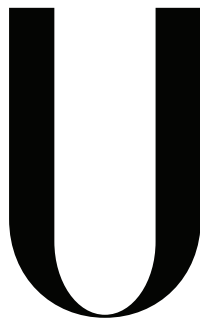


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**ISOLATION, CHARACTERIZATION AND *EX-VIVO*
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DERIVED-MESENCHYMAL STEM/STROMAL CELLS**

Tiago João dos Santos Fernandes Ferro

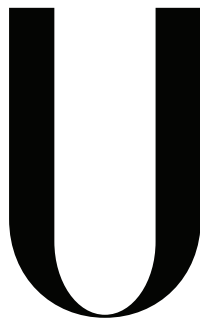
DISSERTAÇÃO

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Dissertação orientada por:

Professor Doutor Joaquim Sampaio Cabral

Professor Doutor Hugo Alexandre Ferreira

2013

FOR ALL THOSE WHO ALWAYS WANTED TO,
BUT NEVER COULD.

RESUMO

As células estaminais mesenquimais ou mesenquimatosas (do inglês '*mesenchymal stem cells*', MSCs) são actualmente alvo de investigação intensa a nível mundial, devido às suas inúmeras propriedades já conhecidas, com potenciais aplicações no campo da medicina regenerativa e na engenharia de tecidos e órgãos artificiais.

A definição actualmente aceite para células estaminais permite definir duas características universais deste tipo de células. Por um lado, apresentam a capacidade de se auto-renovarem de forma ilimitada, exibindo também a capacidade de se diferenciarem num ou mais tipos de células especializadas, de diferentes linhagens.

De acordo com os tipos de células estaminais reconhecidos, as células estaminais mesenquimais são consideradas células estaminais adultas, que residem virtualmente em todos os órgãos do organismo humano. No entanto, embora morfologicamente semelhantes, as células estaminais do mesênquima isoladas de diversas fontes podem ser funcionalmente diferentes, e sabe-se que esta população celular apresenta características distintas consoante a sua localização ou nicho no organismo. No entanto, é globalmente aceite que o seu propósito concentra-se na manutenção fisiológica dos tecidos e órgãos onde estão presentes, com a capacidade de reparar lesões nos tecidos em caso de trauma.

Por outro lado, devido a estudos *in vitro* realizados, o reconhecimento do potencial terapêutico e as possibilidades de utilização destas células foram aprofundados. Actualmente, a sua utilização clínica é reconhecida em várias patologias e doenças, como por exemplo, a doença do enxerto contra o hospedeiro (do inglês '*graft-versus-host disease*', GvHD). As células estaminais do mesênquima têm a capacidade de modelar selectivamente e de forma considerável as respostas imunes do hospedeiro, e é esse o fundamento para a sua utilização no tratamento de patologias auto-imunes.

Através de secreção de factores solúveis (prostaglandinas, várias interleucinas, factores de crescimento, angiogénicos, antifibróticos, antiapoptóticos, por exemplo) e de interacções directas com células do sistema imunitário, apresentam propriedades anti-inflamatórias e imunomoduladoras predominantes, com capacidade de suprimir a actividade de linfócitos T citotóxicos, linfócitos B e linfócitos NK, contribuindo para um estado regulador e supressor das respostas imunes.

Considerando os fins terapêuticos descritos, as células estaminais do mesênquima são normalmente isoladas através de biópsias da medula óssea e do tecido adiposo, no primeiro caso através de procedimentos considerados invasivos e no segundo através de métodos de rotina durante lipoaspirações. Uma fonte alternativa para este tipo de células está actualmente a ser explorada na matriz do cordão umbilical, cujos tecidos são normalmente descartados após o parto.

No entanto, embora o seu potencial clínico seja reconhecido, o número de células estaminais mesenquimais existentes em cada organismo é consideravelmente reduzido. As doses clínicas actualmente aceites estipulam a utilização entre 1 a 5 milhões de células por quilograma de massa corporal do doente. Torna-se assim essencial o desenvolvimento de estratégias que permitam a expansão *ex-vivo* deste tipo de células, mantendo a sua qualidade e ao mesmo tempo assegurando o cumprimento de normas internacionais para a sua utilização clínica.

As células estaminais do mesênquima tradicionalmente isoladas a partir da medula óssea possuem uma elevada capacidade de expansão em cultura, podendo inclusive ser estimuladas para adquirir propriedades específicas. Diversos *cocktails* de citocinas podem ser adicionados aos meios de cultura utilizados, induzindo a proliferação ou a diferenciação numa linhagem particular, originando células especializadas e que possuem as características próprias de um órgão ou tecido. Por outro lado, a utilização de terapias génicas está actualmente a ser analisada, com a utilização de células estaminais do mesênquima como vectores, que expressam um gene de interesse transientemente numa região específica do organismo.

Neste trabalho pretendeu-se analisar e estudar as características particulares de uma nova fonte de células estaminais do mesênquima, os tecidos sinoviais existentes nas cartilagens articulares. É actualmente postulado que as células estaminais mesenquimais derivadas dos tecidos sinoviais poderão apresentar características bioquímicas relevantes (por exemplo, secreção de factores anti-inflamatórios), e um potencial de diferenciação condrogénico superior relativamente às fontes clássicas. Esta possibilidade torna-se particularmente pertinente no tratamento de patologias osteocondrais, uma vez que as terapêuticas actualmente utilizadas visam a atenuação de sintomas e não o tratamento efectivo das causas subjacentes.

As patologias associadas à degeneração da cartilagem, principalmente osteoartrite, tornam-se mais frequentes a nível mundial com o envelhecimento da população. Devido à sua natureza avascular e aneural, a cartilagem apresenta um potencial de regeneração limitado e os procedimentos terapêuticos actualmente aceites consistem na implantação autóloga de condrócitos, recolhidos de um local não sujeito a forças compressivas da articulação, seguido da sua expansão *ex-vivo* em laboratório. Embora praticável, esta técnica não permite responder a todas as limitações e problemas relacionados com a etiologia das patologias em questão.

Neste trabalho foram utilizadas amostras biológicas recolhidas através de biópsias realizadas durante cirurgias artroscópicas de rotina no Centro Hospitalar de Lisboa Ocidental (Hospital de Egas Moniz e Hospital São Francisco Xavier). Foram considerados dadores com degeneração e processos inflamatórios associados à cartilagem, bem como dadores saudáveis, sem patologias permanentes e de longa duração associadas à cartilagem, embora com lesões traumáticas. Foram seleccionados 19 dadores, com idades entre os 20 e 78 anos (9 do sexo masculino). De cada dador foram recolhidas biópsias directas da membrana sinovial, com extracção na bursa suprapatelar. Foram ainda recolhidos os resíduos restantes do sistema *shaver* utilizado durante a artroscopia. Nos dadores com processos inflamatórios e edema da cavidade sinovial procedeu-se igualmente à recolha do fluido sinovial, onde também se encontram células mesenquimais em circulação. É importante salientar que a recolha do fluido sinovial não foi possível em dadores sem processos inflamatórios associados à cartilagem. Nestes dadores, o fluido apresenta apenas um volume residual cuja função é lubrificar as superfícies articulares durante o movimento.

O isolamento das células estaminais mesenquimais a partir das biópsias recolhidas permitiu estimar uma taxa de sucesso entre os 75 e 100%, com eficiências maiores nos dadores mais jovens. O tempo de isolamento na passagem inicial foi consideravelmente longo, comparado com outras fontes de células estaminais mesenquimais, com uma duração entre 21 e 37 dias.

Foram realizadas cinéticas de crescimento em diferentes condições, num regime estático e dinâmico. A densidade inicial de plaqueamento é relevante quando se pretende expandir células em cultura, cujo objectivo em diversas aplicações clínicas (por exemplo, tratamento de doenças e patologias osteocondrais) será a obtenção do maior número de

células no menor intervalo de tempo possível. Uma densidade inicial de 3 000 células/cm² revelou o melhor desempenho entre outras densidades possíveis, não comprometendo uma sinalização intercelular deficitária nem uma inibição de crescimento por contacto directo com células vizinhas.

As condições hipóxicas (2 – 10% O₂) são consideradas uma propriedade biológica presente na maior parte dos órgãos. Foi demonstrado que a cultura de células em tensões reduzidas de oxigénio permite uma expansão mais eficiente, prevenindo ainda vários processos de degeneração e senescência celular, permitindo obter células com características mais próximas daquelas que existem *in vivo*. Cinéticas de crescimento realizadas em hipóxia das células recolhidas a partir dos tecidos sinoviais permitiram obter resultados semelhantes aos existentes na literatura para outras fontes (particularmente medula óssea). Um crescimento num ambiente com 2% de oxigénio permitiu obter uma proliferação mais elevada (42 ± 3) face às condições de normóxia (*fold increase*: 25 ± 5) ao fim de 12 dias.

A expressão dos marcadores celulares recomendados internacionalmente, que permitem a identificação de populações de células estaminais mesenquimais, também foi quantificada por citometria de fluxo. As células isoladas expressaram os marcadores CD73, CD90 e CD105, simultaneamente com expressão negativa de marcadores associados a linhagens hematopoiéticas, entre outras. As capacidades multipotentes destas células foram avaliadas pela indução da diferenciação nas três linhagens clássicas para células do mesênquima: osteogénica, condrogénica e adipogénica.

Os resultados obtidos parecem sugerir que as células mesenquimais derivadas dos tecidos sinoviais apresentam um potencial de diferenciação condrogénico superior face às outras fontes, o que se poderá revelar particular útil em terapias celulares futuras no tratamento de várias patologias osteocondrais.

Para maximizar a expansão *ex-vivo* em condições estáticas foram testados diferentes substratos que permitem aumentar as taxas de adesão e proliferação celular em condições de cultura. A proteína fibronectina, abundante na matriz extracelular, revelou-se o melhor substrato para melhorar as condições de expansão, associado a uma densidade inicial de plaqueamento adequada. A utilização deste substrato de adesão permitiu aumentar o número de células obtidas num período de tempo menor (*fold increase* 28 ± 1 versus 19 ± 4 ao fim de 12 dias).

Um sistema de cultura dinâmico em *spinner flasks*, utilizado *microcarriers* como substrato sólido também foi desenvolvido para maximizar a expansão *ex-vivo* destas células. Utilizando um meio de cultura sem soro e livre de substâncias xenogénicas, foi realizada a expansão de forma eficiente, simultaneamente com a manutenção das propriedades celulares características das células mesenquimais e do seu potencial de diferenciação.

Os *microcarriers* utilizados foram revestidos com um substrato baseado num lisado plaquetário humano, utilizado como substituto de soro em meios de cultura. Um revestimento diferente foi também utilizado, com uma substância disponível comercialmente (CELLStart™) indicada para a expansão de células em condições de cultura sem soro adicionado. Após 12 dias em condições dinâmicas, o *fold increase* máximo obtido foi de 9.9 ± 0.8 . Uma análise ao perfil metabólico das células também foi desenvolvida durante a expansão dinâmica. As taxas de consumo de glucose e produção de lactato calculadas permitiram evidenciar um metabolismo baseado na glicólise aeróbia para a produção de energia.

Estes resultados sugerem que os tecidos sinoviais podem ser considerados uma fonte viável de células estaminais do mesênquima, com aplicações concretas na regeneração e tratamento de patologias osteocondrais. No entanto, as condições de expansão *ex-vivo* ainda necessitam de ser optimizadas, bem como os meios de cultura utilizados que ainda não são totalmente livres de factores xenogénicos. Os processos de isolamento também devem ser optimizados, o que permitirá obter o maior número possível de células a partir de uma única amostra, levando a uma maior eficiência dos processos de expansão.

Palavras-chave: Células estaminais mesenquimais/mesenquimatosas, tecidos sinoviais, isolamento, caracterização, expansão *ex-vivo*, microcarriers, aplicações clínicas

ABSTRACT

Increasing attention is now being given to the use of mesenchymal stem cells (MSCs) in cell therapies and tissue engineering approaches. Articular cartilage is a tissue which can benefit from the clinical application of stem cell research, due to its avascular and aneural nature, that impairs natural healing and regeneration processes. This project aims at the isolation, characterization and *ex-vivo* expansion of human mesenchymal stem cells (hMSCs) derived from both the synovium and the synovial fluid. The comparative characterization to the classical sources of hMSCs, which consists of umbilical cord matrix (UCM), bone marrow (BM) and adipose tissue (AT), is expected to create a sustainable technological platform for the clinical-scale production of hMSCs for cellular therapies with different applications.

Isolation of hMSCs from synovial tissues had a success rate ranging from 75 to 100%, with a higher yield obtained from younger donors. Samples were obtained from 19 donors, age range 20-78 years (9 males). Isolation time and initial passage duration ranged between 21 – 37 days.

Growth kinetics under different initial seeding densities have shown that the largest number of cells is obtained for an initial seeding density of 3 000 cells/cm². Also, the growth in hypoxic conditions was evaluated, confirming previous results for the other sources, with a higher fold increase (42 ± 3) in a low-oxygen environment than in normoxic conditions (fold increase: 25 ± 5). Cell surface markers were identified by flow cytometry, confirming the expression of CD73, CD90 and CD105 while lacking classically defined negative markers for MSCs. The evaluation of differentiation capacity of these cells into three different lineages (osteogenic, chondrogenic and adipogenic) was also performed. Preliminary results have suggested that hMSCs isolated from synovial tissues have a superior chondrogenic differentiation potential, which is particularly useful for the treatment of osteochondral defects and diseases.

For maximizing *ex vivo* expansion potential under static conditions, different surface coatings were tested. Fibronectin proved to be an efficient substrate for improving cell growth, along with an appropriate initial seeding density, which allows achieving higher cell numbers in shorter time periods (*fold increase* 28 ± 1 versus 19 ± 4 after 12 days). A dynamic, serum- and xeno-free culture system (spinner flasks) using microcarriers also

proved to be effective in expanding synovium-derived MSCs, while maintaining desired cell properties and differentiation potential. Beads were coated either with *HPL* or a commercially available cell attachment substrate (CELLStart™), achieving a maximum fold increase of 9.9 ± 0.8 after 12 days of culture. Metabolic profiling was also performed under dynamic culture conditions. Analysis of glucose uptake and lactate production rates showed that cells rely mainly on aerobic glycolysis for the production of energy.

These results suggest that synovial tissues can be seen as a viable source for adult mesenchymal stem cells, especially for tissue replacement strategies on osteoarthritic joints. Nevertheless, there is a need to optimize the *ex-vivo* expansion protocols and xenogenic-free culture media to achieve a considerable cell number for clinical applications. The isolation process must also be optimized, in order to obtain the highest possible number of cells from a single sample.

Keywords: Mesenchymal stem cells, synovial tissues, isolation, characterization, *ex-vivo* expansion, microcarriers, clinical properties

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LIST OF ABBREVIATIONS

AChR	Acetylcholine receptor
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
AM	Amniotic membrane
ASCs	Adult stem cells
AT	Adipose tissue
BM	Bone marrow
CCL-	CC chemokine ligands
CD	Cluster of differentiation
CFI	Cumulative fold increase
CXCL-	CXC chemokine ligands
CXCR-	CXC chemokine receptors
DAPI	4',6-diamino-2-phenylindole dilactate
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DP	Dental pulp
ECM	Extracellular matrix
EPCs	Endothelial progenitor stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
F-CFUs	Fibroblastic colony-forming units
FCS	Fetal calf serum
FGF-2	Fibroblast growth factor-2
FI	Fold increase
FITC-	Fluorescein isothiocyanate
flk-3L	fms-like tyrosine kinase-3 ligand
FLS	Fibroblasts-like synoviocytes
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GT	Gingival tissue

GvHD	Graft- <i>versus</i> -host disease
hESCs	Human embryonic stem cells
HGF	Hepatocyte growth factor
HIM	Hematopoietic inductive microenvironment
hMSCs	Human mesenchymal stem cells / multipotent stromal cells
HPGF	Human platelet growth factor
HPL	Human platelet lysate
HPLC	High-performance liquid chromatography
HSCs	Hematopoietic stem cells
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon gamma
IgA	Immunoglobulin isotype A
IgG	Immunoglobulin isotype G
IgM	Immunoglobulin isotype M
IL-	Interleukin
IMDM	Iscove's modified Dulbecco's medium
iNOS	Induced nitric oxide synthases
ISCs	Intestinal stem cells
LIF	Leukemia inhibitory factor
MACS	Magnetic-activated cell sorting
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MNCs	Mononuclear cells
MPPs	Matrix metalloproteinases
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
MT	Muscle tissue
MTSCC-ISCT	Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy
NIH	National Institutes of Health, USA
NSCs	Neural stem cells
PBS	Phosphate buffered saline
PD	Population doublings

PE-	Phycoerythrin
PGE2	Prostaglandin E2
q_{Met}	Metabolite specific uptake/production rate
SC	Serum containing
SCF	Stem cell factor
SCs	Stem cells
SDF-1	Stromal cell-derived factor 1
SF	Serum-free
sHLA-G5	Soluble human leukocyte antigen G5
SMSCs	Synovium-derived mesenchymal stem/stromal cells
ST	Synovial tissues
Tc	T cytotoxic cell subset
TGF- β	Transforming growth factor beta
Th	T helper cell subset
TIMP-	Tissue inhibitor of metalloproteinase
TNF- α	Tumor necrosis factor alpha
μ_{app}	Apparent specific growth rate
UCB	Umbilical cord blood
UCM	Umbilical cord matrix
VEGF	Vascular endothelial growth factor
VLA-	Very late antigen

I INTRODUCTION

1.1 STEM CELLS

Stem cells (SCs) are unique cells, which have unlimited or prolonged ability to self-renew (to proliferate and maintain a constant pool of cells, in case of asymmetric cell division) and the potential to differentiate into many cell types that are part of an organism (Kondo *et al.*, 2003). These features are inversely related, as the more committed a cell is to a specific lineage, the less the potential to self-renew and vice versa (*Figure 1*) (Polak *et al.*, 2008).

In humans, there are two major types of stem cells: embryonic stem cells (hESCs), isolated from the inner cell mass of blastocysts, and adult stem cells (ASCs), which exists virtually in every tissue and organ. The most common sources for adult stem cells include bone marrow (BM), peripheral blood and adipose tissue (AT). Neonatal tissues such as umbilical cord blood and matrix (UCB/M) are also used as a source for ASCs. Although ASCs have already suffered some type of commitment to a specific cell lineage, they can yet give rise to many cell types (the ones that replenish that organ or tissue, for instance) but this comes with the price of decreasing their ability to self-renew.

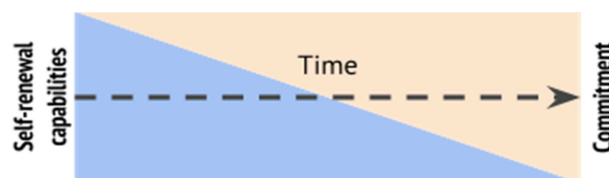


FIGURE 1 Antagonism between self-renewal capabilities and commitment of a specific cell.

For this reason, and from the expansion point of view, hESCs are the best source of stem cells due to their source and earlier origin (Polak *et al.*, 2008). These cells are derived from the inner cell mass of the blastocyst, and present a pluripotent differentiation potential, being able to differentiate into any cell type of all three germ layers: endoderm, mesoderm and ectoderm (Kondo *et al.*, 2003). Currently, research with hESCs and their clinical use in regenerative medicine arises several ethical issues, being highly controlled and legislated, as the harvesting of these cells requires the destruction of human embryos. Furthermore, studies performed using hESCs also induce the irrepressible development of teratomas *in vivo* as well as an increase of genomic

abnormalities related to imprinting, which raises many safety issues still to be addressed (Erdö *et al.*, 2003; Sapienza, 2002).

Relating to these issues, ASCs are becoming the focus of interest for both scientific and clinical communities due to their ability to overcome the problems associated with hESCs (although to some limited extent). These cells reside in specific tissues and organs, and can give rise to any cell type that replenishes that tissue. Examples of these cells include the well-known hematopoietic stem cells (HSCs), endothelial progenitor stem cells (EPCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), intestinal stem cells (ISCs) and others. All of these stem cells are widely dispersed in the organism, populating specific locations and organs (Atala *et al.*, 2008).

The limited ability of certain organs and tissues to regenerate has stimulated interest about the nature of cells involved in injured tissue repair and the processes in which they are involved. The discovery of MSCs and their properties allowed the understanding of some of these processes and led to new possible therapeutic modalities (Marshak *et al.*, 2001). Nowadays, the research and clinical of MSCs is growing because these cells are relatively easy to isolate from various tissues and expand *ex-vivo* under standard culture conditions. Also, MSCs are able to produce a wide range of cytokines and growth factors, besides being hypo-immunogenic, which can lead to their increased use in heterologous transplants for the treatment of many diseases and conditions.

In response to chemical, hormonal or structural stimuli, MSCs can differentiate into cells of mesodermal derived tissues such as osteocytes, chondrocytes, myocytes, adipocytes, bone marrow stroma, tendon, and a variety of other connective tissues. Some authors also reported MSCs ability to differentiate into other tissues (mainly neural tissue, derived from the ectoderm germ layer), adding to these cells even more plasticity which needs to be further investigated (Tropel *et al.*, 2009; Aly *et al.*, 2012). Additionally, MSCs have immunomodulatory effects and can be used *in vivo* as cellular therapies for the treatment of immune diseases (Summer *et al.*, 2008).

1.2 MESENCHYMAL STEM CELLS

In the end of 1960s and beginning of 1970s, the pioneer work of Alexander J. Friedenstein described for the first time the isolation and some properties of a new

population of stromal cells derived from the bone marrow of guinea pigs (Friedenstein *et al.*, 1976).

These cells were first identified as non-hematopoietic and adherent, after seeding whole bone marrow in plastic culture dishes. After 4 hours the non-adherent suspension was washed out and the remaining cells showed the ability to form fibroblastic colonies *in vitro*. This population was firstly termed as fibroblastic colony-forming units (F-CFUs) due to results obtained (Friedenstein *et al.*, 1968), while the term Mesenchymal Stem Cells (MSCs) was only introduced by Caplan in the late 1980s (Caplan, 1988). Due to this, bone marrow derived MSCs are still the most frequently studied cell type and often designated as the gold standard source.

The original *in vitro* progeny of CFU-Fs cultured by Friedenstein and colleagues were later found to have the ability to differentiate along osteogenic, adipogenic and chondrogenic pathways, both *in vitro* and also when implanted *in vivo*. This ability to differentiate into many mesoderm-specific lineages remains one of the most important features of MSCs, and is considered as one criterion (amongst others) to identify a true population of mesenchymal stem cells (Ohgushi & Caplan, 1999).

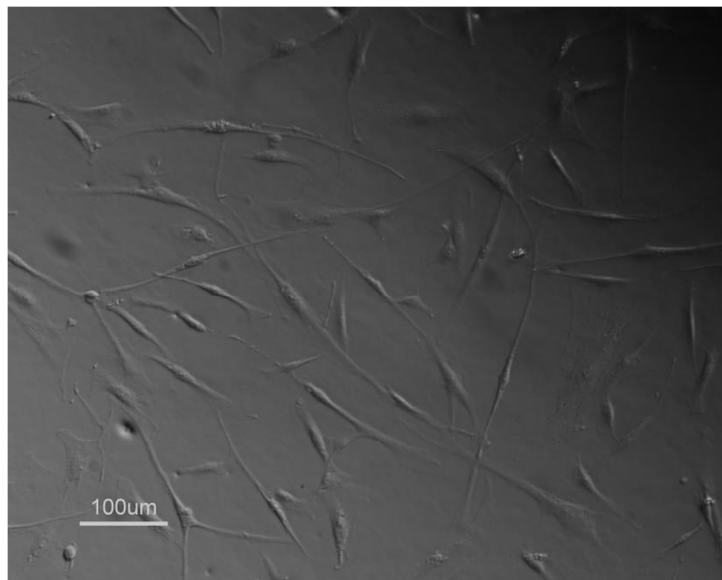


FIGURE 2 Human MSCs from bone marrow under standard culture conditions.

