NANOG Plays a Hierarchical Role in the Transcription Network Regulating the Pluripotency and Plasticity of Adipose Tissue-Derived Stem Cells (ASCs)

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Abstract: The stromal vascular cell fraction (SVF) of visceral and subcutaneous adipose tissue (VAT and SAT) has increasingly come into focus in stem cell research, since these compartments represent a rich source of multipotent adipose-derived stem cells (ASCs). ASCs exhibit a self-renewal potential and differentiation capacity. Our aim was to study the different expression of embryonic stem cell markers NANOG, SOX2 and OCT3/4 and to evaluate if there exists a hierarchal role in this network in ASCs derived from both SAT and VAT. ASCs were isolated from SAT and VAT biopsies of 72 consenting patients (23 men, 47 women; age 45 ± 10; BMI between 25 and 30 range) undergoing elective open-abdominal surgery. Sphere-forming capability was evaluated by plating cells in low adhesion plastic. Stem cell markers CD90 and CD105 were analyzed by flow cytometry and stem cell transcription factors NANOG, SOX2 and OCT3/4 were detected by immunoblotting and Real-Time PCR. NANOG, SOX2 and OCT3/4 interplay was explored by gene silencing. ASCs from VAT and SAT confirmed their mesenchymal stem cell (MSC) phenotype expressing the specific MSC markers CD90 and CD105 and NANOG, SOX2 and OCT3/4. NANOG silencing induced a significant OCT 3/4 (70% ± 0.05) and SOX2 (75% ± 0.03) down-regulation whereas SOX2 silencing did not affect NANOG gene expression. Adipose tissue is an important source of MSC, and siRNA experiments endorse a hierarchical role of NANOG in the complex transcription network that regulates pluripotency and plasticity.

Keywords: adipose derived stem cell (ASC); regenerative medicine; embryonic stem cell marker network

1. Introduction

Both visceral (VAT) and subcutaneous adipose tissues (SAT) represent an alternative source of mesenchymal stem cells (MSCs). Adipose-derived stem cells (ASCs) have been proposed in accordance with the standard nomenclature of the International Fat Applied Technology Society as a plastic-adherent, proliferative, multipotent cell population isolated from adipose tissue [1,2]. ASCs are fibroblastic in morphology and possess the properties of MSCs traditionally isolated from bone marrow [3,4]. Furthermore, ASCs have a remarkable potential to differentiate *in vitro* towards

the osteogenic, adipogenic, myogenic and chondrogenic lineages when treated with established lineage-specific factors [5]. Nowadays there is great interest in understanding more in detail the cellular and molecular mechanisms of ASCs, which are known to modulate self-renewal and differentiation properties. In fact, the latter characteristics have made it possible to consider them as the preferable candidate for the employment in regenerative medicine. The role of the pluripotency factors in the regulation of self-renewal and differentiation is well known in embryonic stem cells [6,7] but their expression and role in MSCs are not well identified and are still controversial [8]. A complex transcription network, the core of which includes the stem cell-specific factors Nanog, Oct4, and Sox2, governs pluripotency [9, 10]. Nanog occupies a central position in this network, but the mechanisms regulating its expression are unclear. Hyslop and colleagues suggested that Nanog acts as a gatekeeper of pluripotency in human embryonic development (11). In this case, down-regulation of Nanog in human ESCs induces up-regulation of endoderm- and trophectoderm-associated genes [11,12]. Our aim was to study the different expression of the embryonic stem cell markers NANOG, SOX2 and OCT3/4 in ASCs derived from SAT and VAT and to explore whether NANOG possesses a more prominent role in this network.

2. Results

2.1 *Isolation of Adipose derived stem cells (ASCs)*

After enzymatic digestion of biopsied human adipose tissue from 72 consenting patients (23 men, 47 women; age 45 ± 10; BMI between 25 and 30) undergoing elective open-abdominal surgery, fresh-isolated stromal vascular cell fraction (SVF) was a compounded cell population with a spindle, triangular, polygonal or round shape, which contains fibroblasts and adipocytes at different differentiation stages. These cells adhered to a flask without a substrate. After 48 hours, the cells were small and not extensional (Fig. 1. A) when observed under a light microscope. After 5 days (fig.1.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. 1.C) colonies of fibroblastic-like cells were observed, although fibroblasts mixed in ASCs were still found. Both these cell types reached confluence after about 15 days. Crystal violet staining revealed that MSCs derived from visceral adipose tissue (V-ASCs) have large nuclei and extended cytoplasms (fig. 1D) while those derived from subcutaneous adipose tissue (S-ASCs) have large nuclei and large cytoplasms (fig.1E). Both S-ASCs and V-ASCs formed cell aggregation at the 3rd passage (Fig. 1F and 1G) and in low-adhesion culture conditions formed spheres, suggesting the stem origin of the cells (Fig.1H and 1I).

