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PhD Thesis

**Effect of industrial GMP human plasma derived supplement on
hMSC behaviour**

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....ai miei splendidi genitori Aldo e Giusi

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Abstract

Human Mesenchymal Stem cells (hMSC) are an heterogeneous set of adult stromal cells that can be isolated from different tissues. They have excellent capacity as regard differentiation potential and immunomodulatory role. For these reasons, hMSC have been used as therapeutic agents for a wide number of human diseases. However, independently of the source, hMSC are scarce in human body, therefore cell expansion *in vitro* before therapeutic applications is mandatory. Foetal Bovine Serum (FBS) is commonly added in cell culture as medium supplement. However, it carries different drawbacks namely the risk of xeno-antigen, zoonoses and viruses transmission as well as ethical and economic issues. Therefore, FBS use in hMSC clinical grade production is seriously discouraged. Furthermore, being hMSC advanced therapy products, their production should follow Good Manufacturing Practices (GMP) guidelines.

The principal aim of this thesis was to optimize a pharmaceutical grade human plasma fraction (Supplement for Cell Culture - SCC) for its use as FBS substitute during hMSC *in vitro* expansion. SCC is obtained in GMP conditions from human plasma pools constituted from over 1000 donation, which ensures lots homogeneity in opposite to FBS and the other available alternatives.

A new formulation, herein called as “SCC-based xeno-free medium” was developed. It makes uses of two growth factors supplementation together with Fibronectin (Fn) coating. hMSC expanded along 4 passages in SCC-based xeno-free medium displayed 4 times faster expansion rate as compared to FBS medium and commercially available xeno-free medium. Importantly, cell phenotype, differentiation capacity and genetic stability were not compromised, in any of the tested conditions. Therefore, we concluded that SCC-based xeno-free medium is a valid alternative to FBS for a safer and rapid hMSC *in vitro* expansion.

Being hMSC attachment dependent cells, the characteristics of the cell culture surface strongly affect cell behaviour, thus surface chemistry may be tailored in order to influence cell *in vitro* growth. In fact, SCC-based xeno-free medium includes a Fn coating of tissue culture plates. Two different surface modification approaches were applied in this thesis to evaluate their influence on hMSC behaviour.

First, we investigate the influence of hydrophobic ($-\text{CH}_3$) and hydrophilic neutral ($-\text{OH}$), positive ($-\text{NH}_3^+$) and negative ($-\text{COO}^-$) surface charged groups on hMSC *in vitro* growth. In FBS medium, cells were able to grow better on hydrophilic than on hydrophobic

surfaces. This effect can be related with serum proteins adsorption that occurs in more favourable conformations for cell presentation on hydrophilic surfaces rather than hydrophobic. In SCC-based xeno-free medium without Fn-coating, cells growth was significantly decreased in all samples tested, in comparison with tissue culture polystyrene surfaces (TCPS). However, upon Fn coating, cells growth was increased being surfaces exposing charged groups more favourable than hydrophobic ones. These results highlight the importance of surface chemistry on hMSC *in vitro* growth, even in the presence of a Fn coating.

Secondly, we improved hMSC *in vitro* culture using an heparin-binding surface. In fact, heparin related molecules (proteoglycans, PG) are abundant in the extracellular matrix and exert a relevant function in growth factors and adhesive molecules presentation to cells. To exploit heparin capacities on hMSC *in vitro* culture, an heparin binding peptide (pKL) was immobilized onto model surfaces. In FBS medium, hMSC cultured on pKL surface displayed a better cytoskeleton organization and increased proliferative capacity as compared to TCPS.

In a future perspective, pKL surfaces will be applied in SCC-based xeno-free medium in an attempt to avoid the need for Fn coating or growth factors addition.

Together, the evidences obtained during this thesis can be applied to improve the quality, efficiency and safety of *in vitro* expanded hMSC for clinical applications.

Resumo

As células estaminais mesenquimais humanas (hMSC) são um conjunto heterogêneo de células adultas do estroma que podem ser isoladas de diferentes tecidos. Estas células têm um excelente potencial de diferenciação e uma elevada capacidade imunomoduladora. Por estas razões têm sido aplicadas como agentes terapêuticos em diversas de doenças. No entanto, independentemente da fonte, as hMSC são escassas no corpo humano e, portanto, necessitam de ser expandidas *in vitro* antes da sua aplicação terapêutica. O soro bovino fetal (FBS) é normalmente adicionado na cultura celular como suplemento do meio. No entanto, este soro apresenta vários inconvenientes, tais como a possibilidade de transmissão de xeno-antígenos, zoonoses e vírus, assim como questões éticas e económicas. Sendo assim, a utilização de FBS na cultura de hMSC para aplicações clínicas é seriamente desencorajada. Além disso, como produto para terapias avançadas, a sua produção deve seguir as diretrizes de boas práticas de fabricação (GMP).

O objetivo principal desta tese foi a otimização de uma fração de plasma humano de grau farmacêutico (Suplemento para Cultura Celular - SCC) para a substituição do FBS durante a expansão *in vitro* de hMSCs. O SCC é obtido em condições GMP a partir de “pools” de plasma humano constituídos por mais de 1000 doações, o que garante homogeneidade entre lotes em oposição ao FBS e às outras alternativas disponíveis.

Uma nova formulação, aqui denominada "SCC-based xeno-free medium", foi desenvolvida. Esta formulação utiliza dois suplementos de fatores de crescimento em conjunto com um revestimento de Fibronectina (Fn). As hMSC expandidas ao longo de 4 passagens nesta formulação, exibiram uma taxa de expansão que foi 4 vezes mais rápida do que quando expandidas em FBS ou num meio xeno-free comercialmente disponível. Para além disso, o fenótipo celular, capacidade de diferenciação e estabilidade genética destas células não foram comprometidos, em nenhuma das condições testadas. Sendo assim, concluímos que o "SCC-based xeno-free medium", é uma alternativa válida ao FBS para uma expansão segura e rápida das hMSC *in vitro*.

Sendo as hMSC dependentes de ancoragem, as características da superfície da cultura celular afetam fortemente o seu comportamento, podendo a química da superfície ser adaptada para influenciar a sua expansão *in vitro*. Na verdade, o meio “SCC-based xeno-free” inclui um revestimento das placas de cultura com Fn. Duas diferentes abordagens

foram utilizadas nesta tese, utilizando modificações de superfície para estudar a sua influência no comportamento das hMSCs.

Primeiro, investigamos a influência dos grupos hidrofóbicos ($-\text{CH}_3$) e hidrofílicos neutros ($-\text{OH}$), positivos ($-\text{NH}_3^+$) e negativos ($-\text{COO}^-$), na coltura *in vitro* das hMSCs. Em meio FBS, a quantidade de células foi maior nas superfícies hidrofílicas do que nas hidrofóbicas. Este efeito pode estar relacionado com as proteínas do soro que podem ser apresentadas às células de maneira mais favorável quando adsorvidas em superfícies hidrofílicas.

Em “SCC-based xeno-free médium” sem o revestimento com Fn, a quantidade de hMSC diminuiu significativamente em todas as amostras testadas, em comparação com o TCPS. No entanto, após o revestimento com Fn, o número de hMSC aumentou, sendo as superfícies com grupos carregados mais favoráveis para o crescimento das hMSC do que as superfícies hidrofóbicas. Esses resultados destacam a importância da química da superfície no crescimento *in vitro* de hMSC, mesmo na presença de um revestimento com Fn.

Em segundo lugar, melhoramos a coltura *in vitro* das hMSCs usando uma superfície com capacidade para ligar heparina. De facto, as moléculas baseadas em heparina (proteoglicanos, PG) são abundantes na matriz extracelular e exercem uma função relevante na apresentação dos fatores de crescimento e das moléculas adesivas às células. Para explorar as capacidades de heparina na coltura *in vitro* das hMSC, um péptido de ligação à heparina (pKL) foi imobilizado em superfícies do modelo. Em meio com FBS, as hMSC expandidas nas superfícies com pKL apresentaram uma melhor organização do citoesqueleto e um aumento da sua capacidade proliferativa em comparação com as superfícies das placas de cultura.

Numa perspetiva futura, as superfícies de pKL irão ser avaliadas em “SCC-based xeno-free médium” na tentativa de evitar a necessidade do revestimento com Fn ou do suplemento com fatores de crescimento.

Em conjunto, as evidências obtidas durante esta tese podem ser aplicadas para melhorar a qualidade, eficiência e segurança das hMSC expandidas *in vitro* para aplicações clínicas.

Publications

- *Xeno-free strategies for safe human mesenchymal stem/stromal cells expansion: supplements and coatings.* Cimino M, Gonçalves RM, Barrias CC, Martins MCL. Stem Cell International (under review).
- *The optimization of the use of a pharmaceutical grade xeno-free medium for in vitro expansion of human mesenchymal stem/stromal cells.* Cimino M, Gonçalves RM, Bauman E, Barroso-Vilares M, Logarinho E, Barrias CC, Martins MCL. Journal of Tissue Engineering and Regenerative Medicine (accepted in June 2017).
- *Effect of surface chemistry on hMSC growth in xeno-free conditions.* Manuscript in preparation.

Abbreviations list

A

Å

AD

ALS

Ang-1

AIED

Armstrong

Alzheimer's disease

Amyotrophic Lateral Sclerosis

Angiogenin-1

Autoimmune inner ear disease

B

B19

BM

BMM

BE

BSA

BSE

BVDV

Parvovirus

Bone Marrow

Bemberg Microporous Membrane

Blood Establishment

Bovine Serum Albumin

Bovine Spongiform Encephalopathy

Bovine Viral Diarrhea Virus

C

CBF

CD

CFU-F

CI

CJD

CLP

CMP

CWD

Clarified Brain Homogenate

Crohn's Disease

Colony Unit Fibroblast

Confidence Interval

Creutzfeldt-Jakob disease

Common Lymphoid Progenitor

Common Myeloid Progenitor

Chronic Wasting Disease

D

DC

DMEM

DNA

Dendritic Cells

Dulbecco's Modified Eagle's Medium

Deoxyribonucleic acid

E

ECM

EGF

EMA

EMCV

ESCs

EtOH

EU

Extracellular Matrix

Epidermal Growth Factor

European Medicine Agency

Encefalomyocarditis virus

Embryonic Stem Cells

Ethanol

European Union

F

FBS

FC

FGF

FFI

Fn

Foetal Bovine Serum

Flattened Cells

Fibroblast Growth Factor

Fatal Familial Insomnia

Fibronectin

G

GLP
GMP
GPI
GSS
GvHD

Good Laboratory Practice
Good Manufacturing Practice
Glycosylphosphatidylinositol
Gertsman-Straussler-Scheuker syndrome
Graft-versus-Host Disease

H

HAV
HBV
HD
HIV
HGF
HLA
HLA-G
HSCs

Hepatitis A Virus
Hepatitis B Virus
Huntington's disease
Human Immunodeficiency Virus
Hepatocyte Growth Factor
Human Leucocyte Antigen
Human Leukocyte G protein
Hematopoietic Stem Cells

I

IBMX
ICAM
ICH
ICM
IDO
IFN
Ig
IGF
IL
iPSC
IRRAS
ISCT

Isobutylmethylxanthine
Intracellular Adhesion Molecule
International Council on Harmonisation
Inner Cell Mass
Indoleamine 2,3- Dioxygenase
Interferon
Immunoglobulin
Insulin Growth Factor-1
Interleukin
induced Pluripotent Stem Cells
Infra-Red Reflectance-Adsorption Spectroscopy
Mesenchymal and Tissue Stem Cell Committee

J

JP

Japanese monograph

K

KGF

Keratinocyte Growth Factor

L

LPS

Lipopolysaccharides

M

MCT
MEV
MHC
MMPs
MS
MSC

Mercury-Cadmium-Telluride
Mouse Encephalomyelitis Virus
Major Histocompatibility Complex
Metalloproteinases
Multiple Sclerosis
Mesenchymal Stem Cells

N

NAT

Nucleic acid testing

NBH	Normal Brain Homogenate
NK	Natural Killer
NO	Nitric Oxide
NSCs	Neuronal Stem Cells
O	
OA	Osteoarthritis
OCA	Optical Contact Angle
P	
P/S	Penicillin/Streptomycin
PB	Phosphate-Buffered Saline
PCE	Cytopathic Effect
PCSA	Polarizer-Compensator-Sample-Analyser
PD	Parkinson Disease
PDGF	Platelet Derived Growth Factor
Ph.Eur	Pharmacopoeia Europa
PGE-2	Prostaglandin E2
PK	Proteinase K
pKL	polyLeucineLysine
PRV	Pseudorabies virus
PPV	Parvovirus
PrP ^{sc}	Scrapie prion protein
PrP ^c	Cellular prion protein
PVDF	Polyvinylidene difluoride
Q	
QA	Quality Assurance
QC	Quality Control
R	
RF	Reduction Factor
RNA	Ribonucleic acid
RS	Rapidly self-renewing
S	
SAM	Self-Assembled Monolayer
SCC	Supplement for Cell Culture
SC	Spinal cord injury
S/D	Solvent/Detergent
SDF-1	Stromal cell Derived Factor-1
SOP	Standard Operating Procedure
SS	Spindle-Shaped
T	
TCID	Tissue Culture Infectious Dose
TCPS	Tissue Culture PolyStyrene
TGF	Transforming Growth Factor
TMP	Transmembrane Pressure
TNBP	Tri-n-butyl phosphate

TNF
TSE

Tumor Necrosis Factor
Transmittable Spongiform Encephalopathy

U
UK
US

United Kingdom
United States

V
VEGF
VCAM

Vascular Endothelial Growth Factor
Vascular Cellular Adhesion Molecule

X
XPS

X-ray Photoelectron Spectroscopy

W
WB
WBU
WHO
WNV

Western Blot
Western Blot Unit
World Human Organization
West Nile Virus

Aim and Structure of the Thesis

Regenerative medicine has received strong interest in the last decades and is moving fast towards new insight in healing and replacement of tissue and organs damaged by age, trauma, diseases or congenital defects. In fact, preclinical and clinical data raise hopeful prospects in the treatment of chronic diseases and acute injuries, including dermal wounds, cardiovascular diseases and treatments for some types of cancer [1]. The main strategy consists in the implantation of freshly isolated or cultured cells into the site of injury. The infused cells are most of the times stem cells, unprogrammed cells in the human body with ability to self-renew and to differentiate along multiple lineage pathways which make them directly responsible for generating tissues and organs [2]. Among stem cells, human Mesenchymal Stem Cells (hMSC) have generated great interest because of their facility to be isolated, extensive expansion rate, systemic engraftment capabilities, immunomodulatory properties and differentiation potential. hMSC can be derived from different tissues such as adult adipose tissue, mobilized peripheral blood and bone marrow (BM), as well as from placenta and umbilical cord blood, being BM the most common source in clinical use. However, hMSC prevalence in all these tissues is low, and the total amounts of isolated cells are often insufficient for clinical applications. The number of required MSCs depends on the type of disease to treat, but in general around 1 to 5 million cells/patient body weight are required for successful transplantation [3]. Thus, isolated hMSC must be first expanded *ex vivo*, using effective and safe methods that maintain their key properties. A hot topic in this field is the lack of common standard protocols for hMSC expansion. The subject of controversy is the choice of media and supplementary factors during cell *in vitro* culture. In attempt to improve safety of clinical trials, animal derived supplements, such as Foetal Bovine Serum (FBS), are not anymore accepted, thus the development of xeno-free strategies is mandatory. Moreover, being hMSC attachment-dependent cells, culture surfaces are often coated with extracellular matrix (ECM) proteins or other commercially available cell adhesion factors, generating an additional element of discontinuity among expansion protocols. Finally, being hMSC considered as advanced therapy products, cells expansion must comply with Good Manufacturing Practices (GMP) guidelines and every step of cell manipulation must be defined in Standard Operating Procedures (SOP).

The principal goal of the work described in this thesis was to develop a xeno-free strategy to expand hMSC *in vitro*, namely using a human plasma derived product of the pharmaceutical industry Grifols called Supplement for Cell Culture (SCC). Being this an industrial PhD project, half of the work was performed in Grifols (Barcelona, Spain) where safety and efficacy of the final product were assessed regarding the most common human infectious agents (viruses and prions), for the development of plasma derived products.

The second part of the project was instead developed in academic setting, in the installation of INEB/I3S (Porto, Portugal) where expansion of hMSC in xeno-free conditions and surface modification for cell culture were developed.

This thesis is divided in 4 main parts:

- Section 1 regards the industrial part of this work, thus it describes quality assurance requirements in plasma product manufacture and the regulatory activities associated with production of plasma protein therapeutics (Chapter I), with special focus in one safety study performed in Grifols (Annex I).
- Section 2 deals with the academic part of this project and is divided in 2 general introductory chapters about: 1) Stem cells in regenerative medicine, with a special focus on hMSC (chapter II); 2) Surface modifications for fundamental studies in cell culture and the most used surface characterization techniques in this ambit (chapter III).
- Section 3 includes the research developed during this project that was, mostly, published/submitted in international journals: 1) a review of the xeno-free strategies described until now for hMSC *in vitro* expansion, including comparisons between culture media, supplements and surface modifications (chapter IV); 2) a new xeno-free strategy for hMSC expansion using SCC as FBS-substitute (chapter V); 3) a work regarding the effect of surface chemistry on hMSC culture (chapter VI); 4) an additional experience that inquires the effect of a surface immobilized heparin-binding peptide [4] on hMSC growth *in vitro* (chapter VII).
- Section 4: consists in an overall analysis of the results presented in this thesis, highlighting the most remarkable findings and discussing future prospects for hMSC *in vitro* expansion and application in regenerative therapies (chapter VIII).

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SECTION 1

SECTION 1

**SAFETY ON INDUSTRIAL HUMAN PLASMA DERIVED
PRODUCTS**

CHAPTER I

REQUIREMENT FOR PLASMA PRODUCTS MANUFACTURE

I.1 INTRODUCTION

Development of any pharmaceutical product requires a restricted regulatory strategy, to assure and protect public health, safety and welfare. Human plasma products are directed by the same regulatory lines than conventional medicinal products.

Plasma is a special raw material since it is not manufactured, it is instead collected by voluntary donors, thus it is considered an ethical starting material. All the steps of plasma proteins production (collection of plasma, fractionation, and extraction of proteins) are regulated by specific guidelines. Those rules guarantee that standards are pre-served, avoid the risk of infection transmissions and ensure the purity and integrity of the final product.

All the aspects of the production of plasma derived products are highly regulated. The entire process is strictly regulated to assure the maximum level of safety for the donor and the recipient of the product. Both industrial groups and government regulatory contribute with the same effort in the production of guidelines.

A number of international authorities control plasma manufacturing industries: World Health Organization (WHO), European Union (EU), United States (US) are the major ones followed by many others national regulatory entities operating in different countries. Moreover in different commercial regions specific books govern the production of pharmaceutical products such as the Pharmacopoeia Europa (Ph.Eur) or the Japanese monograph (JP). Unfortunately there is a lack of a universal accepted legislation and this remains a tricky goal to be reached. The International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) tries to merge the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three areas in an attempt to harmonize the numerous guidelines.

Thus, along this chapter, the regulatory activities that ride the development of plasma protein therapeutics will be discussed, with a special focus in pathogen safety.

I.2 PLASMA MANUFACTURING

The origin of plasma manufacturing is dated during the Second World War, when high quantity of blood serum products was demanded to treat soldiers suffering of burns and shocks, in particular albumin (55-60% of total plasma protein) was separated from plasma for injured soldiers. Cornerstone of proteins separation from plasma was Dr. Edwin Cohn (Harvard University) who gave name to the “Cohn fractionation process,” [1] that is still the basis of plasma fractionation in the modern industry. Cohn fractionation process consists in modification of pH, ethanol concentration and temperature to separate precipitates from plasma into five fractions (I-V). Table I.1 reports the original Cohn Fractionation process and the major protein extracted. Nowadays improvements of the method consent to extract a higher number of plasma proteins. For instance, Fraction I (or “cryoprecipitate”) contains coagulation factors (VII and IX) and fibrinogen, fraction II carries immunoglobulins, thrombin and plasminogen, fraction III antithrombin, alfa 1 antitrypsin and Factor IX complex, fraction IV globulins, transferrin and butyrylcholinesterase, while fraction V is rich in albumin.

Table I.1 Original Cohn Fractionation method

(adapted from http://www.veritastk.co.jp/attached/392/Proliant_White Paper.pdf)

Plasma Fraction	Conditions	Major protein extracted
Plasma	8% EtOH, pH 7, -3 °C	
Fraction I precipitate	25% EtOH, pH 7, -5 °C	Fibrinogen
Fraction II & III precipitate	18% EtOH, pH 5.2, -5 °C	IgG, Thrombin, Plasminogen
Fraction IV-I	40% EtOH, pH 5.8, -5 °C	Globulins
Fraction IV-4 precipitate	25% EtOH, pH 4.8, -5 °C	Transferrin
Fraction V precipitate		Albumin

In the modern industry, plasma manufacturing is a well-defined process made by three basic steps: plasma collection, manufacturing process and release of the final product.

Specific regulations control the whole process, from the donor selection to freezing and storage of the final product. WHO furnished many documents regarding the quality control of blood donations, plasma fractionation and inspections. EMA regulatory guidelines consist in European Directives and guidelines [2, 3] and a monograph from European Pharmacopoeia about “human plasma fractionation”. In USA the guidelines are contained mainly in the Code of Federal Regulations (CFR) 21[4].

I.2.1 Plasma collection

Collection of plasma follows different rules around the world.

First of all, the source of plasma is different between Europe and US. In Europe most of plasma production belongs from recovered plasma, which means that plasma must be separated from the blood cellular component, with a certain risk of cellular contamination and with differences in the content of protein among plasma of different sources [5]. Also the frequency of whole blood donations is only six/year for male and four/year for female. Instead in US most of plasma is collected by plasmapheresis centres, thus risk of cellular contamination is lower and the number of possible donations is higher, since until 33 donations can be made by an individual per year, with a frequency of twice per week [6].

Secondly, the centre where plasma is collected, the blood establishment (BE) is responsible for the safety of plasma, thus serological testing and nucleic acid testing (NAT) are performed in BE installations. Then, plasma freezing and transportation conditions can influence the quality and are under the BE responsibility. Therefore BE must follow specific guidelines that can vary according to the country or the commercial area.

Third, when plasma is collected, epidemiology is very important. An area with high incident of human immunodeficiency virus (HIV), Hepatitis B Virus (HBV) or Hepatitis A Virus (HAV) has increased the risk of the plasma being unsuitable as starting material for plasma product production, than an area with a lower incidence of those viruses. Thus, legislation regarding virus detection can be more or less restricted depending on the BE geographic position.

Fourth, there are countries that import plasma donations because are not self-sufficient and others whose legislation does not allow the entrance of foreign plasma (e.g. USA and Iran).

Fifth, some countries as France, Spain and Belgium do not accept remunerated plasma donations because of ethical considerations, others like USA do accept.

Finally, a common important rule regarding plasma donations is “hemovigilance” which assures the traceability of every plasma donation departing from the donor and arriving to the patient. In compliance with the Directives 2002/98/EC and 2005/61/EC, “traceability records for blood and blood components must be kept at least 30 years after the time of the donation”. However in some countries (e.g. European countries), the identity of the donor is protected since the database that records donors details can’t be shared and all the documents that assure traceability are anonymous.

I.2.2. Manufacturing process

After the plasma is collected, it must be processed to extract plasma proteins through fractionation. Fractionation must follow strict rules for the production of products intended for human use, thus it must be performed under Good Manufacturing Practices and comply with the guidelines designed for plasma-derived medicinal products. In addition to virus specific controls (NAT), the manufacturing process needs to include specific virus inactivation/removal capacity steps. Beyond the safety of the final product, the manufacturing process must guarantee also: maximum protein yield possible, integrity of the proteins activity and purity, since contamination with other plasma proteins can cause adverse reactions such as thrombogenic effects [3].

I.2.3 Release of plasma proteins

In many countries the release of the final product is first performed by the fractionation centre and secondly by the competent authorities, who additionally reviewed all the manufacturing process made by the fractionator and may repeat some safety test.

I.3 QUALITY ASSURANCE AND QUALITY CONTROL

Quality Assurance (QA) and Quality Control (QC) are two of the most important concepts in the pharmaceutical industry. QA is a group of activities that aims to assure that all the steps of the product manufacture follows a set of standards that ensure safety and efficacy. It is then a state of mind as well as an understanding of the guidelines that rules pharmaceuticals production.

QC refers to a system of inspection and controls over the production, evaluation and distribution of the product. By definition, QA regards defect prevention, while QC deals with defect identification. Thus QA is a group of activities that aims to assure quality in the manufacturing processes while QC is an aspect of QA that finds and corrects eventual defects in the finished product before it is released. While QA is responsibility of all the individuals working in the development of the product, QC is the responsibility of a specific team that test the final product to inquire if it has defects. In the industry a specific QC department operates as supervisor and coordinator of all the aspect of quality insurance since it is responsible of organizing inspections to control all the steps of the manufacturing process as well as of the development and storage of all the documentation.

I.4 GMP and GLP

In order to ensure high quality and integrity of products, the pharmaceutical industry must follow “good” practices systems, as Good Manufacturing Practices (GMP) and Good Laboratory Practices (GLP). Regulatory agencies in United States, Europe as well as in other countries supervised GMP and GLP. In EU GMP have been defined in 2010 by European Commission in the Volume 4 of the document “The rules governing medicinal products in the European Union: EU Guidelines for Good Manufacturing Practices for Medicinal Products for Human and Veterinary Use”, where annex 14 is specific for products derived from human plasma or blood. In USA, GMP are described in the document 21CFR and are updated every year, thus are named current GMP (cGMP). GLP principles have been stated by the European Council in two basic Directives: Directive 2004/10/EC and Directive 2004/9/EC. In United States GLP rules have been described by FDA in 21CFR58”.

GMP provide guidelines for manufacturing and testing, assuring that all the products are produced and controlled in agreement with quality standards and ideated according to the product use and specification. Therefore, complying with GMP is mandatory in all pharmaceutical manufacturing. GMP regard for example manufacturing facilities hygienic conditions, controls and validation of manufacturing processes, training of personnel employed, records of manufacture, instruments plant, machinery and packaging processes.

GLP is a set of specific quality procedures applied during experimental (non-clinical) research area, that ensure uniformity, consistency, reliability, reproducibility, quality, and integrity of non-clinical safety/toxicology studies. GLP concerns with lab testing, procedures, equipment used, data recording and maintenance, testing facilities and quality control of testing. Instrument of GLP are Standard Operating procedures (SOPs), documents that furnish detailed instructions for every step of the operations to be done in the laboratory. SOP must be clear, consistent and easy to follow, thus laboratory workers can respect them without any possibility of subjective interpretation.

I.5 VALIDATION AND QUALIFICATION

The WHO applies the word “Validation” to the quality controls of all the processes involved in the development of the product. Qualification is a sub-category of validation, since regards the control of equipment, facilities and technical systems inside the process. In validation studies for pathogen safety the process is performed in a reduced scale in laboratory (*scale down*), mimicking the conditions of the industrial scale, to demonstrate the efficacy, suitability and safety of the step analysed. Effectiveness of the process is given in terms of yield of the product with high quality and biological activity. Data arising from a validation study came from a relevant set of analytical methods and are carefully documented and recorded.

In the case of plasma derived product, a special focus is directed for the validation of production process steps with pathogen clearance capacity.

At the time of protein purification, control of ethanol precipitation conditions (concentration, temperature pH, protein concentration), qualification of chromatographic conditions (pH, resin features, flow rate, temperature) and control of filters conditions are examples of qualification. In pathogen removal/inactivation capacity production process steps, qualification regards control of different parameters that can affect pathogen clearance, for instance control of filters conditions in filtration or controls of lipid content in Solvent /Detergent treatment. Finally, in the final product, demanded tests include: endotoxin and sterility tests, integrity and septicity of containers, analytical methods to check pyrogens and sterility.

I.6 VIRAL SAFETY OF PLASMA PRODUCTS

Human plasma may contain infectious agents as bacteria, endotoxin, viruses and prions. Regarding viruses, the ones that historically have been transmitted by plasma donations and thus require careful screening are: human immunodeficiency virus (HIV), hepatitis A, B, C (HAV, HBV, HCV) and parvovirus (B19). Viruses may originate from their host organism (endogenous virus) or could come from outside origins. Adventitious contaminants are viruses that are not detectable in the source material but are added inadvertently or deliberately during production steps [7]. For instance, they could be introduced in additives (growth factors, serum) or in process operations (serum or albumin for tripsinization).

I.6.1 Virus detection

In order to detect eventual virus, several methods are available (Table I.2). The main features of a virus test must be adequate sensitivity and reproducibility, therefore sufficient controls and statistical significance are needed.

Table I.2: Methods for virus detection

TEST	DESCRIPTION	ADVANTAGES	DISADVANTAGES	TYPE OF METHOD
<i>Infectivity assay</i>	Injection of cell cultures and observation of cytopathic effect (PCE)	Sensitivity	Slow Technically demanding Only for viruses that cause cytopathic effect	Quantitative
<i>Experimental animals</i>	Induction of antibodies in susceptible animals and later detection in serum	Use to study pathogenesis	Expensive Slow	Qualitative
<i>Molecular techniques</i>	DNA or RNA detection	Rapid Sensitive	Expensive Can detect non-infectious virus	Semi-quantitative
<i>Electron microscopy</i>	Observation of cell cultures	Rapid Sensitive Identification of new agents	Requires high concentration of viral particles	Quantitative
<i>Biochemical assays (antigen detection)</i>	Detection of agents	Rapid For virus that cannot be cultured	Equipment expensive Technically demanding	Semi-quantitative

I.6.2 Quantification of virus

Infectivity assay is one of the most common methods applied to calculate virus titre in terms of “*infectious dose*”. This test is based on the fact that when a virus infects a cell, it lyses cells membrane, generating a single macroscopic area of cytopathology denominated “*plaque*”. During the test, a monolayer of cells in a multi well plate is infected with various dilutions of virus and covered with a semi-solid medium (e.g. agar) to avoid that the virus spread uncontrolled on the cultures. If a single cell is infected with a single virus particle, new viruses coming from the initial infection contaminate the enclosing cells, creating a big plaque, an area of infected cells surrounded by non-infected one, perceivable by optical microscopy. Plaque formation occurs in 3-14 days, depending on the type of virus. Then plaques are counted for each virus dilution and the results are treated to calculate the virus titre in the sample.

I.6.2.1 TCID₅₀

The most common way to express the virus amount is “Tissue Culture Infectious Dose (TCID₅₀)”: the virus dilution that infects 50% of the cells in culture on infectivity assay. Replicate wells of cells infected with serial dilutions of the virus, are visualized to score each well as negative or positive for infection. Then, virus titre is calculated according to the Spearman Kärber formula[8, 9]:

$$\log_{10} TCID_{50}/ml = L + [d(S - 0.5)] - \log_{10} v$$

where, L is the $-\log_{10}$ of the first dilution of the sample assayed

d is the $\log_{10}$ of the dilution factor

S is the sum of the proportion of positive wells with respect to the total

v is the volume (ml) of sample spiked per replicate.

The viral titres of the samples are expressed as $\log_{10}$ TCID₅₀/ml or total $\pm$ 95% confidence interval (C.I._{95%}).

The C.I._{95%} is calculated from the standard error (SE):

$$C.I._{95\%} = \pm 1.96 \cdot S.E..$$

with :

$$S.E. = \sqrt{\frac{d^2}{n(n-1)} \left[r - \frac{1}{n} \left(\sum_{j=1}^j r_j^2 \right) \right]}$$

where, d is the $\log_{10}$ of the dilution factor

n is the number of wells spiked for each dilution

r is the total number of infected wells in all the dilutions assayed

r_j is the number of infected wells for the corresponding dilution.

When no virus is detected in the volume assayed, it is possible to calculate the probable concentration of viruses according to the Poisson distribution, with a 95% confidence limit in samples. In the event that a larger sample volume (Bulk Titration) is tested at the same time as the normal titration procedure for any of the sample dilutions, the Bulk titration is used to calculate the titre in this case.

Viruses in suspension (not aggregated) follow the Poisson probability distribution law.

$$P[X = k] = \frac{e^{-\lambda}}{k!} * \lambda^k$$

If $k = 0$ (virus not detected)

Then

$$P[0] = e^{-\lambda}$$

where:

$$\lambda = cv$$

c = concentration of infectious particles per ml

v = volume spiked per dilution in ml

λ = mean of infectious units

For a 95% confidence limit $P[0] = 0.05$

$$0.05 = e^{-cv}$$

$$c = \frac{\ln 0.05}{-v}$$

Infectious units are converted to TCID₅₀.

Thus, the lowest concentration detectable in TCID₅₀ with a 95% confidence limit will be:

$$C = \frac{\ln 0.05}{-\ln 2 * v}$$

$$C = \text{TCID}_{50}/\text{ml}$$

v = total volume of sample per dilution.

I.6.3 Virus clearance

Viral clearance is defined as the ability to remove or inactivate viral contaminants during the manufacturing process of a biological product [10]. In order to minimize the virus load of plasma pools intended for the production of plasma products for human use, three elements have been settled:

1. Selection of donors
2. Screening of donations

3. Specific viral inactivation/removal capacity steps during the manufacturing process

1. Selection of donors:

Qualified donors are selected by a confirmation of their general health status and by repeated tests specific for the most common pathogens. Importantly, plasma of the first donation is always discarded because it may contain viruses from an early stage infection not still detectable. This procedure reduces the virus load of 1 log₁₀ or more[11].

2. Screening of donations:

All plasma donations that enter in the pool and lately the pool itself are screened for the relevant viruses listed above by NAT testing. Moreover once/year every plasma centre must deliver to the EMA a plasma master file, which specify the tests done, the inspections and the epidemiological data of all the donations.

3. Virus inactivation/removal steps

Specific guidelines require that some steps in the manufacturing process are capable of eliminating/inactivate potential virus contamination[12; 4].To accomplish this, a laboratory –scale experiment is used to deliberately spike virus into the material, in procedure so called as spiking experiments (Fig.I.1).

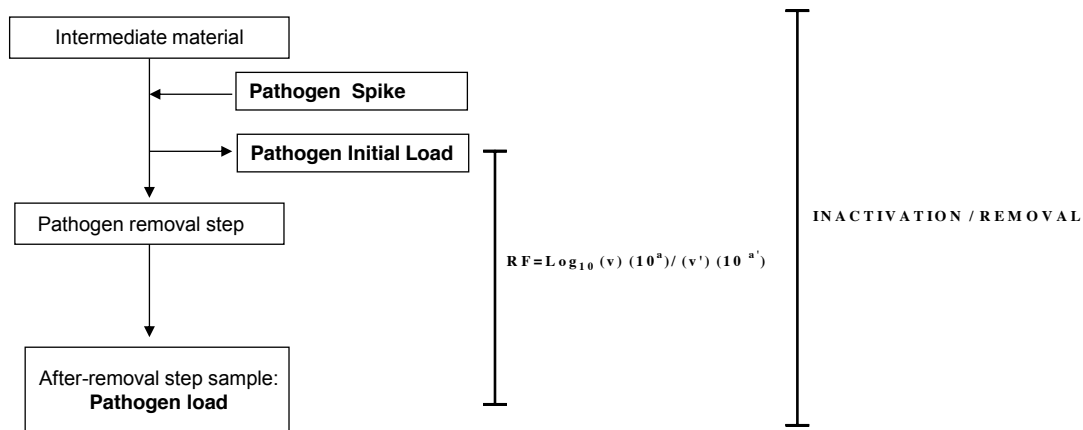


Figure I.1. Typical spiking experiment scheme.

v =volume of the “pathogen initial load”; a = pathogen concentration in the “pathogen initial load”; v' = volume of the “pathogen final load” sample; a' = pathogen concentration on the “pathogen final load” sample.

The quality of the viral clearance method is determined by comparison of the virus titer before and after the treatment, which is expressed on a logarithmic scale, defined as “logarithmic titer reduction” or “reduction factor (RF)”.

In general a multiple approach of virus removal/inactivation is used: manufacture of a single product usually include more than one step of virus removal/inactivation, to implement the mechanism of action of the single one and to guarantee that if viruses escape the first removal/inactivation step, they will be cached in the following one. Consequently, the overall capacity of the whole process to reduce the virus titer, is given by the sum of the RF of the single clearance step or by the summation of the lowest single RF and the highest single RF [13]. Depending on the degree of clearance efficiency of every single step, the overall virus reduction in a manufacturing process, given by the sum of each single step RF, can reach very high values. For instance values of $RF > 20 \log_{10}$ for HIV and HBV have been achieved by the combination of pasteurization, solvent/detergent treatment and nanofiltration [14].

The virus spiked into the starting material can be either the relevant one (as in the case of HIV and HAV) or a model for blood-borne viruses that can’t grow in cell culture. Table I.3 resumes a typical pattern of viruses used in a virus validation study. A good panel of test should include DNA and RNA viruses, different dimensions and enveloped or not

enveloped viruses since this correlates with different physic-chemical characteristics that may affect the efficiency of the clearance step.

