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DOTTORATO DI RICERCA IN MEDICINA SPERIMENTALE
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"Adipose-derived stem cells (ASCs) in regenerative medicine: epigenetic and molecular approaches to optimize the therapeutic potential mediated by cells and secretome"

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1 INTRODUCTION

1.1 Stem Cells

Stem cells are primitive, non-specialized cells with the ability to transform into several other types of cells in the body through a process called "multilinear cell differentiation". They are also characterized by the ability to self-renew. At the basis of self-renewal is the cells' ability to divide asymmetrically, giving rise to two daughter cells that will meet two different fates: one will be identical to the mother cell, keeping the number of stem cells constant; the other will be able to originate a "committed" cell with proliferation, growth and differentiation capabilities, important in the development and regeneration of tissues [1] (Figure 1).

However, the asymmetrical division system is not the only one that can be adopted by a stem cell. Sometimes stem cells can divide through a process called symmetrical division, which allows to produce two stem cells identical to the mother cell ("symmetric self-renewal"). As an alternative, they can also originate two "committed" cells different from the mother cell, but identical to each other, both capable of undergoing differentiation ("symmetric differentiation") (Figure 1). In the first case there is a substantial increase in the stem cell pool, while in the second case there is a greater proliferation of differentiated progenitors.

Establishing a perfect balance between the two types of division makes it possible to achieve an adequate self-maintenance of the stem component in each tissue.

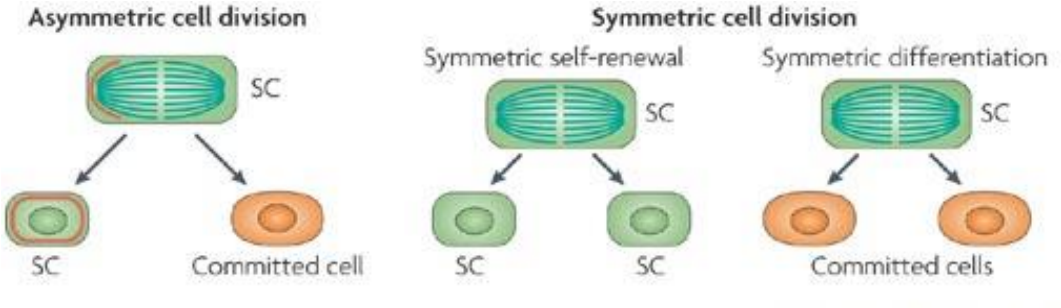


Figure 1. Stem cells division mechanisms. Asymmetric division gives rise to two different daughter cells, one identical to the parent cell and a "committed" one. Symmetric division generates two identical daughter cells, which can both be stem cells identical to the mother cell

("symmetric self-renewal"), or both capable of undergoing differentiation ("symmetric differentiation") [2].

Although stem cells can divide unlimitedly, they are also able to remain in a state of quiescence for an indefinite time. This occurs thanks to their ability to enter and exit the G0 phase of the cell cycle, remaining in an undifferentiated state.

According to the environmental stimuli they are subjected to, stem cells be able to undertake a symmetrical division, characteristic of the embryonic stage, or an asymmetrical replication, typical of the adult phase.

Stem cells are generally classified based on their differentiation capacity:

- **Totipotent stem cells** which are present in the early stages of embryonic development and originate all the cells of an organism (including those of the extraembryonic adnexa such as placenta and umbilical cord).
- **Pluripotent stem cells** which generate all the cells of an organism, except for those belonging to extra-embryonic tissues.
- **Multipotent stem cells** which generate several cell types all linked to a particular function.
- **Unipotent stem cells** which can originate a single tissue while maintaining the capacity for self-renewal (Figure 2) [3].

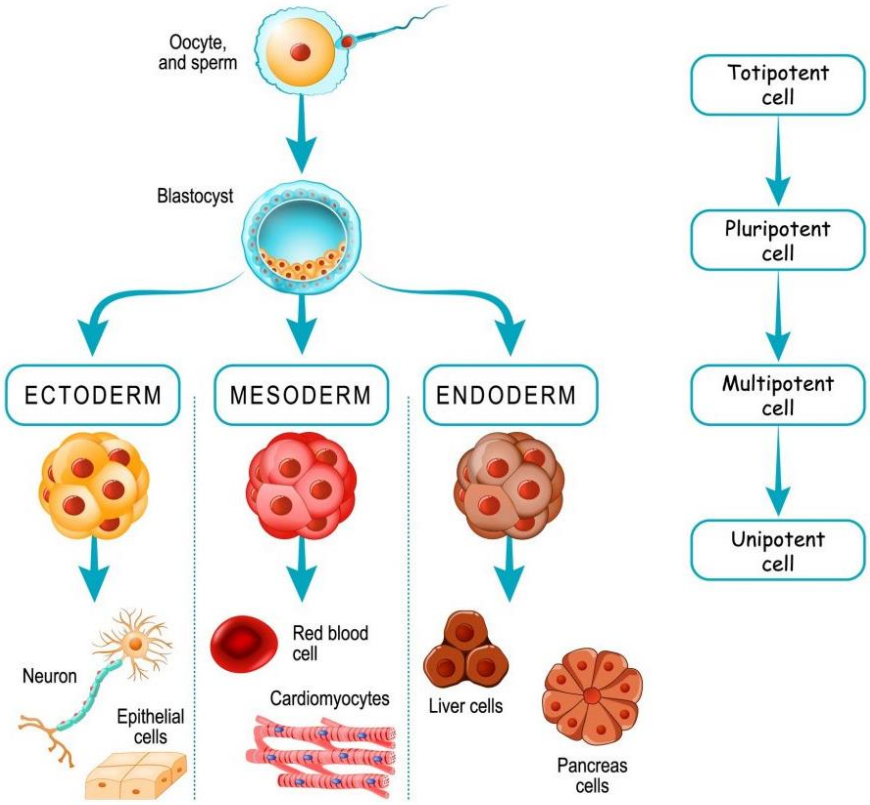


Figure 2. Stem cells classification based on differentiation capacity. Embryonic (totipotent) stem cells are able to originate all the cells of an organism; pluripotent cells can generate all cells except those of extra-embryonic tissues; multipotent cells can only differentiate into some specific cell types; finally, the unipotent cells give rise to a single cell type.

Another classification is based on stem cell site of origin (Figure 3) [4]:

- **Embryonic stem cells**, which are pluripotent cells located in the "inner cell mass" of the blastocyst; they are involved in the initial phase of embryonic development [5]. Their isolation and use require the destruction or the strong manipulation of the embryo and for this reason their use represents an ethically controversial debate.
- **Adult stem cells**, which are multipotent or unipotent cells, contained in all tissues and organs of an adult organism. Their role in the maintenance of an adequate number of cells in the organism is carried out by replacing dead cells or regenerating a damaged tissue. Adult stem cells include fetal stem cells (FSCs) and neonatal stem cells (NSCs), which can be isolated from the umbilical cord and amniotic fluid.

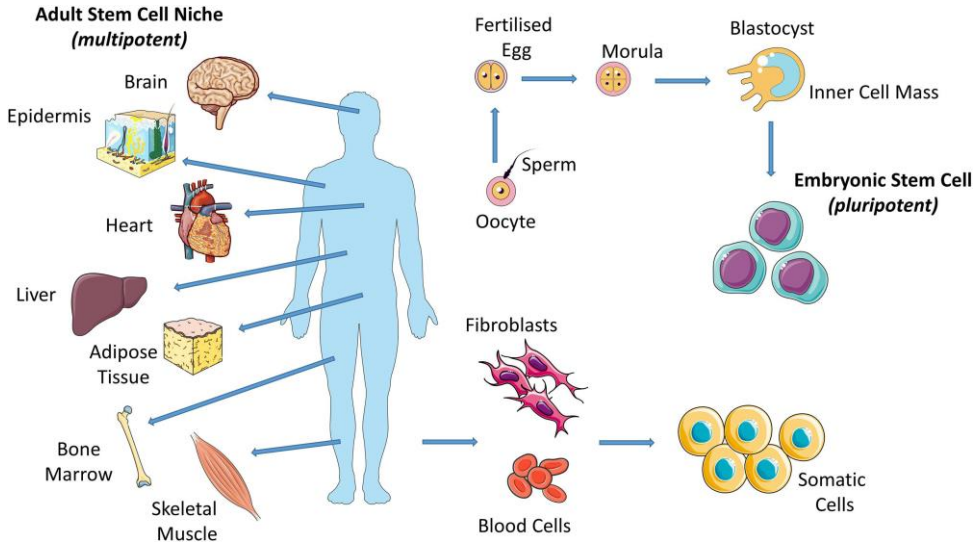


Figure 3. Stem cell classification based on the site of origin. Pluripotent embryonic stem cells are found in the "inner cell mass" of the blastocyst; adult stem cells, multipotent or unipotent, are contained in organs and tissues of the adult individual (modified by [4]).

The main differences between these two types of cells reside in their differentiation capacity and role: in fact, embryonic cells can develop into a whole organism while adult stem cells can regenerate damaged tissues (Figure 4) [6].

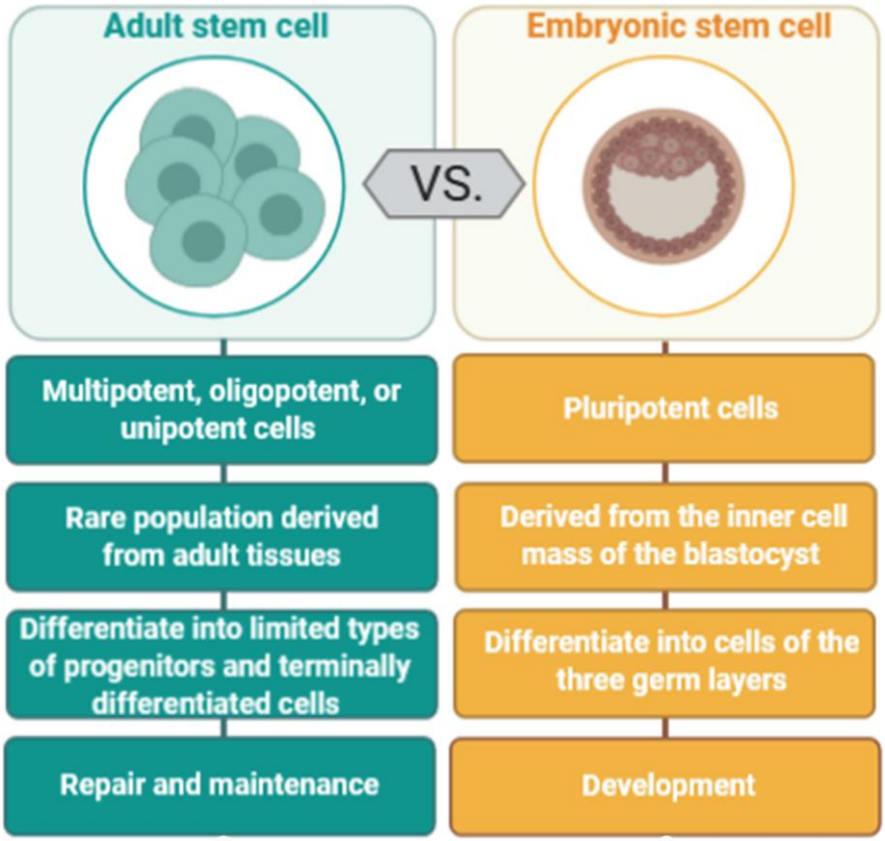


Figure 4. Main differences between embryonic and adult stem cells. The former are pluripotent cells that can originate cells of the three embryonic layers and are responsible for the development of the whole organism. The latter are multipotent cells, which can differentiate into a limited number of progenitors and are responsible for the maintenance of adult tissues and regeneration following damage (modified from [6]).

Despite the multipotent characteristic of adult stem cells, some of them are able to assume morphological and functional characteristics belonging to cells of different embryonic origin. For example, mesodermal bone marrow stem cells can originate ectodermal (neurons or glial cells) or endodermal cells (hepatocytes or pneumocytes), highlighting a pluripotency almost comparable to that of embryonic stem cells [7, 8]. This feature allows these stem elements to migrate to different tissue districts, contributing to regeneration phenomena.

Indeed, the differentiation potential and the lack of ethical implications related to the use of adult stem cells make them more suitable for therapeutic and experimental applications, compared to embryonic cells.

Parkinson's disease, Alzheimer's, burns, heart disease, metabolic diseases (diabetes), rheumatoid arthritis and osteoarthritis are examples of pathologies in which adult stem cells could give a revolutionary contribution in therapy. To this end, a continuous deepening and greater understanding of the biology and the different factors regulating their characteristics and functions are necessary.

Although little is still known, the regulation of the molecular pathways involved in the biological processes of stem cells, i.e., the self-renewal, the maintenance of the G0 state and the capacity for symmetrical or asymmetrical division, is undoubtedly influenced by both genetic and epigenetic factors, able to activate or to switch off specific gene loci.

Regarding the stem cell niche, where the maintenance of a perfect balance between stemness and differentiation is fundamental, a notable contribution is also given by cells intrinsic factors, such as regulatory and cytoskeletal proteins. These proteins are differentially distributed at the poles of the cell, determining the symmetrical and asymmetrical division. Extrinsic factors, on the other hand, are represented by the components within the "stem niche", i.e. the microenvironment within which the stem cells are located. The niche provides structural support and signals that regulate self-regeneration and differentiation processes.

Wnt, TGF-β, Notch, BMP, Shh, as well as β-catenin and integrins, which regulate cell-cell and cell-extracellular matrix interactions, are just some of the factors found in the cellular microenvironment involved in stem cell biological processes. The identification of new molecules involved in specific development programs is a fundamental requirement for the progress of therapies aimed at tissue regeneration, using both endogenous and expanded and/or differentiated stem cells *in vitro*.

1.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a population of multipotent non-hematopoietic progenitor cells. It has been initially isolated from the bone marrow, and it is capable of self-renewal and differentiation into mesodermal cell lines (Figure 5) [9].

MSCs can differentiate into tissues of mesenchymal origin, such as medullary stroma, adipose tissue, bone, cartilage, skeletal muscle and visceral mesoderm [7-10]. In addition to their mesodermal origin, MSCs can also derive from parts of the other two embryonic layers,

such as the ectoderm of the neural crest and the endoderm of the prechordal plate [11]. This gives these cells the ability to differentiate also into neurons, hepatocytes, epithelial, endothelial, renal and lung cells [12].

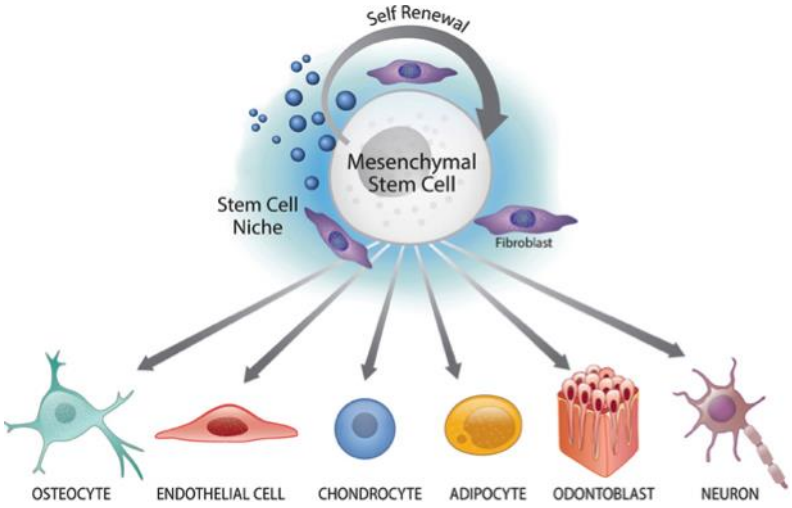


Figure 5. Mesenchymal stem cells (MSCs). These multipotent non-hematopoietic progenitor cells are capable of self-renewal and differentiation in mesodermal cell lines (osteocytes, chondrocytes, adipocytes) but also in ectodermal (neurons) or endodermal (endothelial) cells (modified from [13]).

Mesenchymal stem cells do not possess a unique phenotype. On the contrary, they share markers with other cell types. Despite the functional and phenotypic differences between the various types of MSCs from different tissues, it is possible to base their identification on three criteria established by the International Society for Cellular Therapy (ISCT) (Figure 6), namely:

- ability of adhesion to plastic in standard culture conditions
- expression of mesenchymal markers (CD29, CD44, CD73, CD90 and CD105)
- negativity for hematopoietic/endothelial markers (CD14, CD34 and CD45, CD11b, HLA-DR and CD31)
- ability to differentiate towards mesenchymal lines (osteocyte, chondrocyte and adipocyte) in suitable culture conditions [14].

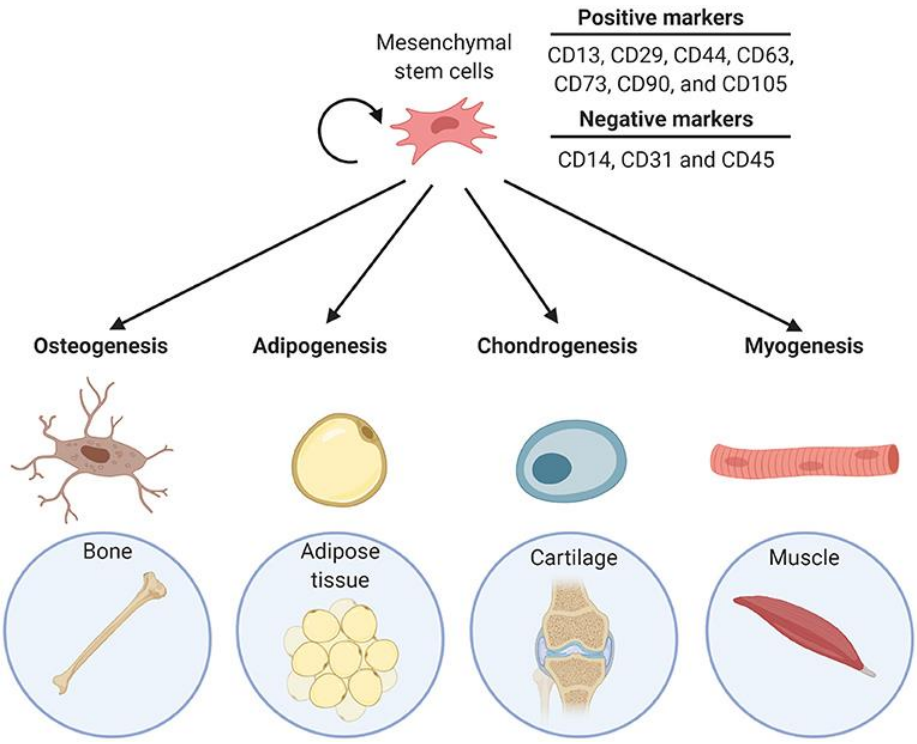


Figure 6. Criteria for the identification of mesenchymal stem cells (MSCs). The expression profile of the surface markers predicts positivity for mesenchymal markers and negativity for hematopoietic endothelial markers. Differentiation capacity must include adipocytic, osteogenic and chondrogenic lineage [15].

1.2.1 Differentiative Plasticity of MSCs

It has been believed that MSCs played an important role in maintaining homeostasis exclusively of the tissue they belonged to. However, recent evidence has emerged regarding the potential ability of MSCs to differentiate into distinct cell types other than the tissue of origin and which may also belong to embryonic layers other than the mesodermal one, if placed in certain microenvironmental conditions (Figure 7).

This ability is defined as "plasticity" of adult stem cells and implies that different cell lineages must be able to derive from a single initial cell. The differentiated cell types must show functionality *in vitro* and *in vivo* and their engraftment must be robust and persistent in the presence and absence of tissue damage.

To date, this phenomenon has not been fully clarified and data are controversial. However, it seems that MSCs, when cultured *in vitro* under appropriate conditions, have the ability to differentiate into striated, smooth and cardiac muscle cells [16], expressing specific muscle

proteins, such as myoD, myogenin, desmin, sarcomeric α-actin etc. They would also be able to move towards an endothelial lineage [17], with the ability to assume structural characteristics resembling capillaries on a Matrigel layer (matrix of the solubilized basement membrane) and to produce lineage-specific proteins such as von Willebrand factor. Other differentiation capabilities would concern the possibility of assuming neuronal characteristics [18], with the presence of tissue-specific proteins and with the acquisition of a neuronal morphology; finally, MSCs have the potential to differentiate towards hepatocytic lineage [19, 20], with the adoption of morphological and functional characteristics belonging to hepatic cells (uptake of DiAc-LDL, albumin release).

Evidence emerged from some studies suggests the possibility that cellular plasticity, observed *in vitro*, may also be present *in vivo*. For example, bone marrow cells could assume a phenotype belonging to cells deriving from different embryonic layers such as neuroectodermal [21, 22] or endodermal ones.

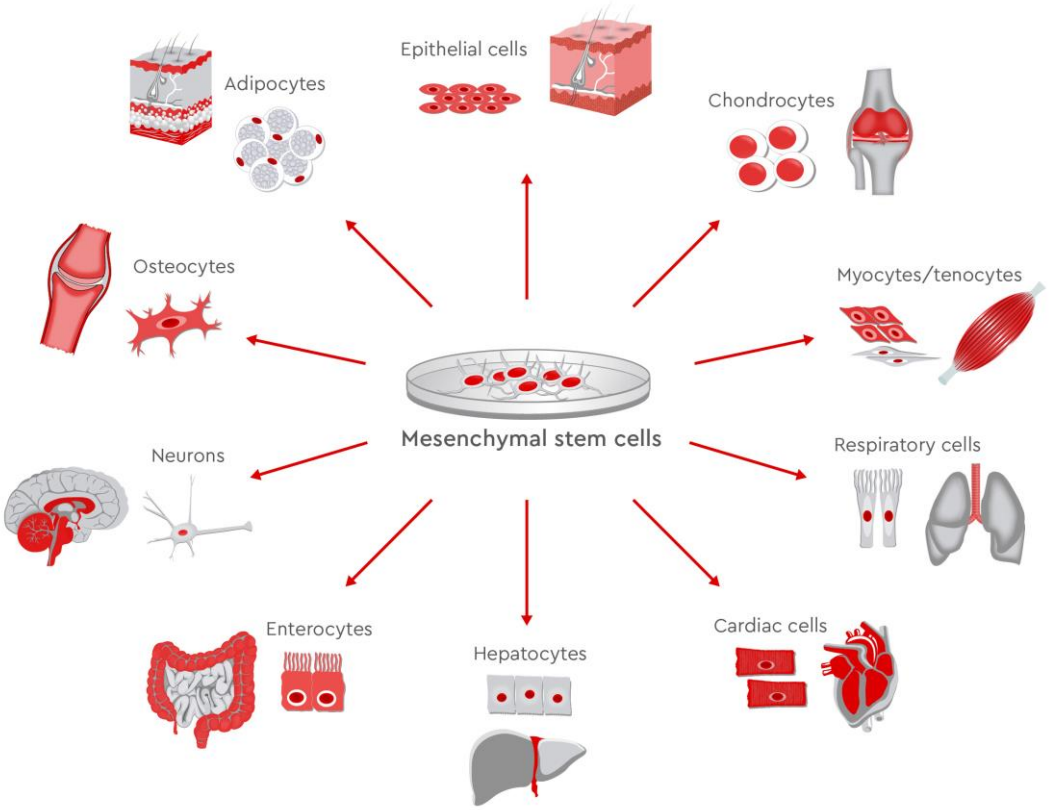


Figure 7. Plasticity of MSCs. MSCs are also able to differentiate into cell types belonging to embryonic layers other than the mesodermal one (hepatocytes, neurons, epithelial cells, respiratory system cells**).**

Although today MSC plasticity remains a debated phenomenon, there are some hypotheses that might explain its fundamental mechanisms [23]. Among these, trans-differentiation (Figure 8A) consists of a cellular switch from an initial differentiation program to a completely different one, often skipping mitotic events. This phenomenon involves the activation of specific genes otherwise inactive: such event can be observed, for example, in pancreatic cells that are able to activate a trans-differentiation program towards a hepatic cellular phenotype *in vitro*, in appropriate conditions. Another model, called "de-differentiation" (Figure 8B), explains how a specialized cell can go back in the differentiation process, acquiring stem or primitive precursor characteristics, and subsequently starting a differentiation path other than the previous one. These two models have not been clearly and unambiguously proven yet and may not be implicated in the *in vivo* plasticity phenomena of MSCs. A further existing paradigm is the trans-determination event (Figure 8C). It represents the possibility of a stem cell or precursor already determined towards a particular function to generate a progeny with a different tissue determination. The change towards other differentiation fates could be explained by the presence, within various tissues, of pluripotent stem cells not yet "committed" that can originate different cell types (hierarchical model) (Figure 8D). An example could be represented by some cells in the bone marrow, called MAPCs (multipotent adult progenitor cells), which are negative for CD34, CD44, CD45, c-kit and MHC class I and II and are able to originate both the hematopoietic and epithelial tissues (liver, lung, intestine) [7].

However, when discussing the enormous differentiation potential of these cells, it is necessary to avoid vain speculation. In fact, the plasticity events could not be directly associated with an intrinsic capacity of the cells, but rather be related to the culture conditions, or influenced by contamination events. Furthermore, some studies have emerged linking apparent plasticity events to other phenomena such as cell fusion (Figure 8E). This phenomenon, although rare, can occur in cells belonging to tissues in which a polyploid condition is observable (hepatocytes, skeletal and cardiac muscle, Purkinje cells), and is also important in the formation of osteoclasts starting from cells of the monocyte/macrophage line.

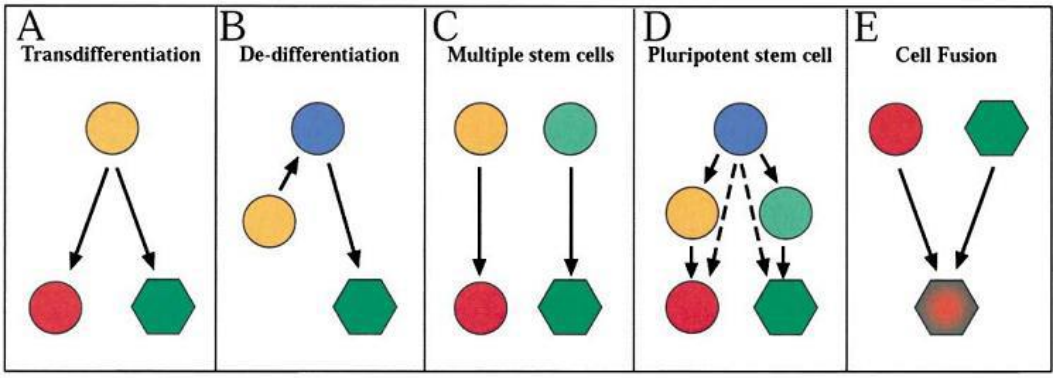


Figure 8. Hypothetical mechanisms underlying MSC plasticity. A. Trans-differentiation; **B.** De-differentiation; **C.** Trans-determination; **D.** Hierarchical differentiation; **E.** Cell fusion [23].

Plasticity and fusion are two rather controversial events, which take into consideration experimental observations that validate or refute both events [24, 25]. It is clear that, when we intend to experimentally investigate plasticity, an important look must be turned towards the development of rigorous and adequate procedures. An important end point concerns the genetic and functional characterization of the cells being examined. This is a necessary procedure to avoid involving excessively heterogeneous populations, or populations presenting chromosomal anomalies, risking the invalidation of the data.
Although there are still many unknown aspects of the phenomenon, the observations conducted to date allow to hypothesize extraordinary therapeutic possibilities, never considered before.

1.2.2 Immunoregulatory Properties of MSCs

Among the most interesting features of MSCs there are their low antigenicity and the robust suppressive immunomodulatory ability (Figure 9) [26].
In fact:

- They are able to inhibit the activity of T lymphocytes, thanks to the absence of costimulatory molecules such as CD80 (B7-1), CD86 (B7-2) and CD40;
- They have a reduced expression of HLA class I molecules and a total lack of HLA class II histocompatibility antigens, which confer them a reduced antigenicity they are able to inhibit the proliferation of T lymphocytes induced by both mitogenic agents and alloantigens that determine graft rejection [27, 28];

- They perform an important inhibitory activity on dendritic cells necessary for antigenic presentation [29];
- They promote the formation of regulatory T cells and reduce the proliferation of B lymphocytes, consequently decreasing the production of antibodies [30];
- They exert an inhibitory action on natural killer cells, both in terms of proliferation and reduction of the expression of activator receptors associated with them (NKp44, NKp30, NKG2D), and they also inhibit their cytotoxic activity and cytokines production [31].

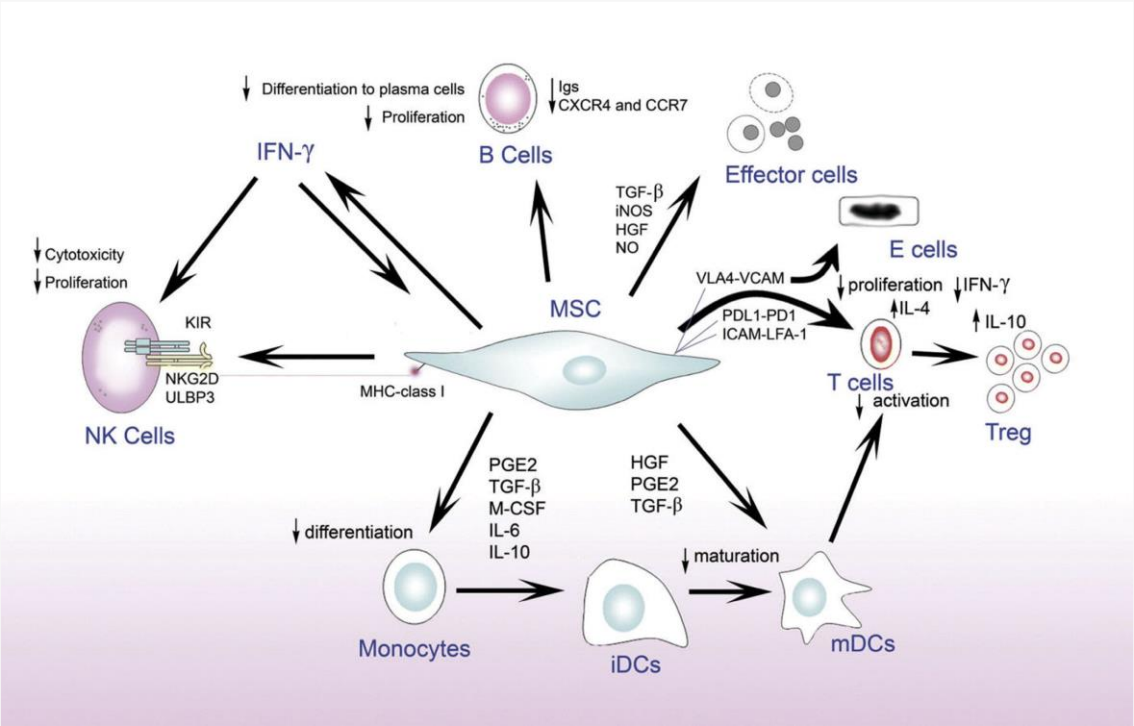


Figure 9. Mechanisms underlying the immunomodulation of MSCs. Immunomodulation by MSCs occurs at multiple levels: **1)** Suppression of naive T cells and memory cells; **2)** Inhibition of the proliferation of B lymphocytes and their differentiation into plasma cells, resulting in a reduction in the formation of antibodies; **3)** Suppression of NK cell proliferation and cytotoxic potential against target cells through the production of several soluble factors, including IFN-γ; **4)** Inhibition of monocyte differentiation into immature dendritic cells (iDCs) and their further maturation into mature DCs (mDCs); **5)** Stimulation of regulatory T cells [26].

All these immunoregulatory properties, combined with the secretion capacity of anti-inflammatory and anti-apoptotic molecules [32], and the typical plasticity of MSCs, make

these cells interesting and potentially useful from a therapeutic point of view, especially for immune-mediated and inflammatory diseases.

