Obesity negatively affects the biological functions of mesenchymal stromal cells derived from bone marrow, subcutaneous and visceral fat of young mice

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Abstract

White adipose tissue (WAT) is distributed in several depots that have distinct metabolic and inflammatory functions. In our body there are subcutaneous (sWAT), visceral (vWAT) and bone marrow fat depots (BFAT). Obesity affects size, function and inflammatory state of WATs. This process can alter the stem cell niches present in these tissues and affect the functions of stem cells residing within. In particular, obesity may affect the activity of mesenchymal stromal cells (MSCs) present in WAT. MSCs are an heterogenous population containing stromal cells, progenitor cells, fibroblasts and stem cells that are able to differentiate in adipocytes, chondrocytes, osteocytes and other mesodermal derivatives.

We performed a comparison of the effects of obesity on MSCs obtained from sWAT, vWAT and BFAT. Our study evidenced that obesity affected mainly the biological functions of MSCs obtained from bone marrow and vWAT with a decrease in proliferation rate, reduced percentage of cells in S phase and trigger of senescence. The onset of senescence was confirmed by expression of genes belonging to RB and P53 pathways.

Our study evidenced that negative consequences of obesity on body physiology may be related also to impairment in the functions of stromal compartment present in the several adipose tissues. This finding provides new insights on the targets that should be considered for an effective treatment of obesity-related diseases.

1. Introduction

Mesenchymal stromal cells (MSCs) are an heterogenous population containing stromal cells, progenitor cells, fibroblasts and stem cells. These latter ones are able to differentiate in adipocytes, chondrocytes, osteocytes and other mesodermal derivatives. MSCs contribute to the homeostatic maintenance of many organs through secretion of many cytokines and growth factors, which exert paracrine and autocrine functions. MSCs have been isolated from stromal component of several tissues and organs, such as bone marrow, adipose tissue, cord blood and dental pulp [1]. The biological properties of stem cells, including those present in MSCs, are regulated by niche, which is a specialized microenvironment composed of cells and extracellular components. Niche houses stem cells and control their self-renewal and progeny production by providing structural and trophic support, topographic information and physiological cues. Several pathological conditions, such as obesity, may heavily modify the niche microenvironment and hence affect stem cell functionality [2].

Obesity is a complex disease that causes white adipose tissue (WAT) dysfunction and is associated with cardiovascular, chronic inflammatory and other metabolic pathologies [3]. In physiological conditions, WAT has a key role in body energy homeostasis by storing fatty acid and releasing them when fuel is required. It contributes to regulation of glucose metabolism and exert endocrine functions by secreting dozens of adipocyte-derived factors (adipokines), which are involved in inflammation, regulation of food intake, energy expenditure, reproductive function, pro- and anti-apoptotic activity [3,4]. WAT is distributed in several depots that have distinct metabolic and inflammatory functions. In our body there are subcutaneous (sWAT) and visceral (vWAT) depots. Obesity determines also alterations in bone marrow microarchitecture by increasing the proportion of bone marrow fat (BFAT), which plays a key role within bone microenvironment, mainly as source of adipokines that regulate hematopoiesis and bone homeostasis [5].

Obesity affects size, function and inflammatory state of WATs and, as consequence, it alters the stem cell niches present in these tissues. These changes induce remodeling of stem cells present in the niches. In particular, MSCs, which contains precursors of adipocytes, could modify their biological properties. This phenomenon may have profound consequences on health given the role of MSCs in bone, cartilage and fat tissue regeneration and in body's homeostasis.

In this scenario, we aimed to evaluate how obesity may affect the *in vitro* biological functions of MSCs derived by sWAT, vWAT and bone marrow (BM). Indeed, there are several studies aiming to

evaluate the consequences of obesity on MSC functions but there an overall analysis that compares the obesity effects on MSCs obtained from different districts is still lacking [6-9].

2. Material and Methods

2.1 Animals

C57BL/6 inbred male mice of 6 weeks of age were purchased from Charles River (MA, USA). The animals were handled in compliance with the protocols that were approved by the Animal Care and Use Committee of Luigi Vanvitelli Campania University (#317/2016PR). After arrival, mice were divided into two groups and were fed either a high-fat diet (Research Diets, NJ, USA) or with a normal diet for 10 weeks. At the end of this treatment, animals were sacrificed and tissue samples were harvested for experiments indicated below.

The high-fat diet consisted of 60% fat from lard, 20% carbohydrate, and 20% protein (total 5.21 kcal/g), whereas the normal diet contained 10% fat, 70% carbohydrate, and 20% protein (total 3.82 kcal/g). Food intake and body weight were measured once a week till the end of experiments.

2.2 Glucose measurement

At the end of high and normal fat treatments, blood glucose levels were determined in fasted animals by tail bleeding using Contour Blood Glucose meter (Ascensia Diabetes Care, NJ, USA) according to manufacturer's instruction.