Table I.3: Relevant and model viruses for virus validation studies

Test Virus	Model for	Enveloped	Genome	Particle Size (nm)
Pseudorabies virus (PRV)	Herpes virus	Yes	DNA	120-200
HIV-1	Relevant virus	Yes	RNA	80-100
Bovine Viral Diarrhea Virus (BVDV)	HCV	Yes	RNA	40-60
West Nile Virus (WNV)	Relevant virus	Yes	RNA	40-60
Encephalomyocarditis virus (EMCV)	HAV	No	RNA	27-30
HAV	Relevant virus	No	RNA	25-32
Mouse encephalomyelitis virus (MEV)	HAV	No	RNA	25-32
Parvovirus (PPV)	B19V	No	DNA	18-24

The characteristics of an ideal clearance method are:

- robustness: any variable should not affect the viral clearance efficiency. To demonstrate the reliability and the reproducibility of the clearance step, specific robustness studies are required. In those studies the worst case condition of the manufacturing process is evaluated for its efficiency in virus clearance;
- high level of viral clearance: 4-6 levels of logarithmic titer reduction must be achieved. The EMA guideline defined clearance step as “*not significant*” ($RF \leq 1 \log_{10}$), “*indicative /contributable*” ($RF = 1-2 \log_{10}$); “*moderate*” ($RF = 2-4 \log_{10}$) and “*high*” ($RF > 4 \log_{10}$);
- broad spectrum of viral clearance: valid against different kind of viruses;
- well-known mechanism of action: inactivation and/or removal;
- non-contaminating: the method must not impact in the product functionality;
- scalability and validate: amenable to scale down and in process testing;
- integrity of the final product: maintain the stability and functionality of the product.

There are basically two kinds of mechanism for virus clearance: inactivation and removal. Inactivation looks for the irreversible loss of viral infectivity, so the virus may remain in the final product but in non-infective form. It is achieved by physical and chemical methods (lyophilization, UV radiation, low pH treatment, solvent/detergent treatment, heat treatment). Removal aims at the reduction of the number of viruses in the product and is performed by filtration, cold ethanol precipitation and chromatography.

I.6.4 Virus inactivation procedures

- Physical Inactivation:

1. Pasteurization (60 °C for 10 h): it is able to inactivate either enveloped or not enveloped viruses, has been proved to be effective against human Parvo B19V, since it destroys its genome and its proteins[15]. It is used in the manufacture of albumin [11].
2. UV radiation: UV radiation at 254nm is able to inactivate viruses by incorporation of dimers of thymidine in their genome or by promotion of viral genome-protein cross linking [16]. It is included in the production of serum preparations [17].

- Chemical Inactivation:

1. Solvent Detergent (S/D) treatment: effective only against enveloped viruses, is the most frequent chemical virus inactivation method, since it is a highly robust method, being effective under different conditions of temperature, pH and protein concentration [18]. Usually is performed by using tri-n-butyl phosphate (TNBP) as solvent in combination with a detergent (for example Triton X100 or Tween 80) [19].
2. pH treatment: extreme values of pH can be used in order to inactivate viruses. Some of them are inactivated by low pH solution composed of glycine, citrate or acetate, while others are susceptible to high pH solutions, such as Sodium hydroxide (NaOH). Low pH inactivates effectively enveloped viruses and some of non-enveloped, depending on pH, incubation time and temperature [11].

Various chemical substances: for instance β -propiolactone treatment and sulfitolysis are used in the production of immunoglobulins [11].

I.6.5. Virus removal procedures

Removal is defined as the physical separation of the virus from the therapeutic component with the goal of the reduction of viral particles in number. Removal methods are the following:

- Precipitation: the most common methods of precipitation are cryoprecipitation and cold ethanol fractionation. The last has been amply used in the production of immunoglobulins and albumin [20]. In this procedure, eventually present viruses are segregated in one of the precipitate fractions that will be washed out.
- Chromatography methods: Affinity chromatography, ion exchange chromatography, size exclusion chromatography and hydrophobic interaction chromatography separate viruses from the product stream. They are used in particular for coagulation factors production since they do not tolerate treatments involving hard temperatures or pressures levels [11].
- Filtration: is a nontoxic, noninvasive, nondestructive method that requires the passage of a protein solution through a nanometer pore size filter that holds viruses while the protein passes into the stream. It is a flexible method since is possible to choose the size of pore in the nanometer range and it is able to remove both enveloped and non-enveloped viruses. It has been amply used for virus removal in the manufacture of small molecules such as immunoglobulins [21], antithrombin [22] and factor IX [23], but also bigger ones as fibrinogen [24, 25]. There are several nanometer filters in the market, so efficient that it is possible to reach a RF up to 6 log₁₀ by a single step of nanofiltration; pore size until 15 nm are available allowing the elimination of the smallest viruses of interest (PPV). A practical example of virus removal involving nanofiltration is illustrated in Annex I.

I.7 PRIONS SAFETY OF PLASMA PRODUCTS

The term Transmissible Spongiform Encephalopathy (TSE), also known as *prion disease*, includes a family of neurological disease that affect animals, principally cows as Bovine Spongiform Encephalopathy (BSE) but also other animals (sheep, cow, deer, elk) with variants as Chronic Wasting Disease (CWD). In humans, these diseases are rare and defined as Creutzfeldt-Jakob disease (CJD) which can be sporadic (sCJD) familiar (fCJD), iatrogenic (iCJD) or variant CJD (vCJD). Other variants of those diseases in

humans are fatal familial insomnia (FFI), Kuru and Gertsmann-Straussler-Scheuer syndrome (GSS)[26] .

These diseases are connected with mutations of the prion protein gene (PRNP) and are neurodegenerative conditions that cause spongiform degeneration of the brain and neural loss, with fatal outcome. The nature of the disease is not well understood and is still case of debate. However the most accepted theory assumes as principal agent a self replicative misfolded “scrapie protein” (PrP^{Sc}) derived from mutation on the wild type “cellular prion protein “(PrP^c) who aggregates and deposit in the brain, causing loss of activity [27]. PrP^c is a 209 amino acid glycoprotein attached to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety. Indeed it can be mono-di or not glycosylated. PrP^{Sc} has the same amminoacidic composition and glycosylation of PrP^c but a different conformation that confers to PrP^{Sc} lower solubility, high tendency to aggregate and higher resistance to proteolysis [28].

vCJD was first described in United Kingdom in March 1996 when has been linked to the dietary exposure to BSE-contaminated beef products. It is in the United Kingdom where the immense majority of cases of vCJD have been found, 178 as at 6th February 2017 of a total of 231 cases worldwide. The rest are distributed as follows: 2 cases in Canada, 27 cases in France, 4 in Ireland, 3 in Italy, 1 in Japan, 3 in the Netherlands, 2 in Portugal, 1 in Saudi Arabia, 5 in Spain, 1 in Taiwan and 4 in the United States(<http://www.cjd.ed.ac.uk/>)[29]. The Taiwanese, Japanese and one of the Canadian cases were either residents in or had visited the United Kingdom during the BSE peak. With regard to the four cases in the US, in two cases they were exposed to the agent in the United Kingdom, and the third case in Saudi Arabia. Regarding the fourth case, it was a citizen born outside the Americas and the infection occurred before he moved to the United States; the patient had resided in Kuwait, Russia and Lebanon (The National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU), Scotland, UK). In the UK, the country with the majority of cases of vCJD, it appears that there is a downward trend in deaths (Figure I.2) and, in parallel, in new cases.

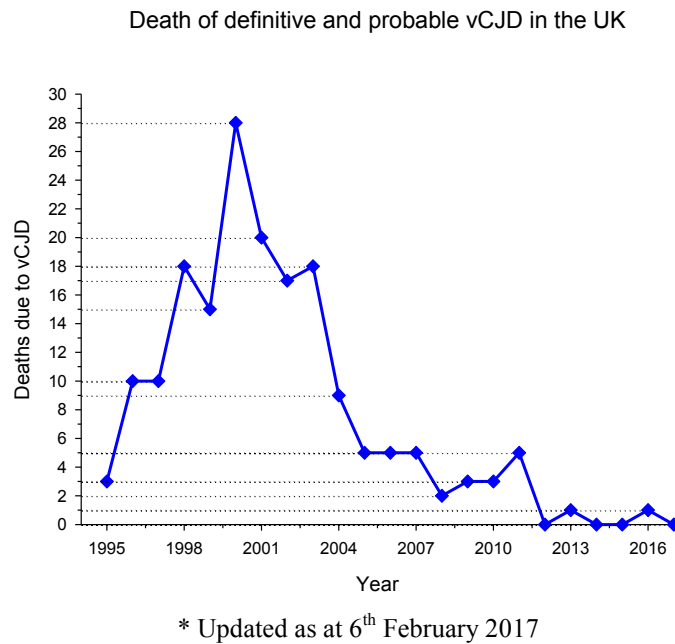


Figure I. 2 Death of definitive and probable vCJD in the UK

As of 6th February 2017, 178 deaths from vCJD have been announced (<http://www.cjd.ed.ac.uk/>), with a general peak of vCJD patients from 1996 to 2000 and an encouraging decline of new cases afterwards. However, cautions against vCJD agent must be still taken, since TSE infections can endure undetected for many years, thus vCJD worldwide presence may be underestimated. Regarding its infectivity, it can occur either by homologous transmission (between individual of the same species) or heterologous (between different species as in the case of the dietary exposure). But the most alarming feature of vCJD is that it is a blood-borne disease: a patient, who received in 1996 red cells from a donor later died of vCJD, also develops symptoms of vCJD 6 years after infusion and died after 6 months [30]. Furthermore, experiments of Houston and Hunter describe how vCJD can be transmitted by blood transfusion [31, 32]. However, only one data [33] reports vCJD transmission by plasma-derived products: an individual was involved with vCJD after administration of plasma-derived Factor VIII manufactured by plasma donations of another individual who developed vCJD 11 years after donations.

The authorities developed EMA/410/01 [34] which states the need of careful prion investigational studies in the plasma manufacturing industry. The risk assessment of prion proteins is more tricky than virus one because donor selection is not efficient. Symptoms of vCJD may be not visible for years and the titre of PrP^{Sc} in blood are very low so it escapes the normal screening procedures. In USA a post donations follow up for

vCJD is demanded. Therefore prevention of vCJD agent transmission in plasma product is achieved by standard prion titre reduction studies.

I.7.1 Prions validation studies

Laboratory scale studies have demonstrated that different steps in the production of biological products are effective in the removal of TSE-causing. The potentially most effective ones are those based on precipitation, nanofiltration, chromatography and deep filtration. The efficacy of these steps in removing TSE-causing agents is evaluated, in a validated laboratory-scale model, by experiments where the pathogenic agent is spiked in the intermediate product of the process prior to the step and determining its removal during the step by western blot and/or bioassay.

I.7.1.2 Western Blot (WB)

As previously mentioned, prion diseases are associated with the conversion of the α -helix rich prion protein (PrP^c) into a β -structure-rich insoluble conformer (PrP^{Sc}). Proteinase K (PK) digestion is the most common way to discriminate between PrP^c and PrP^{Sc} since PK totally degrades PrP^c but cleaves PrP^{Sc} only partially, leaving a truncated 28KDa band, denominated PrP^{TSE} , that can be easily visualized on a acrylamide gel by Western Blot (Fig. I.3).

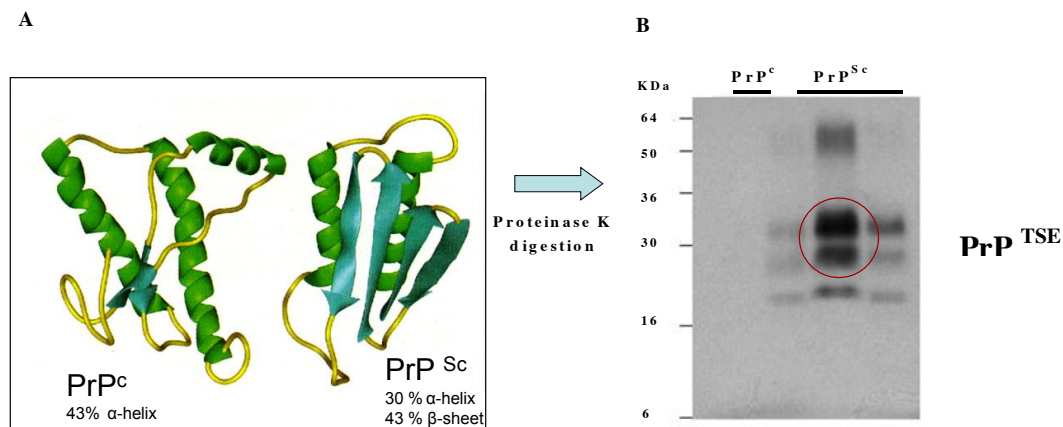


Figure I.3 A. PrP^c and PrP^{Sc} structure: (adapted from [35]). B. Typical western blot pattern after proteinase K digestion (adapted from [36]).

Briefly in a typical prion validation study by WB the starting material is deliberately spiked with a PrP^{Sc} solution and the PrP^{Sc} load is checked after the spiking. Upon the prions removal step, PrP^{Sc} titre is quantified in the final sample. For the quantization of PrP^{Sc} , each study sample to be titrated is digested with PK, serial dilutions ($0.5 \log_{10}$) of each sample are loaded in a polyacrylamide gel, separated by electrophoresis under reducing conditions (SDS-PAGE) and proteins are transferred to a polyvinylidene difluoride (PVDF) membrane. Then, membrane is incubated first with the monoclonal antibody 3F4 produced in mice, specific for PrP, and then with a secondary mouse anti-IgG antibody labeled with peroxidase. Finally, the antigen-antibody complexes are detected by means of a chemiluminescent system. The existence or not of PrP^{Sc} in the sample is determined on the basis of the presence/absence the PrP^{TSE} . The PrP^{Sc} titre of each sample is calculated as the $\log_{10}$ of the inverse of the first dilution where no PrP^{Sc} is

detected and expressed as $\log_{10}$ Western Blot units/ml ($\log_{10}$ WBU/ml). Depending on the weight/volume of the sample, the PrP^{Sc} titers are expressed as $\log_{10}$ total WBU.

Similarly to the virus validation studies, the prion removal capacity of a process is shown by means of RF calculated as the ratio between the PrP^{Sc} load detected in the spiked starting material divided by the quantity of PrP^{Sc} detected in the resulting sample at the end of the process, expressed in $\log_{10}$.

The most common prion strain employed as a spike in this kind of study is the 263K hamster-adapted Scrapie strain. In order to cover the widest possible spectrum of physicochemical forms of the prion, different preparations of PrP^{Sc} obtained from the brains of Scrapie-infected hamsters that showed obvious signs of TSE are employed:

- CBH (clarified brain homogenate):** The infected brain is homogenized in Phosphate-Buffered Saline (PBS) and the suspension clarified by centrifugation. The resulting supernatant is the clarified brain homogenate preparation. This preparation is characterized by high levels of PrP^{Sc} in different physico-chemical forms, a large proportion of which are membrane bound.

- **SARK (detergent-soluble preparations):** After the infected brain has been homogenized in PBS, the preparation is obtained by solubilizing the membrane-associated PrP^{Sc} with detergent (Sarkosyl). This preparation is characterized by high levels of PrP^{Sc} , mainly non-membrane-associated.

A clarified brain homogenate preparation obtained from the brains of healthy hamsters (PrP^{C}) is also used as negative control:

- NBH (clarified normal brain homogenate):** the healthy brain is homogenized in PBS and the suspension is clarified by centrifugation. The resulting supernatant is the clarified normal brain homogenate preparation.

I.7.1.3 Bioassay

The detection of PrP^{Sc} by the Western Blot technique can then be confirmed by an *in vivo* infectivity bioassay [25, 37]. Aliquots of the samples titrated are intracerebrally injected in hamsters following dilution series, to investigate their infectivity *in vivo*. Injected hamsters follow up occurs along 360 days upon injection, monitoring typical vCJD symptoms such as trembling, abnormal walking way, ataxia and head swinging. Finally animals are sacrificed by cervical dislocation. The brains are homogenized and the confirmation of TSE infectivity is performed by histopathology study or PrP^{Sc} WB

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detection of the hamsters' brains. . The calculation of the PrP^{Sc} infection dose is again calculated according to the Spearman-Kärber mathematic expression and the result expressed as $\log_{10} DI_{50}$ (logarithm of the Infectious Dose ₅₀: TSE infectivity titer that affects 50% of animals).

ANNEX I

Study of the elimination of PPV through the nanofiltration step of thrombin process production at different process conditions

Introduction

Planova filters (Fig. I.4) have been developed in 1987 by Asahi Chemical. Those filters are composed by a Bemberg Microporous membrane (BMM), hollow fiber membrane with a multilayered structure consisting in large, bulky void pores connected by capillary pores. Thus, when protein solutions pass through the filter, viruses are impeded to pass through the capillary pores and are retained in the bulky void pores. In this way, a size exclusion mechanism removes viruses from the purification stream.

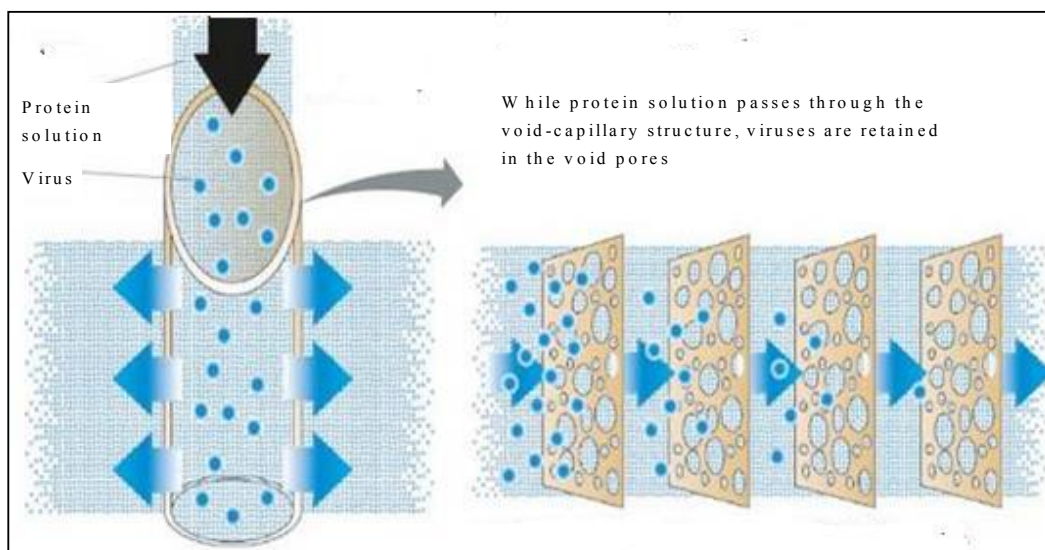


Figure I.4 Planova Filters structure. (Adapted from <https://www.ak-bio.com/products/virus-removal/planova-15n-20n-35n/ordering-info/>).

Nanofiltration by Planova filters it's very efficient in eliminating viruses in different processes and with variable conditions, including pH, ionic strength, protein concentration and temperature.

The efficiency of a nanofiltration step in thrombin production was evaluated, in laboratory scale, through spiking experiments and found to be really efficient in removal of different non-enveloped viruses, including the smallest one: PPV (RF=6.6 logs).

However, it was considered important to assess how the variability of certain nanofiltration process parameters affects the virus removal capacity of the nanofiltration step. Accordingly, the most relevant process parameters in this virus removal capacity study with PPV were modified (Table I.4).

This kind of studies must be performed according to GLP rules by the following steps:

- a. Study proposal and design
- b. Laboratory scale experiments
- c. Mock spike experiments
- d. Redaction and approval of the protocol (GLP)
- e. Viral spike experiments
- f. Redaction and approval of the final report.

a. Study proposal and design

In this phase, the virus removal capacity step is analyzed in an attempt to identify the most relevant parameters for virus removal and thus choose the worst conditions for virus removal. Critical limits values for these nominal conditions will be the parameters of the study.

In the study for Thrombin nanofiltration the following parameters were identified as important for virus removal:

- Protein activity;
- Filters transmembrane pressure (TMP);
- Temperature (T^a);
- System depressurization;

Four independent experiments were planned as described in Table I.5 In addition, a fifth experiment in nominal conditions (into the industrial limits) was performed.

Table I.4 Conditions of experiments for validation study of Thrombin nanofiltration at different process conditions.

Different conditions experiments					Nominal experiment
Parameters analysed	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
Thrombin activity	low	nominal	nominal	low	nominal
Process T ^a	nominal	high	nominal	high	nominal
TMP of nanofiltration system	low in the 2 nd P15N	nominal in the 1 st P15N	low in the 2 nd P15N	low in the 2 nd P15N	nominal
System depressurization	post-washing	not performed	-before post-washing -post-washing	post-washing	post-washing

b. Scale down experiment

Laboratory experiments were performed without virus spiking, since they are used just to inquire whatever the worst conditions proposed in the design are feasible in terms of kinetics of the process and protein recovery.

In the study at different process conditions for Thrombin nanofiltration, all the conditions were found to be attainable under the parameters' value chosen, however conditions with lower TMP in the 2nd P15N were identifies as worst cases among the others.

c. Mock spike experiments

In those experiments, cellular extracts are spiked into the material in order to determine if the virus (PPV) inoculation ratio proposed in the design has any influence on the process. Higher inoculation rates improve Reduction Factor values, but may affect the nanofiltration process therefore a balance between inoculation rate and nanofiltration process performance must be found. Mock spike experiments are performed using the parameters of the worst case experiments, as defined by the laboratory scale experiments.

In the study at different process conditions for Thrombin nanofiltration, a PPV inoculation rate of 1/100 was defined as feasible since it did not affect the process in terms of kinetics neither protein recovery.

d. Redaction and approval of the protocol (GLP)

Once identified the worst process conditions and the virus spike ratio feasible for the study, a high detailed protocol is redacted and must be approved by the Study director, Viral Safety Division, Quality Assurance department, and Research and Development Management.

A typical GLP protocol contains the following points:

- introduction;
- aim of the study;
- definition of director of the study, department and physical location where the study will be performed;
- materials: detailed information such as lot number, origin, reference number of all the materials used in the study (starting material, type of virus and cells) as well as all the equipment and reagent used;
- methods: identification of the specific SOPs numbers for: materials preparation, virus suspension preparation, cell culture methods, virus titration (TCID₅₀);
- experimental design: here details are furnished regarding the conditions used in the industry, in the previous virus validation study and the conditions chosen for the current study. Furthermore a diagram I.1 describes the overall design;
- results interpretation: mathematic equations used to analyze the results of virus removal are defined.

e. Viral spike experiments

Only after the approval of the protocol, virus spiking experiments can be performed.

In the study at different process conditions for Thrombin nanofiltration, 5 experiments with the condition illustrated in table I.4 have been performed and samples have been extracted according to the following diagram, illustrated in the protocol of the study:

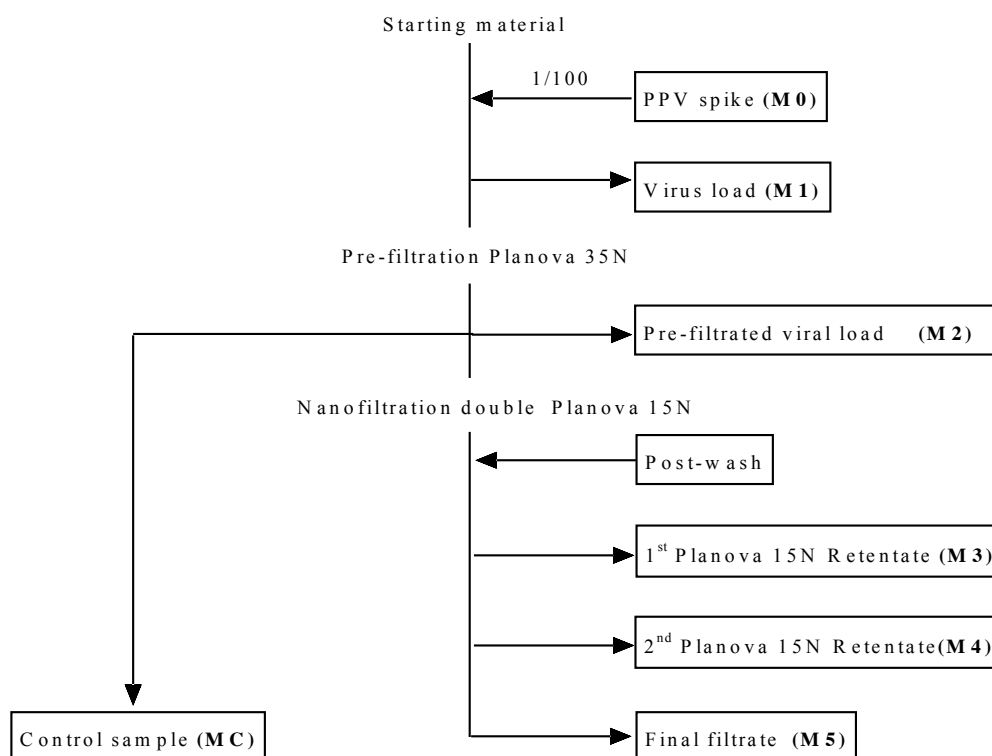


Diagram I.1 Spiking experiment for the validation study of Thrombin nanofiltration at different process conditions

Briefly, the virus (MV) was spiked into the material at dilution 1/100 giving rise to a 1% spike, as defined by mock experiments. An aliquot of the sample was drawn out in order to titer the viral load in the spiked material (M0). The remaining volume of spiked material was pre-filtered through Planova P35N to assure monodispersed viruses (for PPV virus which as a size of 18-20 nm , P35N serves only as a pre-filter). Two aliquots were drawn out from the prefiltered volume. One of them was immediately titrated to determine the prefiltered viral load in the material (M1), whereas the other one, identified as hold sample (MC) was stored under the same temperature conditions as the process until the end of the procedure. The remaining volume of prefiltered spiked material was challenged to the P15N filtration (according to the parameters in table I.4) and sample of the 1st (M3) and the 2nd (M4) Planova 15 N retentate and the final filtrated sample (M5) were collected .All the samples were titrated by an infectivity assay and TICD₅₀ of every sample was defined. Finally RF of the process is calculated as the difference between the viral load in MV sample and M5 sample.

f. Redaction of the final report

In conformity with GLP rules, at the end of the study, a detailed final report was redacted by the Study director and approved by the same approvals of the protocol. QA also organized inspections during all the study (protocol, titrations, experiments, final report) producing the respective audit reports.

In the final report, a table similar to table I.5, illustrates the results:

Table I.5 Results of the study of the removal of PPV through the nanofiltration step of thrombin process production at different process conditions.

Sample	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Prefiltered viral load	5.66	5.95	N/D	6.42	6.23
Control sample	5.53	5.73	6.08	6.38	6.54
Retentate 1 st Planova 15N	4.47	5.76	4.99	5.68	6.01
Retentate 2 nd Planova 15N	≤ 2.45	≤ 2.43	≤ 2.46	≤ 2.46	≤ 2.44
Final filtrate	≤ 0.90	≤ 0.84	≤ 0.92	≤ 0.95	≤ 0.87
Reduction Factor (log₁₀)	≥ 4.8	≥ 5.1	≥ 5.2*	≥ 5.5	≥ 5.4

Titres in log₁₀ TCID₅₀ total. *Value calculated from the hold sample.

The results show that the nanofiltration step through double Planova 15N of the thrombin production process, under different conditions, effectively removes PPV, with RF values found of between $\geq 4.8 \log_{10}$ and $\geq 5.5 \log_{10}$, with no residual infectivity detected in any of the experiments.

Most of the viruses from the pre-filtered viral load are found in the sample 1st P15N filter retentate, with no residual infectivity detected in the 2nd P15N filter retentate.

No differences were observed between the removal results obtained after conducting the experiments under different conditions for thrombin activity, temperature, transmembrane pressure and timing of system depressurization.

The results obtained in the experiments conducted show that the nanofiltration through 15 nm pore size filters (double Planova 15N) of the thrombin production process is robust and is capable of effectively removing PPV. These results demonstrate the virus removal

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capacity of the step, even with process parameters out of industrial specifications. The first P15N filter retains most of the viruses, with no virus detected in the 2nd P15N retentate, demonstrating the high degree of safety from using two consecutive P15N filters.

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SECTION 2
BACKGROUND

CHAPTER II

HUMAN ADULT STEM CELLS AND REGENERATIVE MEDICINE APPLICATIONS

II.1 PRINCIPLES OF REGENERATIVE MEDICINE

The aim of regenerative medicine is to repair or replace tissues or organ function lost due to age, damage, disease or congenital defects. Although the huge advance in this area during last years, there are still many problems to be solved. To improve the potential of regenerative medicine, it is essential to better understand the mechanisms that are on the basis of the natural regeneration processes. Thus, this chapter aims at the elucidation of tissue repair process with a dissertation upon involved cells and will focus mainly on human Mesenchymal Stem Cells and their application in regenerative medicine.

II.1.1 Tissue repair

Human body has the capacity to maintain and regenerate tissues and organs. In fact, human tissues can be ideally divided into three classes: continuously dividing tissues, quiescent tissues and non-dividing tissues. Cells in *continuously dividing tissues* are constantly in proliferation to replace dead cells. Some examples are skin, gastrointestinal epithelium and hematopoietic tissue. *Quiescent tissues* contain cells that do not constantly divide but can enter in cell cycle in response to a signal, such as cell injury. Some of these tissues are liver, kidney and pancreas. Finally *non-dividing tissues*, such as cardiac and skeletal muscle, comprise cells that do not divide since have exit from cell cycle.

Normally, after injury or degeneration, human body can re-establish homeostasis by two processes: regeneration and replacement. *Regeneration* occurs when a new growth replaces completely part of damaged tissues. *Replacement* happens when non-regenerable tissues are repaired by connective tissue in a process commonly known as *scarring* [1]. As a matter of fact, regeneration happens very rarely in humans. Except for continuous dividing tissues, such as gastrointestinal epithelium where new cells coming from stem cell niche replace old ones, pathological examples of pure regeneration are very few. After a severe injury in non-dividing tissues, regeneration is actually impossible for humans. Tissue is

instead repaired by deposition of fibrous tissue, “patching” tissue components more than restoring them.

Tissue repair is an integrated and elaborated process that involves different kinds of cells and a large number of regulatory molecules including growth factors and cytokines. More precisely after wound healing, tissue repair can be characterized by four overlying steps: 1) an initial response to maintain homeostasis; 2) inflammatory response to prevent inflammation; 3) proliferative phase to reconstitute the damaged tissue; 4) remodeling phase to restore tissue strength and function.

During homeostasis phase, platelets form a fibrin clot to seal the wound site and avoid spread of infection to the body. Vasoconstriction around the wound site also isolates it. However, this process is soon followed by vasodilation to allow the required cells to reach the wound site. During the inflammatory phase, immune cells are attracted to the wound site to remove debris and kill eventual bacteria. Proliferative phase consists in: i) formation of blood vessels, ii) deposition of temporary extracellular matrix (ECM) constituted of collagen and fibronectin, iii) re-epithelialization and contraction of the size of the wound. Finally, during the last phase of remodeling, cells recruited to the site of injury are degraded, while temporary ECM is remodeled into a robust and stable structure [2].

Different kinds of cells are involved in this story: cornerstones of regeneration process are stem cells (discussed below in this dissertation) however fibroblasts, endothelial cells and macrophages are also indispensable. In fact, during wound healing, fibroblasts deposit an acellular fibrous tissue, made by collagen, to replace the region of lost cells. Endothelial cells are crucial for the formation of new blood vessels that transport nutrients and cells to the new tissue. Finally macrophages release the growth factors that stimulate both fibroblast and endothelial cell and are responsible of other numerous functions [3].

Other important elements during tissue repair are growth factors, soluble factors whose signaling directs the behavior of cells implied in regeneration. Among them Transforming Growth Factor (TGF), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF) play crucial roles. TGF has many functions but the most important is to promote fibrosis since it attracts and stimulates fibroblast to secrete collagen and inhibits ECM degradation by metalloproteinases (MMPs) [4]. FGF regulates fibroblast, monocytes and epithelial cells migration, stimulates angiogenesis and wound contraction [5]. PDGF secreted by macrophages, endothelial cells and muscle cells, during the early stage of tissue repair, attracts fibroblasts to the wound area, induces the production of MMPs, hyaluronic acid and fibronectin and induces wound

contraction [6]. Finally, as the name suggest, VEGF acts in endothelial cells inducing blood vessel formation and some MMPs synthesis, as collagenase [7].

Last but not least, an element of crucial importance is the tissue upon which all the tissue repair system works: ECM, a complex insoluble polymer network composed by structural molecules (collagen and elastin), adhesive molecules (fibronectin and laminin) and elastic components (proteoglycans and hyaluronan). It can confer different physical characteristic from elasticity in soft tissues to stiffness in bone and is storage of growth factors. Moreover, it serves as scaffold for cells involved in tissue repair, being a degradable physical environment where neovascularization and remodeling can occur. Finally, ECM constitutes a background for all the signaling-exchange needed for repair [8].

II.1.2 Tissue engineering and scaffolds

Tissue engineering is a field of regenerative medicine that aims to induce tissue repair by the use of freshly isolated or cultured cells that can be implanted into the site of injury. However, without adequate supply, only few numbers of cells migrate in the site of injury and will survive. Also, infused cells may not maintain their function after infusion or may cause immunological rejection. Therefore, tissue engineering aims the development of scaffolds that mimic the biological structure and functionality of native ECM and thus, will induce cell proliferation, differentiation and tissue regeneration as ECM does. Following this approach, two strategies can be applied.

One approach consists in the creation of bioengineering constructs made by the patient's own cells in a combination with a biomaterial scaffold: when the construct is implanted *in vivo* the scaffold works as a delivery service for proper signal molecules such as soluble growth factors, that recruit the cells in in the site of injury and stimulate them to form new tissue. The second approach makes use of biomaterial scaffolds that are implanted directly into the injured tissue and stimulate the body's own cells to promote local tissue repair, in a processed so called as "*in situ tissue regeneration*" [9].

During the design of a scaffold for tissue engineering some considerations are important:

- 1) Biocompatibility: the scaffold may not induce an immune reaction.
- 2) Biodegradability: the scaffold is not intended to be permanent, but only a support for the infused cells or the own cells of the body to produce new ECM. After that, scaffold should be degraded. Also, the products of its biodegradation cannot be toxic and must be able to exit from the body without interfering with organs.

- 3) Suitable mechanical properties: scaffolds should have mechanical properties that simulate the tissue where it will be implanted, but at the same time must be robust enough to function from the time of installation to the end of the remodeling process.
- 4) Suitable architecture: scaffold must have a well interconnected pore structure to allow cells penetration, diffusion of growth factors and nutrients inside the scaffold and removal of waste products [10].

Two major categories of scaffold can be described:

- human tissue derived scaffolds: carry the advantage of their good biocompatibility. However, they also have disadvantages that are related with the few available natural sources, the possibility of human diseases transmission and with immunogenicity. Most of them are decellularized ECM obtained from human fibroblast [11], dermis, intestinal mucosa, mesothelium [12] and cardiomyocyte [13].
- Synthetic scaffold: can be composed by a wide variety of materials, such synthetic polymers, ceramics, metallic and composites. Those materials are designed to mimic the three-dimensional features of autograft tissue while maintaining viable cell population.

During the last decades, remarkable advances have been made in the fabrication of biocompatible and biodegradable scaffolds (reviewed in [14]), cell seeding techniques [15], and implantation protocols.

II.2. STEM CELL

Stem cells are unprogrammed cells in the human body with unique characteristics: *i)* the ability of self-renewal that can occur either by symmetric replication (one stem cells gives raise to two identical daughter stem cells able to self-renewal) or asymmetric replication (one daughter cell differentiate while the other remains a self-renewing stem cell); *ii)* the capacity to generate more than one cell type. Therefore they are responsible for regenerating tissue and organs and are considered relevant tools in tissue engineering and regenerative medicine. They can be found in embryos at very early stages of development (embryonic stem cells) and in some adult organs (adult stem cells). Embryonic stem cells are pluripotent since they are able to generate cells from any tissue. Adult (somatic) stem

cells instead are multipotent since they can give rise only to a restricted array of cell type. Adult stem cells are found in abundance in continuously dividing tissues such as skin, cornea, hematopoietic tissue, gastrointestinal epithelium, however they are present also in some quiescent tissues such as liver, pancreas and adipose tissue and central nervous system [16]. In the tissue, adult stem cells are localized into the niche, micro-environments constituted by both stem cells and non-stem cells [17].

Ideally, a stem cell for regenerative medical application, should meet the following criteria: *i.* can be found in generous quantities, *ii.* Can be harvested by a minimally invasive procedure; *iii* Can be differentiated along multiple cell lineage pathways in adjustable and reproducible manner; *iiii* Can be safely and effectively transplanted to either an autologous or allogeneic host; *iiiii* Can be manufactured in accordance with current GMP guidelines.

