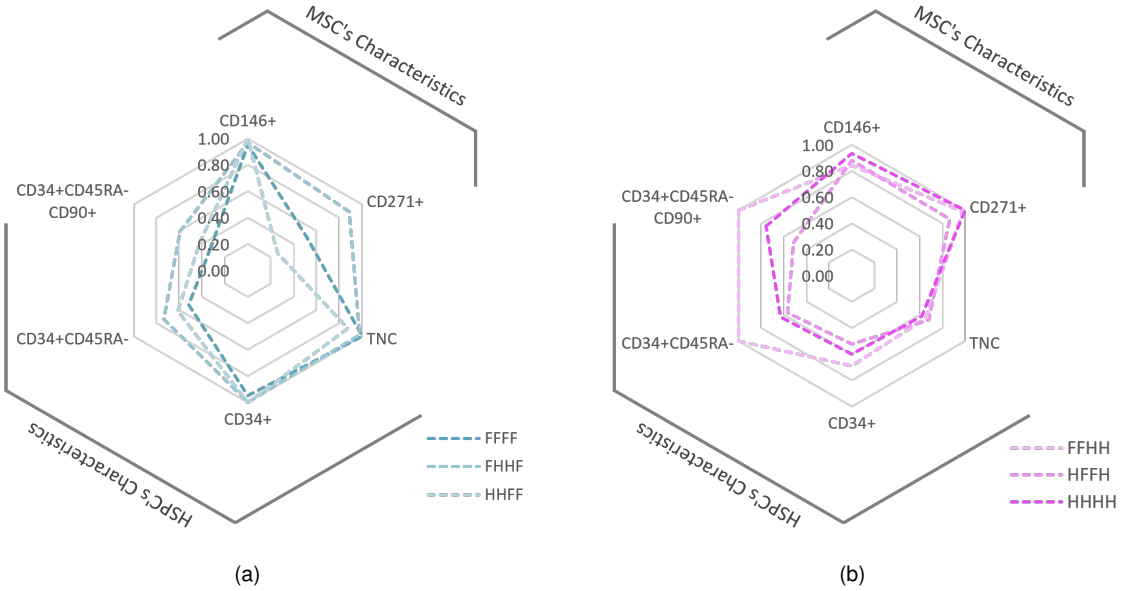


Figure 3.21: Radar chart to depict the relationship between MSC's surface markers (CD146 and CD271) and the main results of the hematopoietic cell expansion (TNC, and expression of $CD34^+$, $CD34^+CD45^-$, and $CD34^+CD45^-CD90^+$ cells). Average values for the 3 MSC donors were normalized by the maximum value for each parameter. (a) feeder-layers with DMEM/FBS as the last culture medium (b) feeder-layers with DMEM/hPL as the last culture medium

centage of $CD34^+CD45^-CD90^+$ HSPC for donor 2 had a strong correlation (r=0.76). Such results were not unexpected since the expression of this surface marker was lower than 10% on all the conditions for Donors 1 and 3, and even for donor 2, it was lower than 30%. Interestingly, previous authors have identified an upregulation of genes related to hematopoiesis in $CD271^+$ MSC. By instance, Kuçi *et. al* identified that in comparison with plastic adherent MSC, isolated $CD271^+$ MSC expressed, between other characteristics, increased levels of extracellular matrix, cell adhesion genes, and hematopoiesis-related genes (CXCL12, FLT3L, IL-3, TPO, KITL) [142]. Furthermore, Ghazanfari *et. al* also identified higher levels of hematopoiesis supporting genes in $CD271^+/CD140a^{-/low}$ MSC in comparison with $CD271^-/CD140a^{-/low}$ MSC [143]. Also, regarding the *in vivo* potential of $CD271^+$ MSC, it has been shown that this population has the ability to promote HSPC engraftment [136]. In order to confirm our results, and better understand if the $CD271^+$ subpopulation could identify a subpopulation of MSC more prone to support the *in vitro* expansion of HSPC, the co-culture system should be done with FL enriched for $CD271^+$ MSC and $CD271^-$ MSC.

On the other hand, the expression of CD146 had some parameters that could be considered to have strong correlations with it, however, they were not coherent between donors. For instance, the number of TNC showed to be strongly related to CD146 expression for Donor 1 and 3 (r=0.82 and 0.76, respectively), contrarily, for Donor 2 the correlation is weak and negative (r=-0.39, which means that higher number of $CD146^+$ MSC could originate a lower amount of TNC). Similarly, a correlation between CD146 expression and the percentage of $CD34^+$, $CD34^+CD45^-$, and $CD34^+CD45^-CD90^+$ was very variable from donor to donor. As an example, the percentage of $CD34^+$ had a strong positive correlation with the percentage of CD146+ MSC for Donor 1 (r=0.91), a weak negative correlation for Donor 2 (r=-

Table 3.8: Correlation coefficient (r) between expression of CD146 on the last passage before establishment of the FL and the parameters to evaluate the hematopoietic cell expansion (TNC, $CD34^+$, $CD34^+CD45^-$, and $CD34^+CD45^-CD90^+$ populations). First three columns depict r per donor and the last one represent mean r ± SEM. The same correlation was calculated between CD271 and the parameters mentioned before.

	Correlation with CD146 expression			
	Donor 1	Donor 2	Donor 3	Average±SEM
TNC	0.82	-0.39	0.76	0.40±0.53
$CD34^+$%	0.91	-0.40	0.64	0.39±0.52
$CD34^+CD45^-$%	-0.35	-0.48	0.80	-0.01±0.54
$CD34^+CD45^-CD90^+$%	-0.40	0.11	0.86	0.19±0.45

	Correlation with CD271 expression			
	Donor 1	Donor 2	Donor 3	Average SEM
TNC	-0.03	-0.58	0.67	0.02±0.43
$CD34^+$%	0.12	-0.56	0.42	-0.01±0.37
$CD34^+CD45^-$%	0.23	0.65	0.27	0.38±0.18
$CD34^+CD45^-CD90^+$%	0.35	0.76	0.42	0.51±0.17

0.40), and a moderate positive correlation for Donor 3 (r=0.64). When analyzing the correlation factors per donor, it is possible to conclude that while Donor 2 did not have any strong correlation between the expression of CD146 on MSC and the parameters of the hematopoietic cell expansion, Donor 1 seem to have a strong correlation between the number of TNC and the expression of $CD34^+$, and Donor 3 appears to have a strong correlation with all the parameters (except for the $CD34^+$ expression, with which it has a moderate correlation). It is important to notice that only Donor 1 had conditions with percentages of $CD146^+$ MSC lower than 70% (HHHH=47.3%, HFFH=33.2%, FFHH=38.4%), being the only one with marked differences on the expression of this surface marker. Overall the differences between donors were considerable, so it was not possible to conclude that expression of CD146 on the MSC surface was correlated with their ability to enhance the *in vitro* expansion of HSPC.

Contrary to what was observed here, Corselli *et. al* verified that $CD146^+$ cells support the long-term expansion of HSPC, while unfractionated MSC and $CD146^-$ cells led to early differentiation of HSPC, compromising their *ex vivo* expansion [70]. In another work, Sacchetti *et. al*, demonstrated that $CD146^+$ MSC not only express regulatory genes related to hematopoiesis (CXCL12 and Angiogenin-1) but also have the ability to generate a niche able to support hematopoiesis *in vivo* when transplanted to immunodeficient mice [141]. Lastly, Sorrentino *et. al* verified that $CD146^+$ MSC had an improved performance on long-term culture (over 8 weeks) of HSPC compared to unfractionated MSC. However, when looking at the results of the hematopoietic cell expansion after the end of the first week (7-days) the results for $CD146^+$ and unfractionated MSC FL are very similar, which may be the reason why it was not possible to find strong correlations in the current study [172].

Chapter 4

Conclusion

MSC are a very complex and heterogeneous population, that has a broad range of functions and multifaceted mechanisms of action. Different donors, sources, isolation methods, or culture conditions can originate MSC populations with different characteristics that can enhance or diminish given functions. Lately, the identification of markers to isolate stricter MSC subpopulations, with tendency for more specific functions has been a growing field of research. Despite the lack of definition that still exists, MSC are one of the most promising cell type for cell-based therapies and the focus of an increasing number of clinical trials. GMP compliant production and transport systems are indispensable, and their impact on MSC's properties needs to be studied.

Short term cell storage for MSC-based therapies is still a major problem. Therefore, the first goal of this work was to understand if Nutristor - a cold storage solution in beta testing - could be a suitable candidate for MSC storage. It was concluded that Nutristor appears to have the advantage to be able to keep MSC viable for up to 5 days (longer than other options), which is important since it gives some leeway during the transportation and administration process. Although MSC adhered to the culture flasks after 5 days of storage with Nutristor, further thorough characterization tests would be required to ensure MSC quality after storage. Moreover, a sizeable portion of the cells were lost during the procedure, which is problematic because therapeutic doses make use of a large number of cells at once. Note that around 40% of the cells were lost in the first 24h of storage, understanding the reason why such loss happened could be useful to develop an optimized version of the storage medium. In the end of the day, Nutristor seem to be a good option to be investigated, but it would be advantageous to test it for other biological replicates.

As stated before, another problem regarding MSC *ex vivo* expansion, is the acquisition of different characteristics upon expansion with different culture conditions. Hence, we evaluated the impact of using a coating substrate (Biolaminina-521 coating) and different culture media supplements (FBS vs hPL) on MSC expansion and ability to support HSPC expansion.

Regarding the influence of laminin-521 coating, it could be observed that while the proliferation capacity of MSC was enhanced by the coating presence, their ability to support the hematopoietic cell expansion was barely affected. Despite the enhanced proliferation of BL-MSC, leading to the reduc-

tion of the culture time, a cost-benefit study would need to be done to understand if this decrease on the culture time would be advantageous. Note that other MSC functions have not been investigated, so more studies should be be conducted to guarantee maintenance of other MSC functions, like their immunomodulatory capacity.

Contrarily, the culture medium with which MSC were expanded affected several MSC properties, between them their capacity to support the expansion of HSPC. MSC cultured with DMEM/hPL had a different phenotype from the ones cultured in DMEM/FBS. When cultured with DMEM/hPL MSC had higher proliferation capacity, presented different morphology (more spindle-shaped and smaller), and it seemed that they had an enhanced expression of the CD271 surface marker. It is interesting to note that, once cells where put in contact with a different culture medium, they readily acquired characteristics of cells expanded with that culture medium. Finally, upon expansion in the presence of FL lastly cultured with DMEM/hPL, HSPC had a diminished proliferation capacity and were more differentiated. It is important to note that these results were very coherent between the three different MSC donors.

