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Osteo Growth Induction titanium surface treatment reduces ROS production of mesenchymal stem cells increasing their osteogenic commitment

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ABSTRACT

Surface characteristics play a special role for the biological performance of implants and several strategies are available to this end. The OGI (Osteo Growth Induction) titanium surface is a surface, obtained by applying a strong acid onto the blasted surface. The aim of this in-vitro study is to evaluate in vitro the osteoproperties of OGI surfaces on Mesenchymal Stem cells derived from dental pulp. Our results confirm that this treatment exert a positive effect on mitochondrial homeostasis, as shown by a decrease in ROS production related to environmental stress on the mitochondria. Morphological and molecular biology analyses confirmed more over that the DPSC cultured on the OGI surfaces appeared more spread in comparison to those grown on control titanium surface and real time PCR and biochemical data clearly demonstrated the increase of osteoconductive properties of the OGI treatment. In conclusion, our results suggest that mesenchymal stem cells sensitively respond to surface properties related to OGI treatment enhancing their osteogenic activities.

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1. Introduction

Implant dentistry has undergone a slow but steady growth during the last 40 years. When teeth are missing for caries, periodontal disease or agenesis, dental implants, such as dentures, are used either to replace the missing elements or to support complex prostheses [1].

The first generation of successfully used clinical titanium implants, which were machined with a smooth surface texture; now approach 40 years in clinical use. The second generation of clinically used implants underwent chemical and topographical modifications, usually resulting in a moderately increased surface topography. Many of these oral implant systems now approach 25 years of clinical use [2].

Surface characteristics play a special role for the biological performance of implants. Whereas mechanical properties such as Young's modulus and fatigue properties are mainly determined by the bulk of the material, chemical and biological interactions between the material and the host tissue are closely associated with the material surface properties [1]. Characteristics such as surface composition, surface topography, surface roughness, and surface energy affect the mechanical stability of the implant/tissue interface [3,4,5]. It was found that cell attachment and proliferation were surface roughness sensitive, and increased as the roughness of Ti-6Al-4V increased [6]. Many works have been carried out on surface treated commercial titanium implants to enhance the osteointegration function [7]. Experimental evidence from in vitro and in vivo studies strongly suggests that some types of surface modifications promote a more rapid bone formation than do machined surfaces. This could depend on an altered surface chemistry and or an increased texture on the micrometer scale [1,8,9]. Methods for altering surface texture can be classified as either techniques that add particles on the biomaterial, creating a surface with bumps (additive methods), and techniques that remove material from the surface, creating pits or pores (subtractive methods) [10].

The additive methods employed the treatment in which other materials are added to the surface, either superficial or integrated, categorized into coating and impregnation, respectively. Meanwhile, the subtractive techniques are the procedure to either remove the layer of core material or plastically deform the superficial surface and thus roughen the surface of core material. The common subtractive techniques are large-grit sands or ceramic particle blasts, acid etch, and anodization [1,11].

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The OGI (Osteo Growth Induction) titanium surface is a surface, obtained [13] by applying a strong acid onto the blasted surface. This treatment combines blasting with large-grit sand particles and acid etching sequentially to obtain macro roughness and micro pits to increase the surface roughness as well as osteointegration [7,14,15].

The aim of this in-vitro study is to evaluate in vitro the osteoproperties of OGI surfaces on Mesenchymal Stem cells derived from dental pulp; namely: Dental Pulp Stem Cell (DPSC) with a great attention to evaluate even long term cultures could affect the efficiency of the commitment of the cells. We'll here focus our effort also in evaluation on the anti-aging properties of the surfaces eventually exerted through variation on ROS production.

2. Material and methods

2.1. Biomaterial

This study cell activity on 2 implant surfaces was evaluated. We utilized implants with grit-blasted surfaces (control) and OGI surfaces (CLC implant, Italy). OGI samples are obtained by surface treatment by large grit sandblasting followed by double acid etching and relevant cleaning cycles.

The materials involved sandblasting medium is corundum and the double acid etching involves a mixture of mineral acids. In this process, the acids used in the double acid etching steps that follow sandblasting remove sandblasting particles

All experimental surfaces were cleaned and sterilized by γ-rays.

2.2. Analysis of the surface chemistry by XPS (X-ray photoelectron spectroscopy)

XPS analysis was performed using a Perkin Elmer PHI 5400 ESCA spectrometer. This is equipped with an X-ray source with an Mg anode, maintained at 20 kV with a nominal power rating of 200 W. The depth analyzed is approx. 5 nm. The pressure inside the analysis chamber has been maintained at approx. 10–9 Torr. The analysis results are expressed in atomic percentages. Two treated and cleaned samples were tested by XPS

2.3. Analysis of surface topography by SEM (scanning electron microscopy)

The surface topography of the implants was evaluated by scanning electron microscope. Analysis was conducted using an EVO MA 10 SEM (Zeiss). The electron acceleration voltage was maintained at 15 kV, the working distance at 15 mm. These parameters are reported in the images, along with the level of magnification (MAG) and the kind of detector utilized (Signal A = SE1 or CZ BSD).

Images were acquired in both conventional mode (Signal A = SE1) and in backscattered electron mode (Signal A = CZ BSD), allowing improved contrast between different chemical elements.

Stereo-SEM (SSEM), using dedicated software to convert conventional SEM images into three-dimensional data (Mex 4.2, Alicona Imaging), evaluated roughness quantitatively. In particular, this evaluation exploits the basic principle of stereovision. Basically, two images of the same field of view are acquired after eucentric rotation by a given angle. This is obtained by changing the angle between the sample and the electrons, by tilting the table that holds the sample. The tilting angle is set and controlled by the instrument control software. The couple of images obtained (stereopair), the size of the field of view and the tilting angle are the incoming data, that the software converts into a single three-dimensional image, where each data point is characterized by the values of the x, y, z coordinates. The image obtained by this process allows then to measure height profiles (roughness profiles) and to calculate the different roughness parameters defined by relevant literature and standards.

2.4. Dental pulp extraction and differentiation

Human dental pulps were extracted from healthy molar teeth, which had been extracted because of mucosal inflammation (impacted teeth with pericoronitis) or for orthodontic reasons from adult subjects aged 16 to 0.66. The pulps were classified into 6 age groups (12 teeth per group). Each subject gave informed written consent for the use of his or her donor of dental pulps. The Ethical Committee of Padua Hospital approved the research protocol. Before extraction, each subject was checked for systemic and oral infections or diseases. Only disease-free subjects were selected for pulp collection. Each subject was pretreated for 1 week with professional dental hygiene. Before extraction, the dental crown was covered with a 0.3% chlorexidine gel (Forhans, New York, NY) for 2 min. After mechanical fracturing, dental pulp was obtained by means of a dentinal excavator or a Gracey curette. The pulp was gently removed and immersed for 1 h at 37uC in a digestive solution: 100 U/mL penicillin, 100 mg/mL streptomycin, 0.6 mL of 500 mg/mL clarithromycin, 3 mg/mL type I collagenase, and 4 mg/mL dispase in 4 mL of 1 M PBS. Once digested, the solution was filtered through 70 mm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ) [15].