The knowledge on stem cell properties has greatly expanded since their discovery and now includes various kinds of stem cells classified according to their differentiation potential. A system of nomenclature is described in Table II.1

Table II.1. Stem cells classification (adapted from [18])

DENOMINATION	DIFFERENTIATION POTENTIAL	EXAMPLES
Totipotent	All embryonic and extraembryonic tissues	Zygote
Pluripotent	All embryonic tissues	Inner Cell Mass (ICM), Embryonic Stem Cells (ESCs), induced Pluripotent Stem Cells (iPS)
Multipotent	All lineages of a tissue/organ	Mesenchymal Stem Cells (MSC) Hematopoietic Stem Cells (HSCs) Neural Stem Cells (NSCs)
Oligopotent	Several but not all lineages of a tissue/organ	Common Myeloid Progenitor (CMP) Common Lymphoid Progenitor (CLP),
Unipotent	Single lineage of a tissue/organ	Macrophages progenitor

II.2.1 Pluripotent Stem Cells

Historically, Embryonic stem cells (ESC) cells were described in 1981 by Evans and Kauffman as “pluripotent cells present in a mouse embryo that show ability to take part in the formation of chimeric animals and to form terato-carcinomas as well as able to differentiate either *in vitro* or after inoculation into a mouse as a tumor *in vivo*”[19].

hESCs are found at the early development in the inner cell mass (ICM) of blastocysts [20] and have been isolated first from mouse ICM [19] and later from human ICM [21]. ESCs have tremendous ability to differentiate in all derivatives of the three primary germ layers (ectoderm, endoderm and mesoderm) therefore they can give rise to any cell type of the adult body. Moreover, they can be maintained in culture for several months without loss of differentiation and self-renew potential.

Due to their wide plasticity and unlimited capacity for self-renewal, hESC have been proposed as a source of replacement cells in regenerative medicine. In fact, they have been successfully derived in cardiomyocytes [22], retinal pigment epithelium cells [23], neurons[24] and hepatocytes [25] among others cell types. Cells derived by hESC have been applied in some clinical trials, especially for treatment of rare retinopathies [23], but also spinal cord injury, type I diabetes, severe heart failure [26].

However, there are several drawbacks related to the use of hESC for cell therapy. Since hESC isolation requires the destruction of human blastocysts, serious ethical concerns are related to their use. Because of this limitation, in the last decades strong efforts have been done for the production of induced Pluripotent Cells (iPSC) from adult tissue cells (i.e. fibroblasts) by retro-virus transfection of hESC pluripotency associated factors (Sox 2, Klf4, C-Myc and Oct3/4). This technology gives rise to cells that possess the same pluripotent characteristics of hESC [27], but do not carry ethical issues. However, also regarding iPSC application in clinics there are some hesitations: hESC are known to proliferate without control and create teratomas once implanted into immuno-deficient mouse [28]. Since iPSC have the same telomerase activity of hESC, they are also believed to be tumorigenic [29]. Moreover, other concerns regard the loss of genome integrity in iPSC and hESC [30].

For these reasons, applications of hESC and iPSC on clinical trials for regenerative medicine have been very limited until now.

II.2.2 Adult stem cells

In 1961[31], James Till and Ernest A. McCulloch gained the recognition of “fathers of stem cell” due to their discoveries with adult stem cell: upon transplantation of syngeneic bone marrow (BM) cells into recipient mice, they observed the formation of cellular colonies in the spleens of the mice, suggesting that BM cells have ability to generate different kinds of myeloerythroid cells and to self-replicate. These evidences introduced the concept of adult stem cells as undifferentiated cells in human body that proliferate by cell division to regenerate or repair damaged tissues and put the basis for the concept of bone marrow transplantation.

In the last decades, consistent advances have been reached in adult stem cells research: they can be isolated from several tissue sources, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis [32]. It is known that they reside in a specific area of each tissue called "stem cell niche" where they remain quiescent for long periods of time until they are activated by a physiological need for tissue renewal (e.g. skin) or by a stimuli such as an injury or disease [17].

Usually, adult stem cells enter into a differentiation pathway to generate the specialized cell type of the tissue where they are located. Therefore, hematopoietic stem cells differentiate into all type of blood cells, neural stem cells into neurons and glia, bone marrow mesenchymal stem cells into cells of mesodermal lineage, skin stem cells into keratinocytes and so on. However, nowadays it is known that adult stem cells can have high “plasticity” a term that indicate the ability of some adult stem cells to cross lineage barriers and to adopt the expression profiles and functional phenotypes of cells unique to other tissues [33]. For instance it was reported that neural stem cells can generate blood [34] and skeletal muscle cells [35], adult stem cells isolated from mammalian skin can differentiate into neurons, glia, smooth muscle cells and adipocytes [36] and bone marrow stromal stem cells give rise to a wide range of cell types including liver [37], kidney [38] and pancreas cells [39].

Adult stem cells are considered very promising tool in regenerative medicine as they represent a substitute to ESC that does not carry ethical concerns and risk of teratome formation. However, a big drawback in the use of these cells is that, being the number of adult stem cells very low in each tissue, it is necessary to expand them in laboratory prior to their use as therapeutic agents. This expansion step can be problematic since cells have

limited capacity to divide after isolation. Moreover, there are also some concerns regarding possible cells contamination and alteration of their phenotype during culture.

II.3 MESENCHYMAL STEM/STROMAL CELLS

Human Mesenchymal Stem/Stromal Cells (hMSC) were first isolated in 1970 by Friedenstein and colleagues, who reported the isolation of marrow-derived fibroblast-like cells, able to adhere to the plastic substrate of the cell culture plate. Since those adhered cells formed separated colonies, they were initially referred as colony unit fibroblast (CFU-F) [40]. More precisely, they collected the whole bone marrow aspirate, cultured it into plastic culture surfaces and, after 4 hours, removed non-adherent cells to eliminate hematopoietic ones. The remaining bone-marrow adherent cells were characterized by splinded-shape and formed colonies able to differentiate in bone-similar structures. Later in 90' the term MSC was introduced [41] and generally agreed [42] as indication of pluripotent progenitor cells from mesoderm able to multiple cell divisions and to give rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, connective tissue. During the following years the knowledge about MSC has increased notably and a controversy rose regarding the nomenclature of these cells. In fact, even the stem cell nature of hMSC has been debated [43, 44] and because they were not found only in bone marrow but in all the post-natal tissues with similarity to stromal cells such as fibroblasts a more appropriate definition of human Mesenchymal Stromal Cells (hMSCs) has been introduced. Nowadays they are mostly defined as *human mesenchymal stem/ stromal cells* (hMSCs). The major problem in MSC research is their high heterogeneous nature *in vitro*, which impedes a universal characterization. At the present day, striking argumentations still regard their identity, function and clinical potential [45]. However, in general hMSC generated worldwide great interest for cell therapy based approach because of their facility to be isolated, extensive expansion rate, systemic engraftment capabilities and differentiation potential.

II.3.1 Sources and Isolation of hMSC

MSCs have been isolated initially from bone marrow (BM), but nowadays it is well known that they can be isolated also from other tissues such as adipose tissue [46], endometrium and menstrual blood [47, 48], umbilical cord [49], placenta [50], trabecular and compact bone [51, 52], fetal liver [53], synovial membrane [54], dental pulp [55] periodontal ligament [56], brain [57] among others.

Different efforts have been made to isolate MSC from alternative sources of bone marrow. Many studies investigate whatever one tissue may be a more advantageous source and inquire whether MSC isolated from different tissue present identical characteristics. For instance according to one report MSC derived from adipose tissue, bone marrow and umbilical cord blood have the same morphology, differentiation potential, expansion rates and surface markers [58]. However, other studies state that umbilical cord blood MSC have less adipogenic differentiation potential [59] and adipose derived MSC have more immunosuppressive properties [60]. Actually, it is rather difficult to compare since different parameters can influence the quality of MSC upon *in vitro* culture.

BM remains currently the most used source for MSC. Here, hMSC are located at the exterior surface of sinusoids in the subendothelial position, forming a part of the three-dimensional perivascular stromal compartment [45]. In BM, MSC are responsible of the integrity of blood vessels, therefore they are inactivated in normal condition but following an injury they become active, evade the perivascular niche and act to regenerate the damaged tissue [61]. However, nowadays it is general accepted that hMSC reside in a vascular niche in the wall of the vessels of every organ and tissue of human body, where pericytes are [62].

Briefly, to isolate MSC, BM is collected by the donor's posterior superior iliac spine by aspiration in a heparin containing syringe and then the whole marrow sample is subjected to fractionation on a density gradient solution such as Percoll. Cells are plated at densities ranging from 1×10^4 to 5×10^4 /cm² and generally cultured in basal medium such as Dulbecco's modified Eagle's medium (high glucose) in the presence of 10% foetal bovine serum (FBS). Primary cultures are usually maintained for 12–16 days to deplete non-adherent hematopoietic cell fraction. Then, due to their high proliferative capacity, MSCs can easily be expanded up to 50 population doublings [63].

II.3.2 Characterization of MSC

Different studies have investigated the properties of hMSC isolated from different tissues. However, there is still great inconsistency of results and, because of their high heterogeneity, uniformity of hMSC characterization remain an entangle aspect in hMSC research. [64].

The Mesenchymal and Tissue Stem Cell Committee of the ISCT [65] has proposed a set of standards to characterize human MSC for their application in scientific investigation and in the clinics.

In principle, three criteria are used to define hMSC:

- Morphology: adherence to plastic surface;
- Immunophenotype: specific marker antigen expression;
- Tri-lineage differentiation.

II.3.2.1.Morphology

hMSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks [65]. Figure II.1 shows typical hMSC splinded shape morphology. However, it has been reported that hMSC can assume at least three morphological subpopulations: i) rapidly self-renewing cells (RS cells); ii) elongated, fibroblastic-like, spindle-shaped cells (SS cells) and iii) slowly replicating, large, cuboidal or flattened cells (FC cells). RS cells [66] present the highest multipotentiality while larger, spindle-shaped cells manifest the higher tendency to differentiate into cartilage [67]. Finally, FC cells are mature hMSC that tend to differentiate into osteoblastic precursors [68].

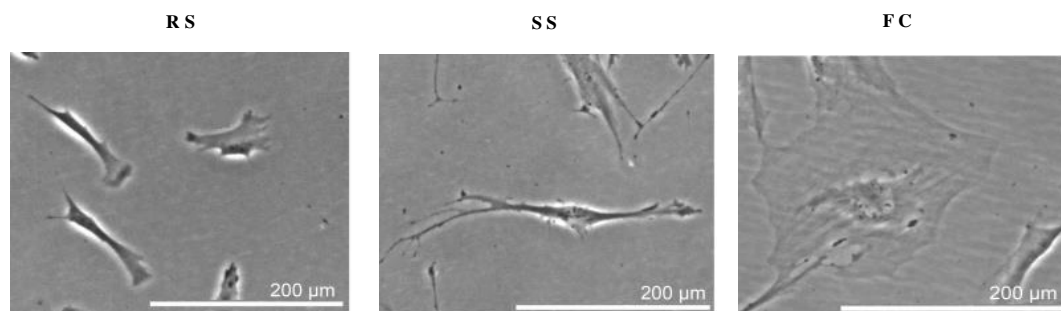


Figure II.1. hMSC morphology (adapted from [66]).

II.3.2.2.Immunophenotype

To define hMSC quality, a set of basic specific surface antigens must be defined by flow cytometry (Table II.2). Briefly, most of the hMSC population may express CD105, CD73 and CD90 while they must lack expression of markers of hematopoietic cells that may contaminate hMSC pools: CD45, CD34, CD14, CD11b, CD79a or CD19 and Human Leucocyte Antigen (HLA). Beside this basic panel of markers, other surfaces antigens (CD44, CD29, CD13, CD146, CD106, CD54, CD166, CD11b, CD49d, CD10 and CD31) have been used to define hMSC identity and results from various studies have been reviewed by Mafi and colleagues [69]. However, there are some discrepancies among results since the expression of some of those markers varies depending on the donor or *in vitro* culture conditions (e.g. seeding density) [70]. Thus, hMSC characterization only based on antigen expression is not enough due to the lack of specific and unique markers.

Table II.2. Panel of markers for hMSC characterization.

Marker	Percentage of expression	Description	Role	Reference
CD105 (“endoglin”)	> 95%	TGF-beta receptor III	TGF-beta signaling during MSC chondrogenic differentiation	[71]
CD73	> 95%	Ecto-5'-nucleotidase	BM stromal interactions, MSC migration, immunomodulatory properties	[71-73]
CD90	> 95%	Thy-1 antigen	cell-cell interactions	[74]
CD45, CD34, CD14, CD11b, CD79a, CD19, HLA II	<2%	Hematopoietic cells markers	---	[69]

II.3.2.3.Tri-lineage differentiation

An additional criterion to define hMSC identity, is their capacity to differentiate into osteoblasts, adipocytes and chondrocytes under standard *in vitro* differentiating conditions [42].

Osteogenesis is verified incubating the cells for up to 21 or 28 days in basal medium (typically DMEM low glucose + 10% FBS) plus osteogenic supplements (100nM dexamethasone, 10mM β -glycerophosphate, 0.05×10^{-3} M ascorbic acid). The medium must be replaced twice per week and osteogenesis can be demonstrated by staining with Alizarin Red or von Kossa [75, 76].

Adipogenesis is conducted incubating the cells in basal medium with adipogenic supplements (1×10^{-4} M dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX) in 0.5 M KOH, 10 μ g/mL insulin in 0.02M HCL, 100 μ M indomethacin). After 2-3 days, adipogenic medium is replaced by insulin medium (basal medium + 10 μ g/ml insulin). In the following 7 days insulin and adipogenic medium are switched every 2-3 days. Then,

cells are incubated in insulin medium for one additional week. Finally, adipogenesis is demonstrated by Oil Red O staining [77].

Chondrogenic differentiation is performed by growing MSC along 21 days in spontaneously formed pellets. Medium is constituted by DMEM with high glucose (4.5 g/L) plus 50 µg/mL ascorbic acid, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, 100 µg/mL Insulin Transferrin Selenium (ITS) and chondrogenic differentiation factors: 0.1 µM dexamethasone and 10 ng/mL TGF-β3. Medium change is performed twice for week and chondrogenic differentiation is evaluated with Toluidine Blue or Alcian Blue or Sefranin O staining [78]. In addition histochemical staining for collagen type II is recommended.

However, differentiation commercial media are already available in the market (e.g. Promocell).

II.3.3.Karyotype screening

Beyond the three general MSC characteristics defined by ISCT, some additional verification is needed, in particular if the cells are intended for medical use. Especially after extensive passaging in culture, cells may undergo genomic aberration including potentially transforming events [79]. Therefore, analysis of karyotype integrity is also strongly recommended.

II.3.4. MSC immunomodulatory properties

One of the most interesting properties of MSC and the reason why they are considered particularly suitable as therapeutic target for tissue repair is their capacity to escape from the immune system detection and even inhibit immune responses, in certain contexts. In fact, MSC are immune-privileged and hypo-immunogenic since they express low level of Major Histocompatibility Complex (MHC) class I alloantigens and do not express HLA Class II neither costimulatory molecules such as CD80, CD86, CD40 [80]. Due to this, MSC are believed to reduce the risk of graft rejection after transplantation.

MSC stimulate tissue regeneration by endorsement of the action of fibroblast, endothelial cells and tissue progenitor cells, since they release epidermal growth factor (EGF), FGF, PDGF, TGF-β, VEGF, hepatocyte growth factor (HGF), insulin growth factor-1 (IGF-1), angiopoietin-1 (Ang-1), keratinocyte growth factor (KGF) and stromal cell derived factor-1 (SDF-1)[81].

But when MSC arrive into the site of injury, some factors released by leucocytes or toxins or infectious agents (TNF- α , IL-1, IFN- γ and LPS), stimulate MSC to produce some immunomodulatory molecules that suppress immune reaction. MSCs inhibit proliferation and activation of both cells of adaptive (T and B cells) and innate immune system (natural killer (NK) and dendritic cells (DC)). MSC can inhibit T cell response by both cell-cell contact and paracrine activity, upon IFN- γ activation. T cell inhibition by cell-cell contact is due to the fact that MSC express on their membrane integrins α 1, 2, 3, 5, 6, V and β 1, 3 and 4, molecules of intercellular adhesion (ICAM.1 and ICAM.2), vascular cell adhesion protein (VCAM)-1, CD72, and CD58 (LFA-3) that bind T lymphocytes [82] and interfere with their proliferation *in vitro* [83]. Indeed, neutralization of CXCR3, a receptor for the T-cell chemokines CXCL9, -10, and -11, inhibits MSCs immunosuppression, demonstrating the importance of cell contact for hMSC immunomodulatory action towards T cell activation [84].

However, it seems that MSC can influence T cell reactivity independently from cell contact by the release of trophic factors, namely TGF- β , HGF, prostaglandin E2 (PGE-2), indoleamine 2,3- dioxygenase (IDO), Nitric Oxide (NO), human leukocyte G protein (HLA-G) and IL-10 that suppress T lymphocyte memory, proliferation and activation [85]. Moreover, it has been reported that MSC produce galectins that increase the activity of T regulatory cells [86].

The mechanism by which MSC inhibit B cell activation is still argument of debate, since the results are controversial, probably due to different experimental settings [87]. It was reported that MSC arrest B cells cycle in B0/B1 by inhibition of their secretion of IgM, IgG and IgA [88] or that MSCs inhibit the caspase 3-mediated apoptosis of peripheral CD19⁺ B-cells by direct cell–cell interaction [89]. However, contradictory results showed that MSC may promote B cell activity by IgG production via IL-6 release [90, 91].

Regarding the innate immune system, MSC inhibit both NK cells and DC. The action of MSC on NK cells is complicated, since NK cells attack cells that lack of MHC I expression, thus MSC should be vulnerable to their action. However, it seems that MSC interact with some MHC I -inhibitory receptor in NK cells that may protect MSC from lysis and suppress NK activation by production of TGF β and PGE2 [92]. However, it was also reported that MSC production of IL-2 and IL-15 actually stimulate NK proliferation, and that MSCs can be killed by activated NK cells [93].

Regarding DC cells, MSC interfere with their maturation because they block the differentiation of monocyte in DC by PGE2 production [94]. Also, it has been reported that

DC co-cultured with MSC in presence of stimulatory factors fail in expression of maturation markers and induce activation of T regulatory cells, inducing a more immune-suppressive environment [95].

Importantly some differences in the immune-suppressive ability of hMSC have been noticed, depending on different passages in culture [96], different culture expansion protocols, different sources, or donor variability [97]. Moreover, MSC from different species produce different immunomodulatory molecules, for instance mice MSC produce high level of NO and poor level of IDO whereas human MSC release much more IDO than NO [81].

In conclusion, even if many studies report controversial findings regarding the role of MSC in immunosuppression, it is nowadays well accepted that MSC can modulate immune responses at multiple levels (Fig.II.2), inhibiting inflammation and stimulating tissue regeneration.

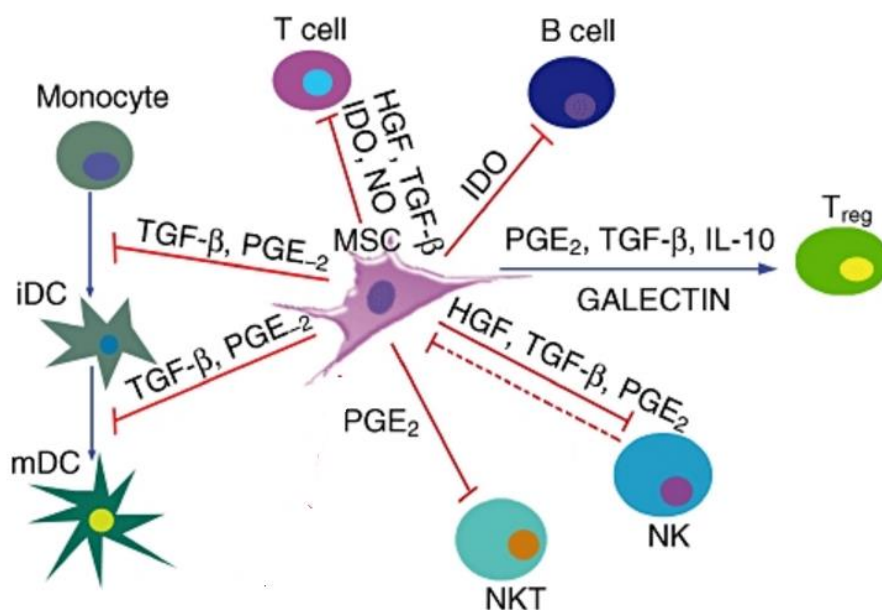


Figure II.2. Schematic representation of hMSC immunomodulatory properties (Adapted from [98]).

II.3.5. hMSC in clinical trials

As in May 2017, 732 clinical trials tested MSCs as therapeutic agents for a wide range of human diseases (<http://www.clinicaltrials.gov>). As mentioned before, MSC are considered particularly convenient in clinical practices, because they are easily to isolate, do not carry ethical problems and can be expanded *in vitro* to achieve therapeutic doses. Most

importantly for clinical settings is their lack of immunogenicity which allows allogenic cell transplantation without the need of immunosuppression.

The success of MSC therapy depends on i) the capacity of the injected MSC to migrate, survive, differentiate and release paracrine factors; ii) the ability of the host tissue to positively react to the injected MSC and to the factors produced by them; and iii) the integration between the exogenous (injected MSC) and endogenous (tissue host) for repair mechanism. Evidently the outcome of therapy depends on the type of MSC used in terms of gender, age and health of the donor [99].

Different strategies can be used for hMSC isolation, manipulation and administration in clinical practice. First, isolation source (e.g. bioptic tissue, peripheral blood) from donors (allogenic) or from the patient itself (autologous) is collected to isolate MSC. Second, being MSC in very low number in the tissue of origin, cells need to be expanded in laboratory to achieve therapeutic doses. Third, MSC can be administrated by intravenous or intra-arterial infusion and they can be vehiculated in injectable products. Moreover, cells can be loaded directly into the target tissue or transplanted during surgery by loading into implantable scaffolds. Among those administration ways, intravenous is the most common since it guarantees a systemic distribution of the cells. However, it carries the risk of embolism in the lung [100-102].

Concerning possible risks of the use of hMSC as therapeutic agents, almost none side effects were reported in clinical trials. However, some concerns have been registered:

- i) some studies have shown that hMSC may be not immunoprivileged, since MSC infused into immunocompetent mice were rejected following an immune-response [93];
- ii) if animal serum is used during cells expansion *in vitro*, severe concerns arise regarding the possible transmission of prions or zoonoses or immune responses to animal proteins attached to the cells surface (see Chapter IV);
- iii) few studies reported the risk of formation of mesenchymal tissue in ectopic sites, following MSC infusion: for instance, bone was developed into the myocardium after MSC infusion in a rat model for myocardial infarction [103].

The beginning of hMSC clinical trials is dated in 1995, when autologous MSC were subministrated to 15 patients with hematologic diseases [104]. At the moment, hMSC are implied in different clinical settings: graft-versus-host disease, autoimmune diseases, neurodegenerative diseases, bone and cartilage regeneration, Crohn's disease, cardiovascular repair, liver diseases, skin transplantation and plastic surgery, cancer therapies.

Graft-versus-host disease (GvHD):

Graft-versus-host disease is a frequent complication that develops after allogeneic hematopoietic stem cells transplantation caused by the natural anti-graft response of the host immune system. The acute form usually appears 100 days after cell transplantation and carries a high risk of mortality. Since the principle mechanism of GvHD is an allogeneic cytotoxic reaction of the donor lymphocytes, the pillar of GvHD treatment is immunosuppression, thus, due to their inhibitory effects on the proliferation and cytotoxic activity on immune system cells, MSC are considered promising therapeutic agents. Actually FDA already admitted the use of MSCs in pediatric acute GvHD. MSC therapy for this disease began in 2004 when Le Blanc and colleagues [105] administered MSC to a 9-year-old boy with severe GvHD and the patient recovered after 1 year. Kurtzberg et al., obtained 64% of response rate in 59 children suffering of severe steroid-refractory GvHD, after 28 days of allogeneic MSC infusion [106]; Prasad and colleagues also reported 58% of positive response, 17% of partial positive response in children affected by grade III and IV gut GvHD and complete resolution of symptoms in 75% of patients [107]. Many other encouraging results have been reported in clinical setting for GvHD (reviewed in [108]). However, the outcome of MSC therapy for GvHD is dependent on the severity of the disease, the source of MSC obtained from single or multiple donors and the culture media used to expand cells, as well as the age of the patient since children in general respond better than adults [108].

Autoimmune diseases: once again, due to their properties to modulate the immune system, MSC have been used as therapeutic agents for autoimmune diseases.

In 2011, Chan Ra and colleagues [109] reported the treatment of different autoimmune diseases with adipose-derived hMSCs: Autoimmune inner ear disease (AIED), Multiple Sclerosis (MS), Polymyositis, Atopic Dermatitis and Rheumatoid Arthritis. hMSC were isolated from small amounts of adipose tissue and expanded to reach a dose of sub administration of 1×10^9 cells. Positive outcomes were found without differences on donor age-dependent efficacy. Allogeneic MSC implied in a clinical trial against lupus erythematosus, succeeded to remit 4 patients 12 and 18 months after treatment [110]. Finally, various pre-clinical and initial clinical studies have indicated MSCs as successful therapeutic agents for diabetes-associated microvascular and secondary diabetic complications [111].

Neurodegenerative diseases: it has been reported that MSC have the capacity to differentiate into mature neural cells, specially neurons, and glia [36]. Considering that

brain as a little regenerative capacity, the challenge is nowadays to use MSC as therapeutic agents for neurological disorders. Their action in this field is due principally to the release of trophic factors such as anti-inflammatory cytokines and chemokines more than their differentiation potential. MSC are believed as efficient therapeutic agents for Parkinson Disease (PD), since in 2010, a clinical trial in advanced PD patients who received a transplantation of BM-MSC results in modest progress such as improvement in facial expression, gait, and freezing episodes and no side effects [112].

Spinal cord injury (SCI) is a traumatic injury to spinal cord that often results in severe disability. Autologous MSC transplantation has been proven to be safe and successful in a number of studies [113, 114], for instance in 2012 among 11 patients transplanted at thoracic level with autologous BM-MSC, 5 (45.5%) improved neurological function [115]. Clinical trials involving MSC are currently undertaken for Alzheimer's disease (AD) Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease (HD). Finally MSC are been investigated as delivery vehicles as they can be genetically modified to carry neural or glial-derived neurotrophic factors in the brain [116].

Bone and cartilage regeneration: Due to their natural tendency to differentiate in osteoblast and chondrocytes, MSC are obviously considered very powerful tools to correct bone and cartilage defects, thus this field has been historically the first one in which MSC have been implied as therapeutic agents. In 1999, Horwitz and co-workers, reported the positive effect of BM-MSC transplantation in children with osteogenesis imperfecta [117] and since then, MSC have been applied for a wide range of bone and cartilage diseases [118]. In orthopedics applications, MSC are often combined with scaffolds to supply mechanical support to the damaged bone. The choice of the right scaffold is crucial, since its features (e.g. stiffness and porosity) can influence cells differentiation potential [119]. MSC delivered in porous hydroxyapatite scaffolds improve the conditions of three patients with large bone defects [120] as well as four patients with large bone diaphysis defects [121]. In some case the entire bone marrow is applied: Hernigou *et al.* reported that percutaneous autologous bone marrow grafting is effective for the treatment of atrophic tibial diaphyseal nonunion [122].

Importantly, MSC release of paracrine factors induces bone and cartilage regeneration by the host tissue itself. For example, secretion of BMP-2 by MSC migrated to the site of injury induces cartilage regeneration and plays a key role in osteoblast differentiation [123]. Moreover, MSC release angiogenic factors, such as VEGF, PDGF-BB and Ang-2

that enhance the formation of vessel and thus, oxygen arrival to the damaged tissue which in turns benefits osteogenesis [124].

Osteoarthritis (OA), a degenerative disease of the connective tissue, has also been successful treated with BM-MSC, because of their chondrogenic differentiation potential, release of trophic factors that protect the cartilage from tissue destruction, induction of progenitor cells differentiation and enhancement of expansion of chondrocytes *in situ* [125]. Furthermore, MSC are currently highly investigated for the treatment of intravertebral disc regeneration *in vitro* and in large animal models [126]. Moreover, various clinical trials are ongoing [127].

Crohn's disease (CD): CD is a type of inflammatory bowel disease, a chronic inflammation of the intestinal mucosa, involving any part of the gastrointestinal tract from oral cavity to perianal region. Many clinical trials indicate MSC as therapeutic target for this disease either by systemic [128] and local injection [129]. In general many promising results without side effects have been reached for the treatment of CD in phase I/II clinical trials [118].

Cardiovascular repair: MSC therapy for cardiovascular disease is a very exciting edge of research since many cardiovascular diseases such as ischemic cardiomyopathy and congestive heart failure are still mortal and donor heart transplantation is not easily accessible. MSC transplantation is a promising way to furnish a renewable source of cardiomyocytes by MSC trans-differentiation [130] and may also support the formation of new blood vessel that support growth of new cardiomyocytes by MSC paracrine action. Various clinical studies reported success in the treatment with BM-MSC of patient with myocardial infarction [131], chronic coronary artery disease [132] or advanced heart disease [133]. The delivery of MSC in the damaged myocardium can be performed by peripheral vein infusion, regional coronary vascular infusion or local direct myocardial injection and the choice of one of those modalities seems to be crucial for the success of the therapy [134].

Liver disease: MSC are currently investigated for their role in different contexts in liver disease. They are believed to reduce oxidative stress, to release important paracrine signal to hepatocytes, to suppress the immune response and to reduce the level of fibrosis. MSC are currently under investigation in pre-clinical trials for the treatment of hepatitis B and C, cirrhosis and ischemic liver injury. However robust phase 2/3 clinical trials have not yet performed [135].

Skin transplantation and plastic surgery: Human skin is furnished of stem cells that can rebuild epidermis. However, in the case of deep injury, when the follicle budge where skin stem cells reside is disrupted, skin grafting is needed [136]. MSC have been first implied in the treatment of burns due to ionizing radiation, since no treatment was available. In this settings MSC were able to reduce local inflammation, mainly because of their paracrine activity and growth factor release [118]. Nowadays, MSC are frequently used in chronic wound, where a prolonged inflammatory phase impairs the healing process, blocking completely the cicatrization or causing extensive scarring. In this case MSC can be just administrated on the top of the wound, injected into the surrounding tissue or included in the skin substitute. However, different results have been achieved according to the age of the patient [137]. MSC have found widely applications in plastic surgery, where are frequently transplanted together with fat and muscle cells [138]. Furthermore, some studies investigated the combination of MSC with acellular dermal matrix, since it seem that MSC decrease the atrophy of the tissue and assure the graft long term follow up [139].

Cancer therapies: Factors released from MSC can have anti-tumor properties, since *in vitro*, they reduced glioma and hepatoma [140, 141]. Moreover, MSC can be engineering to be used as therapy vehicle for cancer treatment. For instance they can be genetically modified to express therapeutic factors to treat cancer such as onco-suppressors and anti-oncogenes, antiangiogenic proteins, pro-apoptotic agents, various cytokynes. Alternatively MSC can be used as vehicle for anti-cancer drugs to selectively kill tumor cells in situ. [32].

In conclusion since their discovery in 1999, MSC have raised tremendous interest as therapeutic agents. These cells are easy to isolate, are able to differentiate to multiple cell types and have pronounced paracrine activity and immunomodulatory properties. Application in clinical trials for a wide range of human diseases already gave very promising results, marking the beginning of a new era, where organ transplantation in not anymore necessary since body own regeneration can be stimulated by MSC activity upon cells implantation.

However, the complex nature of these cells still demand investigation and standardization of procedures applied for their use, since there is still high variability in the ways they can be isolated, expanded, characterized and finally implanted in the human body.

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CHAPTER III

SURFACE MODIFICATION FOR CELL CULTURE AND CHARACTERIZATION TECHNIQUES

Cell *ex vivo* expansion for hMSC application in the clinic is a mandatory and critical step. During hMSC culture, surface chemistry strongly influences cell behavior, mainly by differential protein adsorption and subsequently ligand exposition to cell membranes (chapter IV). Furthermore, the accomplishment of favorable outcomes of MSC application in regenerative medicine depends on the specific control of biomaterial surface properties. In fact, the final outcome of a biomaterial is determined by the events occurring at the bio-interface, intended as the interaction between the surface and the biological system. For these reasons, several efforts have been done to develop strategies to control or modify the surface properties of tissue culture systems and/or biomaterials.

III.1 Self-Assembled Monolayers (SAMs)

One of the most widespread and simple technology for surface modification, includes the use of self-assembled monolayers (SAMs). SAMs are surfaces functionalized by long-chain organic molecules containing, in one of its terminal side, free anchor groups such as thiols, disulphides, amines, silanes or acids [1], that, after binding to an appropriated material surface, will exposure to the biological interface the functional group that is in the other terminal side (Fig.III.1).

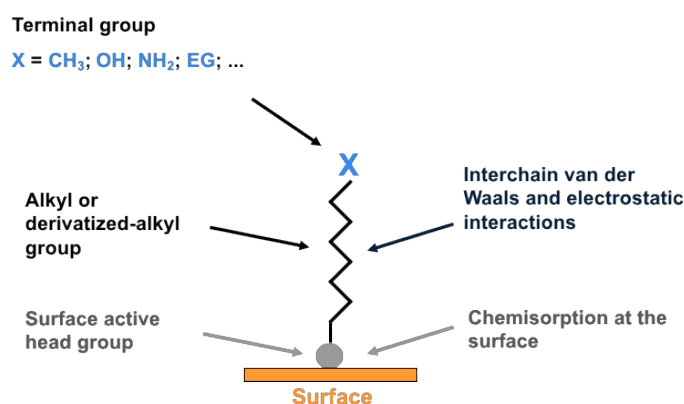


Figure III.1. SAMs general structure

The principle at the basis of SAMs preparation is “self-assembly”, a natural process in which components of a disordered pre-existing system spontaneously organize into a specific arrangement, by non-covalent interactions among the molecules of the system itself. It is indeed the mechanism that in nature drives the organization of peptide chains into proteins, single strand DNA in double helix or the assemble of phospholipids into the cell membrane [2]. Similarly, SAMs are given by the self-organization of long chain molecules on the surface of appropriate substrates, such as metal surfaces (Gold, Silver, Copper, Silicon, Titanium).

SAMs technology acquired success for its numerous advantages:

- i) the process that lead to SAMs formation is relatively simple, does not require expensive equipment neither considerable experience.
- ii) SAMs are highly stable systems.
- iii) their terminal head group can be changed, altering dramatically the macroscopic properties of the surface (wettability, charge distribution, interaction with specific proteins or cells..).

Thus, SAMs are highly ordered model surface systems that can be used to present specific functionalities and to study biological interactions such as cell signaling, cell adhesion, and protein interactions.

For these reasons, SAMs found application in a wide range of fields such as molecular electronics, biosensors, biological assays, engineering biomaterials and in scientific research.

III.1.1. SAMs of alkanethiols on gold

SAMs of alkanethiols on gold are the most used type of SAMs in biological studies. They are particularly advantageous since gold is an inert material, is resistant to oxidation [3] and is an ideal substrate for the most useful techniques applied to analyze surface properties and protein adsorption. Importantly gold is compatible with cells.

They found origin in 1983 when Nuzzo and Allara, reported that alkyl disulfides and alkanethiols spontaneously form organized monolayers on gold [4]. In fact, Au (111) strongly interacts with the sulphur atoms of thiol groups on alkane chains forming a thiolate group (Au-S). Moreover, Van der Waals forces between the alkane chains, form highly compact monolayers [5] (Fig. III.2).

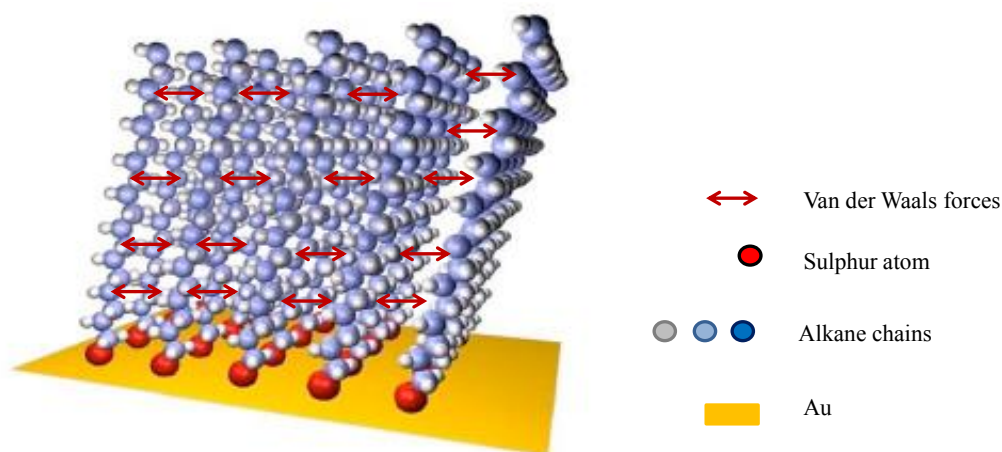


Figure III.2. Schematic view of SAMs prepared by alkanethiols on gold.

