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# *"3D in Suspension versus 2D in Adhesion: molecular profiles in stemness and mesenchymal differentiation of Spheroids from Adipose-derived Stem Cells"*
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# INDEX

# Abstract

**Purpose**: Adipose stem cells (ASCs) represent a reliable source of stem cells with a widely demonstrated potential in regenerative medicine and tissue engineering applications. New recent insights suggest that three-dimensional (3D) models may closely mimic the native tissue properties; spheroids from adipose derived stem cells (S-ASCs) exhibit enhanced regenerative abilities compared with those of 2D models. Stem cell therapy success is determined by "cell-quality"; for this reason, microRNA profiles, the involvement of stress signals and cellular aging need to be further investigated.

**Material and Methods:** Adipose tissue was collected from healthy individuals, 44 females and 17 males, after signing informed consent. Mean age was 50, 25 years (range: 18-77). Lipoaspirate samples were harvested from different body areas such as abdomen, breast, flanks, trochanteric region, and knee. Here, we performed a comparative analysis, molecular and functional, of miRNA expression pattern profile "stemness and differentiation associated", genes connected with stemness, aging, telomeric length and oxidative stress, of adipose stem cells in three-dimensional and adhesion conditions, SASCs-3D and ASCs-2D cultures.

**Results:** We have demonstrated that Spheroids from Adipose-derived Stem Cells (SASCs-3D) present express high level of the typical miRNAs and mRNAs of iPS cells, such as miR-142-3p and SOX2/POU5F1/NANOG, in canonical and in long term *in vitro* culture condition, express low level of the early and late miRNAs and mRNAs typical of chondrocytic, adipocytic and osteoblastic lineages in canonical and in long

term *in vitro* culture condition. The expression levels of stemness-related markers and anti-aging Sirtuin1 were significantly up-regulated ($P < 0.001$) in SASC-3D while gene expression of aging-related p16INK4a was increased in ASCs-2D ($P < 0.001$). We found that 3D and 2D cultures also presented a different gene expression profile for those genes related to telomere maintenance (Shelterin complex, RNA Binding proteins and DNA repair genes) ($P < 0.01$ and $P < 0.001$) and oxidative stress (aldehyde dehydrogenase class1 and 3) ($P < 0.05$, $P < 0.01$ and $P < 0.001$) and presented a striking large variation in their cellular redox state.

**Conclusion**: Based on our findings, we propose a "cell quality" model of SASCs, highlighting a precise molecular expression of microRNA pattern profiles, several genes involved with stemness (SOX2, POU5F1 and NANOG), anti-aging (SIRT1), oxidative stress (ALDH3) and telomeres maintenance.

# Summary

Recently, in the field of oncology, stem cell biology, and tissue engineering, the scientific community has considered artificial two-dimensional cell cultures, as cells express their specific-tissue characteristics only when they reside within a "niche" 3D micro-environment as *in vivo*. It has been shown that cells lose tissue specificity when they are separated from the niche but through many different techniques can be possible to re-establish the three-dimensionality and cell-cell contacts. Cells in a three-dimensional environment give rise to aggregates in suspension, the so-called "spheroids" (Mueller-Klieser, 1997). In the last few years, different techniques have been developed for spheroid isolation but the term “spheroids” cannot be associated to a unique conceptual biological identity. For this reason, further molecular investigations are needed to clearly define the biological characteristics of such spheroids. The principal methods for the isolation of spheroids are spinner flasks, hanging-drop, coated-supports (Iwai et al., 2016). A recent study has shown that it is possible to differently obtain Spheroids from Adipose derived Stem Cells (S-ASCs) in suspension directly from lipo-aspirated samples when the Stromal Vascular Fraction (SVF) portion is cultured with serum free and ultralow-adhesion conditions ((Di Stefano AB 2015). We tried to answer the question of whether real stem cells are those in suspension (3D) or adhesion (2D). Furthermore, we wanted to better understand if the application of S-ASCs could be a feasible strategy in various fields of regenerative medicine. To do this,

a deeper molecular characterization will be required, from genotypic and phenotypic analysis to determine and highlight specific targets that specifically identify adipose stem cells in a lipoaspirate sample.

CHAPTER 1

# Introduction

## 1.1 Stem Cell History

In 1909 Alexander A. Maximow was the first to discuss about stem cells on a text entitled "The lymphocyte as a stem cell, common cells to different blood elements in embryonic development and during the post-fetal life of mammals." The term "stem" (ie stem, trunk), has found diffusion thanks to “On the Origin of Species” by C.Darwin, and subsequently thanks to Haeckel, who associated with the term "stammzelle" both the single-cell organism (parent of all species), both the fertilized egg cell (Ramalho-Santos and Willenbring, 2007). In the following decades, the term “stem” continued to be used in embryological studies (Maehle, 2011), as well as in haematology (Pappenheim and A., 1907), and histology, giving multiple connotation in different contexts: of development, reproduction and differentiation. At the same time, tumor pathogenesis studies have indicated as causative agent of cancer a group of embryonic cells called "embryonic rudiment", who had remained in the adult organism by mistake (Cohnheim and J, 1877). Until the first half of the twentieth century, therefore, the term "stammzellen" has maintained a double meaning: the first of normal development and the second as a causative factor of diseases. In 1978 hematopoietic stem cells were discovered in the human umbilical cord, twenty years later it was discovered they had an important role in leukemia, therefore there was the first association between stem

cells and cancer.

### 1.2 Main Features

The stem cell is a non-specialized immature cell from which it can originate a single tissue, multiple tissues or the whole organism. It must have two characteristics:

- Self-renewal, i.e. a mother cell gives rise to two identical daughter cells, by symmetrical division or by asymmetric division in which one of the two daughter cells undertakes a differentiation path (BECKER et al., 1963), .

- Differentiation potential, or the ability that a cell possesses to specialize towards a tissue line through genetic and protein modifications.

Stem cells are classified according to their differentiation capacity:

• Totipotency: totipotent cell called zygote gives rise to the whole organism including the extra-embryonic appendages (trophoblast and placenta)

• Pluripotency: pluripotent cell gives rise to the three embryonic sheets, endoderm, mesoderm and ectoderm, but is unable to generate the whole organism

• Multipotency: multipotent cell gives rise to only specific tissues, for example hematopoietic stem cells are able to generate only blood cells. These cells are called adult because they are found only in adult and non-embryonic tissues.

• Unipotency: unipotent cell is able to differentiate into a single histological type.

### 1.3 Source of stem cells

Stem cells are further classified according to their origin into:

- Embryonic cells, are pluripotent at the blastocyst stage. Their use in research is prohibited in most countries for religious, ethical and political reasons.

- Fetal cells, obtained from embryonic tissues in advanced stages, and from fetal tissues, blood, bone marrow, etc.

- Stem cells of embryonic adnexa: obtained from the umbilical cord, placenta and amniotic sac.

- Adult stem cells: they are multipotent or unipotent cells, essential for the cellular homeostasis of adult tissues, they have no limitations and they are used for clinical purposes.

### 1.4 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are adult multipotent cells present in the mesenchyme of many tissues such as: bone marrow (Majumdar et al., 1998), adipose tissue (Zuk et al., 2002), placenta (In 't Anker et al., 2004), umbilical cord blood (0848804) and dental pulp (Gronthos et al., 2000). Mesenchyma is a ubiquitous tissue with a regulatory and structural function that originates from mesodermal sheet. In the 1970s, Friedestein and colleagues isolated very rapidly proliferating fibroblast-like cells from the bone marrow (Friedenstein et al., 1976). Currently it has been shown that MSCs, in addition to differentiating into tissues of mesodermal origin, can also differentiate into cells of ectodermal and endodermal tissues such as epithelial cells, cells of the digestive system, liver and lung (Jiang et al., 2002), (Kotton et al., 2001)).

### 1.5 Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

Hematopoiesis have shown the presence of a different population of stem cells in addition to hematopoietic stem cells, localized in the stroma. *In vitro*, the BM-MSCs have been isolated and separated from hematopoietic cells thanks to their ability to adhering to the substrate. Flow cytometric analysis showed the presence of the membrane markers CD105 (SH2), CD73 (SH3 / 4), ICAM-1 (intercellular adhesion molecules), CD29 (Haynesworth et al., 1992), (Conget and Minguell, 1999), (Le Blanc et al., 2003) and the absence of CD45 , CD34, CD14 or CD11. However, although BM-

MSCs play a fundamental role in organogenesis, remodeling and tissue repair, but their use has several disadvantages; drawing bone marrow from iliac crest is painful, invasive and hazardous (Bianco, 2011), volume obtained under local anesthesia is about 40 ml, for larger volumes total anesthesia is required. In addiction the mesenchymal component is a small fraction of the total bone marrow cell population.

### 1.6 Adipose derived stem cells (ASCs)

In regenerative medicine, specific requirements must be satisfied to obtain application benefits. Gimble et al, list the main requirements (Gimble et al., 2007):

- be available in large quantities (billions of cells),
- be collected using minimally invasive techniques,
- be able to differentiate in different cell lines in a reproducible way,
- be treated according to current guidelines.

About this, adipose tissue possesses the requisites listed above, it is homogeneously distributed along the body surface, representing 10-30% of the weight of a healthy person. Its physiological abundance and its easy surgical accessibility have defined it, in recent years, the new primary source of mesenchymal cells (Zuk et al., 2002). Adipose tissue derives from mesodermal sheet and is made up of adipocytes (the largest share) and the vascular stromal fraction (SVF), a heterogeneous population composed of preadipocytes, fibroblasts, smooth muscle vessel cells, leukocytes and adipose stem cells (Weisberg et al., 2003). Flow cytometric analysis showed the presence of the membrane markers such as CD271, CD90 (Thy-1) , CD105 or endoglin; CD29 or β-subunit of the fibronectin receptor, CD44 or receptor III of the extracellular matrix and CD73 or SH3-SH4 (Majumdar et al., 2003) and the lack of typical antigens of the hematopoietic lineage, such as CD45 or CD34. It is known that the molecular pathways of stem cells are controlled both by changes in gene expression, mechanisms to activate

or repress transcription of specific groups of genes, both epigenetic and post-trascriptional modifications. Recently, transcription factors involved in the regulation of the pluripotency state, such as Nanog, Oct-4 and SOX-2, have been investigated. They play a central role in maintaining ESC self-renewal, expressing similar regulatory roles in multipotent MSCs (Jaenisch and Young, 2008), (Pierantozzi et al., 2011). Differentiations towards mesenchymal lines are multi-step processes of molecular and functional modifications that occur in the presence of certain substances. About adipocyte lineage, the culture media must contain insulin, IGF-1, triiodiothyronine and transferrin (Deslex et al., 1987), as well as hydrocortisone or dexamethasone. After a short period there is a gradual accumulation of droplets containing triglycerides, various molecules such as the lipoprotein lipase (LPL) and the activated receptor gamma peroxisome proliferator (PPAR-gamma or PPARG). Osteoblastic differentiation is a multi-step process mediated by ascorbic acid, vitamin D3 glucorticoids or bone morphogenic proteins (BMP) (Zhou et al., 2004), typical markers of osteoblastic differentiation are osteopontin (OPN), alkaline phosphatase (ALP) or RUNX family transcription factor 2 (RUNX2) (Halvorsen et al., 2001). In the extracellular matrix, calcium phosphate minerals accumulate, detectable by von Kossa staining. Chondrogenic differentiation requires ascorbic acid, dexamethasone and TGFb1, SOX9 and Col10A, typical differentiation markers, are expressed after a few days of incubation (Koga et al., 2009). The synthesized hyaluronic acid is detectable by the alcian blue assay.

### 1.7 Spheroids of adipose derived stem cells (S-ASCs)

Two-dimensional (2D) cell cultures are a very useful tool to perform *in vitro* studies and experiments in the field of oncology, stem cell biology, and tissue engineering, but it has been recently questioned whether they properly represent the original tissue. This

is leading to a progressive replacement of 2D cultures in favour of three-dimensional (3D) cell cultures. While cells grown in monolayer lose their tissue specificity, 3D cultures show patterns of protein expression and intercellular junctions that are more faithful to conditions *in vivo* and therefore are a very attractive system for the scientific community. (Abbott, 2003). Even though cells lose their structural characteristics when they are separated from which they originate, there are different techniques that enable one to re-establish three-dimensionality and cell–cell contacts. A 3D environment stimulates cells to grow in suspended aggregates that are defined as "spheroids" (Di Stefano AB 2015), there are several method to obtain them, such as the spinner flasks (Mueller-Klieser, 1987), hanging-drop (Naderi et al., 2014) , and coated-supports (Iwai et al., 2016) techniques. Di Stefano et al, developed a technique for the isolation of spheroids of adipose stem cells (SASCs) from freshly processed tissue implementing a direct *in vitro* spheroid culture method that maintain stemness properties in 3D conditions and also, shown SASCs, with this method, express specific mesenchymal stem cell (MSC) (e.g. CD29, CD9, CD73, CD90, and CD271) and display enhanced ability to differentiate towards MSCs lineages (Di Stefano AB 2015).

### 1.8 "Small regulatory RNA" MicroRNA

MiRNAs are endogenous small noncoding RNAs involved in the regulation of several biological pathways, and they could have a critical role in regulating self-renewal and MSC differentiation (Kang and Hata, 2015). MicroRNAs have been discovered in different points of the genome, 50% of them are intragenic, both at the intronic level, both at the exon-intron level, and on the complementary strand. The remainder is found within non-coding intergenic sequences. The microRNAs are transcribed by RNA Polymerase II, the first transcript, called pri-miRna, consists of 100-100bp and has a stem and loop shape with a cap to 5'P and a poly-Adenine tail to 3'OH, the maturation

process has two steps: Cropping (nuclear process) -> pre-miRna composed of about 70 bp maintains the stem and loop shape with 2-3 nt protruding single helix at the 3'OH end and Dicing (cytoplasmatic process) that formed final double-stranded miRna of 18-22 nt. Between two filaments, only one plays its role, called “guide”, the other called "passenger" is degraded. Due to their small size, a miRNA is complementary to the 3'UTR ranges of different messengers and vice versa, i.e. the same messenger can be the target of different miRNAs that recognize different positions of the 3'UTR region. Over the years several algorithms have been developed, the best known are: Diana-microT, Miranda, MiRtarbase, TARGETMINER, TARGETSCAN-VERT, PICTAR-VERT and many others.

### 1.9 Adipose Tissue: the role of microRNAs

Differentiation of MSCs into specific types of mature cells is regulated by a complex system where growth factors, transcription factors, and signalling pathways play a crucial role. Recent evidence report that microRNAs (miRNAs) are actively involved in regulating cell stemness through a negative, post-transcriptional, gene expression regulation (Ambros, 2004) (Bartel, 2009). Recent studies have shown that miRNAs play an important role on the proliferation and differentiation of adipose stem cells through the modulation of various factors such as receptors or ligands. Numerous pathways implicated in mesenchymal differentiation of ASCs are modulated by miRNAs, including the Bone Morphogenetic Protein (BMP), small mother against decapentaplegic (SMAD), and WNT pathway. In this regard, the BMP signalling pathway has been seen to be modulated by miR-125b, or the SMAD1 protein is found to be down-regulated, by miR-199. Several miRNAs regulate the WNT pathway, in particular, miR-29 which targets some antagonist molecules of the Wnt pathway such as Dkk1, Kremen2 and sFRP2 (Chen et al., 2014). miR-142-3p and miR-25 contribute to maintaining stemness, miR-142-3p seems to play a crucial role in maintaining iPSC immaturity (Abdul Razak et al., 2014) and thus it can be considered a stemness marker. The latter is associated with an indirect modulation of Yamanaka factors through the regulation of some factors like WWP2, fbxw7 (Lu et al., 2012).

