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# Mesenchymal Stem Cells from human adipose tissue

Characterization and Immunomodulatory Properties of Tissue and Cellular Products

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"All men by nature desire knowledge."
— Aristotle, *On Man in the Universe*


"The future belongs to those who believe in the beauty of their dreams."
— Eleanor Roosevelt

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# INTRODUCTION

The histologist Alexander Maksimov in 1908 at a hematology congress in Berlin utilized for the fist time the word 'stem cells', postulating the existence of hematopoietic stem cells. It has been necessary to wait until the '60s for the first studies on these cells: in 1963 McCulloch and Till demonstrated the existence of self-renewing cells in the mouse bone marrow (Siminovitch L et al, 1963; Becker AJ et al, 1963) and the following year Kleinsmith showed the presence of stem cells in teratocarcinoma, able to remain undifferentiated in culture until the use of a specific medium allow them to differentiate in different cellular lineages (Kleinsmith LJ et al, 1964). After only four years was performed the first blood transplant obtained from bone marrow, containing hematopoietic stem cells, to treat severe combined immunodeficiency (SCID) (Gatti RA et al, 1968). But only when in 1998 James Thomson, at the University of Wisconsin-Madison, developed a technique to isolate and grow five independent cell lines derived from human embryonic stem cells that a lot of hope grew around this field and many research groups have undertaken this path of study.

Two characteristics distinguish stem cells from other cell types: the ability to self-renew and to differentiate into multiple lineages (plasticity).

On the basis of the plasticity can be distinguish 4 types of stem cells (Lovell-Badge R et al, 2001) (Figure 1):



- Totipotent cell: can differentiate in all cell lineages present in the body and also in extra-embryonic tissue cells, as the placenta. The zygote has this characteristic.

- Pluripotent cell: can originate all cells belonging to an adult human being, but not in extra-embryonic tissue cells.

- Multipotent cell: is a cell already committed toward a specific lineage and that is the only differentiation pathway it can follow. For instance, hematopoietic stem cells can originate corpuscular elements of the blood.

**Figure 1.** From totipotent to multipotent stem cells.

- Unipotent cell: it can generate only a single cell type. For instance, spermatogonium can originate only spermatozoon.

Stem cells can classify also for origin: embryonic stem cells and adult stem cells.

## 1. Embryonic stem cells

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst during early embryogenesis (Evans M.J. et al., 1981, Martin G.R. et al, 1981). ES cells are pluripotent so, they are able to differentiate into all derivatives of three primary germ layers: ectoderm, mesoderm and endoderm. This property discern ES cells from adult stem cells in the adult body, which are multipotent and can generate only a limited number of cell types. Under defined conditions, embryonic stem cells are capable of propagating themselves indefinitely and this allows them to be employed as useful tools for both research and regenerative medicine. There are three types of pluripotent ES cells that can be defined: ES cells properly called, Embryonic Germ Cells (EG cells) and Embryonic Carcinoma cells (EC cells) (Figure 2):
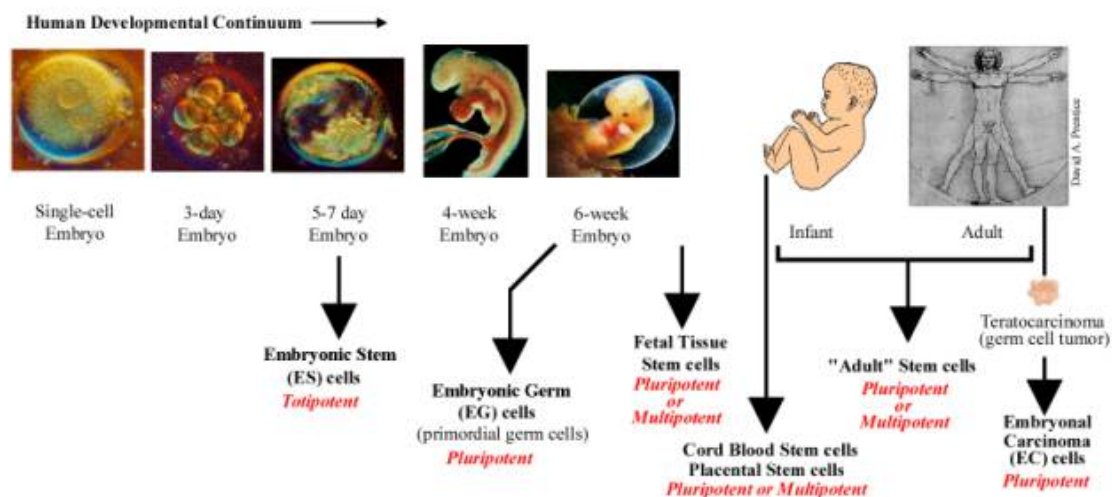


**Figure 2**. Stem cells in development

Plasticity and their unlimited capacity of self-renewal make them a putative tool for regenerative medicine and tissue replacement after disease or injury. They could help also to investigate early human development and study genetic diseases. Several new studies have started to address this issue and this has been done either by genetically

manipulating the cells, or more recently by deriving diseased cell lines identified by prenatal genetic diagnosis. This approach may very well prove invaluable at studying disorders such as Fragile-X syndrome, Cystic fibrosis, and other genetic disorders that have no reliable model system.

Current research focuses on differentiating ES into a variety of cell types for eventual use as cell replacement therapies. Some of the cell types that have or are currently being developed include cardiomyocytes, neurons, hepatocytes, bone marrow cells, islet cells and endothelial cells.

On January 23, 2009, Phase I clinical trials for transplantation of oligodendrocytes derived from human ES cells into spinal cord-injured individuals received approval from the U.S. Food and Drug Administration (FDA), marking it the world's first human ES cell human trial. Hans Keirstead and colleagues at the University of California, Irvine, conducted the study leading to this scientific advancement. A previous experiment had shown an improvement in locomotor recovery in spinal cord-injured rats after a 7-day delayed transplantation of human ES cells that had been pushed into an oligodendrocytic lineage (Keirstead HS et al, 2005). In the proposed phase I clinical study, about eight to ten paraplegics who have had their injuries no longer than two weeks before the trial begins, were selected, since the cells must be injected before scar tissue was able to form. Based on the results of the rodent trials, researchers announced restoration of myelin sheathes, and an increase in mobility is probable. The trial had been put on hold in August 2009 due to concerns made by the FDA regarding a small number of microscopic cysts found in several treated rat models. The major concern of the FDA with the transplantation of ES cells into patients is their ability to form tumors, including teratoma (Knoepfler PS, 2009), but the hold has been lifted as of July 30, 2010.

In October 2010 researchers enrolled and administered ES cells transplant to the first patient at Shepherd Center in Atlanta. The makers of the stem cell therapy, Geron Corporation, estimate that it will take several months for the stem cells to replicate and for the therapy to be evaluated for success or failure. In November 2011 Geron announced it was dropping out of stem cell research for financial reasons, but in 2013 BioTime Company acquired all of Geron's stem cell assets, with the stated intention of re-starting Geron's embryonic stem cell-based clinical trial for spinal cord injury.

The main strategy to enhance the safety of ESCs for potential clinical use is to differentiate the ES cells into specific cell types (e.g. neurons, muscle, liver cells) that have reduced or eliminated ability to cause tumors. Following differentiation, the cells can be subjected to sorting by flow cytometry for further purification.

## 2. Adult Stem Cells

Ethical considerations, technical challenges, and governmental regulations have hindered the ES cells use (Frankel M.S. et al., 2000). As a result, the study of somatic or adult stem cells, which does not generate the same ethical concerns, has increased dramatically.

Adult stem cells originate from embryonic stem cells; during gastrulation three germinal layer are formed and the progeny of embryonic stem cells is separated into distinct groups of precursors, which gradually mature into those that will be considered stem cell organ-and tissue-specific (somatic).

Somatic stem cells can be isolated from various mammalian tissues throughout the period of fetal development and from adult tissues, even though their number gradually decreases until reaching minimum values in adulthood. They have been localized to many tissues including mesenchymal (Pittenger M.F. et al., 1999), neural (Gage F.H. et al., 2000), gastrointestinal (Potten C.S. et al., 1998), hepatic (Alison M. and Sarraf C., 1998), gonadal (Margolis J.and Spradling A., 1995, Conrad S. et al., 2008), and hematopoietic (Weissman I.L., 2000).

Their predominant function is to contribute to the maintenance of tissue homeostasis, generating a progeny of differentiated cells to replace mature cells lost due to injury or physiological renewal. The extent to which stem cells perform this function can change significantly by tissue type; for example tissues such as epidermis, epithelium of small intestine and hematopoietic system are subject to a continuous cell renewal.

Adult stem cells remain in a non-proliferating, quiescent state during most of their lifetime until stimulated by the signals triggered by tissue damage and remodeling (Fuchs E. et al., 2004, Hirao A. et al., 2004). Upon stimulation, adult stem cells re-enter the cell cycle to replenish the stem cell pool, as well as to generate progenitor cells, which then give rise to a variety of differentiated cell types for tissue regeneration and homeostasis.

The number of stem cells within a particular tissue is not regulated at the level of single cell but to the cell population. Stem cells perform symmetrical divisions in which the two daughter cells are identical (division expansive) or, alternatively, different from the mother cell stem (division differentiative).

The self-maintenance is guaranteed by numerical balance between the two types of symmetric division with the population.

A second model, called deterministic, provides that a single stem cell given rise, each mitotic division, to two daughter cells of which at least one is identical of the parent cell. This type of division is said asymmentric because the two daughter cells follow different paths of development. This model guarantees the retention of a stable number of stem cells and the production of differentiated cells. Most of the stem cells using both methods and the balance between them is controlled by the stage of development of the organism and by environmental factors (Morrison SJ and Kimble J, 2006).

Cytokines, growth factors, adhesion molecules, and extracellular matrix components in the stem cell microenvironment play important roles in stem cell fate determination, working as the driving forces to switch from a self-renewal to a differentiation stage (Roelen B.A. et al., 2003, Kratchmarova I. et al., 2005, Mannello F. et al., 2006, Gregory C.A. et al., 2005, Kortesidis A. et al., 2005). However, the downstream intracellular effectors in these processes are still largely unknown.

For many years it was argued that adult stem cells were tissue-specific 'entities' and their differentiation potential was limited to the generation of mature cell lines of the tissue/organ of residence. But in recent years many publications have shown one of the most amazing functional characteristics of adult stem cells: the ability to transdifferentiate, defined as the capability to switch from their specific developmental lineage into another cell type of a different lineage, sometimes across embryonic germ layers. For example, mesenchymal stem cells (MSCs) can be induced to become nonmesodermal cells, including functional neurons, astrocytes, oligodendrocytes, and endothelial cells, by appropriate extrinsic stimuli both *in vitro* (Cho K.J. et al., 2005, Wislet-Gendebien S. et al., 2005, Keene C.D. et al., 2003, Jiang Y. et al., 2002) and *in vivo* (Sato Y. et al., 2005).

The first isolated and studied adult stem cells were hematopoietic stem cells resident in the bone marrow, but in the '70s another cell population was found in rats and

guinea pig bone marrows and they were called stromal fibroblasts of mesenchymal origin, hereafter said mesenchymal stem cells (MSCs) (Friedenstein AJ et al, 1970).

### 3. Mesenchymal Stem Cells

Friedenstein and colleagues found a rare cell population in the bone marrow (0,001% - 0,01% of the total) with fibroblast-like morphology and they were able to adhere to the plastic surface of culture plates. Those colonies were called CFU-F (colony-forming unit- fibroblast) and the constituting cells are able to grow without any particular nutritional exigency, except for the presence of fetal bovine serum (Friedenstein AJ et al, 1970; 1974). Ten years later they were isolated from human bone marrow (Castro-Malaspina H et al, 1980). The name 'Bone Marrow Stromal Fibroblasts' has been replaced by 'Mesenchymal Stem Cells' (MSCs) for the fist time by Arnold Caplan  (Caplan AI, 1991).

MSCs population isolated from the bone marrow are still considered as the gold standard for MSC applications. Nevertheless the bone marrow has several limitations as source of MSCs, including MSC low frequency in this compartment, the painful isolation procedure and the decline in MSC characteristics with donor's age. Thus, there is accumulating interest in identifying alternative sources for MSCs. To this end, for instance, MSCs obtained from the Wharton's Jelly (WJ) of umbilical cords have gained much attention over the last years since they can be easily isolated, without any ethical concerns, from a tissue, which is discarded after birth. Furthermore WJ-derived MSCs represent a more primitive population than their adult counterparts, opening new perspectives for cell-based therapies (Batsali AK et al, 2013).

In the umbilical cord, hematopoietic and mesenchymal stem cells co-exist. It begins to form in the first month of fetal life, when morula's cells (development stage when the zygote consists of 8 to 16 cells) that will give rise to the embryo differ from those that will form placenta and the fetal adnexa (amniotic sac and the umbilical cord). Inside the umbilical cord there are three blood vessels: two arteries and one vein. The portal vein brings to the baby oxygen and nutrients, which come from the placenta, while arteries allow the child to eliminate catabolites into the placenta. These three channels are covered with a gelatinous material called Wharton's jelly, seat of the MSCs.

It is now know that MSCs are virtually ubiquitous, representing a component of the stroma of many tissues, such as bone marrow, skin, digestive epithelium, dental pulp, hair follicles, brain, amniotic fluid, placenta, etc. (Johe K.K. et al. 1996, Johnstone B. et al. 1998).

According to the International Society of Cell Therapy (ISCT), MSCs are defined as being (a) plastic-adherent in the standard cell culture condion, (b) multipotent differentiation potential (adipogenic, chondrogenic and osteogenic) and (c) positive for CD73, CD90 and CD105, and negative for CD11b or Cd14, Cd19 or CD79α, CD45 and HLA-DR in their cell surface immunophenotype (Horwitz EM et al, 2005; Dominici M et al, 2006).

In addition, hMSCs are able to maintain their multidifferentiation potential after commitment (Figure 3). As demonstrated by Song L. et al. (2004) osteoblasts, chondrocytes, and adipocytes differentiated from human mesenchymal stem cells (hMSCs) can transdifferentiate into other cell types in response to extrinsic factors, likely through genetic reprogramming. However, the molecular mechanisms behind the transdifferentiation process are poorly understood.
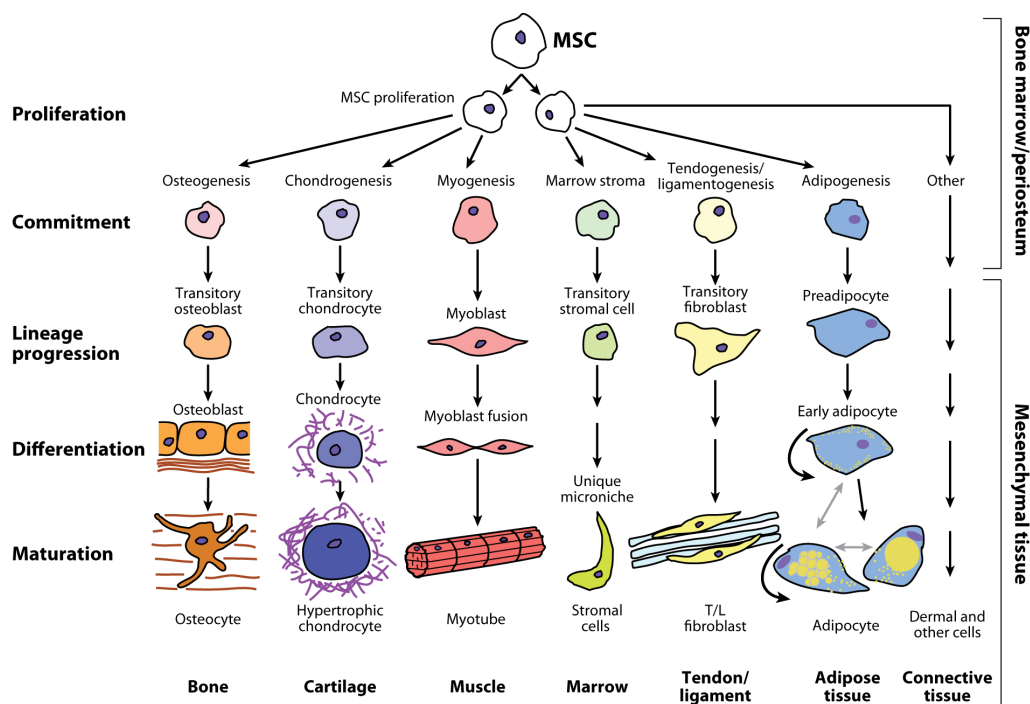


Singer NG, Caplan AI. 2011.
Annu. Rev. Pathol. Mech. Dis. 6:457–78

**Figure 3.** Mesenchymal stem cells multilineage differentiation potential (Caplan A et al, 2011)

Even if hMSCs are isolated by density-gradient fractionation, they remained a heterogeneous mixture of cells with varying proliferation and differentiation potentials. For this reason many attempts have been made to develop a cell-surface antigen profile for the better purification and identification of hMSCs. Particulary important is whether hMSCs isolated from different tissues are identificable by the same immunophenotype.

A further characterization of hMSCs is given by identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency.

Leukemia inhibitory factor (LIF) (Jiang Y., et al., 2002, metal D. 2003), fibroblast growth factors (FGFs) (Tsutsumi S. et al., 2001, Zaragosi L.E. et al., 2006) and mammalian homologues of *Drosophila* wingless (Wnts) (Kleber M and Sommer L., 2004, Boland G.M. et al., 2004), among other growth factors and cytokines, have been implicated in MSC 'stemness' maintenance. MSCs from a variety of mammalian species also express the embryonic stem cell gene markers *oct-4*, *sox-2*, and *rex-1*, among others (Izadpanah R. et al., 2006). Chromatin immunoprecipitation array studies suggest that some Polycomb chromatin-associated proteins are involved globally in maintaining the repression of differentiation genes (Boyer L.A. et al., 2006).

The identification of specific signaling networks and 'master' regulatory genes that govern unique MSC differentiation lineages remains a challenge. The ability to modulate biological effectors to maintain a desired differentiation program, or possibly to prevent spurious differentiation of MSCs, is needed for effective clinical application, as in tissue engineering and regeneration.


## 3.1 Physiological role of Mesenchymal Stem Cells

After years of investigating MSCs out of their native context, little has been learned regarding the identity and function of their precursors in vivo. It is important to note that the fundamental biological properties of mesenchymal stromal cells are likely to be altered by culture conditions and thus should not be directly ascribed to their presumed in vivo counterpart as has often been done in the published literature (Nombela-Arrieta C et al, 2011). Progress in our understanding of MSCs largely relies in having the capacity to recognize progenitor cells in situ, prospectively isolate them and finally assay their multipotency and self-renewal capacity *in vivo*.

***In vivo* perivascular localization.** A key aspect for assessing MSC function *in vivo* is to define their micro-anatomical localization in situ in diverse organs. Efforts to track the identity of tissue-resident MSCs have consistently suggested that these cells lie adjacent to blood vessels (Corselli M et al, 2010). Evidence for such association, came from initial observations that pericytes (also known as Rouget cells or mural cells), which are defined by their perivascular location and morphology, display MSC-like features (Hirschi KK et al, 1996). Pericyte-derived cultures are similar to mesenchymal stromal cell cultures in terms of morphology and cell-surface antigen expression, and can be induced to differentiate into osteoblasts, chondrocytes, adipocytes, but also smooth muscle cells and myocytes under appropriate conditions (Doherty MJ t al, 1998; Collett GD et al, 2005). Conversely, MSC-like cultures were generated from cells enriched directly from tissues based on expression of pericyte-specific markers (Schwab KE et al, 2007). However, evidence that pericytes and MSCs are biologically equivalent has remained indirect for a long time. A recent study identified a combination of markers, such as NG2 (also known as CSPG4), CD146, and PDGFRβ, that seemed to specifically label pericytes in a range of human organs, including fetal and adult skin, pancreas, heart, brain, lungs, bone marrow and placenta. Long-term cultures derived from prospectively isolated pericytes directly from these organs based on specific expression of those marker, displayed similar morphological features to those of cultured mesenchymal stromal cells, as well as trilineage potential in vitro and osteogenic potential *in vivo* (Crisan M et al, 2008).

