1. Activation of adipose tissue and related mesenchymal stem cells: promotion of anti-inflammatory properties through SOX2 transcription factor and HuR RNA-binding protein modulation

Regenerative medicine is an emerging field that combines the knowledge of many disciplines with the aim of curing impaired function in the body. Commonly, it is described as the creation of tissues that can repair, replace, or restore biological structures and/or functions that may be lacking due to congenital defects, aging, disease, or destruction (Haseltine 2003; Gutmann et al. 2005, Greenwood et al., 2006). Mesenchymal stem cells (MSCs) are multi-potent, self-renewing cells with the capacity to differentiate, in vitro, into cells of mesenchymal origin, including osteoblasts, adipocytes, and chondrocytes, and give rise to bone, fat, cartilage, and muscle tissues in vivo (Pittenger et al., 1999). Recent studies have shown that the potentiality of tissue regeneration can be enhanced using adipose derived stem cells (hADSCs) (see Gimble et al., 2011). Stem cell frequency appears to be significantly higher in adipose tissue than in other tissues such as bone marrow (Bieback et al., 2004). Indeed, autologous transplant of adipose tissue is an established therapeutic procedure used for the repair of a variety of tissue damage.

To minimize discrepancies and inconsistencies, and allow comparison of data generated in different laboratories, the International Society of Cellular Therapy (ISCT) has provided guidelines for the definition of MSCs based on (a) their plastic adhesion properties, (b) immunophenotype, and (c) multi-potent differentiation potential (Bourin et al., 2013; Dominici et al., 2006). Furthermore, several recently published reports claim that hADSCs are capable under appropriate stimulation of differentiating into other different cell types such as neurons, cardiomyocytes, hepatocytes and pancreatic cells (Schäffler et al., 2007; Strem et al., 2005) and possess clinical potential for vasculogenesis (Madonna et al., 2009), osteogenesis (Shenaq et al., 2010) and models of neuronal repair (Wakabayashi et al., 2010).

We have recently reported that mechanical manipulation of human adipose tissue without modification of tissue composition, structure and histology, that was totally comparable to that of the original source (see Experimental procedure for details), increased significantly the number of stromal cells bearing the expression of beta-tubulin III. We have suspected that mechanical forces may be of key relevance in adult tissue plasticity as it occurs during embryogenesis, tissue remodeling and mitotic and motile behavior of cells (Vogel et al, 2006). Cells sense and respond to external applied forces, and to forces exerted upon the cell matrix and cell–cell contacts. Stretch-activated ion channels, growth factors, cytoskeleton filaments, extracellular matrices, focal adhesions, and tyrosine kinases appear involved in the conversion of such mechanical stimuli into biochemical signals within the cells (mechano-transduction) leading to downstream events and, then, driving key fate messages to the nucleus (El Haj et al, 1999, Na et al, 2008; Walker et al,

2000, Venkatesan Iyer et al. 2012, Chen et al 2013). Also other authors have postulated the ability of mechanical forces in enhancing tissue engineering applications and regenerative medicine strategies (Wang et al 2009; El Haj et al, 1999).

Our discoveries here reported indicate a clear method of tissue activation that stimulates anti-inflammatory properties in human fat recovered by liposuction with an accompanying reduction in the production of known inflammatory cytokines such as TNFalfa. Fat tissue accumulation is, as a matter of fact, considered an inflammatory process. Such induced anti-inflammatory properties can be activated in a force and time dependent manner by applying a specific movement with an apparatus now technically developed and ready for patent protection.

Pro- and anti-inflammatory factors were assessed in lipoaspirate tissue obtained from the same group off patients and subjected to such specific mechanical force for a variable number of minutes. The untreated fat lipoaspirate acted as control. The results show that the application of a 97 x g force eliminates the typical adipose tissue expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha). Differently, this manipulation increases markedly its natural inhibitor TSG6 and interleukin 15, that are present at marginal level in fat (Figure 1). Such a mechanically induced modification of the fat tissue inflammatory status might have much broader implications as it is suggested by the dramatically enhanced leptin expression(Figure 1), the food desire suppressor produced by fat and this suggest further in vivo application of such force to body surfaces where such an hormone plays a crucial role.

Data on figure 1b show that TSG6 maximal expression has been achieved within 6 and 10 minutes of 97 x g mechanical activation (Figure 1b), and TNF alpha production is inhibited within 6 minutes under the same conditions (Figure 1c). Results reported in panel 1d show that beta-Tubulin III maximal expression can be achieved in 20 minutes of 97 x g mechanical activation (Figure 1d). Lower forces are marginally effective and the effect may vary with different subjects.

