Estabilidad fenotípica de células madre derivadas de tejido adiposo expandidas *in vitro* con plasma autólogo enriquecido con factores de crecimiento

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2020

UNIVERSIDAD NACIONAL DE COLOMBIA

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Tesis o trabajo de grado presentada(o) como requisito parcial para optar al título de:
Magister en Ciencias - Biotecnología

Director(a):
Ph.D Neil Aldrin Vásquez Araque

Grupo de Investigación:
Biotecnología animal

Universidad Nacional de Colombia
Facultad de Ciencias, Escuela de Biociencias
Medellín, Colombia
2020

Even if the open windows of science at first make us shiver after the cosy indoor warmth of traditional humanising myths, in the end the fresh air brings vigour, and the great spaces have a splendour of their own.

Bertrand Russell

Acknowledgments

To my mother and my friends whom I must express my very profound gratitude for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

To my laboratory colleagues, for their valuable collaboration without which this work would not have been possible and to my thesis advisor Neil Aldrin Vásquez at Universidad Nacional de Colombia, Sede Medellín for being open whenever I ran into a trouble spot or had a question about my research or writing.

To the genetics laboratory for providing the necessary infrastructure and the use of facilities for the development of the project.

Thank you.

Valentina Ospina Salazar.

Resumen

Las células madre mesenquimales se conocen ampliamente como una terapia alternativa para varias enfermedades debido a su capacidad de proliferación y regeneración de tejido dañado. Esta capacidad de regeneración podría atribuirse a diferentes mecanismos como la liberación de factores solubles que juegan un papel esencial en la vasculogénesis, la proliferación, el metabolismo y la angiogénesis que al final promueven la reparación del tejido diana. Otros factores involucrados son las citocinas y los factores de crecimiento que están implicados en el metabolismo, la respuesta de defensa, la diferenciación de tejidos, la hematopoyesis y el desarrollo esquelético.

Incluso si las células madre son capaces de promover la regeneración, necesitan la presencia de factores de crecimiento para proliferar y diferenciarse, haciendo necesario el uso de expansión in vitro para usarlas como terapia. El suplemento más común para promover la supervivencia de las células es el suero fetal bovino (FBS) del cual se conocen algunos de sus componentes como proteínas, factores de crecimiento, hormonas, nutrientes y metabolitos, lípidos, minerales que ayudan con la fijación celular, la viscosidad, efecto mitogénico, absorción de glucosa y aminoácidos necesarios para la proliferación de las células.

Entre las desventajas de cultivar las células madre mesenquimales con FBS está la variabilidad fisiológica porque la composición no está completamente determinada y varía lote a lote, lo que hace necesario realizar pruebas exhaustivas antes de ser usado en cultivo. También puede contener contaminantes que generan enfermedades zoonóticas que pueden implicar un peligro en la terapia celular. Esto hace necesario el uso de alternativas libres de xeno para la producción de células madre mesenquimales como el plasma rico en factores de crecimiento (PRGF). En esta investigación se encontró que las células obtenidas del tejido adiposo y sembradas en frascos de cultivo expresaban los marcadores de superficie CD90, CD73, CD105 y eran negativas para CD11b, HLADR, CD45, CD34 y CD19,conservaban su potencial de diferenciación, cinética de proliferación y abundancia relativa del transcripto CXCR7. Sin embargo, la expresión del marcador de superficie CD105 era significativamente menor en las ADSC cultivado con PRGF lo que puede estar relacionado con la disminución de la abundancia relativa del transcripto CXCR4. Esto resalta la necesidad de una mayor investigación para determinar si puede usarse para la expansión *in vitro* de células madre mesenquimales derivadas de tejido adiposo.

Palabras clave: células madre mesenquimales derivadas de tejido adiposo, plasma rico en factores de crecimiento, caracterización fenotípica, tiempo de duplicación de la población, homing

Abstract

The mesenchymal stem cells are known widely as an alternative therapy for several diseases due to their capacity of proliferation and regeneration of damage tissue. This regeneration capability could be attributed to different mechanisms like the release of soluble factors that play an essential role in vasculogenesis, proliferation, metabolism, angiogenesis that in the end promote the repair of the target tissue. Other factors involved are cytokines and growth factors that are implicated in metabolism, defense response, tissue differentiation, hematopoiesis, and skeletal development.

Even if the stem cells are capable of promote regeneration, they need the presence of growth factors to proliferate and differentiate themselves, making the use of expansion in vitro necessary to use them as therapy. The most common supplement to promote the survival of the cells is Fetal bovine serum (FBS) from which there are known some of its components like proteins, growth factors, hormones, nutrients and metabolites, lipids, mineral that help with cell attatchment, viscosity, mitogenic effect, uptake of glucose and aminoacids neccesary for the proliferation of the cells.

Between the disadvantages of culturing the mesenchymal stem cells with FBS are the pysiological variability because the composition is not fully determined and variates batch to batch which makes necessary extensive testing to use it in culture. It also can contain contaminants that generates zoonotic diseases which can be a hazard in cell therapy. This makes neccesary the use of xeno free alternatives for the production of mesenchymal stem cells like plasma rich in growth factors (PRGF). In this investigation was found that the cells obtained from adipose tissue and seeded in culture flasks expressed the surface markers CD90, CD73, CD105, and were negative for CD11b, HLADR, CD45, CD34 and CD19, preserved their differentiation potential, proliferation kinetics and relative abundance of the transcript CXCR7. However the expression of the surface marker CD105 was significantly lower in the ADSC cultured with PRGF can be related to the decrease on the relative abundance of transcripts for CXCR4. This enlightens the need of further investigation to determine wheter it can be used for in vitro expansion of adipose derived mesenchymal stem cells.

Keywords: Adipose derived mesenchymal stem cells, Plasma rich in growth factors, phenotypic characterization,population doubling time, homing

Index

Acknowledgments vi

List of figures xi

List of tables xi

List of Acronyms xii

1 Introduction 2

1.1 Stem cell overview . . . 3
1.2 Sources of Mesenchymal stem cells . . . 3
1.2.1 Embryonic stem cells . . . 3
1.2.2 Bone Marrow . . . 4
1.2.3 Umbilical Cord . . . 5
1.2.4 Dental tissues . . . 5
1.2.5 Induced Pluripotent Stem Cells (iPSC) . . . 5
1.2.6 Adipose Tissue . . . 6
1.3 Homing of Stem cells . . . 9
1.4 In vitro expansion of ADSC . . . 9
1.5 Justification . . . 11

2 Aims 12

3 Methods 13

3.1 Ethical considerations . . . 13
3.2 Isolation of PRGF (Plasma Rich in Growth Factors) by clot retraction . . . 13
3.3 Isolation and expansion of Mesenchymal stem cells from adipose tissue . . . 14
3.4 Phenotypic characterization . . . 15
3.5 Population Doubling time . . . 15
3.6 Differentiation potential efficiency . . . 15
3.6.1 Adipogenic differentiation . . . 16
3.6.2 Chondrogenic differentiation . . . 16
3.6.3 Osteogenic differentiation . . . 16

3.7 Relative abundance of transcripts CXCR4 and CXCR7 17
3.8 Statistical analisys 17

4 Results 18
4.1 Preparation of PRGF and isolation of ADSC 18
4.2 Phenotypic characterization of the ADSC 19
4.3 Population doubling time 22
4.3.1 Population change in time 22
4.4 Differentiation potential 25
4.5 Relative abundance of transcripts CXCR4 and CXCR7 27

5 Discussion 30

6 Conclusions and recommendations 36

References 37

List of Figures

1-1 Differentiation potentail of iPSC . . . 6
1-2 Cell populations present in adipose tissue . . . 7
1-3 Differentiation potential of ADSC . . . 7

3-1 Scheme of the different plasma fractions obtained after centrifugation of the blood . . . 14

4-1 PRGF produced by clot retraction . . . 18
4-2 Isolation of ADSC from Stromal vascular fraction obtained from adipose tissue 19
4-3 Adhesion of mesenchymal stem cells derived from adipose tissue . . . 20
4-4 Effects of the PRGF in ADSC adherence when cultured in vitro . . . 21
4-5 Isotype control of the flow cytometry . . . 22
4-6 Surface markers expression by flow citometry . . . 23
4-7 Surface markers expression of ADSC obtained from cultured with different supplements (FBS or PRGF) studied in passages 2 (light grey) and 5 (dark grey) of culture . . . 25
4-8 Population fold-change of the ADSC cultured with the supplements fetal bovine serum or plasma rich in growth factor . . . 26
4-9 Differentiation potencial of ADSC . . . 27
4-10 Relative abundance of transcripts CXCR4 and CXCR7 in adipose derived mesenchymal stem cells cultured with FBS or PRGF in the first passage (inicial) and in passage 3. . . . 28

List of Table

3-1 Primer sequences used for RT-qPCR 17

4-1 Population doubling time of adipose derived mesenchymal stem cells 24
4-2 Fold Change in normalized population of ADSC cultured in vitro with different suplements 24
4-3 Comparison of means in the relative expression of CXCR4 of adipose derived stem cells cultured with FBS or PRGF. Tukey test with p<0.05 29
4-4 Comparison of means in the relative expression of CXCR7 of adipose derived stem cells cultured with FBS or PRGF. Tukey test with p<0.05 29

List of Acronyms

ADSC Adipose derived mesenchymal stem cells.

ECM Extracellular Matrix.

FBS Fetal Bovine Serum.

FGF Sibroblast growth factor.

GF Growth Factors.

GMP Good Manufacturing Practice.

hPL Human Platelet lysate.

IGF Insulin Growth Factor.

ISCT International Society for Cellular Therapy.

KGF Keratinocyte Growth Factor.

PBS Phosphated-buffered saline.

PDGF Platelet derived grwoth factor.

PDT Population Doubling Time.

PRGF Plasma Rich in Growth Factors.

ROS Reactive Oxygen Species.

SC Stem Cell.

SVF Stromal Vascular Fraction.

TGF Transforming Growth Factor.

VEGF Vascular Endotelial Growth Factor.

1 Introduction

Regenerative medicine represents a huge opportunity for treatment of chronic diseases in humans being the treatment with mesenchymal stem cells one of the most studied and used. The administration of this cells can be local with direct injection in the affected organ however, the novel administration is by venous infusion in which the cells have to have the capacity to migrate to the target tissue (homing) due to the expression of membrane receptor (CXCR4 and CXCR7) for the chemotactic factor SDF-1 (or CXCL12) secreted at the injury site, once there, the mesenchymal stem cells can differentiate and secrete soluble factor that promotes the proliferation and repair of the damage tissue [Chen et al., 2015]. According to the origin of the stem cells, they can be classified as embryonic, hematopoietic and mesenchymal. The mesenchymal stem cells express surface markers like CD73, CD90 and CD105 and have the capacity to differentiate into different cellular lineages. This kind of cells can be found in bone marrow, umbilical cord, adipose tissue and others, but the mesenchymal stem cells obtained from adipose tissue are known widely by the facility to obtain without greater risks of morbidity and in greater numbers [Frese et al., 2016]. However, according to regulations the number of cells that need to be use in a treatment is higher than the number obtained from the different tissues, making this an additional limitation to the incorporation in clinic applications.