Collectively, these results strongly suggest that the precursors of cultured mesenchymal stromal cells preferentially reside close to blood vessels *in vivo*, a trait that is not unique to MSCs but pertinent to other multipotent stem or progenitor cells present in adult tissues. In this respect, hematopoietic stem cells (HSCs) as well as other ill-defined tissue progenitors such as white fat progenitor cells and skeletal muscle stem cells, have been reported to reside in perivascular spaces of bone marrow, adipose tissue and skeletal muscle microvessels, respectively (Kiel MJ et al, 2005; Dellavalle A et al, 2007; Tang W et al, 2008;Traktuev DO et al, 2008). Nevertheless, it is important to note that the terms pericyte and MSC are not equivalent or interchangeable. Although widely used to refer to cells surrounding blood vessels, the word pericyte strictly refers to cells adjacent to capillaries and post-capillary venules (Hirschi KK et al, 1996); however, multipotent MSC-like precursors

have been isolated from the walls of other vascular types, including arteries and veins (Tintut Y et al, 2003; Hoshino A et 2008). Furthermore, because pericytes show an extensive tissue distribution along diverse microvascular beds and have many proposed functions (including vessel stabilization, phagocytosis and regulation of vascular integrity and tone) it is likely that functionally heterogeneous, non-equivalent cell subsets are included under the vague term pericyte. Thus, despite being perivascular, not all MSCs can be referred to as pericytes, and not all pericytes exhibit MSC-specific properties.

**MSCs and hematopoiesis.** The stromal compartment of bone marrow (BM) was the first biological material from which MSCs were isolated. Since then, BM-derived MSCs have been the most widely studied and are the best characterized and are thought to be key regulators of BM physiology. During adulthood, the sustained production of blood cells occurs primarily in the BM. MSCs have long been proposed to be the in vivo precursors of some of the non-hematopoietic components of the BM that regulate hematopoiesis, such as osteoblasts, adipocytes and fibroblastic reticular cells (Friedenstein AJ et al, 1966). Consequently, MSCs are likely to contribute to the homeostasis of the haematopoietic compartment in vivo through the regulatory properties of their mature progeny (Figure 4).



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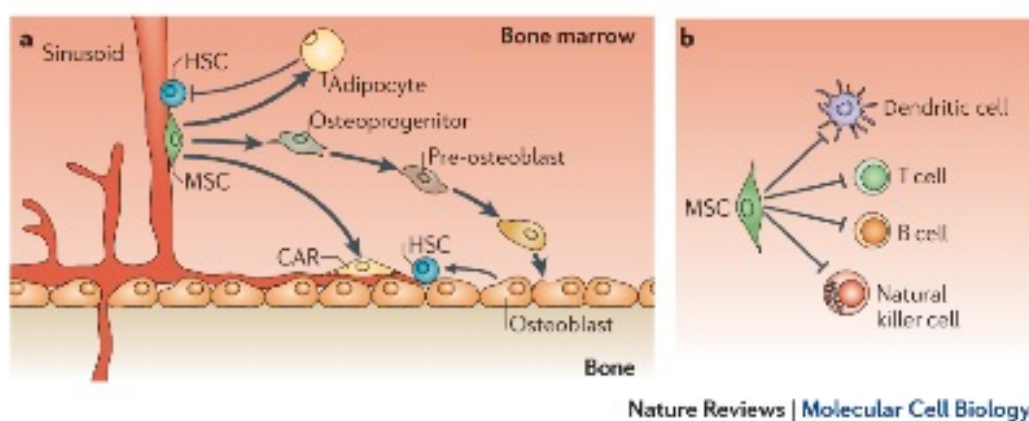
**Figure 4.** Proposed biological functions of BM-resident MSCs *in vivo*. a) MSCs differentiation to provide the supportive environment for hematopoietic development. b) MSCs perivascular localization makes them potentially associate with cells of the immune system. (Nombela-Arrieta C et al, 2011)

Multipotent immature BM-resident MSCs have long been proposed to provide modulatory signals to hematopoietic progenitors based on the fact that mixed cultures

derived from the adherent fraction of BM stoma promote survival and proliferation of HSCs ex vivo (Dexter TM et al, 1977). In addition, MSCs are isolated from all fetal hematopoietic sites even before the HSCs colonization of those tissues (Mendes SC et al, 2005; Campagnoli C et al, 2001). It is therefore thought that MSCs have a key role in the organization of HSCs niches, either through direct interaction or, as proposed (Bianco P et al, 2008), through their reported ability to organize vascular networks, which are key structural and functional components of hematopoietic sites (Sacchetti B et al, 2007; Malero-Martin JM et al, 2008). Recent study, which identified MSCs as Nestin^{+} cells associated with HSCs at the perivascular sites in the bone marrow (Mendez-Ferrer S et al, 2010), has shed light on the issue. Nestin[+] MSCs express high levels of CXCL12, a chemokine that regulates the migration of HSCs. CXCL12 binds to CXCR4 and provides a signal to retain HSCs in the bone marrow (Broxmeyer HE et al, 2005). It has been observed that a small number of HSCs continuously exit to the bloodstream during a hemostatic state (Wright DE et al, 2001; Abkowitz JL et al, 2003). Circulating HSCs survey peripheral tissues and replenish peripheral hematopoietic cells through in situ differentiation, and some of them eventually home to the bone marrow (Massberg S et al, 2007). The release of HSCs into the bloodstream is negatively correlated with the expression of CXCL12 in the bone marrow and is regulated by clock-controlled signals transmitted from the brain to the bone marrow via the sympathetic nervous system (Mendez-Ferrer S et al, 2008). Nestin[+] MSCs, which are enriched in CXCL12 expression and can directly respond to signals from the sympathetic nervous system, are proposed to regulate the trafficking of HSCs. Indeed, depletion of Nestin[+] MSCs leads to increased HSC egress and reduced homing of hematopoietic progenitors to the bone marrow (Mendez-Ferrer S et al, 2010).

**MSCs modulation of immune cells activity.** Immunomodulatory function of MSCs has been proposed because human MSCs (hMSCs) inhibit the proliferation and activation of T cells *in vitro* (Di Nicola M et al, 2002; Le Blanc K et al, 2003). The inhibition is not MHC restricted, and is largely mediated by secreted factors, such as transforming growth factor-β (TGF-β), prostaglandin $E_2$, hepatocyte growth factor (HGF), nitric oxide, interleukin-10, human leucocyte antigen G5 (HLA-G5) and indoleamine 2,3-dioxygenase (IDO) (Meirelles LS et al, 2009; Di Nicola M. et al, 2002, Sato K. et al, 2007, Nasef A. et al, 2007, Meisel L. et al, 2004). The hMSCs

also inhibit B-cell proliferation in vitro (Glennie S et al, 2005; Corcione A et al, 2006). Additionally, in mixed culture hMSCs inhibit the maturation of monocytes into dendritic cells (Jiang XX et al, 2005; Nauta AJ et al, 2006; Ramasamy R et al, 2007), and suppress the pro-inflammatory potential and antigen presentation function of dendritic cells (Jiang XX et al, 2005; Aggarwal S et al, 2005, Beyth S et al, 2005).

Human MSCs attenuate the activation of neutrophils in vitro by inhibiting respiratory burst (Raffaghello L et al, 2008). They also suppress the proliferation of natural killer cells, and restrain their cytotoxic activity (Spaggiari GM et al, 2008; Sotiropoulou PA et, 2006; Poggi A et al, 2005). Given the numerous reports, it seems that hMSCs down-regulate the activities of most, if not all, immune cells in vitro (Figure 5).
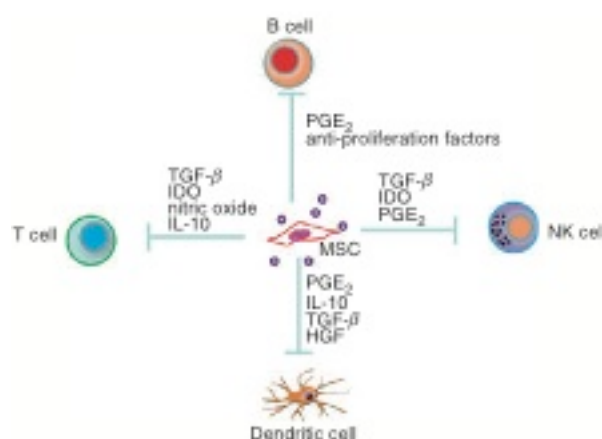


**Figure 5.** hMSCs Suppressive activity against immune cells (Shi C et al, 2012)

The immunomodulatory effects of MSCs are also indicated in animal models, where MSCs are infused to treat pathogenic inflammations. In mice, MSC infusion has been demonstrated to effectively ameliorate autoimmune disorders, including experimental autoimmune encephalomyelitis (Zappia E et al, 2005), diabetes (Urban VS et al, 2008; Fiorina P et al, 2009) and autoimmune bowl disease (Ko JK et al, 2010; Gonzales-Rey E et al, 2009). Infusion of MSC also prolongs the life of a transplanted skin graft in baboons (Bartholomew A et al, 2002). In humans, an immunosuppressive effect of infused MSCs has been reported in acute, severe graft-versus-host disease (GVHD) of a 9-year-old child (Le Blanc K et al, 2004); however, subsequent trials of MSC-based therapies for treating adult GVHD failed to show a significant improvement in mortality (Parekkadan B et al, 2010). The inconsistency of MSCs'

efficacy in preventing GVHD has been observed in preclinical studies (Tolar J et al, 2011). The reasons for this varied efficacy remained unclear because of the incomplete understanding of the mechanisms by which MSCs suppress immune responses. It has been suggested that transplanted MSCs exert their function mainly through secreting soluble mediators, given the fact that there is little evidence showing the engraftment of MSCs after infusion, and that the immunosuppressive effect can be recapitulated by administration of MSC-secreted factors alone (Parekkadan B et al, 2010). The ability of MSCs to produce these factors can be affected by the procedures to isolate the population and expand it *ex vivo* before transplantation. This might explain the variation in difference studies. Future investigation is needed to identify the secreted factors that are essential for the therapeutic effect of MSCs for a specific disease.

It is important to note that most of the studies showing immunomodulatory effects of MSCs were performed on cultured MSCs or transplanted MSCs that had been expanded *in vitro*. Those results might not reflect the physiological function of MSCs *in vivo*. It is possible that the immunological property of MSCs is altered by culture conditions. It remains unclear whether MSCs are immunosuppressive *in vivo* during homeostatic states or during infection. The MSCs express functional Toll-like receptors (TLRs) (Pevsner-Fischer M et al, 2007), which recognize pathogen-associated molecules and other danger signals. Treating MSCs with TLR ligands alters the proliferation, differentiation and migration of the MSCs and their secretion of chemokines and cytokines (Pevsner-Fischer M et al, 2007; Tomchuck SL et al, 2008), with outcomes depending on the nature and context of TLR stimulation. For example, TLR2 and TLR4 ligands enhance the osteogenic differentiation of MSCs, whereas TLR3 ligand inhibits it (Hwa Cho H et al, 2006). Human MSCs primed with TLR4 produce pro-inflammatory mediators, whereas TLR3-primed hMSCs express mostly immunosuppressive ones (Waterman RS et, 2010). It has also been shown that MSCs lose the ability to inhibit T-cell proliferation after triggering of certain TLRs (Liotta F et al, 2008). Therefore, it is possible that stimuli from pathogen-associated molecules during infection can reverse the suppressive feature of MSCs or even skew their function toward promoting immune responses. Indeed, a recent study suggests that MSCs are a critical component of the innate immune system, being able to directly sense the TLR ligands in the circulation and contribute to host defense by

promoting the recruitment of immune cells (Shi C et al, 2011). Additionally, MSCs can function as antigen-presenting cells when primed at a low level of interferon-γ, indicating a protective role against infection in the early stage of the immune response; however, their antigen-presenting function is progressively lost as interferon-γ concentrations increase (Chan JL et al, 2006). This suggests a mechanism that switches the function of MSCs from immunostimulatory to immunosuppressive during the course of infection. The effect of MSCs on the immune system is therefore likely to be context-dependent.

In detail, MSCs mediate their suppressive effect through cell-cell contact and the secretion of various soluble factors (listed above). While MSCs strongly inhibit T cells proliferation via these mechanisms, they preserve the fuction of $CD4^+CD25^+CD127^-$forkhead box P3 $(FoxP3)^+$ regulatory T cells (Treg) (Engela A.U. et al, 2012). Beyond this, *in vitro* studies and studies in animal models have indicated that MSCs have the capacity to generate Treg (Casiraghi F. et al 2008). Intravenous administration of autologous MSCs post-transplant led to a proportional increase of $CD4^+CD25^+CD127^-FoxP3^+$ T cells. Despite their regulatory phenotype, it remained essential to investigate the characteristics and function of these cells. The MSCs-mediated generation of cells with an immunosuppressive function is of particular importance if one considers the fate of MSCs after infusion; Eggenhofer et al. showed recently that after the intravenous administration into mice, MSCs survive no longer than 24 hour. This evidence of short lifespan of MSCs in connection with their proven ability to prolong graft survival prompts further investigation to reveal how MSCs accomplish long-term immunosuppression, and which mediators and mechanisms are involved in this phenomenon.

A hurdle in studying the physiological role of MSCs *in vivo* is the lack of genetic or pharmacological tools that allow for gain-of-function and loss-of-function studies. The work by Mendez-Ferrer et al. (Mendez-Ferrer et al, 2010) and Omatsu et al. (Omatsu Y et al, 2010) has demonstrated that MSCs can be identified, with reasonable specificity, by the expression of a single gene and genetic approaches can be used to deplete those MSCs *in vivo*. It would be interesting to test whether depletion of MSCs in homeostatic conditions will break immune tolerance given the suppressive effects of MSCs.

**MSCs Immunogenicity.** MSCs are considered to be immune-privileged because of their absent or low expression of major histocompatibility complex class II and other co-stimulatory molecules. MSCs have also been found to have an immunosuppressive role. Thus, MSCs have been assumed to be a powerful therapeutic tool that could be used regardless of the major histocompatibility complex identity between donor and recipient. However, recent research has revealed that MSCs can stimulate immune responses under certain conditions. Depending on the IFN-γ level, MSCs can exhibit antigen-presenting properties (Chan JL et al, 2006). At low IFN-γ levels, MSCs can up-regulate the expression of major histocompatibility complex II and gain the ability to act as an antigen-presenting cell (Chan JL et al, 2006). IFN-γ-treated MSCs have also been demonstrated to induce ovalbumin-specific immune responses (Stagg J et al, 2006). The IFN-γ-treated syngeneic MSCs could process the ovalbumin antigen peptide, present it on major histocompatibility complex II molecules, and activate ovalbumin-specific T cells (Stagg J et al, 2006). MSC immunogenicity has also been demonstrated *in vivo* models. The presence of allogeneic MSCs in a non-myeloablative transplantation setting resulted in a significantly increased graft rejection (Nauta AJ et al, 2006; Eliopoulos N et al, 2005; Sbano P et al, 2008).

Furthermore, the administration of allogeneic MSCs induced T-cell responses in naive immune-competent host mice. It appears that MSCs can engraft immune-compromised hosts, but have limited capacity to elicit an immune response in an immune-competent host. Many aspects of the immunogenic properties of MSCs remain to be elucidated; therefore, further studies should validate the efficacy and clinical consequences of the use of MSCs.

**Cell trafficking regulation.** Cultured MSCs constitutively secret a wide range of chemokines, including CCL2, CCL3, CCL4, CCL5, CCL7, CCL20, CCL26, CX3CL1, CXCL1, CXCL2, CXCL5, CXCL8, CXCL10, CXCL11 and CXCL12 (Meirelles LS et al, 2009; Shi C et al, 2012). Immune cells as well as their precursors express the receptors for these chemokines. It is possible that by secreting chemokines MSCs regulate cell trafficking. Studies, which involve the transplantation of MSCs as a regenerative therapy, have suggested that injected MSCs home to the site of tissue injury and continue to express some of the chemokines. Monocytes and macrophages might thereby be attracted and contribute to wound healing. However, it is unclear

whether the expression of chemokines by cultured MSCs is a consequence of *in vitro* activation or the use of specific culture conditions. The expression of chemokines by MSCs *in vivo* has not been well characterized, because of the lack of markers to identify them.

Mesenchymal stem cells also regulate the trafficking of immune cells in the bone marrow. A recent study on monocyte recruitment provides some insights (Shi C et al, 2001). Ly6C$^{hi}$ monocytes are a subset of circulating monocytes that are essential for host defense against a variety of infections (Shi C et al, 2011). They are generated in the bone marrow, and can be rapidly recruited to the peripheral tissues during infection or an inflammatory response. The release of Ly6C$^{hi}$ monocytes from the bone marrow is mediated by CCR2 (C-C chemokine receptor type 2 or CD192), which responds to chemokine CCL2 (chemokine (C-C motif) ligand 2 or monocyte chemoattractant protein-1). Circulating TLR ligands can induce local production of CCL2 in the bone marrow. Using CCL2 reporter mice, CCL2-expressing cells were identified at the perivascular sites with morphology similar to pericytes. *Ex vivo* characterization of these CCL2-expressing cells has revealed their ability to differentiate into osteoblasts, adipocytes and chondrocytes, indicating that CCL2-expressing cells in the bone marrow are MSCs. These cells express TLRs, and they are enriched in CXCL12. Taking advantage of the fact that BM-MSCs are Nestin$^{+}$, CCL2 was specifically deleted from MSCs using Nestin-Cre. The deletion of CCL2 from MSCs resulted in diminished monocyte emigration from the bone marrow, and consequently an impaired immune response against infection (Shi C et al, 2011). This study has demonstrated that bone marrow MSCs can detect microbial products in the circulation and orchestrate innate immune responses by promoting the emigration of monocytes.

The observation that MSCs produce pro-inflammatory chemokines appears to be contradictory to their immunosuppressive effects. However, the ability of MSCs to secrete CCL2 together with other immunomodulatory factors actually provides a unique advantage for MSCs to orchestrate an immune response in the bone marrow.

During infection, immune cells, such as monocytes, are recruited to sites of infection and become activated in the peripheral tissues. The activated monocytes not only control infection but also cause tissue damage. As the bone marrow is rarely a site

harboring infectious pathogens, the destructive activity of immune cells there should be kept in check. It is speculated that MSCs, which are located in close proximity to the sinusoid, attract monocytes to the abluminal aspect of the endothelium by expressing CCL2, and, at the same time, secret regulatory cytokines to suppress the activity of these cells temporally before they traffic to infection sites.

For many years, bone marrow-derived stem cells were the primary source of stem cells for tissue engineering applications (Caplan 1991; Pittenger et al. 1999; Caplan 2007), but a few years ago, Zuk *et al*. (2001, 2002) described a putative stem cell population within the stromal-vascular fraction (SVF) of adipose tissue named processed lipoaspirate (PLA) cells.

## 4. Human Adipose Derived Stem Cells (hADSCs)

Until the year 2000, adult stem cell articles seemed to be limited to the HSCs, the BM-MSCs, the NSC (neural stem cell) and the muscle satellite cell. However, 2001 saw the addition of another adult stem cell to the roster: the adipose-derived stem cell (ASC). In the journal *Tissue Engineering* Zuk PA et al (Zuk PA et al, 2001) first used the term processed lipoaspirate (PLA) cells, owing to their isolation from human lipoaspirates, and proposed that the ASC was a multilineage stem cell population that could be isolated from the stromal vascular fraction of adipose tissue.

The conversion of adipose tissue to calcified bone has been observed in several diseases including lupus, subcutaneous fat necrosis (Shackelford et al.*,* 1975) and Paget's disease (Clarke and Williams, 1975). This conversion should not be possible by the resident, unipotent preadipocyte precursor population. Also, adipose tissue is derived from the embryologic mesenchyme and possesses a well-described stoma that like bone marrow could feasibly contain a mesenchymal stem cell population. The initial results published in *Tissue Engineering* seemed to support this theory.