1.2.3 Localization of MSCs

Since the late 1960s, when MSCs were identified and isolated for the first time within the bone marrow, several advances have been made in defining their localization. To date, they are known to be present in various tissues, such as skeletal muscle, dental pulp, amniotic fluid, umbilical cord, adipose tissue and various fetal tissues [33, 34]. The presence in numerous districts is associated with their perivascular localization [35, 36]. MSCs belonging to different sites have different molecular characteristics, which translate into greater or lesser potential *in vivo*. However, they share some peculiarities belonging to MSCs of bone marrow origin, such as the modality of interaction with the plastic material, the fibroblastic morphology, the formation of CFU-F (units forming fibroblastoid colonies), the expression of some superficial markers and the differentiation potential into osteogenic, adipogenic and chondrogenic lineages following appropriate stimuli.

There are significant obstacles related to the use of bone marrow-derived mesenchymal stem cells (hBMSC), mainly concerning the invasiveness of the sampling technique, which is painful for the donor and with a rather low cell yield. To obtain a more effective supply method, it is therefore important to investigate in depth the alternative tissues source of MSCs, among which the most studied to date are those of fetal and adipose derivation.

1.3 Adipose-Derived Stem Cells

Adipose tissue is a type of connective tissue which is poor in water and rich in triglyceride. It is one of the most abundant tissues in adults and it possesses trophic and mechanical functions, while also playing a role in the production of numerous hormones and molecules. Like the bone marrow, the adipose tissue originates from the embryonic mesoderm as well, and it is essentially composed of adipocytes, vesicular cells with a flattened nucleus essential in the synthesis of triglycerides and their release in the form of glycerol and fatty acids. These cells are located within the so-called Vascular Stromal Fraction (SVF), which forms a network of connective tissue (stroma) and vessels with a supporting function for fat cells. Several cell populations, including MSCs, can be found on the wall surrounding the

vascular tree of adipose tissue [37]. Multipotent stem cells isolated from adipose tissue SVF can be referred to as adipose-derived stem cells (ASCs), as established by the International Fat Applied Technology Society (IFATS) in 2004. These cells share several features with bone marrow derived MSCs, including unlimited self-renewal capacity, immunosuppressive and immunomodulatory properties [38]. They also share the ability to undertake multiple mesenchymal differentiation fates (osteocytes, chondrocytes, adipocytes, myocytes) and the neural lineage [39, 40]. Not only that, but ASC plasticity is such that they can originate smooth, striated-skeletal [41], cardiac [42] and endothelial [43] muscle cells, demonstrating their similarity with bone marrow MSCs (Figure 10). All these characteristics make ASCs important in the processes of organogenesis, remodeling and tissue repair.

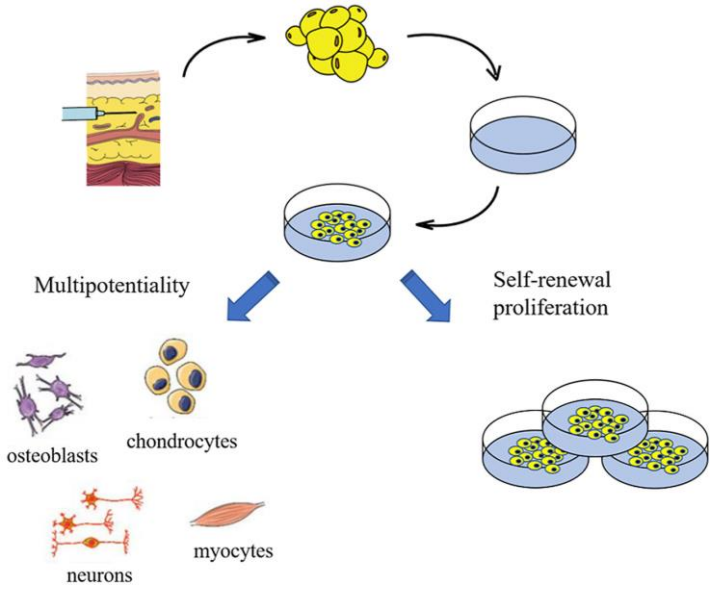


Figure 10. Adipose-derived mesenchymal stem cells (ASCs). These cells are widely found in adipose tissue and can easily be obtained by liposuction. Like bone marrow-derived mesenchymal stem cells (BMSCs), ASCs are also multipotent, capable of differentiating into osteocytes, chondrocytes, neurons and myocytes [44].

In addition to the differentiation characteristics, ASCs share some markers with BMSCs, such as CD105, CD90 and CD44. However, ASCs do not express CD49 and CD106 markers. Furthermore, ASCs express transcription factors typical of the pluripotent state, such as Nanog, Sox2 and Oct4. The different features described so far make ASCs interesting for the

study and treatment of several pathologies, including cardiovascular, neurological and orthopedic diseases, but they can also be considered an optimal stem cell source thanks to their easier accessibility and abundance. Moreover, these cells represent a useful and interesting model for investigating the molecular mechanisms responsible for the development of adipose tissue and the onset of related diseases, such as obesity.

1.4 Adipogenesis

Through adipogenesis, mature adipocytes originating from precursor stem cells develop and accumulate, forming adipose tissue. This initial differentiation step is known as "determination", and requires ASCs to differentiate into preadipocytes, which possess morphological characteristics similar to the former. Unlike ASCs though, preadipocytes can only mature and terminally differentiate into adipocytes. Each step of adipogenesis is finely regulated and coordinated by more than 2000 genes and by different transcription factors that cooperate in shaping adipocyte morphology and physiology (Figure 11) [45].

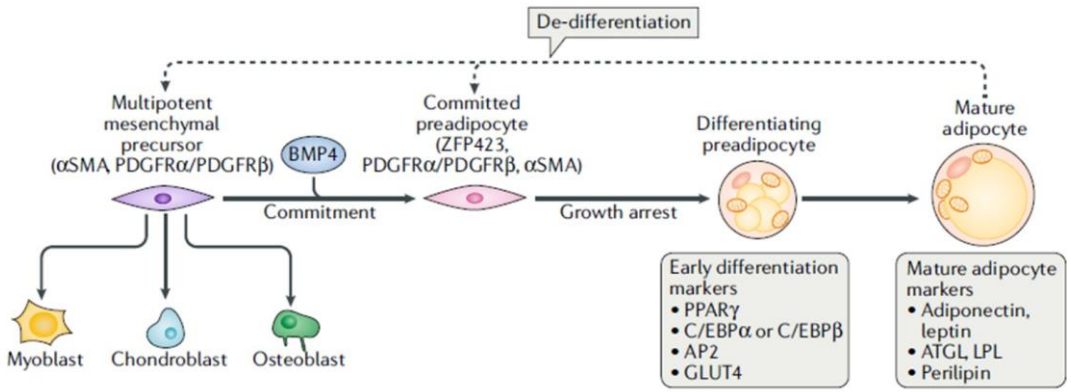


Figure 11. Adipogenesis. Fibroblasts-like multipotent mesenchymal precursors (expressing αSMA and PDGFRα and/or PDGFRβ receptors) can produce myoblasts, osteoblasts and chondroblasts, but also originate preadipocytes, thanks to the signaling of the BMP protein that determines the "commitment" to the adipocyte lineage, with the expression of the transcription factor ZFP423. When the "committed" preadipocyte stops its growth, it activates the main regulators of adipogenesis, namely PPARγ, C/EBPα and C/EBPβ. The accumulation of lipids drives the expression of adipocytic markers in the early stages of differentiation (AP2 and GLUT4). Upon completion of differentiation, mature adipocytes will express all specific markers, including adiponectin, leptin, ATGL, lipoprotein lipase (LPL) and perilipin 1. Adipocytes can also undergo de-differentiation, returning to a preadipocyte phenotype [46].

Although extensively studied *in vitro*, there are still some gaps regarding the factors that regulate adipogenesis and the intermediate cellular phases in the transition from mesenchymal stem cell to mature adipocytes. In fact, most of the available studies concern mouse preadipocyte cell lines, such as 3T3-L1 cells, particularly useful for shedding light on the main adipogenic metabolic functions such as lipolysis, insulin-mediated glucose uptake and lipogenesis. Such studies, however, do not apply to human cells.

Therefore, the genes and factors responsible for adipogenic development have not yet been fully identified. Nevertheless, mature adipocytes seem to play a pivotal role in the secretion of signals useful for the recruitment of new cells [47, 48]. Some of the factors identified are part of the BMP, Wnt and Hh families, important in addressing or inhibiting ASCs towards the adipogenic lineage [49, 50]. Clearly, further investigations are still needed to fully understand the formation of mature adipocytes starting from ASCs.

The use of mouse models has so far made it possible to obtain useful information about the different steps concerning the transition from preadipocytes to adipocytes. The various phases concern the growth stop, clonal expansion, early differentiation and late differentiation. In humans though, it seems that the differentiation process occurs in the absence of the clonal expansion phase.

During the *in vitro* clonal mitotic expansion phase, the preadipocytes grow on the culture plate until contact inhibition is reached. Cell growth then stops by promoting cell cycle exit and maintenance in the G0/G1 phase. At this point, cells begin to accumulate triglycerides and enzymes useful for the biosynthesis of fatty acids and triacylglycerols [51]. This phase is reversible by providing adequate signals so that the cells will re-enter the cell cycle synchronously, dividing further.

At the end of the clonal mitotic expansion phase, cells enter the so-called GD state, necessary to the subsequent differentiation. In the early stages of differentiation, the preadipocytes retain the ability to go back and resume the mitotic phase. However, when key factors such as C/EBPα and PPARγ (trans-activators of adipocyte-specific genes) are expressed, the cells are induced to maintain the adipocyte phenotype [52]. Once the differentiation program has started, the cells undergo significant transformations both at the transcriptional and morphological level. In terms of morphological characteristics, cells begin to acquire a spherical shape typical of adipocytes instead of the more premature fibroblastoid shape.

Cytoskeletal modifications and extracellular matrix rearrangements follow, and once the terminal phase of differentiation is reached, the storage and internal accumulation of triglycerides is put in place. In addition, there is an increase in insulin sensitivity and production of proteins, known as adipokines, with both autocrine and paracrine activities, which contribute to the regulation of energy homeostasis [53].

1.4.1 Molecular Mechanisms of Adipogenesis

The key factors mainly involved in the transition from preadipocytes to adipocytes are transcriptional trans-activators, such as PPARγ (peroxisome proliferator activated receptor) and some members of the C/EBP (CAAT enhancer binding protein) family. PPARγ is strongly involved in the adipogenic program. In fact, it is considered the main regulator of the process, which is able to promote even on its own [45, 52]. In the absence of this factor, the adipocytic phenotype cannot be expressed by the cells. Another function associated with PPARγ concerns the maintenance of the differentiation state. Its suppression, in fact, involves the reduction of the lipid accumulation capacity and the expression of adipogenic markers [54].

The presence of different promoters on the same gene results in the expression of two different isoforms, PPARγ1 and PPARγ2. These isoforms can be found in all types of adipose tissue, but only PPARγ2 is a tissue specific marker [55] and plays a role in promoting insulin sensitivity.

Once activated by specific ligands, PPARγ is also able to bind the retinoic acid receptor RXR (Retinoid X Receptor), forming a complex capable of promoting the release of co-repressors on one hand, and the entry of co-activators on the other hand, acting on specific responsive elements of nuclear DNA [56]. This concerns the transcriptional regulation of genes involved in the metabolic processes of proliferation, adipocyte differentiation, lipogenesis, thermogenesis, glycemic homeostasis and insulin sensitivity (Figure 12) [57].

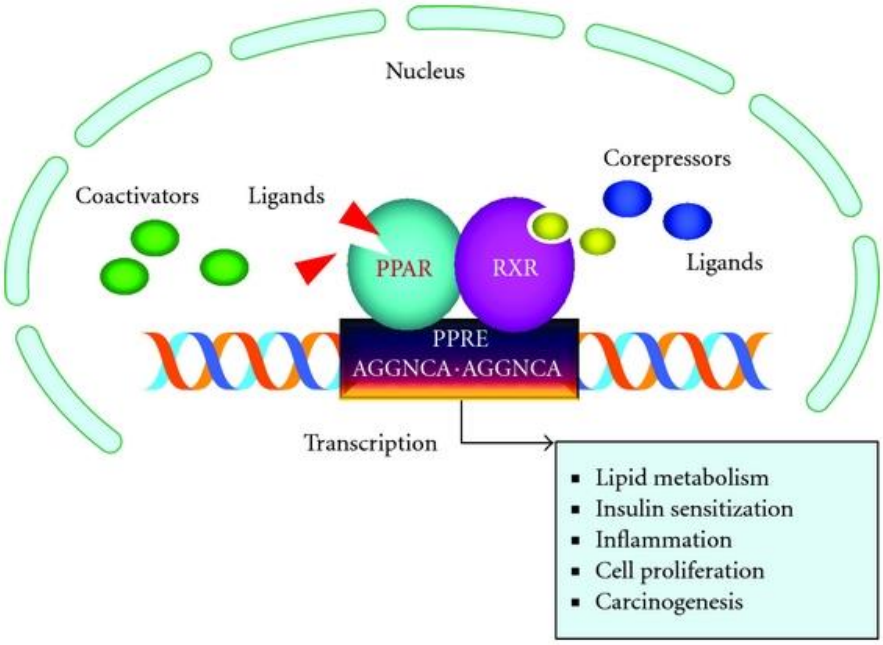


Figure 12. Role of PPARγ in the regulation of lipids and carbohydrates metabolism. When activated by the ligand, PPARγ binds to the retinoic acid receptor RXR, forming a complex capable of promoting the release of corepressors and the entry of co-activators, acting on specific responsive elements of nuclear DNA and triggering transcription of specific genes [57].

Another key regulator of adipogenesis is C/EBPα (CCAAT/enhancer-binding protein alpha). It is a transcription factor involved not only in the process of formation of new fat cells and in the storage of lipids within them, but also in the differentiation of some blood cells and in the metabolism of glucose and lipids in the liver [58]. Furthermore, it has been observed that this protein is able to bind to the promoter that regulates the expression of the gene coding for leptin, a protein important in the modulation of body weight homeostasis. C/EBPα is part of a family consisting of five members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ and CHOP. They are expressed at different times during differentiation: the β and δ forms are expressed earlier than the others and are in turn able to modulate the expression of C/EBPα and PPARγ (Figure 13) [59].

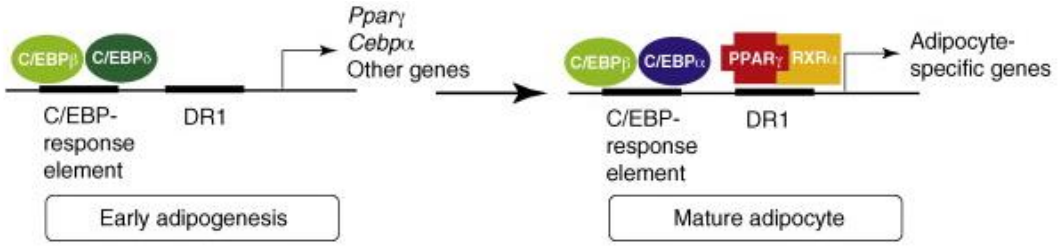


Figure 13. Model of the transcriptional action of PPARγ and C/EBPs during adipogenesis. During the early stages of adipogenesis, C/EBPβ and δ activate the expression of PPARγ, C/EBPα and possibly other adipogenic genes. Downstream genes of terminal differentiated adipocytes are regulated by the binding of PPARγ as a heterodimer to RXRα [59].

In fact, C/EBPβ and C/EBPδ lead to a reduced efficiency in generating adipose tissue if suppressed in mouse models in a single or associated manner [60]. As for the α form of C/EBP, its presence appears to be necessary and sufficient for adipogenesis. In fact, the ectopic expression of C/EBPα in fibroblasts alone is able to induce adipogenesis [61], while its deletion leads to an absence of subcutaneous, perirenal and epididymal adipose tissue. However, breast fat development is normal, with brown adipose tissue (BAT) hypertrophy [62].

So, PPARγ and C/EBPα are both crucial for the formation of adipose tissue, nevertheless PPARγ seems to play a major role in this process. In fact, the overexpression of PPARγ in combination with a gene knock-out of C/EBPα, leads to the restoration of gene expression. This phenomenon has been observed in murine embryonic fibroblasts. However, this event does not occur if one operates in the opposite direction [63]. Nonetheless, the potential compensatory role of other C/EBPs on C/EBPα knock-down cannot be excluded. This ability could be attributed, for example, to C/EBPβ, which exhibits DNA binding properties similar to C/EBPα. The β form is also able to remain transcriptionally present and active in mature adipocytes [64], with maximum expression levels reached in the early stages of adipogenesis, when it promotes the expression of PPARγ and C/EBPα [45].

Such studies on C/EBPs and PPARγ highlight their importance in the transcription of adipocyte genes. However, the molecular mechanisms underlying their cooperation still remain to be identified and clarified.

The intrinsic complexity in the adipogenesis process provides for a level of regulation that also involves other genes in addition to those already described. An important role is

reserved for SREBP-1c (sterol regulatory element binding protein-1c), a membrane-bound transcription factor that functions at the interface between sterol and fatty acid metabolism [65]. SREBP-1c is able to activate several genes coding for various enzymes, such as acetyl CoA carboxylase, fatty acid synthase (FAS), lipoprotein lipase (LPL) and others [66]. It is also able to induce the expression of PPARγ [67, 68], ultimately exercising a pro-adipogenic role.

The activation of the main adipogenic regulators seems to rely on the PI3K/Akt signaling pathway. Once activated by specific signals, this pathway leads to the activation of mTOR, a downstream signaling protein capable of regulating various cellular responses, including adipogenic differentiation.

The PI3K/Akt pathway plays an important role in regulating not only MSC differentiation, but also their proliferation, apoptosis and migration. This transduction pathway can be activated by different molecules, among which there are growth factors, hormones and cytokines or even by hypoxic conditions, and it plays key roles in the physiology and pathophysiology of many cell types, including ASCs. The signaling activation mechanism involves the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3), thanks to the activity of PI3K, the key enzyme of the process. PIP3 binds both Akt and PDK1 (phosphoinositide-dependent protein kinase-1), allowing the latter to phosphorylate Akt. The activation of Akt causes a cascade of responses on multiple downstream targets with the aim of modulating different cellular functions (Figure 14). For example, Akt regulates cell migration via Rac1 and RhoA, increases cell survival via Bcl-2, improves angiogenesis via VEGF and increases cell proliferation and differentiation via the activation of mTOR [69].

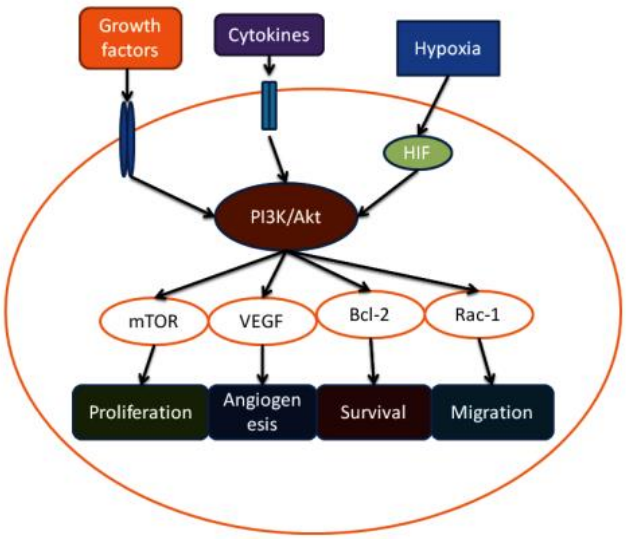


Figure 14. PI3K/Akt pathway in mesenchymal stem cells (MSCs). The activity of PI3K/Akt pathway in MSCs can be stimulated by growth factors/cytokines or by hypoxia. Activation of Akt promotes MSC proliferation, angiogenesis, survival and migration via specific downstream target proteins, such as mTOR, VEGF, Bcl-2 and Rac-1 [69].

Akt role is to phosphorylate a GTPase-activating protein, called TSC2, causing its inhibition. The latter is part of a complex that inhibits Rheb, a GTPase related to Ras, which in its active form is capable of inducing the activation of mTOR. The inhibition of the TSC complex therefore allows Rheb to promote the activity of mTOR, which at this point is free to act on its own substrates (Figure 15).

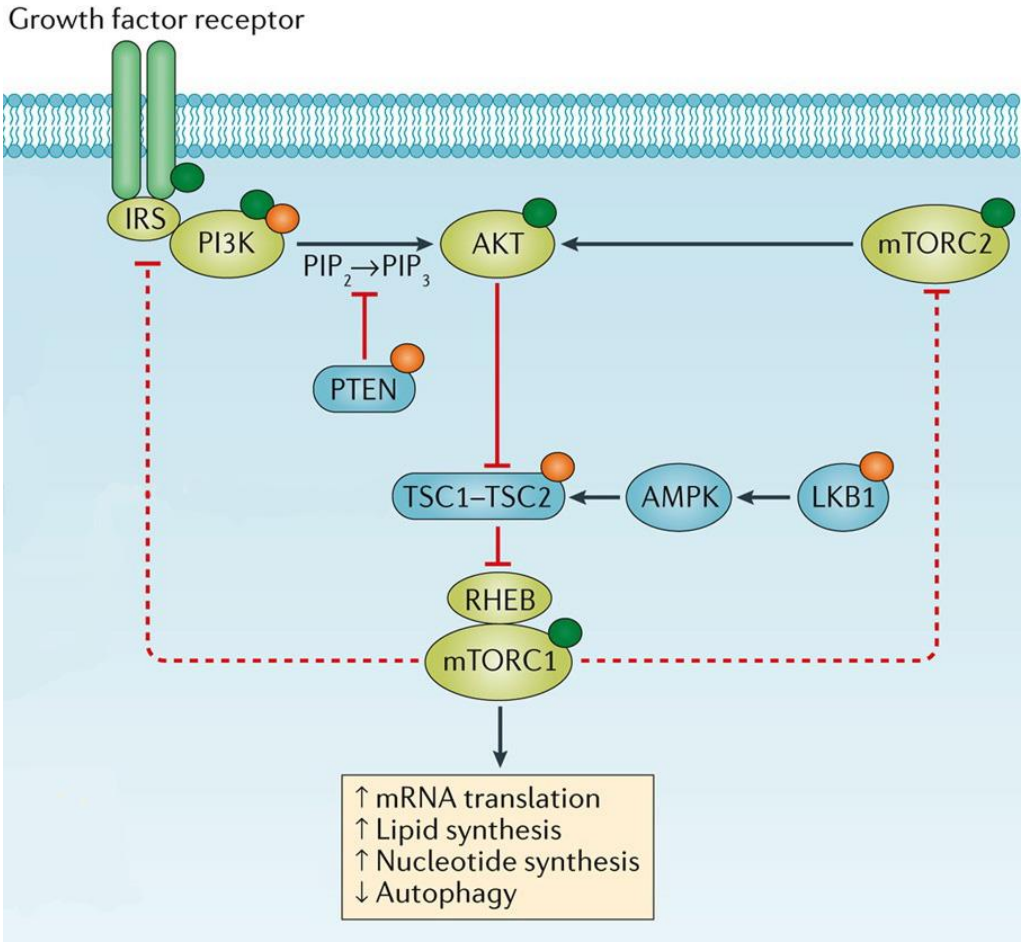


Figure 15. Activation of PI3K/Akt/mTOR pathway by growth factor receptors. PI3K proteins are recruited to the plasma membrane by adapter proteins (IRS) and interact with activated receptors leading to phosphatidylinositol 4,5-bisphosphate (PIP2) phosphorylation to generate phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 is a second messenger that activates AKT kinases, which can phosphorylate the TSC1 and TSC2 proteins, and thus dissociate the TSC1-TSC2 complex, which negatively regulates the activity of the mTOR kinase. Therefore, AKT causes activation of the mTOR 1 complex (mTORC1), resulting in the promotion of related cellular processes. In particular, mTORC1 is involved in a negative feedback loop that serves to prevent overactivation of AKT (red dashed lines) [70].

Regarding adipogenesis, the activation of mTOR seems to be sufficient to promote this process, thanks to its up-regulatory activity towards PPARγ and C/EBPα (Figure 16).

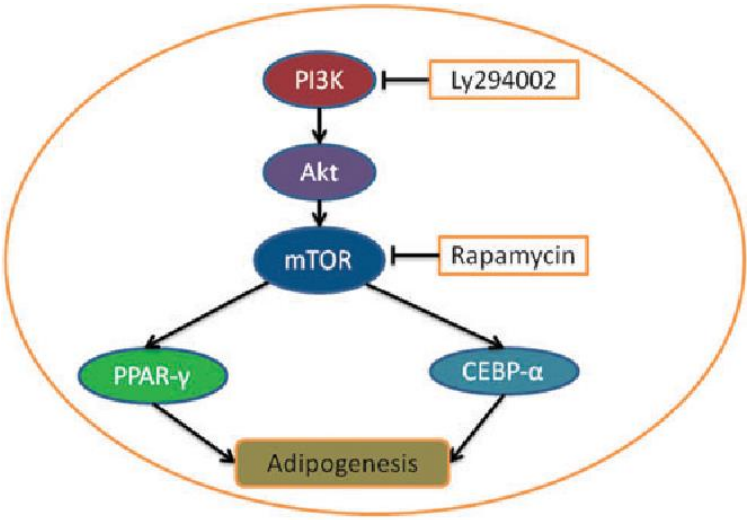


Figure 16. PI3K/Akt/mTOR axis in adipogenesis. PI3K/Akt pathway can regulate adipogenesis via mTOR, which can increase PPARγ and C/EBPα levels. The specific inhibitor of PI3K, LY294002, and the inhibitor of mTOR, rapamycin, reduce the level of adipogenesis [69].

However, the PI3K/Akt/mTOR axis is known to play a controversial role in this process. In fact, on one hand, Yu et al. showed that following the inhibition of this pathway, adipogenesis is decreased [71]. On the other hand, some studies report a promoting response on the phenomenon. It follows the need to conduct new research to ensure greater clarity.

In contrast to the positive regulatory pathways on adipogenesis, there are molecular signals that operate negatively on this process. This is the case of the Wnt/β-catenin pathway, in which some components of the Wnt (wingless-type MMTV integration site) family determine the mobilization of β-catenin in the nucleus by binding the Frizzled receptors [51, 72]. In the absence of stimulation of the upstream signal, the cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3b (GSK3β) in a complex containing Axin and APC (Adenomatous Polyposis Coli) and consequently directed towards proteosome-dependent degradation, mediated by ubiquitin. The stimulation of the pathway through receptor interaction with the ligand leads to the inhibition of both phosphorylation and subsequent degradation of β-catenin, which can then translocate to the nucleus and bind to the transcription factors of the TCF (T-cell factor)/LEF (lymphoid enhancing factor) family, leading to the activation of the responsive genes of the Wnt pathway. Some of these genes interfere with the induction of PPARγ and C/EBPα, having thus a negative effect on

adipogenesis. On the contrary, the inhibition of the Wnt pathway causes the downregulation of Axin2, a constitutive target gene of β-catenin, whose protein product interact with GSK3β, ensuring its retention in the cytoplasm. Decreased Axin2 levels cause GSK3β to be released and undergo nuclear translocation, which allows it to phosphorylate C/EBPβ and Snail. The latter has been shown to be a negative regulator of adipogenesis, inhibiting the expression of PPARγ [73]. The phosphorylation of C/EBPβ by GSK3β results in its increased DNA binding activity, while the phosphorylation on SNAIL decreases its own stability. Therefore, the activation of the β-catenin/Axin2/GSK3β cascade can lead to an increase in Axin2 levels, which would block adipogenic differentiation (Figure 17).

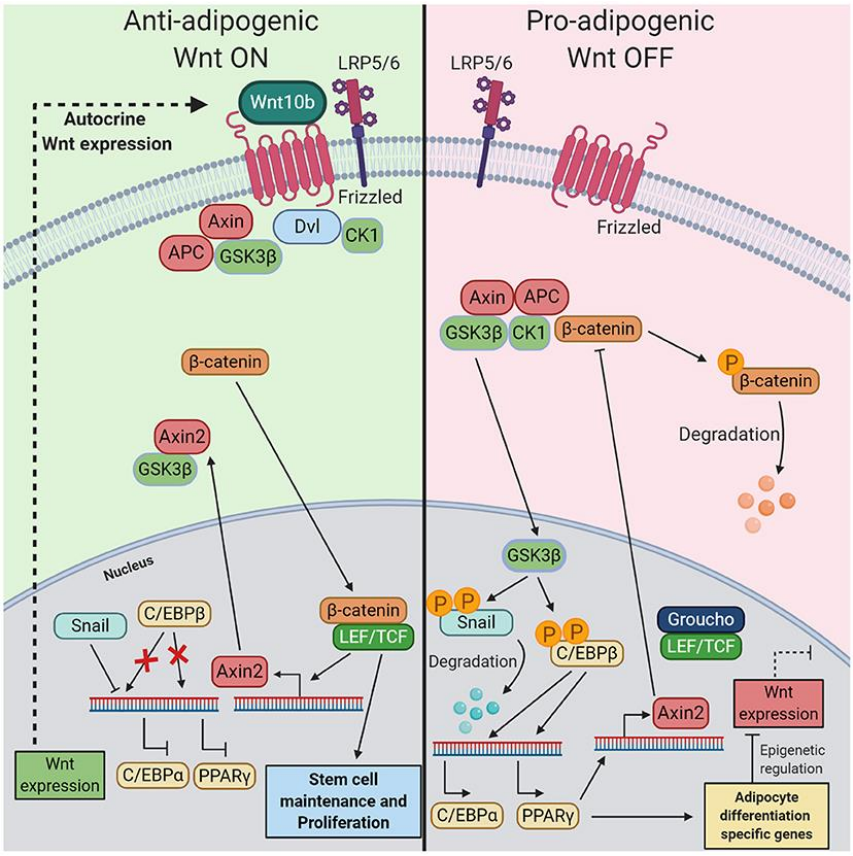


Figure 17. Role of the Wnt/β-catenin pathway during adipogenesis. The activation of the canonical Wnt pathway by Wnt10b leads to the nuclear localization of the β-catenin in the nucleus, where it can bind LEF/TCF. This complex of transcriptional factors initiates the expression of Axin2 and other genes related to the maintenance and proliferation of stem cells. Axin2 binds with GSK3β in the cytoplasm, thus preventing migration into the nucleus. In this state, inactive C/EBPβ prevents the expression of the transcription factors C/EBPα and PPARγ. Furthermore, the Snail factor inhibits the expression of C/EBPα. The inactivation of Wnt pathway results in β-catenin degradation in the cytoplasm, with lack of nuclear translocation. Without

Axin2, GSK3β localizes to the nucleus, where it degrades Snail and activates C/EBPβ. These events initiate the expression of PPARγ and C/EBPα, both crucial for the initiation of the final phase of adipogenesis [15].

Also, Cyclin D1, one of the genes that are expressed following the activation of the Wnt pathway [74] can interact directly with PPARγ and inhibit its activity [75], thus acting as a negative regulator of the adipogenic process.

Furthermore, by inducing a constitutive activation of Wnt signaling in preadipocytes, adipogenesis is inhibited, while the presence of dominant negative mutations for TCF involves a spontaneous differentiation [76]. Consequently, the inhibition of the Wnt pathway and the induction of PPARγ and C/EBPα are fundamental prerequisites for the differentiation of preadipocytes, both human and murine [77, 78].

The role of the MAPK pathway in the differentiation of adipocytes is contradictory. This signaling can be activated by numerous growth factors and cytokines, with modulatory effects on cell growth and differentiation. Elimination of ERK1/2 by antisense oligonucleotide strategy or inhibition of ERK1/2 phosphorylation by MEK inhibitors has been reported to prevent insulin-induced differentiation of 3T3-L1 cells into adipocytes. This suggests a positive role of the ERK pathway in adipogenesis. Conversely, other studies have shown that ERK activation inhibits adipocyte differentiation. To explain these apparently contradictory results, a temporally controlled modulation of ERK activation, that could have opposite effects during adipogenesis, has been hypothesized. In this scenery, ERK must be activated at the beginning of adipocyte differentiation, to allow the clonal expansion phase to be fulfilled, but in subsequent times the kinase activity must be stopped to prevent the inactivation of PPARγ and allow the completion of the differentiation process [79].

In fact, it has been reported that the transcriptional activity of PPARγ can be negatively regulated by phosphorylation mediated by ERK activation, this preventing adipogenic differentiation. The expression of PPARγ with a non-phosphorylatable mutation in a consensus site of MAPK (serine-112) produces cells with greater sensitivity to ligand-induced adipogenesis and resistance to inhibition of differentiation by mitogens [80].