2.5. Immunocytochemical staining

DPSCs were layered over cover slip, fixed with absolute acetone for 10 min at room temperature and cryopreserved at −20C° until use. The following markers were visualized with immunofluorescence: SH2, SH3, SH4, CD14, CD34; CD45 (monoclonal mouse anti-human; SIGMA). Briefly, after non-specific antigen sites were saturated with 1/20 serum in 0.05 M maleate

TRIZMA (Sigma; pH 7.6) for 20 s, 1/100 primary monoclonal anti-human Ab (Sigma) was added to the samples. After incubation, immunofluorescence staining was performed with fluorescein (anti-mouse) secondary antibody.

2.6. Karyotype analysis

After 45 days of culture on DPSc were exposed to colchicine (Sigma-Aldrich, St. Louis, MO, USA) for 6 h, washed in PBS, dissociated with trypsin (Lonza S.r.l), and centrifuged at 300 g for 5 min. Colchicine is need to perform karyotyping analyses of the cells, because we need to stop cell mitosis in the metaphase stage. It is during this stage of nuclear division that the chromosomes are most condensed and, as a result, visible with a light microscope. After the cells have been arrested in this stage, they are then placed into a hypotonic solution, which causes water to enter and enlarge the cells. The cells are then placed into a chemical fixative to maintain this condition. Following this procedure, the cells can be “splatted” onto microscope slides, stained, and viewed microscopically.

The pellet was carefully suspended and incubated in 1% sodium citrate for 15 min at 37 °C, then fixed and spread onto −20 °C cold glass slides. Metaphases of cells were Q-banded and karyotyped in accordance with the international system for human cytogenetic nomenclature recommendations. Twenty-five metaphases were analyzed for three expansions.

2.7. Growth curve and doubling time

Cells were seeded into the implants at an initial density of 5×10^4. When cells reached confluence, they were detached, counted and re-seeded at a density of 5×10^4. The PDT of the cells was calculated according to the formula:

$$PDT = (T0-T1)\log 2/(\log N_t - \log Nt_1)$$

where PDT represents the cell doubling time, t represents the duration

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Table 1
Human primer sequences.

Sequence (5′-3′)	FOR	REV	Product length (bp)
OSTEOCALCIN	GCAGCGAGGTAGTGAAGAGAC	AGCAGAGCGACACCCTA	193
OSTEONECTIN	TGCATGTGTCTTAGTCTTAGTCACC	GCTAACTTAGTGCTTACAGGAACCA	183
OSTEOPONTIN	TGGAAAGCGAGGAGTTGAATGG	GCTCATTGCTCTCATCATTGGC	192
COLL I	TGAGCCAGCAGATCGAGA	ACCAGTCTCCATGTTGCAGA	178
RUNX2	AGCCTTACCAAACAACACAACAG	CCATATGTCCTCTCAGCTCAGC	175
GAPDH	TCAACAGCGACACCCAC	GGGTCTCTCTCTTCCTCTTGTG	203

of cell culture, and N0 and Nt represent the cell number after seeding and the cell number after culturing for t hours, respectively [16].

2.8. MTT assay

To determine the proliferation rate of cells growth on titanium disks with or without treatment, the MTT-based (methyl thiazolyl-tetrazolium) cytotoxicity assay was performed according to the method of Denizot and Lang with minor modifications [Denizot]. The test is based on mitochondria viability, i.e., only functional mitochondria can oxidize an MTT solution, giving a typical blue-violet end product. After harvesting the culture medium, the cells were incubated for 3 h at 37 °C in 1 mL of 0.5 mg/mL MTT solution prepared in PBS solution. After removal of the MTT solution by pipette, 0.5 mL of 10% dimethyl sulfoxide in isopropanol (iDMSO) was added for 30 min at 37 °C. For each sample, absorbance values at 570 nm were recorded in duplicate on 200 μL aliquots deposited in 96-well plates using a multilabel plate reader (Victor 3 Perkin Elmer, Milano, Italy). All samples were examined after 15 and 30 days of culture [17].

2.9. Senescence-associated beta-galactosidase staining

Beta-galactosidase staining was performed using a senescence-associated β-galactosidase (SA-β-Gal) staining kit (Cell Signaling Technology, Danvers, MA, USA) for 12 h. Briefly, human adipose-derived stem cells were cultured with 5, 10, 15, and 20 passages with or without REAC exposure in 6-well plates (3 × 103 per well) for 12 h, fixed with a fixative solution, and then processed according to the kit instructions. All of the experiments were repeated three times, and one representative set of results is shown. The cells were then photographed under an inverted microscope at 100× magnification for a qualitative detection of SA-β-Gal activity. The numbers of positive (blue) and negative cells were counted in five random fields under the microscope (at 200× magnification and bright field illumination), and the percentage of SA-β-Gal-positive cells was calculated as the number of positive cells divided by the total number of cells counted [19].

2.10. ROS measurements

The OxiSelect™ ROS Assay Kit is a cell-based assay for measuring hydroxyl, peroxyl, and other ROS activity within a cell. The assay employs the cell-permeable fluorogenic probe DCFH-DA, which diffuses into cells and is deacetylated by cellular esterases into the non-fluorescent DCFH. In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent DCF, and the fluorescence is read on a standard fluorometric plate reader.

2.11. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted with RNeasy Mini Kit (Qiagen), including DNase digestion with the RNase-Free DNase Set (Qiagen) from implants cultured with DPSCs for 15 and 25 days. The RNA quality and concentration of the samples was measured using the NanoDrop™ ND-1000 (Thermo Scientific).

For the first-strand cDNA synthesis, 200 ng of total RNA of each sample was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen), following the manufacturer's protocol.

2.12. Real-time PCR

Human primers were selected for each target gene with Primer 3 software (Table 1). Real-time PCRs were carried out using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: 15 min denaturation at 95 °C; followed by 40 cycles of 15 s denaturation at 95 °C; annealing for 30 s at 60 °C; and 20 s elongation at 72 °C. Differences in gene expression were evaluated by the 2ΔΔCt method, using DPSCs cultured onto non treated titanium disks for 15 days as control. Values were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal reference, whose abundance did not change under our experimental conditions. Experiments were performed with 3 different cell preparations and repeated at least 3 times.