These previous problems have been solved by the implementation of the culture in vitro of stem cells in mono layer in an essential culture media that contains fetal bovine serum FBS as the source of growth factors and nutrients that promote cell proliferation . However, in therms of translational medicine, the in vitro expansion of the adipose derived mesenchymal stem cells ADSC can not contain xenogenetic components according to the Good Manufacturing Practice GMP therefore there is the need to test xeno free supplements for the culture of stem cells . Some xeno free culture media like MesenCult-XF (Stemcell Technologies, Canada), StemPro MSC SFM (Life Technologies, USA), MSC Nutristem XF (Biological Industries, Israel) and BD Mosaic (Becton Dickinson, USA) have been implemented showing that are safe and that they do not affect the stemness of the cells, however the cost of this culture media and the lack of scientific evidence about the performance of the cells in differentiation and homing limits their use in clinic [Sun et al., 2014].

1.1. Stem cell overview

Stem cells SC are undifferentiated cells that are ubiquitous on the body, present the capability to self renewal and differentiation into other cellular lineages [Itoh et al., 2017]. The SC can be classified according to their origin like embryonic stem cells (ESC) or adult stem cells (ASC) [Lorna Valencia, 2019] or according to the potential like totipotent, those that are capable to originate an entire organism; pluripotent, those with the ability to differentiate into every type of cells including the germinal cells like the ESC and the cells from the inner mass of the blastocyst; multipotent that generates cells from different tissues like the hematopoietic stem cells (HSC) and the mesenchymal stem cells (MSC); and finally, unipotent, those that can only generate one kind of cells according to the tissue of provenance [Bissels et al., 2016].

Initially it was thought that the SC were only capable to differentiate into the cellular lineage from the origin tissue, but different studies proved that SC are capable to differentiate into multiple cellular lineages due to their property of "plasticity"which allows them to cross the barriers between the lineages and adopt the phenotype, biochemistry and the functional properties of the cells from another tissue [Crivelli et al., 2017, Debnath and Chelluri, 2019]. The SC can be obtained from different sources as bone marrow, umbilical cord, adipose tissue among others. The adipose derived mesenchymal stem cells are one of the most promissing populations due to the ease obtention without invasive protocols, low morbility and high quantities comparing them with other sources of mesenchymal stem cells which promotes their use in research and regenerative therapy.

1.2. Sources of Mesenchymal stem cells

The SC as mentioned before, are known for being ubiquitous among the human body to assure its regeneration over time. Between the most known sources for their use in regenerative medicine are:

1.2.1. Embryonic stem cells

This kind of cells are derived from the inner cell mass (ICM) from the embryo in the stage of blastocyst. In this stage of development the embryo is characterized by a cavity know as Blastocele and two types of layers: the outer layer known as trophoectoderm and the inside by the ICM. The extern layer participates in the formation of the placenta while the inner cells develop the germ layers of the embryo like the endoderm (lungs, intestines), mesoderm

(muscle, bones, blood, urogenitals) and ectoderm (epidermic tissue and nervous system) in order to produce a complete organism [Felmer and Arias, 2009]. An embryonic stem cells is known to be able to give rise to all the cells and tissues of an organ except the extra embryonic tissue, which classify them as pluripotent.This cells have numerous application in clinic like development biology, drug discovery, cellular therapy, genetic manipulation, cloning and cellular replacement therapy, but the number of SC obtained is really low, they have high ethical restrictions due to the risk of teratoma formation due to the low capacity of control about their proliferation and differentiation and this process can be very time consuming and expensive [Bissels et al., 2016, Giraldo et al., 2003].

1.2.2. Bone Marrow

This is a type of connective tissue that is located in the space between the bones. There are two types of bone marow: Red bone marrow that originates all blood cells, specifically the HSC and yellow bone marrow that is the adipose tissue that is located in the medular channels of the long bones containing also MSC that produce the bone support cells such as osteocytes, chondrocytes and adypocytes [Rehfeld et al., 2017]. From this tissue the MSC have been of particular interest due to their plasticity and the capacity to form colonies with morphology similar to fibroblasts besides the expression of surface markers that acts as important receptors and ligands between the cells allowing to identify their phenotype. Between the most common surface markers are CD29, CD73, CD90, CD105 and the lack of CD31, CD45 and CD133 [Weyand et al., 2013]. It has been demostrated that the MSC obtained from BM are capable of differentiate not only into osteocytes, adypocites and chondrocytes, but also in hepatocytes, neurons, and pancreatic cells. This cells have been used in regenerative medicine for replacement of the damage tissue in patients with osteoarthritis, spinal cord, neurological, cardiovascular and immunological diseases.

Traditionally, both HSC and MSC have been obtained from bone marrow, from which regenerative medicine treatments can be carried out, but MSCs only make up 0.001-0.01 % of the population of nucleated cells that can be obtained from the bone marrow which implies a low number of cells and therefore would require cell expansion to be used as therapy [Fluxá and Silva, 2017] . Despite the number of cells obtained, MSCs obtained from BM have been used in clinical studies to restore bone marrow that has been destroyed by high doses of chemo and radiation therapy, regardless of the process of extraction of MSCs in bone marrow that are highly traumatic [Hui et al., 2011].

1.2.3. Umbilical Cord

Umbilical cord blood (UCB) was long considered a waste product; however, this changed after it was discovered that it contained hematopoietic stem cells (HSC) that could be used instead of bone marrow transplantation, however, the low cellularity contained in the UCB is a problem for bone marrow transplantation in adults, due to ratio of the number of cells to the body weight of the patient. In 80 ml obtained from umbilical cord blood there are approximately 1.9-3.8x107 nucleated cells, of which the cells presenting CD34 + (HSC) are between 0.7-1.4x105 cel [Horwitz et al., 2014]. Cryopreservation of SCUs is a service widely used in our country today, which consists of freezing and storing HSCs when the baby is born to dispose of them in the future if necessary. To counteract this cell number hurdle, procedures such as double umbilical cord (heterologous) and in vitro expansion are currently used. Furthermore, recent research in regenerative medicine has discovered their cellular potential to regenerate damaged bone marrow and other tissues, which has promoted the use of these cells in different pathologies [Alatyyat et al., 2020].

1.2.4. Dental tissues

MSCs have been identified in a diverse number of dental tissues including follicle, pulp, and periodontal ligament, the source used being DPSC (Dental Pulp Stem Cells), characterized by the expression of CD90, and CD117 with absence of CD44. Due to the ability to differentiate into osteoblasts, neuroblasts and can form the tooth root in a scaffold, it has been suggested that these cells can participate in periodontal and bone regeneration. However, although it is a good source of cells with high plasticity, the number of stem cells, although it has not been disclosed, is presumed to be very low but reachable if expanded *in vitro*[Hussein et al., 2020].

1.2.5. Induced Pluripotent Stem Cells (iPSC)

These are a type of stem cell obtained from adult somatic cells, to which specific gene products associated with pluripotence have been introduced. These cells are similar to natural cells in that they express certain genes and proteins, chromatin methylation patterns, embryonic tissue formation, teratoma formation, viable chimera formation, and potentiality and differentiation [Hui et al., 2011, Weyand et al., 2013].

The generation of iPSC cells has been a source of interest since it allows obtaining pluripotent stem cells from any cell without the controversy of the use of embryos and avoiding the tissue versus host problem given that these cells would be derived directly from the

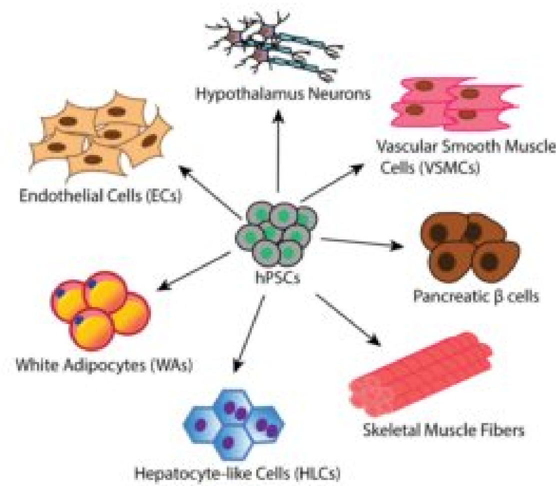


Figure 1-1: Differentiation potentail of iPSC [Yu and Cowan, 2016]

patient being completely autologous. This type of cells have allowed the development of cell models for drug evaluation, due to the ability to differentiate into different cell types such as endothelial cells, pancreatic β cells, hepatocytes, adipocytes, and neurons, among others [Yu and Cowan, 2016].

1.2.6. Adipose Tissue

There are two main types of adipose tissue, brown adipose tissue and white adipose tissue. Brown adipose tissue is less abundant, has greater vascularization and its main function is thermogenesis. While white adipose tissue is the most abundant and is found throughout the body, particularly in subcutaneous and visceral adipose tissue associated with excessive energy storage in the form of triacylglycerols and their hyperplasia causes obesity and metabolic dysfunction. In adipose tissue we find different types of cells such as adipocytes, to which tissue function is attributed, but it also has a stroma composed mainly of blood vessels, muscle cells, pericytes, preadipocytes and a population of stem cells mesenchymal (Fig **1-2**). This last population can be selected by cultivating the Stromal Vascular Fraction (SVF), because it is an adherent population [Zuk, 2013].

Although mesenchymal stem cells derived from adipose tissue (ADSC) encapsulated within the stroma can be obtained from the two types of fatty tissue, the subcutaneous white adipose tissue of the abdomen, thigh and arm has become in the most relevant clinical source, due to its abundance in the human body and the large number of isolated ADSCs [Zuk, 2013]. However, ADSCs obtained from the periumbilical region are more resistant to apoptosis than ADSCs from other parts of the body [Mizuno et al., 2012] and have characteristics that allow

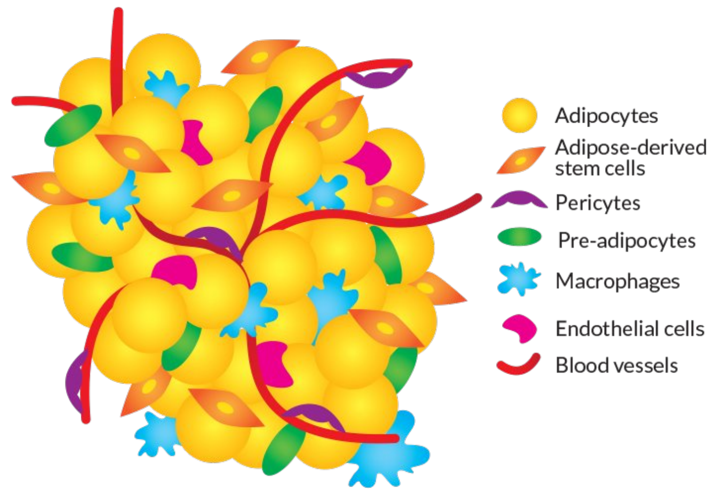


Figure 1-2: Cell populations present in adipose tissue [Vollenstee et al., 2016]

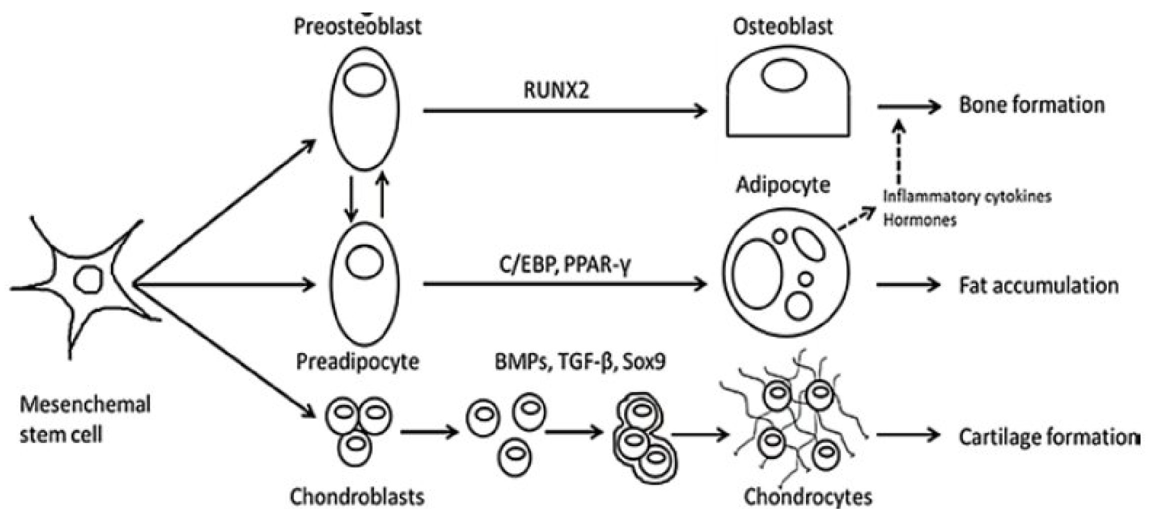


Figure 1-3: Differentiation potential of ADSC [Vollenstee et al., 2016]

cell expansion to a number suitable for tissue engineering and applications in regenerative medicine as well as its potential for differentiation to osteocyte, adipocyte and chondrocyte in vitro (Fig **1-3**) [Chao et al., 2014].