Currently, there are no defined criteria to phenotypically identify MSCs populations unambiguously. Although these cells express an array of surface markers, not a single marker that specifically defines the true *in vivo* MSCs from all sources could be identified.

Therefore, positive and negative phenotypic staining is usually performed, which results in a loose phenotypic definition with significant controversies in this area.

To address this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (MTSCC-ISCT) proposed minimal criteria that define true populations of human MSCs (Dominici *et al.*, 2006). ISCT proposed also that human fibroblast-like, plastic-adherent cell populations, as the ones identified by Friedenstein, should be termed “multipotent mesenchymal stromal cells” instead of the more restrictive name specified by Caplan (Horwitz *et al.*, 2005).

The first criterion defined by MTSCC-ISCT to identify human MSCs is their plastic adherence, when the cells are cultured in standard conditions. The second criterion relates to cell surface receptors expression: MSCs populations must express (>95% positive) CD105, CD73 and CD90, as measured by flow cytometry and must lack the expression (<2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. The last criterion relies on the biologic property that most uniquely identifies MSC, the capability for trilineage mesenchymal differentiation (cells must give rise to osteoblasts, adipocytes and chondroblasts under standard *in vivo* differentiation conditions) (Dominici *et al.*, 2006).

1.3 SOURCES

Mesenchymal stem cells are multipotent stem cells originated from the mesoderm germ layer and which exist in the perivascular sites of nearly all adult tissues and organs. The most well-known sources for MSCs include BM, AT and UCM. These are known as “classical sources”, as they were first characterized and are the most widely used in clinical research and applications. Dental pulp (DP), muscle tissue (MT) and amniotic membrane (AM) are currently accepted as alternative sources for MSCs. New sources under investigation for these cells include synovial tissues (ST) and gingival tissue (GT) (Samad, 2010; Paracchini *et al.*, 2012).

MSCs from different sources differ in terms of easiness of isolation and potential to proliferate and differentiate. The collection method and the risk of viral contamination also vary, as well as the diversity of cytokines and chemokines that are produced by cells isolated from different tissues (Klingemann *et al.*, 2008; Monteiro *et al.*, 2010). Since MSCs isolated from BM, UCM and AT are the most studied, these are the ones that will be discussed further herein, as well as the synovial tissues.

1.3.1 BONE MARROW

Bone marrow is the soft tissue that fills the interior of bones. It is a particularly specialized tissue, with several biological functions incorporating the production of the majority of blood cells in adults. The red bone marrow consists mainly of hematopoietic tissue, controlling the constant production of blood cells, while yellow bone marrow is made of fibroblasts, osteoblasts, osteoclasts, adipocytes, endothelial cells, macrophages, involved by an intricate 3D extracellular matrix, which altogether are responsible for producing colony stimulating factors and creating the favorable environment for hematopoiesis (HIM). MSCs represent only 0.001 to 0.01% of the bone marrow cell population, supporting hematopoiesis mainly by providing extracellular matrix components, cytokines and growth factors (Atala *et al.*, 2008; Moore *et al.*, 2006).

The collection method involves a bone marrow biopsy, usually taken from the iliac crest, which is considered an invasive procedure. BM-MSCs tend to decrease their frequency and lose their proliferative capacity with the age of the donor, and there seems to be a significant decline in their ability to differentiate after age 20 (Klingemann *et al.*, 2008).

1.3.2 ADIPOSE TISSUE

Adipose tissue is a loose connective tissue composed by fibroblasts, macrophages, endothelial cells, but mainly by adipocytes. This tissue also has MSCs embedded within. In humans, adipose tissue is located beneath the skin, around internal organs and breast tissue, and can easily be obtained from liposuctions, perhaps the simplest harvesting procedure of all adult sources (Junqueira, 2004; Gilbert, 2003).

1.3.3 UMBILICAL CORD MATRIX

The umbilical cord establishes the connection between the embryo or fetus and the placenta, being composed by two arteries and one vein embedded within Wharton's jelly and surrounded by an epithelium layer (Qiao *et al.*, 2008). The Wharton's jelly is a mucous connective tissue composed by fibroblasts and occasionally mast cells surrounded by an amorphous matrix rich in collagen (type I, III, IV, VI and VII) and glycosaminoglycans (GAGs) (Majore *et al.*, 2011; Fan *et al.*, 2011).

While the blood that remains in placenta and umbilical cord after birth (UCB) is a main source for HSCs, the Wharton's jelly or UCM is a source of MSCs. The attempts to isolate MSCs from the UCB were ineffective (34-63% success when in UCM it reaches 100%) (Shetty *et al.*, 2006; Kern *et al.*, 2006). The isolation procedure of MSCs from UCM is non-invasive, for both mothers and children.

1.3.4 SYNOVIAL TISSUES

Synovial joints are the most common joints in mammals, and are responsible for the movement and contact of articulating bones. Articular or hyaline cartilage is the main tissue involved in the movement and mobilization of these joints, which is lubricated by the synovial fluid inside the articular capsule.

The articular cartilage appears pearly bluish in color, with firm consistency and considerable collagen (mainly type II) associated with hyaluronic acid and proteoglycans (Junqueira, 2004). These charged molecules are responsible for the high hydration of this tissue. Furthermore, synovial tissues contain no nerves or blood vessels, and its structure is relatively simple, with chondrocytes buried deep into the extracellular matrix of articular cartilage. Each cell makes no direct contact with others. Interestingly, MSCs are also present, as their existence reported firstly for the synovial membrane (De Bari *et al.*, 2001) and later also for the synovial fluid (Jones *et al.*, 2004).

MSCs from synovial tissues can be collected from the synovial fluid, from a synovium direct biopsy or from the filtrate material obtained by using a shaver system during routine arthroscopies, a procedure considered less invasive than bone marrow biopsy (Yokoyama *et al.*, 2005).

1.3.5 A PERIVASCULAR ORIGIN FOR ALL MESENCHYMAL STEM CELLS

Although there are many isolation sources now being used, as stated before, mesenchymal stem cells still lack a detailed and comprehensive true identity characterization, which is also impaired by the lack of knowledge of their innate distribution over the body. MSCs are heterogeneous and only defined subpopulations of these are able to sustain hematopoietic stem cells, regulate immune responses, or differentiate into mesodermal cell lineages. Recent literature supports the evidence that mesenchymal stem cells are found in perivascular sites (adjacent to blood vessels, both

arteries and veins – pericytes, see *Figure 3*), for almost every organ in the body (Crisan *et al.*, 2008).

Further research concluded that pericytes are undistinguishable from populations of mesenchymal stem cells isolated from bone marrow donors, as isolated pericytes exhibit a panel of cell surface markers that are identical to those expressed by MSCs and vice-versa (Caplan, 2008).

This fact led Caplan to speculate if all mesenchymal stem cells have a perivascular origin and are therefore pericytes. If most or all MSCs are indeed pericytes, this feature could lead to new options regarding the physiology and therapeutic actions of mesenchymal stem cells in many conditions and clinical applications, mainly due to cell migration to sites of injury.

Pericytes do not have the capacity of differentiating into hematopoietic or neural phenotypes in adults, although the perivascular site of hematopoietic and neural stem cells in early fetal tissue (Kiel *et al.*, 2005; Hirshi & D'Amore, 1996) implies that other types of stem cells inhabit the perivascular niche. Although many (if not all) of MSCs can be in fact pericytes, this observation led to the clue that the inverse of Caplan speculation is not true, so not all pericytes can be classified as MSCs as the blood vessels are surrounded by many cell types with highly differentiated functions.

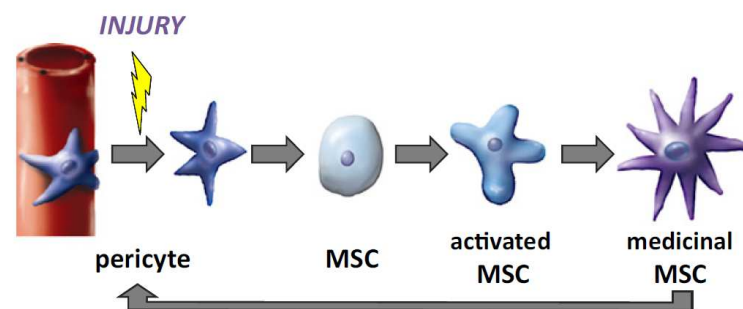


FIGURE 3 Model of pericyte activation and mobilization into active mesenchymal stem cells following injury or trauma on site, which releases cells from contact with blood vessels (Caplan, 2011).

Another interesting fact that could support the perivascular origin of MSCs relates to the expression of context-specific molecules on cell surface. Using a murine model of brain ischemia injury, MSCs lying close to blood vessels were shown to recruit and support neural precursors by expressing SDF-1 and angiopoietin-1 (Ohab *et al.*, 2006). The expression of these two markers is characteristic of pericytes, which could have driven the

observed recruitment and support of neural progenitors (Seo *et al.*, 2009; Sundberg *et al.*, 2002).

1.4 DIFFERENTIATION POTENTIAL

MSCs have multipotent differentiation properties *in vitro* and *in vivo*. (Muraglia *et al.*, 2000; Aslan *et al.*, 2006). Although initial studies were more focused on the differentiation potential of MSCs into the traditional mesodermal pathways (osteogenic, adipogenic and chondrogenic), Caplan proposed that MSCs are able to differentiate into any mesodermal associated tissue, including myogenic and tendon lineages (see *Figure 4*) (Caplan, 2011).

Even though biochemical characteristics of cells differentiated *in vitro* do not necessarily translate to *in vivo* applicability, the most common methods for differentiating MSCs into osteocytes, adipocytes and chondrocytes are discussed herein.

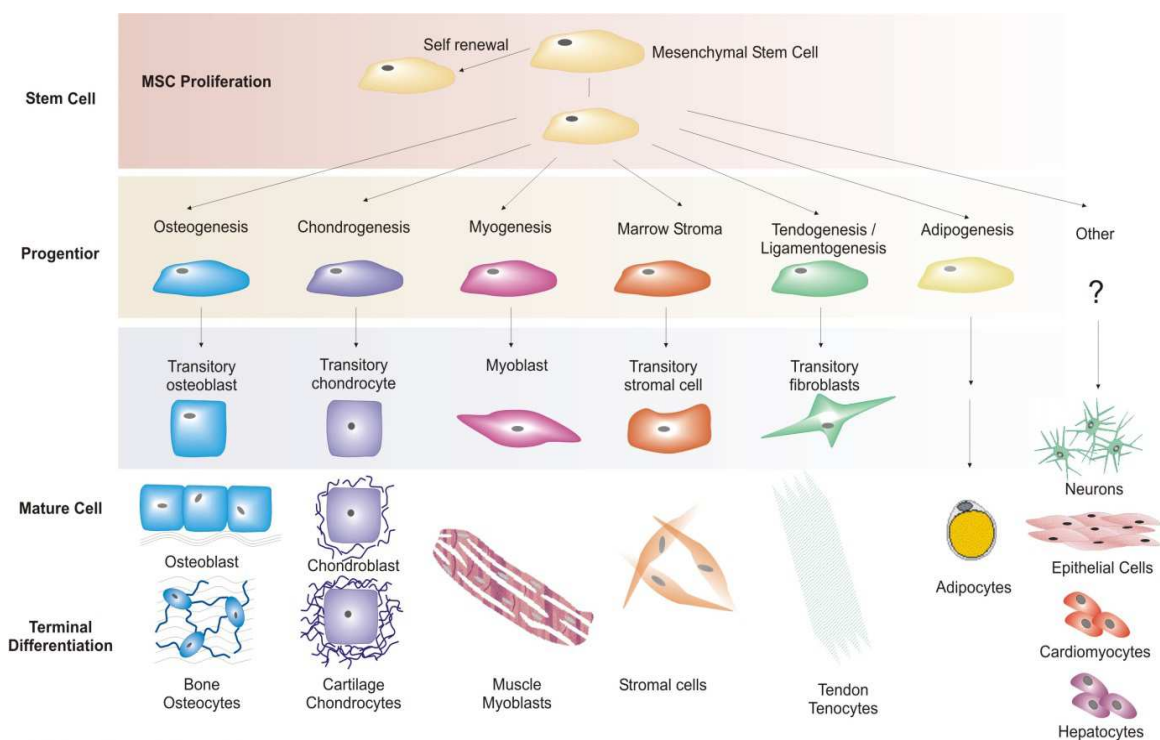


FIGURE 4 Overview of MSC multilineage differentiation potential. MSCs are able to extensively self-proliferate maintaining and undifferentiated phenotype prior to commit into a range of mesoderm-derived tissue and cell types, including bone, cartilage, muscle, marrow stroma and adipose tissue. There is also evidence of a higher degree of plasticity which allow these cells to differentiate into liver, heart, skin and neural tissue (Caplan & Bruder, 2001).

Osteogenic differentiation is usually achieved *in vitro* using a cocktail of various growth factors and cytokines which include dexamethasone, ascorbic acid-2-phosphate and beta-glycerophosphate (Okamoto *et al.*, 2002). Mineralization deposits are visible after one week of constant stimulation by these molecules, but the maximum results are obtained only after three weeks. The evaluation of this process is done by fixing and

staining the cells with silver nitrate for the von Kossa staining which highlights calcium deposits, marking later stages of differentiation. Also, the enzymatic activity of alkaline phosphatase is evaluated, as this enzyme is particularly abundant during active bone formation, staining bone progenitor cells (Jackson *et al.*, 2007).

For adipogenic differentiation, MSCs are cultured in a growth medium supplemented with dexamethasone, isobutylmethylxanthine and insulin for two weeks, with media change twice weekly (Sottile *et al.*, 2002). After some days, cells start to change their appearance to a round morphology and after 5 - 8 days start to accumulate lipids intracellularly in the form of lipid droplets. The evaluation of adipogenic differentiation is made staining the cells with Oil Red-O, a dye which binds to lipid vacuoles inside the cells. Quantitative measurements can be obtained by assessing the enzymatic activity of glycerol-3-phosphate dehydrogenase, a marker of mature adipocytes (Neubauer *et al.*, 2005).

Chondrogenic differentiation requires that the cells become more clustered and so is traditionally achieved by forcing the aggregation of 200 000 to 300 000 MSCs, creating a micromass pellet. Most culture media consist of dexamethasone, ascorbic acid phosphate, insulin, transferrin, selenous acid, linoleic acid and serum albumin. Further additives to this medium include sodium pyruvate, proline, glutamine and TGF- β (Suva *et al.*, 2004), the latter being a central growth factor inducing chondrogenesis *in vivo* (Emans *et al.*, 2007). After two weeks in culture, micromass pellets can be stained with Safranin-O or Alcian blue to highlight glycosaminoglycans, as well as the various extracellular matrix molecules produced, which include collagen (type II and XI), aggrecan, perlecan and syndecan (Derfoul *et al.*, 2006).

1.5 IMMUNOMODULATORY PROPERTIES

The immunomodulatory effect of MSCs mainly emerges from: (i) their ability to secrete a great variety of soluble molecules and (ii) direct cell interactions. The suppression of several immune functions exerted by diverse immunocytes such as T-, B-, and NK cells is an important property of MSCs (Hass *et al.*, 2011).

Mesenchymal stem cells are thought to be low-immunogenic and capable of modifying and controlling the immune response of an organism. These cells typically present low expression levels of the Major Histocompatibility Complex I (MHC-I), non or negligible

levels of MHC-II (Le Blanc *et al.*, 2005) and also lack co-stimulatory molecules (namely CD80/86). By not expressing MHC molecules, allogeneic MSCs are not detected and/or selected by T cells (virtually, the cells cannot be “seen” by the organism), and therefore no immune response is established against them.

This ‘immunoprivileged status’ allows MSCs to be used in transplants without leading to immunological diseases like graft-versus-host disease (GvHD) (Atala *et al.*, 2008; Monteiro *et al.*, 2010). However, this immunoprivileged status is unstable, as recent data of *in vitro* and *in vivo* studies revealed that cellular differentiation of MSC causes a shift from an immunoprivileged to an immunogenic phenotype, inducing cellular cytotoxicity or immune rejection (Huang *et al.*, 2010). Conversely, MSCs effects on the immune system may not be strictly suppressive, as a low dose of MSCs can stimulate dendritic cells prone to promoting T cell activation, whereas a high dose of MSCs were shown to cause the opposite effect (Le Blanc *et al.*, 2003). Furthermore, low amounts of IFN- γ elicit MSCs to express MHC-class II molecules, thus becoming professional antigen-presenting cells (Chan *et al.*, 2006).

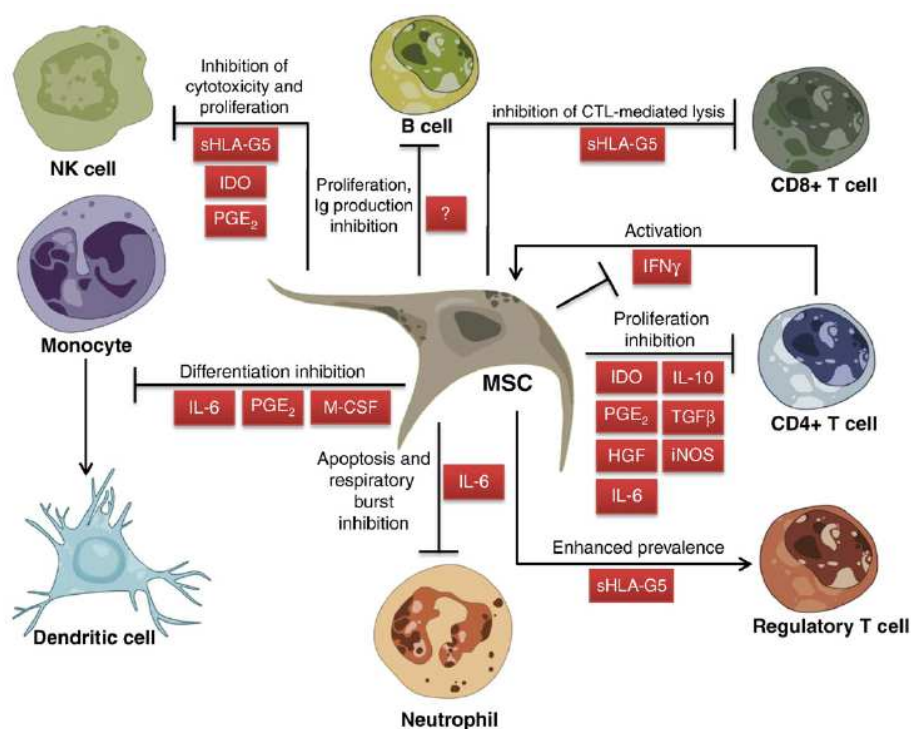


FIGURE 5 Summary of the soluble molecules and cell-surface proteins that mediate the effects of hMSCs and provide information about their local environment. Abbreviations: IFN gamma (interferon γ), IDO (indoleamine 2,3-dioxygenase), IL-10 (interleukin 10), IL-6 (interleukin 6) prostaglandin E2 (PGE2), transforming growth factor beta (TGF β), hepatocyte growth factor (HGF), induced nitric oxide synthases (iNOS), soluble human leukocyte antigen G5 (sHLA-G5), macrophage colony stimulating factor (M-CSF) (Ben-Ami *et al.*, 2011).

Another important feature of MSCs comprises trophic effects mediated by paracrine agents and soluble molecules (see *Figure 5*). These cells constitutively or upon stimulation secrete large amounts of cell signaling factors such as interleukin(IL)-1, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-14, IL-15, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), fms-like tyrosine kinase-3 ligand (flk-3L), CCL2, tissue inhibitor of metalloproteinase (TIMP) 2, transforming growth factor (TGF) beta, CXCL1, CXCL2, CXCL6, vascular endothelial growth factor (VEGF), and fibroblast growth factor-2 (FGF2) (Haynesworth *et al.*, 1996; Albini *et al.*, 1991; Di Nicola *et al.*, 2002; Patel *et al.*, 2010; Lama *et al.*, 2007; Ren *et al.*, 2008).

It is important to notice that some of the cytokines secreted by MSCs have a pivotal role supporting hematopoiesis both *in vivo* and *in vitro*, and this ability involves the constitutive secretion of soluble factors mentioned before, such as SCF, LIF, IL-6, and macrophage colony-stimulating factor (M-CSF). Furthermore, hematopoietic support can be further augmented by IL-1 α -induced secretion of G-CSF and GM-CSF (Majumdar *et al.*, 1998; Haynesworth *et al.*, 1996).

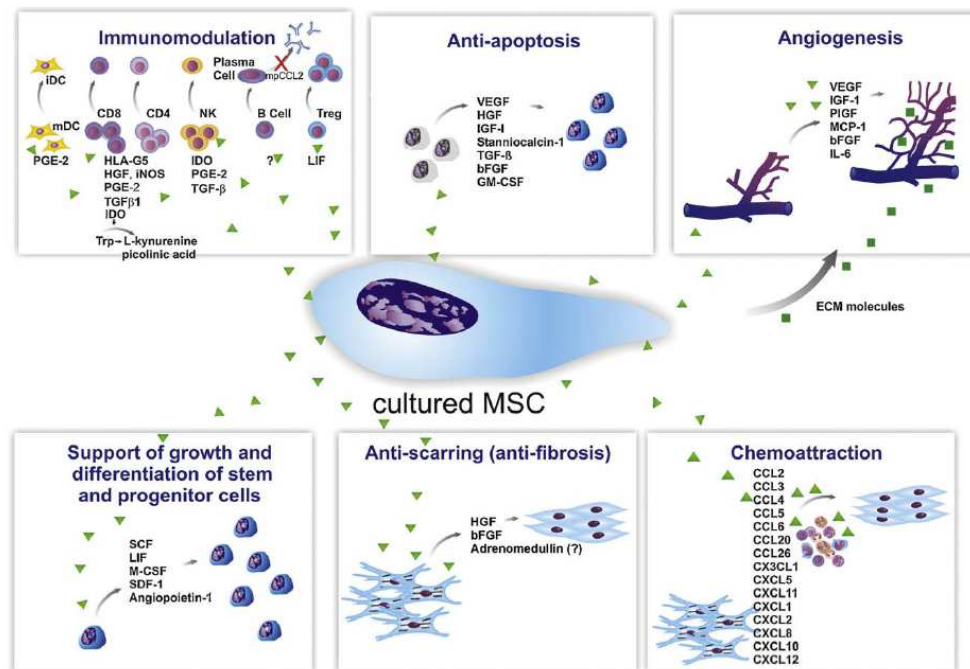


FIGURE 6 Overview of paracrine effects and secretome of mesenchymal stem cells. Soluble factors secreted by MSCs are thought to be essential in establishing cell interactions and responses. Active molecules can be classified into six main categories depending on their actions: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction (Meirelles *et al.*, 2009).

MSC paracrine effects (see *Figure 6*) can be divided into trophic, immunomodulatory, anti-scarring and chemoattractant. The trophic effects of MSCs can be further subdivided into anti-apoptotic (Togel *et al.*, 2007), supportive (stimulation of mitosis, proliferation and differentiation of organ-intrinsic precursor or stem cells) and angiogenic (Meirelles *et al.*, 2009).

These abilities to influence cells of the immune system seem to decrease or even disappear during *in vivo* differentiation. Considering this, MSCs seem to have an intrinsic capacity to promote an immunosuppressive environment, monocyte maturation and secretion of TNF- α , avoid allorecognition and reduce B-cell proliferation, while interfering with T-cell functions (Ankrum *et al.*, 2010; Atala *et al.*, 2008). The molecules secreted, when combined, may form an intricate biochemical environment, with some molecules exhibiting redundant and pleiotropic actions. Also, the differential depletion of each individual molecule does not seem to reduce the suppressive activity of the MSCs (Singer & Caplan, 2011).

1.5.1 INTERACTIONS WITH T CELLS

MSCs can modulate many T-cell functions including cell activation, proliferation and energy. These actions may be exerted directly by MSCs or may occur indirectly via modulatory effects on antigen-presenting cells such as dendritic cells (DCs), resulting in altered cytokine expression profiles and impaired antigen recognition, avoiding TCR signaling activation (Cutler *et al.*, 2010; English *et al.*, 2009).