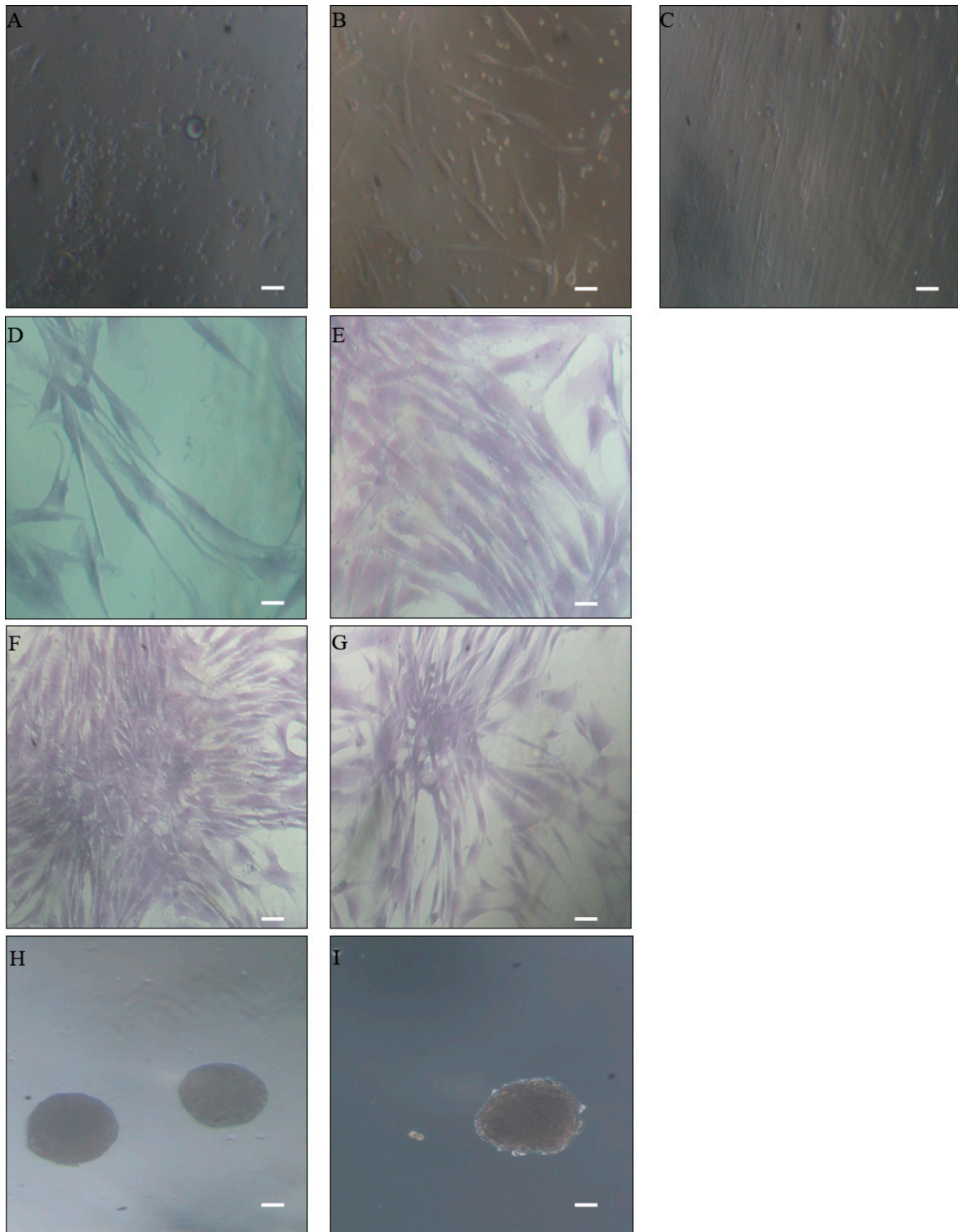



Figure 1: Morphology of adipose-derived stem cells (ADSCs) under light microscopy (10x) with phase contrast with a Nikon DS-FI1 CCD camera. SVF morphological characteristics on day 2 (A), day 5 (B) and day 10 (C) of expansion cultures. Crystal violet staining shows visceral adipose tissue mesenchymal stem cells (V-ASCs) having large nuclei and extended cytoplasms (D) in comparison to subcutaneous adipose tissue mesenchymal stem cells (S-ASCs), which have large nuclei and large cytoplasms (E). F and G show similar aggregation of

MSCs from V-ASC and S-ASC in adhesion cultures. H and I show spheres from V-ASC and S-ASC in low adhesion cultures. An experiment representative of 72 samples studied is shown.

2.2 *Cell cycle analysis*

Cell cycle analysis at passage 3 showed no significant different distribution of cells in the G1, G2 and S phases in S-ASC and V-ASC. S-ASC in the G1 phase was 70.68 ± 2.32%, and V-ASC was 56.01 ± 3.43%.The proliferation index (PI), expressed as % G2 + % M, was 33.3 ± 5.7% and 25.46% ± 6.14% respectively for S-ASC and V-ASC (Fig.2 A and B).