2.3 Mouse MSC isolation and CFU assay

We harvested MSCs from the bone marrow of the femurs and tibias of mice by inserting a 21-gauge needle into the shaft of the bone and flushing it with alpha-MEM. The cells from one animal were plated onto two 100-mm dishes with alpha-MEM containing 15% FBS. After 48 hours, we discarded the nonadherent cells and washed the adherent cells with PBS 1X. We verified by immunocytochemistry that MSCs expressed the surface antigens CD73, CD90 and CD105 as reported [10].

We then incubated the cells for 7 to 10 days in a proliferating medium in order to reach confluence (P0). The cells were then trypsinized and were seeded for the acid beta galactosidase assay.

We collected MSCs from 500 mg sWAT surrounding the hips of animals and from 1 g of vWAT obtained from epididymal area. Tissues were digested in a DMEM solution containing collagenase type II (1mg/ml) for 1 hours at 37°C. Samples were filtered on cell strainers (70 μm mesh), centrifuged and washed three times with PBS 1X. Cells were plated onto 100-mm dishes with

alpha-MEM containing 15% FBS. We verified by immunocytochemistry that MSCs expressed the surface antigens CD73, CD90 and CD105 as reported [10].

We then incubated the cells for 7 days in a proliferating medium in order to reach confluence (P0). The cells were then trypsinized and were seeded for the acid beta galactosidase assay.

An aliquot of cells at P1 were used for the CFU assay. In brief, 1,000 cells were plated in 60 mm plates and were incubated for 15 days without a medium change. The plates were collected, fixed, and stained with 0.5% crystal violet. The stained colonies were identified under a light microscope and were counted.

2.4 Adipogenic differentiation

MSCs were treated for 15 days in mesenchymal stem cell adipogenic differentiation medium (PT-3004- KT - Lonza, MD, USA). The medium contains insulin (recombinant), dexamethasone, indomethacin and 3-isobuty-l-methyl-xanthine (IBMX). Lipid droplets were revealed by staining with Oil Red O.

2.5 Osteogenic differentiation

MSCs were treated for 15 days in mesenchymal stem cell osteogenic differentiation medium (PT-3002- KT-Lonza). The medium contains dexamethasone, ascorbate and glycerophosphate. Staining with Alizarin red revealed calcium deposits in differentiated osteocytes.

2.6 Chondrogenic differentiation

MSCs were seeded as a pellet in 96 round bottom multi-wells and cultured in a chondrogenic medium composed of DMEM, 1% FBS, 50 nM ascorbate-2-phosphate (Sigma-Aldrich, MO, USA), 0.1 mM dexamethasone (Sigma-Aldrich, MO, USA), and 10 ng/mL human transforming growth factor (hTGF)-β1 (Preprotech, UK). After 21 days, Alcian blue staining was performed.

2.7 Cell proliferation assay

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8) colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays (Dojindo Molecular Technology, Japan). We seeded 5,000 cells in 96-wells and CCK-8 were added. The viability was detected by a microplate reader at 450 nm 24 hrs, 48 hrs, and 72 hrs after the incubation.

2.8 Cell cycle analysis

For each analysis, $5x10^4$ cells were collected by trypsin treatment and then, after the PBS washes, were fixed in 70% ethanol overnight at -20°C. The samples were then washed with PBS 1X and finally were dissolved in a hypotonic buffer containing propidium iodide (Sigma-Aldrich MO, USA).

The samples were acquired on a Guava EasyCyte flow cytometer (Merck Millipore MA, USA) and analyzed with a standard procedure using EasyCyte software.

2.9 In situ senescence-associated acid beta galactosidase assay

Cells grown in flasks were fixed using a solution of 2% formaldehyde and 0.2% glutaraldehyde for 5 min at RT. After that, the cells were washed with PBS 1X (MicroGem, Italy) and then incubated with SPiDER-ßgal kit a (Dojindo Molecular Technology, Japan). The samples were acquired on a Guava EasyCyte flow cytometer (Merck Millipore MA, USA) and analyzed with a standard procedure using EasyCyte software.

2.10 Apoptosis detection

Apoptosis was detected using a fluorescein-conjugated Annexin V kit (Dojindo Molecular Technology, Japan) on a Guava EasyCyte flow cytometer (Merck Millipore MA, USA) following the manufacturer's instructions.

2.11 Treatment with DNA-damaging agent and immunocytochemistry for γ-H2AX detection

MSC Cultures were treated for 30 min with 300 µM H_2O_2. Following treatment, the medium was removed, and a complete medium was added. Cells were then collected for data analysis 1, 6, and 48 hrs later.

γ-H2AX (2577, Cell Signaling, MA, USA) was detected according to manufacturer's protocol. DAPI staining was performed, and then cells were observed through a fluorescence microscope (Leica Italia, Italy). The degree of H2AX phosphorylation (γ-H2AX) was evaluated by counting the number of gamma-H2AX immunofluorescent foci per cell. Foci number was determined for 200 cells.