This isolated cell population showed the same surface markers as bone marrow derived MSCs (Zuk PA et, 2001; De Ugarte D.A. et al., 2003, Boquest A.C. et al., 2005) (Figure 6). One year after, in 2002, the same team undertook a more extensive molecular and biochemical analysis of the ASC (i.e., the PLA cell) (Zuk PA et al, 2002). This article not only confirmed their earlier work that the ASC is capable of

differentiating into multiple mesodermal cell types: adipogenic, chondrogenic, osteogenic, and myogenic, but utilized additional approaches such as the expression of multiple lineage–specific genes and functional biochemical assays to confirm this property. Combining these approaches, the data appeared to fulfill one important requirement of a stem cell: differentiation capacity. However, they also fulfilled another important requirement specific to adult stem cells, that of clonogenicity. One of the most obvious hurdles for adult stem cell identification is the heterogeneity of their origin tissue.
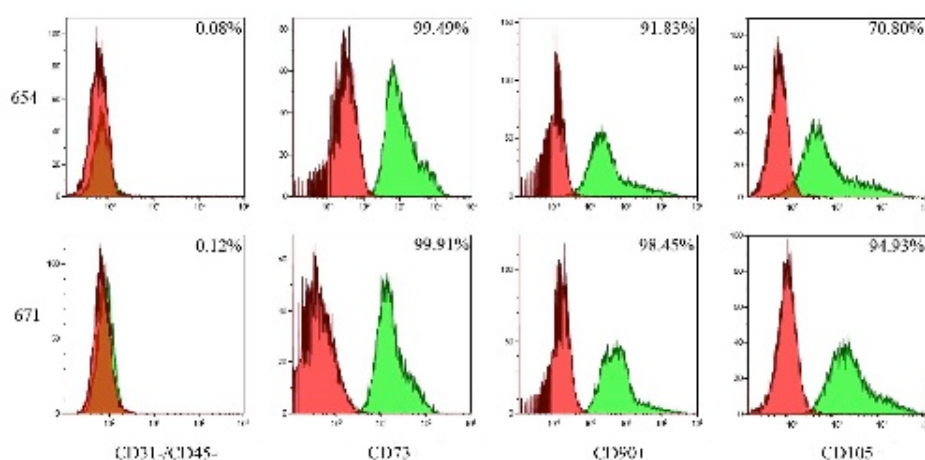


**Figure 6.** Surface markers expression in human adipose derived stem cells (Pendleton C et al, 2013)

Because of this, the observed multilineage differentiation by ASCs may simply be due to the presence of multiple precursor populations, each completing their development. One way to circumvent this would be the isolation of a stem cell, combined with proof of its multipotency. Since 2002, many groups have confirmed that proposal in both human and animal ASC populations. The ability of both human and animal ASCs to undergo mesodermal differentiation at the *in vivo* level has also been presented using a wide variety of animal model systems, but what has become more exciting is the potential of ASCs beyond the mesodermal lineage. Many studies examined the ability of ASCs to differentiate to neuronal-like cells of the ectodermal lineage (Safford KM et al, 2002; Ashjian P et al, 2003). Today, the ability of ASCs to form cells consistent with neurons (Kang S et al, 2004), oligodendrocytes (Safford et al., 2004), functional Schwann cells (Kingham PJ et al, 2007; Xu et al, 2008), and cells of the epidermal lineage (Trottier V et al, 2008) have added credence to the theory that ASCs may be pluripotent rather than multipotent. Not surprisingly, studies

describing the endodermal differentiation of ASCs have also appeared, with ASCs being induced to form hepatocytes and pancreatic islets (Seo MJ et al, 2005; Timper K et al, 2006). The theory that ASCs, like ES cells, may be pluripotent and capable of forming multiple cell types within all three germ layers was proposed (Zuk PA et al, 2010).

The adipose tissue comprises one of the largest organs in the body. Even lean adult men and women have at least 3.0–4.5 kg of adipose tissue, and in individuals with severe obesity, adipose tissue can constitute 45 kg or more of body weight. The adipose organ is complex, with multiple depots of white adipose tissue involved in energy storage, hormone (adipokine) production and local tissue architecture, as well as small depots of brown adipose tissue, required for energy expenditure to create heat (non-shivering thermogenesis) and located primarily in the neck, mediastinum and interscapular areas (Figure 7).
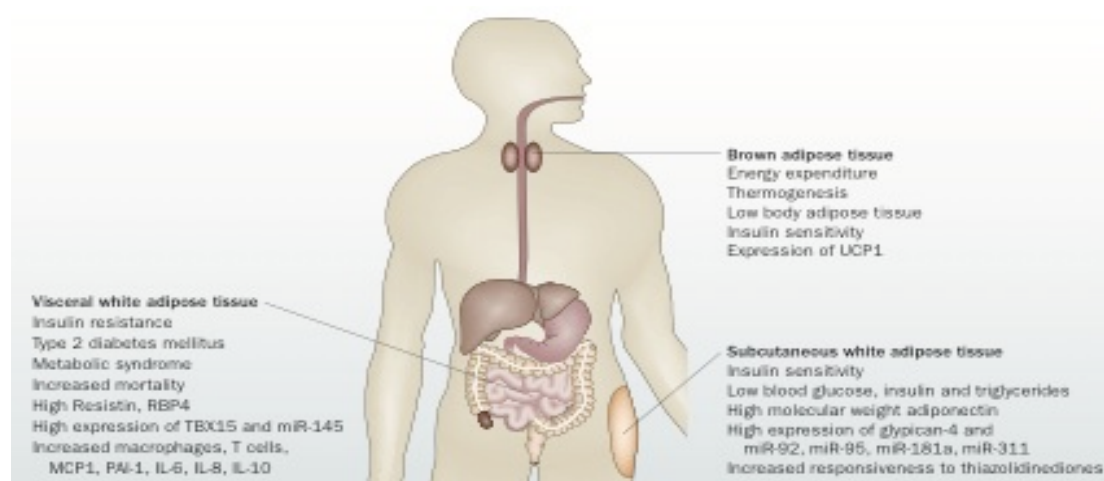


**Figure 7.** Adipose tissue in human. Visceral and subcutaneous white adipose tissue and brown adipose tissue (Tran TT and Kahn CR, 2010)

Although studies are limited, adipose depot specific differences appear to exist with respect to stem cell content (Gimble JM et al, 2007). Whereas multipotent stem cells are abundant within murine white adipose tissue, their numbers and differentiation potential are reduced in brown adipose tissue (Prunet-Marcassus B et al, 2006). In humans, differences in stem cell recovery have been noted between subcutaneous white adipose tissue depots, with the greatest numbers recovered from the arm as compared with the thigh, abdomen, and breast. Furthermore, it is well established that differences exist with respect to preadipocyte and endothelial cell numbers between

subcutaneous and omental white adipose depots in human subjects (Van Harmelen V et al, 2004). It remains to be determined as to which human adipose tissue depot should be harvested for optimal stem cell recovery.

The most used to isolate human adipose derived stem cells is the subcutaneous white adipose tissue, that provides a clear advantage over other MSCs sources due to the ease with which adipose tissue can be accessed (under local anesthesia and with minimum patient discomfort) as well as to the ease of isolating stem cells from the harvested tissue (Casteilla L. et al, 2005, Oedayrajsingh-Varma M.J. et al, 2006). Stem cells frequency is significantly higher in adipose tissue than in bone marrow (Bieback K. et al, 2008).

On the basis that MSCs are associated with blood vessels, Péault team at UCLA, working on ASCs, hypothesized that the pericytes are the component of blood vessels at the origin *in vitro* of conventional MSCs. Culture expanded pericytes displayed a typical MSC immunophenotype and were able to differentiate, *in vivo* or *in vitro*, into all mesodermal lineages, demonstrating, for the first time, a perivascular origin of MSCs (Crisan M et al, 2008). Chen et al. (Chen CW et al, 2013) further showed that pericytes could also promote ischemic heart repair, principally in a paracrine fashion. All pericytes uniformly expressed CD146 and a number of additional studies have reported the existence of perivascular MSCs expressing CD146, among other pericyte markers, in brain (Paul G et al, 2012), adipose tissue (Zanettino AC et al, 2008), placenta (Castrechini NM et al, 2010), endometrium (Schwab KE et al, 2007), bone marrow, and dental pulp (Shi S et al, 2003). Feng et al. (Feng J et al, 2011) used genetic lineage tracing to demonstrate that pericytes indeed differentiate into osteoblasts in a model of incisor growth or in response to incisor damage. However, not more than 15% of the newly formed osteoblasts were generated from pericytes, suggesting the existence of what was defined at that time as a non-pericyte MSC progenitor. The existence of an MSC progenitor distinct from pericytes was suggested by Zimmerlin et al. (Zimmerlin L et al, 2010). The authors used a thorough multicolor flow cytometry approach, combined with immunohistochemical analysis, to define $CD34^+$ $CD31^-$ $CD146^-$ adventitial cells in human adipose tissue. These cells, located in the outmost layer of large vessels (tunica adventitia), were described as MSC progenitors, immunophenotypically and anatomically distinct from $CD146^+$ $CD34^-$

CD31$^-$ pericytes. Similar CD34$^+$ CD31$^-$ MSC progenitors have also been prospectively derived from adipose tissue, saphenous vein and multiple human fetal tissues in independent studies (Traktuev DO et al, 2008; Corselli M et al, 2012; Campagnolo P et al, 2010). Similar to pericytes, adventitial cells are multipotent mesenchymal progenitors able to differentiate into mesodermal lineages and to participate in angiogenesis and heart repair (Campagnolo P et al, 2010; Katare R et al, 2011). Altogether, the advancement in multicolor flow cytometry eventually led to the identification and prospective purification of two distinct progenitors of MSCs, namely pericytes and adventitial cells.

**Functional and therapeutic potential.** The capacity for multi-lineage differentiation allows conversion of ASCs to specialized cells of interest (e.g. insulin-secreting cells and hepatocytes), which would be useful for tissue and cell replacement therapy (Lindroos B et al., 2011).

Alternatively, undifferentiated ADSCs can be administered either via the systemic circulation or directly to the site of intended tissue regeneration for subsequent *in vivo* differentiation into appropriate cell types. Systemically administered ADSCs by default tend to be retrieved in organs such as liver, lungs, heart, and brain (Katz A and Mericli A, 2011). ADSCs express some of the chemokine receptors and ligands and may be directed to sites of injury and inflammation in a mechanism similar to the trans-endothelial migration and diapedesis of leukocytes (Katz A and Mericli A, 2011).

Apart from the application of ADSCs as precursors of differentiated cells for cell replacement, the unique immunobiology and secretome of ADSCs are increasingly appreciated for their therapeutic potential (Figure 8). Like MSCs from other sources, ADSCs are immunoprivileged due to lack of expression of class II major histocompatibilty complex (MHC-II) and co-stimulatory molecules on the cell surface (Lindroos B et al., 2011). This potentially allows allogeneic transplantation of ADSCs into immunocompetent recipients with minimal immune reactions in the host, as exemplified by an *in vivo* study using a rat spinal fusion model (McIntosh KR et al., 2009). In addition, ASCs are also immunomodulatory and can promote tissue repair through immunosuppressive effects exerted via direct cell-to-cell interaction or secreted factors such as prostaglandin E2 (PGE2), leukemia inhibitory factor (LIF)

and kynurenine (Salgado AJ et al., 2010; Cawthorn WP et al., 2012). The immunosuppressive property can be exploited to prevent and treat acute graft-versus-host disease (GVHD) in allogeneic stem cell transplantation, autoimmune diseases and inflammatory diseases such as Crohn's disease, sepsis and rheumatoid arthritis (Lindroos B et al., 2011; Cawthorn WP et al., 2012).
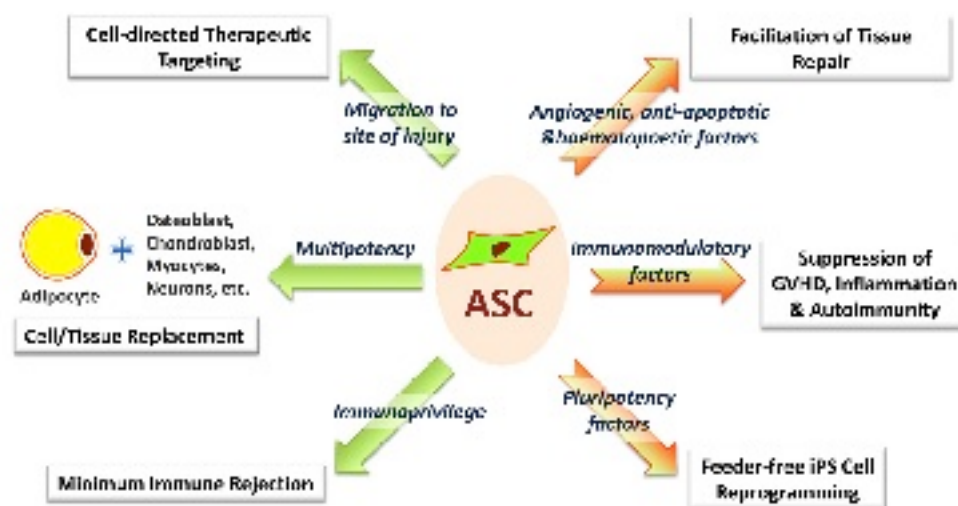


**Figure 8.** Biological properties and therapeutic potential of hADSCs (Ong WK and Sugii S, 2013).

Besides immunosuppressive molecules, ASCs also secrete an array of soluble factors that promote tissue regeneration at the injury site via a paracrine mechanism. The secretome includes angiogenic factors [e.g., vascular endothelial growth factor (VEGF)], anti-apoptotic factors [e.g., insulin-like growth factor-1 (IGF- 1)], hematopoietic factors [e.g., colony stimulating factors and interleukins] and hepatocyte growth factor (HGF) that is hematopoietic, angiogenic and promotes mammary epithelial duct formation (Kilroy GE et al., 2007; Salgado AJ et al., 2010; Baer and Geiger, 2012). It is gaining appreciation that the therapeutic effects of ASC therapy in vivo are largely attributed to the paracrine and immunomodulatory effects of ASCs rather than the cell replacement per se (Katz A and Mericli A, 2011).

ADSCs are also ideal for reprogramming into induced pluripotent stem (iPS) cells. The reprogramming efficiencies of ASCs are substantially higher than those reported for fibroblasts from humans (up to 100-fold) and mice (~5-fold) (Sugii S et al., 2010). Remarkably, the process can be performed without the requirement for feeder cells (Sugii S et al., 2010, 2011). This is possible because ADSCs intrinsically secrete a

high level of self-renewal supporting factors such as FGF2, LIF, fibronectin and vitronectin, and thus can serve as feeders for both autologous and heterologous pluripotent stem cells (Sugii S et al., 2010).

***In vivo* applications.** Today, the proposed uses for ASCs in tissue repair/regeneration are quite impressive. Active areas of research include ischemia revascularization, cardiovascular tissue regeneration, bone/cartilage repair, and urinary tract reconstruction (Table 1).

Work by di Summa et al (di Summa et al, 2009) has suggested that rat ASCs may stimulate peripheral nerve repair, whereas Ryu HH et al (2009) has observed functional recovery upon their transplantation into dogs with acute spinal cord damage. Liver injury repair may also be possible with transplantation of rat ASCs, decreasing key liver enzyme levels and increasing serum albumin (Liang L et al, 2009). Even diabetes may be a target for ASC therapy, with murine ASCs reducing hyperglycemia in diabetic mice (Kajiyama H et al, 2010). Most recently, researchers have begun to explore the potential uses of "reprogrammed" ASCs as iPS (induced pluripotent stem) cells and have suggested that the ASC may be easier to reprogram than the fibroblast (Sun N et al, 2009).

Table 1. Current application of ASCs: a summary

| | References |
|---|---|
| **ASC research activity** | |
| Adipose tissue | Mauney *et al.*, 2007 |
| Bone tissue | Cowan *et al.*, 2004; Dudas *et al.*, 2006; Yoon *et al.*, 2007 |
| Cartilage | Dragoo *et al.*, 2003; Guilak *et al.*, 2004 |
| Skeletal muscle | Bacou *et al.*, 2004; Goudenege *et al.*, 2009 |
| Smooth muscle | Rodriguez *et al.*, 2006 |
| Neural | Ashjian, 2003; Safford *et al.*, 2004; Erba *et al.*, 2009; Nakada *et al.*, 2009 |
| Vocal fold/laryngeal tissue | Kumai *et al.*, 2009; Long *et al.*, 2009 |
| Bladder/urethra | Jack *et al.*, 2005 |
| Cardiovascular tissue/vascular tissue | Miranville *et al.*, 2004; Heydarkhan-Hagvall *et al.*, 2008; Froehlich *et al.*, 2009; Okura *et al.*, 2009c |
| Hematopoietic | Cousin, 2003; Puissant *et al.*, 2005 |
| Pancreatic tissue/islet development | Timper *et al.*, 2006; Okura *et al.*, 2009a; Kajiyama *et al.*, 2010 |
| Hepatic tissue | Seo *et al.*, 2005; Banas *et al.*, 2007; Okura *et al.*, 2009b |
| Epithelial/skin tissue | Brzoska *et al.*, 2005; Jeong, 2009 |
| Kidney tubule epithelial cell | Li *et al.*, 2009 |
| **ASCs in disease and injury** | |
| Intervertebral disc repair | Hsu *et al.*, 2008 |
| Spinal cord injury | Ryu *et al.*, 2009 |
| Peripheral nerve regeneration | di Summa *et al.*, 2009 |
| Glioblastoma treatment | Josiah *et al.*, 2010 |
| Huntington's | Lee *et al.*, 2009 |
| Multiple sclerosis | Riordan *et al.*, 2009 |
| Stroke | Kim *et al.*, 2007 |
| Urinary incontinence | Lin *et al.*, 2010 |
| Erectile dysfunction | Lin *et al.*, 2009a |
| Liver repair | Liang *et al.*, 2009 |
| Diabetes | Lin *et al.*, 2009b |
| Colitis | Gonzalez-Rey *et al.*, 2009 |
| Ischemia | Kondo *et al.*, 2009 |
| Rheumatoid arthritis | Gonzalez-Rey *et al.*, 2010 |
| Antiaging | Park *et al.*, 2008 |
| Wound healing/skin regeneration | Trottier *et al.*, 2008 |
| Cleft palate | Conejero *et al.*, 2006 |
| Tendon repair | Uysal and Mizuno, 2009 |
| **ASC human trials** | |
| Calvarial defect | Lendeckel *et al.*, 2004 |
| Crohn's disease | Garcia-Olmo *et al.*, 2009; Taxonera *et al.*, 2009 |
| Urinary incontinence | Yamamoto *et al.*, 2009 |
| Graft vs. host disease | Fang *et al.*, 2007 |

**Table 1.** Current application of MSCs from adipose tissue (Zuk PA et al, 2010)

The transplantation of human ASCs into a murine model of Huntington's appears to slow progression of the disease, inducing the expression of neuroprotective genes by the host (Lee ST et al, 2009). Human ASCs have recently been used to deliver myxoma virus to experimental gliomas in nude mice, making the ASC a possible vector for oncolytic viral treatment of brain tumors (Josiah DT et al, 2009). Human ASCs engineered to convert 5-fluorocytosine to the antitumor drug 5-fluorouracil have also been used to inhibit prostatic tumor growth. Finally, the ability of ASCs to suppress specific aspects of the immune system (Puissant B et al, 2005) has created another exciting research avenue encompassing everything from organ antirejection to the amelioration of autoimmune diseases (Gonzalez E et al, 2009; Riordan et al, 2009). Nothing seems to be out of the realm of possibility, with work by Park and colleagues investigating whether the secretory products from ASCs can act as anti-wrinkle agents, promoting dermal thickness (Kim JM et al, 2009). Even the popular topic of erectile dysfunction may be solved with the transplantation of ASCs (Lin D et al, 2009).

Moreover, there are emerging clinical applications of the ASC, which started in 2004 with the combination of ASCs and bone grafts to treat extensive craniofacial damage in a 7-year-old girl (Lendeckel S et al, 2004) to a recently completed stage II clinical trial for Crohn's disease (Garcia-Olmo D et al, 2009). ASCs have also been applied in trials for urinary incontinence (Yamamoto T et al, 2009) and graft versus host disease (Fang B et al, 2007).

Although the ES cell with its proven self-renewal capacity and pluripotency would seem to be an appropriate stem cell to use clinically, the recent work on ASCs would suggest that this adult stem cells population may prove to be a powerful weapon in regenerative medicine, since they can be easily harvest from adipose tissue, they are not limited to mesodermal tissue but their differentiation potential is extends to both ectodermal and endodermal tissues and, they have strong immune-modulatory capabilities.