III.1.2 Structure and formation mechanism

A long chain alkanethiol molecule is illustrated in Fig.III.3.

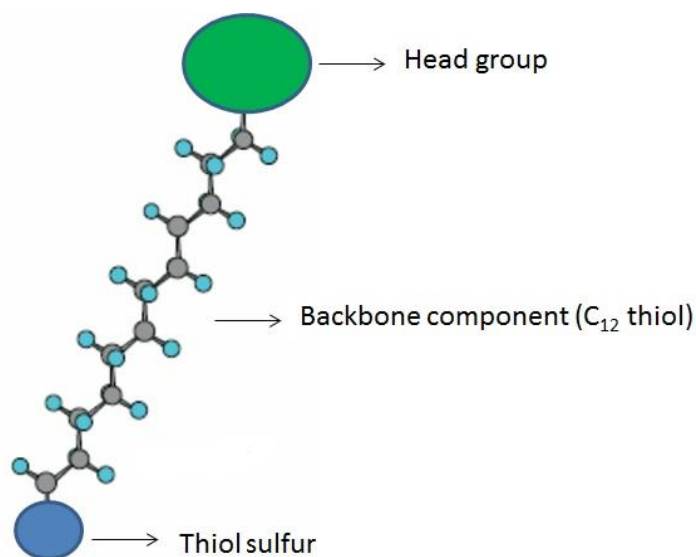


Figure III.3. Alcanethiol molecule structure

It is defined by three chemical entities:

1. Thiol sulfur: it generates the initial driving force for the assembly due to its interaction with the gold at the surface.
2. Backbone component: is a chain of methylene carbons. Van der Waals forces between these methylene carbons provide the force that drives the mechanism of self-assembly.

3. Head group: its nature determines the surface properties of the assembled monolayer since it is the group exposed at the exterior surface.

The interaction between these three elements inside the alkanethiols, dictates the stability and order of the resulting self-assembled monolayer.

SAMs preparation requires the immersion of an extremely clean gold substrate in alkanethiol solutions diluted in ethanol (typically 1 to 0.1 mM). Within a few seconds, a disordered monolayer is formed. During the following 20 hours van der Waals forces between the hydrocarbon chains induce the packaging of alkanethiols into a well-ordered, crystalline layer. Moreover, during this phase, adventitious contaminants and solvents are removed from the monolayer while packing rises by the increase of the alkanethiols on the monolayer [6, 7]. The final assembled monolayers present an hexagonal close crystal lattice structure [7], as described at Fig. III.4. When packaging reaches 100%, the intermolecular spacing is $4.97 \text{ \AA}$, corresponding to the distance between neighboring sulfur atoms, three times that of sulfur atom ($1.85 \text{ \AA}$), which exclude the possibility of S-S interactions [4]. Furthermore, alkyl chains are tilted with an average angle of 27° - 35° with respect to the surface (Fig. III.4). This inclination enhances the intermolecular interaction and balances the headgroup-substrate interaction, minimizing the surface energy [8].

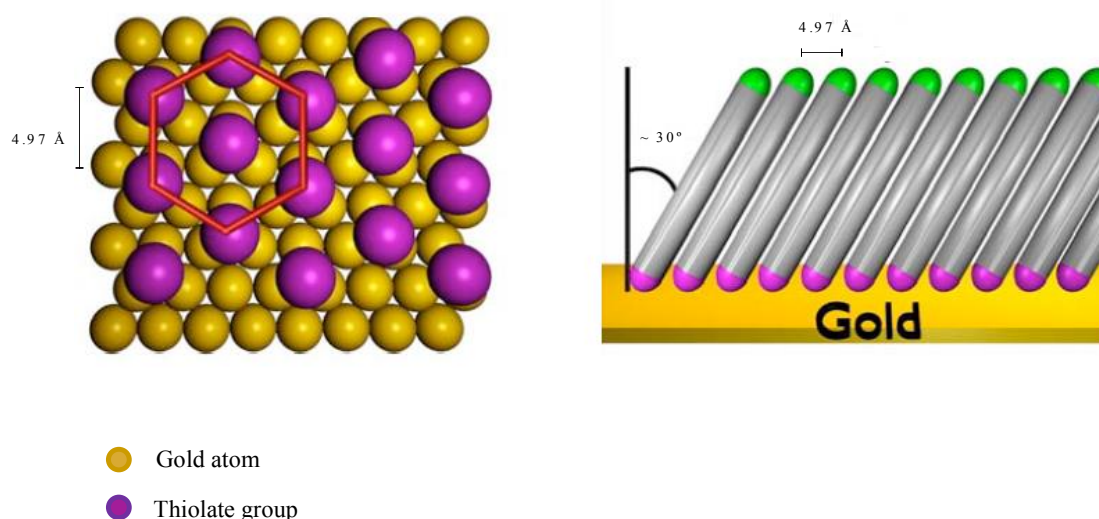


Figure III.4 Structure of SAMs. A: Hexagonal crystal lattice structure. B: tilt of alkanethiol chains (adapted from <http://www.aseblon.com/>).

The final order and quality of the assembled monolayer depends on different factors:

- size of head group: steric hindrance interferes with molecular packing. Thus, alkanethiols that present voluminous head group cannot form well-ordered monolayers. When bulky head group are required, it is convenient to use mixed self-assembled monolayers where alkanethiols with smaller head group behave as separators between alkanethiols with bulky head groups [7];
- length of the alkanethiols chain: it determines the level of order and stability of the monolayer since it was demonstrated that the longer the chain is, the more Van der Waals forces are present and thus, the more ordered is the monolayer [9];
- cleanliness and purity of the gold surface: “fresh” clean gold must be prepared at the moment of SAMs preparation. In fact, as soon as the gold surface is exposed to laboratory environment it may be coated with a layer of contaminants such as hydrocarbons. Alkanethiol solutions must be pure and prepared in an inert environment, such in presence of nitrogen or argon, to avoid thiol oxidation;
- immersion time: diluted solutions of alkanethiols need longer time to pack in ordered monolayers than more concentrated solutions, being the latest more risky for perfect monolayer formation [10];
- type of solvent: ethanol is the most common solvent used to prepare SAMs. However, it has been reported that non-polar solvents such as heptane led to faster SAM formation than ethanol, but with a lower degree of order [11]. In general, the kind of solvent is very important for the monolayer formation since interactions between the solvent and the substrate can compete with the adsorption of thiols. However, the way the solvent acts in SAM formation is not fully understand.

III.2. Techniques for surface characterization

Every study that involves surface modification requires the use of different surface characterization techniques. For the development of the work of this thesis contact angle goniometry, ellipsometry, Infrared Reflection-Absorption Spectroscopy (IRRAS) and X-ray photoelectron spectroscopy (XPS) were used. In this section the basic principle of these methods are described, with the scope of explain the basis for results interpretation more than to describe the techniques in details.

III.2.1 Contact angle Goniometry

Contact angle goniometry is a technique used to obtain information regarding the wettability of a surface.

When a liquid drop is deposited on a solid surface, the contact angle is defined as the angle formed by the interception of the solid-liquid interface and the liquid vapor interface (Fig.III.5). The simplest method to calculate contact angle is the static sessile drop technique: when a liquid can spread on a surface, a small contact angle (less than 90°) is observed, indicating that the wetting of the surface favors the spreading over a large area. Thus if the liquid is water, it indicates a certain hydrophilicity of the surface. On the other hand, a greater contact angle (more than 90°) means that the properties of the surface interfere with the spreading of the fluid on it, thus the fluid forms a compact liquid droplet, indicating when the liquid is water, hydrophobicity of the surface.

Surface tension of the liquid determines the shape of a liquid droplet, since the interaction between a liquid and a solid surface result in a balance between the force of attraction of the liquid molecules to each other (cohesive force) and the attraction of the liquid molecules to the molecules of the surface (adhesive force). Young equation describes this equilibrium as follows [12]:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta$$

where

γ_{sv} = solid surface energy

γ_{lv} = liquid vapor interface tension

γ_{sl} = solid/liquid interface tension

θ = contact angle.

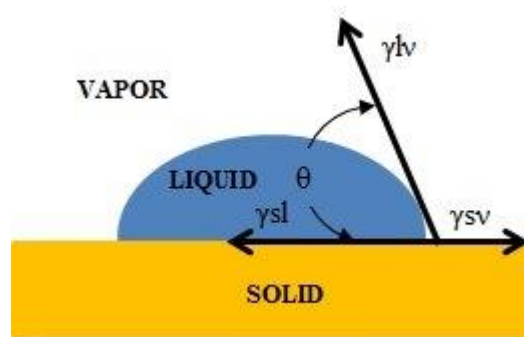


Figure III.5. Schematic view of a liquid drop onto a solid surface showing the forces that are in the base of the drop geometry and are part of the Young equation.

Contact angle goniometry is a powerful technique to detect surface modifications (for instance SAMs preparation and adsorption of proteins and peptides) and to estimate biological interactions with surfaces [13].

III.2.2 Ellipsometry

Ellipsometry is a non-invasive and non-destructive light optical technique which measures the change in polarization of a beam of light upon reflection from a surface. It is based on the fact that when a polarized light beam reflect on a surface it will change to elliptical, thereby the name of the technique. In this technique, light will be decomposed into p and s components, being p -component oscillating parallel and s perpendicular to the plan of incidence (Fig.III.6).

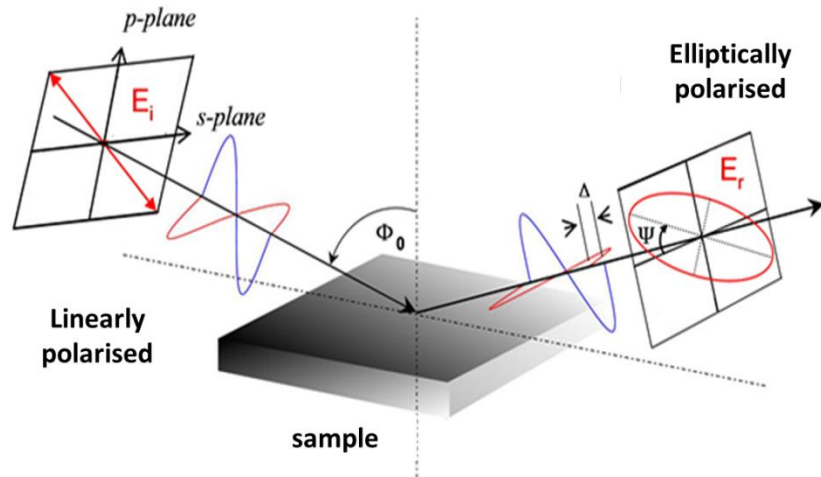


Figure III.6. Schematic illustration of the principle of ellipsometry. Delta is the difference between the phase changes of the *p* and *s* components of the reflected light and Psi is an angle which tangent gives the ratio of amplitude changes for the *p* and *s* components of the reflective light [14].

Thus, ellipsometry measures the ratio (ρ) between the intensity of *s* and *p* components (R_p/R_s) by the following fundamental equation:

$$\rho = R_p/R_s = \tan \psi e^{i\Delta}$$

where:

Δ = “relative phase change” or the phase difference between *p* and *s* components;

ψ = “relative amplitude change”: change in amplitude ratio due to reflection.

Since ψ and Δ depends on the optical characteristic of the surface, departing from this data the software is able to extrapolate the extinction coefficient (*k*) and the refractive index (*n*) of the sample to be analyzed.

After surface modification, namely by SAMs formation or protein adsorption, alterations in thickness will be detected by changes in ψ and Δ values. Therefore, this technique is particularly valuable to determine surface modifications by deposition of additional layers on reflecting surfaces, as gold substrates.

III.2.3 Fourier-Transform Infra-Red Reflectance-Adsorption Spectroscopy (IRRAS)

IRRAS is a vibrational spectroscopy technique that gives information about the molecular structure of a sample by the identification of functional groups and chemical bonds of molecules. IRRAS is used for characterization of thin films deposited on reflective surfaces.

Briefly, an infrared beam of light is emitted and polarized by a polarizer. Then, the polarized light reaches the sample at grazing angle, increasing the sensitivity of the upper surface layers of the sample. The resulting wave is maximal for p-polarization thus enhances the signal of the molecules perpendicular to the surface and provokes molecular vibrations at specific frequencies [15]. If the frequency of the radiation matches the vibrational frequency of the molecules in the sample then radiation will be absorbed, causing a change in the amplitude of molecular vibration. A detector receives the reflected beam and reflected frequencies are correlated to the incident frequencies resulting in those absorbed by the surface of the sample (Fig.III.7)

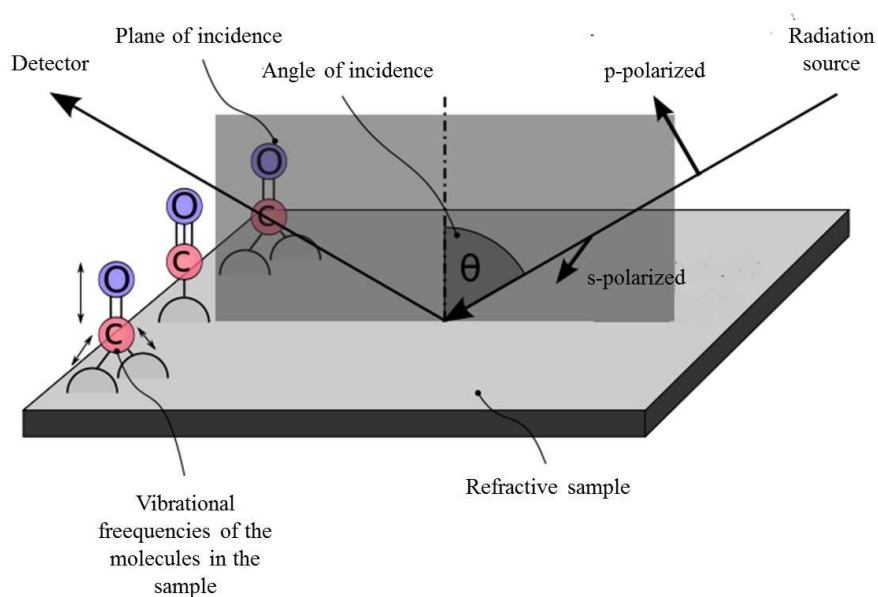


Figure III.7. IRRAS principles for light polarization

Results are presented as a spectrum that depicts absorption bands vs wavelength. Since the absorbed frequencies coincide with the molecular vibrations of specific chemical bonds and functional groups, IRRAS can detect introduction/removal or increase/decrease of specific groups on a surface [15, 16]. Thus, it can be used for the analysis of surface

modification, such as proteins and peptides adsorption/immobilization on refractive surfaces.

III.2.4 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy is a semi-quantitative spectroscopy technique that allows identifying the atomic composition of a sample (Fig.III.8). Experimentally, the sample is irradiated with an X-ray beam (200-2000 eV) in an ultrahigh vacuum chamber. The x-rays cause photoelectric effect, thus emission of photoelectrons from the sample. The spectrometer measures the kinetic energy of the emitted photoelectrons (E_k). Knowing the energy of the incident x-ray beam ($h\nu$), information is displayed by plotting the binding energies of the photoelectrons (E_B) on a spectrum. Data are given from Einstein equation:

$$E_k = h\nu - E_B$$

Since the binding energies of electron orbitals are known and specific of each elements, XPS allows the identification of the atomic composition of the sample surface [17]. For instance, increase of the amount of N may be the evidence of the immobilization of a peptide on a determine surface.

Furthermore, because chemical bounds affect the binding energy of the electron orbital, “shifting” the kinetic energy of a photoelectron, XPS can also give information about the chemical nature (e.g. atomic oxidation state, bound to more electronegative atoms) of the sample surface.

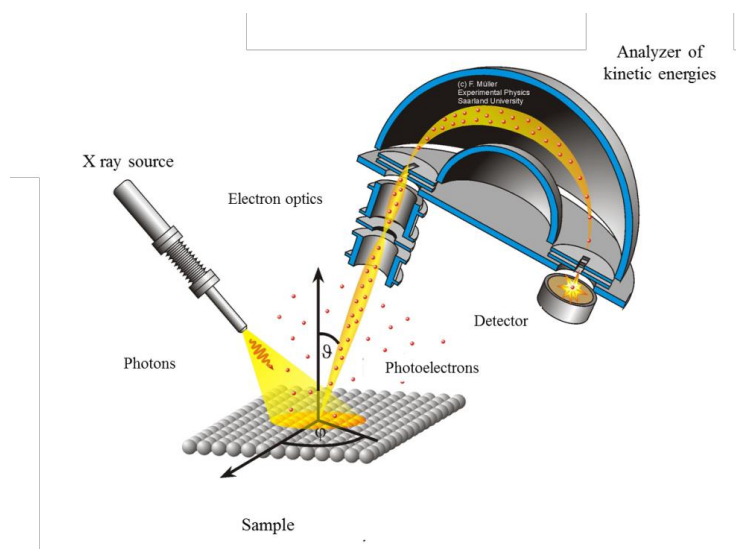


Figure III.8 Schematic representation of ol XPS setup with photon source (X-ray), a sample, electron optics, an energy dispersive analyser and a detector Adapted from <http://jacobs.physik.uni-saarland.de/english/instrumentation/uhvl.htm>

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SECTION 3

RESEARCH WORK

Chapter IV

This chapter is based on the following publication:

Xeno-free strategies for safe human mesenchymal stem/stromal cells expansion: supplements and coatings

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ABSTRACT

Human mesenchymal stem/stromal cells (hMSC) have generated great interest in regenerative medicine mainly due to their multidifferentiation potential and immunomodulatory role. Although hMSC can be obtained from different tissues, the number of available cells is always low for clinical applications, thus requiring *in vitro* expansion. Most of the current protocols for hMSC expansion make use of fetal bovine serum (FBS) as nutrient-rich supplement. However, regulatory guidelines encourage novel xeno-free alternatives to define safer and standardized protocols for hMSC expansion that preserve their intrinsic therapeutic potential. Since hMSCs are adherent cells, the attachment surface and cell adhesive components also play a crucial role on their successful expansion. This review focuses on the advantages/disadvantages of FBS free media and surfaces/coatings that avoid the use of animal serum, overcoming ethical issues and improving the expansion of hMSC for clinical applications in a safe and reproducible way.

IV.1 Introduction

Regenerative medicine aims to repair or replace tissue or organ functions, compromised due to aging, physical damage, congenital defects or diseases. Cell-based therapies are based on the transplantation of freshly isolated or cultured cells into the site of injury. Those cells are frequently stem cells, which have the ability to self-renew and differentiate along multiple lineage pathways, and thus contribute to tissue repair/regeneration [1].

Among stem cells, human mesenchymal stem/stromal cells (hMSC) have generated great interest because they are relatively easy to isolate, can be extensively expanded, and present multiple differentiation potential, namely into bone cells (osteocytes), cartilage cells (chondrocytes) and fat cells (adipocytes). Therefore, they are good candidates for cell-based therapeutic approaches towards several kinds of pathologies, such as myocardial infarction [2], graft-versus-host disease [3] Crohn's disease, neurodegenerative and muscle degenerative diseases [4], cartilage and meniscus repair [5], or stroke and spinal cord injury [6]. According to a study from Hart and colleagues [7], in February 2014, 457 clinical trials involving hMSC were registered worldwide being China the leader in this ranking. At the time of writing the number of clinical trials raise until 706 (www.clinicaltrials.gov).

Human MSC can be derived from different tissues such as bone marrow (BM-hMSC), adult adipose tissue (AT-hMSC), mobilized peripheral blood, as well as from placenta and umbilical cord blood (UC-hMSC), being BM the most common source in clinical use. However, hMSC prevalence in all these tissues is low, and the total amounts of isolated cells are insufficient for clinical applications. For example, BM contains approximately 1 in 3.4×10^4 bone cells [8], with total numbers generally decreasing with donor age [9]. The number of required BM-MSCs depends on the type of disease to treat, ranging, for example, from 2×10^6 cells/Kg in graft-versus-host-disease to 8×10^6 cells/Kg in cardiomyopathy, to 10×10^6 cells/Kg in respiratory distress syndrome (www.clinicaltrials.com). Thus, in order to have sufficient cell numbers for successful transplantation, isolated hMSC must be first expanded *ex vivo*, using effective and safe methods that maintain their key properties in a shorter period of time in order to avoid cell aging and possible contaminations [10].

Several controversies are related with the lack of common standard protocols for hMSC *in vitro* expansion. This is critical, since culture conditions may have an impact in the

transcriptome, proteome and cellular organization of hMSCs, which will affect their engraftment and performance upon transplantation [11]. Discrepancy among laboratories includes the choice of basal media and the addition of supplementary factors. Moreover, being hMSC anchorage-dependent cells, culture surfaces are often coated with extracellular matrix (ECM) proteins or other commercially available cell adhesion factors, generating an additional element of discontinuity among expansion protocols. Finally, to reduce variability between pre-clinical trials, cell culture experiments must comply with Good Manufacturing Practices (GMP) guidelines and every step of cell manipulation must be defined in Standard Operating Procedures (SOP).

In this context, considerable efforts have been made to improve the *ex vivo* expansion of hMSC for clinical applications, at different levels. This review highlights the disadvantages associated with the use of fetal bovine serum (FBS) as a nutrient-rich medium supplement, and focuses on the advantages/disadvantages of different xeno-free and/or serum-free supplements and surfaces/coatings for hMSC expansion.

IV.2. Foetal bovine serum as supplement for hMSC *in vitro* expansion

MSC growth *in vitro* must be supported by the addition of a basal media such as Dulbecco's modified Eagle's medium (DMEM), α -modified minimum essential medium (α -MEM) [12] or media combinations such as 50:50 (v/v) of DMEM: HAM's nutrient mixture F12,[13], among others, that generally includes: *i*) biosynthetic precursors for cell anabolism, *ii*) catabolic substrates for energy metabolism, *iii*) vitamins and trace elements, and *iv*) inorganic ions to maintain the pH and osmolarity of the culture [14]. This basal medium is further supplemented with FBS, a highly-rich supplement that contains a cocktail of cell attachment proteins, growth factors and other important biomolecules.

According to the description by Lalu *et al.* in 2012 [15], from 36 clinical studies involving hMSC, 27 used FBS as media supplement, 5 used human serum, while 4 did not specify the kind of supplement used. Table IV.1 describes some successful clinical trials, almost without side effects, where hMSC were expanded in FBS-supplemented medium.

Table IV.1. Successful clinical trials involving hMSC expanded *in vitro* with FBS-containing medium

Type of disease	Treatment	Reference
Luminal Chron's disease	Allogenic hMSCs	Forbes 2014 [101] (*)
Ischemic stroke	Autologous MSCs	Lee 2010 [102] (**)
Stroke injury	Autologous MSCs	Bang 2005 [103]
Amyotrophic Lateral Sclerosis	Intraspinal cord implantation	Mazzini 2008 [104]
Grade IV acute graft-versus-host disease	Haploidentical MSC	Le Blanc 2004 [105]
Brest cancer	Autologous MSCs	Koç 2000 [106]
Acute myocardial infarction	Autologous MSCs	Chen 2004[107]

* being Australia a country with no incidence of BSE and vCJD the use of certified-FBS for hMSC *ex vivo* expansion is still accepted.

** No adverse events in terms of zoonoses transmission was observed, however since the vCJD latency period may last many years, long-term follow up is needed.

Even though, there are many concerns regarding the use of FBS, in terms of scientific, ethical and economical disadvantages. These are summarized, together with FBS advantages, in Table IV.2.

Table IV.2. FBS use advantages and drawbacks

Use of FBS as supplement for cell culture	
Advantages	Disadvantages
Furnishes a cocktail of growth factors required for <i>in vitro</i> cell growth	Ill-defined
Universal: suitable for all cell types	Lot to lot variability
Do not require laborious cell adaptation protocols	Possible contamination of the cell surface with xenogenic compounds that may influence cell behavior Possible microbiological contamination (virus, prions bacteria, endotoxins, fungi)
	Economical: worldwide availability
	Ethical problems: requires the painful death of bovine fetuses

FBS is an *ill-defined* supplement, with high inconsistency in terms of the quality and quantity of bioactive compounds [16]. Because of the great variability among different FBS batches, pre-selection of specific lots is often required, which is costly, time consuming and also hampers comparisons between different research groups [17]. For example, Knepper *et al.* showed that FBS from three different commercial sources vary on the relative amounts and apparent molecular weights of some transcription factors [18], while Zheng *et al.* showed that different lots of FBS had varying concentrations of proteins such as growth stimulatory and inhibitory factors, with obvious implications in cell growth rates [19].

When FBS is employed in hMSC expansion for cell therapies, there is also a strong concern regarding contamination with xenogenic compounds and microbiological contaminants, such as viruses, prions, bacteria, fungi and endotoxins. Directive 2004/23/EC [20] and its recent implementation [21] specify safety measures to be taken into account during donation, procurement, testing, processing, preservation, storage,

distribution and use of human cells and tissues intended for human applications. A notable concern is the potential cross-specific or zoonic transmission of unknown pathogens related to the exposition of cells in culture to animal-derived compounds. Zoonoses bring the risk of virulent diseases such as anthrax, Q-fever and Creutzfeldt-Jakob disease (CJD), where a special concern regards the risk of Bovine Spongiform Encephalopathies (BSE) transmission and its relation to the new variant CJD (vCJD) [22]. Recently, genetic material from Bovine Viral Diarrhea (BVD) virus [23] and new viruses has been detected in bovine serum by massively parallel sequencing [24]. Moreover, bovine species of micoplasma contaminants, often present in FBS, have been frequently isolated from cell cultures [25, 26], but these can be difficult to detect. In fact, batches of contaminated serum may effectively pass the test for mycoplasma as negative samples, as recently reported [27].

Regarding ethical concerns, FBS is harvested from bovine foetuses taken from pregnant cows during slaughter, by cardiac puncture and without any form of anaesthesia [28]. During this procedure, foetuses undergo anoxia, a strong oxygen deficiency, and are exposed to pain and/or suffering in a procedure which may be considered ethically inhumane. Finally, the use of FBS in cell culture raises logistic and economic problems: around 1 to 3 foetuses are needed to produce just a litre of serum, implying high costs related to animal feeding, installation and maintenance of the necessary infrastructures. Recently, an imminent increase of the FBS cost has been notified and justified according to the reduction in supply and the increased demand from life science and pharmaceutical customers. Considering the extensive spread of cell therapy industry, the demand of serum is likely to significantly exceed the amount of maximum worldwide serum availability [29].

IV.3. FBS-free media formulations for hMSC *in vitro* expansion

Suitable alternatives to FBS for hMSC expansion should ideally guarantee a well-defined composition, a reduced degree of contaminants, low production costs, extended shelf life, and easy availability. Different alternatives have been described in the literature, which can be broadly divided into: 1) chemically defined media and 2) media supplemented with human blood derivatives (Table IV.3).

Table IV.3: Xeno-free supplements for cell culture.

Human blood derivatives				Not human origin
<i>Human serum (HS)</i>	<i>Human platelet lysate (HPL)</i>	<i>Human Umbilical Cord Serum (HUCS)</i>	<i>Human plasma fractions (Supplement for Cell Culture-SCC)</i>	<i>Chemically defined media (CD)</i>
<i>Advantages</i>				
No ethical problems	No ethical problems	No ethical problems	No ethical problems	No ethical problems
No risk of xenogenic compounds transmission	No risk of xenogenic compounds transmission	No risk of xenogenic compounds transmission	No risk of xenogenic compounds transmission	No risk of xenogenic compounds transmission
Easy and inexpensive procedure	Contains high level of GF	Easy and inexpensive procedure	GMP-compliant, compatible with pharmaceutical grade advanced therapies	No risk of transmission of human diseases
<i>Disadvantages</i>				
Variability between individual donations	Variability between individual donations	Variability between individual donations	Ongoing: further investigation will help to a better characterization	Cell specific
Possibility of transmission of human diseases	Possibility of transmission of human diseases	Possibility of transmission of human diseases		Ill-defined because lacks of information provided by the companies
Low availability in terms of amount of donation	Low availability in terms of amount of donation	Low availability in terms of amount of donation		Development may be expensive and time consuming
Discrepancy of results in allogenic settings	Not well defined composition in terms of GF content			Cell-adaptation may be necessary
Quality in autologous settings may not be ideal	Quite laborious and expensive procedure			
Often requires the addition of cytokines and GF				

IV.3.1. Chemically defined media

Chemically defined (CD) media include only components of known composition. Common strategies for the preparation of CD media have been amply described and validated [13]. In general, formulations for MSC culture consist of a basal media, as already discussed, to which different kinds of supplements are added. Until 15-30 years ago, supplements used in CD media, as highly purified hormones or growth factors, were often obtained from human or animal serum but, nowadays, with the advance of recombinant technologies, it is possible to produce a wide range of human proteins that allow the development of completely xeno-free CD media. Since hMSCs can be isolated from different sources and vary among different donors, the optimal CD media may have different specific requirements concerning medium supplements, which turns the development of a “universal” CD media into a challenging task.

Different CD media (serum free and/or xeno free) for hMSC are nowadays commercially available. For example TheraPEAK™ MSCGM-CD™ (Lonza) and PowerStem (PAN Biotech GmbH) are able to support hMSC expansion and differentiation, however CD105 expression may be significantly reduced after culture in those CD media in comparison with serum-supplemented medium [30]. Many of the currently available CD media require pre-coating of culture substrates with ECM proteins to support cell attachment (section IV.4). For example, Stemgro hMSC Medium (Corning), when used in conjunction with the Corning CellBIND Surface (section IV.4.6), enables hMSC attachment and growth comparable to that of serum-containing cultures, including maintenance of MSC multipotency. Another study showed that proliferation of human BM-MSCs was successful on four different commercially available CD xeno-free media (MesenCult-XF, STEMPRO MSC SF, TheraPEAK™ MSCGMCD and Corning Stemgro hMSC Medium) and in particular upon seeding on a proprietary surface (Corning Synthemax Surface). The Corning medium, associated with the synthetic surface, was able to promote significantly higher long term cell expansion than traditional serum containing medium on tissue culture polystyrene (TCPS).

There are however some disadvantages associated with the use CD media that might limit their wider application in clinical scale hMSC expansion. For example, even if databases of commercially available CD media are accessible online [14], their exact compositions are not specified by the suppliers. Cell-doubling times can vary among different CD media [31], and in some cases cell morphology and size may differ and alterations in cells vacuoles may arise. Also, the quality/activity of biological compounds in the CD media

formulations can generate variability among different batches compromising experiments reproducibility. The use of ECM-like coatings, often mandatory, may also raise some concerns, because of batch-to-batch variability, poor information about their protein content, potential immunogenicity of their components and time consuming coating procedures. Often, guidelines for cell adaptation protocols are not provided, which can be a laborious, time-consuming process. Finally, as already stated, this type of media may result in non-optimal cell growth, as compared to medium supplemented with serum or other animal-derived supplements, as reported in the literature in a study where the performance of different commercially available CD media was compared [32].

IV.3.2. Human blood derivatives

Several “humanized” supplements derived from human blood have been proposed, namely: 1) autologous or allogeneic human serum (*hS*), 2) human platelet lysate (*hPL*), 3) umbilical cord blood serum (*hUCBS*) and, more recently, 4) industrial GMP-human plasma derivatives (supplement for cell culture - *SCC*). The use of each of these supplements will be described in more detail in the following sections.

IV.3.2.1. Human serum (*hS*)

Human serum (*hS*) can be collected from autologous or allogeneic whole blood donations. Autologous *hS* has been widely described for its positive effects on cell expansion. For example, medium supplemented with 10% v/v autologous *hS* was shown to be as efficient as the same amount of FBS for hMSC isolation and expansion, and even better as an inducer of osteogenic differentiation [33]. Yamamoto *et al.* [34] demonstrated that a patient's autologous *hS* could be used to expand bone marrow hMSC without compromising their potential for osteogenic differentiation. However, it may be problematic to collect a sufficient amount of autologous serum to propagate hMSC for clinical application. Moreover, depending on the patient, the quality of autologous serum may be not optimal, especially in those undergoing other types of therapies [11]. Also, a negative correlation between donor age and the outgrowth of cells from human trabecular bone was demonstrated in cultures supplemented with autologous serum [35] suggesting that the use of autologous serum is not suitable for elderly patients [36]. To try to overcome these drawbacks, Spees and co-workers demonstrated that, when cells are expanded in medium containing FBS and afterwards transferred to AHS⁺ (autologous human serum supplemented with growth factors), the amount of FBS contaminants can be

reduced in 99.99%, which could be used as a strategy to circumvent the low availability of autologous serum [37]. Nevertheless, Martin *et al.* demonstrated that hMSC cultured FBS were contaminated with xeno-derived carbohydrate N-glycolylneuraminic acid (NeuGc) [38, 39]. The subsequent replacement of FBS by hS was insufficient for achieving complete decontamination, since cells express bovine NeuGc on their surface, even after long periods of culture without FBS [40].

On the other hand, hS from allogenic donations can be associated with donor variability. Nevertheless, Witzeneder *et al.* suggested that pools of 6 donors are sufficient to reduce the variability among human blood samples [41]. However, results from stem cells expansion with allogenic hS still remains controversial, with both positive and negative outcomes described in the literature. For example, autologous serum plus recombinant basic fibroblast growth factor (bFGF) and three recombinant cytokines (Thrombopoietin, Stem Cell Factor and FL or “Fms-related tyrosine kinase 3 ligand”) has been shown to support expansion of primary marrow stromal cells [42]. Adipose tissue derived-MSC expanded with human allogenic serum pooled from 5 donors exhibited a more spindle-shaped morphology and increased motility than the same cells cultured on FBS, and a significantly higher proliferative rate [43]. Moreover it has been demonstrated that human serum contains factors that, in comparison to FBS, exert dramatic effects on BM-MSC cell differentiation increasing the osteogenic and adipogenic activity of dexamethasone [44]. However, Anselme *et. al* [45] observed less formation of fibroblastic colony-forming units (CFU-F) when hMSC were cultured in hS as compared to FBS supplemented media. Nevertheless, BM-MSC cultured in hS before transplantation were more efficient in the production of in vivo bone formation than the same cells cultured in FBS [46]. Aldamash and colleagues [47] did not observe any significant differences in growth rates of an immortalized hMSC line during short-term (10 days) and long-term (100 days) culture in media supplemented with allogenic hS in comparison with FBS. Recently Choi *et al.* compared the effects of different concentrations of autologous serum (1, 2, 5, and 10%) on expansion and adipogenic differentiation of AT- MSC using 10% FBS as a control. They found that cell isolation was successful without difference among media, while the proliferation potential follows the trend: 10% autologous serum > 10% FBS = 5% autologous serum > 2% autologous serum = 1% autologous serum [6].

IV.3.2.2. Human Platelet Lysate (hPL)

Human platelet lysate (hPL) can be obtained from blood platelets using different procedures, among which the most common is the freeze/thaw technique developed by Holmqvist and Westermarck [48]. This procedure for platelet isolation from blood preserves their α granules, which contain a wide range of growth factors such as bFGF, Epidermal Growth Factor (EGF), Hepatic Growth Factor (HGF), Insulin Growth Factor-1 (IGF-1), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor- β 1 (TGF- β 1) and Vascular Endothelial Growth Factor (VEGF). In comparison to human serum, hPL contains higher levels of strong mitogens (e.g. EGF, PDGF and TGF- β 1), whereas the levels of IGF-1 and the protein content are lower due to the removal of immunoglobulins and albumin in the washing procedure [49]. In some studies, hPL has been more efficient than FBS regarding the expansion of hMSC, maintaining their differentiation potential as well as their immunosuppressive properties, namely in the case of hMSC derived from adipose tissue [50]. Importantly, no difference between the use of freshly-prepared and expired platelets concentrates has been observed regarding the proliferation potential and osteogenic differentiation of hMSC [51], suggesting that the bioactivity of this type of supplement is quite stable. The addition of exogenous growth factors has been commonly employed in FBS-supplemented culture to increase cell proliferation rates [52]. However, even if higher proliferation rates have been obtained in cultures supplemented with FBS combined with bFGF, as compared to FBS only, those rates remained lower than the ones obtained in medium supplemented with 10% hPL [53]. Interestingly, hPL without the addition of anticoagulant forms a soft hydrogel, providing a three dimensional (3D) scaffold, which has been described to increase *in vitro* expansion of hMSC by mimicking a more natural 3D context than the commonly used 2D polystyrene surfaces [50]. Finally, platelets derivatives have been already tested in clinical trials with good results for the treatment of tendinopathies and osteoarthritis [54, 55] and in hearth failure [56].