Therefore, albeit controversial, the combination of the MAPK pathway with the other signaling pathways is crucial to maintain a balance between growth and differentiation of fat cells.

1.5 Clinical Application of ASCs

Since the discovery of ASCs more than 15 years ago, there have been major strides in the attempt to use them as a therapeutic tool. The focus of the scientific community towards ASCs continuously shifts between their capacity for mesenchymal differentiation and trans-differentiation (ability to differentiate along alternative lines with respect to the one of origin), up to the evaluation of their potential immunomodulatory and paracrine effects. In this regard, an even greater interest lies in the secretome of these cells, i.e. the set of soluble proteins, exosomes and micro-vesicles secreted by ASCs (Figure 18) [81].

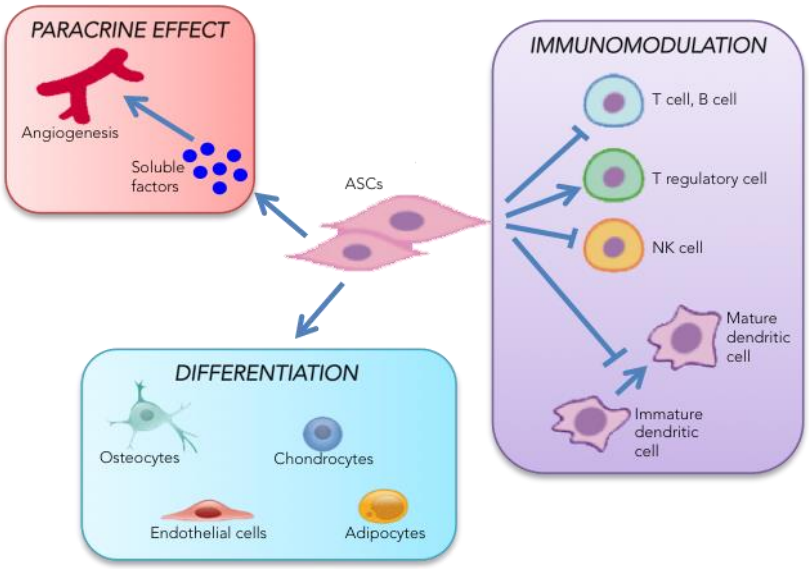


Figure 18. Mechanisms underlying the therapeutic efficacy of ASCs. 1) Multi-differentiation potential; **2)** Immunomodulatory capacity; **3)** Paracrine effects.

The potential clinical use of ASCs, which are in part favored over other stem cell types, lies in some of their positive aspects. In fact, they have several advantages, such as the absence of ethical problems and a lower risk of formation of teratomas at transplant, compared to embryonic stem cells [82]. Moreover, when compared to bone marrow derived MSCs, they show a higher post-collection yield and a less invasive collection procedure.

Within the application areas of ASCs, there are various methods of administration, both systemic and local, which are potentially usable. The effectiveness of the treatments exploited by systemic introduction is influenced by the "homing" capacity of the cells, which is the ability of MSCs, including ASCs, to migrate to the damage site thanks to chemotactic signals secreted by the inflamed tissue [83], as demonstrated in mouse models of allergic rhinitis [84]. Parenteral injections are the methods of choice for systemic administration. These methods are useful in the treatment of various autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis [85]. Intravenous administration, however, could determine the accumulation of cells in various organs [83], generating dangerous cellular aggregates potentially responsible for pulmonary emboli, heart attacks or blood flow disorders [86]. Furthermore, of all the cells administered, the amount of those that actually manage to migrate to the target site still remain uncertain.

As for local administration of ASCs, this occurs mainly by *in situ* injection or topical application on damaged tissue, resulting particularly useful in the treatment of wounds and improving the efficiency of cell engraftment in the target site [87]. This system limits the risks discussed above and is not affected by cellular homing systems as the cells are applied directly to the target tissue. However, this procedure can still be invasive, and its efficacy might be hindered since cells are directly injected into a potentially incompatible microenvironment (Figure 19) [88]. Therefore, the routes of administration of ASCs should be carefully evaluated in order to achieve optimal therapeutic efficiency.

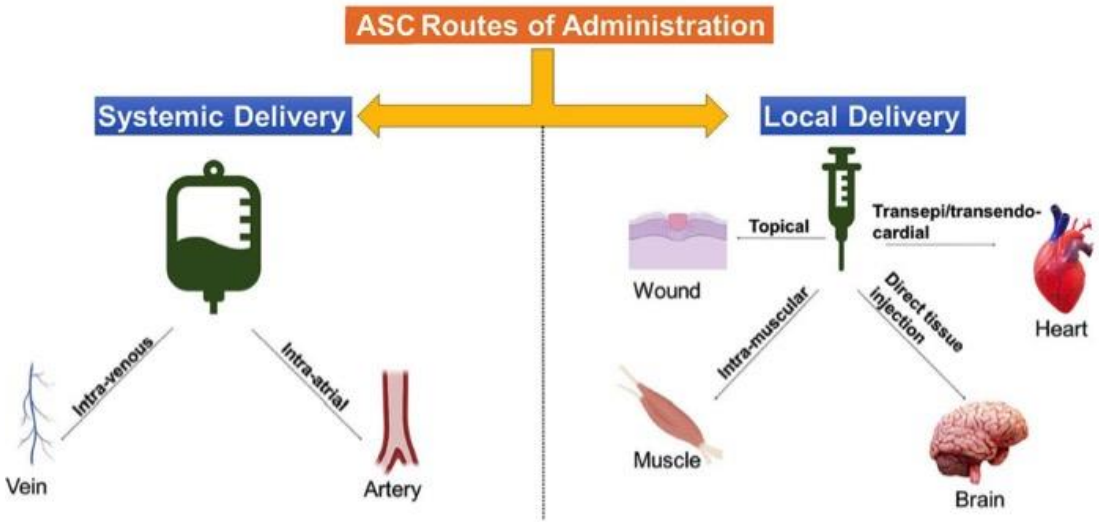


Figure 19. Common routes of administration for ASC-based therapies. The main methods of administration to target tissues are represented by systemic approaches (intravenous or intra-arterial injections) or by local approaches (direct injection into the affected tissue) (modified from [89]).

Although ASC differentiation capacity along the mesenchymal line initially led to a strong interest in the clinical use of these cells, in recent years the research scope of ASCs has significantly expanded.

A very interesting ability of stem cells, which a large part of their clinical efficacy would depend on, justifying the wide field of application, is the so-called "bystander effect". This phenomenon occurs at the time of their transplant and involves the modulation of the host environment thanks to the paracrine secretion of anti-inflammatory and cytoprotective molecules. This effect is so robust that the use of a cell-free conditioned culture medium can reproduce the same effect as the ASC transplant [90]. In particular, extracellular vesicles released from the endosomal compartment that contain biomolecules, proteins and genetic material such as mRNA and miRNA, possess a therapeutic potential useful in cell-free therapy for a large variety of inflammatory diseases (Figure 20).

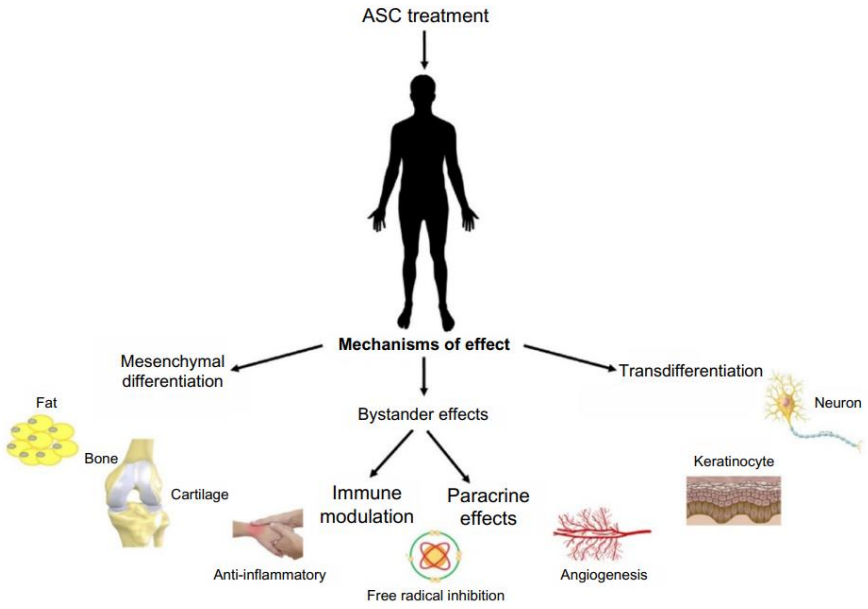


Figure 20. Potential mechanisms underlying ASC clinical efficacy. 1) Differentiation into the cell type of the mesenchymal tissue of origin; **2)** Transdifferentiation into cell types of other tissues

(nervous, epithelial); **3)** "Bystander" effect, mediated by soluble factors capable of determining a paracrine effect [91].

In the clinical application of ASCs, numerous studies witness their ability to differentiate into adipocytes to generate adipose tissue [92]. This type of tissue, in fact, is used in soft tissue reconstruction operations, breast augmentation, facial rejuvenation or as a filler, to name a few [93]. Specifically, some clinical conditions are characterized by large tissue void within the subcutaneous fat layer, caused from traumatic injuries, tumor resection or congenital anomalies [94]. Autologous adipose tissue transfer is commonly used for reconstructive purposes, but its effectiveness is frequently compromised by loss of the implant, due to insufficient vascularization of the transplanted tissue, and volume loss, due to central necrosis of autologous adipose tissue [95].

This often involves the implementation of additional surgical procedures on the patient to achieve the desired effect. To overcome this problem, current research focuses on improving the differentiation and survival of transplanted adipose tissue [96]. In this regard, the culture and subsequent differentiation of ASCs before reinjection is recommended to ensure the presence of viable preadipocytes exclusively [97]. An alternative strategy is "lipotransfer", consisting in the extraction of the SVF, where ASCs reside, from half of the collected adipose tissue. The isolated cells are then added to the untreated portion of the lipoaspirate, resulting in an ASC-rich fat that is re-injected. This would promote long-term survival of the transplant [98, 99].

However, such strategies are not completely efficient. So, developing protocols that are successful in modulating the adipogenesis of ASCs is now regarded as an alternative way to enhance their therapeutic efficiency in regenerative medicine, and thus reaching a favorable outcome in reconstructive surgery.

ASCs could find application also in the event of damage to the skeletal system, especially in clinical conditions in which physiological repair is delayed or absent. This is the case when dealing with unfavorable biomechanics, an extensive damage or a microenvironment failing to heal. The first positive report on the use of ASCs in bone regeneration dates to 2004, when a 7-year-old child underwent stem cell treatment following a head injury [100]. Although the treatment was clinically effective, the use of integral SVF in combination with traditional

bone graft and fibrin glue makes it difficult to establish how much the presence of ASCs contributed to the final clinical outcome.

Several studies have also highlighted the ability of ASCs to differentiate into cartilage-producing cells [44, 101], introducing their potential use in cartilage osteoarthritis, a degenerative joint disease that causes pain, disability and shortening of working life of adults. Therefore, the regeneration of relatively small areas of cartilage using ASCs could lead to significant social and financial benefits.

The applications of ASCs also find place in tissue wound healing. Published results show that the use of ASCs promotes tissue healing, both through cell differentiation and through paracrine effects, mediated by the production of trophic and anti-inflammatory substances [102, 103]. This is the case even in patients with tissue damaged by radiotherapy [104] or from scald burns [105].

There is also evidence that ASCs can transdifferentiate into keratinocytes, opening the doors to epidermal tissue engineering and contributing to a therapeutic future in the treatment of severe burns. In 2007, Rigotti et al. were the first in Italy to publish their experience in the field of radiotherapy-induced skin damage, reporting the results of a cohort of 20 patients with chronic chest wall ulceration due to radiation, successfully treated with fat injections at the serious injuries [106]. To date, ASCs, or more precisely fat injections, are used both for the treatment of damage from late radiotherapy and as a pre-conditioning treatment to decrease rates of surgical complications in elective surgery [107, 108].

ASCs would also find application in cardiovascular diseases. Indeed, they have demonstrated an improvement in ventricular function following intramyocardial injection in rat models with induced myocardial infarction [109]. Good results have also been obtained in phase I and II clinical trials in the treatment of patients with acute myocardial infarction. Phase III clinical trials are underway to further clarify the real efficacy of this therapy.

Recently, ASC-based therapies have been studied for the treatment of neural disorders, for their positive contribution both through the bystander effect and through the trans-differentiation process. In this regard, it has been shown that ASCs are able to transdifferentiate *in vivo* into Schwann cells (SC), neural cells of the peripheral nervous system important in nerve regeneration and repair after injury [110-112]. Furthermore, there is evidence that supports the ability of ASCs to differentiate into neuronal cells [113] with a

differentiation potential similar to BMSCs, but with a higher proliferative capacity [114]. The use of ASCs has also been evaluated in Parkinson's disease, caused by the loss of dopaminergic neurons in the substantia nigra, showing improvements in memory impairment through both intravenous and trans-cerebral injection [115]. The exact mechanisms of therapeutic effect are not fully understood, but three factors are taken into consideration:

- Migration and differentiation in neuronal cells;
- Bystander effect with production of anti-inflammatory cytokines;
- Activation of microglia cells that secrete neurotrophines;

Other ASC applications concern diseases such as diabetes mellitus, in which model rats showed an improvement in weight and insulin tolerance [116]. Furthermore, the immunomodulatory effects of ASCs have been exploited for the treatment of graft versus host disease (GvHD) and graft rejection [117, 118]. The absence of major histocompatibility complex II (MHCII) expression and the immunomodulatory properties of ASCs allow for allogeneic transplantation, limiting common issues about host compatibility, malignant transformation and loss of function that might occur in cell therapy. An allograft combines the advantages of easier management and simpler standardization of the therapeutic product.

An important consideration to be done regarding ASC clinical efficacy is linked to the so-called "Hayflick limit", namely the fact that ASCs enter a state of replicative senescence (i.e., *in vitro* aging) after repeated serial passage in culture, when the cells stop dividing after a certain number of population doublings (PDs) [119]. Senescent ASCs show typical phenotypic features *in vitro*, such as heterogeneity in the proliferation potential, with the presence of subpopulations morphologically described as small, round, rapidly proliferating cells and other subpopulations of slowly dividing, large, flattened cells [120] Moreover, senescent cells display some altered biological properties, like a decrease of the number of colony-forming unit (CFU) cells and of cell migration ability. They also show functional changes such as a reduction of differentiative potential and the acquisition of the so-called senescence-associated secretory phenotype (SASP), which can affect the secretion of proteins that can negatively affect the immunomodulatory potential of ASCs [121, 122] (Figure 21).

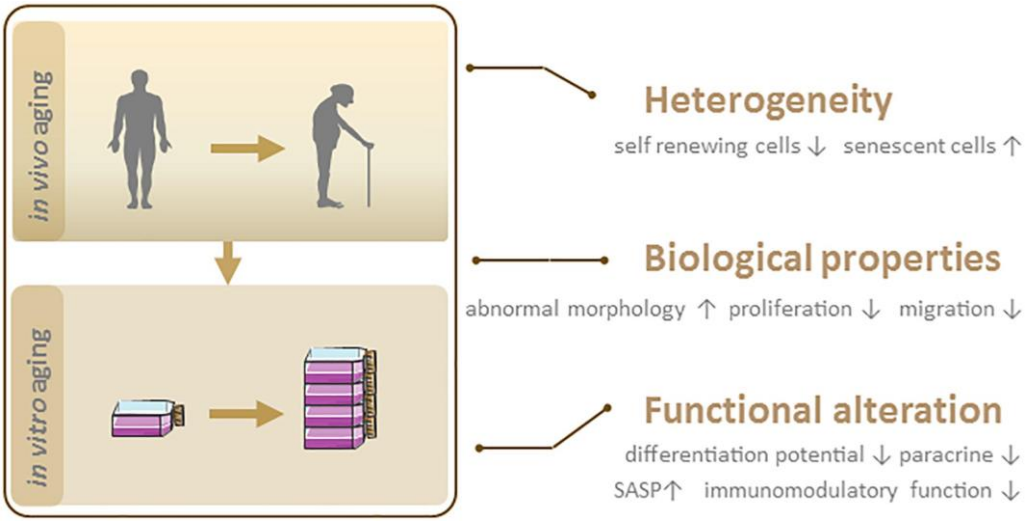


Figure 21. Phenotypic features of senescent ASCs. *In vitro* aging lead to ASC senescence, which is characterized by heterogeneity, biological and functional changes [123].

Given the fact that prolonged *in vitro* expansion can negatively affect ASC immunomodulatory and differentiation capacities, leading to reduced efficacy following administration, at present low-passage cultures are recommended for clinical-scale expansion of cultures. However, in order to overcome this limitation, it becomes imperative both to carefully monitor ASC senescence through the introduction of novel markers and to understand the molecular basis of ASC aging, this allowing the design of strategies aimed at delaying senescence onset.

To obtain the best possible clinical result and increase the efficiency of stem cell therapy, current research focuses on the development of strategies that can improve delivery and engraftment in target tissues. To this end, pre-conditioning strategies that enhance the homing of ASCs can be useful. One could be the use of specific culture conditions with cytokines that induce the expression of migratory receptors (CXCR4). Pre-conditioning, as well as chemical, physical or genetic manipulation and molecular stimulation, can improve cell survival, increase the paracrine effects and the differentiation potential, thus enhancing the therapeutic effect of cell products. Currently, the lack of understanding of the behavior and mechanisms of action of ASCs, combined with the lack of standardization in the isolation and preparation protocols of the cells, represent the main obstacles to the clinical use of cell therapies.

1.6 5-Azacytidine (5-aza)

The multilinear differentiative potential of ASCs implies a delicate balance between self-renewal and differentiation. The orientation towards a specific lineage leads to a series of morphological and functional modifications on the cells, which are influenced not only by specific molecular factors, but also by specific gene expression patterns [124]. Therefore, regulation of gene expression is crucial for the proper functioning of all cellular processes. It occurs also through epigenetic mechanisms, such as chromatin remodeling and DNA methylation, which influence the access at certain gene loci to transcription factors and the transcription apparatus, consequently inhibiting or activating gene expression (Figure 22).

Regulation of the methylation profile relies on members of the methylase family, in particular DNA methyltransferase 1 (DNMT1), which recognizes the hemi-methylated strand and faithfully restores the methylation pattern after each cycle of DNA replication, and DNA methyltransferase 3 (DNMT3A, DNMT3B) which deals with *de novo* methylation during early embryogenesis by methylating specific chromosomal sequences [125].

Methylation is generally associated with transcriptional inhibition and mainly occurs on cytosines followed by a guanine in the so-called "CpG islands", regions where the CG dinucleotide is particularly frequent.

The mechanism of this reaction is driven by DNMTs, which transfer a methyl group in position 5 of the cytosine forming the 5-methylcytosine. The transfer takes place in the presence of a donor, such as S-adenosyl-methionine (SAM or AdoMet).

This process involves the formation of a covalent bond between a cysteine residue in the catalytic site of the DNMT, and the carbon 6 of cytosine (Cyt) in the DNA [126], leading to the activation of the C5 position and to the transfer of the methyl group to the base, followed by a deprotonation of the C5 atom. This in turn resolves the covalent bond between the enzyme and the base in a beta elimination reaction [127]. This allows the release of the enzyme from the DNA, which will now present the methylated Cyt.

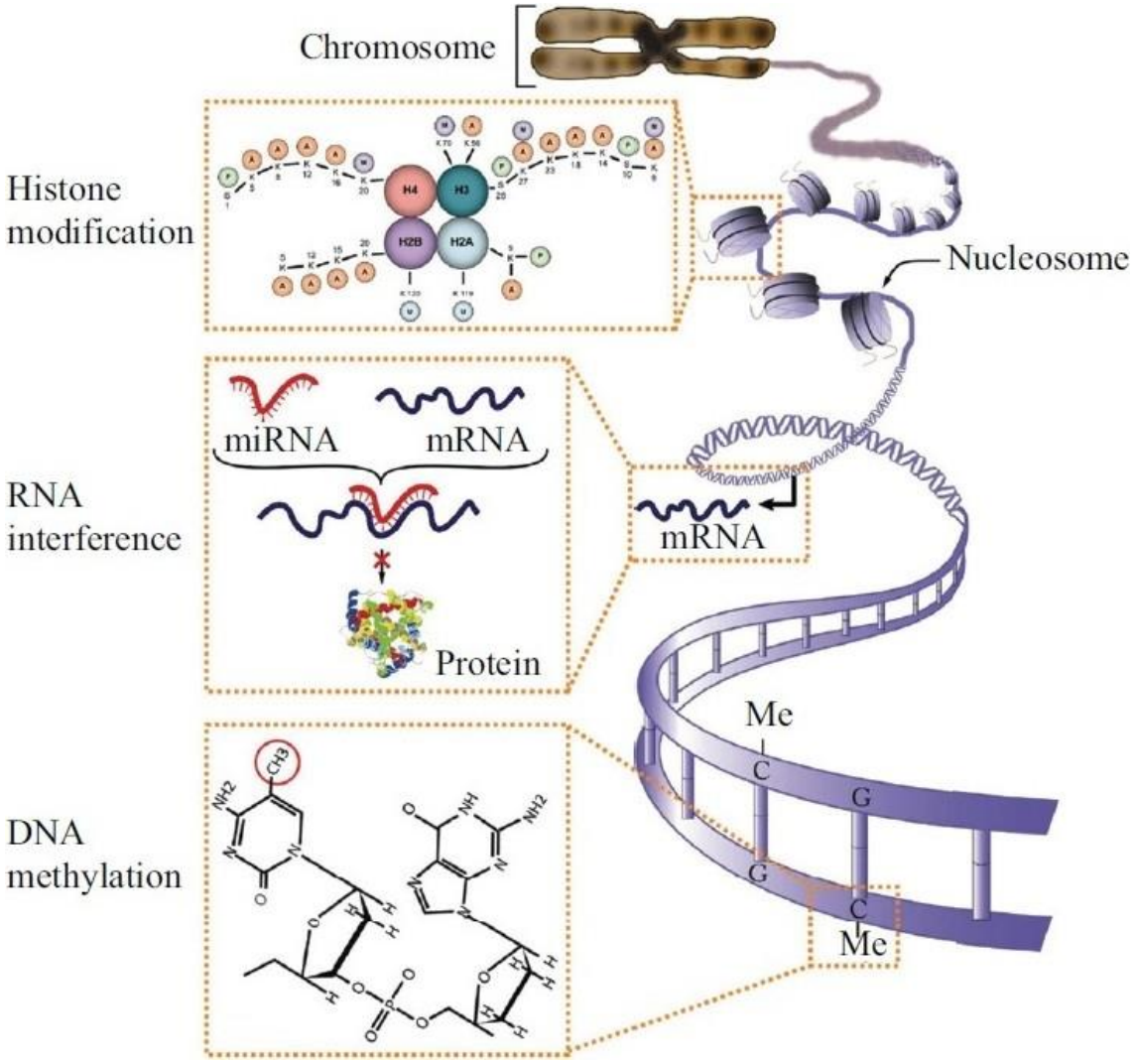


Figure 22. Different levels of epigenetic regulation. 1) Histones modifications; **2)** Post-transcriptional regulation by microRNAs; **3)** DNA methylation by DNA methyltransferases [128].

5-azacytidine (5-aza, Vidaza®) is a nucleoside analogue of cytosine, from which it differs only in the presence of a nitrogenous group in position 5 of the heterocyclic ring. 5-aza was synthesized for the first time as a cytotoxic agent and since the 1980s it is also recognized as having a powerful hypomethylating activity that occurs following its incorporation into the replicating DNA (Figure 23) [129].

This hypomethylating activity made it interesting from an oncological point of view, as it is potentially useful in those neoplastic settings characterized by a high degree of methylation of tumor suppressor genes. 5-aza is therefore currently approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for the treatment of different types of leukemia [130].

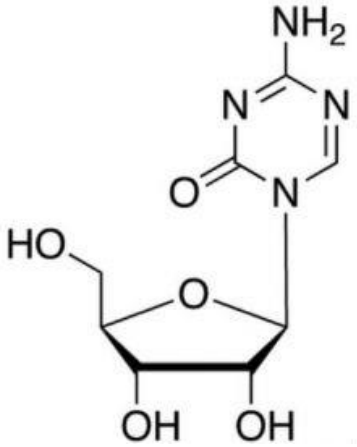


Figure 23. 5-azacytidine. Note the presence of a nitrogenous group in position 5 of the heterocyclic ring, which distinguishes it from its analogue, cytosine.

Azacytidine is part of a group of "epipharmaceuticals" (epigenetically active molecules) defined as "DNMT inhibitors", due to their ability to hinder the normal functioning of DNA methyltransferases. Being a nucleoside analogue, it is able to integrate into both DNA and RNA. In the first case, by interfering with the correct replication of the methylation pattern. In the second case, by producing a ribosomal disassembly with possible prevention of the translation of oncoproteins [129].

Although the mode of action of this inhibitor remains controversial, several mechanisms have been proposed [130]. After being transported into the cells by several nucleoside transporters, including the human concentrative nucleoside transporter 1 (hCNT1) [129], 5-aza can be phosphorylated by several kinases, converting it to its active triphosphate form, namely 5-aza-20-deoxycytidine-50-triphosphate. The latter can be incorporated into DNA to replace cytosine during the replication phase [130]. At this point the DNMT intervenes on azacytosine-guanine dinucleotide as if it were a normal CpG portion and catalyzes the methylation reaction. This results in a covalent bond between the carbon-6 atom of the cytosine ring and the enzyme. However, azacytosine, having nitrogen instead of carbon in position 5, does not allow the beta-elimination necessary to resolve the covalent bond to be carried out. Consequently, the enzyme remains covalently linked to the DNA and its methyltransferase activity is blocked [131]. The presence of the enzyme/5-aza complexes irreversibly bound to DNA triggers damage signaling leading to azacytidine-dependent

degradation of trapped DNA methyltransferases [131] and limiting the amount of enzyme available for the generation of 5-methylcytosine (5-MeC) [132]. Consequently, there is an overall reduction in DNA methylation levels during replication.

The phenomenon of demethylation induced by 5-aza seems to be dose dependent, in fact it is more evident at low drug concentrations, while at higher concentrations 5-aza produces cytotoxic effects.

DNA demethylation through DNMT inhibitors, such as 5-aza, has been shown to have positive effects in driving adult stem cells to different cell lineages, triggering processes such as osteogenesis, myogenesis, hepatic and cardiac differentiation [133, 134]. Recently, data has emerged to support the fact that treatment with DNA demethylating agents is able to positively influence ASC immunosuppressive properties, making them more stable regardless of the number of passages, thus improving their therapeutic applications [135]. Epigenetic modifications induced by methylation on DNA and histones are essential characteristics in determining ASC aging and senescence processes [136, 137]. Furthermore, the use of epipharmaceuticals that promote hypomethylation has been linked to the improvement of the self-renewal capacity of ASCs, the maintenance of their multipotent state and the reduction of cellular senescence processes associated with prolonged *in vitro* cultures [138].

Epigenetic modifications and their alterations are also considered crucial in tumorigenesis, in which gene silencing induced by hypermethylation of tumor suppressors and other cancer-related genes play a fundamental role in the human neoplastic process. Hence, the inhibitors of DNA methyltransferase (such as 5-aza) play an important role in restoring the gene silencing induced by hypermethylation and in re-establishing the control of proliferation and sensitivity to apoptosis [131]. Several reports indicate 5-aza treatment as a known inducer of senescence through activation of the p53 pathway or regulation of telomere activity [139, 140]. It also acts as an inducer of caspase activity and promoter of apoptosis, thus suggesting its use in cancer therapy for acute myeloid leukemia (AML) and myelodysplastic syndrome [141]. While there is much evidence that 5-aza may be particularly important in regulating the diverse biological functions of stem cells, much remains to be discovered about how the DNA methylation mechanism fully defines ASC biology.

1.7 Secretome

The limitations of cell-based therapies concern the practical steps necessary for the isolation, expansion and characterization of stem cells, as well as the appropriate quality and safety controls of the final product to be injected or transplanted [142]. The quality of stem cell samples, in fact, can be easily compromised, leading to the loss of cellular and molecular features fundamental for the clinical use of these cells. Cellular senescence, decreased proliferative ability and loss of differentiative capacity are just some of the factors that make stem cells unsuitable for clinical application [143].

In this context, it is easy to understand the importance of finding and setting up an acellular alternative that allows to exploit the potential of the cells overcoming the limitations related to the cells themselves. Therefore, stem cells paracrine activity has emerged as a promising therapeutic cell-free resource in several conditions, such as wound healing [144] and tissue repair [145], thanks to the production of bioactive molecules (including growth factors, cytokines, chemokines, enzymes, extracellular matrix) secreted directly in the stem cell microenvironment or encapsulated within the extracellular vesicles, which can be classified into three groups: apoptotic bodies which form during cell apoptosis, exosomes as the product of endosome maturation, and microvesicles generated by outward budding of the plasma membrane (Figure 24).

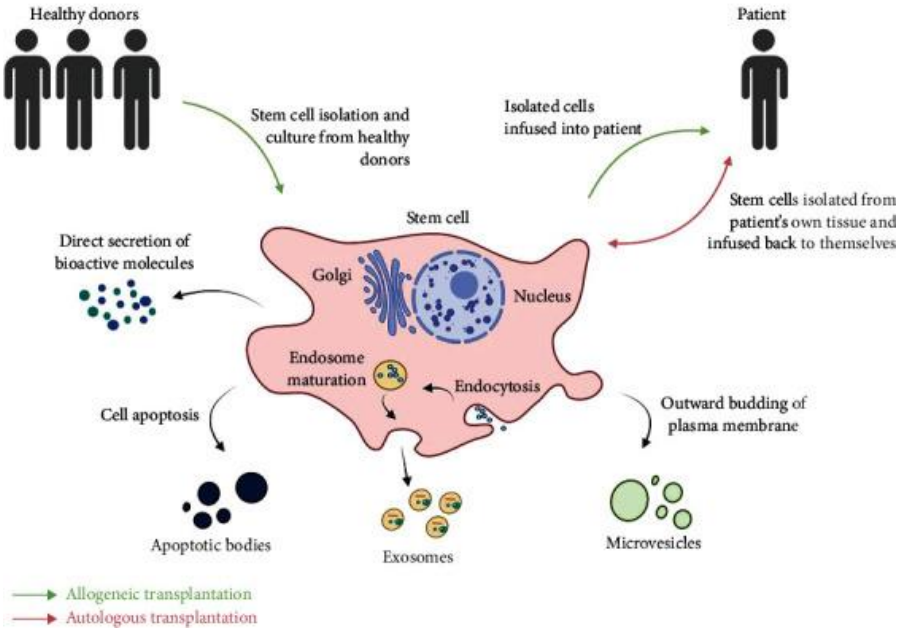


Figure 24. Stem cell therapy and stem cell-derived paracrine mediators. Autologous stem cell transplantation involves the isolation of stem cells from the patient and infusion back to the same patient during treatment. In allogeneic stem cell transplantation, stem cells from single or multiple healthy donors are given to the patient [146]. Stem cells can be exploited also for their production of bioactive molecules, secreted directly or encapsulated within apoptotic bodies, exosomes and microvesicles.

It is now recognized that ASCs promote tissue repair/regeneration through the release of a plethora of soluble factors, including cytokines, growth factors and microRNAs, and extracellular vesicles collectively known as ASC secretome [147] (Figure 25).