The cytokine secretion profile of the different T-cell populations is shown to be affected by the presence of MSCs. When cultured with activated T-cell subsets, a substantial decrease in the production of the pro-inflammatory cytokines interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-17 is observed, followed by increased expression levels of anti-inflammatory cytokines (mainly IL-4 and IL-10) (Ben-Ami *et al.*, 2011).

The proliferation of the two major subsets of T cells ($\alpha\beta$ and $\gamma\delta$) was shown to be directly inhibited *in vitro* by MSCs. Although these cells bear MHC class I molecules on their surface, which could theoretically stimulate T cells, they lack other co-stimulatory molecules needed to fully activate lymphocytes. By using the '3-signal model' for T cell activation, the absence of CD80/CD86 molecules on MSCs will drive the lymphocyte into

an anergic state by default (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Martinet *et al.*, 2009).

The suppressive capacity of MSCs was shown to occur in a dose-dependent fashion and equally affects the proliferation of CD4⁺ (Th, or T helper) and CD8⁺ (Tc or T cytotoxic) T-cell subsets (Najar *et al.*, 2010). This effect is irreversible upon removal of the MSCs, indicating that lymphoid populations become permanently biased towards a resting state after interacting with MSCs in specific conditions (Glennie *et al.*, 2005).

1.5.2 INTERACTIONS WITH B CELLS

There are some contradictory experiments relating to whether MSCs can increase or decrease B cell activation and proliferation. As with other cell interaction studies, MSCs actions seem to be 'context-dependent'. Therefore, the precise biochemical and physiological environment may be the major responsible for such responses.

Some studies have shown an increased proliferation and IgG production of B-cells in direct co-culture with MSCs. B-cell modulation is shown to occur by direct cell-cell interactions as well as soluble factors (mainly IL-6) secreted by MSC (Rasmusson *et al.*, 2007). Also, MSCs promoted the proliferation and differentiation of effectors from naïve B cells, by strongly enhancing their differentiation into plasma cells or memory B-cell populations. This effect was also observed in response to polyclonal stimulation of B cells isolated from pediatric patients with an autoimmune disease (systemic lupus erythematosus) (Traggiai *et al.*, 2008).

In another way, B-cell proliferation was shown to be inhibited by hMSCs through an arrest in the G0/G1 phase of the cell cycle, making them quiescent, and not through the induction of cell death. Conversely, MSCs also inhibit B-cell differentiation because synthesis and secretion of antibody types IgM, IgG, and IgA is significantly impaired (Corcione *et al.*, 2006).

Transwell experiments similarly revealed that MSCs cultured together with B cells, but separated from them, are sufficient to provide maximal inhibition of B-cell proliferation. This indicates that soluble factors secreted by MSCs are responsible for modulating B cell behaviors, on a direct cross-talk fashion, as supernatants from a pure culture of MSCs did not inhibit B-cell proliferation (Corcione *et al.*, 2006). Therefore, it is suggested that MSCs require stimulus signals from B cells to exert their suppressive action.

B-cell chemotaxis is also impaired when cultured with MSCs, mediated by a down-regulation of chemokine receptors CXCR4 and CXCR5. Migration of B lymphocytes to and positioning in lymph nodes is a tightly regulated process and CXCR4 plays a major role for homing and retention of stem cells in the bone marrow (Zou *et al.*, 1998; Ma *et al.*, 1998), as well as trafficking of memory B cells to the T-cell areas of secondary lymphoid organs (Okada *et al.*, 2002; Reif *et al.*, 2002). A down-regulation of these receptors may lead to loss of interaction between T and B-cells, which can prove to be clinically relevant in some contexts. Also, by impairing antibody production, MSCs may have a pivotal role on new cell therapies targeting autoimmune diseases (e.g. myasthenia gravis or IgA nephropathy).

1.6 ISOLATION METHODS FOR MESENCHYMAL STEM CELLS

Although a wide number of protocols exist for the isolation of MSCs, until the present date there is none established universal procedure for the successful isolation of these cells from all known sources, thus making the data obtained non-comparable (Pountos *et al.*, 2007).

As mentioned before, the lack of defined universal surface markers that unambiguously identify hMSCs can compromise the successful isolation of these cells. The initial primary cell populations obtained in culture are intrinsically heterogeneous, and often contaminated with other cell types (e.g. hematopoietic cells). BM was the first tissue where MSCs were isolated from, and a number of different methods have been used over the last decades to increase the number of cells obtained from this tissue, while reducing other cell side-contaminants (Jung *et al.*, 2012).

The most common method for isolating BM-derived hMSCs is the density centrifugation of BM aspirates. Briefly, this technique involves the use of solutions of high density with low viscosity and low osmotic pressure (eg. Ficoll, Percoll) to separate the various fractions of cells in a density gradient. Contamination of samples with other cell types like hematopoietic stem cells or blood results in heterogeneity of the preparation, and the goal is to obtain the mononucleated fraction of BM, which is rich in MSCs (Lennon & Caplan, 2006).

Since MSCs are fibroblastic adherent cells (see *Figure 1*) which require an adequate substrate for attachment, this fraction is then plated in plastic culture dishes. The most commonly used substrate is treated polystyrene (T-flasks), and the initial cell adherence is

enhanced when the cells are in contact with serum, usually fetal bovine serum (FBS), since it provides hormones, growth factors, binding (fibronectin), transport proteins and other supplemental nutrients (Kern *et al.*, 2006) as described in the next section.

After 48-72 hours in culture, the media is renewed and a primary population of spindle-shape, adherent cells is visible under the microscope. However, the initial number of hMSCs obtained can be increased for more than 35% by simple replating the initial waste media washed out during the first feeding, which still contains adherent cells in suspension (Wan *et al.*, 2006).

However, other sources of hMSCs are now routinely used to collect these cells and which may reveal to be easier to access than BM besides requiring less invasive procedures. Solid tissues and organs, such as fat, umbilical cord (namely Wharton's jelly), muscle or synovial membrane are other common sources for the isolation of mesenchymal stem cells. However, isolation protocols differ slightly from those used for semi-liquid BM aspirates, mostly because of the tissues solid nature.

The initial isolation of cells from these sources requires both mechanic and enzymatic treatment. Mechanic digestion is often used with tools that mince the tissue into smaller fragments, after removing parts of non-interest (e.g. blood vessels). After this step, an enzymatic treatment with collagenases is often performed, which cleaves the peptide bonds in the triple helical collagen, thus disrupting the extracellular matrix (ECM) which supports the cells. Unfortunately, there is not a standardized protocol for collagenase digestion of tissues, resulting in a variety of concentrations and digestion times, which may lead to problems when trying to compare different studies (Robey, 1995; Wakitani *et al.*, 1994).

Besides providing an increased yield of the primary cells obtained (Sakaguchi *et al.*, 2004), some studies claimed that enzymatic treatment of tissues could potentially result in changes of the metabolic profile of MSCs, and thus further screening is required in order to know what the main consequences of its use are (Thomas *et al.*, 2004).

Recently, as the phenotypic characterization of hMSCs evolved with the knowledge of cell surface receptors, isolation of these cells could theoretically become easier and faster. Magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) techniques are now being used for enriching initial primary cell populations of MSCs (Inokuchi *et al.*, 2005; Baumgarth & Roederer, 2000). Magnetic bead sorting uses

antibodies coated with magnetic beads, targeting epitopes positively expressed on MSCs (e.g. CD105, endoglin). An external magnetic field is applied separating the positive (antibody labeled) from the negative cells (see *Figure 7*).

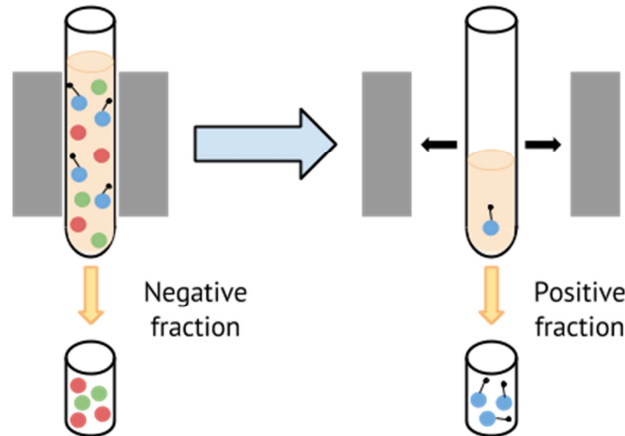


FIGURE 7 Basic principle of cell sorting using magnetic labeled antibodies. Unlabeled cells are eluted after passing through the column, becoming the negative fraction. Cells of interest are retained in the column, until the magnets (grey) are removed. Labeled cells are then collected, as an enriched positive population of selected marker.

Fluorescence-activated cell sorting (FACS) is an alternative method to sort cells of heterogeneous populations, using at least one monoclonal antibody tagged with a fluorescent dye. The method is similar to MACS, though the cells are characterized and separated based on the intensity of fluorescence emitted by the specific antibody.

1.7 OUTLINE OF CULTURE PRACTICES FOR MESENCHYMAL STEM CELLS

After isolation, cells start to proliferate and routine culture methods are used to keep cells growing in optimal conditions. Many factors affect the expansion of MSCs in culture, which can either be donor dependent or technique dependent. Donor dependent factors include the age and the sex of the donor, the presence of trauma on the site of biopsy or the presence of systemic diseases (Seebach *et al.*, 2007; Stenderup *et al.*, 2003).

Culture conditions include incubation in a controlled environment, with a temperature of 37°C and a humidified atmosphere with 5% CO₂ in normoxic conditions. The culture medium is changed every 3-4 days. When cells in culture become confluent (usually 70-80%), a cell detachment solution (commonly Accutase™ or trypsin) is used to harvest them and seed new flasks. Initial seeding density should be between 3 000 and 6 000 cells/cm². These values allow good cell growth kinetics and do not compromise the exchange of factors mediating autocrine signaling of the culture. For some studies,

however, there is a need to use lower density values, with seeding densities as low as 10 cells/cm² (e.g. clonogenic studies).

Even in the presence of oxygen, proliferating and stem cells primary rely on aerobic glycolysis (the Warburg effect) to generate energy, instead of using oxidative phosphorylation of glucose (see *Figure 8*) (Dos Santos *et al.*, 2010). Through this pathway, cells produce lactate which accumulates in the culture medium leading to a decrease in the pH and impairing cell growth and proliferation for values higher than 35mM. The metabolism of glutamine produces ammonia which accumulates intracellularly, leading to cell growth inhibition for values as low as 2mM (Schop *et al.*, 2009).

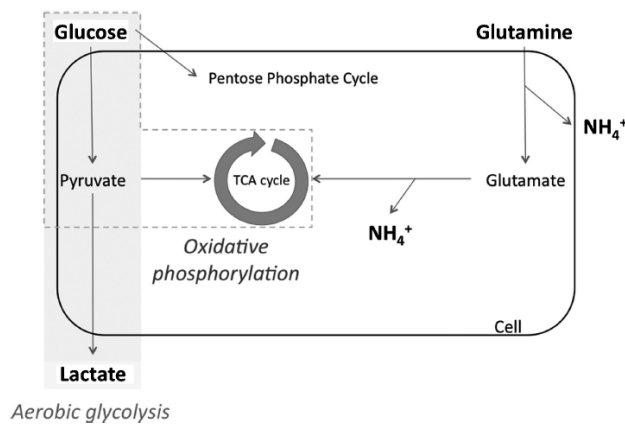


FIGURE 8 Main metabolic pathways of animal cells. In order to generate energy, proliferating hMSCs rely primarily on aerobic glycolysis rather than oxidative phosphorylation (Dos Santos *et al.*, 2010).

Within the appropriate tissues *in vivo*, stem cells like MSC are usually present in stem cell niches under hypoxic conditions. Indeed, bone marrow tissue is hypoxic in nature, with an oxygen tension of approximately 1–7% (Harrison *et al.*, 2002). Therefore, *in vitro* culture of MSCs in a normoxic atmosphere (21% O₂) can be considered an exposure to superior oxidative stress (Bertram & Hass, 2008). This generates metabolic radicals or reactive oxygen species that result in early onset of senescence, as well as the reduction in population doublings in comparison to cells cultured under hypoxia (Grayson *et al.*, 2007; Dos Santos *et al.*, 2010). This information led to the routine use of conditions that promote hypoxic environments when culturing MSCs. Furthermore, cells cultured in low-oxygen tensions reveal an increase of stemness genes Oct4 and Rex-1 expression and telomerase activity, as well as improved extracellular matrix (ECM) formation and organization patterns (Grayson *et al.*, 2006, 2007). This potentially suggests that cells are cultured in more close conditions to their *in vivo* niche, which could improve or maintain cell quality in culture over longer periods of time.

Conventional culture media for expanding hMSCs consists of a liquid basal medium containing glucose (source of carbon), amino acids as a source of nitrogen (glutamine is particularly important in many metabolic pathways) and other molecules, supplemented with fetal bovine or calf serum (FBS or FCS) at 10–20% (v/v). FBS is known to contain a high content of attachment and growth factors, as well as nutritional and physiochemical compounds required for cell maintenance and growth (Jung *et al.*, 2012).

Growth factors and hormones, vitamins (cofactors) and inorganic salts are also part of culture media. The most common ions used in culture media are sodium, potassium, magnesium, calcium, chloride, sulfate and bicarbonate, which altogether simultaneously adjust the pH and the osmotic balance of the cells in culture. Antibiotics, usually penicillin (0.025µg/mL) and streptomycin (0.025U/mL), are also routinely added to the medium to prevent contamination by microorganisms.

However, the use of FBS is not entirely risk-free. Several safety related concerns are raised due to the presence of xenogeneic components, in addition to its inaccurate defined composition (also showing lot-to-lot inconsistency of performance, which does not allow reproducible results under different experimental setups) (Jung *et al.*, 2012). Thus, the development of a more defined serum-free MSC culture medium, which can be tracked and proved harmless for clinical applications, is now becoming absolutely essential (Kuçi *et al.*, 2012).

Autologous or allogeneic human blood-derived substances, including human serum, plasma, platelet derivatives (e.g., platelet lysate), and cord blood serum, are currently under analysis for their clinical utility as an alternative culture media supplement for the growth of MSCs. Human platelet growth factors (HPGF), which are prepared by mechanical disruption or chemical lysis of cell membranes from human virally inactivated platelets, are achieving emerging interest for their use in serum replacement strategies (Tekkatte *et al.*, 2011; Burnouf *et al.*, 2010). However, some studies reported data that showed a reduction of osteogenic or adipogenic differentiation potential when hMSCs were cultured in HPGF-based media (Lange *et al.*, 2007; Gruber *et al.*, 2004).

The ultimate media should be made of chemically defined constituents that support the attachment and growth of hMSCs primary cultures, as well as passaged cultures, while maintaining their therapeutic properties. StemProMSC SFM™ from Invitrogen© represents the first commercial serum-free (SF) medium that allows the successful isolation and

expansion of hMSCs from BM. This SF medium and has recently been cleared by the FDA as a medical device for clinical trials in the United States, ideal for expanding hMSCs to be used in a range of clinical applications (<http://www.invitrogen.com>).

When comparing the phenotype of cells expanded in serum-free media and on an FBS-based reference media, most of the hMSC-specific surface antigens were expressed on both cell populations, although some receptors were expressed in different levels (Agata *et al.*, 2009). This result could suggest that the morphology and molecule expression on the surface of the cells is highly dependent on the chemical composition of the media, thus requiring further screening to optimize culture conditions in order to obtain cells of the best quality for specific and definite uses.

1.8 *EX-VIVO* EXPANSION OF MESENCHYMAL STEM CELLS

As described before, monolayer culture is the basic and the most economical technique for the *ex-vivo* expansion of hMSC (Pountos *et al.*, 2007). Several clinical applications require a significant expansion of the cells prior to its use, although the optimal dosage of hMSCs for their use in therapeutic applications is still unclear and should be dependent upon the type of desired objective (Schallmoser *et al.*, 2008).

Usually, the number of MSCs infused in patients depends largely on body weight, a typical dose between 1 to 5 million cells per kilo being generally suggested (Ankrum *et al.*, 2010; Subbanna, 2007). However, the number of cells collected and isolated from the different sources is much lower and the expansion methods of MSCs are not yet fully optimized, which can impair the achievement of successful therapeutic outcomes.

The frequency of hMSCs in body tissues is very low, being estimated that these cells represent only 0.01% to 0.001% of human BM mononuclear cells (MNCs) (Castro-Malaspina *et al.*, 1980). It was also demonstrated that hMSCs comprise approximately 1 in 10 000, 100 000, and 250 000 BM-MNCs of newborns, teens, and adults, respectively, indicating that the hMSC frequency declines along with age and possibly by the presence or trauma or chronic diseases (Caplan, 2007).

The clinical scale *ex-vivo* expansion of MSCs is traditionally performed under static conditions, using tissue culture flasks (T-flasks). Although these cells can be expanded massively up to 40 generations maintaining their multipotent potential in a relatively short period of time (up to thousand-fold increase after two to three weeks), extensive

subcultivation gradually reduces maximal differentiation capabilities and other potential benefits of MSCs. As expected, during culture their mitosis rate diminishes and this results in cellular senescence, associated with growth arrest and apoptosis (Monteiro *et al.*, 2010).

One of the problems with the culture technology currently used (serial passaging in plastic flasks) is that it is inefficient, costly and time consuming albeit feasible. To overcome this issue, many dynamic systems have been developed to scale-up the expansion of MSC in shorter periods of time. The use of spinner flasks, rotating wall vessels, concentric cylinder bioreactors, and perfusion bioreactors are examples of alternative strategies to the static expansion of MSCs. Some of the features provided in dynamic systems include adequate shear stress, microgravity effects, efficient nutrient supply and metabolite removal, including non-limitation of surface area (Zhao & Ma, 2005).

Spinner flasks can be considered “reduced-scale” bioreactors. These are plastic or glass containers composed of a central magnetic stirrer shaft and side arms for cell seeding, media and gases exchange, allowing an efficient mass transport and diffusion of molecules within the cells (see *Figure 9*) (Schop, 2010).



FIGURE 9 Spinner flask model from Chemglass Life Sciences (<http://www.chemglass.com/>).

The cultivation of adhesion-dependent cells under stirred conditions requires an adequate solid substrate for cell growth and proliferation (Grinnell, 1978). In the late 1960s, a system termed ‘microcarriers’ for growing anchorage-dependent cells was described by van Wezel (van Wezel, 1967). Over the last years, MSCs were shown to be

successfully expanded in spinner flasks or bioreactors using microcarriers as a surface attachment structure (Frauenschuhr *et al.*, 2007; Schop *et al.*, 2008; Sart *et al.*, 2009).

Microcarriers are spherical beads with a diameter of 125-250 micrometre, being commercially available in many compositions and configurations, also providing a high surface-to-volume ratio for cell growth. Differences in porosity, specific gravity, optical properties, presence of animal components or surface treatments makes them highly versatile and particularly suitable for a range of applications (see *Figure 10*). Typical compositions include dextran (Cytodex™, GE Healthcare©), collagen (Cultispher™, Percell®), and polystyrene (SoloHill Engineering®).

Several authors have shown that in the early stage of expansion using microcarriers, successful cell attachment appears to be a crucial step in promoting cell proliferation. Low cell adhesions often imply an increase in the lag phase of growth kinetics (Eibes *et al.*, 2010), thus increasing the time to reach the desirable number of cells. For this reason, the initial adherence of cells still remains a parameter that needs to be improved and several approaches have been tried to optimize it, such as different surface coatings of microcarriers. Interestingly, a 100% cell attachment was achieved by Eibes and colleagues, by pre-coating the microcarriers with undiluted FBS, although this approach associated with the limitations of FBS use in clinical applications (Eibes *et al.*, 2010; Kim *et al.*, 1992).

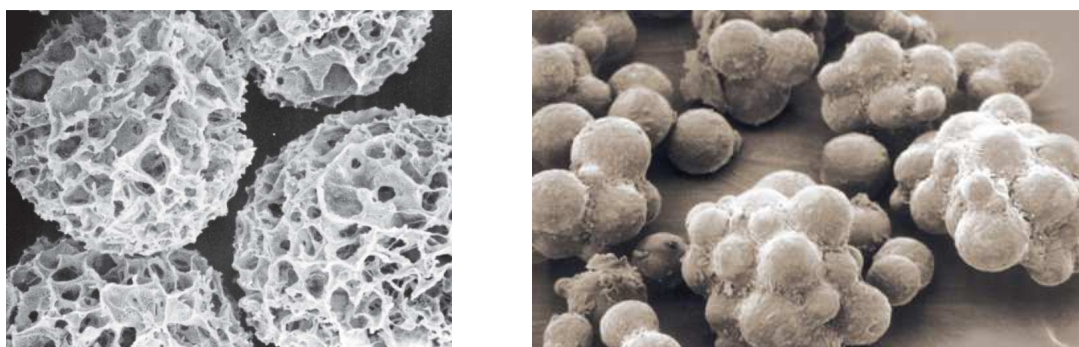


FIGURE 10 SEM picture of empty Cytopore™ macroporous microcarriers (left) (<http://www.gelifesciences.com/>) and aggregated SoloHill Engineering plastic microcarriers (right) (<http://www.solohill.com/>).

Spinner flask agitation is an important parameter, essential to maintain an adequate mass transport of oxygen and nutrients to the cells in culture. Microcarriers are also kept in suspension due to the constant motion inside the container. However, cells cultured in stirred conditions are exposed to hydrodynamic shear stresses and other physical forces, which may affect cell proliferation, growth and differentiation.

The hydrodynamic shear stresses experienced by cells in these culture systems have been shown to specifically influence and up regulate the expression of osteogenic genes in osteoblasts, while also increasing the deposition of a mineralized extracellular matrix (Burdick & Vunjak-Novakovic, 2009). Chondrocyte proliferation was also shown to be induced by shear stresses in stirred culture systems (Malaviya & Nerem, 2002).

Shear forces, provided both by agitation and impeller movement, need to be low enough in order to not decrease the viability and differentiation potential of cells in culture, as stated before (King & Miller, 2007). Although experimental evidence indicates that bead-to-bead interactions are the main source of cell damage in microcarrier culture systems, the turbulent (Kolmogorov) microscale is postulated to be the key criterion for determining whether cell damage will occur (Croughan *et al.*, 1987; Hewitt *et al.*, 2011).

It is also well known that in agitated bioreactors, in the absence of bubbles, liquid flow is transiently turbulent, depending on the prevailing Reynolds number (Nagata, 1975). Therefore, the largest 'eddies' are generated near the main axis impeller region, and are of a similar size to the impeller-blade width. These 'eddies' start a cascade of smaller 'eddies', decreasing in size down to the smallest ones, known as Kolmogorov-scale 'eddies', which are approximately microcarrier size and are dependent of the characteristics of each dynamic system. An 'eddy' precludes precise definition, but it is generally conceived to be a turbulent motion, localized over a region of space (Papoutsakis, 1991).

Damage to cells in aggregates, microcarriers or in suspension is thought to occur when the turbulent Kolmogorov-scale 'eddies' have the same order of magnitude and size of the particles of interest. Since increasing agitation makes Kolmogorov-scale 'eddies' smaller, cells on microcarriers or in aggregates are affected at much lower agitation rates than single cells. The hydrodynamic shear stresses also increase with increasing impeller diameter, stirring speed and also depend on the impeller geometry and location (King and Miller, 2007).

Overall, microcarrier based systems provide a first step platform for the scale-up of MSCs culture for clinical applications. While reducing time and costs for expanding cells, there is a need to develop more efficient culture systems for the fast amplification of MSCs. The full understanding of MSCs biology should allow more efficient engineering

solutions to reduce variability amongst studies, also achieving higher efficiency and safety.

1.9 CLINICAL APPLICATIONS AND FUTURE DIRECTIONS

As of July 2013, the NIH website (<http://clinicaltrials.gov>) lists 349 clinical studies involving mesenchymal stem cells-based therapies. The majority of these studies try to address problems related to tissue damage or autoimmune diseases.

