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# GENOMIC AND PROTEOMIC EVALUATION OF VISCERAL AND SUBCUTANEOUS ADIPOSE DERIVED STEM CELLS AND FUNCTIONAL ROLES OF PLURIPOTENCY TRANSCRIPTION FACTORS

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# INTRODUCTION

## THE STEM CELLS

Stem cells are characterized by two properties: the ability to self-renewal maintaining their undifferentiated state (symmetric division), and the ability to produce cells identical to the mother cells and to differentiated daughter cells with different characteristics (asymmetric division) (1) (Fig. 1).

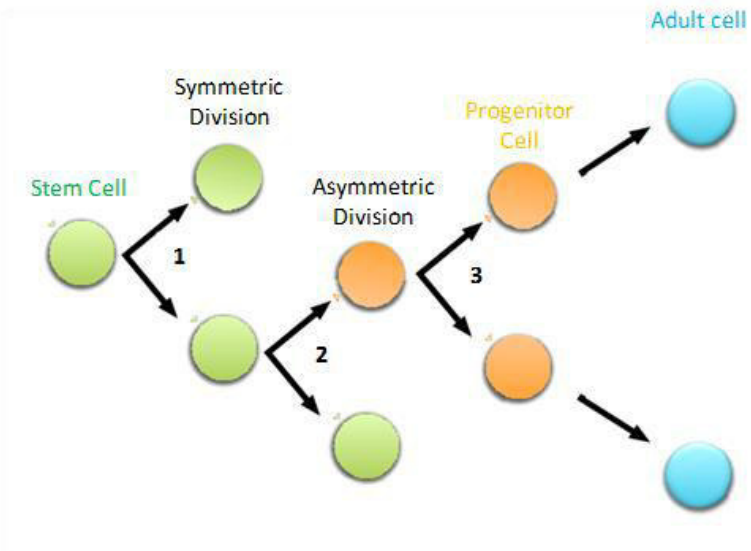



**Figure 1.** Stem cells division. Green dots: stem cells; orange dots: progenitor cells; light blue dots: differentiated cells. 1. Symmetric stem cells division; 2. Asymmetric stem cells division; 3. Progenitor division and terminal differentiation (modified by [Weiss and Troyer, 2006]).

Stem cells are distinguished by their ability to produce mature cells belonging to different genealogies (Fig. 2):

*Totipotent Stem Cells* able to differentiate into embryonic and extra-embryonic cell types. Such cells can give rise to a complete, viable organism. These cells are produced from the fusion of an egg and sperm cell.

*Pluripotent Stem Cells,* which originate from the inner cell mass of the blastocyst and can give rise to any fetal or adult cell type of the three germ layers (mesoderm, endoderm and

ectoderm). These cells are not able to generate an adult organism, because the do not have the potential to contribute to the extra-embryonic cell types.

*Multipotent Stem Cells* able to differentiate into a limited number of cells, but only those of a closely related family of cells, even though it has been demonstrated that these cells can trasndifferentiate into cell lineages of different embryonic origin.

*Unipotent Stem Cells* able to differentiate into only one type of tissue/cell type.

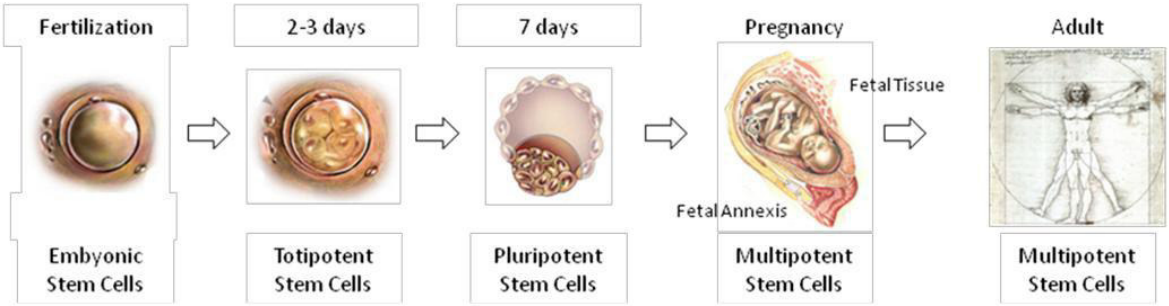



**Figure 2.** Source of stem cells (modified by http://www.sciencecases.org/superman_ethics/primer.asp).

At the end of embryogenesis each tissue is characterized by heterogeneous populations of cells at different stages of maturation, including a population of undifferentiated cells called adult stem cells (2). Adult stem cells are responsible of the maintaining of tissue homeostasis (3), and replacing of terminally differentiated cells lost during senescence or due to trauma and injury (4). These cells are located in a specialized compartment defined "stem cell niche", which is an anti-proliferative and anti-differentiative environment, with the ability to recall and reprogram themselves (5-7). Stem cells are characterized by a high proliferation rate that permit to protect the integrity of the stem cell compartment, and are also able to produce committed progenitor stem cells, which can give rise to mature and functionally differentiated cells (8).

Adult stem cells, in the presence of specific chemical and physical stimuli, can give rise to cells of the three primary germ layers: mesoderm, ectoderm and endoderm (9). For example, it has been observed that neural stem cells can give rise to blood and skeletal muscle cells when transplanted into an ectopic site (10). Another study has shown that mesenchymal stem cells (MSCs) isolated from adipose tissue can differentiate into pancreatic-β cells (11). Therefore, adult MSCs may be considered suitable candidates for applications in tissue engineering and regenerative medicine.

# CHAPTER 1

# MESENCHYMAL STEM CELLS

The presence in the bone marrow of non-hematopoietic stem cells, was suggested by Friedenstein in 1968. These cells, after several passages in culture, showed a typical fibroblast-like morphology able to give rise to different cell lineages such as osteoblasts, chondrocytes and adipocytes (12). The bone marrow is still the main source of MSCs, but several studies have identified these cells in other tissues such as periosteum, dental pulp, skeletal muscle, synovial membrane, trabecular bone tissue, and adipose tissue (13-17).

One of the features that makes the use of MSCs interesting in a clinical setting, is their ability to migrate to the damaged tissue or toward inflammatory sites after intravenously administration (18). This ability has been demonstrated in bone fractures (19), myocardial infarction and cerebral ischemia (20-22). The mechanism by which MSCs migrate across the endothelium to the injury site has not yet been understood, but it is reasonable to assume that the damaged tissue expresses receptors or ligands that facilitate the transport, adhesion and transmigration of MSCs, like the recruitment of lymphocytes to the inflammation site. Essential molecules for the transmigration of leukocytes from the blood to the tissue, such as integrins, selectins and chemokine receptors are also expressed by MSCs (23,24).

Several studies have shown that MSCs have immunomodulatory properties, for example they are able to inhibit the function of most cells involved in immune response, such as B and T lymphocytes, dendritic cells and natural killer cells (25,26). Up to now, the mechanisms underlying the immunosuppressive activity of MSCs, have not been elucidated, but both the direct contact between MSCs and cells of the immune system, and also the release by MSCs of soluble factors such as TGF-β, HGF, and prostaglandin E2 (PGE-2) seem to be important for these functions (27).

Recently, the International Society for Cellular Therapy (ISCT) have defined the minimal criteria to be met for cells to be considered human MSCs (28).

1. Adherence to plastic in standard culture conditions
2. Phenotype positive (≥ 95%+) for CD105, CD73, CD90; phenotype negative (≤ 2%+) for CD45, CD34, CD14 or CD11b, CD79 $\alpha$ or CD19 and HLA-DR
3. *In vitro* differentiation into osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture).

Such criteria show as the presumptive identification of the MSCs remains mostly functional, lacking till now a specific pattern of markers to prospectively and univocally isolate MSCs. Indeed, the basic question of how to define a MSC is still a point of discussion and controversy because MSCs are a heterogeneous population, and some authors tried to find a new panel of markers (29) and to characterize the different subpopulations (29). The most comprehensive set of unique stem cell markers has been defined and associated with embryos and embryonic stem cells (ESCs). In embryos and ESCs, maintenance of a pluripotent state is conferred by a core of developmentally regulated transcription factors including the homeodomain proteins, octamer-binding transcription factor 4 (OCT4), homeobox protein, NANOG, and the high-mobility group transcription factor, SRY-box containing gene 2 (SOX2) (30,31).

One of the important properties of MSCs is also the tropism for cancer cells, which makes them as cellular vehicles for the release of anticancer agents. The MSCs can be engineered to release IFN-β, cytosine deaminase, or oncolytic viruses. These approaches have been evaluated in preclinical models and have demonstrated significant antitumoral efficacy (32,33). Some studies have reported that MSCs can promote tumor growth. The subcutaneous co-injection of adipose derived stem cells (ASCs) with lung carcinoma cells (H460) or glioma cells (U87MG), in nude mice, can lead to an increase in tumor size (34). In conflict with the studies reporting a role of MSCs in promoting tumor growth, other studies show that MSCs are able to inhibit tumor expansion (35). The effect of MSCs on tumor progression can be explained considering the heterogeneity of MSCs, the injected dose, the patient variability and the time of cells administration. In early tumor development studies, the ability of MSCs to support tumor growth may depend on both their immunosuppressive effect and on their ability to promote angiogenesis, a key event in cancer development and in the process of metastatization.

# CHAPTER 2

# ADIPOSE DERIVED STEM CELLS

Adipose tissue is a remarkably complex organ with profound effects on physiology and pathophysiology, but it has not always been viewed in this light. Until the late 1940s, adipose tissue was characterized as a form of connective tissue containing lipid droplets. This gradually began to change with the realization that adipose tissue plays a major role in nutrient homeostasis, serving as the site of calorie storage after feeding and as the source of

circulating free fatty acids during fasting. In the late 1980s to mid 1990s came the discovery of adipose-derived serum factors like adipsin, TNF-a, and leptin (36). Suddenly, adipose tissue was regarded as an endocrine organ at the centre of energy homeostasis. From this point, studies on the developmental, functional, and pathophysiological aspects of adipose tissue have expanded markedly.

There are mainly two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), which are morphologically and functionally different. BAT is much less abundant than the white one. It can be found in the neck and mediastinum in adults, and in the interscapular areas in neonates. The appearance of BAT is literally brown. Brown adipocytes are multilocular, retain small lipid vacuoles compared to white adipocytes and their function is thermogenesis (37,38). Vascularization is obvious because BAT requires much more oxygen consumption compared to other tissues. BAT contains a large number of mitochondria and expresses uncoupling protein 1 (UCP1). UCP1 is a BAT-specific marker, not expressed within WAT. UCP1 is expressed in the inner membrane of mitochondria, mainly regulated by adrenergic signaling through sympathetic innervations, and this signaling is responsible for thermogenesis (37,38). BAT is activated by thyroid hormone, cold temperatures, and thiazolidinediones. Activated BAT is inversely correlated with body mass index, adipose tissue mass and insulin resistance.

On the other side, WAT is found throughout the body. The appearance is yellow or ivory. White adipocytes are unilocular and contain large lipid vacuoles. WAT function is to store excess energy in the form of triglycerides, and its hyperplasia causes obesity and dysfunction of metabolic pathways as insulin resistance. WAT includes two representative types: visceral WAT (VAT) and subcutaneous WAT (SAT). VAT is located under the skin, provides insulation from heat or cold and prevents the deposition of excess lipids in other organs. It is distributed around organs and provides protective padding. Its hyperplasia is linked with pathological metabolic profiles due to its lipolytic nature (39).

Recently, beige adipocytes have been discovered within WAT, especially in the inguinal area (40). Beige adipocytes have the characteristics of both brown and white adipocytes. Beige adipocytes contain both unilocular large and multiple small lipid vacuoles. Their function is the adaptive thermogenesis. In response to cold temperature exposure, beige cells transform into cells which have BAT-like characteristics, such as UCP1 expression and small lipid vacuoles (41). It is still controversial whether the beige adipocytes arise through the transdifferentiation of white adipocytes or by de novo adipogenesis from a subgroup of precursor cells (42).

WAT contains a supportive stroma that is easily isolated in relatively large amounts. Stromal cells that have preadipocyte characteristics can be isolated from adipose tissue of adult subjects in the stromal vascular fraction (SVF), spread *in vitro* and induced to differentiate into adipocytes (15,43-45). These stromal-derived cells were firstly defined as "preadipocytes", even though they were shown to be capable of forming not only fat, but also bone, cartilage and muscle (46-48). In 2004, the International Fat Applied Technology Society adopted the term "ASCs" to define plastic-adherent cells with multilineage capacity isolated from SVF of adipose tissue (49).

Apart from adipocytes and multipotent ASCs, there are other cell types in WAT including the committed adipocyte precursor cells (pre-adipocytes), haematopoietic cell types (e.g., erythrocytes, monocytes and macrophages), endothelial cells and smooth muscle cells (Fig. 3).

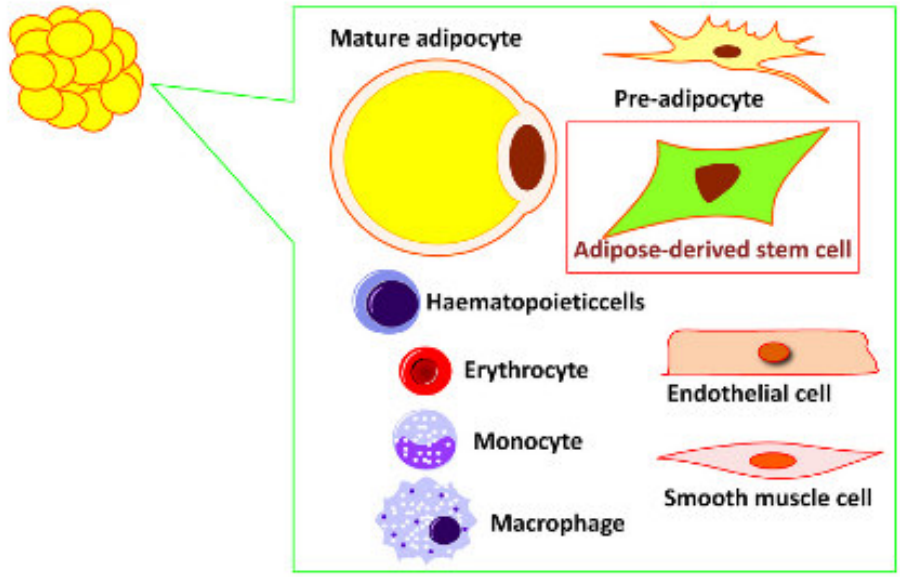



**Figure 3**: Cell types in adipose tissue. A subpopulation of adipose cells is ASCs that possess properties of MSCs and can commit to become pre-adipocytes and subsequently differentiate into mature adipocytes. Other cells include haematopoietic cells (erythrocytes, monocytes and macrophages), endothelial cells and smooth muscle cells. From Ong WK et al Int J Biochem Cell Biol. 2013.

ASCs seem to be proliferating progenitors that are already committed, either prenatally or early postnatal life (50), which may reside in the mural cell compartment of the adipose vasculature, as a subset of both perycites and capillary endothelial cells, but not in the vasculature of other tissues. Therefore, the adipose vasculature appears to function as a progenitor niche and may provide signals to adipocyte development (51).