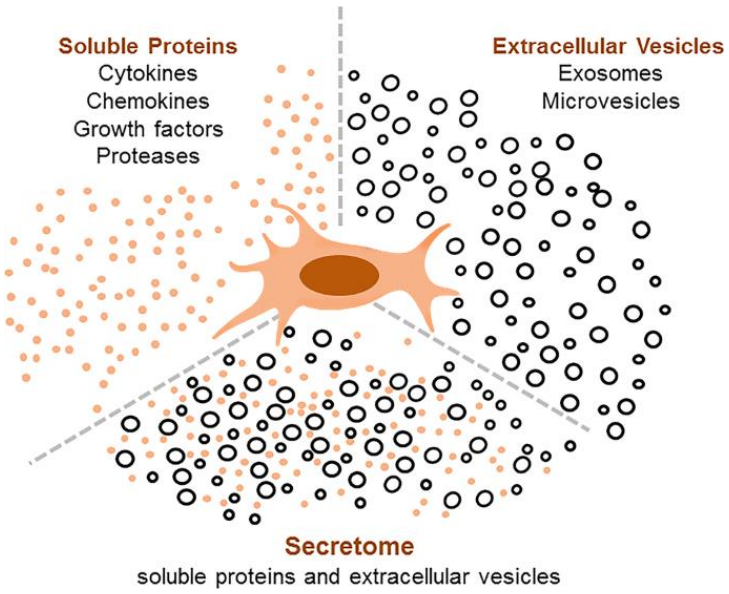


Figure 25. Schematic overview of ASC secretome. The secretome comprises both a soluble fraction and a vesicular fraction. Among the soluble factor there are biologically active molecules such as cytokines, chemokines and growth factors, as well as nucleic acids like microRNAs. The vesicular fraction includes exosomes and microvesicles [148].

The secretory profile of ASCs has been extensively studied and has recently begun to be characterized at the mass spectrometry level. Such studies revealed that ASCs secrete different cytokines and growth factors, such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF-β), insulin-like growth factor (IGF)-1, fibroblast growth factor (FGF), interleukin-6 and -8 and adiponectin [149-151]. Indeed, ASC-based therapies exploit the trophic action of the cytokines and

growth factors contained in the secretome to exert their beneficial effects in a paracrine fashion [152], acting directly on cell survival (protection against cell death or induction of cell migration and proliferation) or on specific processes related to different target systems, such as heart, muscle, central nervous system and immune system. ASC secretome alone is able to promote angiogenesis, through stimulation of endothelial cells proliferation and inhibition of their apoptosis mainly mediated by VEGF, HGF and IGF-1. At the immune system level, also, some reports highlighted ASC immunosuppressive effect not to be cell-to-cell contact dependent. Indeed, the soluble factors secreted by ASCs, rather than their differentiative potential, are considered the main responsible for their immunomodulatory and anti-inflammatory efficacy when transplanted into local areas [153]. As for the central nervous system, the production of specific factors by ASCs has been shown to play a role in neuroprotection and neuronal differentiation [154]. Furthermore, IFG-1 secreted by ASCs has been demonstrated to protect cardiomyocytes from apoptosis, this opening the way to a possible application of ASC secretome in heart regenerative processes [155, 156]. Beside the release of growth factors and cytokines, ASC secretome may also contain nucleic acids, both mRNAs and miRNAs. The stability of such extracellular RNAs secreted by ASC is guaranteed through their inclusion within secretory particles including exosome and microvesicles, which protect them from degradation by ribonucleases. Several studies proved that miRNAs secreted from ASCs can be delivered to target cells where they are taken up and induce substantial changes in gene expression. So, it can be envisioned a novel mechanism of intercellular communication mediated by secretory miRNAs [157]. Indeed, there is growing evidence that secretory miRNAs play a role in a variety of cellular events, such as proliferation, differentiation, angiogenesis, inflammation and immune response, and transfer of genetic information between ASCs and adjacent cells appears to be an important mechanism responsible for ASC paracrine effect in tissue repair/regeneration [158, 159].

The microenvironment in which they reside is fundamental in determining ASC biochemical and physical characteristics, influencing also their secretome and consequentially their therapeutic immunomodulatory capacity [160, 161]. So, ASCs derived from different donors will possess different biochemical and immunomodulatory characteristics, as well as different secretome composition. Conversely, secretome profile is ultimately influenced by *in vitro* culture conditions [162] and exposure to different agents

that can modify the levels and nature of soluble factors secreted by these cells. Some reports indicate the possibility to increase the release of some cytokines involved in hematopoiesis and angiogenesis by exposing cells to basic fibroblast growth factor (bFGF) [163]. Indeed, it has been observed that the administration of ASCs in inflammatory or ischemic areas results in the active secretion of growth and anti-inflammatory factors, thus promoting wound healing and tissue repair. Hence, the observation that therapeutic applications and responses of ASCs and their secretome can be regulated by specific modifications of their culture conditions highlights the importance of culture protocols, in addition to the abundancy of primary source of ASCs, i.e. adipose tissue, and the easiness of isolation, to achieve a successful ASC clinical application. Furthermore, ASC conditioned medium may have a therapeutic potential in selected pathologies requiring immunosuppressive rather than differentiative burst. In such cases, it would be of particular importance to assess how the secretome profile changes according to the number of passages, in the attempt of standardizing its clinical efficacy. Indeed, boosting ASCs features prior to administration represents an attractive strategy to overcome the limited efficacy of naïve ASCs. This can be achieved by modifying culture conditions, pre-conditioning the cells with specific molecules and/or defined media that specifically trigger the expression of the desired factors, or modulating genes of interest by silencing or transfection. Importantly, composition of MSC-vesicles can be also modulated by preconditioning *in vitro*, enabling the generation of disease-specific MSC-based immunosuppressive products, which could be used as a new remedy in cell-free treatment of autoimmune and inflammatory diseases. Moreover, even in the case of a direct application of ASC secretome, rather than naïve cells, optimizing culture conditions remains a suitable strategy to enhance the therapeutic efficiency and to expand the applications of ASCs, and their secretome, to a wider range of regenerative medicine protocols.

1.8 MicroRNAs

Micro-Ribonucleic Acids, universally called "miRNAs", are a class of small, non-coding, evolutionarily conserved RNAs, usually 18-25 nucleotides long. They exert their function as crucial modulators of gene expression acting at a post-transcriptional level and regulating

the stability and the translation of mRNAs. Therefore, they are involved in almost every biological process, such as cell division, differentiation, growth and apoptosis [164].
MiRNAs seem to have a binding site in the 3′ untranslated region (3'UTR) of more than 60% of all mRNAs. This implies that they are strictly regulated and that miRNAs play a role in both normal cellular homeostasis and in pathological states [165]. Additionally, several miRNAs have been demonstrated to target hundreds of mRNAs, suggesting a complex and combinatorial mode of action in messenger RNA regulation [166]. An active regulation of miRNAs homeostasis may be an important factor in maintaining an efficient immune response as miRNAs have critical roles in normal physiology and development, as well as in pathological processes [167]. Insulin signaling, immune-mediated inflammation, adipokine production, adipogenesis, lipid metabolism and food intake modulation are all regulated by miRNAs. These molecules are present in numerous human biofluids (plasma, serum, urine, tears, saliva, colostrum, amniotic, cerebrospinal and seminal fluid) and they may be used as disease biomarkers and predictors of metabolic disease [168]. However, their origin may vary: they can be released from dead cells with no specific function [169] or they can be actively secreted, acting as mediators for intercellular communication [170].
In recent years, miRNAs have been widely recognized as important regulators of the differentiation processes of MSCs.
Regulating miRNAs expression, and thus exploiting their regulatory effects, could support the development of new therapeutic approaches in regenerative medicine, while helping to better understand the molecular pathways underlying MSC differentiation in humans. For example, some miRNAs modulate the balance between MSC adipogenesis, osteogenesis and chondrogenesis, thus controlling the fate of these cells [171].
Considering the great importance of miRNAs as regulators of stem cell biology, synthetic molecules like miRNA mimics or inhibitors may represent effective tools in regenerative medicine and tissue engineering.

1.8.1 Biogenesis of miRNAs

MicroRNAs are initially generated in the nucleus, where RNA polymerase II transcribes their genes into a pri-miRNA - a primary miRNA transcript - longer than 1 kb. Pri-miRNA consists of a stem loop encoding for one or more mature miRNAs, and for some terminal, single-stranded segments with a 7-methyl-guanosine (m7G) cap at the 5′ end and a poly-A

tail at the 3‘ end [172]. The pri-miRNA is then processed into pre-miRNA by the microprocessor complex, consisting of an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha. The DGCR8 subunit recognizes the pri-miRNA, and the subunit Drosha cleaves it to produce a pre-miRNA about 70 nucleotides long. This process can be promoted or repressed by additional RNA binding proteins. Pre-miRNAs are exported to the cytoplasm by the Exportin-5 (EXP5)-Ran-GTP complex, and further processed by the multidomain endoribonuclease III Dicer. Dicer binds and removes the pre-miRNA stem loop region to produce a mature miRNA duplex. The activity of Dicer is regulated by some RNA-binding proteins to ensure the stability and specificity of the Dicer complex and stimulate the cleavage. The miRNA duplex contains both the 5p strand that arises from the 5’ end of the pre-miRNA hairpin and the 3p strand that originates from the 3’ end. Both strands are loaded onto the Argonaut family of proteins (AGO 1-4 in humans) to form the RNA Induced Silencing Complex (RISC).

The assembly of RISC consists of two separate steps. The first step is an ATP dependent process requiring Hsc70/Hsp90 chaperon complex to load the miRNA duplex onto the AGO proteins to form the pre-RISC. In the second step, which is ATP independent, the miRNA duplex unravels inside the AGO proteins, then the “guide” strand is selected while the “passenger” strand is discarded. In humans, the choice between the 5p or 3p strand is made according to the thermodynamic stability and nucleotide composition at the 5‘ end. The strand which contains more Uracil at the 5‘ end, and thus is less stable, is chosen as the guide strand. The more stable strand, containing Cytosine at the 5‘ end, will be the passenger strand and will be degraded by a RISC endonuclease (Figure 26) [173, 174].

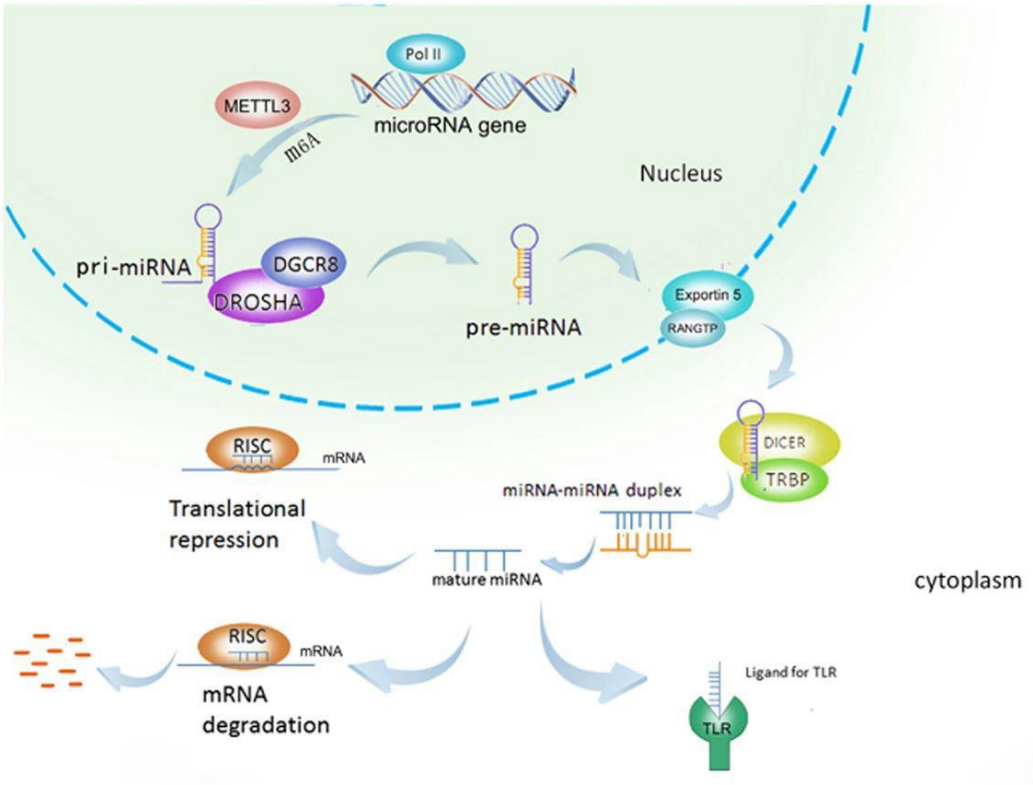


Figure 26. Schematic representation of miRNAs biogenesis. miRNAs processing starts in the nucleus where they are transcribed from genomic DNA as pri-miRNAs. Pri-miRNAs are then cleaved to generate pre-miRNA. Hairpin-shaped pre-miRNAs are actively transported from the nucleus to the cytoplasm, where they are cleaved into miRNA duplexes. The "passenger" strand (orange) is degraded, while the "guide" strand (blue) is incorporated into the RISC and serves as a functional, mature miRNA [175].

Activated RISC binds the mRNA target through base pairing between the 5' end of the miRNA guide and the 3'UTR of the target. The fate of the target mRNA depends on the base-pairing complementarity, and it generally results either in reduced translation or degradation of the mRNA transcript [176].

1.8.2 Mechanism of action

MiRNAs exert their biological functions by binding to their target mRNA and negatively regulate its expression. Evidence supports two distinct silencing mechanisms depending on the binding between the miRNA seed sequence and the target gene, namely mRNA cleavage and translation repression. The first is mediated by endonuclease cleavage of target mRNA by Ago2 (the only member of the AGO family endowed with endonucleolytic

activity) and requires a perfect base-pairing between miRNA and mRNA target. The majority of miRNAs, though, are not completely complementary to their transcripts. Hence, partial complementarity prevents the cleavage activity of Ago2, but RISC complex can still silence target genes by recruiting additional effector proteins, which induce translational repression (Figure 27). In this case, in presence of RISC, ribosomes fail to assemble on a targeted transcript, thus impairing initiation of translation [177].

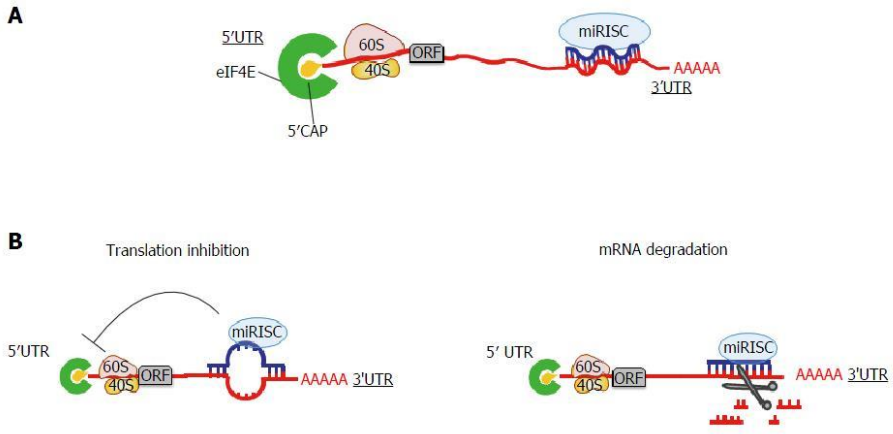


Figure 27. Mechanisms of action of microRNAs. A) miRNAs bind the 3'UTR of a target gene by base pairing. The binding between the miRNA seed sequence (nucleotides 2-8 at the 5'end of the miRNA sequence) and the miRNA regulatory element (MRE) at the 3'UTR of a target gene determines the specific type of regulation; **B)** miRNAs act as inhibitors of translation when the binding at the 3'UTR of target genes is only partially complementary. Instead, when the binding complementarity is perfect, miRNAs induce mRNA degradation. ORF: Open reading frames (Modified by [178]).

The miRNA-mediated mRNA degradation can be achieved also in case of partially matched mRNA, through a process of mRNA decay independent of endonucleolytic cleavage. This is achieved through a poly(A) shortening of target mRNA by the two deadenylase complexes CCR4–NOT and PAN2-PAN3. After deadenylation, target mRNAs undergo degradation in the 5'- 3' mRNA decay pathway. Then, RISC complex directly promotes removing of the 5' cap structure by recruiting decapping enzymes onto the target mRNA. Finally, the exonucleolytic mRNA decay is achieved by the exoribonuclease XRN1 (Figure 28) [177].

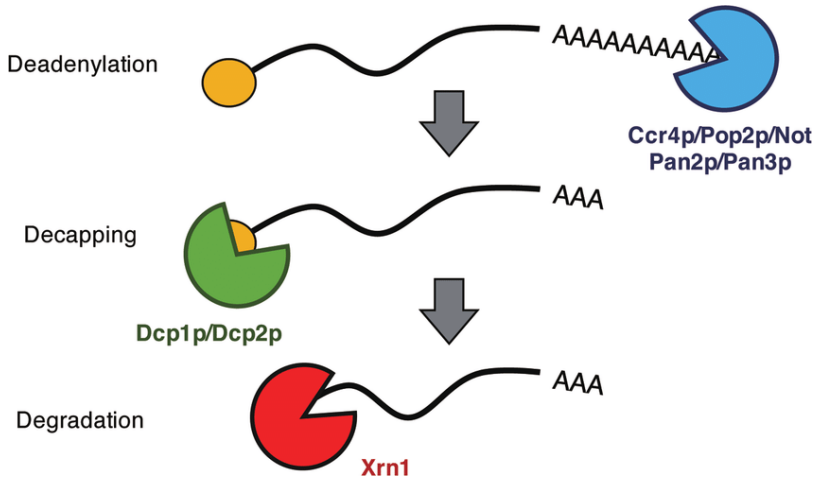


Figure 28. 5′-3′ exonuclease-mediated mRNA decay pathway. The process begins with shortening of poly(A) by the CCR4-NOT/PAN2/PAN3 complex. After the decapping of 5′ cap structure (orange circle), the body of mRNA is degraded with 5′-to-3′ polarity by the exonuclease XRN1 [179].

1.8.3 Therapeutic potential of miRNAs in ASC-based strategies

Recent studies investigated ASC miRNA expression profiles and their influence on the intrinsic stem cell properties of self-renewal and pluripotency [180]. In particular, the discovery of differential miRNA signatures in progenitors and terminally differentiated cells suggested a role of miRNAs in ASC differentiation [159] (Figure 29).

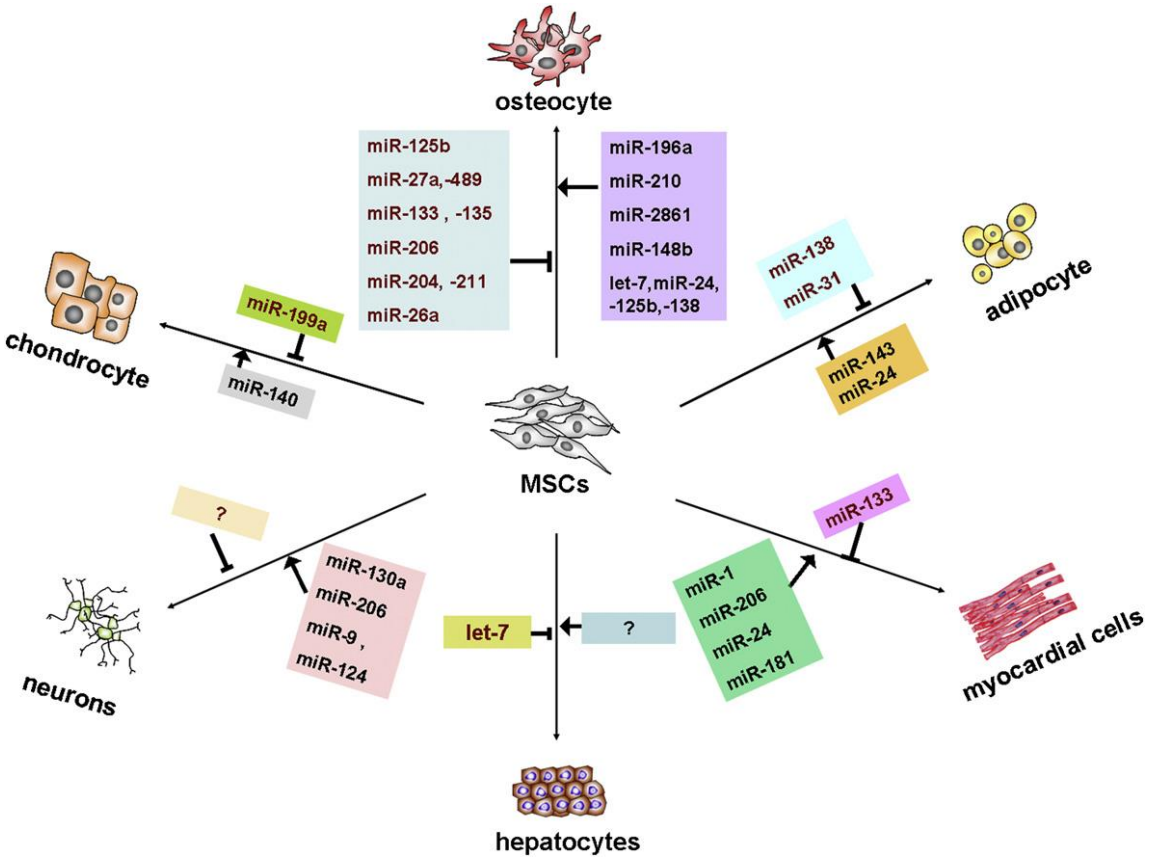


Figure 29. The role of miRNAs in MSC differentiation. Specific sets of miRNAs can be involved in the regulation of both ASC differentiation into mesodermal cell lineages, including osteoblasts, chondrocytes, adipocytes and myocytes, and differentiation into cells of non-mesodermal origin, including hepatocytes and neurons [159].

Given the importance of ASC proliferation and differentiation in the setting of tissue repair, the evaluation of a panel of miRNAs able to regulate these cellular events could be extremely useful prior to clinical application. At the same time, since *ex vivo* expansion is an indispensable step to acquire sufficient amounts of ASCs for clinical therapies, the analysis of miRNAs potentially involved in replicative senescence and ASC aging has been considered a fundamental step to design novel strategies aimed at boosting ASC efficacy [181, 182]. The regulatory action of miRNAs is also exerted through immunomodulation and in the regulation of inflammation. Indeed, several studies have shown that miRNAs are able to regulate macrophage polarization and subsequently the inflammatory process [183]. Several studies proved that miRNAs secreted from ASCs can be delivered to target cells where they are taken up and induce substantial changes in gene expression. So, it can be envisioned a novel mechanism of intercellular communication mediated by secretory

miRNAs [184]. Indeed, there is growing evidence that secretory miRNAs play a role in a variety of cellular events, such as proliferation, differentiation, angiogenesis, inflammation and immune response, and transfer of genetic information between ASCs and adjacent cells appears to be an important mechanism responsible for ASC paracrine effect in tissue repair/regeneration [159, 185].

Since microvesicles released by MSCs protect their cargo from degradation, an innovative avenue for cell-free therapy is represented by the delivery of therapeutic bioactive molecules, in particular miRNAs, through MSC secretome.

1.8.4 MiR-125b-5p

The members of miR-125 family have two different variants of mature miRNAs: the 5p variant and the 3p variant. Both originate from the same pre-miRNA, although miR-125-5p is generally more expressed compared to miR-125-3p [186]. In humans, three homologs compose the miR-125 family: hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2 [187], with mature miR-125b originating from the last two homologs. MiR-125b-5p is located on chromosomes 11q23 and 21q21 loci.

This miRNA has been shown to possess a variety of biological properties, both in physiological and pathological conditions/settings [188]. MiR-125b-5p has also been studied in tumor cells, for example acting as an onco-suppressor in breast cancer cells where it regulates proliferation [189] or in hepatocellular carcinoma, inhibiting metastasis formation [190].

In addition to its action on cancer cell proliferation, miR-125b-5p has also been studied in relation to stem cell proliferation and differentiation. Indeed, it has been shown to inhibit MSC osteoblastic differentiation through the downregulation of cell proliferation [191, 192] and to induce rat BMSCs differentiation into neuron-like cells [193]. Furthermore, miR-125b-5p has been shown to be upregulated during human adipogenesis, suggesting its role as a potential regulator of adipocyte differentiation [194]. So, miR-125b-5p represents an excellent example of a microRNA able to regulate both proliferation and differentiation. Moreover, it has been also investigated for its effects on macrophage polarization and subsequent modulation of inflammation and immune response [195].

As previously described, ASC can release a large number of vesicles carrying miRNAs to target organs, and such genomic information transfer is responsible for some of the known paracrine effects of ASC secretome. Several reports demonstrated the ability of MSCs to secrete miR-125b-1-3p in exosomes, and to regulate through this miRNA some key events related to their therapeutic efficacy. Indeed, exosomal miR-125b is known to ameliorate ischemic acute kidney injury and promote renal tubular repair by targeting the p53 pathway [196]. Similarly, BMSC-derived exosomes carrying miR-125b protect against myocardial ischemia reperfusion injury by targeting SIRT7 [197] and improve autophagic flux determining beneficial effects after myocardial infarction [198]. MiR-125b also plays a central role in the perivascular environment, allowing intercellular communication between MSCs and vascular smooth muscle cells (VSMCs). Specifically, exosomal transfer of miR-125b from MSCs to VSMCs is able to inhibit VMSCs proliferation and migration *in vitro* and neointimal hyperplasia *in vivo* by repressing Myo1e, indicating that miR-125b may be a therapeutic target in the treatment of vascular diseases [199].

Given the key role of miR-125b in mediating the effects of MSC secretome, several strategies have been introduced to potentiate the release of this miRNA. For example, miR-125b-enriched vesicles produced in ASCs genetically modified with a lentiviral vector expressing miR-125b have been shown to reduce hepatocellular carcinoma cell proliferation *in vitro* by modulating the p53 signaling pathway [200]. Alternatively, MSC preconditioning can be adopted as a tool to increase miR-125b secretion. Indeed, exosomes secreted from hypoxia-conditioned MSCs were enriched with miR-125b-5p and showed an increased antiapoptotic effect on ischemic cardiomyocytes *in vitro* and *in vivo* via suppressing p53 and BAK1 [201]. Alternatively, IFNγ treatment has been demonstrated to increase the levels of miR-125a and miR-125b in exosomes, improving their ability to attenuate colitis through repression of Th17 cells differentiation [202].

1.9 Neuropilin 1

Neuropilin 1 (NRP1), with the NRP2 isoform, is part of an important receptors family, in particular for class III semaphorins (Sema3) and the growth factor VEGF [203, 204].

On a structural level (Figure 30), NRP1 is composed by:

- An intracellular cytoplasmic domain without an intrinsic enzymatic activity, but capable of interacting with several proteins, including myosin heavy chain proteins, focal adhesion (FA) proteins and PDZ proteins. The latter are important in the formation of the signal complex and in maintaining the structural integrity of transmembrane proteins, such as NRP1 [205].It is also believed that the cytoplasmic domain is important in the pro-angiogenic activity of NRP1, for example through the binding of FA proteins such as Filamin-A (FlnA);
- A transmembrane domain involved in dimerization, essential for the activity of NRP1 co-receptor itself and for the definition of the ligand [206];
- An extracellular domain that consists of two homologous domains to the complement (CUB or a1/a2 domains) and two homologous to coagulation factors V and VIII (b1/b2 domains), all essential for the definition of specificity to the different Sema3 [203, 204].

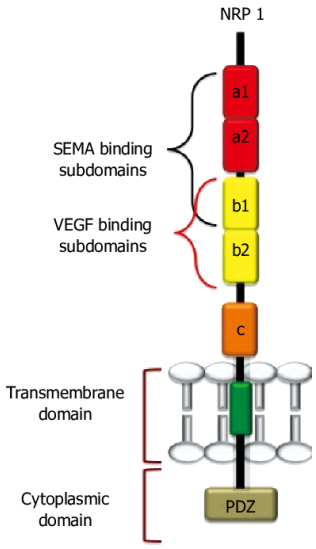


Figure 30. Structure of NRP1.

NRP1 is expressed in a wide variety of cells, including endothelial cells, neurons, pancreatic cells, hepatocytes, melanocytes, osteoblasts [207] and epithelial cells [208]. Furthermore, NRP1 is also expressed by MSCs, where it regulates PDGF-mediated signaling, important for MSC recruitment for vascular development and tissue remodeling [209]. Finally, NRP1 is also expressed by adipocytes, where it regulates their biological activity and influences

their ability to modulate hematopoiesis [210, 211]. The main role of NRP1 is as co-receptor that mediates the activation of the receptor by the specific ligand by interacting simultaneously with both proteins. It has been hypothesized that NRP1 concentrates soluble ligands on cell membrane, increasing their availability for interaction with their respective receptors. However, an alternative hypothesis suggests that NRP1 contributes to the endocytosis of the receptor complex, which in some cases stimulates signal transduction [212, 213].

Moreover, it has recently been demonstrated that NRP1 is also able to bind both free extracellular miRNAs and circulating Ago2-bound miRNAs, and to mediate their internalization without compromising their functionality (as frequently observed for some miRNAs due to degradation or enclosure in an organelle following translocation into the cytoplasm) [214].

Thanks to this function, NRP1 plays an important role in intercellular signaling mediated by miRNAs, although this type of cell communication is necessarily limited to NRP1 expressing cells. Such considerations open the way to novel strategies based on the modulation of NRP1 expression to permit or deny miRNA-mediated regulation of important cellular processes, such as proliferation and differentiation.

2 AIM OF THE THESIS

ASCs are multipotent stem cells that can be isolated from the SVF of adipose tissue. Unlimited self-renewal, immunomodulatory, differentiative and trans-differentiative abilities are characteristics that make these cells important in the processes of organogenesis, remodeling and tissue repair. Since their discovery, more than 15 years ago, significant strides have been made in the attempt to use these cells as a therapeutic tool. Among the various application areas, the most common ones concern regenerative therapy and reconstructive surgery, which may be necessary in the clinical conditions characterized by a large tissue void inside the subcutaneous fat layer, caused by traumatic injuries, tumor resection or congenital anomalies [93, 94]. However, an in-depth analysis of the mechanisms that regulate their biological processes and an improvement of cell expansion protocols is still needed because they would help to achieve fully efficient ASC-based treatments. Therefore, being able to enhance and modulate the characteristics of ASCs *in vitro* is particularly important for overcoming these complications, allowing to be able to manage cell cultures of adipose derivation in an easier and standardized way in the future. Aiming at shedding light on these aspects, my research has focused on verifying the possible ameliorative effects on ASCs using an epigenetic pharmacological approach. Epigenetic mechanisms are crucial for the regulation of gene expression in adult stem cell biology. Therefore, we investigated the effects of a demethylating agent, 5-azacytidine (5-aza), on adipogenic proliferation, migration and differentiation of human ASCs, trying to identify the molecular pathways involved in these processes. This epipharmaceutical with hypomethylating action was tested as an epigenetic modifier on ASCs isolated from lipoaspirate samples, taken from a heterogeneous group of healthy donors. Following a careful phenotypic and morphological characterization of the cells, we carried out various analysis obtaining useful data that allow us to attribute an inhibitory role to 5-aza on cell proliferation and migration, combined with a remarkable inducing effect on adipogenic differentiation. This approach would allow to obtain *in vitro* a cell population more suitable for *in vivo* transfer, avoiding uncontrolled proliferation and anomalous migration phenomena, in favor of the differentiation ones, more useful in reconstructive surgery contexts.

While evaluating the effects of the above mentioned epipharmaceutical on various biological processes of ASCs, my research has also focused on the study of their secretome. Considering the limitations both as regards standardized culture protocols, and the regulatory procedures to be followed for cell-based therapies, the possibility of exploiting a secondary product of ASCs, i.e. their secretome, represents a precious opportunity. Indeed, setting up cell-free treatments would allow to overcome the complications related to the *in vitro* culture processes of ASCs, such as the acquisition of a senescent state after several passages *in vitro* or the need for an initial high number of cells in preparation of a transplant, as well as the limitations due to their use *in vivo*, such as low engraftment yield or migration to the target site once transplanted/injected, the potential formation of cell aggregates depending on the method of administration, or the possible uncontrolled proliferation, while, at the same time, maintaining the powerful immunomodulatory effect of these cells. Furthermore, the possibility of modulating ASCs secretome profile in order to enhance its anti-inflammatory or immunoregulatory capacity, for example, would help to increase the effectiveness of ASCs therapeutic potential through a paracrine effect on the surrounding microenvironment once administered. Another key aspect to consider regarding the paracrine effect of ASC is the important role of microRNAs in influencing stem cell properties, such as self-renewal and pluripotency, and their regulatory action on immunomodulation and inflammation. Several studies, in fact, proved that miRNAs secreted from ASCs can be delivered to target cells and induce changes in gene expression. So, microRNAs can be considered as players in a novel mechanism of intercellular communication between ASCs and surrounding cells responsible for their paracrine effect in tissue repair/regeneration. Therefore, we analyzed the immunomodulatory effects of a miR-125b-enriched secretome of human ASCs, trying to determine the modulation of its cytokines profile. This enriched secretome was tested on co-coltures with PBMCs in order to evaluate potential changes in their activation. Moreover, through silencing experiments we aimed to determine the mediator for circulating miR-125b cellular uptake. The data obtained allow us to attribute a key role as miR-125b receptor to Neuropilin 1, a co-receptor expressed in a wide variety of cells. Moreover, we demonstrated an enhanced immunomodulatory effect of a miR-125b-enriched secretome.