These cells are also characterized by expressing CD-specific surface antigens, being positive for CD44, CD73, CD90, CD105 and HLA, and negative for most hematopoietic antigens such as CD45, CD31 and CD14 [Linero et al., 2014]. Although initially CD34 + mesenchymal stem cells (described for hematopoietic stem cells) were removed, to select cells for expansion; some studies have shown that ADSCs express the CD34 + marker, recovering a significant cell population in regenerative therapy [Weyand et al., 2013].

These surface proteins or membrane markers have been associated with different functions in stem cells:

- CD34. Its function is not yet fully described, but it has been associated with the promotion of cell proliferation and differentiation, as well as resistance to tumor formation. It has also been shown that it promotes the anti-adhesiveness of cells, facilitating their role in the response to chemotactic signals, improving their migration [Scherberich et al., 2013].
- CD44. It is a glycoprotein that plays an important role in cell-cell interaction (paracrine effect), modulating cell adhesion and migration functions.
- CD73 (Ecto-52 nucleotidase). It acts as a catalyst for the dephosphorylation of ribo and deoxyribonucleotides to their corresponding nucleosides, and has also been associated with the immunomodulatory response of mesenquinmal stem cells [Chen et al., 2016].
- CD90 (Thy-1). It is a potential regulator of stem cell differentiation, so its decrease makes the cell more susceptible to differentiation [Moraes et al., 2016].
- CD105 (Endoglin). It acts as the regulatory component of the TGF-β complex (Transforming Growth Factor beta) and mediates the cellular response to it in processes such as cellular proliferation, angiogenesis, differentiation, migration, and cellular apoptosis [Vollenstee et al., 2016, Zuk, 2013].

Taking into account the different sources of stem cells, it should be noted that it is possible to obtain up to 2000 times more MSC in 50 ml of lipoaspirate, compared to 10 ml of bone marrow [Fluxá and Silva, 2017], thus, it can be said that approximately 6 million nucleated cells per milliliter are obtained from bone marrow, of which only 0.001 % are MSCs [Debnath and Chelluri, 2019], approximately 2 million of nucleated cells can be obtained from one gram of adipose tissue, of which 10 % belong to ADSCs. Proving that ADSCs are easier to obtain and in greater quantity for eventual clinical applications.

Preclinical and clinical studies have been performed, evaluating the safety of intravenous infusion of MSCs derived from adipose tissue (ASC) expanded in vitro for the treatment of diseases such as osteogenesis imperfecta, metachromatic leukodystrophy, acute myocardial infarction, disease of graft against host, autoimmune diseases and spinal cord damage [Mizuno et al., 2012], demonstrating that its administration does not affect vital signs, nor does it generate the appearance of adverse effects, up to 12 months post-injection of the stem cells [Ra et al., 2011], so its application in regenerative medicine is considered safe.

1.3. Homing of Stem cells

Stem cells express specific chemokine receptors on their cell surface, migrate to the site that is detected, and bind to chemokines at the site. The main cytokine that has been shown to be important for the recruitment of MSCs is SDF-1 (Stromal Derived Factor-1) also called CXCL12, which belongs to the CXC family of chemokines and was identified as a stimulator of vascularization and stem cell recruitment, interacting with CXC chemokine receptors. In addition to its critical role in facilitating tissue regeneration, SDF-1 is known to be secreted by tumors and it is used in clinic as a tumor marker, but there are no previous reports describing SDF-1-induced malignancy yet [Stuermer et al., 2015].

CXCR4 was primarily identified as the only SDF-1 receptor belonging to the G protein-binding transmembrane proteins. This is expressed by a wide variety of cells such as MSCs, including ADSCs, and plays an important role in their migration capacity [Chen et al., 2015]. Recently, a new SDF-1 receptor, belonging to the CXC family, was identified in MSC derived from bone marrow (BMSC) and in endothelial progenitor cells (EPCs). This receptor, called CXCR7, plays an important role in tumor angiogenesis and metastatic spread and this in vitro blockade has been shown to compromise EPCs migration to the same extent as CXCR4 blockade [Naderi-meshkin et al., 2015]. Strategies to improve chemokine-mediated MSC homing to wounds are primarily aimed at the interaction between SDF-1 and CXCR4 [Hocking, 2015].

1.4. In vitro expansion of ADSC

The dose of MSC derived from adipose tissue, to be applied in humans for a treatment in regenerative therapy, must contain approximately 1-2*10^6hMSC per Kg per dose (in a 80Kg person the dose increases to 160*10^6hMSC) [Salzig et al., 2016], requiring in vitro expansion of these cells to reach this number. MSCs are conventionally expanded in vitro in culture media such as DMEM supplemented with fetal bovine serum (FBS) [Li et al., 2015, Nimura et al., 2008]. FBS is essential during culture, since it contains amino acids, growth factors, hormones, among others, that play a fundamental role in the proliferation of cells in culture. This supplement is of animal origin, which makes it undesirable in clinical application due to the potential transmission of animal pathogens and the risk of developing antibodies against FBS, which could lead to rejection of the transfused cells, in fact, between 20 and 50 % of commercial FBS are virus positive [Li et al., 2015, Mangum et al., 2017]. In addition to presenting differences in terms of quality and reproducibility of in vitro data due to their batch-to-batch variation, there are doubts about the welfare of the animals from which this supplement is obtained. Therefore, it is necessary to implement serum-free

media or to search for alternatives to this supplement that can reduce the risks of zoonosis, which are highly worrisome when talking about therapy and regenerative medicine [Van der Valk and Gstraunthaler, 2017].

With this scenario, it is necessary to use alternatives to FBS such as human serum enriched with growth factors (PRGF) as a supplement, which consists of a limited volume of serum from human peripheral blood enriched with platelets which has a potential regenerative and fully biocompatible anti-inflammatory activity [Orcajo et al., 2011, Anitua et al., 2011] thus increasing the safety of human application of MSCs derived from adipose tissue expanded in vitro [Li et al., 2015, Mangum et al., 2017].

Among the studies that have been carried out, it has been possible to have alternatives for growth sources and to compare their advantages and disadvantages with respect to the use of SFB in in vitro culture. These alternatives are:

- Platelet Rich Plasma (PRP): This consists of the component of peripheral blood called plasma, which contains high levels of platelets and some growth factors. Cells demonstrate growth and maintenance of their phenotype, morphology, and increased proliferative capacity when compared to SFB. Despite being a good alternative, some studies show that there is less differentiation efficiency and that large amounts of blood are also needed to obtain it

- Human Platelet Lysate (HPL): Consists of a portion of plasma with platelet lysate by means of thermal shock. It contains a greater number of growth factors, it manages that the cells expanded with it maintain their immunophenotype, their differentiation capacity, their cloning efficiency and their viability, but it shows great variation between individuals

- Human serum (HS): It is a portion of blood that has been allowed to clot in the absence of an anticoagulant. Contains low levels of platelets. This can be obtained autologously and manages to improve the proliferative capacity of adherent MSC cells. It is suggested to be better than PRP.

Each of these alternatives has both advantages and disadvantages, therefore studies have been carried out in which the portion of (plasma) human serum rich in growth factors PRGF as described above has been found to be the best alternative and is due to the fact that there are great number of platelets that release growth factors, improving conditions for in vitro culture, facilitating the expansion of stem cells and their application in regenerative therapy [Mangum et al., 2017, Vollenstee et al., 2016].

1.5. Justification

Mesenchymal stem cells derived from adipose tissue, they possess several advantages with respect to other stem cells of somatic origin, such as easy to obtain, high proliferation capacity, they present genetic and morphological stability in long-term cultures (10 passages) [Ra et al., 2011], are cryotolerant and have a high differentiation potential to adipocytes, chondrocytes, osteocytes [Krähenbühl et al., 2015], including neurons [Hristova et al., 2017]. However, the amount of stem cells needed for a therapy generates the need for expansion and the search for growth factor supplementation strategies to stimulate cell proliferation.

Although defined culture media have been formulated for the expansion of these cells, the cost is about 4'000,000 per liter of medium (Gibco, Life Thechnologies), which increases the cost and availability of this technology. This commits us to search for new supplements, such as human serum enriched with platelet-released growth factors (PRGF) that has been used in tissue regeneration with an implementation in different areas of human health, such as dentistry, maxillofacial surgery and dental implants, ligament tendon regeneration in orthopedics, and lastly in plastic surgery and cosmetology [Simental Mendía et al., 2015]. This makes PRGF an option of great therapeutic value, by replacing the use of supplements of animal origin (fetal bovine serum), avoiding infectious risks or immunological responses such as allergies in regenerative therapies [Illouz and Sterodimas, 2011].

Therefore, the study of the different parameters of the cells derived from adipose tissue expanded in the presence of PRGF, such as the kinetics of cell proliferation, the phenotypic stability of cells, determined through the profile of specific proteins (markers), the expression of chemotactic receptors and their differentiation potential, would allow the development of a stem cell culture strategy for the initiation of preclinical studies in regenerative medicine.

Thus, the question is whether replacing fetal bovine serum with human serum enriched with growth factors will favor the proliferation and phenotypic stability of the stem cells derived from adipose tissue expanded in vitro, maintaining their differentiation potential.

2 Aims

This thesis has the aim to evaluate the phenotypic stability of the adipose derived mesenchymal stem cells expanded in vitro, in presence of human serum enriched with growth factors. In order to accomplish this, the following objectives are defined:

1. Determine the surface markers profile on the mesenchymal stem cells cultured in vitro with human serum enriched with growth factors.

2. Evaluate the proliferation kinetics of the mesenchymal stem cells cultured in vitro with human serum enriched with growth factors.

3. Evaluate the differentiation potential of the mesenchymal stem cells cultured in vitro with human serum enriched with growth factors.