Human adipocytes differentiated *in vitro* from ASCs, obtained from VAT or SAT biopsies show depot-specific differences for genetic, biochemicals and metabolic endpoints (52-54).

ASCs, as general MSCs, show the ability to maintain their undifferentiated state and to proliferate during *in vitro* expansion (55). The doubling time of ASCs is directly related to several factors, such as donor age (56), donor site (subcutaneous or visceral), surgery, and also culture conditions (57,58).

In addition, ASCs are able to differentiate in mesenchymal-derived tissues:

*Adipocytes*. A specific medium, characterized by dexamethasone, indomethacin, insulin, and isobutyl-methylxanthine, induces ASCs differentiation towards the adipogenic lineage with lipid vacuoles accumulation (59).

*Chondrocytes*. A pellet culture maintained in specific inductive medium characterized by transforming growth factor β (TGF-β), ascorbic acid, and dexamethasone, induces ASCs chondrogenic differentiation able to secrete extracellular matrix proteins, such as collagen type II type II, VI and proteoglycans and expressing biochemical markers of mature chondrocytes (60,61).

*Osteoblasts*. In presence of ascorbic acid, β-glycerolphosphate, dexamethasone and vitamin D3, ASCs express osteogenic markers (15, 44, 62, 63).

*Cardiomyocytes*. When exposed in culture with 5'-azacytidine, ASCs express specific proteins of cardiac myocytes, such as troponin I (64). This suggests that ASCs can be used in the treatment of ischemic damage.

*Smooth Muscle Fibrocells*. Specific inductive media, characterized by 1% FBS and heparin, induce ASCs differentiation with an up-regulation of smooth muscle α-actin and caldesmin (65).

*Myocytes*. When exposed to horse serum (HS), ASCs express early markers of myogenic differentiation such as myoD and myogenin (66,67).

ASCs are also able to trans-differentiate in cell types of the non-mesodermal origin, such as neurons. Human and murine ASCs cultured in the presence of antioxidants and in the absence of serum, acquire a bipolar morphology expressing nestin, intermediate filament, and fibrillary glial protein M (68,69). In addition, ASCs are also able to differentiate into endothelial and epithelial cells, hepatocytes and pancreatic-β cells (11,70,71) (Fig. 4).

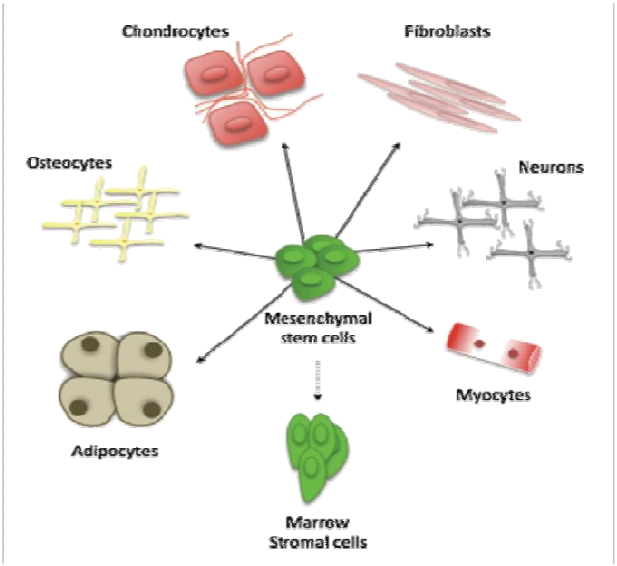



**Figure 4.** Mesenchymal stem cells can differentiate into several mesenchymal tissues such as bone, cartilage, muscle, bone marrow, adipose tissue and tendon

Great effort has been applied to identify specific surface markers on ASCs for their identification *in vivo* and *in vitro*. ASCs express CD9, CD10 (membrane metalloendopeptidase), CD13 (aminopeptidase), CD29 (β1 integrin), CD44 (receptor that binds hyaluronic acid), CD49d (α4 integrin), CD54 (intracellular adhesion molecule 1 or ICAM-1), CD55, CD59, CD71 (transferrin receptor), CD73 (lymphocyte-vascular adhesion protein-2), CD90 (thymocyte differentiation antigen 1), CD105 (endoglin), CD106 and CD166 (Activated leukocyte cell adhesion molecule). In addition, ASCs express class I histocompatibility antigen HLA-ABC, while they do not express class II molecules HLA-DR. These cells do not express hematopoietic markers such as CD14, CD31 and CD45 (15, 72,73).

## 2a. MOLECULAR PATHWAYS INVOLVED IN OSTEOGENIC DIFFERENTIATION

The cells committed to the osteoblastic phenotype are called osteo-progenitors. Continuous recruitment, proliferation and differentiation of cells within bone tissue is regulated by the expression of genes providing the characteristics to the bone phenotype. A precise pattern of the expression of genes encoding the osteoblast phenotype has been shown (74,75). It can be subdivided in three chronologically related distinct stages:

*Proliferation phase*: high mitotic activity that is accompanied by the expression of cell-cycle genes, including genes that encode for histones and cell growth genes (*c-MYC, c-Fos, and c-Jun*). During this period, genes associated with the formation of extra-cellular

matrix (ECM), such as collagen type I, osteopontin (OPN) and fibronectin are actively expressed.

*Matrix development phase*: ECM composition and organization is widely modified, providing a favourable environment for the mineralization and an increase in alkaline phosphatase (ALP) activity.

*Mineralization phase*: coordinated by the osteoblast activity and by the deposition of calcium phosphate hydroxyapatite.

An overview of this modification is shown in Fig. 5

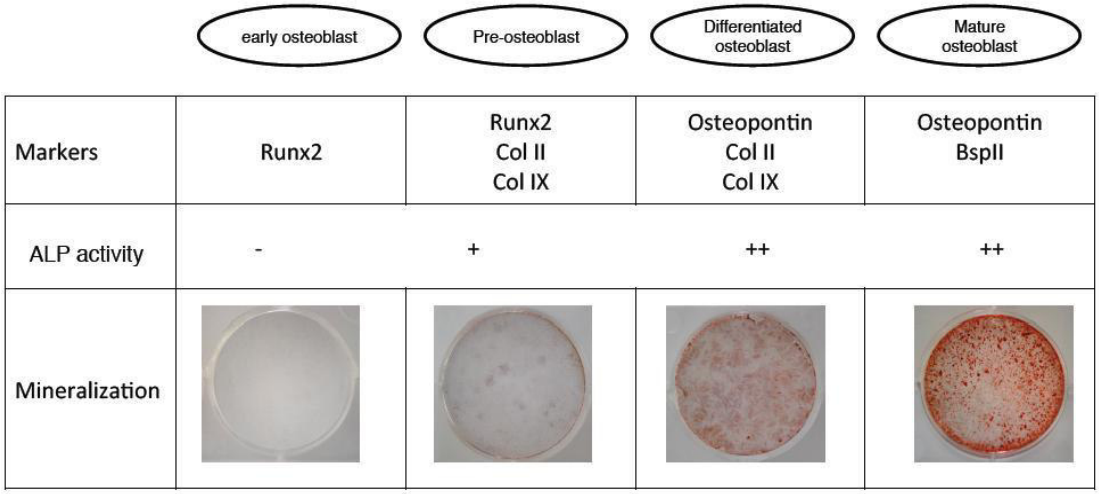



**Figure 5.** Markers modification during MSCs differentiation through osteoblast lineage.

Osteoblast differentiation, recruitment, function and maturation is promoted and regulated by the secretion of lipid-modified glycoproteins of the Wnt family, bone morphogenetic proteins (BMPs) and several transcriptional factors (76).

*Wnt signaling pathway*. Wnt proteins are glycoproteins that bind the Frizzled transmembrane receptor (FZD), activating two distinct signal transduction pathways: the canonical and non-canonical Wnt pathway. The first involves the formation of a complex between the Wnt proteins, FZD, and the co-receptor LRP5 or LRP6. In the non-canonical pathways, Wnt binds the FZD receptor and activates heterotrimeric G proteins, inducing an increase of intracellular calcium by protein C-dependent mechanisms.

Wnt proteins regulate growth, differentiation, function and cell death, and have a key role in the bone biology. The binding of Wnt proteins with the complex FZD/LRP5/6 induces a signal transduction, that involved Dishevelled, Axin and Frat-1 proteins causing the inhibition of glycogen synthase kinase 3β (GSK3). Thus, β-catenin phosphorylation and subsequent ubiquitin-mediated degradation are inhibited, and β-catenin translocates into the nucleus, where it cooperates with T-cell transcription factor/lymphoid enhancer factor (TCF/LEF) in the regulation of target genes expression (77,78) (Figure 6).

Canonical Wnt pathway is involved in bone formation and specific bone markers expression. It is likely that β-catenin activity is required for specific transcription factors activation such as Runx2, that plays a key role in osteogenic and chondrogenic differentiation (77,78).

*Transforming growth factor (TGF)-β signaling*. Several members of the TGF-β superfamily, such as BMPs, have potent osteogenic effects. BMPs transmit signals through Smad-dependent and Smad-independent pathway, including ERK, JNK and p38/MAPK transduction pathways. The BMPs-Smad pathway activates the expression of Distal-less homeobox 5 (Dlx5), which in turn induces the expression of Runx2 and Osterix (Osx) in osteoprogenitor cells.

*Hedgehog (Hh) signaling*. The protein Indian Hh (Ihh) is produced by hypertrophic chondrocytes and it has a direct effect on osteoblast progenitors. The signaling mediated by Ihh regulates the timing and spatial early osteoblastic commitment.

*Notch signaling*. Since Notch receptors and their ligands (Delta 1, 3, 4 and Jagged 1, 2) are transmembrane proteins, the signal transduction is activated upon cell-cell interaction. Notch 1 and Notch 2 are expressed into osteoblasts, while Notch 3 and Notch 4 were identified in subgroups of osteogenic lineage. Notch signaling is able to positively regulate the expression of genes of osteoblastic differentiation and to suppress the osteoblasts maturation induced by BMPs, through Runt-related factor (Runx) 2 inhibition.

*Mitogen-activated protein kinase (MAPK) signaling*. The MAPK are serine/threonine kinase proteins involved in cellular regulation. The MAPK pathway is activated by several growth factors, such as fibroblast growth factor (FGF), Platelet-Derived Growth Factor (PDGF), insulin-like growth factor (IGF), and TGF-β involved into osteogenesis. Extracellular stimuli cause the activation of signal transduction consisting of MAPK, MAPK kinase (MKK or MAP2K) and MAPKK kinase (MAP3K or MKKK).

*Transcription factors*. A central regulator of bone formation is Runx2, also known as *Core-binding factor α1* (Cbf-α1), a member of the Runx family of transcription factors. Runx2 is expressed in MSCs and it is important during the osteogenic differentiation by the activation of genes such as osteonectin, osteopontin and collagen type I (79). Moreover, Runx2 serves as an initial marker of osteogenic cell lineage (80). During osteoblast differentiation, Runx2 and canonical Wnt signalling, play essential roles in the commitment of pluripotent mesenchymal cells to the osteoblastic lineage (79). After commitment into the osteoblastic lineage, the osteoblasts express bone matrix proteins at different levels depending on the maturation grade of the cells.

Immature mesenchymal cells and pre-osteoblasts weakly express collagen type I, and its expression is up-regulated in immature osteoblasts (81). Immature osteoblasts also express osteopontin (Spp1) and sialoproteins (Ibsp). Mature osteoblasts strongly express osteocalcin (bGlap) (82). Mature osteoblasts are embedded into the bone matrix and finally become osteocytes, which express dentin matrix protein 1(Dmp1) (83) (Fig. 6).

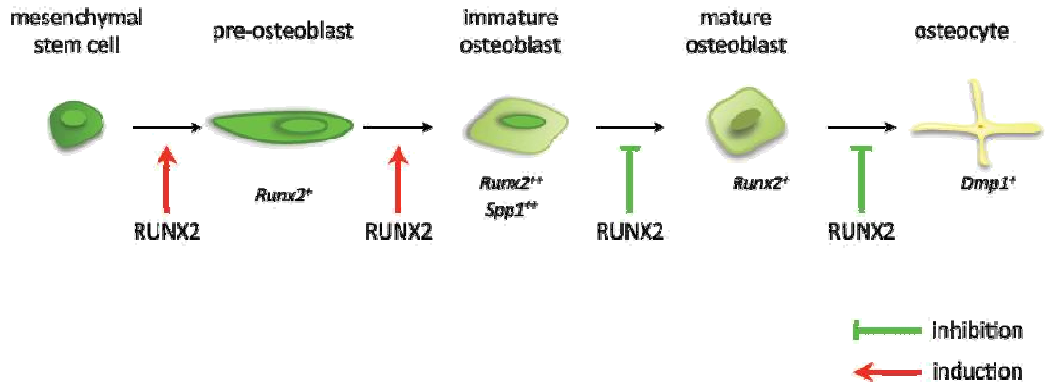



**Figure 6.** RUNX2 directs pluripotent mesenchymal cells to the osteoblast lineage, increases the number of immature osteoblast, but inhibits osteoblast maturation. Pre-osteoblasts express Runx2. Immature osteoblasts express Runx2 and Spp1. Mature osteoblast Runx2 expression is down regulated. Osteocytes express Dmp1+. The transition of immature osteoblasts to osteocytes occurs at an early stage of bone development.

Targeted disruption of Runx2 results in the complete lack of bone formation by osteoblasts (84). Runx2 has been designated as the most pleiotropic regulator of skeletogenesis (85). It functions as an inhibitor of proliferation of progenitor cells (86), and it is also required for osteoblast function and differentiation (87,88).

## 2b. MOLECULAR PATHWAYS INVOLVED IN CHONDROGENIC DIFFERENTIATION

Chondrogenesis of ASCs could be achieved by modulating ASC interactions with specific growth factors (GF). Effect of GFs on chondrogenesis may differ depending on the GF dose, cell type and cell stage. Most researched GFs for chondrogenesis include TGF-β, BMPs, IGF, and FGF. Stimulating ASCs with costly GFs usually requires high concentration and repeated treatments of GFs, and may cause side effects (89). Despite these disadvantages, GFs are inevitably necessary for chondrogenesis of ASCs and MSCs (Fig. 7).