# AIM

Mesenchymal stem cells are powerful sources for cell therapy in regenerative medicine because they can be isolated from various tissues, expanded, and induced into multiple-lineages.

The MSCs population most studied are isolated from bone marrow, but in the last decade cells with similar phenotypic and differentiative features have been obtained from several tissues, including mesenchymal (Pittenger M.F. et al., 1999), neural (Gage F.H. et al., 2000), gastrointestinal (Potten C.S. et al., 1998), hepatic (Alison M. and Sarraf C., 1998), gonadal (Margolis J.and Spradling A., 1995, Conrad S. et al., 2008), and hematopoietic (Weissman I.L., 2000) tissues.

The purpose of this thesis project was establish a standardized new mechanical, non-enzymatic method to obtain mesenchymal stem cells from the human subcutaneous adipose tissue, by use of the Lipogems® device without the need for enzymatic digestion with proteases.

This instrument allows three micro-fragmentation steps of the lipoaspirate adipose tissue, in order to increase the stem cells bioavailability, which resides on the stromal vascular portion of the tissue.

The resulting cell population was characterized to define its "stemness", in term of i) ability to grow in plastic adhesion, ii) multipotency and iii) expression of specific mesenchymal surface markers. Moreover, in order to have a full characterization their growth kinetic, their karyotype after culture and their cell cycle were evaluated.

Besides to the MSCs potential in regenerative medicine, because of their ability to differentiate not only into mesoderm but also endoderm and neuroectoderm, another important potential of MSCs is immune-related property. In a number of *in vitro* studies immunosuppressive effects of MSCs on T cell proliferation have been well described (Engela AU et al, 2013; Lee JM et al, 2012; Najar M et al, 2013).

These immunomodulatory properties have been further confirmed in *in vivo* studies and are being evaluated in clinical trials in disease such as refractory graft-versus-host disease and Chron's disease.

Studied were conducted to evaluate the immunosuppressive properties of the Lipogems® product and MSCs.

# MATERIALS AND METHODS

**Ethical disclosure**

The ethical committee of our Institution approved the design of this study. 59 subjects (males and females, between the age of 30 to 60 years) were enrolled at Istituto Image (Milan, Italy) and Body Sculptique (Weston, FL, USA). The informed consent was obtained from all the patients. All specimens were confirmed for HIV (Human Immunodeficiency Virus 1 and 2), HCV (Hepatitis C Virus), HBV (Hepatitis B Virus) and cytomegalovirus.

**Lipogems®device**

The Lipogems® device is a technological apparatus registered (PCT/IB2011/052204), which allows to process the lipoaspirate adipose tissue, through a mechanic, non-enzymatic, micro-fragmentation.

Processing consist on a progressively reduction in size of the adipose tissue fragments, while eliminating oil residues from the final product suspension, through 3 different steps (Figure 9):

1. fragmentation of lipoaspirate adipose tissue by a  $4mm^2$ mesh;
2. emulsification and removal of oil and blood residues by a saline flow through the device;
3. fragmentation of emulsified lipoaspirate adipose tissue by a $1mm^2$ mesh.
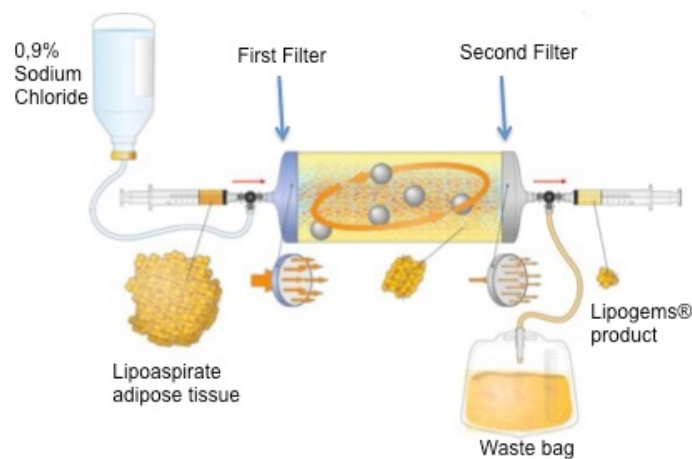


**Figure 9.** Lipogems® device

The procedure takes less the 30 minutes and allow to process about 180 ml of lipoaspirate adipose tissue, obtaining approximately 90 ml of Lipogems® product.

## Isolation and culture of human adipose tissue-derived mesenchymal stem cells (hADSCs)

Human adipose tissue samples were obtained from elective liposuction procedures under local anesthesia. The procedure involved an infiltration step, in which a solution of saline and the vasoconstrictor epinephrine (2μg/ml) was infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells. Subsequently, is provided an inspiration step (lipoaspirate) performed by a 10cc luer lock syringe connected to a disposable 19 cm blunt cannula (3mm OD), with 5 oval holes (1x2mm). A few strokes using a standard liposuction technique are enough to harvest 6 to 10 ml of fat tissue.

*hADSCs from lipoaspirate.* Stem cells from lipoaspirate were purified following previously published methods to obtain cells from stromal vascular portion (SVP) in presence of enzymatic digestion with collagenase I (Sigma) in PBS for 30 min at 37° C (Katz AJ et al, 2005). Enzyme activity was neutralized with an equal volume of Dulbecco's modified eagles medium (DMEM Low Glucose) (Euroclone, Milan, Italy) supplemented with 10% Fetal Bovine Serum (FBS) and the infranatant was centrifuged for 10 min at 2000 rpm (Ahamadi N et al, 2012). Cellular pellet was dissolved in αMEM (Euroclone, Milan, Italy) supplemented with 20% fetal bovine serum (FBS, Euroclone, Milan, Italy), antibiotics (1% Pennicilin/Streptomycin, 0,3 % Amphotericin B). The resulting cell populations were termed Processed Lipoaspirate cells (PLA).

*hADSCs from mechanically micro-fragmented lipoaspirate.* The micro-fragmentation operated by Lipogems® device consist in:

1. Fill 10 ml syringes with lipoaspirate (maximum 18 syringes =180 ml of lipoaspirate);

2. Connect one by one each 10 ml syringes to the unused end of the three way stopcock related to the blue side (4mm$^2$ mesh) and load up the device, keeping

open the exit into the waste bag; in this way the lipoaspirare amount replace the same volume of saline solution;

3. Keeping the two three way stopcock in a position such to create a flow of saline solution through the device, shake until all visible red blood cells and oil residues are removed from the device into the waste bag;

4. Connect a 10 ml syringe at the blue side and fill it with saline solution;

5. Connect an empty 10 ml syringe at the grey side;

6. Keeping close the saline bag and the waste bag entrance into the device push the syringe's plunger allowing the entrance into the device of the saline solution and the exit at the grey side of the Lipogems® product (filling up the empty 10 ml syringe), maintaining the grey side up (Figure 10);

7. Repeat point 4, 5 and 6 until all the Lipogems® product has been collected; leave the sirynges in an upright position to decant for 2 to 5 minutes and carefully remove the excess saline solution.
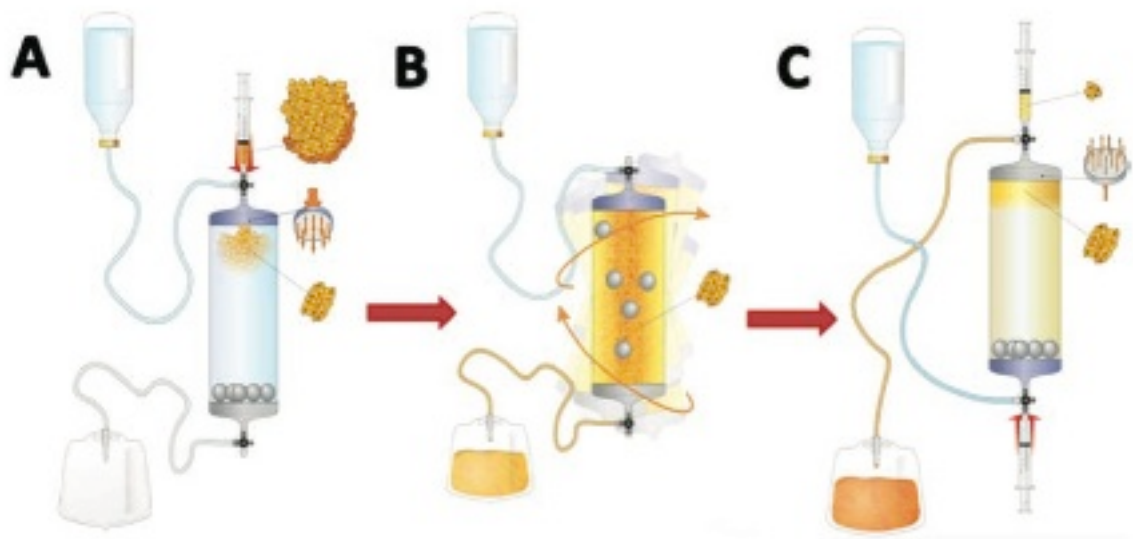


**Figure 10.** Schematic representation of the Lipogems® procedure (Bianchi F. et al 2012).

Stem cells from Lipogems® product were obtained either by centrifugation (10 min at 2000 rpm) or direct seeding of Lipogems® product. Cellular pellet or Lipogems®

product were dissolved in the same media used for PLA cells. As control, a portion of all samples Lipogems® product were treated with Collagenase I, as described before and the pellet was dissolved in the same culture media used above. The resulting cell populations were called Lipogems® hADSCs, Lipogems® product hADSCs and Lipogems® - collagenase hADSCs, respectively. In general all called Lipogems® - derived stem cells.

All cell cultures were maintained at 37° C in humid atmosphere containing 5% $CO_2$. After 2 weeks, the non- adherent fraction was removed and the adherent cells were cultured continuously, while the medium was changed every 3 days. To prevent spontaneous differentiation, cells were maintained at a sub-confluence level; therefore when cells reach 85% confluence, they were detached from the plate by 0.05% Trypsin/EDTA solution, collected by centrifugation (1500 rpm x 5 min) and expanded in culture or cryopreserved at liquid nitrogen in presence of 10% dimethyl sulfoxide (DMSO). After 4 weeks (or later up to 12 months) cells were recovered and kept in culture media.

**Viability and counts after isolation**

Lipogems®- derived stem cells and PLA cells were isolated from four different samples of Lipogems® product and starting lipoaspirate, respectively. Pellets were treated with ACK lysis buffer (Life Technologies) for 10 minutes at 37°C to remove all red blood cells. BD™ Cell Viability Kit was utilized to assay cell viability and to count the final cell number. Following the manufacturer's protocol, have been added 4.0 μL of Thiazole Orange (TO) and 2.0 μL of Propidium Iodide (PI) solution to 2 mL of cell suspension. The final staining concentrations are 84 nmol/L for TO and 4.3 μmol/L for PI. Vortex and incubate for 5 minutes at room temperature. Samples were analyzed by BD Accuri™ C6 flow cytometer using an FSC threshold for mammalian cells, and an SSC threshold for microbial cells. Gate cells using scatter and FL2. TO fluoresces primarily in FL1 and FL2; PI fluoresces primarily in FL3. Therefore, the best discrimination of live and dead populations is on an FL1 versus FL3 plot.

**Lipoaspirate and Lipogems® product cryopreservation**

The major steps of cryopreservation process can be summarized as follows: (1) add cryoprotective agents to cells/tissues before cooling, (2) cool the cells/tissues in a

controlled rate toward a low temperature at which the cells/tissues are stored, (3) warm the cells/tissues, and (4) remove the cryoprotective agents from the cells/tissues after thawing. The optimum-cooling rate for cell survival should be slow enough to avoid intracellular ice formation but fast enough to minimize the cell damage.

Two cryoprotective solution were tested:

1. Dimethyl sulfoxide (DMSO, Sigma) 10% in culture medium. DMSO is a permeable agent that can reduce cell injury due to the intracellular ice formation and "solution effects". It has been widely used as an effective cryoprotective agent in cryopreservation of living cells or tissues. The concentration of DMSO, when used alone as a cryoprotective agent, is usually 10%. Because this agent is tissue toxic at normal body temperature, it should be removed from the previously cryopreserved cells or tissues after thawing.

2. DMSO 0.5M + Trehalose 0.2M in culture medium. Trehalose can dehydrate cells and thus reduce the amount of water present before freezing. It also can stabilize cellular membranes and proteins during freezing and drying. A combination of trehalose, a non-permeable cryo- protective agent, with DMSO, a permeable cryoprotective agent, may significantly enhance the protective effect of adipose tissue during cryopreservation through a possible synergistic mecha- nism but details of the mechanism remain uncertain. Therefore the concentration of DMSO can theoretically be reduced when it is used in combination with trehalose. In Pu LL and colleagues (Pu LL et al, 2009) preliminary studies the optimal combination was determined to be 0.5 Molar (3.3%) DMSO (Sigma) and 0.2 Molar (7.6%) trehalose (Sigma).

Fresh lipoaspirate and Lipogems® product after preparation were put into a vial and mixed with the solution 1 or the solution 2 in a one to one ratio. After adding cryoprotective agents, the vial was placed in room temperature for 10 minutes and then placed into a methanol bath (Mr Frosty Freezing container, Nalgene). The freezing system was programmed to cool at 1~2°C per minute from 22°C to -30°C without artificial ice formation. The vial was then transferred to liquid nitrogen (-196°C) after it reached -30°C for long-term preservation.

Before thawing, the vial containing cryopreserved adipose aspirates was taken from

the liquid nitrogen tank and placed at room temperature for 2 minutes to let the liquid nitrogen vapor out of the vial. The vial was then dropped into a stirred 37°C water bath until the preserved adipose aspirates were thoroughly thawed.

**Measuring of the fragments sizes**

Samples of Lipogems® product and starting lipoaspirate adipose tissue obtained from three different donors were collected in 35mm cell culture dishes with 2mmx2mm grid in PBS. Pictures were taken by use of Canon Powershot SD 1200 IS camera. All tissue fragments area were measured using MetaMorph® NX Microscopy Automation & Image Analysis Software and the statistical analysis was performed with GraphPad Prism 6, with One way ANOVA Turkey's multiple comparisons test.

**Immunohistochemical Analysis**

Histological analyses were performed on fresh or cryopreserved Lipogems® product and starting lipoaspirate adipose tissue. Formalin-fixed paraffin-embedded tissue samples were processed for conventional histopathological examination and immunohistochemistry. Standard 4 μm thick tissue sections stained with hematoxylin and eosin (H&E) were examined by direct wide field light microscopy. For immunohistochemistry, samples were sectioned (4μm-thickness), de-paraffined, and re-hydrated in xylene and graded concentrations of ethanol to distilled water. Then they were rinsed with PBS, treated with blocking solution (PBS supplemented with 1% fetal bovine serum, FBS) for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. After treatment with primary antibodies, the sections were washed with PBS and incubated with appropriate secondary antibodies. Antigen retrieval slides were placed in a water bath containing ethylenediaminetetraacetic acid (EDTA) 1mM, pH 9.0 for 30 minutes at 95C°. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 10 min. Staining was performed with 3,3' diaminobenzidine (DAB) as a chromogen (DAKO EnVision detection kit). In control determinations, primary antibodies were omitted and replaced with equivalent concentrations of unrelated IgG of the same subclass. The following primary antibodies were used: Vimentin (1:5000; Dako Cytomatic); S100 (1: 2000; Novocastra); Microtubule-Associated Protein 2 (MAP-2) (1:500; Chemicon), Glial Fibrillary Acidic Protein (GFAP) (1:500; Dako Cytomatic), β-Tubulin III (1:6000; Covance); human Nestin (1:2000; Millipore);

Nanog (1:1000; Novus). Immunoreactivity was evaluated and positive cells were quantified considering 50 high magnification histological fields (40X). Results were expressed as number of positive cells per histological power field (hpf). For immunofluorescence studies, the sections (4 μm thick) were rinsed with PBS, treated with blocking solution (as above) and incubated with primary antibody anti β-Tubulin III (1:6000; Covance), anti- Vimentin (1:5000; Dako Cytomatic) and anti- FABP4 (1: 200, Cell Signalling Technology) overnight at 4°C. After treatment with primary antibody, sections were washed with PBS and incubated with secondary antibodies (Alexa Fluor® 555 goat anti-mouse or Alexa Fluor® 488 goat anti- rabbit 1:1000, Molecular Probes®, Invitrogen Life Technologies Italia, Monza, Italy) for 2 hours at room temperature. Sections were washed in PBS, nuclei were stained with DAPI (Hoechst 1/1000) and then mounted using the FluorSave Reagent (Calbiochem, Merck Chemical, Darmstadt, Germany) and analyzed by confocal microscopy. As negative reference for the confocal analysis we used a consecutive section that was stained by omitting primary antibodies and replacing them with equivalent concentrations of unrelated IgG of the same subclass. The zero level was adjusted on this reference and used for all the further analyses (we used a new zero reference for each new staining). Results were expressed as percent of positive cells per histological power field (hpf). DAPI supplied the total number of cells being a nuclear staining. This quantification was performed with sections from three different patients.

**Determination of Proliferation Kinetics**

Analysis was performed by cell counting the proliferative capacity of Lipogems®- derived stem cells and PLA cells from 6 patients, which were maintained in culture. The analyses were performed for cells at culture passage 2, fresh and cryopreserved for 1 month or more. Cells were seeded into 48-well culture plates and maintained in culture in growth medium. Live cells were counted by Trypan blue exclusion at 85% confluence. Cell doubling times (DT) and numbers (CD) were calculated from haemocytometer counts and cell culture time (CT) for each passage by the following formula: CD= ln(Nf/Ni)/ln2 and DT=CT/CD, where DT is the cell doubling time, CT the culture time, CD the cell doubling number, Nf the final number of cells, and Ni the initial number of cells. The experiments were performed at least three times for

each point reported in the curve (Sabapathy V et al, 2012; Tchoukalova YD et al, 2012, Christodoulou I et al, 2012).

**Cell Cycle Analysis**

Using Propidium Iodide (PI) incorporation method (Paulis M et al, 2007) were performed cell cycle analyses. Briefly, $5 \times 10^5$ cells were collected, fixed for 15 min at 4°C in ice cold (-20°C) 70% ethanol. The fixed cells were resuspended and centrifuged. The cell pellet was treated with RNase (Sigma) to avoid the RNA contamination, and stained with 25 μg/ml PI at 37°C for 1 hour in the dark. DNA content was analyzed by Cytomics FC 500 (Beckman Coulter). Under these conditions, quiescent cells (G0/G1) were characterized by the minimal RNA content and uniform DNA content. The results of the study were expressed as mean ± SEM.

**G-banding karyotype Analysis**

Cytogenetic analyses were performed on "in situ" cultures obtained by inoculating Lipogems®- derived stem cells directly into a coverslip inside Petri dishes containing 2 ml of media. Cells were treated with Colcemid (0.02 μl/ml) for 90 minutes, hypotonic solution (1:1 Na citrate 1%: NaCl 0,3%) and fixative solution of 3:1 methanol:acetic acid, replaced twice. At least twenty-five QFQ banding metaphases were observed for each sample. The images were acquired using a fluorescence microscope (BX 60 Olympus) and analyzed with Powergene PSI system.