It was hypothesized that the expression of CD146 and CD271 could be related with MSC capacity to support the expansion of HSPC. However, the relationships that were found between these surface markers expression on MSC, and their ability to support hematopoiesis, were very variable between the three MSC donors. Hence, no significant relationship was identified.

Overall, the influence of the culture medium that is used to expand MSC on the outcome of hematopoietic cell expansion was very coherent between different donors from different genders and ages, and proves that despite the need of xeno-free protocols for MSC expansion, it is important to be aware of the influence of culture conditions on MSC functions. Nevertheless, the mechanism behind this effect of hPL on MSC functionality is yet to be determined. Samples of RNA were extracted from MSC at all passages, for the three MSC donors. The next step will be to analyse MSC's transcriptomic profiles for the different conditions and try to understand, at a transcriptomic level, why did the culture medium with which MSC were expanded affect so greatly their hematopoietic supportive function, being the final goal to allow the engineering and selection of an MSC population more prone to support the expansion of HSPC.

As a final remark, understanding the mechanisms behind the differences that are induced due to the production process of MSC-based therapies can be very useful for the production of MSC populations with disease-specific strengths, having the potential to enhance therapeutic outcome and to decrease variability between studies.

Bibliography

[1] A. J. Friedenstein, R. K. Chailakhjan, and K. S. Lalykina. The development of fibroblast colonies in marrow and spleen cells monolayer cultures of guinea-pig bone. *Cell Tissue Kinet*, 3:393–403, 1970. doi: 10.1111/J.1365-2184.1970.TB00347.X.

[2] A. J. Friedenstein, R. K. Chailakhyan, N. V. Latsinik, A. F. Panasyuk, and I. V. Keiliss-Borok. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. *Transplantation*, 17:331–340, 4 1974.

[3] A. J. Friedenstein, R. K. Chailakhyan, and U. V. Gerasimov. Bone marrow osteogenic stem cells: in nitro cultivation and transplantation in diffusion chambers. *Cell Tissue K i m*, 20:263–272, 1987.

[4] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak. Multilineage potential of adult human mesenchymal stem cells. *Science*, 284:143–147, 4 1999. ISSN 00368075. doi: 10.1126/science.284.5411.143.

[5] A. I. Caplan. Mesenchymal stem cells. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, 9:641–650, 1991. ISSN 0736-0266. doi: 10.1002/JOR.1100090504. URL `https://pubmed.ncbi.nlm.nih.gov/1870029/`.

[6] S. Viswanathan, Y. Shi, J. Galipeau, M. Krampera, K. Leblanc, I. Martin, J. Nolta, D. G. Phinney, and L. Sensebe. Mesenchymal stem versus stromal cells: International society for cell gene therapy (isct®) mesenchymal stromal cell committee position statement on nomenclature. *Cytotherapy*, 21:1019–1024, 10 2019. ISSN 1465-3249. doi: 10.1016/J.JCYT.2019.08.002.

[7] M. Dominici, K. L. Blanc, I. Mueller, I. Slaper-Cortenbach, F. C. Marini, D. S. Krause, R. J. Deans, A. Keating, D. J. Prockop, and E. M. Horwitz. Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy*, 8:315–317, 8 2006. ISSN 14653249. doi: 10.1080/14653240600855905.

[8] S. Viswanathan, R. Ciccocioppo, J. Galipeau, M. Krampera, K. L. Blanc, I. Martin, K. Moniz, J. Nolta, D. G. Phinney, Y. Shi, Z. M. Szczepiorkowski, K. Tarte, D. J. Weiss, and P. Ashford. Consensus international council for commonality in blood banking automation–international society for cell gene therapy statement on standard nomenclature abbreviations for the tissue of origin of mesenchymal stromal cells. *Cytotherapy*, 23:1060–1063, 12 2021. ISSN 14772566. doi: 10.1016/j.jcyt.2021.04.009.

[9] A. Ribeiro, P. Laranjeira, S. Mendes, I. Velada, C. Leite, P. Andrade, F. Santos, A. Henriques, M. Grãos, C. M. Cardoso, A. Martinho, M. Pais, C. L. D. Silva, J. Cabral, H. Trindade, and A. Paiva. Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood b, natural killer and t cells. *Stem Cell Research and Therapy*, 4:1–16, 10 2013. ISSN 17576512. doi: 10.1186/SCRT336/FIGURES/3. URL https://stemcellres.biomedcentral.com/articles/10.1186/scrt336.

[10] S. Bucar, A. D. de Matos Branco, M. F. Mata, J. C. Milhano, Íris Caramalho, J. M. Cabral, A. Fernandes-Platzgummer, and C. L. da Silva. Influence of the mesenchymal stromal cell source on the hematopoietic supportive capacity of umbilical cord blood-derived cd34+-enriched cells. *Stem Cell Research and Therapy*, 12:1–16, 12 2021. ISSN 17576512. doi: 10.1186/S13287-021-02474-8/FIGURES/6. URL https://link.springer.com/articles/10.1186/s13287-021-02474-8https://link.springer.com/article/10.1186/s13287-021-02474-8.

[11] M. Gnecchi and L. G. Melo. Bone marrow-derived mesenchymal stem cells: isolation, expansion, characterization, viral transduction, and production of conditioned medium. *Methods in molecular biology (Clifton, N.J.)*, 482:281–294, 2009. ISSN 1064-3745. doi: 10.1007/978-1-59745-060-7_18. URL https://pubmed.ncbi.nlm.nih.gov/19089363/.

[12] H. E. Gruber, R. Deepe, G. L. Hoelscher, J. A. Ingram, H. J. Norton, B. Scannell, B. J. Loeffler, N. Zinchenko, E. N. Hanley, and H. Tapp. Human adipose-derived mesenchymal stem cells: direction to a phenotype sharing similarities with the disc, gene expression profiling, and coculture with human annulus cells. *Tissue engineering. Part A*, 16:2843–2860, 9 2010. ISSN 1937-335X. doi: 10.1089/TEN.TEA.2009.0709. URL https://pubmed.ncbi.nlm.nih.gov/20408770/.

[13] D. Ponnaiyan, K. M. Bhat, and G. S. Bhat. Comparison of immuno-phenotypes of stem cells from human dental pulp and periodontal ligament. *International journal of immunopathology and pharmacology*, 25:127–134, 2012. ISSN 0394-6320. doi: 10.1177/039463201202500115. URL https://pubmed.ncbi.nlm.nih.gov/22507325/.

[14] I. Ishige, T. Nagamura-Inoue, M. J. Honda, R. Harnprasopwat, M. Kido, M. Sugimoto, H. Nakauchi, and A. Tojo. Comparison of mesenchymal stem cells derived from arterial, venous, and wharton's jelly explants of human umbilical cord. *International journal of hematology*, 90:261–269, 9 2009. ISSN 1865-3774. doi: 10.1007/S12185-009-0377-3. URL https://pubmed.ncbi.nlm.nih.gov/19657615/.

[15] K. P. ROBB, J. C. FITZGERALD, F. BARRY, and S. VISWANATHAN. Mesenchymal stromal cell therapy: progress in manufacturing and assessments of potency. *Cytotherapy*, 21:289–306, 3 2019. ISSN 14772566. doi: 10.1016/j.jcyt.2018.10.014.

[16] M. Gimona, M. F. Brizzi, A. B. H. Choo, M. Dominici, S. M. Davidson, J. Grillari, D. M. Hermann, A. F. Hill, D. de Kleijn, R. C. Lai, C. P. Lai, R. Lim, M. Monguió-Tortajada, M. Muraca, T. Ochiya, L. A. Ortiz, W. S. Toh, Y. W. Yi, K. W. Witwer, B. Giebel, and S. K. Lim. Critical considerations

for the development of potency tests for therapeutic applications of mesenchymal stromal cell-derived small extracellular vesicles. *Cytotherapy*, 23:373–380, 5 2021. ISSN 14772566. doi: 10.1016/j.jcyt.2021.01.001.

[17] F. J. Vizoso, N. Eiro, L. Costa, P. Esparza, M. Landin, P. Diaz-Rodriguez, J. Schneider, and R. Perez-Fernandez. Mesenchymal stem cells in homeostasis and systemic diseases: Hypothesis, evidences, and therapeutic opportunities. *International Journal of Molecular Sciences*, 20, 8 2019. ISSN 14220067. doi: 10.3390/IJMS20153738. URL /pmc/articles/PMC6696100//pmc/articles/PMC6696100/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC6696100/.

[18] H. Kawada, J. Fujita, K. Kinjo, Y. Matsuzaki, M. Tsuma, H. Miyatake, Y. Muguruma, K. Tsuboi, Y. Itabashi, Y. Ikeda, S. Ogawa, H. Okano, T. Hotta, K. Ando, and K. Fukuda. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood*, 104:3581–3587, 12 2004. ISSN 0006-4971. doi: 10.1182/BLOOD-2004-04-1488. URL https://pubmed.ncbi.nlm.nih.gov/15297308/.

[19] S. M. Devine, C. Cobbs, M. Jennings, A. Bartholomew, and R. Hoffman. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood*, 101:2999–3001, 4 2003. ISSN 0006-4971. doi: 10.1182/BLOOD-2002-06-1830. URL https://pubmed.ncbi.nlm.nih.gov/12480709/.

[20] M. D. Nicola, C. Carlo-Stella, M. Magni, M. Milanesi, P. D. Longoni, P. Matteucci, S. Grisanti, and A. M. Gianni. Human bone marrow stromal cells suppress t-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*, 99:3838–3843, 5 2002. ISSN 0006-4971. doi: 10.1182/BLOOD.V99.10.3838.

[21] A. Shabbir, D. Zisa, H. Lin, M. Mastri, G. Roloff, G. Suzuki, and T. Lee. Activation of host tissue trophic factors through jak-stat3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair. *American journal of physiology. Heart and circulatory physiology*, 299, 11 2010. ISSN 1522-1539. doi: 10.1152/AJPHEART.00488.2010. URL https://pubmed.ncbi.nlm.nih.gov/20852053/.

[22] A. Corcione, F. Benvenuto, E. Ferretti, D. Giunti, V. Cappiello, F. Cazzanti, M. Risso, F. Gualandi, G. L. Mancardi, V. Pistoia, and A. Uccelli. Human mesenchymal stem cells modulate b-cell functions. *Blood*, 107:367–372, 1 2006. ISSN 0006-4971. doi: 10.1182/BLOOD-2005-07-2657. URL https://ashpublications.org/blood/article/107/1/367/21754/Human-mesenchymal-stem-cells-modulate-B-cell.