4. Determine the effect of the in vitro expansion of the mesenchymal stem cells with human serum enriched with growth factors over the relative abundance of the chemotactic factors CXCR4 and CXC7.

3 Methods

3.1. Ethical considerations

This study was approved by the ethical committee of the Universidad Nacional de Colombia-Sede Medellín under the study code CEMED-098 of June 19, 2018. The Plasma Rich in growth factors (PRGF) and the adipose derived mesenchymal stem cells (ADSC) were obtained from healthy, non smokers and without previous history of diseases donors between the ages of 25 and 40. This donors were informed with the risks of each procedure and informed consent was obtained.

3.2. Isolation of PRGF (Plasma Rich in Growth Factors) by clot retraction

Blood from one healthy young female donor was collected after informed consent into 9 mL tubes (6 tubes were taken) with ACD solution A anticoagulant (BD Vacutainer). Samples were centrifuged at 580g for 8 minutes at room temperature in a PRGF-Endoret system centrifuge (BTI Biotechnology Institute, S.L., Minano, Alava, Spain).

The blood was separated in different fractions as can be seen in Fig **3-1**. The fractions 2 and 3 (corresponding to serum rich in platelets) were collected in a 20ml syringe with the help of an spinal needle 18G (1.2mm) making sure to take most of the buffy coat fraction.

To activate the liberation of growth factors from platelets there was added 0,5ml of Calcium gluconate 10 % for each 10ml of plasma, then it was mixed by slow inversion and activated with laser during 10 minutes. After this time it was incubated at 37°C during 2 hours to promote the clot formation. The released supernatant (serum) was collected by aspiration in a new syringe and passed in a new centrifuge tube. Then it was centrifuged at 1500 rpm for 10 minutes to sediment the red and white blood cells, the obtained supernatant after centrifugation was stored at 4°C until the next day.

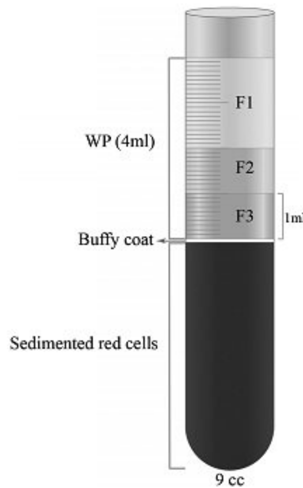


Figure 3-1: Scheme of the different plasma fractions obtained after centrifugation of the blood [Anitua et al., 2011]

The obtained serum was then inactivated during 30 minutes at 56°C. After that, the serum was allowed to reach room temperature and stored at -20°C until use. This inactivation process was also followed with the FBS.

3.3. Isolation and expansion of Mesenchymal stem cells from adipose tissue

Human adipose tissues were obtained by simple liposuction from the abdominal subcutaneous fat with signed informed consent of two young healthy donor between the ages of 25 and 40. The Stromal Vascular Fraction (SVF) cells were isolated using a method modified from that described before by [Ra et al., 2011] and [Sun et al., 2013].The adipose tissue was diluted 2 fold with phosphate-buffered saline solution (PBS) to eliminate red blood cells, and then digested with collagenase type IIS 0.1 % during 20 minutes at 37°C. The digested tissue was centrifuged at 500g for 10 min to generate the cell pellet (SVF, Stromal Vascular Fraction), the obtained SVF was centrifuged at 500g for 5 min and then filtered through a 100μm nylon mesh to remove cellular debris. The pellet was finally resuspended in 5ml of PBS and activated with laser during 20 minutes. Each ml of the (SVF) were cultured in 25cm^2 flasks containing modified Eagle′s medium with High Glucose (H-DMEM) containing L-Glutamine, 10 % of Fetal Bovine Serum FBSor PRGF and 1 % of antibiotic at 37°C, 98 %RH and 5 % CO2. After the detection of adherent cells by inverted microscope, the non-adherent cells were removed by washing with PBS. The cells were subcultured in 75 cm 2 tissue culture flasks when reach 80-90 % of confluence (Passage 0). Adipose derives

mesenchymal stem cells (ADSC) from passage 2 and 5 were used in this study.

3.4. Phenotypic characterization

For the characterization of cell surface phenotype, the ADSC were cultured in 75 cm 2 tissue culture flasks at an initial density of 2000 cells/cm^2 in normal conditions (5 % de CO_2, 37°C and 98 % HR). Trypsinized ADSC (20000 cells) were suspended in 200 μL of PBS and send for analysis by flow cytometry at Prolab laboratories. The cells were analyzed for the cluster of differentiation positive CD73, CD90, CD105, and negative CD11b, HLADR, CD45, CD34 and CD19 [Dominici et al., 2006]. This antibodies were stained with APC, FITC, PerCP-Cy5 and PE fluorochromes, respectively. The negative CD were all into a cocktail studied with the fluorochrome PE and the immunophenotype of ADSC was analyzed using Flow Jo software.

3.5. Population Doubling time

For the analysis of the duplication time of ADSC in different supplements (FBS or PRGF) the cells were cultured in 25cm^2 tissue culture flasks at an initial density of 3000 cells/cm2 during three days. In each flask were delimited 3 areas of 1cm^2 each, the cells were counted in each area every 24 hours during 72 hours in order to obtain the doubling time as [Mehrabani et al., 2016]:

$$PDT = \frac{T}{3.32(Log_{10}(N_t) - Log_{10}(N_0))}, \quad (3\text{-}1)$$

where PDT means population doubling time, T refers to the time for which the cells were analyzed, N_t and N_0 were the final and the initial cell densities, respectively [Núñez Cortés et al., 2014]. The cell counter was performed in the Software Image J.

3.6. Differentiation potential efficiency

20000 ADSCs from passage 5 were cultured with DMEM and the respective supplement (FBS or PRGF) in 24 well plates [Sun et al., 2014], once they reach 60 % confluence the media

were change into the specific media to induce the differentiation following the manufacturer indications .

3.6.1. Adipogenic differentiation

Cells were cultured with StemPro Adipogenesis Differentiation Kit media (GIBCO-Invitrogen, A1007001) during 21 days, refreshing the media every four days. The cells were incubated in normal conditions (5 % de CO_2, 37°C and 98 % HR). After 21 days, the cells were fixed with 4 % glutaraldehyde for 30 minutes and stained with Nile Red dye 0,5 % during 60 minutes, washed twice with PBS and observed under inverted microscope.

3.6.2. Chondrogenic differentiation

The Cells destined to differentiate into chondrocyte were cultured initially in microdrop in each well. They were incubated for four hours, after that the StemPro Chondrocyte Differentiation Kit media (GIBCO-Invitrogen) was added, the cells were mantained in the differentiation media during 21 days and the media was refreshed every three days. The cells were incubated in normal conditions (5 % de CO_2, 37°C and 98 % HR). After 21 days, the cells were washed twice with PBS fixed with 4 % glutaraldehyde for 30 minutes and stained with toluidine blue 0,05 % during 30 seconds, then washed with PBS and observed under inverted microscope.

3.6.3. Osteogenic differentiation

Cells were cultured with StemPro Osteogenesis Differentiation Kit media (GIBCO-Invitrogen, A10072-01) during 21 days, refreshing the media every four days. The cells were incubated in normal conditions (5 % de CO_2, 37°C and 98 % HR). After 21 days, the cells were fixed with 4 % glutaraldehyde for 30 minutes and stained with Alizarin REd 2 % during 10 minutes, washed twice with PBS and observed under inverted microscope.

3.7. Relative abundance of transcripts CXCR4 and CXCR7

In order to determine the expression of the genes related to the capacity of migration of ADSC (CXCR4 and CXCR7) the cells on passage 0 (initial) and 3 were collected once they reach confluence and then the RNA was extracted and purified by RNA TRIzol Plus RNA kit (Thermo Fisher). The cDNA was obtained by the kit SuperScript III cDNA Synthesis System (Invitrogen) using primers oligodT. The quantification of the cDNA obtained by the RNA extracted was measure using NanoDrop at 260nm. Finally, the quantitative PCR was performed by the Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher) kit on the real time thermocycler Rotor Gene Q (Qiagen) using the primers described on Table **3-1** at a final concentration of 0,2mM. The annealing temperature for the primers was determined by conventional PCR using the PCR Supermix kit (Invitrogen) with a temperature gradient between 55 and 62°C.

Table 3-1: Primer sequences used for RT-qPCR [Stuermer et al., 2015]

Gen	Primer Forward	Primer Reverse
CXCR4	5′-TCATCAAGCAAGGGTGTGAG-3′	5′-GGCTCCAAGGAAAGCATAGA-3′
CXCR7	5′-AGAAGATGGTACGCCGTGTCG-3′	5′-TCTTCCGGCTGCTGTGCTTCTC-3′
RPL27	5′-TGAAACCTGGGAAGGTGGTGC-3′	5′-TCTTGGCGATCTTCTTCTTGCC-3′

As a reaction control and data normalization, the expression of the constitutive gene Ribosomal RPL27 was analyzed. The data obtained from the analysis of the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method with the correction factor of amplification efficiency for each gene.

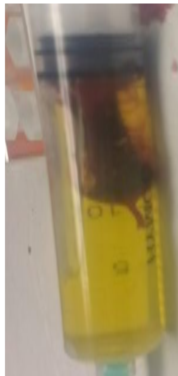
3.8. Statistical analisys

The results presented in this project are for each specific case of donor and were expressed as mean±std (Standard deviation), the statistical analysis was carried out by each donor to those data with a n=3 in the software Minitab 18. After it was demonstrated by the Ryan-Joiner normality test that the data was not normal, the non-parametric test Kruskal Wallis was carried out considering statistical differences when $p<0.05$. For the specific case of the study of the relative abundance of transcripts CXCR4 and CXCR7 the Tukey test was applied.

4 Results

4.1. Preparation of PRGF and isolation of ADSC

Platelet rich preparation constitute a relatively new biotechnology for the stimulation and acceleration of tissue healing through the liberation of different growth factors and cytokines. Based on this, it was proposed in 1999 the use of plasma rich in growth factor (GF) like TGF-B1, VEGF, and IGF; to promote functions such as directed cell migration (chemotaxis), cellular differentiation and proliferation, all of which are key events in repair and regeneration processes. The biological effects of the different GF are not yet defined in terms of a specific concentration but there has been some studies that measure the quantities of each GF [Molina-Miñano et al., 2009].