2.13. Lactate dehydrogenase activity (LDH activity)

LDH activity was measured using a specific LDH Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. All conditions were tested in duplicate. The culture medium was reserved to determine extracellular LDH. The intracellular LDH was estimated after cells lysis with the assay buffer contained in the kit. All samples were incubated with a supplied reaction mixture, resulting in a product whose absorbance was measured at 450 nm using a Victor 3 multi-label plate reader.

2.14. ALP activity measurements

The alkaline phosphatase (ALP) activity was measured up to weeks of cell culture to evaluate the initial differentiation of ADSc into preosteoblasts. Abcam's alkaline phosphates kit (colorimetric) has been used to detect the intracellular and extracellular ALP activity. The kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate, which adsorbed at 405 nm when dephosphorylated by ALP. According to the manufacturer protocol, the culture medium from each sample group was collected and pooled together. At the same time, cells were washed with PBS and then homogenized with ALP Assay Buffer (300 μL in total for each group) and centrifuged at 13,000 rpm for 3 min to remove insoluble material. Different volumes of samples (medium and cells) were then added into 96-well plate, bringing the total volume in each well up to 80 μL with Assay Buffer. 80 μL of fresh medium was also utilized as sample background control. Thereafter, 50 μL of 5 mM pNPP solution was added to each well containing test samples and background control and incubated for 60 min at 25 °C, protecting

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the plate from the light. A standard curve of 0, 4, 6, 12, 16, and 20 nmol/well was generated from 1 mM pNPP standard solution bringing the final volume to 120 μL with Assay Buffer. All reactions were then stopped by adding 20 μL of Stop solution into each standard and sample reaction except the sample background control reaction. Optical density was read at 405 nm in a microplate reader (Victor). The results were normalized subtracting the value derived from the zero standards from all standards, samples and sample background control. The pNP standard curve was plotted to identify the pNP concentration in each sample. ALP activity of the test samples was calculated as follow:

$$\text{APL activity } (\text{U/mL}) = \text{A/V/T}$$

where:

A is the amount of pNP generated by samples (in μmol).
V is the amount of sample added in the assay well (in mL).
T is the reaction times (in minutes).

2.15. Scanning electron microscopy (SEM)

Quantitative evaluation of surface roughness has been conducted in accordance with ISO 4287, providing values for all the parameters defined in the standard. As described before, data were obtained by StereoSEM, generating three dimensional images from a stereo pair made up by two SEM images of the same field of view, obtained at 2000× with a tilting angle of 5°.

For SEM imaging, DPSCs grown on control and treated Ti surfaces for 15 and 25 days were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, then progressively dehydrated in ethanol. Control and treated Ti surfaces without cells were also examined. The SEM analysis was carried out at the Interdepartmental Service Center C.U.G.A.S. (University of Padova, Italy).

2.16. Determination of scavenging effect on DPPH radicals

250 mL of 1, 10-diphenyl-2-picrylhydrazyl (DPPH, Sigma) solution (0.1 mM, in 95 wt% ethanol) was added into Ti control or OGI samples, and deionized water as the control. The mixture was shaken and kept at room temperature in the dark. After 30 min, the absorbance was measured at 517 nm by a spectrophotometer. The DPPH radicals scavenging effect (%) of Ti control surfaces and OGI can be calculated using the equation (34):

$$\text{Scavenging effect } (\%) = \frac{\text{Acontrol} - \text{Asample} \times 100}{\text{Acontrol}}$$

2.17. Statistical analysis

One-way analysis of variance (ANOVA) was used for data analyses. The Levene's test was used to demonstrate the equal variances of the variables. Repeated-measures ANOVA with a post-hoc analysis using Bonferroni's multiple comparison was performed. *t*-Tests were used to determine significant differences (p,0.05). Repeatability was calculated as the standard deviation of the difference between measurements. All testing was performed in SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) (license of the University of Padua, Italy).

3. Results

3.1. XPS analysis

The XPS analysis method was used to evaluate the surface composition: no aluminum from blasting residuals was detected suggesting that all residuals were removed in processing and cleaning steps.

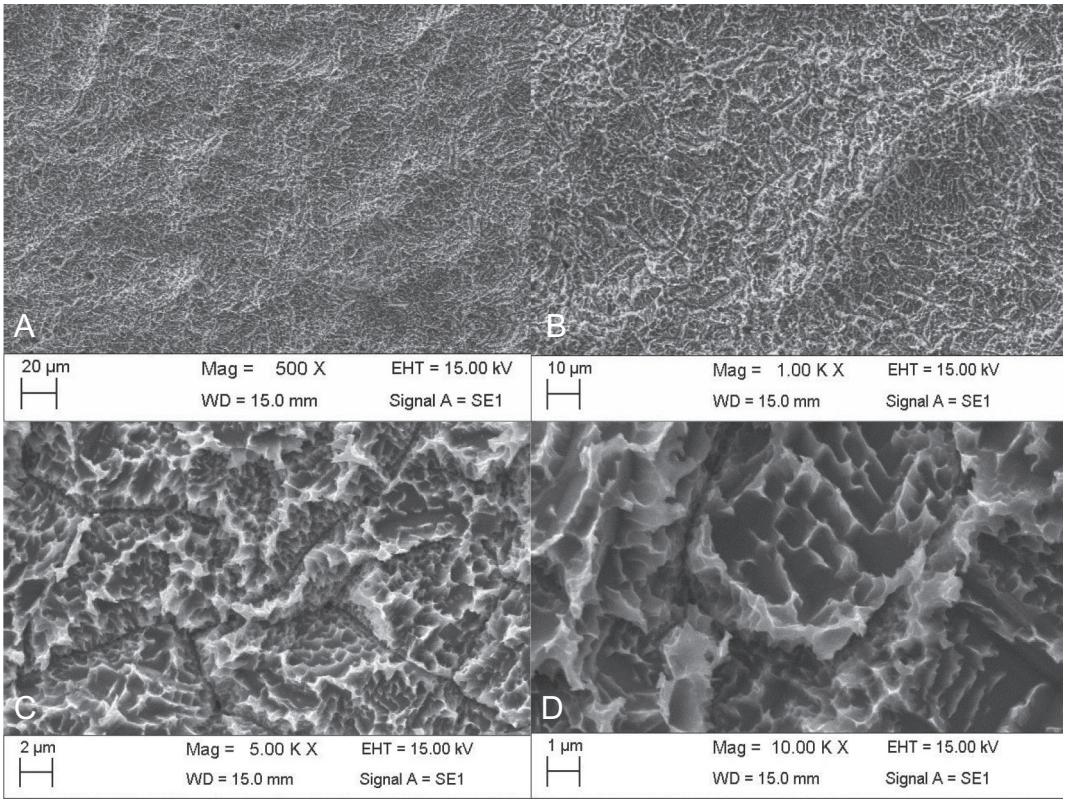

Fig. 1. Fig. 1a–d shows microscopic views of the OGI surface. They confirm that the surface is free from dirt and particles, as suggested by XPS data, in particular from blasting residuals.