We hope that the results obtained from this research will help to better understand the effects induced by demethylating agents, such as 5-aza, on the biology of ASCs. The introduction of such treatments into culture protocols could be useful for the expansion of stem cell populations with specific characteristics, depending on the clinical area of interest. We also hope that our results will contribute to the development of new cell-free, secretome-based strategies to harness the paracrine potential of ASCs.

.

3 MATERIALS AND METHODS

3.1 Ethics Approval and Consent to Participate

The use of clinical samples of adipose tissue for ASCs isolation complied with the Declaration of Helsinki 1975, revised in 2008, and the study methodologies have been approved by the Institutional Review Board of the Department of Experimental Medicine of Sapienza University of Rome. Eight healthy donors (age range 45-63 years) who underwent elective abdominal surgery (liposuction) for generally aesthetic reasons were enrolled. None of the donors had systemic disease or diabetes or were taking drugs that could affect fat mass and metabolism. All donors were asked to sign an informed consent containing all the information regarding the intervention and the intended use of the donated biological material. The patient's right to withdraw his consent to the use of biological material at any stage of the study was also reaffirmed, without any impact on the therapeutic course.

Blood donations for PBMCs isolation were obtained from five screened healthy volunteers under informed consent for research use, approved by the Institutional Review Board of the Department of Experimental Medicine of Sapienza University of Rome.

3.2 ASC Isolation and Culture

Liposuction aspirates of enrolled donors were transferred to the laboratory and processed under sterile conditions within 24 h. Isolation of ASCs was performed as follows (Figure 31). Liposuction aspirates were washed extensively with sterile phosphate-buffered saline (PBS; Aurogene, Rome, Italy) containing 2% penicillin/streptomycin and minced. The extracellular matrix was digested with 0.075% collagenase Type I (Gibco, Paisley, UK) for 30–60 min at 37°C and 5% CO_2. The suspension was filtered to remove debris and centrifuged for 5 min at 2000 rpm. The pellets of stromal vascular fraction (SVF) containing ASCs were washed with PBS, then resuspended in the culture medium and transferred to a T75 culture flask coated with collagen Type IV (Sigma-Aldrich, Milan, Italy). ASCs were self-selected out of the SVF since they were adherent to the plastic tissue culture-ware (Figure 31). ASC were cultured in DMEM-Ham's F-12 medium (vol/vol, 1:1) (DMEM/F12; Gibco) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2

mM L-glutamine, and maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere, with medium change twice a week. When reaching 80–90% confluence, cells were detached with 0.5 mM EDTA/0.05% trypsin (Euroclone, Milan, Italy) for 5 min at 37°C and then replated. ASCs were expanded and cell viability was assessed by using the trypan blue exclusion assay. Cell morphology was evaluated by phase contrast microscopy. Experiments were conducted between passage numbers 3 and 6, unless otherwise specified. Absence of mycoplasma contamination was confirmed by PCR with specific primers.

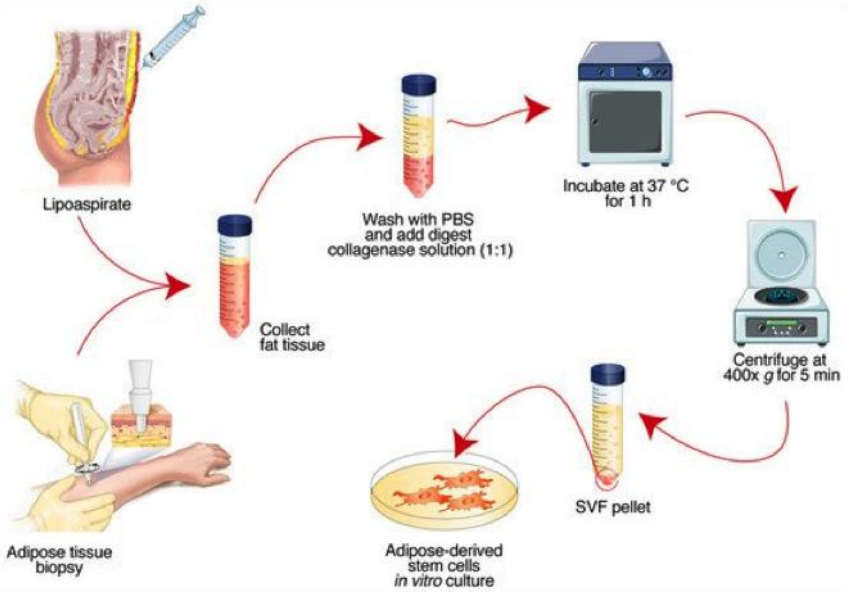


Figure 31. ASC isolation protocol from lipoaspirate. The tissue is washed and enzymatically digested with collagenase, then centrifuged to obtain SVF. Through the subsequent passages in culture, ASCs are selected thanks to their ability to adhere to the substrate [137].

3.3 Cell Treatments

The DNA methyltransferase inhibitor 5-Azacytidine (5-aza) was purchased from Sigma-Aldrich and was reconstituted at 10 mM using dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA). For 5-aza pretreatment, the medium was changed to a freshly made culture medium containing 10 μM 5-aza. After 24 h, the 5-aza containing culture medium was refreshed for additional 24 h (total treatment 48 h). DMSO alone was used as control at 0.1% (v/v) concentration. Cells pretreated with 5-aza or DMSO were cultured in standard medium for 72 h and then subjected to analyses, unless otherwise specified.

When indicated, 5-aza pretreatment was performed in the presence of the mTOR inhibitor rapamycin (0.5 mM; Sigma-Aldrich) or of human recombinant Wnt3a (100 ng/ml; R&D Systems, Minneapolis, MN, United States).

3.4 Cell characterization and apoptosis by flow cytometry

A classic test for the identification of a cell population is phenotyping by looking for cellular markers called clusters of differentiation (CD). To identify the phenotypic profile of ASCs, cells at passage three were subjected to flow cytometric analyses by using a FACSCalibur cytometer (BD Biosciences, San Jose, CA, United States). Briefly, cells were harvested, centrifuged, and fixed for 30 min in ice-cold 2% paraformaldehyde. The single-cell suspensions were washed in flow cytometry buffer containing PBS, 2% FBS and 0.2% Tween 20, then incubated for 30 min with the following monoclonal antibodies, conjugated to fluorescein isothiocyanate, phycoerythrin, or phycoerythrin-Cy5 (BD Biosciences): PE-Cy5 Mouse Anti-Human CD29 (Cat. No. 559882), PE Mouse Anti-Human CD34 (Cat. No. 555822), FITC Mouse Anti-Human CD44 (Cat. No. 560977), FITC Mouse Anti-Human CD45 (Cat. No. 561865), PE-Cy5 Mouse Anti-Human CD90 (Cat. No. 561972), and PE Mouse Anti-Human CD166 (Cat. No. 560903). All monoclonal antibodies were of the IgG1 isotype. Nonspecific fluorescence was determined by incubating the cells with conjugated mAb anti-human IgG1 (DakoCytomation, Glostrup, Denmark).

Flow cytometric analysis was also used for the evaluation of apoptosis. During the initial phases of the apoptotic process phosphatidylserine (PS) translocates from the inner side of the membrane to the outer one. PS can be identified by flow cytometry using labeled Annexin V, a protein capable of binding with high affinity to PS. Using Annexin V staining in combination with a nucleic acid binding dye that is able to discriminate between living and dead cells, such as 7-Amino-actinomycin D (-7AAD), it is possible to obtain the following profile: viable cells are negative for both markers, those in the early stages of apoptosis are positive for Annexin V and negative for 7-AAD and, finally, those in late apoptosis are positive for both markers. Apoptosis was analyzed by using Annexin A5 FITC/7-AAD Kit (Beckman Coulter), following the manufacturer's instructions. Briefly, approximately 2×10^5 cells were stained with Annexin A5 FITC and 7-AAD for 15 min at room temperature in the dark. Fluorescence intensities were collected with a CytoFLEX flow cytometer (Beckman Coulter, Germany). Quadrant analysis was performed using the

Kaluza software (Beckman Coulter) to quantify viable cells (Annexin A5-negative/7-AAD-negative), early apoptotic cells (Annexin A5-positive/7-AAD-negative), and late apoptotic cells (Annexin A5-positive/7-AAD-positive).

3.5 Immunofluorescence (IF) Analysis

Immunofluorescence analysis was used for further phenotypic characterization, to assay the expression of two specific cluster of differentiation markers (CD). IF was performed as follows. Cells grown on coverslips onto 24-well plates were fixed in 4% paraformaldehyde for 30 min at room temperature, followed by treatment with 0.1 M glycine (Sigma-Aldrich) in PBS for 20 min and with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for additional 5 min to allow permeabilization. Cells were then assayed for the expression of specific cluster of differentiation (CD) markers by incubation with primary antibodies to CD29 (Cat. No. 303001; 1:100 dilution; BioLegend, San Diego, CA, United States) and CD166 (Cat. No. 397802; 1:20 dilution; BioLegend).

Immunofluorescence analysis was also conducted to visualize actin cytoskeleton stress fibers and focal adhesions, by incubation with TRITC-Phalloidin (Cat. No. P1951; 1:100 dilution; Sigma-Aldrich) and with antibodies to vinculin (Cat. No. sc-73614; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, United States), respectively.

Also, to assess β-catenin localization and Lamin B1 expression, cells were processed as described above and incubated with antibodies to β-catenin (Cat. No. sc-6973; 1:20 dilution; Sigma-Aldrich) or Lamin B1 (Cat. No. ab65986; 1:100 dilution; Abcam, Cambridge, UK). To evaluate DNA damage, cells were fixed and permeabilized as previously described and incubated with antibodies to phospho-histone H2A.X (Ser139) (γH2AX) (Cat. No. 2577; 1:100 dilution; Cell Signaling Technology, Danvers, MA, United States).

Finally, we performed immunofluorescence to assess the expression of adipogenic markers. Cells seeded on coverslips onto 24-well plates and subjected to adipogenic differentiation were fixed after 3, 7, 14 and 21 days from adipogenic induction, processed for IF as described above and then incubated with antibodies to FABP4 (Cat. No. 967799; 1:10 dilution; R&D Systems). After washing in PBS, primary antibodies were visualized using FITC-conjugated goat anti-mouse IgG (Cappel Research Products, Durham, NC, United States) or Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch

Laboratories, West Grove, PA, United States). Nuclei were visualized using 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). Nonspecific fluorescence was determined by omitting primary antibody. The single stained and merged images were acquired with a Zeiss ApoTome microscope (40x magnification) using the Axiovision software (Carl Zeiss, Jena, Germany). The percentage of FABP4-positive cells was determined by counting cells in at least six random microscopic fields for each condition.

3.6 Proliferation Assays

3.6.1 Growth Curve Analysis

To evaluate the 5-aza effect on ASC growth, cells pretreated or not with 5-aza for 48 h were seeded into 24-well plates at a density of 1×10^4 cells/well and incubated at 37°C and 5% CO_2 for 4 days. The number of cells was counted daily (three wells in each time per group). The mean number of cells at each counting time was plotted. The population doubling time (PDT) was calculated using the formula: PDT=T×log2/(logN1-logN2), where T is the number of days for incubation, N1 and N2 are the cell numbers determined at the beginning and end of the incubation time, respectively.

3.6.2 Colony Formation Assay

The clonogenic assay is useful for establishing the toxicity of a certain substance by measuring its effect on cell growth. The method involves plating a small number of cells and letting them grow forming clones after being incubated with the substance under analysis. So, cells pretreated or not with 5-aza were seeded in 6-well plates in triplicate at a density of 1×10^3 cells/well and incubated at 37°C and 5% CO_2 for 14 days to allow colonies to grow, with medium change every 3 days. Colonies were fixed with methanol, stained with 0.1% crystal violet for 15 min at room temperature (RT) and photographed. Then, crystal violet was solubilized in 30% acetic acid in water for 15 min at RT, and absorbance was measured using the Biochrom Libra S22 UV/VIS spectrophotometer (Biochrom, Berlin, DE) at a wavelength of 595 nm. 30% acetic acid in water was used as blank control. Colony formation capacity in 5-aza-treated cells was calculated in comparison to control samples (DMSO), arbitrarily set to 1.

3.7 Migration Assays

3.7.1 Scratch Test

Cells were seeded in a 6-well plate at a density of 1.0 x 10^5 cells/well and grown until confluence, then treated with 10 µM 5-aza or DMSO for 48 h. Scratch test was performed as follows. A standardized cell-free area was introduced by scraping the confluent monolayer with a sterile tip. After intensive wash, cells were incubated for 24 h in serum-free DMEM/F12. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. Some plates were fixed and photographed immediately after scratching representing the T0 control sample. Migration was quantitated by a measure of the % recovered scratch area after 24 h, performed using ImageJ software (v. 10.2).

3.7.2 Transwell Assay

Cell migration was assayed with 8-µm-pore size Transwell migration chambers (Corning, Corning, NY, USA) in 24-well plates. DMEM containing 10% FBS was added in the lower chambers. Cells were trypsinized and seeded in the upper chambers at a density of 1 x 10^4 cells/upper chamber, maintained in 200 µL of α-MEM containing 2% FBS. Cell migration was allowed to proceed for 6 h at 37°C and 5% CO_2. Cells attached to the membrane in the upper chamber were gently removed with a cotton swab, whereas cells attached to the membrane in the lower chamber were fixed with ice-cold methanol for 20 min at -20 °C and stained with 0.1% crystal violet for 5 min at room temperature (RT). Dried membranes were cut out, mounted on glass slides and photographed. Migrating cells were counted in at least six high power fields for each experimental condition.

3.8 Adipogenic Differentiation

When reaching 100% confluence, cells were treated as described above and then incubated for 3, 7, 14 or 21 days with adipogenic differentiation medium (StemXVivo® Osteogenic/Adipogenic Base Media; R&D Systems) supplemented with the respective media supplement (StemXVivo® Adipogenic Supplement; R&D Systems) to induce adipogenesis.

3.9 Oil Red O Staining

Cells were fixed in 10% formalin for 30–60 min at room temperature, incubated in 60% isopropanol for 5 minutes and stained with Oil Red O solution (cat. No. O0625, Sigma-Aldrich) for 5 min. The images were acquired with AxioVision software (Carl Zeiss, Jena, Germany) using a 20x objective lens. The stained oil droplets were then treated with isopropanol to elute Oil Red O dye, and the absorbance was quantified at 490 nm. Oil red O staining in 5-aza-treated cells was calculated in comparison to control samples (DMSO), arbitrarily set to 1.

3.10 Senescence Associated β-Galactosidase Staining

Cellular senescence was visualized and quantified by measuring the activity of senescent-associated β-galactosidase (β-Gal) with Senescence β-Galactosidase Staining Kit (Cell Signaling Technology), according to the manufacturer's instructions. Briefly, cells in a 6-well plate were washed with PBS and fixed with the Fixative Solution for 15 min at room temperature, followed by staining with the β-Gal Staining Solution. Cells were incubated for 24 h at 37°C in a dry incubator and then photographed. The percentage of β-gal positive cells was calculated by counting cells in three images from each of triplicate wells for each experimental condition.

3.11 Protein Extraction and Western Blot Analysis

Cells were processed for protein extraction through the following procedure: after washing in PBS, cells were lysed in buffer containing 50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, supplemented with inhibitors protease and phosphatase (Roche, Diagnostics, Monza, MB). After incubation on ice for 30 min and centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant containing the total protein extract was recovered for each sample. The Bradford assay was used to determine the protein concentration. Total proteins (30–100 µg) were resolved under reducing conditions by 7–15% SDS-PAGE and transferred to Immobilon-FL membranes (Millipore, Billerica, MA, United States). The membranes were incubated overnight at 4°C with primary antibodies to PPARγ (Cat. No. 2443S), phospho-Akt (Cat. No. 9271S), Akt (Cat. No. 9272S), phospho-ERK (Cat. No. 4370P), ERK2 (Cat. No. 9102), γH2AX (ser139) (Cat. No. 2577) (1:1000 dilution; Cell Signaling),

phospho-mTOR (Cat. No. sc-293133), β-catenin (Cat. No. sc-7963), Cyclin D1 (Cat. No. sc-20044), p53 (Cat. No. sc-126), p21 (Cat. No. sc-6246), cleaved PARP1 (Cat. No. SC-56196), NRP1 (A-12) (1:200 dilution; Santa Cruz), Lamin B1 (Cat. No. ab65986; 1:4000 dilution; Abcam). Primary antibodies were followed by the appropriate horseradish peroxidase (HRP)-conjugated anti-rabbit (1:10000 dilution; Advansta, San Jose, CA, United States) and anti-mouse (1:40000 dilution; Bethyl Laboratories, Montgomery, TX, United States) secondary antibody. β-actin (Cat. No. sc-47778; 1:5000 dilution; Santa Cruz) was used as internal control. Bound antibody was detected using the WesternBright ECL HRP substrate kit (Advansta) according to the manufacturer's instructions. Densitometric analysis was performed with Quantity One Program (Bio-Rad Laboratories S.r.l., Segrate, MI, Italy).

3.12 RNA preparation and quantitative Real-Time PCR (qRT-PCR)

ASCs were harvested and total RNA was extracted using TRIzol reagent (Invitrogen, Milan, Italy). Briefly, Trizol was added to each cell pellet; subsequently, chloroform was added in order to allow, after centrifugation at 12,000 rpm at 4°C, the separation of the solution into an organic and an aqueous phase. The aqueous phase, containing the RNA, was recovered, and the RNA itself was precipitated by adding absolute isopropanol. Then, a wash with 75% ethanol was performed and the precipitated RNA was resuspended in RNase-deprived water, to avoid degradation. Quantity and quality of the extracted RNA were assessed by NanoDrop (Thermo Fisher Scientific, Monza, Italy). Quantitative real-time PCR assays (qRT-PCR) were conducted in triplicate on an ABI 7500 Real Time instrument (Applied Biosystems by Life Technologies), as follows. 1mg of total RNA was reverse transcribed using the High-Capacity RNA to cDNA Kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was diluted 1:5 and then the abundance of specific mRNAs and microRNAs was quantified using the following TaqMan gene expression assay probes (Applied Biosystems by Life Technologies): PPARγ (Hs01115513_m1), c/EBPα (Hs00269972_s1), sFRP-1 (Hs00610060_m1), Axin2 (Hs00610344_m1), p21 (Hs00355782_m1), p53 (Hs01034249_m1), Bcl-2 (Hs04986394_s1), PAI-1 (Hs00167155_m1), IL-6 (Hs00174131_m1), NRP1 (Hs00826128_m1). The amplification was performed in a final volume of 25 µl, using 25 ng

of cDNA as template. A total of 2 µl/well of template was added to the samples together with a 1x concentration of Taqman Universal PCR master mix and water to the volume of 25 µl/well. The temperature conditions for amplification were: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min, for 40 cycles. GAPDH mRNA (Hs02758991_g1) was used as endogenous control.

For microRNA analysis, total RNA from ASCs conditioned medium was extracted using the miRNeasy Serum/Plasma Kit (Cat. N°: 217184, Qiagen, Valencia, CA, USA) following manufacturer's instructions. For miR-125b-5p analysis, 40 ng of RNA was reverse transcribed with miR-125b-5p specific primers (Thermo Fisher Scientific). The expression of miR-125b-5p was analyzed using sequence specific TaqMan MicroRNA Assays (has-miR-125b-5p, 000449; Applied Biosystems). Nuclear RNA (U6) levels were used as an internal control and miR-21 (has-miR-21-5p, 477975_mir; Applied Biosystems) was used as extracellular control.

3.13 Collection of ASC-Conditioned Medium (ASC-CM)

ASC were seeded in a 100 mm dish and treated with DMSO or 5-aza for 48 h in growth media. The medium was replaced with serum-free medium. After 72 h, the supernatant was collected, centrifuged for 10 min at 1500 rpm, filtered to eliminate any debris and stored at -20°C for further analyses. Then, ASC-CM was used at varying dilutions (0, 10, 50 and 100%).

3.14 Transfection with miR-125b-5p Mimic

ASCs were plated in a 6-well plate at a density of 1.5 x 10^5 cells/well. After 24 h, cells were transfected in triplicates with 40 nM of mimic miR-125b-5p or mimic control oligonucleotides, purchased from Sigma Aldrich. Compounds were delivered into the cells with DharmaFect Duo transfection reagent (GE Dharmacon), according to the manufacturer's instructions. Medium was changed after 24 h. After 48 h, cells were harvested for further experiments and the miR-125b-enriched ASC-CM was collected as previously described and stored at -20°C for further experiments. The transfections were repeated at least three times.

3.15 NRP1 Silencing by Specific siRNA

Interfering RNA that specifically reduces NRP1 gene expression (siNRP1) and control interfering RNA, which does not target any known mRNA (siCTRL) were purchased from Santa Cruz Biotechnologies. Cells were plated in 6-well plate at a density of 1.5 x 10^5 cells/well and transfected with siRNA to a final concentration of 50 nM, using HiPer-Fect transfection reagent (Qiagen) according to the manufacturer's instructions. The NRP1 silencing efficiency was evaluated by Western Blot and qRT-PCR.

3.16 Cytokine Array

ASC-CM obtained from cells treated or not with 5-aza, as previously described, was tested for protein secretion by Human XL Cytokine Array Kit (Cat. No. ARY005B; R&D Systems). Membranes were treated and analyzed according to manufacturer protocol. Densitometric analyses were performed with Quantity One Program (Bio-Rad Laboratories S.r.l., Segrate, MI, Italy).

3.17 PBMC Isolation and Culture

Whole blood samples (5 mL) of healthy donors were collected and PBMCs were isolated as follows. Whole blood samples were diluted 1:1 (v/v) with PBS, and Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada) was carefully added. The solution was centrifuged for 25 min at 1800 rpm at room temperature in order to separate red blood cells in a lower phase, Lymphoprep™ containing peripheral blood mononuclear cells (PBMCs) in an interphase, and serum in an upper phase. The serum contained in the upper phase was discarded and the interphase containing PBMCs was collected, washed with PBS and centrifuged for 5 min at 1500 rpm. The supernatants were discarded, and the pellets were washed with PBS and centrifuged for 5 min at 1500 rpm three times. Then, the pellets were resuspended in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine.

3.18 Evaluation of cytokines production in PBMCs

PBMCs were plated in 24-well plates in RPMI with 10% FBS at a density of 5 x 10^5 cells/well. Cells were left untreated or stimulated with either 1 μg/mL of Purified Mouse Anti-Human

CD3 (Cat. No. 550368; BD Pharmingen™, San Jose, CA, United States) in PBS and 3 µg/mL of Purified Mouse Anti-Human CD28 (Cat. No. 555726; BD Pharmingen™) in PBS. PBMCs were then cultured with naïve ASC-CM or with miR-125b-enriched ASC-CM at 37°C and 5% CO_2 for 48 h. After this period, cells were stained with APC Mouse Anti-Human CD4 (Cat. No. 561840, BD Pharmingen™) and Pacific Blue™ Mouse Anti-Human CD8 (Cat. No. 558207, BD Pharmingen™) for 30 min at 4°C. Then, cells were permeabilized with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD 554715, Fisher Scientific, Rodano, Italy) following manufacturer's instructions, to accumulate most cytokine proteins in the Golgi complex and enhance cytokine staining signals. Intracellular staining was then performed for 30 min at 4°C using FITC Rat Anti-Human IL-2 (Cat. No. 559361, BD Pharmingen™) and PE-Cy™7 Mouse Anti-Human IFN-γ (Cat. No. 560924, BD Pharmingen™). Cells were fixed with 1% paraformaldehyde (PFA) and acquired on a CytoFLEX flow cytometer (Beckman Coulter, Germany). The acquisition was performed both on samples stimulated with anti-CD3/anti-CD28 and on non-stimulated samples, in order to estimate the residual intracellular expression of the cytokines.

3.19 Statistics Analysis

Data were analyzed on Prism 8.0 (GraphPad Software, La Jolla, USA) and are shown as mean ± SD from three independent experiments conducted in triplicate, with cells obtained from at least two different donors. Two-tailed unpaired Student's t test was used for statistical analysis. P values < 0.05 were considered statistically significant.

4 RESULTS

4.1 Phenotypic Characterization of ASCs

In this thesis work I used an *in vitro* model represented by cultured ASCs obtained from adipose tissue. Cultured ASCs were selected on the basis of their adherent property and subsequently characterized in order to assess their purity and to exclude the presence of contaminating elements, such as hematopoietic stem cells. Taking into consideration the current lack of identification methods specific for ASCs, we referred to the newest joint declaration of the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy [215] which suggests ASC identification through assessment of tissue origin, cellular morphology, surface markers and multi-differentiation potential. Particularly important is selecting and identifying the surface markers of ASCs, as the surface markers of stem cells transform over successive generations of cells. Therefore, we selected some markers according to a number of global experimental reports [216, 217]. Among them, CD34 and CD45 have commonly been used as surface markers for hemopoietic stem cells; thus, a negative result is useful to rule out this type of stem cells. CD29, CD90, CD166 and CD44 are stable markers for ASCs; so, they should be highly expressed in these cells. Indeed, prior to experimental use, ASCs were phenotypically characterized by flow cytometry. FACS analysis of selected CD markers confirmed that 99.46, 97.82, 99.70 and 96.63% of ASC expressed the mesenchymal markers CD29, CD166, CD90 and CD44, respectively, while only 2.3 and 9.5% of ASC were positive for the expression of the hematopoietic markers CD34 and CD45, respectively (Figure 32A). We also evaluated morphologically ASCs obtained from adipose tissue. They displayed a typical fibroblast-like, spindle-shaped morphology *in vitro* (Figure 32B). The expression of CD29 and CD166 was also confirmed by IF analysis (Figure 32C).

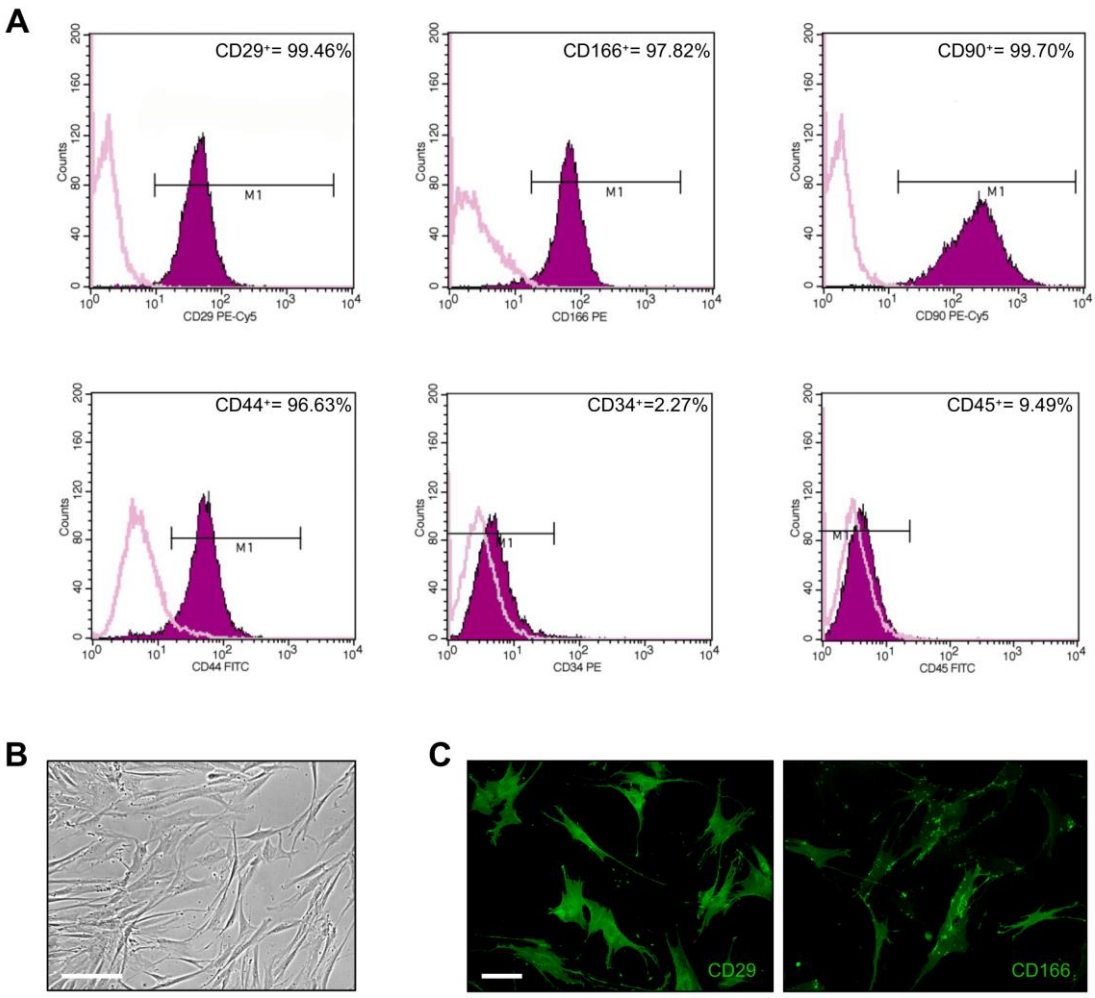


Figure 32. Phenotypical characterization of ASCs. (A) Flow cytometric analysis of ASC cells stained with monoclonal antibodies directed against the mesenchymal markers CD29, CD166, CD90, and CD44, or the hematopoietic markers CD34 and CD45. Purple areas represent patterns obtained with antibodies against the indicated markers, whereas pink lines represent the isotype-matched monoclonal antibody that served as a control. **(B)** Phase-contrast photomicrograph showing the fibroblast-like morphology of ASCs. Scale bar: 200 µm. **(C)** Representative images of CD29 and CD166 expression in ASCs by IF analysis. Scale bar: 50 µm.

These data confirmed that, through our standardized protocol, it was possible to isolate cells from adipose tissue with self-renewal characteristics, properties of adhesion to the plastic material, ability to differentiate into different cell types (data not shown) and presence of markers specific to the stem-mesenchymal line, and therefore defined as ASC. Furthermore, it has been demonstrated that ASC cultures are represented by a pure and homogeneous population free of contaminating cellular elements such as hematopoietic, endothelial or epithelial cells.

4.2 Effects of 5-aza on ASC viability, proliferation, clonogenicity and migration potential

In order to investigate the effects of the demethylating agent 5-aza in ASCs, cells were pretreated with 10 μM 5-aza or DMSO for 48h, and then cultured for 72 h in standard medium prior to further analyses. ASC density after pretreatment was evaluated by phase contrast microscopy. We noticed a slightly decreased confluence in ASCs pretreated with 5-aza with respect to DMSO-treated cells (Figure 33A). When comparing morphological features of ASCs treated with DMSO or 5-aza we observed that both ASCs exhibited a fibroblast-like morphology, but in the 5-aza group some cells showed enlarged nuclei (arrow) and flat, irregular shape (arrowhead) (Figure 33A, enlargements).

To better assess the impact of 5-aza pretreatment on cell morphology, we evaluated actin cytoskeleton organization by fluorescence staining of F-actin filaments with TRITC-Phalloidin (Figure 33B, red). Such analysis revealed a consistent number of stress fibers, forming long linear bundles, which extended throughout the cell (arrow) in DMSO-treated cell (Figure 33B, enlargements). Treating ASCs with 5-aza, actin stress fibers were significantly decreased in the cell body, and their size was clearly decreased in the cortex (arrowheads) (Figure 33B, enlargements), although the morphology of the cells was not markedly altered. Immunofluorescence staining with Vinculin to visualize focal adhesions [218] (Figure 33C, green) revealed a high number of vinculin-positive focal adhesion complexes on the cell membrane (arrowheads) and particularly on the cell edges (arrow) (Figure 33C, enlargements) of DMSO-treated cells. In contrast, 5-aza-treated cells showed very few focal adhesion complexes. So, cells pretreated with 5-aza showed less actin stress fibers and a reduced number of focal adhesions. Such observations might suggest that 5-aza is able to rearrange cell cytoskeleton towards a less invasive and less organized shape, this potentially favoring a differentiation phenotype rather than a migratory one.