However, the use of hPL also presents some disadvantages. First, it is a supplement for cell culture that, similarly to FBS, is not precisely defined. A high variability exists between individual hPLs, as their composition is strongly dependent on donor-related factors, such as age, gender, blood group and platelet individual counts [57]. Nevertheless, pooling different donations can reduce variability. Schallmoser *et al.* demonstrated that pool of 15 donations can improve standardization [58]. Also, hPLs isolation process may affect platelets degranulation and, consequently, growth factors content of hPLs. Additional variability may result from the filtering procedure, storage typology or the content in

heparin or anticoagulants [55]. Moreover, as observed for other human derived media supplements, there is a concern regarding the possibility of immunological reactions, in allogenic settings, and transmission of human diseases. However, Castiglia *et al.* [59] recently described a method named *ihPL* (inactivated hPL), which ensures efficient generation of safe hPL batches, by virus inactivation through UVA light.

IV.3.2.3. Umbilical cord blood serum (hUCBS)

Human umbilical cord blood serum (hUCBS) can be easily obtained following normal delivery, thus normally screened for the most common bacterial or viral contamination and its use does not raise any ethical issues. It contains high levels of soluble growth factors and more than 60 proteins, namely albumin, transferrin and fibronectin (FN) in high concentrations, with different roles in cell growth and stem cells differentiation [12]. Phadnis *et al.* [60] demonstrated that BM-hMSC displayed a 32-fold increase in cell number 5 days after seeding in UCBS against a 10-fold increase in FBS culture conditions. Moreover, 10% UCBS has been proved to be more efficient in promoting hMSC osteogenic differentiation than 10% FBS because of its enhancement of osteocalcin promoter expression [61].

In general, UCBS can be considered advantageous as cell culture supplement considering the easy availability of this allogeneic source, the easy and inexpensive isolation procedure [62] and the near absence of contaminants. On the other hand, some limitations must be considered, such as the high lot-to-lot variability, associated to donor-related features and the presence of adventitious agents that may eventually escape routine screening procedures.

IV.3.2.4. Industrial GMP- human plasma derivatives (supplement for cell culture-SCC)

A new type of supplement cell culture (SCC) has been recently described for *in vitro* cell expansion [63] consisting on a GMP pharmaceutical grade xeno-free human plasma-derived supplement. SCC is obtained from human plasma through cold-ethanol industrial fractionation [64]. Once plasma is collected from healthy donors at USA-based FDA-licensed plasmapheresis centres, plasma pools from over 1000 different donors are assembled in a single plasma unit. Every individual donation is tested for viral markers and all plasma is tested, using nucleic acid techniques, for the presence of DNA or RNA from relevant pathogenic agents, in agreement with the European and American legislations. In

addition, SCC production procedure includes a specific viral inactivation step (gamma irradiation) besides others purification steps with additional pathogen removal capacity [63].

SCC has already been successfully used as a supplement for *in vitro* cell culture to basal medium that supports culture of stem cells such human embryonic stem cells (hES), induced pluripotent stem cells (iPSC) derived from human dermal fibroblast [65] and hMSCs [63]. When hMSC were expanded in SCC (15% SCC reconstituted in basal medium (DMEM: HAM's nutrient mixture F12) containing a cocktail of growth factors and other elements such as insulin, sodium selenite and ethanolamine, cell yield was similar to the one obtained for hMSC expanded in commercial medium obtained from hMSC suppliers (Lonza Group and PromoCell). Moreover, SCC supplemented medium maintained hMSC in an undifferentiated state during expansion and kept their capacity of adipogenic, chondrogenic and osteogenic differentiation, under inducing conditions [63]. However, being SCC a supplement in very recent development, only few data are available in the literature regarding its potential use as hMSC medium supplement. Thus, further investigations will help to a better characterization and improve additional applications. Its introduction in the market as other alternative to FBS will show its potentiality as supplement for *in vitro* cell expansion.

IV.4. Surfaces and coatings to promote hMSC attachment and growth

Anchorage-dependent cells such hMSCs, are supported *in vivo* by the ECM, an assembly of many proteins and glycosaminoglycans (GAGs) that provides a 3D environment for their organization into tissues. Specific interactions between cell surface receptors and ligands on the ECM, mediate intracellular signalling pathways and control gene expression, cytoskeletal organization and cell morphology that are involved in key cellular activities such as cell adhesion, migration, proliferation and differentiation [66].

When cells are cultured with FBS supplemented medium, a protein layer readily adsorbs to the tissue culture polystyrene (TCPS) surface, the material generally used in the commercial cell culture flasks. This protein layer contains several adhesive proteins, such as fibronectin (FN) and vitronectin (VN) that are essential to mediate early stages of cell attachment [67], before cells are able to produce their own ECM [68]. In contrast, hMSC cultured in the absence of FBS do not properly attach to TCPS [69]. A common strategy for hMSC expansion in serum-free conditions is their pre-incubation in FBS as a transient

adaptation step to serum-free medium [69]. This process can confer to the cells the necessary elements for attachment. However, this is not acceptable in a GMP setting, demonstrating the need of identifying defined cell attachment factors, and also designing new culture surfaces that support hMSC adhesion and proliferation, while maintaining its multipotency, in the absence of FBS.

IV.4.1. Surface coating with cell-adhesive proteins

The simplest strategy to improve the attachment of hMSC in culture cell consists in pre-coating TCPS surfaces with purified adhesive proteins that can be isolated from human plasma or synthesized by recombinant technologies. Surface modification with a FN coating is probably the most widely used strategy for improving hMSC adhesion and proliferation in serum-free conditions. FN is a multifunctional, ECM glycoprotein that contains several functionally and structurally distinct domains, including cell-binding domains such as the RGD (Arg-Gly-Asp) sequence that are recognized by cell surface integrins, namely $\alpha 5\beta 1$ and $\alpha v\beta 3$ [71]. Other coating proteins, such as laminin (LN), collagen type I (COL I) and IV (COL IV) and gelatine also showed to improve hMSC adhesion and growth in serum-free medium [69]. In contrast, the number of attached hMSC on poly-D-lysine pre-coated surfaces was very low even when studies were performed in the presence of FBS [69]. Moreover, cells did not show their typical fibroblast-like spindled shape morphology, which could be related with their low spreading capacity in this type of coating [71]. Ode *et al.* also reported that TCPS pre-coated with FN as well as COL I, II, III and IV, fibrinogen (FG), but not with LN, induces hMSC adhesion and proliferation in serum reduced conditions (0.1% v/v FBS) [72]. LN, an ECM protein especially abundant within the basal lamina of epithelial and endothelial tissues, has been described as an important mediator of cell attachment and migration [73]. Yet, the effect of LN coated surfaces on hMSC adhesion and growth is not clear since opposite outcomes have been reported [69, 72, 74, 75]. Differences in hMSC behaviour on LN-coated surfaces could be related with i) protein conformation, which is dependent on the type of substrate, LN concentration and incubation time; ii) type of LN, as different forms have been identified and iii) disparities in terms of cell culture protocols, including the type of medium used.

Ogura *et al.* [76] also demonstrated that hMSC attachment and spreading on FN-coated dishes was increased when compared to albumin-coated dishes. This is not surprising since

albumin, the protein that exists in higher concentration in serum and the first to reach and adsorb to most of the surfaces, is known by its non-adhesive characteristics [77].

Besides affecting hMSC attachment and proliferation, ECM protein coatings can also influence cell differentiation into specific lineages. For example, it is known that FN plays a pivotal role in hMSC osteogenic differentiation, while inhibiting adipogenesis [78]. VN and COL I coatings were also shown to induce MSC osteogenic differentiation [74, 78] through interaction with specific COL I receptor $\alpha_1\beta_1$ and VN receptor $\alpha_v\beta_3$ integrins. This fact was observed even in the absence of soluble osteogenic inducers [74]. LN-1 and LN-5 were shown to promote hMSC osteogenic differentiation in xeno-free conditions (human plasma and platelet extract) [79], but LN-5 suppress chondrogenic differentiation [75]. Since surface chemistry will influence the amount and conformation of the adsorbed protein layer and thus, the exposure of specific integrin binding sequences, the type of substrate used will affect hMSC differentiation profile even when the same protein coating is used [80].

TCPS coated with keratins, isolated from human hair, was recently suggested as an alternative to other most common adhesive proteins for hMSC *in vitro* expansion [81]. This protein contains the LDV (Leu-Asp-Val) cell adhesion motif recognized by cell membrane integrin $\alpha_4\beta_1$. Moreover, human hair keratins can be easily obtained in abundance even from an autologous source. However, more studies need to be performed in order to further validate its suitability for this kind of applications.

IV.4.2. Surface modifications to promote adequate adsorption of adhesive proteins

The type of underlying substrate used for cell culture also influences hMSC adhesion and proliferation, since it dictates the nature, conformation and orientation of adsorbed proteins and, consequently, the exposure of active ligands (cell binding domains) for cell recognition and binding. Commercial cell culture flasks are commonly made of TCPS. These surfaces are obtained by modifying hydrophobic polystyrene surfaces, often by gas-plasma treatment, to turn them hydrophilic and negatively charged. This promotes the adsorption of serum attachment proteins such FN and VN, in relation to other proteins such as albumin [82], providing better surfaces for subsequent cell adhesion.

In fact, it is currently well known that cell-binding domains of FN are hidden when the protein is in its native structure, becoming exposed upon adsorption on surfaces with adequate wettability and charge [83]. So, the effect of adsorbed FN on cell attachment largely depends on the properties of the underlying substrate. For example, Dolatshahi-

Pirouz *et al.* demonstrated that although FN adsorption was higher on gold (Au) surfaces than on hydroxyapatite (HA) surfaces, the morphology of attached hMSC was more regular on HA. This was explained by the higher exposure of cell binding domains on FN adsorbed on HA, which was confirmed using monoclonal antibodies directed against FN cell-binding domains [84]. The influence of surface wettability (hydrophilic vs. hydrophobic surfaces) on selective adsorption of FN and VN from FBS and respective exposure of cell binding domains was also studied using self-assembled monolayers (SAMs) prepared by mixing different ratios of OH (hydrophilic) and CH₃ (hydrophobic) terminated alkanethiols [67]. hMSC adhesion and proliferation increased with the increase of surface hydrophilicity (increase of OH/CH₃) which was directly correlated with the increased exposure of cell-binding domains on adsorbed FN and VN [68]. However, Curran *et al.* [85] did not find differences in hMSC adhesion and viability among -CH₃, -OH, -NH₂, -SH and -COOH glass silane modified surfaces in the presence of FBS. Nevertheless, they demonstrated that surface chemistry were able to modulate hMSC potential of differentiation. They reported that only -CH₃ surfaces were able to maintain MSC phenotype, since cells were expanded without losing their differentiation skills under appropriated stimulus. -NH₂ and -SH surfaces promoted osteogenesis differentiation while -OH and -COOH surfaces support chondrogenic differentiation even in basal conditions [85].

Although all these studies were performed in the presence of FBS, they highlight the importance of surface chemistry on the amount and conformation of adsorbed adhesive proteins, an essential step in the design of substrates for serum-free or xeno-free-hMSC cultures.

IV.4.3. Surface chemical modifications with cell-adhesive peptides

Since the process of surface coating by simple protein adsorption does not provide a high level of control over the presentation of cell-binding domains, different strategies have been idealized to covalently immobilize cell-binding motifs (e.g. RGD) on a substrate [86, 87]. With this strategy, problems associated with surface coatings with proteins from animal or human origin can be overcome, since cells may directly bind to the functionalized substrate. Other advantages of chemically modified synthetic surfaces are their higher stability during storage and their low batch-to-batch variability.

An array with different RGD densities in a bio-inert background was developed using different concentrations of the immobilized peptide sequence RGDSP (Arg-Gly-Asp-Ser-

pro) on ethylene glycol-terminated SAMs. Results demonstrated that higher peptide densities induce higher MSC spreading and focal adhesion formation in the presence of FBS [88]. In fact, it is well known that although surfaces functionalized with RGD motifs can increase hMSC attachment and spreading, its efficacy strongly depends on its surface density [87] and patterning [89]. Surfaces functionalized with RGDs for xeno-free hMSC culture are already commercialized by BD Biosciences (section IV.4.6).

IV.4.4. Surface coatings with decellularized ECM

Single-protein coatings as the ones described above lack the complexity of cells-secreted ECM. Coatings with decellularized ECM can be used as an alternative to single proteins, to improve cell growth and proliferation *in vitro* without the loss of their stem cell properties [90]. To obtain these coatings, hMSC are cultured on tissue culture plates (or other substrate of interest) for enough time to allow cells to produce their own ECM, after which the coating is decellularized through appropriate processing, leaving only the ECM components. The decellularized ECM coating can be maintained in the original substrate, where subsequent hMSC populations can be directly cultured, or can be collected and transferred to other substrate without losing its instructive potential [90].

Lai *et al.*, described the characteristics of an ECM coating produced by hMSC before and after decellularization. Using confocal microscopy it was possible to visualize the localization of COL I and III, FN, small leucine-rich proteoglycans such as biglycan and decorin, and major components of basement membrane such as the large molecular weight proteoglycan perlecan and LN [91].

A coating with ECM derived from human foetal MSC (fMSC) improved adult hMSC proliferation maintaining their multipotency, in comparison with ECM derived from adult MSC and TCPS. This fact could be related with the higher proliferation capacity of fMSC with associated higher amounts of ECM production [92]. These results are very promising for *ex vivo* expansion of hMSCs, however they were not performed in xeno-free conditions. Moreover, the available amount of fMSCs associated with ethical issues could be a concern for this strategy.

The most commonly used decellularization processes are based on the detachment of intact cells using enzymatic processes or the lysis of cells using detergent compounds. However, all these processes have disadvantages, namely the use of proteases such as trypsin, which can damage the ECM components, and the lysis of cells that can contaminate the resultant

ECM with intracellular components. Pattabhi *et al.* described a new decellularization protocol based on cold-EDTA to remove intact cells from ECM without enzymes and detergents and with minimal ECM damage and contamination. They demonstrated that ECM derived from hMSCs obtained using this process enhances the proliferation of naïve hMSC maintaining their potential for osteogenesis and adipogenesis [93].

However, all these assays were performed in standard medium containing FBS since their aim is the development of protocols that can enhance hMSC proliferation maintaining their multipotency. Thus additional experiments are needed to adapt these strategies to a xeno-free MSC cultures.

IV.4.5. Surface modification with GAGs

GAGs are ECM polysaccharides that are involved in a variety of extracellular and intracellular functions, and are being widely explored for tissue engineering applications, both when used as surface coatings [94] or as 3D scaffolds for hMSC culture [95, 96].

Heparin is a linear GAG containing several sulphate and carboxyl groups, with a high negative charge density and with several well-characterized binding domains to different growth factors and ECM proteins, such as FGF-2, FN, VN, LN, COL and bone morphogenetic proteins (BMP) [97, 98].

Heparin-functionalized surfaces may be engineered to attract and bind soluble growth factors, concentrating them on the surface, which avoids the need of using extra high concentrations of soluble growth factors during *in vitro* hMSC expansion. However, besides promoting hMSC adhesion and proliferation, these surfaces also induce osteogenic differentiation in standard culture medium [95]. This fact was associated with heparin ability to sequester from the culture medium not only proteins essential for hMSC adhesion (e.g. FN, VN) and proliferation (e.g. FGF2), but also proteins involved in osteogenic differentiation (e.g. BMPs) [99].

In a different approach, surfaces can be engineered to specifically sequester serum-borne heparin to cell-material interface and thus, to attract and bind soluble growth factors. Heparin-binding surfaces were prepared by covalent immobilization of a small heparin-binding peptide (GGGKRTGQYKL) and the integrin binding peptide (RGDSP) on bio-inert SAMs. RGDSP was included to improve cell adhesion to bio-inert SAMs. In the presence of FBS, these surfaces were able to enhance hMSC proliferation and osteogenic

differentiation by amplifying the activity of endogenous FGF-2 and BMP, respectively [99].

However, it was also suggested that the up regulation of hMSC osteogenic differentiation could be related with heparin-induced alterations on FN conformation. Recent evidences pointed out that heparin induce a more extended conformation of fibrillar FN included in ECM scaffolds. Extended FN may expose hidden binding sites for many growth factors (e.g. FGF-2, BMP-2 and VEGF) that are fundamental on hMSC osteogenic differentiation [100].

IV.4.6. Commercial coatings and modified surfaces for MSC culture

Different xeno-free protein-based coatings or modified culture surfaces for hMSC culture have been developed and are currently commercially available. Their use is nowadays widespread for hMSC *in vitro* expansion, especially when in combination with serum-free or chemically-defined media. Some examples are described in Table IV.4, which is intended to be illustrative rather than all-inclusive

Table IV.4. Commercially available coatings strategies for hMSC *in vitro* culture.

NAME	COMPANY	COMPOSITION	VALIDATION (CELL TYPES)
SOLUBLE COATINGS			
CELLstart™	Thermofisher	Not disclosed	hMSC, human embryonic stem Cells (hESC), and human neural stem cells (hNSC)
Nutristem MSC Attachment Solution	Biological Industries	Affinity-purified human plasma fibronectin	BM-hMSC, AT-hMSC, UC-hMSC.
Xuri MSC attachment solution	GE Healthcare	Affinity-purified human plasma fibronectin	Validated for human BM-hMSC, AT-hMSC and UC-hMSC
MODIFIED PLASTIC WARE			
BD PureCoat™ ECM Mimetic Cultureware: Collagen and Fibronectin	Corning	Synthetic animal-free peptides covalently linked to a proprietary surface: - Fibronectin-derived RGD peptide - Collagen I-derived peptide	BM-hMSC and AT-hMSC Human umbilical cord blood-derived MSC (isolation)
Corning® Synthemax® Surface	Corning	Peptide acrylate coating functionalized with a vitronectin-derived peptide	Validated for hPSCs and other adult stem cell types

IV.5. CONCLUSIONS

With impressive biological properties, hMSC are nowadays considered one of the most promising cell types for cellular therapies, already demonstrated in the many clinical trials using hMSC. Nevertheless, hMSCs are scarce and to achieve sufficient numbers for cell transplantation, an *in vitro* expansion step is required. This procedure requires improvements in order to guarantee a safe preparation of hMSC as therapeutic products. Specific challenges concern the substitution of animal components such as FBS during hMSC *in vitro* expansion, since its uses raises scientific, economic and ethical issues. To improve clinical use of hMSC for advanced therapies, cell culture/expansion under xeno-free conditions should be encouraged. Current strategies include the replacement of FBS with chemically defined media or human plasma derivatives such as hS, hUCBS, hPLs and a more recent GMP-compliant Supplement for Cell Culture (SCC). Beyond soluble components, recent investigations consist also in the development of refined culture surfaces for hMSC *in vitro* culture: in order to augment the adsorption of adhesive and/or growth promoting agents, surfaces can be functionalized with adequate chemical groups and modified by immobilization of peptide ligands or whole ECM proteins such as GAGs and FN. However, most of these studies are being conducted in the presence of serum and additional experiments are needed to transpose these strategies to a xeno-free MSC cultures.

In recent years, different promising experimental settings have been proposed towards clinical safety of hMSCs *ex vivo* expansion and illustrated herein. However there is still a lack of standardized protocols for this intent thus, high quality translation research and exchange among the numerous research groups interested in the field are demanded.

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CHAPTER V

This chapter is based on the following publication:

The optimization of the use of a pharmaceutical grade xeno-free medium for *in vitro* expansion of human mesenchymal stem/stromal cells

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ABSTRACT

Human bone marrow (BM)-derived mesenchymal stem/stromal cells (hMSCs) are considered promisor therapeutic agents in the field of cell therapy and regenerative medicine, mainly due to their relatively facility to be isolated, multi-differentiation potential and immunomodulatory role. However, their application in clinics requires a crucial step of *in vitro* expansion. Most of the protocols for hMSCs *in vitro* culture use foetal bovine serum (FBS) as medium supplement that, being from animal origin, presents several safety concerns and may initiate xenogeneic immune responses after cells transplantation. This work reports the optimization of a pharmaceutical-grade xeno-free strategy for hMSCs *in vitro* expansion based on the supplementation of basal medium with a pharmaceutical-grade human plasma-derived supplement for cell culture (SCC) and two human growth factors (bFGF and TGFβ1), plus a coating of recombinant human fibronectin (Fn). After 4 weeks in culture, this strategy improves hMSCs expansion yield about 4.3-fold in comparison with FBS supplementation and 4.5-fold compared to a commercially available xeno-free medium. hMSCs expanded in SCC-based formulation

maintained their phenotype and differentiation capacity into osteogenic, adipogenic and chondrogenic lineages, without alterations in cell karyotype. Overall, the SCC-based medium appears to be an excellent alternative for the xeno-free expansion of hMSCs as therapeutic agents for clinical applications.

V.1. INTRODUCTION

Human mesenchymal stem cells (hMSCs) have a tremendous therapeutic potential in cell therapies and regenerative medicine due to their high regenerative potential, capacity to differentiate into different cell lineages (such as osteoblasts, adipocytes and chondrocytes), immunomodulatory role, ability for systemic engraftment and ease isolation [1]. For all these reasons, hMSCs were introduced into clinics for the treatment of several diseases such as graft-versus-host disease [2], Crohn's disease [3], neurodegenerative and muscle degenerative disease [4], cartilage and meniscus repair [5], stroke and spinal cord injury [6]. Moreover, several treatments using hMSCs are currently in different phases of clinical trials (<http://www.clinicaltrials.gov/>).

Although hMSCs can be isolated from different body tissues including bone marrow (BM), fat, umbilical cord, Wharton's jelly and peripheral blood, bone marrow-derived MSCs (BM-hMSCs) are historically the most used. However, the number of available cells is always insufficient for clinical applications ($1-5 \times 10^6$ hMSCs/Kg body weight [7]), which imply that after isolation cells need to be expanded *in vitro* for several passages. hMSCs therapeutic use follows the European guidelines that specify safety measures for donation, testing, processing, storage and distribution of human cells and tissues intended for human use [8; 9]. Therefore, Good Manufacturing Practices (GMP)-compliant strategies for hMSCs expansion *in vitro* before infusion into a patient, are strongly demanded.

The behaviour of attachment-dependent cells, such as hMSCs, is mediated by soluble factors present in the medium and by cell adhesive proteins that adsorb to the culture surface [10]. Conventional medium for isolation and expansion of hMSCs consists in a defined basal medium (typical Dulbecco's modified Eagle's medium (DMEM)), which has to be supplemented with foetal bovine serum (FBS) since it contains a cocktail of stimulating factors required for *in vitro* cell growth, as well as adhesive proteins, such as fibronectin, essential for hMSCs surface attachment. However, FBS presents serious safety concerns, namely the possibility to transfer animal proteins (xenoantigens) [11], and unknown pathogens (such as virus and prions) to patients [12]. Moreover, FBS presents

poorly defined composition, lot-to-lot variability [13] and associated ethical [14] and economic concerns [15], which also limit its clinical application.

The progress of recombinant technologies gave rise to several chemically defined media [16, 17] which have already been introduced in the market and are quite widespread for clinical grade hMSCs production. However, these products have also some drawbacks, namely high cost and unknown composition. Moreover, xeno-free media may need further supplementation with biomolecules that are usually present in FBS [18], namely growth factors [19] and adhesive proteins [10] that need to be produced using recombinant technologies or isolated from human sources.

In alternative, human–blood derivatives such as human serum [20], human platelet lysate [21], and human umbilical cord blood [22] have been investigated as alternatives to FBS, however those media also presents some drawbacks especially lot to lot variability [23].

Human plasma derived supplement for cell culture medium (SCC), produced by the pharmaceutical industry Grifols (Parets del Valles, Spain), was recently described as a potential candidate for xeno-free supplementation of hMSCs expansion media [24]. Besides being a pharmaceutical-grade product, SCC have the advantages of: i) providing uniformity between batches, since it is obtained from human plasma pools of over 1000 different donors and ii) be safe, which is assured by every individual donation be tested for viral markers and pathogenic agents and by its production process be subjected to steps with viral clearance capacity [24, 25]. Moreover, SCC is a GMP compliant product, being every step of its development well-defined and validated under Standard Operating Procedures (SOPs). This is rather important since hMSCs are considered advanced therapy medicinal products (ATMP), and thus cell production for clinical applications must follow GMP requirements according to FDA and EMA authorities.

This work aimed to optimize the use of a pharmaceutical grade xeno-free strategy, based on SCC supplemented medium [24] to improve the expansion of hMSCs for clinical applications.

V.2 MATERIALS AND METHODS

V.2.1. Reagents

Recombinant human transforming growth factor beta 1 (TGFβ1; Ref. 240-BT) and 3 (TGFβ3; Ref. 243-B3) were purchased from R&D System and human basic fibroblast growth factor bFGF (Animal Free, Ref. AF-100-18B) from Peprotech EC. Human insulin solution (Ref. I9278-5ML), selenium powder (Ref. 10112), ethanolamine solution (Ref.

E9508), fibronectin solution from human plasma (Fn; Ref. F0895), rabbit Anti-Fn mAb IgG (F3648) and dimethyl sulfoxide (DMSO, Ref. 472301) were purchased from Sigma-Aldrich. L-glutamine (Ref 25030-024) was from Life Technologies and tetramethyl benzidine solution (TMB11x; Ref. 31080) from BioRad.

Flow cytometry (FC) antibodies, mouse IgG1PE (Ref. 21275514), mouse IgG1 FITC (Ref. 21275513), mouse IgG1APC (Ref. 21275516), mouse IgG2a FITC (Ref. 21335023), HLA-DR-FITC (Ref. 21278993S), CD45-APC (Ref. 21270456), CD34-FITC (Ref. 21270343), CD19-PE (Ref. 212701945), CD14-PE (Ref. 21620144S) and CD105-FITC (Ref. 21271053) were purchased from Immunotools; CD73-PE (Ref. 550257) from BD Becton Dickinson SA, Anti-human CD90 APC (Thy-1) (Ref. 17-0909-41) from LabClinics SA. Alexafluor-conjugated phalloidin (Ref. A12379 Molecular Probes) was obtained from ThermoFisher Scientific and 4, 6-Diamidina-2-phenylin (DAPI; Ref D8542) from Sigma-Aldrich.

Osteogenic (Ref. C-28013), adipogenic (Ref. C-28011) and chondrogenic (Ref. C-28012) differentiation media were obtained from PromoCell.

V.2.2. Cells and media

SCC is a freeze-dried product in development at Grifols S.A. (Parets del Valles, Barcelona) derived from human plasma [24] through Cohn-Oncley fractionation [26]. Xenofree SCC based medium was prepared by the reconstitution of SCC at 15% in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 *DMEM/F-12*, no glutamine (Ref. 21331-020, ThermoFisher Scientific) and the addition of 1% of other compounds such as: Insulin, Selenium and Ethanolamine (ISE) and Penicillin/Streptomycin (Pen/Strep). FBS medium was prepared by the reconstitution of MSCs certified FBS (ThermoFisher Scientific, Ref. 10094563) at 10% in DMEM, low glucose, GlutaMAXtm supplement pyruvate (Ref. 21885-108, ThermoFisher Scientific) plus 1% Pen/Strep. SFM medium is a FDA approved commercial MSCs xeno free medium (<http://www.prnewswire.com/news-releases/fda-grants-510k-clearance-to-life-technologies-stem-cell-growth-medium-117920569.html>) composed by serum free media (CTS StemPro® MSCs SFM; Ref. A10332-01), supplement mix (Ref. A10333) and substrate coating solution (CELLstart, Ref. A10142-01) purchased from Alfacene Lda. hMSCs isolated from bone marrow of healthy 21-year-old black male (Lonza PT2501, VWR International, Radnor, Pennsylvania) were used in this study. Being cells

commercially available, no patient consent or approval from Ethics Committee was needed. Briefly, cells were maintained in culture at 37 °C and atmosphere of 5% CO₂ in FBS, SCC or SFM media. Full culture media change was performed twice a week and cells were split by trypsinization when they reached 70-80% confluence. Cells between passage 4 and 9 were used in this study.

V.2.3. Optimization of xeno-free medium supplemented with SCC

V.2.3.1. Quantification of adsorbed Fn from SCC and FBS solutions

The amount of Fn adsorbed on TCPS (tissue culture polystyrene) from FBS and SCC solutions was quantified using an adapted Enzyme Linked Immunosorbent Assay (ELISA) test. For that, 96-well TCPS plates were coated with FBS or SCC solutions (100 %, 20 %, 10% and 5%) diluted in PBS (phosphate buffered saline; pH 7.4) at 37 °C for 1 h. Wells were washed 3 times with PBS and blocked with 3% BSA (bovine serum albumin)/PBS during 1 h at 37 °C. Then, wells were incubated with Rabbit Anti-Fn mAb IgG diluted 1:10000 in BSA/PBS 3% during 1 h at room temperature. Wells were washed with 0.05% Tween 20 in PBS and incubated with an HRP-coupled anti-rabbit IgG secondary antibody, diluted in 3% BSA/PBS solution. After 3 washes with 0.05% Tween 20/PBS, wells were incubated for 10 min at room temperature with TMB solution in phosphate citrate buffer 0.05 M + 0.03% H₂O₂. Enzymatic reaction was stopped by adding 100 µl/ well of H₂SO₄. Supernatants were read at 450 nm and 630 nm (to exclude plate Abs).

V.2.3.2. Effect of Fn coating on hMSCs cytoskeleton organization

To evaluate the effect of Fn coating on hMSCs morphology, 24-well plates (TCPS) were firstly coated with 20 µg/ml of human recombinant Fn in PBS for 1 h at 37 °C. Then plates were washed 3 times with PBS to remove non-adsorbed proteins.

hMSCs were incubated in FBS or SCC media on plates with or without Fn coating. After 24h, cells were washed twice with pre-warmed PBS, fixed in 4% v/v paraformaldehyde for 15 min, incubated in permeabilising buffer for 5 min, and blocked with 1% w/v BSA in PBS at room temperature. To evaluate cytoskeleton organization, actin filaments were stained with Alexafluor-conjugated phalloidin (1:40 in 1% w/v BSA/PBS). Nuclei were counterstained with 20 µg/mL DAPI for 10 min. Samples were washed with PBS, mounted

with Vectashield (Vector) in glass slides and photographed with an inverted fluorescence microscope (Axiovert M100; Carl Zeiss).

V.2.3.3 Effect of Fn coating and growth factor supplementation on hMSCs metabolic activity

For metabolic activity evaluation, hMSCs (P5) were plated at 3 000 cells/cm² in 24-well plates (TCPS) with and without Fn coating and cultured in FBS medium, SCC medium or SCC medium supplemented with bFGF (20 ng/ml) or TGFβ(1 or 3) (10 ng/ml), alone or in combination. At days 1, 3, 7, 10 and 14 cell metabolic activity was assayed by Resazurin assay as described elsewhere [27]. All assays were performed in triplicate.

V.2.4. Evaluation of optimized xeno-free medium supplemented with SCC

V.2.4.1. Expansion

Frozen hMSCs at P4 were quickly thawed in a 37 °C water bath, plated onto a 75 cm² tissue culture (T75) flask in FBS medium and incubated at 37°C and 5% CO₂.

When cells were subconfluent (*ca.* 80% confluent), they were rinsed with PBS and trypsinized. Cells were resuspended in DMEM and 3000 cells/cm² were seeded in duplicate in 25 cm² tissue culture (T25) and cultured under four different conditions: *a)* 10% FBS-supplemented medium; *b)* 15% SCC-supplemented medium + 20 ng/ml bFGF + 10 ng/ml TGFβ3 on 20 µg/ml fibronectin coating (1h, 37 °C); *c)* SCC medium + 20 ng/ml bFGF + 10 ng/ml TGFβ1 on 20 µg/ml fibronectin coating (1h, 37 °C); *d)* SFM: CTS StemPro® MSCs SFM+ 1% L-200mM L-glutamine + 1% Pen/Strep in 1:100 in PBS CELLStart coating (according to manufacture indications).

Cell split was performed at the same time point for all conditions, at the moment when one of them reached confluency. From each T25 flask ½ cell suspension was subcultured while ½ cells suspension was used for FC analysis. From each T25 flask, 75000 cells were subcultured at 3000 cells /cm², while the remaining cells were used to perform adipogenic, osteogenic and chondrogenic differentiation. Full media change was performed every 3 days. Cell morphology was monitored by microscopic examination. Cells numbers and viability were measured on a hemacytometer using the Trypan Blue exclusion method (two counts for each flask). Finally, hMSCs P9 expanded in FBS or SCC medium, have been frozen in FBS or SCC medium + 10% sterile DMSO.

V.2.4.2. Immunophenotype

The expression profile of typical hMSCs markers (CD105⁺, CD90⁺, CD73⁺, HLADR⁻, CD34⁻, CD45⁻ and CD19⁻) was analysed through FC. hMSCs cultured using the four media formulations were harvested at P6, P7, P8 and placed in 15mL conical tubes. Cells were centrifuged (1200 rpm, 7 min), supernatants were removed and cells were resuspended and aliquoted into multiple 15mL falcon tubes within approximately 50 µl FC buffer (0.01% sodium azide/0.5% BSA in PBS). Cells were stained with the following conjugated mouse anti-human monoclonal antibodies: anti CD105-fluorescein isothiocyanate (FITC), anti CD34-FITC, anti HLA-DR-FITC, anti CD14-phycoerythrin (PE), anti CD73-PE, anti CD90-Allophycocyanin (APC), anti CD45-APC or isotype-matched anti IgG-FITC, anti IgG-PE, anti IgG-APC. After incubation for 30 min, in the dark, on ice, cells were washed twice with PBS, centrifuged (1200 rpm, 7 min) and transferred to round-bottom FC tubes. Analysis was performed on a FACS (Fluorescence Activated Cell Sorting) Calibur (BD Biosciences) acquiring 10000 events per sample. Data were analysed with FlowJo software.

V.2.4.3. Differentiation potential

For *in vitro* differentiation assay cells were incubated in specific commercial differentiation media according to the manufacturer's instructions.

hMSCs at P6, P7, P8, P9 cultured in different media formulations (along respectively 1, 2, 3 and 4 passages) were harvested and seeded into i) 24-well plates (6×10^4 cells/well) for osteogenic and adipogenic differentiation and into ii) 15 ml conical tubes at 2.5×10^5 cells/tube, that after centrifugation (150 g/5 min) and formation of a pellet (24h), were incubated for chondrogenic differentiation.

Osteogenic differentiation was assessed after 21 days of incubation by specific staining of alkaline phosphatase activity. Adipogenic differentiation was evaluated after 14 days by observation of cell morphology and specific staining of lipid droplets with Oil Red O.

Chondrogenic differentiation, were estimated after 21 days cells incubation with specific differentiation medium with TGFβ3 (10 ng/ml) with full media change every 3 days. For that, pellets were fixed with 4% paraformaldehyde and embedded in paraffin, and sections (3 µm thickness) were prepared and analysed qualitatively by histology. Proteoglycans secreted by induced cells were stained with 1% toluidine blue 1% sodium borate solution to determine differentiation.

V.2.4.4. Karyotype evaluation

Genome integrity was analysed by fluorescence in situ hybridization (iFISH) assay. Frozen hMSCs P9 previously cultured along 4 passages in FBS, frozen hMSCs P9 previously cultured along 4 passages in SCC + bFGF +TGF β 1 on Fn and frozen hMSCs P4 (input cells) were quickly thawed in superfrost ultra plus slides (Thermo Scientific, Life Technologies, CA, USA) and cultured along 24 h. FISH interface analysis was performed according to standard protocols, with probes direct against chromosomes 7, 12, 17.

V.2.5. STATISTICS

To determine statistical significance of ELISA test, Resazurin assays and expansion assay, a Two-Way ANOVA test for multiple comparisons was performed.

For karyotype analysis a χ quadratic test was applied.

V.3. RESULTS

V.3.1. Optimization of xeno-free medium supplemented with SCC

V.3.1.1. Effect of Fn coating

hMSCs were firstly cultured in basal medium supplemented only with FBS and xeno-free SCC based media. Figure V.1A demonstrate that for short culture times (24h), hMSCs cultured in SCC medium exhibited a slightly different morphology than the one observed in FBS culture where an elongated spindle shape was more evident. Since Fn was described as one of the essential adhesive proteins for initial cell adhesion [28] and is present in FBS, we decided to evaluate the amount of Fn that adsorbs onto TCPS from FBS or SCC at different percentages (5, 10, 20 and 100% solution in PBS) using ELISA (Fig. V.1B). Results demonstrate that the amount of Fn adsorbed from FBS was always higher than from SCC, irrespectively of the concentration used, being this difference statistically significant for the concentration of 10% (canonical serum percentage for hMSCs culture). Moreover, when tissue culture polystyrene (TCPS) plates were pre-coated with 20 μ g/ml of human recombinant Fn (SCC+Fn), cells exhibited the typical hMSCs morphology, namely elongated cells with an organized cytoskeleton and well assembled actin stress fibres (Fig. V.1A). We then analysed cells metabolic activity along 6 days of culture in FBS, SCC medium or SCC+Fn, which showed that hMSCs metabolic activity in SCC was also significantly improved when a Fn coating was used (Fig.V.1C).