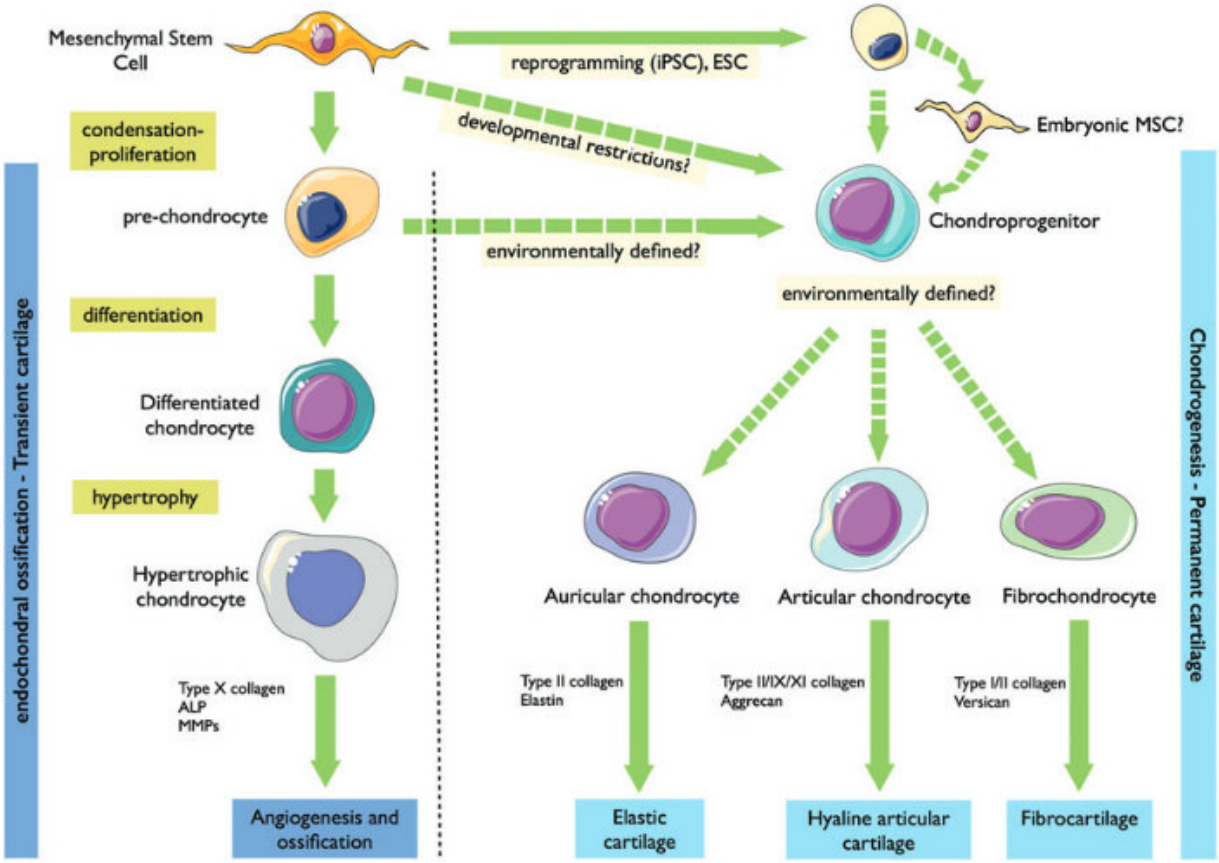



**Figure 7**: Chondrogenic lineage progression of mesenchymal stem cells (MSCs). MSCs appear to be limited to follow an endochondral ossification program, which will end in vascular penetration, marrow deposition, and ossification of this cartilaginous tissue. Differentiation toward the stable hyaline cartilage phenotypes is theoretically restricted. However, it ispossible that reproducing the complex environmental signals that are required for pathway switching has not been discovered. Bypassing these restrictions may be possible using reprogramming technology (induced pluripotent stem [iPS]) or embryonic stem cells (ESCs) as a new start state. Dashed arrows indicate possible routes, although evidence is lacking about the detailed differentiation pathway. Figure from Somoza R et al, Tissue Engineering 2014.

*TGF- β*. The TGF-β family is associated with the regulation of MSC proliferation, differentiation and ECM synthesis. TGF-β1, β2, and β3 are secreted as inactive forms and are activated when they are separated from a latency-associated peptide (90). TGF-β attaches to type I and II receptor serine/threonine kinases and activates R-Smad proteins (91). R-Smad combines with Co-Smad, and then the activated complex is translocated into the nucleus, where it regulates gene expressions as a transcriptional factor (92). TGF-β is a significant factor in chondrogenic differentiation of ASCs (93). Large amount of latent TGF-β is already present in articular cartilage, and even a tiny amount of active TGF-β is considered a potent stimulator for proteoglycan and type II collagen synthesis. When properly treated with TGF-β, the number of chondrocytes differentiated from ASCs and their viability increase

prominently (94). It is well documented that TGF-β induces the expression of a transcriptional factor Sox9, an early gene of chondrogenesis, in its signaling pathway (95). In addition, mRNA expression of collagen type II, an important marker of hyaline cartilage, is significantly enhanced by TGF-β. Aggrecan also shows similar tendency in presence of TGF-β. On the other hand, the expression of collagen I turns out to be much lower, showing another function of TGF-β to maintain hyaline cartilage phenotype of ASC-derived chondrocytes (96, 97).

*BMPs.* BMP plays an important role in forming bone and cartilage, inducing synergistic and overlapping effects each other (98). BMPs interact with cellular membrane receptors and trigger cascades in signal transduction through Smads, enhancing development of cartilage and bone (99). Mutations in BMP genes cause severe problems in skeletal development, such as murine brachypodism and human chondrodysplasia (98). In addition, BMP deficient mice show low viability or severe appendicular skeletal defects (98). Among them, BMP -2, -4, -6, -7, -13, and -14 are known to stimulate chondrogenesis of MSCs and to induce specific gene expression for chondrogenic phenotype. Particularly, BMP-7 accelerates remodeling of chondrocytes and repair of full-thickness cartilage defects in the rabbits. The healing of full-thickness cartilage was also enhanced by combining BMP-7 and microfracture (100). BMP-2, 4, and 6 promote both transcription of collagen type II mRNA and differentiation of ASCs into chondrocytes (101). BMP-2 with Wnt-3A also enhances ASC chondrogenesis, while Wnt-7A induces dedifferentiation (102). BMP-2 can stimulate repair of lesions in cartilage in deeper hypoxic zones (103). In addition, ex vivo retrovirally transduced stem cells with BMP-4 show enhanced chondrogenesis and improved repair in articular cartilage (104). BMPs, implanted in ectopic localizations, may lead to terminal ASC differentiation into hypertrophy and subsequent ossification (105). As a solution for reducing this problem, the Nogging delivery can be used to hinder the ossification triggered by BMP-4 (106). Therefore, proper injection site of BMPs and appropriate regulation of signaling pathway should be considered to improve efficacy in cartilage tissue engineering.

*IGF.* IGF, a protein with high similarity to insulin, acts as the communication tool for the cells to interact with their environmental settings. IGF family includes two ligands IGF-1 and IGF-2, two cell-surface receptors IGF1R and IGF2R, six kinds of IGF-binding proteins (IGFBPs), and IGFBP proteases. IGF-1 and -2 are derived from insulin-like pre-propeptides and have a C-peptide bridge between the α and β-chains in their molecular structures (107). IGF-1 binds to IGF1R and propagates its signal through the MAPK, extracellular signal-regulated kinase (ERK) 1/2, and phosphatidylinositol (PI)-3-kinase-Akt pathways (108).

Generally, IGF-1 is associated with promoting biosynthetic and anabolic reactions. Natural mutations in IGF-1 gene, caused by the deletion of exons 4 and 5, result in severe retardation in skeletal development in both mice and human (109). Particularly in chondrogenesis, animal studies have shown that IGF-1 enhances synthesis of aggrecan, proteoglycans, and collagen type II (110). Studies on the same model showed that the combined use of chondrocytes and IGF-1 not only formed collagen type II rich matrix, but also enhanced the overall continuity and consistency of the repaired tissues (111). IGF-1 and its receptor are expressed by chondrocytes. They are the essential mediators of homeostasis in cartilage, promoting viability and proliferation of chondrocytes (112). With collagen type II matrix, cartilage defects filled with IGF-1 can be repaired more efficiently (113). IGF also shows synergistic effects with TGF-β, enhancing chondrogenesis of ASCs (114). While ASCs treated with TGF-β3 alone produce both collagen type I and II, the production of collagen I in the ASCs treated with both TGF-β3 and IGF-1 is minimal. BMP also has an effect similar to IGF-1 and minimizes the expression of collagen type I (115). However, ASC-derived chondrocytes, however, may respond less to IGF-1 according to age- or osteoarthritis-associated factors. They seem to be partly related to over-expression of IGFBPs, since *in vivo* cartilage repair is dependent on the amount of IGFBPs produced. In addition, the dose of IGF and its combination with other factors should be optimized for better results (111).

*FGF*. FGF includes 22 proteins in human and its molecular weight spans from 17kDa to 34kDa. FGFs are heparin-binding proteins and play an important role in the differentiation and proliferation of a wide range of cells (116). FGF interacts with one of the tyrosine kinase FGF receptors (FGFRs), activating PI3K, Src, MAPKs, ERK, and p38 (117). Mutations of FGFRs usually lead to dysplasias, especially dwarfing chondrodysplasias. FGF attaches to perlecan in pericellular matrix of cartilage, and acts as a mechanotransducer of chondrocytes (118). FGF-2 is related to the proliferation and maintenance of multilineage differentiation capacity of ASCs. ASCs replicate more rapidly and differentiate into chondrocytes in medium with FGF-2 (111). FGF-2 and FGF-18 can regulate cartilage matrix homeostasis. Calcium ions are involved in a signaling pathway leading to the expression of FGF-18, which promotes differentiation of ASCs while suppressing their proliferation. However, FGF-2 can block the synergistic effect between BMP-2 and sonic-hedgehog-transfected progenitors of chondrocytes (119). Co-treatment of FGF-2 with BMP-6 offsets the chondrogenic capacity of BMP-6 (120). FGF-2 may inhibit TGF-β, hence weakening induction of chondrogenesis in murine MSCs (121). In addition, FGFR-2, one of the early

genes up regulated during limb development, negatively regulates chondrogenesis and proliferation of ASCs through MAPK and STAT1 pathways (122).

## 2c. MOLECULAR PATHWAYS INVOLVED IN ADIPOGENIC DIFFERENTIATION

The adipogenic capability of ASCs depends on their WAT depot origin and donor characteristics such as age, sex and metabolic status (123,124). For example, ASCs from SAT differentiate better into mature adipocytes than those from VAT by the standard *in vitro* adipogenesis protocol (125).

Adipogenesis occurs in two stages, the commitment of ASCs to unipotent pre-adipocytes and the terminal differentiation of pre-adipocytes into mature adipocytes (Fig. 8).

Several factors have been identified that commit or inhibit the conversion of pluripotent stem cells to the adipocyte lineage.

These include the BMP family members, such as BMP4 and BMP2 (126,127), Wnt (128,129), and Hh signaling pathways (130-132). BMP4 and BMP2 have an activating role, whereas Hh signaling has an inhibitory role, and Wnt appears to have both an activating role in commitment (129) and an inhibitory role in adipocyte differentiation (133).

Mounting evidence indicates that lineage determination is regulated by a network of extracellular signaling factors that ultimately impinge on the promoters of lineage-specific transcription factors. The balance of these signaling molecules favours the developmental pathway, often simultaneously promoting one pathway, while inhibiting another (133-135).

*BMPs.* BMP4 and BMP2 have been implicated in the commitment of pluripotent stem cells to the adipocyte lineage (127,136).

*Wnt signaling pathway*. A linkage between Wnt signaling and adipogenesis was first recognized through the finding that Wnt10b decreased dramatically during adipocyte differentiation (128). Consistent with this finding, forced expression of Wnt10b prevented adipocyte differentiation by blocking the expression of the key adipogenic transcription factors, peroxisome proliferator-activated receptor (PPAR) γ and C/EBPα. Surprisingly, the Wnt acts at two points in the adipose development program (Fig. 8), both early in the program as an activator of lineage commitment (129) and late in the program as an inhibitor of adipocyte differentiation (128), perhaps through the actions of different Wnt proteins.

The canonical Wnt pathway functions early in the lineage commitment process. In this pathway, cytosolic β-catenin is embedded in a “destruction complex” containing adenomatous

polyposis coli, axin, and GSK-3β (137). In the absence of Wnt stimulation, GSK-3β phosphorylates β-catenin, priming it for ubiquitination and proteasomal degradation (137). Conversely, activation of Wnt signaling through binding of Wnts to their cell-surface receptor, frizzled, and the associated low-density lipoprotein receptor-related coactivator 5/6 promotes dissociation of the destruction complex, thereby allowing β-catenin to accumulate and translocate to the nucleus (138). Thus, during Wnt signaling, β-catenin accumulates in the nucleus, where it activates the transcription factors lymphoid enhancer factor and/or T-cell factor (Fig. 8), triggering the transcription of downstream genes (including c-MYC and Cyclin D1) (139).

Late in the adipogenic program, the canonical Wnt signaling pathway regulates the balance among myogenic, osteoblastogenic, and adipogenetic fates (128) and, by doing so, decreases adipogenesis. Myoblasts in cell culture retain their plasticity in developmental potential because they can be induced to undergo myogenic, adipogenic, or osteoblastogenic differentiation (140). β-catenin-dependent signaling has been reported to promote both myogenesis (128) and osteogenesis (134) while inhibiting the differentiation of preadipocytes into adipocytes (129). Consistent with the inhibitory effect of Wnt on adipogenesis, myoblasts isolated from Wnt10b-null mice exhibit increased adipogenic potential (140). In addition, activation of the Wnt signaling pathway enhances myogenesis and inhibits adipogenesis in cultured MSCs (128).

*Hh signaling pathway.* Although it is generally agreed that expression of Hh has an inhibitory effect on preadipocyte differentiation, the mechanisms linking Hh signaling and adipogenesis remain poorly defined.

No consensus has been reached regarding molecular markers of the pre-adipocyte. One of the first few markers suggested for pre-adipocytes is CD34. Several studies demonstrated that CD31− CD34+ SVF cells, from humans and mice, differentiate better than CD31− CD34− cells *in vitro*, suggesting the correlation of CD34 expression with pre-adipocyte commitment (124). Friedman's group demonstrated that Lin− CD24+ CD29+ CD34+ Sca-1+ CD105− CD117− SVF subpopulations are capable of *in vivo* WAT reconstitution in lipodystrophic mice, indicating that these additional surface markers define pre-adipocytes (141). Another group showed that, like ASCs, pre-adipocytes reside in the adipose vasculature in mice and express pericyte markers and proteins such as CD140 (PDGFR), chondroitin sulfate proteoglycan (NG2) and alpha-smooth muscle actin (-SMA) (50). Zinc-finger protein-423 (Zfp423) has been proposed to be a functional determinant of pre-adipocyte commitment as

Zfp423 was found highly expressed among most adipogenic mouse cell lines like 3T3-L1 by comprehensive transcription factor profiling (142).

Ectopic Zfp423 expression potentiates non-adipogenic cell lines to undergo adipogenesis, while its deficiency markedly diminishes adipogenic capability *in vitro* and *in vivo*. The subsequent events that mediate terminal differentiation into adipocytes are relatively well studied with availability of 3T3-L1 as the pre-adipocyte cell model. Essentially, the process involves a cascade of transcriptional events mediated by PPAR, members of CCAAT/enhancer binding proteins (C/EBPs) and other transcription factors (143,144). PPAR is the master regulator in this regard as all pathways that promote adipogenesis converge to activate PPAR, which in turn upregulates adipogenic genes such as adipocyte fatty acid binding protein (aP2), leptin, adiponectin, CD36 and glucose transporter type 4 (GLUT4) (144).