(a) Clot formation after the activation of the serum

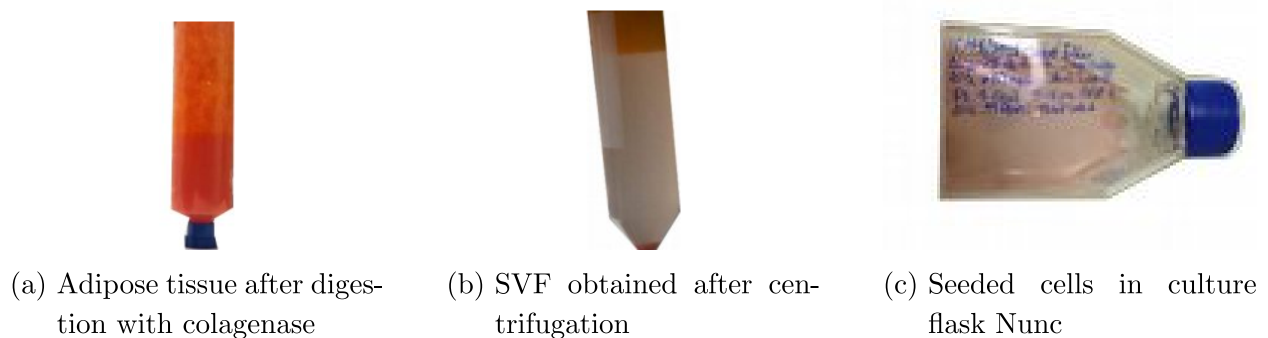
(b) Supernatant obtained after the clot formation

(c) PRGF free of red blood and white blood cells

Figure 4-1: PRGF produced by clot retraction

This procedure makes the human serum production successful due to the generation of the clot **4-1**a that can gather most of the white, red blood cells and the sticky plasma proteins such as fibrin, fibronectin and vitronectin [Anuita et al., 2004] which can be counterproductive in the culture of the mesenchymal stem cells. The clot produced was discarded and the supernatant collected was centrifuged again to eliminate the remaining white blood cells **4-1**b obtaining the human serum rich in growth factors called PRGF as seen in Fig **4-1**. The

PRGF was obtained constantly due to the small amount of blood that could be taken from the donor.



(a) Adipose tissue after digestion with colagenase

(b) SVF obtained after centrifugation

(c) Seeded cells in culture flask Nunc

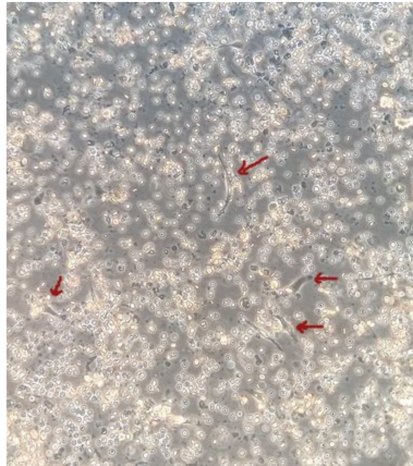
Figure 4-2: Isolation of ADSC from Stromal vascular fraction obtained from adipose tissue

According to the process previously described to obtain the ADSC, in the Fig **4-3** can be seen that the cells were seeded in culture flasks with their respective supplements (FBSor PRGF). After 24 hours there was presence of attached cells to the substrate confirming the first criteria to classify them as mesenchymal stem cells according to the International Society for Cellular Therapy (ISCT). As it can be seen in the Fig **4-3**c once the remaining of the SVF were eliminated, the ADSC were able to proliferate until reach confluence. Two samples of adipose tissue were obtained in order to describe the possible effect of the human serum PRGF in the stemness characteristics of the ADSC.

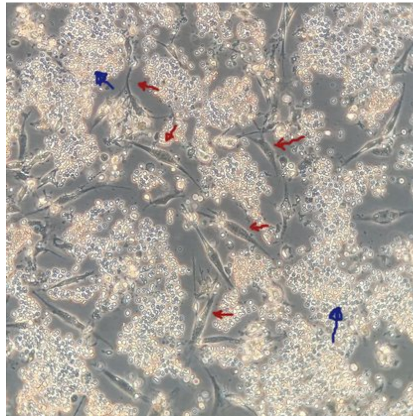
Once the adherents cells (ADSC according to the first criteria from de ISCT) from the SVF were identified in the culture flasks by inverted microscope and reached confluence, the other criteria like expression of surface markers and the differentiation potential were evaluated. However, when mesenchymal stem cells were cultured with PRGF, they started to loss adherence capability in the latest passages like seen in Fig **4-4** and started to form cumules. This behaviour was observed in ADSC obtained from both donors.

4.2. Phenotypic characterization of the ADSC

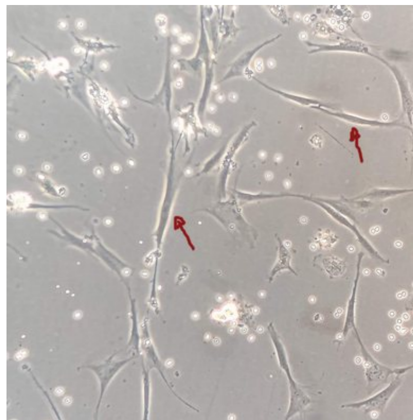
The ADSC that were obtained from human adipose tissue were cultured with different supplements (FBS or PRGF) to evaluate the expression of the surface markers: CD73, CD90, CD105, CD11b, HLADR, CD45, CD34 and CD19. These samples were analysed by flow cytometry during the expansion of the cells. Initially it was obtained the isotype control of the fluorescence **4-5**.



(a) SVF and ADSC (red arrows) after 24 hours of culture



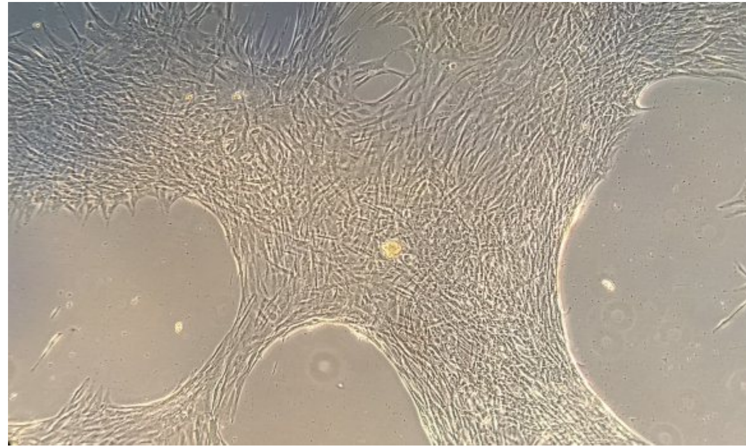
(b) SVF (blue arrows) and ADSC (red arrows) after 48 hours of culture



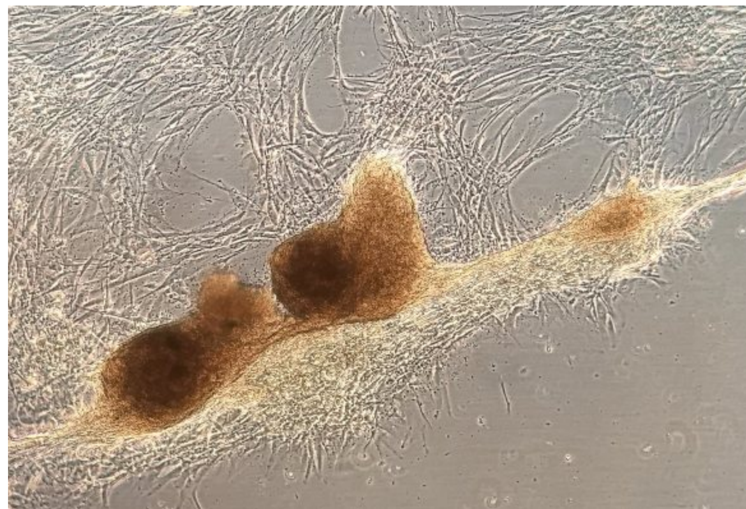
(c) ADSC (red arrows) after elimination of the remainings of SVF

Figure 4-3: Adhesion of mesenchymal stem cells derived from adipose tissue

As can be seen in Fig **4-6** the expression of the positive surface markers is maintained, and the marker cocktail (CD11b, HLADR, CD45, CD34 and CD19) remains negative until passage 5



(a) Lose of adherence properties of ADSC culture with PRGF



(b) Cumule formation due to the lose of adherence in ADSC cultured with PRGF

Figure 4-4: Effects of the PRGF in ADSC adherence when cultured in vitro

(represented in the graphic as "NO MSC"), which can mean that the protocol of adherence for purification of the ADSC is efficient and can isolate adipose derived mesenchymal stem cells.

As can be seen in the Fig **4-7** the expression of the surface markers CD73 y CD90 retained a similar expression over time despite the supplements or the donor. The most drastic changes were seen in the expression of the surface marker CD105 in both donors when the ADSC are cultured with PRGF in which the expression drop 28 % and 76 % when ADSC cultured with FBS in donors 1 and 2, respectively, and 94 % and 96 % when the stem cells were cultured with PRGF donors 1 and 2, respectively from passage 2 to passage 5.