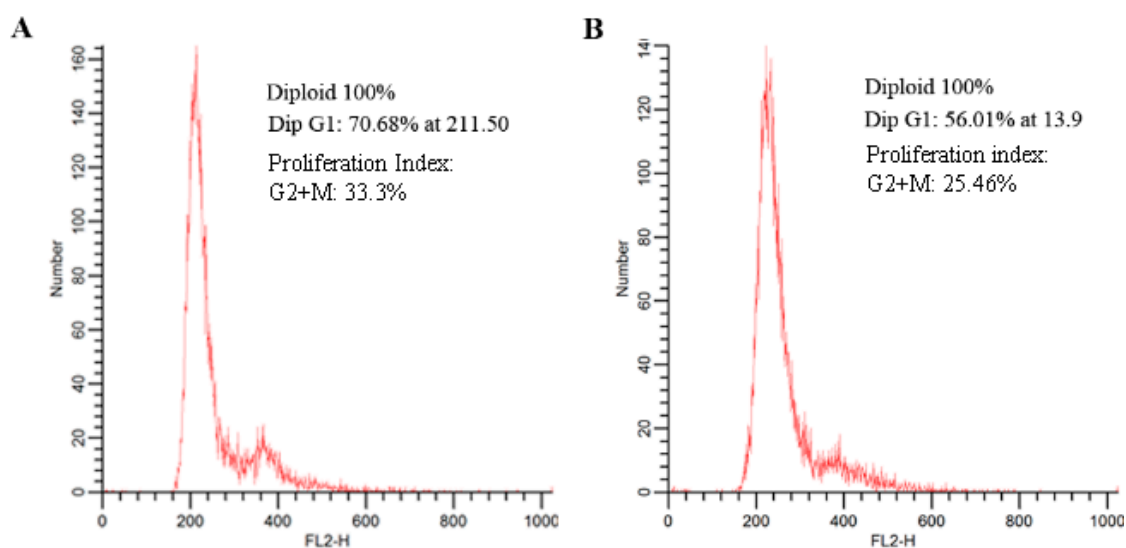



Figure 2: **A**. Cell cycle distribution of S-ASC. **B.** Cell cycle distribution of V-ASC. Both cell samples were analyzed according to Nicoletti's protocol.

2.3 *Flow cytometry stem cell phenotype characterization*

The cell-surface antigenic characteristics of S-ASCs and V-ASCs were analyzed by flow cytometry. At passage 3 both populations showed almost no expression of CD31, CD45 and CD146 (<1%) (Fig.3A) and the 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. 3 B).

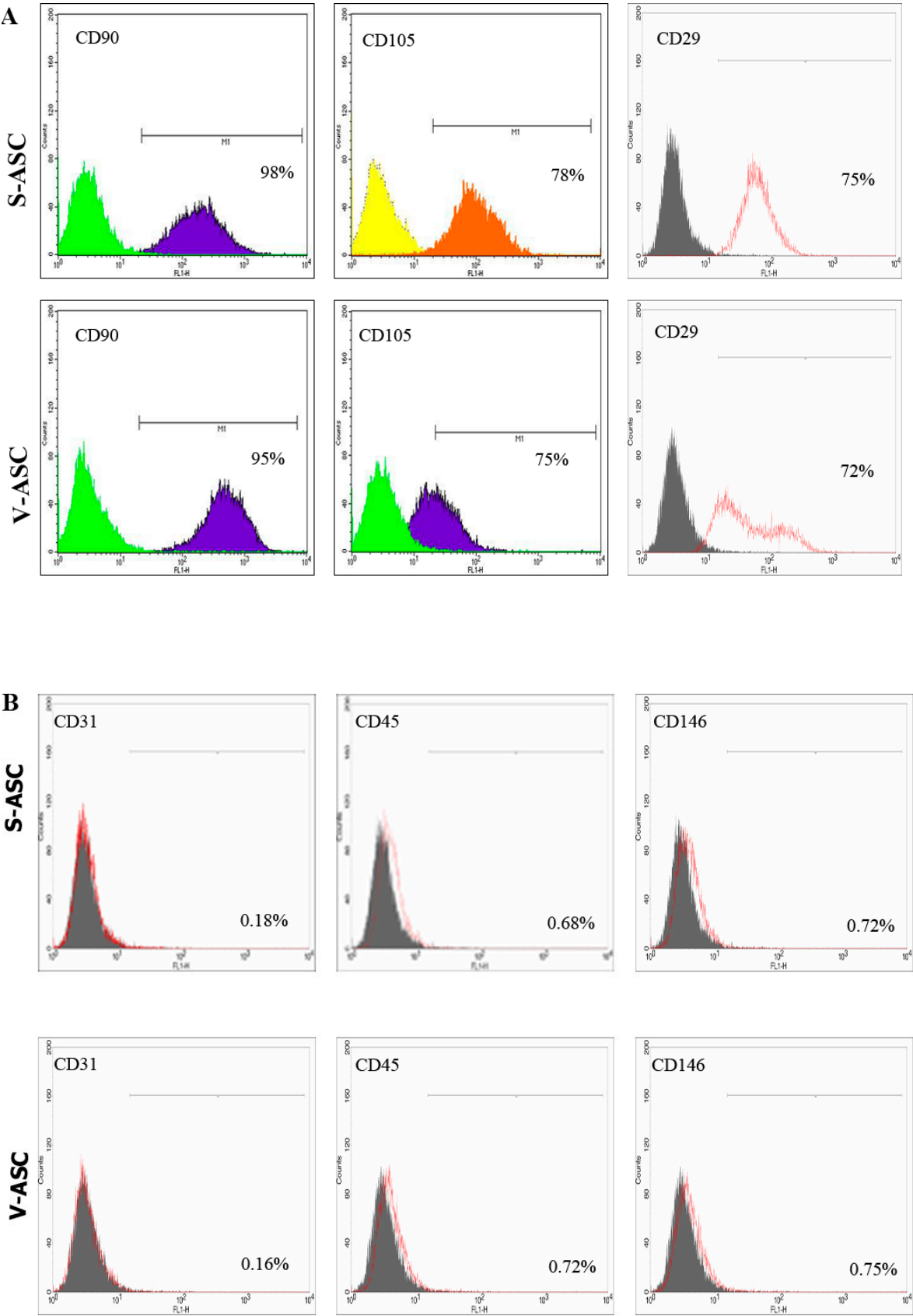



Figure 3 A: Cytofluorimetric assay in S-ASC and V-ASC for CD90 (95 ± 3.6% and 98 ± 3.9% respectively), CD105 (C and D) (75 ± 2.7% and 78 ± 4.2% respectively) and CD29 (89% ± 2.6% and 88% ± 3.2% respectively). B: The cells are negative for CD31, CD45 and CD146. All fields are representative of one S-ASC and V-ASC sample out of at least 12 independent experiments.