Tissue engineering consists of a relatively new field of regenerative medicine, which brings together basic cell biology knowledge and engineering approaches to address problems involving organ and tissue damage. The ideal outcome following tissue damage is regeneration, however, during evolution, mammals have lost the tissue regenerative capacity of invertebrates and vertebrates such as salamanders and so the ability to naturally restore structural and functional tissue properties is somewhat impaired (Brookes *et al.*, 2001). Mesenchymal stem cells are, along hematopoietic stem cells, one of the most promising and studied sources of raw material that can be used to treat and influence many diseases and conditions on the regenerative field.

MSC-based therapies for several pathologies including nonunions (Hernigou *et al.*, 2005), *osteogenesis imperfecta* (Pountos *et al.*, 2007), cartilage damage (Centeno *et al.*, 2006) and myocardial infarction (Yokoyama *et al.*, 2006) are now arising as more clinical data is obtained and proved to be successful in addressing such issues.

Currently, the clinical uses of MSCs depend on four main strategies for using these cells. These approaches consist of the use of: (i) unfractionated fresh bone marrow cells, (ii) culture and expansion of MSCs, (iii) MSCs differentiation, and (iv) genetically modified cells that express key growth factors or are prone to express tissue specific markers (Pountos *et al.*, 2007; Peterson *et al.*, 2005).

Although MSCs are quite heterogeneous by the many sources they can be isolated from, a set core gene expression profile is conserved (Tsai *et al.*, 2007). Even though bone marrow-derived MSCs are considered the gold standard for clinical use, currently there are no studies or data showing an advantage of a particular tissue origin of MSCs for tissue-specific or other clinical applications (Maxson *et al.*, 2012).

As mentioned previously, the availability, differentiation potential and immunomodulatory effects of MSCs make them an emerging tool in various medical fields. In fact, it seems that the potential clinical applications for MSCs are limitless.

The clinical applications of MSCs can be divided into two large groups: (i) cellular therapy and tissue engineering and (ii) treatment of immune-diseases (Summer *et al.*, 2008; Klingemann *et al.*, 2008).

1.9.1 TISSUE ENGINEERING AND WOUND REPAIR

Direct tissue repair is a challenging task which requires a large interplay of cells, molecules, extracellular matrix (ECM) interactions and an intricate biochemical environment. This kind of scenario is difficult to achieve *ex vivo* and therefore other strategies were originally used due to their simplicity.

The simplest approach to deliver MSCs to a specific site is by BM aspiration followed by immediate injection to the site. One current stem cell-based orthopedic therapy includes BM-MSC transplantation for severe *osteogenesis imperfecta*, a genetic disorder in which osteoblasts do not synthesize or synthesize defective collagen type I molecules. It has been found that allogeneic transplant of BM-MSCs might engraft in multiple skeletal sites, due to the ability of these cells to migrate and colonize the injured sites, and effectively improve bone growth velocity (Marshak *et al.*, 2001).

Due to their distinctive properties, MSCs also represent an innovative approach for cardiac regeneration and myocardial infarction treatment. Also, bone fracture, ischemic cerebral disease (Wang *et al.*, 2002) and spinal cord injury (Urdzikova *et al.*, 2006) are conditions where these beneficial properties are demonstrated. Similarly, suspended MSCs injected intra-articularly into the knee joint following injury appeared to engraft and regenerate damaged meniscus and cartilage (Murphy *et al.*, 2003).

Usually, the delivery of MSCs by direct injection into the blood stream produces a systemic effect, rather than an organ/tissue specific response. Although these cells are capable of migrating to sites of injury, only a partial and loose clinical response is achieved as only a small percentage (often <1%) of the infused cells reach the target tissue. Some clinical studies also revealed that homing and migratory capabilities may be impaired by anatomical constraints of the host. After an intravenously delivery of MSCs, several reports claimed entrapment of donor cells in capillaries within the liver, spleen

and lung (microcirculation vessels) (Phinney & Prockop, 2007). This is most likely explained because expanded MSCs in culture media containing xenogeneic molecules provided by FBS become relatively large and start to express many adhesion molecules. However, the number of cells entrapped can be decreased by using vasodilator drugs (e.g. sodium nitroprusside) (Gao *et al.*, 2001). No studies were performed to assess the influence of platelet lysates as a substitute of FBS on cell morphologies and dimensions, for *in vivo* infusions.

More oriented strategies for the repair of specific tissues rely on the 3-D properties of the normal *in vivo* organ of interest. The understanding of the intricate network and the three-dimensional architecture of the ECM are now thought to be crucial for replicating the natural environment of MSCs and drive their successful actions to clinical targets.

The development of artificial three-dimensional (3-D) matrices that mimic the natural ECM are now being considered as a major step to further improve MSCs clinical outcomes. The ECM encodes a myriad of mechanical and biochemical stimuli playing active roles in the regulation of cell behavior, which requires sophisticated approaches in terms of biomaterials design.

Many hydrogels have already been proposed as ECM analogues, alginates being the most studied candidates, due to their extreme versatility as biomaterials and likely to find increasing utility in a wide range of biomedical applications in the near future (Fonseca *et al.*, 2011).

MSCs have a great affinity to mediate cell-cell interactions, due to the constitutive expression of various integrin molecules, such as $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, αv , $\beta 1$, $\beta 3$, and $\beta 4$ (Van Linthout *et al.*, 2011). One of the most common factor of adhesion is mediated by the integrin very late antigen (VLA)-4, ($\alpha 4 \beta 1$, CD49d) which targets a specific peptide sequence ('RGD') of the abundant ECM protein fibronectin (Rüster *et al.*, 2006). This fact can be used to favor MSCs culture in alginate structures, by loading the scaffold hydrogels with 'RGD' motifs, thus providing support and adhesion for the cells.

Furthermore, other possible modifications of scaffolds can include a range of peptides known to interact directly or indirectly with MSCs. More recently, attention has been given to protease-labile crosslinking peptides, due to the ability of matrix metalloproteinases (MMPs) to cleave them. MMPs comprise a large family of enzymes, mainly proteases, that are primarily recognized for their capacity to cleave most components of the ECM, and

hence play a central role in morphogenesis, wound healing, tissue remodeling and cell migration (Page-McCaw *et al.*, 2007).

In a recent study, alginate hydrogels containing motifs of adhesion ('RGD') plus MPP-sensitive peptides ('RGD/PVGLIG'-alginate) were shown to promote MSCs culture in native 3-D conditions found in many tissues of the body (see *Figure 11*). This allows seeded cells to dynamically interact with the hydrogel matrix and neighbor cells, thus mimicking *ex vivo* the biological properties of ECM (Fonseca *et al.*, 2011). Cells expanded in these hydrogels can be further differentiated into the classical mesenchymal lineages, providing patient-specific grafts for bone or cartilage injuries.

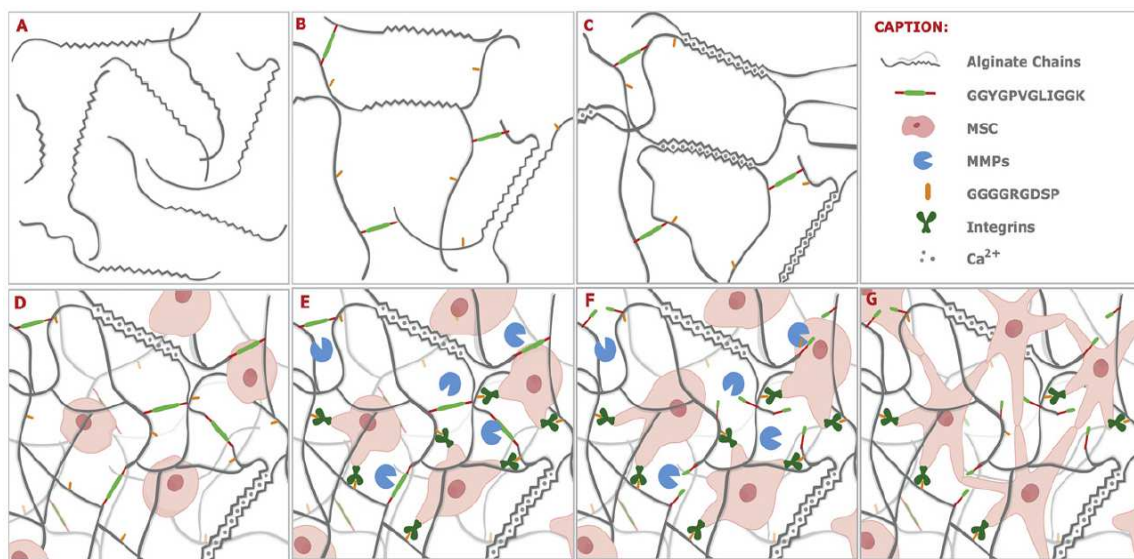


FIGURE 11 Rationale of cell-hydrogel interactions on functionalized alginate 3-D structures. Alginate chains (A) are loaded with cell adhesion and MMP-sensitive motifs (B) before chain crosslinking with Ca²⁺ ions (C). After MSCs seeding (D), adhesion to 'RGD' peptides mediated by integrins and cleavage of 'PVGLIG' MPP-sensitive peptides occur (E-F), allowing entrapped cells to dynamically interact with the hydrogel, becoming less constrained and able to migrate (G) (Fonseca *et al.*, 2011).

Also, the cellular therapy using autologous chondrocyte transplantation for articular cartilage repair may be improved with the use of MSCs. Carticel® (from Genzyme Corporation©, USA) is a commercially available product which promotes the *in vitro* expansion of harvested chondrocytes from a non-load bearing region of the knee, followed by the infusion into an articular cartilage defect and posterior coverage with a periosteal flap. In this case, the use of MSCs prone to differentiate into chondrocytes loaded into an hydrogel structure built with patient-specific dimensions could eliminate the process of chondrocyte harvesting.

Other approaches can be followed, as for example, Osiris Therapeutics® (USA) is testing Chondrogen™ that consists in the insertion of an implant and injection of MSCs in the site of injury for meniscal cartilage repair. For the European market, ChondroCelect® is the only approved cell-based product, developed by Tigenix® (Belgium) for cartilage regeneration in the knee that involves the implantation in the joint of a suspension of characterized viable autologous MSCs.

1.9.2 TREATMENT OF AUTOIMMUNE DISEASES

Many autoimmune diseases could benefit from the clinical use of MSCs, due to their very potent immunomodulatory effect. The list of MSC-related candidate applications includes bone marrow transplant, graft versus host disease (GvHD), juvenile diabetes, rheumatoid arthritis, lupus, inflammatory bowel disease, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) amongst others (Caplan & Correa, 2011).

Beneficial effects for MSC-based treatment have also been reported in studies of experimental autoimmune rheumatoid arthritis and myasthenia gravis, conducted with both mice and rats. In these studies, reduced clinical symptoms, mediated by the reduced expression of inflammatory cytokines and proliferation of AChR-specific plasmocytes have been reported, as well as prevented severe tissue damage and improved body weight (Kong *et al.*, 2009; Yu *et al.*, 2010).

The most studied clinical application of MSCs is the suppression of the adverse effects of graft-*versus*-host disease (GvHD) after hematopoietic stem cell transplantation. Le Blanc and colleagues have shown that intravenous MSCs infusions are effective in treating cases of severe, steroid-refractory GvHD in humans. Even when the expanded cells belong to third-party HLA-mismatched donors, they prove to be equally effective (Le Blanc *et al.*, 2004).

In another study, autologous MSCs derived from adipose tissue were shown to be elective for the treatment of fistulas in patients suffering from Crohn disease. Following five patients with this condition, the external opening of six of eight fistulas could be closed by inoculating them with autologous lipoaspirate-derived MSCs (García-Olmo *et al.*, 2005). Although the results obtained do not allow the demonstration of effectiveness, they might give motivation to undertake *in vivo* studies with autologous adipose-derived MSCs in patients suffering from wound healing defects and ulcers.

Although many MSC-based therapies have shown promising results, currently there are no effective methods to demonstrate both efficacy and full safety of these procedures. This issue can be attributed, in part, to an incomplete understanding of the fate of MSC following infusion, as well as the mechanisms through which they impact host biology.

Following this comprehensive review on the use of human MSCs, a new source for these cells is presented in the next sections. hMSCs isolated from synovial tissues were expanded *ex-vivo* under static and dynamic conditions, followed by characterization studies, comparing the results obtained with others previously published for BM-, AT- and UCM-MSCs.

II AIMS OF THE STUDY

Since the discovery of alternative sources of hMSCs, the scientific community shows an increasing enthusiasm in building a complete catalogue listing the tissues these cells can be isolated from, as well as the pros and cons of choosing different sources, keeping in mind different objectives and applications for using these cells. As synovial tissues are a new source of hMSCs with promising results for clinical applications (e.g. treatment of osteoarthritic diseases), a complete characterization is needed to verify this possibility.

Therefore, there are currently 3 objectives to be explored and achieved. This work aims to develop appropriate responses to each one:

- To isolate and characterize human MSCs from synovial tissue samples, as well as their *ex-vivo* expansion under controlled conditions (static and dynamic).
- To differentiate synovium-derived MSCs into the three mesenchymal lineages, particularly chondrocytes, and prove their clinical potential for the personalized treatment of osteoarthritic diseases.
- To establish, compare and optimize protocols for the isolation and expansion of these cells and to compare the results with the ones obtained with MSCs from different sources, namely BM, AT and UCM.

The results obtained at the end of this work are expected to increase the range of knowledge about mesenchymal stem cells, by evidencing and relating the intrinsic properties of different sources, thus increasing the data available for the isolation and clinical uses of these cells.

III MATERIALS AND METHODS

3.1 HUMAN SAMPLES

Synovial tissue samples (obtained from direct biopsy of synovial membrane, shaver blade filtrate residues and synovial fluid) were collected during routine arthroscopic surgery undertaken by patients at Centro Hospitalar de Lisboa Ocidental (Hospital de São Francisco Xavier and Hospital Egas Moniz), after informed donor consent.

Direct biopsy samples are specifically collected on the suprapatellar bursa, a region where synovial membrane is particularly rich and widely present, although supported by adipose tissue.

Samples are collected and stored in PBS (phosphate buffered saline) supplemented with antibiotics (penicillin and streptomycin) until further processing at Instituto Superior Técnico Stem Cell Bioengineering & Regenerative Medicine Laboratory, located in TagusPark.

3.2 ISOLATION OF MESENCHYMAL STEM CELLS FROM SYNOVIAL TISSUES

Samples of synovial tissues were obtained from both healthy donors and patients with various degrees of joint disease, namely traumatic inflammation and osteoarthritis, after informed consent at Centro Hospitalar de Lisboa Ocidental, Portugal.

The isolation of MSCs from the synovium direct biopsy or filtrate samples starts by its disinfection, rinsing tissues at least 3 times with IMDM (Gibco®, Carlsbad, CA) supplemented with antibiotics and antimycotics, discarding the washout media. Fragmentation of tissues into small pieces is then performed using a scalpel. Synovial fluid samples, due to its nature, do not require this step.

Tissue fragments and synovial fluid samples are then digested using a solution of collagenase type IV (Sigma, St. Louis, CA) and DMEM (Gibco®) at 37°C with strong agitation. Different enzyme concentrations and digestion times were performed, ranging between 0.02% - 0.2% (w/v) for an overnight or 4 hours incubation period, respectively. The activity of collagenase is then stopped by adding IMDM + 10% FBS supplemented with antibiotics in a proportion of 1 : 6. The suspension containing tissues and cells is then filtered and centrifuged for 7 minutes at 1250 rpm. Total cells obtained are plated

and cultured in T-flasks (Falcon BD Biosciences, San Jose, CA) in the presence of DMEM + 10% FBS supplemented with antibiotics.

Synovial fluid samples are processed either with the enzymatic digestion procedure described before, using the minimal enzyme concentration, or by direct plating in T-flasks, using the same culture media described.

3.3 CRYOPRESERVATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

For cell banking, expanded cells from all samples were cryopreserved through the initial four passages (P1 to P4) after isolation. Cryopreservation is performed by storing cells using media supplemented with a cryoprotectant substance, such as dimethyl sulfoxide (DMSO). This compound prevents cell death and damage during freezing procedures by avoiding the formation of ice crystals. Cells were stored in freezing vials (Nunc tubes) using either FBS + 10% DMSO or a commercially available freezing solution, the Recovery™ Cells Culture Freezing Media (Gibco®).

For cryopreservation, cells were centrifuged for 7 minutes at 1250 rpm, resuspended in the appropriate freezing medium and distributed by each freezing vial (not exceeding 10⁶ cells / mL). The cells were then stored at -80°C, establishing the routine working bank. Long term storage (above 2-3 months) was performed by transferring previously cryopreserved samples to liquid nitrogen.

Thaw of samples and cell retrieval was performed by quick immersion of the vial on a water bath of 37°C, followed by the addition of IMDM (Gibco®) with 20% FBS in a 1:5 proportion. The cells were then centrifuged for 7 minutes at 1250 rpm and resuspended using the expansion culture medium.

3.4 CULTURE MEDIA

3.4.1 *EX-VIVO* EXPANSION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

Many culture media can be used for the culture and expansion of MSCs. A major difference among them is the presence (serum containing – SC) or absence of serum (serum free – SF).

The primary culture media used for the expansion of synovium-derived MSCs (SMSCs) was DMEM (Gibco®) supplemented with 10% MSC-qualified FBS (Gibco® or Thermo Fisher, Waltham, MA), which does not require prior testing to certify MSCs research. For

dynamic expansion of SMSCs, the culture medium used was the commercially available StemPRO® MSC SFM XenoFree (Gibco®), that besides being SF it is also free from xenogenic factors. Antibiotics were routinely added to culture media (0.025 µg/mL of penicillin and 0.025 U/mL of streptomycin).

Cells expanded using SF culture media require an additional attachment substrate, due to the absence of adhesion factors and proteins provided by serum. For this purpose, the surface where the cells were cultured was coated for 1 hour at 37°C with either a commercially available xeno-free substrate named CELLstart™ (Invitrogen®, Carlsbad, CA), or new viral inactivated human platelet lysate supplement named *HPL*, both diluted in a proportion of 1 : 100. CELLstart™ is a patented defined substrate that contains only components of human origin, while *HPL* is demonstrated to be a new, serum and xeno-free effective replacement for FBS which supports MSCs culture by providing many platelet derived growth and mitogenic factors (Da Silva & Dos Santos, 2013 – unpublished data).

3.4.2 *IN VITRO* DIFFERENTIATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

The *in vitro* differentiation process of SMSCs was achieved using the commercially available StemPRO™ Adipogenesis, Osteogenesis and Chondrogenesis differentiation kits and culture media. The differentiation media were prepared according to the manufacturer's indication and induction was performed as described.

Briefly, after 15 days of culture in specific conditions (presence of the differentiation media), cells were exposed to different staining methods to evaluate their differentiation. Osteogenic differentiation was evaluated by two staining protocols: Alkaline Phosphatase (ALP) assay, which evaluates the activity of early bone progenitors and Von Kossa (VK) staining, which detects calcium phosphate deposits. The adipogenic and chondrogenic differentiation were evaluated respectively by Oil Red-O staining (for the detection of intracellular vacuoles of lipids) and the Alcian blue staining (that detects cartilage mucopolysaccharides and glycosaminoglycans).

3.5 *EX-VIVO* EXPANSION OF SYNOVIUM-DERIVED MSCs

3.5.1 EXPANSION UNDER STATIC CONDITIONS

After isolation, cells were expanded on static culture systems (T-flasks, Falcon BD Biosciences©) at 37°C and 5% CO₂. A surface area of 25 or 75 cm² was routinely used (T-25 or T-75 flasks). The medium is changed every 3 to 4 days. When cells in culture reach 70 to 80% confluence, they are washed twice with PBS and harvested with 1x Accutase™ for 5-7 minutes at 37°C. The enzymatic action is stopped by dilution, with the addition of IMDM + 10% FBS in a proportion of 1:3. The cells are then centrifuged and resuspended in media for replating (DMEM + 10% FBS) at a density of 3 000 cells/cm², becoming the subsequent passage. The number of viable and dead cells can be determined by counting them in a hemocytometer under an optical microscope using Trypan Blue dye exclusion method.

The fold increase (FI) at the end of each passage was calculated as the ratio between the number of viable cells harvested and the number of viable cells at the beginning (plating):

$$FI = \frac{\# \text{ viable cells harvested}}{\# \text{ viable cells plated}} \quad (1)$$

Although a universal formula is still difficult to obtain, population doublings (PD) were calculated by dividing the logarithm base 10 of the fold increase (FI) by the logarithm base 10 of 2:

$$PD = \frac{\log_{10} FI}{\log_{10} 2} \quad (2)$$

Also, the cumulative fold increase (CFI) was calculated since the initial starting of sample culture as:

$$CFI = \prod FI \text{ (end of each passage)} \quad (3)$$

The doubling time (DT) of cells in culture during passages was calculated as:

$$DT = \Delta t \times \frac{\log_{10} 2}{\log_{10} FI} \quad (4)$$

Where Δt is the passage duration time and FI is the fold increase at the end of the same passage.

3.5.2 EXPANSION UNDER DYNAMIC CONDITIONS

Spinner flasks of 80mL working volume (Bellco Biotechnology, Scientific Industries Inc., Bohemia, NY) equipped with 90° paddles (normal paddles) were used for the stirred culture of MSCs. Plastic microcarriers (20g/L) (SoloHill Engineering Inc., Ann Arbor, MI) were used as substrate for cell grow.

Microcarriers were prepared and coated accordingly to the manufacturer's indications. Briefly, the beads were sterilized and washed before incubation with CELLstart™ or HPL with proper dilution in PBS, for 1 hour at 37°C. Cycles of 2 minutes agitation followed by 10 minutes rest were performed using a ThermoMixer (Eppendorf, Hamburg, Germany). Microcarriers were then gently washed several times with PBS and finally with a minimum volume of culture media StemPRO® MSC SFM XenoFree (Gibco®).

Synovium-derived MSCs at passage 4, previously expanded under static conditions for 1 passage in StemPRO® MSC SFM XenoFree (Gibco®), were seeded (4×10^6) on pre-coated plastic microcarriers in 15mL of the same medium. Pre-warmed medium was then added until reaching half of the final volume (40mL), and the cell suspension was transferred to the spinner flask. Incubator settings were the same as described previously.

The culture was started running 4 hours with low agitation (25 rpm) followed by 11 hours statically, in order to promote the maximum adhesion of cells. After that, continuous mixing (40 rpm) was set. At day 3, StemPRO® MSC SFM XenoFree (Gibco®) medium was added up to final working volume of 80mL. From that moment, 25% of the volume of the culture media was replaced daily.

One condition tested under stirred conditions was the addition of new beads after day 8, for maximizing expansion capacity. For this, plastic microcarriers were prepared as described before, equilibrated in the appropriate culture medium and added to the spinner flask, reaching a final concentration of 30g/L.

Cell growth kinetics in the spinner flask culture were evaluated daily. Two samples (0.5mL) were taken independently from each spinner flask and then incubated with 1x pre-warmed TrypLE™ for 7-10 minutes at 37°C with strong agitation (750 rpm) in the ThermoMixer. The enzymatic action was stopped by the addition of IMDM + 10% FBS in a proportion of at least 1:3. The cells were then isolated from the microcarriers suspension through a Cell Strainer (100 µm) (Falcon BD Biosciences®), centrifuged and resuspended in PBS. The number of viable and dead cells was determined by counting them in a

hemocytometer under an optical microscope using the Trypan Blue exclusion method. The fold increase and cumulative fold increase were calculated as previously described.

On a daily basis, supernatant samples were also collected. Samples of 1mL were collected before and after media change on each spinner flask. Supernatants were centrifuged *eppendorf* tubes for 10 minutes at 1500 rpm and then and stored at -20°C on additional *eppendorf* tubes. The samples were analyzed in an HPLC system Hitachi 7000 Series to determine the concentration of nutrients (glucose) and metabolites (lactate), using the method described by Stoll and colleagues (Stoll *et al.*, 1994).