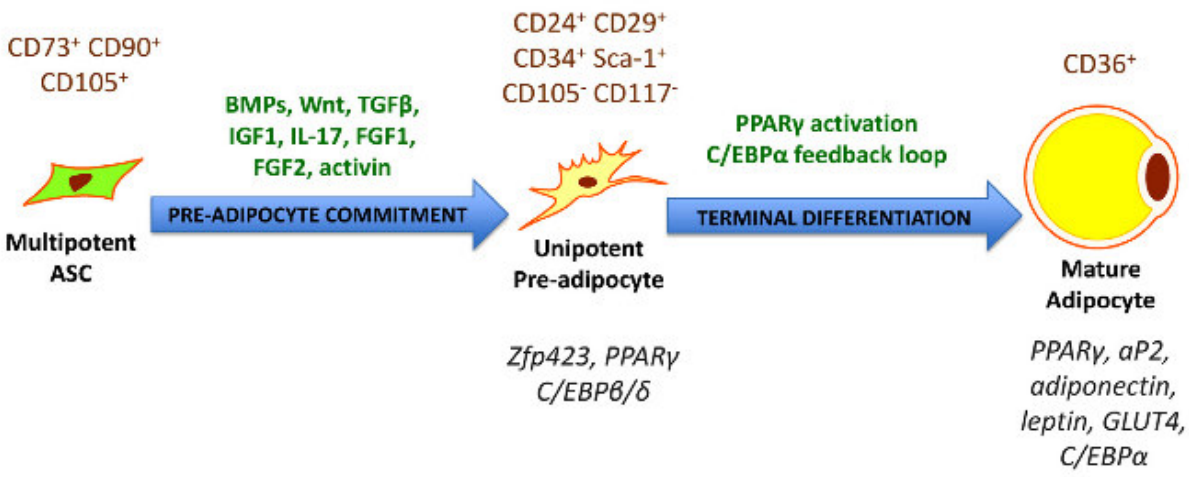



**Figure 8**: Adipogenesis of ASC. Expression of the most representative cell surface markers (top) and genes (bottom) in the ASC, pre-adypocyte and mature adipocyte is shown. From Ong WK et al Int J Biochem Cell Biol. 2013.

# CHAPTER 3

## EMBRYONIC STEM CELLS AND PLURIPOTENCY FACTORS

### *Embryonic stem cells (ESCs)*

ESCs are derived from the inner cell mass (ICM) of the pre-implantation embryo (145-147) and can give rise to all tissue lineages of the three primary germ layers, a property known as

pluripotency. Due to their pluripotency, ESCs hold great promise for basic studies of tissue formation and cell replacement therapy. To achieve the ultimate goal of clinical application, ECSs need to be differentiated efficiently into a specific lineage and undifferentiated ESCs have to be eliminated from the differentiated cells. Another property of ESCs is their continuous self-renewal, which requires that the unique transcriptional profile of the pluripotent state be maintained. In contrast, to differentiate into various cell lineages, ESCs must shift to alternative transcriptional profiles. Both transcriptional and epigenetic regulations play pivotal roles in maintaining the existing transcriptional profile and controlling the plasticity of the transcriptional profile. Additionally, micro-RNAs (miRNAs), novel regulators of gene expression, are emerging as key regulators of pluripotency.

***Pluripotency factors***

In early experimental approaches to address the molecular mechanisms of pluripotency, it was hypothesized that pluripotent cells express a unique set of factors that underlie their 'stemness'. These so called pluripotency factors not only serve as markers of pluripotent cells, but also are functionally important for pluripotency maintenance. Early global transcriptional profiles and genetic studies have identified several critical transcription factors that are required for the pluripotency of ESCs, such as OCT4, SOX2, NANOG, Foxd3, Id and others (30, 148). Given their function in ESCs, expression of OCT4, SOX-2, and NANOG has been also proposed to play a similar role in adult multipotent cells, including MSCs (150-152).

*OCT4* (also known as Oct3), a POU domain-containing transcription factor, was one of the first transcription factors identified as essential for both early embryo development and pluripotency maintenance in ESCs (153,154). The expression of OCT4 is activated at the 8-cell stage and is later restricted to ICM and germ cells in early mouse embryogenesis *in vivo* (154-156). OCT4 is highly expressed in both human and mouse ESCs, and its expression decreases when these cells differentiate and lose pluripotency. OCT4 regulates a broad range of target genes including Fgf4, Utf1, Opn, Rex1/ Zfp42, Fbx15, SOX2 and Cdx2 (157-159). Repression of OCT4 activity in ESCs upregulates Cdx2 expression, leading to ESCs differentiation into trophectoderm (160). OCT4 is also known to activate downstream genes by binding to enhancers carrying the octamer–sox motif (Oct–Sox enhancer), for synergistic activation with SOX2. In contrast with its target genes, little is known about OCT4 upstream regulators. The OCT4 promoter contains conserved distal and proximal enhancers that can either repress or activate its expression depending on the binding factors occupying these sites (161,162). The exact level of OCT4 is important for ESCs fate determination. Loss of OCT4

causes inappropriate differentiation of ESCs into trophectoderm, whereas overexpression of OCT4 results in differentiation into primitive endoderm and mesoderm (163,164).

*SOX2* is an HMG-box transcription factor that is detected in pluripotent cell lineages and nervous system (165-167). Inactivated SOX2 *in vivo* results in early embryonic lethality due to the failure of ICM maintenance (166). SOX2 can form a complex with the OCT4 protein to occupy Oct–Sox enhancers to regulate target gene expression. Oct–Sox enhancers are found in the regulatory region of most of the genes that are specifically expressed in pluripotent stem cells, such as OCT4, SOX2, NANOG, Utf1, Lefty, Fgf4 and Fbx15 (157,158, 168-170).

*NANOG* is another homeobox-containing transcription factor that is specifically expressed in pluripotent ESCs. The essential role of NANOG in maintaining the pluripotency of ESCs is highlighted by the facts that NANOG-deficient ESCs are prone to differentiation, whereas forced expression of NANOG partially renders ESCs self-renewal potential in the absence of LIF (31,171). How NANOG regulates stem cell pluripotency remains entirely unknown. Studies have indicated that NANOG may maintain ESCs pluripotency by downregulating downstream genes essential for cell differentiation such as Gata4 and Gata6 and activating the expression of genes required for self-renewal such as Rex1 and Id (31,171). Although it is widely accepted that NANOG, like OCT4 and SOX2, plays a central role in pluripotency maintenance, this dogma has been challenged by a subsequent report that NANOG protein levels are undetectable in a fraction of ESCs that express OCT4, and the pure populations of NANOG−/− ES cells can be propagated without losing expression of other pluripotency markers (172).

Little is known about the mechanism by which NANOG is regulated in ESCs. Recently, Suzuki et al showed that NANOG expression was upregulated by BrachyuryT and STAT3 in mouse ESCs (173). In human ESCs, Vallier et al reported that Activin/Nodal signaling stimulated expression of NANOG, which in turn prevents FGF-induced neuroectoderm differentiation (174). In addition, several studies indicated that the OCT4/SOX2 complex was directly bound to the NANOG promoter to regulate target gene expression (30). Genomic studies have revealed that OCT4, SOX2, and NANOG frequently bind the same regulatory regions in undifferentiated mouse and human ESCs, and that these binding sites are often in close proximity to one another (30). These results indicate that OCT4, SOX2, and NANOG may physically interact with each other and coordinately regulate target genes in some cases. Additionally, Goke and colleagues reported that combinatorial binding sites of the OCT4/SOX2/NANOG were more conserved between mouse and human ESCs than individual binding sites were (175-177).

# CHAPTER 4

## INDUCED PLURIPOTENT STEM CELLS (IPSc)

Induced pluripotent stem cells (iPSc) are differentiated non-pluripotent stem cells returned to a state of pluripotency through the introduction, in an adult somatic cells, of reprogramming factors, generally transcription factors which regulate gene activity. These cells are similar to natural pluripotent stem cells, in many aspects, such as the expression of stem cell genes and proteins, chromatin methylation patterns, and differentiative ability, but the full extent of their relation to natural pluripotent stem cells is still being assessed (Fig. 9).

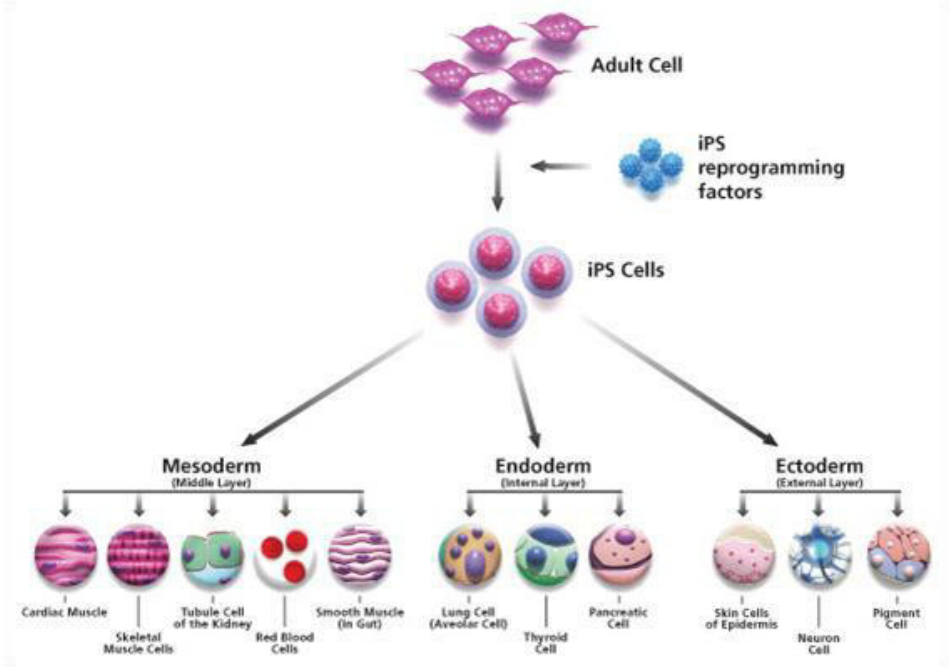



**Figure 9**. Adult cells are used for the derivation of induced pluripotent stem cells (iPSCs), followed by directed differentiation of these cells into cells that have a crucial role in the disease (http://www.sigmaaldrich.com/life-science/stem-cell-biology/ipsc.html).

Takahashi and Yamanaka who identified OCT4, SOX2, KLF4 and c-MYC, as important players to re-establish a pluripotent state in mouse embryonic fibroblasts (MEFs) (Fig. 10), made the first step in the discovery of IPSc (178).

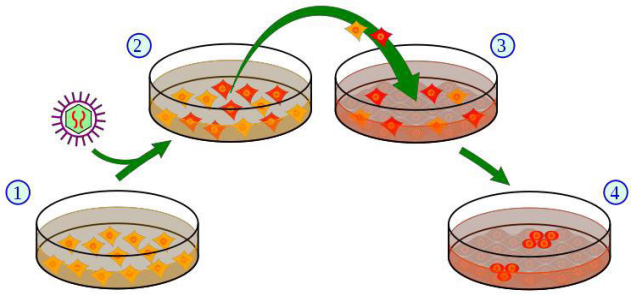



**Figure 10.** Generation of induced pluripotent stem cells. (1) Isolate and culture donor cells. (2) Transfect stem cell-associated genes into the cells by viral vectors. Red cells indicate the cells expressing the exogenous genes. (3) Harvest and culture the cells according to ES cell culture, using mitotically inactivated feeder cells (lightgray). (4) A small subset of the transfected cells become iPS cells and generate ES-like colonies.

The iPSc that were selected based on expression of Fbx15 could not be considered as fully pluripotent because no live chimeric mouse was obtained from them (178). In contrast, iPSc that were selected based on OCT4 or NANOG expression (178-180) had a global transcriptional profile and epigenetic pattern more similar to mouse ESCs. Importantly, NANOG iPSc and OCT4 iPSc could form viable chimaeras and are transmitted through the germ line (178-180). In subsequent experiments, Takahashi et al., (181) and Park et al., (182) demonstrated that the application of the same four factors, OCT4, SOX2, KLF4 and c-MYC, was also able to induce human iPSc from fetal, neonatal and adult somatic cells.

Despite the great potential of iPSc in regenerative therapy, inherent problems could limit its application. For example, tumorigenicity provoked by the reactivation of retroviral-transduced c-MYC (178) makes iPSc unsuitable for transplantation. In this context, Nakagawa et al., (183) have recently reproduced the mouse iPSc derivation by introducing only OCT4, SOX2 and KLF4. Going one-step further, Yu et al., (184) extended the feasibility of carrying out human reprogramming without both KLF4 and c-MYC. In their system, KLF4 and c-MYC could be replaced with NANOG and LIN28 with apparent success in generating human iPSc (184). LIN28 is an RNA binding protein that is downregulated during ESCs differentiation (185), which appeared to have a modest effect on human reprogramming (184). Moreover, expression of LIN28 is absent from several iPS clones recovered by Yu et al. (184), confirming that it is neither essential for the initial reprogramming process, nor is it required for the stable expansion of reprogrammed iPSc.

Nakagawa recently showed that some members of the same protein family could replace KLF4 and c-MYC (183). In an independent study, Jiang et al., (186) showed that Klf2, KLF4 and Klf5 shared an extensive overlap in their genomic targets, and had similar DNA binding

properties. On the other side, the single or pair-wise depletion of Klf2, KLF4 and Klf5 resulted in no morphological changes in mouse ESCs, while the triple knockdown induced differentiation (186).

Therefore, it was hypothesized that Klf2, KLF4 and Klf5 functions redundantly regulating self-renewal and pluripotency of mouse ESCs. Notably, these Klfs were found to bind to numerous genomic loci, and the genes that are co-bound by these Klfs encoded regulators of different cellular functions such as DNA replication and cell cycle (186). The functional redundancy and wide regulatory roles of these Klfs may explain why Klf2 and Klf5 could replace KLF4 as a reprogramming factor (183). Another key finding by Jiang et al., (186) was that Klf2, KLF4 and Klf5 shared many common targets with NANOG, linking these Klf transcription factors to the NANOG transcriptional network. It is likely that one possible function of KLF4 in reprogramming is to act synergistically with OCT4 to restart the ESC-specific gene expression program.

c-MYC is an oncogenic protein known to elicit transformation (187), while KLF4 can function both as a tumor suppressor and as an oncogene (188). Forced expression of KLF4 and c-MYC alone resulted in the formation of tumor cells that are not pluripotent (178). It is believed that OCT4 and SOX2 are responsible for directing the iPSc towards pluripotency (183). Nevertheless, the requirement for KLF4 and c-MYC in iPS generation suggests some common mechanisms between the process of transformation and the induction of self-renewal. KLF4 promotes cell proliferation by suppressing p53 (188).