2.4 *"Spheres" from S-ASC, S-ASC and V-ASC characterization*

Pluripotency-associated genes play important roles in maintenance of self-renewal and multi-differentiation potential of ESCs and the level of these factors determines a pluripotent state. To find out the pluripotent state of ASCs from visceral and subcutaneous adipose tissue we detected some of the embryonic stem cell 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 4A). 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 highly

expressed when compared with both adherent S-ASC and V-ASC cells (p<0.01) (Fig

4B).

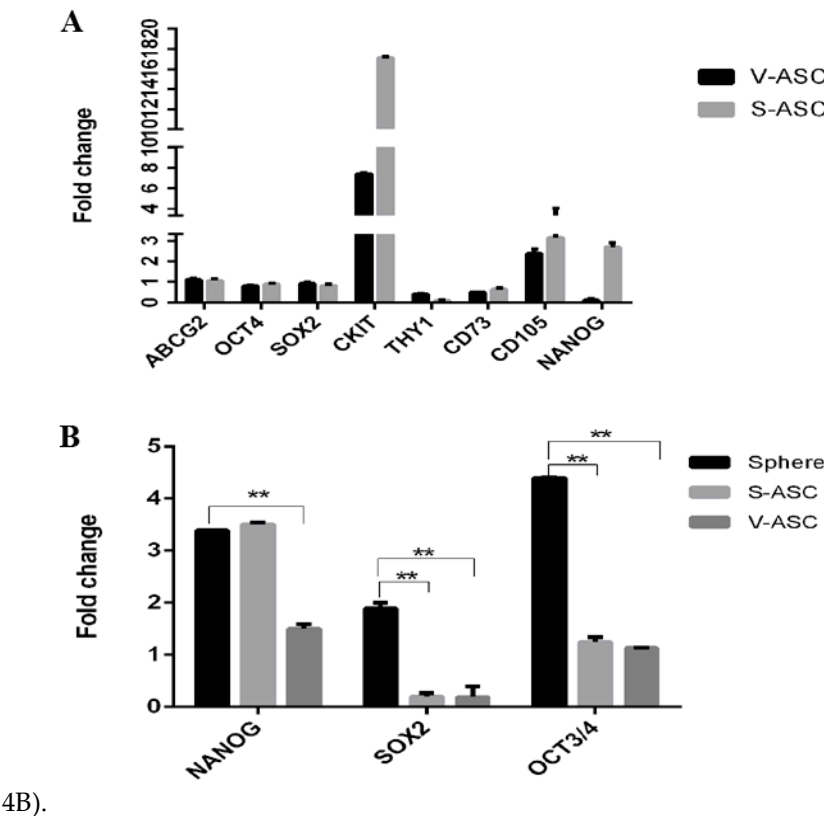



Figure 4 A. qRT-PCR analyses in S-ASC and V-ASC cells. Data are representative of three independent experiments. Relative expression levels for ABCG2, OCT4, SOX2, CKIT, THY1, CD73, CD105, and NANOG were assessed using the $2^{-\Delta\Delta Ct}$ method. Values are shown as mean ± SE, *p<0.05. The data shown are relative to an endogenous control (beta-Actin), with fold change compared to expression levels in commercial bone marrow–mesenchymal stem cells (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 more highly expressed in ASCs isolated from SAT than from VAT (p<0.01) (Fig 5A and B).

