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EXPLORING STEM CELL FATE FROM ADIPOSE TISSUE:

NOVEL APPROACHES TO MODULATE STEM CELL SIGNATURES

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Exploring stem cell fate from adipose tissue: novel approaches to modulate stem cell signatures.
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A Nonno

ABSTRACT

Stem cells (SCs), undifferentiated elements able to acquire specific phenotype upon stimulation, represent an important source for regenerative medicine, restoring function of compromised organs. The purpose of regenerative biology is to identify the cellular and molecular differences that distinguish normal tissue turnover from scar repair, in order to create an ideal microenvironment suitable for regeneration in damaged adult tissues. Stem cell differentiation is a complex process controlled by signaling pathways and molecular mechanisms, acting to maintain tissue homeostasis. A wide range of natural molecules and compounds, known as nutraceuticals or functional foods, are widely used for their therapeutic or preventive effects. These natural and synthetic molecules exert their action via epigenetic modulations of a specific molecular differentiation program and gene expression of lineage-specific markers. Within this context, unraveling the cellular mechanisms involved in the activation and differentiation of the adipose resident stem cells, could help in identifying innovative and preventive tools to counteract obesity and its related diseases. The aim of this project was to evaluate cell behavior in the presence of conditioned media, drugs or natural molecules, in the attempt to counteract the molecular mechanisms involved in inflammatory-associated adipogenesis. Understanding the molecular mechanisms involved in the decision of this fate could lead to the development of drugs capable of influencing stem cell behavior, for future in vivo clinical applications.

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INTRODUCTION

Regenerative medicine identifies the area of research and therapies based on the use of stem cells for tissue regeneration [1]. Several diseases lead to loss of functionality or aging of tissues and organs [2]. In the last years, the interest of researchers has been oriented toward regenerative medicine, with the aim of restoring function of compromised organs [3]. The aim of regenerative biology is to identify the cellular and molecular differences that distinguish normal tissue turnover from scar repair, in order to create an ideal microenvironment suitable for regeneration in damaged adult tissue [4][5]. Regenerative medicine encompasses a set of interdisciplinary activities, including the possibility of inserting growth factors in the damaged site, or alternatively the development of biomaterials for tissue engineering [5]. Most of these, as biomimetic polymers and bioactive three-dimensional scaffolds, are capable of inducing specific cellular responses and the formation of new tissues to be implanted *in vivo* [6][7]. Stem cells (SCs) represent an important source for regenerative medicine, to ameliorate genetic and degenerative diseases, as cardiovascular, musculoskeletal and neurological [8][9]. To be considered ideal for tissue regeneration, SCs must be easily expandable *in vitro*, multipotent and able to permanently restore tissue function avoiding neoplastic transformation [10][11]. The extra cellular matrix (ECM) plays a crucial role in stem cell differentiation [12]. External stimuli coming from the microenvironment in which they reside, known as niche, finely regulate stem cell fate in order to maintain homeostasis and remodel tissues in physiological and pathological conditions [13][14]. SCs could then be harnessed for patients with serious disease, with a high yield and minimizing the risk of rejection [15][16]. Transplantation of bone marrow-derived SCs has been applied for the treatment of celiac disease and chronic inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis (UC)[17], but also for diabetes mellitus (DM) [18]. Novel therapeutic strategies have been proposed for the treatment of DM, such as the administration of growth factors, transplantation of pancreatic islands and infusion of SCs to replace dysfunctional beta-cells [19]. Nevertheless, a possible side effect of SCs based therapies consists in their possible neoplastic transformation [20].

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Unravelling the cellular mechanisms governing stem cell behavior could lead to the development of new therapeutic approaches for tissue repair and other clinical applications.

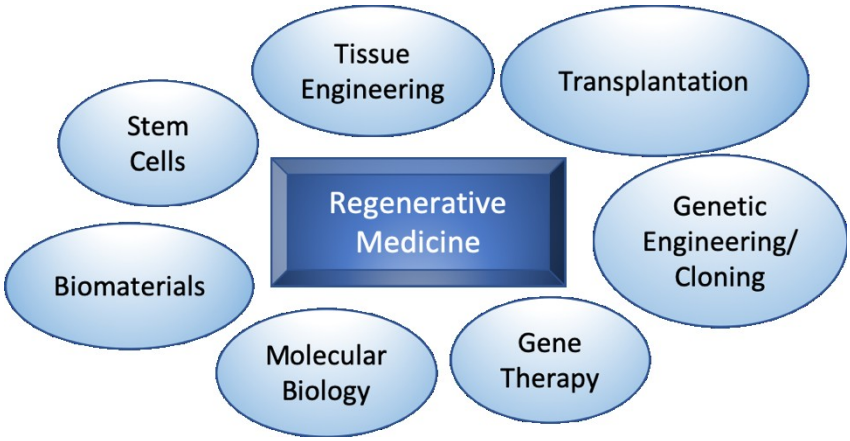

Figure 1. Regenerative medicine in biological reestablishment of tissue functions. Tissue engineering, gene and cell therapies, and stem cell research are some of the methodological approaches commonly used.

1. Stem cells

The origin of the term "stem cells" can be traced back to the late nineteenth century, in 1868 precisely, when Ernst Haeckel, a major supporter of Darwin's theory of evolution, used the term "stem cells" to describe the unicellular ancestor organism from which all multi-cellular organisms presumably evolved [21][22]. Stem cells are in fact undifferentiated primitive cells, with the unique ability to differentiate in the approximately 200 different cell types that form the body: neurons, skin cells, muscle cells, bone cells, liver cells, starting from the embryo and throughout the life span of each individual [23][24]. Therefore, they can be described as cells whose fate has not yet been decided. Four criteria are used to define a stem cell. First of all, the cell must be able to undergo multiple and sequential cell divisions of self-maintenance, a prerequisite for supporting the cell population [25][26]. Subsequently, from each cycle of replication by asymmetrical division, two daughter cells are generated, one of which may differentiate toward a particular cell line, while the other retains the features of stem cells. This is involved in replacing damaged elements in origin tissue and thus

maintaining tissue homeostasis [27][28]. The third criterion for defining stem cells deals with the ability to repopulate tissues when transplanted into a damaged recipient site, a property widely demonstrated for hematopoietic stem cells and more recently for progenitors of liver cells and nervous stem cells [29][8]. A last criterion is that stem cells must contribute to a differentiated progeny *in vivo* even in the absence of tissue damages. They can undergo differentiation, giving rise to specialized cells through a process that depends on signals inside and outside the cell [30]. Internal signals are controlled by specific genes, while external signals include physical contact with adjacent cells and specific molecules of their microenvironment, called "niche" [13][31]. The process of cellular specialization is genetically controlled by the cell nucleus and it is fundamental to allow the development of tissues [32]. According to the level of specialization, stem cells can be classified as totipotent pluripotent, multipotent, oligopotent and unipotent [33]–[35]. In mammals, only the zygote, produced by the fusion of an egg with a spermatozoon, and the cells originating from the first divisions of the fertilized egg, the blastomeres, are totipotent and represent the founder cells of all the cells in the body [36]. By successive divisions, embryonic stem cells originating from the zygote, are capable of generating all types of cells in the body, both *in vivo* and *in vitro*, but are uncapable of generating extra-embryonic tissues [37]. Then, following the process of gastrulation, the three germinative leaves and differentiated cells are generated, including tissue-specific or somatic stem cells, which are responsible for the formation of the residing tissue [38]. These cells have the function of maintaining the structural and functional integrity of the tissues through the replacement of damaged mature cells [39].

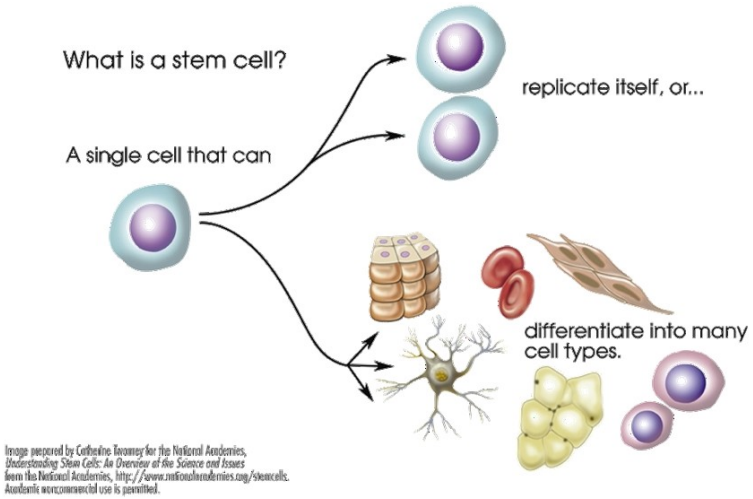

Figure 2. Characteristics of stem cells [40].

1.1 Embryonic stem cells

Embryonic Stem Cells (ESCs) derive from the internal mass (Inner Cell Mass - ICM) of the blastocyst and can be easily isolated from the degradation of an embryo of 5-7 days [41]. The first lines of embryonic stem cells were isolated from mouse blastocyst at the beginning of the '80s, by Martin Evans and Gail Martin, followed by studies carried out in the late 1990s by James Thomson [42][43]. They are cells that can not only be expanded indefinitely in culture while maintaining an undifferentiated state, but are also able to give rise to all adult cell types if injected into the blastocyst [44]. Human embryonic stem lines were obtained from cells of the internal cellular mass that were surgically isolated, dissociated and grown on a layer of murine fibroblasts, which act as "feeder" [45][46]. The term indicates a cell type capable to supply the nutrients or trophic factors necessary for the maintenance of cells in an undifferentiated state [47]. ESCs can generate disorganized masses of mature cells of ectodermal, endodermal and mesodermal origin, called embryoid bodies (EB), and give rise to teratomas if injected into immune depressed mice [48][49].

This ability, defined as pluripotency, make them extremely interesting as a possible therapeutic tool for any type of disease, but, at the same time, highlights their tumorigenic potential for uncontrolled differentiation [50]. ESCs grown *in vitro* maintain self-renewal, telomerase activity, also retaining a stable diploid karyotype. The key genes implicated in the mechanisms of proliferation and self-maintenance are known as "Oct4-like" [51]. Oct4 is the main transcription factor of ESCs, expressed by the zygote during segmentation, being downregulated in the blastocyst and silenced in adult tissues [52]. FoxD3 is another transcription factor that presents the same pattern of expression as Oct4 [53]. Other genes involved in self-renewal are Sox2 and Nanog. Nanog is a transcription factor belonging to the family of homeobox proteins and its expression seems instead limited to the cells of the internal mass and the primordial germ cells [54]. In addition, there are also enzymes used as markers for human ESCs, such as alkaline phosphatase and telomerase [55].

1.2 Adult stem cells

The use of human embryonic stem cells arises ethical implications, as isolation of stem cells from blastocyst occurs only after destroying embryos. For these ethical concerns, somatic stem cells attracted the attention of scientists [56]. Somatic or adult stem cells (Adult Stem Cells-ASCs) are capable to acquire a specific phenotype, according to the features of mature cells in their residing tissue. They show reduced levels of telomerase activity and a limited capability to differentiate, if compared to embryonic cells [57][58]. Indeed, adult stem cells undergo an irreversible senescence process that involves a progressive differentiation toward the mature stage. For these reasons they are defined multipotent stem cells [34][59].

It has been demonstrated that each tissue in the organism, can be a source of stem cells that could be used in autologous transplants, for the ability to differentiate into mature phenotypes [60]. Bone marrow- derived stem cells (BM-MSCs), for example, can differentiate into hematopoietic line, glia, skeletal muscle or bone cells [61]. Stem cells from dermis can differentiate into skeletal muscle, adipose tissue or cartilage. This ability to make different lineages has been defined as "plasticity" of adult stem cells [62]. The degree of plasticity suggests their possible use in the treatment of many diseases, even if their pluripotency does not reach that of embryonic stem cells [8].

The first adult cells extensively characterized are hematopoietic stem cells (HSC). They support hematopoiesis, giving rise to all the blood elements [63].

They were isolated for the first time in mice, based on the antigenic characteristics of the surface: they express CD45, Thy1, c-kit and Sca-1 while not express lineage (Lin) and CD34 antigens [64]. Even human HSCs do not express lineage and CD38 antigens, while they express CD45, c-kit and Thy1 and, unlike mouse antigens, are present in the CD34+ fraction of bone marrow, peripheral and cord blood [63]. Recently, these criteria have been applied to identify adult stem cells from other sources, for example neural stem cells, discriminated by a combination of CD133 and CD24 [65].

1.2.1 Mesenchymal stem cells

Human adult mesenchymal stem cells (hMSCs) can contribute to the maintenance of normal homeostasis in all tissues through the replacement of the degenerated elements [66]. Their definitive fate is regulated by specific signals, as biological molecules and biophysical factors [24]. The presence of stem cells has been identified also at the level of the bone marrow stroma. The interest of researchers has thus focused no longer on the role of stroma as a support for hematopoiesis but on the potential of stem cells located there [67]. Alexander Friedenstein and Maureen Owen were the first to use *in vitro* cultures and transplants in animal to characterize the cells that compose the bone marrow stroma [68]. The cells isolated from the stroma are also called marrow stromal cells, mesenchymal stem cells (mesenchymal stem cell-MSC) or mesenchymal progenitor cells (MPC) [10]. They have a "fibroblast-like" morphology with very little cytoplasm, few mitochondria and a poorly developed Golgi apparatus, can be easily expanded *in vitro* and show differentiating potential towards various cell types: osteocytes, chondrocytes, adipocytes, astrocytes [69][70]. The classical model of mesenchymal cell is derived from the stromal fraction of bone marrow. The isolation procedure involves the surgical removal of the portion of the matrix that is subsequently mechanical disintegrated to obtain from 0.001 to 0.01% of mononuclear cells to put in culture [71].

In vitro studies have shown that only some colonies derive from mesenchymal stem cells, while the others would originate from more mature precursors with limited

differentiation potential. Hence characterizing and identifying the real mesenchymal stem cells actually represent a crucial issue [72].

Since 1992 the existence of a precursor common to mesenchymal and hemopoietic progeny on fetal liver cells, characterized as CD34+, CD38-, HLA DR-, was hypothesized [73]. In the spinal cord microenvironment, stromal cells produce the growth factors needed to promote the activation, proliferation and differentiation of responsive stem cells. Most of these cells are quiescent (G0 of cell cycle) in their niche and only a small fraction respond to paracrine messages, differentiating into CD34+ or mesenchymal stem cells [74][75]. The CD34 marker, identified as a surface glycoprotein, belonging to the sialomucin family, and involved in the cell-cell and cell-matrix adhesion mechanisms, is expressed in hematopoietic progenitors and vascular endothelial cells but cannot be found on *ex vivo* expanded MSCs [76]. In general, MSCs express numerous molecules important for cell adhesion and interaction with the microenvironment of the niche [13]. They also express receptors for chemokine growth factors, integrins and markers shared with other cytotypes while they exhibit negative staining for typically hematopoietic markers such as CD45, expressed on myeloid cells, and CD14, receptor of bacterial lipopolysaccharide, found on monocytes [77][78]. Three antigens have been identified whose simultaneous presence on MSCs, is commonly used for their characterization. First of all the antigen SH2, epitope of the endoglin CD105 or TGF beta-IIIR [79][80]. It plays a key role in endothelial cell interactions and vessel development, mediates interactions between MSCs and hematopoietic cells in the marrow and could play a key role in chondrogenic differentiation by signal transduction [81].

On the other hand, the monoclonal antibodies SH3 and SH4 recognize two different epitopes of CD73, an antigen present in many cell types and in particular in lymphoid tissue but not in hematopoietic precursors [82]. In the spinal cord microenvironment, CD73 could mediate cell-cell interactions, representing a common element between stromal and lymphocyte development [83]. Moreover, its expression can be related to the immunomodulatory activity of mesenchymal cells. Another interesting marker is the CD166 glycoprotein, also involved in osteogenic differentiation, although the precise mechanism of this process has to be clarified yet [84][85].

It is well known that MSCs bind monoclonal antibodies directed against the low affinity receptor for the nerve growth factor, also known as CD271 [86]. This property has recently been successfully exploited to develop a method for cell selection by immunomagnetic sorting.

Due to the low rate of availability and the invasive method to isolate BM-MSCs, new sources of mesenchymal stem cells have been recently evaluated: the human body hosts multipotent stem cells within several niches, including dental pulp, dermis, Wharton jelly of umbilical cord and adipose tissue [87][88].

1.2.2 Adipose-derived stem cells

Since the discovery in 2001 of a population of mesenchymal stem cells within its stromal compartment, human adipose tissue has been evaluated as an important suitable source for regenerative medicine [89]. These cells, called adipose derived stem cells (ADSCs), have been shown to be available for adipose tissue generation, the creation of tissue banks, being also potentially cryopreserved for a long time [90]. ADSCs are multipotent stem cells obtained from the vascular stromal fraction (SVF) of adipose tissue, consisting of CD34+/CD31- adipocyte precursors, CD34+/CD31+ endothelial cells and CD14+/CD31+ macrophages [91][92]. According to several authors, ADSCs isolated from SVF can probably be considered "pericytes" or "perivascular progenitor cells" [93]. Like BM-MSCs, ADSCs generally show a fibroblast-like morphology and have properties and features equivalent to multipotent cells isolated from other tissues. The characterization of the ADSCs surface markers shows positivity towards the epitopes CD13, CD49a, CD49b, CD49d, CD90, CD105, STRO1, CD166, CD117 and CD29, CD44, CD49 and CD54, CD34, CD133, CD144 [25][94].