### 1.10 Long Term *in vitro* culture and replicative senescence

In regenerative medicine, maintenance of stem cell properties is crucial. Aging is considered to be the cause of the decrease in stem cell potential. Human mesenchymal stem cells (hMSCs) are expanded *in vitro* before implantation. However, the relationship between stem cell expansion and genetic stability is poorly understood. All primary human cells, including hMSCs, undergo only limited number of cell divisions

under standard culture conditions, in a process called cellular replicative senescence (HAYFLICK and MOORHEAD, 1961). MSCs become senescent during prolonged "long-term culture", as indicated by their reduced differentiation potential, shortening of the mean telomere length and morphological alterations (Izadpanah et al., 2006), (Bonab et al., 2006). Traditional adherent cells do not grow in serum-free conditions and are therefore susceptible to cell aging, modifying their genetic and protein structures during the long-term *in vitro* culture condition (Zhao et al., 2015). The cell cycle is a process that embodies the central dogma of molecular biology. It is a genetically controlled process, consisting of a series of coordinated and mutually dependent events on which the correct proliferation of eukaryotic cells depends (Israels and Israels, 2001). Primary somatic mammalian cells can replicate approximately 50 cumulative doublings *in vitro,* after which the cells stop dividing (HAYFLICK and MOORHEAD, 1961). Before Leonard Hayflick's discovery, it was believed that cells divide indefinitely. Hayflick demonstrated fibroblasts, isolated from individuals, have a limited proliferative capacity *in vitro* (Hayflick, 1965). After n-cycles of replication they enter in a state called *senescence*. It is assumed there is an internal clock capable of both counting the number of divisions and blocking proliferation once the predetermined threshold of possible divisions has been reached. It is well-known that life span of MSCs is limited, and that during *in vitro* culture they lose differentiation potential and progress to *replicative senescence* (Trivanović et al., 2015), (Allsopp et al., 1992) (Stewart and Weinberg, 2006).

### 1.11 Cellular Aging

Aging (see Glossary) is a gradual deterioration of physiological functions leading to reduced fitness, increased susceptibility to pathologies and increased mortality rate.

Cancer, diabetes, cardiovascular disorders, immune decay and neurodegenerative diseases are among the main ageing-associated pathologies. These phenotypes refer to cellular and molecular changes (Sames, 2005) which defined aging as "the sum of the primary restrictions in the regenerative mechanisms of multicellular organisms". Campisi 2000 associates the term aging with Hayflick's concept of replicative senescence, defining it as "a substantially irreversible arrest of cell division" (Campisi, 2000). Therefore senescent cells, unlike apoptotic ones, are alive but with functional imbalances (Itahana et al., 2001). In general, senescent cells exhibit enlarged and flattened morphology and are blocked in G1 phase, meaning genes that drive cell cycle progression are repressed and cell cycle inhibitors such as p53 / p21 and p16 / RB are up-regulated. Increased cell size is often associated with senescence condition (Dimri et al., 1995), *in vitro* MSCs aged are larger (Baxter et al., 2004), (Mauney et al., 2004) compared to their younger counterparts; exhibit more podiums and contain more actin stress fibers (Stenderup et al., 2003). Senescent cells show altered gene and biochemical processes, (SIRT1) is an NAD1-dependent lysine deacetylase involved in regulating various cellular processes, including cellular senescence, bone homeostasis, and metabolic pathways, SIRT1 contributes to long-term growth through the delay of senescence during prolonged cell passages (Yuan et al., 2012). In absence of SIRT1, MSCs prematurely lose their capacity for self-renewal and show an up-regulation of cell cycle inhibitors, anticipating replicative senescence (Yoon et al., 2014). Yoon et al demonstrated that SIRT1 deacetylase positively regulate the SOX2 expression in the bone marrow-MSCs (BM-MSCs), which is consequently overexpressed. SIRT1 decreases during differentiation processes, regulates osteoblastic differentiation process by deacetylating b-catenin and Runx2 (Simic et al., 2013), (Shakibaei et al., 2012). CDKN2A, also called p16INK4, is a small ankyrin-repeat protein of 16 kDa encoded by INK4a in the INK4/ INK4a ARF locus, has emerged in the past 10 years as a valuable

candidate biomarker, is a key effector of cell senescence (Sorrentino et al., 2014), (Serrano et al., 1995) has been previously associated to senescence and apoptosis (Martin et al., 2014). p16 inhibits and binds to the cyclin-dependent kinases CDK4 and CDK6, inducing cell cycle arrest in G1 (Serrano et al., 1993). Notably, the expression of this gene is reported to increase as mesenchymal stem cells age and undergo senescence (Burrow et al., 2017). However, these studies show that increased p16INK4a expression upon ageing causes loss of self-renewal capacity in multiple tissues. Therefore it is considered an important pro-aging marker, as its expression is a reflection of the metabolic and physiological state of cells .

### 1.12 Telomere, Sheltering Complex and Telomerase

Telomeres are nucleoprotein complexes located in the terminal portions of chromosomes and consist of species-specific, non-coding and highly repeated sequences in tandem, associated with different proteins (Sugimoto, 2010), (Sethe et al., 2006). The 3 'and 5' ends of the DNA double strands are extremely susceptible to nuclease degradation, and highly subject to genic fusion and recombination phenomena (Haber and Thorburn, 1984).Telomeres act as a protective "cap"of linear chromosomes preventing progressive degradation of DNA and conferring genetic stability. Furthermore, telomeres regulate the recognition and separation of chromosomes during mitosis, facilitate DNA replication positioning and anchoring chromosomes to the nuclear machinery (Blackburn, 1991), and influence the transcription of genes near to chromosomal ends. “The end replication problem”, first described by Watson in 1972, concerns the synthesis mechanism of the ends of linear chromosomes. DNA polymerase is unable to replicate the 5 'ends of the lagging strand. The DNA polymerase is capable of proceeding only in the 5'→ 3' direction so, while the guide strand (3'→5' leading strand) is synthesized entirely and continuously, the slow strand (5' → 3' lagging strand)

is synthesized in a discontinuous way starting from small RNA primers, capable of provide to DNA polymerase the "primer 3'OH". OKAZAKY's small fragments are linked each other by DNA ligases. The problem occurs when at the distal end of the lagging filament, DNase removes the 3'OH primer, its degradation leaves a gap at the 5 'end which is thus shorter than the 3' end of 50-100 base pairs (Levy et al., 1992). For this reason, at each cell division cycle, telomeres shorten up to the Hayflick limit (minimum critical length) which signals the onset of replicative senescence. In humans, telomeric DNA consists of nucleotide sequences 5'-TTAGGG-3 'in the leading strand and 3'-CCCTAA-5' in the lagging strand, repeated for approximately 9-15 kb (Moyzis et al., 1988). The 3 'end of the leading filament ends with single-stranded 50-300 G (G-strand overhang) (Zhu et al., 2011). The protruding strand of G folds into a loop and slips into the double helix of telomeric DNA, which in turn opens to form a triple helix structure called the D-loop (displacement loop, minor loop), the larger loop generated by the folding of the "protruding ends it is defined T-loop (telomere loop) (Figure 1B) (de Lange, 2005), (O'Sullivan and Karlseder, 2010). "Shelterin Complex", is a multi-protein complex, TRF1, TRF2, TIN2, RAP1, TPP1 and POT1, involved in the formation and maintenance of telomere architecture and in the regulation of their length (Figure 1B). Shelterin complex binds to the TTAGGG nucleotide sequence, stabilizes and protects the ends of chromosomes by mediating the formation of the T-loop (de Lange, 2005). It is difficult to have a clear picture of each function of Shelterin components, for example many *in vitro* studies have shown that TRF1 determine the T-loop conformation (telomeric repeated binding factor 1) and TRF2 (repeated binding factor TTAGGG) favors the insertion of the G-strand overhang into the telomeric dsDNA, protecting it from mechanisms of repair, degradation, recombination and fusion (Burge et al., 2006), (van Steensel et al., 1998). TRF1 protein appears to be involved in regulation of telomere length (van Steensel and de Lange, 1997) and TRF2

protein hinders the fusion of chromosomal ends and increases their stability (Broccoli et al., 1997).TRF2 also interacts MRE11 (Meiotic Recombination-11) complex, composed of MRE11, Rad50, and the NBS1 (Nijmegen Breakage Syndrome-1) protein, which is implicated in the cellular response to agents that damage DNA, localizes to the telomere. TERF2IP (Repressor / Activator Protein 1) is a TERF2 associated protein, protects telomeres from NHEJ, (Non-homologous End-joining) a DNA repair mechanism, acts as a transcription regulator when associated with TERF2 or other factors (Nandakumar and Cech, 2013), (Martínez and Blasco, 2018), and also has a stabilizing function (Sfeir, 2012). POT1 (Telomere Protection 1) possesses high affinity for the single-stranded TTAGGG region of telomeric DNA (Patel et al., 2015), protecting it from the action of nucleases (Greider, 1999). TPP1 is a POT1 associated protein, loss of TPP1 leads to reduced POT1 function (Červenák et al., 2017) when telomeres need to be elongated, TPP1 is involved in telomerase recruitment (Bejarano et al., 2017). TIN2 (nuclear protein 2 interacting with TRF1 and TRF2) is a stabilizing protein, acts as a bridge between the units attached to the double stranded DNA, TRF1 and TRF2, and the units attached to the single stranded DNA TPP1-POT1 (Méndez-Pertuz et al., 2017). Telomerase is an enzyme, a telomere-specific reverse transcriptase, which adds telomeric repeats (5'-TTAGGG-3) to the ends of linear chromosomes, ensuring their integrity. Telomerase is composed by different proteins, in particular a catalytic subunit with reverse transcriptase function (Telomerase Reverse Transcriptase, TERT) synthesizes DNA from RNA, and RNA component (Telomerase RNA Component, TERC or TR) which acts as a template (Autexier and Lue, 2006), (Wenz et al., 2001)). In normal somatic cells, telomere sequences are lost during replication *in vitro* and *in vivo* due to "*The end replication problem*" phenomenon (Bodnar et al., 1998), (Martens et al., 2000). In contrast, cancer cells, immortalized cells and embryonic stem cells bypass the end replication problem thanks to reverse

transcriptase activity (Kim et al., 1994), (Amit et al., 2000). Unlike most adult somatic cells that are telomerase negative, mild and moderate activity of the enzyme, has described in multipotent stem cells. There are conflicting opinions regarding the proliferative capacity of multipotent, for many telomerase activity has not been detected in multipotent stem cells (Zimmermann et al., 2003), for the others such as Pittenger et al, MSCs possess a moderate active telomerase (Pittenger et al., 1999).

### 1.13 Oxidative stress and micro cellular environment

The term oxidative stress indicates a state of alteration of macromolecules into cells and tissues when these are exposed to an excess of oxidizing agents (McCord, 2000). In all aerobic organisms there is a delicate balance, called redox, between the production of oxidizing substances, including reactive oxygen species (ROS), and the antioxidant defence system that has the task of preventing and / or repairing possible damage produced. ROS, free radicals centred on oxygen, are the main by-products formed in the cells of aerobic organisms (Rahman, 2007), more precisely, they initiate dangerous autocatalytic chain reactions. The main targets of ROS are phospholipid components of bio-membranes and plasma lipoproteins (Halliwell and Gutteridge, 1990), (Kowaltowski and Vercesi, 1999). To be attacked are mainly the polyunsaturated fatty acids (PUFA), which undergo a chain process, known as *lipid peroxidation*, which involves their transformation into lipid hydroperoxides (LOOH) and secondary aldehyde compounds, such as malondialdehyde (MDA) and 4-hydroxinonal (4-HNE). Aldehydes, and their associated toxicity, are involved in many pathological processes including neurodegenerative diseases, cancer etc. (Yoritaka et al., 1996). The aldehyde dehydrogenase (ALDH) superfamily is represented in all three taxonomic domains (Archaea, Eubacteria and Eukarya), it represents a significant metabolic pathway of the

aldehyde detoxification, it oxidizes aldehydes to carboxylic acids. The human genome contains 19 ALDH genes, mutations in which are the basis of several diseases. In particular, members of the ALDH 3 family (ALDH3A1, ALDH3A2, ALDH 3B1 and ALDH 3B2) appear to have unique roles in cellular defence against oxidative stress and aldehyde toxicity (Marchitti et al., 2010).

### 1.14 ROS and ALDH in adipose stem cells

The intracellular redox environment critically affects all major cellular processes, such as proliferation, self-renewal, apoptosis, senescence and differentiation; any alteration of these processes, in mesenchymal stem cells, determines their malfunction. Some reports demonstrated that MSCs cultured under hypoxia showed a diminished capacity to differentiate into adipocytes and osteocytes, supporting the notion that low oxygen tension promotes an undifferentiated state (Müller et al., 2011), (Holzwarth et al., 2010). In particular, excessive production of reactive oxygen species (ROS) compromises the redox homeostasis of stem cells, which leads to serious dysfunctions such as replicative senescence and loss of stem properties during long-term *in vitro* culture (Liang and Ghaffari, 2014), (Holmström and Finkel, 2014) ]. Furthermore, ASCs have been shown to accumulate ROS during adhesion expansion *in vitro* (Suh and Lee, 2017). Under physiological conditions, stem cells keep ROS levels low to preserve their stemness and continue to be functional, remain quiescent in mammals (Holmström and Finkel, 2014), (Shi et al., 2012). ALDH family is one of the most important family of enzymes that impart resistance to oxidative stress on MSCs. High ALDH activity is one of the attributes of many stem cells and contributes significantly to cell survival, i.e. through the ability to eliminate toxic and xenobiotic aldehydes in humans (Balber, 2011). They are involved in a variety of important stem cell functions including regulation of self-renewal ability, differentiation potential, and oxidative stress response, cellular

expansion (Muramoto et al., 2010), (Zhou et al., 2015), (Tomita et al., 2016), (Balber, 2011) In Najar's work, they have used the fluorescence-activated cell sorting (FACS) technology to isolate ASC sub-populations based on their ALDH activity. They have obtained ASCs with a different enzymatic activity of ADLH, $ALDH^{+}$, and $ALDH^{-}$. Results showed that $ALDH^{+}$ ASCs demonstrated a more significant increased expression of proliferation genes, in particular High ALDH3A1 expression. In general, they proposed ALDHs as stemness markers, but also as self-protection, immunity, differentiation markers (Najar et al., 2018).

### 1.15 TGF-ß Cytokine in stem cells

TGF-ß plays a key role in cell differentiation and proliferation especially in stem cells. Proteins of the TGF-ß family play an essential role in maintaining the pluripotency of human ESCs (James et al., 2005). The same is true for multipotent stem cells, TGF-ß produced in an autocrine way, or by factors in their microenvironment. The TGF-ß family is encoded by 33 genes. TGF-ß family ligands include TGF-ßs, activins, nodals, "growth and differentiation factors" (GDF) and bone morphogenetic proteins (BMPs) (Shi and Massagué, 2003). In human MSCs, proliferation is stimulated by Wnt or TGF-ß signaling (Boland et al., 2004), (Jian et al., 2006). TGF-ß1 induces Smad3-dependent nuclear accumulation of ß-catenin in MSCs, which is required for cell proliferation. TGF-β also plays an important role in MSC differentiation (Roelen and Dijke, 2003). For example, BMPs induce the differentiation of mesenchymal cells into chondroblasts or osteoblasts *in vitro*. TGF-β and activin are required in the differentiation of chondroblasts in the early stages.