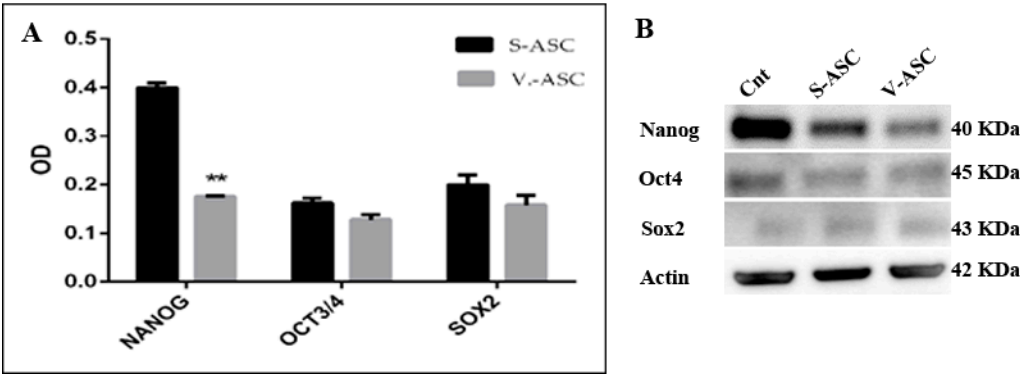
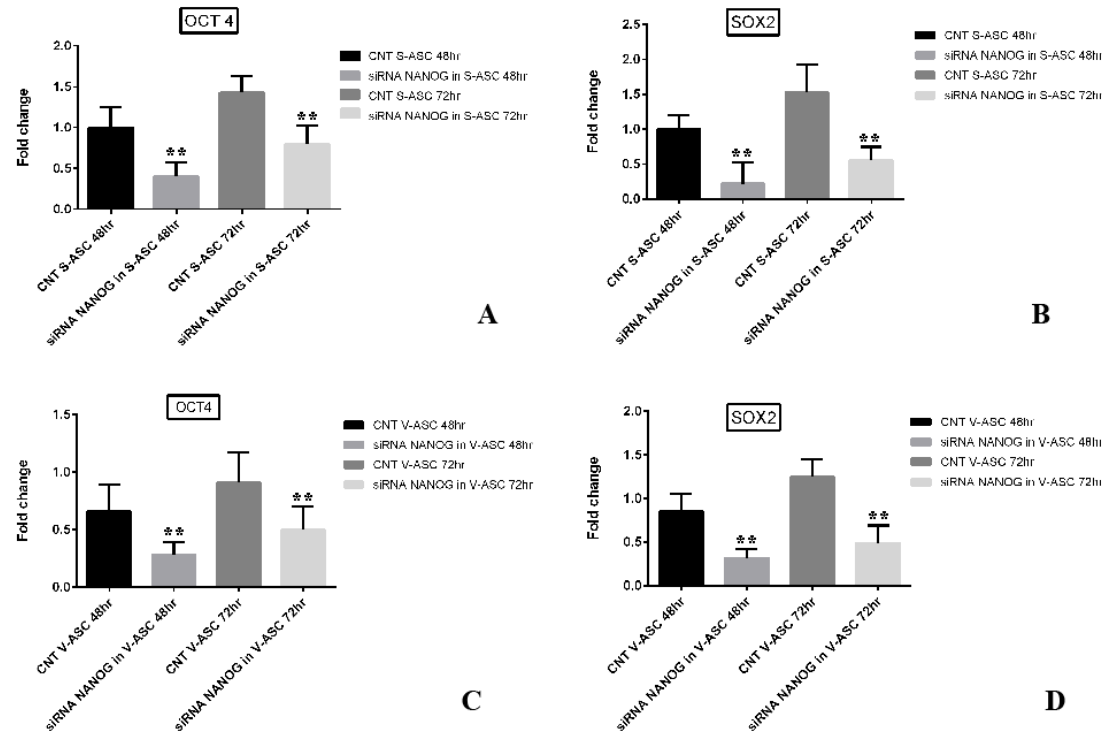



Figure 5 A and B. Western blot analysis in the S-ASC and V-ASC cells of NANOG, OCT3/4 and SOX2. The data are representative of three independent experiments. The values are shown as mean ± SE, **p<0.01. OD, optical density.

2.5 NANOG and SOX2 silencing in S-ASC and V-ASC

To establish the relationship between SOX2, OCT-4, NANOG, we evaluated their expression after NANOG silencing. NANOG silencing caused down-regulation of OCT3/4 (70% ± 0.05, p<0.01) and SOX2 genes (75 ± 0.03%, p<0.05) in S-ASC (Figure 6 A and B) and in V-ASC (figure 6 C and D).

Figure

6: Analysis of NANOG silencing was assessed using the $2^{-\Delta\Delta Ct}$ method. qRT- PCR analysis in S-ASC (A and B)

and V-ASC (C and D) of OCT4 and Sox2 gene expression after Nanog silencing with stealth siRNA (siNANOG) *vs.* siCONTROL treated cells (CNT). The data are representative of three independent experiments. The values are shown as mean ± SE, **p<0.01.

By contrast, SOX2 silencing after 48 hours showed no effect on NANOG expression (Figure 7 A and B)

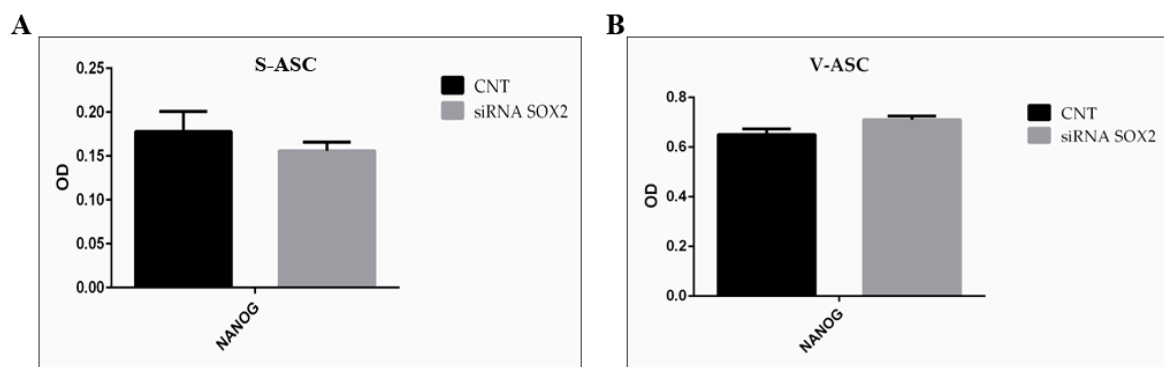



Figure 7 A: Analysis of Sox2 silencing was assessed using the $2^{-\Delta\Delta Ct}$ method. qRT-PCR analysis in S-ASC (A) and V-ASC (B) of Nanog gene expression after Sox2 silencing with stealth siRNA (siRNASox2) *vs.* siCONTROL treated cells (CNT). The data are representative of three independent experiments.