2.12 Reactive oxygen species detection

The intracellular reactive oxygen species (ROS) level was analysed by using the H2DCFDA test (Thermofisher Italia, Italy) according to the manufacturer's instructions. The ROS derivatives are quantified on a Guava EasyCyte flow cytometer (Merck Millipore MA, USA) following the manufacturer's instructions.

2.13 Western Blot (WB) analysis

Cells were lysed in a buffer containing 0.1% Triton (Bio-Rad, CA, USA) for 30 min in ice. 20 µg of each lysate was electrophoresed in a polyacrylamide gel and electroblotted onto a nitrocellulose membrane. We used the following primary antibodies: RB1 (AV33212) and GAPDH (G8795) were from Sigma-Aldrich (MO, USA), RB2/P130 (R27020) was from BD Biosciences (CA, USA), $p27^{KIP1}$ (3686) was from Cell Signaling (MA, USA), while p107 (sc-318), p53 (sc-126), and $p21^{CIP1}$ (sc-397) were obtained from Santa Cruz Biotechnology (CA, USA), and $p16^{INK4A}$ (ab54210) was from ABCAM

(Cambridge, UK). Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (ImmunoReagents, NC, USA) and reacted with ECL plus reagent (Merck Millipore, MA, USA). All of the antibodies were used according to the manufacturer's instructions.

The mean value was quantified densitometrically using Quantity One® 1-D analysis software (Bio-Rad, CA, USA).

2.14 Qualitative RT-PCR

Total RNA was extracted from cell cultures using Omnizol (Euroclone, Italy). The mRNA levels were measured by RT-PCR with 5X ALL-IN-ONE RT MASTERMIX (ABM, Canada) as reported previously [11]. PCR cycles were adjusted to include linear amplification for all of the targets. Each RT-PCR reaction was repeated at least three times. A qualitative analysis of mRNA levels was performed with the "GEL DOC UV SYSTEM" (BioRad Company, CA) as already reported [11].

2.15 Statistical analysis

Statistical significance was determined using one-way ANOVA and post hoc tests by JASP software (https://jasp-stats.org). All statistical analysis data are in supplementary figure 1.

3. Results

3.1 In obese mice the visceral fat and bone marrow derived MSCs showed reduction in proliferation rate and increase in senescence

The high fat diet (HFD) induced a significant increase of mice weight (Fig. 1A), with presence of abundant vWAT depots. As reported HFD treatment caused hyperglycemia as determined with blood glucose measurement (Fig. 1B). We isolated and cultivated MSCs from BM, sWAT and vWAT of obese and normal mice and determine their *in vitro* properties.

In samples obtained from obese animals, the proliferation assay evidenced a reduction in proliferation rate of BM- and vWAT-MSCs, while those obtained from sWAT did not show changes compared with controls (Fig. 2A). This data agreed with cell cycle profile analysis, which evidenced a reduction of S-phase cells in BM- and vWAT-derived cells (Fig. 2B).

We then evaluated apoptosis and senescence by annexin V and acid-beta-galactosidase assays, respectively (Fig. 3A, B). MSCs obtained from obese animals did not evidence change in apoptosis levels compared with controls. In opposition, the percentage of senescent cells was greater in MSCs from obese mice compared with those obtained from normal animals (Fig. 3B). In particular, senescence level in vWAT-MSCs was significantly higher in obese samples than in normal control. An increase in the production and release of reactive oxygen species (ROS) is a typical feature of

senescent cells [12,13]. In MSC cultures obtained from obese animals we detected a significant increase in intracellular ROS levels (Fig. 3C).

3.2 RB and P53 pathways are activated in MSCs obtained by obese mice compared with controls

The decrease in cell proliferation along with the trigger of senescence were consistent with an increase in the levels of RB1, P21, and P16, which play a key role in regulation of cell cycle and senescence (Fig. 3D) [14]. Of note, in vWAT- and sWAT-MSCs from obese mice the cell cycle exit and senescence were not related to P53, as we did not observe an increase of its expression. On the contrary, the P53 protein level was significantly upregulated in BM-MSCs obtained from obese animals (Fig. 3D).

3.3 DNA damage repair following stress

The presence of senescence phenomena in MSCs may suggest that DNA repair system cannot cope all genotoxic injuries occurring during lifetime. We determined the effectiveness of DNA repair machinery by inducing DNA damage with peroxide hydrogen (H_2O_2) treatment and evaluated the expression level of histone γ-H2AX in order to assess the repair capacity of MSCs following genotoxic stress (Fig. 4). DNA damage induces the activation of ATM that promotes H2AX phosphorylation. The phosphorylated isoform (γ-H2AX) contributes to recruit and/or retain of DNA repair proteins on DNA damage foci. The γ-H2AX foci in nuclei are signs of damaged DNA that are being repaired. Soon after DNA damage events, the presence of these foci is a sign of active repair, while foci persistence

several hours or days after stress evidence the presence of unrepaired or misrepaired DNA [15,16].