### 1.16 Rationale and Objectives

Recently our group research has demonstrated spheroids from ASCs (S-ASCs) can be

directly obtained from lipo-aspirated samples, using the stromal vascular fraction (SVF) portion cultured in serum-free and ultra low-adhesion conditions. Thus, we have further genotyped and phenotyped this cell population *in vitro* and *in vivo*, demonstrating S-ASCs express typical MSC markers, such as CD29, CD44, CD9, CD73, CD90, and CD271. Moreover, S-ASCs show more stemness properties than ASCs, as demonstrated by the higher expression of stemness-associated messengers RNA (mRNA). Accordingly, S-ASCs express a lower level of mesenchymal differentiation marker such as bone morphogenetic protein. We have also investigated their regenerative potential in animal models, reporting that cells grown on scaffold, such as Integra (Di Stefano et al., 2020b) , and afterward implanted in a laminectomy site are able to actively participate in bone tissue regeneration (Di Stefano AB 2015) .

The main aim of this project is to answer the fateful question whether real stem cells are those in suspension (3D) or in adhesion (2D). Moreover, if S-ASCs application could be a feasible strategy in various fields of regenerative medicine, from bone or tendons lesions, nervous or endothelial (vascular) regeneration. To do this, a deeper molecular characterization will be needed, from genotypic and phenotypic analysis to determine and highlight specific targets that specifically identify the adipose stem cells in a lipoaspirate samples.

For that purpose, we compared 2D versus 3D cultures analyzing:

- Morphology and viability tests;
- microRNAs profiling and focusing on mRNAs involved in stemness maintenance and mesenchymal differentiation at different time points of culture;
- telomere length, telomerase activity, aging;
- oxidative environment and redox homeostasis;
- Cytokine production.

CHAPTER 2

# Materials and Methods

## 2.1 Adipose Tissue Patients

Adipose tissue was collected from healthy individuals, 44 females and 17 males, after signing informed consent at the Department of Plastic and Reconstructive Surgery of the University Hospital of Palermo. Mean age was 50, 25 years (range: 18-77). The hospital ethical committee approved the study, and from each patient an informed consent was collected. Lipoaspirate samples were harvested from different body areas such as abdomen, breast, flanks, trochanteric region, and knee.

## 2.2 Cell culture

Lipoaspirate samples were digested with collagenase (150 mg/ml; Gibco, Carlsbad, CA) through gentle agitation for 30 min at 37°C. The digested sample was centrifuged at 1200 rcf for 5 min, and the SVF portion was plated with serum-free stem cell-specific media with bFGF; 10 ng/ml; Sigma, St. Louis, MO) and epidermal growth factor (EGF; 20 ng/ml; Sigma). In these conditions, S-ASCs, or called also SASCs-3D, grew as floating spheroids in ultralow culture flasks (Corning, NY) and placed at 37° C in a 5% CO2 humidified incubator (Di Stefano et al., 2015). The SVF was also resuspended in DMEM complemented with 10% FBS for growth under standard adherent (AD.) conditions ASCs-2D. Cell media was replaced twice a week. ASCs were the commercially available STEMPRO® human adipose-derived stem cells, plated as

recommended by the manufacturer (Invitrogen, Carlsbad, CA).

### 2.3 MicroRNA Profiling

miRNAs were extracted from cultured cells using the miRNeasy® Mini Kit (Qiagen, Germantown, MA) and quantified using a NanoDrop spectrophotometer. Reverse transcription (RT) was performed using a TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and Megaplex RT primers (Human Pool A; Applied Biosystems) following the manufacturer's instructions. RT was performed on a 9700 thermocycler (Applied Biosystem) with the following cycling conditions: 40 cycles at 16°C for 2min, 42°C for 1min, and 50°C for 1 s, followed by a final step of 85°C for 5 min to inactivate reverse transcriptase. MegaplexTM RT products were mixed with RNase-free water and TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2× (Applied Biosystem) and then loaded into TaqMan Human MicroRNA Array A (Applied Biosystems). Realtime polymerase chain reaction (PCR) based microfluidic card with embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs and controls was performed on an ABI PRISM 7900HT system (Applied Biosystems) using the 384-well TLDA default thermal-cycling conditions: 50°C for 2 min, 94.5°C for 10 min, and 97°C for 30 s, followed by 40 cycles at 95°C for 30 s and 59.7°C for 1 min. The cycle threshold was automatically calculated using SDS 2.4 software (Applied Biosystems) and miR-191 was used as an endogenous control. Human MicroRNA Arrays were all performed in triplicate on three different samples. The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method, and fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta Ct}$ .

### 2.4 Single miRNA assay

RT was performed for each miRNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). A total of 15 μl reaction was incubated for 30

min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was performed on ABI Prism 7900HT System (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The quantitative reverse-transcriptase PCR reactions were all performed in triplicate on three different samples. The predesigned Taqman primers (Thermo Fisher) were: MIR142-3P 000464, MIR221 000524, MIR100 002142, MIR495 001663, MIR140 001187, MIR30C 000419 , MIR143 002249.The cycle threshold was automatically calculated using SDS 2.4 software (Applied Biosystems) and miR-191 002299 . was used as an endogenous control. The relative expression levels of individual miRNAs were calculated using the comparative ΔΔCt method, and the fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta Ct}$ .

**2.5 mRNA expression profile**

Total mRNAs were extracted from cells using an RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop spectrophotometer. RT was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in 350 ng of RNA. A total of 50 μl reaction was incubated for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. RT-PCR was performed on ABI Prism 7900HT System (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The RT-PCR reactions were all performed in triplicate on three different samples. The predesigned Taqman primers (Thermo Fisher) were: SOX2 Hs01053049_s1; POU5F1 Hs00999632_g1; NANOG Hs04399610_g1; RUNX2 Hs01047975_m1; ALPL Hs01029144m1,SOX9 Hs00165814_m1; COL10A Hs00166657_m1 ;PPARG Hs01115513_m1; LPL Hs00173425_m1; SIRT1 Hs01009006_m1; CDKN2A Hs00923894_m1; TINF2 Hs00173291_m1; TERF2 Hs00194619_m1; TERF2IP Hs00430292_m1; TERT Hs00972650_m1, GAPDH Hs02758991_g1; TGF-β1 Hs00998133_m1. Primer sequences of to detect ALDHs isoforms were as follows: hALDH1A1 fw-: TTGGAAATCCTCTGACCCCA,rv

CCTTCTTTCTTCCCACTCTC; hALDH1A2 fw- CATTGGAGTGTGTGGACAGA, rv- GGAGCTATTTTCCAGGCA; hALDH1A3 fw- TTTTCATCGACCTGGAGG, rv-GACGTTGTCATCTGTGGG; hALDH1B1 fw-ACTTGGCCTCACTCGAGA, rv-CCAGCAAAGTACCGATAC; hALDH3A1 fw- CACATCACCTTGCACTCTCT, rv-AGCTCTTCTTGCCATGGT; hALDH3A2 fw-TAGCTTTTGGTGGGGAGA, rv-CTTGCATCACCTTGGTTT; hALDH3B1 fw-TATCTAATCACGGGCCA, rv-AGCTGCTTGTTTTCTTGC; hALDH3B2 fw TTCTCCAACAGCAGCCAG, rv-CGGACAGCAGAGATATGTAG.

The cycle-threshold was automatically calculated using SDS 2.4 software (Applied Biosystems), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The relative expression levels of mRNAs were calculated using the Livak method by the equation $2^{-\Delta\Delta Ct}$.

### 2.6 Digital PCR

The QuantStudio™ 3D Digital PCR (dPCR) platform (Thermo Fisher Scientific) was used to confirm the miRNA expression pattern obtained with real-time PCR. The analyses were conducted in triplicate using miRNAs isolated from both S-ASCs and ASCs (n = 3). The dPCR was performed in a final volume of 15 µl as follows: 2.25 µl of complementary DNA (concentration 2 ng), 8 µl of QuantStudio™ 3D dPCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA), 4 µl of nuclease-free water, and 0.75 µl of TaqMan MicroRNA Assay (Thermo Fisher Scientific) for miR-142-3p. The sample mix was loaded on a chip using the QuantStudio 3D Digital PCR Chip loader (Thermo Fisher Scientific), and then PCR reaction was performed on ProFlex™ 2× Flat PCR System (Thermo Fisher Scientific) with the following cycling conditions: 96°C for 10 min, 40 cycles (2 min at 56°C and 30 s at 98°C) and a final hold at 60°C for 2 min. After PCR amplification steps, QuantStudio™ 3D Digital PCR chips were first read through QuantStudio™ 3D Instrument and then analyzed through QuantStudio™ 3D

Analysis Suite Software (Thermo Fisher Scientific).

### 2.7 Cell viability

Cell viability was quantified with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega); 50.000 cells/well were plated in biological and replicative triplicate. The absorbance at 490nm was analysed at 24h, 14 and 28 days of culture condition, respectively, in ultralow 96-well plate for SASCs-3D and in adherent 96-well plate for SASCs-3D AD and ASCs-2D. Detection of cell death was performed using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were collected and washed twice in PBS, resuspended in 1X Binding Buffer and stained with 5µl of fluorescein isothiocyanate (FITC)-conjugated Annexin V for 20 minutes at room temperature in the dark. Then they were washed once with PBS and stained with 7-aminoactinomycin D (7-AAD) Viability Staining Solution (eBioscience) for 5 minutes. All samples were analysed by FACS ARIA flow cytometer (BD Bioscience, USA) and also analysed using FlowJo software (Tree Star, version 10.5.3).

### 2.8 Cytochemical and Immunofluorescence staining

The cytoblocks, which were 4-µm-thick sections, were cut, deparaffinized, rehydrated, and evaluated with hematoxylin and eosin staining. Sections were analyzed under the Axio Scope A1 optical microscope (Zeiss, Jena, Germany), and microphotographs were collected using the Axiocam 503 Color digital camera (Zeiss).

For immunofluorescence staining, the sections were were incubated with primary antibodies: rabbit polyclonal anti-human SOX2 (SP76, catalog number 760-4621; Ventana, Roche, CA), rabbit polyclonal anti-human NANOG (N3C3, catalog number GTX100863; Genetex, Irvine, CA), and rabbit polyclonal anti-human Oct-4A (C30A3, catalog number 2840; Cell Signaling Technology, Danvers, MA). After Fc blocking, primary antibodies binding was revealed by fluorochrome-conjugated secondary antibodies: Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L, A11008 Lot

1583138; Invitrogen Molecular Probes, Carlsbad, CA). The slides were counterstained with DAPI nucleic acid stain (Invitrogen Molecular Probes). Confocal analysis was used to acquire fluorescence staining.

### 2.9 Mesenchymal differentiations

Mesenchymal differentiation was performed with a specific cell medium to induce chondrogenic, adipogenic, and osteogenic differentiation by means of the STEMPRO® Differentiation Kit (Invitrogen), and S-ASCs were cultured in adhesion conditions until 28 days. Cell viability, adhesion, and differentiation were assessed by daily observation using optical microscopy.

### 2.10 Telomere length analysis

Chromosomal DNA from the samples was extracted using the QIAGEN DNeasy kit for blood and tissues according to the manufacturer's protocol. After quantification with NanoDrop 2000, DNA (35ng/reaction) was analysed according to Cawthon method (Cawthon, 2002). Telomere (T) gene and single copy gene (S) were quantified with different SYBRgreen RT-PCR condition (18cycles for T and 30cycles for S) in separate 96-well plates. The primer sequences were: tel 1, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT (270 nM); tel2 TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA (900 nM); 36B4u CAGCAAGTGGGAAGGTGTAATCC (300 nM); 36B4d CCCATTCTATCATCAACGGGTACAA (500 nM). The reaction volume was 20 µl/well. T/S ratio, that is the relative expression level of the target/single copy gene in DNA samples, was calculate using Livak method ($2^{-\Delta\Delta CT}$)(Livak and Schmittgen, 2001).

### 2.11 mRNA array of human telomere complex genes

The human telomere complex genes expression profile was analysed using the TaqMan Array 96-well Plate Human Telomere Extend by Telomerase (Thermo Fisher

Scientific), a comprehensive collection of predesigned, pre-formulated primer and probe sets to perform quantitative gene expression in 96-well plate standard. Of 28 genes in triplicate, 19 are Telosome-associated genes and 9 are endogenous controls, which were analysed by quantitative real-time PCR (qPCR). Total mRNAs were extracted from cells using RNeasy Mini Kit (Qiagen) and quantified using a spectrophotometer NanoDrop 2000. 450ng of total mRNA were retro-transcribed in cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The qPCR reactions were performed on an ABI PRISM 7900HT system (Applied Biosystems), in triplicate on three different samples SASCs 3D and ASCs 2D using 20µ l of cDNA in 96-well Plate with embedded TaqMan primers and probes for 28 human gene. -Reaction mixtures for amplification and thermal condition were set according to the manufacturer's protocol. Relative expression was normalised to the mean of the reference beta-2-microglobulin (B2M) gene, using the Livak's method.

### 2.12 Lipid peroxidation and ROS assay

Lipid peroxidation was measured using the Lipid Peroxidation Sensor BODIPY™ 581/591 C11, following manufacturer's instructions (Life technologies). Briefly, $2x10^5$ SASC-3D and ASC-2D cells were incubated with 10µM BODIPY in a 1 ml volume of PBS for 30 minutes at 37°C and analysed using a FACS ARIA flow cytometer (BD Bioscience, USA). Cellular ROS production was measured using the total Reactive Oxygen Species (ROS) Assay kit (Invitrogen). $3x10^5$ cells from 2D and 3D cultures were treated with 1X ROS fluorescence probe in a 1mL volume of PBS for 60 minutes and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. ROS levels were assessed by flow cytometry.

### 2.13 Cytokine analysis

To study intracellular IL-10 and TGF-β expression, cells were harvested, washed twice in PBS cointaning 1% FCS and fixed with Inside Fix (Inside Stain Kit, Miltenyi Biotec,

Auburn, CA, USA), containing 3.7% formaldehyde for 30 minutes at room temperature protected from light. Fixation was followed by permeabilization with Inside Perm (Inside Stain Kit, Miltenyi Biotec, Auburn, CA, USA), containing a detergent and 0.05% sodium azide. Staining of intracellular cytokines was performed by incubation of permeabilized cells with PE-labelled anti-IL-10 (clone JES3-9D7, Miltenyi Biotec, Auburn, CA, USA) and APC-labelled anti-TGF-β1 (clone CH6-17E5.1, Miltenyi Biotec, Auburn, CA, USA) for 20 minutes at room temperature in the dark. Following two additional washes in PBS containing 1% FCS, cells were analyzed by FACS ARIA flow cytometer (BD Bioscience, USA). Viable cells were gated using forward and side scatter criteria and the analysis were performed on 100.000 acquired events for each sample by using FlowJo software (Tree Star, version 10.5.3).