In order to obtain a quantitative profile of the culture system, specific metabolic uptake/production rates (q_{Met}) were calculated for every time interval using the following equation:

$$q_{\text{Met}} = \frac{\Delta \text{Met}}{\Delta t \bar{X}_V} \quad (4)$$

Where ΔMet is the difference of nutrient/metabolite concentration during the time interval Δt and \bar{X}_V is the average viable cell number for that time period. The apparent yield of production of lactate from glucose ($Y'_{\text{lactate/glucose}}$) was calculated as the ratio between $q_{\text{lactate}}/q_{\text{glucose}}$.

$$Y'_{\text{lactate/glucose}} = \frac{q_{\text{lactate}}}{q_{\text{glucose}}} \quad (5)$$

At days 1, 4, 7, 9 and 12, samples of cell-containing beads (0.5mL) were gently washed twice with PBS (500 μL) and fixed with 500 μL of 2% paraformaldehyde (Sigma) for 20 min at room temperature. Excess PFA was removed and beads were washed once again with PBS. After washing, beads were protected from light and incubated with 500 μL of 4',6-diamino-2-phenylindole dilactate (DAPI, 1.5 $\mu\text{g/mL}$ in PBS) for 5 min at room temperature. Excess DAPI was removed and beads were rinsed three times with PBS. Stained nuclei (blue color) were pictured under a fluorescence microscope. The staining of cells with DAPI, a fluorescent stain, marks specifically genetic material, allowing a qualitative assessment of cellular proliferation, attachment to microcarriers and viability.

3.6 CHARACTERIZATION OF THE EXPANDED SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS FROM DIFFERENT DONORS

3.6.1 PROLIFERATIVE CAPACITY AND GROWTH KINETICS

Synovium-derived MSCs from passage 3 were plated on 12 well plates (Falcon BD Biosciences®) with different initial seeding densities.

Initial seeding densities of 100, 500, 1 000, 3 000 and 6 000 cells/cm² were used. Cells were cultured for 14 days at 37°C and 5% CO₂, using DMEM + 10% FBS supplemented with antibiotics.

At culture days 3, 5, 7, 10, 12, 14, 17, 19 and 21, the medium from 2 wells was removed and the wells were washed twice with PBS, before harvesting them with 1x Accutase™ for 5-7minutes at 37°C, as previously described. Cell suspension was recovered to a conical tube, centrifuged for 7 minutes at 1250 rpm and resuspended in PBS. The number of viable and dead cells was determined by counting them in a hemocytometer under an optical microscope as previously described.

The apparent specific growth rate for each growth kinetic (μ_{app}) during exponential phase was calculated based on:

$$\mu_{app} = \frac{\ln\left(\frac{X_f}{X_i}\right)}{\Delta t} \quad (6)$$

Where X_f and X_i are respectively the number of viable cells at the end and at the beginning of a given time interval Δt .

The use of hypoxia was also evaluated for proliferative analysis of synovium-derived MSCs. A C-Chamber connected to a Proox Model 21 controller (BioSpherix, Redfield, NY) was used to establish a hypoxic environment (2% O₂). Cell harvesting was performed as described before. Comparisons between hypoxia and normoxia experimental results were determined by using a two-sided paired sample Wilcoxon signed rank test. A p-value less than 0.05 was considered statistically significant.

3.6.2 GROWTH KINETICS UNDER DIFFERENT SURFACE COATINGS

As previously described, synovium-derived MSCs from passage 3 were plated on 12 well plates (Falcon BD Biosciences®) coated with different adhesion-promoting factors. For this purpose, porcine gelatin and human fibronectin were used independently.

Porcine gelatin (Sigma®) was dissolved in PBS for a final concentration of 2%, autoclaved and sterile filtered before use. Well plates were coated with this solution at 37°C for 20 minutes. Excess fluid was then removed.

Human fibronectin (Sigma®) was prepared as described by the manufacturer's instructions, dissolved in PBS and sterile filtered before use. Well plates were coated with this solution, for a final surface concentration of 2.5µg/cm² and incubated for 45 minutes at room temperature. Excess fluid was then removed.

Cells were resuspended in the culture media used, DMEM + 10% FBS, and seeded at 1 000 cells/cm² on previously prepared well plates of each coating. At culture days 3, 5, 7, 10 and 12, cells were collected and counted from two wells independently as previously described.

3.6.3 CLONOGENIC POTENTIAL

To evaluate the clonogenic potential of synovium-derived MSCs, by its capacity to form colonies (CFUs-F – colony forming units-fibroblast), cells from P1 to P10 were plated at a very low density (10 cells/cm²) on T-12.5 flasks (Falcon BD Biosciences®), cultured with DMEM + 10% FBS and maintained for 14 days at 37°C and 5% CO₂ without medium change.

After 14 days, cells were washed once with PBS and incubated with 0.5% Crystal Violet solution in methanol (Sigma®) for 30 minutes at room temperature. The stained colonies were washed four times with PBS and once with distilled water. After drying at room temperature, the number of small (5 to 25 cells), medium (25 to 50 cells) and large (more than 50 cells) colonies was counted. The average number of CFUs-F was calculated for each cell passage.

3.6.4 IMMUNOPHENOTYPE ANALYSIS

At the beginning of the expansion (cells at passage 2), SSMSCs were evaluated for immunophenotypical expression of different surface markers.

Flow cytometry using a panel of mouse anti-human monoclonal antibodies (PE- and FITC-conjugated) was performed for all samples. The phenotypic characterization included the evaluation of surface proteins using antibodies against CD14, CD19, CD34, CD45, CD73 (Becton Dickinson Immunocytometry Systems), CD80, CD90 (Biolegend),

CD105 (BD Pharmingen) and human leukocyte antigen (HLA)-DR (Becton Dickinson Immunocytometry Systems).

For immunophenotype analysis, cells at passage 2 were centrifuged for 7 minutes at 1250 rpm, resuspended in PBS and split onto FACS tubes (with a minimum of 10^5 cells per tube). The respective antibody (5 μ L) was added to each tube and incubated for 15 minutes at room temperature protected from light. To remove the excess of antibody, 2 mL of PBS was added and the cells were centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the cells were resuspended and fixed in 2% paraformaldehyde (PFA) (Sigma®) before storage at 4°C. Isotype controls were also prepared for every experiment. The cells were analyzed by flow cytometry (FACSCalibur equipment, Becton Dickinson®) for quantification of the expression of each surface marker. A minimum of 10 000 events was collected for each sample and the CellQuest (Becton Dickinson) or Flowing software (University of Turku, Finland) was used for acquisition and analysis.

3.6.5 MULTILINEAGE DIFFERENTIATION ABILITY

The ability of MSCs to differentiate into osteoblasts, chondrocytes and adipocytes *in vitro* is a relevant parameter for the characterization of these cells.

SMSCs were evaluated for their multilineage differentiation ability at passage 3. Cells were plated on 12 well plates (Falcon BD Biosciences®) and the differentiation protocol for each lineage was followed, according to instructions provided by the commercially available differentiation kits used. The cells were cultured at 37°C and 5% CO₂ during 15 days, followed by specific staining protocols.

For osteogenic and adipogenic differentiation, cells were initially plated at a high density (6 000 cells/cm² per well) in the presence of DMEM + 10% FBS and expanded until reaching 80-100% confluence. After that, the StemPRO® Osteogenesis or Adipogenesis differentiation culture medium was added, with media change every 3-4 days.

For chondrogenic differentiation, cells were previously concentrated to a density of 10^7 cells/mL in StemPRO® MSC SFM XenoFree medium. Droplets of 10 μ L of this suspension were plated on the surface of each well and incubated for 1 hour under high humidity conditions at 37°C and 5% CO₂. Excess fluid was removed and the StemPRO™ Chondrogenesis differentiation culture medium was added. Medium was carefully changed every 3-4 days in order to not disturb the pellets containing cells.

Cells expanded under stirred conditions were also evaluated for multilineage differentiation capacity. Upon 12 days of culture in the spinner flask, synovium-derived MSCs were retrieved from the microcarriers (as previously described) and the three lineage differentiation assays were performed.

3.6.5.1 OSTEOGENIC DIFFERENTIATION

After osteogenic induction, cells were prepared for alkaline phosphatase (ALP) assay and von Kossa staining. For ALP assay, cells were rinsed with PBS and fixed in 4% paraformaldehyde (Sigma) for 10 minutes. After fixing, cells were incubated with a 0.1M solution of Tris-HCl (Sigma-Aldrich) containing Naphtol AS MX-PO₄ (0.1mg.mL⁻¹) (Sigma) in dimethylformamide (Fischer Scientific) and 0.6mg/mL of Red Violet LB salt (Sigma) for 45 minutes. The excess staining was removed by washing three times with distilled water. The cells were kept in distilled water and observed under the optical microscope.

For von Kossa staining, the cells were rinsed once with PBS and stained with 2.5% (w/v) of silver nitrate solution (Sigma®) for 30 minutes at room temperature. Excess fluid was removed and both stained cells and deposits were rinsed once with distilled water. Finally cells were observed under the optical microscope (Leica Microsystems).

3.6.5.2 ADIPOGENIC DIFFERENTIATION

The assessment of differentiation toward an adipogenic phenotype was performed based on the intracellular storage of lipids. Cells were washed with PBS and fixed with 2% PFA solution for 30 minutes at room temperature. After this step, cells were rinsed once with PBS and incubated with 0.3% Oil Red-O solution (Sigma®) for 1 hour at room temperature. After removal of excess staining, cells were washed twice with distilled water and observed under the optical microscope.

3.6.5.3 CHONDROGENIC DIFFERENTIATION

Chondrogenic differentiation was evaluated by staining specific molecules produced in cartilage tissues, such as glycosaminoglycans. Differentiation media was carefully removed, keeping the cell pellets in the well plates. Pellets were rinsed once with PBS and fixed in 2% PFA for 30 minutes at room temperature. Excess fluid and pellets were washed with PBS. Staining was performed with 1% Alcian Blue solution (Sigma), prepared previously in 0.1N HCl for 30 minutes. After removal of staining solution pellets were

rinsed three times with PBS and then kept in distilled water for visualization under optical microscope.

IV RESULTS AND DISCUSSION

4.1 ISOLATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

Mesenchymal stem cells isolated from the synovial tissues may prove to be a promising alternative to the use of hMSCs from other sources in specific clinical applications. BM- or AT-hMSCs still represent the most studied sources for these cells, although there is no evidence that choosing a particular tissue source demonstrates to have better advantages and clinical properties.

Furthermore, SMSCs may have a superior chondrogenic potential due to its origin, which may prove to be important for tissue engineering approaches on cartilage degeneration and future treatments. Expanded cells isolated from synovial tissues could also be routinely used for patient-specific drug screening and testing, mainly due to the easiness of isolation as the cells are commonly collected by non-invasive procedures.

The isolation of synovium-derived MSCs was done using mechanic fragmentation of solid tissues followed by enzymatic digestion using collagenase IV. A total of 19 donors (age range 20 – 78 years, 9 males) were selected for the isolation of cells from direct biopsy samples, shaver blade filtrate residues and synovial fluid. A total of 36 samples were individually processed. Average weight and volume per sample are presented in *Table 1*.

Sample type	Average weight \pm SEM	Average volume \pm SEM
Synovium direct biopsy	1.01 \pm 0.159 g	N/A
Shaver blade filtrate	1.83 \pm 0.031 g	N/A
Synovial fluid	N/A	4.50 \pm 1.15 mL

TABLE 1 Weight of solid samples and volume for synovial fluid samples collected. Values presented as mean \pm SEM.

Average isolation success rates were of 75% (12/16) for synovium direct biopsy, 80% (12/15) for shaver blade filtrate residues and 100% (5/5) for synovial fluid. Interestingly, it was not possible to obtain synovial fluid samples from younger and/or donors with non-diseased joints. Normal joints only have a minimum volume of synovial fluid, which reduces friction between articular cartilages during movement. In diseased joints, fluid is overproduced supported by inflammation, leading to tissue swelling but allowing its collection. Similar results were obtained by Sekiya and colleagues (Sekiya *et al.*, 2012).

Sample type	Isolation time (hours per sample)	Number of MSC-like cells \pm SEM (cells/mL)
Synovium direct biopsy	8 \pm 4	7.3 \times 10 ⁴ \pm 1.4 \times 10 ⁴
Shaver blade filtrate		1.3 \times 10 ⁵ \pm 0.21 \times 10 ⁵
Synovial fluid		4.2 \times 10 ⁴ \pm 1.1 \times 10 ⁴

TABLE 2 Each sample was isolated using enzymatic digestion methods. Mean time of digestion and number of cells collected at the end are displayed. Values presented as mean \pm SEM.

The average number of isolated MSCs-like cells and cells counted at the end of the initial passage (P₀) are present in *Tables 2* and *3*. Isolated samples were rich in red blood cells and other contaminants. After 7 days of culture without media change, non-adherent cells were removed and the culture was maintained with routine medium changes every 3-4 days until reaching 70-80% confluence. For some samples, a new replating of the first waste media was performed, leading to the isolation of adherent-cells on a longer time period. The culture of fresh harvested cells was performed in DMEM + 10% FBS supplemented with antibiotics.

Sample type	Initial passage duration (P ₀) (days)	Number of MSC-like cells \pm SEM (cells / mL)
Synovium direct biopsy	37 \pm 6	6.6 \times 10 ⁵ \pm 0.52 \times 10 ⁵
Shaver blade filtrate	21 \pm 2	6.5 \times 10 ⁵ \pm 0.49 \times 10 ⁵
Synovial fluid	32 \pm 5	7.5 \times 10 ⁵ \pm 0.28 \times 10 ⁵

TABLE 3 Initial passage duration for each sample type, and respective number of cells harvested at the end. Values presented as mean \pm SEM.

Cell morphology was accessed during culture. Overall, cells presented a standard morphological MSC phenotype with a long, fibroblastoid, appearance (see *Figure 12*). Differences were mainly observed for cells isolated from synovial fluid, which seemed to have a more compact morphology, lacking spreading membrane processes for some donors.

The success and efficiency of the isolation process seems to be highly dependent of donor conditions. The amount of biopsy sample also seems to influence the total number of cells obtained. While smaller solid samples provide best results (ratio #isolated cells / mass), synovial fluid samples seem to have an opposite performance. Inflamed joints also seems to be a key factor, as the highest number of cells obtained from synovial fluid correlates with local degenerative diseases, thus increasing the cells circulating in the fluid. Shaver blade filtrate derived cells seem to have the fastest isolation period,

measured by the initial passage duration. This may be due to a more efficient mechanic processing of the samples by its nature (shaver blade), which allows more cells to be extracted per sample.

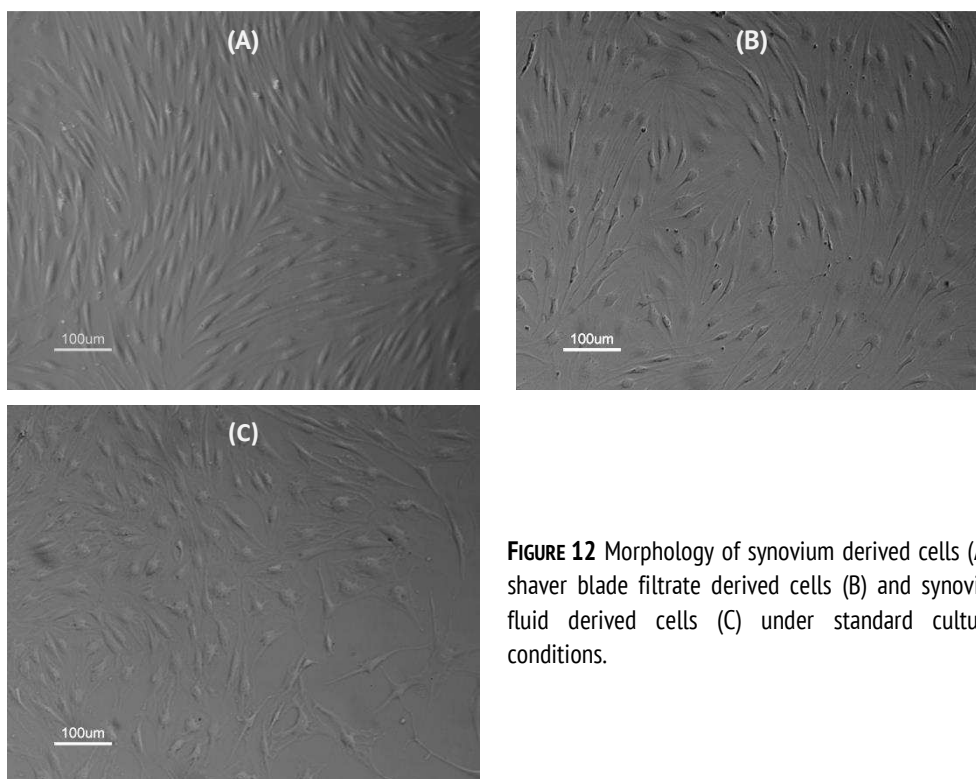


FIGURE 12 Morphology of synovium derived cells (A), shaver blade filtrate derived cells (B) and synovial fluid derived cells (C) under standard culture conditions.

The optimization of the isolation protocol may lead to the increase of the number of cells obtained per sample. New directions for this would possibly include a density gradient centrifugation method using Ficoll for synovial fluid samples, which would permit an enrichment of mononuclear cells isolated while discarding biological contaminants such as red blood cells. For solid samples, new enzymatic digestion methods should be optimized, possibly including other ECM-specific proteolytic enzymes combined with collagenase.

4.2 STORAGE AND CRYOPRESERVATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

Following isolation, cells were expanded in static conditions using T-25 and T-75 flasks. For storage and cell bank creation purposes, isolated cells from each donor and sample type were cryopreserved from passage one (P1) to four (P4) at -80°C.

Cells never exceeded a number of 10^6 stored in each cryovial. A total number of 5.92×10^7 cells were cryopreserved during this work.

4.3 *EX-VIVO* EXPANSION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

The *ex-vivo* expansion of synovium-derived MSCs was performed in two different culture systems, static (T-Flasks and well plates) and stirred (spinner Flasks).

4.3.1 *EX-VIVO* EXPANSION IN STATIC CONDITIONS

After cell isolation, the expansion of synovium-derived hMSCs isolated from the different donors was performed throughout ten passages (P0 to P10) in T-Flasks or well plates, using DMEM + 10% FBS. The cells showed a considerably slow growing rate, when comparing with the other sources (BM: 6-8, AT: 5-6 and UCM: 4-5 days, respectively) (Simões *et al.*, 2013) reaching 70-80% confluence in an average of 15 ± 3 days for synovium isolated cells, 19 ± 5 days for cells from synovial fluid and 13 ± 1 days for the shaver filtrate cells. Synovium-derived cells seem to have a rather constant time between passages, even for higher ones. On the other hand, the average time between passages for synovial fluid tends to increase over time, maybe due to cellular senescence at an early stage in culture (see *Figure 13*). The average time between passages is highly dependent both on donor condition and the yield of cells obtained upon isolation.

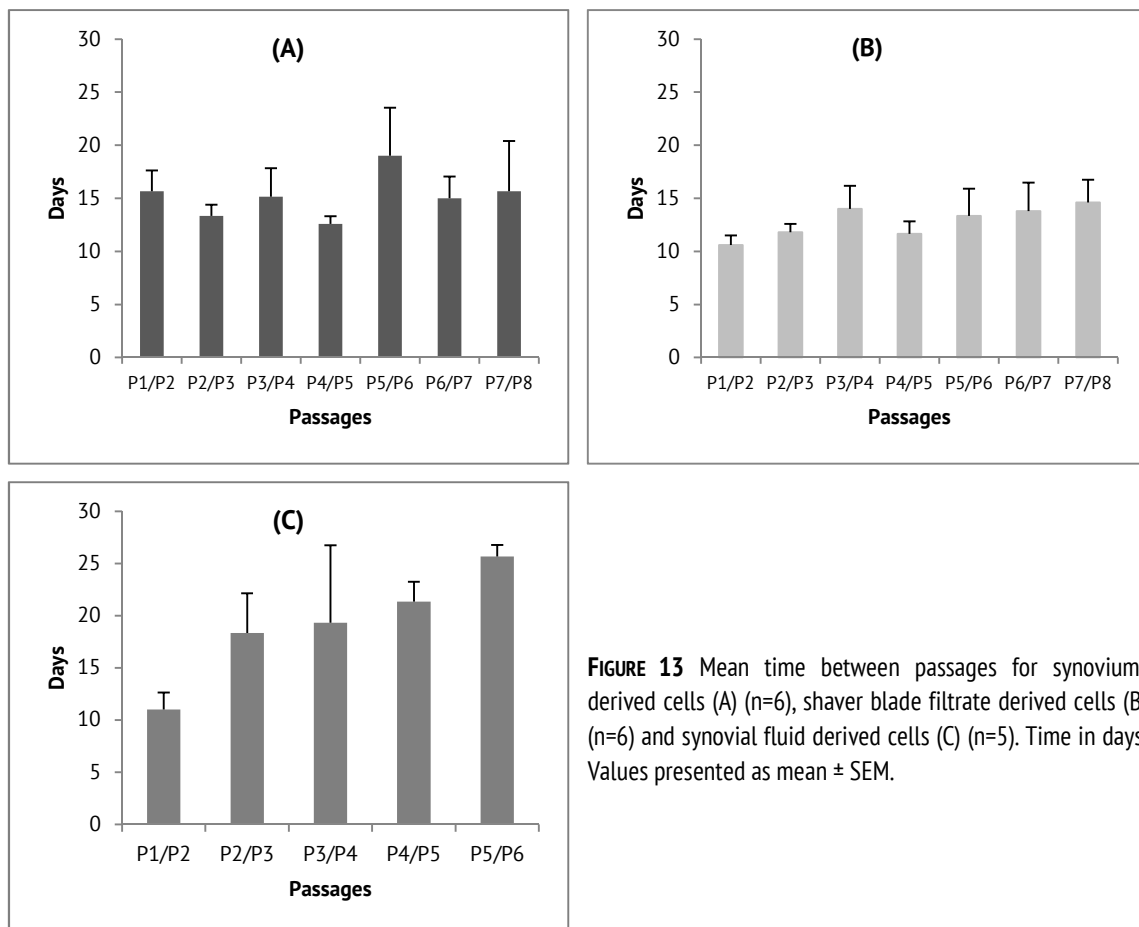


FIGURE 13 Mean time between passages for synovium-derived cells (A) (n=6), shaver blade filtrate derived cells (B) (n=6) and synovial fluid derived cells (C) (n=5). Time in days. Values presented as mean \pm SEM.

Doubling time of the first three passages was also assessed. Shaver filtrate derived cells seem to have the lowest doubling times of the samples evaluated, at 120 ± 18 hours. Synovium-derived cells show a doubling time under the same conditions of 162 ± 33 hours and synovial fluid derived cells present with the highest doubling time, at 176 ± 52 hours. These values contrast significantly with previous results obtained for the other sources of MSCs.