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Table 2
A typical three-dimensional view of the OGI surface.

Parameter	OGI surfaces	Control
Ra	1.61 +/− 0.31	1.72 +/ 0.19
Rq	1–86 +/− 0.41	1.90 +/− 0.41
Rz	11.24 +/− 0.31	10.54 +/− 0.44
Rp	6.24 +/− 0.21	6.40 +/− 0.65
Rv	5.00 +/ 0.51	4.13 +/− 0.43
Rc	5.21 +/− 0.11	5.63 +/− 0.67

Where the definition of the various parameters are as follow:

Parameter	
Ra	Average of roughness profile
Rq	Root mean square roughness of profile
Rz	Maximum height of roughness of profile
Rp	Maximum peak height of roughness of profile
Rv	Maximum valley depth of roughness profile
Rc	Mean height of profile irregularities of roughness

3.2. SEM analysis

Fig. 1a–d show microscopic views of the OGI surface. They confirm that the surface is free from dirt and particles, as suggested by XPS data, in particular from blasting residuals. The whole surface is homogeneously treated. The peculiar features of the OGI surface are highlighted by the typical topography, which shows both big "holes" due to large-grit sandblasting on which the microroughness due to acid etching is superimposed. Figures show the typical nice tri-dimensional topography, which imparts to these surfaces spongy-like characteristics. Short peak-to-peak distance, of the order of about 1 μm, are also present. This combination of long-range (large grit sandblasting) and short range (acid etching) roughness is a feature of OGI surfaces (Table 2).

A typical three-dimensional view of the OGI surface in shown in Fig. 2, that once again shows the co-existence of long-range and short range roughness.

3.3. Dental pulp extraction and differentiation

Fig. 3a illustrates the phenotypic characterization of culture-expanded human MSCs (hMSC) by immunofluorescence analysis. Cells were consistently positive for β1 integrin SH2 (93.22%), SH3 (96,63%) and SH4 (89,35%). Specific hematopoietic markers such as CD 14, CD 34 and CD 45 were consistently negative. The chromosomal stability of DPSc cultured in vitro up to 45 days was analyzed by means of karyotyping. As reported in Fig. 3b, no chromosomal alterations are present in DPSc after long term in vitro cultures.

3.4. Proliferative activity

In order to evaluate proliferation activity of MSC on OGI titanium surfaces we have performed both Population doubling time (PDT) and MTT test. PDT is usually used to evaluate the ability of the cell to duplicate in number and is therefore a direct marker of the proliferative ability of the cell. In this experiment, we analyzed the PDT of MSC cultured in the titanium surfaced treated with OGI and in control (titanium surfaces not treated with OGI). PDT was evaluated at 5 different in vitro passages (p) of the cultures after 3, 7, 14, 21, 28 days. As reported in Fig. 4a, we observed well-defined cell growth for each passage in both surfaces. On each sample moreover proliferative ability decreased in time and during in vitro aging and during the osteogenic commitment. Proliferative ability has been moreover evaluated by the MTT test (Fig. 4b). The rising in MTT values confirmed that the cells were alive. When cells started the commitment, about after 14–18 days, the MTT value reach a steady state strongly related to the commitment in an adult phenotype of stem cell.

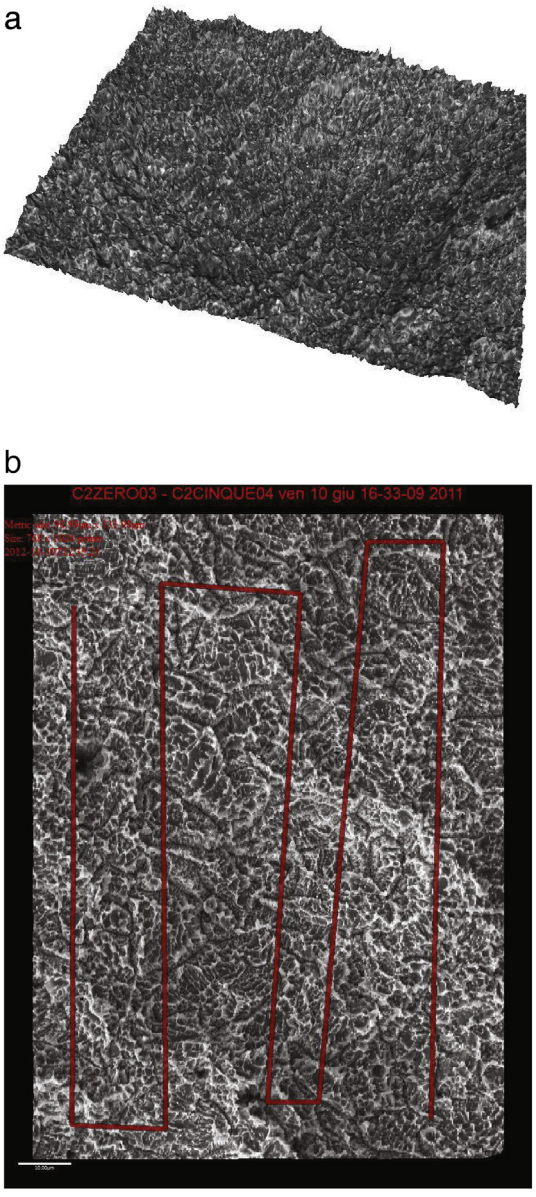

Fig. 2. A typical three-dimensional view of the OGI surface in shown in Fig. 2.

3.5. Senescence and ROS production

Normal somatic cells invariably enter a state of irreversibly arrested growth and altered function after a finite number of divisions. This process, is thought to be an underlying cause of aging. Studies on cells cultured from donors of different ages, genetic backgrounds, or species suggest that senescence occurs in vivo and that organismic lifespan and cell replicative lifespan are under common genetic control. However, senescent cells cannot be distinguished from quiescent or terminally differentiated cells in tissues. Thus, evidence that senescent cells exist and accumulate with age in vivo is lacking. There is a simple test that is based on the evidence that several human cells express a beta-galactosidase, histochemically detectable at pH 6, upon senescence in culture. This marker was expressed by senescent, but it was also absent from immortal cells. In our samples there was an in vitro-age-dependent increase in this marker in MSC. This marker provides in situ evidence that senescent cells may exist and accumulate with age in vitro. This senescence activity of stem cell due to their in vitro cultures has been evaluated by β-galactosidase staining detected at fixed time points of (3, 7, 21, 12, and 28 days) into both surfaces (Fig. 5a). In particulars