We performed a follow up of γ-H2AX foci 1, and 24 hrs following 300 μM H_2O_2 treatment of MSC cultures. BM-MSCs from control mice showed a strong increase of γ-H2AX staining 6 hours after stress and then a decline of damage foci. In samples from obese animals the peak of DNA damage foci was detected 1 hour after stress and then the signal intensity decreased. Of interest in sWAT- and vWAT-MSCs from both control and obese mice we noticed a persistence of γ-H2AX foci even at 24 hrs time point suggesting the presence of unrepaired/misrepaired DNA.

3.4 Stemness and differentiation properties

Senescence could affect the MSC properties, such as self-renewal and multipotential differentiation properties. CFU assay, however, did not show significant changes in the clonogenic potential of MSCs from obese samples compared with control (Fig. 5A). Moreover, the MSC differentiation into adipocytes, chondrocytes and osteocytes, following incubation in induction media, was not impaired in obese mice (Fig. 5B).

MSCs are an heterogenous population containing both stem cells and lineage committed progenitors for production of osteocytes, chondrocytes and adipocytes. Hence, in absence of external differentiation cues, in proliferating MSC cultures it is possible to detect markers of lineage commitment. The identification of such markers is an indication of which lineage committed progenitors are more abundant and/or are already in an advanced differentiation status in the MSC population. In proliferating MSCs from BM, sWAT and vWAT of control animals we observed the expression of adipogenic (LPL, PPARG) and osteogenic markers (Osteopontin/SPP1, Osterix/Osx), while we did not detect the expression of chondrogenic markers (Aggrecan/Acan, Col1A1) (Fig. 5C). The obesity status did not affect the expression of these markers (Fig. 5C).

4. Discussion

There are several studies showing that MSCs reside at the outer surface of the sinusoids in the bone marrow stroma. This finding supports the hypothesis that multipotent stromal cells could be present in the subendothelial region of small blood vessels present in the stromal component of nearly every organ. Indeed, MSCs can be isolated stromal component of adipose tissues and play a key role in adipose tissue development, differentiation and maintenance as well as in organismal homeostasis [1]. Impairment of MSC physiological activities can have profound consequences on body's functions. We investigated how alteration of extracellular environment, as it occurs in obesity status, may affect MSC activities. Indeed, many studies have evaluated the pathophysiological consequences of obesity on the biology and functions of adipocytes [17,18]. Some others addressed the effects of obesity on MSC functions but there are no comprehensive studies that compare the consequences of such a disease on MSCs obtained from different districts [6-9].

We performed a comparison of the effects of obesity on MSCs obtained from sWAT, vWAT and BFAT of young male mice. We selected only young male animals to reduce variability, since chronological aging and estrogen fluctuation may affect MSC functions [19-21]. Further studies should be performed to evaluate the effects of age and sex on MSC dysfunction associated with obesity.

Our study evidenced that obesity affected mainly the biological functions of MSCs obtained from BM and vWAT with a reduction in proliferation rate, reduced percentage of cells in S phase and trigger of senescence. The onset of senescence was confirmed by expression of genes belonging to RB and P53 pathways.

The MSC obtained by sWAT appeared less affected by obesity status even if an increase in senescence phenomena were detected. The onset of senescence may be related to ROS production in a self-sustaining loop: ROS may induce senescence and then senescent cells produce ROS [22]. Of note, we observed a significant increase of intracellular ROS concentration in MSCs obtained from visceral and bone marrow depots of obese animals compared with controls. The increase in ROS may be one of the way obesity may affect MSC functions: high levels of circulating lipids and glucose and can produce an excess of energy substrates for cellular metabolic pathways, which in turn increase the ROS production. Obesity did not increase ROS production in MSCs obtained from sWAT. This phenomenon may be to high expression of genes involved in redox regulation as evidenced by Zhang and co-workers [23].

The DNA repair capacity of MSCs is not significantly affected by obesity status in BM-MSCs. At the opposite, in MSCs obtained from sWAT and vWAT of both normal of obese mice we observed a permanence of unrepaired DNA foci following genotoxic stress, suggesting that MSCs coming from these fat depots are less proficient in coping DNA damage stress than BM-MSCs. This result should be further investigated also for the therapeutic implications it may have. Indeed, in recent years, sWAT- is used as alternative source to BM to obtain MSCs for cell therapy treatments. A note of caution should be sounded on this alternative if it will be proved that MSCs obtained from sWAT are less effective in repairing DNA compared with BM-MSCs.

Senescence phenomena observed in MSCs obtained from obese mice did not affect their clonogenic potential and differentiation properties. This observation may be at odds with the onset of senescence phenomena. It remains to be established if MSC differentiation that is not affected in obese mice can produce mature and functional adipocytes, osteocytes and chondrocytes. This investigation requires further *in vivo* studies along with physiological analysis on differentiated cells.