Western blot analysis of SOX2 and OCT3/4 after NANOG silencing with stealth siRNA after 72 hr in S-ASC (optical density (OD)= 0.8 ± 0.1 and 0.24 ± 0.01, p< 0.01) and in V-ASC (OD= 0.5 ± 0.01 *and* 0.22 ± 0.01) caused down-regulation of the OCT4 gene in S-ASC (OD= 1.16 ± 0.17 *vs.* 0.38 ± 0.12; p<0.01) and in V-ASC (OD=1.09 ± 0.16 *vs.* 0.39 ± 0.12, p< 0.01). At the same time down-regulation of the SOX2 protein was observed in S-ASC (OD: 0.49 ± 0.05 *vs.* 0.20 ± 0.02; p<0.01) and in V-ASC (OD 0.5 ± 0.01 *vs.* 0.01 ± 0.001; p<0.01) (Figure 8).

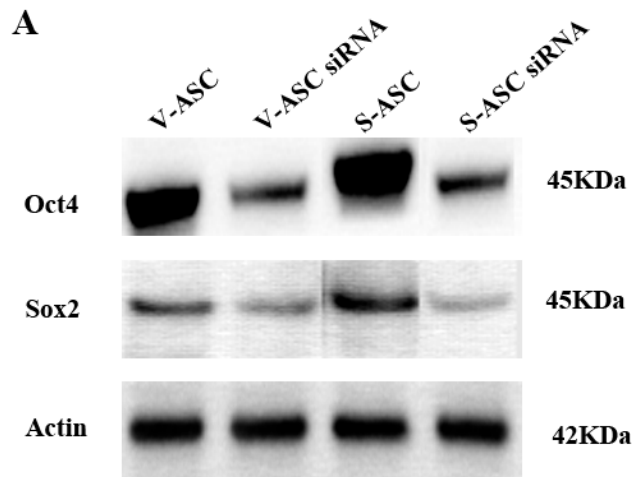



Figure 8: Western blot of Oct4and Sox2 with proteins extracted from V-ASC and S-ASC cells after NANOG

silencing with stealth siRNA vs. siCONTROL treated cells.

By contrast, SOX2 silencing in S-ASC (OD= 1.3 ± 0.1 *vs*. 0.23 ± 0.06, p<0.01) and in V-ASC cells (OD=

1.16 ± 0.013 *vs*. 0.56 ± 0.04; p < 0.01) was not able to affect NANOG protein levels in S-ASC (OD=

1.17 ± 0.1 *vs*. 1.2 ± 0.01, p NS) and in V-ASC (OD= 0.7 ± 0.2 *vs*. 0.68 ± 0.1; p NS) (Figure 9)

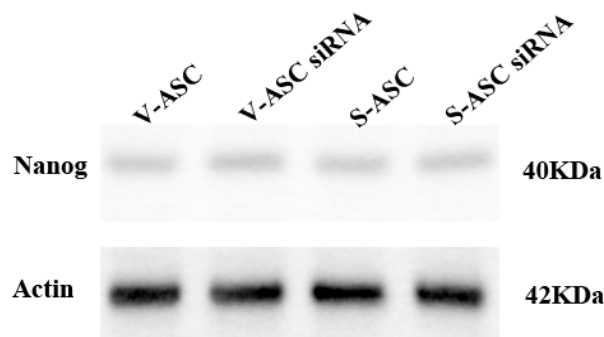



Figure 9: Analysis of Sox2 silencing. Western blot of NANOG with proteins extracted from S-ASC and V-ASC

cells after SOX2 silencing with stealth siRNA *vs*. siCONTROL (CNT) treated cells. The results were normalized

with beta actin. The data are representative of three independent experiments.

3. Discussion

Adipose tissue represents an interesting source of multipotent stem cells, [13,14] and is considered

fundamental for comprehension of adipose tissue biology under normal physiology. In addition,

interest in disease state conditions is also developing, even though limited information is available

on visceral and subcutaneous ASCs (Adipose derived stem cells) and the relationship between *in vitro* and i*n vivo* adipogenesis.

The purpose of this work was to study the different expression of the embryonic stem cell markers NANOG, SOX2 and OCT3/4, and the role of Nanog in this network, evaluated in both S-ASCs and V-ASCs isolated from SAT and VAT in a large series of obese and non-obese subjects, who underwent elective open-abdominal and laparoscopy surgery. When isolated MSC were cultured without any substrate [15], we observed morphological differences in V-ASC and S-ASC cell lineages which included cell shape and cell size in accordance with previous observations [16]. ASCs derived from subcutaneous adipose tissue were characterized by large nuclei and cytoplasms and formed cell aggregation (fusiform shape), whilst ASCs derived from visceral adipose tissue showed a large nuclei and extended cytoplasms (classical fibroblast-like). Our data confirm that cells are capable of forming sphere clusters in serum-free medium supplemented with b-FGF and EGF [17] and these spheres expressed all the ESC markers evaluated, SOX-2, OCT4 and NANOG, more markedly than SAT and VAT, demonstrating that the spheres mostly express stemness. Interestingly, during the time of floating culture, the spheres maintained expression of stem cell markers. The finding that floating cell cultures maintain and even increase the stemness potential of stem/progenitor-derived cells, probably inhibiting differentiation patterns, represents another significant observation of this study. Consequently, the proposed technique can be used to maintain stem characteristics for a longer time than traditional adhesion cultures. Immunophenotyping of MSCs derived from subcutaneous and visceral adipose tissue demonstrated that cell populations expressed well defined MSC- associated surface markers CD29, CD73, CD90 and CD105 [18,19]. Western blot analysis confirmed the presence of stem cell markers; both ASCs were positive for SOX2, OCT3/4 and NANOG, which are the principal transcription factors that regulate pluripotency and plasticity. Our results indicate that the stemness of ASCs should be defined by their ability to differentiate into multiple lineages coupled with their expression of the pluripotent stem cell-related genes Oct-4, SOX2, and NANOG in order to functionally distinguish ASCs as more stem cell-like [20]. We found that the embryonic stem cell marker NANOG is over-expressed in MSCs derived from adipose tissue, confirming recent studies that have demonstrated a central role of NANOG in embryonic stem cells [21].