[23] B. Zhang, R. Liu, D. Shi, X. Liu, Y. Chen, X. Dou, X. Zhu, C. Lu, W. Liang, L. Liao, M. Zenke, and R. C. Zhao. Mesenchymal stem cells induce mature dendritic cells into a novel jagged-2–dependent regulatory dendritic cell population. *Blood*, 113:46–57, 1 2009. ISSN 0006-4971. doi: 10.1182/BLOOD-2008-04-154138. URL https://ashpublications.org/blood/article/113/1/46/24398/Mesenchymal-stem-cells-induce-mature-dendritic.

[24] A. Pradier, J. Passweg, J. Villard, and V. Kindler. Human bone marrow stromal cells and skin fibroblasts inhibit natural killer cell proliferation and cytotoxic activity. *Cell Transplantation*, 20: 681–691, 6 2011. ISSN 09636897. doi: 10.3727/096368910X536545/ASSET/IMAGES/LARGE/10.3727_096368910X536545-FIG2.JPEG. URL https://journals.sagepub.com/doi/10.3727/096368910X536545.

[25] J. G. Rasmussen, O. Frøbert, L. Pilgaard, J. Kastrup, U. Simonsen, V. Zachar, and T. Fink. Prolonged hypoxic culture and trypsinization increase the pro-angiogenic potential of human adipose tissue-derived stem cells. *Cytotherapy*, 13:318–328, 2011. ISSN 14772566. doi: 10.3109/14653249.2010.506505.

[26] A. Andrzejewska, S. Dabrowska, B. Lukomska, M. Janowski, A. Andrzejewska, S. Dabrowska, B. Lukomska, and M. Janowski. Mesenchymal stem cells for neurological disorders. *Advanced Science*, 8:2002944, 4 2021. ISSN 2198-3844. doi: 10.1002/ADVS.202002944. URL https://onlinelibrary.wiley.com/doi/full/10.1002/advs.202002944https://onlinelibrary.wiley.com/doi/abs/10.1002/advs.202002944https://onlinelibrary.wiley.com/doi/10.1002/advs.202002944.

[27] J. M. Ryan, F. P. Barry, J. M. Murphy, and B. P. Mahon. Mesenchymal stem cells avoid allogeneic rejection. *Journal of Inflammation*, 2:1–11, 7 2005. ISSN 14769255. doi: 10.1186/1476-9255-2-8/TABLES/1. URL https://link.springer.com/articles/10.1186/1476-9255-2-8https://link.springer.com/article/10.1186/1476-9255-2-8.

[28] E. Klyushnenkova, J. D. Mosca, V. Zernetkina, M. K. Majumdar, K. J. Beggs, D. W. Simonetti, R. J. Deans, and K. R. McIntosh. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *Journal of Biomedical Science 2005 12:1*, 12:47–57, 1 2005. ISSN 1423-0127. doi: 10.1007/S11373-004-8183-7. URL https://link.springer.com/article/10.1007/s11373-004-8183-7.

[29] U. N. L. of Medicine. Home - clinicaltrials.gov, 2019. URL https://clinicaltrials.gov.

[30] Q. Zhao, H. Ren, and Z. Han. Mesenchymal stem cells: Immunomodulatory capability and clinical potential in immune diseases. *Journal of Cellular Immunotherapy*, 2:3–20, 3 2016. ISSN 2352-1775. doi: 10.1016/J.JOCIT.2014.12.001.

[31] M. E. Bernardo, N. Zaffaroni, F. Novara, A. M. Cometa, M. A. Avanzini, A. Moretta, D. Montagna, R. Maccario, R. Villa, M. G. Daidone, O. Zuffardi, and F. Locatelli. Human bone marrow–derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Research*, 67:9142–9149, 10 2007. ISSN 0008-5472. doi: 10.1158/0008-5472.CAN-06-4690. URL https://aacrjournals.org/cancerres/article/67/19/9142/533802/Human-Bone-Marrow-Derived-Mesenchymal-Stem-Cells.

[32] M. Feng, Y. Li, Q. Han, X. Bao, M. Yang, H. Zhu, Q. Li, J. Wei, W. Ma, H. Gao, Y. An, R. C. Zhao, C. Qin, and R. Wang. Preclinical safety evaluation of human mesenchymal stem cell transplantation in cerebrum of nonhuman primates. *International Journal of Toxicology*, 33: 403–411, 9 2014. ISSN 1092874X. doi: 10.1177/1091581814545244/ASSET/IMAGES/LARGE/10.1177_1091581814545244-FIG2.JPEG. URL https://journals.sagepub.com/doi/full/10.1177/1091581814545244.

[33] Y. Wang, Z. B. Han, J. Ma, C. Zuo, J. Geng, W. Gong, Y. Sun, H. Li, B. Wang, L. Zhang, Y. He, and Z. C. Han. A toxicity study of multiple-administration human umbilical cord mesenchymal stem cells in cynomolgus monkeys. *https://home.liebertpub.com/scd*, 21:1401–1408, 9 2011. ISSN 15473287. doi: 10.1089/SCD.2011.0441. URL https://www.liebertpub.com/doi/10.1089/scd.2011.0441.

[34] Annex i summary of product characteristics, . URL https://www.ema.europa.eu/en/documents/product-information/alofisel-epar-product-information_en.pdf.

[35] . Ministry of food and drug safety - cell therapy product: Anterogen, . URL https://www.mfds.go.kr/eng/brd/m_30/view.do?seq=71337&srchFr=&srchTo=&srchWord=&srchTp=&itm_seq_1=0&itm_seq_2=0&multi_itm_seq=0&company_cd=&company_nm=&page=1.

[36] . Ministry of food and drug safety - cell therapy products: Pharmicell co., ltd., . URL https://www.mfds.go.kr/eng/brd/m_30/view.do?seq=70957&srchFr=&srchTo=&srchWord=&srchTp=&itm_seq_1=0&itm_seq_2=0&multi_itm_seq=0&company_cd=&company_nm=&page=1.

[37] Cartistem®. URL https://www.medi-post.com/cartistem/.

[38] Medsafe product detail. URL https://medsafe.govt.nz/regulatory/ProductDetail.asp?ID=15063.

[39] Product monograph - prochymal®, Mar 2014. URL https://pdf.hres.ca/dpd_pm/00024994.PDF.

[40] Ministry of food and drug safety - cell therapy products: Corestem, inc. URL https://www.mfds.go.kr/eng/brd/m_30/view.do?seq=70956.

[41] Rd / production — jcr pharmaceuticals co., ltd. URL https://www.jcrpharm.co.jp/en/site/en/biopharmaceutical/global.html.

[42] Critical-limb-ischemia — stempeutics. URL https://www.stempeutics.com/critical-limb-ischemia.

[43] Review reports: regenerative medical products — pharmaceuticals and medical devices agency, . URL https://www.pmda.go.jp/english/review-services/reviews/approved-information/0004.html.

[44] M. Jackson and A. Krasnodembskaya. Analysis of mitochondrial transfer in direct co-cultures of human monocyte-derived macrophages (mdm) and mesenchymal stem cells (msc). *BIO-PROTOCOL*, 7, 2017. ISSN 2331-8325. doi: 10.21769/BIOPROTOC.2255.

[45] C. Moriscot, F. D. Fraipont, M. Richard, M. Marchand, P. Savatier, D. Bosco, M. Favrot, P. Benhamou, M.-J. Richard, and P.-Y. Benhamou. Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro. *Stem Cells*, 23:594–603, 4 2005. ISSN 1066-5099. doi: 10.1634/STEMCELLS.2004-0123. URL https://academic.oup.com/stmcls/article/23/4/594/6415021.

[46] L. H. Silva, M. A. Antunes, C. C. D. Santos, D. J. Weiss, F. F. Cruz, and P. R. Rocco. Strategies to improve the therapeutic effects of mesenchymal stromal cells in respiratory diseases. *Stem Cell Research Therapy*, 9, 2 2018. ISSN 17576512. doi: 10.1186/S13287-018-0802-8. URL /pmc/articles/PMC5828113//pmc/articles/PMC5828113/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5828113/.

[47] C. Sun, K. Zhang, J. Yue, S. Meng, and X. Zhang. Deconstructing transcriptional variations and their effects on immunomodulatory function among human mesenchymal stromal cells. *Stem Cell Research and Therapy*, 12:1–16, 12 2021. ISSN 17576512. doi: 10.1186/S13287-020-02121-8/FIGURES/6. URL https://stemcellres.biomedcentral.com/articles/10.1186/s13287-020-02121-8.

[48] T. Ma, K. Gong, Q. Ao, Y. Yan, B. Song, H. Huang, X. Zhang, and Y. Gong. Intracerebral transplantation of adipose-derived mesenchymal stem cells alternatively activates microglia and ameliorates neuropathological deficits in alzheimer's disease mice. *Cell transplantation*, 22 Suppl 1, 2013. ISSN 1555-3892. doi: 10.3727/096368913X672181. URL https://pubmed.ncbi.nlm.nih.gov/24070198/.

[49] D. Offen, Y. Barhum, Y. S. Levy, A. Burshtein, H. Panet, T. Cherlow, and E. Melamed. Intrastriatal transplantation of mouse bone marrow-derived stem cells improves motor behavior in a mouse model of parkinson's disease. *Journal of neural transmission. Supplementum*, pages 133–143, 2007. ISSN 0303-6995. doi: 10.1007/978-3-211-73574-9_16. URL https://pubmed.ncbi.nlm.nih.gov/17982886/.

[50] A. Schwerk, J. Altschüler, M. Roch, M. Gossen, C. Winter, J. Berg, A. Kurtz, and B. Steiner. Human adipose-derived mesenchymal stromal cells increase endogenous neurogenesis in the rat subventricular zone acutely after 6-hydroxydopamine lesioning. *Cytotherapy*, 17:199–214, 2015. ISSN 1477-2566. doi: 10.1016/J.JCYT.2014.09.005. URL https://pubmed.ncbi.nlm.nih.gov/25457280/.

[51] G. Tang, Y. Liu, Z. Zhang, Y. Lu, Y. Wang, J. Huang, Y. Li, X. Chen, X. Gu, Y. Wang, and G. Y. Yang. Mesenchymal stem cells maintain blood-brain barrier integrity by inhibiting aquaporin-4

upregulation after cerebral ischemia. *Stem cells (Dayton, Ohio)*, 32:3150–3162, 12 2014. ISSN 1549-4918. doi: 10.1002/STEM.1808. URL https://pubmed.ncbi.nlm.nih.gov/25100404/.