This could counteract the effects of c-MYC in inducing p53-dependent apoptosis in primary fibroblasts (187). On the other hand, KLF4 can suppress proliferation by activating p21, while c-MYC exerts an opposing effect by repressing p21 (187). Based on these observations, it was postulated that the balance between KLF4 and c-MYC might be important in regulating growth and self-renewal of the iPSc (179). Recent studies showed that NANOG could substitute the roles of KLF4 and c-MYC in human somatic cell reprogramming (184). KLF4 promotes cell proliferation via the mediation of p53 suppression (188). Since p53 is a negative regulator of NANOG (189), KLF4 may have a functional role in the activation of NANOG during reprogramming. Therefore, overexpression of NANOG could negate the need for activation through the KLF4/p53 pathway. In addition, KLF4 can cooperate with OCT4 and SOX2 to activate the Lefty1 core promoter in mouse ESCs (190). However, NANOG hase been found to physically interact (191) with OCT4 and to cooperate extensively with OCT4 and SOX2 in the core-transcriptional network (30,192). As a result, NANOG may be able to replace some function of KLF4 in the initiation of reprogramming

via its interaction with OCT4 and SOX2. Previous genome-wide studies have revealed that NANOG binds to KLF4 and Klf5 in mouse ESCs (192), while KLF5 is co-bound by OCT4, SOX2 and NANOG in the human ESCs (30). Thus, it is tempting to speculate that the activation of KLF5 by OCT4, SOX2 and NANOG in the human system is sufficient to mitigate the lack of exogenous KLF4. Interestingly, c-MYC is a target of NANOG in the mouse ESCs (192). NANOG may regulate and recruit the downstream endogenous expression of KLF4 and c-MYC during human reprogramming.

This suggests a possibility for NANOG to act as a downstream effector of the KLF factors in human reprogramming. As a result, retroviral transfection of NANOG is sufficient to induce reprogramming in the absence of the introduced KLF factors (193,194).

It has been shown that the tumour suppressor p53 binds to the promoter of NANOG and suppresses its expression after DNA damage in mouse ESCs. p53 can thus induce differentiation of ESCs into other cell types which undergo efficient p53-dependent cell-cycle arrest and apoptosis. Deletion of NANOG causes early embryonic lethality, whereas constitutive expression enables autonomous self-renewal of ESCs (31,171).

One of the characteristic features common to the various studies is the invariable requirement of OCT4 as a reprogramming factor. Recent genome-wide studies have identified the downstream targets of OCT4, which include genes encoding for self-renewal factors, lineage-specific factors, signaling molecules and DNA damage response sensors (191). Thus, OCT4 is implicated in a broad spectrum of cellular processes that collectively specify the self-renewal state of the ESCs. By introducing OCT4, KLF4 and c-MYC, iPSc that are somewhat similar to ESCs in terms of morphology and proliferative rate could be obtained (178). However, the iPSc without SOX2 failed to differentiate and lacked the property of pluripotency (178). This finding suggests three possibilities pertaining to the roles of OCT4 and SOX2. Firstly, OCT4 and SOX2 together are required to activate common downstream target genes that specify the pluripotent state. Secondly, OCT4 alone is not sufficient for proper cell fate specification and differentiation. Lastly, SOX2 may not be required for the initiation of reprogramming. The role of SOX2 as a co-factor of OCT4 in regulating downstream target genes in ESCs is well recognized. Genome-wide ChIP-on-chip analyses revealed the widespread co-operative nature of the two transcription factors in regulating genomic targets (30). However, it is likely that SOX2 also functions independently of OCT4 in cell fate determination and during the differentiation process. Unlike OCT4, SOX2 expression is not restricted to pluripotent ESCs but is also expressed in extraembryonic ectoderm and neuroectoderm (166). Moreover, SOX2-null mice die at a later stage during the

time of implantation due to a failure of epiblast formation, as compared to OCT4-null embryos that cannot form the ICM. Interestingly, while Oct1 and Oct6 cannot replace OCT4 as a reprogramming factor (183), Sox1 and Sox3 can substitute SOX2 in the derivation of iPSc (183). Taken together, it appears that SOX2 may not be necessary for the process of reprogramming, but the activity of SOX2 is critical for pluripotency and proper differentiation of the reprogrammed iPSc.

The above-mentioned studies suggest that OCT4, SOX2 and NANOG may function in distinct pathways but operate inter-dependently to coordinate the specification of the ESCs state. Understanding the interactions among these transcription factors and their regulation of downstream genomic targets will be instrumental in delineating their specific roles in governing the maintenance of pluripotency and driving cell fate decision during the differentiation process.

# AIM

Aim of the project was the phenotypical and functional characterization and differentiation of human ASCs derived from SAT and VAT. In addition, the project was aimed to the evaluation of the different expression of ESC markers NANOG, SOX2 and OCT4 in ASCs derived from SAT and VAT and the characterization of NANOG role in the pluripotency network.

# MATERIALS AND METHODS

Human SAT and VAT biopsies were obtained from 42 obese subjects undergoing elective open-abdominal and laparoscopy surgery. The protocol was approved by the Independent Ethical Committee at the Azienda Ospedaliero-Universitaria Policlinico P. Giaccone, Palermo, Italy. All patients gave their written informed consent.

**Adipose tissue biopsies**

On the day of surgery, general anaesthesia was induced and adipose tissue specimens were obtained from the subcutaneous and omental depots. Approximately 1 g adipose tissue was

taken from each fat depot. All biopsies were handled under sterile conditions and immediately used for subsequent preadipocyte isolation.

**ASC Isolation**

Tissue specimens were immediately transported to the laboratory in Hanks' balanced salt solution (HBSS; pH 7.4), dissected from fibrous material and visible blood vessels, cut into little fragments and incubated in $PBS^{C2++}$/Mg supplemented with 1 mg/ml collagenase type I (Sigma Chemical, St Louis, MO, USA), with vigorous shaking (100 cycles/min) for 1 h at 37ºC. Resulting material was filtered through 250 mm mesh, and adipocytes and free oil were separated from the SVF components by centrifugation at 1,200 rpm for 5 min at room temperature. The SVF pellet was resuspended in a growth medium consisting of DMEM/Ham's F12 1:1 supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5% FCS, 1 ng/ml FGF2, and 10 ng/ml EGF.

**Adipogenic, osteogenic and chondrogenic differentiation**

For adipogenic differentiation, confluent ASCs at passage 4 were differentiated in a chemically defined serum-free medium containing antibiotics, 2 nMtriiodotyronine (T3), 100 nM human insulin, 100 nM dexamethasone, and 1 mM rosiglitazone, as previously reported by Perrini et al. (54). For the first 4 days of the differentiation period, 0.5 mM of methyl-isobutylxanthine was also added. Osteogenic and chondrogenic differentiations were induced by StemProOsteogenesis Differentiation Medium and StemProChondrogenesis, respectively.

**Histochemical Staining of Differentiated Cells**

Differentiation of cells into adipocytes was assessed through morphological analysis and Oil-Red-O staining. Cells in three wells were fixed with 3.7% (vol./vol.) formaldehyde in PBS, and their triacylglycerol content was stained with 0.3% (wt/vol.) Oil-Red-O in 60% (vol./vol.) isopropanol. After repeatedly washing with water, the differentiated adipocytes were estimated by direct counting under light microscope.

Osteogenic differentiation was evaluated through alizarin red staining. After 21 days or longer under differentiating condition, cells were fixed with 4% formaldehyde solution for 30 minutes. After fixation, they were stained with 2% Alizarin Red S solution (pH 4.2) for 2 to 3 minutes and rinsed with distilled water. Complete osteogenesis differentiation was visualized

under light microscope and the images and pictures were taken on wide-field microscopes (Zeiss) with a color CCD camera (Nikon).

Chondrocytes differentiation was evaluated by Alcian Blue Stain Analysis. After 14 days or longer, under differentiating conditions, cells were fixed with 4% formaldehyde solution for 30 minutes. After fixation, cells were stained with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 minutes and, to neutralize the acidity, they were rinsed three times with 0.1 NaOH. Blue staining showed the synthesis of proteoglycans by chondrocytes. Complete chondrogenesis differentiation was visualized under light microscope and the pictures were taken on awide-field microscopes (Zeiss) with a color CCD camera (Nikon).

**Sphere culture**

SVF cells were seeded at $1x10^2$ cell/$cm^2$ in ultra low adherent flasks (Corning, Avon, France) in defined culture medium which consisted of DMEM/F12 supplemented with L-glutamine (2 mM), non essential amino acids (1×), B27 (1×) (Invitrogen), glucose (0.6%, Sigma), human bFGF (10 ng/ml), human EGF (20 ng /ml). Cells were incubated at 37 °C under 5% CO2 and half of the medium was changed once a week. To test if spheres containing cells could revert to monolayer growth, they were dissociated with Accutase (Sigma) and plated in flasks treated for cell culture (TPP) in ASC expansion medium.

**RNA Isolation and Quantitative RT-PCR (qRT-PCR)**

mRNA from ASC populations isolated from VAT and SAT human biopsies was isolated by using a RNeasy kit (Qiagen, Hamburg, Germany). Two hundred and fifty ng of RNA from ASC and 2ug of RNA from VAT and SAT biopsies "*in toto"* were reverse-transcribed with standard reagents (Promega). One microliter of each reverse-transcription reaction was amplified by using SYBR Green PCR master mix from Qiagen (Quantitectsybr green master mix), using the RotorGene PCR system (Qiagen). For each gene, mRNA expression was normalized for the housekeeping gene β-actin. Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. PCR primers, Oct 4, Nanog, Thy-1 (CD-90), CD105 and CD 73 were purchased from Qiagen (QuantiTect® Primer Assays, Qiagen), primer for Sox2 was purchased from MWG and primer for β-actin was purchased from Invitrogen.

**Flow cytometry analysis**

The cells were harvested and filtered through a 40-µm filter mesh and suspended at the concentration of $1x10^6$ cells/ml. Then 100 µl of cell suspension containing $1x10^5$ cells was used for each flow cytometric test.

**Immunophenotyping**

Anti-CD31, anti-CD45, anti CD146, anti CD29, anti- CD90 and anti-CD105 mouse monoclonal antibodies were tested on s-ASC and v-ASC. The incubation conditions were in accordance with the manufacturer's instructions. Cells were washed twice with PBS/BSA 5% and incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 1 h in the dark. Data were acquired on a FACS Calibur and analyzed using CELL Quest Pro software (Becton Dickinson).

**Analysis of Cell Cycle Status of MSCs**

Single cell suspensions of S-ASC and V-ASC were obtained and seeded at a density of $2x10^3$ cells/$cm^2$ (passage 3) and DNA content was performed, according to Nicoletti's protocol. Briefly, $1x10^6$ cells were fixed in 70% ethanol, rehydrated in PBS and then re-suspended in a DNA extraction buffer (with 0.2 M NaHPO4 , 0.1% Tritonx-100 and, pH 7.8). After staining with 1 µg/mL of propidium iodide (PI) for 5 minutes, fluorescence intensity was determined by analysis on a FACS Calibur flow cytometer (Becton-Dickinson, New Jersey, USA). Data acquisition was performed with CellQuest (Becton Dickinson) software, and the percentages of G1, S, and G2/M phase cells were calculated with the MODFIT-LT software program (Verity Software House, Inc.).

**siRNA transfection**

siRNA transfection in ASCs was performed using INTERFERinTM transfection agent (Polyplus-Transfection, Illkirch, France), according to the manufacturer's instructions. Briefly, cells were seeded into six-well plates at a density of 250,000 cells/well or 96-well plates at a density of 3000 cells/ well. The transfection agent and the siRNA complex were added to the cells and incubated for 72 hours for expression analysis and 96 hours for protein detection. The final concentration of SOX2 siRNA was 100 nM for mRNA analysis and 150 nM for protein detection and 40 nM for NANOG siRNA. Each assay was performed in triplicate in at least three independent experiments. SOX2 was silenced using Stealth SiRNA SOX2 HSS144045 (Invitrogen, Milan, Italy). As control, siCONTROL Stealth siRNA

Negative Control was used (Invitrogen, Milan). NANOG was silenced by NANOG siRNA (h) (Santa Cruz Biotechnology), and control siRNAs were used as a no-target control (Santa Cruz Biotechnology).

**Western Blot analysis**

Proteins were extracted from cultured cells using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40), supplemented with a protease inhibitor cocktail (Complete mini, Roche Diagnostics GmbH) and phosphatase inhibitors. Protein content was determined according to Bradford's method. Proteins were separated using 4-20% gradient polyacrilamyde gels (BioRad), electrotransferred to nitrocellulose membrane, and blotted with the following primary antibodies: rabbit antihuman SOX2 (Poly6308, BioLegend, San Diego, CA), mouse antihuman OCT4 (sc-5279, Santa Cruz Biotechnology), goat antihuman NANOG (sc-30331, Santa Cruz Biotechnology), mouse anti b-actin IgG1 (A5441, Sigma-Aldrich). Secondary antibodies were goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology), goat antimouse IgG-HRP (sc-2031, Santa Cruz Biotechnology), and donkey anti-goat IgG-HRP (sc-2033, Santa Cruz Biotechnology). Antigen–antibody complexes were visualized using the ECL prime (Amersham) on a CCD camera (Chemidoc, BioRad, Milan, Italy). Western blot bands were quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

# RESULTS

**Isolation of ASCs**

The fresh-isolated SVF was a compounded cell population with the shape of spindle, triangle, polygon and round, containing fibroblasts, adipocytes at different differentiation stages. These cells adhered to flask without substrate. After 48 hours cells were small and not extensional (Fig.11 A) when observed under a light microscope. After 5 days (Fig.11 B), these cells extended and grew gradually. After 7 days, the number of some mature cells, such as mature adipocytes, decreased and the morphology of most cells tended to be uniform. After 10 days, (Fig.11 C) colonies of fibroblastic-like cells were observed, although fibroblasts mixed in ASCs were still found. Both these cell types reached confluency after about 15 days. Crystal violet staining revealed that visceral (v)-ASCs had large nucleus and extended cytoplasm

(Fig.11 D), while subcutaneous (s)-ASCs had large nucleus and large cytoplasm (Fig.11 E). Both s-ASCs and v-ASCs formed a cell aggregation at the third passage (Fig.11 F and G).