In vitro and *in vivo* studies have also shown a high degree of plasticity of these cells. ADSCs can be committed towards mesodermal derived tissues, as adipocytes, chondrocytes, osteoblasts, muscle cells, and towards non-mesodermal tissues, as angiogenic, hepatic, pancreatic, epithelial and neurogenic phenotypes [95][96].

The adipose tissue can be collected from different sources through various methods of isolation. The first application of adipose tissue for self-/homo-grafts belongs to the end of the 19th century [97].

The introduction of the modern liposuction technique at the end of the 1970's, allowed the removal of subcutaneous adipose tissue through the use of cannula of variable diameter, through small incisions in the area of interest [98].

This technique has the advantage of obtaining large amounts of adipose tissue with minimally invasive procedure, simplifying isolation and making it less traumatic [99].

In the following years, several improvements were made in the instrumentation, techniques and devices used. The isolation of the cellular component residing in the adipose tissue involves mechanical trituration followed by enzymatic digestion [100]. The recovery of ADSCs is higher than that of other classical adult sources such as bone marrow: the ADSCs constitute 2% of the lipoaspirate cells, while the MSCs in the marrow represent less than 0.1% [101]. An innovative technique to minimize the manipulation of any cellular product, in order to maintain the native characteristics of the tissue, was developed by Tremolada and collaborators. It is based on non-enzymatic processing of lipoaspirates, called Lipogems®, to obtain a minimally manipulated preparation containing vital ADSCs, with elimination of the oil and blood residues [102][103]. *In vitro* tests revealed the differentiating and immunomodulatory capability of these cells, representing an excellent candidate in regenerative medicine approaches. Moreover, unlike other lipoaspirates, this product can be frozen without losing the vital and functionally active stem cell population [104][102].

2. Adipose Tissue

Adipose tissue (AT) is a connective tissue responsible for different physiological functions [105]. It is composed of adipocytes, surrounded by a stroma of connective fibers, intercellular matrix, fibroblasts, immune system cells and blood vessels [106].

For many decades, the adipose tissue has been viewed as a passive organ dedicated to lipids storage during excessive caloric intake, capable of releasing energy when needed by other organs [107][108].

It represents an important energy reserve for the body, prevents the dispersion of internal heat, protects and supports the internal organs [109]. Adipose tissue exerts also important endocrine function, secreting hormones as leptin, adiponectin and resistin [110].

Adiponectin is involved in regulation of glucose and lipid metabolism and energy homeostasis, while resistin is responsible for insulin resistance and inhibits the activity of AMPK (AMP-activated protein kinase) in liver and skeletal muscle [111][112]. On the other hand, leptin is related to the inhibition of food intake and stimulation of energy consumption [113].

The distribution of adipose tissue in human body depends on age and gender differences. In men, fat is mainly stored around the waist, while fat accumulation in woman is around the hips [114][115]. Moreover, with aging, changes in body composition are associated not only with modifications in total adiposity and body weight, but also with increase in bone marrow fat content, leading to lower bone mineral density and the risk of elderly fractures [116]. Adipose tissue can be classified in subcutaneous adipose tissue (SCAT), with mechanical and protective functions [117], and visceral adipose tissue (VAT), a hormonally active component of total body fat presents in the abdominal cavity [118]. Accumulation of adipose tissue is also formed between muscles and in the connective sepia that separate the various bundles. The subcutaneous adipose tissue affects the entire body surface and above all the deep layers of skin [119]. The 50% of the fat is located in the subcutaneous panniculus, where it performs both an insulating and a mechanical function; 45% in the abdominal cavity, forming the visceral adipose tissue, and, finally, for 5% in the muscle tissue, where supports muscular activity [120]. SCAT and VAT show difference in their structures and in endocrine system regulation. In mammals, VAT contains greater numbers of large APCs than SCAT, which generally contains small adipocytes but a higher lipolytic activity [110][121]. In addition, non-adipocyte resident cell population secrete higher levels of proinflammatory cytokines in VAT then in SCAT, responsible for increased risk of developing insulin resistance and metabolic syndromes [122]. Adipocytes are responsible of storing triglycerides during calories intake and to mobilize these reserves in the form of fatty acids, according to the tissue needs [123]. Mature adipocytes retain a large number of enzymes and regulatory proteins crucial for lipolysis and lipogenesis [124].

Some of these molecules, such as adipokines, are secreted in the blood, and are able to regulate the biological activity of other cells, including central nervous system,

pancreas, liver, skeletal muscle tissue, kidneys, endothelium and immune system [125][126].

Therefore, adipose tissue plays a key role in homeostatic regulation of the energy balance, insulin sensitivity and vascular-endothelial function [109][110]. Alterations in distribution of adipose tissue and its endocrine function are related to insulin resistance, increased risk of developing type 2 diabetes mellitus, atherosclerosis and cardiovascular disease [127].

The molecular mechanisms involved in this kind of mutations are not fully known yet. It is believed that these alterations are mainly related to a dysregulation in VAT activity and adipokines secretion [128][129].

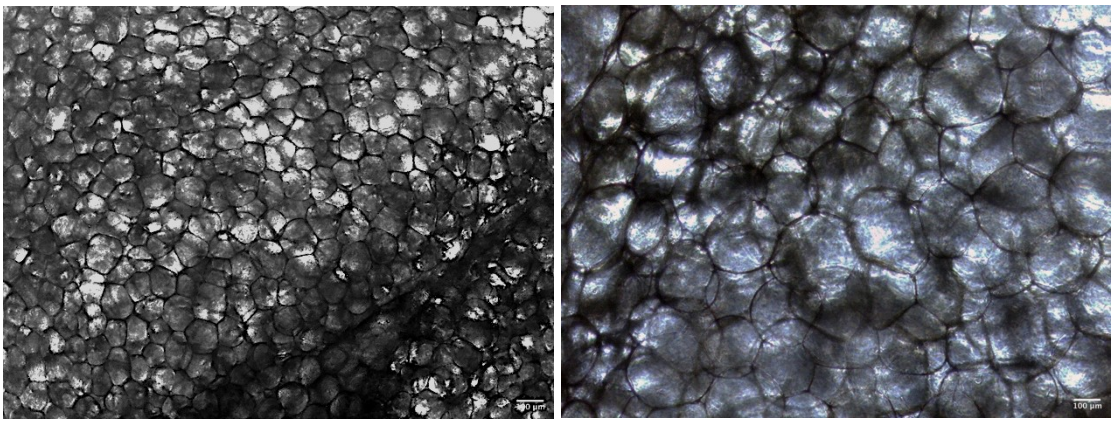

Figure 3. Structure of adipose tissue. Mature adipocytes are the main population composing adipose tissue. Images are acquired by optical microscope. Scale bar=100µm.

2.1 Types of adipose Tissue

In mammals, the fat is organized as white (WAT) and brown (BAT) adipose tissue, having typical structures and functions [130]. WAT stores energy as triacylglycerol (TAG), whereas BAT dissipates energy as heat by acid fatty metabolism [131][132].

In addition, an inducible thermogenic fatty tissue type has been identified, known as beige [133].

2.1.1 Beige adipose tissue

Beige adipose tissue, also called "brite", derives from the differentiation of precursor cells in mature adipocytes or from the transformation or trans-differentiation of pre-existing white adipocytes [134]. This kind of cells, classified as beige/brite or brown-induced, have intermediate features between WAT and BAT but are able to respond to thermogenic stimuli [135][136].

WAT, following certain stimuli, can transdifferentiate from a WAT phenotype toward a BAT-like one, modifying its morphology, gene expression and mitochondrial activity. At the beginning, they exhibit large lipid drops and the absence of UCP1[137][138]. When they receive thermogenic stimuli, the adipocytes changes their morphology to brown like features: multilocular lipid drops, with a low expression of UCP1 [139][140]. The process of WAT transforming into beige is called "browning"[141]. This transformation may depend on various stimuli, including hormonal signals as catecholamines. These are hormones released in stressogenic situations and may have relevant therapeutic applications for obesity and obesity-related syndromes in humans [142]. The factors able to induce the darkening of the WAT are classified in the class of activators of the SNS [143]. This mechanism leads to the activation of lipolytic phenomena and fatty acid oxidation, and is also associated with over-expression of genes involved in mitochondrial function and thermogenesis typical of brown adipocytes [144]. Among the activators of the sympathetic nervous system, we distinguish the β-adrenergic agonists and the endogenous signal neuropeptides. Transcription factors as peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein beta (C/EBPβ), and FOXC2, growth factors, as Bone morphogenetic protein 7 (BMP7), FGF21, BDNF, and BMP8B, enzymes and proteins associated with lipid drops, as perilipin, are also included [145]–[147]. Understanding the cellular mechanisms controlling the plasticity of WAT-BAT could lead to the design of future treatments and drugs for type 2 diabetes and obesity, also improving the outcome of tissue transplantation and gene therapy [148].

2.1.2 White adipose tissue

The WAT, also called unilocular tissue, consists of cells in close contact with each other, with a poor extracellular matrix [149].

The adipocytes are very huge and can reach 150-200 µm in diameter. The adipocytes are arranged to form aggregates, between which numerous blood vessels are placed [150].

The lipid drop is one, unilocular, which lodges almost the entire intracellular space, while the cytoplasm is reduced and located on the periphery of the cell. This tissue is constantly exposed to mechanical stresses [151][152]. The principal functions of WAT are the storage of lipids during fasting and the release of fatty acids through β-oxidation process, providing adenosine triphosphate (ATP) for cellular biological processes [153]. The white adipose tissue is also known as an important secretory organ, producing cholesterol, retinol, steroid hormones, prostaglandins and adipokines [154][155]. Alterations or inadequate release of these molecules, can lead to the onset of diseases, as obesity, insulin resistance, increased risk of metabolic syndrome, cardiovascular diseases and other more [156][157]. WAT can be distinguished in subcutaneous, under the skin, or omental, that can be localized inside the abdomen (gonadal, mesenteric, omental, and perirenal), with a less innervation than BAT [158].

2.1.3 Brown adipose tissue

The brown adipose tissue, also called multilocular, consists of adipocytes widely distributed and significantly smaller than the WAT. However, adipocytes form aggregates with a glandular-like appearance, with a real endocrine function [159][136]. BAT is a highly vascularized and innervated tissue, and brown adipocytes possess multilocular lipid droplets and a high number of mitochondria, whose cytochromes are responsible not only for vascularization, but also for the characteristic brown color of the tissue [160][161].

In fact, a special transmembrane channel protein called thermogenin is placed in the internal membrane of the mitochondria, which allows the passage of protons to the matrix without involving the complex of ATP synthase [162][163].

This tissue is responsible for the metabolism of fatty acid, thanks to the presence of mitochondria uncoupling protein 1 (UCP1). Activation of UCP1 stimulates the uptake of lipids and glucose from circulation to support thermogenesis [131][164].

The thermogenesis of BAT is physiologically stimulated by noradrenaline released by the nerve fibers of the sympathetic system (SNS) [165].

The transduction of the thermogenesis signal occurs mainly through the β-adrenergic receptors present on the membrane of brown adipocytes [166]. The activation of adenylate cyclase (AC, adenylyl cyclase) leads to increased levels of cytosolic cyclic adenosine monophosphate (cAMP). Increased cAMP induces hydrolysis of triglyceride reserves through the activation of protein kinase A (PKA) [167][168]. Through this process, the fatty acids are released and act as a substrate for mitochondrial oxidation and, at the same time, activate the UCP1 [169]. BAT is typical of rodents and hibernating animals. In humans it provides thermoregulation in the first years of life. The amount of BAT tends to decrease with age and persists in adulthood in a variable way in the perirenal, cervical, mediastinal and mesenteric areas [170][171].

2.2 Features of cells in adipose tissue

Adipose tissue is a heterogeneous cell population, including mature adipocytes, endothelial cells, macrophages, pericytes, and pre-adipocytes or adipose-derived stromal/stem cell [172]. Mature adipocytes originate from precursor cells differentiation placed in the connective tissue and capable to differentiate into adipocytes, chondrocytes, osteoblasts, and myocytes [10]. These precursor cells are called lipoblast or preadipocyte or adipoblasts [173]. During development, these cells progressively accumulate lipid drops in their cytoplasm, becoming globular, with a very reduced cytoplasm that forms a ring around a single large vacuole rich in lipids [174][175].

WAT formation is the results of increasing adipocyte size and number. The potential to produce new fat cells from lipoblast continues throughout the lifespan [176]. In the course of differentiation, a series of chronological changes in the expression of numerous genes occur. This event is represented by the appearance of mRNAs and protein markers and in the accumulation of triglycerides in the cytoplasm of the cells [177].

Among the various transcription factors involved in adipocyte differentiation, CCAAT/reinforcing binding protein (C/EBP-) and peroxisome proliferator-activated receptor (PPAR- γ) are involved in the activation of adipo-specific genes and in the cell-cycle arrest, required for adipocyte differentiation [178][179].

C/EBP-is expressed slightly before the first phases of gene transcription as compared to PPAR- γ. C/EBP transcription factor interact also with Co-activator-3 steroid receptor (SRC-3) in regulating PPAR2 expression and white adipocytes formation [180][181]. Although the expression of C/EBP-α and PPAR-γ increases dramatically during adipocyte differentiation, the low level of these factors expressed as preadipocytes may be enough to mediate the growth arrest preceding differentiation [182]. Lipoprotein lipase (LPL) is secreted by mature adipocytes, but also by other cell types such as heart muscle cells and macrophages, and is important in controlling lipid accumulation and in catalyzing the hydrolysis of triglycerides [183]. Preadipocyte factor-1 (Pref-1) also participates in the maintenance of the pre-adipogenic phenotype. During the last phase of differentiation, cultured adipocytes significantly increase lipogenesis and become insulin-sensitive [182][184]. Adipocytes also synthesize other specific adipose tissue products not directly related to lipid metabolism. These include aP2, an adipocyte-specific fatty acid binding protein found in adipose tissue and plays an important role in intracellular metabolism and fatty acid transport [185]. Acyl-coenzyme A (CoA)-binding protein (ACBP) is also significantly induced during differentiation, regulating the number of acyl-CoA esters available for various metabolic processes [186].

2.3 Understanding adipocytes features a lesson from the clinic: obesity and obesity-related disorders

Obesity is a multifactorial worldwide disease in adults, as well as among children and adolescents, associated to a great risk of morbidity and mortality due to an abnormal and uncontrolled fat accumulation in adipose tissue [187][188]. Obesity has become a global epidemic in the past decades, associated with high medical costs for its

diagnosis and treatment. Premature mortality, disability, loss of productivity and absenteeism are only a few of the consequences related to it [189]–[191].

Moreover, a large part of the health expenditure for treatment of obesity comes from public insurance. The excess of fat depots is measured according to Body Mass Index (BMI) determination, a parameter derived from the weight in kilograms divided by the square of the height in meters (kg/m2) of the individual [192]. Based on the data from the Global BMI Mortality Collaboration, a BMI of 20.0-25.0 kg/m2 is associated with the lowest mortality rate. According to World Health Organization (WHO) a BMI greater or equal to 30 Kg/m2 is consistent with obesity and a BMI between 25 and 30 Kg/m2 identifies overweight [193][194]. Obesity is classified according to BMI in:

- Normal weight 18-25
- Overweight 30-35
- 1st degree obesity 30-35
- 2nd degree obesity 35-40
- Severe obesity 40-50
- Super obesity >50

The distribution of adipose tissue in the body has important implications for health [195]. Obesity is the result of the intake of excessive calories needed for body activity. In mammals, a complex set of hormones and nerve signals acts to keep energy and nutrient consumption balanced [196].

In maintaining the body's homeostasis, the signal produced in the adipose tissue is able to influence the brain centers that control eating behavior and metabolic activity [197]. Adipokines and in particular leptin secreted by adipose tissue, provide the information on the availability of energy tissue reserves, stimulating the production of the anorexic hormone that suppresses appetite [198]. Obesity is often associated with metabolic syndrome and onset of type II diabetes, followed by cardiovascular disease, kidney failure, blindness, difficulty in healing wounds and, first of all, development of insulin resistance [199][200]. There is also a low-degree of chronic inflammation. With the

increase in adipose tissue, in particular the omental tissue, the number of macrophages, responsible for the elimination of damaged adipocytes [201] also grows. Macrophages in obese subjects control the production of most cytokines, followed by a drastic increase in insulin production [202]. Cytokine production, together with the reduced sensitivity to insulin is the main cause of the complications associated with obesity: cardiovascular diseases (CVD), hypertension and other more, leading in the most serious cases to premature death [127][203]. Not all obese patients show the symptoms of obesity-related disorders. This fraction of patients has a regular insulin sensitivity and reduced amount of visceral fat compared to obese patients with metabolic syndrome [204]. The metabolic syndrome is associated with dyslipidemia, hyperglycemia, hypertension, doubling the risk of coronary heart and cerebrovascular disease [205]. Therefore, obesity depends on body composition rather than on body weight and in particular on the number of adipocytes. The treatment of obesity requires a prolonged and determined effort [206]. Actually, interest of research is aimed at finding methods leading to a decrease in weight by eliminating adipose tissue without affecting the non-lipidic mass [207][208]. Understanding the mechanisms of adipocytes differentiation could pave the way for future therapeutic application and development of new drugs and methodologies to counteract severe obesity in patients [209].

3. Stem cell plasticity

MSCs are able to acquire different phenotypes replacing injured elements under external signals from their microenvironment, acting to maintain tissue homeostasis [210].