Indeed, we performed a preliminary qualitative analysis of differentiation process with cytochemistry and RT-PCR techniques, while Wu and co-workers performed a semi-quantitative analysis of osteo-chondro-adipo differentiation of MSCs from obese and normal mice. They found that obesity greatly affect MSC differentiation [9].

A further comment needs the finding that obesity profoundly affect the functions of BM-MSCs. These cells play a key role in regulation of hematopoietic stem cells self-renewal, proliferation and differentiation. The presence of anaemia in obese people may be a consequence of altered hematopoietic stem cell fitness due to impairment of MSC activities [24,25].

In conclusion, our study evidenced that negative consequences of obesity on body physiology may be related also to impairment in the functions of stromal compartment present in the several adipose tissues. This observation further reinforces the concept that obesity is a disease as designated by American Medical Association (AMA) in 2013. Alteration of stromal component may have profound and deleterious consequences on body functions, since stem cells that promote tissue renewal and homeostasis are located in this stromal component.

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Figure Legends

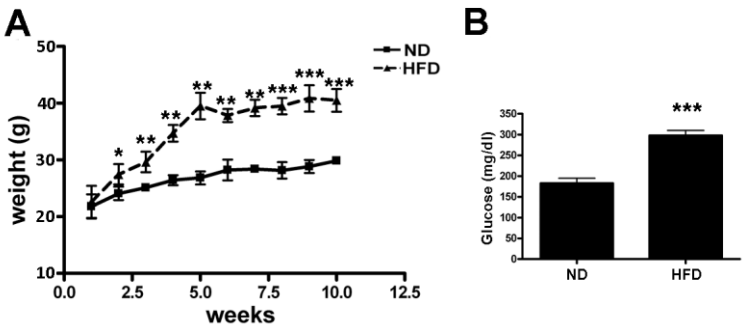

Figure 1 – Treatment of mice with HFD

Panel A: The graph shows the weight of six mice fed with HFD and other six ones with ND for 10 weeks.

Panel B: The graph shows the mean blood glucose levels determined in mice at the end of treatment with either HFD or ND. Data are shown with standard deviation (SD) n=6 *p<0.05.

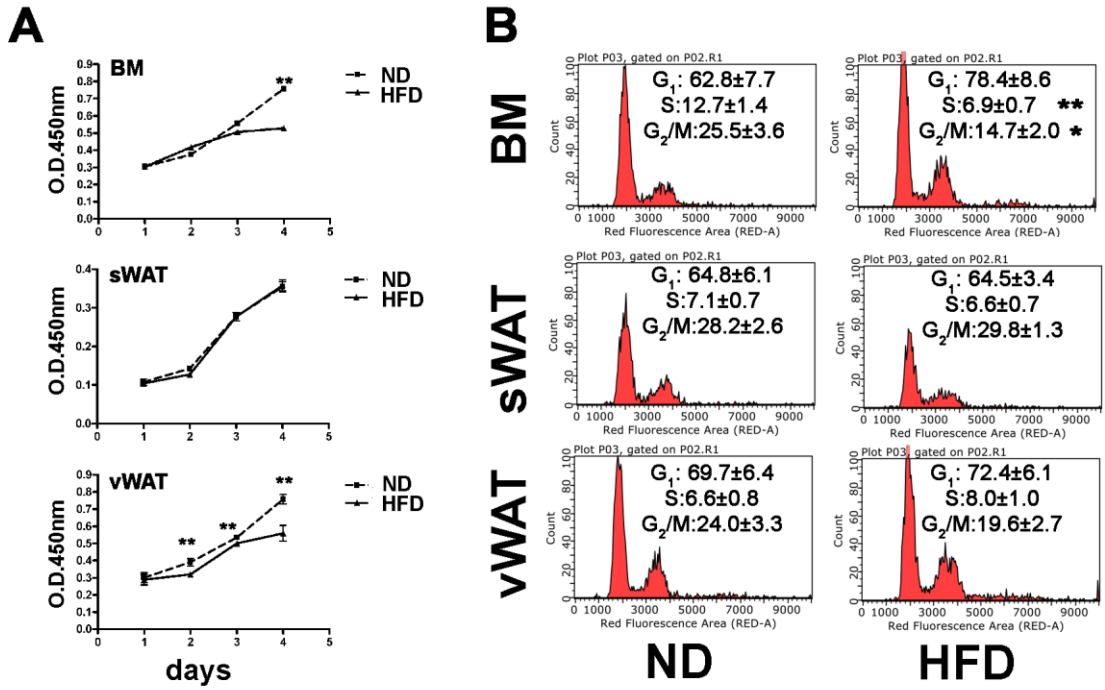

Figure 2 – Proliferation and cell cycle analyses

Panel A: MSC cell proliferation was evaluated by a Cell Counting Kit-8 (CCK-8) colorimetric assays. The graph shows data coming from obese and control samples. Data are shown with standard deviation (SD) n=6 *p<0.05.