**Flow Cytometry Analysis**

Lipogems®- derived stem cells and PLA cells at passage zero (p0) and, after culture, at passage two (p2) were phenotypically characterized by Flow Cytometry. After detachment by means of 0.05% Trypsin/EDTA, cells were washed and $1 \times 10^5$ cells were re-suspended in 100 μl PBS w/o Ca2+ and Mg+ (Euroclone Italy) for staining with the following antibodies: anti-CD90 FITC (BD Biosciences), anti-CD73 Brilliant Violet 421 (Biolegend), anti-CD105 PE (Biolegend), anti-CD146 Alexa Fluor 647 (Biolegend), anti-CD31 biotin (eBioscience), anti-CD34 PE/Cy7 (Biolegend), anti-CD44 Brilliant Violet 421 (Biolegend), anti-CD45 Brilliant Violet 510 (Biolegend), anti-CD19 PE (eBiosciences), anti-CD14 APC (Biolegend), anti-HLA-DR PC7 (Beckman Coulter). Where staining with biotin-conjugated antibodies

was performed the Brilliant Violet 605 streptavidin (Biolegend) was used as chromofore. Dead cells often give false positive results, as they tend to bind nonspecifically to many reagents. Therefore, removing dead cells from flow cytometry data is a critical step, so live and dead cells discrimination was perform by LIVE/DEAD® Fixable Dead Cell Stain near IR (Life Technologies). Samples were fixed with paraformaldehyde 1% and then analyzed by flow cytometry (BD LSRII flow cytometer, BDBiosciences) using BD FACSDIVA™ software (BDBiosciences). The same procedure was utilized in the immunophenotypic characterization of Lipogems® hADSCs and PLA cells after cryopreservation (1 month or later) and Lipogems® hADSCs obtained from Lipogems® product thawed after different periods (1 week, 1 months and two months) and directly seeded in culture. The results of the analysis were expressed as mean ± SEM.

**In vitro differentiation.**

*Adipogenic differentiation.* Lipogems®- derived stem cells and PLA cells were seeded ($6 \times 10^3$ cells/cm²) in adipogenic medium following already published protocols (Kawaji A et al, 2010; Kim CY et al, 2011). The adipogenic medium consisted of DMEM High Glucose supplemented with 10% FBS, 1μmol/L dexamethasone, 500 μM 3-isobutyl-1-methyl-xanthine and 10μM insulin. After two weeks in culture cells were fixed in 10% formaldehyde for 1 hour and stained with Oil red-O (Han KL et al, 2006) solution to show lipid droplets accumulation. For the quantification of intracellular lipid accumulation of Oil Red O, the stained lipid droplets were eluted with 100% isopropanol for 10 min. The optical density was measured at 500 nm by spectrophotometer (Lambda Bio, Perkin Elmer).

*Osteogenic differentiation.* Lipogems®- derived stem cells and PLA cells were seeded in osteogenic medium ($10^4$ cells/cm²) in DMEM Low Glucose, 10% FBS, 10nM dexamethasone, 200μM ascorbic acid, 10mM β-glycerol phosphate (Meyers VE et al, 2005). Cells were maintained in culture for 3 weeks and then fixed in 70% ethanol and stained with 1mg/ml Alizarin Red S dye to detect mineralized matrix (Liao J et al, 2011).

*Neural differentiation.* Three different protocols were tested:

1. Lipogems®- derived stem cells and PLA cells were seeded at the density of

$4 \times 10^4$ cells/cm$^2$ in presence of BD Matrigel™ and basic fibroblast growth factor (bFGF, 10 ng/ml) for 48 hours. For others 5 days the growth factor was removed and FBS 1% was added to the medium (Marfia G. et al, 2011).

2. Lipogems®- derived stem cells and PLA cells were seeded at the density of $6 \times 10^4$ cells/cm$^2$ in presence of collagen bovine type IV (BD Pharmigen) in the NEU medium (Stem Cell Medium (SCM) deprived by mitogens: EGF and bFGF), and 10% of FBS for 2 weeks (Navone S et al, 2012).

3. Lipogems®- derived stem cells and PLA cells were seeded at the density of $1 \times 10^5$ cells/cm$^2$. 24 hours of pre-induction are required, in DMEM, supplemented with 10% FBS and 1mM βmercaptoethanol. Subsequently, the medium was change in DMEM supplemented of 2% DMSO, 1-10 mM βmercaptoethanol and 200 μM butylated hydroxyanisole (BHA) and cells were keep in culture for 5 days (Ahamadi N et al, 2012).

Differentiated cells were characterized by immunochitochemical staining.


**Indirect immunofluorescence**

After seeding ($3.5 \times 10^3$ cells/cm$^2$) onto glass slides and grown until 85% confluence, cells were fixed with 4% paraformaldehyde. After saturation (4% BSA, 0,3% Triton X-100) and permeabilization, cells were incubated overnight at 4°C with primary antibodies against human Nestin (Clone #196908, R&D Systems), Vimentin (Polyclonal, Santa Cruz), GFAP (Polyclonal, Covance), O4 (Clone 81, Chemicon), β-tubulin III (Clone TUJ1, Covance), EpoR (Clone H-194, Santa Cruz). Cells were rinsed and then probed 45 minutes with secondary antibodies Alexa Fluor 488 or 543 anti-mouse, anti-rabbit or anti-goat (Invitrogen, Carlsbad, California). Nuclei were counterstained with DAPI (2 μg/ml in PBS, Roche), and glasses were mounted with FluorSaveTM (Millipore). Images were taken using Leica SP2 confocal microscope with He/Kr and Ar lasers (Heidelberg, Germany). In negative control experiments, primary antibodies were replaced with equivalent concentrations of unrelated IgG of the same subclass. The quantification of positive cells was performed by considering on a minimum of 9 independent fields (3 fields/3 coverslips/treatment) of photomicrographs captured with 20x objective. Total counts of each markers immune-reactive cell were performed and the number of positive cells was expressed

as the percentage to the total cells. DAPI supplied the total number of cells being a nuclear staining.

**PBMCs Isolation and T-lymphocytes selection**

Peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coats of healthy donors (Continental Group Services, Inc, Miami) or umbilical cord blood (New York Cord Blood Bank, New York City) after Ficoll-Paque™ Premium (GE Healthcare Life Sciences) gradient centrifugation. Using the MACS system (Miltenyi Biotec) according to the manufacturer's instructions, $CD3^+$ T-lymphocytes were purified (> 95% purity) by negative selection (Figure 11).
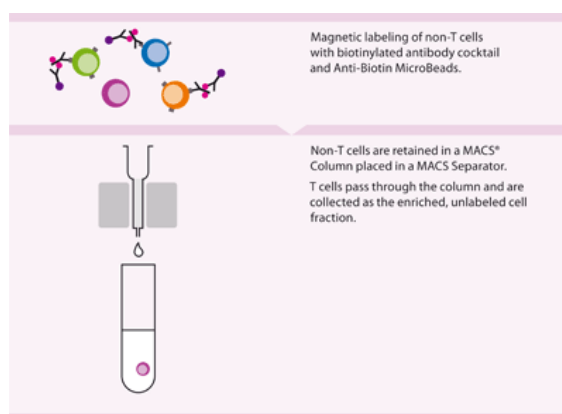


**Figure 11.** Magnetic negative selections of T lymphocytes population

**Co-culture of T lymphocytes with PLA cells and Lipogems®- derived stem cells**

PLA cells and Lipogems®- derived stem cells were plated in round bottom 96well plates at different concentration (from $2x10^6$cells/ml to $2,5x10^4$ cells/ml) in RPMI, supplemented with 10% FBS and βmercaptoethanol. T-cells ($2x10^5$) were plated and activated by Dynabeads® human T activator CD3/CD28 (Figure 12) (1:1 ratio with T lymphocytes, Life technologies).

Lymphocyte proliferation was assessed by flow cytometry using CellTrace™ Violet Cell Proliferation Kit (Invitrogen) labeling. After 3 days, T lymphocytes were collected and assessed for the expression of CD3 (anti CD3 PerCP Cy 5.5, Biolegend), CD4 (anti-CD4 BV605, BD Biosciences), CD8 (anti-CD8 PECy7, BD Biosciences), CD25 (anti-CD25 PE, BD Biosciences), CD127 (anto-CD127 BV711, BD Biosciences) and Forkhead box P3 (anti-Foxp3 APC, eBiosciences) by flow cytometry analysis.

**Figure 12.** Dynadeads® human T activator CD3/CD28 mechanism.

# RESULTS

Human Adipose derived stem cells (hADSCs) were obtained from subcutaneous adipose tissue of 59 donors subjected to elective liposuction procedure under local anesthesia. Each tissue sample was processed by different methods:
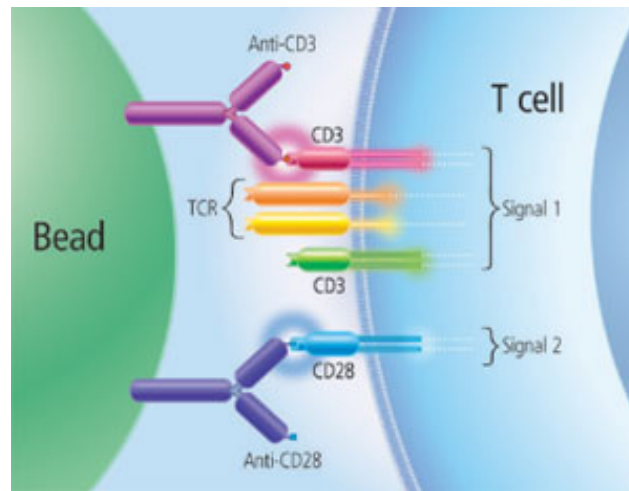
1. Prepared by enzymatic digestion with Collagenase I and the resulting cell populations were termed Processed Lipoaspirate cells (PLA);
2. Lipogems® device followed by enzymatic digestion with Collagenase I, and the resulting cell populations were called Lipogems® - Collagenase hADSCs;
3. Lipogems® device followed by a single centrifugation and the resulting cell populations were termed Lipogems® hADSCs;
4. Lipogems® device and directly seeding of Lipogems® product, where the obtaining cell populations were named Lipogems® product hADSCs.

The hADSCs prepared by these methods were compared in term of viability, yield of cells, surface markers expression, differentiation capability and immunomodulatory properties.

## 1. Characterization of human lipoaspirate adipose tissue and corresponding Lipogems® Product

### 1.1 Evaluation of size reduction of the adipose tissue fragments

In order to assess the dimensions of adipose tissue fragments before and after the use of the device, samples of Lipogems® product and starting lipoaspirate adipose tissue obtained from three different donors and evaluated (Figure 12).
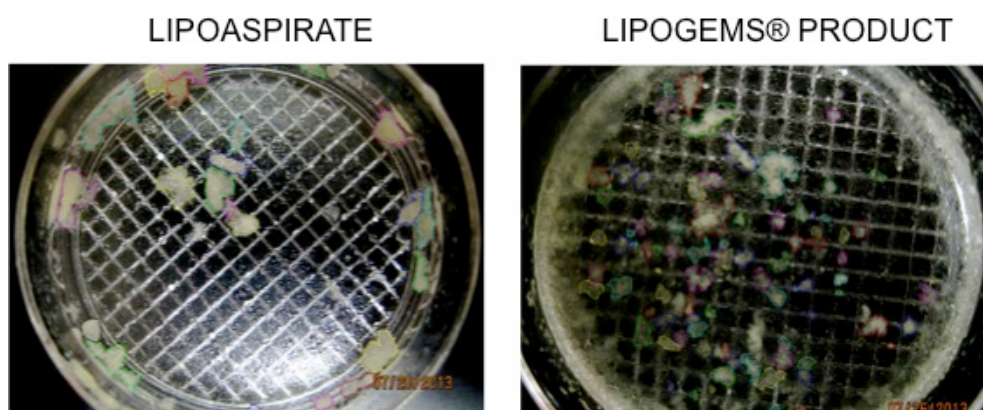


**Figure 12.** Pictures taken by Canon Powershot SD 1200 IS camera and analysed using MetaMorph® NX Microscopy Automation & Image Analysis Software. Grid= 1mm$^2$

Using MetaMorph$^®$ NX Microscopy Automation & Image Analysis Software was used to outline and measure tissue fragment area. The mean area was $4,775 \pm 0,508$ mm$^2$ and $1,036 \pm 0,106$ mm$^2$ for lipoaspirate and Lipogems$^®$ product, respectively (Figure 13), highlighting an 80% size reduction.
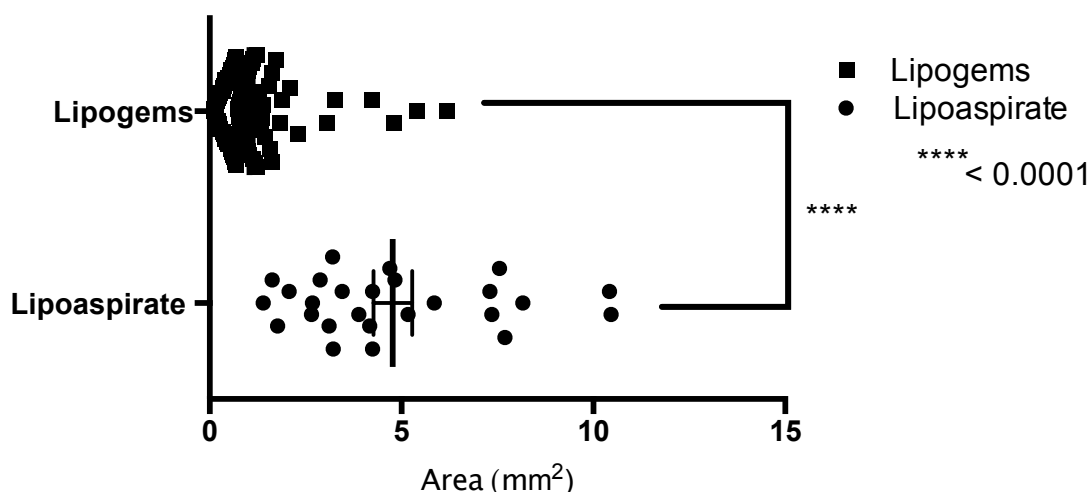


**Figure 13.** One way ANOVA Turkey's multiple comparison test to evaluate the decrease of the fragments size as a result of using of the Lipogems$^®$ device.

1.2 Immunohistochemical features analysis

Formalin fixed paraffin embedded sections of lipoaspirate and corresponding Lipogems$^®$ product were studied by means of immunohistochemistry. Results showed a similar structure and a comparable distribution of the stromal vascular fraction, as evidenced by hematoxylin and eosin (H&E) staining (Figure 14).

Vimentin is a type III intermediate filament protein, that is expressed in mesenchymal cells, as their major cytoskeletal component. In Figure 14 the high expression of Vimentin would give a further idea about the structure of the tissues and the absence of abnormalities after freezing (Figure 14B) or after centrifugation (Figure 14C).

It is becoming increasingly evident that physical forces in the microenvironment play a key role in regulating many important aspects of cell biology. Some authors have suggested that both adipose- and bone marrow- derived MSCs may express nestin and other markers in response to manipulation, such as cytoskeletal distruption and extreme stress (Gimble JM et al, 2006; Lu P et al, 2004; Neuhuber B et al, 2004).

The expression of neural markers such as Nestin and βTubulin III (Tuj1) was investigated in lipoaspirate and Lipogems® product samples (fresh and frozen), and cell pellets obtained by centrifugation (Figure 14). Both tissues and their cell pellet shown positivity for Nestin, which is an intermediate filament protein expressed in dividing cells during the early stages of development in the central nervous system (CNS), peripheral nervous system (PNS), but also it consider a marker for neural stem cells.

Moreover Lipogems® product expressed the neural precursor marker βTubulin III ($0,18 \pm 0,03$ positive cells/hpf), meanwhile lipoaspirate samples resulted negative.

These data suggest an enrichment of cells with neural markers after Lipogems® processing compared to the lipoaspirate.
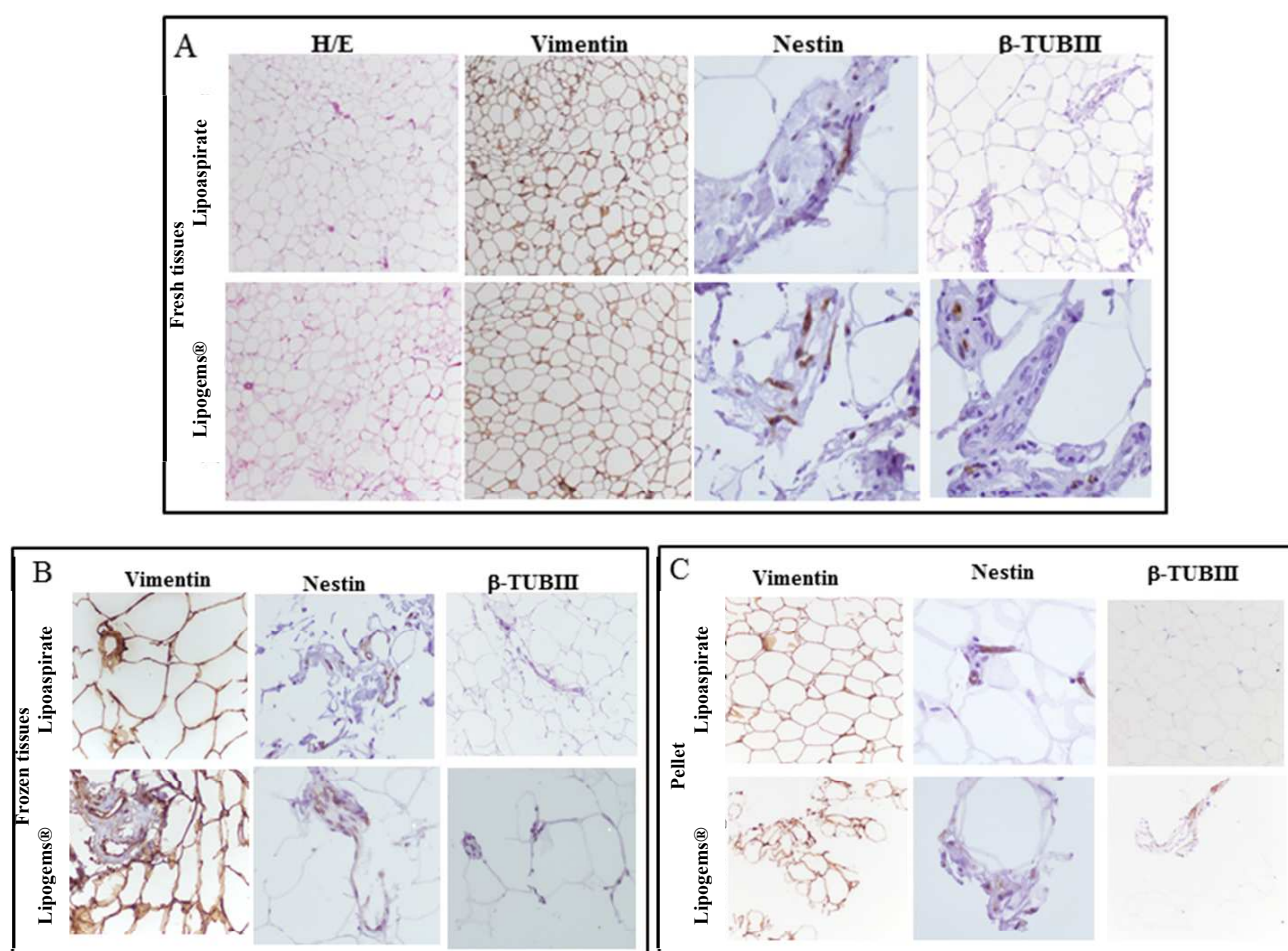


**Figure 14.** Comparative immunohistochemical analysis performed on fresh tissues, after their cryopreservation at -80° for 72 hours, and on the pellet of cells obtained after the fresh tissues centrifugation (2000 rpm x10 min; pellet). Comparative histological analysis was performed on tissue samples obtained from the same donor.