### 2.14 Statistical analysis

Data are expressed as mean ± standard deviation of three independent experiments. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by either a Tukey's or Bonferroni's multiple comparison post hoc test. Significance levels were analysed with GraphPad Prism 5 statistical software and indicated as p values (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

# CHAPTER 3

# Results

## 3.1 miRNA array profiling

To obtain new insights on the stemness potential of S-ASCs, we investigated their miRNA expression profile. In particular, a screening analysis and relative quantification of 377 modulated miRNAs were performed through real-time PCR, comparing S-ASCs with their adherent counterpart ASCs (Figure 2A). miRNAs were analyzed using TaqMan MicroRNA Assays (Panel A Cards; Thermo Fisher Scientific), and miR-191 was used as endogenous control. In the S-ASC population, we reported 26 upregulated miRNAs (14%) and 56 downregulated miRNAs (25%); 46% miRNAs were undetermined as Ct values were higher than 35, whereas no differences were reported for 15% miRNAs (Figure 2B). Differentially expressed miRNAs with statistical significance were identified by volcano plot analysis (Figure 2C). A fold change greater than two was used as cutoff to identify significantly deregulated miRNAs (Figure 2D,E). The analyses were conducted in triplicate on three different biological replicates, and miRNA expression levels were calculated as a mean between the triplicates. Based on further and more detailed literature reviews, we decided to proceed our investigation by focusing on those miRNAs reported to be specifically involved in stemness and early-late chondrogenic, adipogenic, and osteoblastic differentiation stages (Figure 3A).

### 3.2 S-ASCs express a high level of miR-142-3p and stemness genes typical of iPSCs

Among 26 upregulated miRNAs, miR-25, and miR-142-3p were 16- and 20-fold increased, respectively (Figure 2). Interestingly, these two miRNAs are known to be involved in the self-renewal and maintenance of pluripotency in stem cells, in particular in iPSCs, in which they have been found to be highly upregulated. Analysis of their targets by Lu et al. (2012) showed that both the ubiquitin ligases (RBR-E3), WW domain containing E3 ubiquitin protein ligase 2 (Wwp2) and F-box and WD repeat domain containing 7 (Fbxw7), which target OCT4, are regulated by miR-25 (Lu et al., 2012). Similarly, miR-142-3p is also upregulated in iPSCs and is involved in stemness maintenance and proliferation regulation in some mesenchymal lineages (Abdul Razak et al., 2014). Our analysis showed that miR-142-3p is surprisingly upregulated in the S-ASC population (20-fold) compared to the ASCs (Figure 3B). These data were also confirmed with digital PCR, allowing the absolute quantification of miR-142-3p with greater sensitivity (Figure 3C). We proceeded to investigate the expression levels of three typical genes involved in stemness, self-renewals, and plasticity: SOX2, NANOG, and OCT4 (Loh et al., 2006), (Riekstina et al., 2009). The analysis was performed using real-time PCR and demonstrated that the S-ASCs expression levels of SOX2, NANOG, and OCT4 were more than two-fold greater compared with those found in ASCs (Figure 3B). Moreover, we decided to analyze through immunostaining the protein expressions of SOX2, NANOG, and OCT4 in paraffin-embedded S-ASCs, confirming also the expressions of these mRNAs at the protein level (Figure 3D). These important findings support our thesis, according to which S-ASCs have superior stemness features compared to the ASCs.

### 3.3 S-ASCs express a low level of miRNAs and mRNAs related to early and late chondrogenic differentiation

Nowadays, it is well known that the miRNA and mRNA expression levels are subjected to change during the progression from stem cells to differentiated cells (Chen et al., 2014). Accordingly, mRNA and miRNA expression profiles may change along mesenchymal differentiation (Kuosmanen et al., 2017) , and therefore we decided to investigate the most representative miRNAs involved in early and late chondrogenic differentiation stages in 3D S-ASCs. In particular, elevated levels of miR-495 have been reported in the early chondrogenic differentiation stage (Lee et al., 2014), whereas the upregulation of miR-140 is associated with the late stage of differentiation (Karlsen et al., 2014). Therefore, we cultured both S-ASCs and ASCs under chondrogenic condition, and we evaluated the expression levels of miR-495 and miR-140 (Figure 4A). We showed that S-ASCs express a lower level of miR-495 compared with ASCs, suggesting that the adherent counterpart could probably be considered as precursors or progenitors rather than as real stem cells. To confirm the involvement of miR-495 in early stage differentiation, we analysed this miRNA in S-ASC-derived chondrocytes, obtained after 28 days of culture. As expected and in accordance with our thesis, S-ASC-derived chondrocytes express low levels of miR-495, demonstrating that this miRNA is lost in the late differentiation stage. We also analyzed a direct target of miR-495, the SOX9 gene (SRY-box 9), which is known to be involved in chondrogenesis since the earlier stages. As expected, SOX9 mRNA levels appear to be inversely correlated to miR-495. Indeed, SOX9 is downregulated in S-ASCs, whereas it is increased in chondrocytes after 28 days of *in vitro* differentiation (Figure 4A). On the contrary, the levels of miR-140, which is involved in the late chondrogenic differentiation stage, are higher in ASCs and chondrocytes after 28 days of culture compared with both S-ASCs. Mature chondrocytes express high levels of miR-140 as they enter in a state of hypertrophy during chondrogenic differentiation. We also evaluated the

expression of collagen type X α1 chain gene COL10A1 gene, an important late chondrogenic differentiation marker. As expected, no expression of COL10A1 was reported in both S-ASCs and ASCs, whereas higher expression levels were reported in differentiated chondrocytes. For all the experiments here reported, we used miR-191 and GAPDH as endogenous control for miRNA and mRNA analyses, respectively (Figure 4A).

### 3.4 S-ASCs express a low level of miRNAs and mRNAs related to early and late adipogenic differentiation

As previously studied in chondrogenic differentiative lineage, we also aimed to study the molecular changes that characterize the adipogenic differentiation process. From the literature, we know that miR-30c promotes adipocyte differentiation and could be considered as representative of early adipogenic stages (Karbiener et al., 2011). Therefore, we investigated the level of miR-30c in SASCs, ASCs, and S-ASC-derived adipocytes obtained after 28 days of culture under adipogenic condition. In particular, S-ASCs showed a downregulation of miR-30c compared to adherent ASCs, whereas adipocytes showed the highest level of miR-30c. We then evaluated the mRNA level of peroxisome proliferator activated receptor γ (PPARG), which is normally expressed since the earlier phases of adipogenic differentiation and is a target of miR-30c. Unexpectedly, we found a higher expression of PPARG in S-ASCs than in adherent ASCs; this interesting result gives us reason to speculate that S-ASCs are heterogeneous primary cell lines with probably still residual mature adipose cells with respect to ASCs that are pure, homogenous, commercially available cells. Considering the late adipogenic differentiation marker, we chose to investigate miR-143 (An et al., 2016) and lipoprotein lipase (LPL) mRNA. We showed that miR-143 expression strongly increases during the differentiation from S-ASCs to adipocytes after 28 days of culture. Furthermore, according to the literature (Preiss-Landl et al., 2002), LPL mRNA is

absent in S-ASCs and ASCs, whereas it is highly expressed in differentiated adipocytes. The aforementioned results increasingly support the idea of the strong stemness properties of S-ASCs (Figure 4B).

### 3.5 S-ASCs express a low level of miRNAs and mRNAs related to early and late osteoblastic differentiation

In this analysis, we focused mainly on osteogenic differentiation because Di Stefano et al 2015 have previously demonstrated the enhanced osteoinductive properties of S-ASCs in a small-animal vertebral lesion model (Di Stefano AB 2015). Indeed, we showed that S-ASCs grown on Integra and implanted in a laminectomy site are able to actively participate in bone tissue regeneration. With the current study, we wanted to make a step forward in understanding the molecular mechanisms underlying this important process. For this reason, we decided to study miRNA-level changes in osteoblastic cells at several time points, from 7 to 28 days of *in vitro* differentiation. In particular, according to previous published data (Hoseinzadeh et al., 2016), we analyzed miR-221 that is considered a modulator of earlier osteogenic differentiation stages. MiR-221 seems to be overexpressed in ASCs compared to S-ASCs, as well as in all the differentiated osteoblastic populations obtained from S-ASCs after 7,14, 21, 28 days of culture under osteogenic condition. Moreover, the expression level of runt related transcription factor 2 gene (RUNX2), involved since the earlier phase of osteogenesis, increases during differentiation, reaching a peak after 21 days of culture. The last analysed microRNA is miR-100, which is related to the late stage of osteoblastic differentiation (Zeng et al., 2012). Indeed, miR-100 is less expressed in S-ASCs compared with ASCs with an increasing trend during osteoblastic differentiation. Moreover, the expression of alkaline phosphatase (ALP) mRNA is almost absent in both S-ASCs and ASCs, whereas it increases during osteoblastic differentiation (Figure

4C). All these data confirm once again the highly undifferentiated stage of our cell line compared to adherent ASCs.

### 3.6 Molecular analysis during long-term *in vitro* culture in S-ASCs and ASCs

We have also investigated whether the expression profiles of both miRNA and mRNA undergo changes during long-term *in vitro* culture at different time points (1, 14, and 28 days) in S-ASC and ASC models. We showed that miR-142-3p remains stably expressed at high levels in the S-ASC population until 28 days (Figure 5A); accordingly, SOX2, NANOG, and OCT4 expressions are greater in S-ASCs compared with those in ASCs (Figure 6A, B, C). The analysis of the previously discussed early and late differentiation miRNAs showed a similar trend also during long-term culture in S-ASCs and ASCs (Figure 5B, C, D). These interesting data further demonstrate the ability of S-ASCs to keep their stem properties analyzed so far up to 28 days. Overall, we observed an increasing trend of miRNA expression in S-ASCs along the different time points in long-term cultures. However, this miRNA expression behavior still remains definitely lower than ASCs. Moreover, miR-30c, PPARG, and LPL showed an expected permanently upregulated behavior. This result may be due to the heterogeneous nature of the S-ASCs population, and therefore there might be an adipose memory. In ASCs, early miRNA expression levels were maintained or instead progressively increased, whereas tardive miRNAs decreased during long-term culture. Chondrocyte (except for COL10A1) and osteoblastic mRNAs maintained the same miRNA trend in both S-ASCs and ASCs. This could be explained considering the possibility that S-ASCs progress from stem cell to precursor-progenitor cells, whereas ASCs can be considered more as progenitors than pure stem cells that undertake the path of differentiation but not completing it.

### 3.7 Morphology and viability of 2D versus 3D cell culture conditions

Cell cultures of SASCs-3D, established in ultra-low attachment surfaces (U.L.) and which cells are majorly in a quiescent state exhibited a slight decline in MTS enzymatic reduction at 28 days of culture compared to the ASC-2D, which in contrast are known to be in active proliferation due to serum stimulation and adhesion condition (Figure 7A and B). To dissect the sole contribution of plate surfaces to these differences, we plated SASCs-3D in flasks treated to promote cell adhesion (AD.) while maintaining the same stem cell medium and evaluated eventual changes in the growth and spatial distribution of cells. The enhanced attachment condition did not modify SASCs viability and overall, their round shape was preserved; however, the original 3D organization was maintained no longer than 20 days of culturing (Figure 7A). Beyond 20 days, spheroids started to adhere to the flask surface an acquired the typical fibroblastoid shape that is characteristic of ASCs in adhesion. The MTS assay revealed no significant difference in the metabolic activity of SASCs-3D AD. at 1, 14 and 28 days of cultures (Figure 7B). To assess apoptosis, Annexin V/7-AAD staining was use. We found no significant differences between the 3D and 2D culture conditions during the first 14 days (14-21% of early apoptotic events). At 28 days, these events diminished in SASCs-3D (9%) compared to adherent cells (28-30% of total) (Figure 7C). These differences could be inherently related to primary cultures and their lengthy adaptation to different growing conditions.

### 3.8 Comparative evaluation of stemness and aging related genes

Expression profiles analysis of the stemness-related genes SOX2, POU5F1 and NANOG, in SASCs-3D and ASCs-2D at 14 and 28 days of culture, revealed an increase of at least 1.5-fold in the expression of all genes in SASCs-3D compared to ASCs-2D. Consistent with the expression of SOX2 protein in SASCs-3D, the transcript levels of the gene were 2.5-fold and 1.7-fold higher in SASCs-3D compared to ASCs-2D at day 14 and 28 days, respectively, ($P < 0.001$) (Figure 8A,B). *In vitro*, cellular aging is affected by culture-related conditions, especially serum exposure (Søndergaard et al., 2017). Lifespan of mesenchymal stem cells (MSCs) is limited and as *in vitro* culturing is prolonged their differentiation potential diminishes and cells progress to replicative senescence (Wagner et al., 2009). On this basis, we analysed the expression level of the anti-aging marker Sirtuin1, and we found higher expression of this gene in SASCs-3D compared to ASCs-2D ones (14d: $P< 0,001$ and 28d: $P< 0.01$),found opposite expression pattern for the senescence and apoptotic marker CDKN2A, also called p16INK4a, which was significantly down-regulated in SASCs-3D compared to ASCs-2D (4-fold) ($P < 0.001$) (Figure 8 C,D).

### 3.9 Comparative evaluation of telomeric length and telomere-associated genes

To further investigate the aging-related changes between SASCs-3D and ASCs-2D, we performed a comparative analysis of telomeric length, using the qPCR method described by Cawthon (Cawthon, 2002). This test showed that the T/S ratio was similar in SASCs-3D and ASCs-2D, with a fold change of approximately 1 at both time points (Fig. 9A/B). Surprisingly, in both culturing conditions we observed an increase (3-fold) of the T/S ratio at 28 days compared to 14 days. We also quantified 19 genes associated to human telomeres using a Human Array Telomerase card (Invitrogen) that included genes of the Shelterin complex and co-factors (Fig. 9C), RNA binding protein (Fig. 9E)

and DNA repair genes (Fig. 9F) 28 days. We found an increase of TINF2 (~1.5 fold), TERF2 (~3 fold), HNRNP-A1 (~2 fold), HNRNP-F (~2 fold), RAD50 (~3 fold) and XRCC5 (~2 fold) expression levels in SASCs-3D compared to ASCs-2D, while all the others genes, except tankyrase 2 (TNKS2), HNRNP-C and NBN were significantly downregulated. We validated these expression differences for TINF2, TERF2 and TERF2IP members of the Shelterin complex (Fig. 9D). Intringuigly, the expression of HTERT was low or undetected in SASCs-3D and ASCs-2D using both assays, the card and qPCR. This is consistent with a previous report describing undetected HTERT in adipose stem cells (Zimmermann et al., 2003); (Samsonraj et al., 2013); (Trivanović et al., 2015).