Panel B: Representative cell cycle analysis of MSCs harvested from obese and normal mice. Data are expressed with SD (n = 6) *p<0.05.

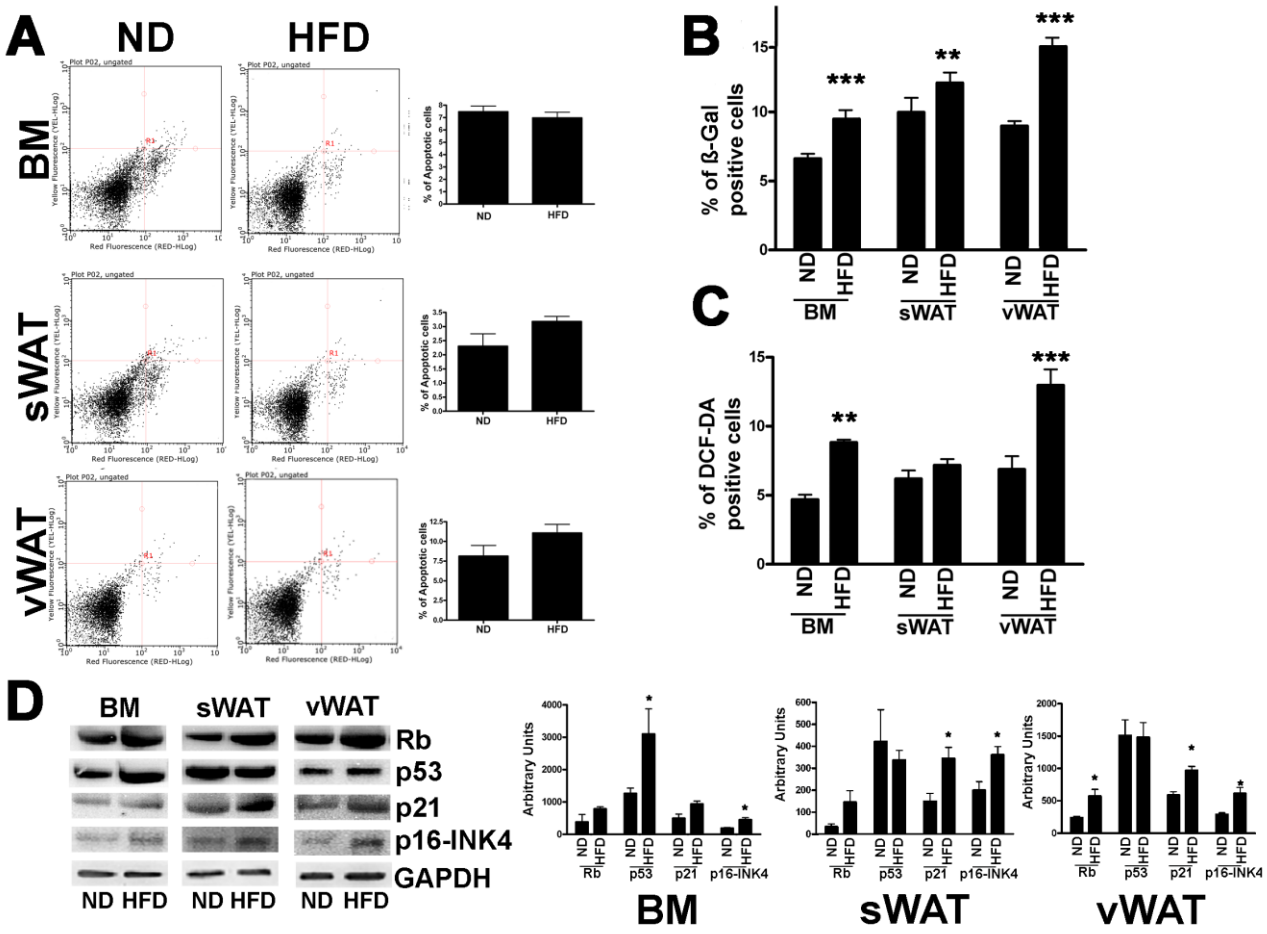

Figure 3 – Apoptosis and senescence in MSCs from obese and control animals

Panel A: Representative FACS analysis of MSC apoptosis. The assay identifies early (Annexin V + and 7ADD −) and late apoptosis (Annexin V + and 7ADD +). Apoptosis is a continuous process and we calculated the percentage of apoptosis as the sum of early and late apoptotic cells. The histogram shows the mean percentage of Annexin V-positive cells. Data are expressed with standard deviation (n = 6).

Panel B: The graph shows mean percentage value of senescent cells determined by SPiDER-ßgal assay. Data are expressed with SD (n = 6) *p<0.05.