[52] W. H. Organization. Cardiovascular diseases (cvds), Jun 2021. URL https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

[53] Y. Guo, Y. Yu, S. Hu, Y. Chen, and Z. Shen. The therapeutic potential of mesenchymal stem cells for cardiovascular diseases. *Cell Death Disease 2020 11:5*, 11:1–10, 5 2020. ISSN 2041-4889. doi: 10.1038/s41419-020-2542-9. URL https://www.nature.com/articles/s41419-020-2542-9.

[54] M. G. S. J. Sutton and N. Sharpe. Left ventricular remodeling after myocardial infarction. *Circulation*, 101:2981–2988, 6 2000. ISSN 00097322. doi: 10.1161/01.CIR.101.25.2981. URL https://www.ahajournals.org/doi/abs/10.1161/01.cir.101.25.2981.

[55] F. V. D. Akker, S. C. D. Jager, and J. P. Sluijter. Mesenchymal stem cell therapy for cardiac inflammation: Immunomodulatory properties and the influence of toll-like receptors. *Mediators of Inflammation*, 2013:13, 2013. ISSN 09629351. doi: 10.1155/2013/181020. URL /pmc/articles/PMC3872440//pmc/articles/PMC3872440/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3872440/.

[56] S. T. Ji, H. Kim, J. Yun, J. S. Chung, and S. M. Kwon. Promising therapeutic strategies for mesenchymal stem cell-based cardiovascular regeneration: From cell priming to tissue engineering. *Stem Cells International*, 2017, 2017. ISSN 16879678. doi: 10.1155/2017/3945403.

[57] A. Tormin, O. Li, J. C. Brune, S. Walsh, M. Ehinger, N. Ditzel, M. Kassem, and S. Scheding. Human primary cd271+/cd45/cd146/low and cd271+/cd45/cd146+ bone marrow cells are developmentally closely-related stroma stem cells with similar functional properties but different in-situ localization. *Blood*, 116:2594–2594, 11 2010. ISSN 0006-4971. doi: 10.1182/BLOOD.V116.21.2594.2594. URL https://ashpublications.org/blood/article/116/21/2594/131604/Human-Primary-CD271-CD45-CD146-Low-and-CD271-CD45.

[58] G. Sheng. The developmental basis of mesenchymal stem/stromal cells (mscs). *BMC Developmental Biology*, 15, 11 2015. ISSN 1471213X. doi: 10.1186/S12861-015-0094-5. URL /pmc/articles/PMC4654913//pmc/articles/PMC4654913/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4654913/.

[59] J. Hur, J. Park, S. E. Lee, C. H. Yoon, J. H. Jang, J. M. Yang, T. K. Lee, J. I. Choi, H. M. Yang, E. J. Lee, H. J. Cho, H. J. Kang, B. H. Oh, Y. B. Park, and H. S. Kim. Human peripheral blood-born hematosphere as a niche for hematopoietic stem cell expansion. *Cell Research 2011 21:6*, 21:987–990, 4 2011. ISSN 1748-7838. doi: 10.1038/cr.2011.69. URL https://www.nature.com/articles/cr201169.

[60] J. Grassinger, D. N. Haylock, B. Williams, G. H. Olsen, and S. K. Nilsson. Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. *Blood*, 116:3185–3196, 10 2010. ISSN 0006-4971. doi: 10.1182/BLOOD-2009-12-260703. URL https://ashpublications.org/blood/article/116/17/3185/27958/Phenotypically-identical-hemopoietic-stem-cells.

[61] X. He, V. Gonzalez, A. Tsang, J. Thompson, T. C. Tsang, and D. T. Harris. Differential gene expression profiling of cd34+ cd133+ umbilical cord blood hematopoietic stem progenitor cells. *https://home.liebertpub.com/scd*, 14:188–198, 5 2005. ISSN 15473287. doi: 10.1089/SCD.2005.14.188. URL https://www.liebertpub.com/doi/10.1089/scd.2005.14.188.

[62] M. T. D. la Morena and R. A. Gatti. A history of bone marrow transplantation. *Hematology/Oncology Clinics*, 25:1–15, 2 2011. ISSN 0889-8588. doi: 10.1016/J.HOC.2010.11.001. URL http://www.hemonc.theclinics.com/article/S0889858810001565/fulltexthttp://www.hemonc.theclinics.com/article/S0889858810001565/abstracthttps://www.hemonc.theclinics.com/article/S0889-8588(10)00156-5/abstract.

[63] long term estimates of cancer incidence and mortality. URL https://ecis.jrc.ec.europa.eu/explorer.php?

[64] Bone marrow aspirate. URL https://allcells.com/resources-and-support/protocols/human-bone-marrow-aspirate/.

[65] M. H. Costa, A. M. de Soure, J. M. Cabral, F. C. Ferreira, and C. L. da Silva. Hematopoietic niche – exploring biomimetic cues to improve the functionality of hematopoietic stem/progenitor cells. *Biotechnology Journal*, 13:1700088, 2 2018. ISSN 1860-7314. doi: 10.1002/BIOT.201700088. URL https://onlinelibrary.wiley.com/doi/full/10.1002/biot.201700088https://onlinelibrary.wiley.com/doi/abs/10.1002/biot.201700088https://onlinelibrary.wiley.com/doi/10.1002/biot.201700088.

[66] I. K. McNiece, J. Harrington, J. Turney, J. Kellner, and E. J. Shpall. Ex vivo expansion of cord blood mononuclear cells on mesenchymal stem cells. *Cytotherapy*, 6:311–317, 2004. ISSN 1465-3249. doi: 10.1080/14653240410004871. URL https://pubmed.ncbi.nlm.nih.gov/16146883/.

[67] N. Alakel, D. Jing, K. Muller, M. Bornhauser, G. Ehninger, and R. Ordemann. Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of cd133+ hematopoietic stem cells during ex vivo expansion. *Experimental hematology*, 37:504–513, 4 2009. ISSN 1873-2399. doi: 10.1016/J.EXPHEM.2008.12.005. URL https://pubmed.ncbi.nlm.nih.gov/19216019/.

[68] D. Jing, A. V. Fonseca, N. Alakel, F. A. Fierro, K. Muller, M. Bornhauser, G. Ehninger, D. Corbeil, and R. Ordemann. Hematopoietic stem cells in co-culture with mesenchymal stromal cells - modeling the niche compartments in vitro. *Haematologica*, 95:542–550, 4 2010. ISSN 1592-8721. doi: 10.3324/HAEMATOL.2009.010736. URL https://haematologica.org/article/view/5549.

[69] F. P. Lampreia, J. G. Carmelo, and F. Anjos-Afonso. Notch signaling in the regulation of hematopoietic stem cell. *Current Stem Cell Reports*, 3:202, 9 2017. ISSN 21987866. doi: 10.1007/S40778-017-0090-8. URL /pmc/articles/PMC5548842//pmc/articles/PMC5548842/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5548842/.

[70] M. Corselli, C. J. Chin, C. Parekh, A. Sahaghian, W. Wang, S. Ge, D. Evseenko, X. Wang, E. Montelatici, L. Lazzari, G. M. Crooks, and B. Péault. Perivascular support of human hematopoietic stem/progenitor cells. *Blood*, 121:2891–2901, 4 2013. ISSN 0006-4971. doi: 10.1182/BLOOD-2012-08-451864. URL https://ashpublications.org/blood/article/121/15/2891/31340/Perivascular-support-of-human-hematopoietic-stem.

[71] Z. Liu, Y. Zhang, H. Xiao, Z. Yao, H. Zhang, Q. Liu, B. Wu, D. Nie, Y. Li, Y. Pang, Z. Fan, L. Li, Z. Jiang, F. Duan, H. Li, P. Zhang, Y. Gao, L. Ouyang, C. Yue, M. Xie, C. Shi, Y. Xiao, and S. Wang. Cotransplantation of bone marrow-derived mesenchymal stem cells in haploidentical hematopoietic stem cell transplantation in patients with severe aplastic anemia: an interim summary for a multicenter phase II trial results. *Bone Marrow Transplant*, 52(5):704–710, May 2017.

[72] Z. Liu, X. Wu, S. Wang, L. Xia, H. Xiao, Y. Li, H. Li, Y. Zhang, D. Xu, D. Nie, Y. Lai, B. Wu, D. Lin, X. Du, Z. Jiang, Y. Gao, X. Gu, and Y. Xiao. Co-transplantation of mesenchymal stem cells makes haploidentical HSCT a potential comparable therapy with matched sibling donor HSCT for patients with severe aplastic anemia. *Ther Adv Hematol*, 11:2040620720965411, 2020.

[73] T. Rostami, N. Maleki, A. Kasaeian, M. Nikbakht, A. Kiumarsi, S. Asadollah Mousavi, and A. Ghavamzadeh. Co-transplantation of bone marrow-derived mesenchymal stem cells with hematopoietic stem cells does not improve transplantation outcome in class III beta-thalassemia major: A prospective cohort study with long-term follow-up. *Pediatr Transplant*, 25(3):e13905, May 2021.

[74] W. Wang and Z. C. Han. Heterogeneity of human mesenchymal stromal/stem cells. *Advances in Experimental Medicine and Biology*, 2019. doi: 10.1007/978-3-030-11096-3_10. URL https://doi.org/10.1007/978-3-030-11096-3_10.

[75] B. Sacchetti, A. Funari, C. Remoli, G. Giannicola, G. Kogler, S. Liedtke, G. Cossu, M. Serafini, M. Sampaolesi, E. Tagliafico, E. Tenedini, I. Saggio, P. G. Robey, M. Riminucci, and P. Bianco. No identical "mesenchymal stem cells" at different times and sites: Human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels. *Stem Cell Reports*, 6:897–913, 6 2016. ISSN 2213-6711. doi: 10.1016/J.STEMCR.2016.05.011.

[76] K. S. Park, E. Bandeira, G. V. Shelke, C. Lässer, and J. Lötvall. Enhancement of therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Stem Cell Research Therapy 2019 10:1*, 10:1–15, 9 2019. ISSN 1757-6512. doi: 10.1186/S13287-019-1398-3. URL https://link.springer.com/articles/10.1186/s13287-019-1398-3https://link.springer.com/article/10.1186/s13287-019-1398-3.

[77] E. Rohde, K. Pachler, and M. Gimona. Manufacturing and characterization of extracellular vesicles from umbilical cord–derived mesenchymal stromal cells for clinical testing. *Cytotherapy*, 21:581–592, 6 2019. ISSN 1465-3249. doi: 10.1016/J.JCYT.2018.12.006.