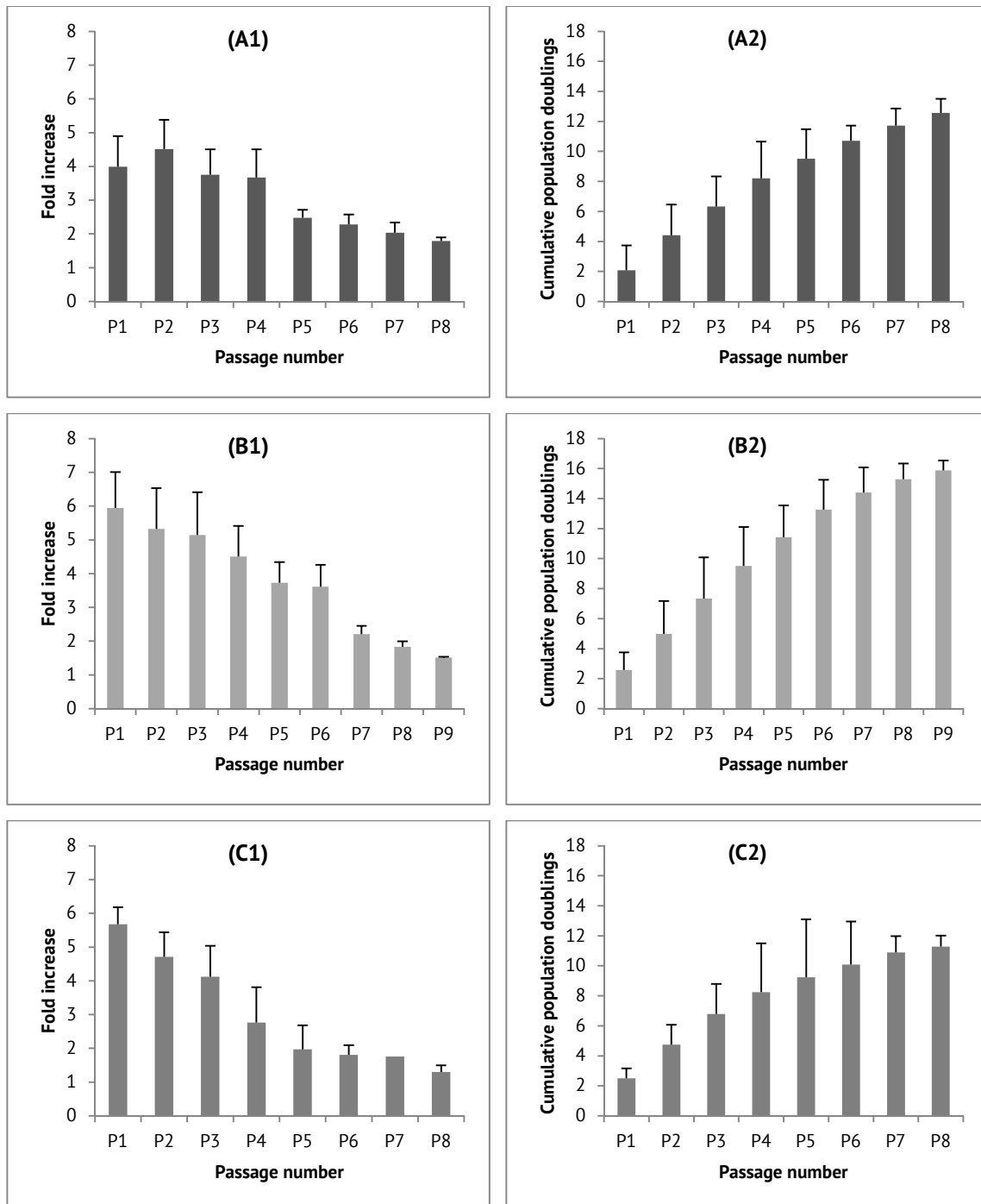


FIGURE 14 Fold increase (FI) at the end of each passage for synovium-derived cells (A1), shaver blade filtrate derived cells (B1) and synovial fluid cells (C1). The respective cumulative population doublings (CPD) are also presented, for synovium-derived cells (A2), shaver blade filtrate derived cells (B2) and synovial fluid cells (C2). Values presented as mean \pm SEM.

Lu and colleagues performed proliferation studies with BM- and UCM-MSCs which revealed that the mean doubling time of the UCM-MSCs in passage 1 (P1) was about 24 hours and remained almost constant up to P10. In contrast, the mean doubling time of BM-MSCs was 40 hours increasing considerably after P6 (Lu *et al.*, 2006). Other study by Peng and colleagues described mean population doubling times of 45 hours for AT-MSCs and 61 hours for BM-MSCs (Peng *et al.*, 2008), which may indicate that the growth of cells may depend largely on donor conditions and site of tissue extraction.

Following this, the fold increase at the end of each passage was also evaluated (see *Figure 14*). The expansion performance of synovium-derived MSCs tends to decrease with increasing passages, showing similar results with the other sources. The average fold increases throughout culture time in the last passages was almost half than the ones obtained in the first passages. Also, as the cell passage number increases, the cells tend to present a longer and thinner morphology and consequently occupy a larger area.

Synovium-derived MSCs showed a lower fold increase for the first passage, compared with shaver filtrate derived- or synovial fluid cells. This may be due to the nature of diseased joints, impairing the proliferative capacity of residing cells. A more detailed study, matching donor age and disease conditions would allow a better understanding of the phenomena affecting cell expansion.

4.3.1.1 GROWTH KINETICS UNDER DIFFERENT INITIAL SEEDING DENSITIES

Following cell growth over time is essential to know how different MSCs evolve in culture. Also, an understanding of such data would allow the development of better strategies to accomplish the maximum *ex-vivo* expansion in a shorter period of time.

Cells were seeded at 100, 500, 1 000, 3 000 and 6 000 cells/cm² in well plates (see *Figure 15*). After 21 days in culture, there were no significant differences between an initial seeding density of 100 or 500 cells/cm². This further demonstrates the clonogenic potential of these cells. Also, no differences were found on the initial seeding densities of 3 000 and 6 000 cells/cm² over the same time period. In fact, the optimum initial density seems to be 3 000 cells, which allows good growth kinetics. Higher cell densities and the beginning may compromise the surface for adhesion, or even start to deliver death cells to nearby cells early, causing an inhibition of growth by direct cell-to-cell contact. Fold increase analysis reveal that the highest cell doubling number in culture was achieved by the smallest seeding density. Conversely, lower doubling numbers were achieved by the

higher initial densities. Although this parameter is useful, it should be used wisely due to many mathematical artifacts arising from its use, as higher fold increases do not necessarily imply higher cell numbers.

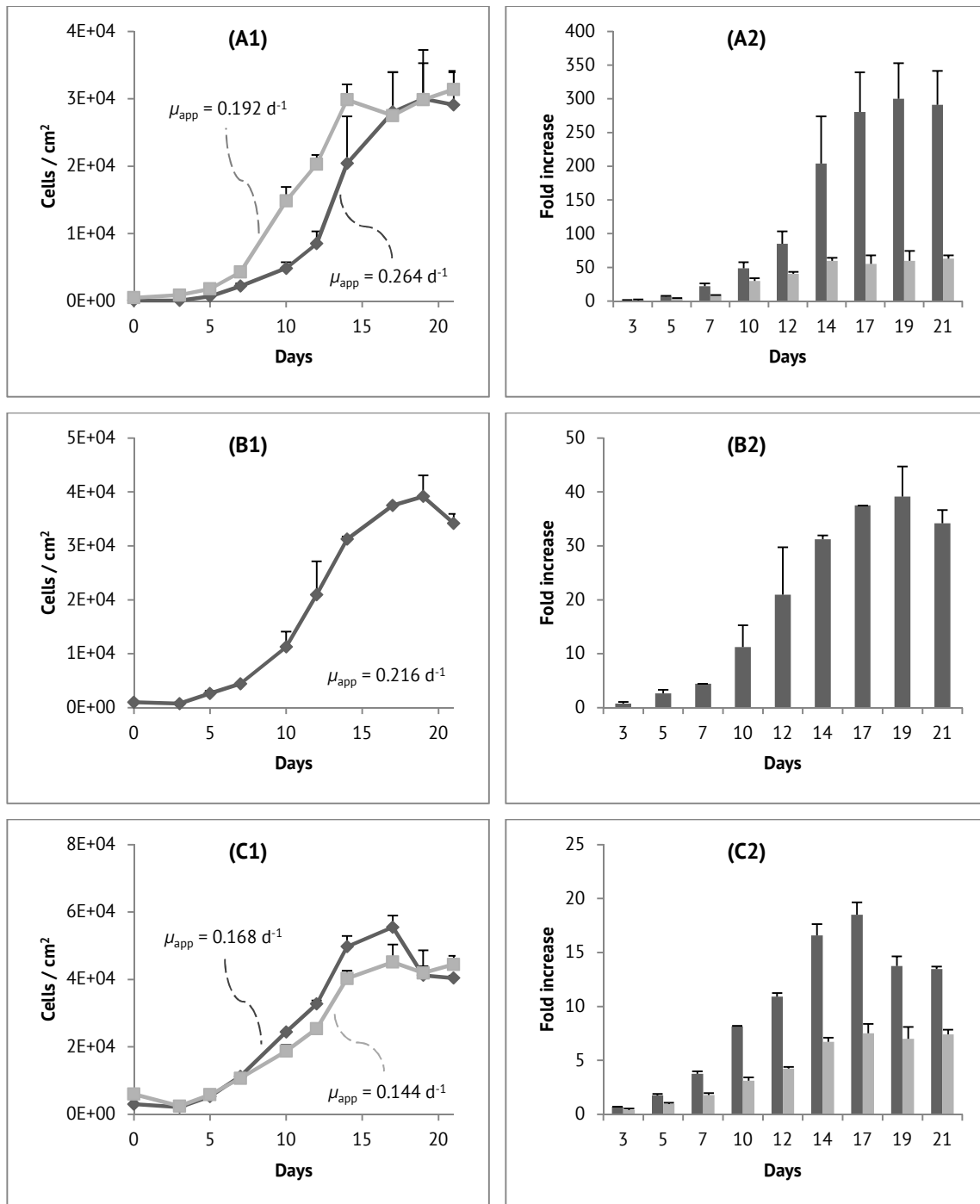


FIGURE 15 (A1) Growth kinetics at initial seeding density of 100 (dark grey) and 500 (light grey) cells/cm², results in the same units, and respective fold increase (A2) over time. (B1) Growth kinetics at initial seeding density of 1 000 cells/cm², results in the same units, and respective fold increase (B2) over time. (C1) Growth kinetics at initial density of 3 000 (dark grey) and 6 000 (light grey) cells/cm², results in the same units, and respective fold increase (C2) over time. Values presented as mean \pm SEM. Time in days.

4.3.1.2 GROWTH KINETICS UNDER HYPOXIC CONDITIONS

Previous results have shown the beneficial effect of hypoxia for the expansion of MSCs from bone marrow. The same effect was evaluated for synovium-derived MSCs using cells originated from the shaver blade filtrate. A 2% hypoxic environment was used for the expansion, which is a typical oxygen tension value for many cell niches in the human body.

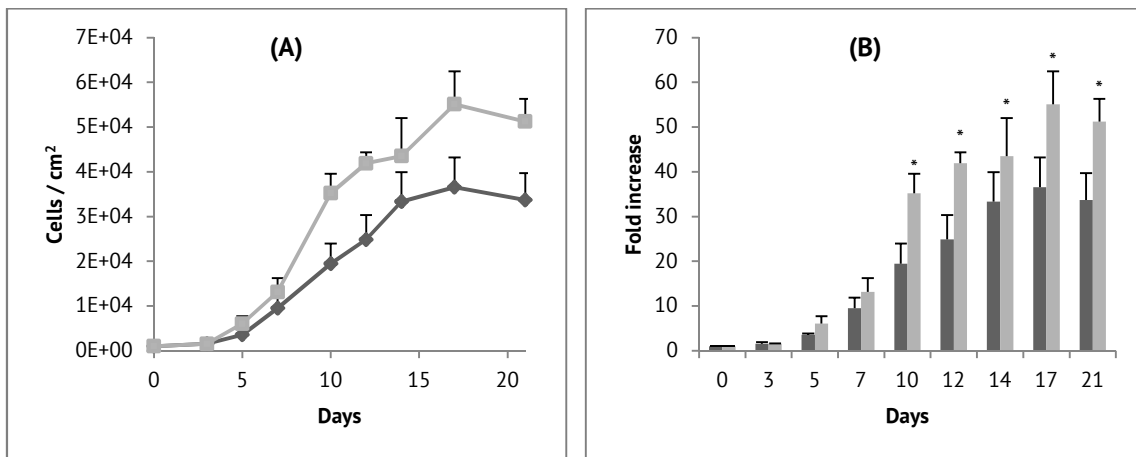


FIGURE 16 Growth kinetics (n=5) over time under 2% O₂ hypoxia (light grey) and normoxia (dark grey). Average values \pm SEM for cells/cm² at each day are shown (A). Fold increase over time for both experiments (B). Asterisk indicates a statistically significant difference in the mean from normoxia samples at that time point ($p < 0.05$).

The results are consistent with previous ones obtained for other sources of MSCs (Dos Santos *et al.*, 2009). Hypoxia promotes an early exponential phase of growth (see Figure 16), as well as a statistically significant higher fold increase upon day 10 (FI: 42 ± 3 vs. 25 ± 5 , after 12 days). This improvement may result from a less damaging environment for the cells, as well as a more effective cell adhesion and ECM formation patterns (Grayson *et al.*, 2007).

Even though these results show the beneficial effects of hypoxia, growing synovium-derived cells under new oxygen tensions would be interesting. Articular cartilage has an intrinsic oxygen tension varying between 1 – 6% for the deep and superficial zone, respectively (Zhou *et al.*, 2004; Silver, 1975), and synovial fluid presents an oxygen tension ranging from 6.5 – 9% (Falchuk *et al.*, 1970; Lund-Olesen, 1970). The use of these cells for clinical applications may benefit from controlled growth at oxygen tensions closer to the ones that exist *in vivo*, as these are shown to promote chondrocyte differentiation with better results, besides improving proteoglycan and hyaluronan synthesis as well as collagen type II, which all combine to form an extracellular matrix of

superior biomechanical properties (Domm *et al.*, 2002; Lennon *et al.*, 2001; Nevo *et al.*, 1998).

Overall, an hypoxic environment results in beneficial outcomes for the expansion of these cells in shorter time periods, with average improvements between 24 - 48 hours of culture time.

4.3.1.3 GROWTH KINETICS USING DIFFERENT SURFACE COATINGS

The adhesion substrate also plays in important role in promoting initial cell seeding and proliferation. By the previous results, SMSCs seem to have an intrinsic long lag phase at the beginning of the expansion. The use of fibronectin or other attachment substrates could improve culture time or the number of cells obtained in static conditions.

Shaver filtrate derived cells were used to test different coatings, evaluating cell growth kinetics over time. As previous results showed, initial seeding density is also a key factor on the successful expansion, thus two different seeding densities (standard 1 000 and 4 000 cells/cm²) were used along gelatin and fibronectin coatings (see *Figure 17*).

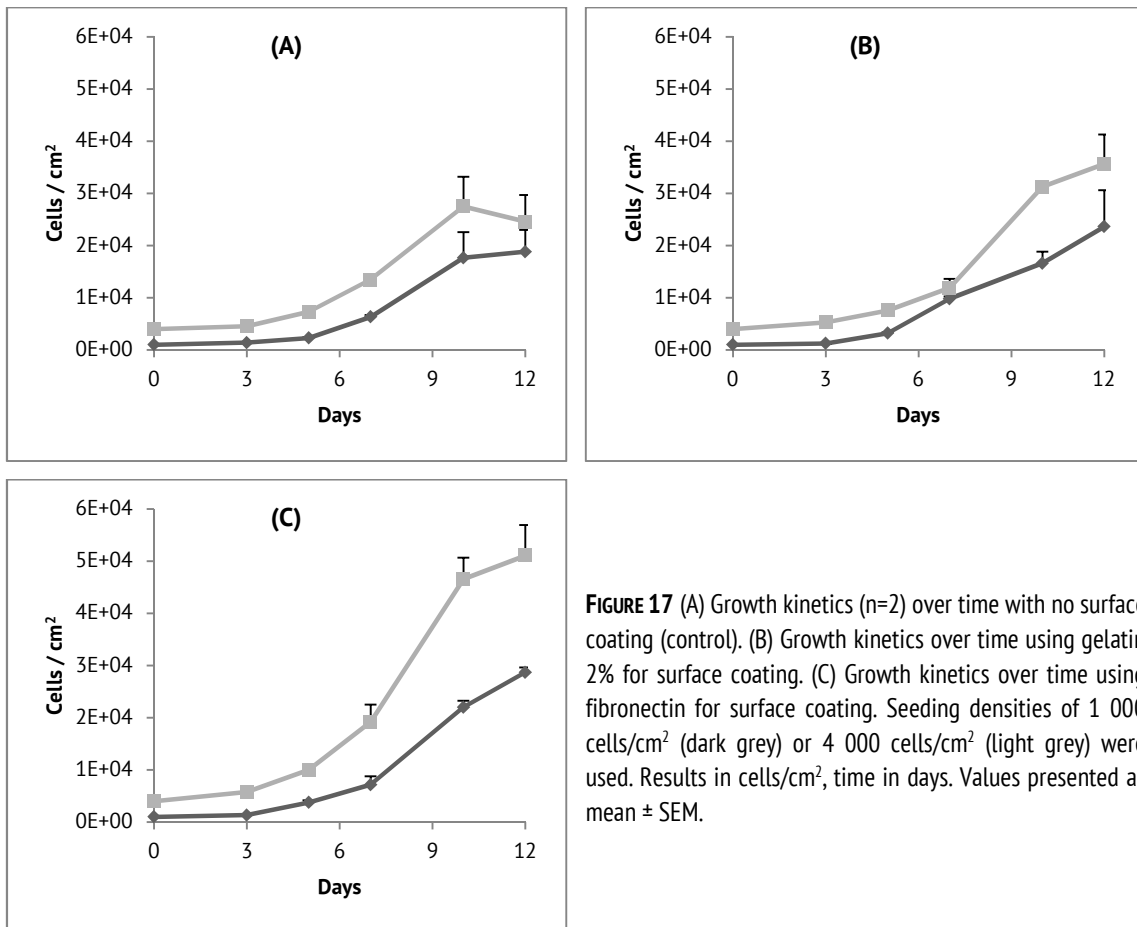


FIGURE 17 (A) Growth kinetics (n=2) over time with no surface coating (control). (B) Growth kinetics over time using gelatin 2% for surface coating. (C) Growth kinetics over time using fibronectin for surface coating. Seeding densities of 1 000 cells/cm² (dark grey) or 4 000 cells/cm² (light grey) were used. Results in cells/cm², time in days. Values presented as mean ± SEM.

Even though the use of gelatin for mimicking the ECM components should be avoided, it improved overall proliferation of the cells, particularly at higher initial densities. Fibronectin proved to be the best substrate when culturing synovium-derived cells at higher initial densities. At the standard initial density of 1 000 cells/cm², no significant differences in the number of cells obtained were found after 12 days in culture (see *Figure 18*). This may suggest a need for stronger autocrine signaling of the cells, which is achieved when the initial seeding density is higher.

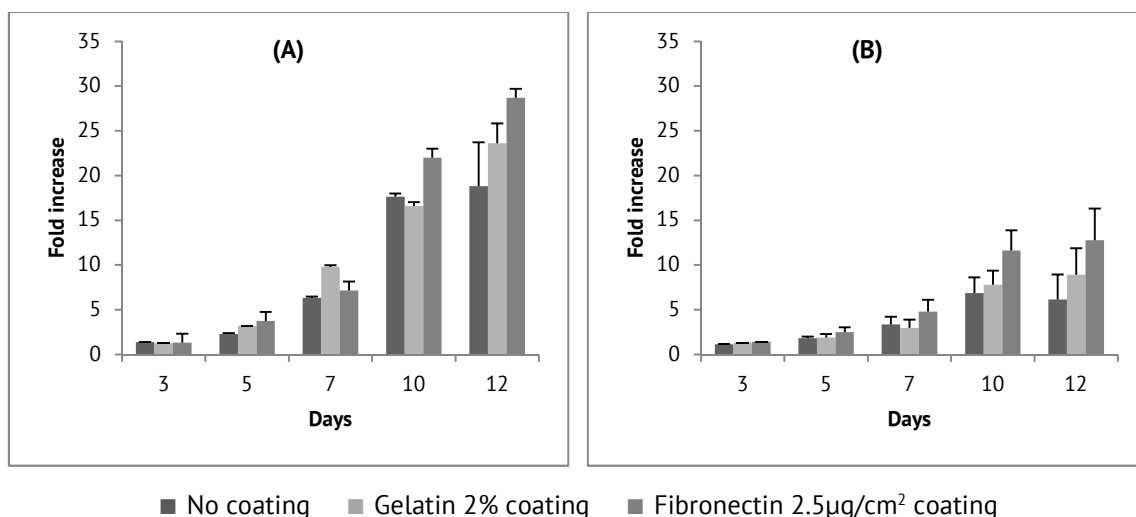


FIGURE 18 Fold increase over time at initial seeding of 1 000 cells/cm² (A) and 4 000 cells/cm² (B), for different surface coatings. Values presented as mean ± SEM.

4.3.1.4 CLONOGENIC POTENTIAL

An important parameter used to characterize hMSCs is the evaluation of their clonogenic potential, which is a direct measure of the ability a single cell has to form a colony (colony forming unit-fibroblast, CFUs) without autocrine signaling from adjacent cells. In order to assess this capacity, the cells were plated at a very low density (10 cells/cm²) and cultured for 15 days without media change.

Synovium derived-hMSCs clonogenic potential was evaluated for each passage (from P1 to P10) in presence of the culture medium DMEM + 10% FBS. Results until passage 7 are shown in *Figure 19*.

The ability to form colonies was maintained under several passages for cells derived both from the synovium and from the shaver blade filtrate. The clonogenic potential to form small colonies (up to 25 cells each) seems to be independent from the passage number, while larger colonies gradually decrease in frequency as the cells are passaged.

This may indicate that senescence is associated with a stronger autocrine signaling demand for proliferation.

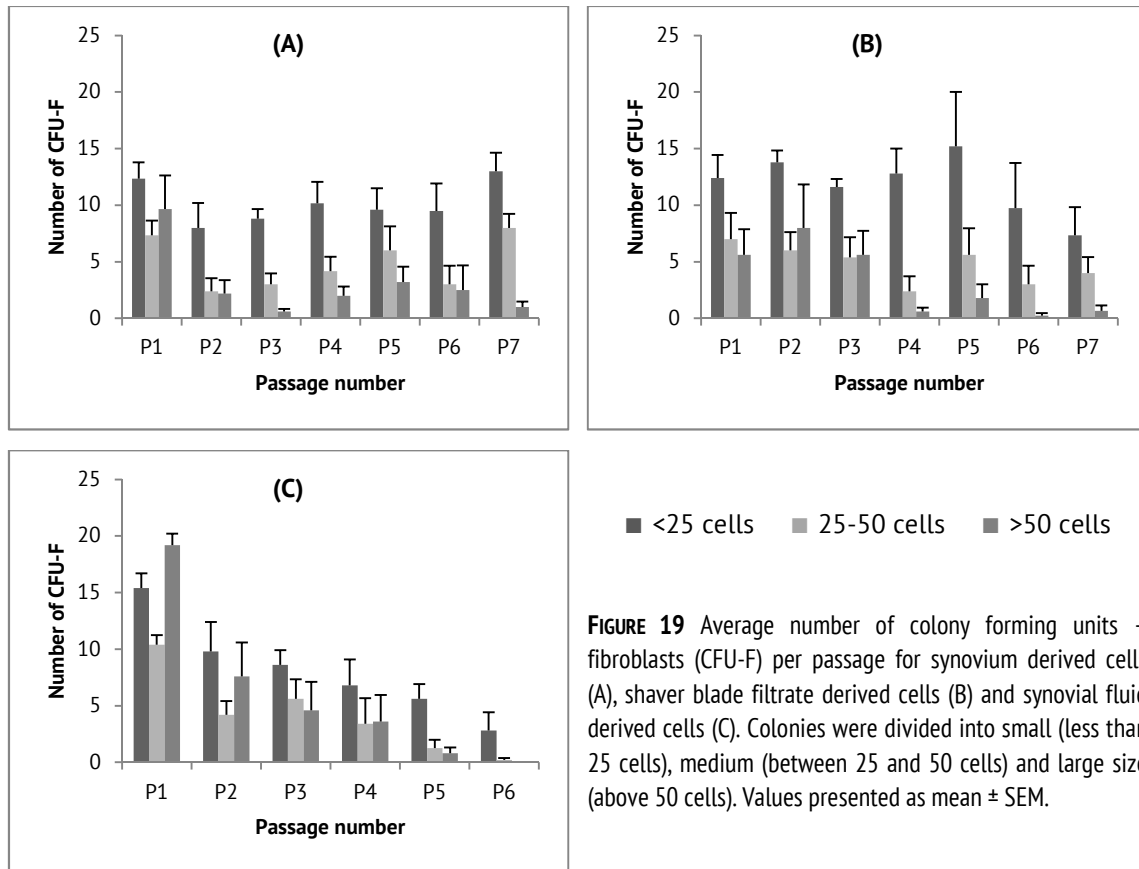


FIGURE 19 Average number of colony forming units - fibroblasts (CFU-F) per passage for synovium derived cells (A), shaver blade filtrate derived cells (B) and synovial fluid derived cells (C). Colonies were divided into small (less than 25 cells), medium (between 25 and 50 cells) and large size (above 50 cells). Values presented as mean \pm SEM.