In the regeneration processes, stem cells residing in tissues not affected by the damage may also contribute to the function recovery after injury [211]. The plasticity of stem cells is the ability of adult tissue stem cells to acquire a new identity. The term plasticity also indicates the potential of stem cells to differentiate into adult cells phenotypically different from those of their original tissues [212][62][213]. In response to different types of signals, stem cells are able to rearrange their membrane and cytoskeleton structure. Cell polarity is a fundamental feature of migrating cells, and

represent the ability to redistribute cellular organelles and proteins for cell division and migration [214][215]. Cell viability, cell proliferation and tissues homeostasis are regulated by a perfect and controlled balance between symmetrical and asymmetrical division of MSCs [27]. The asymmetric division leads to the formation of two different daughter cells. One with self-renewal properties, like the mother cell, and the other one that differentiate toward a specific cell type and has a reduced regenerative potential [28][216]. Alterations in this system are related to the loss of tissue function, as occurs in aging [217], when stem cells lose their regenerative potential by dividing mainly symmetrically, or to uncontrolled cell proliferation, as occurs in cancer progression [218].

4. Epigenetic of self-renewal and differentiation

Stem cell differentiation is a complex process controlled by signaling pathways and molecular mechanisms that influence the expression of the main stem markers Transcription Factor 4 (Oct-4), sex determination region Y-box 2 (Sox-2) and Homeobox Nanog protein (Nanog) [219][220]. These transcription factors are essential in maintaining MSC pluripotency and are also involved in adult somatic cell reprogramming. If these factors are downregulated, cells immediately start the differentiation process [221][221]. These transcription factors are also able to suppress a group of genes needed by the embryo for development. If repressed, these set of genes leads to production of additional transcription factors responsible for the activation of genes needed for differentiation [222]. When the embryo begins to grow, Oct4, Sox2 and Nanog are inactivated and the ESCs lose their pluripotency. These transcription factors could thus be considered positive stem switchers: they regulate the expression of other genes by direct interaction with chromatin or through the presence of other factors, changing the conformation and making certain regions accessible or not for transcription [223][224]. The expression of pluripotency genes influence cell cycle and prevent the spontaneous differentiation of cells even when they are expanding in culture[225].

In vitro studies on MSCs show that an over-expression of Oct4 and Sox2 results in the promotion of proliferation, with an increase in the duration of the S phase of the cell cycle, and in a greater expression of the markers of the adipogenic and osteogenic

differentiation [226][227]. On the other hand, the over-expression of Nanog has been shown to slow down adipogenesis in MSCs [228]. Epigenetics refers to the range of hereditary changes in the structure of chromatin that can influence gene expression and represents the molecular reaction to all environmental changes [229].

These chromatin modifications, involving miRNA, DNA methylation and chromatin remodeling, are orchestrated by different types of enzymes, such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone acetyltransferases (HATs) in a still unclear fashion [230][231]. Epigenetic mechanisms are able to guide the MSCs behavior from the undifferentiated to the differentiated state. Environmental and developmental stimuli influence chromatin structure and the activation of a specific transcription program, playing a central role in maintaining MSC regenerative potential [232][233]. DNA methylation plays a key role in reprogramming somatic cells, through repression of differentiating genes and regulation of the MSC undifferentiated state [234]. Additionally, microRNAs (miRNAs), small non-coding RNAs, have been discovered as regulators of cell pluripotency and reprogramming, suggesting their possible therapeutic application of various disorders, including myocardial infarction, neurodegenerative and muscle diseases [235]–[237]. Nevertheless, further studies and continuous innovations are needed in the attempt to develop novel strategies in the epigenetic control of stem cell fate and tissue homeostasis.

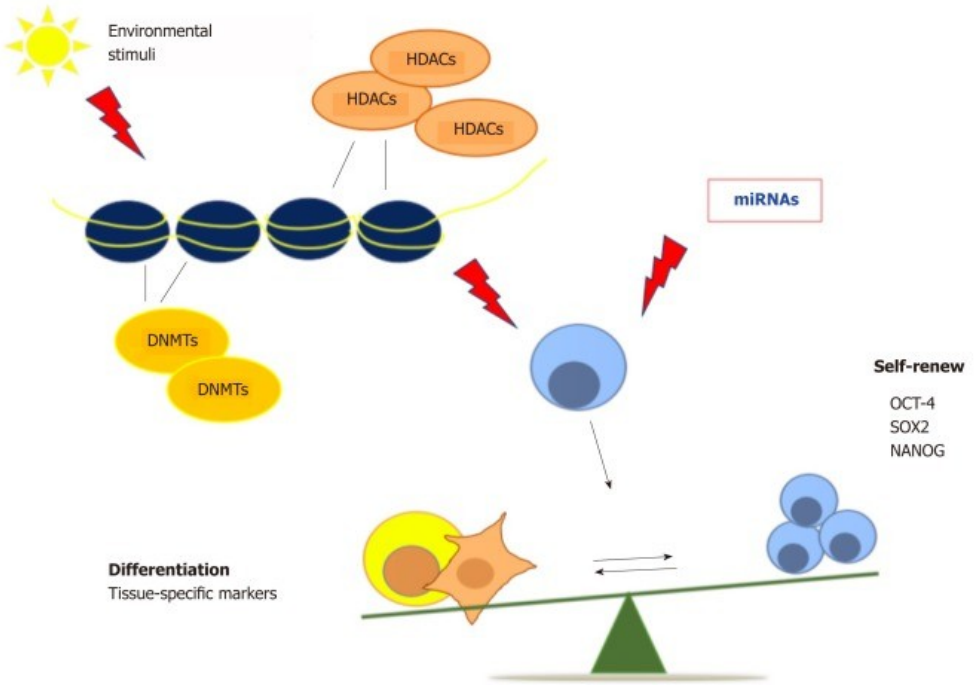

Figure 4. Epigenetic modulators exert a role in modulating stem cell fate, finely tuning stemness related genes and tissue-specific markers [238].

5. Orchestrating stem cell identity

Since the discovery of stromal cells from adipose tissue, several researchers have been interested in the use of molecules in the attempt to modulate stem cell commitment [233][238]. These natural and synthetic molecules exert their action via epigenetic modulations of a specific molecular differentiation program and gene expression of lineage-specific markers [239][240]. ADSCs cultured in the presence of calcium, or ascorbic acid, β-glycerol phosphate and dexamethasone in the growth medium, are capable to differentiate into osteoblasts, inducing an increase in the levels of expression of involved transcription factors [241][242]. Colorimetric tests (e.g. Alizarin Red) and gene expression analysis of the main osteogenic genes, highlight the accumulation of intracellular calcium deposits and reduced expression of stemness genes [243]. Retinoic acid (RA) promotes neural differentiation in mouse embryonic cells [244], or cardiac differentiation where used in combination with butyric acid and hyaluronic acid esters (HBR), increasing the transcription of cardiogenic genes and various growth factors such as VEGF (Vascular Endothelial Growth Factor) and HGF (Hepatocyte Growth Factor) [245]–[247]. More recently, a mixture of HBR and melatonin

has been successfully used to induce an osteogenic phenotype in dental pulp stem cells (DPSCs) [248]. Some authors found that the activin A, bone morphogenetic protein 4 (BMP4), VEGF or Dickkopf-related protein 1 (DKK1), can optimize cardiac development in human stem cell lines and mice [249][250]. Different combination of activin A, nicotinamide and resveratrol are able to induce mouse ADSCs differentiation into Insulin-Producing Cells [251][252]. The addition of dexamethasone, HGF, fibroblast growth factor 4 (FGF4) and other growth factors in the culturing medium induce ADSCs differentiation towards functional hepatocyte-like cells [253][254]. These findings highlight the chance to apply *in vitro* modulation of stem cells potential for future *in vivo* applications with a great impact in regenerative medicine [255], [256].

5.1 Focus on: Melatonin

A wide range of natural molecules and compounds, known as nutraceuticals or functional foods, are widely used for their therapeutic or preventive effects [257].
Melatonin (N-Acetyl-5-methoxytryptamine), synthetized from serotonin by pineal gland during the night, is a neurohormone involved in regulating circadian rhythm and reproduction, and in a number of other pleiotropic effects [258][259]. Melatonin can exert many other functions on mitochondrial activity and on the immune cell production, as well as anti-apoptotic properties, improving the outcome of stem cell transplantation [260][261]. In addition, melatonin also exhibits various effects on stem cells, controlling cell viability and differentiation. It is a molecule greatly preserved with physiological and pathophysiological functions, and whose synthesis decays with aging [262][263]. Melatonin is able to induce neural differentiation of stem cells in transplanted mice reducing the production of reactive oxygen species [264]. Moreover, melatonin can influence the differentiation of MSCs towards osteogenic, chondrogenic, adipogenic and myogenic lineages [265]. These processes involve the activation of Wnt/β-catenin pathway, MAPKs and TGF-β signaling, and the recruitment of AMPK-activated protein kinase (AMPK), and the protein Forkhead box O3 (FOXO3) [266][267]. AMPK activation is also involved in the regulation of the expression of the peroxisome proliferator-activated receptor gamma γ (PPARγ), the main adipogenic orchestrator gene and molecular target of the natural compounds used in the management of obesity [268].

These physiological effects exerted by melatonin, are mediated by the interaction with specific G protein-associated receptors, including MT1 and 2 [269][270] and with epigenetic modulators as HDACs or Sirtuins, a class of proteins involved in a wide range of cellular processes [271][272]. In particular, SIRT 1, 3 5, are mostly involved in metabolic controls, while SIRT2 and SIRT6, are mainly related to aging processes. The ability of melatonin to decrease body weight and inflammation, promoting adipocyte pyroptosis [273] and protecting tissue against mitochondrial dysfunction, suggest an application of this molecule during inflammatory obesity [274][275].

5.2 Vitamin D

Vitamin D is a fat-soluble vitamin with numerous biological properties, capable of influencing the physiology of the body system through the epigenetic regulation of over 200 genes [276]. It is involved in the maintenance of calcium and phosphate homeostasis and in the processes of bone mineralization, and its deficiency determines the onset of various diseases [277]. It is synthetized from a liposoluble prohormone that is exposed to a series of hydroxylation reactions in the skin, liver and kidney [278], starting from a cholesterol precursor, 7-dehydrocortisol, becoming vitamin D2 (25-hydroxyvitamin D3) or ergocalciferol, and vitamin D3 (1,25-dihydroxy vitamin D3) or cholecalciferol, its biologically active form [279][280]. Once synthetized, it is released into the blood where, thought vitamin D-binding protein (DBP), it can be absorbed by adipose tissue or transported to the liver for subsequent activation [281][278][282]. The enzymes responsible for both vitamin D activation and inactivation are part of the cytochrome P450 monooxygenase (CYP) family [283][284]. In humans, the main CYPs associated with vitamin D are CYP24A1, CYP27A1, CYP27B1 and CYP2R1 [285]. CYP27A1, CYP2R1, CYP3A4 and CYP2J3 are mainly located in the liver and are involved in the first phase of pre-vitamin conversion at the c-25 site into 25-hydroxy-vitamin D3 or calcitriol [286]. CYP27B1 and CYP24A1 are mainly located in the renal tubules and complete the activation pathway with hydroxylation at the c-1, producing 1,25-dihydroxyvitamin D3 or biologically active cholecalciferol [287]. In order to perform its function, the 1,25-$(OH)_2$D3 bind to a nuclear receptor, the vitamin D receptor (VDR) [277]. The VDR is a member of the superfamily of the nuclear receptor

and acts as a transcription factor induced by ligand [288][289]. The binding between vitamin D and the VDR induces conformational changes in the activation domain, allowing the formation of the transcriptional complex associated with the retinoid receptor X (RXR) [290]. The heterodimer formed binds to the specific DNA sites called VDRE (Vitamin D response elements), modulating the enzymes responsible for the chromatin modification [280]. 1,25-$(OH)_2$D3 is able to influence chromatin remodeling by modulating HDACs and DNA methylation. VDR is expressed in adipocytes and regulates adipogenic gene expression and apoptosis, and is involved in the control of calcium homeostasis and osteogenic-associated genes (osteocalcin, osteopontin, calbindin, calcium channels) [291][292]. Many genes activated by vitamin D are involved in various other systems, such as the regulation of proliferation and cell differentiation, and in oxidative stress, inflammation and immune response [293][294]. Recent studies highlighted the role of 1,25$(OH)_2$D3 in influencing the gene expression of key molecules involved in adipogenesis, fat oxidation and lipolysis through the activation of the nicotinamide-dependent adenine dinucleotide (NAD)-signaling pathway [182][295][296]. Vitamin D deficiency leads to altered bone development, such as rickets in children and, in adults, to a variety of health problems as osteoporosis, muscle weakness, cardiovascular disease, metabolic syndrome, multiple sclerosis, mental defects, a variety of cancer diseases and many other disorders [297]–[299]. Vitamin D and VDR deficiency has also recently been proposed as a cause of obesity [300][301]. Deficiencies are commonly caused by an insufficient intake or imbalance of enzymes involved in the activation and/or metabolism of vitamin D [302].

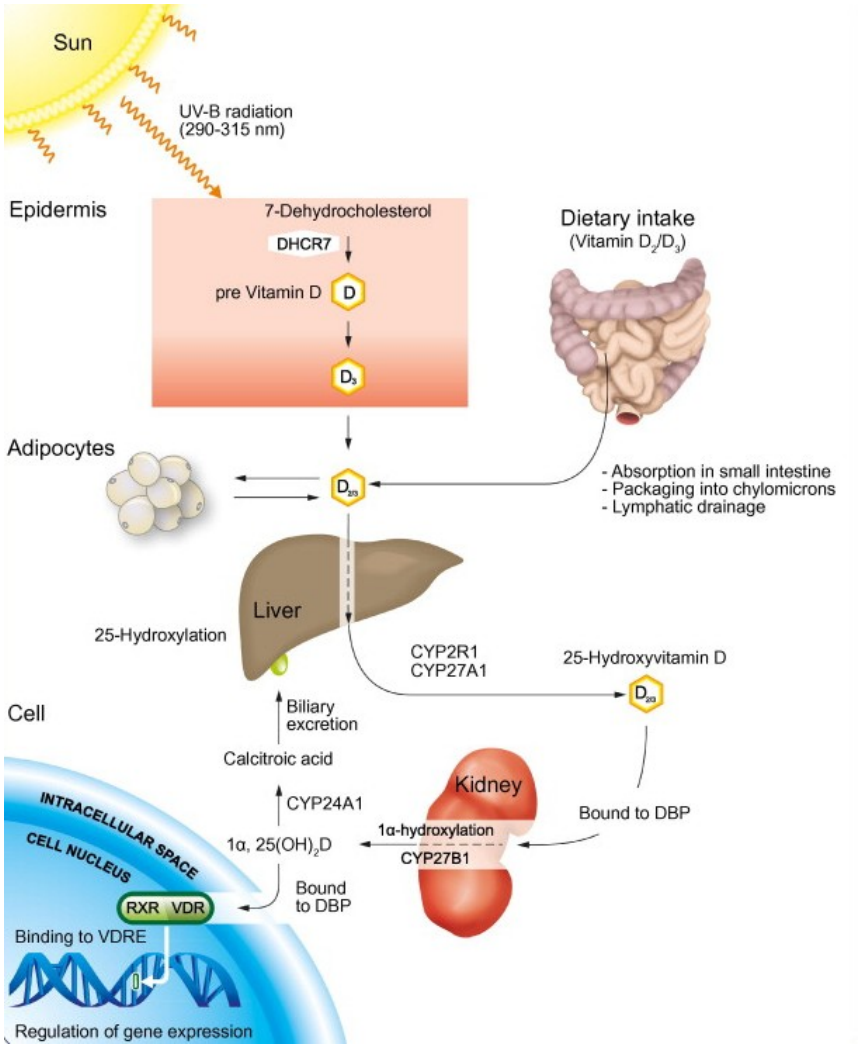

Figure 5. Vitamin D metabolism involving P450 enzymes [303].

5.3 Natural extracts from plants

Nutraceuticals are applied in regenerative medicine for their potential to modulate physiological processes and cure a variety of disorders [304][305]. Medicinal plants are known for their antiseptic, antibacterial and anti-inflammatory properties [306]. They also have antioxidant activities and stimulate tissue regeneration counteracting acute inflammation [307]–[309]. This protective mechanism exerts a key role in restoring tissue function.

Myrtus communis L. belongs to this class of plants, known for the amount of bioactive polyphenols, mainly anthocyanins, in its berries and seeds [310]. Commonly used for the production of liqueur in the food industry [311], it represents an important resource that can be used as a food supplement or extracts with antioxidant and anti-inflammatory activity [312]. Chronic inflammation leads to excessive secretion of proinflammatory cytokines (TNF-α and IL-6) involved in the onset of several diseases [313][314]. The production of reactive oxygen species (ROS) contribute to worsening the fall of the inflammatory state, increasing the release of cytokines, aging, cancer, diabetes, and atherosclerosis [315][316]. Polyphenols, as anthocyanins, gallic acid derivatives and flavonoids, counteract the release of toxic molecules and stimulate physiological defenses [317]. The class III NAD-dependent histone deacetylase sirtuin-1 (SIRT1) regulates various physiological processes and is involved in metabolism, stress response and aging [318]. Several studies have shown that downregulation of SIRT1 promotes inflammatory cytokine secretion, whereas when upregulates protects from premature senescence and oxidative stress [319][320]. In addition, phytochemicals are involved in the heat shock-induced response as a mechanism of self-defense [321]. Heat Shock Proteins (HSPs) are responsible for protein homeostasis and play an important role in aging, also protecting cells from oxidative stress damages [322]. Limited ROS production, together with modulation of TNF-α release, play a critical role in the enrolment of stem cells at injured sites, indorsing cell migration and tissue regeneration [323][324]. For these reasons, natural extracts can be applied in the attempt to drive cell proliferation and activate natural defenses through epigenetic regulations and post-transcriptional modifications [325][326].