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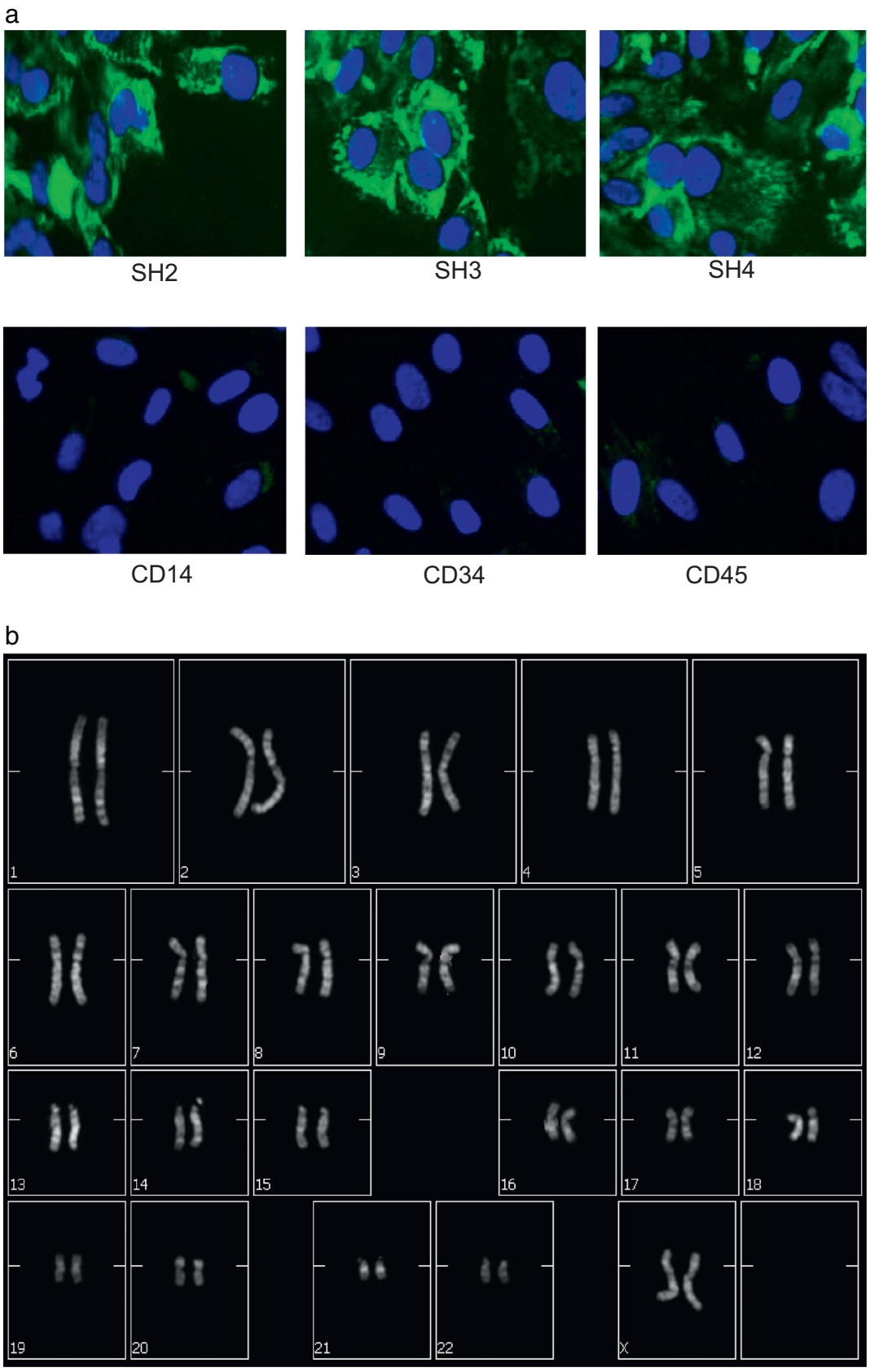

Fig. 3. a) Immunofluorescence analysis of SH2; SH3; SH4; CD 14; CD 34; and CD 45. Cell in green are positive for SH2, SH3 SH4 and negative con CD14, CD34 and CD45 as no green staining is present. Fibroblast is used as negative controls (data not shown). b) Karyotype analysis of DPSCs cultured in vitro for 45 days. No chromosomal alterations are present. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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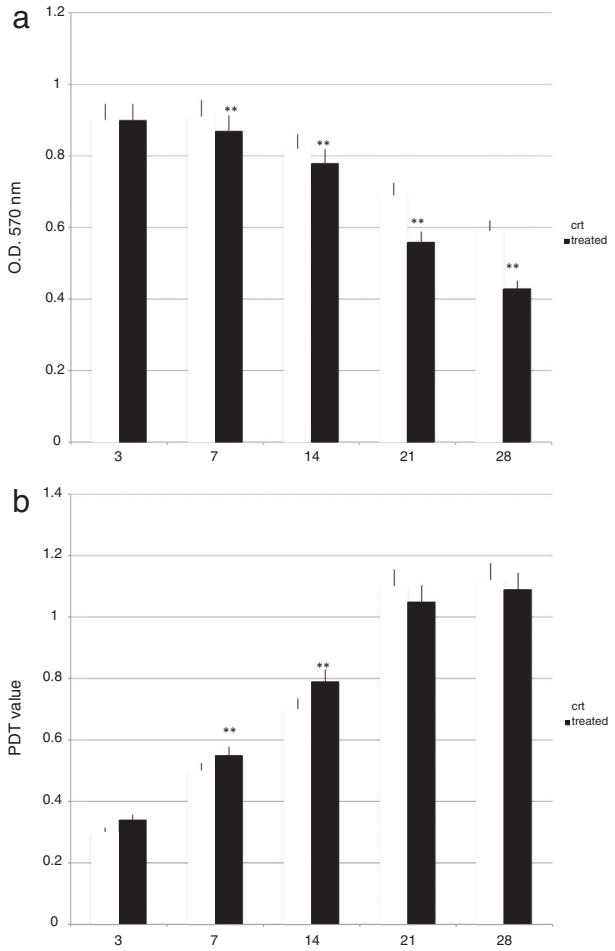

Fig. 4. a) Population doubling time (PDT) of DPSCs cultured in both of surfaces in presence of osteogenic medium. The graph shows in vitro passage and tested at each time point. *t*-Tests were used to determine significant differences (p,0.05). *p,0,05; **p,0,01; ***p,0,001. b) MTT assay of DPSCs cultured on the control and treated Ti surfaces. DPSCs MTT related values are higher on the treated disks in the first periods of in vitro cultures and decrease when cells start the commitment respect to the control one at each time points of detection.