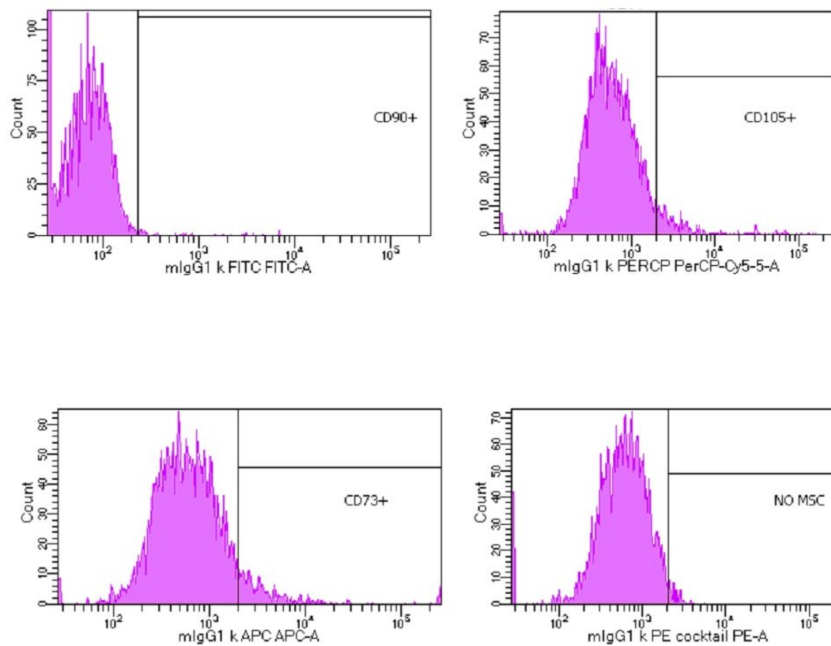


Figure 4-5: Isotype control of the flow cytometry

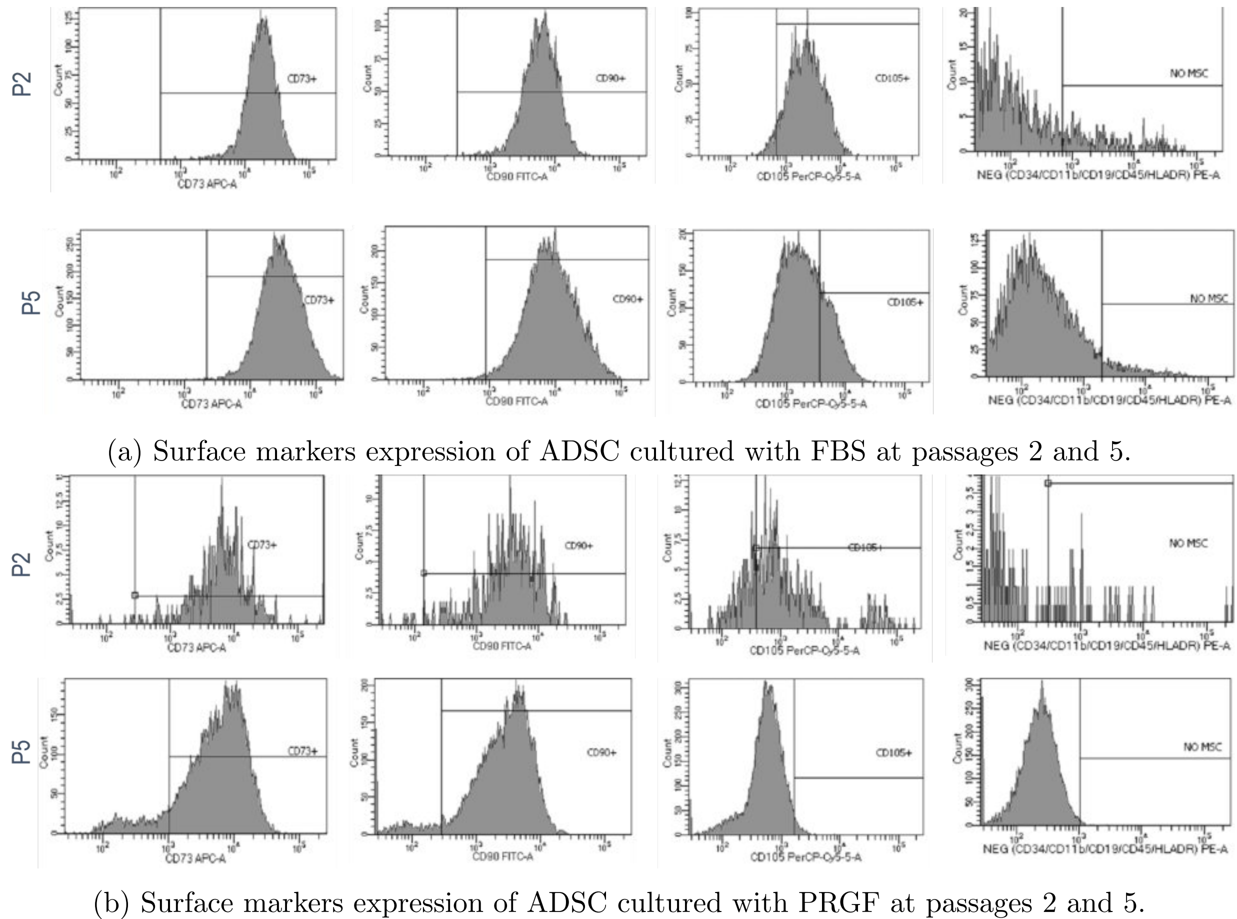
4.3. Population doubling time

ADSC were obtained from the minced adipose tissue and were seeded in $25cm^2$ with three marked areas of $1cm^2$, in each area was counted the number of cells every 24 hours during three days in order to apply the Eq 3-1 and obtain the population doubling time. The ADSC cultured with FBS maintained their needle shape and their adherence to plastic in time, but the cells cultured with PRGF presented less capacity to adherence when they reached passage 5.

The data presented in Table **4-1** analyzed by the non parametric test Kruskal Wallis in the statistic program Minitab18 shows that PDT behavior changes both per supplement and per passage in every donor. In Donor 1 was found a significant difference (p=0,021) in the PDT when ADSC were cultured with PRGF increasing as the passages progressed, in donor 2 was found that the PDT with FBS also increased with a significant difference (p=0,034).

4.3.1. Population change in time

The population change in time was measured by the change between the initial number of cells seeded and the final number of cells obtained after 72 hours of culture. The data was



(a) Surface markers expression of ADSC cultured with FBS at passages 2 and 5.

(b) Surface markers expression of ADSC cultured with PRGF at passages 2 and 5.

Figure 4-6: Surface markers expression of ADSC cultured with different supplements (FBS or PRGF) at passages 2 (P2) and 5 (P5).

normalized with the number of adhere cells after 24 hours of the passage.

In Fig **4-8**a we can notice that the population change in the supplement PRGF was initially higher but while the expansion in culture flasks continues the population decreases almost three times (p=0,021).

Particularly for the donor 2 (Fig **4-8**b), the stem cells cultured with FBS show a significant difference between the passages 2 and 5 (p=0,034) showing that the number of populations decreases when cultured with this supplement, whilst the cells cultured with PRGF the population does not change significantly.

The results for the normalized population in each donor is similar even if the cells from donor 1 were capable of proliferate more at the beggining of the expansion.

Table 4-1: Population doubling time of adipose derived mesenchymal stem cells

Donor	Supplement	Passage	PDT (days)
1	FBS	2	3,34 ± 0,24
		5	2,79 ± 0,14
	PRGF	2	1,91 ± 0,51
		5	8,16 ± 1,12*
2	FBS	2	11,18 ± 0,80
		5	17,12 ± 2,18*
	PRGF	2	3,37 ± 0,87
		5	3,08 ± 0,09

Table 4-2: Fold Change in normalized population of ADSC cultured in vitro with different suplements

Donor	Supplement	Passage	Fold Change in Normalized Population
1	FBS	2	1,87±0,08
		5	2,11±0,08
	PRGF	2	3,23±0,84*
		5	1,30±0,05
2	FBS	2	1,21±0,02*
		5	1,13±0,02
	PRGF	2	1,92±0,27
		5	1,97±0,04

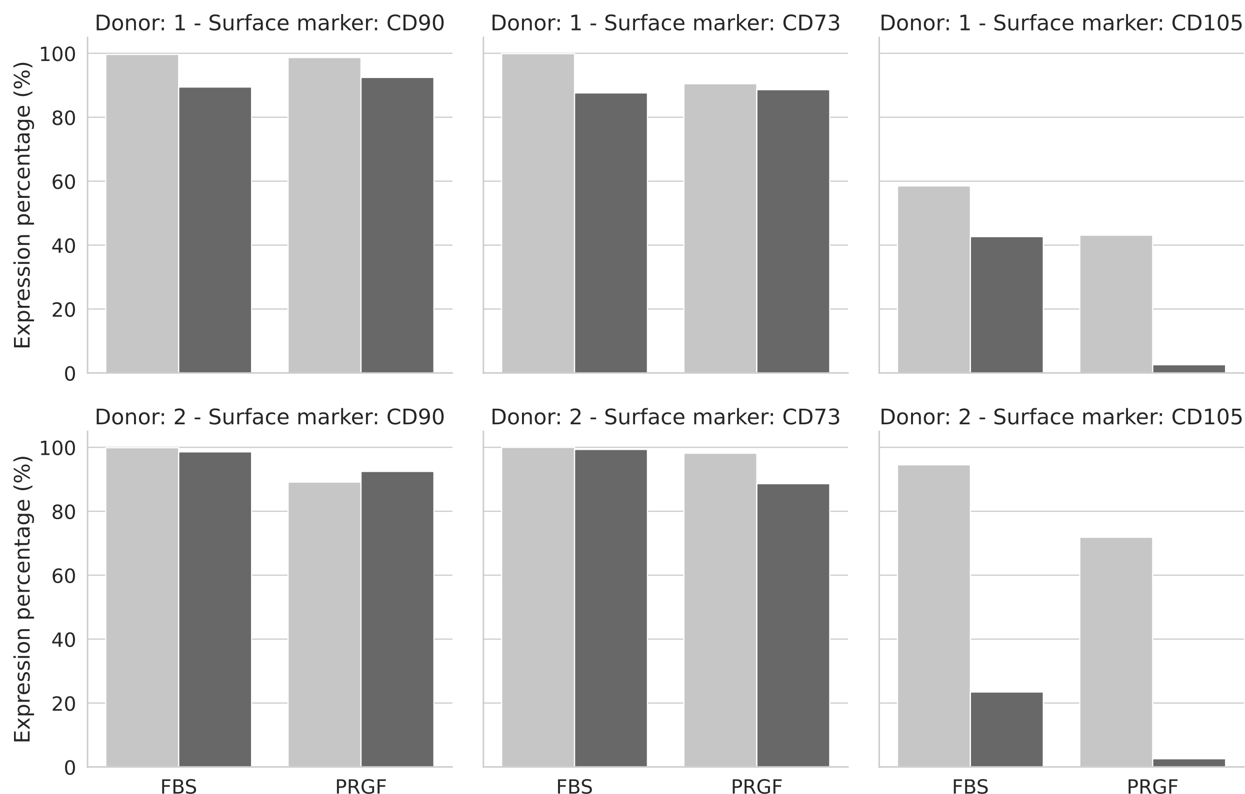
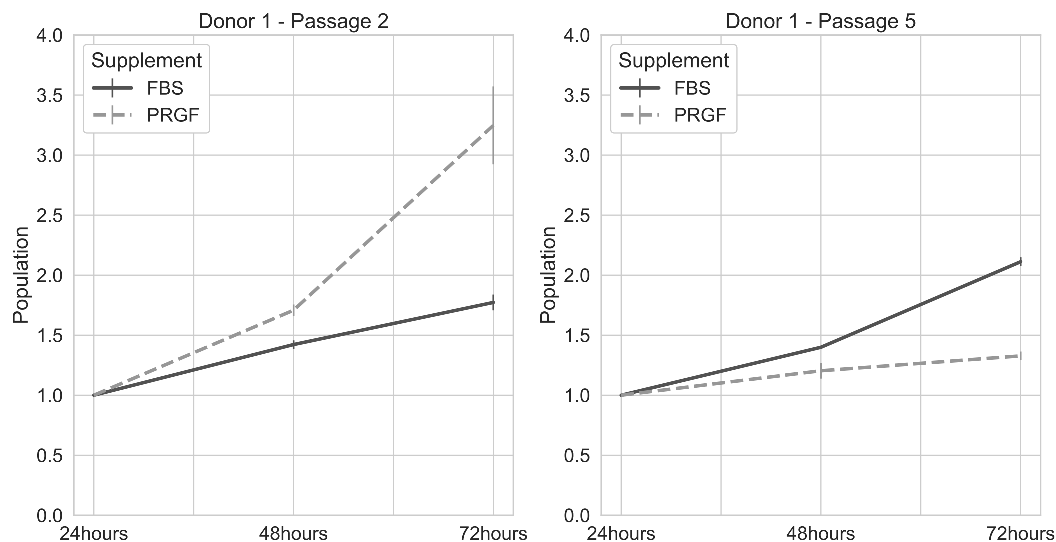


Figure 4-7: Surface markers expression of ADSC obtained from cultured with different supplements (FBS or PRGF) studied in passages 2 (light grey) and 5 (dark grey) of culture

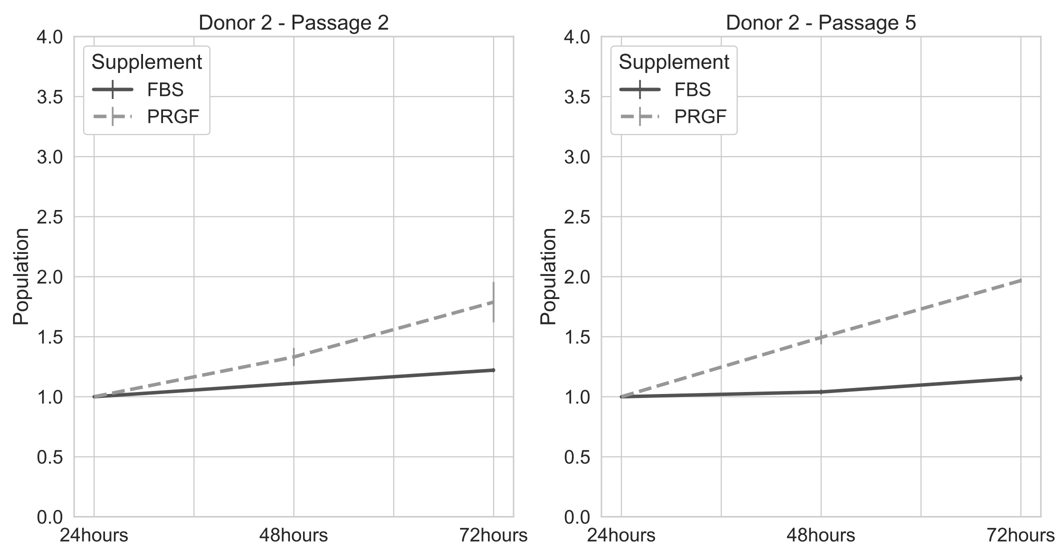
4.4. Differentiation potential

One of the criteria that the stem cells have to fulfill is that the differentiation potential is preserved when cultured in the different supplements. This potential is measured in a qualitative manner so we only detect the presence o absence of the colorant that indicates the lipid drops in the adipocytes (detected by Nile Red coloration) and the proteoglycans present in the chondrocytes (detected by toludin blue coloration). The cells proved to have adipogenic, chondrogenic and osteogenic (data not shown) differentiation potential.