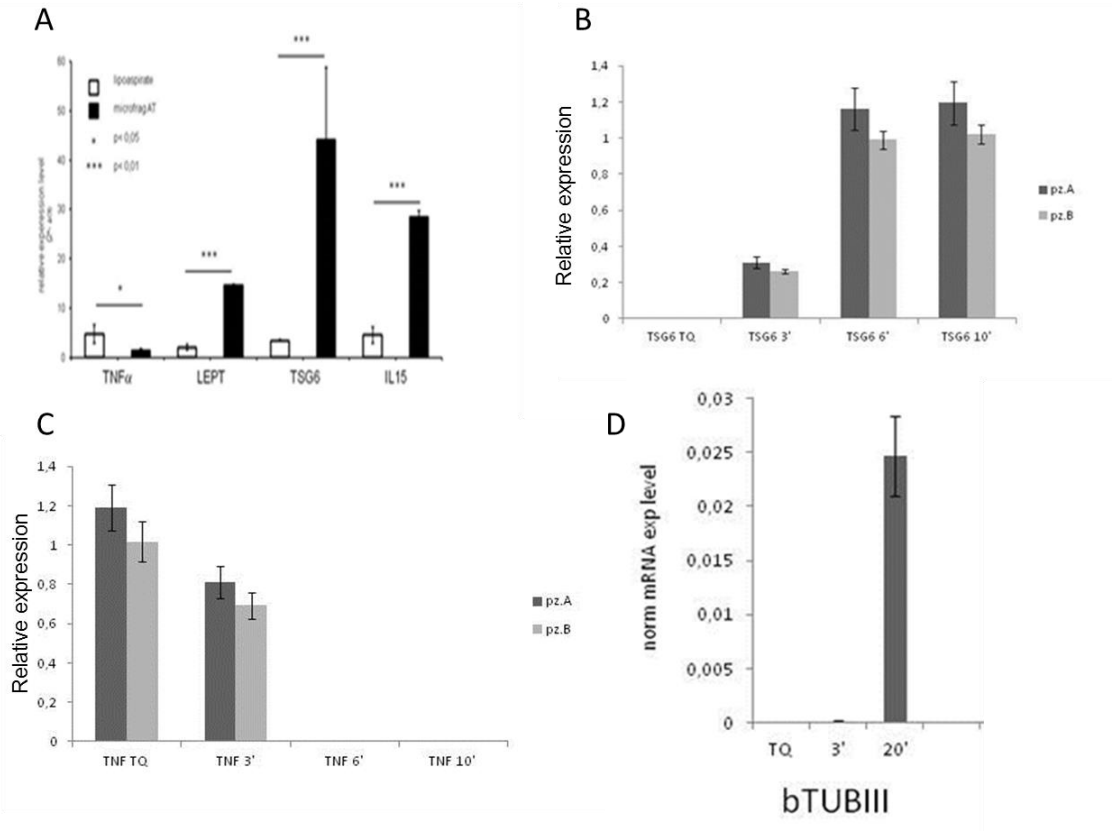

Figure 1. *Cytokine differential expression in lipoaspirated adipose tissue after mechanical activation. Beta tubulin IIII requires 10 - 20 minutes stimulation, while TSG6 is already observable at 3' stimulation and is at maximum within 6 – 10, as IL15. TNF alfa is suppressed in 6 minutes.*

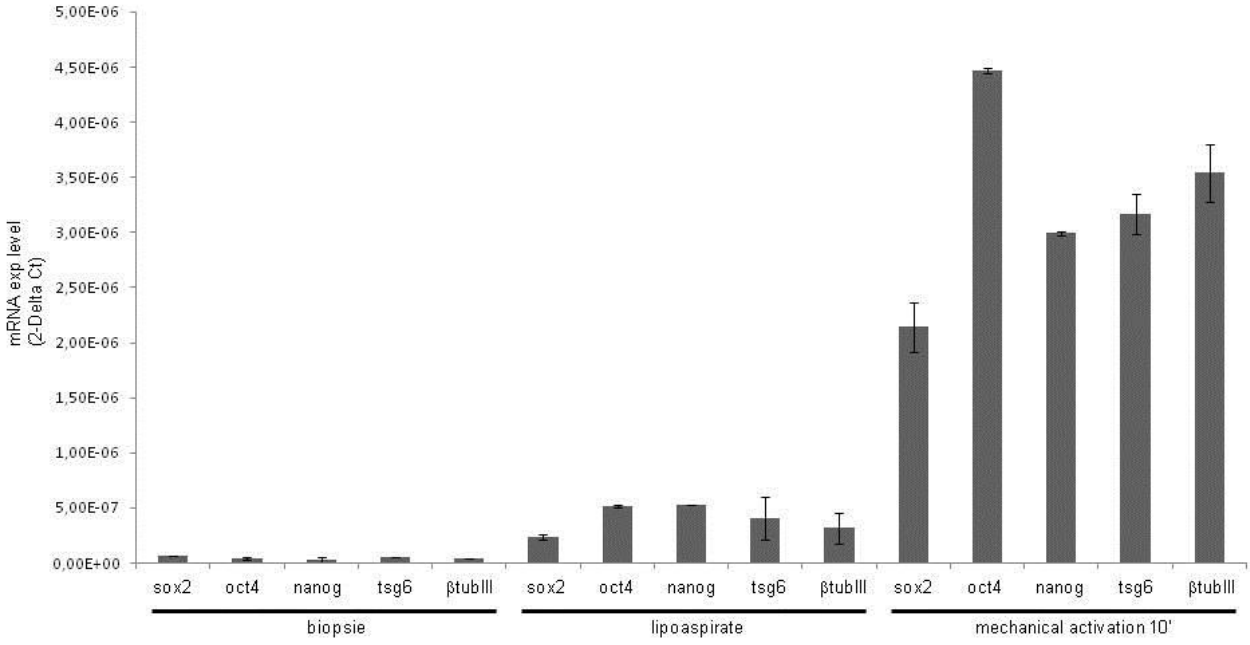

FIGURE 2. Some reports suggest that also human fat lipoaspirate (Coleman technique) has some positive effects when used in a variety of reparatory conditions that may require some antiinflammatiry action. Here we investigated some pluripotency genes (SOX2, OCT4, Nanog), beta-tubulin III and TSG6 mRNA by real-time RT-PCR in biopsy, lipaspirated, and lipoaspirated mechanically activated (10 minutes) from human adipose tissue. It can be seen that all investigated genes are barely active in regular biopsy tissue, and are slightly activated by Coleman's procedure after liposuction (lipoaspirate). However after our specific activation for 10 minutes the up-regulation is uncomparably higher (Figure 2).