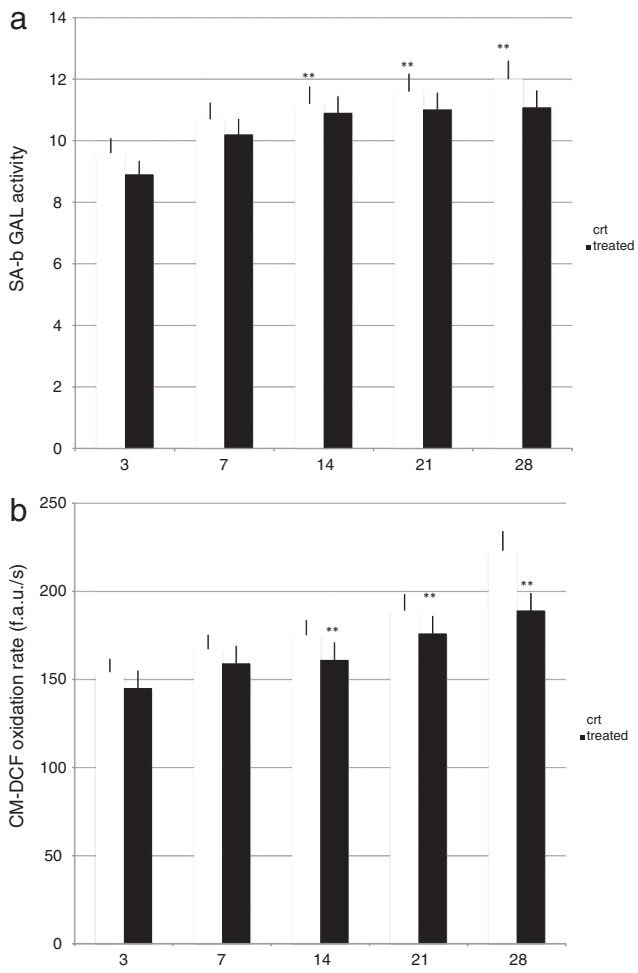

Fig. 5. a) Senescence-associated β-galactosidase (SA β-Gal) staining in DPSc seeded onto both surfaces at fixed time points of 3, 7, 14, 21 and 28 days. A time-dependent increase in metabolic activity related to the in vitro aging is observable in both surfaces, weather the lower values are presents only is DPSc where cultured on OGI surfaces. b) ROS production in cells cultured onto both surfaces due to the in vitro aging of the cells, was observed: *t*-tests were used to determine significant differences ($p < 0.05$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Lower ROS values were detected in presence of OGI treatment.

our results show that cells cultured on OGI surfaces have a significative higher value compared to the cells cultured on the control one, suggesting that a less aging could be occur thanks to OGI treatment. The values of SA-β-Gal-staining were increase for every time point in both conditions and this result became more pronounced as the culture time increased. Moreover, a less significant increase in the intensity of the staining in the cultures on OGI surfaces was observed.

3.6. ROS measurement

To test whether the OGI treatment influence cell aging activity, we assessed ROS generation. Under environmental stress, the cells increased ROS production, leading to an imbalance between ROS generation and its neutralization by anti-oxidative enzymes and low molecular weight antioxidants, such as glutathione. This disturbance in the redox equilibrium is defined as oxidative stress. Under conditions of oxidative stress, the cell accumulates ROS, and the anti-oxidative response that follows involves modifications in signaling pathways. As reported in Fig. 5b a time dependent increase in metabolic activity was observed in cells seeded onto both surfaces. When the MSC were cultured on OGI surfaces a well-defined decrease in ROS production was revealed, and this finding confirms the ability of OGI to influence the aging of the cells through mitochondrial function.

3.7. DPPH assay

DPPH radical scavenging assay is the most widely used method for screening antioxidant activity, since it can accommodate many samples in a short period and detect active ingredients at low concentration. The scavenging ability of antioxidants on DPPH radicals is thought to be due to their hydrogen donating ability. On our samples the DPPH radical scavenging effects (%) of Ti control surfaces and OGI surfaces are very low: 0.37% in control versus 0,41% on OGI surfaces

3.8. Gene expression

Real-time PCR was performed on MSC cultures in both surfaces in presence of osteoinductive factors (Fig. 6) after 28 days of cultures on control and OGI surfaces in presence of osteoinductive medium. The gene expression detected by means of the molecular markers selected can provide information on the mature osteogenic phenotype (osteocalcin; osteonectin; osteopontin; RUNX2; COL1A1; Wnt; Foxo 1; ALP; BMP2; BMP7). As shown in Fig. 6, the presence of osteoinductive medium was able to induce a good osteogenic

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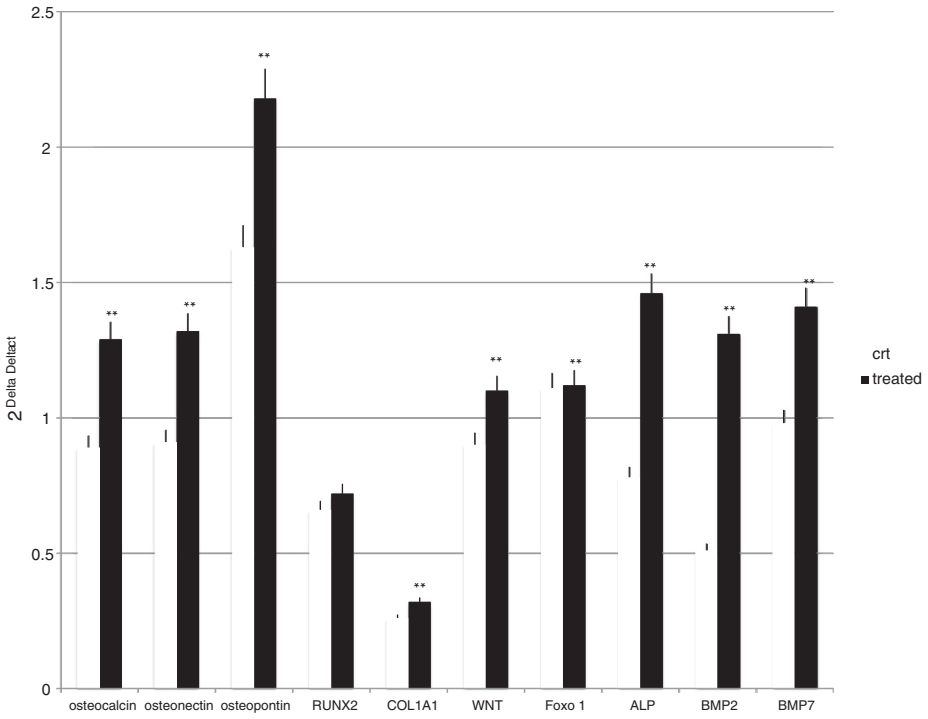

Fig. 6. Gene expression by real-time PCR on DPSCs in control and OGI surfaces monolayers and in nanostructured scaffolds. Markers selected were bone morphogenic protein (BMP) 2, BMP-3, cathepsin B (CTSB), CTSD, collagen type I (Col1A1), collagen type III (Col3A1), Distal-less homeobox (DLX) 1, DLX5, fibroblast growth factor (FGF), transforming growth factor b1 (TGFb1), receptor activator of nuclear factor kappa-B (RANK), osteopontin, osteonectin, and osteocalcin. The results for each experiment are from quadruplicate experiments. Values are expressed as the mean 6 SD. *t*-Tests were used to determine significant differences (p,0.05). *p,0,05; **p,0,01; ***p,0,001.

commitment of the MSC in both conditions. Greater level of expression has been detected Indeed, the expression profile for osteopontin, osteonectin, osteocalcin, runx, FOX, bone morphogenic protein (BMP 2 and 7), and collagen type I is higher when cells are present on OGI surfaces compared to control.