As seen in Fig **4-9**, the cells preserved the capability of differentiation into chondrocytes (A) and adipocytes (C) when cultured in vitro. We could see the formation of the condrocytes and the formation of the lipid drops in the cells cultured with their respective differentiation culture media. As known by the ISCT this is the last requirement to identify the cells as mesenchymal stem cells.



(a) Population fold-change in the ADSC obtained from Donor 1 cultured with different supplements in passages 2 and 5.



(b) Population fold-change in the ADSC obtained from Donor 2 cultured with different supplements in passages 2 and 5.

Figure 4-8: Population fold-change of the ADSC cultured with the supplements fetal bovine serum or plasma rich in growth factor

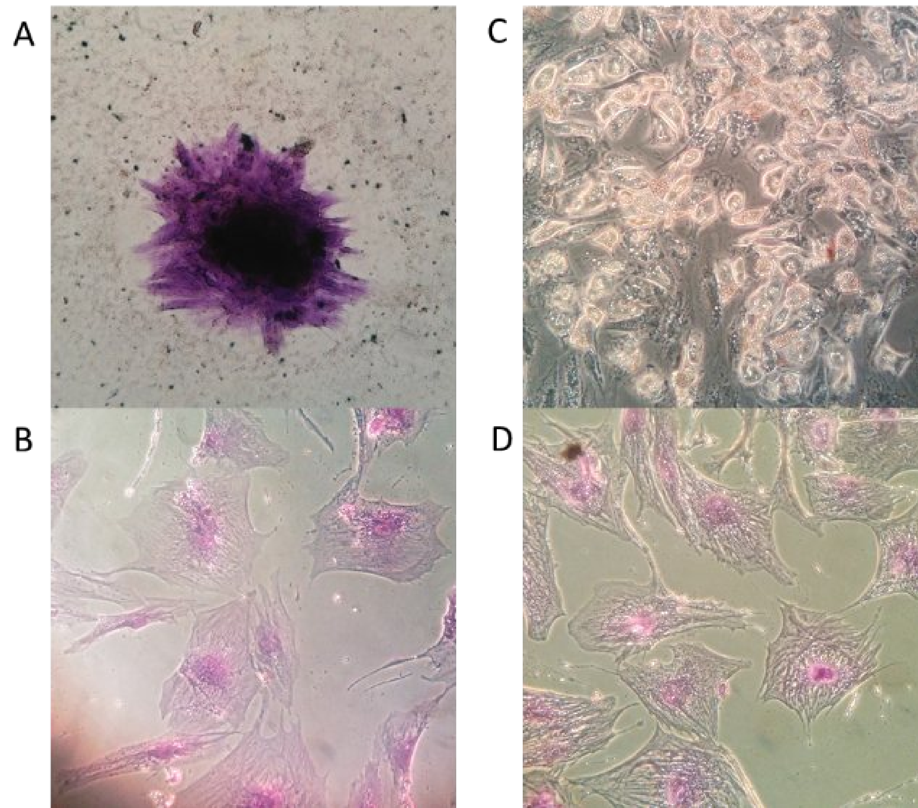
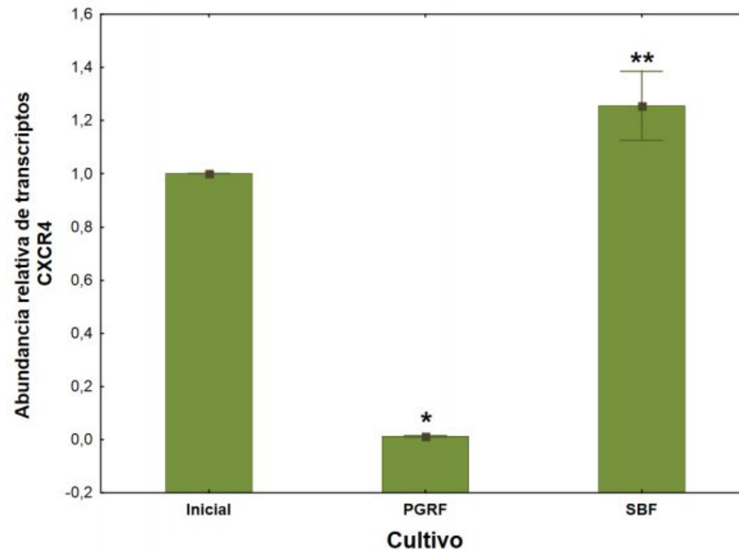


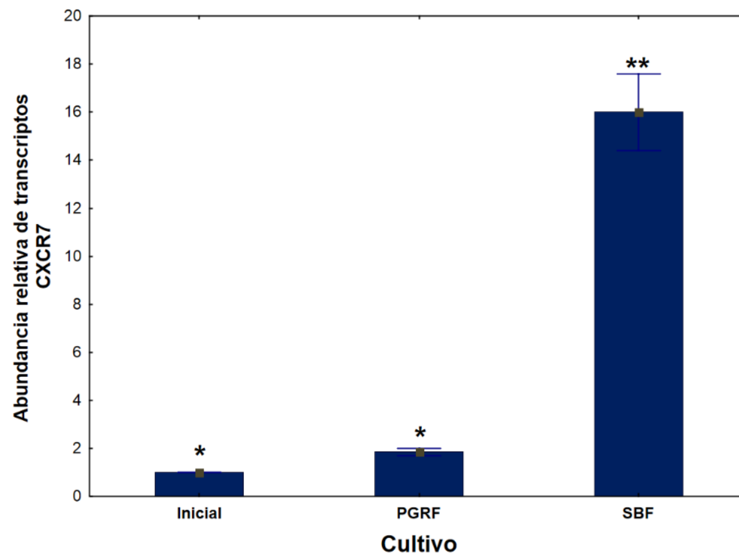
Figure 4-9: Differentiation potential of ADSC (A)Chondrogenic differentiation of ADSC after 21 days in the specific media. (B) ADSC without the differentiation media as control of differentiation. (C) Adipogenic differentiation for ADSC after 21 days of culture in the differentiation media. Red coloration indicates the presence of lipid drops in cells (D) Control of differentiation. The images were taken at 20X by inverted microscope.

4.5. Relative abundance of transcripts CXCR4 and CXCR7

As it can be seen in the Fig **4-10**, initially the ADSC show a expression of the factors, but this expression is significantly different in each supplement noticing that when cultured with PRGF the expression decreases in CXCR4 (Fig **4-10**a) but in FBS is similar to the initial. In the expression of CXCR7, the expression in cells cultured with PRGF is maintained similar at the initial, but in FBS the cells express higher quantities of the chemokine CXCR7.



(a) Relative abundance of transcript CXCR4 in adipose derived mesenchymal stem cells



(b) Relative abundance of transcript CXCR7 in adipose derived mesenchymal stem cells

Figure 4-10: Relative abundance of transcripts CXCR4 and CXCR7 in adipose derived mesenchymal stem cells cultured with FBS or PRGF in the first passage (inicial) and in passage 3.

Table 4-3: Comparison of means in the relative expression of CXCR4 of adipose derived stem cells cultured with FBS or PRGF. Tukey test with p<0.05

	Inicial	**PGRF**	**SBF**
Inicial		0,000227	0,000571
PGRF	0,000227		0,000227
SBF	0,000571	0,000227	

Table 4-4: Comparison of means in the relative expression of CXCR7 of adipose derived stem cells cultured with FBS or PRGF. Tukey test with p<0.05

	Inicial	**PGRF**	**SBF**
Inicial		0,141879	0,000227
PGRF	0,141879		0,000227
SBF	0,000227	0,000227	

5 Discussion

The platelets play a crucial role in the wound healing thanks to their hemostatic function and presence of cytokines and growth factors, that is the main reason why platelet concentrates are used in clinical tests for treatment of chronic skin ulcers due to their role in the wound healing process that consists in two components: regeneration and repair. During the regeneration process, the tissue is replaced by the proliferation of surrounding undamaged cells, while in repair, the tissue defect is replaced by granulation tissue which promotes scar formation. There are several growth factors which are known to be involved in the wound healing process, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGFβ), and keratinocyte growth factor (KGF).

[Tseng et al., 2018] show how these GFs participate in the reparation of the wound by the interaction of different cells and substances that helps to promote the cellular proliferation, migration and differentiation. This process is simulated in the specific protocol for the production of PRGF, in which with the activation of the platelets by calcium gluconate the GF starts to being released activating the cascade of pathways that promote cell proliferation and survival.

As it can be seen in the previous results, the specific protocol that was used to obtain PRGF involves the formation of the fibrin clot and the centrifugation of the serum obtained after the platelet activation. Those are supposed to be different protocols that does not involve to take the buffy coat fraction due to the amount of white blood cells that it can contain, but for this particular case it was decided that the buffy coat fraction was taken in order to collect the most of platelets possible and cells that can release cytokines and GF, elimination of the white and red blood cells remaining is carried out by a second step of centrifugation. According to [Mellado-López et al., 2017] the common protocol to obtain PRGF improves the cell survival, proliferation and decreases the senescence of the mesenchymal stem cells modulated by the degranulation of the α granules in platelets which contain several GFs that act as mitogenics and can bind to G-protein receptors activating the PI3K-Akt signaling pathways [Baudry et al., 2015].

Comparing the results obtained in the population doubling time with the PDT reported in the literature, these are similar times when we cultured the ADSC with PRGF and the reported with FBS, according to [Mitchell et al., 2006] the PDT when ADSC were cultured with FBS was approximately 4.7 days in passage 2, this is comparable with the PDT obtained of ADSC from donor 1 cultured with FBS and ADSC from donor 2 cultured with PRGF. We can see that the PDT of ADSC from donor 1 in FBS was much higher but in PRGF was lower in passage 2.

In other study the population doubling time is studied in ADSC when culture with FBS and it is demonstrated that the cells present a PDT of approximately 4 days [Applegate, 2015], consistent with the behaviour of the cells from donor 1 in which the PDT in FBS was almost 4 days, but when culture with PRGF decreases to almost 2 days. In some other study were the PDT of ADSC are culture with FBS and xeno-free medium the PDT are 14 and 6 days, respectively [Blázquez-Prunera et al., 2017], consistent with the results obtained for donor 2 in which the PDT in FBS was almost 11 days but when cultured with PRGF decreases to almost 4 days.