6. Translation to clinical applications

Adult stem cells have attracted the attention in the field of regenerative medicine for their easily *in vitro* expansion and the ability to differentiate towards different phenotypes, overcoming ethical issues related to the use of ESCs [327]. MSCs are currently used in gene therapy and in the treatment of serious diseases, sometimes representing the only alternative to conventional treatments to improve the quality of life of the patients [328][329]. Transplant of MSCs is currently the most frequently used cell therapy in hematological diseases [330], and the main actor in several clinical

studies for neurological amyotrophic lateral sclerosis (ALS) [331][332]. ALS is characterized by progressive spinal cord motor neurons degeneration, involving toxicity and inflammatory processes associated with resident cell populations [333]. Currently drugs that suppress oxidative stress, can be used in the attempt to maintain motor neuron function [334]. Autologous MSC transplantation represent an alternative to conventional therapy. MSCs are used as therapeutic tools in stem cell transplants for their immunomodulatory properties, secretion of growth factors and regeneration of damaged tissues, especially in patients refractory to conventional therapies [335][336]. Autologous transplants are applied in leukemias, lymphomas, multiple myeloma and other hematological neoplasms [337]. The discovery of a population of ADSCs residents in stromal fraction of adipose tissue, has paved the way for several possible applications of lipoaspirate in the field of regenerative medicine [338]. Stem cells derived from adipose tissue have been shown to improve wound healing, ulcers and skin defects [339]; they can be used for the regenerative treatment of large head and face defects due to trauma or simply age, or used for joint reconstruction in arthritis [340]. The use of adipose tissue is very promising in breast reconstruction in patients who have undergone mastectomy for breast cancer and subsequent radiotherapy [341]. After the infiltration of adipose tissue, a marked improvement was observed due to the deposition of new collagen, skin hypervascularization and dermis hyperplasia in the case of face burns of 2 and 3 degrees [342][343]. Co-transplantation could also play an important role in preventing or contrasting the Graft-versus-Host disease (GvHD), one of the most frequent clinical complications of rejecting allogeneic bone marrow transplants [344][345]. For these reasons, adipose tissue is considered an ideal source of stem cells that can be used for tissue generation and reconstruction and, above all, for autologous transplants [346]. For acute diseases such as heart attack or immunologically based diseases as diabetes, allogenic therapy may be necessary unless cells can be obtained in large quantities from the patients [347]. Nowadays, several clinical trials with MSCs are ongoing, being successful in the treatment of imperfect osteogenesis, in improving the rooting of transplanted HSCs and in the control of autoimmune diseases (multiple sclerosis) and the rejection of transplants, in the repair of metabolic diseases (juvenile diabetes), cardiovascular system (heart attack, stroke) and the nervous system, as Alzheimer and Parkinson [347]–[349]. Unravelling the factors that

govern these processes, should lead to an improvement in the methods for stem cell differentiation, making this phenomenon clinically relevant.

AIM OF RESEARCH

The stromal fraction of adipose tissue is an important source of stem cells exploitable in regenerative medicine. MSCs residing in adipose tissue play an important role in the maintenance of cellular and tissue homeostasis, replacing dead or non-functioning cells. Fat tissue seems to play a central role in inducing inflammation, as excessive nutrition leads to changes in its cellular composition and to the production of pro-inflammatory cytokines. Hence, inflammation occurring in the injured tissue acts by recruiting resident MSCs. Abdominal obesity is associated with a state of chronic inflammation, which leads to insulin resistance and metabolic disorders. Understanding the cellular mechanisms involved in the activation and differentiation of the adipose resident stem cells, could help in identifying innovative and preventive tools to counteract obesity and the related diseases. The aim of this project was to evaluate cell behavior in the presence of conditioned media, drugs or natural molecules, in the attempt to counteract the molecular mechanisms involved in inflammatory-associated adipogenesis. Within this context, we aimed at modulating the epigenetic mechanisms involved in stem cell differentiation and cellular behavior, reducing both the production of pro-inflammatory cytokines and the inappropriate activation of adult stem cells in stressful conditions. The findings obtained here could open up novel strategies for future therapeutic approaches in epigenetic controlling stem cell fate and tissue homeostasis.

7.1 Adipose-derived stem cell isolation

Adipose-derived Stem cells (ADSCs) were isolated from subcutaneous and omental adipose tissue harvested during surgery processes for different reasons, from human adult female and male patients (n = 12, age = 45 ± 15 years, BMI: 22 ± 3 kg/m^2). All the patients participating at the study, approved by the Ethics Committee Review Boards for Human Studies in Sassari (n° ETIC 240I/CE 26 July 2016, Ethical committee, ASL Sassari), signed a written informed consent. Cell isolation procedures were performed by mechanical and enzymatic digestion of fat tissue, which was washed repeatedly with sterile Dulbecco's phosphate buffered saline (DPBS) (Euroclone, Milano, Italy) containing 200 U/mL penicillin and 0.1 mg/mL streptomycin (Euroclone, Milano, Italy), to remove the blood cells. Subsequently, fat sample was mechanically reduced to small fragments by sterile scalpels and then enzymatically digested for 1 hour at 37°C in a solution of 0,1% Collagenase Type I (Gibco Life Technologies, Grand Island, NY, USA). The enzyme activity was neutralized with 10% of fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) and the fat digestion solution was filtered with 70 µm cell strainer (Euroclone, Milano, Italy) and centrifuged at 600× g for 10 min, to separate the two distinct cell fractions of mature adipocytes, that were removed, and the stromal vascular fraction (SVF) that includes adipose-derived stem cells. The SVF in fact, is composed of preadipocytes, endothelial cells, pericytes, fibroblasts, adipose-derived stem cells (ADSCs) and hematopoietic stem cells. After collagenase digestion, mature adipocytes with a high fat content are separated as a floating layer. The adipocytes were transferred into a 12 cm^2 culturing flasks filled with basic Dulbecco's modified Eagle's Medium (DMEM) (Life Technologies Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 200mM L-glutamine (Euroclone, Italy), and 200 U/mL penicillin—0.1 mg/mL streptomycin (Euroclone, Milano, Italy). The flasks were placed upside-down in incubator at 37°C with 5% CO_2. Once attached, the adipocytes were washed and put in incubator with fresh medium.

All cells remaining after the removal of mature adipocytes constitute the SVF. The pellet of SVF was resuspended into a basic medium, plated in 12 cm^2 culturing flasks and transferred in incubator at 37°C and 5% CO2. After 48 h of incubation, the cultures were washed with DPBS and kept in the fresh medium. The culture medium was changed every 3 days. When the cells reached 80–90% confluence, they were harvested using 0.25% Trypsin EDTA (Euroclone, Milano, Italy), counted and passed into new flasks.

7.2 Cells magnetic separation

For isolation of adipose progenitor cells from the stromal vascular fraction (SVF), CD117 MicroBeads (Miltenyi Biotec, Minneapolis, MN, USA) were used. CD117, also known as c- kit, is a stem cell receptor, encoding a 145kD cell surface glycoprotein belonging to the class III receptor tyrosine kinase family [350]. According to the manufacturer's instructions, cells were trypsinized and resuspended in a specific buffer containing phosphate- buffered saline (PBS) pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Cells were then incubated with a primary monoclonal anti-c/kit (CD117) antibody for 1h at 37°C and, after washing, with a secondary antibody directly conjugated to MicroBeads for 15 min at 4°C. Then, the cell suspension was loaded onto a MACS® Column, which was placed in the magnetic field of a MACS Separator. The magnetically labeled CD117+ cells were retained within the column, while the unlabeled cells eluted through. After removing the column from the magnetic field, the magnetically positively selected CD117+ cells can be eluted in another tube, recovered and put in culture for subsequent experiments.

7.3 ADSC characterization by flow cytometry

To evaluate the percentage of mesenchymal markers on isolated population, flow cytometry analysis was performed. A total of 1×10^6 ADSCs were trypsinized, centrifuged and fixed in 1% formaldehyde for 10 min at room temperature. After fixation, cells were permeabilized using a permeabilization buffer (eBioscienceMilano,

Italy) for 30 min at 4◦C, and then washed and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated primary antibodies for 1h at 4°C.

ADSCs were tested for CD73-PE, CD90-PE (BD Biosciences, San Jose, CA, USA), CD105-PE (Santa Cruz Biotechnology, Heidelberg, Germany), CD45-FITC and CD31-FTC (Sigma-Aldrich, Munich, Germany) (all at 1 μg/10^6 cells). After washing, cells were analyzed on a flow cytometer (CytoFlex, Beckman Coulter, Milan, Italy) by collecting 10,000 events. Cells were positive for CD73, CD90 and CD105, and negative for CD31 and CD45 (Table 1). The data analysis was performed using CytExpert Software (Beckman Coulter, California, USA).

Table 1. Cell characterization by CytoFlex Beckman Coulter.

Antigen	Expression
CD31	-
CD45	-
CD73	+
CD90	+
CD105	+

7.4 ADSC culturing and treatment

7.4.1 Culture in the presence of melatonin and Vitamin D

After characterization, ADSCs were grown in a basic medium (BM) and propagated until the quantity required to carry out the subsequent *in vitro* tests has been reached. Cells at passage 5 were thus exposed to various treatments. One group of cells, used as undifferentiated control, was maintained in a growing (BM). Another group of ADSCs was induce to adipogenic differentiation by culturing in a conditioned adipogenic differentiation medium (DM or ADM), composed of basic medium supplemented with 1 μM dexamethasone, 0.5 μM hydrocortisone, 60 μM indomethacine, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX Sigma Aldrich Chemie GmbH, Munich, Germany). Positive control of adipogenic differentiation was represented by mature adipocytes.

One group of cells was exposed to differentiation medium in the presence of 0,01M melatonin (Melatonin-DM or Melatonin+ADM), or 10^{-8}M vitamin D (Vitamin D10^{-8}M-DM) or 10^{-6}M vitamin D (Vitamin D 10^{-6}M-DM), or melatonin plus 10^{-8}M vitamin D (Melatonin+Vitamin D10^{-8}M-DM), melatonin plus 10^{-6}M vitamin D (Melatonin+Vitamin D 10^{-6}M-DM or Melatonin+VitaminD+ADM). As a positive control for osteogenic differentiation, a group of ADSCs was cultured in a previously described osteogenic differentiation medium (ODM) [248], containing DMEM (Life Technologies Grand Island, NY, USA), 20% FBS (Life Technologies, Grand Island, NY, USA), 100 nM dexamethasone, 200 µM L-Ascorbic acid 2-phosphate, 10mM betaglycerol2-phosphate (all from Sigma Aldrich Chemie GmbH, Munich, Germany), 2mM L-glutamine (Euroclone, Milan, Italy), 200 U/mL penicillin-0.1 mg/mL streptomycin (Euroclone, Milan, Italy).

7.4.2 ADSC exposure to natural extracts

ADSCs at passage 5 were cultured in a basic growing medium in the presence or absence of natural *Myrtus* by-products. Pulp and seeds of this Mediterranean plant, residual of industrial liquor production (Industrial by-Product or Ind by-P) or obtained in laboratory from fresh fruit (Laboratory by-Product or Lab by-P), were freeze-dried and lyophilized. To perform the experiments on cells, the freeze-dried *Myrtus* extracts was suspended in cell basic culturing medium at a final concentration of 0,5 mg/ml. The ADSCs were then directly exposed to extracts for 12, 24 and 48h, while control cells were cultured in the basic growing medium only. After extracts exposure, cells were incubated with 100 μM H_2O_2 in basic growing medium for 1h, to induce senescence. ADSCs cultured with 100 μg/ml ascorbic acid (Sigma-Aldrich, Germany) were used as positive control of antioxidant activity and stressful conditions [351].

7.5 Cell viability assay

Cytotoxicity of *Myrtus* extracts was evaluated by the Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Sigma-Aldrich, Germany). Cells, seeded in 96-well plates at a concentration of 10,000 cells/well, were cultured in the presence of 200 μl of different

Myrtus by-Products for 12, 24, and 48 hours. ADSCs were then induced to senescence by H_2O_2 exposure. At the end of incubation, the medium was replaced with 100 μl of 0.65 mg/ml MTT and cell were incubated for 2h at 37°C.

After incubation, formazan was dissolved in DMSO and absorbance detected at 570 nm using Varian50 MPR, Microplate reader. The viability of H_2O_2-senescenct cells precultured with *Myrtus* extracts (treated cells) was calculated as % cell viability referred to untreated control cells as following formula: (OD570 treated cells) × 100/(OD570 control).

7.6 Nitric Oxide Production

To evaluate the nitric oxide (NO) production after treatment with by-Products and induction of senescence, Griess Reagent Kit for Nitrite Determination (Thermo Fisher Scientific, USA) was used. ADSCs were seeded in 96-well plate and incubated with 150 µl of nitrite standard solution for 30 min according to the manufacturer's protocol. The concentration of nitrites at different time point was read in a spectrophotometric microplate reader (Varian50 MPR, Microplate reader) as the absorbance at 548 nm wavelength of each treated sample referred to control.

7.7 RNA extraction and gene expression analysis

Total RNA of control undifferentiated cells and treated ADSCs cultured in the previously described conditions, was isolated at each time point, using the ChargeSwitch total RNA Cell Kits (Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol, and measured by spectroscopy with Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Approximately 1 µg of total RNA from each treatment was reverse transcribed in cDNA using Superscript Vilo cDNA synthesis kit (Life Technologies USA), according to the manufacturer's instructions. Quantitative polymerase chain reaction was performed using a CFX Thermal Cycler (Bio-Rad). Amplification was run in 96-well reaction plates (Applied Biosystems, Darmstadt, Germany) using Platinum® Quantitative PCR SuperMix-UDG Kit (Thermo Fisher Scientific). A 2× SuperMix whit SYBR Green I, 0.1 µM of each primer, and 3 µL cDNA generated from 1 µg of the total RNA template

were mixed in 25 µL volumes and added to each reaction. The qRT-PCR was performed under standard conditions (50 ◦C for 2 min, 95 ◦C for 2 min followed by 40 cycles of 30 secs at 95°C for denaturation, 30 secs at 60-64 for annealing and 1 min at 60°C for extension). Each sample was performed in triplicate wells for each gene, including endogenous control, a non-template control and distilled water control on the same plate. The relative expression of each transcript was determined using target Ct values and normalized to GAPDH, considered as a reference gene, while the mRNA levels of positive controls and ADSCs treated with the different conditioned media were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of the mRNA levels observed in undifferentiated ADSCs at time 0, define as a control. The qRT-PCR was performed for the following genes: octamer-binding transcription factor 4 (Oct-4); Sex determining region Y-box 2 (Sox2); Homeobox protein Nanog (NANOG); adipocyte fatty acid binding protein (aP2); peroxisome proliferator-activated receptor gamma (PPAR-γ); lipoprotein lipase (LPL), and acyl-CoA thioesterase 2 (ACOT2); histone deacetylases class I (HDAC1); histone deacetylases class III or Sirtuins (SIRT 1 and 2); Stanniocalcin 1 (STC1); Osteocalcin (bone gamma-carboxyglutamic acid-containing protein BGLAP); Bone morphogenetic protein (BMP2); Interleukin 6 (IL-6); Tumor necrosis factor alpha (TNF-α) and Heat Shock Protein 90b (Hsp90b). All primers used, designed using "PRIMER BLAST" and "primer3", spanning all exons and highly specific, were from Life Technologies and are reported in Table 2.

Table 2. Primers sequences.

PRIMER NAME	FORWARD	REVERSE
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG
OCT-4	GAGGAGTCCCAGGCAATCAA	CATCGGCCTGTGTATATCCC
SOX2	CCGTTCATGTAGGTCTCGGAGCTG	CAACGGCAGCTACAGCTAGATGC
NANOG	CATGAGTGTGGATCCAGCT	CCTGAATAAGCAGATCCAT
aP2	AGACATTCTACGGGCAGCAC	TCATTTTCCCACTCCAGCCC
PPAR-γ	AATCCGTCTTCATCCACAGG	GTGAAGACCAGCCTCTTTGC
LPL	CAGGATGTGGCCCGGTTTAT	GGGACCCTCTGGTGAATGTG
ACOT2	GAGGTCTTCACACTGCACCA	TCTTGGCCTCGAATGGTATC
HDAC1	ACTGCTAAAGTATCACCAGAGGGG	CACACTTGGCGTGTCCTTTG
SIRT 1	CATTTTCCATGGCGCTGAGG	TGCTGGTGGAACAATTCCTGT
SIRT 2	TTGCTGAGCTCCTTGGATGG	GGGGAGGGAGCTGTAAGAGA

STC1	TTCGGAGGTGCTCCACTTTC	CAGGCTTCGGACAAGTCTGT
BGLAP	GAGCCCCAGTCCCCTACCCG	GACACCCTAGACCGGGCCGT
BMP2	GAAGAAGAGGAGACTTCAAATG	TATCCCCAGCCTTCTTGGGA
IL-6	TCTCAACCCCCAATAA	GCCGTCGAGGATGTA
TNF-α	CCTCAGACGCCACAT	GAGGGCTGATTAGAGAGA
Hsp90b	AGTTGGAATTCAGGGCATTG	TTTCTCGGGAGATGTTCAGG

7.8 Immunofluorescence Microscopy

ADSCs were induced to differentiation in the presence of various conditioned medium, for 21 total days. Cells used as control were cultured in a basic medium to maintain their undifferentiated state. At the end of differentiation, cells were trypsinized and cell suspension transferred at low density in 8-well chamber slides (BD-falcon). Once attached at slide surface, ADSCs were fixed with 100% Methanol (Sigma Aldrich GmbH, Germany) at −20 °C for 30 min and then at −80 °C for 30 min and permeabilized by 0.1% Triton X-100 (Life Technologies, USA)-PBS. Cells were then washed three times in PBS and incubated with 3% Bovine Serum Albumin (BSA)—0.1% Triton X-100 in PBS (Life Technologies, USA) for 30 min. Primary monoclonal antibodies directed against activating signal cointegrator-1 (ASC-1) (Santa Cruz Biotechnology, Heidelberg, Germany), proton-coupled amino acid transporter (PAT2) (Santa Cruz Biotechnology), and transmembrane protein 26 (TMEM26) (Abcam, Cambridge, UK) were added and incubated overnight at 4°C. At the end of incubation, after washing step in PBS, ADSCs were incubated with fluorescence-conjugated goat anti rabbit IgG or TRITC-conjugated anti mouse secondary antibodies at 37 °C for 1h in the dark. Cell nuclei were labelled with 1 µg/mL 4,6-diamidino-2-phenylindole (DAPI). All microscopy analyses were performed with a confocal microscope (TCS SP5, Leica, Nussloch, Germany).