Panel C: The graph shows mean percentage value of cells showing discrete amount of intracellular ROS that are above the threshold detected by H2DCFDA assay. Data are expressed with SD (n = 6) *p < 0.05.

Panel C: Western blot analysis of proteins regulating senescence. The picture shows a representative blot analysis the expression levels of Rb, p53, p21, p16/INK4, Gapdh (loading control). The graph shows mean expression levels (±SD, n = 6 biological replicates, *p<0.05, **p<0.01).

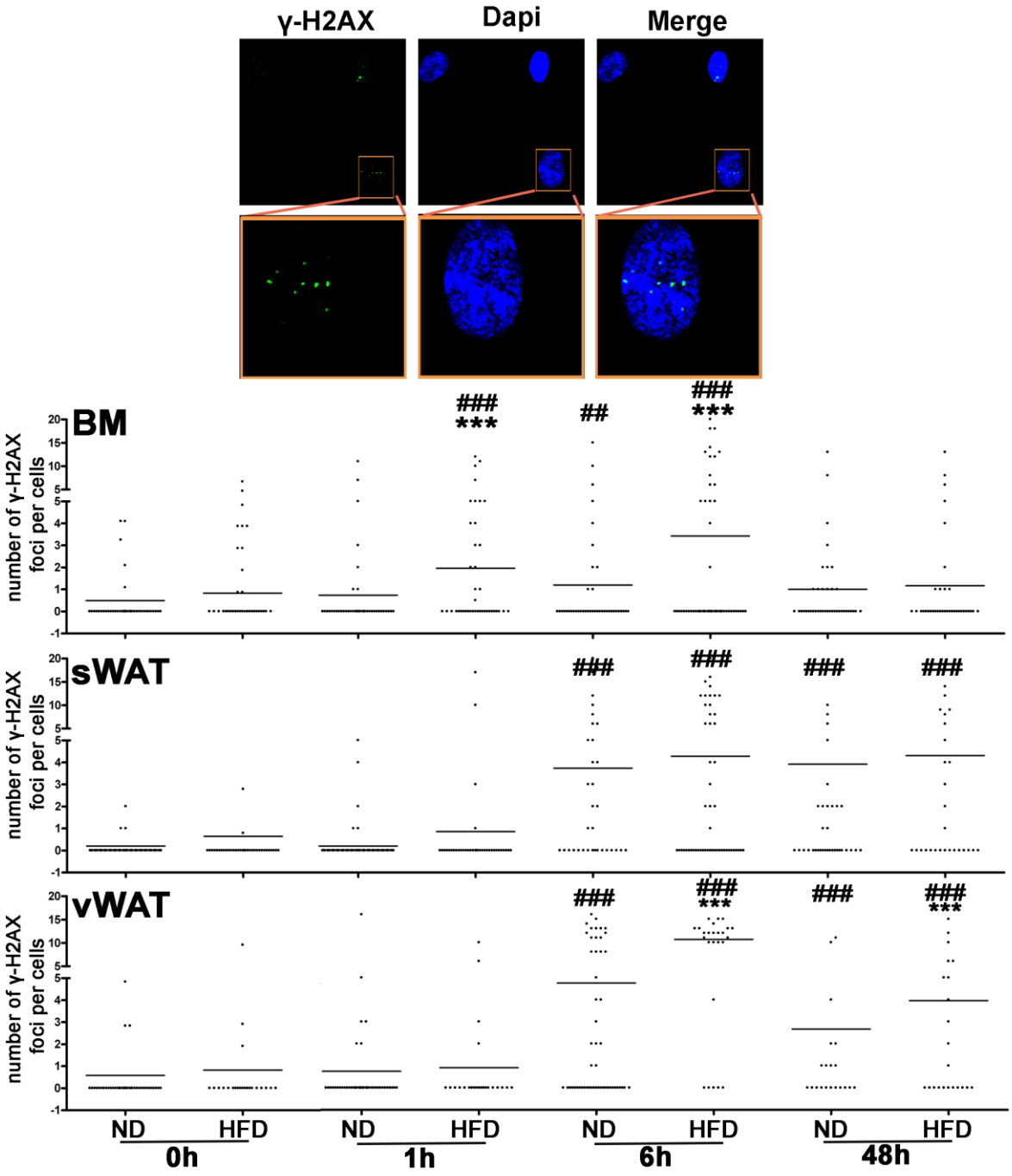

Figure 4 -DNA damage following genotoxic stress in MSCs from obese and control animals

The pictures are representative images of immunocytochemistry performed on MSC samples to detect γ-H2AX (green) and nuclei (blue). Insets, are higher magnifications of the same samples. The column scatter plot indicates the degree of H2AX phosphorylation that was determined by counting the number of γ-H2AX immunofluorescent foci per cell. The foci number was determined for 200 cells. Each dot represents a single cell. Horizontal bars indicate the mean value for each category (n = 6 biological replicates; *p < 0.05).