The pluripotency regulatory genes SOX2, NANOG and OCT4 are fully activated within 6 minutes of 97 x g mechanical activation. Thus in addition to the anti-inflammatory properties also the parameters defining the stemness of cells present in the tissue are increased by the applied mechanical activation in a force and time dependent manner (Figure 3).

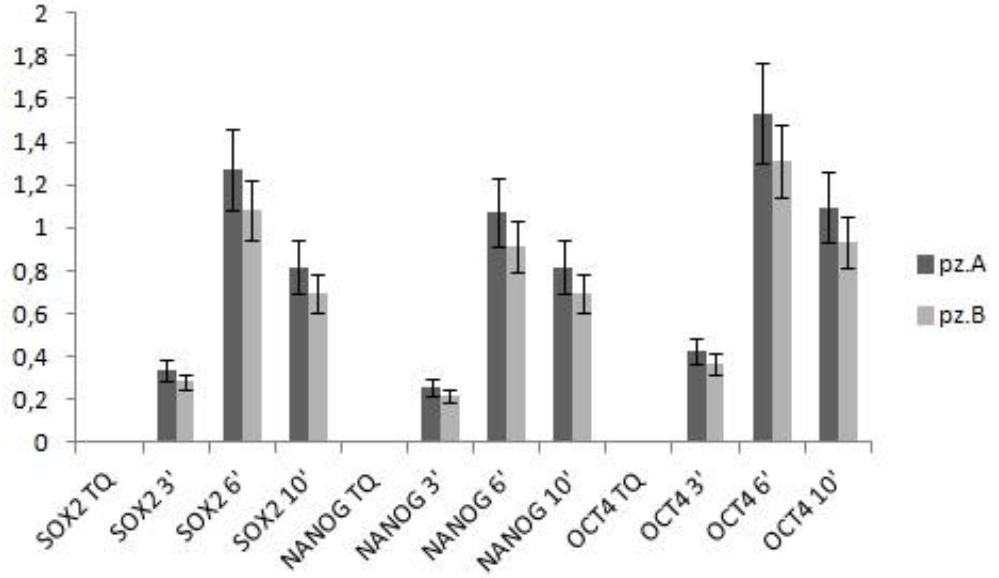

Figure 3. *Quantitative real time PCR measuring mRNA expression levels of pluripotency genes.*

Sox2 has a critical role in the maintenance of embryonic and neural stem cells and holds great promise in research involving induced pluripotency (Nicols et al., 1998; Avilion et al., 2003) Furthermore, Go et al. observed that Sox2 is not only essential for pluripotency and self-renewal of embryonic stem cells, but is also expressed in somatic stem cells with superior expansion and differentiation potential (Go et al., 2008). SOX2 is a transcription factor (34Kda) that contains a HMG box for DNA binding and forms trimeric complexes with Oct4 to control the expression of a number of genes. We tested a direct gene expression regulation lead by SOX2 transcription factor on the promoter region of different genes coding for the above described cytokines. Moreover, it has also been reported that the Chip-seq on SOX2 immuno-precipitate shows a high efficient binding of SOX2 on IL6 promoter in glioblastoma or ES cells (Fang et al 2011). We analyzed *in silico* the -3000bp promoter region of beta tubulin III, TSG6, TNFalpha, IL1b, IL10, IL15 and leptin, looking for SOX2 consensus sequence and we designed ChIP (Chromatin ImmunoPrecipitation) assays to detect the binding of the transcription factor in wet. The Chip assay shows that SOX2 and HuR bind to the promoter region of beta- tubulin III, IL15 and TSG6 and that the mechanical application of 97 x g regulates in a differential manner such interaction (Figure 4). SOX2 does not bind any more beta tubulin III promoter after 20 minutes force application, thus transcription is enhanced; while TSG6 enhanced transcription appear to be regulated by the loss of HuR binding. IL15is likely regulated by other factors in addition to Sox 2 and HuR.

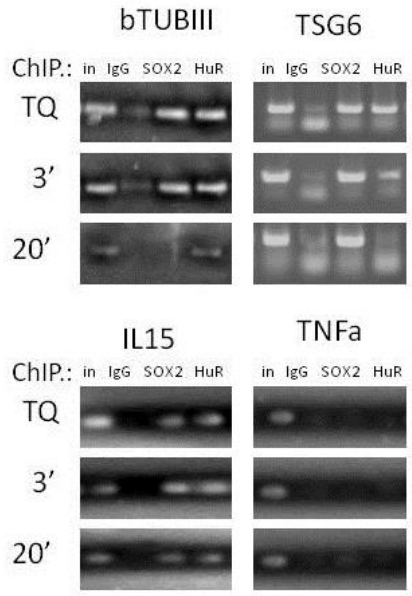

Figure 4. *Mechanical force application to adipose tissue modifies SOX2 interaction with the promoter of specific genes (beta-tub III, TSG6, IL15 and TNF alpha).*

2. Minimal manipulation of mesenchymal stem cells obtained from activated adipose tissue with application in regenerative medicine

Adipose stem cells were purified from human fat harvested through microliposuction under tumescent local anesthesia using Coleman's microcannulas.