1.3 Viktig and count of adipose-derived stem cells after isolation

Lipogems® - derived stem cells and PLA cells were isolated from lipoaspirates, each from a different donor. Starting from 1 ml of lipoaspirate treated with Collagenase I, Lipogems® product digested with Collagenase I, and Lipogems® product processed by centrifugation, we obtained 34189,22 ± 14737,13 cells, 32949,83 ± 25891,37 cells and 1427,31 ± 844,98 cells, respectively. As shown in Figure 15, there was a considerable variability between donors as indicated by the standard error of the mean (SEM).
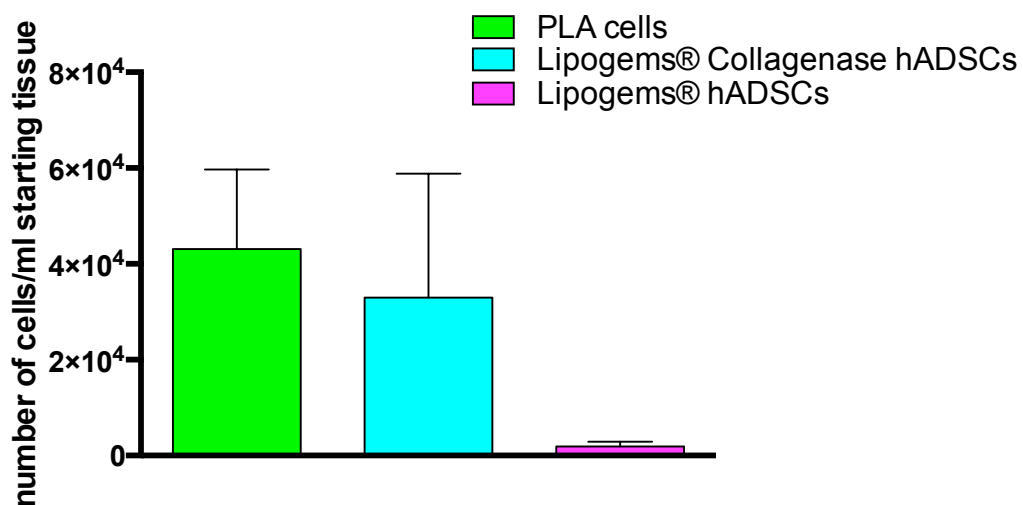


**Figure 15.** Number of cells that could be obtained from 1 ml of starting lipoaspirate and corresponding Lipogems® product, processed by enzymatic digestion or by centrifugation evaluated by use of BD™ Cell Viability kit and BD Accuri™ C6 Flow Cytometer.

Without the enzymatic digestion the yield of cells is much lower. This was further confirmed by culture of the isolated cells that required almost two weeks more time than cells obtained with Collagenase I treatment to reach the confluence.

1.4 Analysis of the immunophenotype of cellular populations contained in lipoaspirate adipose tissue and Lipogems® product

To further understand what is initially present in the two tissue products, Lipogems® Collagenase hADSCs and PLA cells obtained from fresh tissues were analyzed for surface marker expression by flow cytometry immediately after isolation. Considering the very low number of cells that is recovered from Lipogems® product by

centrifugation without enzymatic treatment, we didn't have sufficient cells for flow cytometry analysis.

As is shown in Figure 16, Panel A 16,46% of PLA cells and 20,71% of Lipogems® Collagenase hADSCs were non-hematopoietic cells (CD45⁻), showing a relative enrichment, by the Lipogems® method, of these cells.

Within this population of cells they were further characterized by the expression of mesenchymal markers (CD90, CD73, CD105 and CD44) (Figure 16, Panel B).

The increased expression of these markes in Lipogems® Collagenase hADSCs compared to PLA cells suggest an increased recovery of MSCs after the Lipogems® processing. In this Panel is shown the presence of Pericytes population (CD146⁺ CD31⁻ CD34⁻), and the Supradventitial population (CD146⁻ CD31⁻ CD34⁺) equal to 5,04 ± 2,38 percentage for PLA cells and 11,10 ± 4,75 percentage for Lipogems® Collagenase hADSCs.



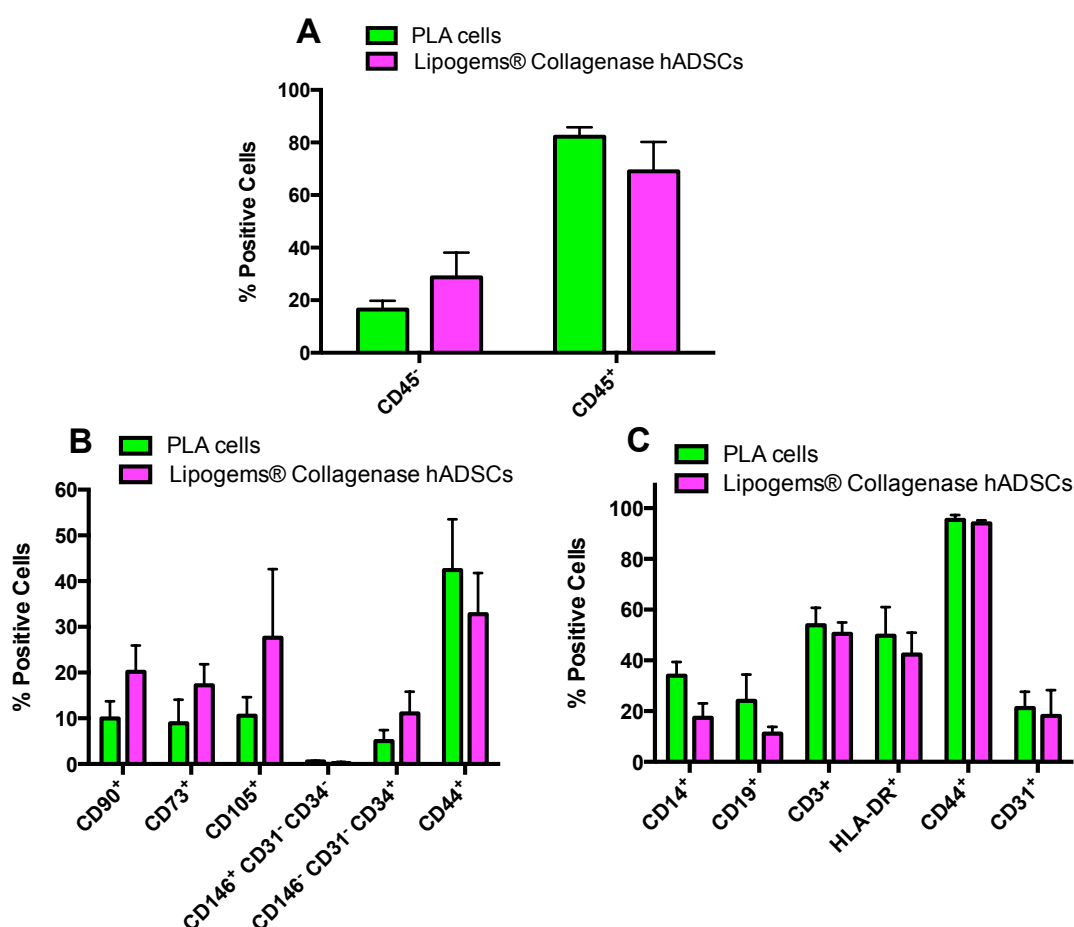**Figure 16.** Flow cytometry data from five different cases, from which have been obtained PLA cells from lipoaspirates adipose tissue and Lipogems® Collagenase hADSCs from the correspondent Lipogems® products. (A) CD45⁻ population; (B) CD45⁺ population and (C) particular of CD45⁻ portion with Pericytes and Supradventitial populations.

CD90 or Thy-1 is a glycophosphatidylinositol (GPI) anchored conserved cell surface protein. It was originally discover as the first T lymphocytes marker but now is used also as marker of a variety of stem cells, including MSCs, and for the axonal processes of mature neurons.

CD73 or 5'-nucleosidase (5'-NT) is a plasma membrane protein that catalyzes the conversion at neutral pH of extracellular nucleotides to membrane-permeable nucleosides. Mesenchymal stem cells result positive for this marker.

CD105 or Endoglin is a part of the TGFβ receptor complex and the presence of CD105, together with CD90 and CD73 can define a MSCs population (Dominici M et al, 2006).

CD44 is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs).

In the Panel C of Figure 16 is shown a consistent population of $CD45^+$ and within it a population of T lymphocytes $CD3^+$ close to 50% for both cell preparations. Moreover, resulted present also other immune system cells types, such as macrophages and neutrophils ($CD14^+$), B lymphocytes ($CD19^+$), antigen presenting cells (APC) and activated T cells ($HLA-DR^+$), monocytes and again some types of T-cells ($CD31^+$).

There was also a very high positivity for CD44 inside $CD45^+$ population, which is an indicative marker of effector-memory T cells.

No statistically significant difference was found between the two cell preparations, using a Two-way ANOVA with Sidak correction for multiple comparisons.

## 2. Isolation and culture of Lipogems® - derived stem cells and PLA cells

Adipose tissue collected during liposuction surgery was processed by means of Lipogems® device. hADSCs were obtained with or without Collagenase I enzymatic digestion. Live morphology (captured by means of EVOS® microscope apparatus, AMG, USA) of these cells (Figure 17, A-D) presented a fibroblast-like phenotype comparable to that of PLA cells obtained from lipoaspirate processed by means of classical enzymatic digestion method (Figure 17, panels G and H). Lipogems® - derived stem cells, obtained with or without enzymatic digestion, are able to grow in mesenchymal classic medium (αMEM) and in neural Stem Cells Medium (SCM).
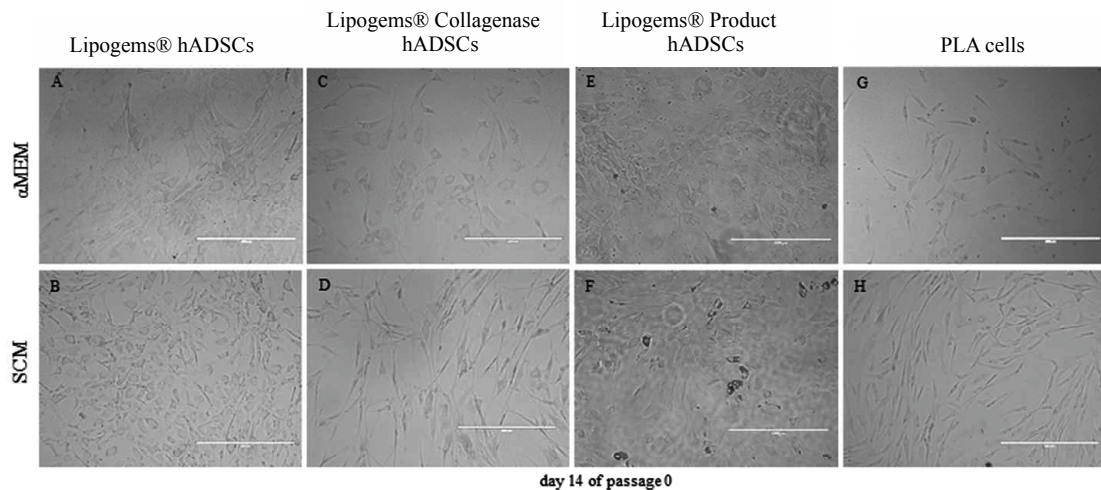
**Figure 17.** Morphological features of Lipogems®- derived stem cells and PLA cells. Scale bar was 400μm.

With very similar efficiency, hADSCs can also be obtained by directly plating the Lipogems® product (Figure 17, panels E and F). In standard conditions (without Collagenase I treatment, but with centrifugation of Lipogems® product) it becomes possible to isolate $5 \times 10^5$ hADSCs from 1 ml. These cells maintained in αMEM are flat, large with few short processes (Figure 17), while those grown in SCM appear slim and elongated with one or few elongated processes (Figure 17).
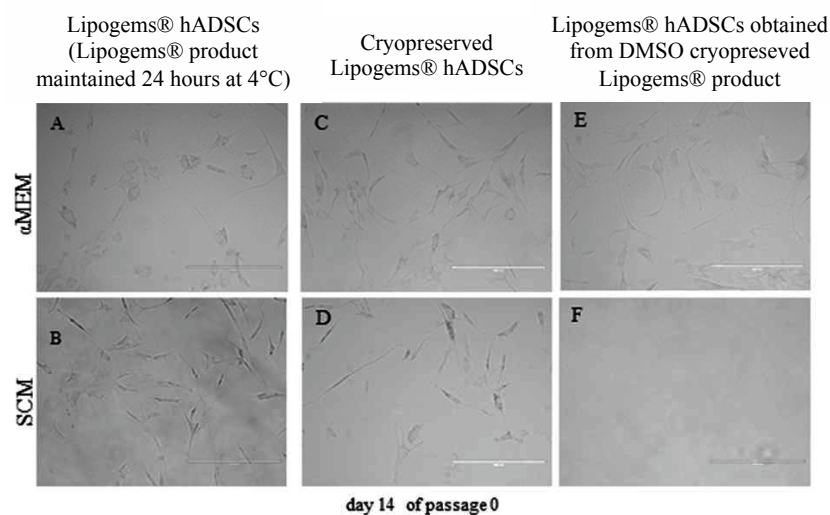


**Figure 18.** Morphologies features after 4°C maintaining for 24 hours and after cryopreservation of Lipogems® hADSCs and Lipogems® product. Scale bar 400 μm.

hADSCs can be obtained and grown in culture also when Lipogems® product has been preserved at 4°C from 24 to 72 hours (Figure 18 panels A and B) or

cryopreserved in liquid nitrogen in presence of cryoprotectant agent (DMSO 10%) even for a long time (at least 12 months). Such cells can be kept in culture without any apparent modifications of their typical features (Figure 18 panels C and D). Differently from fresh tissue it is necessary to use αMEM medium to obtain cells from cryopreserved fragmented specimen (Figure 18 panels E and F). No PLA cells were obtained when lipoaspirate was frozen and stored in liquid nitrogen.

### 3. Characterization of cellular products after culture

3.5 Proliferation features of Lipogems®- hADSCs and PLA cells

In order to investigate the growth capability of purified Lipogems® - hADSCs expansion curves of cell populations obtained from different cases were established. They grew quite well for several passages, and then entered a phase of slower growth (around the twelfth passage). Similar data were also obtained and reported previously by other authors (Yang XF et al, 2011).
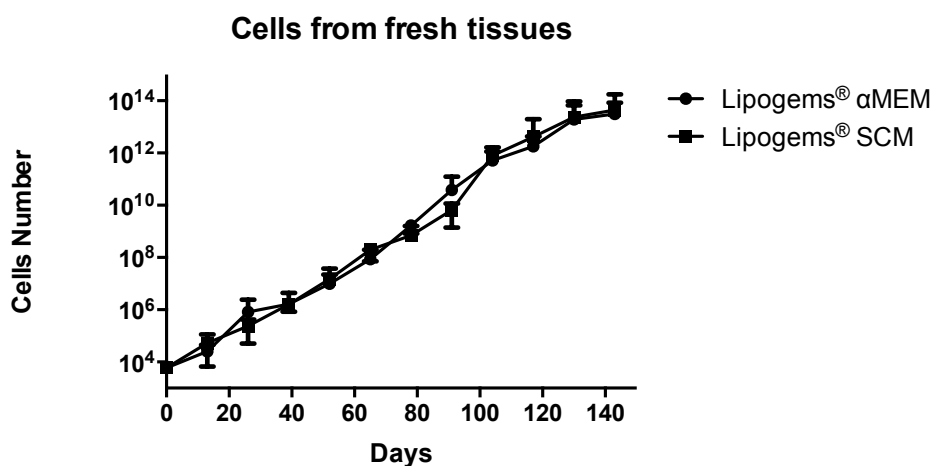


**Figure 19.** Exponential curves of Lipogems® - hADSCs grown in αMEM and SCM.

3.6 Cell cycle and chromosomal stability

The DNA content was analyzed by Cytomics FC 500 and the cell cycle was analyzed with ModFit software. The results show that 15.88 ± 1.09% of that Lipogems® hADSCs cultured in αMEM in S+G2/M (active proliferative phase) and 78.93 ±

0.35% were in G0/G1 phase (quiescent phase). Cells cultured in SCM medium displayed 21.19 ± 1.42% in S+G2/M phase, with the remaining cells (75.52 ± 0.31%) in G0/G1 phase (Figure 20, panel A). Study results demonstrated that less than 0,6% of Lipogems[®] hADSCs grown in both media were in apoptosis.

Lipogems[®] hADSCs maintained in both growth media did not present any chromosomal rearrangement, as assessed by QFQ-banding performed at early (Figure 20, panel B). Chromosome number was normal in all analyzed samples (n=4).



| Phase of Cell Cycle | αMEM | SCM |
|---|---|---|
| Apoptosis | 0,21±0,06% | 0,15±0,08% |
| G0 - G1 | 79,64±0,31% | 75,52±0,31% |
| S | 2,48±0,56% | 3,06±0,37% |
| G2 - M | 13,80±0,53% | 18,13±1,05% |
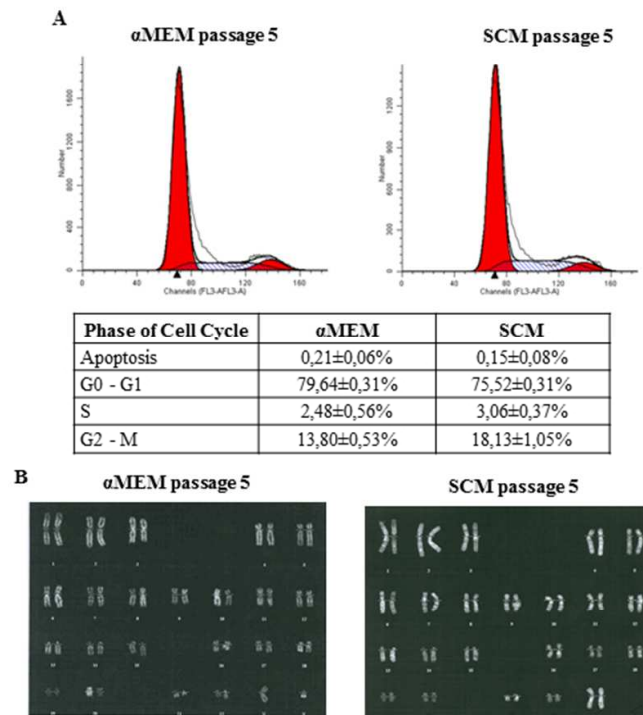
**Figure 20.** (A) Cell cycle patterns were investigated by FACS after propidium iodide staining. Analyses were performed in Lipogems[®] hADSCs obtained from four different cases and the mean ± SEM was reported in the table. (B) Karyotype analyses.

### 3.7 Immunophenotypic characterization

Although many efforts had been made toward the characterization of surface markers of mesenchymal stem cells, a specific marker capable of identifying a uniquely homogeneous mesenchymal cell population is yet to be identified. Many phenotypic profiles useful for the study of cell-cell interactions and cell-environment are however well known (Oswald J et al, 2004). Lipogems[®]- derived stem cells and PLA cells, isolated from five different subjects, and grown in αMEM medium at confluence of the second cellular passage were studied by means of FACS analyses.