[78] K. W. Witwer, B. W. V. Balkom, S. Bruno, A. Choo, M. Dominici, M. Gimona, A. F. Hill, D. D. Kleijn, M. Koh, R. C. Lai, S. A. Mitsialis, L. A. Ortiz, E. Rohde, T. Asada, W. S. Toh, D. J. Weiss, L. Zheng, B. Giebel, and S. K. Lim. Defining mesenchymal stromal cell (msc)-derived small extracellular vesicles for therapeutic applications. *Journal of Extracellular Vesicles*, 8:1609206, 2019. ISSN 2001-3078. doi: 10.1080/20013078.2019.1609206. URL https://www.tandfonline.com/action/journalInformation?journalCode=zjev20.

[79] T. S. Chen, F. Arslan, Y. Yin, S. S. Tan, R. C. Lai, A. B. H. Choo, J. Padmanabhan, C. N. Lee, D. P. V. de Kleijn, and S. K. Lim. Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human esc-derived mscs. *Journal of Translational Medicine*, 9:47, 2011. ISSN 1479-5876. doi: 10.1186/1479-5876-9-47. URL https://doi.org/10.1186/1479-5876-9-47.

[80] T. R. Olsen, K. S. Ng, L. T. Lock, T. Ahsan, and J. A. Rowley. Peak msc-are we there yet? *Frontiers in Medicine*, 5:178, 6 2018. ISSN 2296858X. doi: 10.3389/FMED.2018.00178/BIBTEX.

[81] D. S. Cherian, T. Bhuvan, L. Meagher, and T. S. Heng. Biological considerations in scaling up therapeutic cell manufacturing. *Frontiers in Pharmacology*, 11:654, 5 2020. ISSN 16639812. doi: 10.3389/FPHAR.2020.00654/XML/NLM.

[82] J. Rowley, E. Abraham, A. Campbell, H. Brandwein, and S. Oh. Meeting lot-size challenges of manufacturing adherent cells for therapy. *BioProcess International*, 3 2012. URL https://bioprocessintl.com/manufacturing/cell-therapies/meeting-lot-size-challenges-of-manufacturing-adherent-cells-for-therapy-328093/.

[83] S. R. Caruso, M. D. Orellana, A. Mizukami, T. R. Fernandes, A. M. Fontes, C. A. Suazo, V. C. Oliveira, D. T. Covas, and K. Swiech. Growth and functional harvesting of human mesenchymal stromal cells cultured on a microcarrier-based system. *Biotechnology Progress*, 30:889–895, 7 2014. ISSN 1520-6033. doi: 10.1002/BTPR.1886. URL https://onlinelibrary.wiley.com/doi/full/10.1002/btpr.1886https://onlinelibrary.wiley.com/doi/abs/10.1002/btpr.1886https://aiche.onlinelibrary.wiley.com/doi/10.1002/btpr.1886.

[84] A. Mizukami, A. Fernandes-Platzgummer, J. G. Carmelo, K. Swiech, D. T. Covas, J. M. Cabral, and C. L. da Silva. Stirred tank bioreactor culture combined with serum-/xenogeneic-free culture medium enables an efficient expansion of umbilical cord-derived mesenchymal stem/stromal cells. *Biotechnology Journal*, 11:1048–1059, 8 2016. ISSN 1860-7314. doi: 10.1002/BIOT.201500532. URL https://onlinelibrary.wiley.com/doi/full/10.1002/biot.201500532https://onlinelibrary.wiley.com/doi/abs/10.1002/biot.201500532https://onlinelibrary.wiley.com/doi/10.1002/biot.201500532.

[85] F. D. Santos, A. Campbell, A. Fernandes-Platzgummer, P. Z. Andrade, J. M. Gimble, Y. Wen, S. Boucher, M. C. Vemuri, C. L. D. Silva, and J. M. Cabral. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnology and Bioengineering*, 111:1116–1127, 6 2014. ISSN 1097-0290. doi: 10.1002/BIT.25187. URL https://onlinelibrary.wiley.com/doi/full/10.1002/bit.25187https://onlinelibrary.wiley.com/doi/abs/10.1002/bit.25187https://onlinelibrary.wiley.com/doi/10.1002/bit.25187.

[86] C. C. Barrias, M. C. L. Martins, G. Almeida-Porada, M. A. Barbosa, and P. L. Granja. The correlation between the adsorption of adhesive proteins and cell behaviour on hydroxyl-methyl mixed self-assembled monolayers. *Biomaterials*, 30:307–316, 1 2009. ISSN 0142-9612. doi: 10.1016/J.BIOMATERIALS.2008.09.048.

[87] Y. K. Wang and C. S. Chen. Cell adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation. *Journal of Cellular and Molecular Medicine*, 17:823, 7 2013. ISSN 15821838. doi: 10.1111/JCMM.12061. URL /pmc/articles/PMC3741348//pmc/articles/PMC3741348/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3741348/.

[88] E. Hohenester and J. Engel. Domain structure and organisation in extracellular matrix proteins. *Matrix Biology*, 21:115–128, 3 2002. ISSN 0945-053X. doi: 10.1016/S0945-053X(01)00191-3.

[89] A. Ode, G. N. Duda, J. D. Glaeser, G. Matziolis, S. Frauenschuh, C. Perka, C. J. Wilson, and G. Kasper. Toward biomimetic materials in bone regeneration: Functional behavior of mesenchymal stem cells on a broad spectrum of extracellular matrix components. *Journal of Biomedical Materials Research Part A*, 95A:1114–1124, 12 2010. ISSN 1552-4965. doi: 10.1002/JBM.A.32909. URL https://onlinelibrary.wiley.com/doi/full/10.1002/jbm.a.32909https://onlinelibrary.wiley.com/doi/abs/10.1002/jbm.a.32909https://onlinelibrary.wiley.com/doi/10.1002/jbm.a.32909.

[90] N. V. Andreeva, O. G. Leonova, V. I. Popenko, and A. V. Belyavsky. Controlled formaldehyde fixation of fibronectin layers for expansion of mesenchymal stem cells. *Analytical Biochemistry*, 514:38–41, 12 2016. ISSN 0003-2697. doi: 10.1016/J.AB.2016.09.003.

[91] R. M. Salasznyk, W. A. Williams, A. Boskey, A. Batorsky, and G. E. Plopper. Adhesion to vitronectin and collagen i promotes osteogenic differentiation of human mesenchymal stem cells. *Journal of biomedicine biotechnology*, 2004:24–34, 4 2004. ISSN 1110-7251. doi: 10.1155/S1110724304306017. URL https://pubmed.ncbi.nlm.nih.gov/15123885/.

[92] F. Mittag, E.-M. Falkenberg, A. Janczyk, M. Götze, T. Felka, W. K. Aicher, and T. Kluba. Laminin-5 and type i collagen promote adhesion and osteogenic differentiation of animal serum-free expanded human mesenchymal stromal cells. *Orthopedic Reviews*, 4:36, 11 2012. ISSN 2035-8237. doi: 10.4081/OR.2012.E36. URL /pmc/articles/PMC3626307//pmc/articles/PMC3626307/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3626307/.

[93] C. Somaiah, A. Kumar, D. Mawrie, A. Sharma, S. D. Patil, J. Bhattacharyya, R. Swaminathan, and B. G. Jaganathan. Collagen promotes higher adhesion, survival and proliferation of mesenchymal stem cells. *PLOS ONE*, 10:e0145068, 12 2015. ISSN 1932-6203. doi: 10.1371/JOURNAL.PONE.0145068. URL https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0145068.

[94] S. J. Cifuentes, P. Priyadarshani, D. A. Castilla-Casadiego, L. J. Mortensen, J. Almodóvar, and M. Domenech. Heparin/collagen surface coatings modulate the growth, secretome, and morphology of human mesenchymal stromal cell response to interferon-gamma. *Journal of Biomedical Materials Research Part A*, 109:951–965, 6 2021. ISSN 1552-4965. doi: 10.1002/JBM.A.37085. URL https://onlinelibrary.wiley.com/doi/full/10.1002/jbm.a.37085https://onlinelibrary.wiley.com/doi/abs/10.1002/jbm.a.37085https://onlinelibrary.wiley.com/doi/10.1002/jbm.a.37085.

[95] M. Aumailley. The laminin family. *Cell Adhesion Migration*, 7:48, 1 2013. ISSN 19336926. doi: 10.4161/CAM.22826. URL /pmc/articles/PMC3544786//pmc/articles/PMC3544786/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3544786/.

[96] R. Nishiuchi, J. Takagi, M. Hayashi, H. Ido, Y. Yagi, N. Sanzen, T. Tsuji, M. Yamada, and K. Sekiguchi. Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins. *Matrix biology : journal of the International Society for Matrix Biology*, 25:189–197, 4 2006. ISSN 0945-053X. doi: 10.1016/J.MATBIO.2005.12.001. URL https://pubmed.ncbi.nlm.nih.gov/16413178/.

[97] U. Lindner, J. Kramer, J. Behrends, B. Driller, N. O. Wendler, F. Boehrnsen, J. Rohwedel, and P. Schlenke. Improved proliferation and differentiation capacity of human mesenchymal stromal cells cultured with basement-membrane extracellular matrix proteins. *Cytotherapy*, 12:992–1005, 2010. ISSN 14772566. doi: 10.3109/14653249.2010.510503.

[98] J. Hashimoto, Y. Kariya, and K. Miyazaki. Regulation of proliferation and chondrogenic differentiation of human mesenchymal stem cells by laminin-5 (laminin-332). *Stem Cells*, 24:2346–2354, 11 2006. ISSN 1066-5099. doi: 10.1634/STEMCELLS.2005-0605. URL https://academic.oup.com/stmcls/article/24/11/2346/6401558.

[99] P. A. Sotiropoulou, S. A. Perez, M. Salagianni, C. N. Baxevanis, and M. Papamichail. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *STEM CELLS*, 24:462–471, 2 2006. ISSN 1549-4918. doi: 10.1634/STEMCELLS.2004-0331. URL https://onlinelibrary.wiley.com/doi/full/10.1634/stemcells.2004-0331https://onlinelibrary.wiley.com/doi/abs/10.1634/stemcells.2004-0331https://stemcellsjournals.onlinelibrary.wiley.com/doi/10.1634/stemcells.2004-0331.

[100] M. Guiotto, W. Raffoul, A. M. Hart, M. O. Riehle, and P. G. D. Summa. Human platelet lysate to substitute fetal bovine serum in hmsc expansion for translational applications: A systematic review. *Journal of Translational Medicine*, 18:1–14, 9 2020. ISSN 14795876. doi: 10.1186/S12967-020-02489-4/TABLES/10. URL https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-020-02489-4.