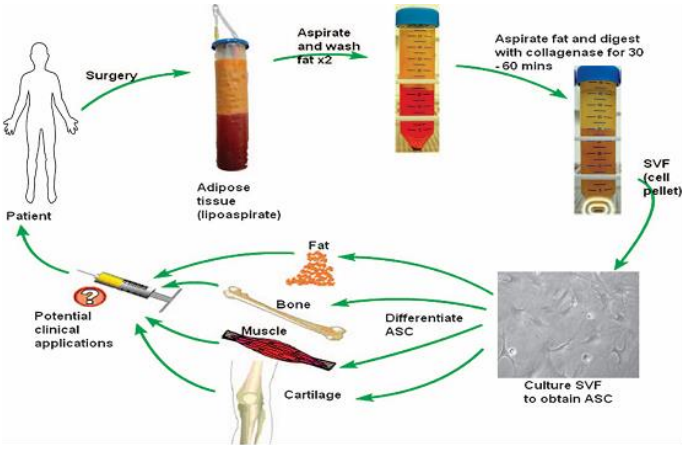

Figure 5.*. Processing of lipoaspirate to obtain hADSCs (modified from Zuk et al 2002).*

Following this procedure lipoaspirate fat was centrifuged in closed sterile system at 2000 rpm for 10 minutes and the pellet was digested with Collagenase for half an hour at 37°C (Figure 5). The digest is centrifuged to separate the floating population of mature adipocytes from the pelleted

stromal vascular fraction (SVF). After 24-hour from plating in standard medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine), non-adherent cells are removed. The plastic-adherent cell population identifies the adipose tissue–derived stromal cells (ADSCs).

A key challenge in regenerative medicine is the minimal manipulation of stem cells, in order to easily translate a specific cell line in cell therapy. Where "minimally manipulation" has been defined as a processing that does not alter the original relevant characteristics of the tissue relating to the tissue's utility for reconstruction, repair, or replacement (www.ema.europa.eu; www.fda.gov). The regulatory agencies (FDA and EMA) and the international society of stem cell research (ISCCR) claim that the cell therapy should be considered as a medicinal product when there is more than a minimal manipulation of cells destined for clinical application or where the intended use of the cells is different to their normal function in the body (www.ema.europa.eu; www.fda.gov; Hyun et al 2008). Regulatory agencies stress that the protection of patients is the core of those rules. In addition to applying the same safety and efficacy rules as for all medicinal products, the quality and manufacturing of cells based products are set out in good-manufacturing-practice (GMP) requirements. These are globally recognized standards for quality assurance in the production and control of medicines. Any use of such cell-based medicines is subject to authorization and controls, including the procedure for their manufacture (www.ema.europa.eu; www.fda.gov). According to regulatory agencies, during *in vitro* cell culture, consideration should be given to ensure acceptable growth and manipulation of the isolated cells. The processing steps should be properly designed to preserve the integrity and control the function of the cells. The procedures for any manipulation should be documented in detail and closely monitored according to specific process controls. The duration of cell culture and maximum number of cell passages should be clearly specified and validated. The relevant genotypic and phenotypic characteristics of the primary cell cultures, of the established cell lines and the derived cell clones should be defined and their stability with respect to culture longevity determined. Consistency and repeatability of the cell culture process should be demonstrated and the culture conditions including the media and the duration should be optimized with respect to the intended clinical function of the cells. For these reasons, the stem cells research applied to regenerative medicine has the obligation to adhere to the fundamental concept of "minimal manipulation".

Starting from adipose tissue subjected to 97 x g force, we were able to set up an effective and reproducible method for isolation and expansion of hADSCs through the modification of methods described by Matsumoto et al., 2007 and Perrini S., et al 2013. The lipoaspirated adipose tissue 97 x g treated is placed in 25-cm^2 culture flasks (NUNC, Roskilde, Denmark) filled completely with growth medium. This allows the tissue adhesion to the ceiling of the plate. After 2 weeks in culture the adipose tissue is removed, the flask is flipped over and cells are maintained in culture. After 30

days the cells reach the 90% confluence. Starting from each plated containing 2 ml of mechanically treated adipose tissue the yield of cells is about 5.5-6 x10^5 cells.
Live morphology (captured by means of EVOS® microscope apparatus, AMG, USA) of these cells shows a fibroblast-like phenotype comparable to that of cells obtained with classical methods (Figure 6).

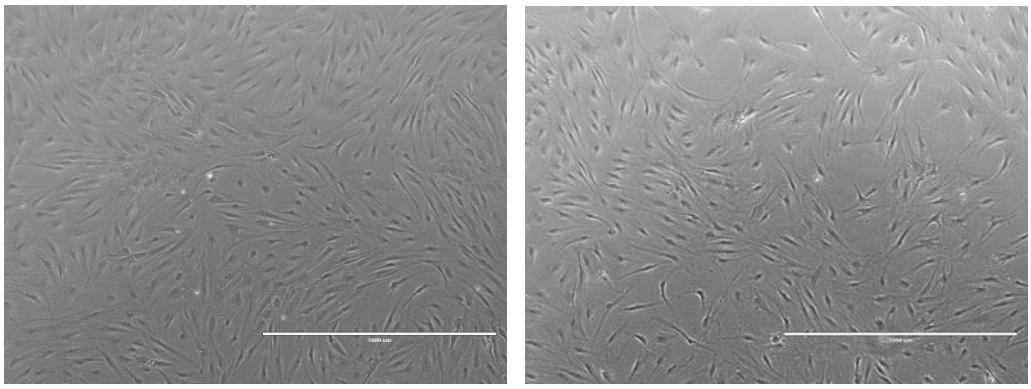

Figure 6. *Live morphology of hADSCs obtained from adipose tissue treated with orbital shaking (6 and 10 minutes, respectively).*

The immunohistochemical features classically show the expression of beta-tubulin III (TUJ1) and vimentin (Figure 7).