CD45 has been used as discriminant, since MSCs are negative for this hematopoietic

marker. Lipogems®-derived stem cells and PLA cells expressed typical mesenchymal markers (such as CD90, CD73, CD105 and CD44) at high percentage, close to 100% (Figure 21, Panel A). CD146, CD31 and CD34 were used to define the pericytes population (CD146$^+$ CD31$^-$ CD34$^-$) and the supradventitial stem cells population (CD146$^-$ CD31$^-$ CD34$^+$) (detail in Figure 21, panel C). The highest percentage of pericytes (22,3% of the total cell population) were obtained by isolating hADSCs with centrifugation of the Lipogems® product and a statistical significance was shown between PLA cells and Lipogems® hADSCs (pvalue ≤ 0,01, analyzed by Two-way ANOVA with Turkey correction for multiple comparison).
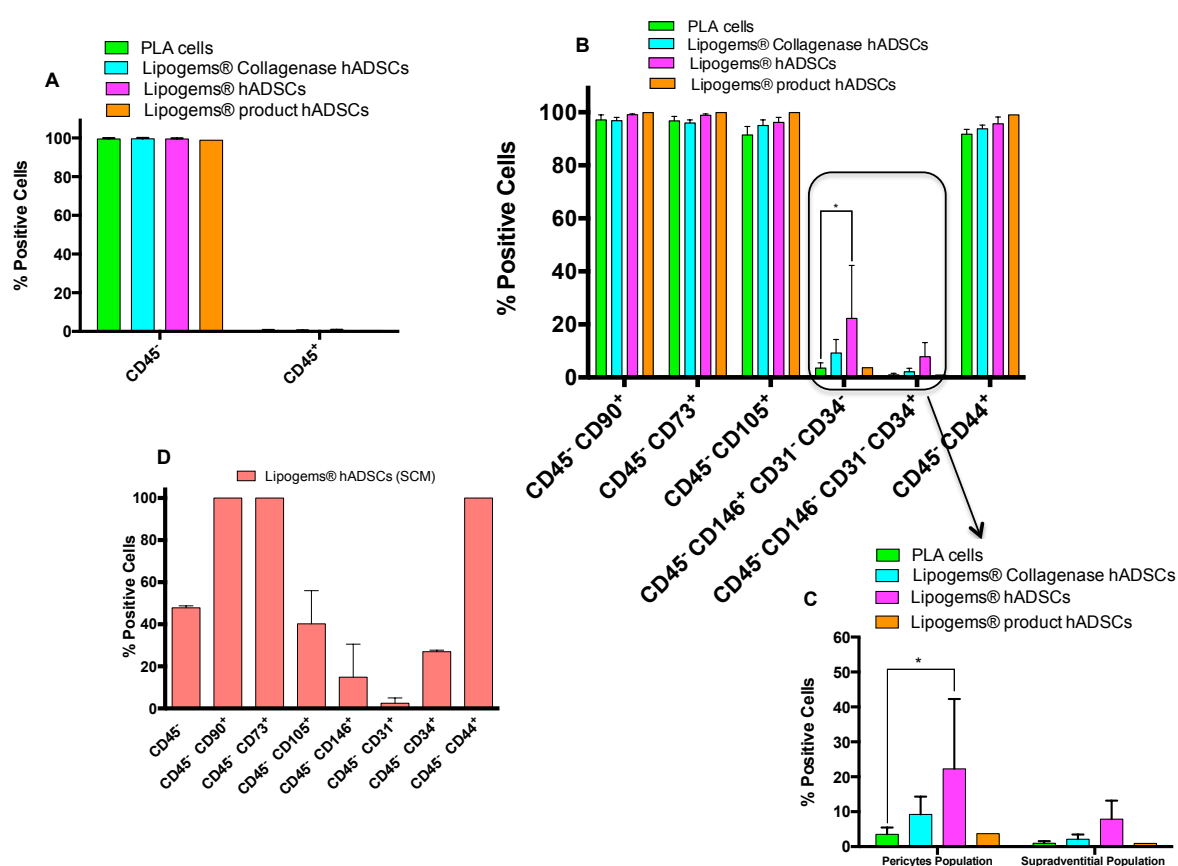


**Figure 21.** Cell surface expression of Lipogems®- derived stem cells and PLA cells

For the few CD45$^+$ cells (Figure 21, Panel B) there was no positivity for CD14, which is a component of immune system, a co-receptor for the detection of the bacterial lipopolysaccharide (LPS). Moreover, there was no expression of CD19 (B-lymphocyte antigen) and HLA-DR, an MHC class II cell surface receptor.

Figure 21, Panel D shows the expression of markers analyzed on three preparation of Lipogems® hADSCs grown in SCM medium, which shown a consistent presence of

CD90, CD73 and CD44 but a population of CD105 positive close to 40%.

Cells obtained from cryopreserved Lipogems® product maintained in liquid nitrogen in presence of DMSO or DMSO and trehalose solutions for 9 days to 1 month were assessed in culture and by Flow Cytometry for the expression of the same surface markers.
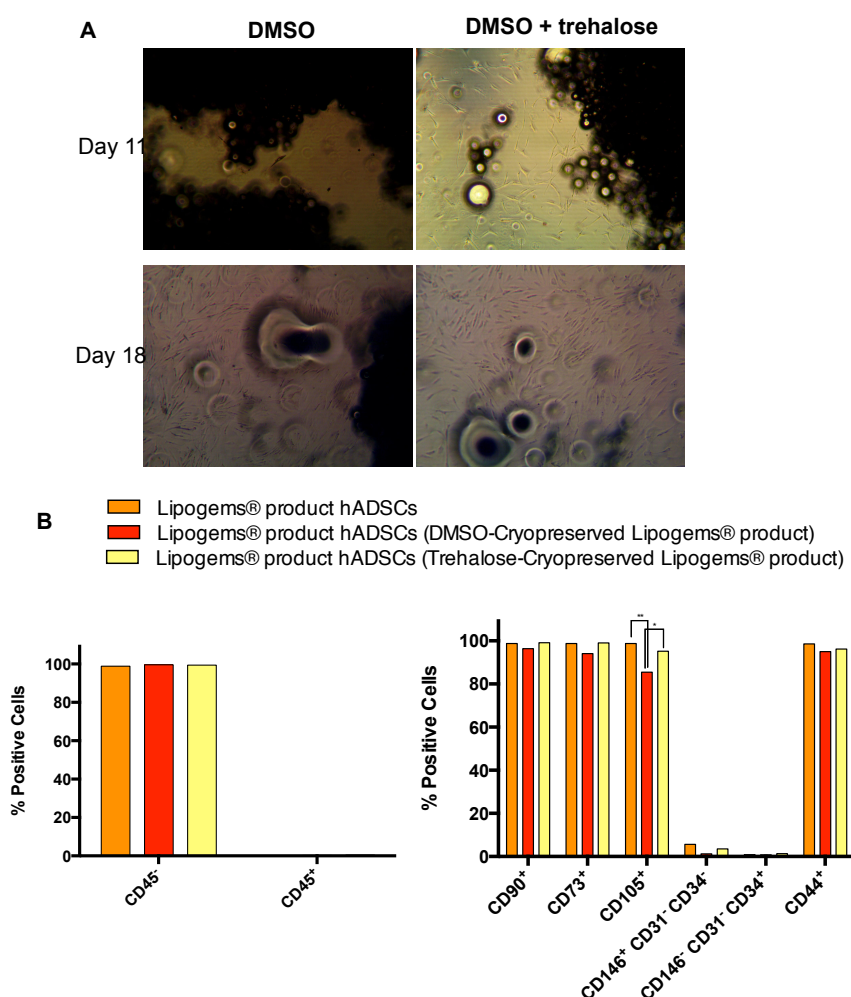


**Figure 22.** Cryopreserved Lipogems® product with DMSO or DMSO/trehalose solutions (A) in culture and (B) analyzed at the second cellular passage by flow cytometry

Cryopreservation with DMSO supplemented with trehalose allowed a good recovery of Lipogems® Product and was superior to Lipogems® Product frozen only with DMSO. In both condition hADSCs can be observed growing from the tissues at the same day (day 4) but in greater number in trehalose/DMSO preserved tissues. Moreover, cultures of cryopreserved Lipogems® product in presence of trehalose shown less presence of debris and oil (Figure 22, panel A).

At confluence of the second cellular passage cells were collected and stained for FACS evaluation. The expression of all markers evaluated did not undergo

modifications as consequence of the two freezing protocol, except for the CD105 expression, for that showed, small, but a statistically significant decrease after cryopreservation (Figure 22, panel B).

### 3.8 *In vitro* differentiation potential

To investigate whether Lipogems® hADSCs exhibited the developmental potential typical of MSCs *in vitro* differentiation experiments were set up for mesenchymal commitment. Differentiation of PLA cells was assessed as control.

By using a classical adipogenic differentiation approach, which lasted for two weeks, we observed that, either starting from cells cultured in αMEM or in SCM, after adipogenic induction for 5 days, the cell morphology changed into a round shape. At the end of the differentiation period (14 days), small bubble-shaped oil red O-positive-lipid droplets appeared in the cytoplasm (Figure 23).
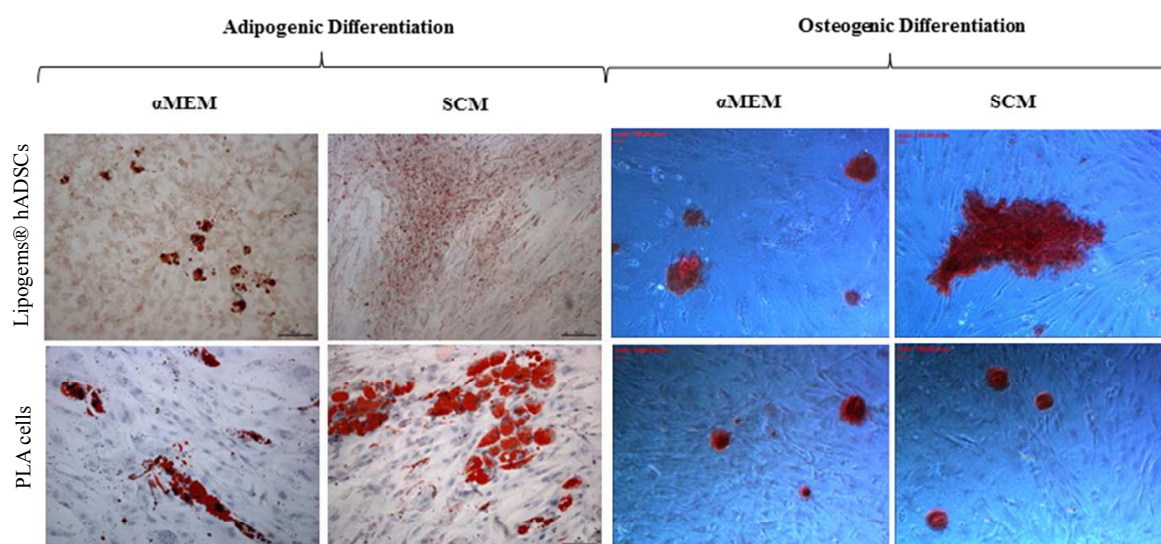


**Figure 23.** *In vitro* adipogenic and osteogenic differentiation of Lipogems® hADSCs and PLA cells. Scale bars are 100 μm for the adipogenic differentiation and 100 pixels for the osteogenic differentiation. Data are representative for 2 different experiments for four cases.

When Lipogems® hADSCs were cultured in osteogenic medium for three weeks, during the first week of incubation cells proliferated and reached almost complete confluence. Later, cellular aggregates were observed and gradually increased until the completion of the differentiation. These aggregates were characterized by the presence of amorphous material deposits, that were positive to the staining with Alizarin red S (Figure 23).

Three different protocols were tried to differentiate Lipogems® hADSCs into cells of neural lineage (Marfia G et al, 2011; Navone S et al, 2012; Ahamadi N et al, 2012), but no one give a complete differentiation.

Curiously, during the development of these experiments we discovered that Lipogems® hADSCs in basal medium (αMEM and SCM) expressed percentages of some neural markers, when evaluated by immune-fluorescence method. These markers include GFAP (Glial fibrillary acidic protein), an intermediate filament protein, which is expressed by numerous cell type of the central nervous system, including astrocytes and ependymal cells; $O_4$ (Oligodendrocytes marker O4), a differentiation marker expressed on the surface of oligodendrocytes and βTubulin III (Tuj1), a neuron specific Tubulin (Figure 24). Figure 24, Panel B shows a quantitation of the expression expressed as the percentage of positive cells and data are the mean ± SEM of three independent experiments with similar results (* p value ≤ 0,05).



| | αMEM | SCM |
|---|---|---|
| Vimentin | 63,56 ± 18,39 | 100 ±0 |
| Nestin | 99,49 ± 0,79 | 100 ±0 |
| GFAP | 56,64 ± 16,36 | 88,09 ± 5,75 |
| $O_4$ | 99,38 ± 0,34 | 95,65 ± 1,94 |
| βTubulin III | 98,47 ± 1,07 | 100 ±0 |
| EpoR | 0,21 ± 0,24 | 37,98 ± 17,30 |

**Figure 24.** Lipogems® hADSCs expression of neural markers

Cell grown in the mesenchymal medium αMEM show a lower expression of the mesenchymal protein Vimentin compared to cells grown in SCM.

Nestin was present in nearly 100% of cells in both, highlighting the presence of neural precursor cells. Markers of ultimate differentiation, such as GFAP, O4 and βTubulin

III are very close to 100%, except for GFAP expression on Lipogems® hADSCs, either grown in αMEM than SCM, whom were 56,64 ± 16,36 and 88,09 ± 5,75 in percentage, respectively.

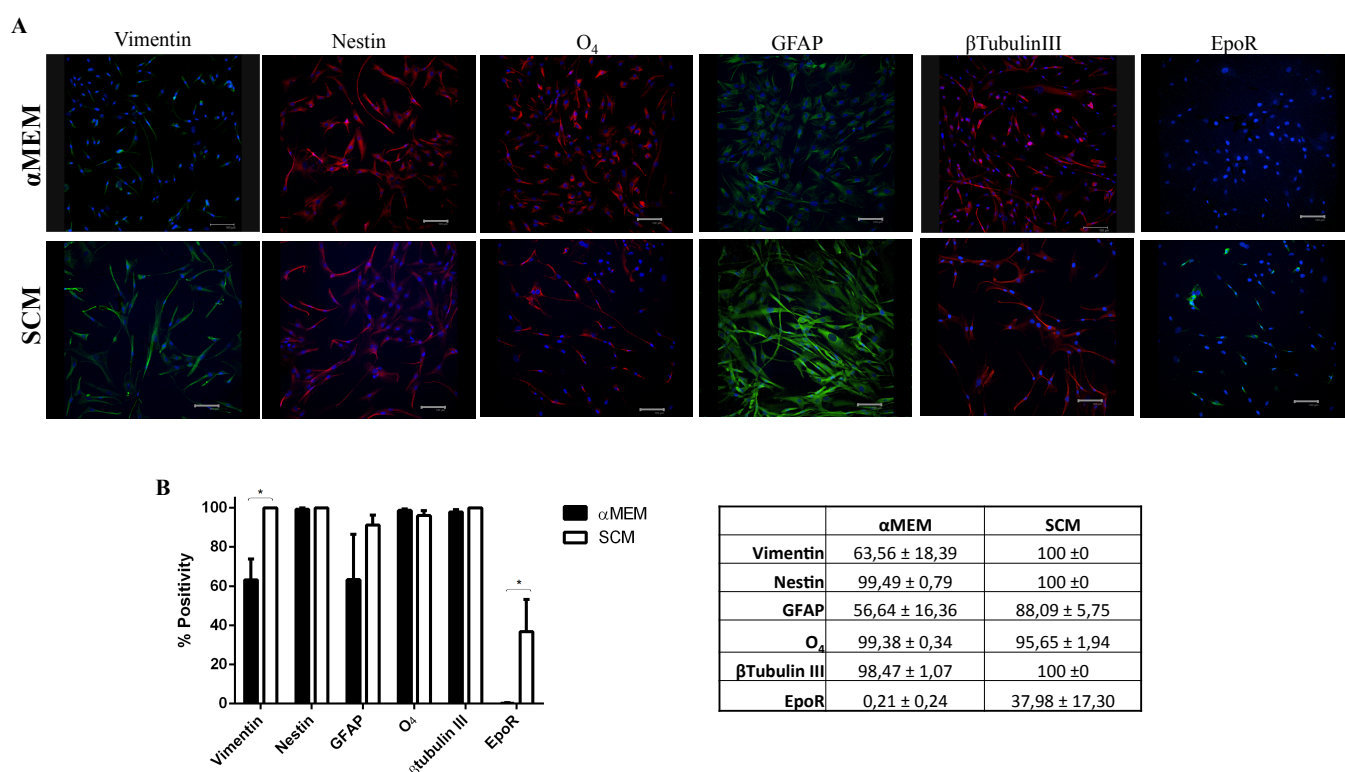Erithropoietin (Epo) exerts its function through the EpoR (Epo Receptor), a member of the class I Cytokine receptor family (Pereira R et al, 2000). Following binding of Epo to its receptor, the receptor forms homodimers and undergoes phosphorylation by physically associating and interacting with the tyrosine kinase JAK2 (Janus Kinase-2). Once phosphorylated, these tyrosine residues allow the recruitment and activation of a number of downstream adaptors and effectors including STAT5 (Signal Transducers and Activators of Transcription factor-5), PI3K (Phosphoinositide-3 Kinase), SHIP (SH2-containing Inositol Phosphatase), the tyrosine phosphatase SHP1 and SHP2 and the ERKs (Extracellular Signal Regulated Kinases), JNK (Jun N-terminal Kinases) and p38 MAPK (Mitogen Activated Protein Kinase) (Iwatsuki K et al, 1997). STAT5 act as a transcription factor, binds to nuclear DNA carrying the signal from the membrane to the nucleus (Barber DL et al, 1997). Epo and EpoR are required for survival, proliferation and differentiation of committed erythroid progenitor cells. Recently has been shown that EpoR is also required for the neuronal differentiation (Marfia G. et al, 2011), so we investigated the expression of Epo Receptor on Lipogems® hADSCs and as appear in Figure 24, about 40% of cells population grown in SCM expressed it, meanwhile cells grown in αMEM expressed it at very low percentage, suggesting that the culture in SCM medium is enriched in putative neural precursor cells.

## 4. Study of immune-modulating properties

The immunomodulatory capabilities of PLA cells and Lipogems®- derived stem cells were studied by co-culture at different ratio T lymphocytes – MSCs in presence of an anti-CD3 stimulus. The proliferation was assessed by labeling with Cell Trace™ Violet, that can be easily diffuses into cells where it is cleaved by intracellular esterases to yield a highly fluorescent compound. This compound covalently binds to intracellular amines, resulting in stable well-retained fluorescent staining that can be fixed with aldehyde fixatives.

Figure 25 show changing in proliferation of CD4$^+$ T lymphocytes in consequence of different co-culture ratios with all cell preparation of hADSCs. For statistical analysis the culture of T lymphocytes in presence of the anti-CD3 stimulus was used as reference, normalized to 100%.

It was possible obtain a suppression of CD4$^+$ T cells population and a dose dependence was evident, where the most consistent suppression was with a 1:1 ratio.

In detail, PLA cells were significantly suppressive starting from the 1:10 ratio (pvalue= 0,01) and the decrease of T cells in proliferation was most significant at lower ratios.

Lipogems$^®$ Collagenase hADSCs showed a linear trend of suppression with an increase in significance starting from the 1:10 ratio arriving until the strongly suppressive 1:1 ratio.

The effects of Lipogems$^®$ hADSCs on the CD4$^+$ T lymphocytes proliferation was less stronger compared to PLA cells and Lipogems$^®$ Collagenase hADSCs, reaching a significant decrease in CD4$^+$ proliferation starting from the 1:3.

Moreover, the Two-way ANOVA analysis showed also a different into the 1:10 ratio between PLA cells compared to Lipogems$^®$ hADSCs and between Lipogems$^®$ Collagenase hADSCs compared to Lipogems$^®$ hADSCs, suggesting that at this ratio PLA cells and Lipogems$^®$ Collagenase hADSCs are more suppressive than Lipogems$^®$ hADSCs.

All of these results suggest that Collagenase cells preparations are more strongly suppressive on the CD4$^+$ T lymphocytes population.

In Figure 26 is shown the effect of different co-culture ratios of the three hADSCs preparation with T cells on the CD8$^+$ T lymphocytes population.

All of hADSCs preparations were significantly suppressive at the ratio 1:1, and Lipogems$^®$ Collagenase hADSCs showed a linear significant trend of suppression starting from the 1:3 ratio.