[101] K. Bieback, B. FERNANDEZ-MUÑOZ, S. PATI, and R. SCHÄFER. Gaps in the knowledge of human platelet lysate as a cell culture supplement for cell therapy: a joint publication from the aabb and the international society for cell gene therapy. *Cytotherapy*, 21:911–924, 9 2019. ISSN 1465-3249. doi: 10.1016/J.JCYT.2019.06.006.

[102] J. van der Valk, D. Brunner, K. D. Smet, F. Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price, M. L. Scarino, and G. Gstraunthaler. Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods. *Toxicology in Vitro*, 24:1053–1063, 6 2010. ISSN 0887-2333. doi: 10.1016/J.TIV.2010.03.016.

[103] M. Bernardi, F. Agostini, K. Chieregato, E. Amati, C. Durante, M. Rassu, M. Ruggeri, S. Sella, E. Lombardi, M. Mazzucato, and G. Astori. The production method affects the efficacy of platelet derivatives to expand mesenchymal stromal cells in vitro. *Journal of Translational Medicine*, 15:90, 5 2017. ISSN 14795876. doi: 10.1186/S12967-017-1185-9. URL /pmc/articles/PMC5412035//pmc/articles/PMC5412035/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5412035/.

[104] C. Capelli, M. Domenghini, G. Borleri, P. Bellavita, R. Poma, A. Carobbio, C. Micò, A. Rambaldi, J. Golay, and M. Introna. Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from small samples of bone marrow aspirates or marrow filter washouts. *Bone Marrow Transplantation 2007 40:8*, 40:785–791, 8 2007. ISSN 1476-5365. doi: 10.1038/sj.bmt.1705798. URL https://www.nature.com/articles/1705798.

[105] C. Doucet, I. Ernou, Y. Zhang, J. R. Llense, L. Begot, X. Holy, and J. J. Lataillade. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. *Journal of Cellular Physiology*, 205:228–236, 11 2005. ISSN 1097-4652. doi: 10.1002/JCP.20391. URL https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.20391https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.20391https://onlinelibrary.wiley.com/doi/10.1002/jcp.20391.

[106] K. Schallmoser, R. Henschler, C. Gabriel, M. B. Koh, and T. Burnouf. Production and quality requirements of human platelet lysate: A position statement from the working party on cellular therapies of the international society of blood transfusion. *Trends in Biotechnology*, 38:13–23, 1 2020. ISSN 0167-7799. doi: 10.1016/J.TIBTECH.2019.06.002.

[107] A. Oikonomopoulos, W. K. V. Deen, A. R. Manansala, P. N. Lacey, T. A. Tomakili, A. Ziman, and D. W. Hommes. Optimization of human mesenchymal stem cell manufacturing: the effects of

animal/xeno-free media. *Scientific Reports 2015 5:1*, 5:1–11, 11 2015. ISSN 2045-2322. doi: 10.1038/srep16570. URL https://www.nature.com/articles/srep16570.

[108] P. Du, X. Tao, K. Liu, J. Lin, Y. Shi, K. Park, H. Y. Chen, C. P. Lin, J. Chang, R. C. Wong, H. Pan, and P. Y. Wang. Human platelet lysate (hpl) alters the lineage commitment and paracrine functions of human mesenchymal stem cells via mitochondrial metabolism. *Applied Materials Today*, 26: 101264, 3 2022. ISSN 2352-9407. doi: 10.1016/J.APMT.2021.101264.

[109] J. Xu, W. Lian, J. Chen, W. Li, L. Li, and Z. Huang. Chemical-defined medium supporting the expansion of human mesenchymal stem cells. *Stem Cell Research and Therapy*, 11:1–11, 3 2020. ISSN 17576512. doi: 10.1186/S13287-020-01641-7/FIGURES/5. URL https://stemcellres.biomedcentral.com/articles/10.1186/s13287-020-01641-7.

[110] S. Kinzebach and K. Bieback. Expansion of mesenchymal stem/stromal cells under xenogenic-free culture conditions. *Advances in Biochemical Engineering/Biotechnology*, 129:33–57, 2013. ISSN 07246145. doi: 10.1007/10_2012_134/COVER. URL https://link.springer.com/chapter/10.1007/10_2012_134.

[111] M. Patrikoski, M. Juntunen, S. Boucher, A. Campbell, M. C. Vemuri, B. Mannerström, and S. Miettinen. 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, 2013. ISSN 17576512. doi: 10.1186/SCRT175. URL /pmc/articles/PMC3707027//pmc/articles/PMC3707027/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3707027/.

[112] J. Y. Kim, W. K. Rhim, H. J. Seo, J. Y. Lee, C. G. Park, and D. K. Han. Comparative analysis of msc-derived exosomes depending on cell culture media for regenerative bioactivity. *Tissue Engineering and Regenerative Medicine*, 18:355–367, 6 2021. ISSN 22125469. doi: 10.1007/S13770-021-00352-1/FIGURES/5. URL https://link.springer.com/article/10.1007/s13770-021-00352-1.

[113] N. Azouna, F. Jenhani, Z. Regaya, L. Berraeis, T. Othman, E. Ducrocq, and J. Domenech. Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: Comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. *Stem Cell Research and Therapy*, 3:1–14, 2 2012. ISSN 17576512. doi: 10.1186/SCRT97/FIGURES/8. URL https://link.springer.com/articles/10.1186/scrt97https://link.springer.com/article/10.1186/scrt97.

[114] K. Mareschi, S. Castiglia, A. Adamini, D. Rustichelli, E. Marini, A. G. S. B. Niclot, M. Bergallo, L. Labanca, I. Ferrero, and F. Fagioli. Inactivated platelet lysate supports the proliferation and immunomodulant characteristics of mesenchymal stromal cells in gmp culture conditions. *Biomedicines 2020, Vol. 8, Page 220*, 8:220, 7 2020. ISSN 2227-9059. doi: 10.3390/BIOMEDICINES8070220. URL https://www.mdpi.com/2227-9059/8/7/220/htmhttps://www.mdpi.com/2227-9059/8/7/220.

[115] S. Palombella, M. Guiotto, G. C. Higgins, L. L. Applegate, W. Raffoul, M. Cherubino, A. Hart, M. O. Riehle, and P. G. D. Summa. Human platelet lysate as a potential clinical-translatable supplement to support the neurotrophic properties of human adipose-derived stem cells. *Stem Cell Research and Therapy*, 11:1–14, 10 2020. ISSN 17576512. doi: 10.1186/S13287-020-01949-4/FIGURES/4. URL https://stemcellres.biomedcentral.com/articles/10.1186/s13287-020-01949-4.

[116] P. Gupta, G. N. Hall, L. Geris, F. P. Luyten, and I. Papantoniou. Human platelet lysate improves bone forming potential of human progenitor cells expanded in microcarrier-based dynamic culture. *Stem Cells Translational Medicine*, 8:810–821, 8 2019. ISSN 2157-6564. doi: 10.1002/SCTM.18-0216. URL https://academic.oup.com/stcltm/article/8/8/810/6403793.

[117] T. Aoyama. Transportation of mesenchymal stem cells for clinical applications. *Mesenchymal Stem Cells - Isolation, Characterization and Applications*, 11 2017. doi: 10.5772/67716. URL undefined/state.item.id.

[118] D. Freimark, C. Sehl, C. Weber, K. Hudel, P. Czermak, N. Hofmann, R. Spindler, and B. Glasmacher. Systematic parameter optimization of a me 2so- and serum-free cryopreservation protocol for human mesenchymal stem cells. *Cryobiology*, 63:67–75, 10 2011. ISSN 00112240. doi: 10.1016/J.CRYOBIOL.2011.05.002.

[119] L. Weng and P. R. Beauchesne. Dimethyl sulfoxide-free cryopreservation for cell therapy: A review. *Cryobiology*, 94:9–17, 6 2020. ISSN 1090-2392. doi: 10.1016/J.CRYOBIOL.2020.03.012. URL https://pubmed.ncbi.nlm.nih.gov/32247742/.

[120] Y. Petrenko, M. Chudickova, I. Vackova, T. Groh, E. Kosnarova, J. Cejkova, K. Turnovcova, A. Petrenko, E. Sykova, and S. Kubinova. Clinically relevant solution for the hypothermic storage and transportation of human multipotent mesenchymal stromal cells. *Stem Cells International*, 2019, 2019. ISSN 16879678. doi: 10.1155/2019/5909524.

[121] A. zy nska, M. S. nska, P. Szpak, N. K. sniak, J. Malejczyk, and I. K. nska. Influence of hypothermic storage fluids on mesenchymal stem cell stability: A comprehensive review and personal experience. *Cells*, 2021. doi: 10.3390/cells10051043. URL https://doi.org/10.3390/cells10051043.

[122] F. T. CELIKKAN, C. MUNGAN, M. SUCU, A. T. ULUS, O. CINAR, E. G. ILI, and A. L. CAN. Optimizing the transport and storage conditions of current good manufacturing practice –grade human umbilical cord mesenchymal stromal cells for transplantation (huc-heart trial). *Cytotherapy*, 21:64–75, 1 2019. ISSN 14772566. doi: 10.1016/j.jcyt.2018.10.010. URL http://www.isct-cytotherapy.org/article/S1465324918306662/fulltexthttp://www.isct-cytotherapy.org/article/S1465324918306662/abstracthttps://www.isct-cytotherapy.org/article/S1465-3249(18)30666-2/abstract.

[123] A. L. Tiago. *Optimization of Mesenchymal Stromal Cell Culture Methodologies Towards the Development of a Cell Therapy for Autoimmune Diseases*. PhD thesis, Dec 2020.

[124] U. Galderisi, G. Peluso, and G. D. Bernardo. Clinical trials based on mesenchymal stromal cells are exponentially increasing: Where are we in recent years? *Stem Cell Reviews and Reports*, 1: 1–14, 8 2021. ISSN 26293277. doi: 10.1007/S12015-021-10231-W/FIGURES/4. URL https://link.springer.com/article/10.1007/s12015-021-10231-w.

[125] G. Siegel, T. Kluba, U. Hermanutz-Klein, K. Bieback, H. Northoff, and R. Schäfer. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Medicine*, 11:1–20, 6 2013. ISSN 17417015. doi: 10.1186/1741-7015-11-146/TABLES/2. URL https://bmcmedicine.biomedcentral.com/articles/10.1186/1741-7015-11-146.