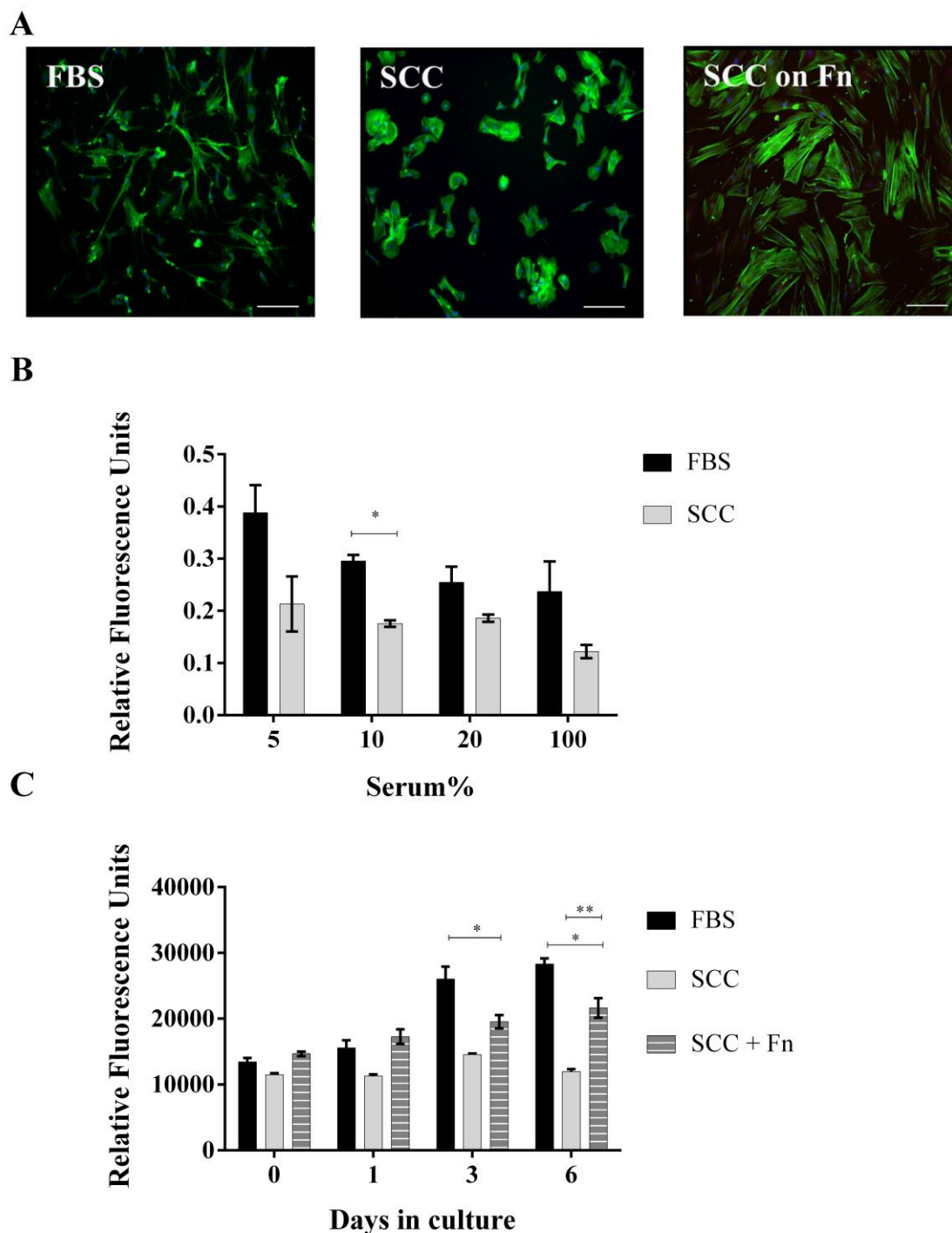


Figure V.1. Effect of Fn coating on hMSC behaviour. **A)** hMSC morphology examined by immunofluorescence detection of F-actin (green) and DNA (blue), 24h after seeding on FBS medium, on SCC and on SCC + Fn coating. Scale bar is 100 μ m. **B)** ELISA quantification of surface-adsorbed Fn from FBS or SCC solutions diluted in PBS at 5, 10, 20, 100 %. Values are expressed as mean $\pm$ SD. * P value < 0.05. **C)** hMSC metabolic activity on FBS medium, SCC and SCC + Fn coating assessed by Resazurin assay at days 0 (4 hours after seeding), 1, 3 and 6. Values are expressed as mean of relative fluorescence units $\pm$ SD. * P value < 0.05; ** P value = 0.01.

V.3.1.2. Effect of Fn coating and growth factor supplementation

It was previously described that, under serum free conditions, hMSCs growth could be enhanced by the addition of 20 ng/ml of bFGF in combination with other growth factors such as PDGF (various concentrations) and TGF β (10 ng/ml) [10]. Also, signalling of those growth factors is considered highly relevant for hMSCs growth and differentiation [29]. Thus, hMSCs metabolic activity was evaluated in the presence of SCC supplementation with the strong mitogens, bFGF and TGF (β 1 and β 3), alone or in combination in the presence of Fn coating (Fig.V.2). When bFGF was added to SCC in combination with TGF (either β 1 or β 3), a substantial increase in metabolic activity was observed, namely in comparison to hMSCs in FBS-supplemented medium after 10 days in culture. This effect was not observed if only one of these growth factors were used individually.

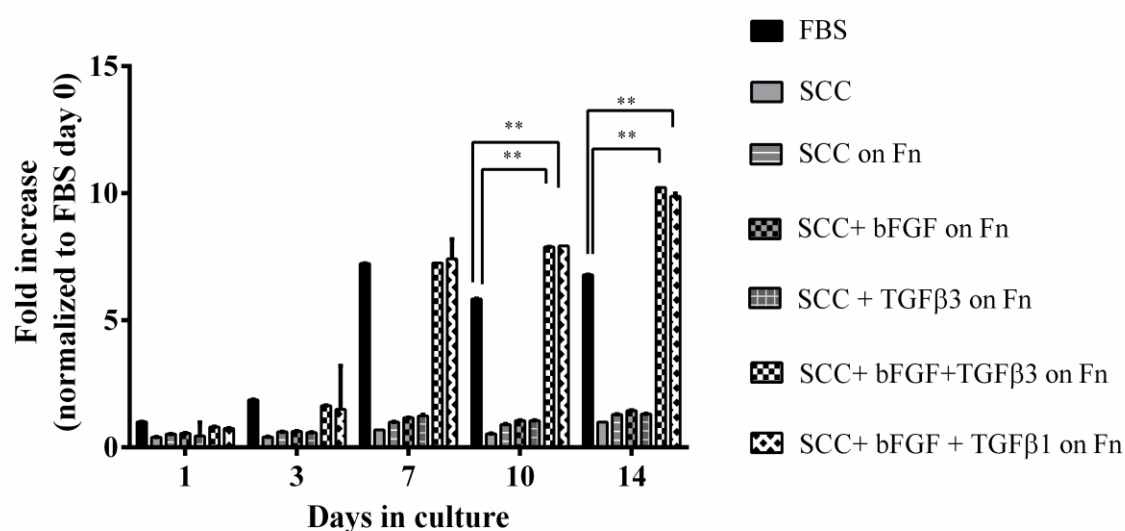


Figure V.2. hMSC metabolic activity along 14 days in media with FBS or SCC, supplemented with additional growth factors. To assess the effect of soluble growth factors, SCC was supplemented with bFGF only or in combination with two isoforms of TGF β (1 or 3) on Fn coating. At days 1, 3, 7, 10 and 14 cell metabolic activity was assayed by Resazurin assay. Values were normalized against FBS medium condition (fold increase). hMSC on SCC + FGF + TGF β 1 or β 3 on Fn, reach levels of metabolic activity higher than FBS canonical medium after 10 days in culture.

V.3.2. Evaluation of the optimized xeno-free medium supplemented with SCC

V.3.2.1. Expansion

hMSCs expansion during longer times in culture (about 30 days) and along 4 sequential passages in the optimized SCC supplemented medium (SCC + bFGF + TGF (β 1 or β 3) + Fn coating) was further addressed and compared with FBS and commercial serum-free medium. For that, hMSCs in passage 4 (BM-MSCs P4) were expanded in 4 different media formulations *a)* canonical FBS containing medium: DMEM+10% FBS (FBS); *b)* SCC + 20 ng/ml bFGF + 10 ng/ml TGF β 3 on Fn coating (SCC+ bFGF + TGF β 3 on Fn); *c)* SCC + 20 ng/ml bFGF + 10 ng/ml TGF β 1 on Fn coating (SCC+ bFGF + TGF β 1 on Fn) and *d)* commercial serum free medium: STEMPRO® MSCs SFM on CELLStart coating (SFM).

In a first experimental setting, cells were spliced when reaching about 70-80% confluence and seeded at 3000 cells/cm² at each passage. After 28 days, cell yield was significantly higher for hMSCs cultured in SCC media (both with TGF β 3 and TGF β 1), as compared to FBS and SFM (Fig. V.3A). After 4 passages, the fold increase in hMSCs total numbers, with respect to the first passage (P5), reached 310-fold for SCC with TGF β 3 and 295-fold for SCC with TGF β 1 against 62-fold for FBS and 66-fold for SFM (****, $p < 0.0001$) for FBS in comparison with either SCC + TGF β (1 or 3) (Fig. V.3B). Moreover, cell viability was evaluated along these 4 passages (Fig. V.3C), revealing a very high percentage of viable cells for the four media formulations, which was always $\geq 92\%$.

In another experimental setting, hMSCs were expanded and half-split in each passage, to better reproduce the cell expansion protocols typically used in clinics. Still, SCC formulation led to faster hMSCs growth rate than FBS or SFM (Fig. V.3D). In this last experiment, cell yield reached 219-fold for SCC with TGF β 3 and 199-fold for SCC with TGF β 1, against 29-fold for FBS and 109-fold for SFM, after 4 passages.

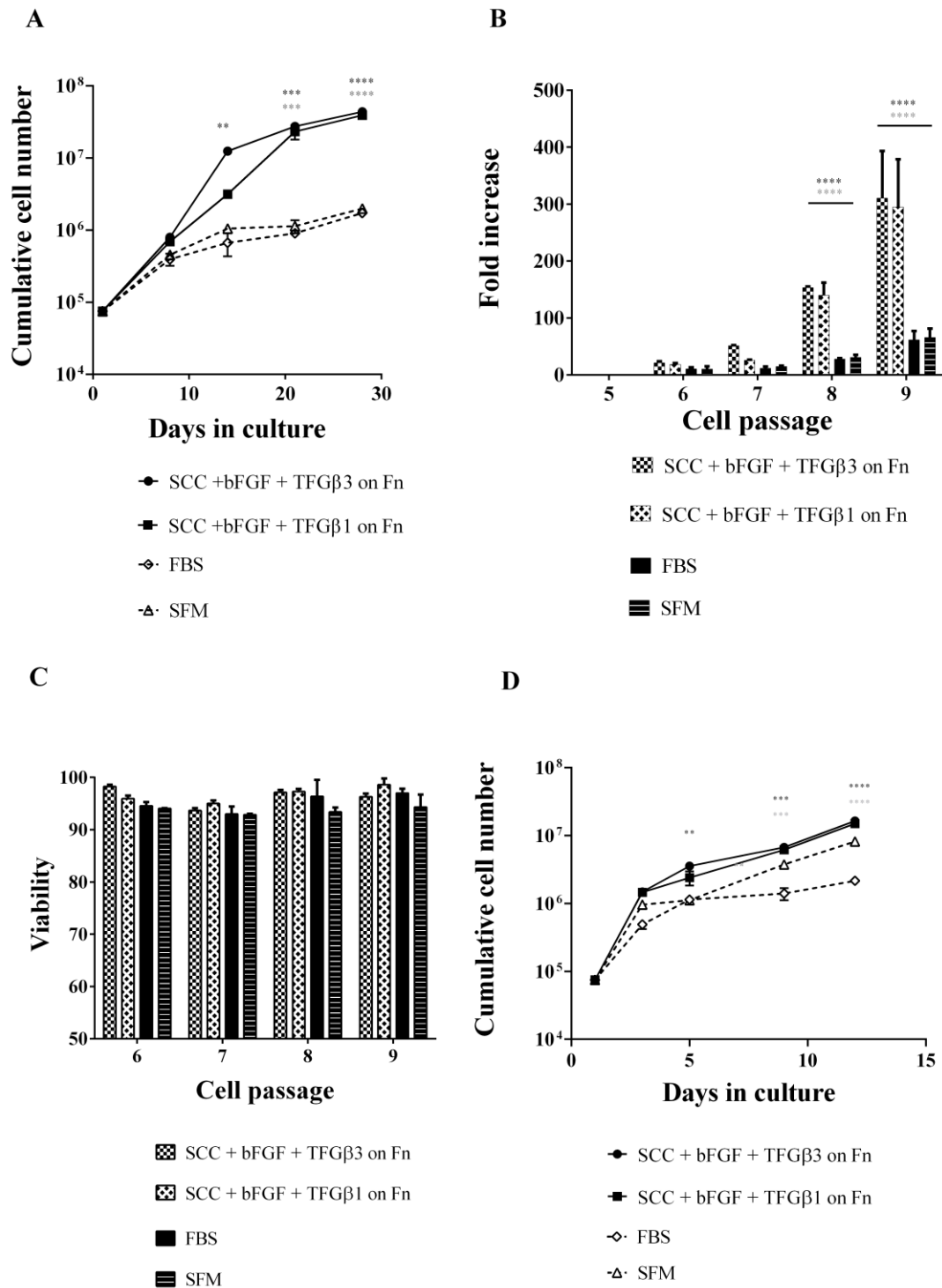


Figure V.3. hMSC expansion along 4 passages in FBS, SCC with TGFβ1 or β3 on Fn coating and commercially available SFM. A) Cell expansion curve: hMSC were seeded at 3000 cells/cm² and split was performed at the same time point for all conditions, when one of them reached confluent. Values are expressed as mean ± SD of two counts for each medium condition.

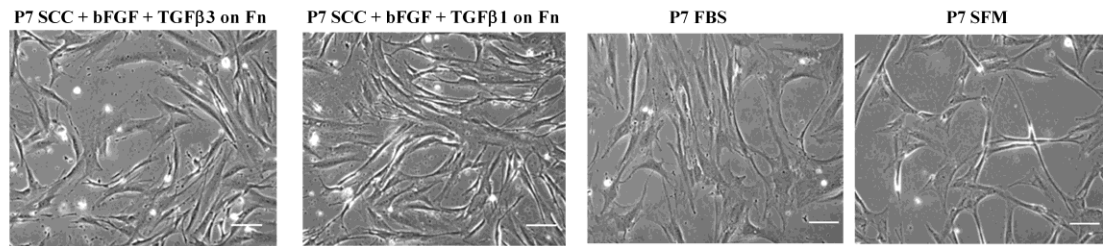
*Statistical significant difference between FBS and SCC with TGFβ3. * Statistical significant difference between FBS and SCC with TGFβ1. ** P value = 0.01; **** P value = 0.0001. Cells on SCC with TGFβ1 or β3 exhibit faster growth kinetics as compared to those on FBS and SFM: B) Fold increase in cell number with respect to the first passage (P5): SCC with TGFβ1 or β3

resulted in higher proliferation rates than FBS and SFM. **C)** Viability was assayed by the Tripa Blue exclusion method at each passage for each medium condition. No significant differences were noticed among different conditions. Data are expressed as mean $\pm$ SD of two counts for each condition. **D)** Theoretical scale up expansion experiment (half-split at each passage). Cells on SCC with TGF β 1 or β 3 have faster expansion rates as compared to FBS and SFM. Values are expressed as mean $\pm$ SD of two counts for each medium condition. * Statistical significant difference between FBS and SCC with TGF β 3. * Statistical significant difference between FBS and SCC with TGF β 1. ** P value = 0.01; *** P value = 0.001; **** P value = 0.0001.

V.3.2.2. Morphology and immunophenotype

The hMSCs expanded in the 4 different media formulations described above presented a normal spindle-shaped morphology [30], as observed by phase contrast microscopy (Fig.V. 4A). The expression profile of typical positive and negative hMSCs markers (CD105⁺, CD90⁺, CD73⁺, HLADR⁻, CD34⁻, CD45⁻ and CD19⁻) was analysed by Flow Cytometry (FC). Figure V.4B shows FC representative histograms hMSCs at P7, and the percentage of positive cells for each marker at passage 6, 7 and 8 for hMSCs expanded in SCC, FBS and SFM formulations is presented in Table V.1. In general, no significant differences were noticed on the expression of the different markers between cells expanded in SCC formulations and the FBS control. Cells in both SCC formulations kept high expression of positive markers (CD90, CD105, CD73), above 90% along P6, P7 and P8, and low expression (under 6%) of negative markers, (CD19, CD14, HLA-DR, CD45), a similar phenotype was observed for cells expanded in FBS. In SFM, although more than 90% of the cells expressed CD90 and CD73 and less than 8% expressed the negative markers, cells presented lower expression of CD105 (< 87%) when compared with FBS (> 90%), at P7 and P8.

A



B

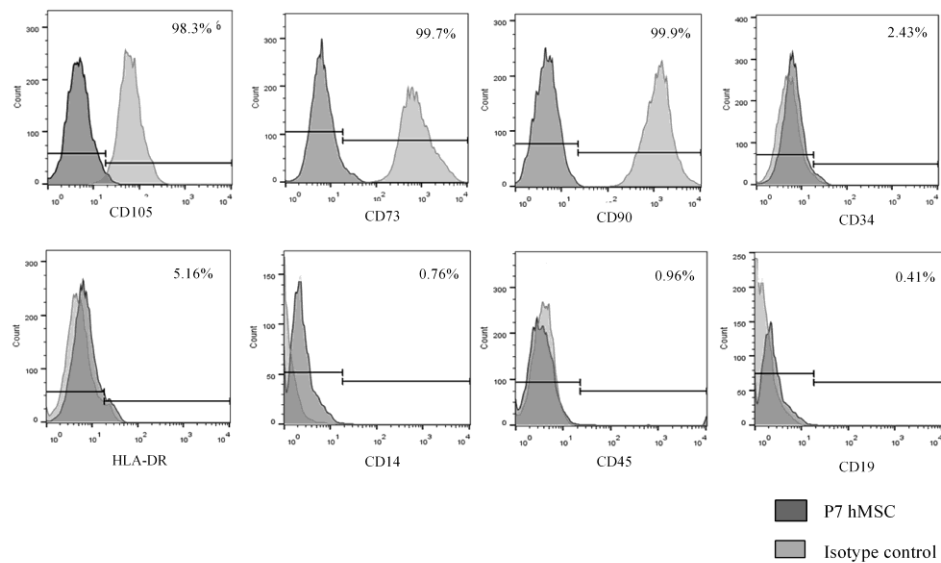


Figure V.4. hMSC characterization during expansion. **A)** Representative images of hMSC morphology at passage 7, monitored by phase contrast microscopy. Scale bar is 100 μ m. **B)** hMSC phenotype determined by flow cytometry. Representative histograms showing expression patterns of different markers for hMSC at P7 in SCC + FGF + TGF β 1 on Fn. Cells presented low or no expression of CD14, CD19, CD34, CD45 and HLA-DR, and high expression of CD73, CD90 and CD105. Control refers to isotype control antibodies.

Table V.1. Flow cytometry results. FC analysis of hMSC expanded along one (P6), two (P7) and three (P8) passages in FBS, SCC + FGF + TGF β 1 or β 3 on Fn and SFM. During three passages, cells presented low or no expression of negative markers CD14, CD34, CD45, CD19 and HLA-DR and high expression of positive markers CD73, CD90 and CD105. The expression pattern was similar among the different media formulation. *na*= not applicable.

Medium Condition	Cell passage	Positive markers for hMSC				Negative markers for hMSC			
		CD105	CD73	CD90	CD14	CD45	CD19	CD34	HLA-DR
SCC + bFGF + TGFβ3 on Fn (% positive cells)	P6	96	95.8	95.4	0.9	1.5	0.3	6.1	2.2
	P7	91.8	93.3	95.0	na	na	na	0.9	0.8
	P8	98.7	96.8	97.8	na	na	na	3.1	0.5
SCC + bFGF + TGFβ1 on Fn (% positive cells)	P6	91.2	98.8	98.6	0.8	0.9	0.4	1.9	2.3
	P7	98.3	99.7	99.9	na	na	na	2.4	5.2
	P8	98.3	96.7	99.9	na	na	na	2.4	0.6
SFM (% positive cells)	P6	86.1	95.6	93.6	0.7	0.6	0.1	7.4	1.0
	P7	91.7	93.3	99.3	na	na	na	0.9	5.7
	P8	85.1	94.7	95.3	na	na	na	4.7	2.7
FBS (% positive cells)	P6	96.7	97.9	95.7	3.1	1.1	0.1	10.1	2.1
	P7	93.1	96.3	99.2	na	na	na	4.9	2.1
	P8	90.7	93.5	96.4	na	na	na	0.8	0.3

V.3.2.3. Differentiation potential

hMSCs ability to undergo adipogenic, chondrogenic and osteogenic differentiation capacity after expansion in SCC with TGF β (3 or 1) and FBS (control), was evaluated after every passage in these media. For that, expanded hMSCs were cultured in commercial differentiation media to induce adipogenesis, chondrogenesis and osteogenesis [30].

Adipogenic differentiation confirmed by Oil Red O staining shows the presence of lipid vesicles (in red), indicators of successful differentiation, and occurred when hMSCs were expanded in the different media (Fig.V.5 a-f). Likewise, toluidine blue staining of cartilaginous matrix (in purple) in hMSCs pellet sections confirmed the chondrogenic

differentiation potential of these cells in all the media tested (Fig.V.5 g-l). In addition, ALP activity staining (pink) shows the osteogenic differentiation potential of hMSCs and indicates that cells previously expanded in FBS and SCC with TGF β 1 were able to differentiate along the osteoblastic lineage (Fig V.5 m-p). However, hMSCs expanded in SCC with TGF β 3 seem to have partially loosened their osteogenic potential after 3 passages (Fig V.5r). This suggests that only the formulation SCC + bFGF + TGF β 1 on Fn can be used to successfully expand hMSCs *in vitro* and in xeno-free conditions without affecting hMSCs differentiation potential.

Importantly, if cultured in any of the SCC media formulations along 14 days, hMSCs did not spontaneously (without induction media) differentiate into none of the three tested lineages (data not shown).

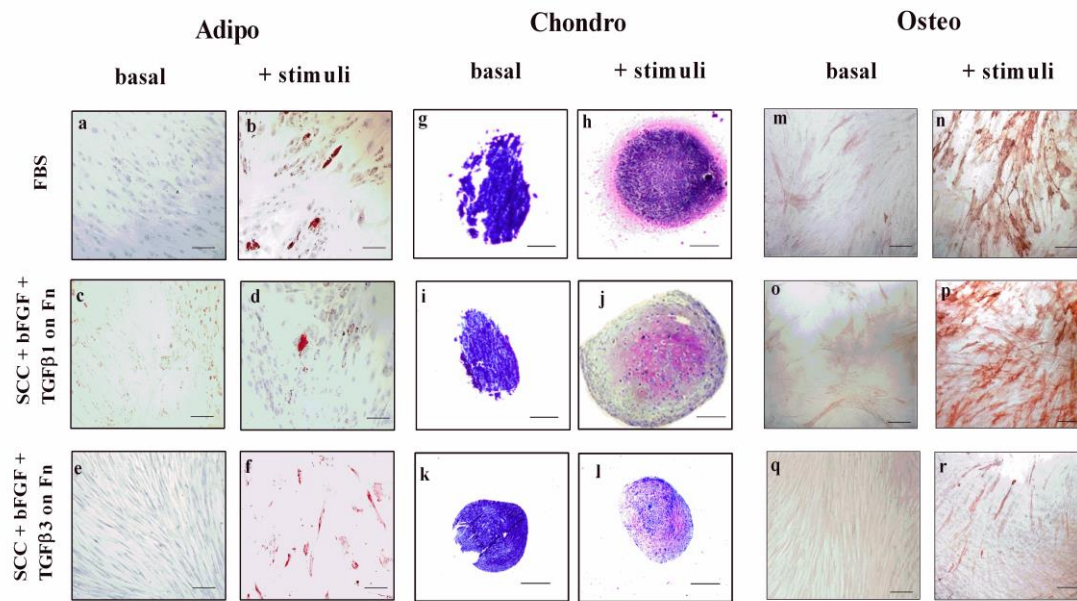


Figure V.5. Tri-lineage differentiation of hMSC cultured in different media. After 4 passages expansion, hMSC ability to differentiate along adipogenic, chondrogenic and osteogenic lineage was assayed. **a-f)** Oil Red O staining of cells previously expanded in FBS medium (a, b) , SCC +bFGF + TGFβ1 on Fn (c, d) and SCC + bFGF + TGFβ3 on Fn (e, f) . Cells were able to differentiate into adipogenic lineage when appropriately stimulated (right column) but not in basal conditions (left column). **g-l)** Toluidine Blue staining of histologically processed pellets of cells previously expanded in FBS medium (g, h) , SCC + bFGF + TGFβ1 on Fn (i, j) and SCC + bFGF + TGFβ1 on Fn (k, l). Cells were able to differentiate into chondrogenic lineage when appropriately stimulated (right column) but not in basal conditions (left column). **m-r)** ALP activity staining of cells previously expanded in FBS medium (m, n) , SCC +bFGF + TGFβ1 on Fn (o, p) and SCC + bFGF + TGFβ3 on Fn (q, r). Cells expanded in FBS or SCC +bFGF + TGFβ1 were able to differentiate along osteogenic lineage when appropriately stimulated (right column) but not in basal conditions (left column). However, after expansion in SCC + bFGF + TGFβ3 on Fn hMSC capacity to differentiate along osteogenic lineage decreased significantly. Scale bar is 100μm.

V.3.2.4. Karyotype evaluation

To confirm that long-term culture in SCC + bFGF + TGFβ1 on Fn did not affect chromosome stability of expanded hMSCs, cytogenetic analysis was performed by interface FISH for the input cells (P4) before expansion and for those obtained after 4 passages in the different media (P9) [31]. Targets of analysis were chromosomes 7, 12 and 18. As shown in Figure V.6, the percentage of polyploidy is very low for all hMSCs tested (< 2.5%). Moreover, no statistically significant differences were detected in terms of polyploidy neither aneuploidy between cells expanded in both media, demonstrating that no chromosome mis-segregation was noticed in hMSCs expanded in SCC + bFGF + TGFβ1 on Fn.

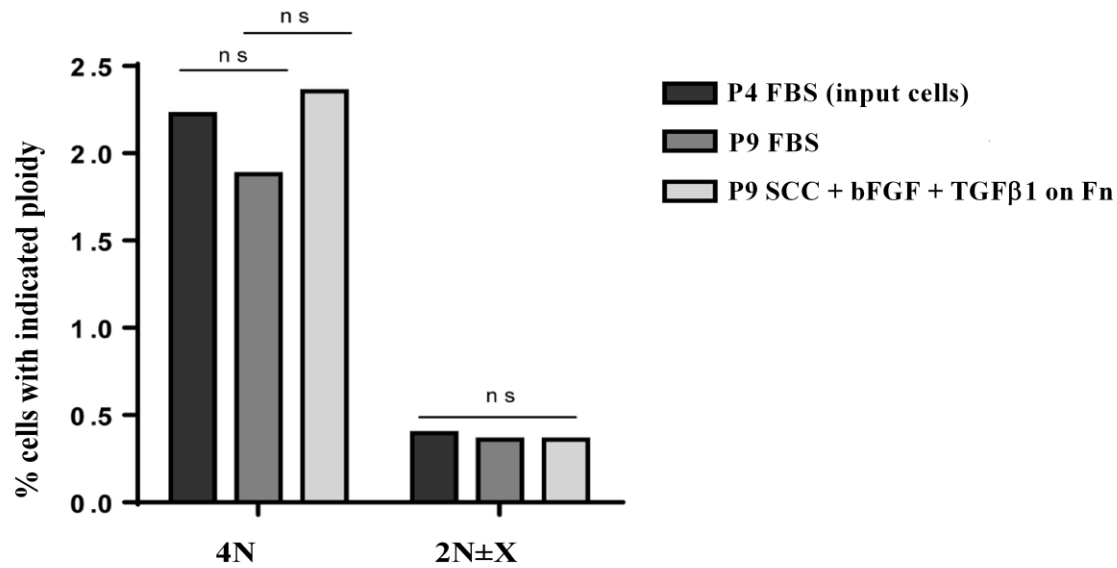


Figure V.6. Analysis of hMSC karyotype. Cells were labelled by a chromosome-specific FISH staining with probes for chromosome 7, 12 and 17. Cells were classified as having gained or lost 1-few chromosomes ($2 \pm X$ signals for each of the three chromosomes analysed) or as tetraploid (4 signals for each of the three chromosomes analysed). Comparing input cells (FBS P4) to cells expanded in FBS (P9 FBS) or in SCC+FGF +TGFβ1 on Fn (P9), no differences were noticed in terms of aneuploidy ($2N \pm 1$) and ploidy (4N).

V. DISCUSSION

The requirement of xeno-free strategies for hMSCs *in vitro* expansion is an urgent need, due to the increasing application of these cells as therapeutic agents. In alternative to FBS, “humanized” supplements and chemically defined media have been described as promising tools and already applied in some clinical trials [32]. Nevertheless, there is still a lack of standardized protocols to expand hMSCs that conform to good manufacturing practices and assure safety of the final product. SCC is a GMP-compliant pharmaceutical-grade supplement for cell culture under development that is safe and presents very low variability between batches. Therefore this work aimed to optimize a SCC-based xeno-free medium for hMSC *in vitro* expansion.

Starting from the observation that hMSCs cultured in medium supplemented only with SCC lose their typical elongated morphology, and knowing that attachment dependent cells, such as MSCs, need to bind to adhesive proteins to proliferate, we decided to test the effect of an adhesion protein coating on hMSCs morphology. Among adhesion proteins, Fn is recognized to play a key role in cell adhesion, through engagement of cell-membrane integrin receptors [33]. Indeed, most of the commercial xeno-free chemically defined

media for hMSCs expansion require pre-coating of culture plates with an ECM protein-enriched solution (e.g. CELLStart). In our study, human Fn coating induced hMSCs attachment improving their morphology in 24 hours (Fig. V.1A), as previously described by others [28]. Our results also showed an increase of hMSCs metabolic activity along 6 days on SCC + Fn coating (Fig. V.1C) when compared to the uncoated surface, where the amount of adsorbed Fn from SCC is probably insufficient, as suggested by the ELISA results (Fig. V.1B).

Furthermore, it is well known that cells in culture are influenced by different soluble factors, many of them present in serum, which bind to cell surface receptors and initiate downstream signalling leading to different cell responses [34]. In fact, most of the protocols for hMSCs expansion under xeno-free conditions include media supplementation with soluble elements such as growth factors and hormones, to improve cell growth, especially those that involve chemically defined media [35] or human serum [36]. Here, the influence of two growth factors as supplements for SCC medium were tested and we observed that bFGF and TGF β (either 1 or 3) acted synergistically on hMSCs growth, as previously described by Ng *et al.*[29] but in combination with PDGF. Our results demonstrated that hMSCs cultured in SCC + bFGF with TGF β (1 or 3, on Fn) reached higher growth rate and cell yields than hMSCs cultured in canonical FBS medium or commercial available serum-free medium (SFM), maintaining high cell viability along 4 expansion passages (Fig V.3).

Moreover, hMSCs phenotype was characterized after expansion along 4 passages in SCC medium formulation, following the criteria established from the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [30]. Flow Cytometry analysis of cells at every passage indicate a homogenous population expressing CD105, CD90, CD73 surfaces markers, but not HLA-DR, CD34, CD14 and CD19. Actually, after one passage expansion (P6) in SCC with TGF β 1 the expression of CD105 was under 95% but at the following passages it increased until 98%, so we do not consider this data particularly alarming. It was surprising to notice that in cells expanded in SFM the expression of CD105 was less than 90%, after one and three passages expansion. Since this result is slightly different from those reported on the commercial brochure of SFM (in which CD105 expression is reported to be 99% after 3 passages), we speculated that our result might be an artefact of our specific culture conditions. Nevertheless, it was already reported that hMSCs displayed lower CD105 expression in commercial available serum free medium as compared to hMSCs expanded in FBS containing medium [37].

Furthermore, hMSCs were able to differentiate along the adipogenic and chondrogenic lineages (Fig.V.5 a-l) when expanded in xenofree SCC based media with TGF β 1 or TGF β 3. They were also able to differentiate in osteogenic lineage in SCC medium supplemented with TGF β 1 (Fig.V.5o, 5p). However, with TGF β 3, a decrease in osteogenic differentiation after 3 passages was noticed (Fig.V.5q, 5r). This observation is in concordance with the findings of Moiola and colleagues [38], according to whom mineral deposition was progressively decreased in hMSCs osteogenic cultures with increasing TGF β 3 concentrations over a 28 days period. In our study, this effect was cumulative, as we observed a decreased expression of ALP activity along subsequent passages. Moreover, it was recently suggested that TGF β 3 prevents phosphate-induced osteogenesis through inhibition of BMP and Wnt/ β -Catenin Pathways [39].

The stability of cell karyotype upon cell expansion in SCC formulations was also evaluated. Diploid human cells frequently accumulate chromosomal abnormalities when cultured under standard condition [40]. Indeed, some studies described accumulation of chromosome instability and generation of carcinoma in immunocompromised mice after MSCs long-term culture [41]. To evaluate genomic stability of hMSCs after expansion in SCC + bFGF + TGF β 1 on Fn, we analysed the karyotype in interphase at passage 4 and passage 9 by iFISH. The application of specific centromeric probes for fluorochrome-labeled chromosome 7, 12, 17 let us distinguish between diploid cells (two signal per cell and probe), or aneuploidy (more or less than two signals per cell and probe). We did not find difference in terms of aneuploidy or polyploidy between input cells and cells expanded either in FBS or in SCC+ bFGF + TGF β 1 on Fn (Fig.V.6). Thus, we could conclude that hMSCs cultured on our optimized formulation (SCC + bFGF + TGF β 1 on Fn) did not experience chromosome mis-segregation and kept balanced karyotype. This is rather important if hMSCs expansion is intended for clinical application since aneuploidy is a hallmark of oncogenic transformation.

In conclusion, we have optimized a suitable strategy for hMSCs expansion in a xeno-free setting by the application of a new, safe, ethical acceptable and consistent cell culture medium under development, containing a GMP-compliant FBS substitute that could broaden the clinical application of hMSCs as therapeutic agents.

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CHAPTER VI

Effect of surface chemistry on hMSC growth in xeno-free conditions

Abstract

Human Mesenchymal Stem cells (hMSC) are adult stem cells with excellent capacities for regenerative medicine being currently applied as therapeutic agents in many clinical trials. However, since in the human body hMSC are in few numbers, cell *in vitro* expansion is necessary. During cell culture, surface chemistry strongly influences cell behavior, mainly by modulation of protein adsorption at the cell-material interface. In this work, self-assembled monolayers (SAMs) were used as model surfaces to investigate the effect of hydrophobic (-CH₃) and hydrophilic neutral (-OH), positive (-NH₃⁺) and negative (-COO⁻) charged groups on hMSC *in vitro* growth. Cells were cultured either in Foetal Bovine Serum (FBS) containing medium or in SCC-based xeno-free medium, with or without fibronectin (Fn) pre-coating. SCC is a pharmaceutical grade human plasma fraction recently described as FBS substitute for hMSC expansion.

Results indicate that in FBS containing medium and without Fn coating, hMSC growth follows the trend OH = COOH = NH₂ >> CH₃, since hydrophilic surfaces promote a better serum-borne proteins adsorption than hydrophobic ones. In SCC-based xeno-free medium, without Fn coating, growth was significantly reduced on all the surfaces tested in comparison with TCPS. Nevertheless, hMSC growth was increased upon Fn coating, being NH₂-SAMs the most favorable surface. These results point out that, although mediated by adsorbed proteins, surface chemistry is very important on hMSC behavior, even for longer times in culture, when cells are producing their own extracellular matrix (ECM).