3.9. ALP activity measurements

To confirm the early differentiation of MSC towards osteoblast phenotype, the ALP activity (expressed as U/mL that is the amount of enzyme causing the hydrolysis of 1 μmol of pNPP per units per mL) was quantified both into cells and in culture medium. Particularly, the intracellular ALP activity was 0.41 U/mL into MSC grown on OGI surfaces and 0.38 U/mL in MP group. Even the extracellular ALP was higher in OGI (0.29 U/mL) than the MP group, where it was slightly lower (0.21 U/mL). No statistical differences were observed between the groups.

3.10. Intracellular and extracellular lactate dehydrogenase activity (LDH activity)

In order to overcome the controversial results of the MTT assay related to the decreasing in time of the value, the LDH activity assay was also performed on the cells. Fig. 7a shows the intracellular LDH activity of the cells seeded on both surfaces: the graph proves that cells were able to produce metabolites, with improved results after seven days from seeding. As reported in Fig. 7b, extracellular LDH activity, generally related to a membrane damage related to the scaffolds, was also measured on the culture medium: the graph confirms that metabolites were secreted by the same cells with no difference between the surfaces.

3.11. Morphological analyses

SEM has evaluated morphological analyses of MSC seeded onto Ti surfaces. In Fig. 8a–d and SEM images revealed how cells attached to the surface for the samples: control (a–c) and OGI (b–d). After 5 day of culture, cells in all cases, were characterized by short and thin filopodia and characterized by a good distribution for both samples. This observation is confirmed by the measurements with Image J software, showing that the area of the cell cultured for 5 day on control surfaces is approximately equal (199.33 μm^2) than that cultured on OGI (189.29 μm^2). It can be moreover observed that MSC appears spread, overlapped, which enable communication with each other and develop well-defined phyllopod (black arrows).

3.12. Discussion

In the present study we have investigated the osteogenic response of dental pulp Mesenchymal Stem Cells to an OGI titanium based surfaces. The surface Nano topography and chemistry would influence the interfacial cellular interaction and then its commitment if they were in a stemness condition. In the current study, proliferation analyses related to stem cells seeded onto the treated surfaces demonstrate some little differences between the OGI surfaces with the control.

In order to evaluate the osteogenic influence of the titanium surfaces, protocol requires a long term in vitro cultures up to 28 days. The aging process related to this condition, has been evaluated and results showed that long-term in vitro cultures was strongly influenced by OGI surfaces. A slow-down in the aging process is indeed closely associated with the presence of OGI treatment. Moreover, this treatment exerts a positive effect on mitochondrial homeostasis,

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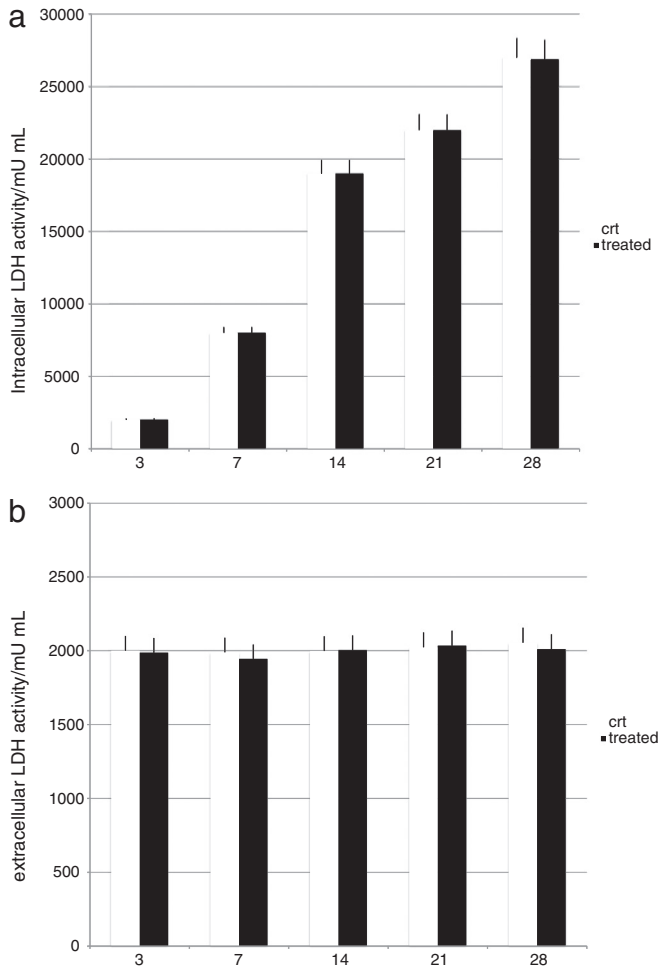

Fig. 7. a) Intracellular LDH activity assay. b) Extracellular LDH activity assay.

as shown by a decrease in ROS production related to environmental stress on the mitochondria. The presence of antioxidant effect of the surfaces as been moreover evaluated by DPPH assay that confirm the surfaces does not exert di per se any direct antioxidant ability at extracellular level.

In brief, the DPSC cultured on the OGI surfaces appeared more spread in comparison to those grown on control titanium surface and had developed longer filopodia, which is an indication that OGI treatment is appropriate materials for cell colonization. In the mean time real time PCR data sets clearly demonstrated the osteoconductive properties of the OGI structure in terms of gene expression. Particularly, osteopontin presented a greater tendency of expression in cells cultured in the presence of OGI compared to the other genes.

These date are is also ascertained by the results of ALP activity test since ALP represents a key enzyme secreted by osteoblast in the early stages of differentiation, which provides high concentration of phosphate at the sites of mineral depositions. The mineralization process, related to matrix and mineral deposit, instead, is a process that occurs in the presence of mature osteoblasts during new bone formation.