[126] Y. Xie, W. Liu, S. Liu, L. Wang, D. Mu, Y. Cui, Y. Cui, and B. Wang. The quality evaluation system establishment of mesenchymal stromal cells for cell-based therapy products. *Stem Cell Research and Therapy*, 11:1–14, 5 2020. ISSN 17576512. doi: 10.1186/S13287-020-01696-6/TABLES/6. URL https://stemcellres.biomedcentral.com/articles/10.1186/s13287-020-01696-6.

[127] E. M. Agency. Committee for medicinal products for human use (chmp) guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer 3rs technical update*. 2006. URL www.ema.europa.eu/contact.

[128] J. Galipeau, M. Krampera, J. Barrett, F. Dazzi, R. J. Deans, J. DeBruijn, M. Dominici, W. E. Fibbe, A. P. Gee, J. M. Gimble, P. Hematti, M. B. Koh, K. LeBlanc, I. Martin, I. K. McNiece, M. Mendicino, S. Oh, L. Ortiz, D. G. Phinney, V. Planat, Y. Shi, D. F. Stroncek, S. Viswanathan, D. J. Weiss, and L. Sensebe. International society for cellular therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. *Cytotherapy*, 18:151, 11 2016. ISSN 14772566. doi: 10.1016/J.JCYT.2015.11.008. URL /pmc/articles/PMC4745114//pmc/articles/PMC4745114/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4745114/.

[129] Fda and Cber. Guidance for industry potency tests for cellular and gene therapy products. 2011. URL http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

[130] Y. Y. Lipsitz, N. E. Timmins, and P. W. Zandstra. Quality cell therapy manufacturing by design. *Nature Biotechnology 2016 34:4*, 34:393–400, 4 2016. ISSN 1546-1696. doi: 10.1038/nbt.3525. URL https://www.nature.com/articles/nbt.3525.

[131] G. Ren, X. Zhao, L. Zhang, J. Zhang, A. L'Huillier, W. Ling, A. I. Roberts, A. D. Le, S. Shi, C. Shao, and Y. Shi. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *The Journal of Immunology*, 184:2321–2328, 3 2010. ISSN 0022-1767. doi: 10.4049/JIMMUNOL.0902023. URL https://www.jimmunol.org/content/184/5/2321https://www.jimmunol.org/content/184/5/2321.abstract.

[132] Z. X. Yang, Z. B. Han, Y. R. Ji, Y. W. Wang, L. Liang, Y. Chi, S. G. Yang, L. N. Li, W. F. Luo, J. P. Li, D. D. Chen, W. J. Du, X. C. Cao, G. S. Zhuo, T. Wang, and Z. C. Han. Cd106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. *PLOS ONE*, 8:e59354, 3 2013. ISSN 1932-6203. doi: 10.1371/JOURNAL.PONE.0059354. URL https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0059354.

[133] M. Pietilä, S. Lehtonen, E. Tuovinen, K. Lähteenmäki, S. Laitinen, H. V. Leskelä, A. Nätynki, J. Pesälä, K. Nordström, and P. Lehenkari. Cd200 positive human mesenchymal stem cells suppress tnf-alpha secretion from cd200 receptor positive macrophage-like cells. *PLOS ONE*, 7:e31671, 2 2012. ISSN 1932-6203. doi: 10.1371/JOURNAL.PONE.0031671. URL https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0031671.

[134] H. J. Kim, K. W. Kim, Y. R. Kwon, B. M. Kim, and Y. J. Kim. Forced expression of cd200 improves the differentiation capability and immunoregulatory functions of mesenchymal stromal cells. *Biotechnology Letters*, 40:1425–1433, 10 2018. ISSN 15736776. doi: 10.1007/S10529-018-2561-0/FIGURES/4. URL https://link.springer.com/article/10.1007/s10529-018-2561-0.

[135] Y. Zhao, G. Su, Q. Wang, R. Wang, and M. Zhang. The cd200/cd200r mechanism in mesenchymal stem cells' regulation of dendritic cells. *American Journal of Translational Research*, 13:9607, 2021. ISSN 1943-8141. URL /pmc/articles/PMC8430165//pmc/articles/PMC8430165/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC8430165/.

[136] S. Kuçi, Z. Kuçi, H. Kreyenberg, E. Deak, K. Pütsch, S. Huenecke, C. Amara, S. Koller, E. Rettinger, M. Grez, U. Koehl, H. Latifi-Pupovci, R. Henschler, T. Tonn, D. von Laer, T. Klingebiel, and P. Bader. Cd271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica*, 95:651–659, 2010. ISSN 1592-8721. doi: 10.3324/HAEMATOL.2009.015065. URL https://pubmed.ncbi.nlm.nih.gov/20179086/.

[137] Z. Kuçi, S. Kuçi, S. Zircher, S. Koller, R. Schubert, H. Bönig, R. Henschler, R. Lieberz, T. Klingebiel, and P. Bader. Mesenchymal stromal cells derived from cd271+ bone marrow mononuclear cells exert potent allosuppressive properties. *Cytotherapy*, 13:1193–1204, 11 2011. ISSN 1465-3249. doi: 10.3109/14653249.2011.605118.

[138] A. C. Bowles, D. Kouroupis, M. A. Willman, C. P. Orfei, A. Agarwal, and D. Correa. Signature quality attributes of cd146+ mesenchymal stem/stromal cells correlate with high therapeutic and secretory potency. *Stem Cells*, 38:1034–1049, 8 2020. ISSN 1066-5099. doi: 10.1002/STEM.3196. URL https://academic.oup.com/stmcls/article/38/8/1034/6409351.

[139] L. Zhang, X. Zhang, Y. Liu, W. Zhang, C.-T. Wu, L. Wang, and R. Article. Cd146+ umbilical cord mesenchymal stem cells exhibit higher immunomodulatory activity and therapeutic efficacy in septic mice. 10 2022. doi: 10.21203/RS.3.RS-2145532/V1. URL https://www.researchsquare.comhttps://www.researchsquare.com/article/rs-2145532/v1.

[140] G. M. Crane, E. Jeffery, and S. J. Morrison. Adult haematopoietic stem cell niches. *Nature Reviews Immunology 2017 17:9*, 17:573–590, 6 2017. ISSN 1474-1741. doi: 10.1038/nri.2017.53. URL https://www.nature.com/articles/nri.2017.53.

[141] B. Sacchetti, A. Funari, S. Michienzi, S. D. Cesare, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P. G. Robey, M. Riminucci, and P. Bianco. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell*, 131:324–336, 10 2007. ISSN 00928674. doi: 10.1016/j.cell.2007.08.025. URL http://www.cell.com/article/S0092867407010872/fulltexthttp://www.cell.com/article/S0092867407010872/abstracthttps://www.cell.com/cell/abstract/S0092-8674(07)01087-2.

[142] S. Kuçi, Z. Kuçi, R. Schäfer, G. Spohn, S. Winter, M. Schwab, E. Salzmann-Manrique, T. Klingebiel, and P. Bader. Molecular signature of human bone marrow-derived mesenchymal stromal cell subsets. *Scientific Reports 2019 9:1*, 9:1–10, 2 2019. ISSN 2045-2322. doi: 10.1038/s41598-019-38517-7. URL https://www.nature.com/articles/s41598-019-38517-7.

[143] R. Ghazanfari, H. Li, D. Zacharaki, H. C. Lim, and S. Scheding. Human non-hematopoietic cd271pos/cd140alow/neg bone marrow stroma cells fulfill stringent stem cell criteria in serial transplantations. *Stem Cells and Development*, 25:1652–1658, 11 2016. ISSN 15578534. doi: 10.1089/SCD.2016.0169/ASSET/IMAGES/LARGE/FIGURE3.JPEG. URL https://www.liebertpub.com/doi/10.1089/scd.2016.0169.

[144] A. Tormin, O. Li, J. C. Brune, S. Walsh, B. Schütz, M. Ehinger, N. Ditzel, M. Kassem, and S. Scheding. Cd146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood*, 117:5067, 5 2011. ISSN 15280020. doi: 10.1182/BLOOD-2010-08-304287. URL /pmc/articles/PMC3109533//pmc/articles/PMC3109533/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3109533/.

[145] S. Pinho, J. Lacombe, M. Hanoun, T. Mizoguchi, I. Bruns, Y. Kunisaki, and P. S. Frenette. Pdgfr and cd51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *Journal of Experimental Medicine*, 210:1351–1367, 7 2013. ISSN 0022-1007. doi: 10.1084/JEM.20122252. URL www.jem.org/cgi/doi/10.1084/jem.20122252.

[146] S. Méndez-Ferrer, T. V. Michurina, F. Ferraro, A. R. Mazloom, B. D. MacArthur, S. A. Lira, D. T. Scadden, A. Ma'ayan, G. N. Enikolopov, and P. S. Frenette. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature 2010 466:7308*, 466:829–834, 8 2010. ISSN 1476-4687. doi: 10.1038/nature09262. URL https://www.nature.com/articles/nature09262.

[147] B. Delorme, J. Ringe, N. Gallay, Y. L. Vern, D. Kerboeuf, C. Jorgensen, P. Rosset, L. Sensebé, P. Layrolle, T. Häpupl, and P. Charbord. Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells. *Blood*, 111:2631–2635, 3 2008. ISSN 0006-4971. doi: 10.1182/BLOOD-2007-07-099622. URL https://ashpublications.org/blood/article/111/5/2631/109938/Specific-plasma-membrane-protein-phenotype-of.

[148] Y. Mifune, T. Matsumoto, S. Murasawa, A. Kawamoto, R. Kuroda, T. Shoji, T. Kuroda, T. Fukui, Y. Kawakami, M. Kurosaka, and T. Asahara. Therapeutic superiority for cartilage repair by cd271-positive marrow stromal cell transplantation. *Cell Transplantation*, 22:1201–1211, 7 2013. ISSN 09636897. doi: 10.3727/096368912X657378/ASSET/IMAGES/LARGE/10.3727_096368912X657378-FIG2.JPEG. URL https://journals.sagepub.com/doi/10.3727/096368912X657378.

[149] T. S. Park, M. Gavina, C. W. Chen, B. Sun, P. N. Teng, J. Huard, B. M. Deasy, L. Zimmerlin, and B. Péault. Placental perivascular cells for human muscle regeneration. *https://home.liebertpub.com/scd*, 20:451–463, 10 2010. ISSN 15473287. doi: 10.1089/SCD.2010.0354. URL https://www.liebertpub.com/doi/10.1089/scd.2010.0354.

[150] C. W. Chen, M. Okada, J. D. Proto, X. Gao, N. Sekiya, S. A. Beckman, M. Corselli, M. Crisan, A. Saparov, K. Tobita, B. Peault, and J. Huard. Human pericytes for ischemic heart repair. *Stem Cells*, 31:305–316, 2 2013. ISSN 1066-5099. doi: 10.1002/STEM.1285. URL https://academic.oup.com/stmcls/article/31/2/305/6408032.