### 3.10 Comparative evaluation of oxidative environment and redox homeostasis

Two functional tests were performed to evaluate the oxidative state of cells in 3D vs 2D conditions. These tests revealed that SASCs-3D have lower endogenous levels of ROS (~2.7 -fold at 14d and 4-fold at 28d) and in general lower values of lipid peroxidation than ASCs-2D (Fig. 10 A and B). An increasing number of studies have identified ALDHs as an important marker of normal and cancer stem cells (Najar et al., 2018); (Vassalli, 2019). ALDHs are known to participate in cell differentiation in which ALDH class 1 plays a central role in modulating the retinoic acid levels dynamically (Vasiliou et al., 2000). Other members of the ALDH family, such as ALDH class 3 enzymes are known to mitigate cellular oxidative stress via oxidation of HNE and MDA formed as secondary products during lipid peroxidation (Black et al., 2012). Consistent with the lower levels of lipid peroxidation exhibited by SASCs-3D, we found that three members of ALDH-3 family (ALDH3A1, ALDH3A2 and ALDH3B2) were up-regulated in SASCs-3D compared to ASC-2D (Figure 10C). Interesting, ALDH1 family members ALDH1A1, ALDH1A3 and ALDH1B1 were significantly down-regulated in SASCs-

3D (Figure 10D).

### 3.11 Comparative evaluation of cytokine production

The production and secretion of cytokines has been reported as an essential function of ASCs (Razmkhah et al., 2011). We analysed the expression of TGF-β1, an important anti-inflammatory cytokine, at both transcript and protein level. We found the cytokine mRNA levels consistently increased (3-fold) in SASCs-3D all along the period of culturing (Figure 11A/B). Importantly, the percentage of cells positive for TGF-β1was also increased in SASCs-3D compared to ASCs-2D (60% and 80% SASCs-3D vs 36% and 33% ASCs-2D at 14d and 28d, respectively) (Figure 11 C/D).

CHAPTER 4

# Discussion

Two-dimensional cell cultures are a very useful tool to perform *in vitro* studies in the fields of oncology, stem cell biology, and tissue engineering. Nevertheless, it has been recently questioned whether the 2D model properly represents the original tissue. Indeed, whereas cells cultured in monolayer lose their tissue specificity, 3D cultures exhibit protein expression patterns and intercellular junctions that are similar to *in vivo* condition. Stem cell therapy success is largely determined by the "cell quality" of the implanted cells (Lindroos et al., 2011). Preserving stem-like features are fundamental for successful treatment. A 3D environment stimulates cells to grow in suspended aggregates that are defined as "spheroids." We have previously demonstrated that S-ASCs can be directly obtained from lipo-aspirated samples, using the SVF portion cultured in serum-free and low-adhesion conditions. Moreover, our S-ASCs showed important regenerative potential, demonstrated by their capacity to actively participate in bone tissue regeneration in a small animal model (Di Stefano AB 2015). We wanted to further characterize SASCs 3D to make a step forward in understanding the molecular mechanisms underlying their stemness properties. We demonstrate that SASCs 3D represent a distinct upstream state from the currently studied ASCs expanded in 2D adherent monolayer cultures. Adherent stem cells have started their initial differentiation by expressing adhesion molecules and therefore should be termed

more generically as mesenchymal stromal cells. In contrast, SASCs 3D are a unique cell population that has not undergone differentiation induced by culture conditions, which their adherent counterparts are subject to (Leto Barone et al., 2013). Indeed, we provide evidence that the 3D environment in which SASCs-3D are cultured seems to increase their capacity to maintain a pluripotent behaviour after multiple passages as compared to MSCs. These important findings provide a robust basis to further investigate the application of SASCs-3D in regenerative medicine.

We identify a number of "cell quality" factors through the evaluation of genes expression involved in stemness, miRNA expression profiles, oxidative stress, aging, and telomere length and maintenance on spheroids from adipose derived stem cells (SASCs) when adipose stem cells are culture in 3D vs 2D conditions. The different behaviour of SASCs-3D and ASCs-2D suggests specific intrinsic properties determined by culturing conditions. About miRNAs profile, after an initial analysis of 380 miRNAs, we focused on miR-142-3p for different reasons. Although miR-142 was first identified in hematopoietic cells (Skårn et al., 2013), there is evidence of its expression also in other cells, such as induced iPSCs, MSCs, colon cancer cells (Shen et al., 2013), and hepatocellular carcinoma cells (Wu et al., 2011). miR-142-3p expression is modulated by DNA methylation of CpG islands located in the enhancer region. DNA methylation determines a transcription repression and indeed in undifferentiated iPSCs CpG motifs of miR-142-3p enhancer region are not methylated; contrariwise, the same region is methylated in fibroblasts determining the complete absence of miR-142-3p in these cells. Using the Genomatix Software Suite, (Abdul Razak et al., 2014) have identified putative binding sites for MYC protooncogene, bHLH transcription factor (MYC), and SOX2 in the genomic region up to 1 kb from the miR-142 mature sequence. Therefore, these transcription factors are involved and cooperate in the full induction of miR-142-3p expression. Furthermore, the physiology of iPSCs can be probably

regulated by transforming growth factor β1 and WNT family member 1 (WNT1) pathways (Abdul Razak et al., 2014). Canonical WNT1 signaling participates also in bone formation, and it was demonstrated that miR-142-3p expression is correlated with osteoblastic differentiation, by repressing adenomatous polyposis coli expression (Hu et al., 2013). We showed, for the first time, miR-142-3p is highly expressed in SASCs-3D compared to ASCs-2D, suggesting that these cells possess similar molecular characteristics to iPSCs. Moreover, and to confirm our hypothesis, we observed an overexpression of three of Yamanaka's factors, such as NANOG, OCT-4, and SOX2 in 3D S-ASCs. These results demonstrate a real difference between two adipose stem cell populations, in suspension and in adhesion, suggesting that probably SASCs maintained an upstream evolutive stage compared to their adherent counterpart. We report further data that endorse this hypothesis since we have also conducted a detailed molecular analysis of miRNAs and mRNAs involved in typical mesenchymal differentiation lineages. We investigate some miRNAs involved in two distinct moments of differentiation, namely, early and late stages of chondrogenic, adipogenic, and osteogenic lineages. The analysis highlighted the downregulation of early stage markers (miR-495, miR-30-c, and miR-221) in S-ASCs compared to ASCs. The late stage miRNAs (miR-140, miR-143, and miR-100) were almost absent in S-ASCs, whereas in ASCs they were already detectable and they strongly increase in mature differentiated cells (chondrocytes, adipocytes, and osteoblasts) in both cell lines. A similar trend was observed among the mRNAs involved in early and late stages, in particular the early markers were downregulated in S-ASCs, except for PPARG (as explained above) and the late are equally absent in both populations. Furthermore, during *in vitro* passages, we showed that the stemness markers, miR-142-3p and SOX2/NANOG/OCT4, were maintained until 28d in S-ASCs, demonstrating the maintenance of the stemness characteristics during long-term cultures (Di Stefano et al., 2018). In 3D cultures, the

secretory activity is greater and cytokines and growth factors reach higher concentrations favouring effective cell communication. More so, each cell has a greater number of contacts with adjacent cells and the extracellular matrix acts as a mesh, scaffold and modulator of cell growth, proliferation, and differentiation (Lee et al., 2018). *In vivo*, MSC spheroids have been shown to secrete more anti-inflammatory and proangiogenic factors to improve tissue repair (McMillen and Holley, 2015). This is in good agreement with the increased production of important anti-inflammatory factors such as TGF-β1 in SASCs-3D culture compared to ASCs-2D as revealed in this thesis and in associated work (Di Stefano et al., 2020a) .Intriguingly, we were unable to detect SASCs-3D or ASCs-2D that produced IL-10 cytokine. This cytokine is well described as to be mainly secreted by immune-regulatory cells but whether this also pertains to adipocytes remains unclear. In a very recent study, Juan R Acosta et al. concludes that macrophages are the predominant source of IL-10 in human white adipose tissues and that this cytokine does not directly affect human adipocyte function (Acosta et al., 2019). In the absence of SIRT1, MSCs prematurely lose their capacity for self-renewal and show an up-regulation of cell cycle inhibitors, anticipating replicative senescence (Yoon et al., 2014). Yoon et al demonstrated that SIRT1 deacetylase positively regulate the SOX2 expression in the bone marrow-MSCs (BM-MSCs), which is consequently overexpressed (Yoon et al., 2014). These experiments concur with our findings of SOX2 and SIRT1 expression changing in the same direction. Consistent with SOX2 and SIRT1 association to self-renewal, we found both genes significantly up-regulated in SASCs-3D compared to ASCs-2D cultures. Also interesting, the expression of CDKN2A, a gene involved in the regulation of the cell cycle, was significantly down-regulated (4-fold) in SASCs-3D compared to ASCs-2D cultures. CDKN2A, also called p16INK4a has been previously associated to senescence and apoptosis (Martin et al., 2014). Notably, the expression of this gene is reported to increase as mesenchymal stem

cells age and undergo senescence (Burrow et al., 2017). Telomeres have the important function of protecting chromosome ends from degradation by DNA repair mechanisms, therefore preventing the formation of chromosomal aberrations. Also, they provide a non-coding DNA pad whose purpose is to prevent a progressive shortening of the ends that occurs with replication, the so-called fine-replication problem. Telomerase is found expressed in highly proliferating cells during embryogenesis, while it is dramatically down-regulated as cells undergo differentiation to the point to be undetectable in many adult somatic cells. Accordingly, high expression levels of TERT is considered one of the landmarks of the pluripotent state (Flores and Blasco, 2010). The analysis on the telomeric length showed similar T/S ratio between 3D and 2D experimental culture conditions. This match with the fact that SASCs and ASCs comes from adult tissues and not from germinal or embryonal stages. Contrary to expectations, we observed a slight increase of T/S ratio in primary cultures at 28 days compared to 14 days. Based on the absence of TERT, this apparent increase is unlikely to be an elongation of telomere length but can be attributed to a negative selection of highly differentiated cells and a concomitant enrichment in stem cells or progenitors/ precursors cells, favoured by the cell culture condition. Molecules of the Shelterin complex such as TERF1, TERF2 and others, and molecules of the non-Shelterin complex such as MRE11 complex and the DNA-activated, catalytic subunit (DNA-PK) cooperate together to stabilize telomeres. TERF2 and TINF2 genes protect the ends of the chromosomes, meaning a down regulation of these genes can lead to a degradation of the telomeric extremities and the triggering of cellular senescence. The reduction of TERF2 expression can have too a negative impact on the structure of the telomere, being TERF2 involved in the formation of the T-loop of the extended DNA strand protecting telomeres from the microenvironment (Pirzada et al., 2019). Comparing the expression levels of TINF2 and TERF2 in SASCs-3D and ASCs-2D, we found TINF2

significantly increased in SASCs-3D at 14 days, while TERF2 mRNA showed predominantly higher expression levels at 28 days, thus suggesting that SASCs-3D preserved a prolonged proliferation capacity. Interestingly, we also found a significant increase of HNRNPA1 (~2-fold), a gene that is suggested to be a positive regulator of stem cells renewal and differentiation in smooth muscle (Huang et al., 2013) and chondrocyte cells (Meng et al., 2017). In general, little is known about the role of HNRNPF in adult stem cells, especially in adipose stem cells. In Embryonal Stem Cells, HNRNPF and HNRNPH were previously described to be highly expressed (Yamazaki et al., 2018)). SASCs-3D also showed significant increased levels of RAD50 and XRCC5, while MRE11 and XRCC6 were downregulated compared to ASCs-2D. Mre11-Rad50-NBN (MRN) complex is well known to controls telomere homeostasis. It interacts with the ATM complex to detect chromosomal double strand DNA breaks (DSBs) and coordinate the overall cellular responses to DNA damage (Stracker and Petrini, 2011). All these genes represent DNA protective factors against DNA lesions, and participates in the ATM, ATR or NHEJ signalling (de Lange, 2005). The telomere end protection is a fine (not a static) controlled process that still remains undefined today due to the difficulty in distinguishing the DSBs from dysfunctional telomeric ends. It seems that the deletion of a single component minimally impact the repair processes activity. Yet, recent studies addressing the complete loss of Shelterin factors concluded that telomere uncapping activates at least six different pathways (Mann and Markham, 1998); (Sfeir and de Lange, 2012). mRNAs analysis showed striking differences between SASCs-3D and ASCs-2D expression pattern of Shelterin complex genes and their associated co-factors, with expression changes occurring in either directions. However, due to the limited information available, the single contribution of each gene is difficult to interpret. Further studies in this direction would help us to clarify the specific DNA repair pathways that are preferentially activated in SASCs-3D.

Another marked observation to emerge from the comparison data is the significantly lower endogenous levels of ROS in SASCs-3D compared to ASCs-2D. Accumulating evidences points to the beneficial role of ROS in proliferation and cell survival as far as its levels are low enough as to not cause concomitant cellular damages (Marengo et al., 2016). Indeed, high levels of ROS production lead to apoptosis and is a mechanism by which many chemotherapeutic agents can act (Perillo et al., 2020). Beside direct actions, ROS also elicit lipid peroxidation, leading to the production of 4-hydroxynoneal (HNE) and malondialdehyde (MDA) (Gaschler and Stockwell, 2017). Not surprisingly, in addition to its higher levels of ROS, ASCs cells also exhibit higher lipid peroxidation. HNE and MDA aldehydes issued from lipid peroxidation are highly electrophilic and their accumulation in cells lead to formation of adducts with DNA and proteins compromising cellular function and eventually leading to cell death (Kong and Kotraiah, 2012). ALDH class 3 subfamily of aldehyde dehydrogenases are capable to detoxifies HNE and MDA efficiently by oxidation into their corresponding non-toxic and excretable carboxylic acids (Muzio et al., 2012). Significantly, three members of ALDH3 subfamily (ALDH3A1, ALDH3A2 and ALDH3B2) are up-regulated in SASCs-3D compared to ASCs-2D supporting the idea that SASCs-3D are better protected against the deleterious effect of aldehydes generated during lipid-peroxidation than ASCs-2D cultures are a similar trend was expected for the ALDH class 1 subfamily of aldehyde dehydrogenases, which are widely used as stem cell markers (Tomita et al., 2016); (Vassalli, 2019). Surprisingly, ALDH1A1 and ALDH1A3 were significantly downregulated in SASCs-3D compared to ASCs-2D. However, given that our findings are based on adipocytes cells of identic origin, the differences in ALDH1 should be interpreted with caution. ALDH1 are the unique members of the ALDH family of enzymes that participates in the metabolism of vitamin A and the production of all-trans retinoic acid (RA), a small molecule that activate gene transcription through its binding

to nuclear retinoic acid receptors (RAR and RXR) (le Maire et al., 2019). As consequence, RA acts in several biological conditions, including embryonic development and cell differentiation. It can thus be suggested that higher expression of ALDH1 in ASCs may result in higher production of RA and consequently a higher commitment towards differentiation. This is in good agreement with the use of RA for the differentiation of embryonic stem cells into motor neurons or Xenopus ectoderm into pancreas (Moriya et al., 2000); (Deshpande et al., 2006). On the basis of our results, we propose a model of SASCs "*cell quality*", which highlights a precise molecular expression profile for several mRNA involved in stemness (SOX2, POU5F1 and NANOG), anti-aging (SIRT-1), oxidative stress (ALDH3A1, ALDH3A2 and ALDH3B2) and telomere maintenance (TINF2, TERF2, HNRNPA1, HNRNPF, RAD50 and XCCR5). All these factors are up-regulated in SASCs compared to the traditional adherent ASCs and many others are instead down-regulated. This study provides a "*transcriptional portrait*" of several relevant genes that could assist in the evaluation of SASCs fitness; nevertheless, further studies will be required to investigate the exact contribution of these genes to the phenotype and functional features of SASCs. In our view, these results represent an excellent initial step towards the implementation and quality monitoring of long-term adipose stem cell cultures aimed to be used in downstream research or in clinical setting, notably in the regenerative medicine field.