7.9 Red Oil O and Alizarin Red staining

To evaluate cell differentiation processes, ADSCs cells were cultured for 21 days on tissue culture 24-wells plate (BD-falcon) in the presence of different conditioned media or basic growing medium for undifferentiated control. For adipogenic differentiation, cells were fixed with 10% formalin for 30 min at RT and then washed twice for 5 min

in H2O and in 60% isopropanol. ADSCs were stained with Oil red O solution for 15 min and after a washing step in H2O, counterstained for 2 min in Mayer's hematoxylin solution (Sigma Aldrich GmbH, Germany).

The level of adipogenic differentiation was evaluated by light microscopy and analysis of lipid accumulation was performed using image analysis software (ImageJ, National Institutes of Health, USA), using mature adipocytes as a positive control.

For osteogenic differentiation cells were fixed with 10% formalin for 15 min at RT. After three washing steps in distilled water (ddH_2O), ADSCs were stained with 2% alizarin red S solution (Santa Cruz Biotechnology) for 20 min at RT. To avoid the excess of solution, cells were then washed several times with ddH_2O and finally observed under a light microscope (Leica, Nussloch, Germany). Cytoplasmatic calcium deposition was analyzed using ImageJ (ImageJ, National Institutes of Health, USA), using cells cultured in osteogenic differentiation medium as positive control of osteogenic phenotype.

7.10 Senescence Associated β-galactosidase Staining

The number of senescent cells in culture was evaluated by the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Germany). ADSCs plated in 6-well plate were cultured in the presence or absence of *Myrtus* extracts for 12, 24 and 48h, and then senescence was induced by exposure to H_2O_2. At each time point, the cells were fixed and stained with X-gal Solution according to the manufacturer's instructions. Cells were then incubated overnight at 37°C and finally observed by light microscope (Leica, Nussloch, Germany). SA-*β*-Gal activity and the number of relative positively blue-stained cells was calculated as the percentage of total number of cells using ImageJ software (ImageJ, National Institutes of Health, USA).

7.11 Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences version 13 software (SPSS Inc., Chicago, IL, USA). Non-parametric Kruskal-Wallis rank sum and Wilcoxon signed-rank test were applied to evaluate the distributions and homogeneity of each group variance at different times of observation, assuming p value <0.05 as statistically significant. The first was used to detect differences between treatments, while the second was applied to evaluate, in the same group, differences (delta Ct) between data collected over a period of observation and the control reference value.

RESULTS

8.1 Features of ADSCs after treatment with conditioned media

Changes in morphology of ADSCs was evaluated by optical microscopy after 21 days of exposure to melatonin and vitamin D conditioned media. Melatonin+vitamin D-treated cells showed a significant different morphology as compared to both cells cultured in adipogenic medium (ADM) alone, exhibiting a morphology typical of mature adipocytes, and with untreated control cells, culturing in a basic growing medium (BM) (Figure 9).

8.2 Biomolecules maintain cell viability reducing nitric oxide production after H_2O_2 exposure

Thiazolyl Blue Tetrazolium Bromide (MTT) assay was used to evaluate cell metabolic activity after extracts exposure and senescence initiation. The extracts tested showed no cytotoxicity in cells, nevertheless a slight increase in cell viability after H_2O_2 exposure was observed (Figure 10). *Myrtus* biomolecules preserved the viability of cells exposed to stressor events for all the evaluated time points, maintaining their mitochondrial activity, as compared to not pre-treated control cells. Moreover, *Myrtus* extracts showed an important antioxidant activity, able to significantly counteract the production of nitric oxide after a strong oxidative stress, as the exposure to H_2O_2. The NO production was considerably reduced after 12 and 24h of treatment with extracts, as compared to control cells. This decrease was maintained even after 48h of *Myrtus* exposure, although being statistically not significant (Figure 11).

8.3 Modulation of stem cells regenerative potential in inflammatory response

ADSCs exposure to *Myrtus* extracts was able to significantly decrease the expression of proinflammatory cytokines, induced by H2O2 stressor, as compared to untreated cells. In particular, Interleukin 6 (IL-6) and Tumor necrosis factor alpha (TNF-α) showed a

significantly downregulation after 12h of treatment (Figure 12) while, being induced for longer period of treatment.

This result could be explained considering the ability of this cytokine to stimulate regenerative properties of stem cells by their recruitment at the injured site. Moreover, also stem cell plasticity was implemented, as demonstrated by the upregulation of the stemness related genes Oct-4, Sox2 and NANOG upon *Myrtus* exposure (Figure 13).

8.4 Cellular response to stress and cell differentiation involve Sirtuin-Dependent Epigenetic Changes

Cellular response to oxidative stress damages is associated with increased levels of HSP90b and SIRT1 mRNA (Figure 14), protecting cells from a premature senescence. Moreover, this upregulation in Sirt1 and 2 gene expression could also be observed during stem cell differentiation. HDAC1 and Sirtuins were significantly upregulated in cells exposed to the adipogenic medium in the presence of melatonin, reaching a maximum after 21 days in culture (Figure 15). This effect was further highlighted when cells were cultured in the presence of both melatonin and vitamin D, as compared to cells exposed to the adipogenic medium alone. In addition, also cells cultured in the presence of the classical osteogenic medium exhibited the same trend (Figure 15), demonstrating a role of the epigenetic regulators HDAC1 and Sirtuins in stem cell osteogenic differentiation.

8.5 Melatonin and vitamin D counteract adipogenesis inducing the molecular pattern of osteogenesis

The combination of the two molecules modulated the expression of the main adipogenic markers during ADSCs differentiation. ADSCs exposed to the adipogenic medium showed an upregulation of PPAR-γ expression after 7, 14 and 21 days of culturing, while the expression of the same gene significantly decreased in cells cultured in the same adipogenic medium plus vitamin D or melatonin or both (Figure 16). AP2 was also actively expressed in cells exposed to adipogenic differentiation medium, showing a significant downregulation when the two molecules were added to the same medium (Figure 16). LPL and ACOT2 showed the same trend (Figure 16). Interestingly, ADSCs cultured in the adipogenic medium alone did not express the osteogenic markers STC1,

BGLAP and BMP2 that were induced when cells were cultured in the same medium but in the presence of melatonin and/or vitamin D (Figure 17).

Similar results were obtained for the adipogenic-related genes ACOT2, LPL and TMEM26.

The expression of these genes was induced when ADSCs were cultured for 21 days in the presence of the adipogenic differentiation medium alone, while was significantly inhibited in the presence of melatonin and vitamin D in the same medium (Figures 18-20).

8.6 Melatonin and vitamin D inhibit intracellular lipid accumulation inducing ADSCs mineralization

Consistent with gene expression analysis, ADSCs exposure to the adipogenic differentiation medium induced intracellular lipid accumulation, that was counteracted in the presence of melatonin or vitamin D (Figure 21). Fat droplet formation was inhibited when melatonin and vitamin D were added simultaneously to the adipogenic differentiation medium, while cytosolic calcium accumulation was induced (Figure 21). The mineralization process was absent in cells grown in an adipogenic differentiation medium alone, while appearing particularly in cells exposed to the same medium plus melatonin and vitamin D, and being superimposable to what observed in cells cultured with the classic osteogenic differentiation medium (Figure 22).

8.7ADSCs survive to premature senescence induced by oxidative stress

ADSCs treated with *Myrtus* by-Products counteracted premature senescence induced by H_2O_2 exposure, as shown by the number of blue senescent cells. The extracts are able to protect cells from oxidative stress damages, significantly counteracting the senescence process (Figure 23). β-gal analysis revealed that this antisenescence activity of extracts was higher for 24 and 48h of treatment (Figure 23).

CONCLUSIONS

The human body possesses the ability to resist to various environmental stressors, by activating adaptation mechanisms responsible for restoring the physiological homeostasis [352][353]. Variations in biological processes lead to pathological condition as aging, diabetes, cardiovascular diseases and cancer [354][355]. Inflammation is one of the first biological responses against injuries, essential for protecting tissue integrity and functionality [313]. Oxidative stress could be responsible for activating inflammatory response, along with pro-inflammatory cytokine secretion and premature cellular senescence [356]. Endogenous biological systems are able to balance ROS production and modulate cellular responses to increase physiological defense processes and cell survival [357]. When this regulation system is compromised, the body loses its ability to counteract stress, leading to irreversible organ and tissue damages [358]. Natural compounds, as flavonoids and anthocyanins also known as phytochemicals, can directly interact with endogenous antioxidant responses, showing anticancer properties and protection against several disorders [359]–[361]. These molecules show immunomodulatory properties, able to counteract the synthesis of enzymes involved in ROS production [362][363]. *Myrtus* contains a large amount of these compounds in its berries that can be employed in reducing inflammation, oxidative stress, also protecting cells from premature senescence[308], [309][364][365]. The pretreatment of ADSCs with different *Myrtus* by-Products decreased the production of nitric oxide after oxidative stress induction (Figure 11) and significantly downregulated the expression of TNF-α and IL-6 in the first hours of H_2O_2 exposure (Figure 12). On the other hand, pro-inflammatory cytokines release is crucial in promoting tissue regeneration through stem cell recruitment [366][367]. *Myrtus* extracts promote tissue regeneration, through modulation of TNF-α and IL-6 levels and above all increasing the expression of stemness markers. In fact, Oct-4, Sox2, and NANOG showed a significative upregulation in ADSCs exposed to *Myrtus* treatment, as compared to control untreated cells (Figure 13). In addition, the treatment with the extracts increased the levels of Hsp90b expression (Figure 14), involved in maintenance of mitochondrial activity and cell viability in stressing conditions [368]. The upregulation of the pluripotency markers is strictly related to the regulation of SIRT1 deacetylase expression, preventing premature senescence (Figure

14) being also involved in cellular stress and inflammatory responses [369]. Furthermore, the NAD-dependent protein deacetylase SIRT1 is associated with regulation of stem cell differentiation processes [370], by inhibiting PPAR-γ expression and the appearance of an adipogenic phenotypes [371]. During adipogenesis, PPAR-γ was significantly upregulated in ADSCs cultured in the presence of adipogenic differentiation medium, as compared to control untreated cells (Figure 16), while the presence of melatonin and vitamin D interfered with its expression, committing cells to the osteogenic phenotype (Figure 17). Epigenetic modulators are frequently used to orchestrate stem cell fate [233][237]. Butyric acid, in combination with retinoic and hyaluronic acids, is able to inhibit HDAC expression, inducing stem cells differentiation toward a cardiac phenotype [247], [372], [373]. HDAC class 1 is involved in the establishment of a specific stem cell fate, while maintaining stem pluripotency upon inhibition [374]. During the first three days of culturing according to the above described conditions (adipogenic conditioned media with melatonin and vitamin D), ADSCs showed a downregulation of HDAC1 (Figure 15), that was then upregulated from 7 to 21 days of culturing in the presence of adipogenic differentiation medium and the combination of melatonin and vitamin D (Figure 15). These changes in HDAC1 level of expression is related to the appearance of an osteogenic phenotype, despite the presence of the adipogenic conditioned medium. Our results can explain the establishment of an adipogenic commitment during the first days of treatment, followed by a loss of stemness and the appearance of an osteogenic phenotype at the end of the differentiation process, as described also by other authors [375]. The presence of melatonin and vitamin D in the adipogenic medium is able to counteract intracellular lipid accumulation and adipogenesis (Figure 21), inducing osteogenesis, as revealed by alizarin red assay (Figure 22).

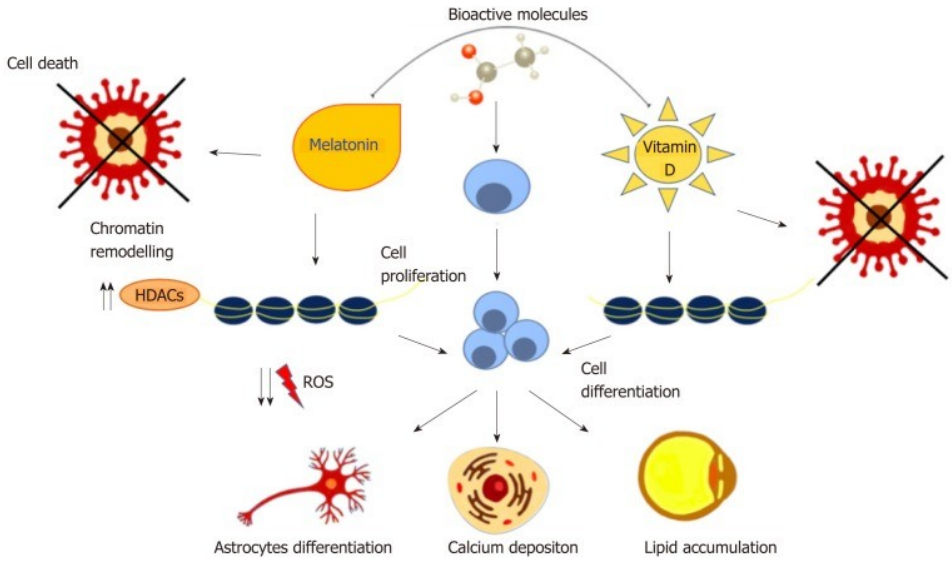

Figure 6. Bioactive molecules modulate stem cell fate inducing epigenetic modifications and influencing the expression of specific differentiation markers [238].

Bioactive molecules can influence stem cell behavior modulating gene expression, and cellular mechanisms responsible for pluripotency and differentiation. It is also demonstrated that ADSCs retain a sort of memory of their belonging tissue, able to drive their fate [376][377]. Understanding the molecular mechanisms involved in the decision of this fate could lead to the development of drugs capable of influencing stem cell behavior, for *in vivo* clinical applications [28]. These results described for the first time a direct association between melatonin and vitamin D and epigenetic modulators in driving stem cell commitment, thus highlighting novel specific tools for stem cell manipulation and regenerative medicine.

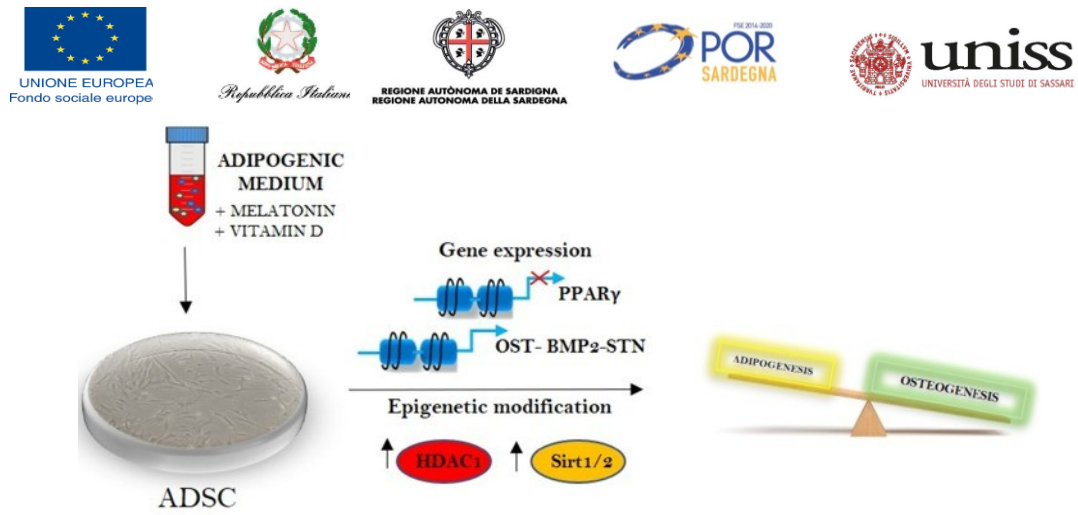

Figure 7. The balance between osteogenesis and apipogenesis involves epigenetic modification influencing the expression of tissue-related genes [256].