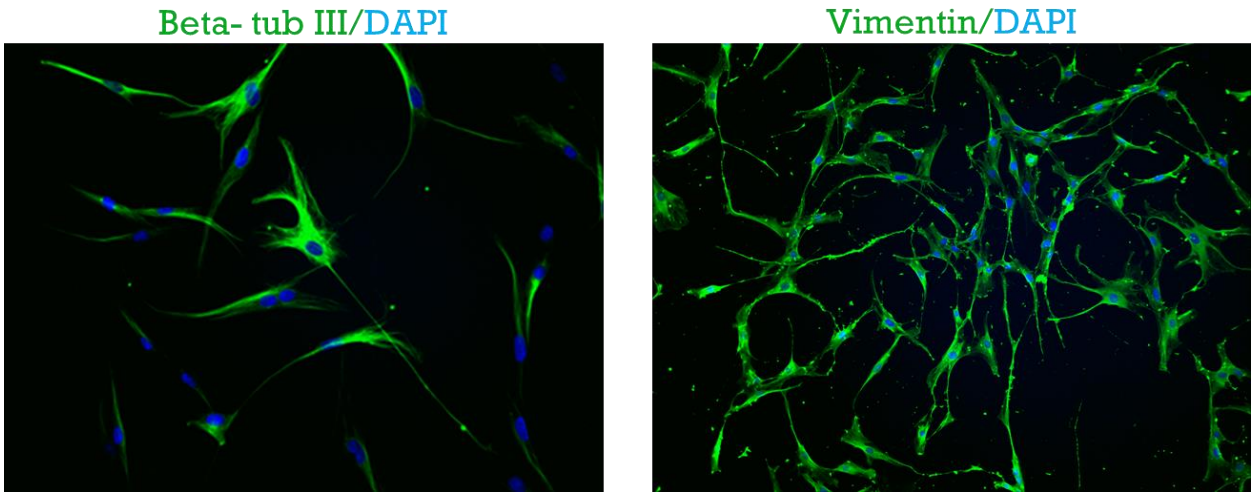

Figure 7. *Beta- tubulin III (TUJ 1) and vimentin expression in hADSCs obtained from adipose tissue treated with orbital shaking.*

The hADSCs isolated with the above described protocol were characterized for the expression of mesenchymal markers by means of FACS analysis (Figure 7). These hADSCs express at high values (about 90-100%) known mesenchymal markers (such as CD44, CD73, CD90, and CD166) (Figure 7). Hematopoietic markers (CD45, CD133, CD56) are very few represented (Figure 7), while endothelial markers (CD31, CD34, CD144, CD146, KDR) are more represented in cells

obtained from 97 x g treated adipose tissue (Figure 8). This could suggest that these cells may be prone also to an endothelial fate.

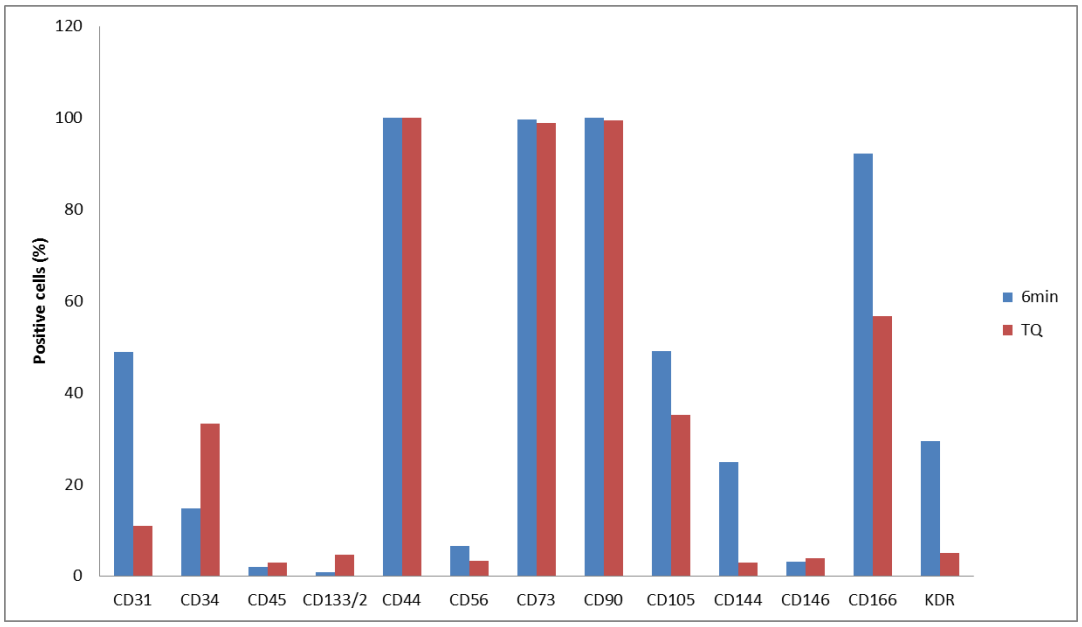

Figure 8. *FACS analysis of mesenchymal marker expression in MSCs obtained from adipose tissue treated or not with 97x g force.*

To investigate the chromosomal stability of hADSCs obtained from mechanically activated adipose tissue we performed a QFQ-banding assay. Cells did not present any chromosomal rearrangement and the chromosome number was normal (Figure 9).

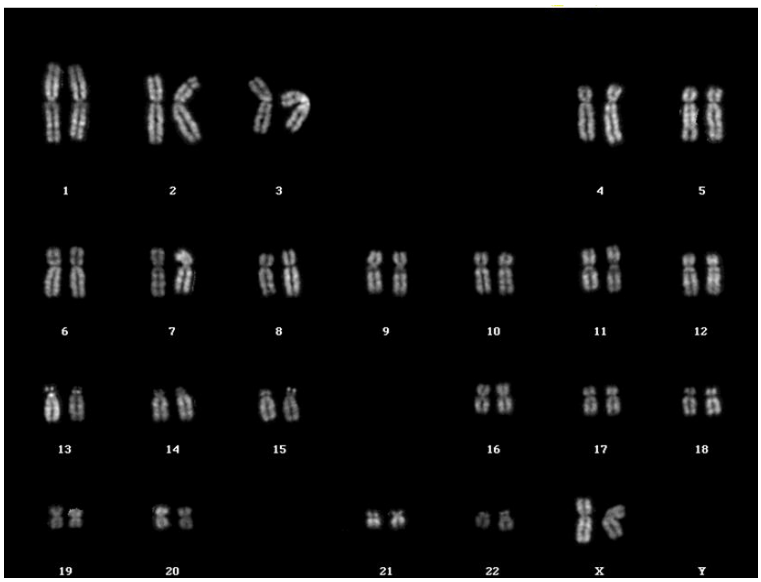

Figure 9. *Karyotype analysis in hADSCs (passage 2) obtained from adipose tissue treated with mechanical force. Micrograph refer to hADSCs of one case and is representative of the same analysis performed in the other three cases.*