CHAPTER 5

# Tables and Figures

**Table 1**. List of genes and their functions annotated in the NCBI-gene database.

| Entrez ID | Genes | Full name gene | Functional description |
|---|---|---|---|
| 6657 | SOX2 | SRY-box transcription factor 2 | Regulation of embryonic development, stem-cell maintenance |
| 5460 | POU5F1 | POU class 5 homeobox 1 | Embryonic development, stem cell pluripotency |
| 79923 | NANOG | Nanog homeobox | Cell proliferation, renewal, pluripotency |
| 860 | RUNX2 | RUNX family transcription factor 2 | Regulatory factors involved in osteoblastic differentiation and skeletal gene expression. |
| 249 | ALP | alkaline phosphatase biomineralization associated | Bone mineralization |
| 6662 | S0X9 | SRY-box transcription factor 9 | Chondrocyte differentiation |
| 1300 | COL10A1 | collagen type X alpha 1 chain | Short chain collagen expressed by hypertrophic chondrocytes during endochondral ossification |
| 5468 | PPARG | peroxisome proliferator activated receptor gamma | Regulator of adipocyte differentiation |
| 4023 | LPL | lipoprotein lipase | Triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake |
| 2597 | GAPDH | glyceraldehyde-3-phosphate dehydrogenase | Catalyzes an important energy-yielding step in carbohydrate metabolism |
| 23411 | SIRT1 | Sirtuin 1 | Intracellular regulatory proteins |

| | | | |
|---|---|---|---|
| | | | with mono-ADP-ribosyltransferase activity |
| 1029 | CDKN2A | Cyclin dependent kinase inhibitor 2A | Cell cycle gene, tumor suppressor gene |
| 26277 | TINF2 | TERF1 interacting nuclear factor 2 | Member of Shelterin complex, assembly regulator |
| 7014 | TERF2 | Telomeric repeat binding factor 2 | Member of Shelterin complex, negative regulator of telomere length, protective role of telomeres |
| 25913 | POT1 | Protection of telomeres 1 | Regulating telomere length, protecting chromosome ends |
| 54386 | TERF2IP | TERF2 interacting protein | Member of Shelterin complex, telomere length regulation |
| 7015 | TERT | Telomerase reverse transcriptase | Enzyme with reverse transcriptase activity |
| 8658 | TNKS | Tankyrase | Telomere length regulation, vesicular traffic |
| 80351 | TNKS2 | Tankyrase2 | Telomere length regulation, vesicular traffic |
| 3178 | HNRNPA1 | Heterogeneous nuclear ribonucleoprotein A1 | Regulation of alternative splicing |
| 3185 | HNRNPF | Heterogeneous nuclear ribonucleoprotein F | Regulate alternative splicing, polyadenylation, pre-mRNA processing, mRNA metabolism and transport |
| 3183 | HNRNPC | Heterogeneous nuclear ribonucleoprotein C | Regulate alternative splicing, polyadenylation, pre-mRNA processing, mRNA metabolism and transport |
| 3181 | HNRNPA2B1 | Heterogeneous nuclear ribonucleoprotein 2B1 | mRNA metabolism and transport |
| 3182 | HNRNPAB | Heterogeneous nuclear ribonucleoprotein AB | Pre-mRNA processing, mRNA metabolism and transport |
| 3184 | HNRNPD | Heterogeneous nuclear ribonucleoprotein D | Regulate alternative splicing, polyadenylation, pre-mRNA processing |
| 10111 | RAD50 | RAD50 double strand break repair protein | DNA double-strand break repair |
| 7520 | XRCC5 | X-ray repair cross complementing 5 | DNA double-strand break repair |
| 4683 | NBN | Nibrin | DNA double-strand break repair, DNA damage-induced checkpoint activation |

| | | | |
|---|---|---|---|
| 4361 | MRE11 | MRE11 homolog, double strand break repair nuclease | Telomere length maintenance, DNA |
| 2547 | XRCC6 | X-ray repair cross complementing 6 | DNA double-strand break repair, Transposition |
| 216 | ALDH1A1 | Aldehyde dehydrogenase 1 family member A1 | Regulation of the metabolic responses to high-fat diet |
| 8854 | ALDH1A2 | Aldehyde dehydrogenase 1 family member A2 | Synthesis of retinoic acid (RA) from retinaldehyde, development of adult tissue |
| 220 | ALDH1A3 | Aldehyde dehydrogenase 1 family member A3 | Aldehyde dehydrogenase enzyme that uses retinal |
| 219 | ALDH1B1 | Aldehyde dehydrogenase 1 family member B1 | Major oxidative component of alcohol metabolism |
| 218 | ALDH3A1 | Aldehyde dehydrogenase 3 family member A1 | Lipid peroxidation |
| 224 | ALDH3A2 | Aldehyde dehydrogenase 3 family member A2 | Alcohol metabolism, lipid peroxidation |
| 221 | ALDH3B1 | Aldehyde dehydrogenase 3 family member B1 | Lipid peroxidation, protection from oxidative stress |
| 222 | ALDH3B2 | Aldehyde dehydrogenase 3 family member B2 | Alcohol metabolism, lipid peroxidation |
| 7040 . | TGFβ1 | Transforming growth factor beta 1 | Cell proliferation, differentiation, multifunctional cytokine |

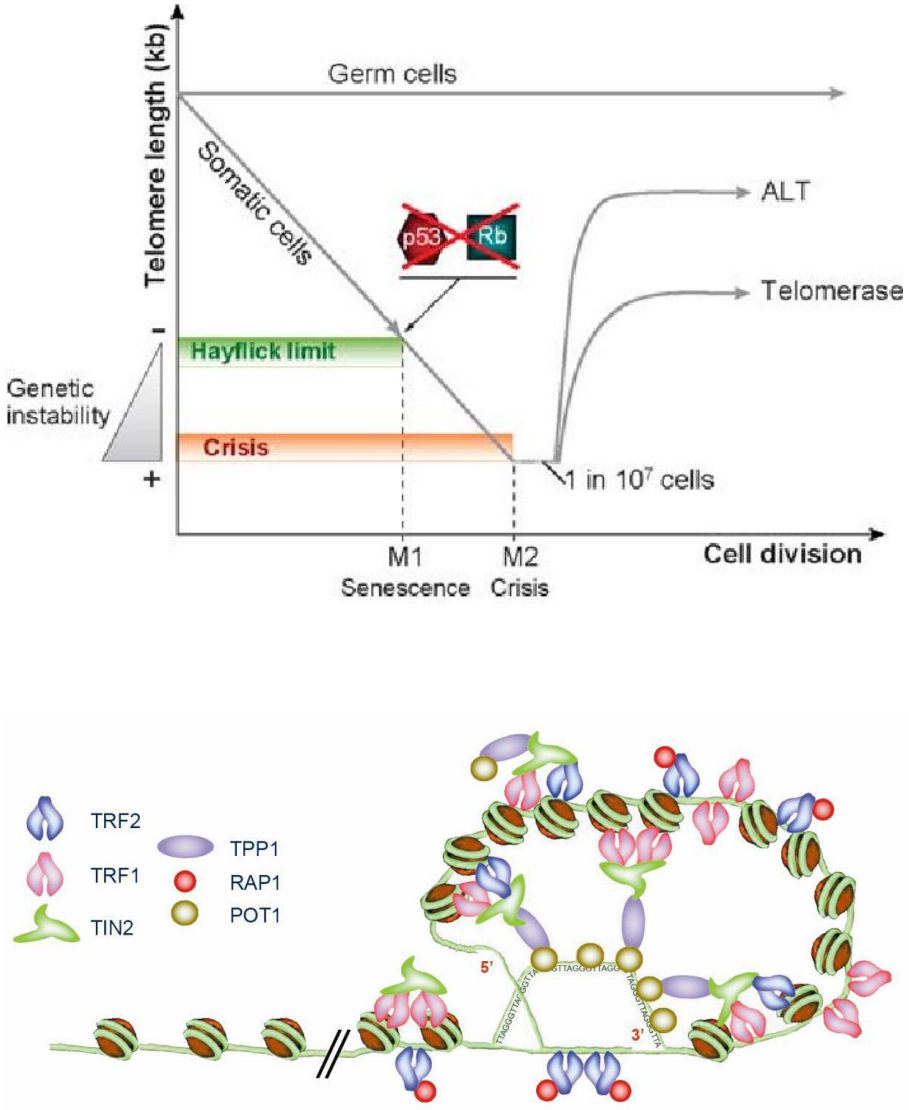


Figura 1. A (Stewart e Weinberg, 2006), B T-loop, D-loop and shelterin protein complex (TRF1, TRF2, TPP1, POT1, TIN2 and TERF2IP o RAP1)

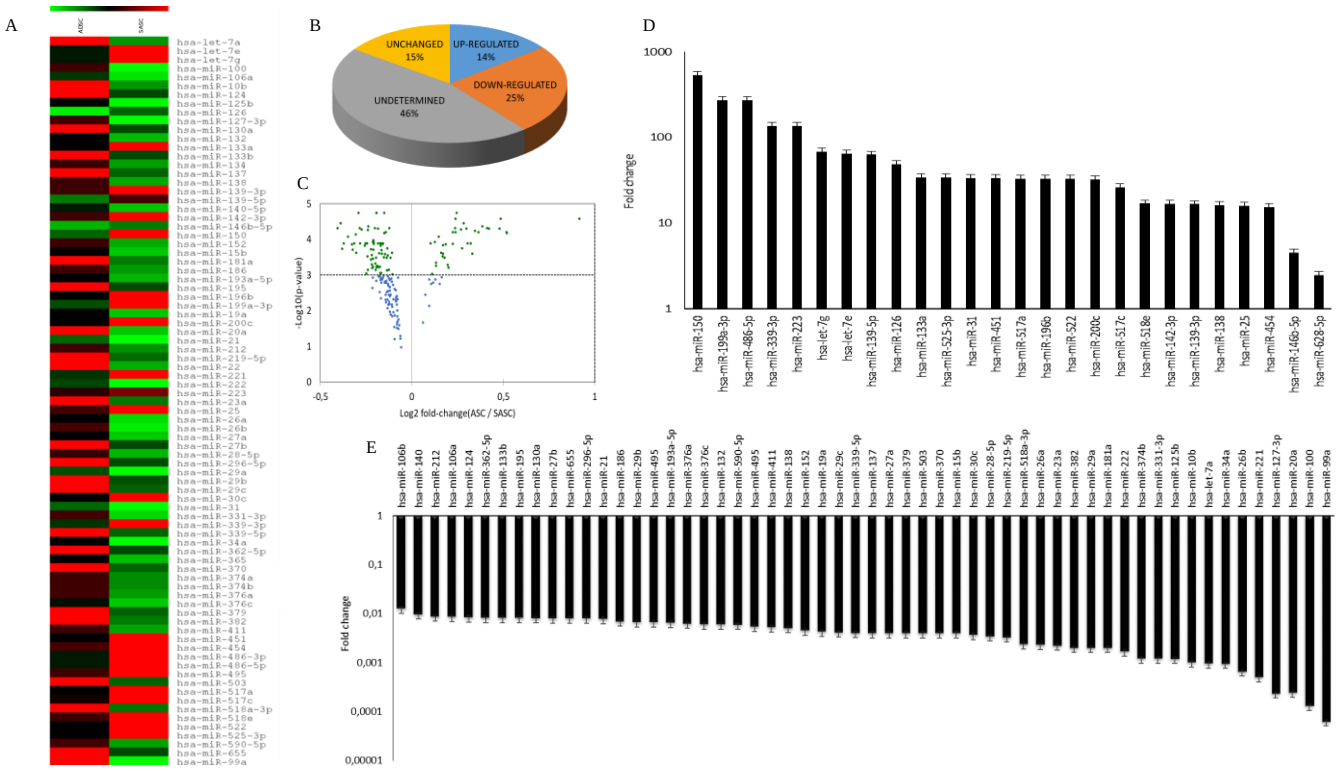


Figura 2. MicroRNA array profiling. (a, b) Clustergrams of 380 miRNAs in 3D S-ASCs and ASCs; the analysis showed 26 upregulated (14%) and 56 downregulated (25%) miRNAs; 46% were undetermined (Ct values were higher than 35), and no differences were reported for 15% miRNAs in S-ASCs versus ASCs. (c) The volcano plot is used for statistical analysis of miRNA arrays; the points above the line represent statistically significant miRNAs (left and right); $p < 0.05$, whereas those below line indicate no significant miRNAs. (d, e) Graphic representation of miRNA fold change. A fold change greater than two was used as cut-off to identify significantly deregulated miRNAs.

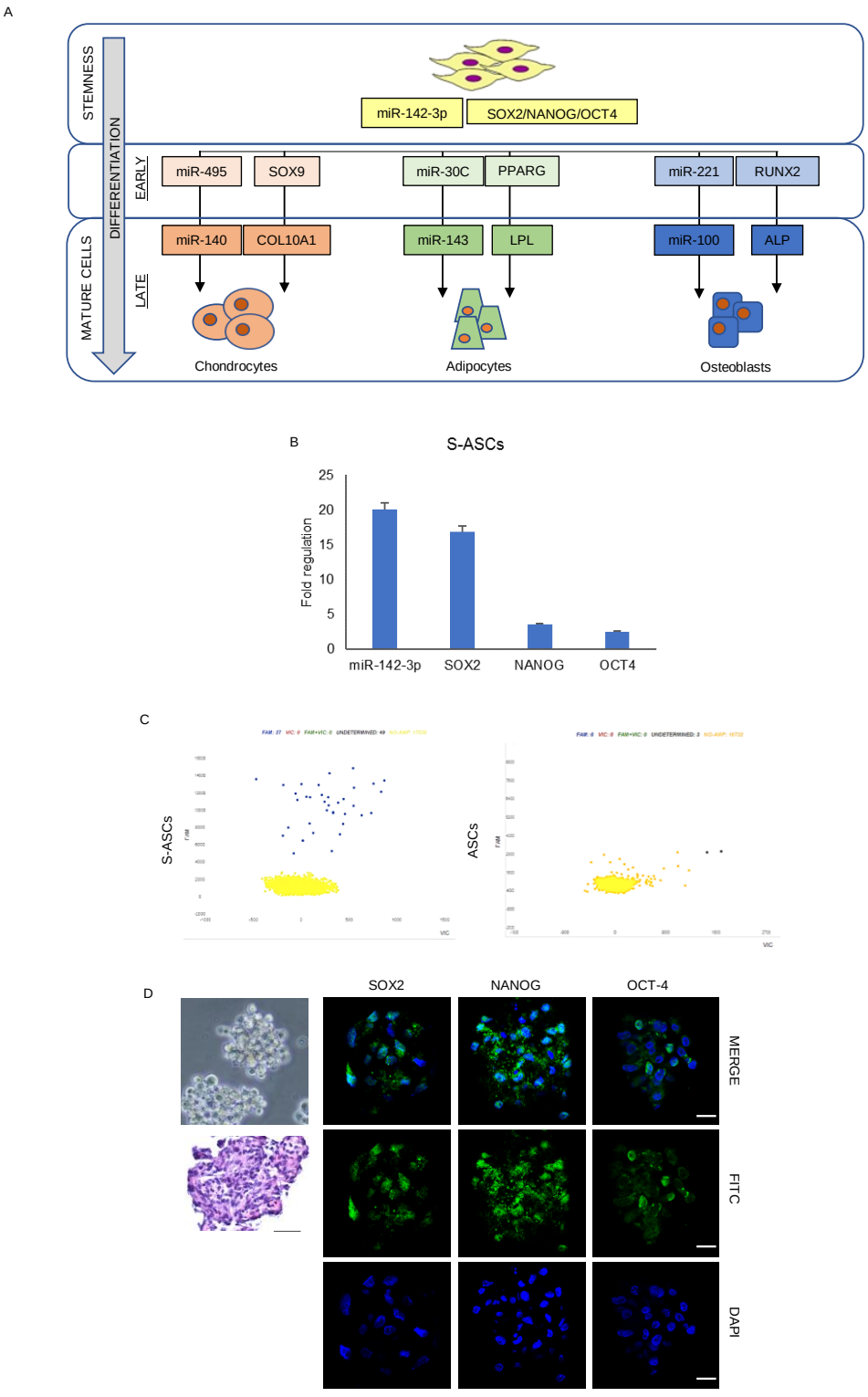


Figura 3. Expression level of miR-142-3p and stemness markers. (a) Summary diagram of microRNAs and messenger RNAs involved with stemness and early and late mesenchymal differentiation analyzed in the study. (b) Fold regulation of miR-142-3p and three Yamanaka's factors (SOX2, NANOG, and OCT4). miR-191 and GAPDH were used as endogenous controls (c) Expression of miR-142-3p through digital PCR in 3D S-ASCs and ASCs. (d) Phase contrast; hematoxylin and eosin staining; nuclear SOX2, NANOG, and OCT4 through immunofluorescent reaction (×40 magnification) on 3D S-ASC paraffin-embedded section.