To evaluate the effects of 5-aza on cell proliferation/viability, we also performed a growth kinetics assay. Cells were counted at several time points (days 1, 2, 3, and 4) after seeding, and their number was plotted against the time points, showing a very similar growth rate between DMSO and 5-aza-treated cells (Figure 33D). So, cell growth kinetics revealed no significant differences in the ASC proliferation potential after 5-aza pretreatment. For both

cell treatments, we calculated the mean population doubling time (PDT) on the basis of the exponential growing phase. 5-aza did not show significant effects on PDT (2.54 days vs. 2.61 days of DMSO; Figure 33E). So, this concentration of the drug seemed to show no deleterious effect on cell viability.

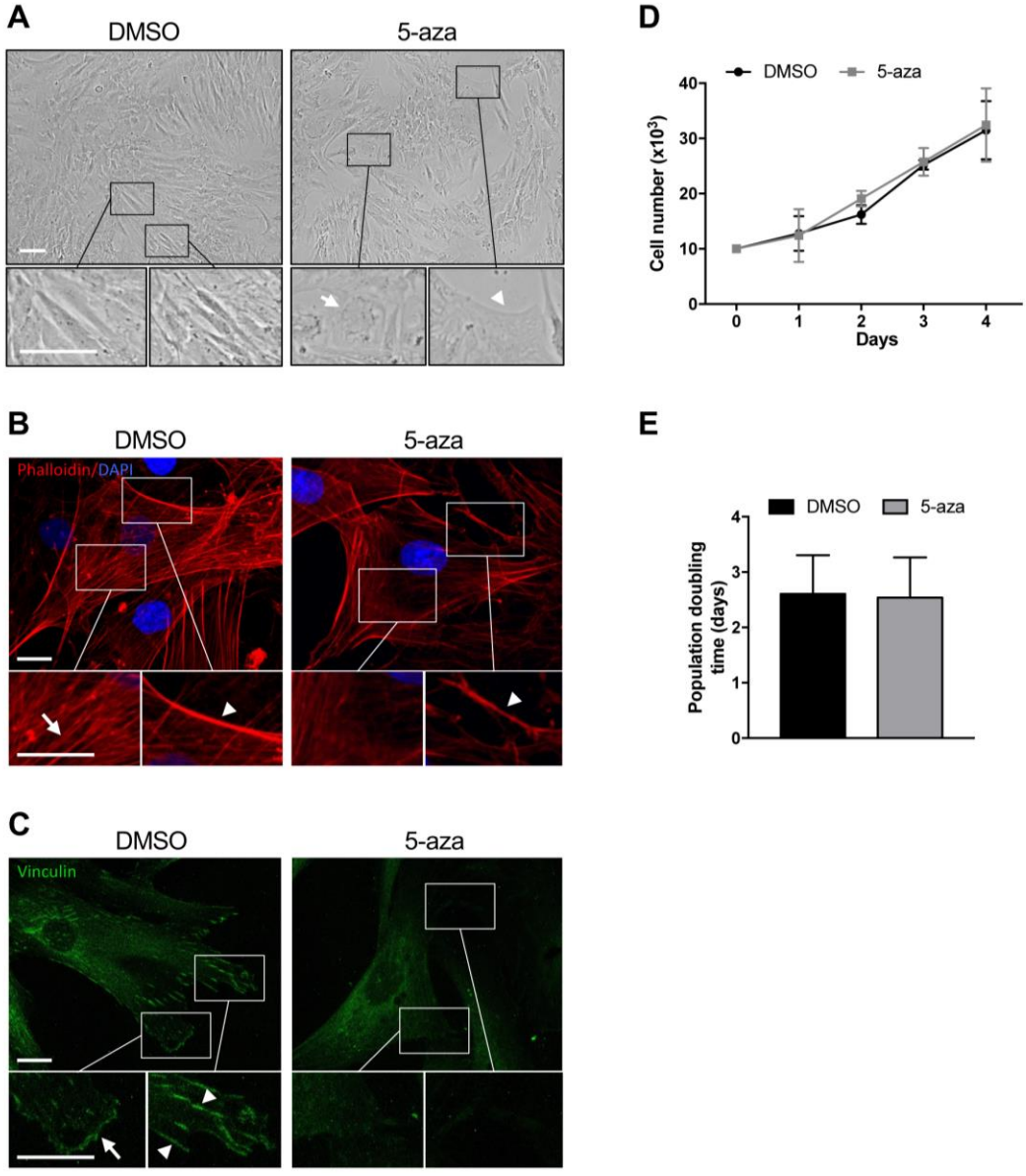


Figure 33. 5-aza affects ASC morphology, actin cytoskeleton distribution and growth. (A) Phase-contrast photomicrographs showing the morphology of ASCs pretreated with 5-aza or DMSO. Representative areas of the full image are presented as enlargements in lower panels. Scale bars: 100 µm. **(B)** Fluorescence images showing phalloidin labeling of actin filaments (red) in ASCs pretreated with 5-aza or DMSO. Nuclei were visualized using 4′, 6-diamido-2-phenylindole dihydrochloride (DAPI) (blue). Representative areas of the full image are presented as enlargements in lower panels. Scale bars: 10 µm. **(C)** Fluorescence images

showing Vinculin labeling of focal adhesions (green) in ASCs pretreated with 5-aza or DMSO. Representative areas of the full image are presented as enlargements in lower panels. Scale bars: 10 µm. **(D)** Growth kinetics of ASCs pretreated with 5-aza or DMSO. **(E)** Population doubling time calculated from the exponential growing phase of ASCs. Bars represent means ± SD of three independent experiments, each performed in triplicate.

The effects of 5-aza on ASC self-renewal capacity were further evaluated through analyzing their ability to form colonies at low-density inoculation, as previously reported [219]. So, we performed a clonogenic assay, which involves plating a small number of cells and letting them grow forming clones after being incubated with DMSO or 5-aza. The cells adhered to the plate were then stained with Crystal Violet, and by measuring the absorbance we could define the quantity of clones that have formed, and calculate the percentage of clonogenicity, verifying whether there are differences between the different treatments. As indicated in Figure 34A, 5-aza pretreatment significantly decreased the amount of ASC colonies, with a 30% reduction in colony forming ability in 5-aza-treated cells relative to DMSO-treated controls.

Then, we decided to assess the effect of 5-aza on ASC migration ability, since migratory potential is strictly related to ASC repair ability *in vivo* [220]. To this aim, we used different migration assays. First, a so-called "scratch assay" was performed for monitoring cell migration. Briefly, a cell-free area was introduced in monolayers of ASCs, pretreated or not with 5-aza, and cells were allowed to migrate from the edge of the scratch for 24 h. As shown in Figure 34B, 5-aza-treated cells showed only a limited migration with respect to control DMSO-treated cells. The graph, representing the percentage of covered scratch area ("wound closure") with respect to 0 h control, highlighted a 25% decrease of migration ability at 24 h in cells subjected to 5-aza pretreatment compared to that in the DMSO-treated cells (Figure 34B).

Then, we analyzed cell migration through transwell chambers. Quantification of cell migration was performed by counting migrated cells, after staining with Crystal Violet. The transwell assay revealed a 46% reduction in the number of migratory cells of the 5-aza group compared to that of the DMSO group within 6 h (Figure 34C), this confirming that 5-aza pretreatment was able to induce in ASCs a reduced response in terms of cell motility.

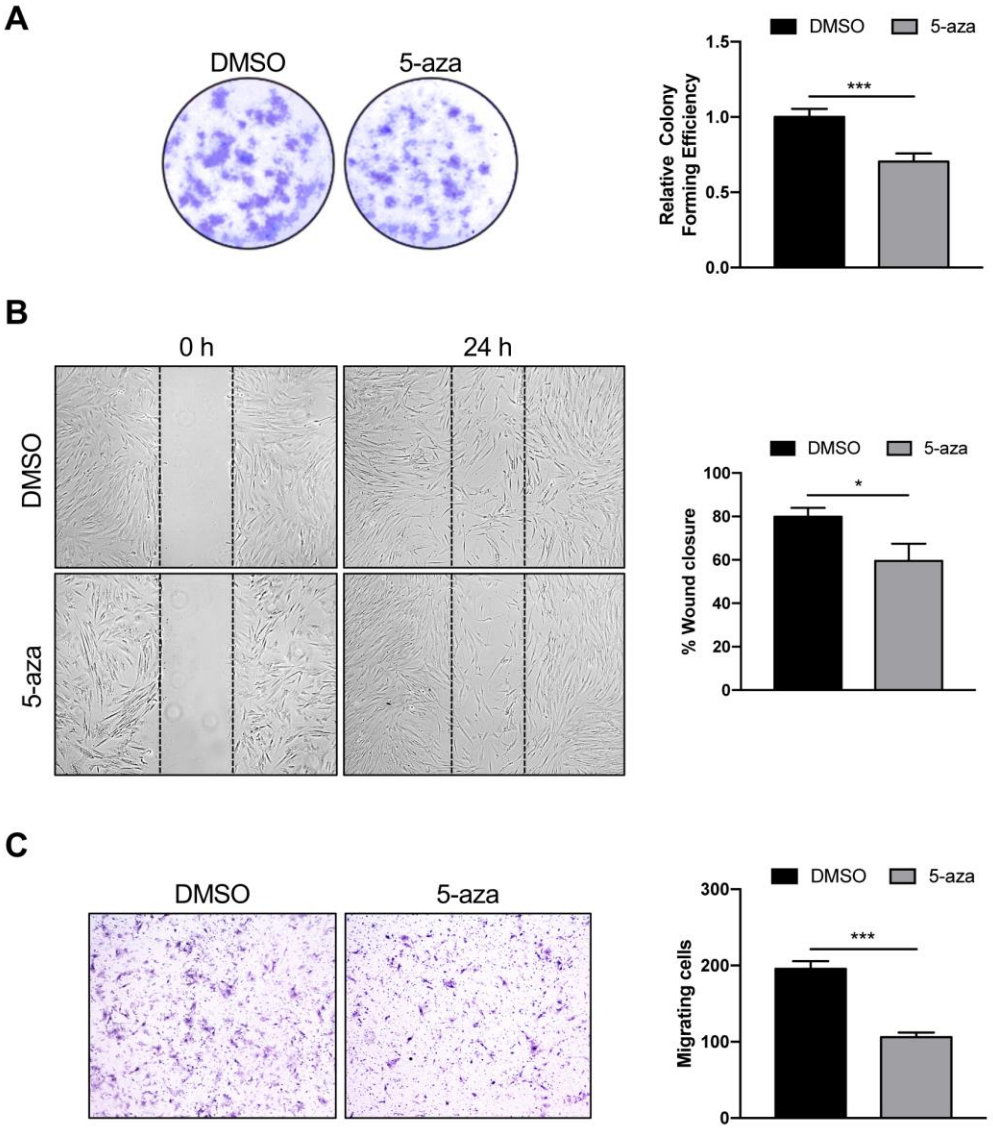


Figure 34. 5-aza affects ASC proliferation and migration. (A) Colony formation assay showing the effect of 5-aza on ASC clonogenic ability. Colonies were stained with crystal violet. Colony forming efficiency was calculated by crystal violet absorbance. **(B)** Scratch test showing the effect of 5-aza on ASC migration. The percentage of recovered area at 24 h was measured by ImageJ software. **(C)** Transwell assay of ASCs pretreated with 5-aza or DMSO. Migrated cells were stained with crystal violet. Migration efficiency was calculated by counting the number of migrating cells. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, ***$p < 0.0005$ vs. DMSO.

4.3 Effects of 5-aza pretreatment on adipogenic differentiation

We investigated the effects of 5-aza on lineage-specific differentiation by evaluating adipogenesis in ASC cultures pretreated or not with 10 μM 5-aza for 48 h before adipogenic

induction. Cells pretreated with DMSO or 5-aza were cultured in Adipocyte Differentiation Medium for 3, 7, 14 or 21 days and differentiation was assessed by evaluation of intracellular lipid accumulation through staining with Oil Red O (Figure 35A) and by the expression of the adipocyte-specific fatty acid binding protein 4 (FABP4) through IF analysis (Figure 35B). 5-aza-treated cells showed a significantly increased accumulation of lipid droplets compared to DMSO-treated cells at Day 3 and Day 7 of induction, as determined by quantification of Oil Red-O staining (+95% and +71% vs. DMSO, respectively) (Figure 35C). The same trend was observed by counting the percentage of FABP4-positive cells at 3 and 7 days (+55% and +49% vs. DMSO, respectively) (Figure 35D). At later times of adipogenic induction, no significant differences between 5-aza- and DMSO-treated cells were observed. Interestingly, 5-aza pretreatment seemed to increase the size of lipid droplets at every time of induction (Figure 35A, inserts).

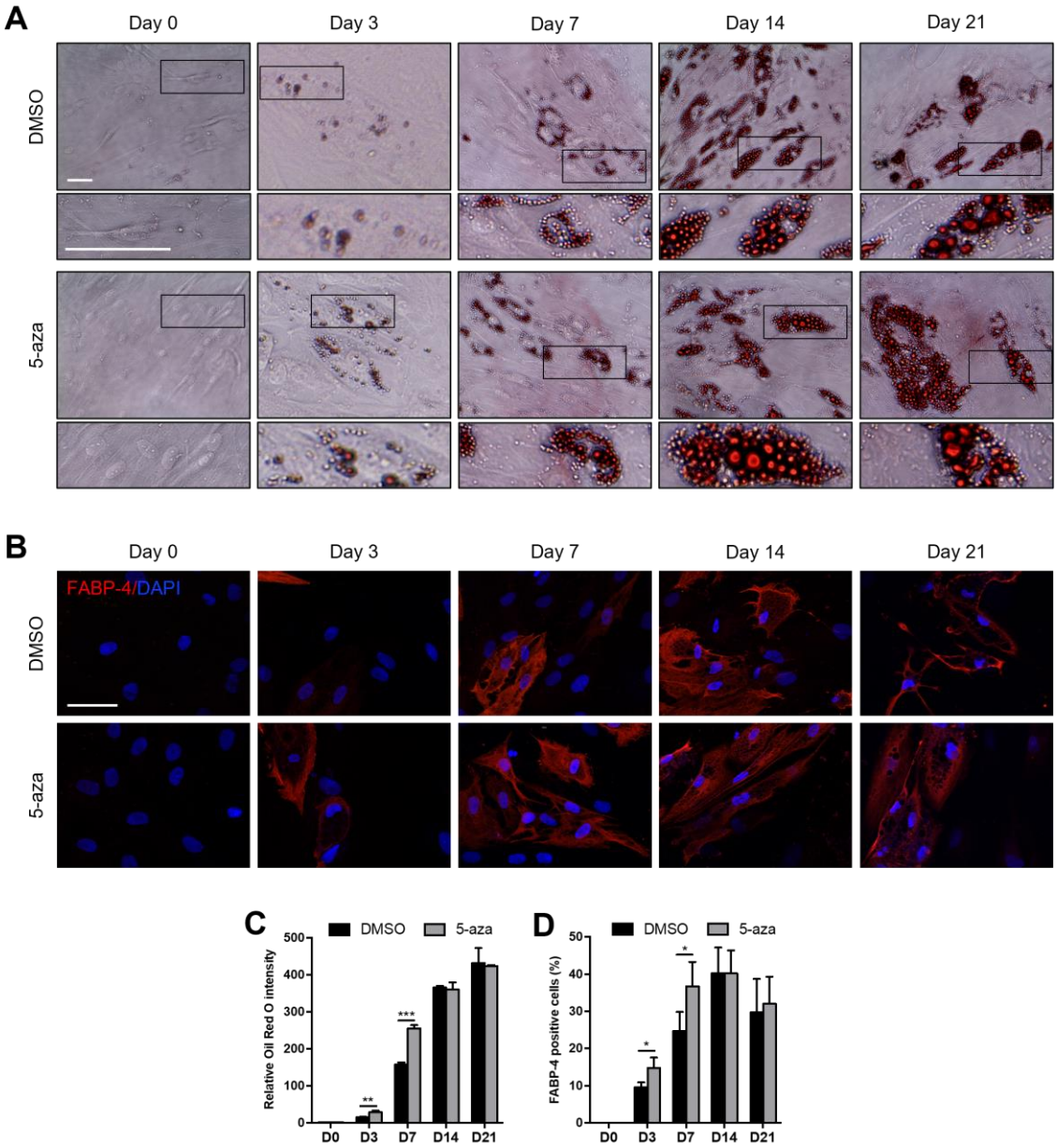


Figure 35. 5-aza affects ASC adipogenic differentiation. (A) Phase-contrast photomicrographs showing ASCs pretreated with 5-aza or DMSO and stained with Oil Red O to visualize lipid accumulation at day 0, 3, 7, 14 and 21 of adipogenic induction (D0-D21). Representative areas of the full image are presented as enlargements in lower panels. Scale bars: 50 µm. **(B)** IF analysis showing the expression of FABP4 (red). Nuclei were visualized using DAPI (blue). Scale bar: 50 µm. **(C)** Quantitative analysis of lipid droplets by measuring Oil Red O absorbance. **(D)** Percentage of FABP4-positive cells determined by counting the number of FABP4-positive cells vs. total number of cells in ten random areas for each condition. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ vs. DMSO.

We then analyzed the expression of the master regulators of adipogenesis, PPARγ and c/EBPα, in response to 5-aza pretreatment. As expected, expression of both PPARγ and c/EBPα mRNA was significantly increased in the cultures induced towards adipogenesis, starting from 3 days, with a maximum at 14 days and then a decrease at 21 days. Interestingly, 5-aza pretreatment was able to significantly increase PPARγ and c/EBPα expression at 0, 3 and 7 days of induction (PPARγ: 2.9-, 2.4- and 2.0-fold vs. DMSO, respectively; c/EBPα: 11.4-, 3.1- and 2.0-fold vs. DMSO, respectively) (Figure 36A). Moreover, 5-aza seemed also to further decrease both the differentiation markers at later times of induction (Figure 36A). PPARγ protein expression was also evaluated by WB analysis (Figure 36B), and densitometric analysis confirmed a significant increase at 0, 3 and 7 days, and a decrease at 21 days. At 14 days, despite the lower expression of PPARγ mRNA observed in 5-aza-treated cells, its protein levels still remain similar to those of DMSO-treated cells, potentially suggesting the intervention of post-transcriptional mechanisms regulating protein turnover. Overall, early ASC adipogenesis was positively affected by 5-aza pretreatment.

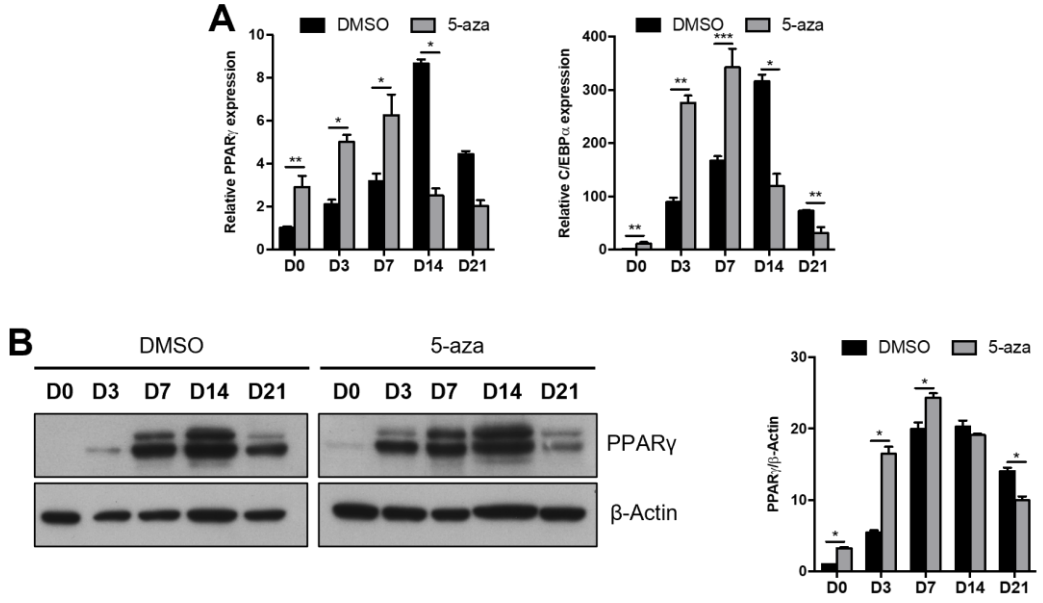


Figure 36. 5-aza modulates the master regulators of ASC adipogenic differentiation. (A) PPARγ and c/EBPα mRNA expression, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. **(B)** Western blot analysis of PPARγ protein expression. β-Actin was used as internal control. Densitometric analysis was reported as relative expression with respect to D0 of DMSO-treated cells. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ vs. DMSO.

4.4 5-aza pretreatment modulates PI3K/Akt/mTOR pathway

Akt/mTOR molecular pathway is a fundamental signaling pathway involved in cell survival and apoptosis, having also has a pivotal role in the regulation of proliferation, migration and differentiation of mesenchymal stem cells [69]. However, a direct effect of 5-aza on this pathway in ASCs has not yet been assessed. So, the activation of this pathway was evaluated by Western blot analysis, by assessing AKT phosphorylation on Ser473. As reported in Figure 37A, the levels of phosphorylated AKT protein (phAKT) relative to total protein are markedly impaired by 5-aza pretreatment for 48 h (0.3-fold vs. DMSO-treated cells), while total AKT levels remained unchanged. Such evidence, due to the central role of the AKT signal transduction pathway in cell migration, is in line with the reduced motility observed in 5-aza-treated cells. We then assessed the activation of the downstream protein mTOR, which is known to induce the two main adipogenic transcriptional factors PPARγ and c/EBPα [71]. Interestingly, we observed that after 5-aza pretreatment mTOR phosphorylation shows a slight increase (1.1-fold vs. DMSO) (Figure 37A). We also confirmed the ability of 5-aza pretreatment to induce PPARγ and c/EBPα mRNA expression (3.1-fold and 9.6-fold vs. DMSO, respectively) (Figure 37B). In light of these results, we sought to demonstrate the existence of an Akt-independent activation of mTOR, which might explain the positive effects of 5-aza pretreatment on adipogenic differentiation. To test this hypothesis, we performed 5-aza pretreatment in the presence of rapamycin, a known mTOR inhibitor. As reported in Figure 37C, PPARγ increase observed upon 5-aza treatment (1.3-fold vs. DMSO) was completely abolished by rapamycin (0.6-fold vs. DMSO), this confirming the central role of mTOR in 5-aza-mediated enhancement of adipogenic induction. Since the MAPK pathway also plays a key role in different cellular functions, including proliferation and differentiation [221], we further investigated the effects of 5-aza on ERK activation. Our results, showing a reduction in ERK phosphorylation upon 5-aza pretreatment (0.7-fold vs. DMSO; Figure 37D), might also explain the reduced clonogenicity in 5-aza-treated cells observed in colony forming assay (Figure 34A).

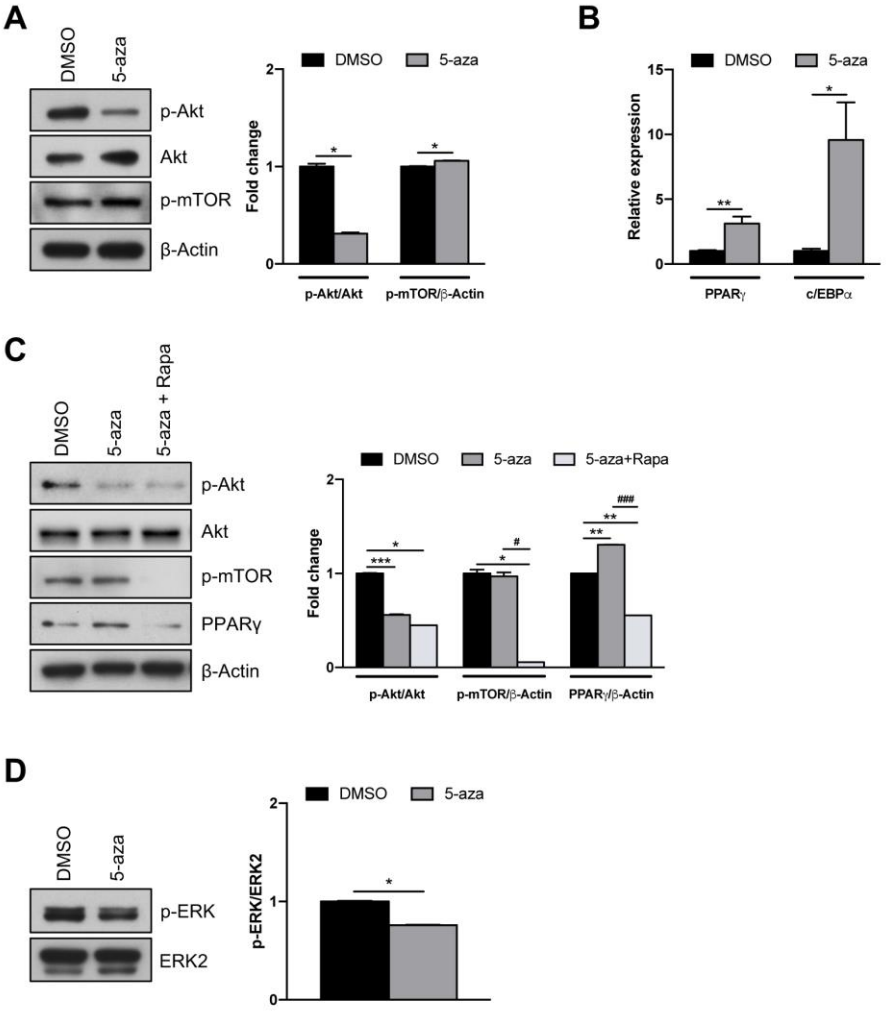
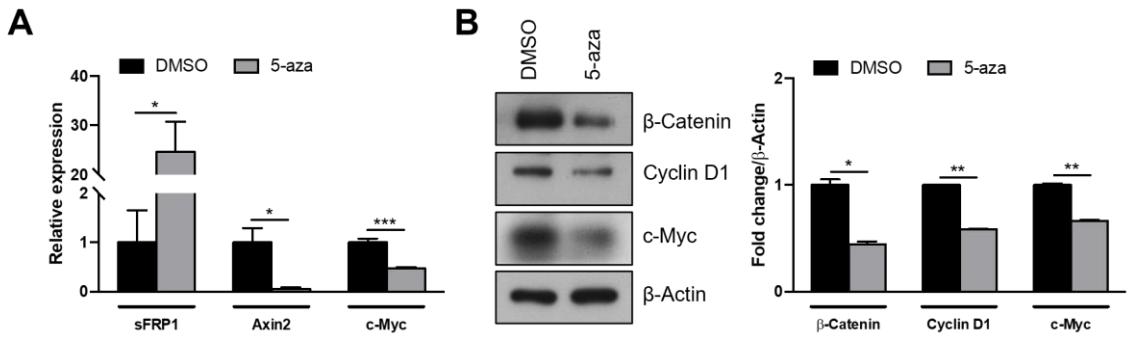


Figure 37. 5-aza effects on Akt/mTOR and MAPK signaling pathways. (A) Western blot analysis of phospho-AKT, AKT and phospho-mTOR protein expression in ASCs pretreated with 5-aza or DMSO. β-Actin was used as internal control. **(B)** PPARγ and c/EBPα mRNA expression, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. **(C)** Western blot analysis of phospho-AKT, AKT, phospho-mTOR and PPARγ protein expression in ASCs pretreated with 5-aza or DMSO in the presence or not of rapamycin. β-Actin was used as internal control. **(D)** Western blot analysis of phospho-ERK and ERK2 protein expression. Densitometric analysis was reported as relative expression with respect to DMSO-treated cells. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ vs. DMSO. #$p < 0.05$, ###$p < 0.0005$ vs. 5-aza.

4.5 5-aza pretreatment inhibits Wnt/β-Catenin pathway

The adipogenic commitment of ASCs is regulated by a complex and highly orchestrated gene expression program and many developmental signaling pathways [222]. To further

explore the mechanisms through which 5-aza regulates adipogenic differentiation, we tested whether 5-aza pretreatment affects the endogenous expression of key adipogenic regulators belonging to the Wnt/β-catenin pathway. First, we focused on the expression of secreted Wnt inhibitors factors, such as the secreted Frizzled-related protein 1 (sFRP1). Intriguingly, after 5-aza pretreatment, the mRNA expression of sFRP-1 was dramatically upregulated (24.6-fold; Figure 38A), this suggesting a shut-down of Wnt signaling. To verify such hypothesis, we focused on the expression of Wnt target genes, such as Axin2, Cyclin D1 and c-Myc. Consistently with a Wnt pathway inhibition, we observed that 5-aza treatment induced the downregulation of Axin2, as assessed by means of qRT-PCR (0.05-fold; Figure 38A), as well as Cyclin D1 and c-Myc, as assessed by WB (0.6-fold and 0.7-fold, respectively; Figure 38B). The canonical Wnt pathway implicate that in the absence of Wnt activation a "destruction complex" consisting of various scaffold proteins phosphorylates the N-terminus of β-catenin, which is then recognized by an E3 ubiquitin ligase subunit, followed by ubiquitination and subsequent proteasome degradation. Destruction of β-catenin prevents its nuclear localization. So, we also analyzed the effects of 5-aza treatment on β-catenin protein expression. WB analysis showed that β-catenin is significantly affected by the treatment (0.4-fold; Figure 38B). Moreover, IF analysis with ApoTome microscope confirmed partial β-catenin degradation and also revealed that its intracellular distribution was significantly altered by 5-aza, with an impairment of nuclear translocation and an increase of membrane signal to the detriment of cytoplasmic one (Figure 38C).
Overall, these data imply that the 5-aza ability of enhancing adipogenic differentiation in ASC cells might be exerted also by inactivating canonical Wnt signaling.



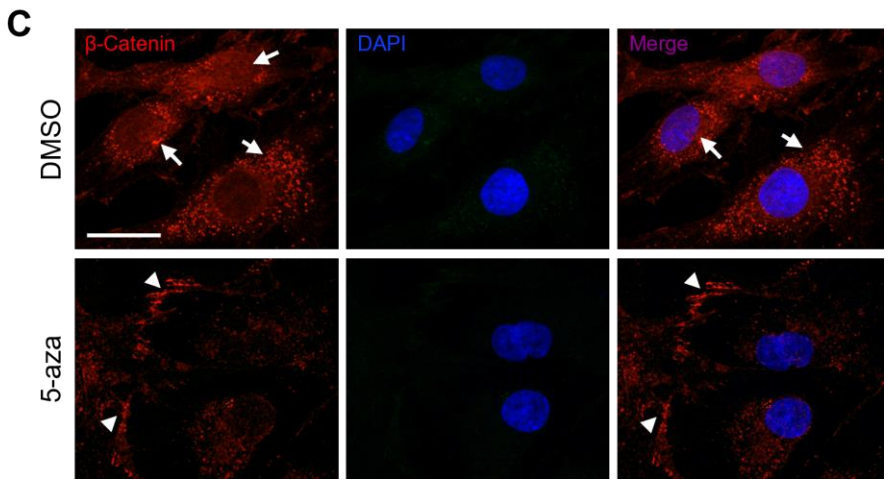


Figure 38. 5-aza effects on Wnt/β-catenin signaling pathway. (A) sFRP-1 and Axin2 mRNA expression in ASCs pretreated with 5-aza or DMSO, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. **(B)** Western blot analysis of β-catenin, Cyclin D1 and c-Myc protein expression. β-Actin was used as internal control. Densitometric analysis was reported as relative expression with respect to DMSO-treated cells. **(C)** IF analysis showing the expression of β-catenin (red). Nuclei were visualized using 4′, 6-diamido-2-phenylindole dihydrochloride (DAPI) (blue). Scale bar: 50 μm. mRNA levels were normalized to GAPDH mRNA expression. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$, ***$p < 0.005$ vs. DMSO; #$p < 0.05$, ##$p < 0.005$, ###$p < 0.0005$ vs. 5-aza.