The generation of spheroids is normally restricted to neural stem cells, here we show that also hADSCs from activated fat form neurospheres.

hADSCs were incubated with serum-free medium normally used for the formation of neurospheres (Gritti et al 2002). The sphere forming assay was performed by plating 5 x 10^3 /cm2. About 50% of the hADSCs obtained from mechanically activated adipose tissue formed large spheroids reaching a mean diameter of 100 µm in 21 days of culture (Figure 10). About 50% of the hADSCs obtained from mechanically activated adipose tissue formed large spheroids reaching a mean diameter of 100 µm in 21 days of culture, and forming an average of 100,95 ± 10,89 spheres/cm2 within 21 days (Figure 9). Mouse adult neural precursors cells obtained from the sub ventricular zone were used as positive control, in this case the formation of spheres was obviously superior (Figure 10).

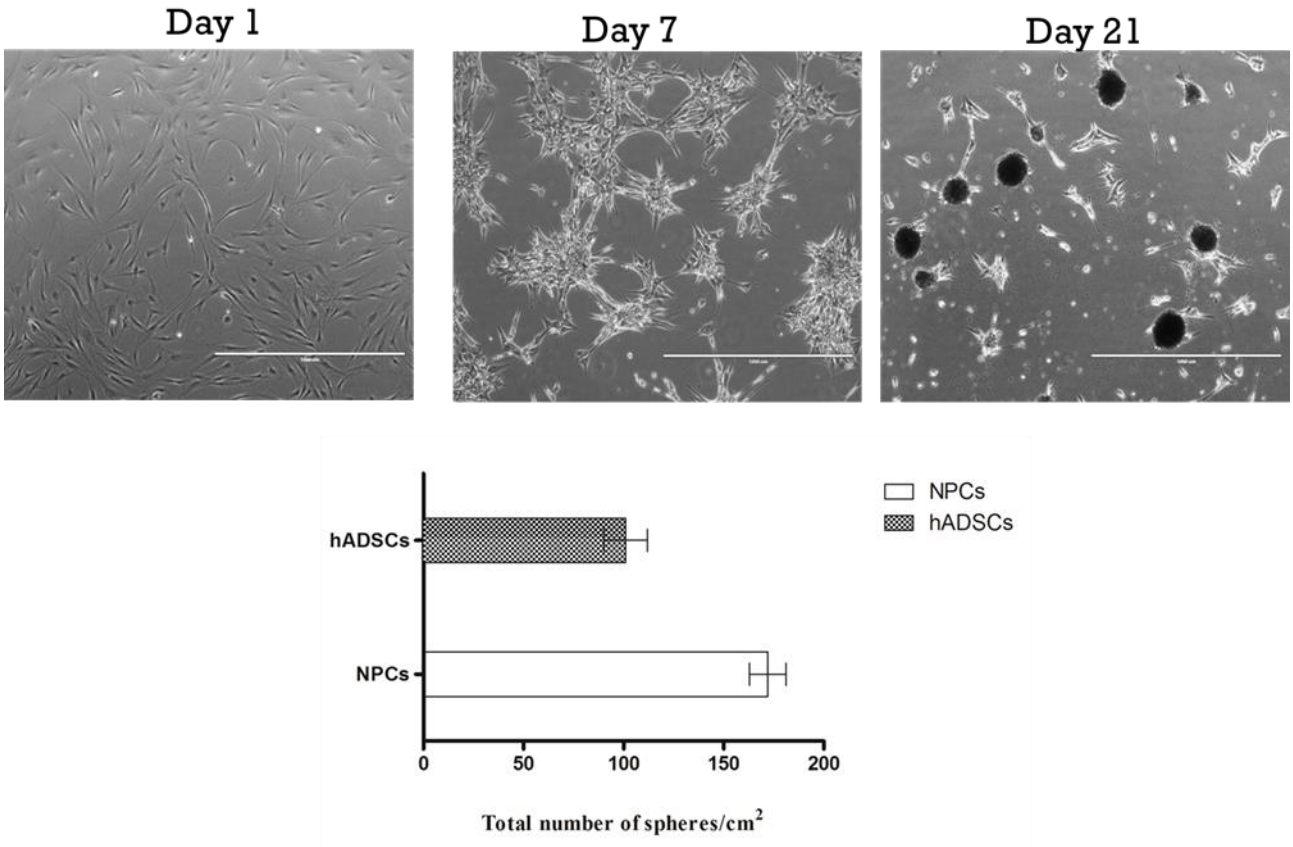

Figure 10. *After being cultivated in neurospheres medium for 21 days hADSCs from mechanically activated adipose tissue formed spheroids.*

The involvement of SOX2 in regulating the expression of above cited cytokines was also investigated in cells isolated from 97x g treated adipose tissue. The Chip assay shows that SOX2 binds to the promoter region of beta- tubulin III, IL15 and TSG6 (Figure 11).