These further results suggest that the most strong suppression, considering CD4$^+$ and CD8$^+$ T lymphocytes populations, was obtained with Lipogems$^®$ Collagenase hADSCs, that were able to significantly suppress T cells starting from 1:5 ratio.
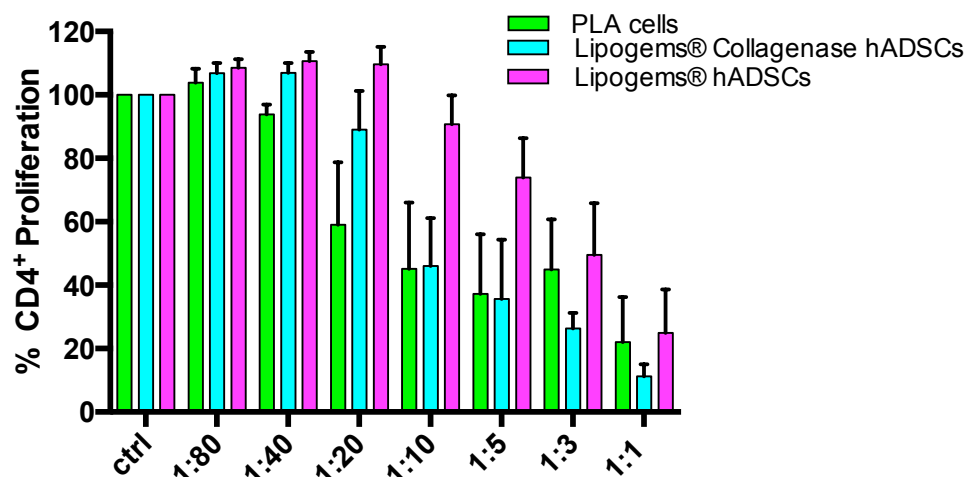
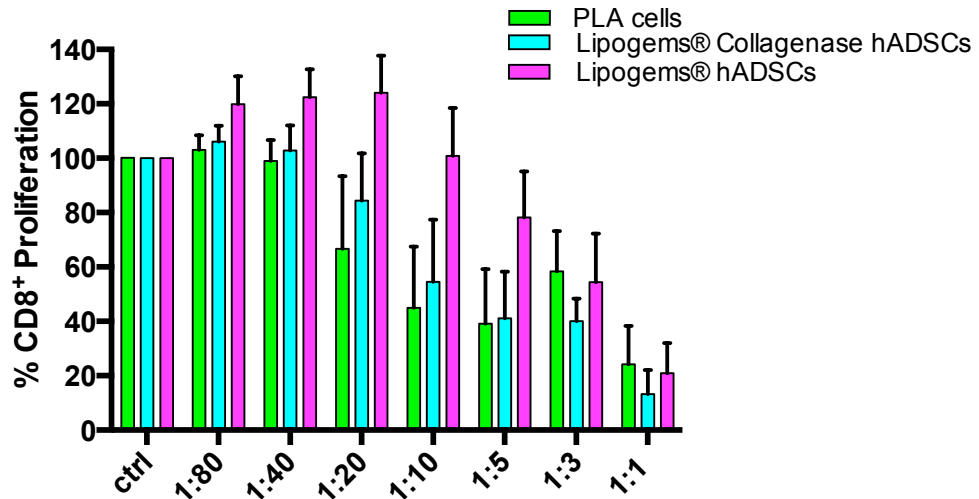| Bonferroni's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| | | | | | |
| PLA cells | | | | | |
| ctrl vs. 1:80 | -3.857 | -49.66 to 41.94 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | 6.122 | -39.68 to 51.92 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | 40.99 | -4.805 to 86.79 | No | ns | 0.1070 |
| ctrl vs. 1:10 | 54.88 | 9.087 to 100.7 | Yes | * | 0.0105 |
| ctrl vs. 1:5 | 62.75 | 16.96 to 108.6 | Yes | ** | 0.0024 |
| ctrl vs. 1:3 | 55.05 | 9.254 to 100.8 | Yes | * | 0.0102 |
| ctrl vs. 1:1 | 77.99 | 32.19 to 123.8 | Yes | *** | 0.0001 |
| | | | | | |
| Lipogems® Collagenase hADSCs | | | | | |
| ctrl vs. 1:80 | -6.834 | -52.63 to 38.96 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | -6.924 | -52.72 to 38.87 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | 10.91 | -34.89 to 56.71 | No | ns | > 0.9999 |
| ctrl vs. 1:10 | 53.98 | 8.182 to 99.78 | Yes | * | 0.0123 |
| ctrl vs. 1:5 | 64.38 | 18.58 to 110.2 | Yes | ** | 0.0018 |
| ctrl vs. 1:3 | 73.67 | 27.87 to 119.5 | Yes | *** | 0.0003 |
| ctrl vs. 1:1 | 88.78 | 42.99 to 134.6 | Yes | **** | < 0.0001 |
| | | | | | |
| Lipogems® hADSCs | | | | | |
| ctrl vs. 1:80 | -8.582 | -54.38 to 37.22 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | -10.64 | -56.44 to 35.16 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | -9.669 | -55.47 to 36.13 | No | ns | > 0.9999 |
| ctrl vs. 1:10 | 9.249 | -36.55 to 55.05 | No | ns | > 0.9999 |
| ctrl vs. 1:5 | 26.04 | -19.76 to 71.83 | No | ns | 0.8168 |
| ctrl vs. 1:3 | 50.46 | 4.661 to 96.26 | Yes | * | 0.0229 |
| ctrl vs. 1:1 | 75.09 | 29.30 to 120.9 | Yes | *** | 0.0002 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| 1:20 | | | | | |
| PLA cells vs. Lipogems® Collagenase hADSCs | -30.08 | -70.51 to 10.34 | No | ns | 0.2132 |
| PLA cells vs. Lipogems® hADSCs | -50.66 | -91.09 to -10.23 | Yes | ** | 0.0095 |
| Lipogems® Collagenase hADSCs  vs. Lipogems® hADSCs | -20.58 | -61.01 to 19.85 | No | ns | 0.6384 |
| | | | | | |
| 1:10 | | | | | |
| PLA cells vs. Lipogems® Collagenase hADSCs | -0.9047 | -41.33 to 39.52 | No | ns | > 0.9999 |
| PLA cells vs. Lipogems® hADSCs | -45.64 | -86.06 to -5.208 | Yes | * | 0.0220 |
| Lipogems® Collagenase hADSCs  vs. Lipogems® hADSCs | -44.73 | -85.16 to -4.303 | Yes | * | 0.0255 |

**Figure 25.** CD4^{+} lymphocytes proliferation analyzed in Anti-CD3 Assay of co-culture with PLA cell, Lipogems® Collagenase hADSCs and Lipogems® hADSCs at different ratios.

| Bonferroni's multiple comparisons test | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| | | | | | |
| PLA cells | | | | | |
| ctrl vs. 1:80 | -2.909 | -60.05 to 54.23 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | 1.113 | -56.03 to 58.26 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | 33.43 | -23.72 to 90.57 | No | ns | 0.7470 |
| ctrl vs. 1:10 | 55.16 | -1.985 to 112.3 | No | ns | 0.0647 |
| ctrl vs. 1:5 | 61.02 | 3.877 to 118.2 | Yes | * | 0.0298 |
| ctrl vs. 1:3 | 41.71 | -15.43 to 98.85 | No | ns | 0.3199 |
| ctrl vs. 1:1 | 75.93 | 18.79 to 133.1 | Yes | ** | 0.0035 |
| | | | | | |
| Lipogems® Collagenase hADSCs | | | | | |
| ctrl vs. 1:80 | -6.070 | -63.21 to 51.07 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | -2.855 | -60.00 to 54.29 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | 15.58 | -41.56 to 72.73 | No | ns | > 0.9999 |
| ctrl vs. 1:10 | 45.51 | -11.64 to 102.6 | No | ns | 0.2093 |
| ctrl vs. 1:5 | 58.89 | 1.752 to 116.0 | Yes | * | 0.0397 |
| ctrl vs. 1:3 | 59.96 | 2.817 to 117.1 | Yes | * | 0.0344 |
| ctrl vs. 1:1 | 86.74 | 29.59 to 143.9 | Yes | *** | 0.0007 |
| | | | | | |
| Lipogems® hADSCs | | | | | |
| ctrl vs. 1:80 | -19.92 | -77.07 to 37.22 | No | ns | > 0.9999 |
| ctrl vs. 1:40 | -22.39 | -79.53 to 34.75 | No | ns | > 0.9999 |
| ctrl vs. 1:20 | -24.03 | -81.18 to 33.11 | No | ns | > 0.9999 |
| ctrl vs. 1:10 | -0.8513 | -57.99 to 56.29 | No | ns | > 0.9999 |
| ctrl vs. 1:5 | 21.74 | -35.41 to 78.88 | No | ns | > 0.9999 |
| ctrl vs. 1:3 | 45.58 | -11.56 to 102.7 | No | ns | 0.2075 |
| ctrl vs. 1:1 | 79.10 | 21.96 to 136.2 | Yes | ** | 0.0022 |

| | | | | | |
|---|---|---|---|---|---|
| 1:20 | | | | | |
| PLA cells vs. Lipogems® Collagenase hADSCs | -17.84 | -68.29 to 32.60 | No | ns | > 0.9999 |
| PLA cells vs. Lipogems® hADSCs | -57.46 | -107.9 to -7.017 | Yes | * | 0.0206 |
| Lipogems® Collagenase hADSCs  vs. Lipogems® hADSCs | -39.62 | -90.06 to 10.83 | No | ns | 0.1717 |
| | | | | | |
| 1:10 | | | | | |
| PLA cells vs. Lipogems® Collagenase hADSCs | -9.652 | -60.09 to 40.79 | No | ns | > 0.9999 |
| PLA cells vs. Lipogems® hADSCs | -56.01 | -106.5 to -5.566 | Yes | * | 0.0248 |
| Lipogems® Collagenase hADSCs  vs. Lipogems® hADSCs | -46.36 | -96.80 to 4.086 | No | ns | 0.0813 |

**Figure 26.** CD8^{+} lymphocytes proliferation analyzed in Anti-CD3 Assay of co-culture with PLA cell, Lipogems® Collagenase hADSCs and Lipogems® hADSCs at different ratios.

We have also analyzed if there was a changing in the T regulatory cells population associated with the suppressive abilities, as suggested in some papers (Casiraghi F. et al 2008; Engela A.U. et al, 2012). Figure 27 show that there is no a significant changing in the percentage of T regulatory cells, suggesting that in this experimental condition another mechanism is involved in the suppression process, even if an increase of the case history could help to better define the effect of PLA cells and Lipogems®-derived hADSCs on the T regulatory cells population.
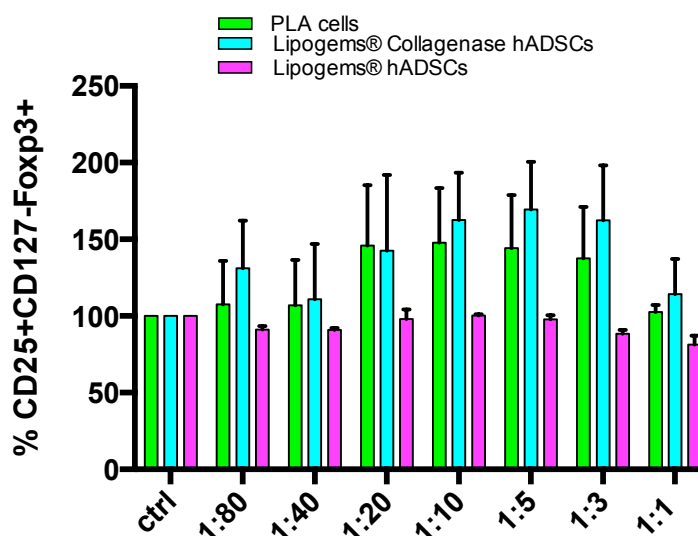


**Figure 27.** T regulatory cells population analyzed in Anti-CD3 Assay of co-culture with PLA cell, Lipogems® Collagenase hADSCs and Lipogems® hADSCs at different ratios.

The same experimental procedure was used to study the effect of lipoaspirate adipose tissue and the correspondent Lipogems® product on T lymphocytes proliferation, trying to reproduce what could be happen *in vivo*. In order to approximate the clinical use of Lipogems® product, a third kind of adipose tissue was tested, that is Lipogems® product passed through a 1cc syringe connected with a 23 needle.

For statistical analysis the culture of T lymphocytes in presence of the anti-CD3 stimulus was used as reference, normalized to 100%.

As shown in Figure 26 there was no statistically significant differences in CD4+ and CD8+ T lymphocytes proliferation and in T regulatory population between the three adipose tissue preparation and T lymphocytes alone in presence of the anti CD3 stimulus. These results suggest that the whole tissues had no effect on T cells population, in term of suppression and stimulation of the T regulatory cell population.

This is likely be due to sub-optimized experimental conditions for to evaluating effects of tissue clusters to evaluate what could happen *in vivo*, because of the likely effects of an hypoxic environment during *in vitro* incubations of relatively large tissue clusters.



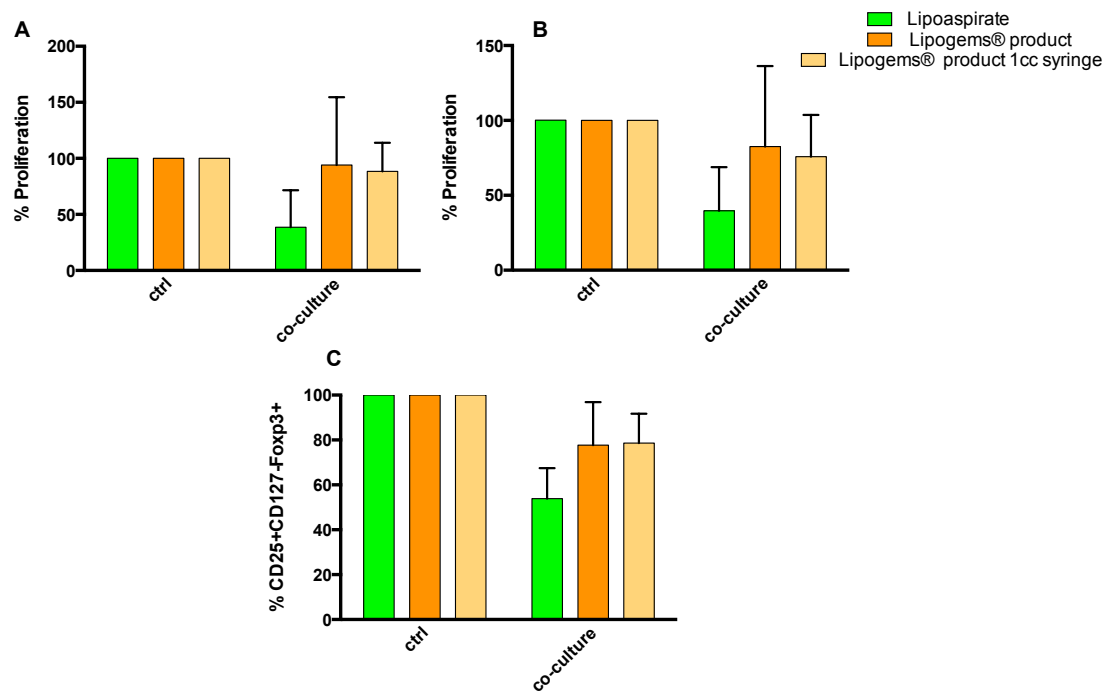**Figure 26.** Anti-CD3 Assay for T lymphocytes in co-culture with lipoaspirate adipose tissue, correspondent Lipogems® product and Lipogems® product 1cc syringe. (A) CD4^{+} proliferation; (B) CD8^{+} proliferation; (C) T regulatory cells population.

# DISCUSSION

Adipose - derived mesenchymal stem cells (ADSCs) as well as the adipose tissue itself represent an abundant source of cells and tissue and their use is currently a major area of research in regenerative medicine. A great advantage is represented by the minimally invasive accessibility to adipose tissue and its ready availability (Safford KM et al, 2005).

In the present study, we report a new method to isolate human ADSCs (hADSCs), which consists of three micro-fragmentation steps of the lipoaspirated subcutaneous adipose tissue by use of the Lipogems® device. The tissue product results in a size reduction equal to 80% and with a consistent decrease of blood and oil residues and other contaminants.

Here, we hypothesized that the mechanical fragmentation of the adipose tissue could be the key factor, which triggers fine intrinsic changes in the tissue source. In fact using a immunohistochemical approach it has been shown that the expression of the neural marker β-tubulin III could be detected after processing, while it was completely undetectable in the starting lipoaspirate of the adipose tissue. Suggesting either an enrichment of cells/tissue expressing this marker or an induction of expression during the processing. The morphological analysis of the fragmented tissue exhibited a remarkably preserved stromal structure that retains its typical constitutive elements at the same density and quantitative distribution compared with the lipoaspirate adipose tissue of origin.

The Lipogems® product could be processed to obtain hADSCs with different approaches, including the classical enzymatic treatment, a single centrifugation or by directly placing in tissue culture. The yield was different with the used method; enzymatic digestion can give 30 times more cells than the only centrifugation.

The cell population composition determined at the time of isolation (i.e. without further expansion), was comparable between lipoaspirate and Lipogems® product, with the presence of mesenchymal stem cells and a consistent component of immune system cell types, such as T lymphocytes, monocytes, macrophages. That latter immune component was lost during cell culture and expansion, which allows the enrichment and selection of the mesenchymal stem cells portion.

The characteristics of Lipogems®- derived hADSCs have been investigated through

the use of different approaches, including flow cytometry and immunocytochemistry. These cells showed growth kinetics similar to the other adult stem cells (Christodoulou I et al, 2012) with a proliferation phase followed by the senescence, which in our experimental conditions occurred close to passage twelve.

Chromosomal rearrangement were evaluated by G-banding karyotype assay and none were found, leading us to conclude that our culture condition avoided genetic modifications.

A stem cells population with the typical characteristics of surface markers of classical MSCs (Bianco P et al, 2008) was obtained when the Lipogems® product was subjected to culture *in vitro*, either by processing through enzyme, centrifugation or by means of direct plating. Flow cytometry analyses showed that Lipogems®-derived hADSCs, expanded in αMEM medium and expressed classical mesenchymal markers including CD90, CD73, CD105 and CD44 (Bianco P et al, 2008; Maurer MH et al, 2011).

While the supradventitial stem cell population was present in all type of hADSCs preparations at the similar very low percentage, a single centrifugation of the Lipogems® product resulted in an enrichment of the CD146 positive cells and yielded the highest percentage of pericytes population compared to conventional adipose tissue cell isolation procedures. Pericytes enveloping microvessels and adventitial cells surrounding larger arteries and veins have been described as possible MSC forerunners (Murray IR et al, 2013).

A hallmark of MSCs is their multipotency and ability to give rise to cell populations of mesenchymal origins, such as osteoblastic, chondrogenic and adipogenic lineages. These features were present in hADSCs obtained from Lipogems® product, although the *in vitro* essays of lipid droplets accumulation demonstrated qualitative differences between Lipogems®- derived hADSCs and Processed Lipoaspirate cells (PLA cells). This observation could support the hypothesis that subcellular changes were triggered during the micro-fragmentation of the adipose tissue, causing a reduced ability to convert in adipose cells and an increase in the ability of forming bones precursors.

In basal growth condition (αMEM and SCM media) was found high expression of typical neural marker, such as GFP, $O_4$ and βtubulin III and the EPO Receptor (EPOR) was expressed when these cells were grown in SCM. These findings are suggestive that micro-fragmentation of lipoaspirate adipose tissue changed some molecular features in the tissue resident cells so that hADSCs obtained in this

condition became more plastic and adaptive to the extracellular signals.

Notably, the above neurogenic profile was reported to a lower extent by other authors in mesenchymal stem cells obtained from different sources, such as bone marrow (Tondreau T et al, 2004), amniotic fluid (Bottai D et al, 2012), nucleus polposus (Navone SE et al, 2012) and adipose tissue (Jang S et al, 2010). The ability of undifferentiated MSC to express immature and mature proteins typical of other tissues without any induction may support their plasticity to differentiate easily in many various tissues (Jiang Y et al, 2002). Our findings suggest the pluripotent nature of Lipogems®- derived stem cells, and highlights their novelty for regenerative studies and those focused on molecular determinants of stemness.

Moreover, MSCs are considered immunologically privileged, since they do not express costimulatory molecules required for complete T cell activation on their surface. Several studies have shown that MSCs exert an immunosuppressive effect on cells from both the innate and acquired immunity systems (de Vasconcellos Machado et al, 2013). MSCs can regulate the immune response *in vitro* by inhibiting the maturation of dendritic cells, as well as by suppressing the proliferation and function of T and B lymphocytes and natural killer cells.

PLA cells and Lipogems®- derived stem cells are suppressive on the T lymphocytes proliferation and a dose-dependence was highlighted. A strong effect was found at the ratio 1 MSCs : 1 T cells, but the mostly suppressive population is Lipogems® Collagenase hADSCs, that have significant effect on the T cells proliferation starting from the 1:5 ratio.

The Lipogems® product that consisted of tissues clusters did not show a suppressive effect on T lymphocytes proliferation, nor did the starting lipoaspirate tissues. This is likely be due to sub-optimized experimental conditions for to evaluating effects of tissue clusters to evaluate what could happen *in vivo*, because of the likely effects of an hypoxic environment during *in vitro* incubations of relatively large tissue clusters.

These special immunological properties make these cells a promising strategy in the treatment of immune-mediated disorders, such as graft-versus-host disease and autoimmune diseases, as well as in regenerative medicine. The understanding of the underlying immune regulation mechanisms of Lipogems®-derived stem cells, and also of the pathways involved in the differentiation of these cells in various lineages is essential for their successful and safe application in different areas of medicine.

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