[151] K. Tamai, T. Yamazaki, T. Chino, M. Ishii, S. Otsuru, Y. Kikuchi, S. Iinuma, K. Saga, K. Nimura, T. Shimbo, N. Umegaki, I. Katayama, J. I. Miyazaki, J. Takeda, J. A. McGrath, J. Uitto, and Y. Kaneda. Pdgfr-positive cells in bone marrow are mobilized by high mobility group box 1 (hmgb1) to regenerate injured epithelia. *Proceedings of the National Academy of Sciences of the United States of America*, 108:6609–6614, 4 2011. ISSN 00278424. doi: 10.1073/PNAS.1016753108/SUPPL_FILE/PNAS.201016753SI.PDF. URL https://www.pnas.org/doi/abs/10.1073/pnas.1016753108.

[152] S. Wislet-Gendebien, F. Bruyère, G. Hans, P. Leprince, G. Moonen, and B. Rogister. Nestin-positive mesenchymal stem cells favour the astroglial lineage in neural progenitors and stem cells by releasing active bmp4. *BMC Neuroscience*, 5:1–12, 9 2004. ISSN 14712202. doi: 10.1186/1471-2202-5-33/TABLES/1. URL https://bmcneurosci.biomedcentral.com/articles/10.1186/1471-2202-5-33.

[153] K. H. Susek, E. Korpos, J. Huppert, C. Wu, I. Savelyeva, F. Rosenbauer, C. Müller-Tidow, S. Koschmieder, and L. Sorokin. Bone marrow laminins influence hematopoietic stem and progenitor cell cycling and homing to the bone marrow. *Matrix Biology*, 67:47–62, 4 2018. ISSN 0945-053X. doi: 10.1016/J.MATBIO.2018.01.007.

[154] H. Qian, E. Georges-Labouesse, A. Nyström, A. Domogatskaya, K. Tryggvason, S. E. W. Jacobsen, and M. Ekblom. Distinct roles of integrins 6 and 4 in homing of fetal liver hematopoietic stem and progenitor cells. *Blood*, 110:2399–2407, 10 2007. ISSN 0006-4971. doi: 10.1182/BLOOD-2006-10-051276. URL https://ashpublications.org/blood/article/110/7/2399/103650/Distinct-roles-of-integrins-6-and-4-in-homing-of.

[155] P. S. Godavarthy, C. B. Walter, C. Lengerke, and G. Klein. The laminin receptors basal cell adhesion molecule/lutheran and integrin 71 on human hematopoietic stem cells. *Frontiers in Cell and*

Developmental Biology, 9:2973, 10 2021. ISSN 2296634X. doi: 10.3389/FCELL.2021.675240/BIBTEX.

[156] Z. Tian, C. K. Wang, F. L. Lin, Q. Liu, T. Wang, T. C. Sung, A. A. Alarfaj, A. H. Hirad, H. H. C. Lee, G. J. Wu, and A. Higuchi. Effect of extracellular matrix proteins on the differentiation of human pluripotent stem cells into mesenchymal stem cells. *Journal of Materials Chemistry B*, 10:5723–5732, 8 2022. ISSN 2050-7518. doi: 10.1039/D2TB01026G. URL https://pubs.rsc.org/en/content/articlehtml/2022/tb/d2tb01026ghttps://pubs.rsc.org/en/content/articlelanding/2022/tb/d2tb01026g.

[157] M. Zaim, S. Karaman, G. Cetin, and S. Isik. Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells. *Annals of Hematology*, 91:1175–1186, 8 2012. ISSN 09395555. doi: 10.1007/S00277-012-1438-X/FIGURES/7. URL https://link.springer.com/article/10.1007/s00277-012-1438-x.

[158] P. Gao, P. Han, D. Jiang, S. Yang, Q. Cui, and Z. Li. Effects of the donor age on proliferation, senescence and osteogenic capacity of human urine-derived stem cells. *Cytotechnology*, 69: 751–763, 10 2017. ISSN 15730778. doi: 10.1007/S10616-017-0084-5/FIGURES/9. URL https://link.springer.com/article/10.1007/s10616-017-0084-5.

[159] J. Khetan, M. Shahinuzzaman, S. Barua, and D. Barua. Quantitative analysis of the correlation between cell size and cellular uptake of particles. *Biophysical Journal*, 116:347–359, 1 2019. ISSN 0006-3495. doi: 10.1016/J.BPJ.2018.11.3134.

[160] E. Flores-Figueroa, S. Varma, K. Montgomery, P. L. Greenberg, and D. Gratzinger. Distinctive contact between cd34+ hematopoietic progenitors and cxcl12+ cd271+ mesenchymal stromal cells in benign and myelodysplastic bone marrow. *Laboratory Investigation 2012 92:9*, 92:1330–1341, 6 2012. ISSN 1530-0307. doi: 10.1038/labinvest.2012.93. URL https://www.nature.com/articles/labinvest201293.

[161] S. Riis, F. M. Nielsen, C. P. Pennisi, V. Zachar, and T. Fink. Comparative analysis of media and supplements on initiation and expansion of adipose-derived stem cells. *Stem Cells Translational Medicine*, 5:314–324, 3 2016. ISSN 2157-6564. doi: 10.5966/SCTM.2015-0148. URL https://academic.oup.com/stcltm/article/5/3/314/6397906.

[162] F. Paduano, M. Marrelli, F. Palmieri, and M. Tatullo. Cd146 expression influences periapical cyst mesenchymal stem cell properties. *Stem Cell Reviews and Reports*, 12:592–603, 10 2016. ISSN 15586804. doi: 10.1007/S12015-016-9674-4/TABLES/1. URL https://link.springer.com/article/10.1007/s12015-016-9674-4.

[163] M. G. Scioli, G. Storti, A. Bielli, M. Sanchez, M. Scimeca, J. M. Gimble, V. Cervelli, and A. Orlandi. Cd146 expression regulates osteochondrogenic differentiation of human adipose-derived stem cells. *Journal of Cellular Physiology*, 237:589–602, 1 2022. ISSN 1097-4652. doi: 10.1002/JCP.30506. URL https://onlinelibrary.wiley.com/doi/full/10.

1002/jcp.30506https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.30506https://onlinelibrary.wiley.com/doi/10.1002/jcp.30506.

[164] M. Barilani, F. Banfi, S. Sironi, E. Ragni, S. Guillaumin, F. Polveraccio, L. Rosso, M. Moro, G. Astori, M. Pozzobon, and L. Lazzari. Low-affinity nerve growth factor receptor (cd271) heterogeneous expression in adult and fetal mesenchymal stromal cells. *Scientific Reports 2018 8:1*, 8:1–11, 6 2018. ISSN 2045-2322. doi: 10.1038/s41598-018-27587-8. URL https://www.nature.com/articles/s41598-018-27587-8.

[165] R. J. Smith, A. Faroni, J. R. Barrow, J. Soul, and A. J. Reid. The angiogenic potential of cd271+ human adipose tissue-derived mesenchymal stem cells. *Stem Cell Research and Therapy*, 12:1–14, 12 2021. ISSN 17576512. doi: 10.1186/S13287-021-02177-0/FIGURES/6. URL https://link.springer.com/articles/10.1186/s13287-021-02177-0https://link.springer.com/article/10.1186/s13287-021-02177-0.

[166] F. A. Abo-Aziza and A. A. Zaki. The impact of confluence on bone marrow mesenchymal stem (bmmsc) proliferation and osteogenic differentiation. *International Journal of Hematology-Oncology and Stem Cell Research*, 11:121, 4 2017. ISSN 20082207. URL /pmc/articles/PMC5575725//pmc/articles/PMC5575725/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5575725/.

[167] R. Mehrasa, H. Vaziri, A. Oodi, M. Khorshidfar, M. Nikogoftar, M. Golpour, and N. Amirizadeh. Mesenchymal stem cells as a feeder layer can prevent apoptosis of expanded hematopoietic stem cells derived from cord blood. *International Journal of Molecular and Cellular Medicine*, 3:1, 2014. ISSN 2251-9637. URL /pmc/articles/PMC3927388//pmc/articles/PMC3927388/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3927388/.

[168] T. Walenda, G. Bokermann, M. S. V. Ferreira, D. M. Piroth, T. Hieronymus, S. Neuss, M. Zenke, A. D. Ho, A. M. Müller, and W. Wagner. Synergistic effects of growth factors and mesenchymal stromal cells for expansion of hematopoietic stem and progenitor cells. *Experimental Hematology*, 39:617–628, 6 2011. ISSN 0301-472X. doi: 10.1016/J.EXPHEM.2011.02.011.

[169] M. Hammoud, M. Vlaski, P. Duchez, J. Chevaleyre, X. Lafarge, J. M. Boiron, V. Praloran, P. B. de la Grange, and Z. Ivanovic. Combination of low o2 concentration and mesenchymal stromal cells during culture of cord blood cd34+ cells improves the maintenance and proliferative capacity of hematopoietic stem cells. *Journal of Cellular Physiology*, 227:2750–2758, 6 2012. ISSN 1097-4652. doi: 10.1002/JCP.23019. URL https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.23019https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.23019https://onlinelibrary.wiley.com/doi/10.1002/jcp.23019.

[170] N. Ketheesan, C. Whiteman, A. B. Malczewski, R. G. Hirst, and J. T. L. Brooy. Effect of cryopreservation on the immunogenicity of umbilical cord blood cells. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for*

Haemapheresis, 30:47–54, 2004. ISSN 1473-0502. doi: 10.1016/J.TRANSCI.2003.05.002. URL https://pubmed.ncbi.nlm.nih.gov/14746821/.

[171] R. Majeti, C. Y. Park, and I. L. Weissman. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell stem cell*, 1:635, 12 2007. ISSN 19345909. doi: 10.1016/J.STEM.2007.10.001. URL /pmc/articles/PMC2292126//pmc/articles/PMC2292126/?report=abstracthttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC2292126/.

[172] A. Sorrentino, M. Ferracin, G. Castelli, M. Biffoni, G. Tomaselli, M. Baiocchi, A. Fatica, M. Negrini, C. Peschle, and M. Valtieri. Isolation and characterization of cd146+ multipotent mesenchymal stromal cells. *Experimental hematology*, 36:1035–1046, 8 2008. ISSN 0301-472X. doi: 10.1016/J.EXPHEM.2008.03.004. URL https://pubmed.ncbi.nlm.nih.gov/18504067/.