Stem cell pluripotency and differentiation are strictly controlled by a coordinated network of transcription factors [22]. Among them, OCT4 and NANOG have long been recognized as crucial transcriptional regulators of stem cell self-renewal during embryogenesis [23, 24]. More recently, it has been shown that both OCT4 and NANOG are also expressed by adult undifferentiated MSCs [25-30]. Nanog is a homeobox-containing transcription factor with an essential function in maintaining the pluripotent cells of the inner cell mass and in the derivation of embryonic stem

cells (ESCs) from these [31]. Furthermore, over-expression of Nanog is capable of maintaining the pluripotency and self-renewing characteristics of ESCs under what normally would be differentiation-inducing culture conditions [32]. Recent genome-wide studies have identified the down-stream targets of OCT4, which include genes encoding for self-renewal factors, lineage-specific factors, signaling molecules and DNA damage response sensors [23]. 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 differentiation and to the loss of pluripotency property [33]. NANOG is accordingly considered a core element of the pluripotent transcriptional network and is required for germline development. Transient down-regulation of NANOG appears to predispose cells towards differentiation but does not mark commitment. Therefore, unlike OCT4 and SOX2, NANOG plays a pivotal role in maintenance of the epiblast and ES cells by repressing differentiation along the primitive endoderm lineage. SOX2 is capable of hetero-dimerizing with OCT4 to mediate the transcription activities of several ES cell specific genes including NANOG [29]. Interestingly, OCT4 and SOX2 are also involved in reciprocal regulation of each other's expression [34]. OCT4 over-expression 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) signaling pathways [35]. As in developmental processes, in adult tissues several factors are engaged in modulating and maintaining the balance between self-renewal and differentiation [18]. Indeed, it has been reported that ASCs possess their own multipotency to reprogram into more primitive stem cells [36]. However, how this process takes place mechanistically remains controversial. Recently, Suzuki et al showed that NANOG expression was unregulated by Brachyury T and STAT3 in mouse ESCs [37]. In human ESCs, Vallier et al. reported that Activin/Nodal signaling stimulated expression of NANOG, which in turn prevents FGF-induced neuroectoderm differentiation [38]. In addition, several studies have indicated that the OCT4/SOX2 complex was directly bound to the NANOG promoter to regulate target gene expression [39]. 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 [39]. 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 [40, 41].

In our experiment we observed that down-regulation of NANOG led to significant down-regulation of *OCT*4 and Sox2 probably consistent with loss of pluripotency. Indeed, in previous

studies down-regulation of NANOG led to significant down-regulation of *OCT4* and loss of ES/EC cell-surface antigens; an up-regulation of several marker genes, including *GATA4*, *GATA6*, *LAMININ B1*, and *AFP* [33,42-44] was reported., Moreover, the trophectoderm specification   is indicated by up-regulation of *CDX2, GATA2*, *hCG-alpha*, and *hCG-beta* [21, 31, 45-48] All these data confirm the use of adipose tissue as a potential source for multipotent cells and above all propose a suitable approach for future regenerative medicine and tissue engineering applications, and constitute a valuable resource in biotechnology. Our data clearly show that it is possible to evaluate the distribution of various adipocyte subpopulations when several flow cytometric parameters and gene expression studies are combined. However, despite our experimental evidence on the fundamental role of Nanog, fully convincing data for ASCs are still missing and in future studies other methods are necessary to investigate NANOG down-regulation in S-ASC and in V-ASC to establish the role of the long-term differentiation of specialized cells for regenerative medicine *in vitro* and *in vivo*.

4. Materials and Methods

Establishment of Adipose derived stem cell cultures

Subcutaneous (SAT) and Visceral (VAT) Adipose Tissue Biopsies were obtained from 42 obese and 28 non-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. On the day of surgery, after an overnight fast, adipose tissue specimens were obtained from the subcutaneous and omental depots. Approximately 1 g of adipose tissue was taken from each fat depot. All biopsies were handled under sterile conditions and immediately used for subsequent preadipocyte isolation. Tissue specimens were immediately transported to the clean room of the laboratory of Regenerative Medicine (ISO 14644 Ec-GMP) in DMEM/Ham's F12 1:1, dissected from fibrous material and visible blood vessels, cut into little fragments and incubated in PBSC2++ /Mg supplemented with 1mg/ml collagenase type I (Sigma Chemical, St Louis, MO, USA), with vigorous shaking (100 cycles/min) for 1 h at 37ºC. The resulting material was filtered through a 250 mm mesh, and adipocytes and free oil were separated from stromal vascular (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, 5% FCS, 1 ng/ml FGF-b, and 10 ng/ml EGF DMEM/Ham's F12, 10% FCS and antibiotics.

Sphere cultures

SVF cells were seeded at $1*10^2$ cell/cm^2 in ultra-low adherent flasks (Corning, Avon, France) in defined culture medium which consisted in DMEM/F12 supplemented with L-glutamine (2 mM), nonessential amino acids (1×), B27 (1×) (Invitrogen), human b-FGF (20 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. Sphere formation was assessed by counting the number of spheres (cells > 3) under an optical microscope. To test if sphere-containing cells can revert to monolayer growth, spheres were dissociated with Accutase (Sigma) and plated in flasks treated for cell culture (TPP) in ASC expansion medium.