FIGURES

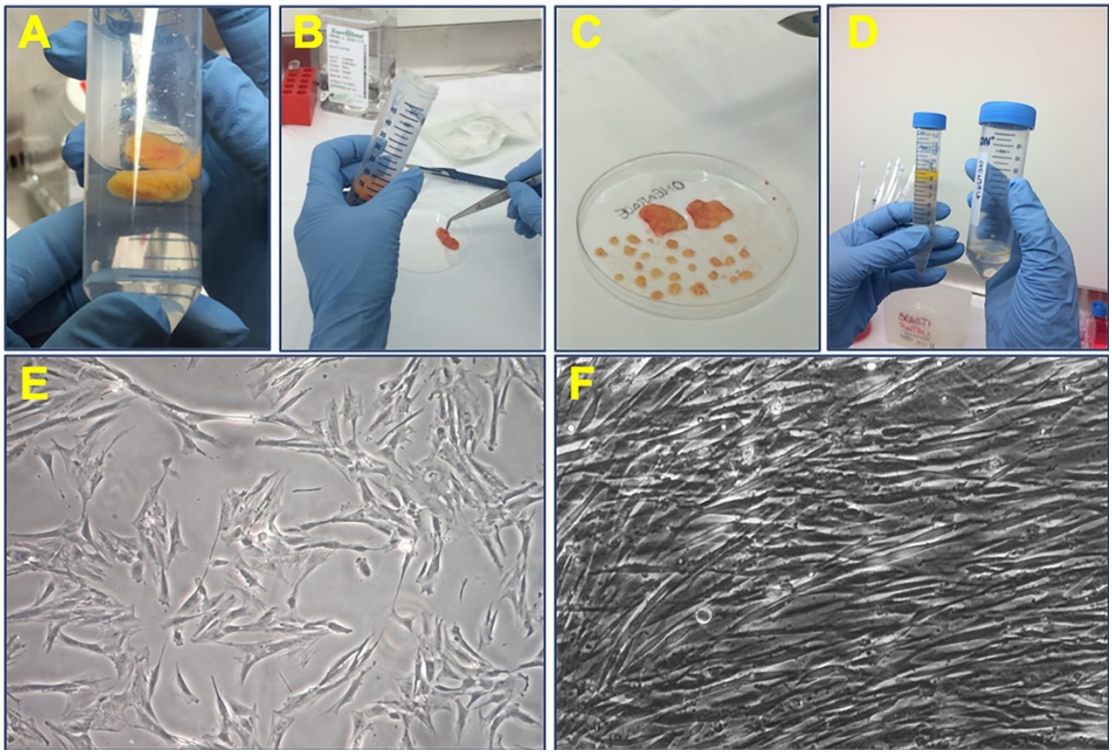

Figure 8. Isolation from adipose tissue. Adipose tissue (A-B) was mechanically fragmented (C) and digested in a solution of Collagenase I. After centrifugation (D), the SVF was put in culture and cells cultured in the presence of basic growing medium (E) until reaching confluence (F).

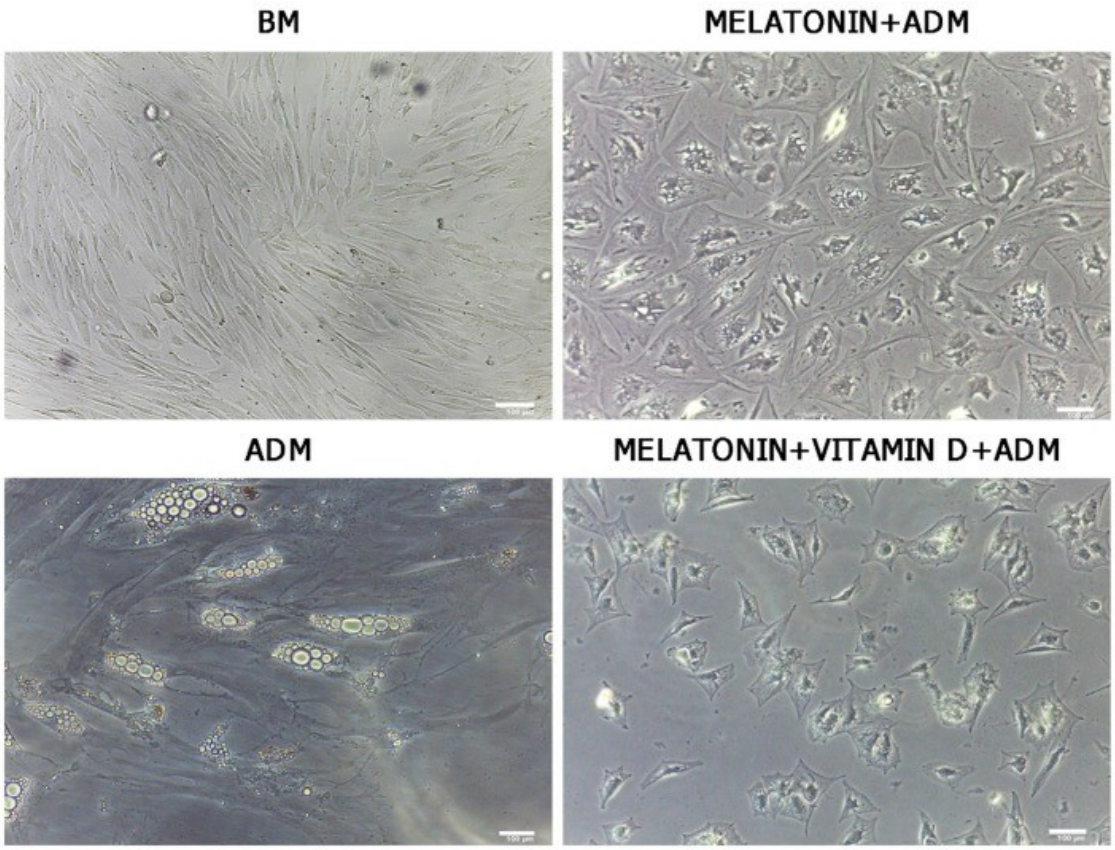

Figure 9. Analysis of cell morphology after culturing in conditioned medium [256]. Cell morphology changed in the presence of melatonin (Melatonin+ADM) or melatonin and vitamin D (Melatonin+VitaminD+ADM) Control cells were maintained in basic medium (BM) while adipogenesis was induce in the presence of differentiation medium alone (ADM). Images were acquired by optical microscope. Scale bar=100 µm.

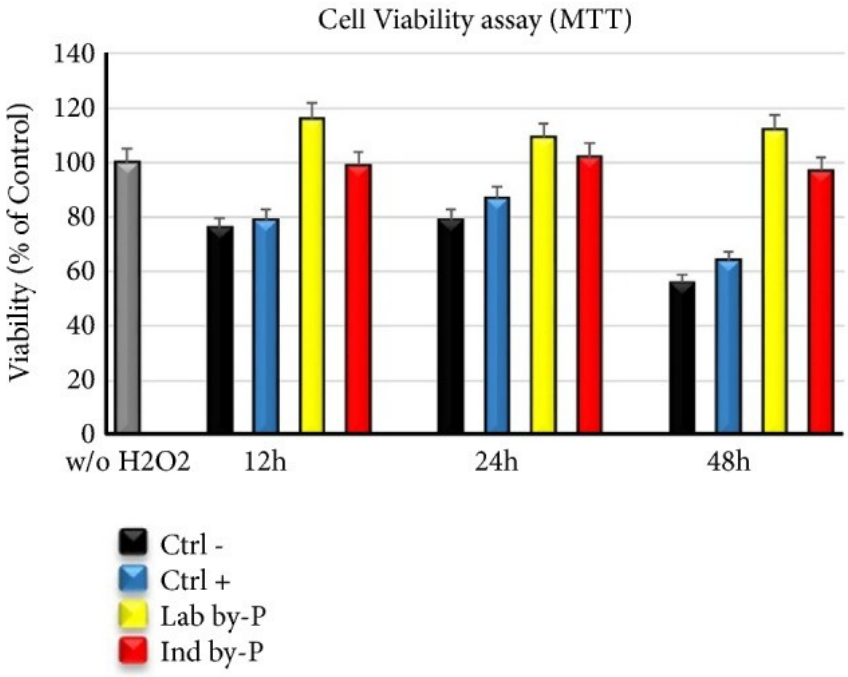

Figure 10. Cell viability assay by MTT [309]. Cell viability of treated cells was expressed as percentage of control cells, considered as 1. Data were analyzed using SPSS version 13 and are expressed as mean± SD considering $p \leq 0.05$ (*) as statistically significant.

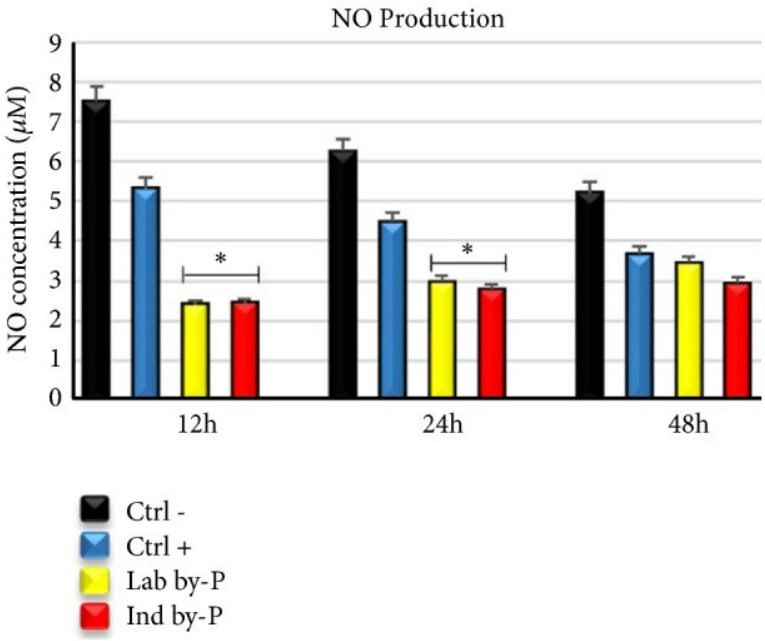

Figure 11. NO concentration after inducing oxidative stress [309] was evaluated in cells pretreated with *Myrtus* extracts (yellow and red bars), as compared to untreated senescent cells (black bars). Positive control for antioxidant activity were cells exposed to ascorbic acid (blue bars). The concentration of NO was read at 548 nm and expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

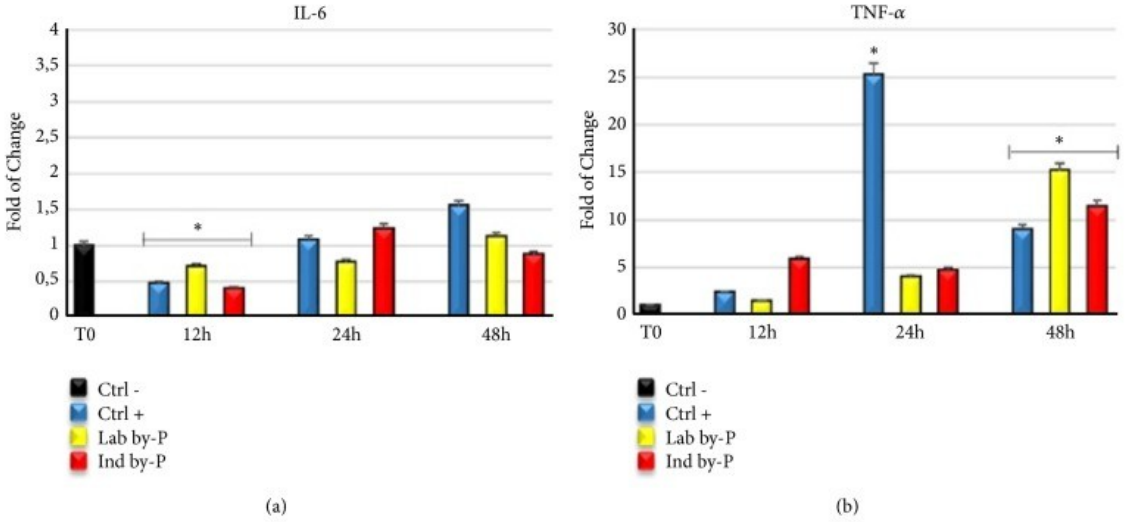

Figure 12. Gene expression levels of proinflammatory cytokines [309]. The expression of IL-6 (a) and TNF- α (b) was evaluated in cells pretreated with the extracts as compared to control cells (black bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs, defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

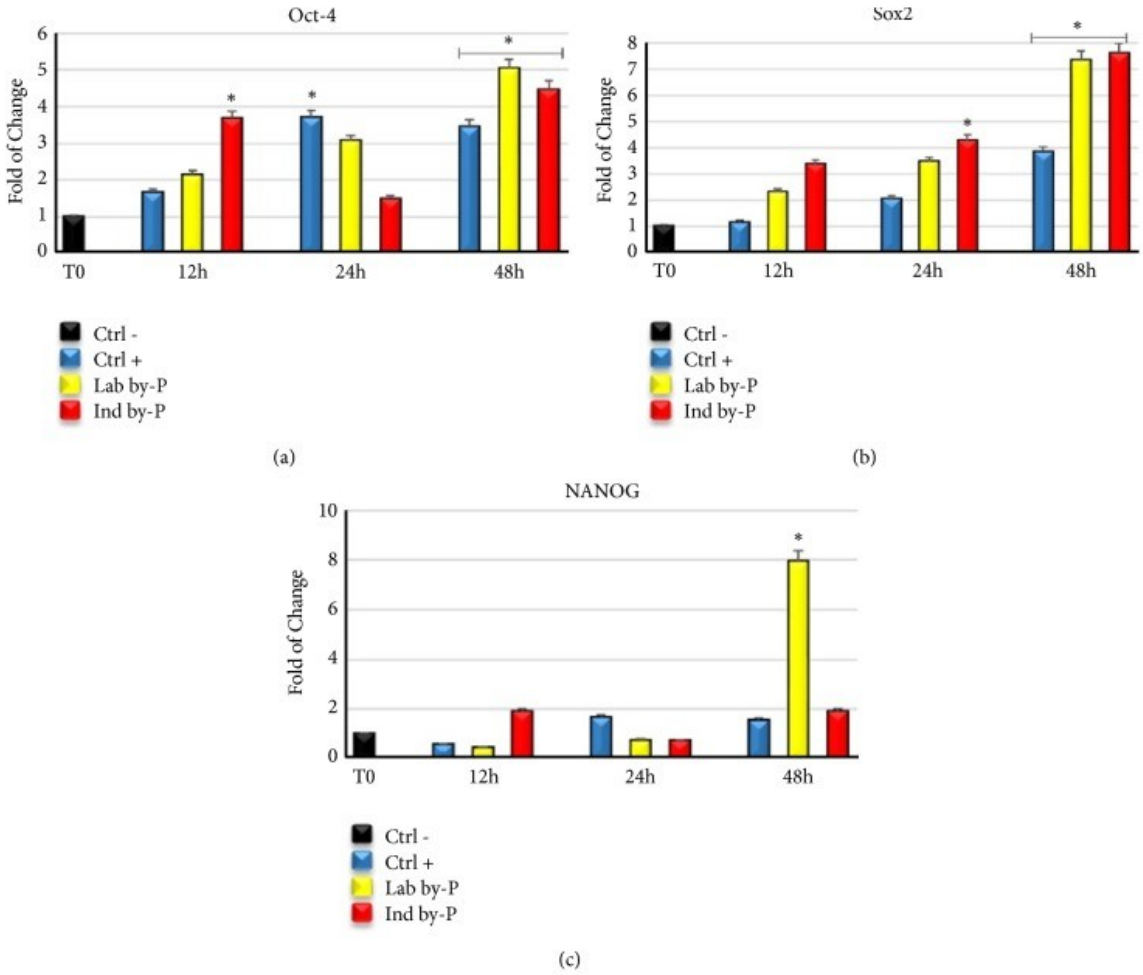

Figure 13. Gene expression levels of stemness related genes [309]. The expression of Oct-4 (a), Sox2 (b) and NANOG (c) was evaluated in cells pretreated with the extracts as compared to control cells (black bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs, defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

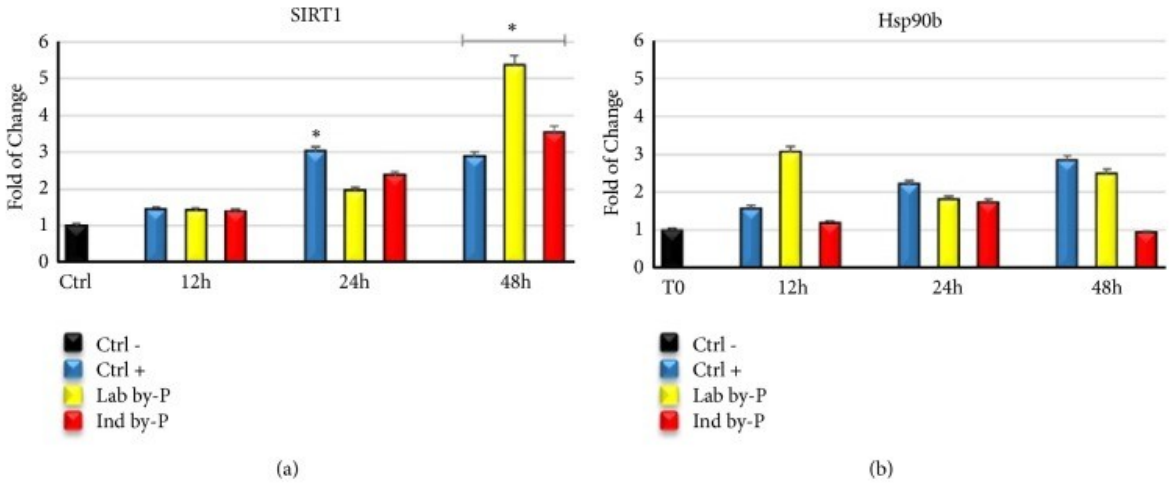

Figure 14. Gene expression levels of SIRT and HSP [309]. The expression of SIRT1 (a) and Hsp90b (b) was evaluated in cells pretreated with the extracts as compared to control cells (black bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs, defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