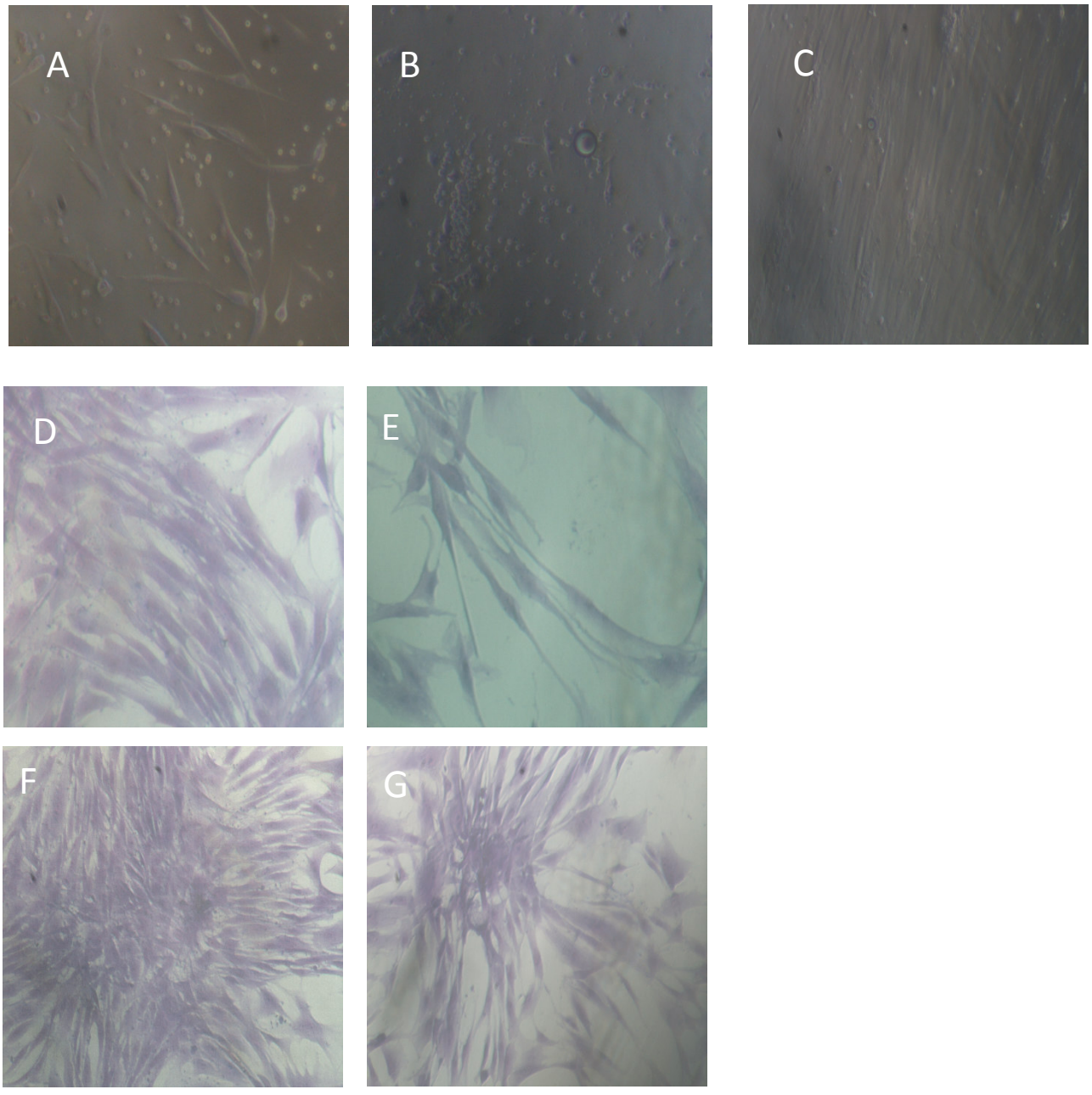



**Figure 11**: Morphology of adipose derived stem cells (ASCs) under light microscopy with phase contrast. SVF morphological characteristics on days 2 (A), 5 (B) and 10 (C) of expansion cultures. Crystal violet staining shows visceral -ASCs having large nucleus and extended cytoplasm (D) in comparison to subcutaneous –ASCs that have large nucleus and cytoplasm (E). F and G show similar aggregation of MSC from v-ASC and s-ASC in adhesion cultures.

## Cell cycle analysis

The cell cycle analysis at passage 3 showed a different distribution of cells in G1, G2 and S phase in S-ASC and V-ASC. S-ASC in G1 phase was 70.68 ± 2.32% , V-ASC was 56.01 ± 3.43% , in G2 phase S-ASC was 0.00% while V-ASC was 43.99 ± 2.5%. In S phase S-ASC was 29.32 ± 3.45% while V-ASC 0.00%. (Fig.12 A and B).

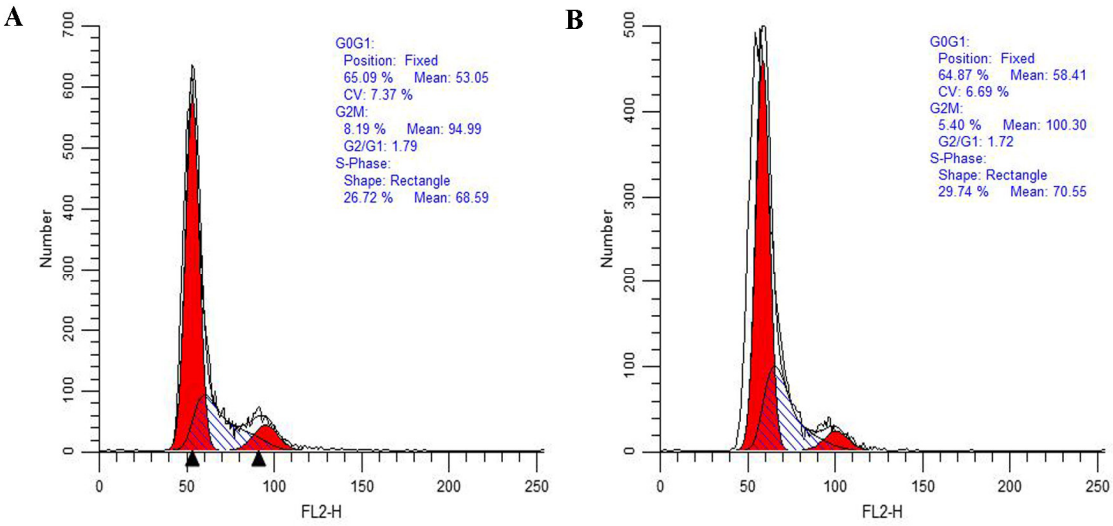



**Figure 12**: In A cell cycle distribution of S-ASC, in fig. 2 B V-ASC cell cycle distribution performed according Nicoletti's protocol.

## ASCc differentiation

ASCs were differentiated into adipogenic, chondrogenic, and osteogenic lineages and confirmed by Oil Red-O, Alizarin red and Alcianblu staining, respectively. Adipocyte differentiation was evaluated by both morphological analysis under light microscopy and Oil-Red-O staining. No lipid droplets were in untreated preadipocytes. However, following exposure to the differentiation medium, cells gradually accumulated large multilocular lipid droplets. Lipid accumulation was evident in subcutaneous and omental adipocytes derived from SVF, and did not differ between the two cell cultures.

Under osteogenic induction conditions, deposition of calcified matrix by the cells was confirmed by positive staining with Alizarin Red and did not differ between the two cell cultures. After 14 days in the chondrogenic induction medium, most of the cells in the culture

were involved in aggregate formation, and empty areas were present in the culture flasks. On day 21 of chondrogenic induction, the cell aggregates became darker in color, which denoted the formation of dense cell aggregates with increased ECM production. Chondrogenic differentiation was confirmed by Alcian blue staining. In all experimental studies, there were no differences between the two cell cultures (Fig. 13).

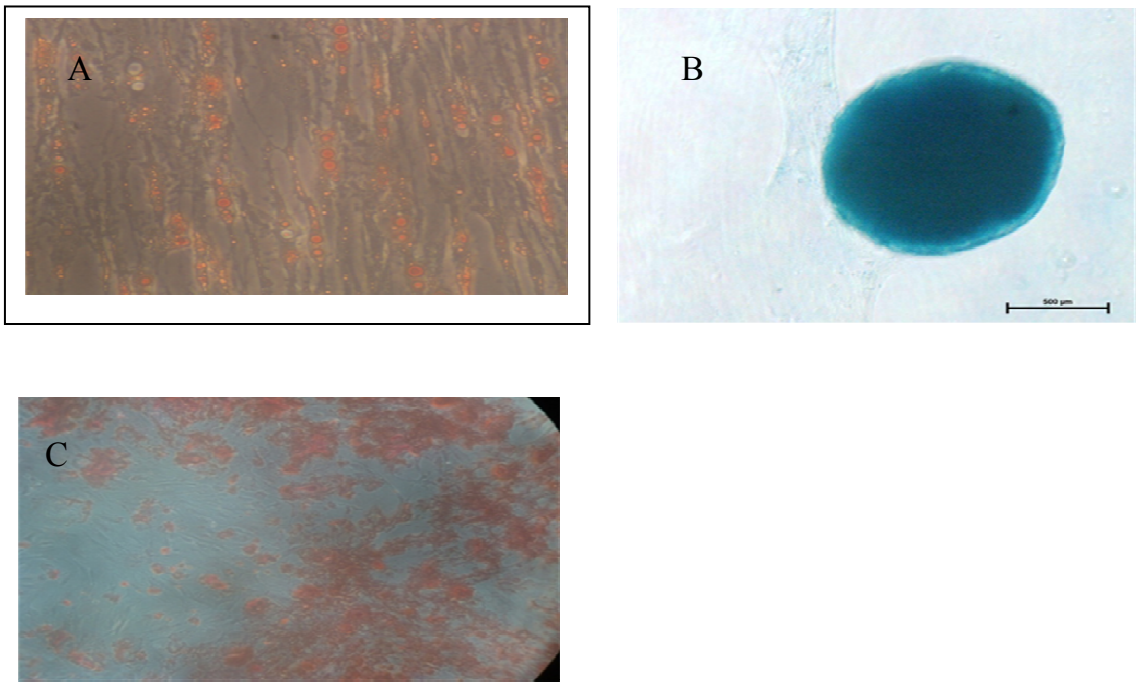



**Figure 13**: ASCs differentiation in adipocytes (A), condrocytes (B) and osteoblasts (C).

**Serum-free culture conditions allowing for sphere formation**

In ultra low adherent flasks, no cell adhered to the bottom and only spheres were obtained in the supernatant (Fig. 14). The cells that composed the spheres appeared homogeneous in size and morphology.

After enzymatic dissociation and culture in ASCs expansion medium, containing fetal calf serum, spheres could revert to monolayers, in flasks treated for adherent cell culture. They re-adheared and morphologically resembled primary cultured ASCs obtained from SVF plating and continued to proliferate. ASCs maintained their multipotentiality when cultured as spheres in defined medium. The expression of SSEA-4, as pluripotency-associated marker was evaluated by immunofluorescence. SSEA-4 expression was significantly higher than that of ASCs cultured in monolayer.

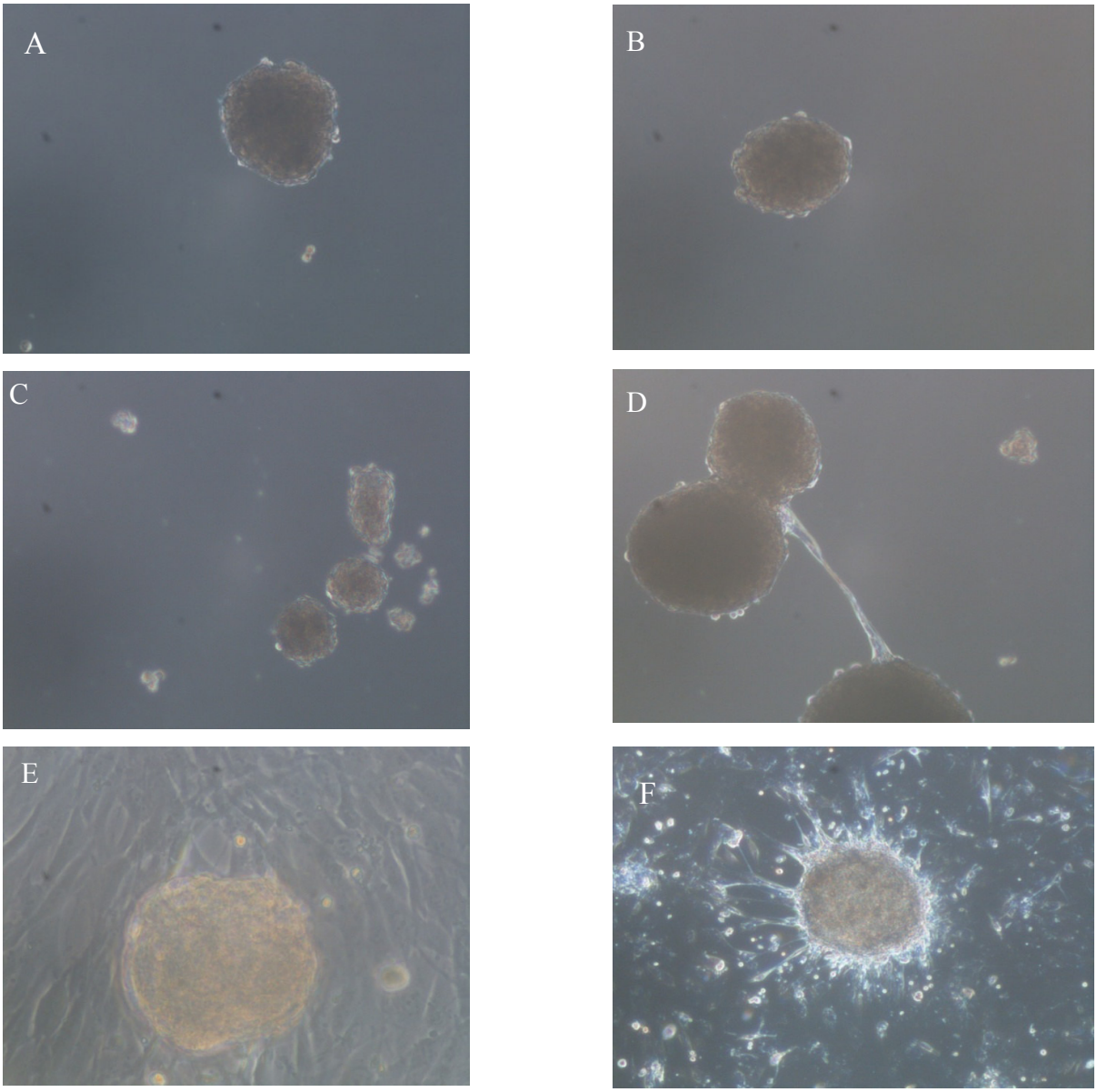



**Figure 14**: Spheres in ultra-low flasks. A, C, E show spheres from s-ASCs. B, D, F show spheres from v-ASCs.

**Analysis of expression of pluripotency-associated genes in ASCs by RT-PCR**

ESC markers, such as ABCG2 (v-ASC 1.11 ± 0.06 *vs.* s-ASC 1.065 ± 0.1), OCT4 (v-ASC 0.8 ± 0.05 *vs.* s-ASC 0.9 ± 0.05), SOX 2 (v-ASC 0.93 ± 0.06 *vs.* s-ASC 0.83 ± 0.07) , C-KIT (v-ASC 7.43 ± 0.07 *vs.* s-ASC 17.13 ± 0.09), THY-1 (v-ASC 0.42 ± 0.005 *vs.* s-ASC 0.10 ± 0.03), CD-73 (v-ASC 0.5 ± 0.003 *vs.* s-ASC 0.64 ± 0.06), CD-105 (v-ASC 2.38 ± 0.2 *vs.* s-ASC 3.16 ± 0.9) and NANOG (v-ASC 0.11± 0.8 *vs.* s-ASC 2.7 ± 0.2) . ASC from VAT and SAT expressed all of the stem cells markers (Fig 15 A). In spheres, SOX2 (1.9 ± 0.1 *vs.* 0.2 ± 0.07 *vs.* 0.195 ± 0.2) , OCT4 (4.4 ± 0.01 *vs.* 1.25 ± 0.09 *vs.* 1.130 ± 0.2) and NANOG (3.39 ±

0.01 *vs.* 3.51 ± 0.04 *vs.* 1.5 ± 0.09) were high expressed when compared with both adherent s-ASC and v-ASC cells ($p<0.01$) (Fig 15 B).