To further confirm the role of both mTOR activation and Wnt/β-catenin pathway inhibition in 5-aza-mediated enhancement of adipogenic differentiation, an experimental setting in which mTOR was inhibited and Wnt pathway was induced has been setup. In detail, we performed adipogenic induction in cells pretreated with 5-aza alone or in the presence of the mTOR inhibitor rapamycin, of the human recombinant Wnt3A protein, a known inducer of canonical Wnt signaling pathway, or both. The effect of such treatments on adipogenic differentiation was evaluated by assessing the expression levels of the master regulators of adipogenesis, PPARγ and c/EBPα. As expected, we observed that the increased expression of PPARγ and c/EBPα induced by 5-aza at 3 and 7 days (PPARγ: 2.9- and 1.6-fold vs. DMSO, respectively; c/EBPα: 3.1-, and 2.0-fold vs. DMSO, respectively) was impaired by either rapamycin (PPARγ: 1.0- and 0.1-fold vs. DMSO, respectively; c/EBPα: 1.1-, and 0.1-fold vs. DMSO, respectively) or Wnt3A (PPARγ: 0.7- and 0.1-fold vs. DMSO, respectively; c/EBPα: 0.3-, and 0.04-fold vs. DMSO, respectively), and much more in the

presence of rapamycin and Wnt3A together (PPARγ: 0.7- and 0.1-fold vs. DMSO, respectively; c/EBPα: 0.1-, and 0.01-fold vs. DMSO, respectively) (Figure 39). These data are in line with our hypothesis of an involvement of the two pathways in mediating 5-aza effect on ASC adipogenesis.

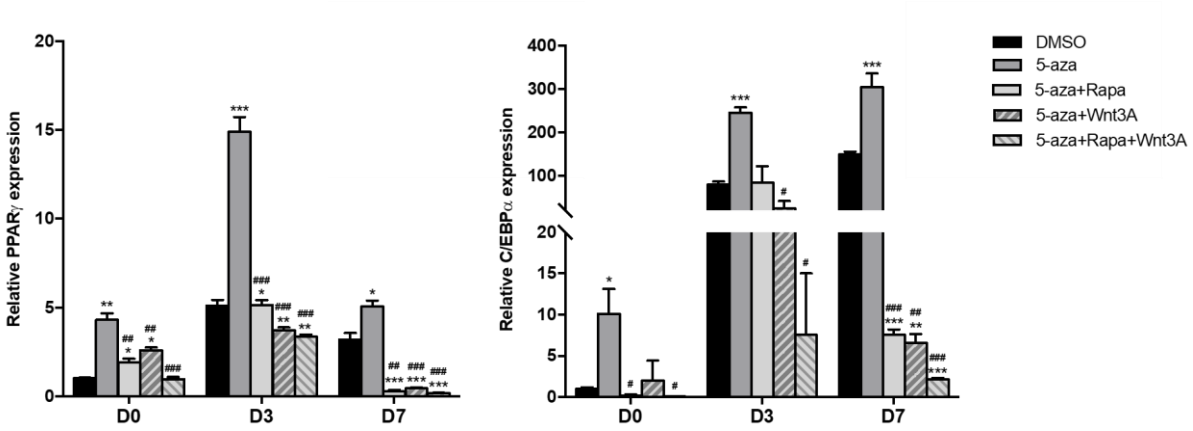


Figure 39. 5-aza effects on Wnt/β-catenin signaling pathway. PPARγ and c/EBPα mRNA expression, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$, ***$p < 0.005$ vs. DMSO; #$p < 0.05$, ##$p < 0.005$, ###$p < 0.0005$ vs. 5-aza.

4.6 5-Aza promotes ASC cell senescence

ASCs inevitably acquire a senescent phenotype after prolonged *in vitro* expansion, and it is known that the proportion of senescent cells is a key factor in determining ASC effectiveness in clinical applications. Given the potential use of 5-aza as a senescence inducer [223], and in light of the above results indicating flattened morphology, reduced colony formation ability and Cyclin D1 downregulation upon 5-aza pretreatment, we wondered whether 5-aza was able to induce ASC senescence. We analyzed ASCs at early, intermediate and late passages. Cells at P2 were treated with 10 μM 5-aza or DMSO for 48 h and then cultured in standard medium. At P4, P8 and P12, 5-aza- or DMSO-treated cells were stained with the Senescence β-Galactosidase Staining Kit and the percentage of senescent cells was calculated. As shown in Figure 40A, 5-aza-treated ASCs at P4, P8 and P12 showed a significant increase of senescent cells with respect to DMSO (14.9% vs. 10.2%, 19.4% vs. 10.3% and 24.9% vs. 17.6% of DMSO, respectively), suggesting that 5-aza pretreatment was able to significantly induce senescence in cultured ASCs. The senescent state is characterized

by cell cycle arrest, which is maintained by the activation of several pathways including p53/p21WAF1 signaling [224]. So, we next analyzed the effects of 5-aza on p53 and p21 gene expression. We observed an increase of both p53 and p21 mRNA levels upon 5-aza pretreatment (1.7-fold; Figure 40B). As shown in Figure 40C, the protein expression of p53 and p21 was also significantly increased in ASCs pretreated with 5-aza compared with ASCs pretreated with DMSO (1.3-fold and 1.5-fold, respectively). We also investigated the expression of Lamin B1, a protein known to be downmodulated in senescent cells [225]. WB experiments shown in Figure 40C demonstrated a significant downregulation of Lamin B1 protein expression upon 5-aza treatment (0.5-fold vs. DMSO), which was further confirmed by IF experiments showing an impairment of Lamin B1 nuclear membrane signal in 5-aza-treated cells (Figure 40D).

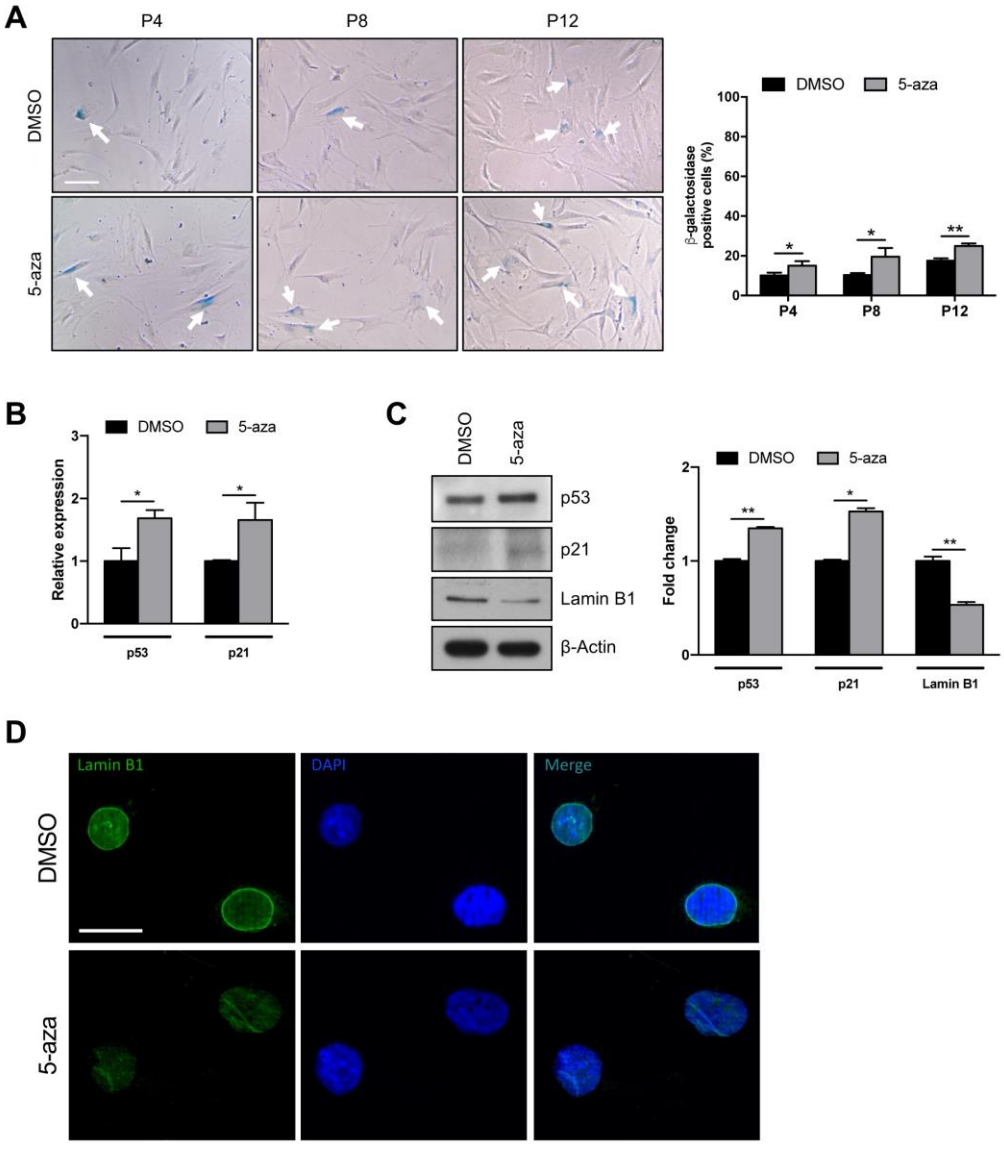


Figure 40. 5-aza effects on DNA damage and apoptosis. (A) Phase-contrast photomicrographs showing ASCs pretreated with 5-aza or DMSO at passage 4, 8 and 12 (P4-P12), stained with β-Galactosidase Staining Kit to visualize senescent cells. The percentage of β-Gal-positive cells was determined by counting the number of blue cells vs. total number of cells in ten random areas for each condition. **(B)** P53 and p21 mRNA expression, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. **(C)** Western blot analysis of p53, p21 and lamin B1 protein expression. β-Actin was used as internal control. Densitometric analysis was reported as relative expression with respect to DMSO-treated cells. **(D)** IF analysis showing the expression of Lamin B1 (green). Nuclei were visualized using DAPI (blue). Scale bar: 50 µm. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.005$ vs. DMSO.

Another feature of senescence is the induction of the senescence-associated secretory phenotype (SASP), which comprises cytokines and soluble factors released from senescent cells. We therefore measured the SASP components in supernatants of ASC cells treated with DMSO or 5-aza using cytokine array. It revealed significantly increased secretion of SDF-1, MIF, PAI-1, IL-6 and IL-8 (Figure 41A). All these molecules are reported to be significantly altered between presenescent and senescent states [226]. The increase in PAI-1 and IL-6 was also confirmed by qRT-PCR analysis (2.5- and 2.4-fold, respectively; Figure 41B). Altogether, these data indicate that 5-aza pretreatment might increase cell senescence of ASCs during *in vitro* expansion.

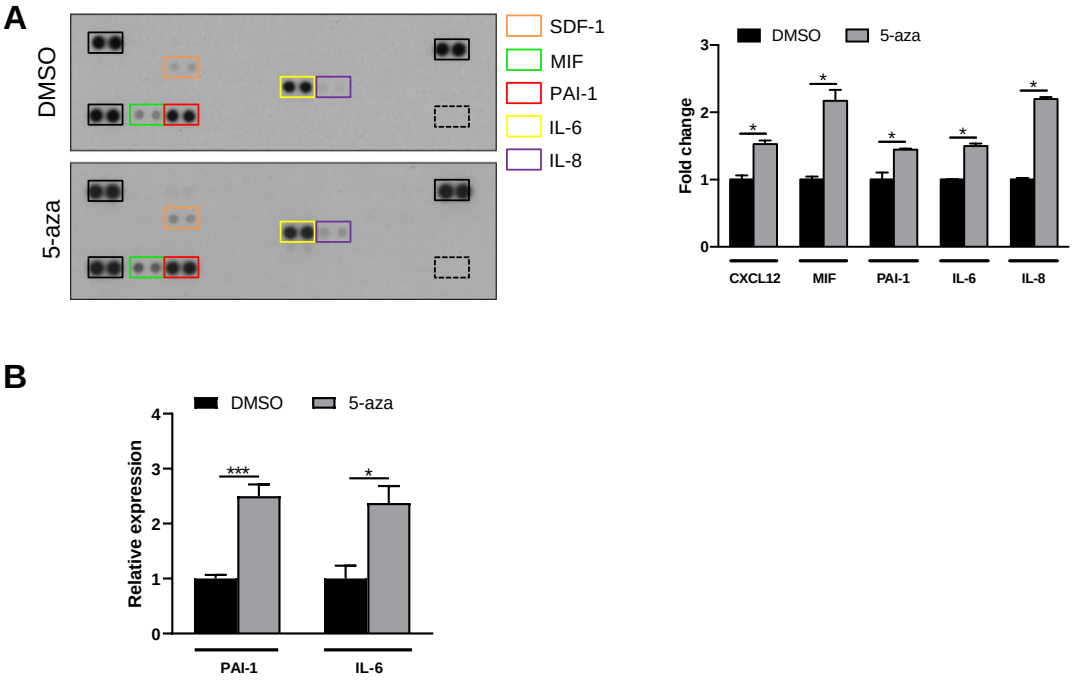


Figure 41. 5-aza induce a SASP phenotype in ASC. A) Human cytokine array on the culture medium of ASCs pretreated with 5-aza or DMSO. Significantly changed cytokines in the presence of 5-aza pretreatment were marked and shown on the right. Black boxes represent positive control; black dotted boxes represent negative control. Densitometric analysis was reported as relative expression with respect to DMSO-treated cells. **B)** PAI-1 and IL-6 mRNA expression in ASCs pretreated with 5-aza or DMSO, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. Bars represent means ± SD of three independent experiments, each performed in triplicate. * $p < 0.05$ vs DMSO.

4.7 5-Aza effects on DNA damage and Apoptosis

Since it is known that senescence can be induced by a variety of stimuli, including DNA damage, we wondered if 5-aza pretreatment was able to increase DNA double-strand breaks (DSBs) in ASCs. Since the efficient repair of DSBs requires a coordinated DNA Damage Response (DDR), which includes phosphorylation of histone H2AX, forming γH2AX, we assessed γH2AX expression by IF analysis in ASCs treated or not with 5-aza. Our results revealed that 5-aza pretreatment, at this dose and timing, does not induce an evident increase of γH2AX foci (Figure 42A). Such observation was further confirmed by WB analysis with γH2AX antibodies, showing no upregulation upon 5-aza treatment (0.9-fold; Figure 42B).

Beyond its contribution to cellular senescence, the transcription factor p53 is known to regulate the expression of target genes involved in apoptosis [227]. So, following our observation of an upregulation of p53 upon 5-aza treatment, we wondered if this effect could also trigger the activation of the apoptotic pathway in ASCs. To evaluated apoptosis, the expression of cleaved PARP1, a known hallmark of caspase activation, was analyzed. WB analysis showed a slightly decreased level of cleaved PARP1 upon 5-aza treatment (0.8-fold; Figure 42B), this suggesting the absence of apoptotic stimuli. To further confirm these data, we performed a flow cytometry quadrant analysis with annexin A5 FITC/7-AAD double staining, showing that 5-aza treatment even reduced the percentage of apoptotic cells (18% in DMSO vs. 13.3% in 5-aza; Figure 42C). Accordingly, we further observed a significant increase in the mRNA expression of the anti-apoptotic factor Bcl-2 upon 5-aza treatment (2.6-fold; Figure 42D), which could be responsible for the determination of

cellular senescence versus apoptosis. These data strongly suggested that 5-aza pretreatment induce a senescent phenotype rather than cell death in ASCs.

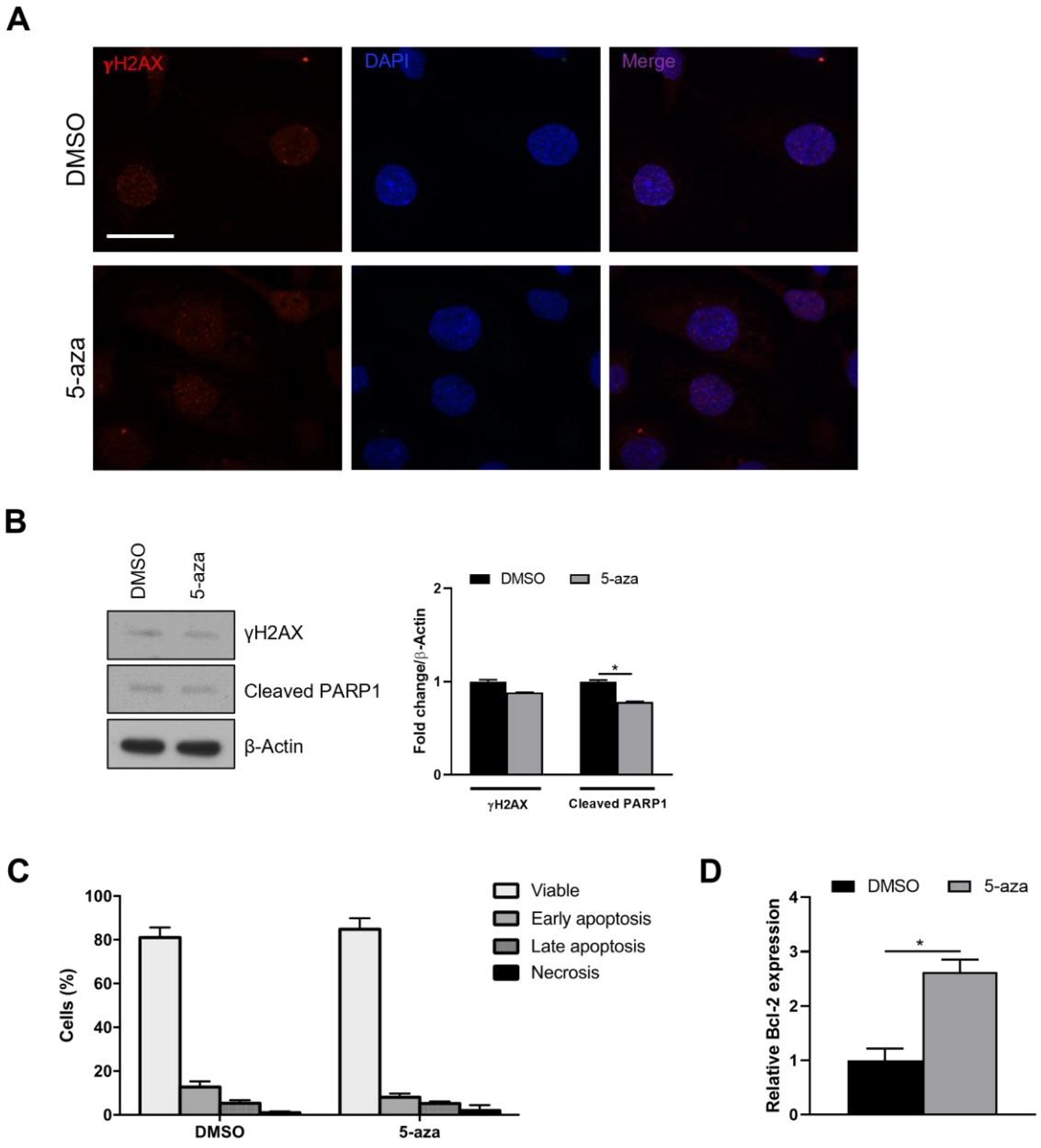


Figure 42. 5-Aza effect on DNA damage and apoptosis. (A) IF analysis showing γH2AX foci (red) in ASCs pretreated with 5-aza or DMSO. Nuclei were visualized using 4′, 6-diamido-2-phenylindole dihydrochloride (DAPI) (blue). Scale bar: 50 µm. **(B)** Western blot analysis of γH2AX and cleaved PARP1 protein expression. β-Actin was used as internal control. Densitometric analysis was reported as relative expression with respect to DMSO-treated cells. **(C)** Flow cytometry quadrant analysis with annexin A5 FITC/7-AAD double staining. The percentages of viable, early apoptotic, late apoptotic and necrotic cells are expressed as histograms. **(D)** Bcl-2 mRNA expression, evaluated by qRT-PCR. mRNA levels were normalized

to GAPDH mRNA expression. Bars represent means ± SD of three independent experiments, each performed in triplicate. *$p < 0.05$ vs. DMSO.

4.8 Generation of a miR-125b-enhanced ASC-CM

Our results indicated that 5-aza pretreatment was able to potentiate ASC differentiation ability towards the adipogenic lineage, opening the way to the adoption of this preconditioning protocol in all the clinical applications requiring new adipose tissue, such as soft tissue defects, wound healing and fibrotic diseases. However, the evaluation of secretome profile of ASC pretreated with 5-aza by cytokine array pointed out an increase in pro-inflammatory factors, such as IL-6 and IL-8, this suggesting that 5-aza preconditioning might not be useful for therapeutic applications of ASC secretome in selected pathologies requiring immunosuppressive rather than differentiative burst. So, given the central role of secreted miRNAs in mediating the paracrine effects of ASCs and the wide literature assessing the therapeutic potential of miR-125b-5p, we decided to produce a secretome enriched with miR-125b-5p and to test its effect on target cells. To this aim, ASCs were transiently transfected with a miR-125b-5p mimic or a mimic CTRL and ASC-CM was collected as previously described. The expression levels of miR-125b-5p were subsequently evaluated by qRT-PCR both at intracellular level and in the ASC-CM. Figure 43A shows a consistent increase in the levels of miR-125b-5p in the cells, as expected (10.4-fold vs. mimic CTRL). Interestingly, a stronger increase in miR-125b-5p levels was observed in the ASC-CM (69-fold vs. mimic CTRL; Figure 43B), thus suggesting that transient transfection was able not only to increase miR expression but also to boost its secretion. So, our strategy to potentiate ASC secretome was confirmed to be efficient.

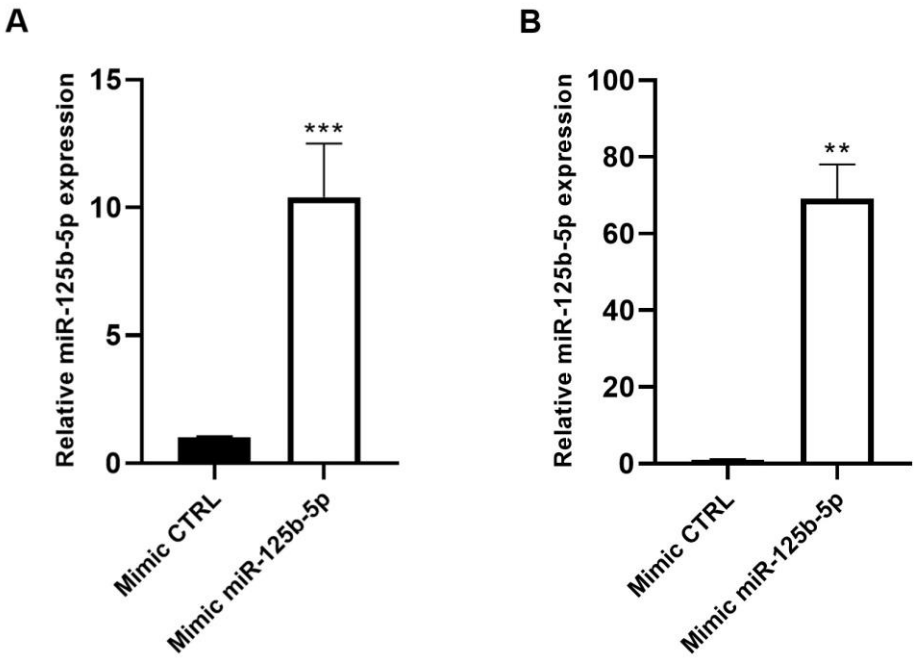


Figure 43. (A) Relative expression levels of miR-125b-5p in ASC after transfection. **(B)** Relative expression levels of miR-125b-5p in ASC-CM after transfection. Bars represent means ± SD of three independent experiments, each performed in triplicate. * $p < 0.05$ vs mimic CTRL.

4.9 NRP1 Silencing decreases miR-125b-5p intracellular levels

The second step to validate our secretome potentiation strategy was to assess if the treatment with miR-125b-enriched ASC-CM is able to transfer this miR to the target cells, where it should exploit its therapeutic action. As previously described, the membrane receptor NRP1 is able to bind and internalize circulating microRNAs [214]. In order to evaluate the potential role of NRP1 in the internalization of miR-125b-5p upon treatment with miR-125b-enriched ASC-CM, we silenced NRP1 expression in ASCs by transient transfection with a specific siRNA (siNRP1). As a control, cells were also transfected with a control siRNA designed not to target any gene (siCTRL). Transfected cells were subsequently cultured with the miR-125b-5p-enriched ASC-CM. The efficacy of NRP1 silencing was confirmed by the robust decrease of NRP1 expression in silenced cells, as assessed by both qRT-PCR (0.14-fold vs. siCTRL; Figure 44A) and Western blot analysis (0.004-fold; Figure 44B). Following treatment with miR-125b-enriched ASC-CM, we observed a significant lower intracellular expression of miR-125b-5p in NRP1-silenced ASCs

with respect to siCTRL cells (0.68-fold vs. siCTRL) (Figure 44C). Such data indicate that the absence of NRP1 causes a decrease in the internalization of miR-125b-5p, this confirming the hypothesis of a role of NRP1 as miRNA receptor.

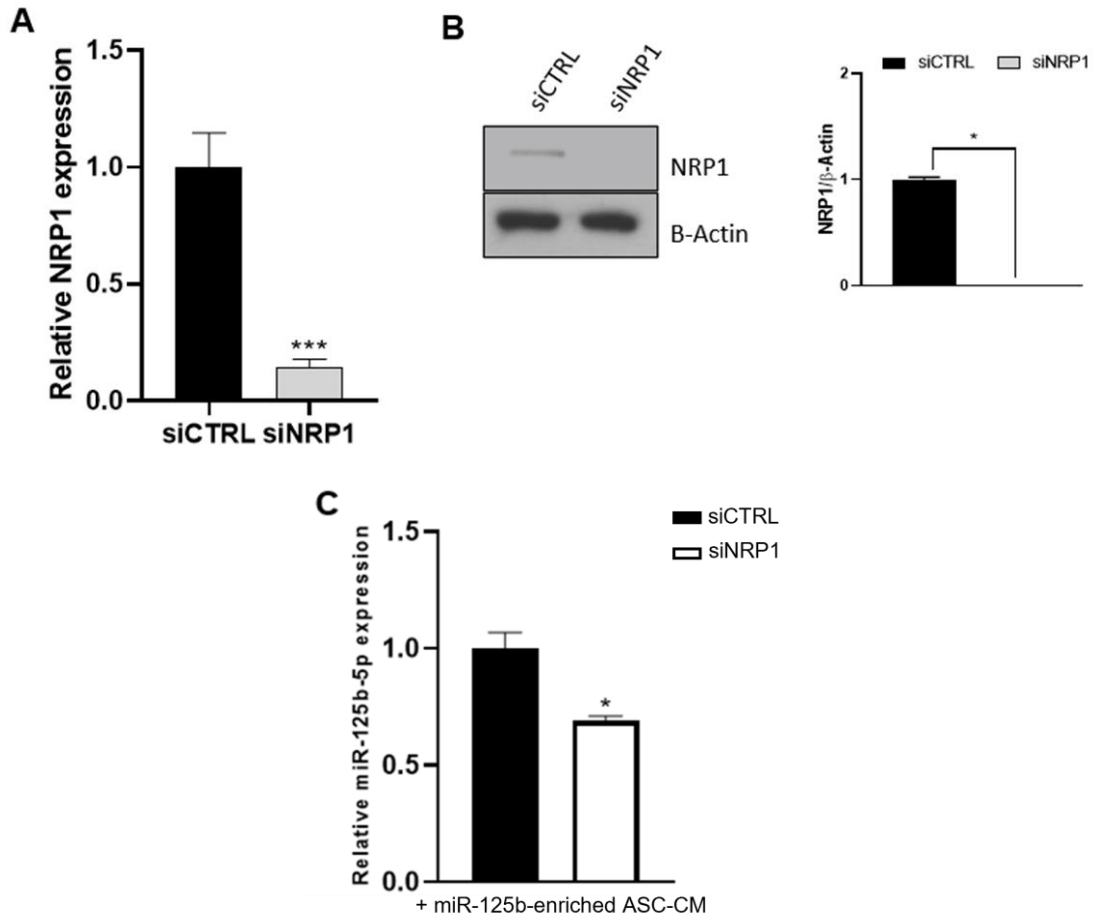


Figure 44. NRP1 silencing decreases miR-125b-5p intracellular levels following treatment with miR-125b-enriched ASC-CM. (A) NRP1 mRNA expression in cells transiently transfected with siNRP1 or siCTRL, evaluated by qRT-PCR. mRNA levels were normalized to GAPDH mRNA expression. **(B)** Western blot analysis of NRP1 protein expression in siNRP1 or siCTRL cells. β-Actin was used as internal control. Densitometric analysis was reported as relative expression with respect to siCTRL cells. **(C)** MiR-125b-5p expression in siNRP1 and siCTRL cells after culturing with miR-125b-enriched ASC-CM, evaluated by qRT-PCR. miRNA levels were normalized to U6 snRNA expression. Bars represent means ± SD of three independent experiments, each performed in triplicate. * $p < 0.05$.

4.10 MiR-125b-enriched ASC-CM shows improved immunomodulatory properties

The last step to finally validate our miR-125b-enriched secretome was to assess its therapeutic potential with respect to the secretome obtained from naïve cells. First, we assessed if the transient transfection with miR-125b, beside boosting miR-125b secretion, was also able to generally modulate ASCs secretome profile. In particular, we were interested in investigating the modulation of factors related to ASC immunomodulatory ability, so we used a cytokine array to measure the expression of the main inflammatory molecules in supernatants of ASC transfected with mimic miR-125b-5p with respect to mimic CTRL-transfected cells. The array revealed a significantly decreased secretion of CXCL12, MIF and IL-18 (Figure 45). All these molecules share a pro-inflammatory role [228-230]. In particular, the downregulation of B-cell chemotaxis to CXCL12 is one of the known mechanisms at the basis of the immunomodulatory potential of MSC secretome [231]. So, the further decrease of CXCL12 in miR-125b-enriched ASC-CM strongly suggests an increased ability of this boosted secretome to reduce the chemotactic properties of B cells. On the other hand, the array analysis highlighted an increased secretion of CXCL1 and CXCL8 (previously known as IL-8), which are responsible for neutrophils attraction during the inflammatory response, through the activation of the CXCR2 receptor [232] (Figure 45). Since neutrophils recruited to the site of injury are also able to regulate the activity of the adaptive immune response, including both T and B cell activation, the effects of miR-125b-enriched ASC-CM on the migration and activation of neutrophils could have important relapses on secretome immunomodulatory effects.

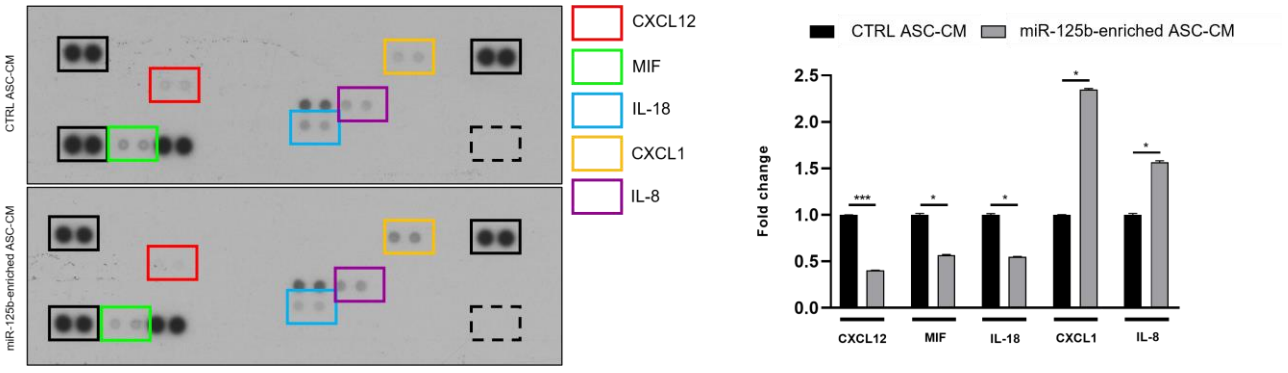


Figure 45. Mir-125b transfection modulates cytokine composition of ASC secretome. Human cytokine array on the culture medium of ASCs transfected with mimic CTRL or mimic miR-125b-5p. Significantly changed cytokines in the presence of mimic miR-125b-5p were marked and shown on the right. Black boxes represent positive control; black dotted boxes represent negative control. Densitometric analysis was reported as relative expression with respect to mimic CTRL transfected cells. Bars represent means ± SD of three independent experiments, each performed in triplicate. * $p < 0.05$ vs mimic CTRL.