VI.1. INTRODUCTION

hMSC are a promising source for regenerative medicine applications. Their use as therapeutic agents requires cells expansion *in vitro* and, frequently, implantation into patients within a biomaterial. Thus, in the recent years many efforts have been directed either for the optimization of cell culture conditions or for the construction of advanced biomaterials for cell delivery. In nature, cell function is strictly dependent on the

interaction with the Extracellular Matrix (ECM), the non-cellular component within all tissues and organs that furnish physical scaffolding for cells and induces biochemical and biomechanical signals modulating tissue morphogenesis, differentiation and homeostasis [1]. Similarly, both substrates for *in vitro* cell culture and surfaces of implant biomaterials should promote cells specific responses in a controllable manner. In fact, many studies demonstrated how behavior of cells is influenced from the inherent physic-chemical properties of the surface, such as roughness, morphology, chemistry (energy, polarity, wettability) and composition [2-5]. Furthermore, it is well known that when a surface enters in contact with plasma or serum, it will be immediately coated by a layer of adsorbed proteins, which will influence cell adhesion and growth (21). Surface properties strongly influence protein adsorption, in terms of quality, quantity, orientation and conformation [5-7]. This in turns, generate different cell responses to different substrate composition [6, 8, 9].

In the last years, several concerns raised regarding the use of Foetal Bovine Serum (FBS) during hMSC *in vitro* expansion since it carries the possibility to transfer animal proteins (xenoantigens) [10] or unknown pathogens to patients [11], among other limitations (Chapter IV).

Our previous results (Chapter V), showed that SCC-based xeno-free medium may be a suitable FBS-substitute for hMSC *in vitro* expansion. SCC is a pharmaceutical grade product derived from human plasma pools and produced in GMP settings. Being hMSC attachment dependent cells, SCC-based xeno-free medium required a pre-coating with human recombinant Fibronectin (Fn) to allow cells to attach on Tissue Culture Polystyrene (TCPS).

This work aims to analyze whatever a different surface chemistry may abolish the need for Fn coating in SCC-based xeno-free hMSC culture. At the same time, we aimed to evaluate if exposition of different groups at the surface may influence hMSC proliferation either in FBS or SCC-based xeno-free medium. To this aim, self-assambled monolayers (SAMs) of alkanethiols on gold were used as model surfaces. SAMs are very powerful tools to study the interface between a material surface and the biological system, since, besides being very easy to prepare, their chemical properties can be controlled at molecular level (12-14). In this work, SAMs terminating with either hydrophobic ($-\text{CH}_3$) and hydrophilic neutral ($-\text{OH}$), positive ($-\text{NH}_3^+$) and negative ($-\text{COO}^-$) charged groups were used.

VI.2 MATERIALS AND METHODS

VI.2.1 Materials

Gold substrates (1x1 cm²) were purchased at INESC-MN, Portugal and were prepared as described elsewhere [12]. Thiols were purchased by Sigma-Aldrich: 11-mercapto-1-undecanol thiol (OH-thiol; HS(CH₂)₁₁OH, 97%; Ref:674249), 1-hexadecanethiol (CH₃-thiol; HS(CH₂(CH₃))₁₅ 99%; Ref:674516), 16- mercaptohexadecanoic acid (COOH-thiol; HS(CH₂)₁₅CO₂H, 90%; Ref:448303) and 11-Amino-1-undecanethiol hydrochloride (NH₂-thiol; HSCH₂(CH₂)₉CH₂NH₂ · HCl, 99%; Ref: 674397).

Fibronectin solution from human plasma (Fn; Ref:F0895) was also purchased by Sigma Aldrich.

hMSCs isolated from bone marrow of healthy 21-year-old black male were purchased by Lonza (PT2501). Being cells commercially available, no patient consent or approval from Ethics Committee was needed.

Supplement for cell culture (SCC) was gently provided from Grifols S.A. (Parets del Valles, Barcelona) and is a product in development derived from human plasma [13] through Cohn-Oncley fractionation [14]. Xeno-free SCC-based medium is constituted by 15% SCC in in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 *DMEM/F-12*, no glutamine (Ref:21331-020, ThermoFisher Scientific) + 1% of Insulin, Selenium and Ethanolamine (ISE) + 10ng/ml Transforming Growth Factor beta 1 (TGFβ1) + 20 ng/ml basic Fibroblast Growth Factor (bFGF) and Penicillin/Streptomycin (P/S). This medium was previously optimized and described by us (Chapter V). FBS medium is constituted by MSCs certified FBS (ThermoFisher Scientific, Ref:10094563) at 10% in DMEM, low glucose, GlutaMAXtm supplement pyruvate (Ref:21885-108, ThermoFisher Scientific) plus 1% P/S.

VI.2.2. Methods

VI.2.2.1 Gold substrates

Just before used, gold substrates were cleaned with fresh “piranha” solution (7 parts concentrated sulfuric acid (95 vol.%, BDH Prolabo), 3 parts hydrogen peroxide (30 vol. % Merck) for 5 min (*CARE: this solution reacts violently with many organic materials and should be handled with care*). They were then rinsed sequentially with ethanol (99.8%,

Merck), deionized and distilled water and ethanol in ultrasonic bath for 2 min. They were dried with a gentle argon stream and then immersed in the thiol solutions.

VI.2.2.2. Monolayer formation

Thiol solutions with a final concentration of 1mM were prepared in ethanol (99.8% Merck) for OH and CH₃-SAMs, basic ethanol (prepared by addition of drops of Ammonium Hydroxyde solution (Sigma) until reach pH~12) for NH₂-SAMs or acidic ethanol (prepared by addition of drops of pure chloric acid (BDH Prolabo) until reach pH~2) for COOH-SAMs [15]. Solutions were prepared in a nitrogen environment.

Freshly cleaned gold substrates (VI.2.2.1) were incubated into thiol solutions along 24 h, at room temperature, in a nitrogen environment. After incubation, monolayers were rinsed 3 times along 2 min in ultrasonic bath with fresh ethanol (neutral for OH and CH₃-SAMs, basic for NH₂-SAMs and acidic for COOH-SAMs) according to [15]. Monolayers were flowed with a stream of argon and kept in a nitrogen-filled container until use.

VI.2.2.3 SAMs characterization

VI.2.2.3.1 Contact angle measurements

Contact angle measurements were performed with a contact angle measuring system from Data Physics, model OCA 15, equipped with a video CCD-camera and SCA 20 software. Measurements were performed with the sessile drop method as describe elsewhere [12]. After 4 μ L drop deposition of MilliQ water, images were taken every 2s over 90s. The contact angle for each time was calculated using the ellipse method. The water contact angle for each SAM was calculated by extrapolating the time dependent curve to zero.

VI.2.2.3.2 Ellipsometry

Ellipsometry measurements were performed using an imaging ellipsometer, model EP3 from Nanofilm Surface Analysis. This ellipsometer was operated in a polarizer-compensator-sample-analyzer (PCSA) mode (null ellipsometry). The light source was a solid-state laser with a wavelength of 532 nm. The gold substrate refractive index (n) and extinction coefficient (k) were determined by using a delta and psi spectrum with a variation of angle between 65 and 75°. These measurements were made in four zones to correct for any instrument misalignment. Results are the average of eight measurements on each of the three samples for each type of SAM.

VI.2.2.3.3 X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed using a Kratos Axis Ultra HSA spectrometer (at CEMUP- Centro de Materiais da Universidade do Porto) using a monochromatic Al ($K\alpha$) X-ray source operating at 15kV (90 W). Analyses were performed at 10 nm from the surface using 70° take-off angle. Survey spectra over a range of 0-1150 eV were collected with analyser pass energy of 80 eV. High resolution C (1s) and S (2p) spectra were collected with analyser pass energy of 40 eV. Spectra were fitted using the CASAXPS software and fitted by setting the maximum of the resolved C (1s) peak to 285.0 eV.

VI.2.2.4 Cell culture

In this study, BM-hMSC were maintained in culture at 37 °C and atmosphere of 5% CO₂ in DMEM + 10% FBS. Full culture media change was performed twice a week and cells were split by trypsinization when they reached 70-80% confluence. Cells passage 6 and 7 were used in this study.

VI.2.2.5 Cell metabolic activity analysis

For metabolic activity evaluation, hMSCs (P6) were split and seeded at 3000 cells/cm² in 24-well suspension culture plates (Sarstedt, ref. 83.3922.500) on TCPS discs, Au surfaces and OH-, CH₃-, COOH- and NH₂-terminated SAMs. For serum containing condition, cells were cultured in FBS medium. For xeno free condition without coating, cells were cultured in xeno-free SCC based medium. For xeno-free condition with coating, surfaces were coated with 20 µg/ml of human recombinant Fn in phosphate buffer saline (PBS, PH 7.4) for 1 h at 37 °C. Then, surfaces were washed 3 times with PBS to remove non-adsorbed Fn and hMSC were seeded and cultured in xeno free SCC based medium.

At days 0 (4 hours after seeding) 1, 3, and 5 cell metabolic activity was assayed by Resazurin assay as described elsewhere [16]. Assays were performed in triplicate.

VI.2.3 STATISTICS

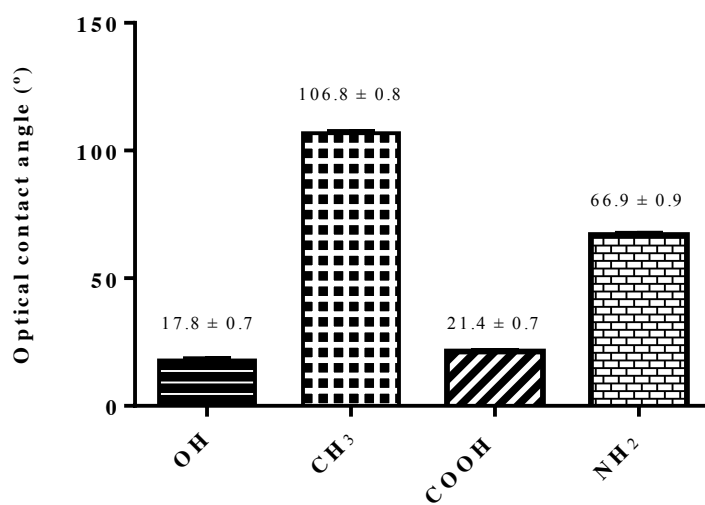
To determine statistical significance of Resazurin assays, a Two-Way ANOVA test for multiple comparisons was performed.

VI.3. RESULTS

VI.3.1 Surface characterization

Surface characterization, performed using water contact angle measurements and ellipsometry, indicates achievement of the desired chemical moieties on the surfaces (Fig. VI.1). In fact, the lower and the higher water contact angle (Fig. VI.1A) obtained for OH- and CH₃-SAMs, respectively, demonstrated the hydrophilicity and hydrophobicity expected for these surfaces [12]. Water contact angles values for COOH-SAMs and NH₂-SAMs are also in agreement with previously published data [7]. The values obtained by ellipsometry (Fig. VI.1B) indicated a higher thickness for CH₃ and COOH-SAMs (1.8 nm) and lower for OH and NH₂-SAMs (1.5 nm), as expected, since OH- and NH₂-terminated thiols present alkane chains of 11 carbons while those of CH₃- and COOH-terminated thiols have 16 carbons.

A



B

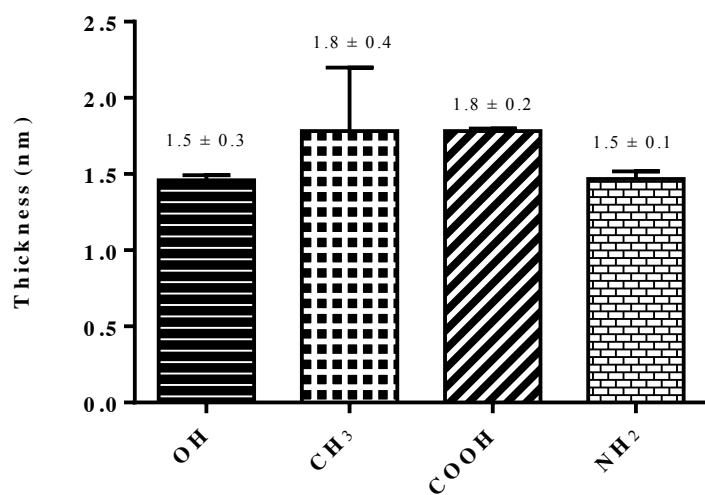


Figure VI.1. Surface characterization of SAMs surfaces as determined by A) water optical contact angle measurement; B) ellipsometry (surface thickness).

Results from XPS survey demonstrated that only the expected elements were detected on SAMs surface indicating that no contamination took place. (Fig.VI.2)

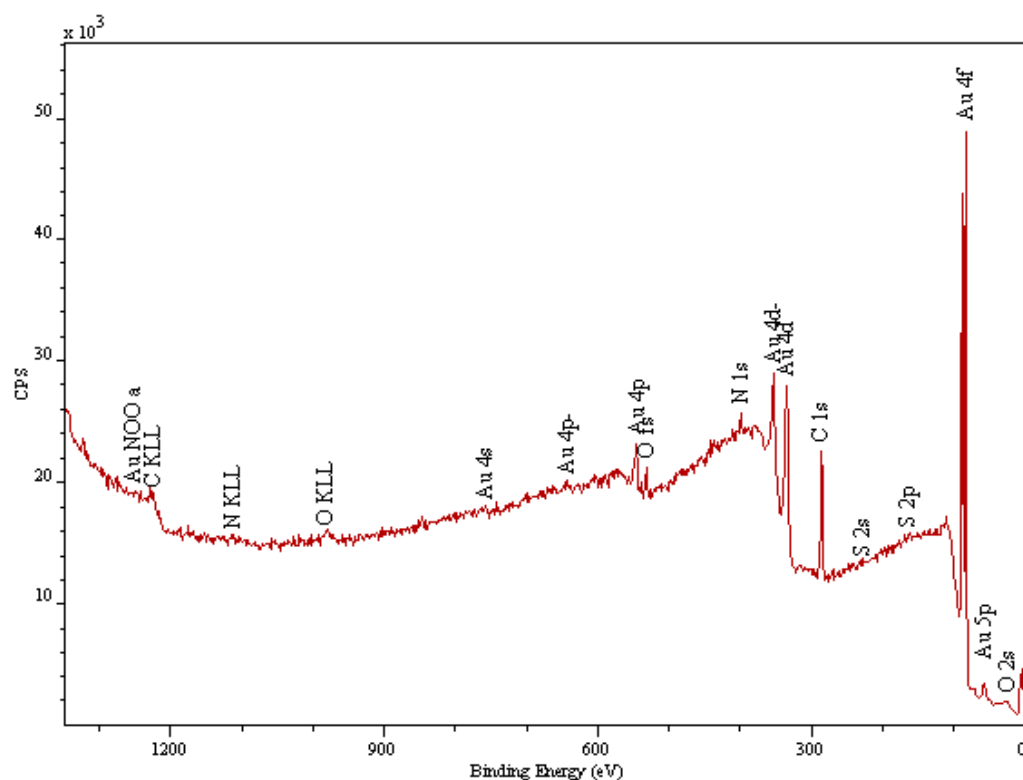


Figure VI.2. Representative spectrum survey of XPS analysis (NH₂ SAM).

Table VI.1. Relative surface atomic composition of SAMs as determined by XPS analysis

Element	Relative Atomic Percentage (%)			
	HS(CH ₂) ₁₁ OH	HS(CH ₂) ₁₅ CH ₃	HS(CH ₂) ₁₅ COOH	HS(CH ₂) ₁₁ NH ₂
C 1s	86	98	83	85
N 1s	ND	ND	ND	5
O 1s	11	ND	15	8
S 2p	3	2	2	2

*ND= not detectable

** All data were calculated after gold percentage subtraction.

Nitrogen (N) content was only detected on NH₂-SAMs confirming the successful immobilization of the desired chemical moiety. Oxygen (O) atomic percentage was higher in COOH-SAMs than OH, very low in NH₂-SAMs while no oxygen was detected in CH₃ – SAMs. Residual values of oxygen are usually found on all SAMs [12]. Also, carbon (C) content was higher on CH₃-SAM since, as a relative percentage, this surface is only composed by carbon and sulphur. Finally, sulphur (S) from thiol groups was present in all samples.

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Relative atomic percentage of the expected elements obtained by XPS spectra of SAMs are described at Table VI.1.

C spectra

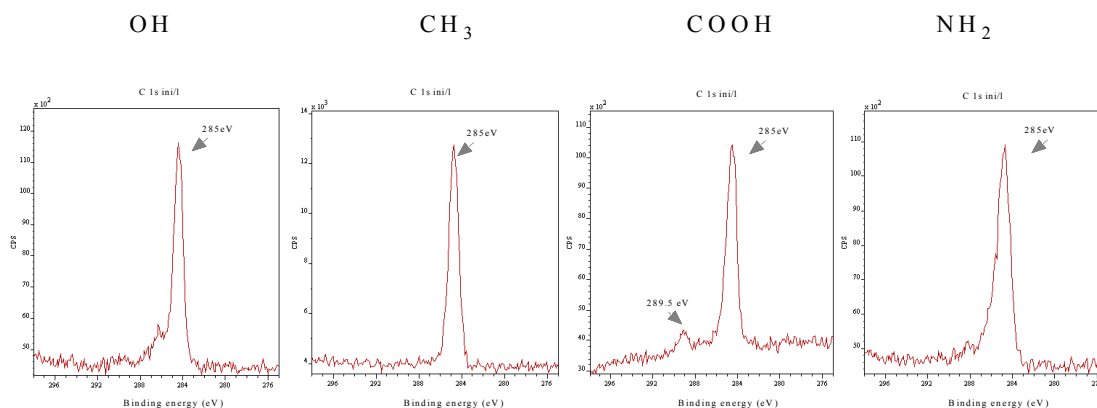


Figure VI.3 C (1s) high resolution spectra for OH, CH₃, COOH and NH₂ SAMs surfaces.

Fig.VI.3 shows XPS high resolution spectra of C1s for all samples. C1s binding energy of the CH₃-SAM was centred at 285 eV, which corresponds to carbon bond to other carbon (C-C). On OH-SAMs and NH₂-SAMs, an additional C1s peak at 286.7 eV that is assigned to C–O and C–N bond was observed. On the COOH-SAM another C1s peak was present at 289.5 eV which corresponds to the O=C–O bond [12].

S spectra

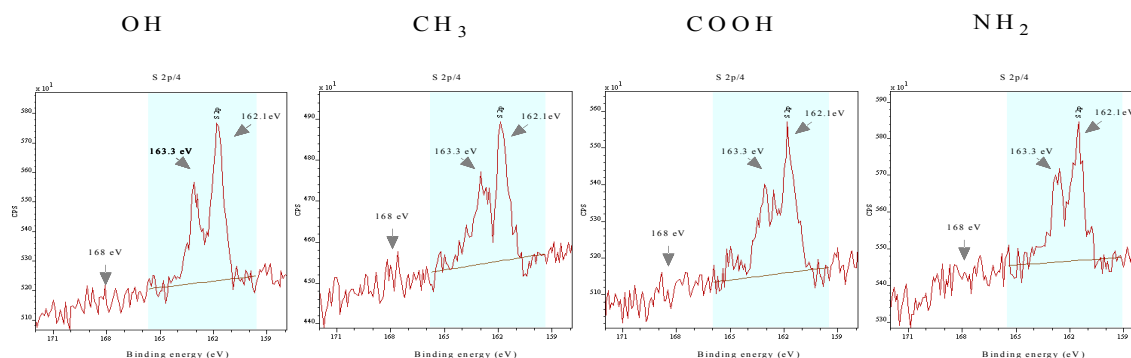


Figure VI.4 S (2p) high resolution spectra for OH, CH₃, COOH and NH₂ SAMs surfaces.

S2p high-resolution spectra are as doublet structure centred at 162.1 and 163.3 eV for S bond to gold [17]. No detectable intensity is present in the Binding Energy

region ~ 168 eV in all the SAMs studied showing that no oxidized sulphur was present in all SAMs and thus, that monolayers were correctly organized/oriented.

VI.3.2 hMSC culture on SAMs

VI.3.2.1. FBS medium

Fig. VI.5 shows metabolic activity of hMSC cultured in FBS medium, on SAMs, and controls (TCPS and Au) without any pre-coating.

A



B

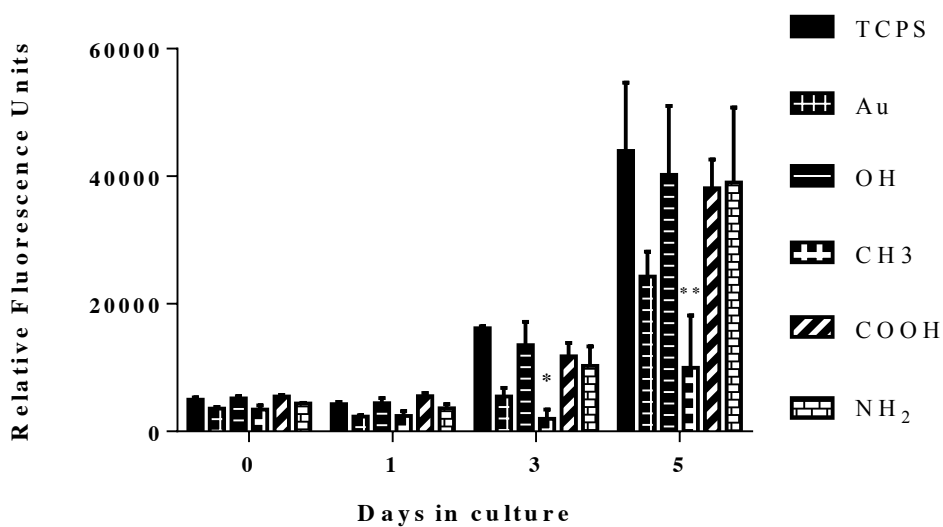


Figure VI.5. hMSC behaviour on different surfaces in FBS medium.

A. hMSC adhesion as assayed by Resazurin assay 4 hours after seeding (day 0).

B. hMSC growth evaluated by Resazurin assay at days 0, 1, 3 and 5. Values are expressed as mean $\pm$ SD of three counts on three samples for each condition. * Statistical significant difference as compared to TCPS. * P value = 0.05; **P value = 0.001

hMSC metabolic activity was not improved on SAMs and Au as compared to TCPS. However, looking at cells metabolic activity on SAMs, 4 hours after seeding (Fig.VI.5A), adhesion follows the trend $\text{OH} = \text{COOH} > \text{NH}_2 \geq \text{CH}_3$. This is in agreement with the literature since hydrophilic (OH) and negative charged (COOH) surfaces promote a superior cell attachment and cytoskeleton organization than hydrophobic ones, as CH_3 -SAMs [18].

As regards cell growth along 5 days in FBS medium (Fig.VI.5B), significant differences were not noticed among TCPS, OH, COOH and NH_2 surfaces. However, cell growth was significantly lower on hydrophobic surfaces (CH_3), as previously reported for osteoblasts and MSC [7, 19].

VI.3.2.2 Xeno-free medium with and without Fn coating

Fig. VI.6 depicts hMSC metabolic activity 4 ours after seeding, in SCC-based xeno-free medium, with and without Fn coating.

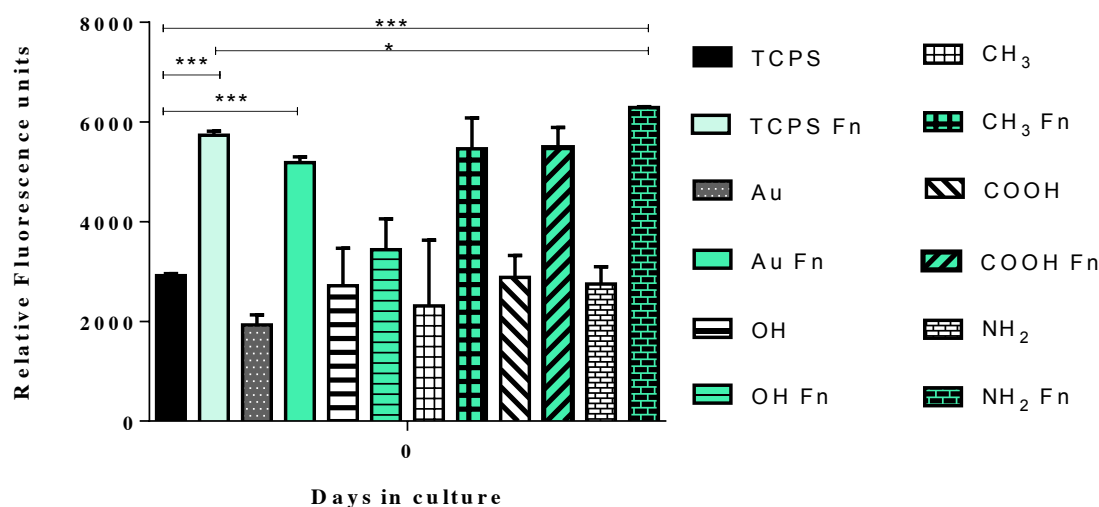


Figure VI.6. hMSC adhesion on different surfaces, with (green background) and without (white background) Fn coating on xeno-free medium as analysed by Resazurin assay 4 hours after seeding (day 0). Values are expressed as mean $\pm$ SD of three counts on three samples for each condition. * Statistical significant difference as compared to TCPS. * P value = 0.05; ** P value = 0.01; *** P value = 0.001.

Regarding cell growth in xeno-free medium, without Fn coating, we did not noticed differences in terms of hMSC adhesion on the different surfaces. When surfaces were pre-

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coated with Fn, adhesion increased as compared to no-coated surfaces in all the surfaces, except on OH-SAMs (Fig.VI.6).

With respect to cell growth, with and without Fn coating, none of the surfaces used improved the effect of TCPS on hMSC growth (Fig. VI.7). Without Fn coating, CH₃-SAMs was even refractory to cells growth at day 3 (Fig.VI.7A).

hMSC growth on Fn coated SAMs followed the trend $\text{NH}_2 \geq \text{COOH} \sim \text{OH} \gg \text{CH}_3$, with positive charged surfaces (NH₂) exerting a much higher proliferative effect as compared to hydrophobic ones (CH₃) (Fig.VI.7B). hMSC growth in OH-SAMs, that was not affected by Fn coating at day zero, was able to recovered in relation to CH₃-SAMs after 3 and 5 days culture.

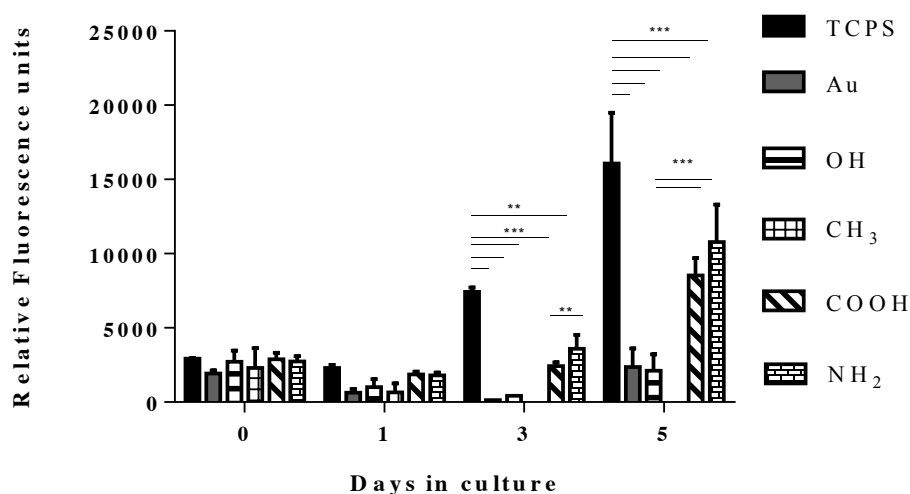
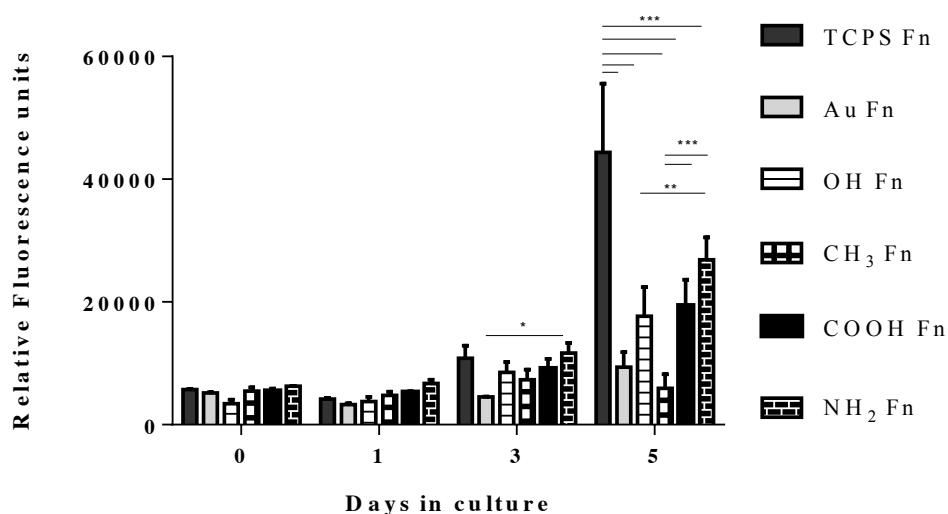
A**B**

Figure VI.7. hMSC growth on xeno-free medium on different surfaces, along 5 days without (A) or with (B) Fn coating. Values are expressed as mean $\pm$ SD of three counts on three samples for each condition. * Statistical significant difference as compared to TCPS. * P value = 0.05; ** P value = 0.01; *** P value = 0.001.

3.1.4. DISCUSSION

The physical/chemical properties of a cell culture surface have dramatical effects on cell behavior [5, 8]. In this work, SAMs were used as model surfaces to investigate the role of different exposed functionalities (hydrophobic and hydrophilic neutral, positive and

negative charged) on hMSC adhesion and growth. Different SAMs functionalities were well characterized using different characterization techniques. SAMs are well defined surfaces that are easy to prepare and whose properties can be tailored to study cell interactions with different chemical moieties or biomolecules [9]. When cells were cultured in FBS containing medium, adhesion follows the trend $\text{OH} = \text{COOH} > \text{NH}_2 \geq \text{CH}_3$, and growth was similar between OH, COOH and NH_2 -SAMs while impeded on CH_3 -SAM. This is probably related to the favorable effect of hydrophilic and charged surfaces on the adsorption of cell adhesive proteins from FBS (e.g. vitronectin and fibronectin). Furthermore, after adsorption and depending on the surface physicochemical characteristics, proteins may adopt a geometrical conformation that expose specific binding sites to cell adhesion receptors, such as integrins [20, 21]. On hydrophobic surfaces (CH_3 -SAM) instead, proteins are adsorbed in denaturated or rigid forms, that impede the exposure of their cell-binding domains or avoid cellular interactions [22, 23]. In fact, in a comparative study, cells adherent to OH surfaces holded the highest levels of protein recruitment for focal adhesions formations (talin, α -actinin and paxillin), NH_2 and COOH surfaces presented intermediate levels while CH_3 surfaces exhibited the lowest level [22]. Moreover, hydrophobic surfaces could adsorb from serum non-adhesive proteins as albumin that could avoid cell-binding to surfaces [24].

However, as compared to TCPS, none of the chemistries studied increased hMSC adhesion and growth. This may be related to the fact that TCPS is already optimized for cell culture in FBS-containing medium.

In parallel, in xeno free culture, we could clearly notice the influence of surface adsorbed proteins on hMSC behavior, since we analyzed cell growth with and without coating with one of the most important ECM protein for cell adhesion: Fn. It was previously demonstrated (chapter V), that hMSC in xeno-free SCC-based medium require a Fn coating to adhere to TCPS. In this study, cell growth without Fn coating was low on all surfaces studied. Instead, when SAMs were Fn-coated, cells adhesion increased in all surfaces used except in OH-SAMs. As a matter of fact, it is in general agreed that Fn adsorption, from a pure solution, is enhanced in surfaces exposing NH_2 , COOH and CH_3 groups and reduced in OH surfaces [8, 9]. Moreover, it has been also reported that surface chemistry alters protein adsorption kinetics, competition and the structure of adsorbed Fn [25], modulating the presentation of Fn integrin binding domain and thus altering cell adhesion strength [7]. Indeed, in this report, departing from a trend of $\text{OH} = \text{COOH} > \text{NH}_2 \geq \text{CH}_3$ (4 hours in FBS medium) cell adhesion changed to $\text{NH}_2 \geq \text{COOH} \sim \text{CH}_3 \gg \text{OH}$, upon

4 hours in Fn coating and in xeno-free medium (where the competition for adsorption with other proteins may be reduced as compared to serum containing medium) and $\text{NH}_2 \geq \text{COOH} \sim \text{OH} \gg \text{CH}_3$ after 5 days. Interestingly, the effect of surface chemistry with and without Fn coating was well visible until day 5, even if surfaces were already covered by ECM produced by hMSCs.

However, none of the surfaces tested had a better influence on cell adhesion/growth than TCPS Fn, confirming the need of Fn pre-coating with SCC based xeno-free medium (Chapter V).

In conclusion, this work highlights the importance of surface chemistry on hMSC *in vitro* culture and/or implantation within a biomaterial. Future perspectives of this study concern the influence of surface functionality on other phenotypic aspects of hMSC, such as differentiation, long-term expansion and gene expression.

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CHAPTER VII

Enhancement of hMSC *in vitro* proliferation by surface immobilization of a heparin-binding peptide

Abstract

In vitro cells expansion is a crucial step for the use of human Mesenchymal Stem Cells (hMSC) as therapeutic agents for clinical advanced therapies. In the last years, research has been conducted to optimize hMSC cell culture protocols by mimicking cell physiological micro-environment. *In vivo*, cell behaviour is dependent on the signals coming from the Extracellular Matrix (ECM). Heparin related molecules, that are abundant in ECM, can bind several growth factors (e.g. basic Fibroblast Growth Factor (bFGF) and adhesive proteins (e.g. Fibronectin (Fn)). In this work, the effect of a heparin binding peptide, poly(Lysine/Leucine) (pKL), on hMSC adhesion and growth, was studied upon its immobilization on self-assembled monolayer (SAMs).

Cell cytoskeleton formation and growth were significantly increased in pKL-surface in comparison with control surface (tissue culture polystyrene - TCPS), demonstrating the high potential of this strategy to improve hMSC *in vitro* expansion.

VII.1 INTRODUCTION

Human Mesenchymal Stem Cells (hMSC) are very promising for applications in regenerative medicine, due to their high multipotency capacity and immunomodulatory properties [1].

However, hMSC *in vitro* expansion is a mandatory step for their clinical application, that still needs to be improved (chapter IV).

Cell behaviour depends on the multiple signals belonging from the environment in which cells are placed, whose main component is the Extracellular Matrix (ECM). Besides its function as mechanical support, ECM plays an important biochemical role by *direct* interaction with cells or *indirectly* by non-canonical growth factors presentation [2]. ECM adhesive proteins (e.g. fibronectin, collagen) contain highly conserved domains that bind to

cell adhesion receptors. Moreover, glycosaminoglycans - GAGs (e.g. heparin, heparan sulphate) recruit heparin-binding molecules, such as adhesive proteins and soluble growth factors, to the proximity of cell-membrane, amplifying signal transduction [3]. Importantly, once bound to the ECM, growth factors may become “solid phase ligands” insoluble, inactive or not bioactive molecules released by remodelling of the matrix, following the action of enzymes such as metalloproteinases [3]. Thus, ECM can also be considered a reservoir of growth factors. This is particularly relevant for stem cells, since *in vivo*, they are located in unique and specialized niche where ECM constitutes the essential player [4]. Thus, one strategy to improve hMSC culture *in vitro*, consists in the use of ECM components as inducers of cell growth and adhesion.

Heparin and heparin sulphate act as co-receptors of growth factors, controlling the stability and retention at the cell surface and mediating their transport via endoglycosidase (heparanase) [5]. For example, they act on Fibroblast Growth Factor (FGF) at two levels: i) regulating the strength and time of FGF signaling by modulation of FGF dimerization and binding to FGF-receptor [6]; ii) defining the diffusion potential of FGF signaling through the ECM [7].

For its capacities, heparin found application in hMSC research both in biomaterial design and in cell culture strategies for expansion. In fact, many biomaterials incorporate heparin (reviewed in [8]), with the scope of attracting growth factors, avoiding their degradation and enhancing their interactions with cell membranes receptors [9].

The effect of heparin as soluble cell culture supplement on hMSC behavior is controversial since either, growth promoting [10] and growth reducing effects [11] have been reported. These discrepancies could be related with differences in heparin concentrations [12].

Alternatively, Chang et al. used hydrogels mimicking heparin moieties to increase long-term expansion of human Pluripotent Stem Cells (hPCS) [13]. Furthermore, covalent immobilization of a small heparin-binding peptide together with an integrin binding peptide onto a cell culture plate enhanced hMSC *in vitro* adhesion and proliferation, by sequestration of serum-borne heparin to cell-material interface [14].

Poly(Lysine/Leucine) (pKL) capacity to bind heparin from plasma, after its immobilization onto Ethylene Glycol-terminated Self-Assembled Monolayer (EG4-SAMs), was previously demonstrated [15].

This work aims to evaluate the effect of pKL surfaces on hMSC adhesion and growth. It is expected that these surfaces would enhance hMSC proliferation by the sequestration of

serum-borne heparin that will concentrate, at cell-material interface, adhesive proteins and growth factors in a bioactive conformation.