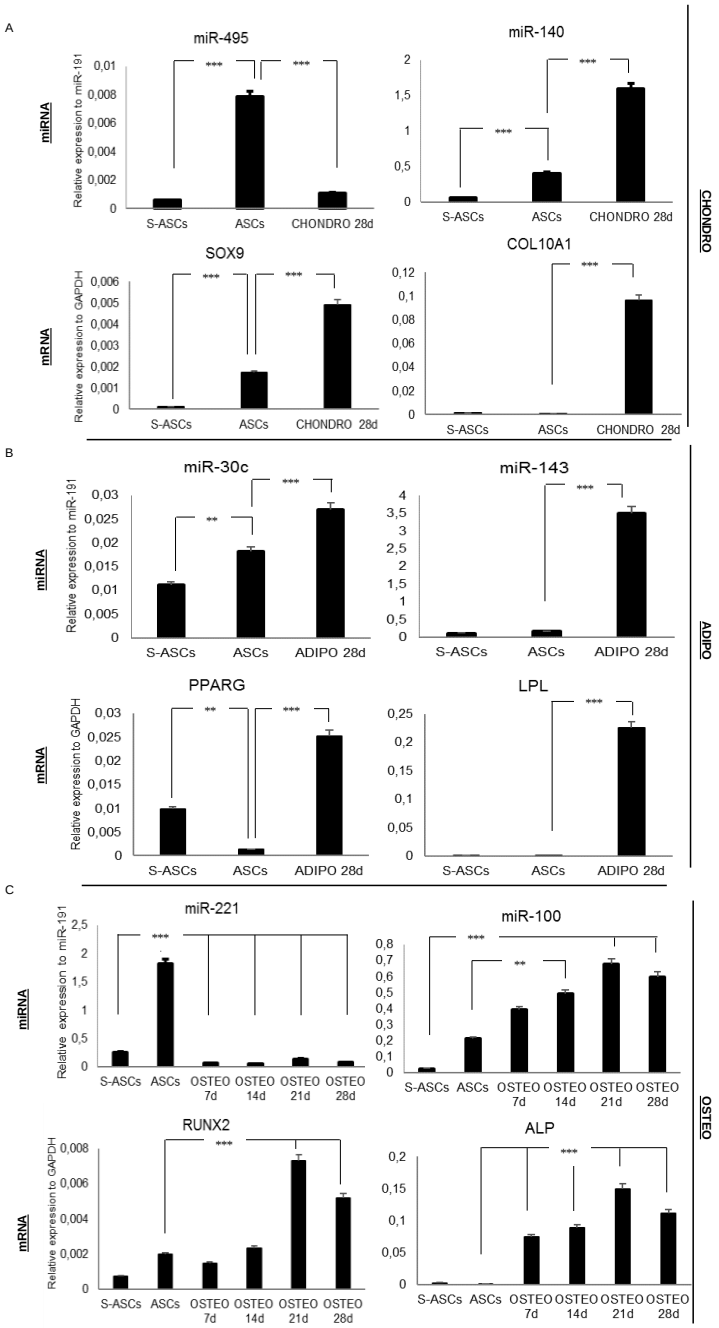


Figura 4. Expression levels of microRNAs (miRNAs) and messenger RNAs (mRNAs) related to early and late mesenchymal differentiation. MiRNA and mRNA expressions in chondrogenic (a), adipogenic differentiation (b), and osteogenic (c) in S-ASCs, ASCs, and S- ASCs differentiated cells after 28 days of *in vitro* differentiation. The error bars indicate SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

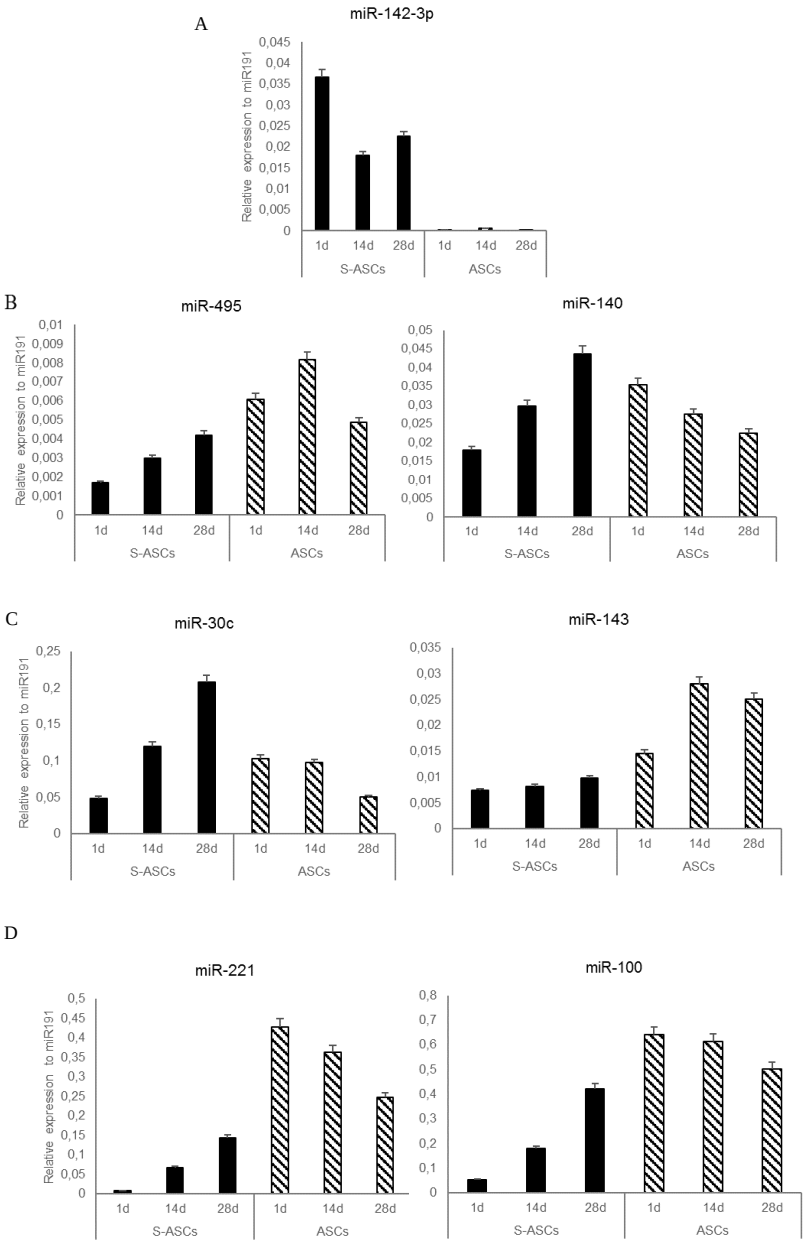


Figura 5. MicroRNAs (miRNAs) analysis during long-term *in vitro* culture. Relative miRNA expression after 1, 14, and 28 days *in vitro* culture in 3D S-ASCs and ASCs, involved in stemness (a), early and late chondrogenic (b), adipogenic (c), and osteogenic differentiation (d). miR-191 was used as endogenous control. The error bars indicate SD (n = 3).

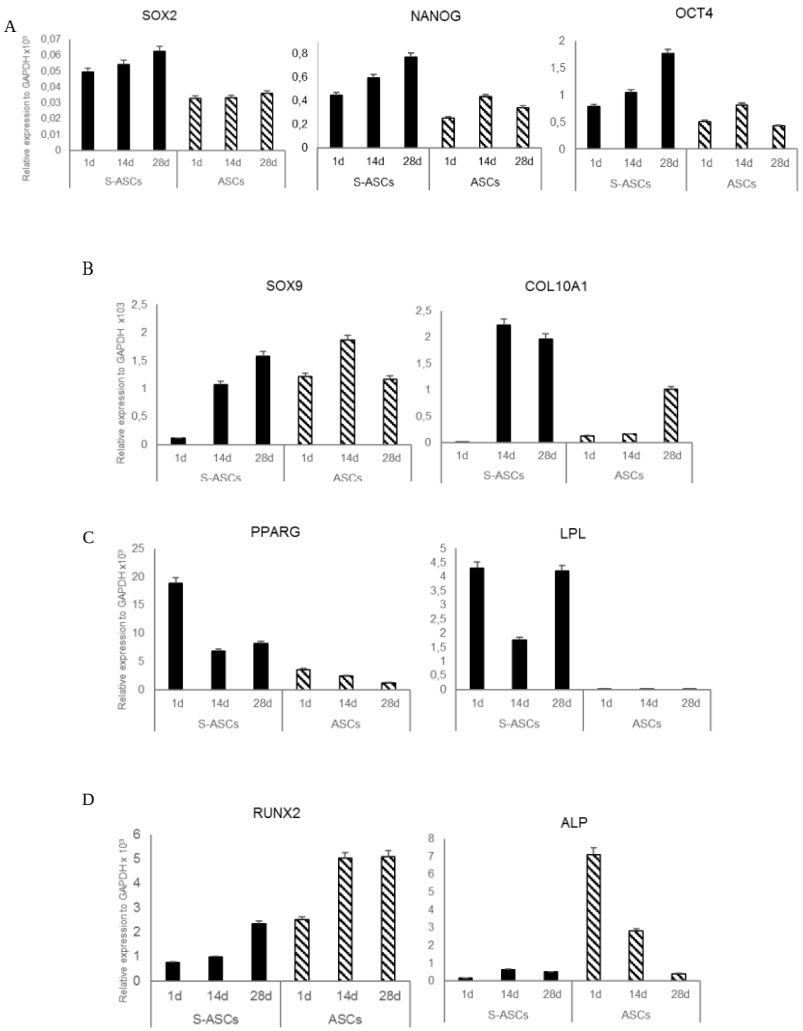


Figura 6. Messenger RNA (mRNA) analysis during long-term *in vitro* culture. Relative mRNA expressions after 1, 14, and 28 days of *in vitro* culture in 3D S-ASCs and ASCs, involved in stemness (a) and early and late chondrogenic (b), adipogenic (c), and osteogenic differentiation (d). GAPDH was used as endogenous control. The error bars indicate SD (n = 3)

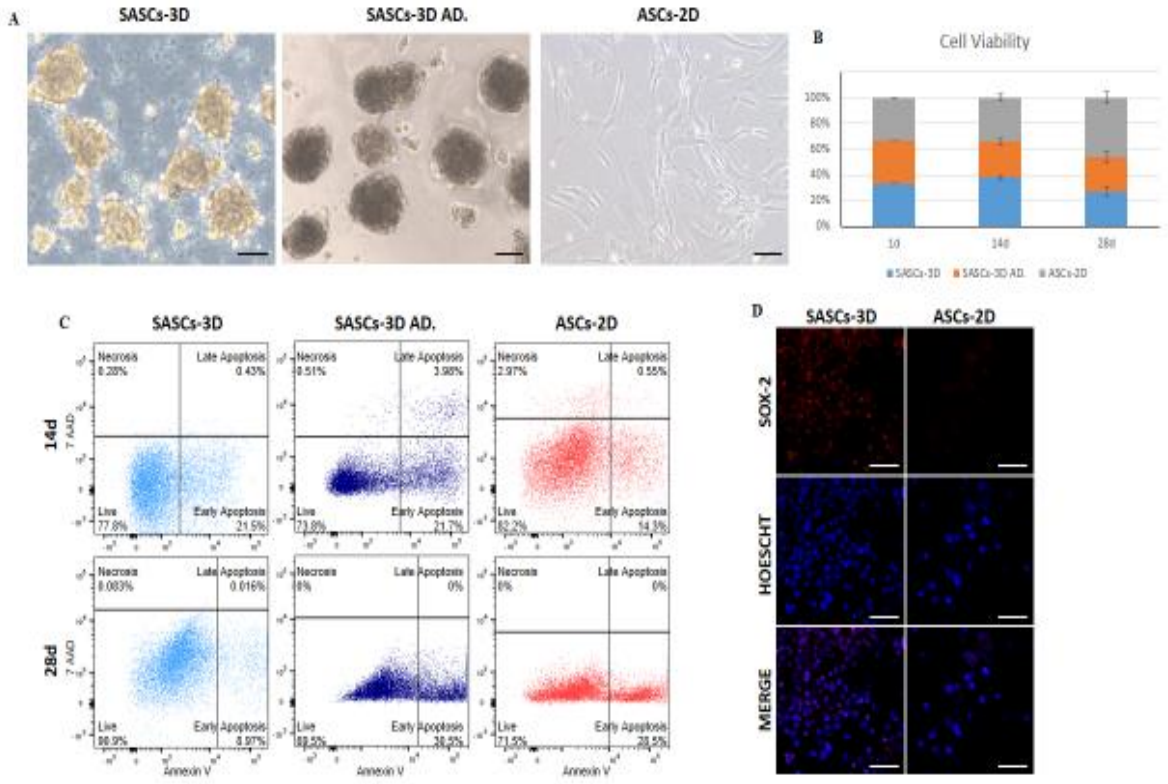


Figura 7. Morphological analysis of cell culture 2D versus 3D. (A) Phase contrast images of SASCs-3D in ultra-low adherent surface, SASCs-3D in adherent condition SASCs-3D AD. and ASCs-2D. Scale bar = 50 µm. (B) Cell viability after 1, 14 and 28 days of *in vitro* culture. (C) Annexin V/7-AAD staining at 14 and 28 days of *in vitro* culture. (D) Immunofluorescent analysis of SOX2 in SASCs -3D and ASCs-2D. Scale bar = 200 µm