Evaluation of morphological characteristics

Cells were fixed with for 15 min at RT in 2% (wt/vol) paraformaldehyde, washed twice in distilled water and stained with crystal violet. Then cells were observed for their morphological features under a Zeiss phase contrast microscope and photographed with a Nikon camera.

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 $5x10^5$ cells was used for each flow cytometric test.

<u>*Immunophenotyping:*</u>

Human anti-CD31, human anti-CD45, human anti CD146, human anti CD29, human anti-CD90 and human anti-CD105 (see table 1) monoclonal antibodies were tested on S-ASC and V-ASC. The incubation conditions were in accordance with the manufacturer's instructions. For anti-CD90 and anti-CD105 cells were washed twice with PBS/BSA 5% and incubated with Alexa Fluor 488 goat

anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) antibody for 1 h in the dark. Data were acquired

on a FACS Calibur and analyzed using CELL Quest Pro software (Becton Dickinson).

Table 1. Monoclonal antibodies used for the characterization of cell phenotypes

Primary antibody/localization marker	Code Number	Diluition	Incubation
CD31, surface	Miltenyi Biotec, 130-092-654	1:100	30 minutes, r.t.
CD45, surface	Miltenyi Biotec, 130-080-202	1:100	30 minutes, r.t.
CD146, surface	Miltenyi Biotec, 130-092-851	1:100	30 minutes, r.t.
CD90, surface	Chemicon, CBL415	1:50	o/n, r. t.
CD105, surface	Biolegend,323202	1:50	o/n, r. t.
CD29, surface	Miltenyi Biotec, 130-101-256	1:100	30 minutes, r.t.
Secondary antibody	Code Number	Diluition	Incubation
AlexaFluor 488	Life Technologies, Z25402	1:50	20 minutes, r.t.

Miltenyi Biotec, Bergisch Gladbach, Germany;Santa Cruz, Dallas, Texas

BioLegend, London, UK

Life Technologies, Carlsbad, CA, USA

o/n overnight, *r.t.* room temperature

Analysis of Cell Cycle Status of MSCs

Single-cell suspensions of S-ASC and V-ASC were obtained and seeded at a density of $2*10^3$ cells/

cm^2 (passage 3) and the DNA content was assessed, 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 NaHPO$_4$, 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 phase cells were calculated with the MODFIT-LT software program (Verity Software House, Inc.).

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

mRNA from ASC populations isolated from VAT and SAT biopsies derived from obese and normal weight patients was isolated using an RNeasy kit (Qiagen, Hamburg, Germany). 250 ng of RNA from S-ASC and V-ASC were reverse-transcribed with standard reagents (Promega). One microliter of each reverse-transcription reaction was amplified 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 (Table 2)

Table 2. Real-time quantitative PCR primers used for gene expression investigation

RNA	PRIMER SEQUENCE	CODE NUMBER
ABCG2		QT00073206
NANOG		QT01844808
OCT3/4		QT00210840
SOX2	FOR: 5'-GGAGACGGAGCTGAAGCCGC-3' REV: 5'-GACGCGGTCCGGGCTGTTTT-3'	

THY1		QT00023569
CD105		QT00013335
CD73		QT00027279
β-ACTIN	FOR:-5'-GGACTT CGA GCA AGA GAT GG-3' REV: 5'-AGC ACT GTG TTG GCG TAC AG-3'	

siRNA transfection

siRNAs transfection in ASC cells was performed using INTERFERin TM 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). siCONTROL Stealth siRNA Negative Control was used as a control (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 by BioRad, electrotransferred to nitrocellulose

membrane, and blotted with the following primary antibodies: rabbit antihuman SOX2 (Poly6308, BioLegend, San Diego, CA), mouse antihuman Oct-4 (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) (see table 3). 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).**Table 3.** Antibodies used for characterization for stem cell markers

Primary antibody/localization marker	Code Number	Dilution	Incubation
Nanog, nuclear and cytoplasmatic	sc-30331, Santa Cruz Biotechnology	1:500	o/n, 4°C
Oct3/4, nuclear	sc-5279 , Santa Cruz Biotechnology	1:500	o/n, 4°C
Sox2 nuclear	Poly6308, BioLegend, San Diego, CA	1:500	o/n, 4°C
Secondary antibody	Code Number	Diluition	Incubation
Goat anti-rabbit IgG-HRP	sc-2030, Santa Cruz Biotechnology	1:2500	90 minutes, r.t.
Goat anti-mouse IgG-HRP	sc-2031, Santa Cruz Biotechnology	1:2500	90 minutes, r.t.

Donkey anti-goat IgG-HRP	sc-2033, Santa Cruz Biotechnology	1:2500	90 minutes, r.t.

Santa Cruz, Dallas, Texas

o/n overnight, *r.t.* room temperature

5. Conclusions

The use of adipose tissue as a potential source for multipotent cells above all proposes a suitable approach for future regenerative medicine and tissue engineering applications, and constitutes a valuable resource in biotechnology. Our experiments evidence the fundamental role of Nanog, but convincing data for ASCs are still missing and in future other methods are necessary to investigate NANOG downregulation in S-ASC and in V-ASC to establish the role of long-term differentiation of specialized cells for regenerative medicine *in vitro* and *in vivo*.

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Conflicts of Interest: The authors declare no conflict of interest

Abbreviations

S-ASC   Subcutaneous- Adipose Stem Cell

V-ASC   Visceral-Adipose Stem Cell

SVF   Stromal vascular fraction

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