VII.2 MATERIALS AND METHODS

VII.2.1 pKL synthesis

Poly(L-lysine, L-leucine) (pKL) was synthesized using ring-opening polymerization of amino acid N-carboxyanhydrides (NCAs) according to [15].

VII.2.2 Gold substrates

Gold substrates (1x1 cm²; INESC-MN, Portugal), were prepared and cleaned according to Martins *et al.* [16].

Briefly, chromium (5 nm) and gold (25 nm) layers were deposited by ion beam from chromium and gold targets (99.9% purity) on silicon wafers (AUREL, GmbH). The choice of chromium is due to the fact that it improves the adhesion of gold to silicon.

Immediately before used, gold substrates were cleaned with fresh “piranha” solution (7 parts concentrated sulfuric acid (95 vol.%, BDH Prolabo), 3 parts hydrogen peroxide (30 vol.% Merck) for 5 min (CARE: *this solution reacts violently with many organic materials and should be handled with care*). They were then rinsed sequentially with ethanol (99.8%, Merck), distilled water and ethanol in ultrasonic bath for 2 min and dried with a gentle argon stream.

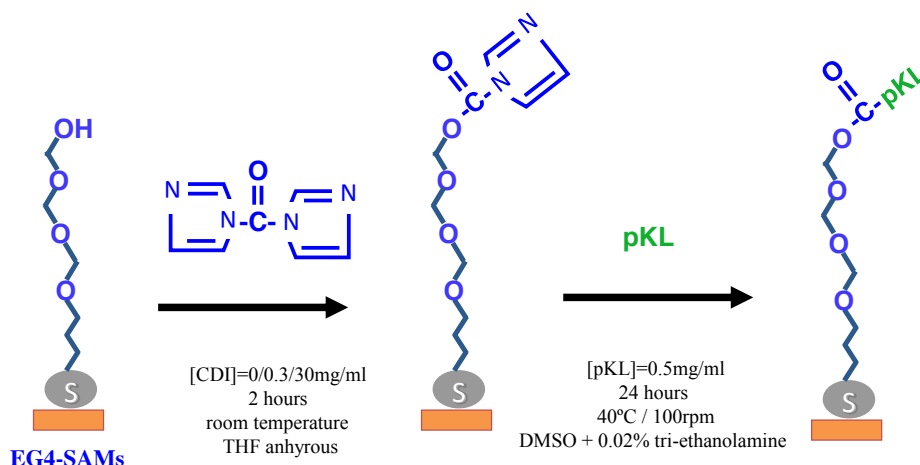
VII.2.3 Preparation of EG4-SAMs

EG4 SAMs were prepared as described elsewhere [15]. Briefly (11-mercaptoundecyl) triethyleneglycol (C₁₇H₃₆O₄S; Sensopath Technology, SPT-0011P4) was diluted with ethanol (99.8%; Merck) to prepare alkanethiol solutions of final concentration of 0.01mM. Gold substrates were immersed in these solutions at room temperature over 24 h in a nitrogen environment. After 24 h incubation, monolayers were washed three times in ethanol and dried with an argon stream.

VII.2.4 Immobilization of a pKL onto activated EG4-SAMs

pKL immobilization was proceeded using two reaction steps (Scheme VII.1):

- 1) activation of OH groups of EG4-SAMs using N,N' carbonyldiimidazole (CDI);

2) Immobilization of pKL through free NH_2 groups.

Scheme VII.1. Covalent binding of pKL on EG4-SAMs via CDI chemistry

To prepare EG4-SAMs with different degrees of activation, SAMs were immersed in solutions with different CDI concentrations: 0, 0.3 and 30 mg/mL, in anhydrous tetrahydrofuran (THF; Merck) as described elsewhere [15].

Activated SAMs were immersed in a solution of 0.5 mg/mL of pKL in dimethyl sulfoxide (DMSO; BDH Prolabo) + 0.02 % tri-ethanolamine (Sigma-Aldrich) during 24 h (100 rpm, 40 °C). As control, the same reaction was performed in buffer (DMSO solution). After immobilization, SAMs were washed with DMSO (3 times x 5 min in an ultrasonic bath) and distilled water (2 times x 5 min in an ultrasonic bath). After washing, SAMs were dried with a stream of pure argon.

VII.2.5 Surface characterization

Fourier transform infrared reflection spectroscopy (FTIR) and Infrared reflection absorption spectroscopy (IRAS)

pKL was analysed using FTIR spectrophotometer from PerkinElmer, model 2000, after KBr pellets preparation by blending 2 mg of pKL with 200 mg KBr (previously dried at 105°C for 24 h). The infrared spectra were obtained using 100 scans at 4 cm^{-1} spectral resolution.

SAMs were analysed by Infrared reflection absorption spectroscopy (IRAS) using a VeeMax II Accessory (PIKE) and a liquid-nitrogen-cooled MCT (Mercury-Cadmium-Telluride) detector coupled to FTIR spectrophotometer. The instrument was continuously

cleaned with dry nitrogen for 5 min before data collection and during measurements to eliminate water vapour absorption. Spectra were collected using the 80° grazing angle reflection mode. The incident light was p-polarized. For each SAM, a gold surface was used as the background. For each sample and reference, 200 scans were collected with 4 cm⁻¹ resolution.

Spectra were all collected under dry nitrogen for 5 min before data collection and during measurements to eliminate water vapour absorption.

Contact angle measurements

Contact angle measurements were performed with a contact angle measuring system from Data Physics, model OCA 15, equipped with a video CCD-camera and SCA 20 software. The equipment contains an electronic syringe unit with a gas-tight 500 µl dosing syringe (Hamilton). SAMs were put in a closed, thermostated chamber saturated with water to prevent evaporation of the liquid from the drop. Measurements were performed using the sessile drop method with distilled and deionized water at 25°C. After 4 µL drop deposition, images were taken every 2s over 90s. The contact angle for each time was calculated according to the ellipse method. The water contact angle for each SAM was calculated by extrapolating the time dependent curve to zero. Three replicates were used.

Ellipsometry

For ellipsometry measurement, an ellipsometer model EP³ from Nanofilm Surface Analysis was used. The ellipsometer operates in a polarizer compensator-sample-analyzer (PCSA) mode (null ellipsometry). As light source, a solid-state laser with a wavelength of 532 nm was used. The gold substrate refractive index (n) and extinction coefficient (k) were determined by using a delta and psi spectrum with a variation of angle between 66° and 76°. To correct for any instrument misalignment, measurements were made in four zones. To determine the thickness of SAMs, the same kind of spectrum was used and n and k for the organic layer were set as 1.45 and zero, respectively. Results are the average of eight measurements on each of the three samples for each type of SAM.

VII.2.6 Cell culture

hMSCs isolated from bone marrow of healthy 21-year-old black male (Lonza PT2501, VWR International, Radnor, Pennsylvania) were used in this study. Being cells commercially available, no patient consent or approval from Ethics Committee was

needed. Briefly, cells were maintained in culture at 37 °C and atmosphere of 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) + 10% Foetal bovine serum (FBS, ThermoFisher Scientific, REF:10094563) + 1% Penicillin/Streptomycin. Full culture media change was performed twice a week and cells were split by trypsinization when they reached 70-80% confluence. Cells passage between passage 4 and 7 were used in this study.

VII.2.7 hMSC cytoskeleton organization

hMSCs were seeded in 24-well suspension culture plates (Sarstedt; ref. 83.3922.500) on Tissue Culture Polystyrene dishes (TCPS), sterilized EG4-SAMs activated with 0, 0.3 and 30 mg/ml CDI with and without immobilized pKL. After 24h, cells were washed twice with pre-warmed Phosphate Buffer Saline (PBS), fixed in 4% v/v paraformaldehyde for 15 min, incubated in permeabilising buffer for 5 min (0.1% v/v Triton-x in PBS) and blocked with 1% w/v BSA (Bovine Serum Albumin) in PBS at room temperature. To evaluate cytoskeleton organization, actin filaments were stained with Alexafluor-conjugated phalloidin (1:40 in 1% w/v BSA/PBS; Molecular Probes, A12379). To analyse focal adhesion formation, vinculin was immunostained with primary mouse anti-human monoclonal antibody for 1h at 37° C (hVIN-1, 1:100 in 1% w/v BSA,PBS, Sigma V-9131), followed by rabbit anti-mouse Alexa fluor 594 conjugated Fab fragments IgG for 1h at 37°C (1:100 in 1% w/v BSA/PBS; Molecular Probes, A-21204). Nuclei were counterstained with 20 µg/mL 4,6-Diamidina-2-phenylin (DAPI; Sigma, D-9542) for 10 min at room temperature. Samples were washed with PBS, mounted with Vectashield (Vector) in glass slides and photographed with an inverted fluorescence microscope (Axiovert M100; Carl Zeiss).

VII.2.8 hMSC metabolic activity

For metabolic activity evaluation, hMSCs (P7) were plated at 3000 cells/cm² in 24-well suspension culture plates (Sarstedt) in EG4-SAMs and TCPS dishes as controls and EG4-SAMs activated with 0.3 and 30 CDI previously immersed on pKL solution or DMSO buffer. Cells were cultured along 7 days in DMEM + 10% FBS + 1% P/S.

At days 0 (4 hours after seeding) 1, 3, 5 and 7 cell metabolic activity was assayed by Resazurin assay as described elsewhere [17]. Assay was performed in triplicate.

VII.3. STATISTICS

To determine statistical significance of Resazurin assay Two-Way ANOVA test for multiple comparisons was performed. For ellipsometry and contact angle measurements a One-Way ANOVA test for multiple comparison was used.

VII.4 RESULTS

VII.4.1 pKL synthesis

FTIR spectra of synthesized pKL were comparable to literature data [18] confirming that monomers were incorporated into pKL giving rise to a chain of 25 L-Lysines and 25 L-Leucines. pKL theoretical molecular weight was around 8500 Da in accordance with the literature [15].

VII.4.2 pKL immobilization on EG₄SAMs

Immobilization of pKL on EG₄ SAMs was analysed through ellipsometry, water contact angle goniometry and IRAS. Figure VII.1A, show the average thickness of the samples analysed. In the absence of pKL, EG₄-CDI activated SAMs displayed thickness ranging from 2.5 ± 0.3 nm (EG₄-SAMs) to 3.51 ± 0.3 nm (30 CDI buffer) with the increase of CDI concentration in solution from 0 to 30 mg/ml. This demonstrated that EG₄-SAMs were activated as previously described [15]. Following pKL immobilization, average thickness varied between 2.8 ± 0.3 nm (0 CDI pKL; adsorbed pKL) to 7.61 ± 1.2 nm (30 CDI pKL) with increasing concentration of CDI.

Figure VII.1B, shows water contact angle measurements. EG₄-SAMs before and after CDI activation (without pKL) presented values of contact angles varying from $38 \pm 2^\circ$ for EG₄-SAMs and $41 \pm 3^\circ$ for 0-CDI buffer to $55 \pm 4^\circ$ for 30-CDI buffer. In comparison, SAMs with immobilized pKL were more hydrophobic with values ranging from $61 \pm 1^\circ$ for 0 CDI pKL (adsorbed pKL) to $66 \pm 3^\circ$ for 30 CDI pKL.

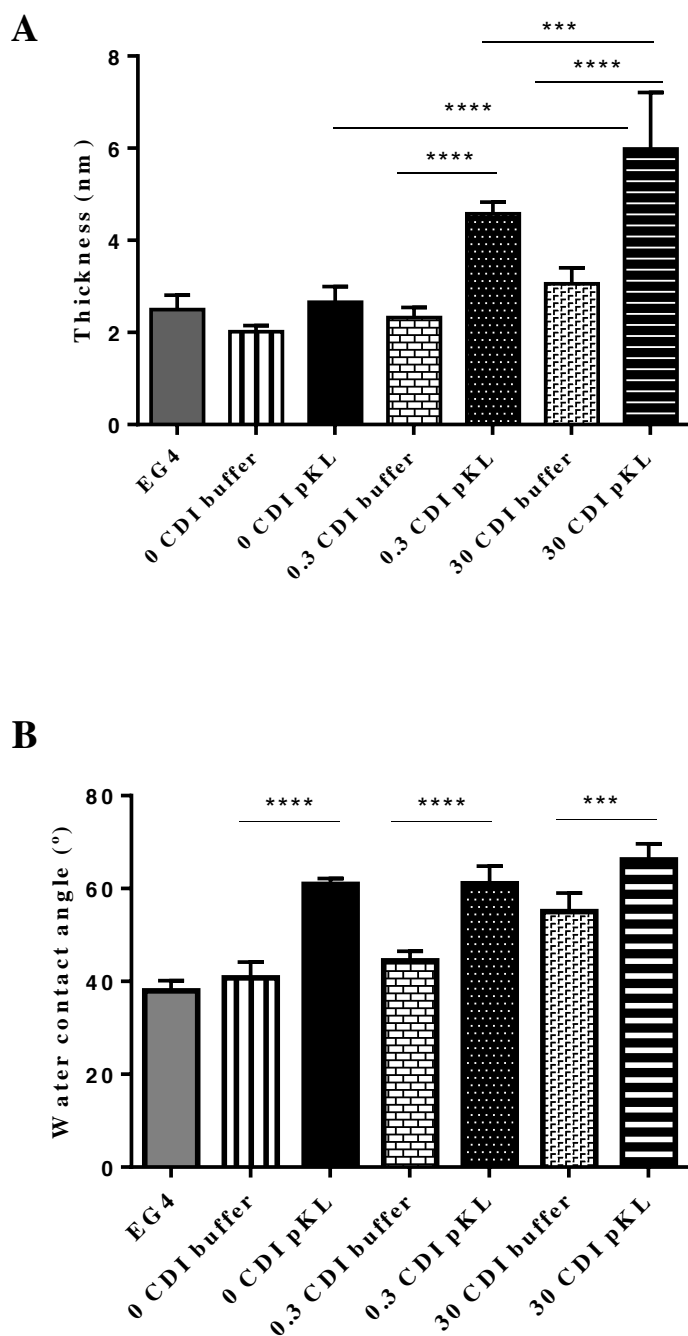


Figure VII.1. A. Ellipsometry of EG4-SAMs immersed in a pKL solution or in buffer with increasing CDI concentrations. Results are averages of three replicates.* Statistical difference:*** p value <0. 001; **** p value< 0.0001. **B. Water contact angle EG4-SAMs samples immersed in a pKL solution or in buffer with increasing CDI concentrations.** Results are averages of three replicates. *Statistical difference :*** p value <0. 001; **** p value< 0.0001.

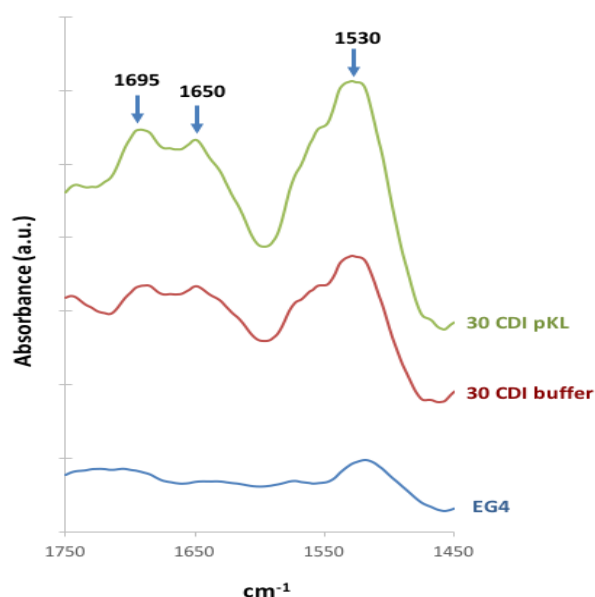


Figure VII.2. IRRAS spectra of EG4 SAMs (EG4), after activation with CDI (30 CDI buffer) and pKL immobilization (30 CDI pKL). a.u.=arbitrary units

Figure VII.2, shows IRAS spectra of EG4-SAMs and SAMs pre-activated with CDI with and without pKL. The characteristic infrared absorption bands of peptides and proteins, namely the amide peaks at $1690 \pm 45 \text{ cm}^{-1}$ (assigned to C=O stretching vibration) and $1540 \pm 60 \text{ cm}^{-1}$ (representing N-H in-plane deformation coupled with C-N modes) [19], can be observed on SAMs with pKL (30 CDI pKL). However, similar absorption bands are present on the spectra of EG4-SAM pre-activated with CDI (30 CDI buffer) although in a lower intensity. This fact is related with the presence of intermediate imidazolyl-carbamate groups after CDI activation (Scheme VII.1) that absorbs infrared radiation in similar region than peptides. Nevertheless, the higher intensity of the absorption bands in 30 CDI-pKL as compared to 30 CDI buffer, suggests that pKL was successfully immobilized [15].

VII.4.3 hMSC morphology on EG4 –pKL surfaces

hMSC morphology on pKL-SAMs and TCPS was evaluated by fluorescent staining of F-actin, vinculin and nuclei 24 hours after cells seeding (Fig.VII.3). hMSC on pKL immobilized surfaces (either 0.3mg/ml or 30 mg/ml CDI) showed a more elongated shape (in green) and more focal adhesions formation (in red), index of better cytoskeleton organization than cells on TCPS and when pKL was only adsorbed on EG4-SAMs (0 CDI pKL).

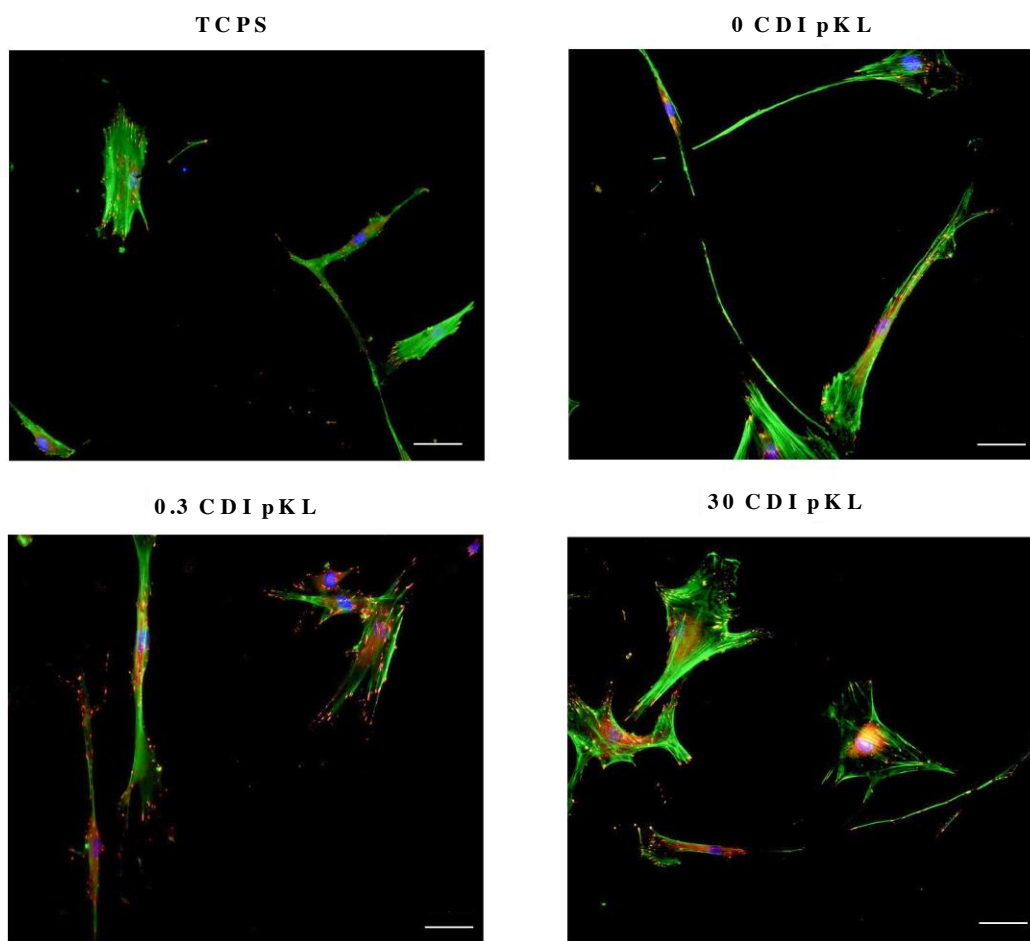


Figure VII.3. Cytoskeleton organization of hMSC 24 h after seeding on control and surfaces containing pKL immobilized after activation with different degrees of CDI. Actin filaments stain in green, vinculin in red and nuclei in blue. Scale bar is 50μm.

VII.4.4 hMSC growth on EG4 –pKL SAMs

hMSC growth on EG4-pKL SAMs and controls was analysed along 1 week. Figure VII.4 shows that cells metabolic activity was significantly higher in surfaces immersed in pKL solution (either 0.3 or 30 CDI) as compared to not activated SAMs (0 CDI pKL; adsorbed pKL), TCPS and SAMs activated with CDI but not immersed in pKL solution (30 CDI buffer). Also, surfaces containing CDI but not pKL (30 CDI buffer) had a better growth promoting effect as compared to TCPS.

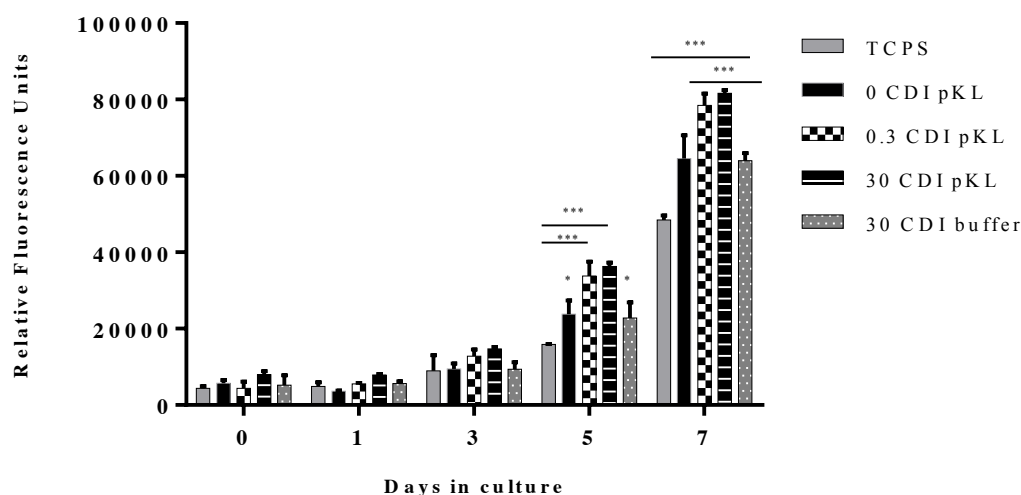


Figure VII.4 hMSC metabolic activity on EG4-SAMs immersed in pKL solution or buffer with and without activation with different concentrations of CDI. Results are averages of three replicates. * Statistical difference as compared to EG4 surface (* p value <0.05; ** p value <0.01; *** p value <0.001)

VII.4.5 DISCUSSION

hMSC are used as therapeutic agents in a wide range of human diseases (www.clinicaltrials.com). However, before transplantation in the human body cells must be expanded *in vitro*. For a better approach in cell culture, we should keep in mind that cell behaviour *in vivo* is directed by multiple signals belonging from the ECM. Soluble molecules such as FGF, transforming growth factor beta (TGF β) bone morphogenetic protein (BMP), sonic hedgehog (SHH) are crucial signalling molecules that diffuse through the extracellular space and promote specific cellular responses, conditioned by their local concentration [20]. Furthermore, ECM components such as fibronectin, heparin and related molecules influence stem cells activity by non-canonical growth factor presentation [2].

In the present work, we immobilized at the cell material interface a synthetic heparin binding peptide (pKL) to enhance hMSC *in vitro* growth. pKL peptide was synthesized using the ring-opening polymerization of α -amino acid NCAs and immobilized on EG4-SAMs by CDI activation according to a protocol published by our group [15]. Surface

characterization demonstrates that pKL was successfully immobilized onto EG4-SAMs (Fig.VII.1 and VII.2).

Upon 24 hours, cells on EG4-SAMs with 0.3 CDI pKL and 30 CDI pKL exhibited a more elongated shape and a better cytoskeleton organization than cells cultured on TCPS (Fig.VII.3). We speculate that this effect could be caused by heparin adsorption at the surface since in a previous work, heparin immobilized on non-cell-adhesive surfaces promotes hMSC adhesion [21].

Since it was previously demonstrated that surface immobilized pKL can bind heparin from plasma, we also speculate that the enhancement of hMSC adhesion on pKL-SAMs could be related with surface binding of serum borne heparin that will mediate the attraction to the surface of adhesive molecules from FBS, such as Fn which is well known to improve cell adhesion *in vitro* [22].

As regards cell growth along 7 days (Fig.VII.4), we found that pKL surfaces increase significantly hMSC metabolic activity comparing to TCPS. Also, since metabolic activity was not enhanced at the same level on 30 CDI-buffer surfaces we could correlate the cell growth promoting effect to the presence of pKL. These data are in agreement with the literature, since hMSC proliferation was promoted in GAG-derivatized chitosan membranes containing heparin [23] or on SAM functionalized with a heparin binding peptide [14].

Future prospective of this work regards the elucidation of the mechanism exerted from pKL surfaces on hMSC behaviour. First, we aim to demonstrate that heparin is adsorbed at the surface as stated in [24]. Second, considering that enhancement in cell growth was visible after 5 days in culture, we speculate that heparin adsorbed on the surface may enhance the production of endogenous growth factors. In fact, for example several studies describe that FGF binds selectively to heparin [25-27]. Moreover, Hudalla and co-workers reported that a heparin-binding SAMs upregulate hMSC proliferation in FGF-dependent manner, since upon the addition of a FGF inhibitor, hMSC proliferation on Hep-binding surface was similar to controls [14]. Thus, to address if FGF is involved in the mechanism of action of pKL surfaces, we plan to inquire whatever FGF adsorption takes place upon heparin attraction at the surface. Also additional experiments are necessary to understand why cell adhesion is improved in pKL surface. To this aim, evaluation of Fn adsorption at the surface may be necessary: pKL was found to be 70% selective for heparin in competitive adsorption studies, in the presence of 1% plasma [24]. In the presence of 10% FBS it may be even less selective and adsorb serum-borne Fn or Vitronectin. This point

will eliminate the need of RGD peptide, required for more specific heparin binding peptide as in [14], marking a nice advantage for immobilized pKL application in hMSC cell culture.

Once the mechanism of pKL action is elucidated, it will be interesting to use pKL surfaces for hMSC expansion in xeno-free settings. The use of FBS is being banned in clinical grade MSC, since it carries several drawbacks in terms of scientific, economic and ethical issues (Chapter IV). Supplement for Cell Culture (SCC) is a pharmaceutical grade product derived from human plasma pools that has been used as FBS substitute in SCC-based xeno-free medium, a new xeno-free strategy for hMSC expansion recently described by our group (Chapter V). Therefore we plan to design further studies aiming at the combination of pKL surface with SCC-based xeno-free medium, for a safe and rapid hMSC *in vitro* expansion.

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SECTION 4
CONCLUSIONS

CHAPTER VIII

CONCLUSIONS AND FUTURE PERSPECTIVES

In the era of regenerative medicine, hMSC are considered the most prominent field of inquiry and consideration because of their biological importance in clinical applications [1]. In fact, being adult stem cells, hMSC do not carry ethical concerns as embryonic stem cells. Moreover, due to their immunogenicity and immunomodulatory role, they are very interesting tools for clinical advanced therapies (chapter II). At the time of writing, 730 clinical trials, using hMSC as therapeutic agents, are in different clinical phases worldwide, showing very promising results for a notable number of human diseases [2-5]. One of the principal concerns, is that hMSC amount harvested from an human body is not enough for medical purposes, thus before transplantation into patients, cells need to be expanded in laboratory. Nowadays the literature presents a bulk of publications regarding hMSC *in vitro* expansion, a crucial step that concerns cell maintenance through several passages maintaining cells undifferentiated state, differentiation potential, phenotype and karyotype (chapter IV). Moreover, the use of hMSCs for cell therapy demands GMP production in accordance with the guidelines for advanced therapeutic medical products (ADMP) [6].

During *in vitro* culture, cells demand a complex medium rich in adhesive proteins and growth factors. Those molecules are commonly furnished through medium supplementation with FBS. However, to avoid possible hMSC contamination with zoonoses and/or xeno-antigens [7, 8], FBS use during MSC clinical grade production must be eluded. As a matter of fact, different alternatives such as chemically defined media and human plasma derivatives have been described (chapter IV). However, there is a lack of consistency among results and the high number of possibilities gives rise to a labyrinth of alternatives instead of a unique standardized solution.

SCC is an industrial product in recent development, extracted from human plasma pools, that has been recently described as alternative to FBS for hMSC and iPSC culture [9, 10]. Being the original plasma pool constituted from over 1000 donation, SCC carries the big advantage to no present lot-to-lot variability, a drawback that regards either FBS and other available alternatives. Specific legislations (section I) regulate plasma collection, manufacturing and release of plasma products, as SCC [6]. Those legislations are applied in effective GMP and GLP guidelines. Importantly, each industry includes a specific

department of quality control, which assures that all those rules are fully respected. Specific attention addresses the viral and prions safety of the final product and thus, pathogens elimination/inactivation steps are deliberately inserted within the production process (chapter I, annex I).

Therefore, the principal aim of this thesis was the optimization of a SCC-based xeno-free medium for hMSC *in vitro* expansion (chapter V). A new SCC formulation was achieved. This is constituted by a mixture of DMEM/F12 media + insulin, selenium, ethanolamine (ISE), + 15% SCC + two human recombinant growth factors (TGF β and bFGF) and includes a pre-coating of the tissue culture plates with human recombinant Fn. Upon expansion in SCC-based xeno-free medium, hMSC yield was increased 4.3-fold as compared to FBS supplemented medium and 4.5-fold in comparison to a commercially available chemically defined medium. Furthermore, cells kept correct phenotype, differentiation capacity and did not present genetic alterations. Overall, SCC-based xeno-free medium can be considered a new effective strategy for hMSC *in vitro* expansion, being faster and safer in comparison to FBS supplementation and carrying less variability as compared to the other human blood derivatives.

In future work, and for future clinical applications, it will be interesting to identify at genetic/protein level the secretome of SCC-expanded hMSC. In fact, it may be relevant to investigate whatever cells expanded in SCC-based xeno-free medium produce angiogenic or immune-modulatory factors and compare the level of expression to cells expanded in FBS. Furthermore, it may necessary to directly isolate MSC with SCC-based xeno-free medium, for a completely xeno-free clinical grade hMSC production. Finally, it may be necessary to analyse the effect of SCC-based xeno-free medium on hMSC isolated from different donors and sources, since it has been demonstrated that these factors affect hMSC behaviour [11].

hMSC are attachment dependent cells and therefore, they need to be connected to a protein scaffold to proliferate, migrate and differentiate [12]. Thus, during cells *ex vivo* expansion, the properties of tissue culture surface influence cells adhesion and growth and can be tailored for better culture strategies. Moreover, surface chemistry is rather important when hMSC are intended for advanced therapies, since most of the time they are transplanted together with a biomaterial scaffold. In this optic, it is necessary to study cell behaviour in relation with biomaterial properties, because those can affect cell proliferation and/or differentiation mainly by the influence on protein adsorption at the bio-interface [13, 14].

Therefore, the scope of this work included also the use of model surfaces (self-assembled monolayers -SAMs) (chapter III), to address different surface modifications for hMSC *in vitro* culture. In addition, we aimed to evaluate if surface chemistry could be used to avoid the need of Fn coating in SCC-based xeno-free medium.

The influence of different surface chemistries (hydrophobic and hydrophilic neutral, positive and negative charged) on hMSC growth, has been investigated in either FBS or SCC-based xeno-free medium (chapter VI). Results demonstrate that when hMSC are grown on FBS containing medium without protein pre-coating, adhesion followed the trend $\text{OH} = \text{COOH} > \text{NH}_2 \geq \text{CH}_3$, while growth was similar between OH, COOH and NH_2 SAMs and impeded on CH_3 -SAM. This confirms the results of the literature [15] since hydrophobic surfaces are usually refractory to cell adhesion because they promote proteins adsorption in non-active forms or in a denaturated states that do not support cell adhesion. Also, they may adsorb non-adhesive proteins as albumin, avoiding cell attachment [16]. On hydrophilic surfaces instead, the favourable effect on the adsorption of cell adhesive proteins from FBS (e.g. vitronectin and fibronectin) lead cells grow, without difference among the different surfaces tested. However, all the surfaces had lower efficiency regarding cell growth when compared to TCPS, probably because polystyrene is already optimized for high efficient protein recruitment at the surface.

In xeno-free settings, cells growth was significantly reduced in the absence of Fn pre-coating in all modified surfaces tested in comparison with TCPS Fn, demonstrating the need of a Fn pre-coating when hMSC are growing in SCC-based xeno-free medium. Upon Fn coating, hMSC growth (first 4 hours) was significantly increased in all the surfaces tested except on the OH-SAMs. This could be related with the amount of adsorbed Fn that, in pure solutions, adsorbs in higher amounts on hydrophobic and hydrophilic charged surfaces than on neutral hydrophilic surfaces [17]. Nevertheless, after 5 days, the trend of hMSC growth was changed $\text{NH}_2 \geq \text{COOH} \sim \text{OH} \gg \text{CH}_3$. The lower hMSC growth on CH_3 -SAMs can be explained by the irreversible adsorption of Fn in a non-active orientation/conformation way in hydrophobic surfaces [18]. This result highlights that the effect exerted by surface chemistry on cell behaviour is mainly dependent on the way the surface modulates proteins adsorption.

The importance of ECM on stem cell behaviour is nowadays well known, since stem cells reside in special niche where ECM proteins not only furnish a biomechanical support but also a biochemical role, by non-canonical growth factor presentation [19]. Heparin, abundant *in vivo* in the ECM as proteoglycans, is known to bind, store and/or present

different growth factors influencing various transduction signals pathways and therefore cells proliferation [20, 21]. On this basis, we engineered a specialized SAMs-based heparin binding surface by covalent immobilization of pKL on PEG-SAMs. Again, we aimed to establish a new surface chemistry for hMSC *in vitro* expansion. hMSC cytoskeleton organization and cell proliferation were significantly increased on pKL surface than on TCPS (chapter VII). A better cell cytoskeleton organization on pKL surface suggest higher amounts of adsorbed Fn than on TCPS or that Fn adsorbed onto pKL is in a conformation/orientation that better promote cell adhesion and proliferation than when adsorbed on TCPS. This fact could be related with the capacity of pKL-SAMs to bind heparin from plasma [22]. Since Fn has multiple heparin binding domains [23], it could be attracted to pKL-SAMs through an heparin coating formed on this monolayer. Moreover, Fn adsorbed through an heparin layer should mimic the natural Fn in ECM, which will favour hMSC behaviour. However, Martins et. al [22] state that pKL is not 100% selective for heparin in presence of plasma and thus this surface can bind other proteins with a 30% rate. Thus, in presence of 10% FBS, other elements could adsorb at the surface, exerting differential effects on cell growth. Finally, the heparin layer may attract either endogenous or serum-borne growth factors. If this hypothesis is correct, pKL surface could be used to abolish the need for supra-physiological growth factor concentrations in hMSC cell culture medium, harnessing growth factors endogenous activity. However, more studies are necessary to evaluate:

- i) if pKL surface binds heparin from serum;
- ii) which adhesive proteins are attracted to pKL surface and if this effect is dependent of heparin adsorption;
- iii) if any growth factor (e.g. FGF) is attracted at the surface upon heparin adsorption;
- iv) the influence of pKL surface on other hMSC properties, such as differentiation potential, surface markers and karyotype.

Moreover, further investigation is needed to explore the properties of this surface in xeno – free settings. In particular, we aim to adapt pKL surface for applications in combination with SCC-based xeno-free medium, since this approach could eliminate either the need for Fn coating or growth factor supplementation.

Another possibility for this heparin-binding peptide (pKL) application is its incorporation on hMSC membranes, before transplantation and after their expansion in SCC-based xeno-

free medium. A recent concern in the field is the possibility, even if rare, of thrombo-inflammation upon hMSC infusion in humans. This problem causes the loss of a relevant number of transplanted cells. It was recently demonstrated that the immobilization of heparin conjugates on hMSC surface protects the cells from damage [24]. Therefore, upon cells implantation in human body, pKL, localized at the cellular membrane, may attract heparin from the patient own blood, avoiding thrombo-inflammation.

In conclusion, the major aim of this thesis was to grow hMSC in xeno free settings, using a pharmaceutical grade human plasma fraction. SCC-based xeno-free medium demonstrated excellent performances to this scope.

Future perspectives include the combination of hMSC expansion in SCC-based xeno-free medium with pKL either binding the surface or the cellular membrane.

SCC-based xeno-free medium may be the subject of further investigations, in an attempt to develop a new strategy that harnesses the excellent potential of hMSC for regenerative medicine.

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