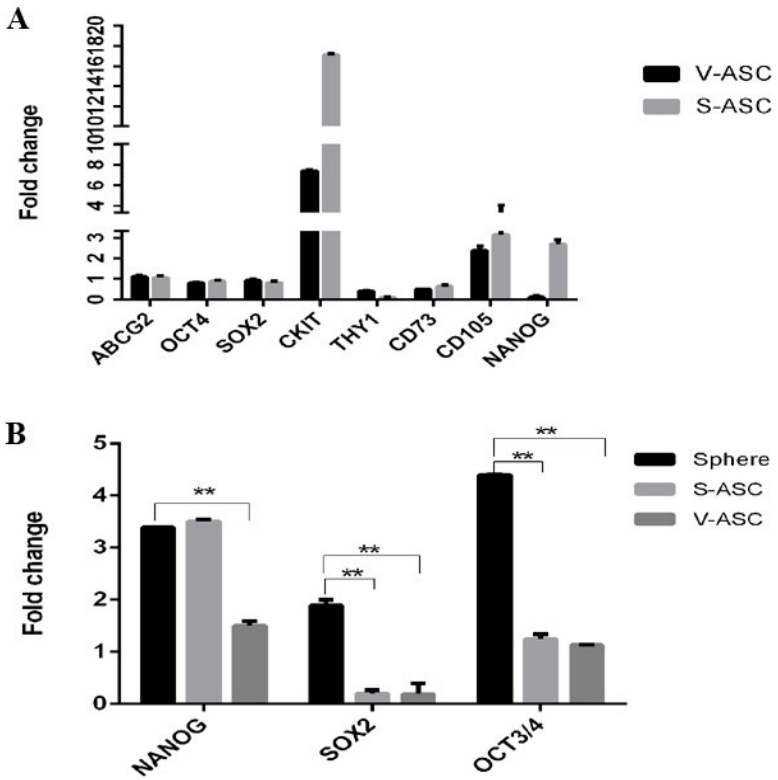



**Figure 15 A**. qRT-PCR analysis in S-ASC and V-ASC primary cells. Data are representative of three independent experiments. Relative expression levels for ABCG2, OCT4, SOX2, CKIT, THY1, CD73, CD105, and NANOG were assessed using $2^{-\Delta\Delta Ct}$ method. Values are shown as mean ± SE, *p<0.05.Data shown are relative to an endogenous control (beta-Actin), with fold change compared to expression levels in commercial Bone Marrow – mesenchymal stem cell (set to 1). **B**. qRT-PCR analysis in spheres from S-ASC and V-ASC primary cells. Data are representative of three independent experiments. Values are shown as mean ± SE, **p<0.01

Comparing SOX2 (OD v-ASC 0.158 ± 0.02 *vs.* s-ASC 0.2 ± 0.02), NANOG (OD v-ASC 0.176 ± 0.001 *vs.* s-ASC 0.4 ± 0.01) and OCT4 (OD v-ASC 0.128 ± 0.01 *vs.* s-ASC 0,163±0,01) protein expression of s-ASC and v-ASC. NANOG was higher expressed in ASC isolated from SAT than from VAT ($p<0.01$) (Fig 16A and B).

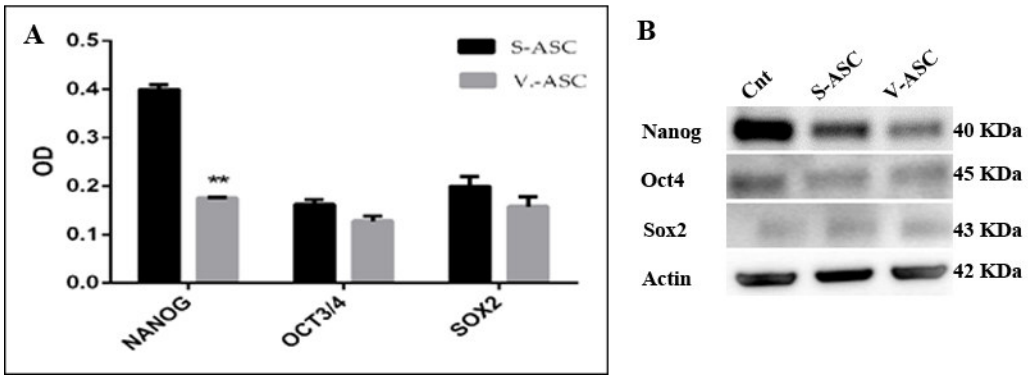
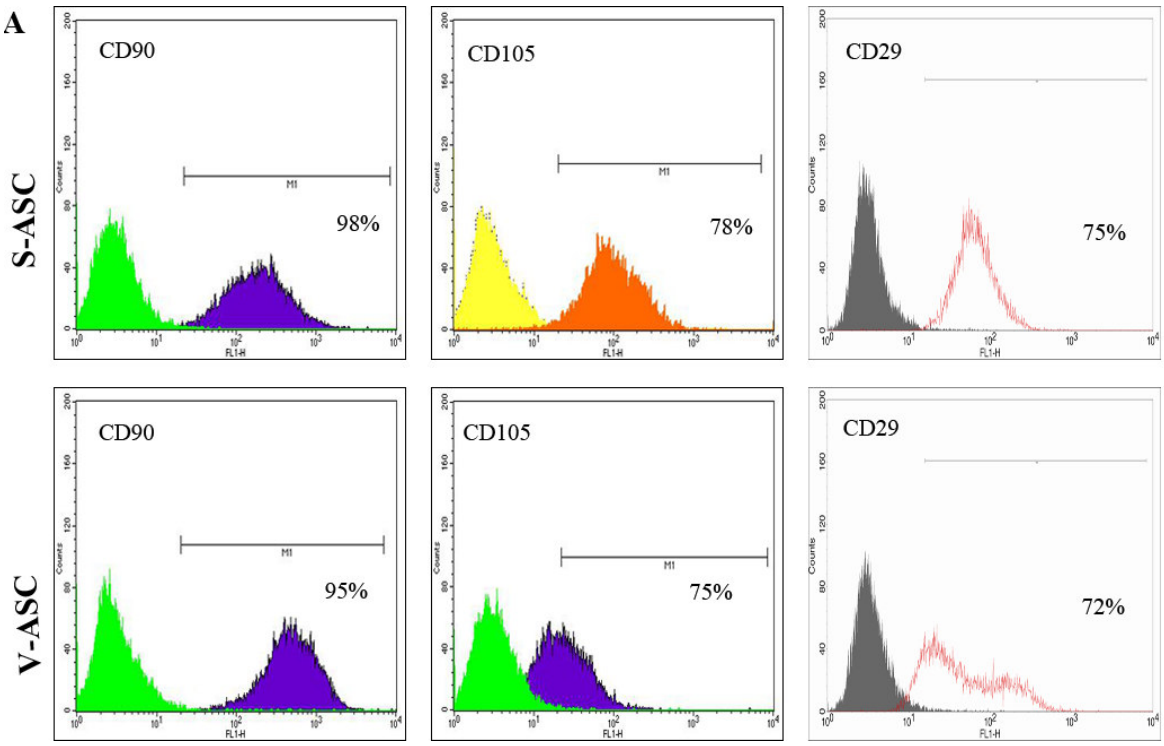



**Figure 16 A and B.** Western blot analysis in the S-ASC and V-ASC primary cells of NANOG, OCT3/4 and SOX2. Data are representative of three independent experiments. Values are shown as mean ± SE, **p<0.01. OD, optical density.

## Flow cytometry analysis

The cell-surface antigenic characteristics of s-ASCs and v-ASCs were analyzed by flow cytometry. Both populations at passage 3 showed almost no expression of CD31, CD45 and CD146 (<1%) (Fig.17 A) and positive markers were found to be CD90 (98 ± 3.9 % and 95 ± 3.6% respectively),CD105 (78 ± 4.2% and 75 ± 2.7% respectively) and CD29 ( 75% ± 2.5% and 72% ± 2.8% respectively) (Fig. 17 B).



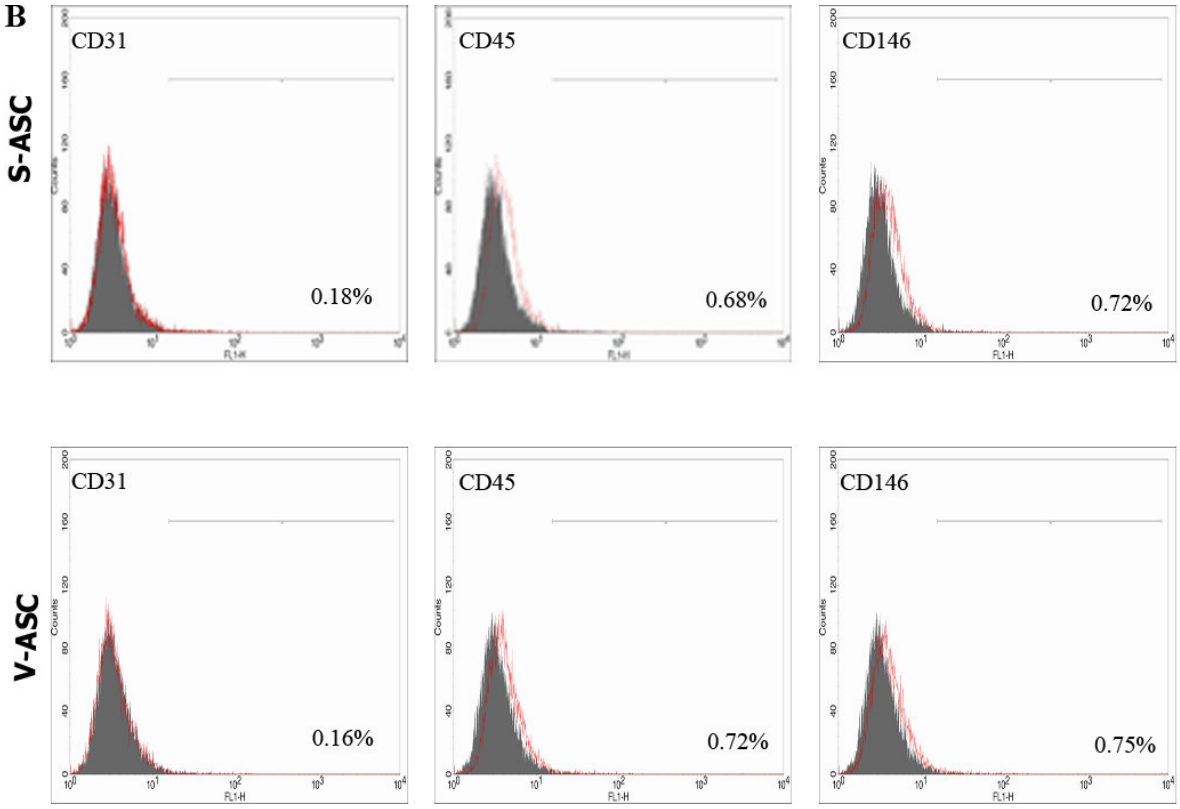



**Figure 17:** A. Cytofluorimetric assay in s-ASC and v-ASC for CD90 (95 ± 3.6% and 98 ± 3.9 %respectively), CD105 (C and D)(75 ± 2.7% and 78 ± 4.2% respectively) and CD29 (89% ± 2.6% and 88% ± 3.2% respectively). B: Cell are negative for CD31, Cd45 and CD146. All fields are representative of one S-ASC and V-ASC sample out of at least 12 independent experiments.

**NANOG and SOX2 silencing in s-ASC and v-ASC**

NANOG silencing caused down-regulation of OCT4 (70% ± 0.05, $p<0.01$) and SOX2 genes (75% ± 0.03, $p<0.05$) in s-ASCs and v-ASCs after 48 hr (Fig. 18). By contrast, SOX2 silencing after 48 hr did not affect NANOG and OCT4 expression (Fig. 19).

Western blot analysis of NANOG silencing with stealth siRNA after 48 hr in s-ASCs (OD 0.8 ± 0.1 vs 0.24 ± 0.01, $p< 0.01$] and v-ASCs (OD 0.5 ± 0.01 vs 0.22 ± 0.01) resulted in a OCT4 down-regulation was confirmed in s-ASCs (OD 1.16 ± 0.17 vs 0.38 ± 0.12 $p<0.01$) and in v-ASCs (OD:1.09 ± 0.16 vs. 0.39 ± 0.12, $p< 0.01$) and in the SOX2 down regulation in s-ASCs (OD 0.49 ± 0.05 vs. 0.20 ± 0.02, $p<0.01$) and v-ASCs (OD 0.5 ± 0.01 vs 0.01 ± 0.001, $p<0.01$) (Fig. 20).

SOX2 silencing in s-ASCs (OD: 1.3 ± 0.1 vs 0.23 ± 0.06, $p<0.01$) and in v-ASCs (OD 1.16 ± 0.013 vs 0.56 ± 0.04) did not affect neither NANOG protein levels in s-ASCs (OD: 1.17 ± 0.1 vs 1.156 ± 0.01, $p= 0.580$) and in v-ASCs (OD: 0.7 ± 0.2 vs 0.68 ± 0.1, $p= 0.480$) nor OCT4

protein levels in s-ASCs (OD: 0.98 ± 0.1 vs 0.92 ± 0.01, p= 0.580) and in v-ASCs (OD: 0.69 ± 0.2 vs 0.60 ± 0.1, p= 0.480) (Fig. 21).