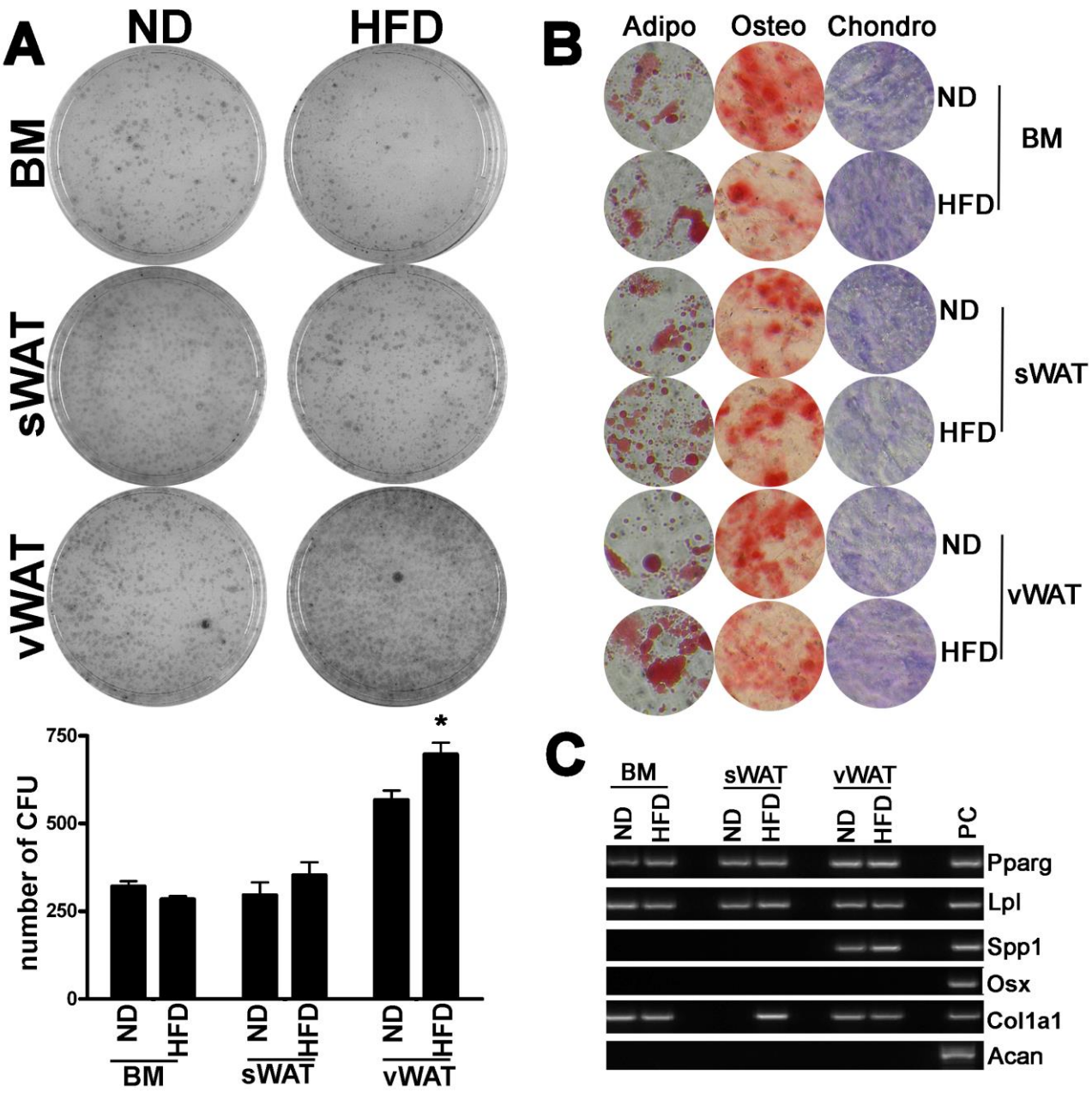

Figure 5 – Clonogenic and differentiation properties of MSCs

Panel A: The pictures show representative crystal violet staining (CFU-assay) of clones obtained after 14 days of incubation, with MSCs obtained from obese and control animals. The mean number of clones per 1,000 cells plated in 100 mm dish (± SD, n = 6, *p < 0.05,**p < 0.01) is indicated in the graph below.

Panel B: Adipocyte, osteocyte, and chondrocyte differentiation of MSCs obtained from obese and control animals. The figure shows representative images of Oil Red Oil (adipocytes), Alizarin Red S (osteocytes), and Alcian blue (chondrocytes) staining for every experimental condition.

Panel C: Qualitative RT-PCR analysis of differentiation markers expressed in proliferating MSC cultures from obese and normal samples. The picture shows a representative gel-electrophoresis

analysis.

Supplementary File 1: Statistical analysis

For experiments described in the main text the results from ANOVA and post hoc tests are provided.