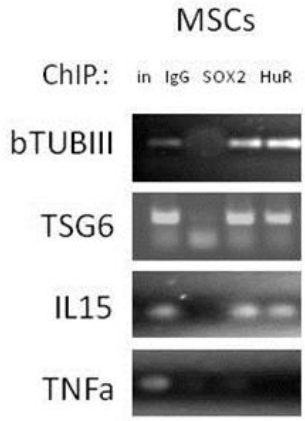

Figure 11. SOX2 interacts with the promoter of specific genes (beta-tub III, TSG6, IL15 and TNF alpha) also in MSCs isolated from 97 x g treated adipose tissue.

Recent reports suggest that MSCs are able to modulate inflammation (Prockop et al., 2013). THP-1 is single, round suspension cells with distinct monocytic markers and resemble primary monocytes and macrophages in morphology and differentiation property (Tsuchiya et al., 1980). Interleukin-1β (IL-1β) is a potent mediator of inflammation and the immune response, produced primarily by activated monocytes (Dinarello, 1994). Stimulation of monocytes with bacterial endotoxin, lipopolysaccharide (LPS) induces IL-1β production via activation of the pro-IL-1 gene, expression of pro-IL-1β mRNA, and subsequent translation of the pro-IL-1β message. Pro-IL-1β is processed to mature, biologically active IL-1β by the cysteine protease interleukin-1 converting enzyme (Kostura et al., 1989). The anti-inflammatory action of hADSCs obtained from mechanically activated adipose tissue was evaluated in a co-culture assay (trans-well, see experimental procedure) with THP1 cells activated with LPS (10 microgr/ml; for 3 hrs). As reported in figure 12 the release of IL-1β is maximal 3 hours after the stimulation with LPS, and the presence of hADSCs obtained from mechanically activated adipose tissue significantly reduced the secretion of the investigated chemokine (Figure 12).

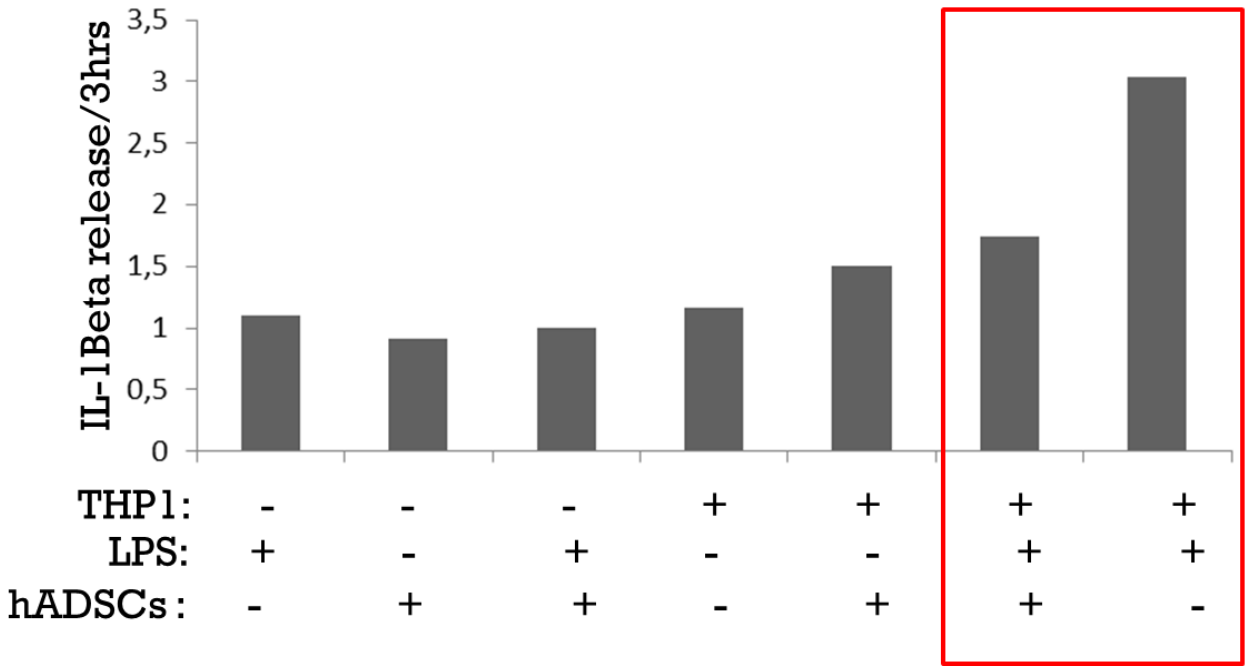

Figure 12. *MSCs isolated from mechanically activated adipose tissue are able to reduce IL-1 beta release from THP1 cells.*

EXPERIMENTAL PROCEDURES

Tissue mechanical activation and cell culture

Human adipose tissue samples have been obtained from elective liposuction procedures under local anesthesia (Lidocaine, AstraZeneca London, United Kingdom). This procedure will involve an infiltration step, in which a solution of saline and the vasoconstrictor epinephrine (2 µg/ml) (Key Customer Solutions S.A.S, Basiglio, Milan, Italy) will be infused into the adipose compartment to minimize blood loss and contamination of the tissue by peripheral blood cells. The final product will be termed lipoaspirate adipose tissue (Lipoaspirate). For mechanical activation 10 ml of washed Liposapirated adipose tissue is collected in 50 ml tube and then is activated by orbital shaking (97 x g) for different times (3, 6, 10 and 20 minutes) at room temperature. Two ml of mechanically activated adipose tissue are seeded in 25 cm2 flasks completely filled with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, Lot 6G2146) and will be incubated at 37°C in 5% CO2. Flask are flipped over and cells will float up and adhere to the top inner ceiling surface of the flask. After 15 days, the medium and the tissue are removed, and the flasks will be inverted so that cells will now be on the bottom. The medium is changed every 4 days until the cells reach the confluence (90-95% after about 30 days).