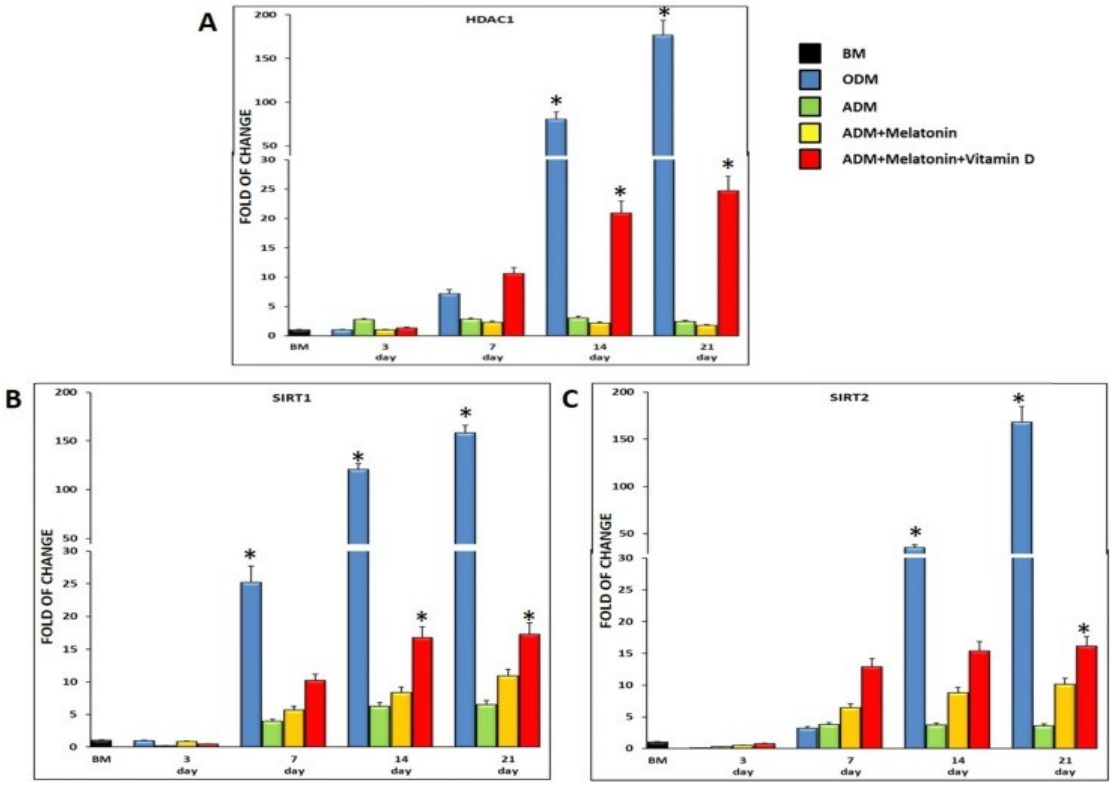

Figure 15. Gene expression levels of SIRT and HDAC in adipogenic conditioned medium [256]. The expression of HDAC1 (A), SIRT1 (B) and SIRT2 (C) was evaluated in cells cultured in the presence of melatonin (yellow bars) or in differentiation medium with melatonin and vitamin D (red bars), as compared to control untreated cells (black bars). Positive control for osteogenesis were ADSCs cultured in osteogenic medium (blue bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs (black bars). defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

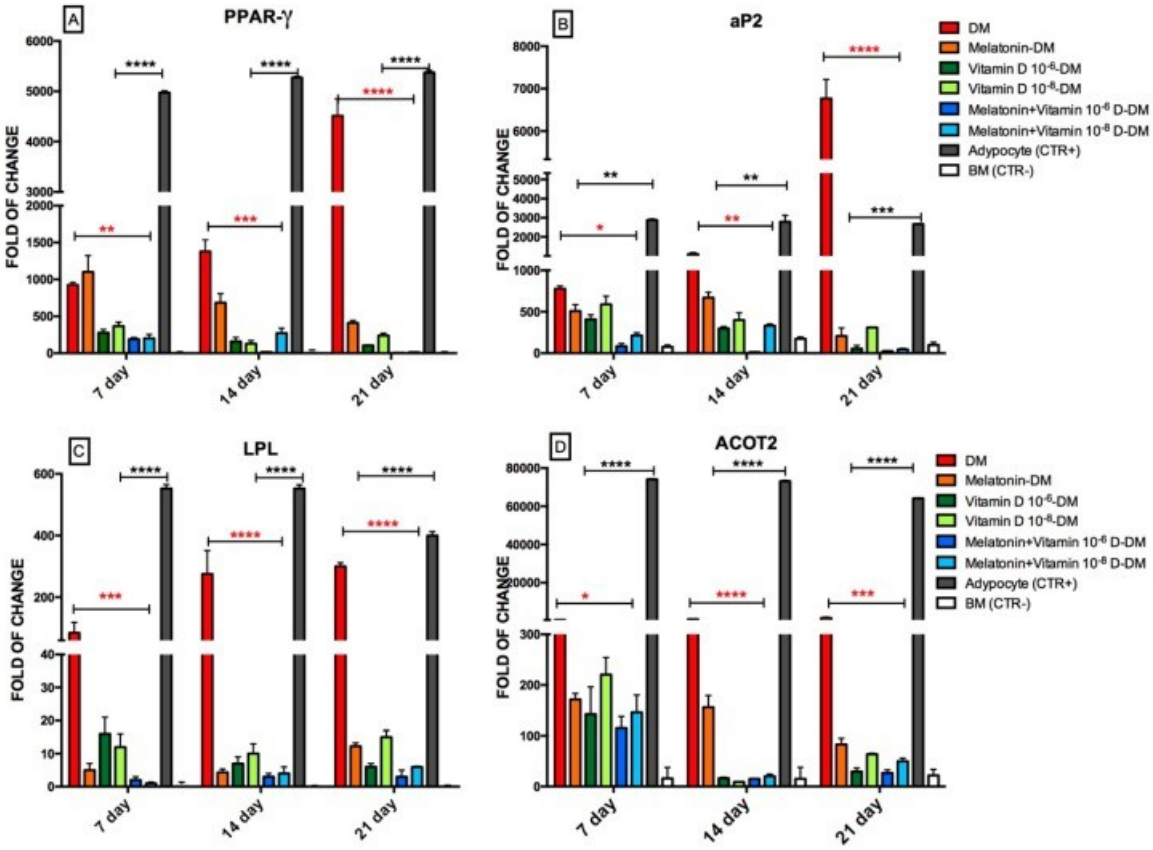

Figure 16. Gene expression levels of adipogenic related genes [255]. mRNA levels or PPAR γ (A), aP2 (B) LPL (C) and ACOT2 (D) were evaluated in cells cultured in the presence of different conditioned media, as compared to control untreated cells (red bars). Positive control for adipogenesis were ADSCs cultured in adipogenic medium (red bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs (white bars) defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant. Significant difference from the differentiation medium is marked by red asterisks, while significant difference from mature adipocytes (black bars) is marked by black asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

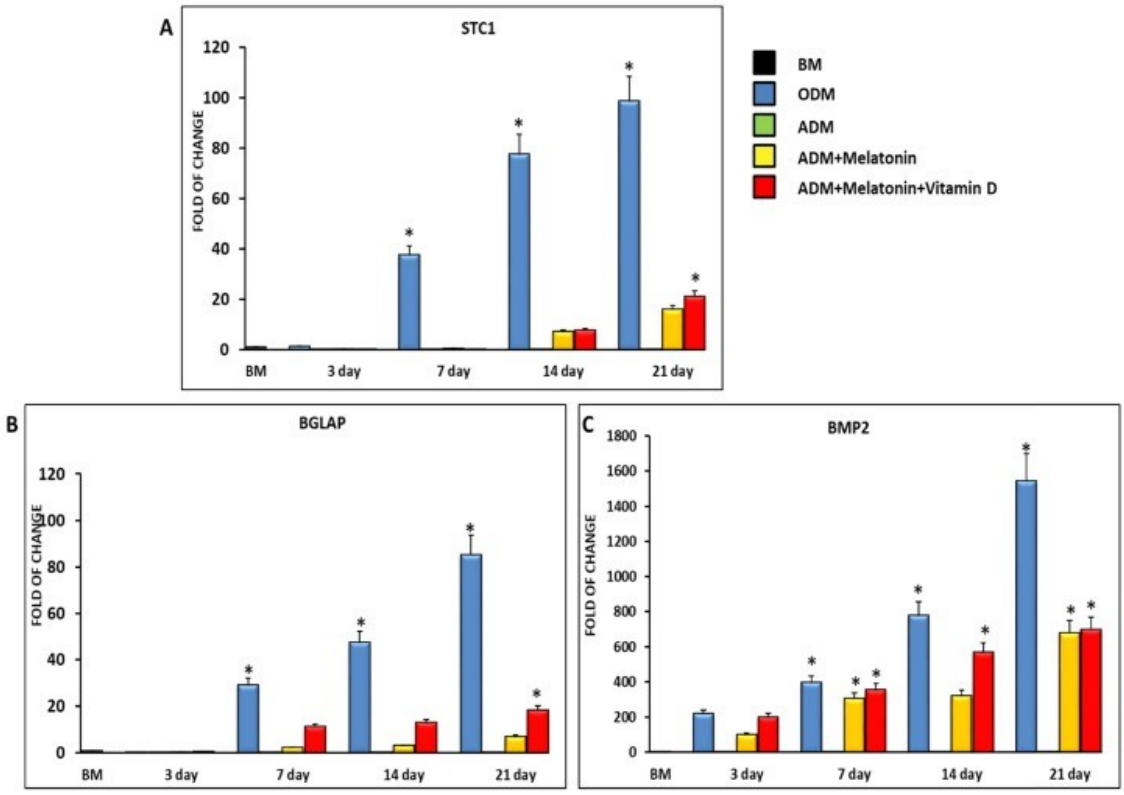

Figure 17. Gene expression levels of osteogenic related genes [256]. The expression of STC1(A), BGLAP (B) and BMP2 (C) was evaluated in cells cultured in the presence of melatonin (yellow bars) or in differentiation medium with melatonin and vitamin D (red bars), as compared to control untreated cells (black bars). Positive control for osteogenesis were ADSCs cultured in osteogenic medium (blue bars). The mRNA levels for each gene were expressed as fold of change ($2^{-\Delta\Delta Ct}$) of mRNA levels of control ADSCs (black bars). defined as 1 and normalized to Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

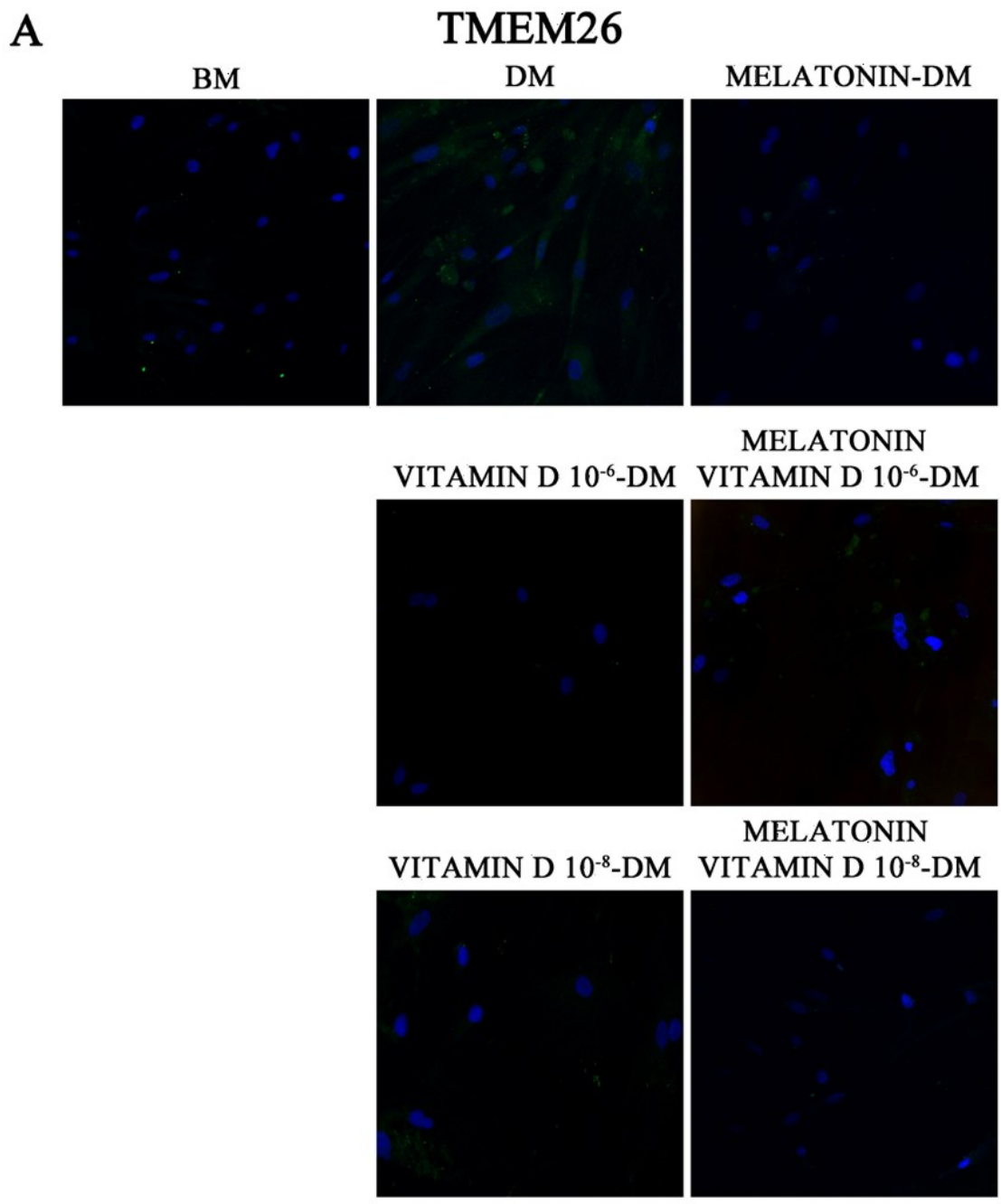

Figure 18. Immunohistochemical analysis of adipogenic related proteins [255]. Expression of TMEM26 was evaluated in cells cultured in the presence of different conditioned media. Nuclei are labelled with 4,6-diamidino-2-phenylindole (DAPI, blue). Scale bars: 40 μm.

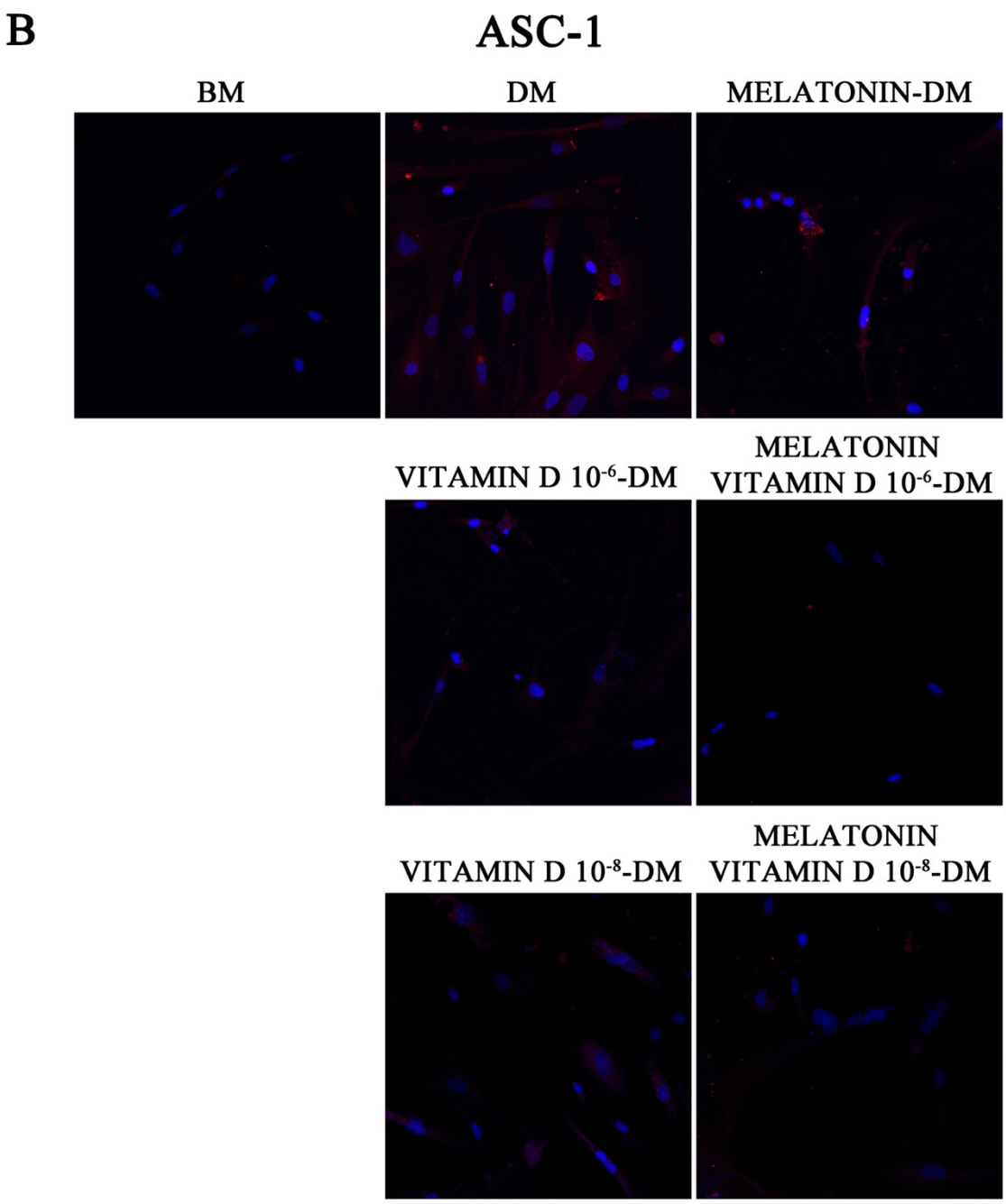

Figure 19. Immunohistochemical analysis of adipogenic related proteins [255]. Expression of ASC-1 was evaluated in cells cultured in the presence of different conditioned media. Nuclei are labelled with 4,6-diamidino-2-phenylindole (DAPI, blue). Scale bars: 40 μm.