4.11 MiR-125b-enriched ASC-CM shows immunomodulatory effects in PBMCs

It is known that modulation of the cytokine balance, decreasing type 1 cytokines, might represent one of the mechanisms involved in the anti-inflammatory and immunoregulatory action of several drugs and treatments. So, using a flow cytometric method, we analyzed the percentage of CD4+ and CD8+ T cells producing the type 1 cytokines IL-2 and IFN-γ in PBMCs after stimulation with anti-CD3 and anti-CD28 monoclonal antibodies, and we compared the results between cells treated with miR-125b-enriched or naïve ASC-CM, obtained from ASCs in the early phase of culture (passage 3). We observed a decrease in the frequency of CD4+ cells producing IL-2 (0.99% vs 1.53%) and IFN-γ (0.11% vs 0.74%) in cells treated with miR-125b-enriched ASC-CM with respect to naïve ASC-CM-treated cells (Figure 46A). An even higher decrease was observed among CD8+ cell population (3.36% vs 7.43% for IL-2 and 0.09% vs 1.54% for IFN-γ) (Figure 46B).

The decreased frequency of subsets of CD4+ and CD8+ T cells producing type 1 cytokines reflects some impairment of T cells induced by miR-125-enriched ASC-CM, this confirming our hypothesis of an immunomodulatory action mediated by this potentiated secretome.

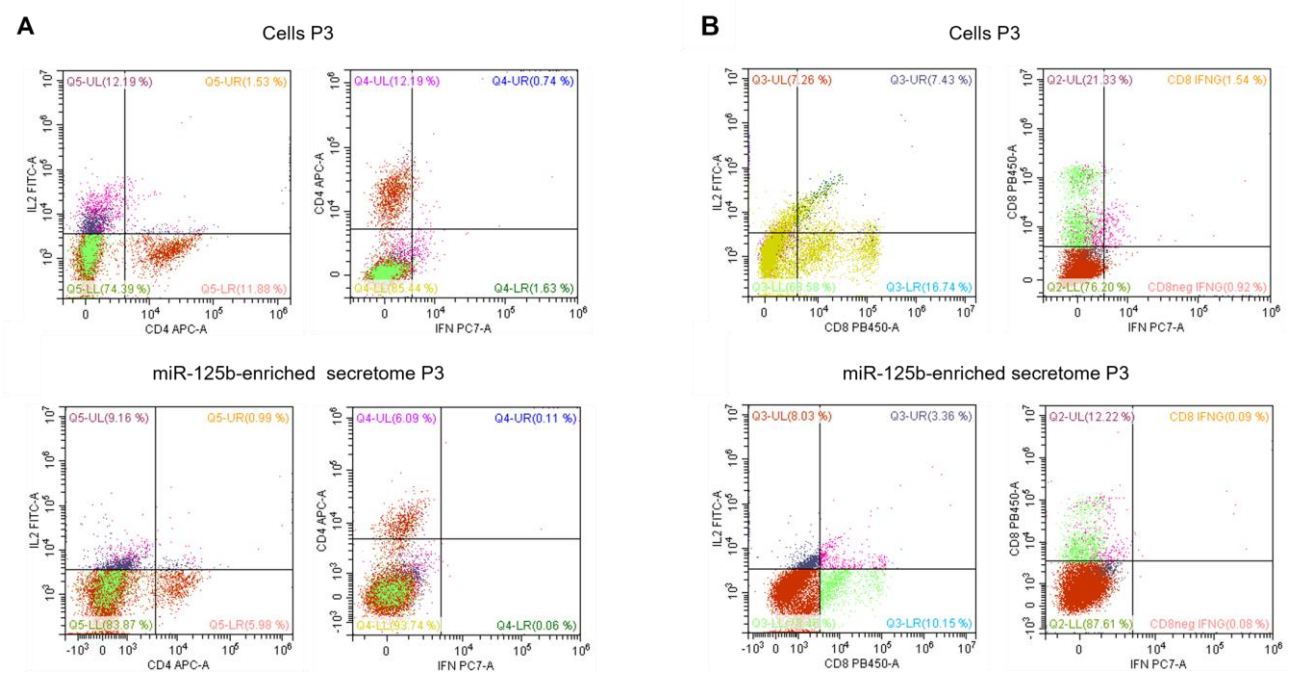


Figure 46. Cytokine production of stimulated CD4+ and CD8+ T cells upon treatment with miR-125b-enriched ASC-CM from early-passaged cells. (A) Percentage of IL-2 and IFN-γ positive CD4+ T cells in PBMCs treated with naïve or miR-125b-enriched ASC-CM obtained from cells at passage 3 (P3); **(B)** Percentage of IL-2 and IFN-γ positive CD8+ T cells in PBMCs treated with naïve or miR-125b-enriched ASC-CM obtained from cells at passage 3 (P3).

To further investigate to what extent the immunomodulatory properties of ASC secretome are influenced by cell culturing due to the onset of replicative senescence, we evaluated the percentage of cytokine-positive cells after treatment with ASC-CM (naïve or miR-125b-enriched) obtained from cells in a later phase of culture (passage 12). We observed no significant variations in the frequency of CD4+ cells producing IL-2 (0.86% vs 0.80%) and IFN-γ (0.23% vs 0.16%) in cells treated with miR-125b-enriched ASC-CM with respect to naïve ASC-CM-treated cells (Figure 47A). Indeed, an immunomodulatory effect of miR-125b-enriched ASC-CM was still evident among CD8+ cell population (3.22% vs 6.84% for IL-2 and 0.24% vs 0.84% for IFN-γ) (Figure 47B).

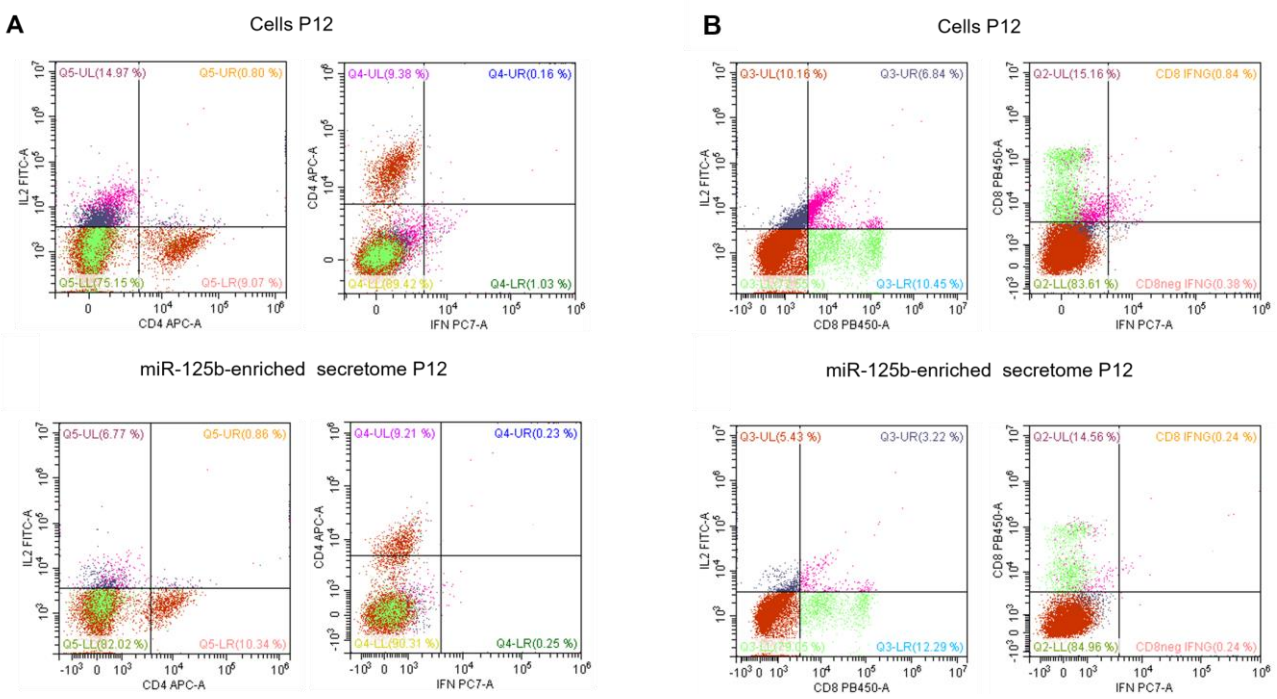


Figure 47. Cytokine production of stimulated CD4+ and CD8+ T cells upon treatment with miR-125b-enriched ASC-CM from late-passaged cells. (A) Percentage of IL-2 and IFN-γ positive CD4+ T cells in PBMCs treated with naïve or miR-125b-enriched ASC-CM obtained from cells at passage 12 (P12); **(B)** Percentage of IL-2 and IFN-γ positive CD8+ T cells in PBMCs treated with naïve or miR-125b-enriched ASC-CM obtained from cells at passage 12 (P12).

All these data suggest that a secretome enriched with miR-125b-5p possesses an increased immunomodulatory ability. So, boosting ASC secretome with miR-125b-5p might be a useful strategy to potentiate these cells' paracrine ability to suppress inflammation, thus developing a useful acellular tool for the treatment of diseases characterized by chronic inflammation.

5 DISCUSSION

ASCs are a multipotent stem cell population that resides in the SVF of adipose tissue. The interesting characteristics of these cells, such as unlimited self-renewal ability, immunomodulatory properties, remarkable differentiation and transdifferentiation capabilities, make these cells important in the processes of organogenesis, remodeling and tissue repair. Over the years ASCs have aroused increasing interest in the scientific community, also in consideration of their greater yield, availability and reduced ethical implications that favor them compared to other types of stem cells. All these factors, combined with the possibility of exploiting the so-called "bystander effect", fundamental in the modulation of the tissue microenvironment, allow ASCs to play an extremely useful role in cell therapies applicable to various clinical settings.

Although ASCs can be used for a wide variety of target diseases, including autoimmune-based pathologies, wound healing in different tissues, neural disorders, metabolic pathologies and cardiovascular diseases, one of the most promising application areas remains the reconstructive surgery.

In fact, transferring autologous adipose tissue is commonly used method in soft tissue reconstruction, in many surgical operations or more generally, as a technique for filling a large tissue void that can often be caused by traumatic injuries, tumor resection or congenital anomalies. However, soft tissue defects represent a complex challenge for the plastic surgeon, especially when considering the high prevalence of diseases like diabetes mellitus, obesity, occlusive disease of the peripheral arteries, chronic venous insufficiency or various complications due to the radiotherapy course [228]. One of the main obstacles of the technique lies precisely in the degree of effectiveness of its use, which is compromised by the frequent loss of the implant, due to insufficient vascularization of the transplanted tissue, and by the loss of volume caused by the central necrosis of the adipose tissue itself [95]. The clinical application of ASCs, administrated in the damaged area, could help overcoming these problems. Indeed, the absence of major histocompatibility complex II (MHCII) expression and the immunomodulatory properties of ASCs enable allogeneic transplantation of these cells, limiting common concerns about host compatibility, malignant transformation and loss of function that might occur in any cell therapy. Therefore, developing protocols aiming at efficiently modulating the adipogenesis of ASCs

in vivo would help to enhance their therapeutic efficacy in regenerative medicine and greatly improve the outcome of reconstructive surgery, provided that the problems related to the survival and differentiation capacity of ASCs are resolved in the transplanted microenvironment [95].

The differentiation process involves a series of epigenetic modifications of the cellular genome [229]. Chromatin remodeling and DNA methylation are among the main epigenetic mechanisms that regulate gene expression, as they are capable of influencing the access of transcription factors and the general transcription apparatus to certain gene loci.

5-azacytidine is a drug capable of acting on cells as an epigenetic modifier; it is labeled as DNMT inhibitor due to its ability to hinder the normal functioning of DNA methyltransferases. Being a nucleoside analogue, it is able to intercalate itself in the replicating DNA, replacing the normal cytosine and thus altering a normal CpG portion. The structure of 5-aza does not allow the release of the DNMTs that interact with these genomic portions in an attempt to methylate them. In fact, during the process, the methyltransferases are kept covalently and irreversibly bound to DNA. This triggers an azacytidine-dependent degradation system of trapped DNMTs, reducing the amount of enzyme available for subsequent methylation processes. The direct consequence of this mechanism is a global and non-specific reduction in DNA methylation levels during replication, which inevitably leads to a different regulation of biological pathways in the cells.

Relying on previous evidence that highlights 5-aza (5-aza, Vidaza®) as an epipharmaceutical potentially useful in regulating important biological processes of stem cells, the aim of the study was to evaluate its modulatory effect on the activity of proliferation, migration and adipogenic differentiation in ASCs. The final goal being the improving of *in vitro* culture protocols and the evaluation of new epigenetic strategies for the enhancement of adipose-derived stem cells. Achieving this aim would significantly improve the therapeutic outcomes related to the clinical application of these cells and obtain new information about the ability of methylation mechanisms to influence cell biology. In order to investigate the effects of the demethylating agent on ASCs, the cells were pretreated with 5-aza 10 μM or DMSO for 48 hours and then cultured for 72 hours in standard medium before proceeding with the analyses.

During our study, we first isolated and phenotypically characterized the ASCs, confirming the high presence of mesenchymal markers CD29, CD90, CD44 and CD166 by FACS analysis. Following pretreatment with 5-aza we analyzed the density and morphology of the cells, which presented a reduced confluence and a fibroblastic morphology, with respect to the DMSO group.

We then evaluated the influence of the treatment on the vitality and growth kinetics of ASCs. Through PDT analysis, no significant differences were detected in the growth potential of the pretreated sample and, similarly, the staining with Trypan blue showed no alteration in the percentage of live cells, thus demonstrating that the action of the demethylating agent is not toxic at the established dose. On the contrary, 5-aza appears to have a significant impact on cell proliferation and migration. In fact, following the clonogenic assay, the group treated with 5-aza showed a clear reduction in the ability to form new colonies when compared to the control sample, thus highlighting a possible reduction in cell proliferation.

Similarly, cell migration potential is also significantly reduced by 5-aza pretreatment, as demonstrated by the scratch test and transwell test analysis.

Since migration, proliferation and differentiation of mesenchymal stem cells are strongly regulated by the PI3K/Akt signaling pathway [69], we wanted to analyze the susceptibility of this pathway to the action of 5-aza in ASCs, evaluating any direct effect on this signaling. Furthermore, since the MAPK pathway is also implicated in various cellular functions, including proliferation and differentiation [221], we tried to detect any alteration on the transduction of this pathway. The results obtained by WB showed an impairment of Akt phosphorylation and a reduction in ERK phosphorylation levels following pretreatment. These elements help us to provide a potential explanation for the reduced cell proliferation and migration observed in functional assays (Figures 34A, 43B and 34C).

One of the most interesting aspects that emerges from our study concerns the observation of a marked promoting activity of adipogenic differentiation, imputable to pretreatment with 5-aza. In fact, the results deriving from the histological and immunophenotypic analysis carried out on ASCs, respectively through Oil Red O staining and IF for the detection of FABP4, confirm a significant intracellular accumulation of lipid constituents and an evident increase in the number of differentiated cells after pretreatment with 5-aza. Furthermore, we observed the production of larger lipid droplets in the pretreated group

with respect to the control group. With this in mind, we wanted to investigate the possible interference of the demethylating agent on the molecular components of the signaling pathways potentially involved. The PI3K/Akt pathway, for example, is strongly involved in adipogenesis, even if its role is controversial. Indeed, Yu et al. reported a decrease in adipogenesis upon inhibition of this pathway [71], in agreement with the evidence that mTOR, a protein activated downstream of this pathway, is able to induce the two main adipogenic transcription factors PPARγ and c/EBPα [71]. On the other hand, Fitter et al. have shown that they can promote adipogenesis by inhibiting PI3K activity through imatinib [230]. In our study, we highlighted a reduction in Akt phosphorylation, accompanied by stimulation of adipogenesis. Since some previous studies have indicated that mTOR activation may be sufficient alone in promoting adipogenesis through upregulation of PPARγ and c/EBPα [69], we wanted to analyze in detail the activation of mTOR after treatment with 5 -aza. Indeed, despite the reduction of Akt phosphorylation that we found, the WB and RT-PCR analysis showed that pretreatment with 5-aza does not negatively influence the phosphorylation of mTOR, which remains activated. We also demonstrated that the upregulation of PPARγ and c/EBPα induced by 5-aza is strictly dependent on mTOR activation, since it is impaired in the presence of the mTOR inhibitor rapamycin. The ability of 5-aza to interfere with the expression of PPARγ and c/EBPα through mTOR explains the positive effect on adipogenic induction that we observed in the early stages of differentiation.

Another important but equally controversial pathway for its role in adipogenesis is the MAPK pathway. Both positive and negative functions, in fact, can be attributed to this signaling pathway within the adipogenic context. The activation of ERK signaling is crucial in supporting mitotic clonal expansion during the early stages of adipogenesis, but the subsequent shutdown of this signaling pathway is necessary to allow the differentiation of the involved stem cells [231, 232]. Previous studies show that the activation of the MAPK signal may be responsible for the inhibition of adipogenesis, probably due to the ERK-mediated phosphorylation of PPARγ, with consequent reduction of its transcriptional activity [79, 80]. Hence, the reduced ERK activity observed in our cells during 5-aza treatment could further contribute to the increased adipogenic differentiation in ASCs.

The influence of 5-aza also seems to extend to another molecular signaling pathway. Following our analysis, we observed an involvement of the Wnt/β-catenin pathway. This

pathway is important both for mitotic activity and for its ability to influence the differentiation programs of stem cells. In particular, previous evidence indicates this path as essential in triggering the differentiation of mesenchymal stem cells towards an osteogenic lineage [233], while it can be considered a negative regulator towards adipogenesis [234, 235]. The antagonistic effect would occur due to an indirect inhibition that some Wnt target genes would carry out on the expression of the main adipogenic regulators c/EBPα and PPARγ. In our case, the effects observed following pretreatment concern the upregulation of the secreted Wnt inhibitor factor (sFRP1), observed by qPCR analysis, and the prevention of nuclear translocation of β-catenin, visible through IF analysis with the ApoTome microscope. This evidence clearly indicates an inhibitory effect of 5-aza on the canonical Wnt pathway, with the consequent downregulation of the constitutive targets of this pathway, such as Axin2, Cyclin D1 and c-Myc, quantified by WB. Axin2 is a protein capable of interacting with GSK3β in the cytoplasm, preventing the kinase from translocating to the nucleus. A previous study showed that the reduced expression of Axin2 could be responsible for the release of GSK3β and the consequent nuclear entry of the kinase, which at this point can activate the transcription of c/EBPα and PPARγ [73]. This is consistent with the data we have obtained on the 5-aza-mediated downregulation of these genes. These conclusions are further strengthened by studies evaluating the ability of 5-aza to decrease the expression of β-catenin and cyclin D1 in endometrioid carcinoma and prostate cancer cells [236, 237]. Furthermore, our data indicating the reduction of Cyclin D1 and stimulation of PPARγ induced by 5-aza are in agreement with the functional antagonism between these two molecules that has been demonstrated in fibroblasts [75].

Aging is another factor that negatively affects MSC migration and homing abilities [219]. It has been shown, in fact, that directed migration toward stimuli is a critical requirement for MSCs to obtain better functional outcomes in cell-based therapy. Inhibition of the pathway involving Activator protein (AP)-1 was found to be associated with a decreased migratory capacity in senescent MSCs, when subjected to pro-inflammatory signals [238].

Cell migration involves the reorganization of the actin cytoskeleton [239]. MSCs derived from old donors exhibit reduced response to biological and mechanical signals because their actin cytoskeleton is less dynamic.

The potential clinical use of ASCs lies in several advantages, such as the absence of ethical problems and a low risk of formation of teratomas at transplant, or a high post-collection yield and a low invasive collection procedure. At the same time, though, the limitations affecting the use of cell-based therapies concern the practical steps necessary for the isolation, expansion and characterization of stem cells, as well as the appropriate quality and safety controls of the final product to be injected or transplanted [142].

As previously stated, part of ASCs clinical efficacy depends on the "bystander effect", which is the modulation of the host environment through the paracrine secretion of anti-inflammatory and cytoprotective molecules. This effect is so robust that the use of a cell-free conditioned culture medium can reproduce the same effect as the ASC transplant [90]. In particular, extracellular vesicles that contain biomolecules, proteins and genetic material such as mRNA and miRNA, possess a therapeutic potential useful in cell-free therapy for a large variety of inflammatory disease. With these premises, it is easy to understand the importance of finding and setting up an acellular alternative that allows to exploit the potential of the cells overcoming the limitations related to the cells themselves. Therefore, stem cells paracrine activity has emerged as a promising therapeutic cell-free resource in several conditions, such as wound healing [144] and tissue repair [145], thanks to the production of bioactive molecules.

Indeed, in addition to ASCs differentiative capabilities, an even greater interest lies in the ability of ASCs to promote tissue repair/regeneration through the release of a plethora of soluble factors, including cytokines, growth factors and microRNAs, and extracellular vesicles collectively known as ASC secretome. In particular, several studies proved that miRNAs secreted from ASCs can be delivered to target cells where they are taken up and induce changes in gene expression. So, secreted microRNAs can be considered a novel mechanism of intercellular communication [157], playing a role in a variety of cellular events. The transfer of genetic information between ASCs and adjacent cells, in fact, appears to be a crucial mechanism responsible for their paracrine effect in tissue repair/regeneration. Several reports demonstrated the ability of MSCs to secrete miR-125b in exosomes, and to regulate through this miRNA some key events related to their therapeutic efficacy thought this miRNA. MiR-125b-5p, indeed, represents an excellent example of a microRNA able to regulate both proliferation and differentiation. Moreover, it has been also investigated for its effects on macrophage polarization, and modulation of

inflammation and immune response [195]. Hence, given the key role of miR-125b in mediating the effects of MSC secretome, several strategies based on the release of this miRNA have been taken into consideration.

The therapeutic applications and responses of ASCs and their secretome can be regulated by specific modifications of their culture conditions, highlighting the importance of culture protocols and *in vitro* manipulation strategies to enhance and optimize ASCs secretome therapeutic efficacy.

In the second part of this research project, we focused on the evaluation of ASCs secretome effects on immunoregulation. So, through transfection experiments with mimic oligonucleotides, we obtained a secretome enriched with miR-125b-5p, confirming that the transient transfection was able to boost its secretion.

In order to validate our secretome enhancing strategy, we assessed if the treatment with miR-125b-enriched ASC-CM was able to transfer this miR to the target cells. As previously mentioned, NRP1 is a widely expressed co-receptor, which has been shown to be able to bind and internalize circulating microRNAs [214]. Hence, in order to evaluate the potential interaction between NRP1 and miR-125b-5p, making this protein a possible mediator for extracellular microRNAs, we silenced NRP1 in ASCs and cultured the silenced cells with the miR-125b-5p-enriched secretome. As hypothesized, we observed a significant lower intracellular expression of miR-125b-5p in NRP1-silenced ASCs, indicating that the absence of NRP1 caused a decrease in the internalization of miR-125b-5p, this confirming the hypothesis of a role of NRP1 as miRNA receptor.

The last steps to validate our miR-125b-enriched secretome was to assess its therapeutic potential with respect to the secretome obtained from naïve cells. Indeed, we focused on investigating the modulation of those factors related to ASC immunomodulatory ability. Hence, to assess if the transient transfection with miR-125b was able to modulate ASCs secretome profile, we first used a cytokine array to measure the potential modulation of expression of the main inflammatory molecules in supernatants of ASC transfected with mimic miR-125b-5p with respect to mimic CTRL-transfected cells. We observed a significantly decreased secretion of the pro-inflammatory molecules CXCL12, MIF and IL-18. In particular, the relevance of the data obtained can be appreciated considering that the downregulation of B-cell chemotaxis to CXCL12 is one of the known mechanisms at the basis of the immunomodulatory potential of MSC secretome [231], this confirming the

increased ability of the boosted secretome to reduce the chemotactic properties of B cells. On the other hand, we observed an increased secretion of CXCL1 and CXCL8 as well. These molecules are responsible for neutrophils attraction during the inflammatory response through the activation of the CXCR2 receptor [232], and since neutrophils recruited to the site of injury are also able to regulate the activation of both T and B cell, it is clear that the effects of miR-125b-enriched ASC-CM on neutrophils could have important relapses on secretome immunomodulatory effects. Lastly, we evaluated the percentage of CD4+ and CD8+ T cells producing type 1 cytokines IL-2 and IFN-γ in PBMCs treated with miR-125b-enriched or naïve ASC-CM, obtained from ASCs in the early and late phases of culture (passage 3 and passage 12, respectively). The decreased frequency of subsets of CD4+ and CD8+ T cells producing type 1 cytokines observed reflects some impairment of T cells induced by the boosted secretome.

All these data suggest that a secretome enriched with miR-125b-5p possesses an increased immunomodulatory ability. Hence, boosting ASC secretome with miR-125b-5p might be a useful strategy to potentiate ASCs paracrine ability to suppress inflammation, thus developing a useful acellular tool for the treatment of diseases characterized by chronic inflammation.

6 CONCLUSIONS

In summary, in this research we studied the effects of 5-aza pretreatment on the proliferation, migration and adipogenic differentiation of human ASCs, which are schematized in Figure 48.

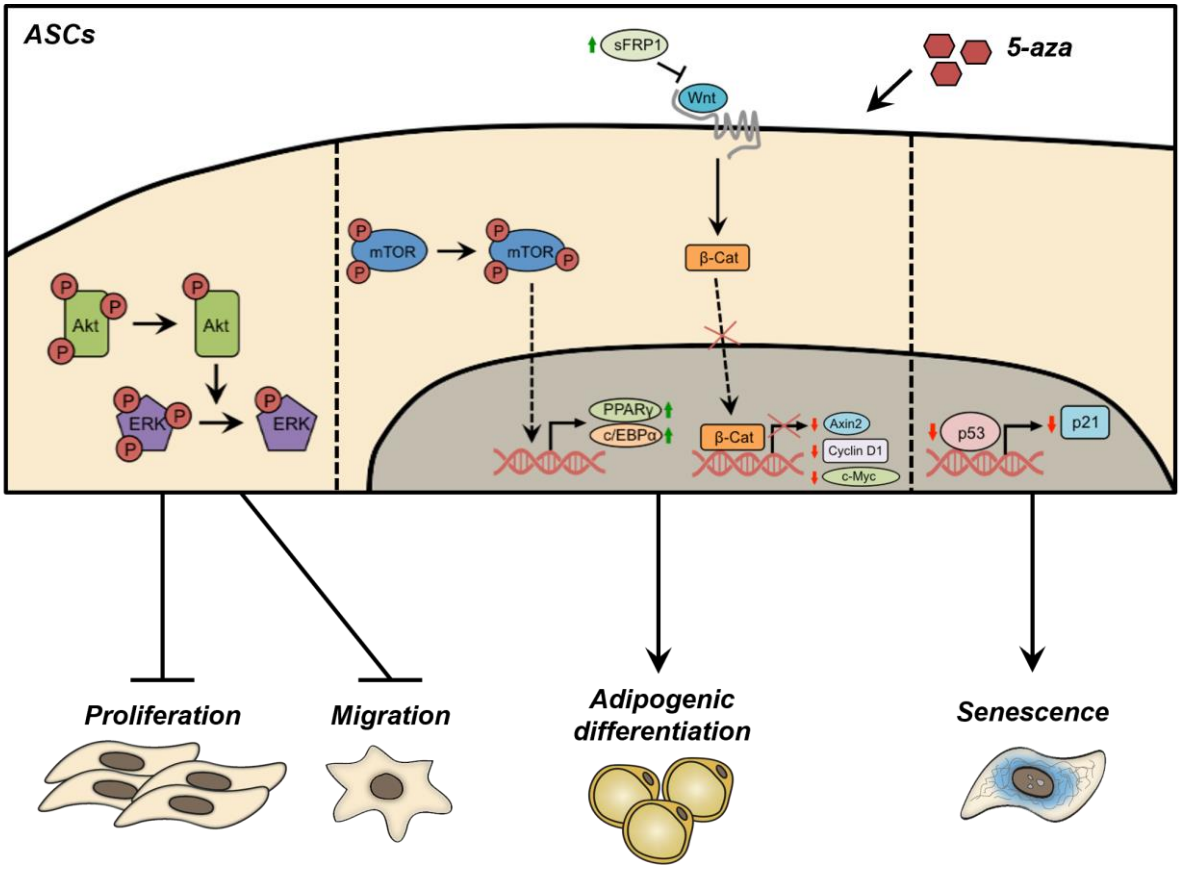


Figure 48. Schematic representation of the effects of 5-aza pretreatment on ASCs. Pretreatment with 5-aza inhibits the phosphorylation of Akt and ERK, reducing cell proliferation/migration. 5-aza treated ASCs maintained an Akt-independent activation of mTOR, allowing the activation of the master genes of adipogenesis, PPARγ and c/EBPα. The treatment with 5-aza also impairs the Wnt/β-catenin signaling, by increasing the expression of the Wnt inhibitor sFRP1, affecting β-catenin expression and nuclear translocation, and reducing the transcription of the Wnt target genes Axin2, Cyclin D1 and c-Myc. Finally, 5-aza pretreatment induces an upregulation of the p53/p21 axis and a decrease of Lamin B1 in the nuclear membrane, a known hallmark of cell senescence.

From the data emerging from our study, it is possible to attribute an inhibitory role to 5-aza in relation to the processes of cell proliferation and migration. We believe that this inhibition

could easily be explained by the decreased levels of two specific proteins, Akt and ERK, which play a key role in two important pathways, PI3K/Akt pathway and MAPK pathway. These signaling pathways, in fact, are notoriously involved in the regulation of different biological pathways, including proliferation and migration. The alterations in their signaling cascades justifies the difficulties encountered by ASCs in carrying out the aforementioned processes.

On the contrary, the analysis of the effect of 5-aza on the adipogenic differentiation program reports a positive impact of the demethylating agent on this differentiation process. To confirm this, in addition to histological and immunophenotypic analysis, which detect an early formation of intracellular lipid vacuoles and the presence of specific differentiation markers, we have reported variations in three signaling pathways: the PI3K/Akt/mTOR pathway, the MAPK pathway and the Wnt/β-catenin pathway, all important in adipogenic differentiation. Overall, the use of 5-aza pretreatment was able to induce Akt-independent mTOR activation, resulting in upregulation of c/EBPα and PPARγ, but also to induce inhibition of Wnt/β-catenin, allowing further activation of the mechanism necessary for the transcription of the main adipogenic regulators. All this, combined with the reduced phosphorylation of ERK, which in its active form would potentially be able to inhibit the action of PPARγ, would help to increase adipogenesis in ASCs.

Moreover, the molecular approach for ASC therapeutic potentiation based on a miR-125b-enriched secretome led to the observation of an enhanced immunomodulatory ability of such acellular tool. The modulation in cytokines expression, along with the decreased activation of T cells, are promising results that confirm that a secretome enriched with miR-125b-5p possesses an increased immunomodulatory efficency. Hence, boosting ASC secretome might be a useful strategy to potentiate ASCs paracrine ability to suppress inflammation, allowing to develop a strategic acellular tool for the treatment of diseases characterized by chronic inflammation.

Taking into consideration the data obtained in our study, we can contribute to shedding more light on the potential risks and advantages of an *in vitro* epigenetic and molecular approaches to improve the existing therapeutic approaches for the clinical use of ASCs.

The possibility of regulating and manipulating certain biological characteristics in specific cells allows to overcome many practical obstacles that occur in standard cell therapies. In our case, we have established that the administration of 5-aza on ASCs is able to provoke

interesting modulatory effects on cell proliferation, migration and differentiation, and that boosting ASC secretome represents a feasible strategy to enhance the paracrine immunomodulatory efficiency of these cells. These data, in addition to shedding light on the molecular mechanisms that regulate the biology of ASCs, may have important translational implications in regenerative medicine. In fact, the functional enhancement of ASCs and their secretome, can contribute to the improvement of therapeutic outcomes in the field of reconstructive surgery and in the treatment of inflammatory/immune disease, and in expanding the range of applicability in various other clinical areas.

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