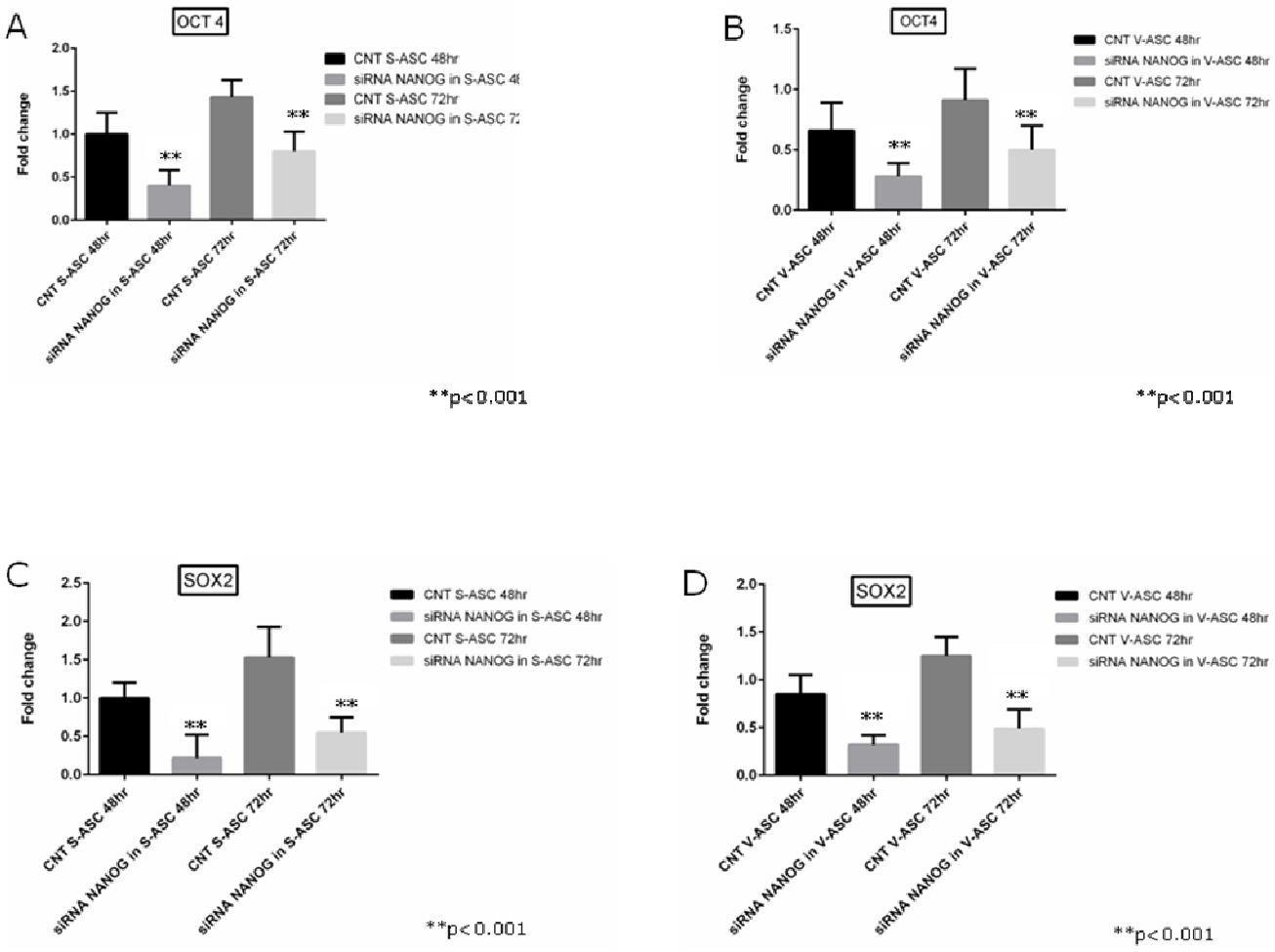



**Figure 18:** Analysis of NANOG silencing. qRT- PCR analysis in s-ASC (A) and v-ASC of (B) OCT -4 and in sASC (C) and vASC (D) SOX2 gene expression after NANOG silencing with stealth siRNA (siNANOG) vs. siCONTROL treated cells (CNT). Data are representative of three independent experiments. Values are shown as mean ± SE, **p<0.001.

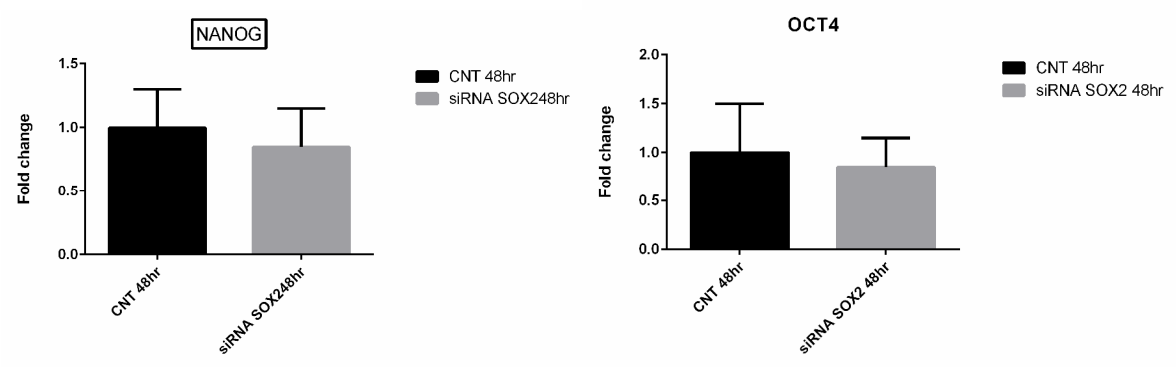



**Figure 19.** Analysis of SOX2 silencing. qRT-PCR analysis in s-ASC. (A) NANOG and (B) OCT4 gene expression after SOX2 silencing with stealth siRNA (siSOX2) vs. siCONTROL treated cells (CNT). Data are representative of three independent experiments. Values are shown as mean ± SE

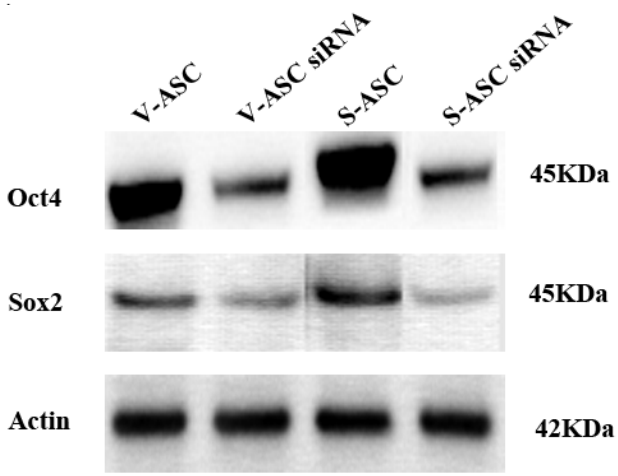



**Figure 20.** Western blot analysis of OCT4, SOX2 and actin with proteins extracted from v-ASC and s-ASC cells after NANOG silencing with stealth siRNA vs. siCONTROL treated cells.

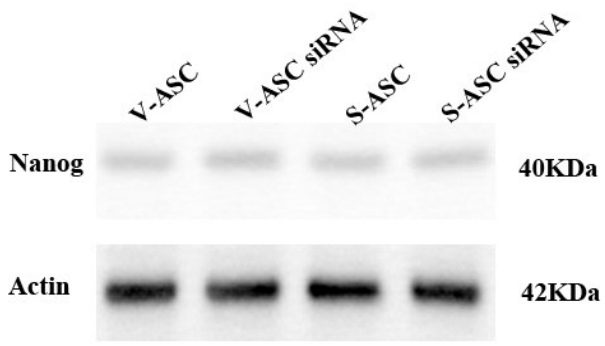



**Figure 21.** Western blot of NANOG and actin with proteins extracted from s-ASC and v-ASC cells after SOX2 silencing with stealth siRNA vs. siCONTROL (CNT) treated cells. Results were normalized with beta actin. Data are representative of three independent experiments. Values are shown as mean ± SE, $p<0.05$

# DISCUSSION

The results of the current study show that both SAT and VAT are composed by a large amount of ASCs and no differences in terms of expression of ESC and MSC markers are observed in the two kind of adipose tissue.

The biological diversity of adipose tissue depots has become a fundamental issue in recent years, in light of its potential impact on human health (195). It is known that VAT is morphologically and functionally different from SAT (196). Depot-related variations have been described for a variety of biological endpoints, such as activation of the insulin-signalling pathway (197), glucose uptake responses (198,199) and adiponectin secretion (200). It has been proposed that extrinsic factors, including depot-specific blood flow, cell density, cell heterogeneity and/or innervation (201) could contribute to distinct gene expression patterns and metabolic profiles in adipocytes of different anatomical regions.

We have isolated ASCs from SAT and VAT of the same donor and systematically characterized the features of these paired ASCs. We showed that both s- and v-ASCs expressed the cell surface markers characteristic of the MSCs. However, they show distinct cell morphology in culture, at least in the early passages, which included the cell shape and cell size in accordance with previous observations (202). ASCs derived from SAT had large nucleus and cytoplasm and formed a cell aggregation (fusiform shape), while ASCs derived from VAT had large nucleus and extended cytoplasm (classical fibroblast-like). Both ASCs derived from SAT and VAT expressed ESC (ABCG2, SOX-2, OCT4 and NANOG) and MSC markers (CD 73, CD 105 and CD90). Immunophenotyping of v-ASCs and s-ASCs demonstrated that more than 95% of cell populations expressed well defined MSC-associated surface markers CD73, CD90 and CD105 (203,204). However, s-ASCs expressed more strongly the NANOG marker than v-ASCs, suggesting that subcutaneous tissue may be a more suitable cell source for regenerative medicine, expressing more “stemness” than the visceral one, even though, this finding needs to be further elucidated.

In the current study, we also evaluated the behavior of the ASCs obtained from spheres. Multiple approaches employ sphere cluster technology to study stem cells (205). In contrast to conventional monolayer cell culture, in which cells grow only in two dimensions on a flat surface of a plastic dish, suspension cultures allow cell growth in all three dimensions. Stem

cells have been grown as spheres in many studies using different protocols (non-adherent plates, methylcellulose semi-solid media, hanging drops and round-bottom 96-well plates) (205,206). The most studied of these models are embryoid bodies, defined as spherical clusters of both pluripotent and committed stem cells that can organize in a developmental-specific manner and give rise to mature cells from any differentiation lineage. The primary goal of growing cells as spheres has been to reveal the basic principles of a normal tissue organization that gives cells the opportunity to arrange in a three dimensional context similar to tissues (207).

ASCs cultured in suspension maintained their proliferative and multipotent differentiation capacity *in vitro*, more than cells grown in adhesion. This lack of adhesion can represent a minor degree of differentiation of these cells, which would therefore upstream of differentiation cascade faced by stem cells during of their life cycle.

Indeed, the use of the culture medium for stem cells, which for its chemical composition is highly specific, precludes the possibility of differentiated cells to growth. In fact, such soil, determines a positive selection of stem cells, causing the death by apoptosis of each other type differentiated cells. Very important is the growth in the absence of fetal bovine serum. The serum contains factors not yet identified, capable of activating or inhibiting the proliferation and the cell differentiation (208). The growth in the non-adhesion, determined by the use of flasks ultralow attachment is a stem cell index, as only these cells are capable of proliferation in the microenvironment. On the contrary, the adhesion to the solid support by molecules of adhesion still not expressed by stem cells is essential for differentiated cell growth. The differentiated cells that do not adhere to the flask, in fact, undergo death for a particular type of apoptosis defined “anoikis” (209). The anoikis (from the Greek ν - "no", οἶκ "house") is a type of programmed cell death that prevents the growth and adhesion of mature cells in an ECM, different from their own (this mechanism instead is lost in cancer cells metastatic). This occurs when cells no longer adhere to their ECM due to the breaks between integrins and their ligands. Generally, these interactions allow cells to proliferate and differentiate, but the loss of these bonds leads differentiated cells to death. Non-differentiated stem cells grow in suspension regardless the interaction between integrins and their ligands and these cells do not have the anoikis (210).

Studies conducted on cancer stem cells from different tissues (thyroid, colon and breast) showed that they share the same biological behavior of ASCs such as, the growth induced by specific GF, the absence of serum and the growth in suspension (211-214).

Interestingly, spheres expressed more strongly all the ESC markers evaluated, SOX-2, OCT4 and NANOG, than SAT and VAT, demonstrating that ADSCs derived from spheres are the cells that express mostly the stemness.

The current study was also aimed to the evaluation of NANOG role in the pluripotency network. The stem cell pluripotency and differentiation are strictly controlled by a coordinated network of transcription factors (215). Among them, OCT4 and NANOG have been long recognised as crucial transcriptional regulators of stem cell self-renewal during embryogenesis (192,216). More recently, it has been shown that both OCT4 and NANOG are also produced by adult undifferentiated MSCs and can be recovered from bone marrow, human cord blood and umbilical cord matrix (182,217-221).

Recent genome-wide studies have identified the downstream targets of OCT4, which include genes encoding for self-renewal factors, lineage-specific factors, signaling molecules and DNA damage response sensors (192). Thus, OCT4 seems to be implicated in a broad spectrum of cellular processes that collectively specify the self-renewal state of the ESCs. On the other side, the role of SOX2 seems to be crucial too. Indeed, the lack of SOX2 leads the ESCs to a differentiation failure and to the loss of pluripotency property (178).

NANOG is accordingly considered a core element of the pluripotent transcriptional network and it is required for germline development. Transient downregulation of NANOG appears to predispose cells towards differentiation but does not mark commitment.

Therefore, unlike OCT4 and SOX2, NANOG plays a pivotal role in the maintenance of the epiblast and ES cells by repressing differentiation along the primitive endoderm lineage. SOX2 is capable of heterodimerizing with OCT4 to mediate the transcription activities of several ES cell specific genes including NANOG (220). Interestingly, OCT4 and SOX2 are also involved in reciprocal regulation of each other's expression (223).

OCT4 overexpression has been shown to induce de-differentiation of ASCs into a more immature status by activating the AKT/phosphoinositide 3-kinase (PI3K) and extracellular signal-related kinase (ERK1/2) signalling pathways (224). As in developmental processes, in adult tissues several factors are engaged to modulate and maintain the balance between self-renewal and differentiation (221). Indeed, it has been reported that ASCs possess their own multipotency to reprogramme into more primitive stem cells (225). However, how this process takes place mechanistically remains controversial.

Recently, Suzuki et al showed that NANOG expression was upregulated by BrachyuryT and STAT3 in mouse ESCs (174). In human ESCs, Vallier et al reported that Activin/Nodal signaling stimulated expression of NANOG, which in turn prevents FGF-induced

neuroectoderm differentiation (175). In addition, several studies indicated that the OCT4/SOX2 complex was directly bound to the NANOG promoter to regulate target gene expression (176). Genomic studies have revealed that OCT4, SOX2, and NANOG frequently bind the same regulatory regions in undifferentiated mouse and human ESCs, and that these binding sites are often in close proximity to one another (176). These studies suggest that OCT4, SOX2, and NANOG can physically interact with each other and coordinately regulate target genes in some cases. Additionally, Goke and colleagues reported that combinatorial binding sites of the OCT4/SOX2/NANOG were more conserved between mouse and human ESCs than individual binding sites were (177-178).

In the current study, the NANOG and SOX-2 silencing both in ASCs derived from VAT and SAT was performed. A significant drop of OCT4 and SOX-2 expression both in SAT and in VAT was observed after NANOG siRNA silencing after 48 and 72 hr. On the other side, when the SOX-2 silencing was performed, no decrease of NANOG and OCT4 proteins expression was found both in SAT and in VAT.

Thus, it appears that SOX2 may not be necessary for the process of reprogramming, even though the activity of SOX2 is known to be critical for pluripotency and proper differentiation of the reprogrammed iPSc.

Taken together, the data of the current study tend to suggest that SOX-2 has not a significant role in determining the NANOG expression, while NANOG appears to play a key role in the process of stem cells self-renewal. Indeed, NANOG appears to have a hierarchical role in the regulation of OCT4 and SOX2 activation, regulating their expression, while SOX2 appears to play a downstream role in the pluripotency network. However, this study has a limit, because the OCT4 silencing was not performed.

In conclusion, the current study shows that SAT and VAT contain a high amount of ASCs. ASCs derived from VAT and SAT appear to be similar regarding the expression of ESC and MSC markers, even though ASCs from SAT express more than ASCs from VAT the NANOG gene. ASCs derived from SAT and VAT are able to cluster in spheres, when grown in suspension, which appear to maintain the stemness ability for several passages and which show, more than the ASCs cultered in adhesion, the ESC and MSC markers. In addition, NANOG may be the primum movens in the activation of the circuitry of self-renewal and may have a hierarchical role in the pluripotency network. However, further studies need to be conducted to confirm our results and to study the effects of OCT4 silencing in NANOG and SOX2 genes expression.

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The thesis is dedicated to Clelia.

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