In contrast, synovial fluid derived cells seem to have a maximum potential on the lower passage. However, the ability to form large colonies decreases considerably over the first passages, succeeded by the loss of potential in forming smaller colonies. After passage 6, a seeding density of 10 cells/cm² proved to be unsuccessful for the proliferation of these cells, possibly indicating a loss of the clonogenic potential.

Although these results can be compared with other sources, further screening of the clonogenic capacity of these cells is needed, as the cells obtained from the synovial fluid are already biased by the disease of respective donors.

4.3.2 EX-VIVO EXPANSION UNDER DYNAMIC CONDITIONS

The use of appropriate numbers for clinical applications is central for the successful use of MSCs in addressing particular scientific problems. The ultimate goal would be their expansion in dynamic systems, providing a fast and reliable system for the scale up of the quantity of cells produced.

To this end, the cells from one donor (shaver filtrate sample) were expanded at passage 4 in spinner flasks in order to evaluate their expansion potential under stirred conditions and compare it with the static cultures. Different coatings were used on the microcarriers, in order to assess which one favors the best adhesion at the beginning of the expansion, while maintaining cell quality over time in culture.

Dynamic expansion was performed using StemPro® MSC SFM XenoFree culture medium for 12 days at 37°C and 5% CO₂. At each day, a sample was taken to determine the number of viable cells and 25% of the medium was renewed every day upon day 3. After 24 hours in culture, the initial adhesion was quantified for the three spinners. Adhesion rates ranged from 36 – 42% for *HPL* and CELLStart™ coatings, respectively. These values are considerably higher than others obtained previously with CELLStart™, for BM- and AT-MSCs, which range from 22 - 23% respectively (Dos Santos *et al.*, 2011). Although this may suggest that synovium-derived MSCs could have a superior capacity of attachment, an improvement of the commercial formulations used for enhancing cell adhesion may not be excluded.

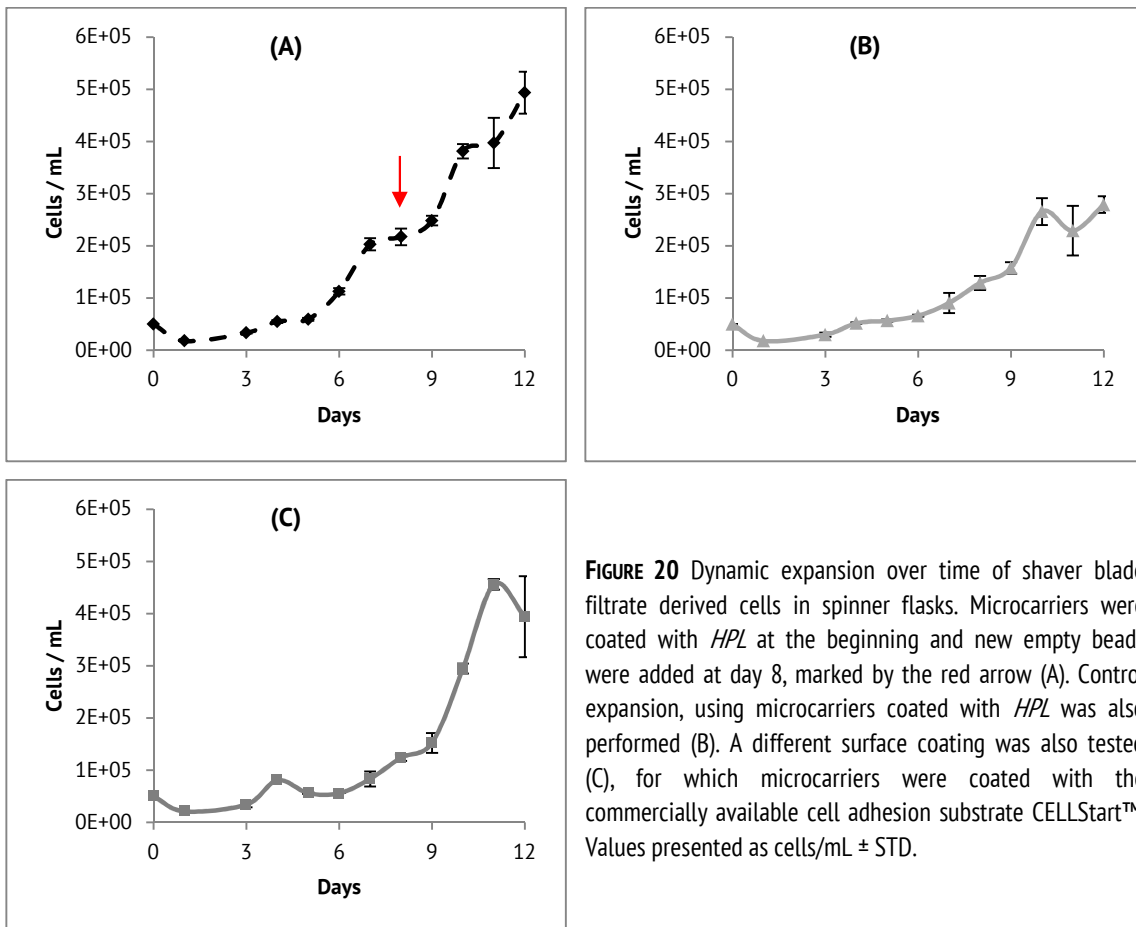


FIGURE 20 Dynamic expansion over time of shaver blade filtrate derived cells in spinner flasks. Microcarriers were coated with *HPL* at the beginning and new empty beads were added at day 8, marked by the red arrow (A). Control expansion, using microcarriers coated with *HPL* was also performed (B). A different surface coating was also tested (C), for which microcarriers were coated with the commercially available cell adhesion substrate CELLStart™. Values presented as cells/mL \pm STD.

Results obtained shown that after day 10 and until the end of culture, there seems to be a steady state on the number of viable cells in culture (see *Figures 21* and *22*). This can be associated to the accumulation of metabolic by-products (e.g. lactate) or even surface limitation. In the spinner flask (A), new beads coated with *HPL* added at day 8 may seem to minimize this issue, and the expansion appears to continue after day 12. In the same way, for this spinner flask the maximum fold increase obtained was approximately 10 ± 0.80 . For the other spinners, maximum fold increase was 6.6 ± 0.32 for the expansion using microcarriers coated with *HPL* but without adding new beads (B), and 9.1 ± 0.20 for CELLStart™ coated microcarriers (C). There is no significant difference for the fold increase obtained between spinners (A) and (C). The values obtained seem to be lower than the expansion results from other sources, namely BM- (18 ± 1), AT- (14 ± 7) (Dos Santos *et al.*, 2011) and UCM-MSCs (21) (Simões, I., unpublished data).

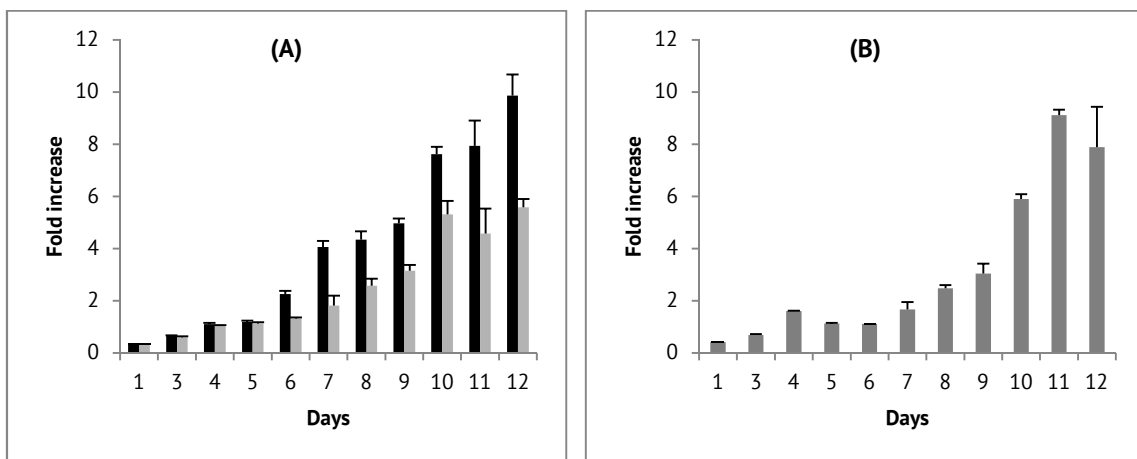


FIGURE 21 (A) Fold increase over time of spinner flasks containing microcarriers coated with *HPL*. Standard culture for control (light grey) and addition of empty microcarriers (black) at day 8 are shown. (B) Fold increase over time of spinner flask with CELLStart™ coating, standard culture. Values presented as mean \pm STD. Time in days.

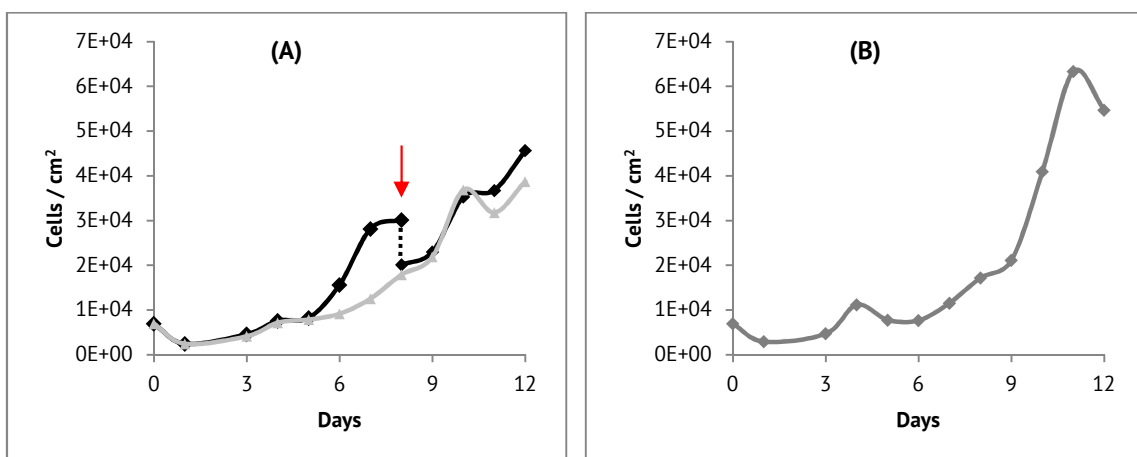


FIGURE 22 Cells per surface area over time in spinners with *HPL* coating (A) or CELLStart (B). On plot (A), black line relates to *HPL* spinner with new beads added at day 8 (addition marked by red arrow) and light grey line relates to standard control culture. Results in cells/cm². Time in days.

DAPI staining was also performed during the expansion of synovium-derived MSCs in stirred conditions, in order to assess cell distribution on the plastic microcarriers in the three different conditions evaluated. In all the spinners it was possible to observe a gradual increase in cells growing attached to the microcarriers. Few beads remained totally empty throughout time in culture, which indicates a good and homogenous distribution of growing cells. For the spinner flask to which new empty beads were added at day 8, phenomena of bead-to-bead cell transfer could also be observed.

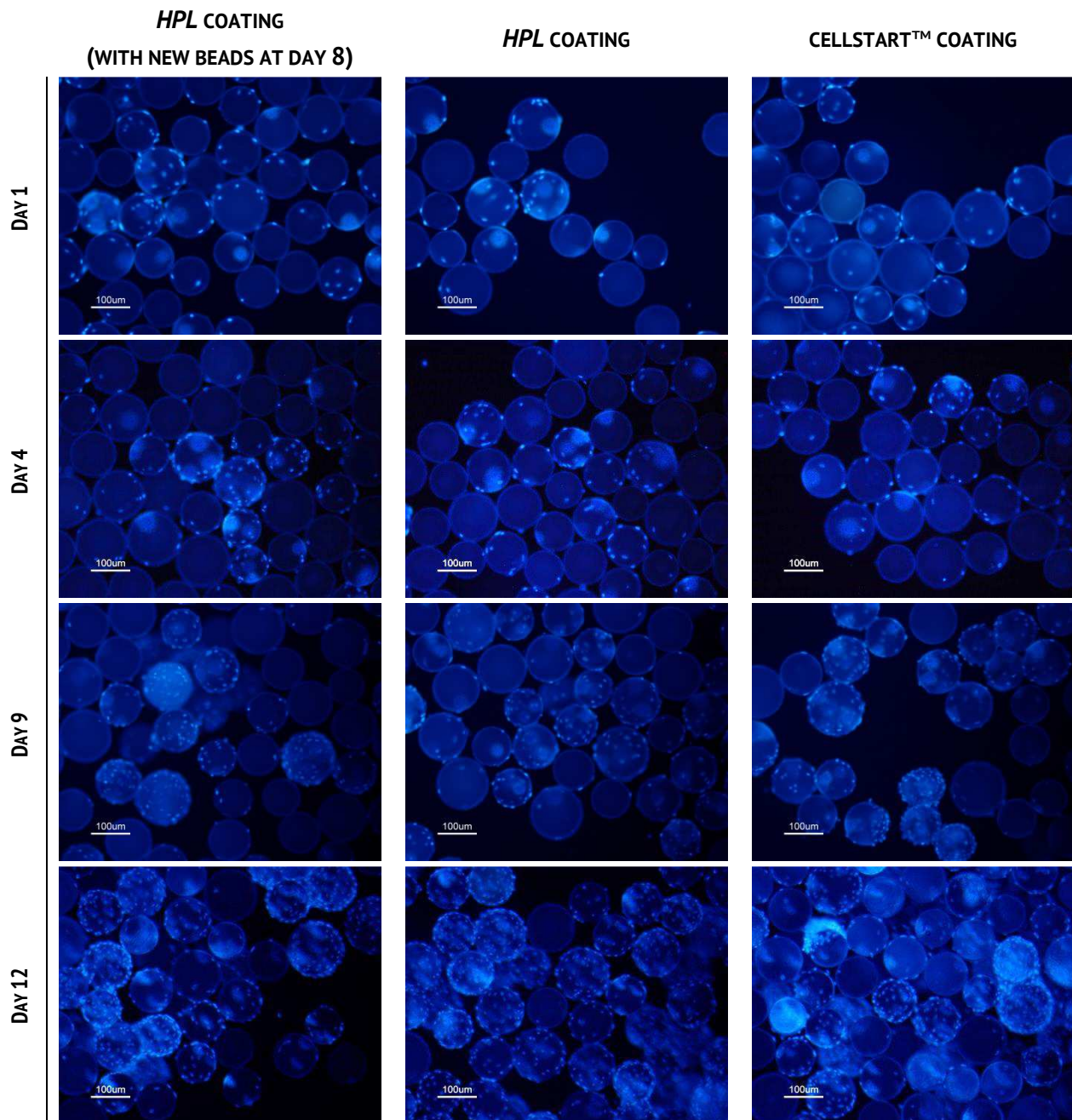


FIGURE 23 DAPI staining of microcarriers containing cells over time in culture, until final day (12). First column depicts microcarriers coated with *HPL*, with new beads added at day 8. Second column depicts *HPL* coated beads, in standard culture conditions. Third column depicts microcarriers coated with CELLStart™. No significative aggregation was observed for the three spinners during expansion.

4.4 IMMUNOPHENOTYPIC CHARACTERIZATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS

Synovium-derived MSCs were immunophenotypically characterized at the beginning of the expansion (passage 2), using the minimum panel of surface antigens proposed by the International Society of Cellular Therapy.

Before expansion under stirred conditions, the expression of the same surface markers was also evaluated, as well as at the end of the expansion on spinner flask.

For static conditions characterization, cells from 3 donors were used for each kind of sample. Less than 2% of all samples evaluated were marked positive for the expression of CD14, CD19, CD34, CD45, CD80 and HLA-DR. As BM-, AT-MSCs and UCM-MSCs, these cells do not express CD34 or CD45. Considering the other markers, the expression of CD73 was above 99% for all samples evaluated. CD90 and CD105 expression on synovium-derived cells was less than expected (< 90%), although shaver filtrate samples show a standard value (>99%) for CD105 (see *Figure 24*).

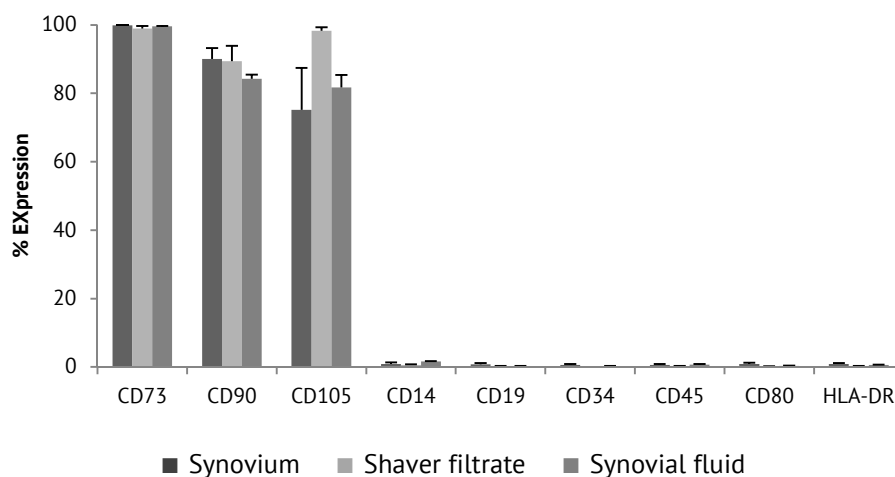


FIGURE 24 Expression of surface membrane markers for synovium-derived mesenchymal stem cells expanded under static conditions. Analysis performed with cells at passage 2 (n=3). Values displayed as mean \pm SEM.

This decrease on receptor expression might be due to several reasons. The use of an inappropriate anti-CD90 or CD105 antibodies conducting to an ineffective labeling may explain the results obtained. Also, the enzymatic or mechanic treatment for harvesting cells before analysis might also have affected these surface receptors in particular (Wiesmann *et al.*, 2006; Brown *et al.*, 2007). It is also known that the culture medium may influence the particular characteristics of cells, mainly due to the heterogeneity of FBS routinely used, leading to variable expression levels of all the receptors evaluated (Tyndall *et al.*, 2007).

A more elaborate explanation for these results may arise from the particular tissue niche present in synovial joints. Synovium is formed by a thin intimal lining layer, which encapsulates synovial fluid. This layer is composed by two cell types in relatively equal proportions: type A or macrophage-like synovial cells and type B or fibroblasts-like synoviocytes (FLS) (Bartok & Firestein, 2010).

Type B synoviocytes, or FLS, are mesenchymal cells that exhibit many characteristics of fibroblasts, including expression of type IV and V collagens, vimentin, and CD90, thus impairing the attempts to distinguish them (Firestein, 2009; 1996). The origin of this population is unclear, but could arise from the migration of true mesenchymal stem cells from the nearby tissues or expansion of a stem cell pool in the synovium when cartilage is injured or other inflammatory processes occur (Chang *et al.*, 2010).

Isolated cells from synovial tissues may prove to be a more exclusive population of mesenchymal stem cells mixed with type B synoviocytes, with proper characteristics different from true mesenchymal stem cells, and a more committed differentiation potential towards osteogenic or chondrogenic lineages. Further screening of these cells is required, which is beyond the scope of this work.

Cells expanded under stirred conditions were also evaluated before and after expansion. Cells were grown for one passage under StemPro® MSC SFM XenoFree culture media before inoculation on spinner flasks. When cells were grown on this media, an upregulation of CD90 and CD105 receptors seems to have occurred (>99%), comparing with previous results obtained for cells expanded using DMEM + 10% FBS (see *Figure 25*).

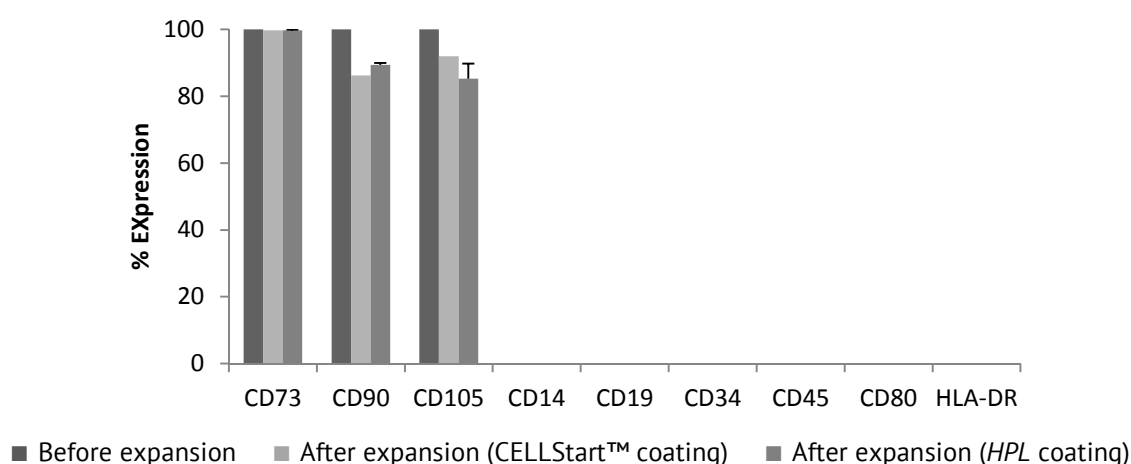


FIGURE 25 Expression of surface membrane markers for shaver blade filtrate derived cells, before and after dynamic expansion on spinner flasks. Values presented as mean \pm SEM when applicable.

Comparing the values obtained at the beginning and end of expansion in the dynamic system, there seems to be a general predisposition for decreasing the expression of all the cell surface markers that were tested. Nevertheless, this slight decrease is not significant and similar results are routinely obtained from MSCs from other sources (namely BM-, AT-, and UCM-) expanded under dynamic conditions. No significant differences on surface receptor expression for different types of microcarriers coating (CELLStart™ or HPL) were found.

4.5 METABOLITE ANALYSIS

In addition to cell growth kinetics, the analysis of cell nutrient consumption (glucose) and metabolite production (lactate) rates were performed. These parameters would allow a better understanding and profiling of stirred culture conditions, relating to the metabolism of synovium-derived MSCs over time (see *Figure 26*).

Glucose is converted to many metabolites, lactate being the most important for cells under standard culture conditions. As mentioned before, inhibitory and toxic concentrations of lactate have been reported for several cell lines (Hassell *et al.*, 1991). For hMSCs, a concentration above 35mM is considered toxic, thus impairing cell proliferation. During culture in dynamic conditions for 12 days, lactate never reached toxic concentrations.

The specific glucose uptake (q_{glucose}) and lactate production (q_{lactate}) rates were determined based on the metabolite concentration for the specified culture period. Also, the apparent yield of lactate from glucose ($Y'_{\text{lactate/glucose}}$) was estimated as described before. This yield has a theoretical maximum value of 2, as no more than two molecules of pyruvate (and consequently lactate) can be obtained from a single molecule of glucose.

Adding new beads during culture time seems to support a shift in the metabolism of cells growing attached to the microcarriers. After the addition of beads, glucose metabolism efficiency decreases, leading to an increase in lactate production. This result may suggest that alternative metabolic pathways are activated, as cells try to expand rapidly, since more surface area is available for growth and adhesion. Bead-to-bead transfer phenomena affecting metabolism and energy production may also not be excluded, as the shear stresses experienced may influence the gene expression profile of the cells growing.