Histological analyses of control and mechanically treated lipoaspirate tissue

Histological analyses were performed on fresh adipose tissue (with or without mechanical treatment) on samples coming from the same subject. Briefly, formalin-fixed paraffin-embedded tissue samples were processed for conventional histopathological examination and immunohistochemistry. Standard 4 µm thick tissue sections stained with hematoxylin and eosin (H&E) were examined by direct wide field light microscopy. Four immunohistochemistry paraffin samples were sectioned (4µm-thickness), de-paraffined, and re-hydrated in xylene and graded concentrations of ethanol to distilled water. Then they were rinsed with PBS, treated with blocking solution (PBS+1% V/V fetal bovine serum for 1hr at room temperature) and incubated with primary antibodies overnight at 4°C. After treatment with primary antibodies, the sections were washed with PBS and incubated with appropriate secondary antibodies. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 10 min. Staining was performed with 3,3’ diaminobenzidine (DAB) as a chromogen (DAKO EnVision detection kit). In control determinations, primary antibodies were omitted and replaced with equivalent concentrations of unrelated IgG of the same subclass. The following primary antibodies were used: Vimentin (1:5000; Dako Cytomatic); S100 (1: 2000; Novocastra); β-Tubulin III (1:6000; Covance); human Nestin (1:2000; Millipore); Nanog (1:1000; Novus).

For immunofluorescence studies, cells were rinsed with PBS, fixed with paraformaldehyde (4% in PBS) treated with blocking solution and incubated with primary antibody anti β-Tubulin III (1:6000; Covance) overnight at 4°C. After treatment with primary antibody, the sections were washed with PBS and incubated with secondary antibody (Alexa Fluor® 555 goat anti-mouse 1:200 Molecular Probes®, Invitrogen, Life Technologies Italia, Monza, Italy) for 2 hours at room temperature. Nuclei were stained with DAPI (Hoechst 1/1000) and then mounted using the FluorSave Reagent (Calbiochem, Merck Chemical, Darmstadt, Germany) and analyzed by immunofluorescence microscopy (Leica 5500). As negative reference for the confocal analysis we used a consecutive section that was stained by omitting primary antibody anti β-Tubulin III and replacing it with equivalent concentrations of unrelated IgG of the same subclass.

RNA extraction and qRT-PCR analyses.

Total cellular RNAs were extracted using TRI Reagent® (Sigma-Aldrich, St. Luis, MO, USA) according to the manufacturer's instruction. RNA purity and quantity were assessed by Nanodrop (Fisher Scientific) (A260/A280 1.8-2 was considered suitable for further analysis), possible contaminating DNA was removed, and cDNA was prepared from 1 µg of RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA).

Gene expression in adipose tissues. Quantitative results were obtained by SYBR-Green real-time assay (Euroclone s.p.a. Milan, ITALY) on total RNA extracted using TRI Reagent® (Sigma-Aldrich, St. Luis, MO, USA) treated with DNase I - RNase free (Ambion inc. Foster City CA, USA) and retrotranscribed using High Capacity cDNA Reverse Transcription Kit (Ambion inc. Foster City CA, USA) as manufacturer recommended. Differentially expressed genes were detected applying a significance threshold on t-test unequal variance.

ChIP on adipose tissue

Chromatin immunoprecipitation was performed on 1 ml of lipoaspirated adipose tissue mechanically treated of not as described in Haim et al 2013.

G-banding karyotype analyses

Cytogenetic analyses were performed on "in situ" cultures obtained by inoculating hADSCs directly onto a coverslip inside Petri dishes containing 2 ml of medium (αMEM). Cells were treated with Colcemid (0.02 µl/ml) (Life Technologies Carlsbad, California, USA) for 90 minutes, hypotonic solution (1:1 Na citrate 1%: NaCl 0.3%) (Sigma-Aldrich St. Louis, MO, USA) and fixative solution of 3:1 methanol:acetic acid (VWR International Radnor, Pennsylvania, USA), replaced twice. At least twenty-five QFQ banding metaphases were observed for each sample. The image was acquired using a fluorescence microscope (BX 60 Olympus) and analyzed with Powergene PSI system.

Spheroid Formation

hADSCs derived from mechanically activated lipoaspirate tissue were grown in alpha-MEM medium with fetal bovine serum (20%) to reach 85% confluence at passage 1. Alpha MEM medium was removed, cells were washed twice with sterile PBS, then neurospheres-medium containing bFGF (20 ng/ml) and EGF (10 ng/ml) in absence of serum was added (Gritti et al 2002).

THP-1 cells culture and activation with LPS

THP-1 cells (202-TIB; ATTC, Rockville, MD) were grown in suspension in RPMI 1640 media supplemented with 10% fetal calf serum (FCS), 50 U/mL penicillin, and 50 µg/mL streptomycin in 150-cm2 tissue culture flasks. Cell culture and all experiments were carried out at 37°C in 5% CO2, 95% room air. Cells were passaged every 3 days and used between passages 3 and 12. The anti-inflammatory action of hADSCs was assayed in trans-well culture system onto 6 well plates. hADSCs were plated in the well below at the density of 1,5 x 10^4 cells/well in alpha MEM medium. Twenty four hours after their adherence THP-1 cells were added into the upper ate the density of 75 x 10^4 cells/well. To induce IL-1 beta release lipopolysaccharide (LPS) was administered at the final concentration of 10 microgr/ml. After three hours of incubation supernatants were collected in ice and immediately frozen. IL- 1beta release was investigated by immunoblotting.

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