There are studies that show that depending on the concentration of PRGF, the PDT can change, being the concentration of 10 % the one that shows a PDT lower (approximately 2.5 days) compared with 10 % of FBS [Van Pham et al., 2016], this is similar to the obtained in the donor 2 (3.37 days) and donor 1 (1.9 days), in which the PDT of the cells cultured with PRGF is lower than the one when cultured with FBS. According to what was found in the first donor when the cells were cultured in PRGF in passage 5, the PDT increases it was found that ADSC can decrease the cell number after 16 days in culture proving that is possible that the PDT increase from passage 2 to passage 5 [Van Pham et al., 2016].

The results shows that the capacity to proliferate can depend on the source of the cells, i.e, the donor and that it can change throughout the *in vitro* expansion. Depending on the age and health of the donor, the cells present with more proliferate activity making important these criteria in order to use adipose derived mesenchymal stem cells in cellular therapy. Some studies have found that when the cells are cultured in an hypoxic environment and in addition of antioxidants, the PDT can decrease improving the proliferate capacity if the adipose derived mesenchymal stem cells [Sun et al., 2013, Sun et al., 2014].

The expression of the surface marker CD105 has been prove to decrease when the ADSC are cultured *in vitro*. [Mitchell et al., 2006] shows that the cells require to be expanded due to the low expression of the surface markers on the SVF, however, the expression of CD105 in passage 2 is 61 %. [Watson et al., 2014, Mark et al., 2013] show that ADSC cultured with FBS in passage 7 have a CD105 expression of 19 %, and the ADSC cultured in this project cultured with PRGF have a higher expression, but when cultured with PRGF show a lower expression in passage 5. Some studies report a low expression of the surface marker CD105

when ADSC are cultured with FBS [Roxburgh et al., 2016] to less than a 10 % and xeno free supplements like human platelet lysate (hPL) [Mangum et al., 2017] by 31 % in passage 2 which may be consistent with the results obtained from each donor that in passage 2 the expression of CD105 decreases to almost 40 %.

According to this it would be coherent to say that a xeno-free supplement like the human serum PRGF used in this project can show similar behaviour of the expression of the surface markers in the mesenchymal stem cells as it has been reported previously [Vanda S et al., 2018, Qu et al., 2020]. As said before, research on the effect of antioxidants and hypoxic conditions prove to improve the expression of this stem cell marker, which is why it would be something to consider for further studies.

Surface marker CD105 also known as endoglin is a type III auxiliary coreceptor of TGFβ complex, this surface marker participates in the regulation of the intracellular Smads via the TGFβ receptor complex, which regulates cell proliferation, adhesion, migration and permeability. The signaling through endoglin induces inhibition of caspases 3 and 8 resulting in inhibition of apoptosis [Valluru et al., 2011].

Knowing that endoglin also participates in the cell adhesion of the mesenchymal stem cells to the ECM, it is important to mention how this surface marker interacts in the process,it is known that CD105 is a member of the TGF-β1 receptor family and stimulates the synthesis of extracellular matrix like proteoglycans, type II collagen, fibronectin, and others [Simental Mendía et al., 2015], although the role of CD105 in this signaling is not entirely understood this surface marker has an anti-apoptotic function (in hipoxyc conditions) being involved in the cell adhesion to the ECM (extracellular matrix) resulting in a novel function for CD105 in modulating the actin cytoskeleton and cell migration. The cytoplasmic tail of CD105 interacts with the LIM domains of ZRP-1 redistributing them at the focal adhesion sites (endpoints of the actin filaments used by the cell to attach to the ECM. ZRP-1 binds to FAK, Src, and Crk playing an important role in cell motility and the turnover of focal adhesion structures. These molecular interactions might allow CD105 to influence focal adhesion composition and thereby, impact cell adhesion.

CD105 triggered adhesion with integrin β1 ligands such as collagen type I, IV and laminin. The affinity of integrins for their substrate can be altered by signals from within the cell (integrin activation) that modulate the association of focal adhesion proteins such as talin with integrin β1 cytoplasmic domains, CD105 positively influences integrin activity by modulating the protein composition of integrin-dependent cell attachment sites, thereby influencing integrin activity by inside-out signaling. CD105 cooperates with integrin β1 to mediated enhanced cell adhesion resulting in enhanced integrin activity [Muenzner et al., 2005, Sanz-Rodriguez et al., 2004]. Knowing that CD105 mediates the TGFβ1 signal [Lee et al., 2017] functioning as a co-receptor to the TGF-β1 receptor complex via ALK1 and SMAD proteins

and that those are related to angiogenesis, cell proliferation, migration and extracellular matriz production. Interaction of Endoglin with TGF-β1 mediated by the type I TGF-β receptor ALK5, upregu- lates cytoplasmic Smad2 levels leading to endothelial nitric oxide synthase (eNOS) expression, which is involved in regulating vascular function. Endoglin also interacts with a scaffolding protein, GAIP-interacting protein C (GIPC), which is mediated by a class I PDZ-binding motif in the cytoplasmic domain. Endoglin co-localizes with GIPC and enhances TGF-β1 induced phosphorylation of Smad1/5/8, enhancing endothelial cell growth and adhesion [Kasprzak and Adamek, 2018].

Cells expanded with allogeneic serum showed reduced UFC and normally didn't reach more of the 60 % of confluence, they also show that the genes related with cytoskeleton and ECM were downregulated in the mesenchymal stem cells cultured with autologus serum [Shahdadfar et al., 2005], this can be compared with the results obtained in this project due to the PDT in FBS was higher than the one in PRGF, and also they show that the cells cultured with human serum show less capacity of adherence which can be related to the down regulation of the pathways related to cell adhesion and the decrease in the expression of the surface marker CD105.

According to the results obtained from this project in relation to the percentage of expression of the CD105 and the previous mention of the interaction between CD105 and cell adhesion, the cells cultured with PRGF in passage 5 **4-7** presented less adherence capacity **4-4** as the number of passages increases. It has been proposed that the surface marker CD105 can be particularly sensitive to enzymatic and cell damage by physical mechanisms [Vanda S et al., 2018], this can also explain the decrease on its expression as the cells are subjected to mechanical damage by trypsin during each passage. Similar studies show that the use of xeno-free supplements like PRGF can affect in an unknown manner the expression of the surface marker CD105 and in consequence the population that show less expression of this marker seemed less capable of attachment and proliferation [Cleary et al., 2016] which is seen in the behaviour of the adipose derived mesenchymal stem cells cultured with the human supplement, they after 3 passages in the *in vitro* culture started to loss adhesion to the surface, generating the cell cumules shown in **4-4**b.

In order to promote the cell adhesion of the mesenchymal stem cells it is recommended the culture in hypoxic conditions. Some authors modify the cell flask substrate to collagen IV, fibronectin, laminin, vitronectina, obtaining better results in adherence with the coating of the culture surface with fibronectin [Salzig et al., 2016], they can also recommend that the cells can be cultured with FGF-2 that is known to enhance the mitotic potential of the mesenchymal stem cells increasing their growth rate and potential for self renewal. There is also another recommendation made in several articles that includes the exposition of the ADSC during the expansion with and hypoxic atmosphere, this characteristic improves the cells proliferation by decreasing the doubling time of the cells

[Sun et al., 2013, Sun et al., 2014, Bidkhori et al., 2016].

There is another factor that can affect the stability of the human mesenchymal stem cells, this factor is the exposition of the adipose derived mesenchymal stem cells to high concentrations of glucose, in this project was used the High glucose DMEM media culture, however some studies report that in high glycemic conditions the Akt signaling pathway that contributes to cell proliferation is suppressed, this condition also leads to senescence resulting in reduced proliferation, also the cells cultured in hiperglycemic conditions are more prone to cell death upon cell detachment from the culture flask. This loss of the anti-apoptotic effect is suggested to be related to the suppression of CD105 [Karina et al., 2019]. This confirms the previous explanations of the reduced proliferation and adherence capacity. So CD105 downregulation plus the high glycemic conditions and the human serum make the cells express less capability of adherence and proliferation through time. The process by which the high glucose affects the cells is related to the production of reactive oxygen species (ROS), when the glucose is high, this species increases inactivating the Akt signaling pathway which leads to the activation of the transcription factor FoxO3a, that when is not phosphorilated by the Akt molecules, it can enter the nucleus and promote the transcription of target genes that promotes the apoptosis by the protein Bim and decreases the anti-apoptotic protein BcLxL [Peng et al., 2013] which leads to decrease in the cell replication ad increase the cells senescence.

The migration of the mesenchymal stem cells towards the tissue that express the SDF-1 factor is principally attributed to the expression of the receptor CXCR4 even more than the expression of the receptor CXCR7 because this receptor presents also functions related to adhesion and survival of the cells [Chen et al., 2015]. Additionally it has been found that the expression of CXCR4 is lost as the expansion *in vitro* is performed [Karp and Leng Teo, 2009, Lin et al., 2017, Bidkhori et al., 2016]. This can explain the different response of the cells isolated in the first passage that had more expression of CXCR4 than the isolated in the third passage with PRGF. The expression of cells cultured with FBS indicates that was an augment in the expression of CXCR7 compared to the first passage and the supplement PRGF, keeping the expression of the CXCR4 receptor compared to the first passage. Some studies have proved that the decrease on the expression of CXCR4 does not affect the migration potential of the ADSC, they continue to express higher quantities of CXCR7 which makes possible to increase homing by interaction of this receptor with the chemotactic factor SDF-1 [Bidkhori et al., 2016]. It has also been found that the expression of both receptors (CXCR4 and CXCR7) can be higher when cultured under hypoxic conditions.

Even if the cells are viable and can proliferate, depending on the donor, these does not preserve the phenotype characteristics showing that the cells can enter a process of senescence when cultured with PRGF and this can have consequences in the clinical application. Some authors recommend the use of CD13 as a adipose derived mesenchymal stem cell marker instead/or as a complement to CD105 [Roxburgh et al., 2016] due to the conflicting reports

of the stability of the last one in the expansion in vitro process, however it is necessary to run more experiments with a higher number of adipose mesenchymal stem cells samples in order to determine the effect in a bigger population of cells. The results of gene expression can be contrasted with the obtained in the duplication time which is clearly the lowest time in the cells cultured with PRGF, indicating a possible inversely proportional relationship between cell migration capacity and in vitro proliferation.

6 Conclusions and recommendations

There is a donor effect in the in vitro expansion of the adipose derived-mesenchymal stem cells meaning that the source of the adipose tissue can change the characteristics of the ADSC under in vitro expansion.

One of the reasons why the cells cultured with FBS had a strong adherence to the cell culture flask while the cells cultured with PRGF presented less capacity of adhesion to the substrate was because these cells presented higher expression of the receptors related to the capacity of adherence, proliferation and cell survival like endoglin (CD105).

The in vitro expansion of the adipose derived-mesenchymal stem cells with PRGF preserve the surface markers profile, promotes the proliferation and maintain the differentiation potential therefore the ADSC maintain the phenotypic stability.

The relative abundance of the transcripts relate with homing (CXCR7) is maintained with PRGF and improved with FBS, the gene expression of CXCR4 decreases in ADSC cultured with PRGF, however they conserve CXCR7.

This project is an exploratory approach to the effects of this particular protocol to obtain PRGF on adipose derived mesenchymal stem cells with no intention to validate it for clinical applications. Taking this into account to future studies it is necessary further studies focused on the expression of surface markers and chemotactic factors with more samples of adipose tissue.

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