Our results suggest that mesenchymal stem cells sensitively respond to surface properties related to OGI treatment enhancing their osteogenic activities. In order to explain how OGI surface could act this increasing on osteogenic commitment trough and anti aging ROS dependent way, we have postulated that the extracellular matrix environment of OGI could affect Wnt family receptor modifying the final commitment of MSC. MSCs, a non-hematopoietic stem cell population are multipotent cells able to differentiate into mature cells of several mesenchymal tissues, such as fat and bone. As common progenitor cells of adipocytes and osteoblasts, MSCs are delicately balanced for their differentiation commitment. This event is important in bone environment. The dysregulation of the adipo-osteogenic balance has been linked to several pathophysiologic processes, osteopenia, osteopetrosis, and osteoporosis. Several investigations have demonstrated that fat-induction factors inhibit osteogenesis, and, conversely, bone-induction factors hinder adipogenesis [18]. Indeed a variety of external cues contribute to the delicate balance of adipo-osteogenic differentiation of MSCs, including chemical, physical, and biological factors. Among these, aging is note to cause a decrease in the number of bone-forming osteoblasts and an increase in the number of adipocytes. It is well known that the generation of reactive oxygen species (ROS) can lead to mitochondrial dysfunction, DNA damage, and the promotion of aging –dependent processes [19].

More over as reported by Galli et al. [24,25] little is known about how surface topography can modulate mesenchymal cell responses to oxygen-related stress occurring with age, or during the early phases of wound healing or inflammation. To antagonize Reactive Oxygen Species (ROS), cells resort to defense mechanisms, relying on β-catenin, a molecular switch between a TCF-mediated pathway, which promotes cells proliferation and commitment, and an alternative one controlled by FoxO, which induces quiescence and defenses against ROS. It is known that the roughness of titanium surfaces affects cell proliferation and differentiation. However, the mechanisms mediating the cellular responses to surface topography are only partially understood. Galli et al. showed that surface roughness modulates the responsiveness of mesenchymal cells to Wnt3a, that this requires the control of β-catenin degradation, and that the control of β-catenin signaling by surface topography is accountable for at least part of the effects of surface on cell differentiation.

The potential for differentiation of MSCs according to age is controversial, but most of the studies have shown a decrease in their capacity to undergo osteogenic differentiation [20]. Recent studies have demonstrated that the Wnt/β-catenin signaling plays an important role in stem cell aging. However, the mechanisms of cell senescence induced by Wnt/β-catenin signaling are still poorly understood. Zang et al. has indicated that activated Wnt/β-catenin signaling can induce MSC aging. In this study, the authors reported that the Wnt/β-catenin signaling was a potent activator of reactive oxygen species (ROS) generation in MSCs [21]. These results indicated that the Wnt/β-catenin signaling could induce MSC aging through promoting the intracellular production of ROS, and ROS may be the main mediators of MSC aging induced by excessive activation of Wnt/β-catenin signaling. There are evidence that, during aging, the status of mMSC changes with respect to both their intrinsic differentiation potential and production of signaling molecules that contribute to the formation of a specific marrow microenvironment necessary for maintenance of bone homeostasis. With aging, the number of mMSC committed to the adipocytic lineage increases, whereas the number of mMSC committed to the osteoblastic lineage decreases. Increased expression of the adipocyte-specific transcription factor PPAR-γ2 and increased production of its activator might be a driving force for pro-adipocytic and anti-osteoblastic changes in the differentiation potential of mMSC [21].

Having said this, it is well established that extracellular matrix (ECM) stiffness plays a significant role in regulating the phenotypes and behaviors of many cell types. However, the mechanism underlying the sensing of mechanical cues and subsequent elasticity-triggered pathways remains largely unknown. Du et al. observed that stiff ECM significantly enhanced the expression level of several members of the Wnt/β-catenin pathway [22].

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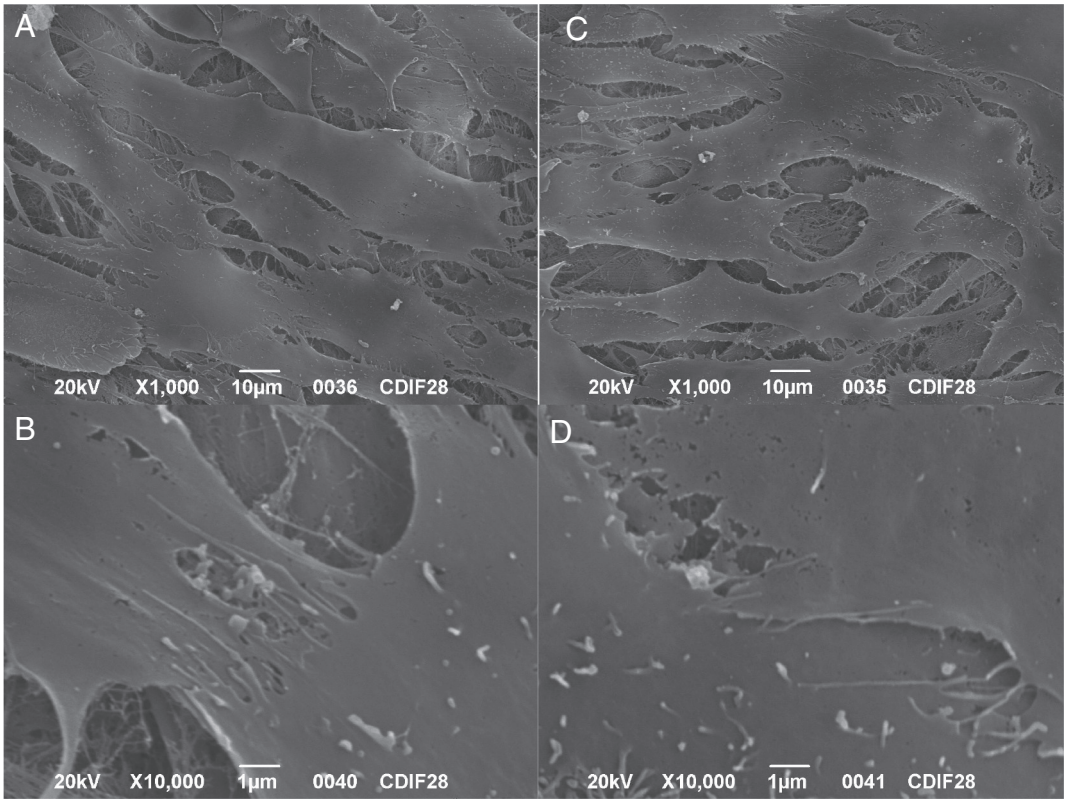

Fig. 8. SEM analyses of MSC seeded onto Ti surfaces. a–b: SEM images of MSC attached to the surface of control surfaces c–d: SEM images of MSC attached to the surface of OGI surfaces. After 5 days of culture, cells in all cases, were characterized by short and thin filopodia and characterized by a good distribution for both samples.

In the end, with propose that OGI could affect the osteogenic commitment of MSC stimulating an ECM environment that reduces the aging of MSC.

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