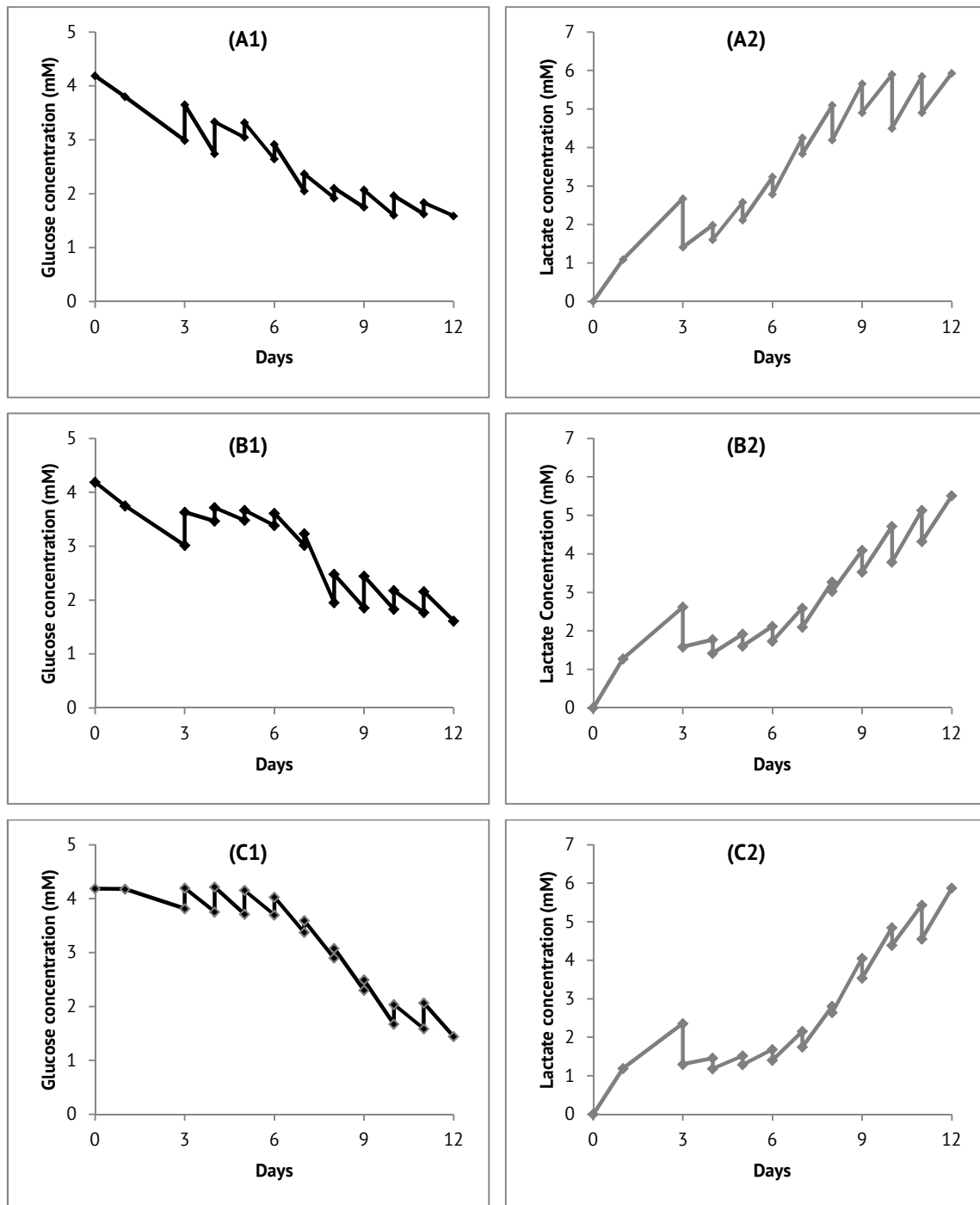


FIGURE 26 Glucose and lactate concentration over time during dynamic culture of shaver blade filtrate derived MSCs. Plots for spinner flask containing beads coated with *HPL* and new beads at day 8 (A1 and A2), *HPL* coated beads standard spinner (B1 and B2) and spinner containing beads coated with CELLStart™ (C1 and C2) are shown. Concentrations in mM. Time in days.

When comparing the efficiency of glucose consumption, cells mainly present a high rate of glycolysis followed by lactate production. Overall, the metabolic processes were found to be efficient by using microcarriers coated with *HPL* under standard culture conditions. When new beads were added at day 8, the apparent yield of lactate obtained from glucose raised above the theoretical value of 2 (see Figure 27).

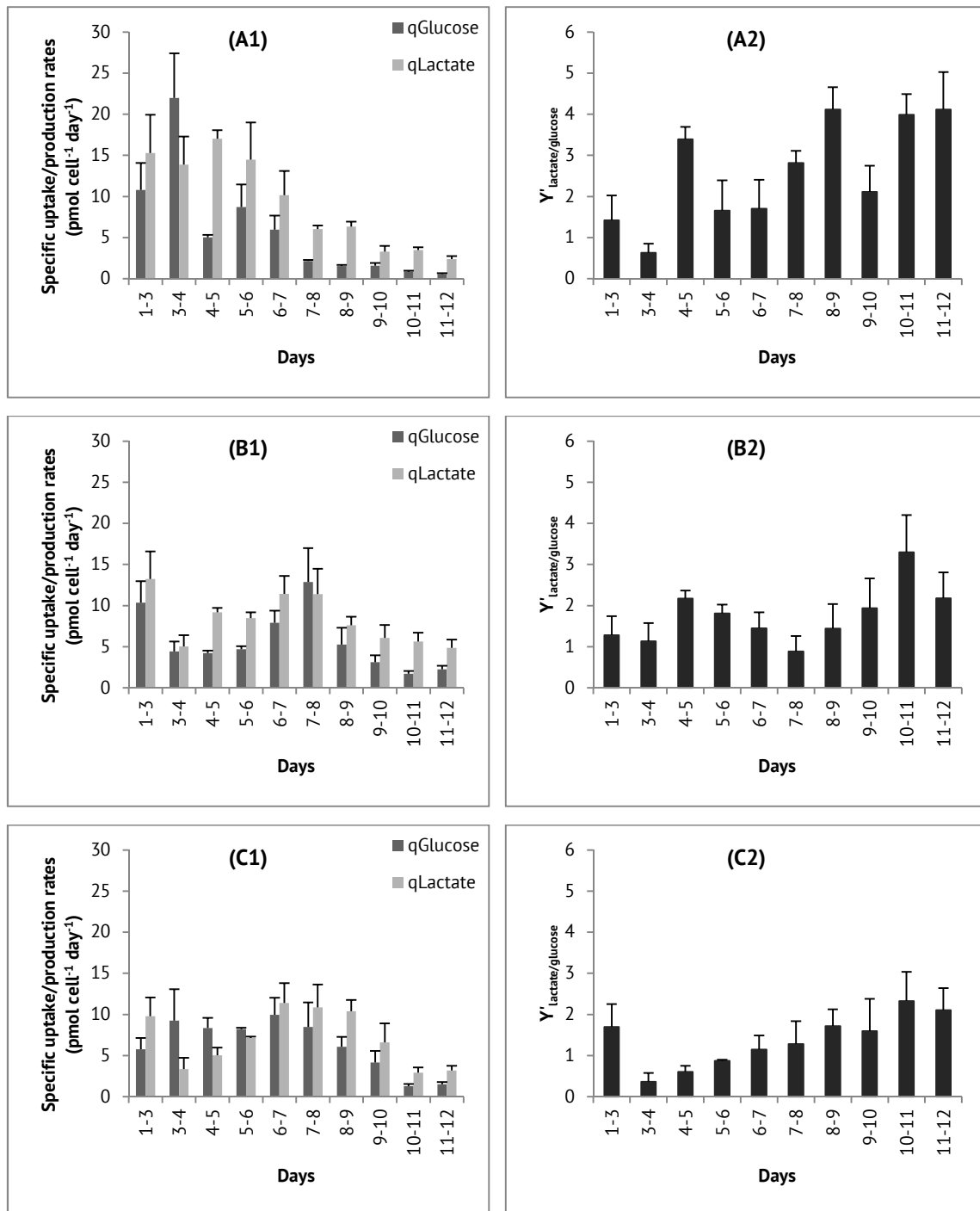


FIGURE 27 Apparent yield of lactate from glucose over time (right) and specific glucose uptake (dark grey) and lactate production (light gray) rates (left) during dynamic culture of shaver blade filtrate derived MSCs. Plots for spinner flask containing beads coated with *HPL* and new beads at day 8 (A1 and A2), *HPL* coated beads standard spinner (B1 and B2) and spinner containing beads coated with CELLStart™ (C1 and C2) are shown. Yield of lactate from glucose in mol_{lactate} mol_{glucose}⁻¹. Uptake and production rates in pmol cell⁻¹ day⁻¹. Time in days. Values presented as mean ± STD.

These results may indicate that cells tend to adapt their active metabolic pathways, preferably leading to glutamine consumption rather than glucose, as the major source of carbon and energy necessary to support growth and expansion. However, ammonia and glutamine concentrations were not evaluated, due to HPLC analytical methods

limitations. In the future, those results would be relevant to better understand the metabolic processes during expansion under stirred conditions.

Dynamic system	$Y'_{\text{lactate/glucose}}$ (before day 8)	$Y'_{\text{lactate/glucose}}$ (after day 8)	$Y'_{\text{lactate/glucose}}$ (overall)	Maximal FI
HPL beads standard	1.45 ± 0.426	2.21 ± 0.679	1.76 ± 0.657	5.6 ± 0.3
HPL new beads (D8)	1.93 ± 0.912	3.58 ± 0.851	2.59 ± 1.20	9.9 ± 0.8
CELLStart™ beads	0.99 ± 0.439	1.94 ± 0.294	1.37 ± 0.603	9.1 ± 0.2

TABLE 4 Apparent yield of lactate from glucose for different time periods under dynamic conditions. Values presented as mean \pm STD in $\text{mol}_{\text{lactate}} \cdot \text{mol}_{\text{glucose}}^{-1}$.

The glucose uptake rate and lactate production rate were also assessed during culture time. Overall, cells attached on HPL coated beads tend to produce more lactate comparing to beads coated with CELLStart™ (see Table 4). This may be due to the mitogen activity of HPL, stimulating energy production, cell metabolism by alternative pathways than aerobic glycolysis and cell division. Nevertheless, values obtained for specific glucose uptake and lactate production rates are not different from previous data available elsewhere on literature. For BM- and AT-MSCs, glucose uptake rates were found to range from 5 – 17 and 10 – 20 $\text{pmol cell}^{-1} \text{ day}^{-1}$, respectively. Lactate production rates for these sources range from 10 – 26 and 10 – 31 $\text{pmol cell}^{-1} \text{ day}^{-1}$, respectively (Dos Santos *et al.*, 2011).

For synovium derived MSCs, specific glucose uptake rate for cells growing on HPL coated beads ranged from 1 – 21 $\text{pmol cell}^{-1} \text{ day}^{-1}$, while lactate production rate for the same beads ranged from 2 – 17 $\text{pmol cell}^{-1} \text{ day}^{-1}$. For cells growing on CELLStart™ coated beads, a decrease of these rates was noticed (glucose uptake rate range 1 – 10 $\text{pmol cell}^{-1} \text{ day}^{-1}$ and lactate production rate range 3 – 11 $\text{pmol cell}^{-1} \text{ day}^{-1}$) (see Table 5).

Dynamic system	q_{glucose}		q_{lactate}	
	before day 8	after day 8	before day 8	after day 8
HPL beads standard	7.40 ± 3.29	3.08 ± 1.36	9.79 ± 1.00	6.03 ± 0.99
HPL new beads (D8)	9.09 ± 6.37	1.14 ± 0.43	12.80 ± 3.67	3.87 ± 1.49
CELLStart™ beads	8.33 ± 1.29	3.25 ± 1.28	7.93 ± 3.00	5.78 ± 3.03

TABLE 5 Specific glucose uptake and lactate production rates during culture under dynamic conditions. Values presented as mean \pm STD in $\text{pmol} \cdot \text{cell}^{-1} \text{ day}^{-1}$.

4.6 MULTILINEAGE DIFFERENTIATION ABILITY

The multilineage differentiation ability into osteocytes, adipocytes and chondrocytes is a proposed standard for the evaluation and identification of mesenchymal stem cells. The

confirmation of this capability qualifies hMSCs to be used in many clinical applications, but do not exclude further testing of cell morphology, proliferative potential and immunophenotype.

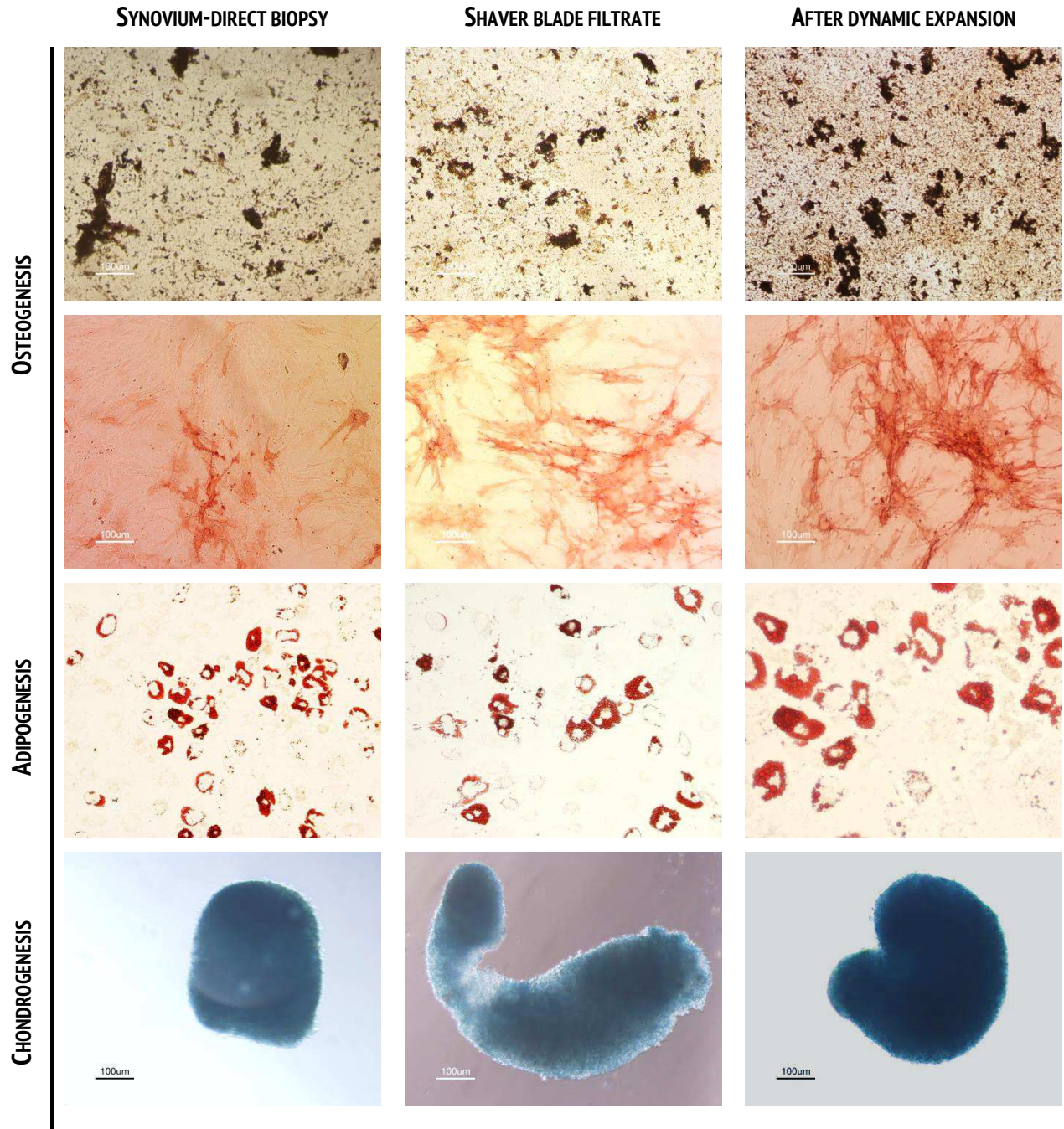


FIGURE 28 Multilineage differentiation of synovium-derived mesenchymal stem cells. Osteogenesis was evaluated by von Kossa staining (first row) and ALP assay (second row). Adipogenesis was evaluated by staining cells with Oil Red-O. Chondrogenesis pellets were stained with Alcian blue for assessing the presence of glycosaminoglycans. Results for synovium-direct biopsy, shaver blade filtrate derived cells and cells expanded under stirred conditions are shown. Synovial fluid cells were not tested for differentiation.

The osteogenic differentiation process was evaluated by two different methods: the von Kossa staining, for detection of deposition calcium and phosphate, after reaction with silver nitrate, and the ALP (alkaline phosphatase) assay, which detects the activity of this enzyme producing a pink or reddish staining, particularly when cells are in early bone progenitor stages, as the activity of this enzyme is mainly higher during active bone formation. After two weeks of osteogenic induction the hMSCs isolated from synovium and shaver blade filtrate demonstrated a distinct osteogenic phenotype, by the positive staining results for the increased activity of ALP and deposits of calcified matrix (see *Figure 28*).

Adipogenesis and differentiation into fat cells is characterized by the presence of intracellular lipid deposits that can be detected by Oil Red-O staining, generating reddish deposits. The cells which were cultured in an adipogenic inductive culture medium revealed a positive staining result when in presence of Oil Red-O, confirming an adipogenic phenotype.

For chondrogenesis, Alcian Blue staining was used to detect the differentiation into chondroblasts, due to its affinity to detect glycosaminoglycans, produced by chondrocytes. The evaluation of the chondrogenic phenotype of hMSCs exposed to chondrogenic differentiation medium demonstrated the deposition of a blue staining, possibly in the extracellular matrix of chondrogenesis pellets, thus confirming the production of glycosaminoglycans.

The multilineage differentiation ability of these hMSCs was evaluated at passage 4, for at least two donors of each sample type. Synovial fluid derived cells were not tested for differentiation. Interestingly, when differentiating cells collected from a donor with a clinical diagnosis of an extensive degenerated joint, chondrogenesis was not achieved. Cells readily differentiated into osteocytes and adipocytes, presenting higher mineralization and lipid accumulation than the average of donors (data not shown). This may further elucidate the fibrotic degeneration of cartilage and adjacent tissues, observed in patients with joint diseases, which impairs natural repairing processes by resident mesenchymal stem cells.

V CONCLUSIONS AND FUTURE DIRECTIONS

Mesenchymal stem cells (MSCs) compose an incomparable population of adult stem cells that proved to have a great clinical value, with applications now arising in several cell therapies and regenerative medicine uses. Additionally, the emerging knowledge obtained over the last few years about the immunological behavior of these cells point them as the newest trend in allogeneic treatments. By systemic infusion, MSCs have a major impact in cell therapies for the treatment of autoimmune diseases such as graft-versus-host disease (GvHD), Chron's disease, multiple sclerosis, rheumatoid arthritis or even systemic lupus erythematosus.

Every day, new insights on research methods, clinical trials and possible new applications and approaches related to the use of MSCs in regenerative medicine increases considerably. For accomplishing this, the major sources of MSCs used have been bone marrow (BM), adipose tissue (AT) and umbilical cord matrix (UCM) derived cells. Besides bone marrow extraction by highly invasive procedures, it has been considered the gold standard source for MSCs. Recently, umbilical cord matrix (UCM) has been explored as a new potential source for MSCs, with advantages relating non-invasive procedures and to the more primitive cells obtained, which are less likely to have been exposed to blood-borne contaminants. Although many sources are now considered for successful isolation of MSCs, none has proved advantages over the others, and the particular clinical applications are expected to be improved by choosing and appropriate source for each use.

Typically, clinical uses of MSCs require a large amount of cells. With cell dosages up to 5 million of cells per kilogram of mass of the patient, while cells collected from a single patient being considerably less, it is mandatory to develop new and efficient methods for the scale up and large scale *ex-vivo* expansion of these cells. Expansion systems now being developed based on fully controlled bioreactors must aim at meeting both safety and cost-effectiveness requirements.

Also, innumerable attempts have been made in order to achieve a safer, clinical-scale and reproducible chemically defined culture medium to expand MSCs *ex-vivo*. In this context, both serum-free media and xenogenic-free medium (StemPro® MSC SFM XenoFree) formulations have been especially developed for the expansion of hMSCs.

The present work aimed at the isolation and expansion of synovium-derived mesenchymal stem cells, extracted by direct biopsy, shaver blade filtrate residues or synovial fluid, while comparing their proliferative capacity, clonogenic potential, immunophenotype, multilineage differentiation ability with hMSCs previously isolated from the bone marrow, adipose tissue and umbilical cord matrix. The main conclusion is that synovium-derived cells can be seen as an alternative to other sources for hMSCs isolation, particularly when planning clinical application focusing on cartilage treatment or other osteoarthritic diseases. A wide range of donors were used, which permitted the setup of a comprehensive cell bank at Stem Cell Bioengineering and Regenerative Medicine Laboratory. Although a considerable number of donors were used, a restricted number of experiments were performed due to the time constraints of this master project. For this reason, all experiments should be repeated with more donors, in order to verify the results obtained and better evaluate their statistical significance.

The isolation of synovium-derived hMSCs was overall successful (above 75%), although initial passage durations were considerably higher comparing with cells derived from other sources. This may be due to inadequate isolation protocols, which require further optimization, or even the low number of cells obtained per biopsy. The cells were successfully expanded in static conditions using serum-containing culture medium (DMEM + 10% FBS) but also in dynamic conditions, using a serum- and xenogeneic-free culture medium (StemPro® MSC SFM XenoFree). The isolated cells were analyzed by flow cytometry, confirming the immunophenotype proposed by the International Society of Cellular Therapy, identifying populations of hMSCs.

As expected, the expansion of synovium derived-hMSCs in dynamic culture system turned out to be more efficient than the expansion in static culture system reaching higher cell numbers in a shorter period of time, while avoiding cell expansion over several consecutive cell passages. hMSCs seem to benefit from the expansion under adequate shear stresses, which could mimic their *in vivo* niche. Also, the clinical properties and multipotent differentiation abilities were maintained after expanding these cells in stirred conditions for 12 days.

The comparative analysis of the isolated synovium-derived hMSCs with the hMSCs from the other sources indicated that these cells are affected by many donor-related conditions, particularly age and/or the presence of trauma. Also, for synovial fluid derived

cells the average time between passages increase as the cells are passaged and enter into senescence.

hMSCs isolated from synovial tissues also generated a lower number of CFUs-F colonies, particularly restricting the ability to form large colonies (over 50 cells) as the passages increase. Also, the immunophenotypic analysis demonstrated no significant differences between hMSCs from the three different sources and all of them demonstrated the ability to differentiate into adipocytes, osteocytes and chondrocytes. Cells derived from donors with cartilage and joint associated pathologies readily differentiated into osteoblasts and adipocytes, but not into chondroblasts, with a possible insight of the non-healing properties of *in vivo* cartilage.

Considering the accomplished results it seems that synovial tissues derived hMSCs can be faced as a viable alternative to other hMSCs, namely BM and AT. Nevertheless, there is a need to further screen and characterize these cells, such as by gene expression analysis and secretome quantification. Also, the development of optimized xenogenic-free culture medium to expand cells for clinical applications must be considered.

An optimization of the isolation process may prove beneficial to obtain a higher number of cells from a single donor. Samples may be centrifuged using a density gradient (Ficoll) for enriching the mononuclear fraction. The dynamic, large scale expansion on spinner flasks also requires optimization, mostly in order to eliminate or diminish the number of cells lost in the first day of expansion due to a poor adhesion to the microcarriers. However, the addition of empty beads during culture time proved to be a practical alternative for maximizing expansion proficiencies, by non-limiting the surface area for cell proliferation.

It would be interesting in the future to use 3-D scaffolds for cell growth and attachment. Many biomaterials can be used for this purpose, even tailor-made with patient specific dimensions and sizes. Further experiences are required to check the feasibility of expanding cells on scaffolds, while being able to differentiate them into chondrocytes and further implantation on cartilage trauma or injury sites.

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