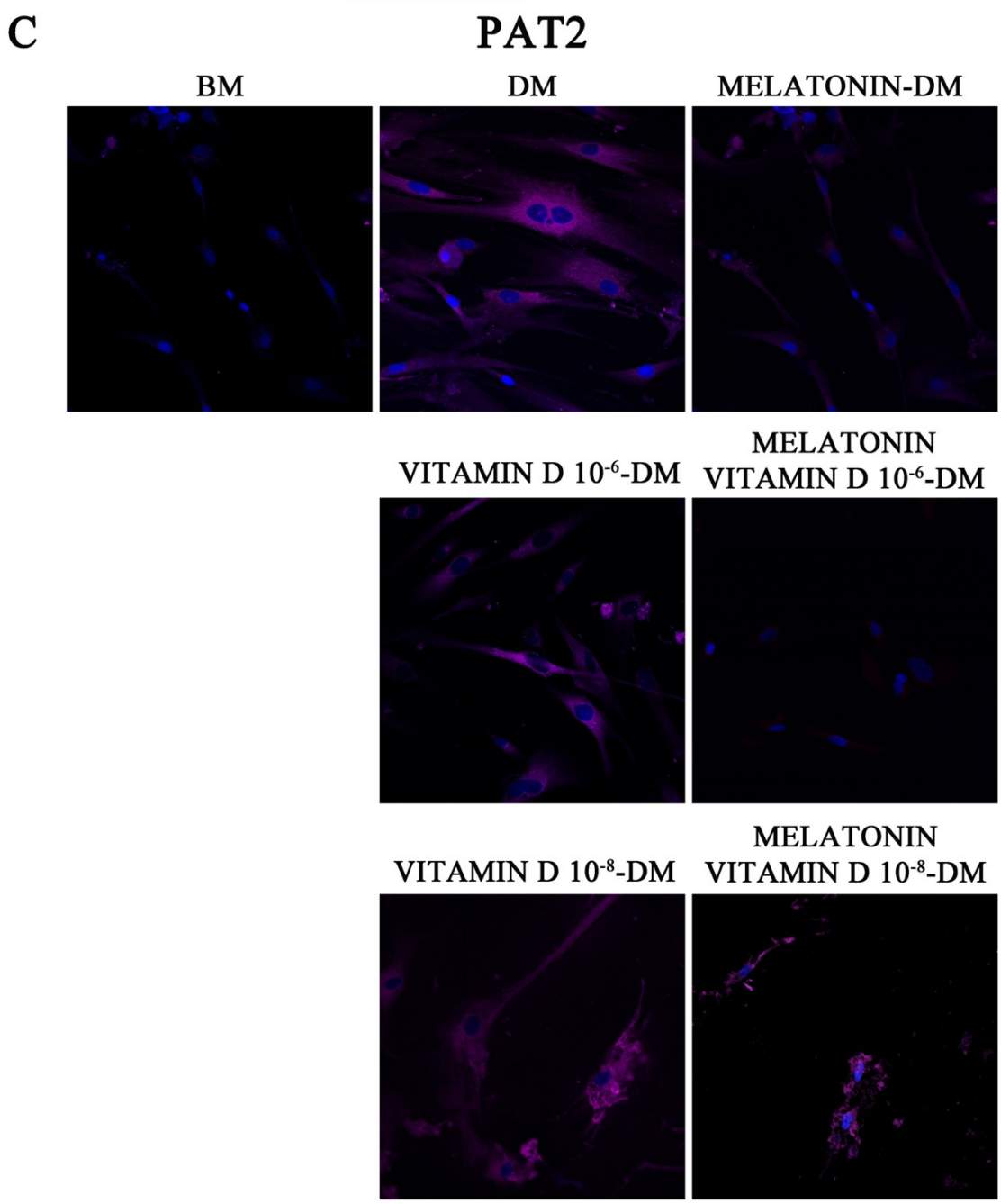

Figure 20. Immunohistochemical analysis of adipogenic related proteins [255]. Expression of PAT2 was evaluated in cells cultured in the presence of different conditioned media. Nuclei are labelled with 4,6-diamidino-2-phenylindole (DAPI, blue). Scale bars: 40 μm.

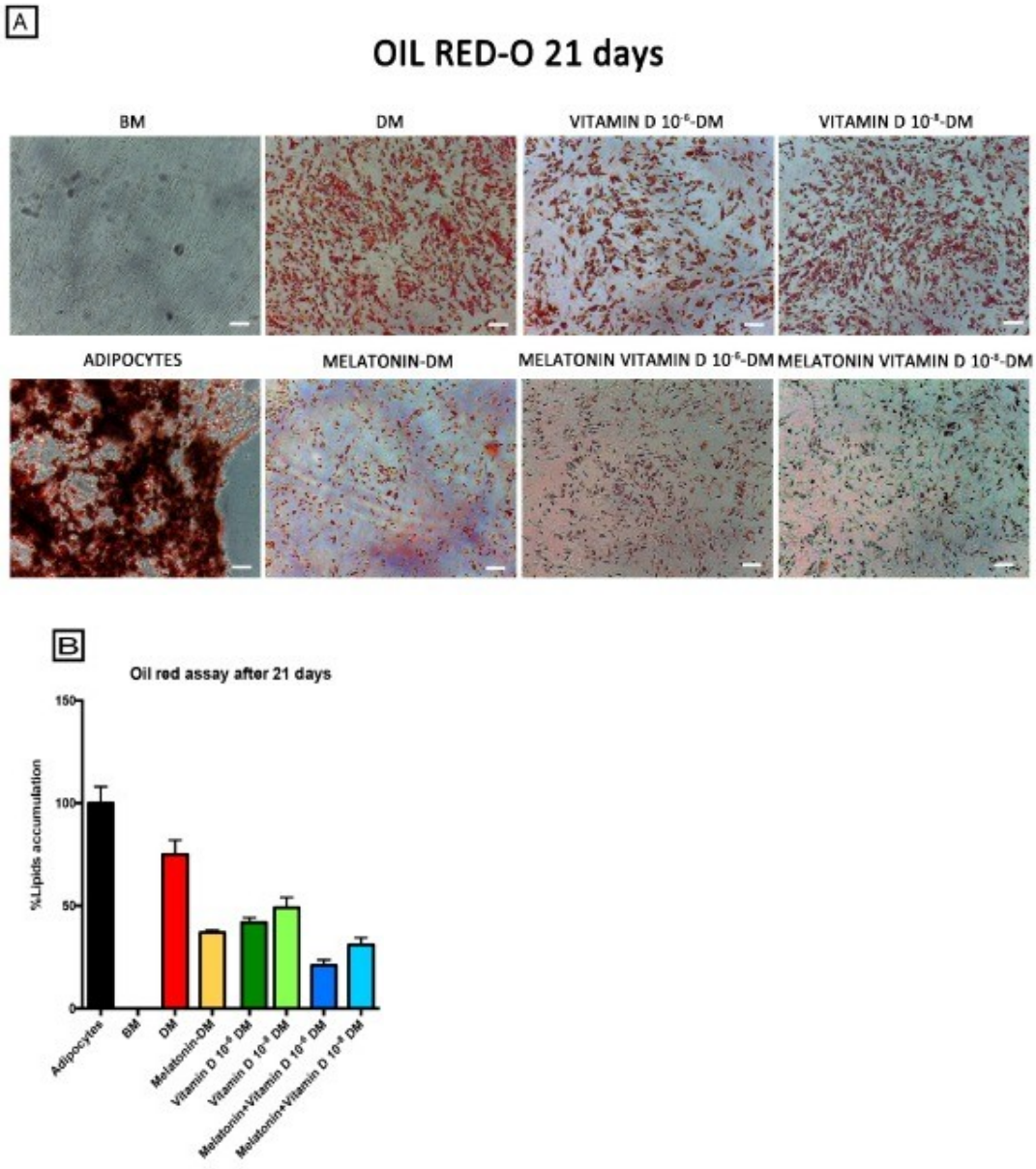

Figure 21. Evaluation of lipid accumulation [255]. Lipid accumulation during adipose differentiation (A) was evaluated in ADSCs cultured in the presence of different conditioned media after 21 days. Scale bar=100 µm. The amount of lipid droplets was calculated using ImageJ (B). Positive control were mature adipocytes (black bars), while negative control for adipogenesis were control untreated ADSCs (white bars). Data are expressed as mean ± SD.

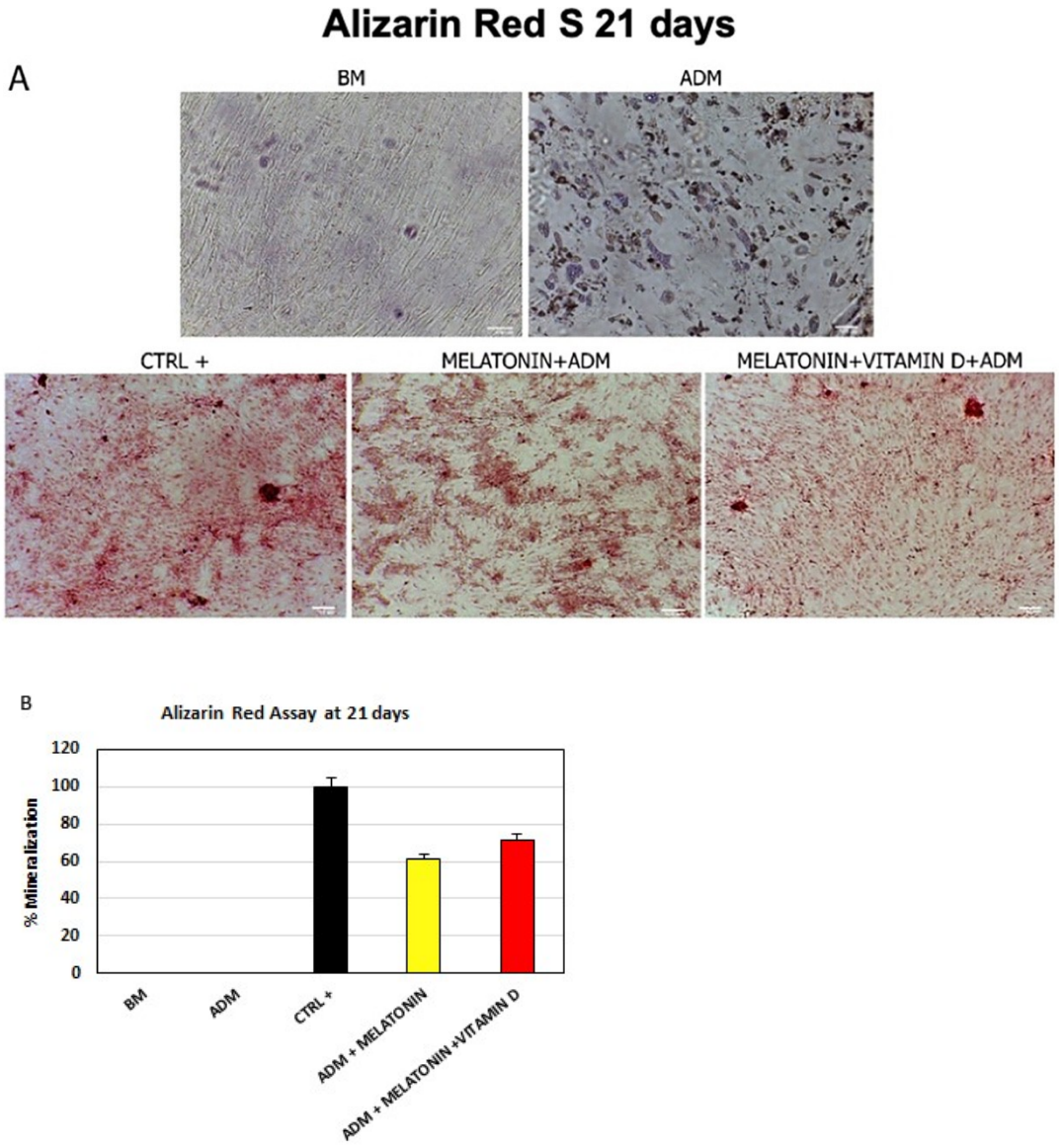

Figure 22. Evaluation of calcium accumulation [256]. Mineralization during differentiation (A) was evaluated in ADSCs cultured in the presence of different conditioned media after 21 days. Positive control (CTRL+) for osteogenesis are ADSCs cultured in osteogenic conditioned medium. Scale bar=100 μm. The percentage of mineralization was calculated using ImageJ (B). Data are expressed as mean ± SD.

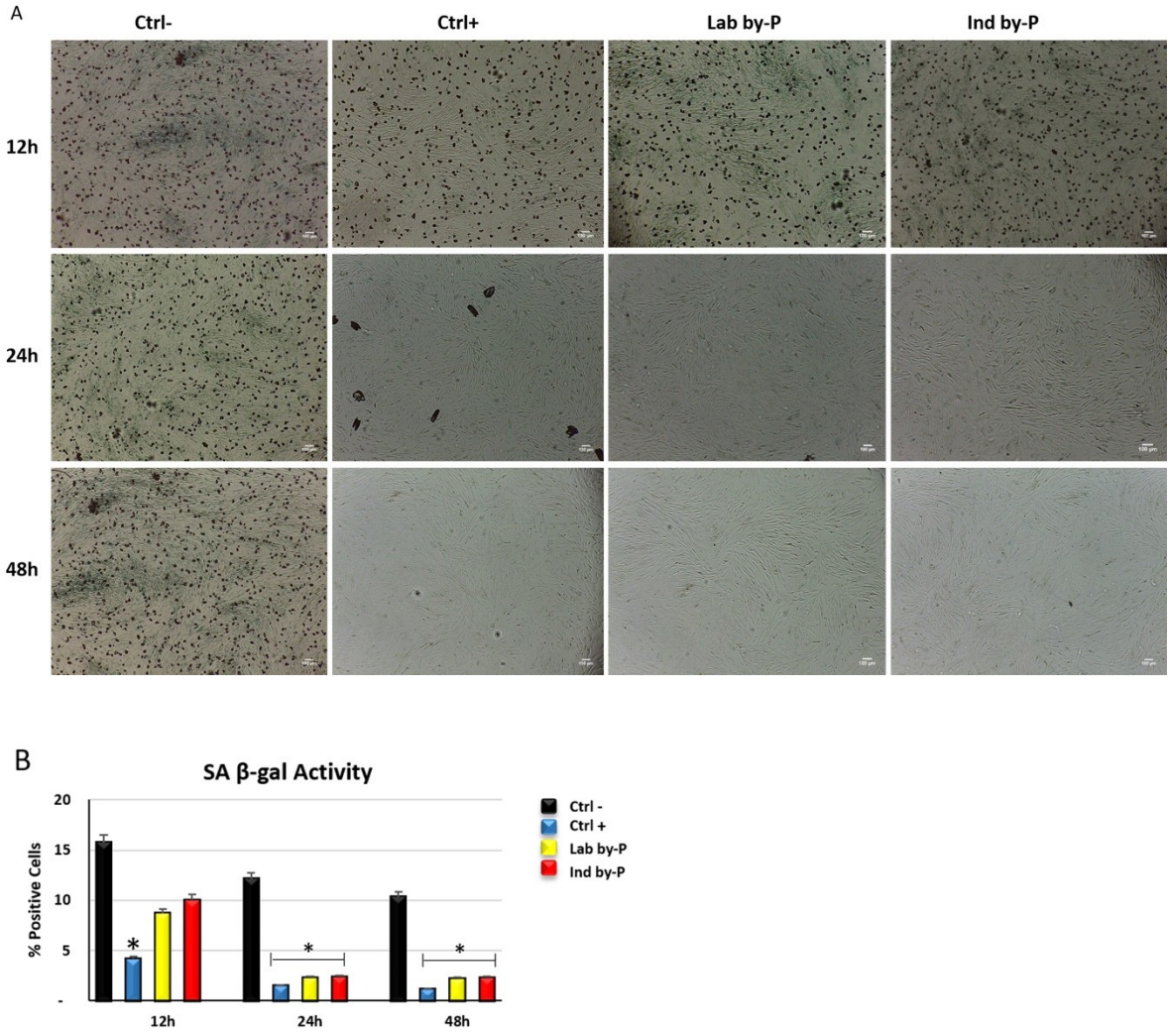

Figure 23. β-galactosidase activity [309]. Senescence-associated β-gal (A) was evaluated in cells pretreated with *Myrtus* extracts (yellow and red bars), as compared to untreated senescent cells (black bars). Positive control for antioxidant activity were cells exposed to ascorbic acid (blue bars). Scale bar=100 µm. The number of senescent (blue) cells was calculated as the percentage of SA-β-Gal-positive cells for each treatment relative to the total number of cells counted using ImageJ software analysis. Data were expressed as mean± SD assuming $p \leq 0.05$ (*) as statistically significant.

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