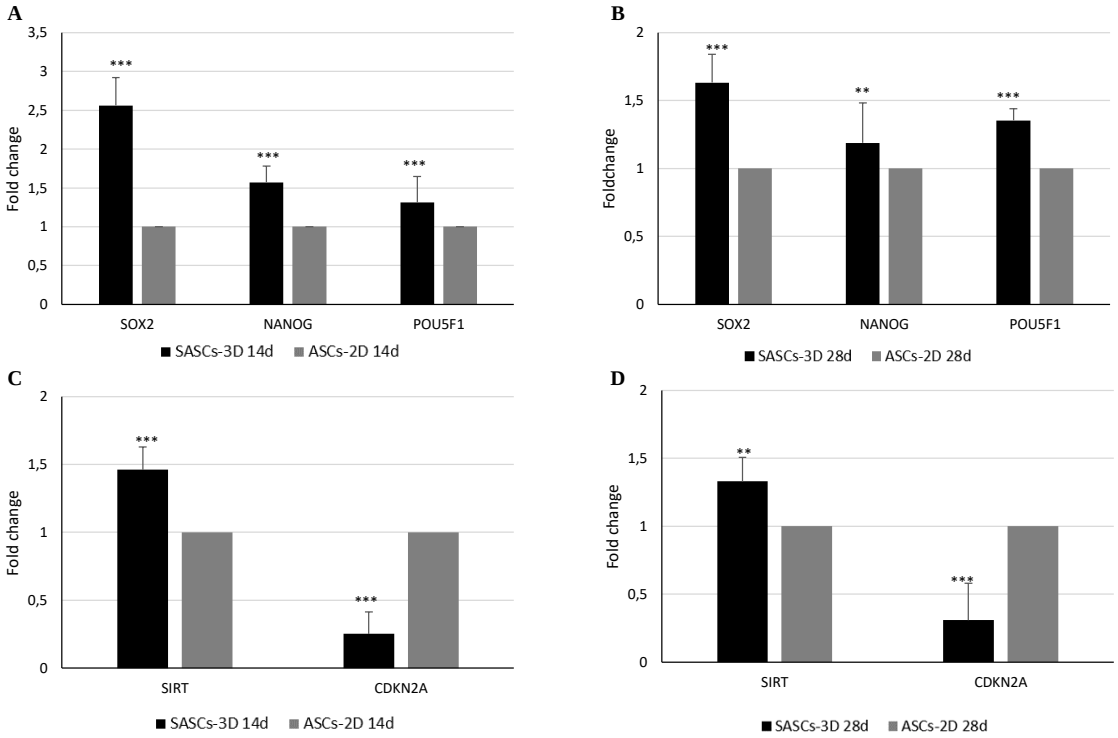


Figura 8. Comparative analysis of mRNAs expression on genes involved in stemness and aging-related processes. Relative gene expression fold change of SOX2, NANOG and POU5F1 in SASCs-3D and ASCs-2D at 14 days (A) and 28 days (B) of cell culture. SIRT1 and CDKN2A expression levels in SASCs-3D and ASCs-2D cells at 14 (C) and 28 (D) days of *in vitro* culture. The error bars indicate SD (n = 3); **p < 0.01, ***p < 0.001.

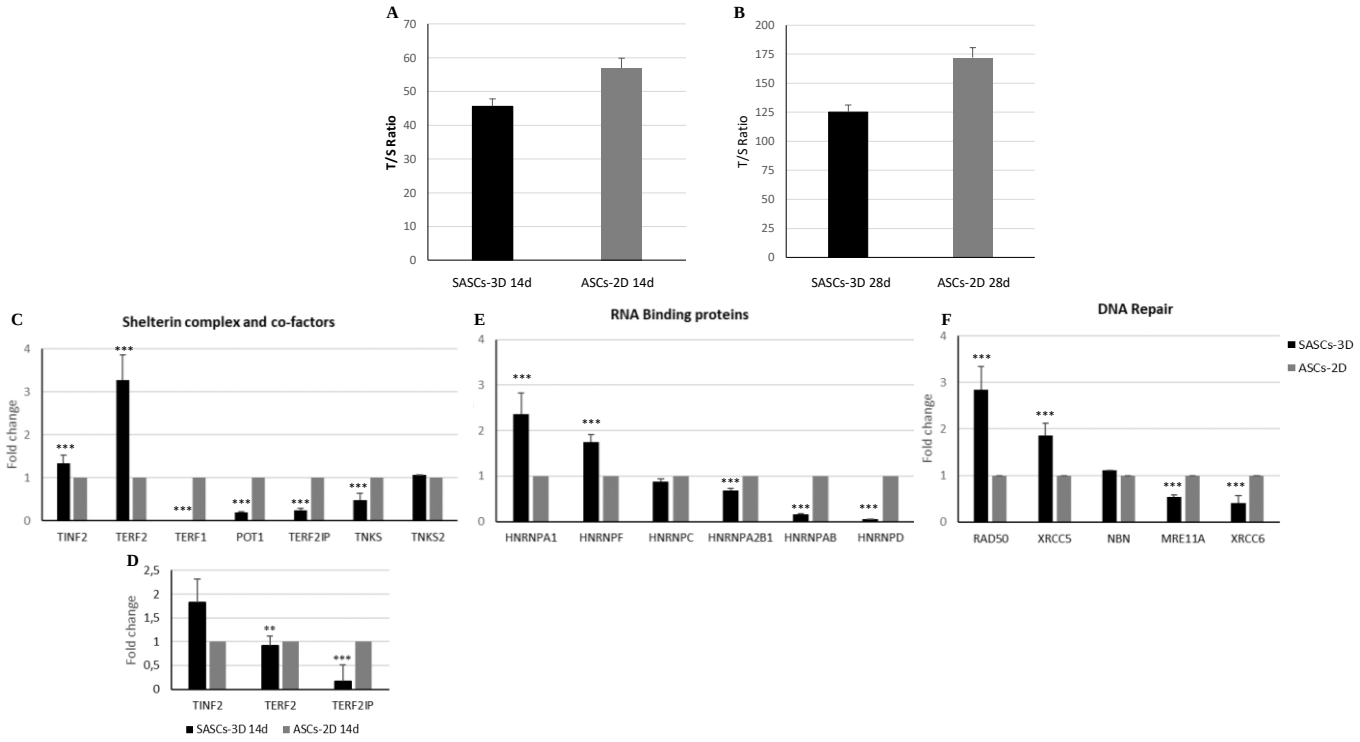


Figura 9. Comparative evaluation of telomeric length and genes associated with telomere maintenance. Telomeric length quantification (T/S ratio) of SASCs-3D and ASCs-2D at 14 days (A) and 28 days (B) and mRNA expression analysis of 19 associated to Shelterin complex (C), RNA binding protein (E) and DNA repair (F) at 28 days of *in vitro* culture. Gene expression of TINF2, TERF2 and TERF2IP (D) in SASCs-3D and ASCs-2D at 14 days of cell culture. The error bars indicate SD (n = 3); ** $p < 0.01$, *** $p < 0.001$

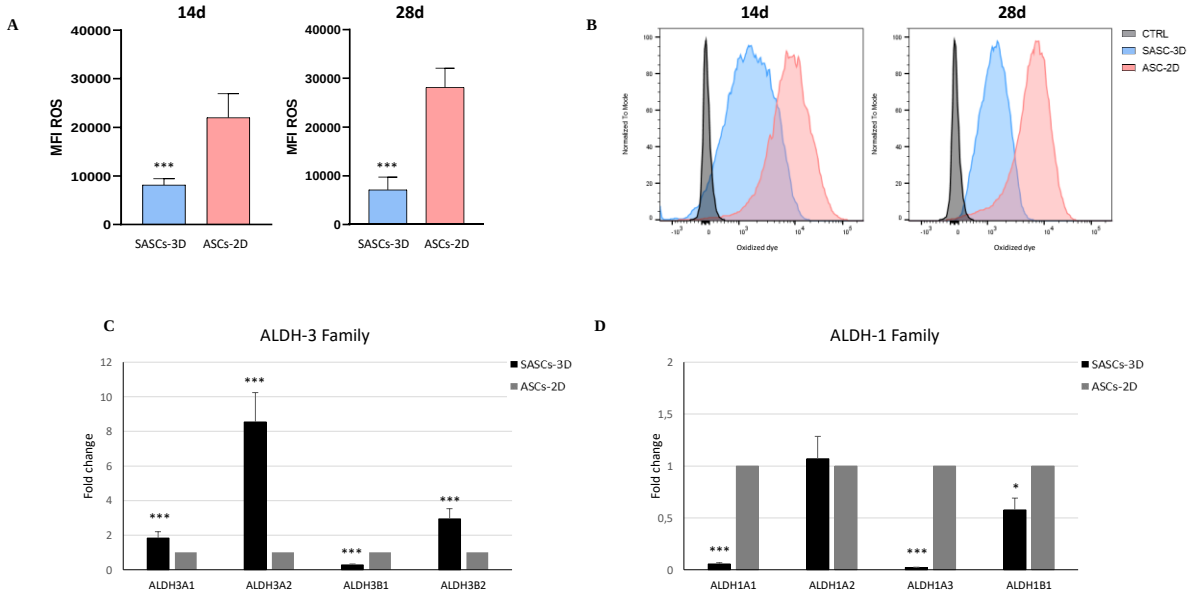


Figura 10. Comparative evaluation of oxidative stress genes. (A) ROS endogenous levels in SASCs-3D and ASCs-2D at 14 and 28 days of *in vitro* culture. The graph represents the mean fluorescent intensity (MFI). (B) Histogram representation of lipid peroxidation in SASCs-3D and ASCs-2D at 14 and 28 days assessed by flow cytometry. Realtime PCR analysis of ALDH class 3 (C) and ALDH class 1 (D) isoenzymes in SASCs-3D and ASCs-2D at 28 days *in vitro* culture. The error bars indicate SD (N = 3); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

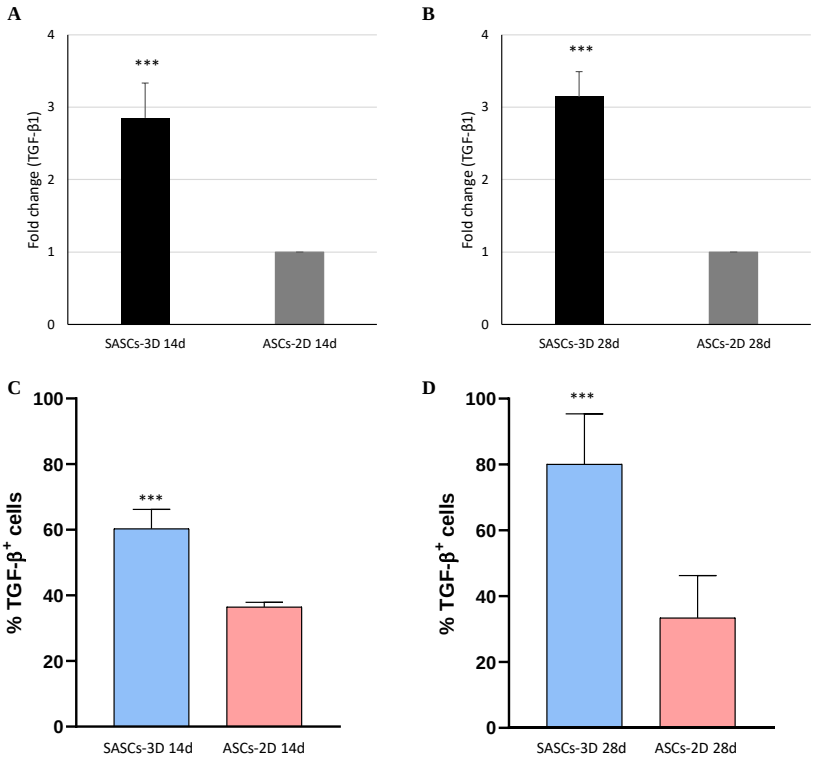


Figura 11. Comparative evaluation of TGF-β1 expression. Relative gene expression fold change of TGF-β1 in SASCs-3D and ASCs-2D at 14 (A) and 28 days (B) of *in vitro* culture. Flow cytometry analysis of TGF-β1 expression in SASCs-3D and ASCs-2D at 14 days (C) and 28 days (D) of *in vitro* culture. The error bars indicate SD (n = 3); ** $p < 0.01$, *** $p < 0.001$.

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## SCIENTIFIC PRODUCTIONS

### Scientific Production

- Anna Barbara Di Stefano*, Federica Grisafi*, Mileidys Perez-Alea, Marta Castiglia, Marta Di Simone, Serena Meraviglia, Adriana Cordova, Francesco Moschella, Francesca Toia. (2020) Cell quality evaluation with gene expression analysis of spheroids (3D) and adherent (2D) adipose stem cells. GENE
- DI STEFANO, A., Massihnia, D., Grisafi, F., Castiglia, M., Toia, F., Montesano, L., et al. (2018). Adipose tissue, angiogenesis and angio-MIR under physiological and pathological conditions. EUROPEAN JOURNAL OF CELL BIOLOGY
- Di Stefano, A., Grisafi, F., Castiglia, M., Perez, A., Montesano, L., Gulino, A., et al. (2018). Spheroids from adipose-derived stem cells exhibit an miRNA profile of highly undifferentiated cells. JOURNAL OF CELLULAR PHYSIOLOGY
- A.B. Di Stefano, M.A. Sabatino, F. Grisafi, E. Muscolino, M. Perez Alea, F. Toia, C. Dispenza, A. Cordova, F. Moschella. Hydrogel scaffolds blends to host Spheroids from human adipose stem cells. Abstract presentation at Tenth International Conference Regenerative Surgery Roma 2019.
- M.A. Sabatino, A.B. Di Stefano, E. Muscolino, F. Grisafi, L.A. Ditta, D. Giacomazza, A. Cordova, F. Moschella, C. Dispenza. Hydrogel scaffolds based on k-Carrageenan/xyloglucan blends to host spheroids from human adipose stem cells. Abstract presentation at Eurofillers Polymerblends. Palermo 2019
- 6. Barbara Di Stefano Ph.D., Federica Grisafi, Luigi Montesano M.D. , Antonio Russo M.D. , Adriana Cordova M.D. , Francesco Moschella M.D. , Angelo A. Leto Barone M.D. Shaking The Ground You Walk On: Are Adipose-derived Stem Cells (ASCs) True Stem Cells? Abstract presentation at IFATS International Federation for Adipose Therapeutics and Science, Miami 2017.
- A. Barbara Di Stefano Ph.D, Federica Grisafi , Alessandro Perez, Marta Castiglia Ph.D. , Antonio Russo M.D. , Francesco Moschella M.D., Angelo A. Leto Barone M.D.and Adriana Cordova M.D. Molecular analysis of Spheroids from Adipose-derived Stem Cells (S-ASCs) during *in vitro* long-term culture. Abstract presentation at Regenerative Surgery Roma 2017.
- Alessandro Perez*, Anna Barbara Di Stefano*, Marta Castiglia, Martina Sorrentino, Daniela Matranga, Federica Grisafi, Cristiano Corso, Gianluca Scoarughi, Gaetano Barbato, Nadia Barraco, Valentina Calò, Florinda Di Piazza, Daniela Massihnia, Angela Listì, Luisa Castellana, Aurelia Ada Guarini, Lavinia Insalaco, Enrico Bronte, Antonio Russo. The effects of LIPUS on ctDNA release in the medium of NSCLC cell lines. Abstract presentation at AIOM Associazione Italiana di Oncologia Medica, Roma 2017.